Prasad, B. V. V., & Balaram, P. (1983) In *Conformation in Biology* (Srinivasan, R., & Sarma, R. H., Eds.) pp 133–139, Adenine, New York.
Prasad, B. V. V., & Balaram, P. (1984) *CRC Crit. Rev. Biochem. 16*, 307–348.
Prasad, B. V. V., Balaram, H., & Balaram, P. (1982) *Biopolymers 21*, 1261–1273.
Rashin, A. A., & Honig, B. (1984) *J. Mol. Biol. 173*, 515–521.
Ravi, A., & Balaram, P. (1984) *Tetrahedron 40*, 2577–2583.
Ravi, A., Prasad, B. V. V., & Balaram, P. (1983) *J. Am. Chem. Soc. 105*, 105–109.
Roberts, G. C. K., & Jardetzky, O. (1970) *Adv. Protein Chem. 24*, 447–545.
Roos, A., & Boron, W. F. (1981) *Physiol. Rev. 61*, 296–436.
Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563–568.
Smith, G. D., Pletnev, V. Z., Duax, W. L., Balasubramanian, T. M., Bosshard, H. E., Czerwinski, E. W., Kendrick, N. E., Mathews, F. S., & Marshall, G. R. (1981) *J. Am. Chem. Soc. 103*, 1493–1501.
Spray, D. C., & Bennett, M. V. L. (1985) *Annu. Rev. Physiol. 47*, 281–303.
Steitz, T. A., & Shulman, R. G. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 419–444.
Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, Freeman, San Francisco.
Sundaralingam, M., Drendel, W., & Greaser, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7944–7947.
Thomas, P. G., Russell, A. J., & Fersht, A. R. (1985) *Nature (London) 318*, 375–376.
Thornton, M. J. (1982) *Nature (London) 295*, 13–14.
Warshel, A. (1981) *Acc. Chem. Res. 14*, 284–290.
Warshel, A., Russel, S. T., & Churg, A. K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4785–4789.
Wold, F. (1981) *Annu. Rev. Biochem. 50*, 783–814.

Isolation and Characterization of a Lectin from the Cortical Granules of *Xenopus laevis* Eggs†

Tatsuro Nishihara,‡ Ron E. Wyrick,§ Peter K. Working,‖ Yee-Hsiung Chen,⊥ and Jerry L. Hedrick*
Department of Biochemistry and Biophysics, University of California, Davis, California 95616
Received December 20, 1985; Revised Manuscript Received June 9, 1986

ABSTRACT: A cortical granule lectin was isolated from eggs of the South African clawed toad *Xenopus laevis*. The lectin was released from the cortical granules by activation of dejellied eggs with the Ca^{2+} ionophore A23187. The lectin was purified by affinity chromatography with its natural ligand, the egg jelly coat, chemically coupled to a Sepharose matrix. The purified lectin was homogeneous by the criteria of isoelectric focusing (pI = 4.6), immunodiffusion, and immunoelectrophoresis but existed in two different molecular weight isomers as determined by sedimentation velocity ultracentrifugation and disc gel electrophoresis. Molecular weights of the isomers were determined by ultracentrifugation, disc gel electrophoresis, and gel filtration and found to be 539 000 and 655 000. Chemically, the lectin was a metalloglycoprotein, composed of 84.0% protein, 15.8% carbohydrate, and 0.19% calcium. No unusual types or amounts of amino acids were present. The carbohydrate moiety was composed of fucose, mannose, galactose, glucosamine, galactosamine, and sialic acid. The monosaccharide specificity of the lectin was investigated with the sugar inhibition of the precipitin reaction in gels. The lectin was specific for D-galactosyl sugars with the configuration at carbon atoms 2–4 of primary importance.

Fertilization in the eggs of the South African clawed toad *Xenopus laevis* results in marked changes in the morphology of the egg surface (Grey et al., 1974). In particular, the extracellular vitelline envelope, VE,[1] is converted to the morphologically distinct fertilization envelope, FE. The FE functions in blocking supernumerary sperm penetration at fertilization, the block to polyspermy reaction. It has been demonstrated that the isolated VE is readily penetrated by sperm whereas the isolated FE is impenetrable by sperm (Grey et al., 1976). Morphologically, the FE differs from the VE by the presence of a distinct ultrastructurally amorphous layer termed the fertilization (F) layer between the VE and the innermost jelly coat layer J_1 (Grey et al., 1974). Chemical and macromolecular analyses of the isolated envelopes, as well as microscopic evidence, indicated that the F layer is formed by interaction of components derived from the cortical granules and the innermost jelly coat layer, J_1 (Grey et al., 1974; Wolf et al., 1976).

We previously suggested that the interaction of the cortical granule and jelly components to form the F layer was, in molecular terms, a metal-mediated lectin–ligand interaction (Wyrick et al., 1974a,b; Nishihara et al., 1975). The purpose

† This work was supported in part by a grant from the USDHS, HD-4906.
‡ Present address: Laboratory of Microbiology, Suntory Institute for Biomedical Research, Mishima-Gun, Osaka, 618 Japan.
§ Present address: Director of Research, Hollister-Stier Laboratory, Spokane, WA 99220-3153.
‖ Present address: Chemical Industry Institute of Toxicology, Research Triangle Park, NC 27709.
⊥ Present address: Graduate Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan, China.

[1] Abbreviations: ACFT, affinity column flow through; CG_{ex}, cortical granule exudate; CGL, cortical granule lectin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FE, fertilization envelope; F layer, fertilization layer; J_1, jelly coat layer; NANA, *N*-acetylneuraminic acid; PAS, periodic acid–Schiff base; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; VE, vitelline envelope.

0006-2960/86/0425-6013$01.50/0

of this paper is to report the isolation of the cortical granule component that interacts with the jelly component, to provide evidence that functionally it is a lectin, and to report some of the cortical granule lectin's physicochemical and sugar binding characteristics.

Materials and Methods

Xenopus laevis Eggs. Sexually mature *X. laevis* were imported through Dart and Howes Limited, Capetown, South Africa, or collected in Orange County, CA. Eggs were obtained from *X. laevis* following injection of human chorionic gonadotropin as previously described (Wolf & Hedrick, 1971), except for injection of pregnant mare serum gonadotropin (35 IU per toad), 3.5–4.5 days prior to human chorionic gonadotropin injection.

Eggs were dejellied with either 45 mM mercaptoethanol in DeBoers solution (110 mM NaCl, 1.3 mM KCl, and 1.3 mM $CaCl_2$, pH 7.2, with $NaHCO_3$) adjusted to pH 9.5 with sodium hydroxide or in 2–5 mM dithiothreitol in DeBoers solution at pH 8.0 as previously described (Wyrick et al., 1974a). A solution of the innermost jelly coat layer J_1 was obtained by first manually dissecting jelly layers J_3 and J_2 and then dissolving the exposed jelly layer J_1 in dithiothreitol solutions as previously described (Yurewicz et al., 1975).

