

Sperm Surface Proteins Persist after Fertilization

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ABSTRACT Certain sperm components labeled with fluorescein isothiocyanate or its radioactive derivative, ^{125}I -diiodofluorescein isothiocyanate (^{125}IFC), are transferred at fertilization to the egg, where they persist throughout early cleavage stages at a localized site in the embryo cytoplasm (Gabel, C. A., E. M. Eddy, and B. M. Shapiro, 1979, *Cell*, 18:207-215; Gundersen, G. G., C. A. Gabel, and B. M. Shapiro, 1982, *Dev. Biol.*, 93:59-72). By using image intensification we have extended these observations in the sea urchin to the pluteus larval stage, in which >60% of the embryos have localized fluorescent sperm components. Because of the unusual persistence of the sperm components in the embryo, a characterization of the nature of the labeled species in sea urchin sperm was undertaken. ~10% of the ^{125}IFC was in sperm polypeptides of $M_r > 15,000$. These proteins were on the sperm surface as shown by their sensitivity to externally added proteases. The remainder of the ^{125}IFC in sperm was in several low-molecular-weight species, none of which was ^{125}IFC -derivatized phospholipid.

To determine if any labeled sperm polypeptides remained intact in the embryo after fertilization, ^{125}IFC -labeled sperm proteins were recovered from one-cell and late gastrula stage embryos by using an anti-IFC immunoadsorbent. Most of the labeled sperm proteins were degraded shortly after fertilization; however, distinct sets of labeled polypeptides were recovered from both one-cell and gastrula stage embryos. Six of the labeled polypeptides recovered from both embryonic stages had identical SDS gel mobilities as labeled sperm polypeptides. Other polypeptides in the embryos appeared to arise from limited proteolysis of sperm proteins. Thus, in this physiological cell fusion system, individual sperm proteins are transferred to the egg at fertilization, and some persist intact or after specific, limited degradation long after gamete fusion, until at least the late gastrula stage.

Fertilization is the process whereby haploid genomes are united and development is initiated. Central to this process is the fusion of gamete membranes, which results in the incorporation of the sperm nucleus as well as other, nonnuclear, sperm components into the egg. Although the insertion of cytoplasmic sperm components during fertilization has been observed in almost every animal phyla, few examples exist in which the fates of these components have been addressed (see bibliographies in references 1, 33, 34). Furthermore, the great dilution of sperm components after gamete fusion (from 10^4 - to 10^{11} -fold depending on the species, [1]) has limited such studies to observations of morphological markers of the sperm, such as the axoneme or the mitochondria, which can be identified after fertilization. The consensus of these investigations is that sperm organelles are degraded during the early cleavage stages, although notable exceptions do exist (see bibliographies in references 33 and 34). In none of these

studies have individual sperm components been pursued at the molecular level.

It is perhaps the reliance on morphological markers that accounts for the paucity of studies on the fate of the sperm membrane after fertilization, for once the sperm membrane fuses with the egg, it loses its identity. In most organisms the entire sperm membrane fuses with the egg and is thought to be incorporated into the zygote membrane (6), analogous to fusion of tissue culture cells (10); in others, notably mammals (2, 3) teleosts (19), and diptera (29), the apical sperm membrane is internalized as a vesicular remnant. Two studies, one in the hamster (40) and one in the surf clam *Spisula solidissima* (24), have indirectly investigated the integration of the sperm membrane by taking advantage of differences in ligand-binding properties of the sperm and egg membranes. In both cases the inability to detect sperm membrane markers shortly after fertilization was interpreted as evidence for rapid inter-

mixing of the two gamete membranes after fusion. Alternative interpretations, such as internalization, were not addressed because of the lack of a positive marker for the sperm membrane.

Because fertilization is the best-understood physiological cell fusion system, an analysis of the fate of specific sperm components in the embryo is of interest in defining the extent to which the integrity of cellular constituents is preserved after membrane fusion. To follow the sperm membrane after fertilization, we have covalently derivatized sperm with fluorescein isothiocyanate (FITC)¹ or its iodinated derivative ¹²⁵I-diiodofluorescein isothiocyanate (¹²⁵IFC) (12). When FITC-labeled sperm were used to fertilize sea urchin or mouse eggs, a localized region, called a "patch," of sperm-derived fluorescence was observed in the embryo and appeared to persist during early cleavage stages (11). This sperm patch was subsequently shown to reside in the interior of the egg (15). Because of the unusual persistence of these labeled sperm components in embryos, we have extended our initial characterization of the labeled species in the sea urchin sperm. In addition, we have developed a purification scheme for the labeled proteins based on the ability to recover ¹²⁵IFC-labeled material using immunoabsorbents prepared with anti-IFC antibodies. This has allowed a biochemical characterization of labeled sperm proteins after their transfer to the egg.

MATERIALS AND METHODS

Materials: ¹²⁵IFC was prepared as previously described (14), except that a 50-fold rather than a 100-fold excess of K¹²⁵I was used during the iodination of fluorescein amine. This resulted in ¹²⁵IFC of roughly twice the specific activity (~50 Ci/mmol). The Na¹²⁵I (>500 mCi/ml) used to prepare ¹²⁵IFC was purchased from New England Nuclear (Boston, MA). Pronase (45,000 proteolytic units/g) and Hoechst dye 33258 (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl) benzimidazole) were obtained from Calbiochem-Behring (San Diego, CA). Trypsin-*n*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (224 U/mg) was from Worthington Biochemical Co. (Freehold, NJ). Nonidet P-40 (NP-40) was from BDH Chemicals and was purchased through Gallard/Schlesinger (New York). FITC was purchased from Baltimore Biological Laboratories, MD.

Gametes of the sea urchin, *Strongylocentrotus purpuratus*, were obtained as previously described (32). All manipulations of the gametes were performed at 10–11°C.

Sperm Labeling: Sea urchin sperm used for labeling with ¹²⁵IFC or FITC were washed two times with Millipore-filtered seawater (MSW, Millipore Corp., Bedford, MA) by centrifugation (1500 *g* for 10 min), and the concentration was approximated by determining the absorbance at 600 nm ($A_{600} = 8 \times 10^7$ sperm/ml). Labeling with ¹²⁵IFC was essentially as described (12), except the concentration of ¹²⁵IFC was from 10 to 30 μ g/ml and an additional wash was performed before applying the sperm to the Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) column. The specific radioactivity of sperm after the labeling procedure was obtained by determining the sperm concentration with a hemacytometer and the radioactivity in a gamma counter (Beckman Instruments, Inc., Fullerton, CA, model 4000). The specific radioactivity of sperm labeled in this manner varied from 0.015 to 0.035 cpm/sperm depending primarily on the specific activity of the ¹²⁵IFC.

Labeling of sperm with FITC was as previously described (15).

Protease Digestions: ¹²⁵IFC-labeled sperm were diluted into MSW buffered with 10 mM HEPES, pH 7.8, so that the final sperm concentration was $2-5 \times 10^8$ sperm/ml. Trypsin-TPCK or pronase was added to 1.0 mg/ml from freshly prepared stock solutions of 20 mg/ml in HEPES-buffered seawater. Controls received an equal volume of HEPES-buffered seawater. The reactions were stopped at various times by placing the incubations on ice and adjusting the reaction mixture to 10% trichloroacetic acid (TCA). >90% of the total radioactivity remained TCA-precipitable in all the incubations. TCA precipi-

¹ *Abbreviations used in this paper:* ATA, 3-amino-1,2,4-triazole; FITC, fluorescein isothiocyanate; ¹²⁵IFC, ¹²⁵I-diiodofluorescein isothiocyanate; MSW, Millipore-filtered seawater; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; TCA, trichloroacetic acid.

tion was chosen to stop the action of the proteases because proteolysis continued if the sperm were washed and then directly disaggregated with SDS solubilization buffer. The TCA precipitates were collected by centrifugation (12,000 *g* for 15 min at 4°C) and after carefully removing the supernatant, the pellets were solubilized in SDS solubilization buffer (4% SDS, 4% β -mercaptoethanol, 100 mM Tris, pH 6.8, 20% glycerol, and 0.2 mM phenylmethyl sulfonyl fluoride [PMSF]), and boiled 5 min. The PMSF used throughout this study was always added from a freshly prepared stock solution in 100% ethanol. The high concentration of SDS and β -mercaptoethanol was needed to effectively solubilize the sperm. When necessary, the pH of the sample was adjusted to 6.8 by adding small amounts of 1 M NaOH. The samples were then analyzed by SDS PAGE (see below).

