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Isolation, Physicochemical Properties, and the Macromolecular Composition of the Vitelline and Fertilization Envelopes from *Xenopus laevis* Eggs†

Don P. Wolf,‡ Tatsuro Nishihara, David M. West,§ Ron E. Wyrick,¶ and Jerry L. Hedrick*

ABSTRACT: As a step toward defining in molecular terms the sperm-triggered block to polyspermy reaction established by the egg at fertilization, vitelline (VE) and fertilization (FE) envelopes were isolated from eggs of the South African clawed toad *Xenopus laevis* and some of their physicochemical properties determined. Envelopes were isolated after lysis of the fertilized or unfertilized eggs by sieving techniques; isolated envelopes retained their in situ morphology as determined by electron microscopy. The isolated envelopes had different solubility properties and, in general, VE was more readily dissolved by aqueous solvents than FE, although both could be completely dissolved by detergents or chaotropic agents. Changes in envelope solubility correlated with the progression of the cortical reaction implicating a role for cortical granule material in modifying the solubility properties of the envelope. The VE and FE were composed of protein and carbohydrate with no lipid components detected. As determined by immunodiffusion experiments, the FE contained the same antigens as the VE plus components derived from the cortical granules and the innermost jelly layer, J_1. The macromolecular composition of the envelopes was determined by sodium dodecyl sulfate gel electrophoresis. The VE contained at least 11 glycoproteins with molecular weights ranging from 125 000 to <16 000 with two components (40 000 and 33 000) accounting for almost two-thirds of the total stainable material. The FE contained ten glycoproteins that had the same molecular weights as those in the VE. One glycoprotein component underwent a reduction in molecular weight from 77 000 to 67 500 when the VE was converted to the FE. This molecular weight change was interpreted as the probable result of limited proteolysis. In addition, the FE gel electrophoresis patterns contained macromolecular components derived from the cortical granules and jelly layer, J_1, consistent with the immunodiffusion experiments. These components were absent when the FE was prepared in the absence of Ca^{2+}, suggesting a role for Ca^{2+} in binding the VE, cortical granules, and J_1 components together. We concluded that the conversion of the glycoproteinaceous VE to FE at fertilization is caused by interaction of the VE with components from the cortical granules and jelly layer J_1. These interactions are of both a chemical and physical nature.

Establishment of a block to polyspermy at fertilization in some animal ova is effected by conversion of an extracellular vitelline envelope (VE) which is penetrable by sperm to a fertilization envelope (FE)[1] which is impenetrable by sperm (for reviews see Piko, 1969; Austin, 1968; Monroy, 1965). The transformation is produced by the so-called cortical reaction that is triggered by the first spermatozoon to contact the egg. The cortical reaction involves exocytosis of granules lying immediately beneath the plasma membrane of the egg. It is accompanied by an elevation or lift-off of the vitelline envelope from the egg plasma membrane (Nishihara and Hedrick, 1976).

In the case of the South African clawed toad *Xenopus laevis,* the conversion of the VE to the FE produces a new structure located between the innermost jelly coated layer J_1 and the former VE (Grey et al., 1974). The new structure has been termed the fertilization layer or F layer. It was postulated that F layer formation was via a precipitin reaction between a macromolecular component of the cortical granules and the innermost jelly coat layer, J_1 (Wyrick et al., 1974a,b; Wolf, 1974a). The FE has been shown to be impenetrable to sperm and to have a greatly reduced binding affinity for sperm in comparison with the VE (Grey et al., 1976), thus establishing its role in a block to polyspermy at fertilization.

Although the morphological alterations in the surface of the *Xenopus* egg have been well defined and their involvement in preventing supernumerary sperm penetration established, our

† From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received November 18, 1975.* This work was supported in part by a United States Public Health Service Grant (HD 4906).

‡ Current address: Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174.
§ University of Colorado, Medical Center, Denver, Colorado 80220.
¶ Hollister-Stier Laboratories, Spokane, Washington 99220.

[1] Abbreviations used are: FE, fertilization envelope; VE, vitelline envelope; J_1, jelly coat layer J_1; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PAS, periodic acid-Schiff base.

understanding of the molecular nature of these events is incomplete. The present investigation was undertaken to provide insight into the molecular basis of the VE to FE transformation by isolation of these extracellular structures and comparison of their physicochemical properties and their macromolecular compositions. A preliminary report of certain aspects of this study has been published (Wolf and Hedrick, 1971a).

Methods

Procurement of eggs and sperm from *Xenopus laevis* was as previously described (Wolf and Hedrick, 1971b).

Preparation of the VE. Unfertilized eggs were dejellied with 0.045 M mercaptoethanol in DeBoers solution at pH 9.5, room temperature with gentle stirring (Gusseck and Hedrick, 1971). The immersion time in the mercaptan solution was less than 2 min to minimize dissolution of the VE. The dejellied eggs were rinsed in DeBoers solution and then lysed by passage through a 10-ml syringe fitted with an 18 or 19-gauge needle. The envelopes were immediately sieved out of the suspension using a nylon screen (102 Nitex bolting cloth, 102-μm mesh opening). Envelopes suspended on the nylon screen were rinsed repeatedly (eight to ten times) with ice-cold distilled water until they were essentially free of contaminating yolk platelets. Jelly adhering to the envelopes after isolation was removed by briefly washing with ice-cold 0.045 M mercaptoethanol, pH 9.5, followed by cold distilled water. The time involved in this procedure was minimized since the VE slowly dissolve in distilled water (see Results). The envelopes were washed from the screen, suspended in a minimal volume of distilled water, collected by centrifugation at 5000g, and stored at 4 °C. Typically, 1 mg of VE protein and 1.3 mg of FE protein were recovered from 1000–1500 eggs. Upon storage, the VE's imbibed water and their subsequent swollen state made collection by centrifugation more difficult. Swelling of isolated envelopes has also been observed by light microscopy (Grey et al., 1976).