The cell-free starting material for purification of the cortical granule lectin (CGL) was a cortical granule exudate (CG_{ex}) prepared by activating dejellied eggs. Eggs (30 000–40 000) were dejellied with mercaptoethanol in DeBoers solution and washed thoroughly with DeBoers solution, and the washed dejellied eggs were suspended in twice their volume of 10 mM Tris-HCl, pH 7.2, in DeBoers solution. To the egg suspension was added the calcium ionophore A23187 (Eli Lilly, 5 mM in ethanol) to a final solution concentration of 1 μM. After 20 min of gentle shaking at room temperature, the medium was decanted from the eggs. The eggs were washed with three portions of the Tris-buffered DeBoers solution, and the exudate medium and washing solutions were combined and centrifuged at 40000*g* for 20 min at 4 °C. The clear centrifugate was dialyzed against distilled water, adjusted to pH 7.5 with Tris, lyophilized to dryness, and dissolved in 10–15 mL of standard buffer (10 mM Tris-HCl, pH 7.8, 154 mM NaCl, 1 mM $CaCl_2$). This solution was then dialyzed against the standard buffer and will be referred to as the cortical granule exudate, CG_{ex}. With care, this procedure resulted in no egg lysis and less than 0.1% lysis when larger numbers of eggs were processed.

For the purification of the CGL, an affinity column was prepared with dithiothreitol-dissolved total jelly (all three jelly coat layers) coupled to cyanogen bromide activated Sepharose 4B. On one occasion, the jelly coupled to the column was a jelly coat layer J_1 fraction enriched in sulfated glycoproteins by cetylpyridinium chloride precipitation (Wyrick et al., 1974a). Sepharose 4B activation and coupling procedures utilized the method of Steinmann and Stryer (1973) with the following modifications. To 133 mL of ice-cold cyanogen bromide activated Sepharose 4B 133 mL of a dialyzed jelly solution (1.29 mg of neutral hexose/mL) in 0.1 M sodium acetate–0.15 M sodium chloride, pH 8.0, with sodium bicarbonate was added and the mixture stirred for several days at 4 °C. The substituted Sepharose was washed with 0.1 M sodium acetate–0.15 M sodium chloride, pH 6.8, until the washed solution showed no absorbance at 280 nm. The yield of the coupling step was 25.8% (44.2 mg) of neutral hexose. The column was prepared from this material (2.0 × 43.8 cm, 138-mL bed volume) and then equilibrated with the standard buffer.

For studies on the ligand specificity of the CGL, galactan oligosaccharides were prepared by fractionating gum arabic (Calbiochem). The mono-, di-, tri-, tetra- and greater than pentasaccharide fractions were prepared by chromatography of the galactans on Sephadex G-15. Identification of the fractions was accomplished by comparison of their K_{av} values with K_{av} values of known oligosaccharides (mono to tetra) and by cochromatography with standard oligosaccharides.

Activity Analysis. The assay for activity (precipitin activity) of the CGL was by the appearance of a precipitin line on an agarose plate (double diffusion in two dimensions) with jelly coat layer J_1 as the precipitating ligand (Wyrick et al., 1974a). Jelly coat layer J_1 was prepared with dithiothreitol as the solubilizing agent (Yurewicz et al., 1975) and used, after dialysis to remove the dithiothreitol, in concentrations from 0.17 to 0.36 mg of neutral hexose/mL. The double-diffusion plate was prepared from 1% agarose in the standard buffer with 3 mm diameter holes 2 mm in depth and 3 mm apart. Serial dilutions of the CGL were allowed to diffuse for 24 h in a moist chamber at 22 °C. Activity of the CGL can be expressed in titer units, e.g., the maximum dilution of the preparation that gives a visible precipitin reaction. The minimum CGL (homogeneous) detectable under these assay conditions (vs. 3.4 μg of J_1 hexose/20 μL) was 0.43 ± 0.13 μg, which corresponded to a specific activity of 2300 titer units/mg of CGL.

For the inhibition studies of the lectin reaction, two methods were used: (1) carbohydrate was added to the agarose solution before the plates were poured, or (2) sugar solutions equal to the volume of the agarose in the plate but at twice the desired final concentration were overlayed on the developed plates, i.e., precipitin line dissolution by the sugars.

Arylamidase activity of CG_{ex} preparations was determined by monitoring the hydrolysis of L-arginine β-naphthylamine fluorometrically (excitation at 335 nm, emission at 410 nm) with a concentration of 0.0125% of the amide in 10 mM Tris-HCl, 154 mM NaCl, and 1 mM $CaCl_2$ at pH 7.0.

Immunological Methods. Antibodies to CG_{ex} or to homogeneous CGL were prepared with female New Zealand white rabbits. Immunization in complete Freund's adjuvant was via the popliteal lymph node in the rear leg by methods previously described (Wolf et al., 1976). Booster immunizations in incomplete Freund's adjuvant were given weekly for a period of 4–6 weeks; a total of 300 μg to 1 mg of material was used for each rabbit. Immunodiffusion in two dimensions was as previously described with unpooled antiserum (Yurewicz et al., 1975). A 1% agarose gel was used containing 10 mM Tris-HCl, pH 8.0, 154 mM sodium chloride, and 0.1% sodium azide with either 1 mM calcium chloride or 5 mM EDTA.

Electrophoresis. Disc gel electrophoresis at 4 °C employed the system of Davis (1964). The effects of specific sugars on the mobility of the CGL were investigated by adding 0.1 M sugar to both the spacer and running gels. The sample of the CGL containing 0.5 M of the sugars was tested.

Isoelectric focusing was performed on a 5% acrylamide gel with 2% ampholytes (Ampholine, LKB Instruments, pH 3.5–10) according to the method of Wellner (1971) or that of Catsimpoolas (1968). The focusing was performed over a period of 24 h at 4 °C, maintaining the total power at less than 0.2 W.

Gels were stained for protein with Coomassie Blue R-250 by the procedure of Fairbanks et al. (1971), for carbohydrate with the periodic acid–Schiff base reaction according to Fairbanks et al. (1971), or for sulfated and/or phosphorylated

components with 1% Alcian Blue in 7% acetic acid. After fixation, the isoelectric focusing gels were rinsed to remove the ampholytes before staining. For the activity stain of the CGL, gels were directly immersed in a jelly coat layer J_1 solution prepared in the standard buffer. Stained gels were scanned at 600 nm (Coomassie Blue and Alcian Blue), 560 nm (periodic acid–Schiff base), or 360 nm (the CGL–J_1 precipitin band) on a Gilford 2400-S spectrophotometer with a linear transport attachment. The R_m of each band was calculated from the scanning pattern. Relative peak areas were determined by cutting and weighing procedures.