The integrity of the sperm during the incubations was assessed with Hoechst dye 33258 as described in Results.

Fractionation of ¹²⁵IFC-labeled Sperm: ¹²⁵IFC-labeled sperm components were solubilized by detergent extraction in the following solution: 3.0% (vol/vol) NP-40, 20 mM HEPES, pH 7.8, 1 mM β -mercaptoethanol, 10 μ g/ml Aprotinin and 0.2 mM PMSF. The ¹²⁵IFC-labeled sperm were added to this solution so that the final sperm concentration was 1×10^9 sperm/ml (MSW was added to adjust the volume) and incubated for 30 min at room temperature. The extraction mixture was then diluted with 2 vol of 20 mM HEPES, pH 7.8, 2 mM EDTA, 1 mM dithiothreitol, 10 μ g/ml Aprotinin and 0.2 mM PMSF, and the solubilized material was separated from the extracted head and tail remnant by centrifugation (12,000 *g* for 30 min at 4°C). The solubilized material is referred to as the NP-40 extract.

Ethanol fractionation of the NP-40 extract was carried out to remove the large amount of low-molecular-weight ¹²⁵IFC-labeled species and to concentrate the ¹²⁵IFC-labeled proteins (see Results). Absolute ethanol (-15°C) was added slowly with stirring to the NP-40 extract on ice and stirred for an additional 30 min at -15°C. Of the various concentrations tested, 30% ethanol (vol/vol) was the lowest that precipitated the ¹²⁵IFC-labeled proteins quantitatively. Precipitates were collected by centrifugation (12,000 *g* for 30 min at -15°C) and either directly resuspended in SDS solubilization buffer and analyzed by SDS PAGE, or during purification of ¹²⁵IFC-labeled components, redissolved in a minimum volume of 0.5% NP-40, 0.1% SDS, 20 mM HEPES, pH 7.8, 1 mM dithiothreitol, 10 μ g/ml Aprotinin, and 0.2 mM PMSF (R buffer) and dialyzed against the same solution without Aprotinin. The SDS increased the recovery of detergent-solubilized ¹²⁵IFC-proteins by the anti-IFC immunoabsorbent (16) and facilitated the resolubilization of ethanol precipitates from embryos.

Fertilization with ¹²⁵IFC-labeled Sperm and Preparation of

Embryo Extracts: Eggs were pooled from several sea urchins and washed three to four times with MSW. The eggs were then resuspended to ~10% (vol/vol) and fertilized with ¹²⁵IFC-sperm at a sperm to egg ratio of 100–120:1. Egg and embryo concentrations were determined by counting in calibrated capillary tubes. 1 min after insemination, 3-amino-1,2,4-triazole (ATA) was added to 2 mM so that the fertilization membrane could subsequently be removed (35). When >90% of the embryos exhibited elevated fertilization membranes (~2 min), they were diluted with 30 volumes of MSW containing 2 mM ATA and 0.1% BSA; BSA reduced the transfer of extra sperm equivalents (see Results) in some experiments. Embryos to be harvested at the late gastrula stage were washed three additional times with MSW, resuspended to 0.5% in MSW containing 50 μ g/ml penicillin and streptomycin, and cultured with gentle stirring. Embryos harvested at the one-cell stage were washed three additional times with MSW containing 2 mM ATA, and then the fertilization membranes were removed according to Showman and Foerder (35). Three additional washes with MSW were performed to remove the released fertilization membranes, and then the embryos were adjusted to 0.1% NaN₃ and allowed to settle on ice. The embryos were then resuspended to ~25% (vol/vol) in Ca⁺⁺-free seawater and extracted with 5 vol of 0.5% NP-40, 20 mM PIPES, pH 6.9, 0.3 M sucrose, 100 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol, 10 μ g/ml Aprotinin, 20 μ g/ml soybean trypsin inhibitor (SBTI), and 0.2 mM PMSF. After stirring for 30 min at room temperature the insoluble material was removed by centrifugation (12,000 *g* for 30 min at 4°C). Late gastrula-early prism embryos (60–70 h postinsemination) were harvested by adjusting the culture to 0.1% NaN₃ and allowing the embryos to settle on ice. After washings (as above) the embryos were resuspended to 50% (vol/vol) (to account for the large volume of the gastrula) and extracted as above.

Ethanol precipitation of the embryo extracts was performed as for sperm.

Purification of ¹²⁵IFC-labeled Components: ¹²⁵IFC-labeled components were purified on an immunoabsorbent prepared by coupling affinity-purified, rabbit anti-IFC IgG to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) to yield a resin with 2 mg IgG/ml resin. Details of the preparation and characterization of this resin are presented elsewhere (16). The immunoabsorbent was washed three times before use with R buffer. For sperm, 100–200 μ l of the dialyzed ethanol precipitate was incubated batchwise with 50–100 μ l of the immunoabsorbent for 2 h at 4°C. The resin was then washed with R buffer (two times), 1 M NaCl, pH 10.3 (three times),

8 M urea (three times) and finally with R buffer (two times). This harsh washing regimen was made possible by the tight binding of IFC components to the resin. Elution of the bound ^{125}IFC -labeled components was achieved as described below.

Because of the larger volumes of the embryo ethanol precipitates (10–20 ml after resolubilization), it was more practical to use the immunoadsorbent as a mini-column (same amount of resin as was used for sperm), passing the extracts over the column twice at a flow rate of 4–5 ml/h. A third pass of the embryo extracts over the column did not increase the recovery. The resin was then transferred from the column to a 1.5-ml conical microfuge tube and washed batchwise as for sperm.

Elution of the bound ^{125}IFC -labeled components from the immunoadsorbent was achieved by adding 1.2 vol of SDS solubilization buffer to the resin and boiling for 5 min. The small amount of radioactivity recoverable from the embryos made it necessary to recover all the material eluted with SDS solubilization buffer in as small a volume as possible. This was achieved by adding the SDS solubilization buffer to the resin and boiling it for 5 min in a plastic, 1.5-ml conical centrifuge tube. After boiling, a small hole was made in the tube which was then placed piggy-back onto a Surety column (Evergreen Scientific, Los Angeles, CA) and centrifuged for 1 min in a clinical centrifuge. Any resin that leaked through the hole was retained on the filter of the Surety column while the eluted material was collected in the sample cup. Approximately 90% of the eluted material was recovered and because no dilution of the sample occurred, it could be directly loaded onto an SDS gel.

Recovery of ^{125}IFC -labeled Components from Supernumerary Sperm: A 30–40% (vol/vol) suspension of washed eggs was fertilized with ^{125}IFC -labeled sperm at a sperm to egg ratio of 200:1. Where indicated, inhibitors of the enzymes released by the cortical granules during fertilization were added just before adding the sperm. Egg activation, as judged by fertilization membrane elevation, was >80% in all incubations. At 10 min postinsemination the eggs were diluted 10-fold with MSW containing the appropriate inhibitor and removed from the sperm by hand centrifugation. The supernumerary sperm were collected from the supernatant by centrifugation (1,500 g for 10 min), resuspended in a small volume of cold (4°C) MSW, and immediately precipitated by adding cold (4°C) TCA to 10%. The TCA-precipitated sperm were processed for SDS PAGE as described above for the protease-digested sperm samples.

Fluorescence Image Intensification Microscopy: Fertilization of eggs with FITC-labeled sperm and subsequent culturing of embryos were as described (11, 15). The image intensification system employed to observe late embryos was also as described (15), except Kodak Technical Pan Film 2415 (Eastman Kodak Co., Rochester, NY) was used for all photography.

Other Methods: SDS PAGE was performed on gradient (7.5–15% acrylamide) slab gels according to Laemmli (22). After electrophoresis, gels were routinely stained with Coomassie Brilliant Blue R-250 (8). To quantify loss of radioactivity during the staining/destaining protocol, radioactivity in 0.5-cm slices from individual lanes was determined before and after staining. Autoradiography of dried, stained gels was with X-Omat AR film (Eastman Kodak Co., Rochester, NY) and a Cronex Lighting-Plus intensifier screen (E. I. DuPont Co., Wilmington, DE) at -70°C . Autoradiograms were scanned on a Joyce-Loebl MKIII recording microdensitometer, and quantitation of peak areas was performed by determining the weight of peaks cut out from the tracings.

