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Radioiodination Studies of the Envelopes From Xenopus laevis Eggs

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To investigate the molecular basis of the observed morphological and biological characteristics of coelomic egg envelopes (CE), vitelline envelopes (VE), and fertilization envelopes (FE) of Xenopus laevis eggs, envelopes were radioiodinated under a variety of conditions: in situ, isolated and intact, or solubilized. The distribution of ^{125}I in envelope components was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each envelope type displayed unique profiles when iodinated in the intact state. A major constituent of VE, the 41,500 molecular weight component, was not labeled in the intact state, although the corresponding component of CE was heavily labeled. After dissociation of the envelope by guanidine-HCl or sodium dodecyl sulfate, all of the components could be radioiodinated. However, when the envelopes (VE and FE) were dissolved by heating and subsequently radioiodinated by lactoperoxidase, the resulting radioactivity profile was similar to that of the intact envelopes, suggesting that in the heat-dissolved envelope, the individual components retain similar structural relations as in the intact envelope. Quantitative but not qualitative differences were found between the inner and outer aspects of VE and FE. The significance of these findings is discussed in relation to what is known about the morphological, biological, and molecular properties of the envelopes.

Key words: fertilization, egg envelopes, glycoproteins, molecular topography

The envelope of the Xenopus laevis egg exists in nature as one of three morphologically and biologically distinct types: the coelomic egg envelope (CE), the vitelline envelope (VE), and the fertilization envelope (FE). An egg recovered from

Abbreviations used: CE, coelomic egg envelope; FE, fertilization envelope; VE, vitelline envelope; VE*, vitelline envelope component of the fertilization envelope; F-layer, fertilization-layer; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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the body cavity is enclosed by the CE, which is not penetrable by sperm [1]. During passage down the oviduct, the envelope is converted to the VE and the egg accumulates three jelly-coat layers. The VE can be penetrated by sperm, but after fertilization of the oviposited egg, the envelope becomes the FE, which cannot be penetrated by sperm. This second conversion results in a block to polyspermy [2,3].

The morphological characteristic that distinguishes a VE from an FE is the presence of the fertilization or F-layer on the outer surface of the FE. Wyrick et al [4] demonstrated that the F-layer is produced by a CA^{++} dependent lectin-ligand interaction that can be inhibited by galactosides. Greve and Hedrick [5] used immunocytochemistry to show that the lectin is sequestered in the cortical granules of unfertilized eggs. After fertilization or egg activation with the calcium ionophore A23187, the cortical granule lectin is found in the F-layer and the perivitelline space. This F-layer forms a macromolecular block and a cellular block because macromolecules such as ferritin cannot penetrate it and because unfertilized eggs incubated in either cortical granule exudate or purified cortical granule lectin exhibit an F-layer and cannot be fertilized ([4]; Nishihara and Hedrick [in preparation]).

We wished to establish the molecular basis for the observed differences in the morphology and sperm penetrability of CE, VE, and FE. Other studies indicated that CE, VE, and FE were composed of very similar glycoprotein components ([7,8]; Gerton and Hedrick [in preparation]). Thus, the possibility existed that the observed ultrastructural and functional differences were due to conformational or positional differences of the various macromolecular envelope components. To probe this possibility, we radioiodinated the envelopes under a variety of conditions, *in situ*, isolated and intact, or solubilized, and analyzed the radiolabeling profiles using SDS-PAGE. We found distinct iodination profiles for intact, isolated CE, VE, and FE. There were also profound differences between the iodination profiles of intact versus dissociated envelopes.

MATERIALS AND METHODS

Materials

VE and FE were prepared according to Wolf et al [8]. CE was prepared as described by Grey et al [1]. Lactoperoxidase was purified from bovine milk [9]. IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was purchased from Pierce Chemical Company. Electrophoresis-grade acrylamide, 2,2'- methylene-bis-acrylamide, and SDS were from Bio-Rad Laboratories. The calcium ionophore, A23187, was a gift from the Eli Lilly Company. Amersham-Searle supplied carrier-free Na ^{125}I. All other chemicals were reagent grade from commercial sources.