Rocket electrophoresis experiments were performed as described by Laurell (1972). The sample of the CGL or the CG_{ex} was electrophoresed into an agarose medium containing antiserum prepared against the CG_{ex}. A 1% agarose plate, 1 mm thick containing 0.025 M sodium barbital, pH 8.6, was exposed to CG_{ex} antiserum for 1 h, and 3–5 μL of CGL (2–10 μg) was added to a 2-mm well. Electrophoresis was conducted at a constant voltage of 270 V at 4 °C until equilibrium between the antigen–antibody reaction was obtained. After formation of the rocket-shaped precipitin band was complete, the agarose slab was washed overnight with water and then stained with 0.1% nigrosine in 7% acetic acid for 20 min. Excess stain was removed with 5% and 10% acetic acid solutions.

Ultracentrifugation. Sedimentation velocity and sedimentation equilibrium experiments were conducted with a Spinco Model E ultracentrifuge. Solutions of the CGL were dialyzed against several changes of the standard buffer for 2 days before centrifugation. The partial specific volume of the CGL was calculated from its chemical composition as described by Cohn and Edsall (1943). The partial specific volumes for carbohydrate residues and calcium were those reported by Gibbons (1966).

Chemical Methods. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Neutral hexose was determined by the phenol–sulfuric acid method with galactose as a standard (Dubois et al., 1956).

Prior to acid hydrolysis for amino acid analysis, solutions of the CGL were thoroughly dialyzed in a plastic container against distilled and deionized water, lyophilized, and dried in vacuo over P_2O_5 for several days at 22 °C. The lectin dried to constant weight was used for amino acid analysis as well as for determination of the absorptivity, the sugar composition, and calcium content. The final dialysis medium was concentrated and used for the controls of these analyses. Weighed lectin samples were hydrolyzed in vacuo in 6 M HCl at 110 °C for 24, 48, and 72 h, and amino acid content was determined by conventional automated column chromatographic methods. Maximum values were taken for the determinations of Ile, Phe, Tyr, and Val. The values of Ser and Thr were determined by extrapolation to zero time of hydrolysis. Cysteic acid was determined after the sample was treated with performic acid and calculated from the cysteic acid/alanine ratio. Tryptophan was determined by the procedure of Edelhoch (1967).

The carbohydrate composition of the lectin was quantitatively determined by gas chromatographic analysis. The sugars were determined as the alditol acetate derivatives after resin-catlayzed acid hydrolysis according to the method of Porter (1975). The retention time and response factor of each sugar were determined with myoinositol as the internal standard. The correction factors of 1.000 and 1.176 for conversion of glucosamine to the *N*-acetylglucosamine and galactosamine to the *N*-acetylgalactosamine derivatives were those of Porter (1975). Sialic acid was determined by the thiobarbituric acid method after hydrolysis with NANA as a standard according to Warren (1959, using eq 2).

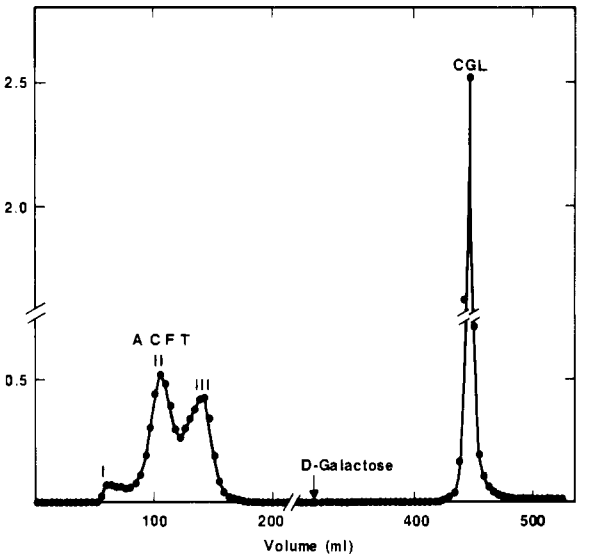

FIGURE 1: Affinity chromatographic separation of CGL: CG_{ex}, 8.9 mL, collected from 30 000 eggs applied to a 2.0 × 43.8 cm column of the jelly–Sepharose 4B affinity material at 4 °C. Arrow indicates elution with 1.0 M D-galactose, at a flow rate of 15 mL/h.

The calcium content of the lectin was determined fluorometrically by the titration of a calcium–calcein complex with EDTA at alkaline pH according to the method of Borle and Briggs (1968).

RESULTS

Lectin Purification. The ionophore A23187 induces the cortical reaction in *Xenopus laevis* (Steinhardt et al., 1974). Egg activation is easily observed at the light microscopic level (e.g., egg rotation, pigment rearrangement, and initiation of cleavage), and the exocytotic release of the contents of the cortical granules is apparent at the electron microscopic level. Lectin activity appeared in the media 2–5 min after the addition of A23187 and reached a maximum within 10 min (Monk & Hedrick, 1986). Ionophore activation effected extensive degranulation of the egg. In addition, the hypotonic extract of activated eggs showed little lectin activity in contrast to that of unactivated eggs. Typically the CG_{ex} from 1000 eggs contained 645 ± 100 μg of protein and 142 ± 16 μg of neutral hexose. Contamination of the CG_{ex} by activated egg lysis could be determined by measurement of the arylamidase activity (arginine naphthylamide hydrolysis) present in the preparation. We observed a very high activity of this enzyme in the cell sap of an ionophore-activated egg; in contrast, the activity was not observed in the hypotonic extract of an unactivated egg. Accordingly, the numbers of lysed eggs in a CG_{ex} preparation could be estimated by measuring the arylamidase activity, a properly prepared CG_{ex} having no arylamidase activity.

