SOME PROPERTIES OF HYALIN

The Calcium-Insoluble Protein of the Hyaline Layer of the Sea Urchin Egg

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ABSTRACT

The principal protein component of the hyaline layer of sea urchin eggs is the calcium-insoluble protein first described by Kane and Hersh. The protein hyalin is abnormally high in acidic amino acids, almost devoid of basic amino acids, and characteristically rich in valine and proline. Essentially all of the cysteine present is found in the disulfide form; no evidence points to intermolecular disulfide linkages. Hyalin from several species has a minimal subunit weight of about 100,000, though evidence exists for a particle three times this weight in urea or guanidine hydrochloride from one species. Optical rotatory dispersion measurements indicate no α-helix content, though the dispersion has unique characteristic features. Addition of small quantities of calcium causes hyalin to gel to a birefringent fibrous form. The fibrous, birefringent form of hyalin is rendered isotropic upon addition of EDTA, but the birefringence is restored with re-addition of divalent cation.

INTRODUCTION

A unique calcium-insoluble protein has been shown to be the major component of isolated "cortical" material from unfertilized sea urchin eggs (Stephens and Kane, 1966) and identified as a major constituent of the hyaline layer (Yazaki, 1968; Kane and Stephens, 1969). This protein was originally isolated from whole egg homogenates and egg acetone powders and forms a clear gel at relatively low divalent ion concentrations (Kane and Hersh, 1959). These gels can be solubilized by removal of the divalent ion and the gelation repeated. This protein is localized primarily in the cortical granules of the unfertilized egg and in the hyaline layer after fertilization. It is unrelated to the calcium-insoluble protein prepared from egg extracts by Sakai (1965), shown later to be immunologically a different material (Yazaki, 1968).

Previous preliminary studies of the protein's properties (Kane and Hersh, 1959; Stephens and Kane, 1966) have indicated it to be extraordinarily acidic, relatively free of α-helix content, and apparently composed of relatively high molecular weight subunits. This present study involves the further physical and chemical characterization of this calcium-insoluble protein—*hyalin*[1]—and a correlation of its properties in solution with its functional properties in the cortical granules and the hyaline layer of the fertilized egg.

MATERIALS AND METHODS

The calcium-insoluble protein hyalin was prepared from acetone powders of eggs (Stephens, 1967) of the

[1] Hyalin is proposed as the name of this protein since it is the main protein constituent of the hyaline layer.

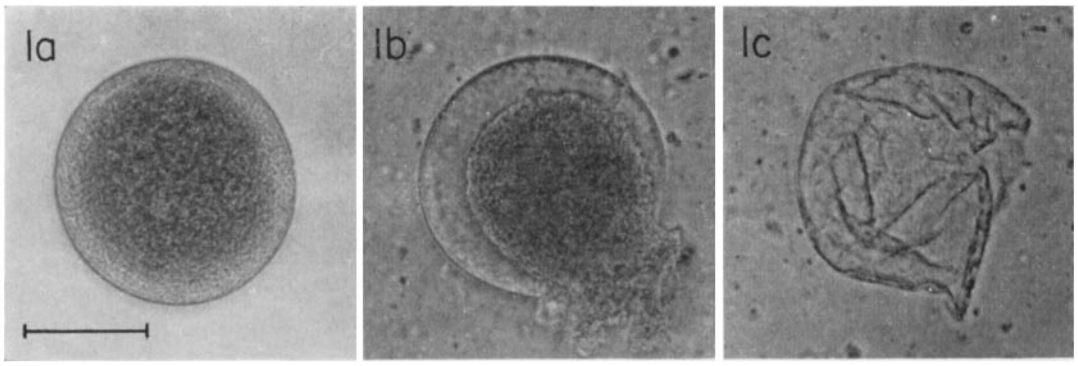

FIGURE 1 Separation of cortical and hyaline material from an unfertilized egg of the sea urchin *Strongylocentrotus droebachiensis*. *a*, Egg in normal seawater. *b*, Perfusion of 0.1 M $MgCl_2$ causes swelling and extrusion of cytoplasm through rupture in the cortex. *c*, Isolated cortex freed of internal cytoplasm by gently tapping the coverglass. Phase-contrast optics. Scale marker = 100 μ. × 160.

sea urchins *Strongylocentrotus droebachiensis*, *S. purpuratus*, *Arbacia punctulata*, *Tripneustes gratilla*, and *Lobocentrotus atratus*. The powders were extracted for 30 min with 10 times their weight of 0.05 M NaCl and 10 mM Tris·HCl, pH 7.5; the residue was sedimented at 35,000 *g* for 30 min; and the protein was precipitated from the supernatant solution with 0.1 M $MgCl_2$ or 0.02–0.05 M $CaCl_2$. The resulting gel protein was dialyzed for 24 hr against at least two changes of the above extraction buffer, sedimented at 35,000 *g* and then re-gelled and re-dialyzed. The final dialyzate was used in the experiments to follow.