Methods

VE* preparation. The F-layer was extracted from FE prepared in the presence of 1.34 mM $CaCl_2$ by suspending the envelopes in 0.5 M galactose, 1.0 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.8) for 2 hr at room temperature. The resulting VE* was pelleted by centrifugation and the supernatant solution (solubilized F-layer components) removed. The envelopes (VE*) were rinsed by centrifugation in fresh galactose buffer, followed by several rinses in ice-cold water to remove the galactose and salts.

Solubilization conditions. For this study, envelopes were solubilized by three procedures: (1) Guanidine-HCl-dissociated envelopes were prepared by solubilization in 6 M guanidine-HCl, 0.02 M sodium phosphate (pH 6.5), followed by extensive

dialysis to remove the guanidine-HCl. (2) SDS-dissociated envelopes were prepared by mixing one part 10% SDS with four parts of a suspension of envelopes. This mixture was heated at 100°C for 90 sec to completely dissolve the envelopes. (3) After extensive rinsing in ice-cold water, intact, isolated envelopes could be dissolved in 50 mM Tris-HCl (pH 9.5) by heating to 70–75°C for 5–10 min [8]. The resulting clear solution was cooled to room temperature and neutralized with dilute HCl. Envelopes solubilized by this last procedure are referred to as heat-dissolved envelopes.

Iodination procedures. Intact isolated envelopes, in situ envelopes, guanidine-HCl-dissociated or heat-dissolved envelopes were radioiodinated by the lactoperoxidase preocedure [10]. Heat-dissolved or guanidine-HCl-dissociated envelopes were either dialyzed or chromatographed on a Sephadex G25 column to remove free iodide from the iodinated protein. After envelope iodination in situ, dejellied eggs were rinsed two to three times in DeBoer's solution (0.11 M NaCl, 1.34 mM $CaCl_2$, 1.32 mM KCl adjusted to pH 7.2 with $NaHCO_3$). Envelopes were isolated from these eggs as described above. Intact, isolated envelopes were rinsed five to six times in ice-cold water after radioiodination. These ^{125}I-envelopes were then dissolved in 2% SDS as described above.

In some cases, IODO-GEN was used for iodinating intact, isolated or SDS-dissociated envelopes [11]. Culture tubes (10 × 75 mm) were plated with IODO-GEN and rinsed with 1 ml of buffer just prior to use. Intact, isolated envelopes (0.2 ml packed volume) were suspended in 2.0 ml of DeBoer's solution containing 10 mM Tris adjusted to pH 7.8 with HCl. The envelope suspension was transferred to a tube plated with 50 μg of IODO-GEN, and the reaction was initiated by the addition of 5 μg of carrier-free Na^{125}I (approximately 100 μCi/μl). The tube was sealed with Parafilm and incubated at room temperature. At the end of 20 min, the envelopes were transferred to a 12-ml conical centrifuge tube and suspended and pelleted four times in 1 mM NaI, 1.34 mM $CaCl_2$, and twice in ice-cold water. The resulting envelope pellet was then dissolved in 2% SDS and stored at −20°C. SDS-dissociated envelopes were iodinated using tubes plated with 10 μg of IODO-GEN. The reaction mixture contained 135 μl of a 3.0 mg/ml solution of SDS-dissociated envelopes, 34 μl of 50 mM Tris-HCl (pH 8.0), and 6 μl of carrier-free ^{125}I (100 μCi/μl). After 15 min at room temperature, the reaction mixture was transferred to a tube containing 19 μl of 2.5 M NaI (final concentration = 0.25 M NaI). Free iodide was then removed from iodinated protein by chromatography on a Sephadex G25 column.

Electrophoresis. SDS-PAGE was carried out according to Laemmli [12]. Gels containing 8.5% polyacrylamide (30:0.8 by weight acrylamide:methylene-bis-acrylamide) were run in glass tubes (0.5-cm inner diameter, 9.0 cm long). Gels were stained with Coomassie blue according to Fairbanks et al [13].

Determination of radioactivity. The cylindrical gels were stained, scanned for Coomassie blue staining at 600 nm, cut into 1 mm sections, and counted in a crystal scintillation counter.