Thin-layer chromatography was performed on LKSD silica gel plates (Whatman Laboratory Products, Inc., Clifton, NJ) in tanks lined with filter paper. The solvent systems used were $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{acetic acid}$ (65:25:4:2) and ethylacetate/pyridine/acetic acid (50:1:1) typically used for separating phospholipids or fluorescein derivatives (21, 36), respectively. Phospholipids were detected with molybdenum blue spray reagent (Sigma Chemical Co., St. Louis, MO). IFC-derivatized phospholipids were prepared by incubating 5 mg/ml phospholipid standards (Sigma Chemical Co.) with IFC (1 mg/ml) at 37°C , overnight. The reaction was performed in 60% CHCl_3 , 5% CH_3OH , and 35% acetone. Derivatization was confirmed by the altered mobility of the derivatized phospholipid on thin-layer chromatography plates and the fluorescence of the phospholipid derivative. Autoradiography on X-Omat AR film was performed by placing the thin-layer chromatography plate on an unopened piece of film at room temperature.

Protein determinations were performed with the Lowry assay (25) modified to remove interfering lipids and reducing agents (37).

RESULTS

Persistence of FITC-labeled Sperm Components in Late Sea Urchin Embryos

Sperm labeled with FITC show a characteristic pattern of fluorescence with the midpiece being the most highly labeled

region (12). When such sperm fertilize eggs, the fluorescent sperm components are transferred to the egg as a clustered patch of material (11) residing in the embryo cytoplasm (15). Although this has been observed in several echinoderms (11, 34, and unpublished data), the mouse (11), and the hydroid, *Aequorea aequorea* (Gundersen, G. G., unpublished data), it is with the sea urchin, *Strongylocentrotus purpuratus*, that we have made our most extensive observations. Using conventional fluorescence microscopy, the sperm-derived fluorescent patch was only occasionally observed in early gastrula stage embryos (11). However, by coupling an image intensifier to a microscope, we have been able to extend our observations to much later stages in sea urchin development. Fig. 1 shows the fluorescent sperm patch in several developmental stages, from the one-cell embryo through the 4-d pluteus larva. Observations with the image intensifier established that, in over 90% of the early cleavage stage embryos (0–18 h at 10°C), the fluorescent patches could be detected; by the blastula stage (24 h at 10°C) the percentage had dropped to 75%, but decreased only slightly through gastrulation and larva formation. It is likely that the percentage of late embryos with the fluorescent sperm patch is even greater, but because these observations were made on fixed embryos, the swelling of the blastocoel and the attendant increase in the volume of the embryo may have obscured fluorescent patches on the underside of these embryos. This was further suggested by videotape recordings of swimming embryos in which all surfaces of the embryo were exposed; the fluorescent patch alternately appeared and disappeared as the embryo rotated (Fig. 2). In these late embryos a potential artifact may result from the binding of supernumerary sperm to the embryos after the protective fertilization membrane is shed at hatching, or from their engulfment by pluteus stage embryos. To control for this, labeled sperm were added to one-cell embryos that had been fertilized with unlabeled sperm; the embryos were then washed and cultured identically to embryos that had been fertilized with labeled sperm. In several hundred such control embryos observed throughout development, none was found with the fluorescent patch. These results suggest that the labeled sperm material in the embryos remains clustered as a patch throughout early development. A biochemical characterization of the labeled species in the sperm and after they are transferred to the egg is presented below.

Protease Sensitivity of ^{125}IFC -labeled Polypeptides

IFC has previously been shown to label primarily the surface of sperm (12); however, direct evidence that intracellular proteins are not labeled with IFC has not been obtained. To address this question, we subjected ^{125}IFC -labeled sperm to proteases under conditions in which the sperm remained intact and then analyzed the digested sperm with SDS PAGE. Digestions were performed at 10°C with either trypsin-TPCK (1 mg/ml) or pronase (1 mg/ml) and stopped by addition of TCA to 10%. A small aliquot was removed from each sample just before the TCA precipitation to determine if the sperm remained intact (see below). The TCA precipitates were resolubilized in SDS buffer and then analyzed by SDS PAGE and autoradiography. Trypsin treatment for 1 h resulted in the degradation of many of the ^{125}IFC -labeled polypeptides (see, for example, bands at M_r of 36,000, 40,000, 52,000, 110,000, and 150,000 in Fig. 3). The region around M_r of 30,000 (observed to have at least three distinct ^{125}IFC -polypeptides in less heavily loaded gels) was also sensitive to

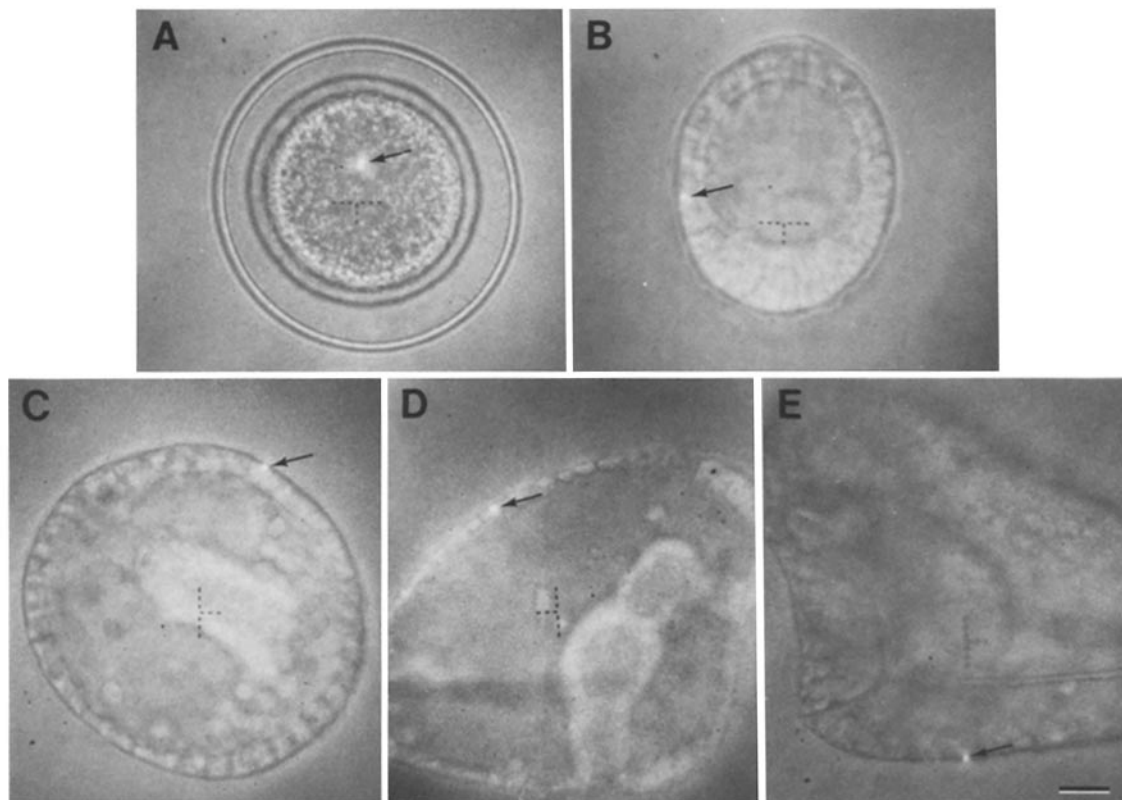


FIGURE 1 Fluorescence image intensification microscopy of sea urchin embryos fertilized with FITC-labeled sperm. The sperm-derived fluorescent patch is shown for several stages of early sea urchin development. Photographs were taken directly off the image intensifier screen with a 35-mm camera. Illumination was with both brightfield and fluorescence sources so that the exposures (15–30 s) would show the fluorescent patch (arrow) as well as some of the embryo morphology. When necessary the embryos were immobilized with 1% NaN₃. A–E are of different embryos. The “T” is a focusing aid for the image intensifier. (A) One-cell embryo (2 h postinsemination [p.i.]); (B) blastula, after hatching (28 h p.i.); (C) late gastrula (50 h p.i.), the archenteron is visible in the center of the embryo; (D) prism (78 h p.i.), the well-developed gut is visible just to the right of center; (E) early pluteus (94 h p.i.), one of the spicules is seen just above the fluorescent patch. Bar, 20 μ m. \times 310.