A typical affinity chromatographic separation of the lectin with the jelly–Sepharose affinity material is illustrated in Figure 1. The column was monitored by A_{280nm} and by disc gel electrophoresis. Three peaks of material were not adsorbed by the column. The first flow-through peak (ACFT-I) emerged at the V_0, gave only PAS-positive components located in the stacking gel after disc gel electrophoresis, and had no detectable absorbency at 280 nm, indicating that the material was probably of a polysaccharide nature. The third zone (ACFT-III) eluted at V_t and showed several faint bands with R_ms from 0.38 to 1.0 when electrophoresed in a 5% poly-

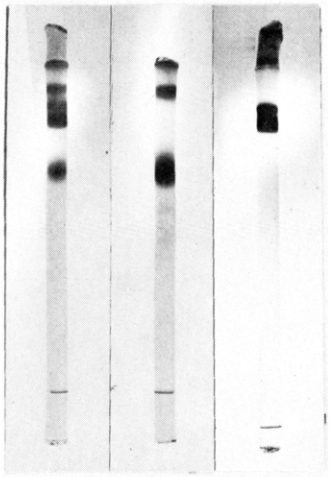

FIGURE 2: Disc gel electrophoresis of CG_{ex} (28 μg), ACFT-II (62 μg), and CGL (26 μg) with the system of Ornstein and Davis in a 5% polyacrylamide gel. A copper wire was inserted at the dye front. The stained proteins are from left to right CG_{ex}, ACFT-II, and CGL.

acrylamide gel. Most of the bands contained both protein and carbohydrate as concluded from their staining characteristics. The relative amounts of these two fractions, ACFT-I and -III, were related to the amount of egg lysis that occurred during CG_{ex} preparation. These two fractions, therefore, were not considered to be true constituents of the CG_{ex}. Disc gel patterns of CG_{ex} uncontaminated by egg lysis had insignificant amounts of ACFT-I and -III (Figure 2). The second fraction ACFT-II contained maximally five glycoproteins (positively staining for protein and carbohydrate) with R_ms of 0.02 (8.9%), 0.07 (29.2%), 0.19 (10.2%), 0.30 (50.9%) and 0.42 (0.9%) in a 5% polyacrylamide gel (Figure 2, the R_m 0.02 and the R_m 0.19 bands were occasionally missing). Precipitin activity with J_1 was not detected in any of these three ACFT fractions when as much as 45 mg of CG_{ex} protein was applied to the column. Additional washing of the affinity column with the standard buffer solution eluted nothing until 1 M D-galactose in the standard buffer was added. The disc gel pattern of the galactose-eluted lectin was distinct from those of the ACFT fractions (Figure 2). Of the precipitin activity applied to the column, greater than 94% was recovered in the galactose-eluted fraction. The recovery of protein was quantitative, and all macromolecules present in CG_{ex} were recovered either in the ACFT fractions or in the galactose-eluted fraction. Typically, 444 ± 88 μg of lectin was recovered from 1000 eggs (four experiments using 27 000–47 000 eggs). From area analysis of the disc gel electrophoretic patterns stained with Coomassie Blue and PAS, the lectin accounted for 77% of the total CG_{ex} material (protein and carbohydrate). The lectin was freed of the galactose used in its elution from the affinity column by dialysis against water adjusted to pH 7.5 with Tris. The galactose-free material was lyophilized, dissolved in an appropriate buffer (usually in the standard buffer unless otherwise noted), dialyzed against the same buffer, and then centrifuged. The purified lectin dissolved in the standard buffer was stable in terms of both precipitin activity and the pattern obtained on disc gel electrophoresis for periods of over 6 months when stored at −20 °C and for periods of over 3 months when stored at 4 °C. Storage at a lower pH, e.g., pH 7, resulted in loss of the precipitin activity and alteration of the pattern obtained on disc gel electrophoresis after prolonged storage.

The purified lectin showed no absorption in the visible portion of the spectrum and possessed an ultraviolet absorption spectrum and fluorescent spectrum typical of proteins containing aromatic amino acids. The intensity and the shape of the ultraviolet spectrum with its maximum at 279 nm was not affected by a range of ionic strength from 0 to 0.2. A linear relationship was obtained between concentration and A_{280nm} with an absorbtivity of $A^{1\%}_{279nm,1cm}$ = 15.4. The A_{260nm}/A_{280nm} ratio was 0.557. The fluorescence spectrum of the lectin in the standard buffer showed an excitation maximum at 291 nm and an emission maximum at 328 nm and with a shoulder at 339–340 nm.

Table I: Effect of Various Treatments on Precipitin Reaction of CGL and J_1[a]

treatment	CG lectin	J_1
35 °C, 50 h[b]	+	+
50 °C, 5 min	+	+
100 °C, 5 min	−	+
6 M urea	+	+
4 M guanidine hydrochloride	−	+
50 mM EDTA–50 mM DTT	−	+
Pronase (70 μg/mL), 35 °C, 50 h[b]	−	+
0.02 M $NaIO_4$, 4 h[c]	+	−

[a] Incubation for 3 h at 22 °C with the standard buffer (pH 7.8) unless otherwise noted. (+) Full activity; (−) no activity. [b] In the presence of 1% toluene. [c] Incubation at 4 °C in the dark in 0.05 M sodium acetate buffer, pH 6.1.

Which Component Is the Lectin? In order to establish which component contains the binding site and which component is the ligand in this two-component system (jelly coat and cortical granule components), both components were treated in ways to establish which component was functionally protein and which component was functionally carbohydrate. Each of the components was individually treated, the reagent or condition removed, and the precipitin activity under the standard assay conditions determined. The conditions of treatments and results are listed in Table I. The component from the cortical granules was, for example, heat-sensitive and inactivated by 4 M guanidine, EDTA–DTT, trypsin, or Pronase treatment. It was resistant to treatment with $NaIO_4$. The J_1 component showed just the opposite effects. The cortical granule component was functionally protein and the J_1 component functionally carbohydrate. Therefore, the cortical granule component is the lectin and the J_1 component the ligand. As will be detailed later, the cortical granule component interacts specifically with D-galactose and D-galactose derivatives and is a glycoprotein with a compositional predominance of protein. Jelly coat layer J_1 on the otherhand is also composed of glycoproteins but with a composition that is predominantly carbohydrate (Yurewicz et al., 1975).

Homogeneity Criteria. (A) Gel Electrophoresis. Disc gel electrophoresis of the purified lectin gave two bands staining coincidentally with Coomassie Blue and PAS, and both bands gave a precipitin reaction with J_1 solutions (Figure 2). The slower moving species was designated CGL-2 and the faster moving species CGL-1. The molecular relationship of these two CGL bands will be the subject of a subsequent paper.

In an attempt to preparatively resolve CGL-1 and CGL-2, the purified lectin was reapplied to the affinity column and galactose-eluted under varying pH (6–9) and ionic strength solutions. However, both CGL-1 and CGL-2 were bound with equal affinity to the jelly–Sepharose column and eluted as a broad symmetrical peak when a linear gradient of D-galactose from 0 to 1 M was used. Both CGL-1 and -2 were bound to a concanavalin A–Sepharose column, prepared in an analogous way to the jelly–Sepharose column reported here, and were coincidentally eluted with 0.02 M D-glucose.