RESULTS

SDS-PAGE of Envelope Components

Figures 1 and 2 show typical densitometric scans of SDS-gels of VE, FE, VE*, and CE. For the purposes of discussion, we will use a shorthand notation to refer to the different components, eg, the 37K component is that component with a molecular

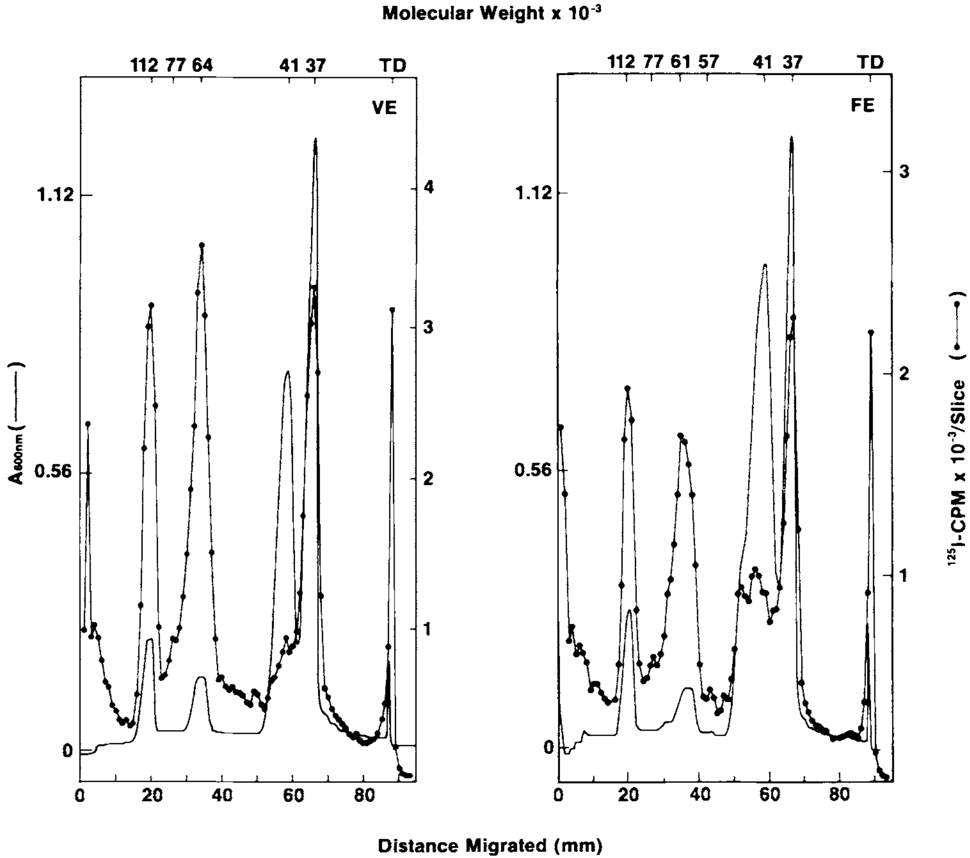

Fig. 1. SDS-PAGE of intact, isolated VE and FE radioiodinated by the lactoperoxidase procedure (8.5% polyacrylamide tube gels.) The stained gels were scanned and the radioactivity per 1-mm gel slice determined as described in the text. The unresolved doublets (eg, 120K/112K) are indicated by the lower molecular weight component of each doublet. TD, tracking dye.

weight of 37,000. The molecular weights used in this study (Table I) are those reported by Gerton and Hedrick (manuscripts in preparation) and differ slightly from those previously reported [8]. Since most of the envelope components are glycoproteins, the differences in the molecular weights determined in these two studies may reflect the different polyacrylamide gel concentrations used [14]. Pairs of protein bands that can be distinguished by eye appear as asymmetric peaks by scanning (eg, the 120K/112K peak). The major differences between the SDS-PAGE profiles of VE and FE were (Fig. 1): (1) the absence in VE and presence in FE of a large macromolecule (peak J) that barely penetrated the running gel; (2) the shift of the 69K/64k peak of VE to a lower molecular weight after the conversion of VE to FE; and (3) the appearance of a shoulder on the higher molecular weight side of the 41K peak ([8]; Gerton and Hedrick [in preparation]). When included in the protein sample analyzed by SDS-PAGE, lactoperoxidase (MW = 77,500) was detected as a peak between the 120K/112K and 69K/64K peaks. When FE was treated with galactose to remove the F-layer, and the resulting VE* analyzed by SDS-PAGE, the contribution