Alternatively, with *C. atratus* or *A. punctulata*, cortices (Fig. 1) were isolated (Kane and Stephens, 1969) and solubilized by 24-hr dialysis against 50 mM NaCl and 10 mM Tris·HCl, pH 7.5, and the resulting protein was purified by re-gelation and dialysis as above.

Hyalin was also prepared by homogenization of *C. atratus* eggs in 0.05 M NaCl, 10 mM Tris·HCl, pH 7.5 and sedimentation of particulate matter at 35,000 *g* for 30 min, and was twice gelled and purified as above. No differences in properties were seen in protein prepared by these three alternative methods.

After purification, the protein preparations responded negatively to anthrone or orcein tests for carbohydrate (see Kane and Hersh, 1959).

Sedimentation velocity studies were performed with a Spinco Model E analytical ultracentrifuge equipped with Schlieren and interference optics. All determinations were carried out at 20° ± 1°C, with aluminum-filled Epon single and double-sector centerpieces. Sedimentation rates at infinite dilution were determined from plots of $1/s$ vs. c and corrected to standard conditions (Schachman, 1959).

Molecular weights of the protein in 0.1 M NaCl, 8 M urea, 5 M guanidine hydrochloride, or 0.1 M Na_2CO_3 were determined by the short-column sedimentation-equilibrium technique of Van Holde and Baldwin (1958), and evaluated from the slope of a plot of log $1/x\ dc/dx$ vs. x^2 (Chervenka, 1969) or by determination of dc/dx at the midpoint of the solution column (Yphantis, 1960), where x is the distance in cm from the center of rotation and dc/dx is the concentration gradient. Concentration dependence of molecular weight was evaluated by use of multichannel centerpieces and methodology described by Yphantis (1964).

Amino acid composition was determined with a Beckman 120B automatic amino acid analyzer. 3–4 mg of protein were hydrolyzed in 6 N HCl in evacuated, nitrogen-flushed tubes for 24, 48, and 72 hr at 110°C. Destruction of serine and threonine was estimated by extrapolation to zero hydrolysis time. Cysteine content was determined by the 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) titration method of Ellman (1959). Tryptophan was estimated spectrophotometrically in 0.1 N NaOH (Goodwin and Morton, 1946).

The partial specific volume of hyalin (0.728) was determined from the amino acid composition (McMeekin and Marshall, 1952).

Polyacrylamide gel electrophoresis was carried out on 5 and 7.5% gels at pH 8.9 in 8 M urea according to methods originated by Davis (1964).

Optical rotatory dispersion (ORD) studies were carried out in the visible and ultraviolet regions with a Cary 60 recording spectropolarimeter. The visible ORD data were evaluated according to Urnes and Doty (1961) to determine b_0, employing a λ_0 of 212 mμ, and a mean residue weight of 123 g.

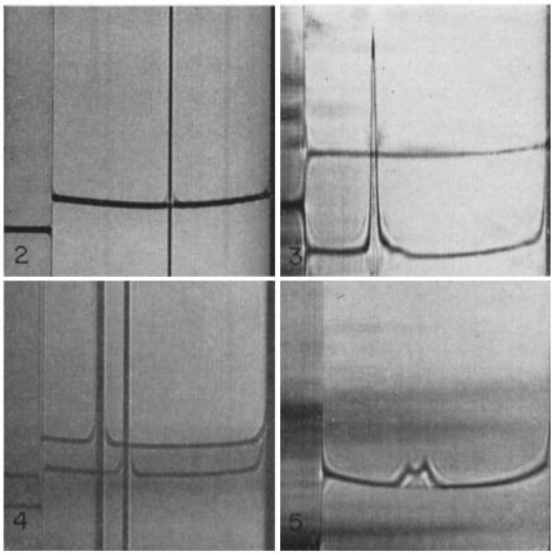

FIGURE 2 Hyalin of *Colobocentrotus atratus* in 0.1 N NaCl, 10 mM Tris·HCl, pH 7.5. 80 min after reaching a speed of 52,640 rpm; 80° bar angle.