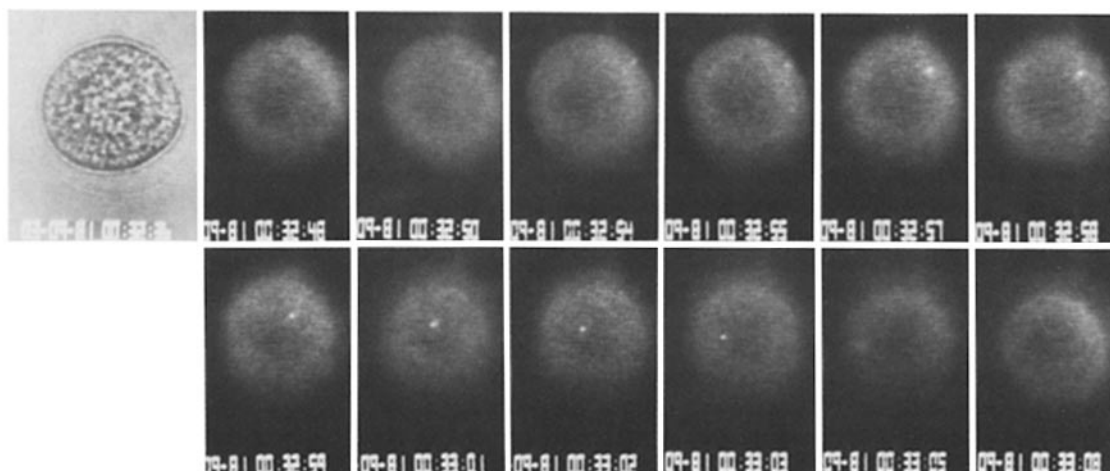


FIGURE 2 Fluorescence image intensification microscopy of a sea urchin blastocyst just before hatching. The sequential photographs are of a living blastocyst constrained within the fertilization membrane, yet rotating before hatching. The photographs were taken from a videotape recording and show the elapsed time (in minutes and seconds) on the lower right of each photograph. The first photograph (time = 32:36), was made with brightfield illumination; all others were illuminated for fluorescence. The sperm-derived fluorescent patch appears on one edge of the embryo (visible as a blur at 32:50) and as the blastocyst rotates, the fluorescent patch rotates with it, so that it appears to traverse the embryo surface (32:51 to 33:05), until it disappears on the backside of the embryo (33:08). Through-focusing of the blastocyst was unsuccessful in locating the fluorescent patch once it had rotated to the backside. \times 180.

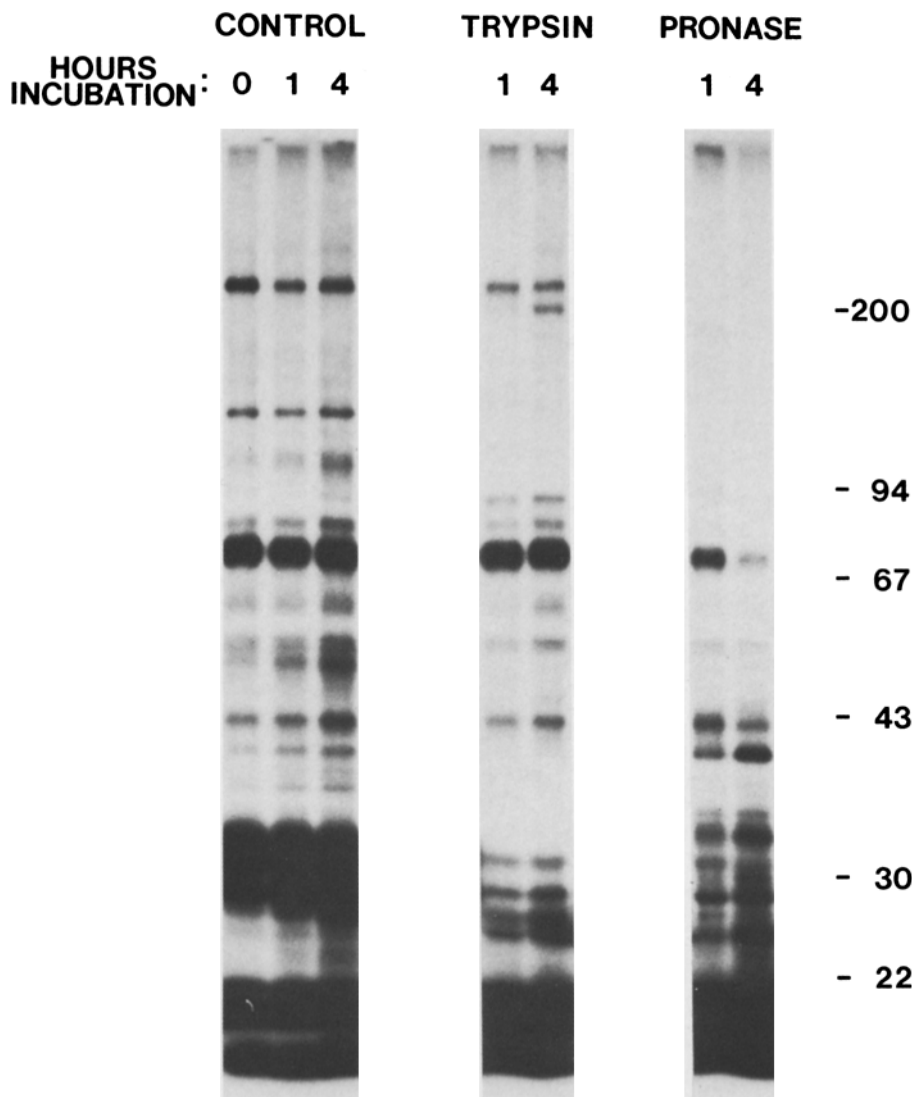


FIGURE 3 SDS PAGE analysis of the protease sensitivity of ^{125}I -IFC-labeled polypeptides in whole sperm. The autoradiogram from a typical experiment is shown. On the left are autoradiograms of samples incubated in MSW without added proteases and on the right are the autoradiograms of samples incubated with the proteases indicated (see Materials and Methods for details). An equal amount of radioactivity was loaded for each sample. Positions of standard molecular-weight markers ($\times 10^{-3}$) are shown on the right.

trypsin treatment, although some residual radioactivity remained. Other labeled polypeptides were resistant to trypsin even after prolonged incubation (for example, bands at M_r of 42,000 and 74,000, Fig. 3). Pronase, a broad spectrum protease, was more effective than trypsin in cleaving the ^{125}I -IFC-labeled polypeptides. A 1-h incubation with pronase resulted in the digestion of all but two of the labeled sperm polypeptides (M_r of 42,000 and 74,000, Fig. 3), and after 4 h even these were partially digested. The labeled species appearing at M_r of 40,000, as well as those species below 35,000 in the pronase-digested sample, presumably represent degradation products of larger polypeptides since they appeared to increase in intensity in the 4-h digestion. Thus, all of the polypeptides of $M_r > 20,000$ were at least partially susceptible to protease action. These results suggest that most of the ^{125}I -IFC-polypeptides (with $M_r > 20,000$) are exposed on the sperm and thus can serve as specific biochemical markers for the sperm surface. This argument presumes that the sperm were not permeant to the protease.

To test this assumption and assess the integrity of the sperm during protease digestions, we developed a simple microscopic assay based on the binding of Hoechst dye 33258 to DNA in the sperm nucleus. Hoechst dye 33258 is nonfluorescent in aqueous solutions but becomes highly fluorescent upon bind-

ing to DNA (23). Live, motile sperm did not take up dye into their nuclei for over 1 h when incubated with 10–100 $\mu\text{g}/\text{ml}$ Hoechst dye at pH 7.5–8.0. However, when the sperm were permeabilized with low concentrations of NP-40 or SDS, 100% of the sperm nuclei became fluorescent. When aliquots from the protease digestions were incubated with 10 $\mu\text{g}/\text{ml}$ Hoechst dye 33258, >95% of the sperm ($n = 100$) remained nonfluorescent, indicating that the sperm remained impermeant during the digestions.