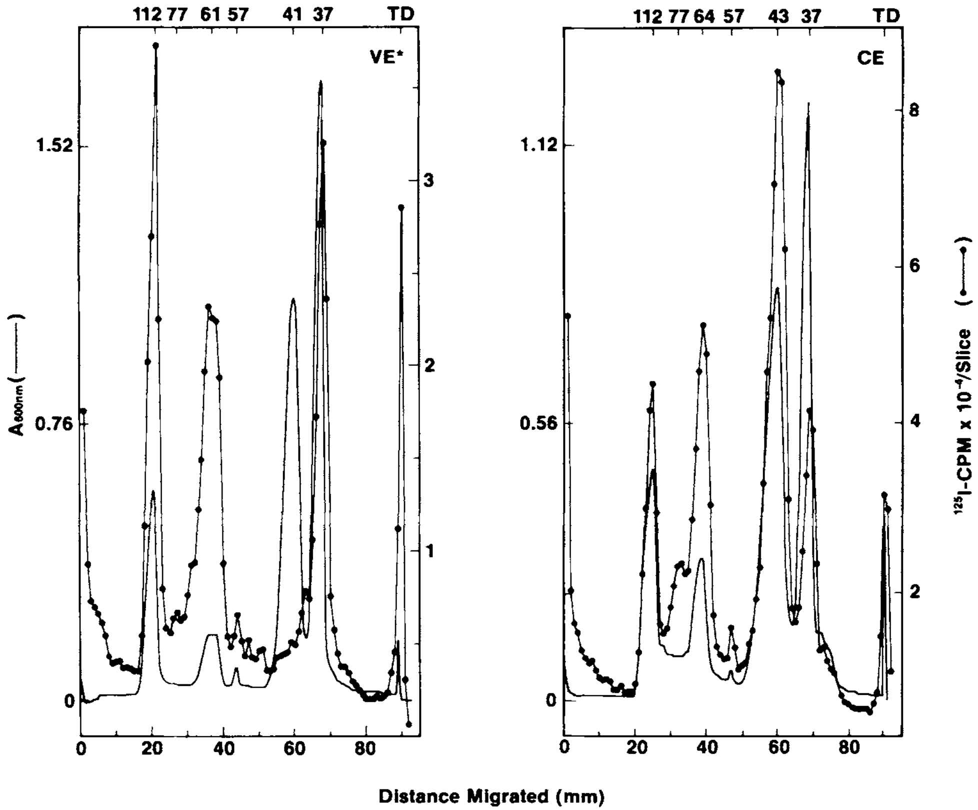

Fig. 2. SDS-PAGE of intact, isolated VE* and CE radioiodinated by the lactoperoxidase procedure. See Figure 1 for more details.

TABLE I. Major Xenopus Envelope Components

	Component molecular weight	
Peak	This paper[a]	Wolf et al [8][b]
I	Top of running gel	Top of running gel
120K/112K	120,000	125,000
	112,000	118,000
69K/64K	69,000 (VE)	77,000 (VE)
	64,500 (VE)	71,500 (VE)
66K/61K	66,000 (FE)	71,500 (FE)
	61,500 (FE)	67,500 (FE)
57K	57,500	60,000
43K	43,500 (CE)	
41K	41,500 (VE,FE)	40,000
37K	37,000	33,000

[a]Determined on 7.5% polyacrlamide gels Gerton and Hedrick (in preparation).
[b]Determined on 10.0% polyacrylamide gels[8].

of peak J to the protein profile was generally greatly reduced or completely eliminated (Fig. 2). The shoulder seen on the 41K peak of gels of FE was not seen in gels of VE*. With 8.5 % acrylamide in the running gel, gels of CE were virtually identical to those of VE, although subsequent experiments have demonstrated the the CE peak corresponding to the 41K peak of VE has a slightly higher molecular weight of 43,500 (Gerton and Hedrick, manuscript in preparation).