FIGURE 3 *C. atratus* hyalin in neutral 0.5 M KCl (upper trace) and in 0.1 M Na_2CO_3 (lower trace). 48 min after reaching a speed of 42,640 rpm; 60° bar angle. Complete aggregation takes place in 0.5 M KCl.

FIGURE 4 Hyalin from *C. atratus* in 8 M urea with (upper trace) and without (lower trace) 2% mercaptoethanol. Also present are 0.5 M KCl and 10 mM Tris·HCl, pH 7.5. 256 min after reaching a speed of 59,780 rpm; 80° bar angle.

FIGURE 5 *C. atratus* hyalin in 10 mM EDTA, 0.05 M NaCl and 10 mM Tris·HCl, pH 7.5. 32 min after reaching a speed of 60,000 rpm; 60° bar angle.

RESULTS

Behavior of Hyalin in Solution

Any comments regarding the solution properties of hyalin should be prefaced with the statement that, under virtually all of the conditions thus far studied, this protein consistently shows a phenomenal tendency for association. The sedimentation rate dependence upon concentration varied greatly from preparation to preparation within a species, but consistently extrapolated to the same value for $S^\circ_{20,w}$. Such an effect is attributable to the viscous nature of the solution; samples dialyzed for long periods of time in order to remove most residual divalent cations showed significantly lower concentration dependence than samples dialyzed simply for solubilizing the protein. Identification of this protein alone or in complex mixtures cannot be made on the basis of the unextrapolated sedimentation rate.

The purified hyalin sedimented at a single, hypersharp boundary (Fig. 2), regardless of species. The extrapolated sedimentation rates for *S. droebachiensis* and *C. atratus* in 0.1 M NaCl at neutrality were found to be 8.9 and 7.2S, respectively. Increase in ionic strength above 0.2 brings about a considerable broadening of the boundary and evidence for considerably higher aggregation. The hyalin from *C. atratus* precipitates at an ionic strength of approximately 0.4, whereas hyalins from the other species give extremely broad boundaries of sedimentation rate severalfold higher than that found at low ionic strength. The molecular weight for *C. atratus* hyalin in low ionic strength at neutrality was found to be in excess of 700,000 (Table I).

In the case of other solvents, evidence exists for breakdown into subunits. At high pH (10.5) in 0.1 M Na_2CO_3 (Fig. 3), the sedimentation rate of *C. atratus* hyalin is reduced to 5.1S, while the molecular weight averages 368,000, roughly half that found at neutrality. In urea or in guanidine hydrochloride (Fig. 4), where a minimal subunit should be observed, the molecular weight is reduced further, averaging 285,000, but the sedimentation rate is somewhat higher: 5.8 and 6.3S for 8 M urea and 5 M guanidine hydrochloride, respectively. The lower sedimentation rate in the alkaline range is quite likely due to the effects of charge repulsion, an effect minimized in the urea and guanidine solvents by neutrality and salt addition.

Representative plots of reciprocal sedimentation rate vs. concentration for *C. atratus* hyalin in neutral salt, alkaline salt, and 8 M urea are given in Fig. 6. Typical short-column molecular weight determinations are illustrated in Fig. 7 for *C. atratus* hyalin in neutral salt and in 5 M guanidine hydrochloride. The protein in either urea or guanidine hydrochloride was always reasonably homogeneous. In neutral salt, both the variability in sedimentation rate dependence upon concentration and the molecular weight plots were indicative of considerable aggregation. This aggregation manifested itself in the latter determinations as a clear pellet at the cell bottom, increasing with time of centrifugation, but had little effect upon the linearity of the plots over most of the solution column.

TABLE I

Physical Parameters of Colobocentrotus atratus Hyalin in Various Solvents

Solvent	Molecular wt.			Sedimentation coefficient
	C_0	$\frac{1}{2}C_0$	$\frac{1}{4}C_0$	$(S^0_{20,w})$
0.1 M NaCl, pH 7.5	763,000	694,000	725,000	7.2
0.1 M Na_2CO_3‡	369,000	366,000	369,000	5.1
8 M Urea*	280,000	279,000	275,000	—
8 M Urea/SH*, ‡	299,000	309,000	345,000	5.8
5 M G·HCl	258,000	262,000	238,000	6.3
	275,000	309,000	—	

Abbreviations: SH, 2% mercaptoethanol; G. HCl, guanidine·HCl; C_0 = 3.0 ± 0.2 g per ml in all cases.