Preparation of the FE. The FE was prepared essentially as the VE with the following differences. Fertilized eggs were allowed to develop 2–4 h at room temperature (morula to blastula stages) before dejellying. After dejellying, vigorous rinsing with DeBoers solution lysed the unfertilized eggs thereby preventing contamination of the FE with VE. After washing away the broken unfertilized eggs, the fertilized eggs were lysed as previously described, collected on a nylon screen and washed with ice-cold 1.34 mM $CaCl_2$. Washing with 1.34 mM $CaCl_2$ rather than distilled water prevented the loss of certain F layer components especially in the presence of mercaptans. The FE also imbibed water upon storage in distilled water at 4 °C but did not swell to the extent that the VE swelled.

Light and Electron Microscopy. In order to determine the time course of the cortical reaction, fertilized eggs were transferred to Smith's fixative at various times after fertilization and prepared for light microscopy as previously described (Grey et al., 1974). Electron microscopy of isolated envelopes used methods previously described (Grey et al., 1974).

Antibody Production. Antisera to sodium dodecyl sulfate solubilized envelopes or heat dissolved envelopes (see Results) were prepared using female New Zealand White rabbits. Samples were emulsified (1:1) in complete Freund's adjuvant just prior to injection for initial immunization; subsequent injections utilized incomplete Freund's adjuvant. Subcutaneous injections at the base of the neck were given weekly for a period of 4 weeks (5 mg total material used). Alternatively, injections directly into the popliteal lymph node in the rear legs were given weekly for a period of 4–6 weeks (C. Furlong and R. Willis, personal communication). A total of 400 μg of material was used for each rabbit. Bleeding and preparation of the antiserum used conventional techniques (Williams and Chase, 1967). Immunodiffusion in two dimensions and immunoelectrophoresis were as previously described using unpooled antiserum (Yurewicz et al., 1975).

Protein. Protein concentrations were determined by the method of Lowry et al. (1951), ninhydrin reactivity after acid hydrolysis, or fluorometrically (Udenfriend, 1962). In the Lowry et al. and fluorometric methods, bovine serum albumin was used as the standard protein. The fluorometric procedure utilized a 0.1 M Tris-HCl–0.05 M NaCl solution, pH 8.5.

Amino Acid Analysis. Envelopes were hydrolyzed in vacuo in 6 M HCl at 110 °C for 24 h, and the amino acid content was determined by conventional automated chromatographic methods. Cysteic acid was determined after treating the sample with performic acid. Tryptophan was determined by the procedure of Edelhoch (1967).

Carbohydrate. Total neutral sugars were determined by the anthrone method using glucose as a standard (Spiro, 1966) or alternatively the phenol sulfuric acid method using galactose as a standard (Ashwell, 1966). Fucose, the hexoseamines, and specific neutral monosaccharides were determined by gas chromatographic analysis. The sugars were determined as the alditol acetates after resin-catalyzed acid hydrolysis of the heat-dissolved and extensively dialyzed envelopes according to the method of Porter (1975). Sialic acid was determined by the thiobarbituric acid method after hydrolysis using *N*-acetylneuraminic acid as a standard (Warren, 1959, using eq 2).

Lipid. The presence of lipid in the envelopes was investigated by gas chromatographic determination of fatty acids as methyl ester derivatives (Kannangara and Stumpf, 1972).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The sodium dodecyl sulfate–polyacrylamide gel electrophoretic procedures described by Weber and Osborn (1969), Neville (1971), and Laemmli (1970) were used. The procedure of Laemmli gave superior separation and the results reported here utilized his conditions. We routinely used electrophoresis grade acrylamide obtained from Bio-Rad Laboratories, Richmond, California. After electrophoresis, gels were stained for protein using Coomassie blue by the procedure of Bickle and Traut (1971) or by Fairbanks et al. (1971) or for carbohydrate using the periodic acid–Schiff base reaction by the procedure of Fairbanks et al. (1971). Gels were scanned at 600 nm (Coomassie blue) or 560 nm (periodic acid–Schiff base) using a Gilford spectrophotometer with a linear transport attachment. The scanning speed was 1 cm/min, the recorder speed 1 min/in., and the slit width was 0.05 mm.

Results

Purity and Structure of Isolated Envelopes. The extent of contamination of isolated envelope suspensions by cellular organelles was determined by microscopic examination. The only organelle contaminants in the envelope preparations were yolk platelets. The majority of the yolk platelets were removed during the sieving and washing procedure, but a small number sometimes adhered to the envelopes and carried over into the final suspension. Uncontaminated envelopes were less dense than envelopes with adhering yolk platelets and could be separated by repeated decantation of envelope suspensions. This resulted in higher purity but lower yields of envelopes. Only yolk platelet free envelopes were used for chemical analyses.