The R_ms of CGL-1 and -2, as determined by disc gel electrophoresis at several different gel concentrations, were

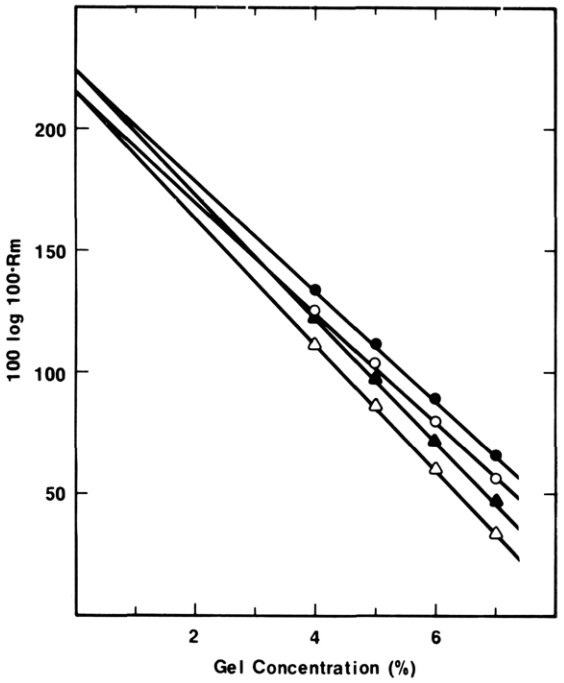

FIGURE 3: Plots of log R_m vs. percent gel concentration: CGL-2 (△, ▲) and CGL-1 (○, ●) with (△, ○) or without (▲, ●) the addition of D-galactose to the system.

determined. Plots of percent gel concentration vs. log R_m (Figure 3) showed that CGL-1 and -2 had identical free-solution mobilities (ordinate intercept) but dissimilar molecular weights (different slopes), indicating that they are size isomers (Hedrick & Smith, 1968). The conclusion that CGL-1 and -2 were size isomers was supported by isoelectric focusing experiments. Under several different conditions of isoelectric focusing, only a single band was ever observed that stained coincidentally with Coomassie Blue and PAS and gave a precipitin line with solutions of J_1. The pI of the CGL was 4.61 ± 0.11 with ferritin (pI = 4.4) and bovine serum albumin (pI = 4.9) as standards.

From area analysis of the disc gel electrophoresis patterns, CGL-2 accounted for 24.9 ± 1.6% of the total Coomassie Blue staining material and CGL-1 for 75.1 ± 1.6% (six determinations using three different CGL preparations). The same relative distribution of CGL-1 and CGL-2 was obtained with the PAS stain or formation of a precipitin band with J_1 solutions. The distribution between the two forms of the lectin was the same when CG_{ex} was electrophoresed. The extent of egg lysis contaminating the CG_{ex} preparation did not affect the CGL-1 to CGL-2 distribution. Thus, the size isomer relation of CGL-1 and CGL-2 was not the result of changes induced in the macromolecules during the purification. Neither CGL-1 nor CGL-2 stained with Alcian Blue, indicating that they were not sulfated or phosphorylated macromolecules.

(*B*) *Ultracentrifugation.* When the purified CGL was characterized by sedimentation velocity ultracentrifugation, two sedimenting species were observed. The $s_{20,w}$ values were not concentration dependent over the range examined (1.41–5.63 mg/mL). The faster sedimenting peak (CGL-2) represented 25% of the total area and had an $s_{20,w}$ value of 19.0 S. The slower sedimenting species (CGL-1) accounted for 75% of the area and had a $s_{20,w}$ equal to 16.2 S.

From analysis of sedimentation equilibrium observations with purified CGL, a straight line relationship between the log of concentration and the square of the radius was found. The slope of this plot for an apparently homogeneous macromolecule corresponds to the molecular weight of CGL-1 as will be detailed later.

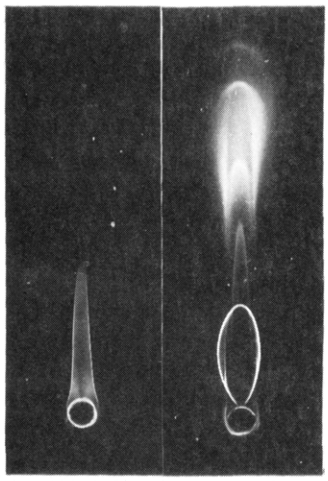

FIGURE 4: Rocket electrophoresis patterns of CGL (left) and CG_{ex} (right) with antiserum against CG_{ex}. Experimental conditions are as described in the text.

(*C*) *Immunodiffusion and Electroimmunodiffusion.* Purified CGL gave a single sharp precipitin line in double-diffusion experiments using rabbit antisera prepared against CG_{ex} or purified CGL. The three fractions not adsorbing to the affinity column (ACFT-I to -III) displayed antigenic nonidentity with the CGL line as evidenced by double-spur formation with antiserum against CG_{ex}. Thus, CGL and ACFT contain immunologically distinct macromolecules. With prolonged diffusion, a second faint precipitin line of CGL and antisera interaction sometimes appeared. The second precipitin line is not due to an immunogenic difference between CGL-1 and -2 as will be detailed in a subsequent paper. The second precipitin line increased in intensity when purified CGL preparations were stored under suboptimal conditions. We cannot, at present, satisfactorily explain this aging phenomenon, but clearly the appearance of the second minor precipitin line is not due to an impurity in the CGL preparation.

Rocket electrophoresis experiments were conducted with antisera against CG_{ex}. One precipitin line was observed for the purified CGL (Figure 4). However, on occasion another faint line was detected similar to that seen in the immunodiffusion experiments. The secondary precipitin line showed a reaction of identity with the primary precipitin line.

Molecular Weight. (*A*) *Gel Electrophoresis.* Molecular weight estimates using disc gel electrophoresis by the method of Hedrick and Smith (1968) used as standard proteins β-lactoglobulin, ovalbumin, bovine serum albumin, lactate dehydrogenase, β-amylase, ferritin, and carboxydismutase. The molecular weight of CGL-2 was 695 000 ± 10 000 (standard deviation), and that of CGL-1 was 571 000 ± 29 000.