Iodination Profiles of Intact, Isolated Envelopes

Each envelope type displayed unique iodination profiles when they were iodinated prior to dissociation with SDS. In the intact state, the 41K component of VE was not radiolabeled by either the lactoperoxidase or IODO-GEN procedures (Fig. 1). Label was found in the corresponding molecular weight region of FE but prior removal of the F-layer by the galactose treatment produced intact VE* in which the 41K component could not be radioiodinated (Fig. 2). In contrast, when intact CE was radioiodinated by the lactoperoxidase procedure, all of the major protein staining bands possessed ^{125}I (Fig. 2).

Iodination Profiles of Dissociated Envelopes

A profound difference existed between the iodination profiles of envelopes iodinated before dissociation and envelopes dissociated before iodination. When envelopes were dissociated by guanidine-HCl, dialyzed to remove the dissociating agent, and subsequently iodinated by the lactoperoxidase procedure, all of the major proteins detected by Coomassie blue staining were radioiodinated (Fig. 3). Similar results were obtained for SDS-dissociated envelopes that were iodinated by the IODO-GEN procedure (data not shown).

Iodination Profiles of Heat-Dissolved Envelopes

When the heat-dissolved VE or FE was radioiodinated by the lactoperoxidase procedure, the resulting iodination profiles were very similar to the profiles obtained when intact, isolated envelopes were radioiodinated (Fig. 1). The presence or absence of calcium in the reaction mixture had no effect on the iodination profiles of heat-dissolved envelopes (data not shown).

Comparison of Inside and Outside Labeling

As demonstrated by electron microscopy, lactoperoxidase does not permeate VE or FE of Xenopus eggs [Nishihara and Hedrick, in preparation]. Therefore, the outer surface of the envelope was specifically radioiodinated when the envelope was in situ around the dejellied egg. The inner surface of the envelope was preferentially radioiodinated by first iodinating the outer surface of the envelope of intact dejellied eggs with nonradioactive ^{127}I, isolating the envelopes, and then iodinating the intact, isolated envelopes with radioactive ^{125}I.

When envelopes (VE and FE) iodinated in this manner were compared, there were quantitative differences between the inner and outer aspects of each envelope type. Qualitatively, the same components were labeled by both the inner and outer labeling procedures. However, if the iodine incorporation per gel slice was expressed as the percentage of the total radioactivity in the gel and the resulting percentages for the inner labeling experiments substracted from the outer surface labeling values (Fig. 4), the 120K/112K and 69K/64K components were more exposed on the outer surface