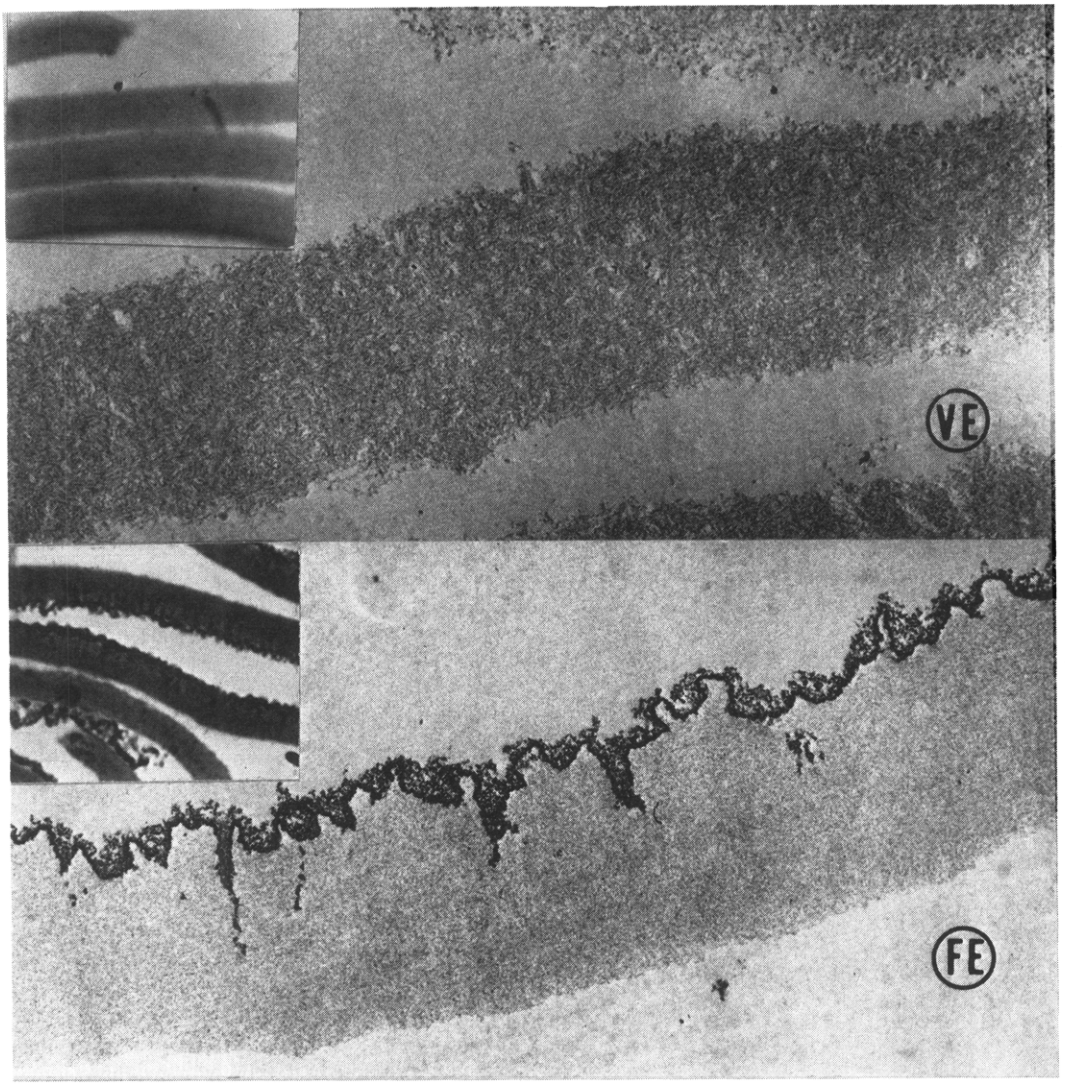

FIGURE 1: Morphology of isolated vitelline (VE) and fertilization (FE) envelopes. Magnifications are: light micrographs, 1000×; electron micrographs, 32 000×.

In addition, the yolk platelet contaminants were not solubilized by some of the procedures used to dissolve the envelopes, as will be discussed later, and could be separated from the envelopes by selective dissolution. The morphology of the envelopes was maintained during the isolation procedure as shown by comparison of the structures of the isolated envelopes with their in situ structures (Figure 1 and Grey et al., 1974).

Solubility. In initial experiments, envelope solubility was determined by microscopic examination (20–50×) during 3–4 h of contact with various solvents at room temperature. In general, the VE was more easily dissolved (i.e., in terms of rate and extent of envelope dissolution) than the FE. The VE was dissolved by 0.045 M mercaptoethanol at pH 8, 0.5 to 10% sodium dodecyl sulfate at pH 8, and slowly by water or solutions buffered close to neutrality. The FE, on the other hand, did not visibly dissolve in water, in solutions buffered close to neutrality or, in 0.045 M mercaptoethanol, pH 8. However, it was dissolved by the following: 0.5 to 10% sodium dodecyl sulfate, pH 8; 6 M guanidine hydrochloride, pH 7; 8 M urea, pH 9.5; 0.5% sodium deoxycholate; 2 M sodium trichloroacetate, pH 7; 0.5% HIO_4, pH 2; and 0.1 M NaOH. It was incompletely dissolved by the nonionic detergents Tween 20 and Triton X-100 at concentrations up to 10%. The relative solubility of the VE and FE was quantitated in solutions of 0.5% sodium dodecyl sulfate, 0.01 M mercaptan at pH 8.5, and in water.

Figure 2 shows the rate and extent of solubilization of the FE in a 0.5% sodium dodecyl sulfate solution at room temperature. Greater than 95% of the ninhydrin positive material in the FE preparations was recovered in the supernatant solution under these conditions. The sodium dodecyl sulfate insoluble material remaining in the pellet was due to yolk platelet contamination as determined by morphological and solubility considerations. Thus, sodium dodecyl sulfate solutions rapidly and completely dissolve the fertilization envelope; the VE was similarly dissolved.