Fractionation of ^{125}I -IFC-labeled Sperm Components

As described above, ^{125}I -IFC appears to label several sperm membrane proteins; however, a large amount of the labeled material, even in undigested sperm, migrates near the dye front of the SDS gel (Fig. 3). To further investigate the nature of this material, we performed a fractionation of the labeled material. Extraction of labeled sperm with NP-40 solubilizes between 90 and 95% of the radioactivity and only 25% of the sperm protein (Table I and [12]). When examined by SDS PAGE and autoradiography, the detergent extract was found to contain most of the labeled polypeptides as well as the labeled material migrating near the dye front (Fig. 4A, com-

TABLE I
Summary of ^{125}IFC -labeled Protein Enrichment in Sperm Fractions

| Fraction | Volume | Total protein | Total radioactivity | Specific radioactivity | Recovery of cpm |
|---------------------|-----------|---------------|-----------------------------|--------------------------------|-----------------|
| | <i>ml</i> | <i>mg</i> | <i>cpm</i> $\times 10^{-6}$ | <i>cpm/mg</i> $\times 10^{-7}$ | <i>%</i> |
| Whole sperm | 0.48 | 0.77 | 13.0 | 1.7 | 100 |
| NP-40 extract | 1.43 | 0.20 | 12.2 | 6.1 | 94 |
| Ethanol precipitate | 0.52 | 0.16 | 3.3 | 2.1 | 25 |

pare lanes 1 and 2). The Coomassie Blue-stained gel showed that only a subset of the major sperm polypeptides was solubilized (Fig. 4B, lanes 1 and 2). Coomassie Blue did not stain the low-molecular-weight material that was extracted along with the ^{125}IFC -labeled polypeptides (Fig. 4, A and B, lane 2). An intact, although slightly swollen, head and tail remnant was recovered in the NP-40-insoluble fraction removed by centrifugation (12,000 *g* for 30 min). Although this fraction contained the majority of the sperm protein, it had only residual amounts ($\sim 5\%$) of ^{125}IFC -labeled material (also, see reference 12).

To further concentrate the ^{125}IFC -labeled proteins in the NP-40 extract, an ethanol precipitation was performed at varying ethanol concentrations. Analysis of the precipitate formed at the lowest ethanol concentration tried (30%) indicated that only 25% of the radioactivity was precipitated. However, when this precipitate was analyzed on SDS PAGE, all of the labeled polypeptides in the NP-40 extract were recovered (Fig. 4A, lane 4) as well as all of the major polypeptides detected by Coomassie Blue staining (Fig. 4B, lane 4). SDS PAGE of the ethanol-soluble fraction showed that almost all of the labeled material in this fraction migrated near the dye front (Fig. 4A, lane 3); the corresponding Coomassie Blue-stained gel confirmed that little protein was recovered in the ethanol-soluble fraction (Fig. 4B, lane 3).

A summary of the enrichment of ^{125}IFC -labeled proteins resulting from these steps is given in Table I for a typical fractionation. The initial extraction gave almost a fourfold increase in specific radioactivity (counts per minute per milligram of protein) due to the selective extraction of proteins from the sperm. This extraction also solubilized the large amount of ^{125}IFC bound to low-molecular-weight material. Removal of the ^{125}IFC -labeled low-molecular-weight material by ethanol precipitation resulted in a decrease in the apparent specific radioactivity, although all of the ^{125}IFC -polypeptides ($M_r > 15,000$) were recovered in the ethanol precipitate.

Although the same amount of radioactivity was applied to the SDS gel for each of the fractions in Fig. 4, the autoradiogram showed more radioactivity in some samples than others (compare, for instance, lanes 3 and 4 in Fig. 4A). We suspected that ^{125}IFC -labeled material was lost from the gel during the staining/destaining protocol, and a determination of the radioactivity in the gel before and after staining confirmed this. The ^{125}IFC -labeled material that was removed from the gel during staining/destaining was of low molecular weight (migrating at the dye front), and accounted for 90% of the total radioactivity present in whole sperm and in the NP-40 extract, and 75% of the protein-enriched ethanol-precipitated fraction. Thus, SDS PAGE was useful for analyz-

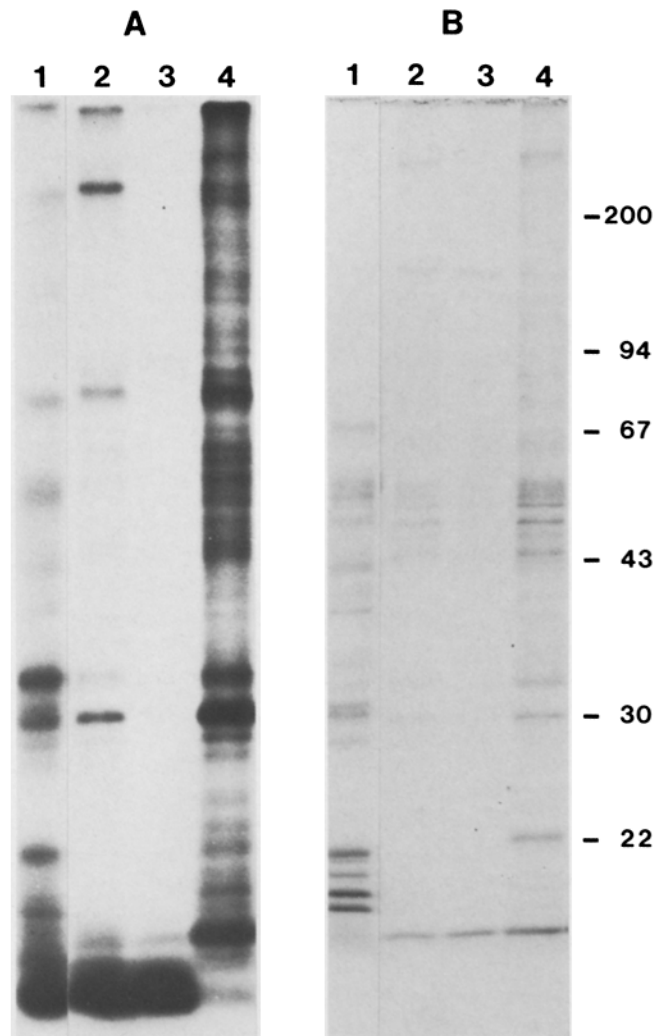


FIGURE 4 SDS PAGE analysis of the steps in the fractionation of sperm ^{125}IFC -labeled polypeptides. A fraction enriched for ^{125}IFC -labeled polypeptides was prepared by extracting labeled sperm with NP-40 and then precipitating the labeled proteins with 30% (vol/vol) ethanol (see text). The autoradiogram (A) and the corresponding Coomassie Blue-stained gel (B) are shown. (Lane 1) whole sperm; (lane 2) NP-40 extract; (lane 3) ethanol-soluble fraction; (lane 4) ethanol-precipitated fraction. An equal amount of radioactivity was applied to the gel for each sample. Positions of standard molecular-weight markers ($\times 10^{-3}$) are shown on the right.

ing the labeled polypeptides in sperm fractions; these polypeptides represented $\sim 10\%$ of the total labeled species.

We have not yet identified the low-molecular-weight material, but have characterized some of its properties. The majority of it did not partition with lipid in a typical $\text{CHCl}_3/\text{CH}_3\text{OH}$ extraction protocol (4); only 10% of the radioactivity was found in the organic phase. Thin-layer chromatographic separation of the low-molecular-weight-labeled fraction (see Materials and Methods) showed that the major amine-containing phospholipids of sea urchin sperm (21), phosphatidyl ethanolamine and phosphatidyl serine, which are both potential targets for ^{125}IFC -labeling, were not labeled when compared with authentic standards of IFC-labeled phosphatidyl ethanolamine ($R_f = 0.48$) and IFC-labeled phosphatidyl serine ($R_f = 0.26$) (data not shown). By similar chromatography we found that $< 10\%$ of the total radioactivity present in the NP-40 extract was free ^{125}IFC , and none of it was ^{125}I diiodoflu-

orescein amine ($R_f = 0.4$) (data not shown), the expected hydrolysis product of ^{125}IFC (20, 26). Thus little ^{125}IFC was noncovalently bound to sperm, and the derivatized species were stable throughout the fractionation procedure.