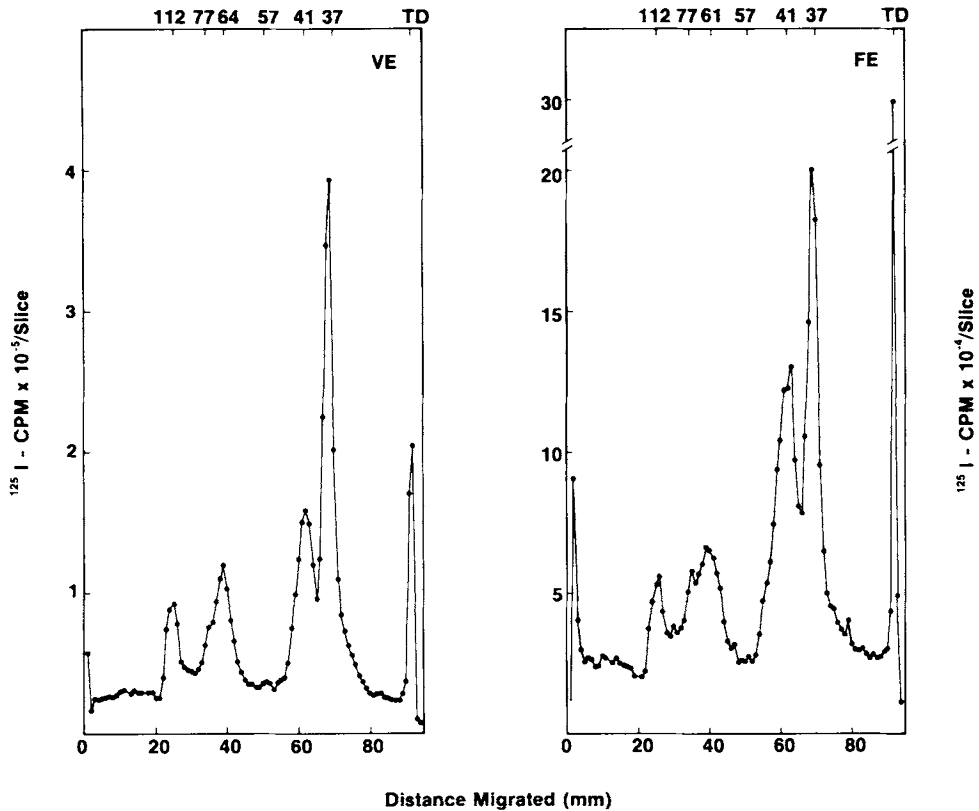

Fig. 3. Plots of the radioactivity per slice of SDS gels of VE and FE dissociated with guanidine-HCl prior to radioiodination by lactoperoxidase.

of VE in comparison with the other envelope components. With FE, the high molecular weight component(s) represented by peak J was very exposed on the outer surface, the 120K/112K and 64K/61K components had an equal exposure on the inner and outer surfaces of FE, and the 37K component was more exposed on the inner surface of the envelope.

DISCUSSION

These radioiodination studies of Xenopus egg envelopes provide additional structural information concerning the different envelope types that are distinguishable in terms of morphology and sperm penetrability. Differences were detected in the iodination profiles of intact, isolated envelopes from coelomic eggs, unfertilized oviposited eggs, and fertilized eggs.

The existence of a characteristic radioiodination profile for CE in comparison to VE is the first known molecular difference between these Xenopus egg envelopes. That the 43K component was readily labeled in intact, isolated CE but that the corresponding 41K component was not labeled in VE without the prior addition of

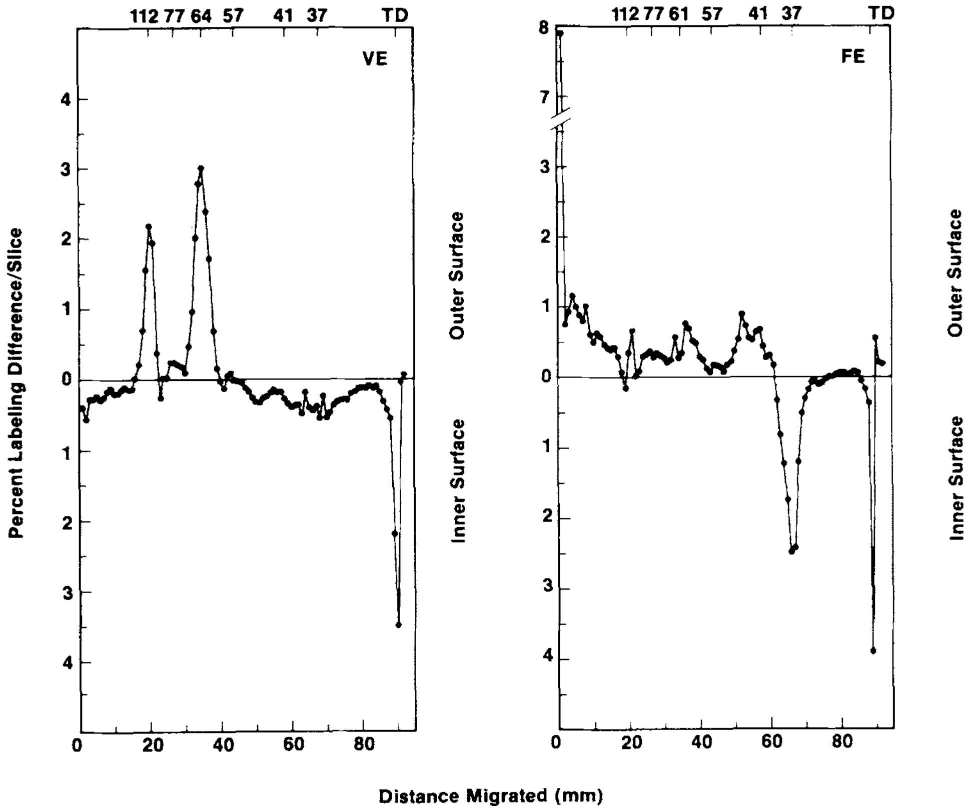

Fig. 4. Plots of the differences between labeling patterns of the inner and outer surfaces of VE and FE. Envelopes were labeled on their outer or inner surfaces as described in the text. The radioactivity of each gel slice was expressed as a percentage of the sum of radioactivity of all the gel slices. The radioactivity percentage for each gel slice from the inner surface labeling experiment was subtracted from the radioactivity percent in the corresponding slice from the outer surface experiment to yield the percent labeling difference per slice.