(*B*) *Gel Filtration.* The molecular weight of CGL was determined by gel filtration on a Sepharose 6B column. The column was calibrated with blue dextran, ferritin, catalase, aldolase, bovine serum albumin, hemoglobin, and ^3H_2O. The CGL eluted from a column equilibrated with the standard buffer as a single asymmetric peak with a M_r of 377 000 (Figure 5). The apparently low molecular weight, as determined by gel filtration in comparison with that determined by disc gel electrophoresis, can be explained by interaction of the CGL with the Sepharose 6B. As will be shown later, the lectin has a specificity for galactosyl residues, and Sepharose (agarose) is a galactose–anhydrogalactose polymer. The interaction between the CGL and the Sepharose polymer can be eliminated by the addition of either 0.5 M D-glactose or

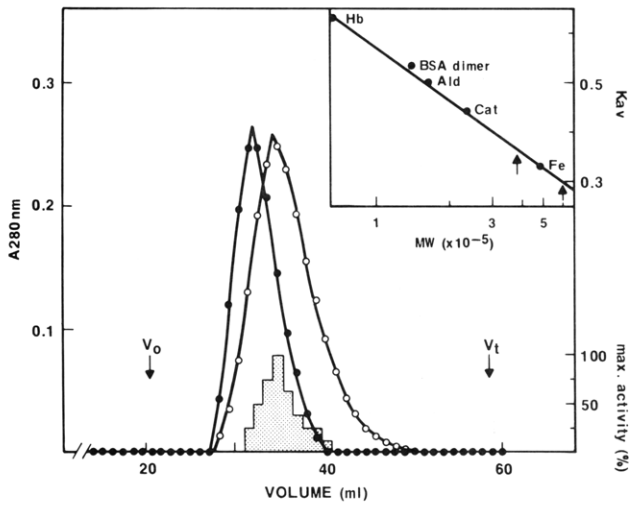

FIGURE 5: Elution profile of CGL from a column of Sepharose 6B. CGL (1.55 mg/0.5 mL) was applied to a 1.08 × 61.5 cm column of Sepharose 6B equilibrated with the standard buffer with (●) or without (○) the addition of 0.5 M D-galactose. Elution was conducted at a flow rate of 6 mL/h. Each fraction collected in the absence of galactose was assayed for the precipitin reactivity against J_1 (stipled bar graph). (Insert) Calibration curve of the Sepharose 6B column with blue dextran, ferritin, catalase, aldolase, bovine serum albumin, hemoglobin, and ^3H_2O. The arrows indicate the K_{av} values of the CGL eluted from a column equilibrated with or without D-galactose.

5 mM EDTA to the eluting buffer. In the presence of galactose, the lectin eluted from a Sepharose 6B column as a single symmetrical peak with a M_r of 576 000 (Figure 5). As will be shown later with disc gel electrophoresis, galactose does not affect a change in molecular weight of the CGL. Due to the higher concentration of CGL-1 compared to CGL-2, the apparent molecular weight corresponds most closely to that of CGL-1.

(*C*) *Sedimentation Equilibrium.* The molecular weight estimates of the CGL by disc gel electrophoresis methods and gel filtration methods are relative methods and required standardization with macromolecules of known molecular weights. We, therefore, determined the molecular weight of the CGL by sedimentation equilibrium measurements. The apparent molecular weight, M_{app}, was calculated from sedimentation equilibrium data at several different protein concentrations and three different rotor speeds, with a partial specific volume of 0.704 mL g^{-1}. From a plot of M_{app}^{-1} vs. protein concentration, the best line was determined by least-squares fitting, and the extrapolated value at zero protein concentration was 568 000. The extrapolated value is a weight-average molecular weight of CGL-1 and CGL-2. The apparent molecular weight of CGL was highly concentration dependent below 1.0 mg/mL. In contrast, at the higher concentrations used to determine the $s_{20,w}$, there was no apparent concentration dependency. In combination with CGL-1 and CGL-2 distribution determined by disc gel electrophoresis, the molecular weights of CGL-1 and CGL-2 were estimated to be 539 000 and 655 000, respectively.

Chemical Composition. Purified CGL is composed of 83.96% protein, 15.85% carbohydrate, and 0.19% calcium. The composition of the CGL in terms of amino acids, monosaccharides, and calcium is presented in Table II. The CGL was composed of the normal spectrum of amino acids with no unusual types or amounts present. The carbohydrate composition of the CGL consisted of fucose, glucosamine, galactosamine, mannose, galactose, and sialic acid. The hexosamine content was calculated as the *N*-acetyl derivative as this is the most common derivative found in glycoproteins, the CGL is

Table II: Amino Acid, Sugar, and Calcium Composition of CGL

amino acid	weight %	mol/10^5 g	mol/mol[f]
Ala	3.50	49.3	280
Arg	4.00	25.6	145
Asx	11.15	96.8	549
Cys/2[a]	2.39	23.1	131
Glx	7.66	59.3	336
Gly	4.62	80.9	459
His	1.09	7.9	45
Ile	3.33	29.5	167
Leu	4.99	44.1	250
Lys	4.81	37.5	213
Met	1.27	9.8	56
Phe	5.77	39.2	222
Pro	3.71	38.1	216
Ser	5.60	64.4	365
Thr	4.68	46.3	262
Trp[b]	3.35	18.0	102
Tyr	8.21	50.3	285
Val	3.83	38.7	219
	83.96[g]		
sugar[c]			
fucose	1.14	7.8	44
GlcNac[d]	6.01	29.6	168
GalNac[d]	2.63	13.0	74
mannose	2.44	15.0	85
galactose	2.73	16.8	95
sialic acid[e]	0.91	2.9	16
	15.85[g]		
calcium	0.19[g]	4.8	27

[a] Performic acid oxidation followed by acid hydrolysis. [b] According to Edelhoch (1967). [c] Determined by gas chromatography of the alditol acetate derivatives. [d] Calculated as *N*-acetylhexosamine. [e] Determined by the thiobarbituric acid method. [f] A M_r of 568 000 was used. [g] Total.

reactive with the plant lectins from wheat germ and *Dolichos biflorus*, which are *N*-acetylhexosamine-specific. As mentioned under Materials and Methods, the calcium content of the CGL was determined by the titration of a calcium–calcein complex with EDTA. Barium and strontium are the only cations that cannot be distinguished from calcium by this procedure. As the existence of barium or strontium in CGL seems remote (Vallee & Wacker, 1970), we interpret these results as a measure of the calcium content of the CGL. About 27 mol of calcium/mol of CGL (M_{app} = 568 000) was found in two separate determinations.