Figure 3 shows the comparative solubility of VE and FE in 0.01 M mercaptoethanol, pH 8.5, at room temperature. Complete solubility was defined by the dissolution of the envelopes in 1% sodium dodecyl sulfate. The VE is rapidly and completely dissolved while the FE is slowly and incompletely dissolved by mercaptoethanol solutions. Similar results were obtained using 0.01 M dithiothreitol solutions under the same conditions.

Figure 4 illustrates the solubility of the VE and FE in distilled water at 0 °C. The VE was slowly (i.e., >5 days) but

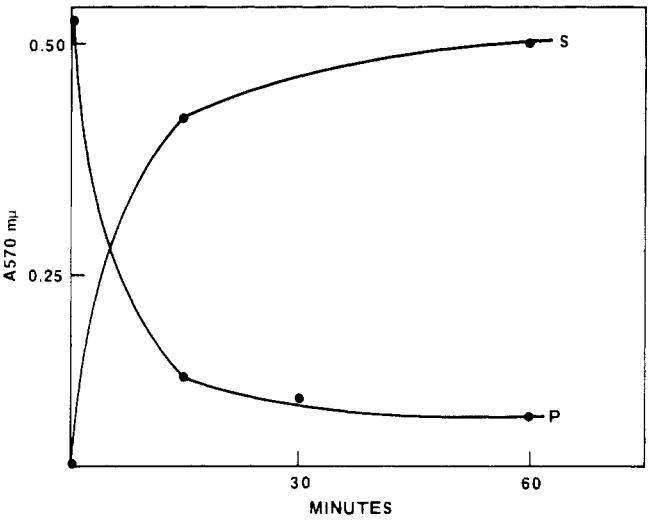

FIGURE 2: Solubility of the fertilization envelope in 0.5% sodium dodecyl sulfate at room temperature. S, supernatant solution; P, pellet. Protein was determined in the supernatant solution and in the pellet by quantitative ninhydrin analyses after 6 M HCl hydrolysis.

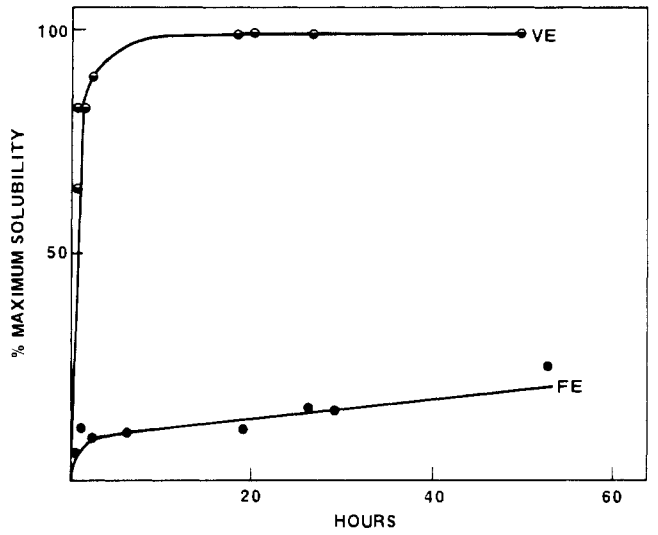

FIGURE 3: Solubility of vitelline and fertilization envelopes in 0.01 M mercaptoethanol, pH 8.5, at room temperature.

completely dissolved under these conditions while the FE was not dissolved to any detectable extent. The differential solubility of VE and FE was not due to bacterial degradation as addition of 50 μg/ml of tetracycline did not affect differential solubility of the envelopes. The solubility of the VE in cold distilled water has obvious implications for its preparation and storage after isolation as mentioned earlier.

The envelopes were easily solubilized in water or very dilute buffers at an alkaline pH when suspensions were heated. Complete dissolution of both the VE and FE was obtained by heating a water suspension of envelopes to 70 °C for 5–10 min at pH 9.5 (adjusted with Tris or NaOH solutions). Subsequent cooling of the solution to 0 °C and adjustment to pH 8.0 resulted in continued solubility for VE but the FE slowly precipitated from solution (after 1–2 days). Precipitation was pH dependent and occurred more rapidly at pH values less than 7 for both the VE and FE. Envelopes solubilized by this procedure will be referred to as heat-dissolved envelopes.

The marked solubility differences in the envelopes suggested

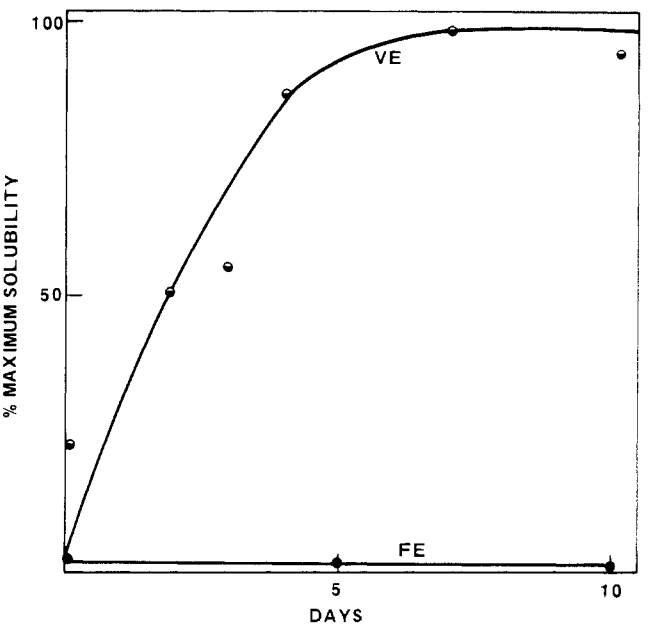

FIGURE 4: Solubility of the vitelline and fertilization envelopes in distilled water at 0 °C.

that solubility may be used as a criterion in determining whether an envelope was a VE or FE. Accordingly, an attempt was made to correlate changes in envelope solubility with the progression of the cortical reaction in fertilized eggs.