Recovery of ^{125}IFC -labeled Sperm Polypeptides from Embryos

As a result of fertilization in the sea urchin, sperm components are diluted $\sim 200,000$ -fold (1), as calculated from the relative volumes of gametes of *Echinus esculentus*. Using the relative specific activities (counts per minute of ^{125}IFC /milligram of protein) of ^{125}IFC -labeled sperm, and of eggs fertilized with labeled sperm, we found a protein dilution factor of $\sim 100,000$ for *S. purpuratus*. This dilution prohibited analysis of ^{125}IFC -labeled sperm proteins after fertilization simply by solubilization of embryos followed by analysis with SDS PAGE. To overcome this problem we developed a method to specifically recover the labeled proteins utilizing an immunoadsorbent prepared with anti-hapten (IFC)-specific antibodies. A detailed description of the preparation and characterization of this immunoadsorbent is presented elsewhere (16).

When ^{125}IFC -labeled sea urchin sperm are used to fertilize eggs, the radioactivity is quantitatively transferred to the embryo and persists throughout early development (11). However, more than one sperm equivalent of radioactivity can be transferred if high sperm-to-egg ratios are used during insemination, even though the embryos develop monospermi-ally (13). In order to purify the labeled sperm components from embryos it was advantageous to label the sperm heavily (see Materials and Methods). When these sperm were used to fertilize eggs at a sperm-to-egg ratio of 100–120:1, greater than one sperm equivalent of radioactivity was occasionally transferred to the embryo. In five experiments in which we attempted to purify ^{125}IFC -labeled sperm proteins, the transfer ratio (sperm equivalents of radioactivity per embryo) was 1.8 ± 1.0 for one-cell embryos and 1.6 ± 1.0 for late gastrula embryos. However, the individual determinations show that these averages were distorted due to two of the five experiments in which the transfer ratios were much greater than one (Table II). In each of these experiments the embryos developed normally, suggesting that only one sperm actually fused with the egg. The high transfer ratio observed in these two experiments was not due to bound supernumerary sperm, because the fertilization membranes were removed, either experimentally or naturally (by hatching), before the determination of the transfer ratio. Moreover, eggs fertilized with FITC-labeled sperm under similar conditions are rarely ob-

served to have bound supernumerary sperm or more than one sperm-derived fluorescent patch as described previously (11, 15) and confirmed here. Although the use of ^{125}IFC -labeled sperm may result in several potential artifacts that could contribute to the high transfer ratios occasionally observed (see Discussion), the high transfer ratio occasionally seen did not affect the pattern of ^{125}IFC -polypeptides recovered from embryos (see below).

Embryos fertilized with ^{125}IFC -labeled sperm were harvested at either the one-cell stage (2 h postinsemination) or at the late gastrula–early prism stage (60–70 h postinsemination). A protein-enriched fraction was prepared by ethanol precipitation of proteins extracted with NP-40. These steps were performed exactly as for sperm, except for a slight modification of the initial extraction protocol to account for the large amount of embryonic material employed (see Materials and Methods). The recovery of radioactivity from sperm and embryos for each step in the protocol is shown in Table III and indicates that no significant difference existed in the ethanol precipitability of the ^{125}IFC -labeled sperm components after they were transferred to the egg.

To purify the ^{125}IFC -labeled components the ethanol precipitate was resolubilized in R buffer and passed over the anti-IFC immunoadsorbent. After washing the resin to remove nonspecifically bound material (see Materials and Methods), $>50\%$ of the radioactivity in the ethanol-precipitated fraction of sperm remained bound to the resin (Table III); the non-binding fraction was shown to contain the same distribution of ^{125}IFC -labeled polypeptides that was present in the bound material (16). The recovery of radioactivity from resolubilized ethanol-precipitated fractions of embryos was less successful; only $\sim 25\%$ of the radioactivity bound to the immunoadsorbent (Table III). This disparity may be due to the larger volume or higher protein concentration of the embryo preparations which were passed over the resin; the recovery of radioactivity was reduced to 35% when resolubilized ethanol-precipitated fraction of ^{125}IFC -labeled sperm was mixed with an ethanol-precipitated fraction prepared from one-cell or gastrula stage embryos (fertilized with unlabeled sperm) and then passed over the immunoadsorbent.

Nearly quantitative elution ($>80\%$) of the ^{125}IFC -labeled polypeptides that bound to the immunoadsorbents was achieved when the immunoadsorbent was boiled directly in SDS sample buffer (16). Although this treatment resulted in the removal of some IgG from the resin, it facilitated the analysis of the small amount of radioactivity recoverable from embryos since the eluted material could be analyzed directly by SDS PAGE. All of the ^{125}IFC -labeled polypeptides present

TABLE II
Transfer Ratio of Embryos Fertilized with ^{125}IFC -labeled Sperm

| Experiment | Transfer ratio* | |
|------------|------------------|------------------------|
| | One-cell embryos | Gastrula stage embryos |
| 1 | 1.4 | 1.1 |
| 2 | 1.1 | 1.0 |
| 3 | 2.2 | 1.5 |
| 4 | 1.0 | 1.0 |
| 5 | 3.4 | 3.3 |
| Average | 1.8 ± 1.0 | 1.6 ± 1.0 |

* The transfer ratio is the number of sperm equivalents of radioactivity per embryo.

TABLE III
Recovery of ^{125}IFC Radioactivity in Fractions from Sperm and Eggs

| Fraction | Recovery* (of cpm) | | |
|--------------------------|--------------------|------------------|------------------------|
| | Sperm | One-cell embryos | Gastrula stage embryos |
| | | % | |
| NP-40 extract | 92 ± 4 | 96 ± 4 | 93 ± 7 |
| Ethanol precipitate | 22 ± 1.8 | 26 ± 1.9 | 19 ± 3.7 |
| Anti-IFC immunoadsorbent | 12 ± 1.7 | 6.2 ± 1.6 | 4.2 ± 0.8 |

* % Recovery was determined from three separate experiments in which ^{125}IFC -labeled sperm were used to fertilize eggs. Preparation of fractions is described in Materials and Methods.

in the resolubilized ethanol precipitate of labeled sperm were recovered with the anti-IFC immunoadsorbent except for several labeled polypeptides of $M_r > 200,000$ (Fig. 5, compare lanes A and B). Similarly, the labeled sperm polypeptides were recovered intact when they were mixed with and purified from an ethanol precipitate of gastrulae (Fig. 5, lane C) or one-cell embryos (not shown) that had been fertilized with unlabeled sperm. Thus, there was no significant proteolysis or alteration of ^{125}IFC -labeled sperm polypeptides during the purification protocol.

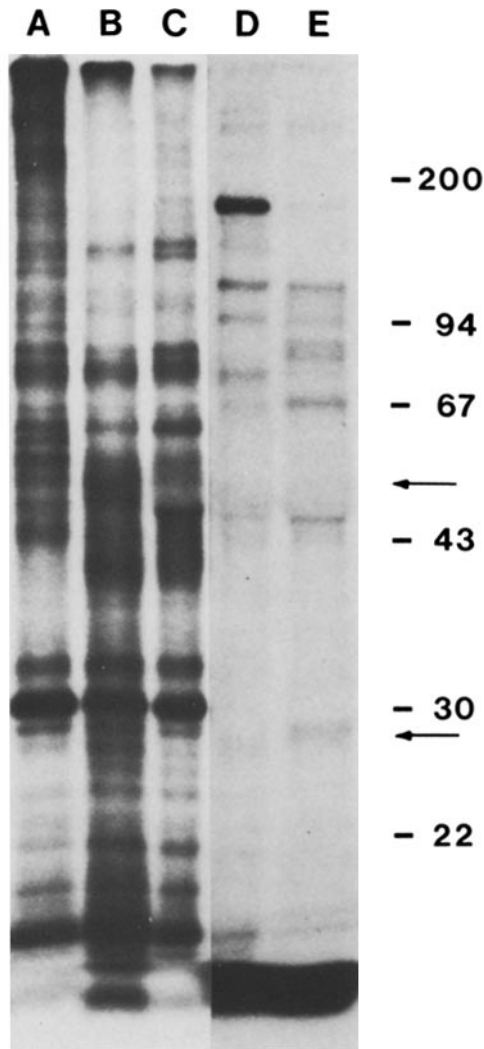


FIGURE 5 SDS PAGE analysis of ^{125}IFC -labeled polypeptides recovered from sperm and embryos. ^{125}IFC -labeled polypeptides were recovered from ethanol precipitates of sperm, one-cell embryos, late gastrula stage embryos, and a mixture of ethanol precipitates from ^{125}IFC -labeled sperm and gastrula stage embryos (fertilized with unlabeled sperm). The autoradiogram from the stained, dried gel is shown. (A) Ethanol precipitate of ^{125}IFC -labeled sperm; (B) ^{125}IFC -labeled polypeptides from sperm recovered by the immunoadsorbent; (C) ^{125}IFC -labeled polypeptides from sperm mixed with a gastrula preparation and then recovered by the immunoadsorbent; (D) ^{125}IFC -labeled polypeptides from one-cell embryos recovered by the immunoadsorbent; (E) ^{125}IFC -labeled polypeptides from gastrula stage embryos recovered by the immunoadsorbent. Positions of standard molecular-weight markers ($\times 10^{-3}$) are shown on the right. Arrows indicate the positions of rabbit IgG heavy and light chains. An equal amount of radioactivity was loaded for each sample.