* Determined in the presence of 0.5 M KCl and 10 mM Tris, pH 7.5.

‡ Determined from dc/dx at the midpoint of cell.

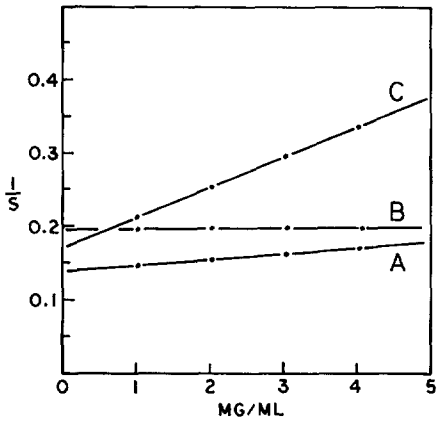

FIGURE 6 Reciprocal sedimentation rate vs. concentration for *C. atratus* hyalin in 0.1 M NaCl, 10 mM Tris·HCl, pH 7.5 (*A*), 0.1 M Na_2CO_3, pH 10.5 (*B*), and 8 M urea with 0.5 M NaCl, 10 mM Tris·HCl, pH 7.5, and 2% mercaptoethanol (*C*).

The minimal subunit value for some other species is about one-third that found for *C. atratus*, while the sedimentation rate for the native protein of two *Strongylocentrotus* species is significantly higher than that for *C. atratus*. These data, which are summarized in Table II, include early values determined by Kane and Hersh (1959) for *S. purpuratus*.

From the data thus far available, one could make an argument for a monomer-trimer-hexamer association, based on a minimal subunit weight of 100,000–120,000. It is not clear why hyalin from *C. atratus* has such a high minimal molecular weight subunit, though the possibility of ester or amide linkages between several chains has not been eliminated.

Addition of EDTA to a solution of hyalin brings about a splitting of the single boundary into two, one of which has a markedly higher sedimentation coefficient than the original particle. The extrapolated sedimentation rates for *C. atratus* hyalin in 1 mM EDTA were found to be 7.8 and 10.4S (Fig. 5). These particles very likely represent the original particle (7.8S) and an aggregate thereof (10.4S), whose interaction has been somehow affected by calcium removal. One might deduce that two chains of different molecular weight are present in hyalin, but the

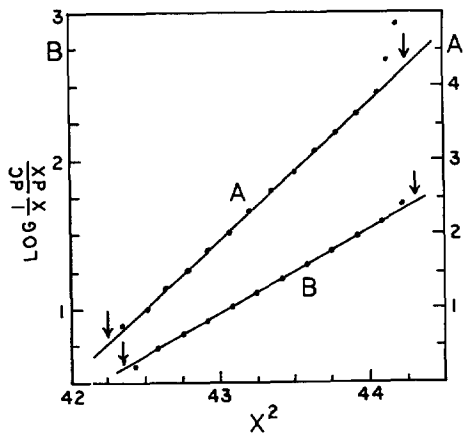

FIGURE 7 Molecular weight determination of *C. atratus* hyalin in 0.1 M NaCl, 10 mM Tris, pH 7.5 (*A*) and in 5 M guanidine hydrochloride and 2% mercaptoethanol (*B*). Arrows indicate menisci.

TABLE II

Physical Parameters of Hyalin from Various Species

Species	Solvent	Molecular wt.	Sed. rate ($S^0_{20,w}$)
S. droebachiensis	0.1 M NaCl, pH 7.5	—	8.9
S. droebachiensis	8 M urea/SH	90,000	3.0‡
T. gratilla	8 M urea/SH	120,000	3.1‡
*S. purpuratus**	0.1 M NaCl, pH 7	355,000	9.3
*S. purpuratus**	0.1 M NaCl, pH 9	102,000	5.3

Abbreviations: SH, 2% mercaptoethanol.

* Values averaged from Kane and Hersh (1959).

‡ Not extrapolated to infinite dilution.

protein is reasonably homogeneous in urea or guanidine and also is shown to be homogeneous by electrophoresis. Depending upon species and varying from preparation to preparation and with time of exposure to EDTA, the EDTA "subunits" differ considerably in their relative proportions in the mixture.

Amino Acid Composition

The hyalins from three representative species show a rather unique composition (Table III). Most notably, they are strikingly low in basic amino acids while aspartic and glutamic acids represent about one-fourth of the residues present. How many of these acidic amino acids are aminated remains to be determined, however.