Correlation of Envelope Solubility and the Cortical Reaction. The time-dependent, in vivo conversion of VE to FE was determined by isolating envelopes from eggs (1000–2000) at various times after addition of a sperm suspension. To stop the conversion reaction, eggs were dejellied in 0.045 M mercaptoethanol–DeBoers solution in 1 min or less and lysed in the usual way. Envelopes were sieved out of the suspension within 30 s of lysis and separated as described in Methods. Envelope solubility in 0.01 M mercaptoethanol, pH 8.5, was measured fluorometrically. The results (Figure 5) represent solubility data from two separate experiments involving 15 000 eggs. The time between insemination and observable sperm penetration to the VE (jelly penetration time) varied between 1.5 and 2.5 min. Fertilization of control eggs exceeded 90%. The number of eggs undergoing the cortical reaction as indicated by the absence of cortical granules as a function of time after insemination was determined microscopically using cytological techniques (Grey et al., 1974). The reaction was terminated at various times by immersing the eggs in Smith's fixative. Each point in Figure 5 representing progression of the cortical reaction was the average of approximately 6 eggs. The change in envelope solubility satisfactorily correlated with the progression of the cortical reaction.

Chemical Composition. The protein, carbohydrate, and lipid content of VE and FE were determined. The percentage protein content of the envelopes (dried in vacuo over P_2O_5) determined either by the Lowry et al. (1951) method or by amino acid analysis was the same on a mass basis. The percentage carbohydrate content of the dried envelopes differed according to the method used. The relative protein and carbohydrate composition of the VE and FE using different methods is shown in Table I. The differences in carbohydrate content by these methods may be caused by the sugar specificity of the method, by the extent of hydrolysis, and/or by the different color values of various monosaccharides (Spiro, 1966; Ashwell, 1966). Nevertheless, all three methods showed a higher carbohydrate

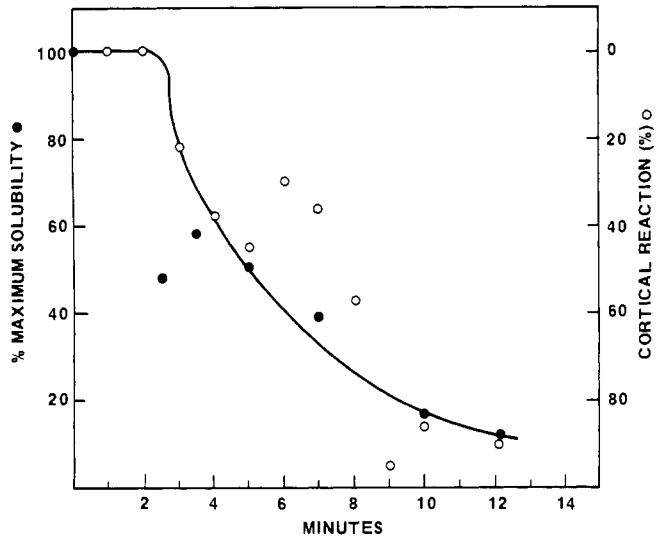

FIGURE 5: Envelope solubility changes and the cortical reaction as a function of postfertilization time. Solubility was determined using 0.01 M mercaptoethanol, pH 8.5, as the solvent. The number of eggs undergoing the cortical reaction was determined by cytological techniques (Grey et al., 1974).

content of the FE than VE. We had the highest confidence in the gas chromatographic methods and subsequent calculations (Tables II and IV) utilized these values.

The ultraviolet absorption spectrum and fluorescence spectrum of the dissolved envelope preparations were typical of those observed for most proteins containing aromatic amino acids. Absorption coefficients of $A_{280nm,1cm}^{1\%} = 8.56$ and 9.32 were obtained for the VE and FE in 6 M guanidine hydrochloride–0.02 M phosphate buffer (pH 6.5). Analysis of the amino acid composition after acid hydrolysis yielded the usual spectrum of amino acids with no unusual types or amounts of amino acids present (Table II). However, the amino acid compositions of the VE and FE were significantly different (e.g., Gly, Lys, Pro, and Tyr) indicating that VE and FE are chemically distinct. The higher absorption coefficient of the FE at 280 nm compared with the VE is consistent with the higher aromatic amino acid content of the FE.

The carbohydrate composition of the envelopes was determined by gas chromatographic analysis of the alditol acetate derivatives and also by chemical methods. Fucose, glucosamine, galactosamine, mannose, galactose, and sialic acid were present in both the VE and FE but were quantitatively different in the two envelope preparations (Table II). On the basis of these sugar analyses, the VE and FE are chemically distinct.

The lipid content of the envelopes was determined by gas chromatographic analysis of methylated fatty acid derivatives. Less than 1 μg of fatty acid per 1 mg of envelope was detected in the VE or FE. This value was within the error of the procedure and was considered insignificant. Additionally, no phosphate was detected in either the VE or FE. These results indicated not only that the envelopes did not possess lipid components, but also that dissolved envelope preparations were not contaminated with membrane or subcellular organelles that contain lipids, e.g., yolk platelets. We conclude from these chemical analyses that the envelopes are composed of glycoproteins.