Carbohydrate Specificity of CGL. (*A*) *Inhibition Studies.* The specificity of the CGL was investigated by inhibiting the CGL–J_1 precipitin reaction with various sugars by the two methods described under Materials and Methods. Both methods showed qualitatively indentical results, but a higher concentration of sugar was required in the overlaying method than in the method where the sugar was initially incorporated into the agarose. The results presented in Table III were obtained by the overlaying method. Serial dilutions of the CGL were diffused against a constant amount of J_1, and a sugar solution equal to the volume of the agarose in the plate at twice the desired final concentration was overlayed on each of the developed plates to dissolve the precipitin line. The minimum CGL giving a visible precipitin reaction after 24-h incubation with the sugar solution was compared with a control plate with same constituents but without the addition of sugar, where 10 μL of CGL contained 64 titer units of activity. Of the biologically important hexoses tested, only D-galactose inhibited the reaction. The pyranose ring of D-galactose was apparently necessary as evidenced by the lack of inhibition with galactitol or γ-D-galactonolactone. The aldose form was needed as the furanose D-tagatose had no inhibitory activity. Tagatose, a ketohexose, has the same configurations as ga-

Table III: Sugar Inhibition of CGL–Jelly Reaction[a]

sugar	% inhibition 25 mM	50 mM	100 mM
monosaccharides			
L-arabinose	0	0	50
D-galactose	0	50	75
methyl α-D-galactoside	0	50	75
methyl β-D-galactoside			0
p-nitrophenyl α-D-galactoside		0	75
p-nitrophenyl β-D-galactoside			0
disaccharides			
melibiose	0	50	75
lactose	50	75	100
lactulose			0

[a] The following monosaccharides were tested and had no inhibitory activity at 100 mM: D-arabinose, L-fucose, *N*-acetyl-D-galactosamine, galactitol, D-glucose, D-glucosamine, glucuronic acid, D-mannose, D-mannosamine, L-rhamnose, and D-tagatose. In addition, raffinose and stachyose did not inhibit at 100 mM.

lactose at C-3, -4, and -5 and is capable of existing in both furanose and pyranose ring forms. However, since it had no inhibitory activity compared to galactose, we assume that under the conditions used tagatose existed primarily in the furanose form. The hydroxyl group on C-2 of D-galactose was important as neither D-galactosamine nor *N*-acetyl-D-galactosamine inhibited the precipitin reaction. The results obtained from use of several epimers of D-galactose suggest that the configuration at carbons 2–4 of D-galactose was of primary importance. This conclusion was supported by the inhibition observed with L-arabinose. Of the pentoses tested, only L-arabinose inhibited the precipitin reaction. D-Galactose and L-arabinose have the same configurations with respect to asymmetric carbon atoms 2–4.

Studies of the anomeric specificity of D-galactosides gave ambiguous results. The methyl or *p*-nitrophenyl α-D-galactosides inhibited the precipitin reaction. The same β-galactoside derivatives, on the other hand, did not. However, with di- and oligosaccharides no anomeric specificity was observed. The disaccharide melibiose [α-galactosyl(1,6)-glucose] was equally inhibitory as D-galactose. The disaccharide lactose [β-galactosyl(1,4)glucose] inhibited at half the concentration of D-galactose. Thus, at the monosaccharide level, the α-derivative was inhibitory whereas the β-derivative was not. At the level of the disaccharides, the β-derivative was apparently more effective than the α-derivative in inhibiting the precipitin reaction.

The linkage between the nonreducing sugar and the penultimate sugar was tested by comparing the inhibition of the disaccharides and some oligosaccharides. Both 1–4 and 1–6 linkages were capable of inhibiting lectin reaction. Of the galactan fractions tested, the trisaccharide was by far the most effective and inhibited the precipitin reaction to the extent of 50% at 10 mM, one-fifth the concentration of D-galactose required for 50% inhibition. From these inhibition studies, we concluded that the specificity of the lectin binding site was for a sugar type of the D-galactose configuration. Galactosides, however, were more effective inhibitors than monosaccharides. The anomeric specificity or the penultimate sugar specificity of the oligosaccharide cannot be dictated at this time. The linkages between the reducing sugar and the penultimate residue also cannot be specified from our results. In addition to the inhibition by certain sugars, EDTA and EGTA totally inhibited the precipitin reaction, thus indicating the involvement of divalent metal ions in the lectin–ligand reaction.

(*B*) *Interaction of CGL with Mono- and Disaccharides: Physical Studies.* The interaction of the CGL with sugars was studied by disc gel electrophoresis. Sugars at 0.1 M were incorporated into both the stacking and the running gels. The sample of the CGL was also made 0.5 M in the sugar. D-Glucose, a noninhibitor, changed the R_m to a small extent, whereas the potent inhibitors galactose and lactose greatly reduced the R_m of both CGL-1 and CGL-2. The binding by the CGL of inhibitory sugars did not change the size of the macromolecule. This was concluded from a study of the variation in the R_m of the CG lectin–sugar complex as a function of gel concentration (Figure 3). The slope of the log R_m vs. gel concentration plot of the galactose–CGL complex was the same as the that of CGL by itself; thus, they had the same molecular size. It was shown previously that an equilibrium binding situation between a protein and a noncharged oligosaccharide can give rise to parallel slopes when the protein and the protein–ligand complex are compared by the methods used here (Hedrick et al., 1969). The monosaccharide–CGL interaction as judged by these disc gel electrophoresis studies showed the same sugar specificity as that in precipitin line inhibition studies. The interaction of CGL with galactosides was also observed in the gel filtration experiments with Sepharose 6B (Figure 5).

Discussion

Although agglutinins (lectins) have previously been reported in eggs, particularly fish eggs, this is the first report of purification of an egg lectin with an established function. The CGL is the major constituent of the egg cortical granules, being 77% of the total glycoproteins in the CG_{ex}. The nature and function of the other five cortical granule constituents is unknown although β-*N*-actetylglucosaminidase activity has been detected and purified (Greve et al., 1985; Prody et al., 1985).

The CGL is a complex molecule in terms of its molecular characteristics. It is puzzling as to why such a seemingly simple function as binding to a carbohydrate ligand requires an oligomeric metalloglycoprotein with a molecular weight of over 500 000. Perhaps the CGL has functions yet to be detected in addition to binding the jelly coat ligand.

A number of galactosyl-specific lectins isolated from plants and animals have been described. In terms of their physicochemical properties, each of the galactosyl binding lectins appears to be unique, or at least no generalities are apparent [for review of plant lectins, see Goldstein and Hayes (1978)]. In terms of molecular properties, the CGL most closely resembles the hepatic membrane protein that binds asialoglycoproteins (Kawasaki & Ashwell, 1976; Hudgin et al., 1974). They are both acidic glycoproteins (p*I* = 4.6 or 4.7) that function in a large oligomeric state (molecular weights of greater than 500 000). The subunit structure is simpler in the case of the hepatic membrane protein (two different molecular weight subunits) than in the case of the CGL (five different molecular weight subunits; T. Nishihara, R. E. Wyrick, and J. L. Hedrick, unpublished results). On the other hand, the oligomeric structure of CGL is discrete with two molecular weight isomers while the hepatic membrane protein is polydisperse exhibiting several molecular weight isomers. In terms of sugar specificity, the CGL shows the same anomalous binding as the β-galactoside binding lectins isolated from calf and chicken tissues (Briles et al., 1979). The methyl α-galactoside is more inhibitory of binding than the methyl β-galactoside, but lactose, a β-galactoside, is a better inhibitor of binding than the methyl α-galactoside. This apparent paradox has yet to be adequately explained.