The proline content, averaging about 8 mole%, if distributed uniformly should allow virtually no α-helix to form (see Szent-Györgyi and Cohen, 1957). Therefore, one might expect the molecule to be globular or random coil or both, depending upon charge.

The cysteine content averages about 2.5 mole% but DTNB titration indicates less than one residue per 100,000 g of protein to be present as free sulfhydryl. Thus, whatever conformation exists must be maintained through disulfide bonds, hydrophobic interactions, or salt linkages.

Tryptophan in hyalin from *C. atratus* was found to represent only 0.13 mole %, corresponding to a minimal molecular weight of 106,000, assuming one tryptophan per polypeptide chain and a calculated mean residue weight of 123.

Acrylamide Gel Electrophoresis

Considering the highly acidic nature of hyalin, one would expect rapid mobility on alkaline acrylamide gels, especially if reduced to minimal subunits with urea. However, the high molecular weights determined for the protein would tend to offset any such effect. Indeed, the mobility of *C. atratus* hyalin on urea-containing 7.5% polyacrylamide at pH 8.9 was found to be 0.06 with respect to the front while that for *S. droebachiensis* was found to be 0.15, but considerable aggregation at the spacer gel-running gel interface was noted. On 5% gels, no aggregation was seen and R_f's of 0.10 and 0.26 were found for hyalin from *C.*

TABLE III

Amino Acid Composition of Hyalin from Several Species of Sea Urchins

Moles/1,000 total moles.

	S. purpuratus	*S. droebachiensis*	*C. atratus*
Lysine	10.1	12.6	15.5
Histidine	4.6	5.5	5.9
Arginine	22.1	20.8	22.3
Aspartic	168.6	130.0	154.0
Threonine	70.5	126.0*	86.2
Serine	15.5	86.9*	19.4
Glutamic	92.7	78.1	90.2
Proline	90.0	72.7	84.3
Glycine	99.1	84.6	92.7
Alanine	72.7	70.0	83.9
Cysteine	26.6	27.9	25.4
Valine	132.7	107.0	126.9
Methionine	4.5	7.2	5.9
Isoleucine	59.2	50.9	58.9
Leucine	43.0	41.9	45.3
Tyrosine	27.6	26.1	25.8
Phenylalanine	60.4	50.7	57.4
Tryptophan	—	—	1.3

* Extrapolated to zero hydrolysis time.

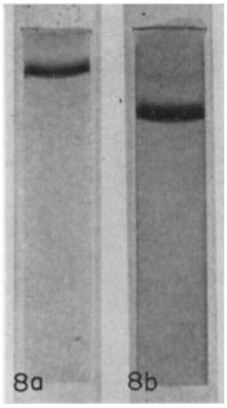

FIGURE 8 Polyacrylamide gel electrophoresis of *C. atratus* (*a*) and *S. droebachiensis* (*b*) hyalin. The 5% gels were 8 M in urea and buffered at pH 8.9.

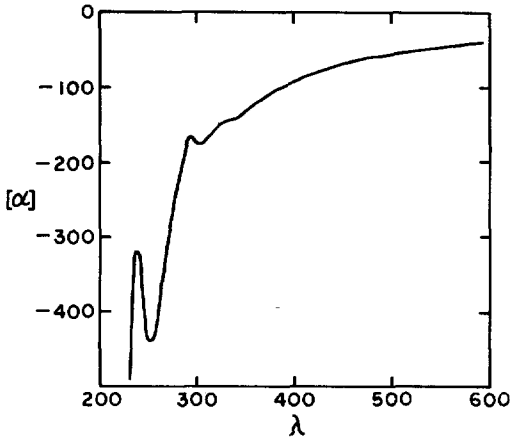

FIGURE 9 Optical rotatory dispersion of *C. atratus* hyalin in 0.05 M NaCl and 10 mM Tris·HCl, pH 7.5, carried out in the visible and ultraviolet regions.

atratus and *S. droebachiensis*, respectively (Fig. 8) These R_f values are in keeping with the inverse relationship that one would expect from the ratio of the minimal subunit molecular weights for these two species, namely about 3:1. In the various species investigated, only single bands were observed, indicative of reasonable homogeneity.