Macromolecular Composition. Characterization of the macromolecular composition of the VE and FE by immunological methods employed immunodiffusion in two dimensions and immunoelectrophoresis. Antisera were prepared against either sodium dodecyl sulfate dissolved or heat-dissolved envelopes. The antigens used in the double diffusion and immunoelectrophoresis experiments were heat-dissolved VE and FE. The results are summarized in Table III. Using antisera prepared against sodium dodecyl sulfate dissolved envelopes, the anti-VE serum gave three precipitin lines against the VE and FE that completely fused at their intersections (Wyrick et al., 1974a). Thus, the VE contained the same antigens as the FE using anti-VE serum. Anti-FE serum, on the other hand, gave

TABLE I: Relative Protein and Carbohydrate Content of VE and FE.

Method Used		Protein (%)	Carbohydrate[a] (%)	Number of Analyses[b]
Anthrone	VE	94.5 ± 0.8	5.5 ± 0.8	3
	FE	92.1 ± 1.9	7.9 ± 1.9	3
Phenol sulfuric acid	VE	90.1 ± 0.2	9.9 ± 0.2	3
	FE	88.8 ± 1.2	11.2 ± 1.2	3
Gas chromatograph	VE	84.6	15.4	2
	FE	83.3	16.7	2

[a] The carbohydrate content includes sialic acid independently determined as described in the text and reported in Table II. [b] Each analysis was of a different envelope preparation.

TABLE II: Amino Acid and Sugar Composition of the VE and FE.

	VE		FE	
	Weight %	mol/10^5 g	Weight %	mol/10^5 g
Amino acid				
Ala	3.36	47.3	3.33	46.7
Arg	5.95	38.1	5.48	35.1
Asp	10.00	86.9	10.02	87.1
Cys/2[a]	3.04	29.5	3.06	29.6
Glu	7.09	54.9	7.21	55.8
Gly	2.33	40.8	2.61	45.6
His	0.95	6.9	1.05	7.6
Ile	4.51	39.7	4.31	38.1
Leu	6.81	60.1	6.44	56.9
Lys	3.24	25.3	3.67	28.7
Met	2.26	17.2	2.15	16.4
Phe	4.80	32.7	5.11	34.7
Pro	5.83	60.1	5.19	53.5
Ser	6.09	69.9	5.54	63.6
Thr	6.37	62.9	5.93	58.6
Trp[b]	1.89	10.1	1.93	10.4
Tyr	4.25	26.1	4.76	29.1
Val	5.90	59.5	5.47	55.1
	84.62%		83.28%	
Sugar[c]				
Fuc	1.05	7.2	1.35	9.3
GlcNAc[d]	3.70	18.2	4.36	21.5
GalNAc	3.80	18.7	3.93	19.3
Man	2.96	18.3	2.79	17.2
Gal	2.68	16.5	3.00	19.5
Sialic acid	1.20	3.9	1.30	4.1
	15.38%		16.72%	

[a] Performic acid oxidation followed by acid hydrolysis. [b] According to Edelhoch (1967). [c] Determined by gas chromatography of alditol acetate derivatives. [d] Calculated as *N*-acetylhexoseamines.

TABLE III: Antigenic Relatedness of the VE and FE Determined by Immunodiffusion.

Anti-serum	Number of Precipitin Lines VE	FE	Cross Reactivity of Precipitin Lines
$NaDodSO_4$ dissolved			
VE	3	3	All three lines of VE and FE gave patterns of identity.
FE	3	4	Three FE lines gave patterns of identity with VE lines; the fourth FE line gave a pattern of identity with a J_1 component.
Heat dissolved			
VE	2	2	Both VE and FE lines gave patterns of identity.
FE	2	4	Two of the FE lines gave patterns of identity with VE lines. One FE line gave a pattern of identity with a J_1 component and one FE line gave a pattern of identity with a cortical granule exudate component.

three precipitin lines with the VE and four with the FE. Three of the lines showed patterns of identity with the VE lines whereas the fourth gave a pattern of identity with a component from jelly layer J_1. Anti-VE serum did not give a precipitin line against J_1. Thus, we conclude from these experiments that the FE contains all the antigens of the VE plus a J_1 antigen.

Immunoelectrophoresis at pH 8.6 in 1% agarose of heat-dissolved VE and FE gave three precipitin lines with the anti-VE serum (sodium dodecyl sulfate dissolved VE) and four precipitin lines with the anti-FE serum (sodium dodecyl sulfate dissolved FE). The three slowly migrating anodic components of the VE gave patterns of identity with the three slowly moving anodic components of the FE. The rapidly migrating component of the FE was likely derived from jelly layer J_1 due to its large mobility (Hedrick et al., 1974; Yurewicz et al., 1975).

Using antisera against heat-dissolved envelopes, anti-VE serum gave two precipitin lines with patterns of identity for both VE and FE. Anti-FE serum gave two precipitin lines with VE, but four with FE. Two of the FE lines were identical with the VE lines, while one appeared to be identical with a J_1 component and one was identical with a cortical granule exudate component (Wyrick et al., 1974b; Nishihara et al., manuscript in preparation). These immunological results suggest that FE contains all of the antigenic components of VE plus antigens from jelly layer J_1 and the cortical granules.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used as a more definitive method for analyzing the macromolecular constituents of the envelopes and was particularly appropriate in view of the solubility of the envelopes in sodium dodecyl sulfate solutions. Heat treatment in the presence of sodium dodecyl sulfate was required for complete dissociation of the envelopes. The macromolecular components of envelopes dissolved by sodium dodecyl sulfate alone or by heat followed by sodium dodecyl sulfate were not completely dissociated as large aggregates were detected by sodium dodecyl sulfate gel electrophoresis, gel filtration, and analytical ultracentrifugation experiments. Reducing agents such as mercaptoethanol or dithiothreitol did not significantly affect the gel patterns but were still routinely used. Standardization of the Laemmli system (1970) employed a 10% polyacrylamide gel and seven proteins with molecular weights ranging from 23 200 to 92 000. Table IV presents the R_m, molecular weight, protein, and carbohydrate composition of the VE and FE components resolved in this system. The VE contained significant glycoprotein components with molecular weights of 125 000, 118 000 (these two components were optimally resolved in 6% or less gels), 77 000, 71 500, 60 000, 40 000, 33 000, 30 500, 29 000, 26 500, and <16 200 (material at the dye front). The components with molecular weights of 40 000 and 33 000 contained almost two-thirds of the total envelope mass.