denaturants suggested that there may be distinct differences in the conformation and the macromolecular compositions of CE and VE. Subsequent results confirming this possibility are discussed in another report concerning the CE to VE conversion [Gerton and Hedrick, in preparation].

Since all of the components of SDS or guanidine-HCl-dissociated envelopes could be radioiodinated, it was surprising that the 41K component of intact, isolated VE was not labeled by either the lactoperoxidase or IODO-GEN procedures. This component is one of the two major components of VE, and we anticipated that it would be iodinated to some extent by these procedures. Richter [15] also found that the 41K component of intact VE was not labeled by lactoperoxidase. Because both lactoperoxidase and IODO-GEN have been utilized as cell-surface-specific labeling reagents, the 41K component may be a nonsurface molecule or be masked by other envelope components.

Results from the digestion of the egg envelope by the Xenopus embryo hatching protease supports this interpretation [16]. This purified enzyme prefers the higher molecular weight components of VE and FE as substrates and only slowly hydrolyzes the 37K component and more slowly hydrolyzes the 41K component. Since the higher molecular weight components were the first to disappear after the hatching-enzyme treatment, it was suggested that these components are the "nuts and bolts" that hold the major structural components together to form the intact envelope.

The integrity of the envelope can be distrupted by heating for a short time at an alkaline pH. This treatment apparently fragments the envelope into soluble supramolecular complexes, but it is not sufficient to completely dissociate the envelope components. Some aspects of envelope structure are maintained after heat dissolution, since the 41K component of VE cannot be radioiodinated by the lactoperoxidase procedure. Apparently heat dissolution breaks the envelope into supramolecular aggregates in which the individual macromolecules retain similar structural relations as in the intact envelope. The aggregates are quite large, since they were excluded from Sepharose 4B which has an exclusion limit of 5×10^6 for globular proteins [7].

Some of the data for the inner and outer surface labeling of VE and FE can possibly be explained by the cortical granule lectin-jelly coat precipitation reaction [4–6]. As noted earlier, the 120K/112K and 69K/64K components of VE are relatively more exposed on the outer surface than the inner surface of the envelope. All other components (except those at the dye front) are evenly distributed throughout the envelope. After the conversion of VE to FE, this preferential exposure of the 120K/112K and 69K/64K components is eliminated, a profound outer surface exposure of peak J is noted, and the 37K component becomes more exposed on the inner envelope surface. This suggests that the VE to FE conversion, perhaps because of masking by the newly-formed F-layer, may decrease the accessibility of the 120K/112K, 66K/61K, and 37K components to iodination from the outer surface. Although the correlation is not understood, it has not escaped our attention that the components represented by these three peaks are the same as those that were most readily attacked in the previously mentioned hatching-enzyme study [16].

The radioactivity associated with the 41K position of FE was not due to any VE* component. This radioactivity and that of peak J are attributable to components of the F-layer, the precipitate of jelly coat macromolecules and cortical granule lectin released from the egg [4,8]. A cortical granule lectin has been purified using a jelly-Sepharose 4B affinity column ([4]; Nishihara et al [in preparation]). When purified cortical granule lectin was analyzed by SDS-PAGE, its subunits comigrated with the shoulder seen on the 41K component of FE gels. The solubilized F-layer components contain cortical granule lectin and a high molecular weight component that is probably the jelly coat ligand for the lectin. Production of VE* by treating intact, isolated FE with galactose removes the cortical granule lectin. Thus, the 41K component of VE* is similar to that of VE and cannot be labeled by the lactoperoxidase procedure.

The procedures used in this study have given us additional information concerning the structure of Xenopus egg envelopes. Conformational and positional changes are associated with changes in the envelope composition. Future experiments should be directed toward understanding how the structural differences between CE, VE, and FE relate to the morphological and functional differences among these three envelope types.

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