Optical Rotatory Dispersion

The optical rotatory dispersion of *C. atratus* hyalin in the visible and ultraviolet regions is illustrated in Fig. 9. Treatment of the data in the region of 400–600 mμ by means of a Moffit-Yang plot (Urnes and Doty, 1961) yields a b_0 of zero for hyalin from *T. gratilla*, *S. droebachiensis*, and *C. atratus*, indicating that the molecules have no α-helix content. The fine structure in the ultraviolet region was reproducible in all preparations examined, the 248-mμ trough being the main characteristic. No trough at 233 mμ, characteristic of α-helix, was observed.

Behavior of Protein Gels

When hyalin is gelled with divalent cations, birefringent fibers are produced (Fig. 10 *a*). Washing such fibers with EDTA causes the loss of nearly all birefringence and a lengthening of the fibers (Fig. 10 *b*). Re-addition of divalent cation causes the fibers to shorten and regain most of their birefringence (Fig. 10 *c*). Thus, the fibers behave as polyanions, shortening and becoming birefringent when charges are neutralized with divalent cations while lengthening and losing birefringence when these ions are removed by chelation with EDTA.

As mentioned above, few, if any, free sulfhydryl groups exist in hyalin. If the protein is reduced by mercaptoethanol in 8 M urea and then dialyzed against Tris buffer free of oxygen, addition of calcium causes no gelation. Bubbling oxygen through the calcium-containing solution causes gel formation at gas-liquid interfaces, apparently as a result of oxidation of the protein to a conformation permitting gelation.

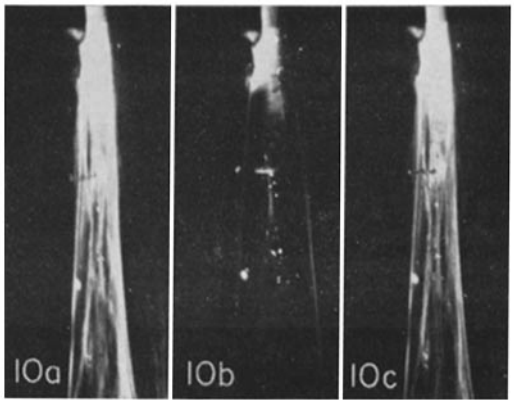

FIGURE 10 Fibers of hyalin from *Arbacia punctulata* in polarized light. Fibers formed in 0.1 M $MgCl_2$ are birefringent (*a*), lose birefringence upon perfusion of 10 mM EDTA (*b*), but regain most original birefringence upon perfusion with 0.1 M $MgCl_2$ (*c*). × 50.

DISCUSSION

Hyalin in the ultracentrifuge is a homogeneous high molecular weight particle with a minimal subunit weight of 90,000–120,000, or, in the case of *C. atratus* hyalin, in excess of 250,000, though the tryptophan content of the latter protein indicates a minimal subunit weight of 106,000. Both proline content and ORD measurements imply a lack of α-helix. Essentially no free sulfhydryl groups are found in hyalin; the protein contains about 2.5 mole % cysteine in the disulfide form which, along with hydrophobic bonds and salt interactions, must determine the conformation. The high acidic amino acid content of hyalin, together with the paucity of basic amino acids, would suggest that the protein might behave as a polyanion. With sufficient divalent cation, hyalin forms birefringent fibers which may be reversed to a nonbirefringent form by EDTA treatment; the conformation may be restored by re-addition of divalent cation.

The hyaline layer is formed by material originating from the breakdown of the cortical granules at fertilization (Endo, 1961). The hyaline substance is refractile and gel-like and insoluble in seawater. The properties of the protein hyalin correlate well with the behavior of the hyaline layer, as the ionic conditions in seawater would cause its gelation and the removal of the hyaline layer in isotonic monovalent salt parallels the solubilization of the protein in such solutions. It seems likely that the protein forms the major component in the isolated hyaline material of Vacquier (1969), although his finding that protein makes up only 45% of this material indicates that other components may play an important role in its formation and function.

The apparent existence of this gel protein in the cortical granules (Yazaki, 1968) in a solated form, followed by its transformation into the hyaline layer on granule breakdown, could be explained as a simple gelation upon contact with the divalent cations of seawater; or, conceivably, the protein may be in a partially reduced state in the granule, only to be oxidized and subsequently undergo gelation upon release.

The question of other roles for this protein in the cell remains open. There is evidence that in some species, at least, a fraction of the hyalin is found within the cytoplasm (Kane and Stephens, 1969). This may serve only as a reserve store of hyalin to replenish the hyaline layer, or may have some more active structural role in the control of cytoplasmic viscosity and gelation, particularly at furrowing. Only further work on intracellular localization of the protein and investigation into possible changes during cell division will answer these questions.

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