Gel patterns of FE prepared by extensively washing the envelopes before dissolution in Ca^{2+}-free medium (distilled water) were essentially the same as VE except for the presence of a 67 500 component, the disappearance of a 77 000 component (these two components were optimally resolved in 4.5% gels) and a concomitant increase in the amount of protein at the dye front ($R_m = 1.00$). From these results, we suggest that proteolysis of the 77 000 component gives rise to the 67 500 component plus the small molecular weight protein of the dye front. The other minor components listed in Table IV varied greatly from preparation to preparation and sometimes were entirely missing. These components were therefore not considered to be true constituents of the envelopes.

Formation of the F layer of the fertilization envelope was shown to involve Ca^{2+} (Wyrick et al., 1974a; Wolf, 1974b). Accordingly, FE prepared by extensive washing with Ca^{2+}-free media may result in the loss of this structure or some of its macromolecular components (Wolf, 1974b; Nishihara and Hedrick, manuscript in preparation). Comparison of gel patterns of FE prepared in the presence of 1.34 mM $CaCl_2$ showed distinct differences from FE prepared in Ca^{2+}-free media. FE prepared with Ca^{2+} media contained the following additional components: (1) an 88 500 glycoprotein component, (2) a 46 000 glycoprotein component appearing as a shoulder on the major 40 000 component, (3) an increase in the amount of a 40 000 component(s), (4) a PAS-positive component in the stacking gel, and (5) a carbohydrate-rich glycoprotein component(s) with a $R_m \sim 0$. There were no differences in the gel patterns of VE prepared in the presence or absence of Ca^{2+}. It was suggested from other work in this laboratory with purified subcellular fractions (Nishihara and Hedrick, manuscript in preparation) that the 88 500, the 46 000, and the increase in the 40 000 component are components contributed to the FE from the cortical granules and the large molecular weight (low R_m) component(s) are components contributed to the FE from jelly layer J_1.

Discussion

To our knowledge this is the first comprehensive report of the isolation, physicochemical properties, and macromolecular composition of an envelope surrounding a vertebrate egg. From chemical analyses and the staining characteristics of its constituent macromolecules as resolved by sodium dodecyl sulfate gel electrophoresis, the envelope appears to be composed of glycoproteins. The macromolecular composition of the VE is relatively simple, with two components accounting for the majority of the envelope mass, and nine or more other components accounting for the remaining mass. These are minimal estimates, however, as individual bands on sodium dodecyl sulfate gel electrophoresis may be composed of more than one substance. FE differs from VE in terms of its macromolecular composition. Both immunological and sodium dodecyl sulfate gel electrophoretic experiments suggest that a jelly layer J_1 component(s) and a cortical granule component(s) are incorporated into the FE. In addition, a VE glycoprotein com-

TABLE IV: 10% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of the VE and FE.[a]

R_m		Mol Wt × 10^{-3}		% Composition			
				VE		FE	
VE	FE	VE	FE	Protein	Carbohydrate	Protein	Carbohydrate
	In stacking gel[b]		ND[b]				0.06[b]
	0.000[b]		ND[b]			ND[b]	ND[b]
0.170	0.170	125	125	} 6.98	} 3.15	} 7.80	} 3.19
0.184	0.184	118	118				
0.198	*0.198*	*111*	*111*	ND	ND	ND	ND
0.226	*0.226*	*102*	*102*	*0.55*	*0.45*	*0.40*	*0.33*
	0.240		88.5[b]			0.44[b]	0.09[b]
0.316		77.0		2.54	1.07		
0.339	0.339	71.5	71.5	2.51	1.29	3.14	1.57
0.356	*?*	*68.5*	*?*	*0.59*		} 4.27	} 1.10
	0.362		67.5				
0.367	*?*	*67.0*	*?*	*1.17*			
0.390	*0.390*	*63.5*	*63.5*	*1.17*			
0.412	0.412	60.0	60.0	2.61[c]		1.24[c]	
0.424	*0.424*	*58.5*	*58.5*	ND	} *0.16*	ND	
0.457	*0.463*	*54.5*	*53.5*	*0.39*		*0.31*	
0.491	*0.486*	*50.0*	*51.0*	*0.59*		*0.38*	
0.508	*0.508*	*48.5*	*48.5*	*0.31*		*0.19*	
0.525	*0.520*	*46.5*	*47.0*	*0.89*		*0.50*	
	0.530[b]		46.0[b]			} 30.3 } (44.4)[b,d]	} 7.20 } (9.32)[b,d]
0.593	0.593	40.0	40.0	28.8	7.26		
0.678	0.678	33.0	33.0	29.8	1.38	30.2	1.44
0.717	0.712	30.2	30.5	0.79	0.11	0.80	0.09
0.735	0.735	29.0	29.0	1.68	0.16	1.60	0.18
0.780	0.774	26.3	26.5	1.38	0.20	1.25	0.14
0.825	*0.825*	*23.7*	*23.7*	*0.38*	*0.04*	*0.28*	*0.06*
1.000	1.000	<16.2	<16.2	1.46	0.16	1.96	0.15
Total:				~84.6	15.4	84.6 (99.1)[d]	15.4 (17.6)[d]