When the ^{125}IFC -labeled polypeptides in the embryo preparations were eluted from the anti-IFC immunoadsorbent and analyzed by SDS PAGE, numerous labeled polypeptides were recovered (Fig. 5, lanes D and E). Examination of Fig. 6 shows that the polypeptides recovered from the embryos can be grouped into two classes: those that co-migrate with labeled sperm polypeptides, and those that are unique to the embryos. Representatives of the first class (e.g., $M_r = \sim 150,000$ [in one-cell embryos], 94,000, 74,000, 52,000, 28,000, 20,000, and 14,000) presumably represent sperm polypeptides that persist intact in the embryo after fertilization. Those of the second class (e.g., $M_r = \sim 180,000$, 110,000, 88,000 [in gastrula embryos], and 67,000) are most likely proteolytic fragments derived from labeled sperm polypeptides. Fig. 5 also shows that in comparison with the labeled polypeptides recovered from sperm or a mixture of ^{125}IFC -labeled sperm proteins and unlabeled embryo extracts, much more of the labeled material recovered from the embryos migrated at the gel front. This is likely the result of proteolysis of the large number of labeled sperm polypeptides that were not found in embryos. The labeled polypeptides shown in Fig. 5 were recovered from one-cell embryos that had a transfer ratio of 2.2 and gastrula stage embryos that had a transfer ratio of 1.5. The same pattern of labeled polypeptides was obtained in two additional experiments in which the transfer ratio was 1.0 and 3.4 for one-cell embryos and 1.0 and 3.3 for gastrulae.

Although precise turnover rates for the sperm proteins once they are incorporated into the egg cannot be determined from just two time points, quantitative densitometry of the autoradiograms from SDS gels indicated that the relative stability of different sperm polypeptides in embryos varied considerably. The data from three experiments are summarized in Table IV. The large spread of values reflects the difficulty in

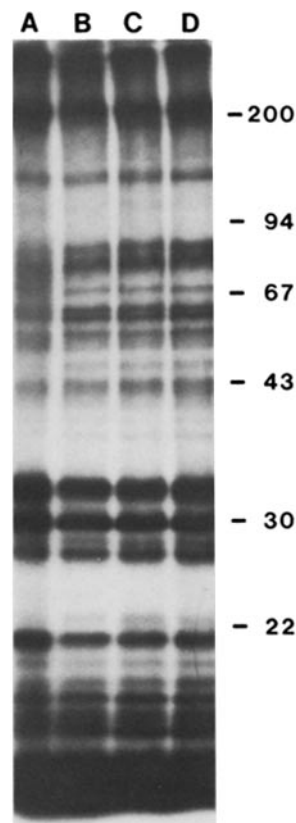


FIGURE 6 SDS PAGE analysis of ^{125}IFC -labeled polypeptides recovered from supernumerary sperm. The autoradiogram of ^{125}IFC -labeled polypeptides from whole sperm is shown in lane A and is compared with ^{125}IFC -labeled polypeptides from supernumerary sperm recovered from insemination mixtures (lanes B–D). The inseminations were done in MSW alone (lane B), or in MSW to which was added 0.5 mg/ml SBTI (lane C) or 2 mM ATA (lane D). An equal amount of radioactivity was loaded for each sample. Positions of standard molecular-weight markers ($\times 10^{-3}$) are shown on the right.

TABLE IV
Quantification of Sperm Polypeptide Levels Found in Embryos

| Polypeptide M_r | Remnant of ^{125}IFC -labeled sperm polypeptide | |
|----------------------|--|----------------|
| | One-cell stage | Gastrula stage |
| | | %* |
| ~180,000* | (100) | 8 ± 3 |
| ~150,000 | 34 ± 24 | ND |
| 110,000* | (100) | 102 ± 35 |
| 94,000 | 88 ± 36 | 76 ± 33 |
| 74,000 | 44 ± 34 | 21 ± 15 |
| 67,000* | (100) | 221 ± 110 |
| 52,000 | 21 ± 14 | 27 ± 13 |
| 28,000 | 13 ± 3 | 17 ± 5 |
| 20,000 | 21 ± 0 | 5.9 ± 2.4 |
| 14,000 | 24 ± 8 | 13 ± 4 |

ND, not detected.

* These polypeptides were unique to embryos, so the level found at the one-cell stage was set to 100%.

† Values are averages of three determinations.

quantifying peak heights from densitometric scans of lanes that had different baselines (e.g., compare lanes *B* and *C* with lanes *D* and *E* in Fig. 5). Most of the sperm polypeptides were found at reduced levels in the one-cell stage, indicating that they were degraded rapidly during the 2-h period after fertilization. The sperm polypeptides that persisted to this stage generally had slower but finite rates of degradation between the one-cell and gastrula stages. An interesting exception to this case is the sperm polypeptide, $M_r = 94,000$, which underwent little degradation either directly after fertilization or during subsequent development. Those polypeptides that were unique to the embryos did not show a typical pattern of degradation: one polypeptide, $M_r = \sim 180,000$, was almost completely lost by the gastrula stage; another, $M_r = 110,000$, was recovered in similar amounts from both embryonic stages, whereas those of $M_r = 67,000$ and $88,000$ actually increased during development; and the latter was found only at the gastrula stage.

A persistent problem in the immunoadsorption protocol we employed with embryo extracts was the elution of IgG from the anti-IFC immunoadsorbent along with the ^{125}IFC -labeled polypeptides. When this eluted material was subsequently analyzed by SDS PAGE, the large amount of IgG altered the mobility of ^{125}IFC -labeled polypeptides that migrated in the same region of the gel as the heavy and light chains of IgG (the arrows in Fig. 5 indicate the positions of the heavy and light chains of IgG). This effect can be seen by comparing the ^{125}IFC -labeled polypeptides in the ethanol-precipitated fraction of sperm (lane *A*) and those recovered from the sperm with the immunoadsorbent (lane *B* or *C*) in Fig. 5.

Recovery of ^{125}IFC -labeled Polypeptides from Supernumerary Sperm

The protease sensitivity of the ^{125}IFC -labeled polypeptides in intact sperm and the apparent early degradation of some of these polypeptides after fertilization raised the possibility that these proteins were altered by one of the enzymes released during fertilization (9, 30). To investigate this possibility, supernumerary sperm were recovered from incubations with eggs in the presence or absence of 2 mM ATA (to inhibit ovoperoxidase, [9]) or 0.5 mg/ml SBTI (to inhibit the cortical

granule protease[s], [30]). The inseminations were performed at high egg densities (30–40%) to maximize the possibility of seeing an enzymatic effect. Activation (measured by fertilization membrane elevation, which is only partial in the presence of SBTI [30]) was >80% in all cases. When supernumerary sperm were solubilized in SDS, and the labeled polypeptides analyzed by SDS PAGE, no difference was found between supernumerary sperm and sperm not exposed to eggs (Fig. 6). Similarly, the addition of SBTI or ATA to the insemination mixture had no effect on the pattern of labeled sperm polypeptides recovered (Fig. 6). This experiment showed that supernumerary sperm could be recovered from egg suspensions without an alteration in their ^{125}IFC -labeled polypeptide pattern, and suggests that the apparent proteolysis of labeled sperm proteins (see above) occurs either during or after sperm–egg fusion.