The CGL is an unusual lectin in that a specific function for it has been described and experimental evidence in support

of its function has been obtained. The CGL is subcellularly relocated at fertilization from the cortical granules to the F layer and perivitelline space (Greve & Hedrick, 1978); when combined with its ligand in the jelly coat to form the F layer, it renders the envelope impenetrable to sperm (Grey et al., 1976); the F layer can be reconstituted in vitro from its isolated component parts (CGL + jelly coat ligand + envelope; Nishihara & Hedrick, 1977a); treatment of a jellied egg with the purified CGL renders the egg unfertilizable (Wyrick et al., 1974a; T. Nishihara and J. L. Hedrick, unpublished results); all these observations support the functioning of the CGL as blocking sperm penetration when the CGL is combined with its ligand in the jelly coat. A role for the CGL in the fertilization process can also be argued on teleogical grounds. Cortical granules exist only in eggs, and their contents are released from the egg (exocytosis) at fertilization in most organisms. Thus, the function of the molecules within the egg cortical granules are likely limited to fertilization or early developmental events.

Lectins have been described or isolated from eggs, oocytes, and embryos of *X. laevis* and other amphibians (Sakakibara et al., 1976, 1979; Roberson & Barondes, 1982; Nitta et al., 1984). However, the physiological functions of such lectins have yet to be determined. Lectins isolated from oocytes and embryos or eggs of *X. laevis* have used methods substantially different from those used here. These lectins have some characteristics in common with those reported here for CGL, e.g., galactose specificity, but also apparently differ in certain of their physicochemical properties, e.g., isoelectric points, molecular weights, and binding to affinity columns (Roberson & Barondes, 1982; Nitta et al., 1984). As stated in Nitta et al., it is possible that heterogeneous lectins may be present in *X. laevis* eggs. The relation between the various lectins isolated from amphibians has yet to be determined.

Understanding the structure–function properties of the lectin–ligand interaction that produces the F layer requires the purification and characterization of the jelly coat ligand as well as the CGL, work that is currently in progress. It has recently been suggested that the ligand for CGL may be synthesized by the pars recta region of the oviduct (Yoshizaki & Katagiri, 1984). The mechanism of the lectin–ligand interaction that prevents sperm penetration of the egg is totally unknown. Perhaps a sperm receptor on the vitelline envelope is somehow reacted with the complex and buried or otherwise covered up by the interaction. Alternatively, the lectin–ligand interaction (F layer) may be resistant to sperm enzymes, such as acrosin, which assist the sperm in traversing the envelope. Additional information is clearly required for a more complete understanding as to the molecular mechanism(s) involved in altering the sperm penetrability of the egg envelope at fertilization.

ACKNOWLEDGMENTS

We are indebted to Dr. R. L. Hamill, Eli Lilly Co., for the gift of the ionophore A23187.

Registry No. Ca, 7440-70-2.

REFERENCES

Borle, A. B., & Briggs, F. N. (1968) *Anal. Chem. 40*, 339–344.
Briles, E. B., Gregory, W., Fletcher, P., & Kornfeld, S. (1979) *J. Cell Biol. 81*, 528–537.
Catsimpoolas, N. (1968) *Anal. Biochem. 26*, 480–482.
Cohn, E. J., & Edsall, J. T. (1943) in *Proteins and Amino Acids, and Peptides as Dipolar Ions*, p 370, Reinhold, New York.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.
Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.
Gibbons, R. A. (1966) in *Glycoproteins* (Gottschalk, A., Ed.) p 61, Elsevier, London.
Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 128–340.
Greve, L. C., & Hedrick, J. L. (1978) *Gamete Res. 1*, 13–18.
Greve, L. C., Prody, G. A., & Hedrick, J. L. (1985) *Gamete Res. 12*, 305–312.
Grey, R. D., Wolf, D. P., & Hedrick, J. L. (1974) *Dev. Biol. 36*, 44–61.
Grey, R. D., Working, P. K., & Hedrick, J. L. (1976) *Dev. Biol. 54*, 52–60.
Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155–164.
Hedrick, J. L., Smith, A. J., & Bruening, G. E. (1969) *Biochemistry 8*, 4012–4019.
Hudgin, R. L., Pricea, W. E., Jr., Ashwell, G., Stockert, R. J., & Morel, A. G. (1974) *J. Biol. Chem. 249*, 5536–5543.
Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 1296–1302.
Laurell, C. B. (1972) *Scand. J. Clin. Lab. Invest., Suppl. 29*(124), 21–37.
Lowry, O. H., Rosebrough, M. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Monk, B., & Hedrick, J. L. (1986) *Zool. Sci.* (in press).
Nishihara, T., & Hedrick, J. L. (1977a) *J. Cell Biol. 75*, 172a.
Nishihara, T., & Hedrick, J. L. (1977b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 811.
Nishihara, T., Wyrick, R. E., & Hedrick, J. L. (1975) *Chem. Biol. 12*, 718–722.
Nitta, K., Takayanagi, G., & Kawauchi, H. (1984) *Chem. Pharm. Bull. 32*, 2325–2332.
Porter, W. H. (1975) *Anal. Biochem. 63*, 27–43.
Prody, G. A., Greve, L. C., & Hedrick, J. L. (1985) *J. Exp. Zool. 235*, 335–340.
Roberson, M. M., & Barondes, S. H. (1982) *J. Biol. Chem. 257*, 7520–7524.
Sakakibara, F., Takayanagi, G., Kawauchi, H., Watanabe, K., & Hakomori, S. (1976) *Biochim. Biophys. Acta 444*, 386–395.
Sakakibara, F., Kawauchi, H., Takayanagi, G., & Ise, H. (1979) *Cancer Res. 39*, 1347–1352.
Steinhardt, R. A., Epel, P., Carroll, E. J., Jr., & Yanagimachi, R. (1974) *Nature (London) 252*, 41–43.
Steinmann, A., & Stryer, L. (1973) *Biochemistry 12*, 1499–1502.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins 5*.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.
Wellner, D. (1971) *Anal. Chem. 43*, 59A.
Wolf, D. P., & Hedrick, J. L. (1971) *Dev. Biol. 25*, 348–359.
Wolf, D. P., Nishihara, T., West, D. M., Wyrick, R. E., & Hedrick, J. L. (1976) *Biochemistry 15*, 3671–3678.
Wyrick, R. E., Nishihara, T., & Hedrick, J. L. (1974a) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2067–2071.
Wyrick, R. E., Nishihara, T., & Hedrick, J. L. (1974b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 1453.
Yoshizaki, N., & Katagiri, C. (1984) *Zool. Sci. 1*, 255–264.
Yurewicz, E. C., Oliphant, G., & Hedrick, J. L. (1975) *Biochemistry 14*, 3101–3107.