[a] Numbers in italics were trace components not considered true components of the envelope. Braces indicate components insufficiently resolved for separate compositional analysis. [b] Additional components in the FE formed when the FE was prepared in the presence of Ca^{2+}. ND means not determined. [c] These values were highly variable from preparation to preparation. [d] The percentage of this component when the FE was prepared in the presence of Ca^{2+}.

ponent is modified in a way consistent with its undergoing limited proteolysis. These limited chemical changes induce marked physical changes in the envelope when it undergoes the VE to FE transition. The VE was readily dissolved by mercaptan solutions and was slowly dissolved by distilled water. In contrast, FE had only limited solubility in mercaptan solutions and was virtually insoluble in distilled water. It has been shown that the disulfide bonds of the VE are readily reduced by mercaptans while the disulfide bonds of the FE are inaccessible to mercaptan reduction (Wyrick, 1974; Wyrick and Hedrick, manuscript in preparation). This suggests marked conformational differences between the envelopes. In addition dissolution by heating suspensions of the envelopes also indicates a physical difference between them. The VE can be dissolved (melted) at much lower temperatures than the FE, an observation consistent with their having comformational differences in their macromolecular components (Wyrick, 1974; Wyrick and Hedrick, manuscript in preparation). The VE and FE also differ in regards to their susceptibility to proteolysis by trypsin (Wolf, 1974a).

The VE to FE transformation is apparently induced by interaction of the VE and J_1 with material(s) released from the cortical granules. The transformation involves chemical and physical (conformational) changes that render the envelope impenetrable to sperm, thereby effecting a block to polyspermy. Further investigation of this process will be focussed on isolation of the jelly and cortical granule components involved and attempts to carry out the in vitro conversion of VE to FE with isolated and chemically defined components.

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Methionyl-tRNA Synthetase from *Escherichia coli:* Active Stoichiometry and Stopped-Flow Analysis of Methionyl Adenylate Formation†

François Hyafil, Yannick Jacques, Guy Fayat, Michel Fromant, Philippe Dessen, and Sylvain Blanquet*

ABSTRACT: Native dimeric methionyl-tRNA synthetase and its monomeric proteolytic fragment are shown to form and to bind 1 mol of methionyl adenylate per polypeptide chain. Moreover, at 25 °C, each monomer of the dimeric native enzyme behaves independently, exhibiting the same parameters for the methionine activation reaction as does the monomeric modified enzyme. These results were obtained using several independent methods including equilibrium and nonequilibrium dialysis, active site and tryptophan fluorescence titrations, and stopped-flow by fluorescence. Stopped-flow resolution of the reversible methionine activation reaction also demonstrates that methionine and ATP-Mg^{2+} react without coupling to form a ternary enzyme·methionine·ATP-Mg^{2+} complex. This complex readily converts to enzyme·methionyl~adenylate·PP-Mg^{2+} with a standard free energy close to zero. It is concluded that the uncoupled enzyme·methionine·ATP-Mg^{2+} complex may resemble the transition state of the reaction at the expense of the additional synergistic binding energy provided by reciprocal coupling, within the site, of the methionine molecule with the adenosine and PP-Mg^{2+} parts of the ATP-Mg^{2+} molecule (Blanquet, S., Fayat, G., and Waller, J. P. (1975), *J. Mol. Biol. 94,* 1).

Recent studies have demonstrated that several aminoacyl-tRNA synthetases exhibit extensive sequence duplication. Enzymes so far shown to possess this property are either monomeric structures composed of a polypeptide chain having twice the normal subunit size (i.e., approximately 100 000 daltons) (Kula, 1973; Koch et al., 1974; Waterson and Konigsberg, 1974; Bruton, 1975a) or else dimeric enzymes composed of two such large protomers (Bruton et al., 1974). The existence of these large protomers, which may have arisen through duplication and fusion of adjacent genes coding for normal sized subunits (i.e., approximately 45 000 daltons), raises the important issue of their functional significance.

In the case of tyrosyl-tRNA synthetase from prokaryotic origin, composed of two normal, 45 000-daltons subunits in noncovalent association, it has been shown that the subunits exhibit anticooperativity for substrate binding to the tyrosine adenylating sites, while on the other hand both subunits are able to synthesize tyrosyl adenylate at different rates (Fersht, 1975; Jakes and Fersht, 1975). In an attempt to extrapolate this property to the synthetases which possess the sequence duplication feature, these authors have performed experiments which tend to indicate that, in spite of the loss of symmetry resulting from covalent associations, remains of this anticooperativity have been preserved during the fusion process. The rationale of the two sets of binding sites for amino acid and ATP in such twice repeated sequences has been proposed in terms of a general mechanism for enhancement of specificity for the amino acid in the esterification of tRNA (Fersht, 1975).

However, the specificity of interaction with tRNA itself plays a role at least as crucial as that involving amino acid in

† From Ecole Polytechnique, Laboratoire de Biochimie, Laboratoire Associé n° 240 du Centre National de la Recherche Scientifique, 91120 Palaiseau, France. *Received December 31, 1975.* This study was supported in part by the Commissariat à l'Energie Atomique (Saclay, France) and by the Délégation Générale à la Recherche Scientifique et Technique.