DISCUSSION

Covalent modification by small reagents has been used in the past to investigate both the structure and function of proteins. In several instances, the introduction of a small reagent has provided a means with which to recover the labeled material by using specific anti-hapten antibodies (38) or other binding proteins (28). We have extended this approach to the cellular level in order to specifically recover sperm components after fertilization. As mentioned earlier, the large dilution of sperm components at the time of sperm–egg fusion and the morphological similarity of the gamete surfaces have made it difficult to determine the fate of sperm surface components within early embryos. However, by labeling the sperm before fertilization with FITC, or its radioactive congener, ^{125}IFC , it is possible to identify the components of sperm that are binding the chromophore, to follow the labeled components after fertilization by fluorescence microscopy and autoradiography (13, 15, 33, 34), and to recover the individual sperm proteins from embryo homogenates.

Labeling of mammalian or invertebrate sperm with FITC (or ^{125}IFC) results in a heterogeneous distribution of label (as visualized by fluorescence microscopy and as reflected by immunoelectron microscopy using antibodies directed against IFC) with the highest density in the midpiece region (12). That ^{125}IFC labels sperm membrane proteins is supported by the data presented above on the protease sensitivity of ^{125}IFC -labeled sperm polypeptides. Under conditions in which the sperm remained intact, the majority of the labeled polypeptides above 20,000 M_r were cleaved by trypsin and almost all were sensitive to pronase. This protease sensitivity, i.e., pronase being more effective than trypsin, of the ^{125}IFC -labeled sperm proteins is similar to that observed for membrane proteins labeled with other vectorial reagents (5, 18, 31) and suggests that almost all the sperm polypeptides labeled with ^{125}IFC are exposed on the sperm surface.

Although ^{125}IFC labels sperm surface proteins, the majority of the ^{125}IFC -labeled species migrated as low-molecular-weight material on SDS gels and were eluted during the subsequent staining/destaining protocol. This material, along with the labeled proteins, was selectively solubilized from the sperm by NP-40. Some of the low-molecular-weight material was separated from the labeled proteins by ethanol fractionation, yielding a preparation, the ethanol-precipitated fraction, that was enriched in labeled proteins. Solubility of the low-molecular-weight material in 30% ethanol and in 45% methanol, 9% acetic acid (SDS gel staining solution) suggested

that it contained lipids or small hydrophobic polypeptide(s). Sea urchin sperm have large amounts of the amino-containing phospholipids, phosphatidyl ethanolamine and phosphatidyl serine (21), although the relatively high pK of their amino groups (7) would be expected to reduce their reactivity towards isothiocyanates. The small amount of radioactivity extracted with $\text{CHCl}_3:\text{CH}_3\text{OH}$, and the lack of detectable ^{125}IFC -labeled material co-migrating with IFC-labeled phosphatidyl ethanolamine or phosphatidyl serine standards on thin-layer chromatograms, indicate that labeled phospholipids are only minor components of the low-molecular-weight species labeled with ^{125}IFC . TLC analysis did reveal a small amount of free ^{125}IFC associated with sperm in addition to several other species that migrated in the two solvent systems used. Further analysis of the low-molecular-weight labeled material is being performed to determine its composition.

In studying the ^{125}IFC -labeled sperm components that are transferred to the embryo, we have focused on the polypeptides with $M_r > 15,000$, because they can be enriched by ethanol precipitation and analyzed by SDS PAGE after affinity purification. The affinity purification of IFC-labeled proteins was accomplished using an immunoadsorbent prepared by coupling affinity-purified, rabbit anti-IFC antibodies to Sepharose. A small amount of the immunoadsorbent (50–100 μl) was used to recover very dilute solutions of IFC-labeled proteins from relatively large volumes of extract (10–20 ml) and was thus particularly well suited for recovering ^{125}IFC -labeled sperm proteins after fertilization. The apparent high affinity of this immunoadsorbent has a drawback; the bound ^{125}IFC -labeled proteins were released only by elution conditions harsh enough to remove IgG from the immunoadsorbent. IgG was a contaminant of the purified ^{125}IFC -labeled material, but it did not greatly interfere with subsequent examination of the purified polypeptides on SDS gels (some distortion is observed for labeled polypeptides migrating in the same region as the heavy chains of IgG; see Fig. 6).

Most of the ^{125}IFC -labeled sperm polypeptides were not found even in one-cell embryos, suggesting that they were degraded shortly after fertilization. This hypothesis is supported by the increase in labeled material recovered from embryos that runs near the dye front of SDS gels (Fig. 5). Nonetheless, numerous labeled polypeptides of the sperm were recovered from both one-cell and gastrula stage embryos, including several ($M_r = 94,000, 74,000, 52,000, 28,000, 20,000,$ and $14,000$) that co-migrated with labeled polypeptides recovered from the sperm. The polypeptides of $M_r = 94,000, 74,000$ and $52,000$ that remained in gastrulae are of similar size to species in the sperm that were sensitive to proteases, although the polypeptide of $M_r = 74,000$ was only slowly cleaved by pronase (see Fig. 3). These sperm polypeptides were found as late as the gastrula stage, although quantitative densitometry suggested that all of them, except the polypeptide of $M_r = 94,000$, were slowly turned over during development (Table IV). An interesting class of proteins is found in the larger polypeptides that do not co-migrate with labeled sperm polypeptides, and thus presumably arise from them by limited proteolysis (see Fig. 5). That limited proteolysis is generating these "embryonic forms" of sperm polypeptides is supported by the finding that the polypeptide of $M_r = 67,000$ actually increased during development, and another polypeptide, $M_r = 88,000$, was only observed at the gastrula stage. Another of these polypeptides, the 110,000 polypeptide, appears to be generated at the one-cell stage (i.e., it is not found in the sperm) and then persists without further

degradation until the gastrula stage. Thus, although the levels of most of the sperm polypeptides or their cleavage products undergo changes during development, two of them, the 94,000- and the 110,000-polypeptides, remain at about the same level in both the one-cell and gastrula stages.

One of the assumptions made in this study is that the ^{125}IFC -labeled proteins recovered from embryos represent sperm proteins transferred to the egg at fertilization, and that any modifications in these labeled proteins reflect normal events in the course of development. It is thus worth considering some potential artifacts that could result from the ^{125}IFC -labeling protocol we employed. Artfactual loss of ^{125}IFC -labeled proteins recoverable with the anti-IFC immunoadsorbent could be the result of degradation of the thiourea derivative (i.e., that formed by the reaction of isothiocyanates with amino groups); however, the extremes in pH and temperature necessary to degrade these derivatives (20, 26) are not likely to be encountered in embryos. Aryl halides, like ^{125}IFC , are generally very stable (27); nonetheless, the ovoperoxidase released at fertilization (9) could provide an enzymatic means of deiodinating the ^{125}IFC . Although this would effectively reduce the amount of ^{125}IFC -labeled protein species, the recovery of all the labeled proteins from supernumerary sperm exposed to ovoperoxidase released at fertilization (Fig. 6) suggested that little deiodination is occurring. Another potential problem is occasioned by the presence of a small amount of unreacted ^{125}IFC in sperm. This free ^{125}IFC appears to be tightly bound to the sperm because the labeling procedure incorporates several washes by centrifugation as well as a step in which the labeled sperm are passed over a column of Sephadex G-25 (12). In principle, this free ^{125}IFC could label egg proteins during insemination, and these would then be purified along with the labeled sperm proteins. However, several lines of evidence suggest that the small amount of free ^{125}IFC in sperm does not contribute to the pattern of ^{125}IFC -labeled proteins recovered from the embryos. First, numerous ^{125}IFC -labeled polypeptides recovered from embryos co-migrated with species labeled in the sperm, and it seems unlikely that egg proteins of similar molecular weight would be labeled fortuitously by free ^{125}IFC contributed from sperm. Second, when extracts of ^{125}IFC -labeled sperm proteins were mixed with cold embryo extracts and subsequently purified, all the recovered ^{125}IFC -labeled proteins corresponded to those present in the original sperm extract. Third, in experiments in which we incubated several marker proteins with ^{125}IFC -labeled sperm, the exogenously added proteins did not become labeled (e.g., see Fig. 6, lane C; the SBTI [$M_r = 22,000$] did not become labeled). This suggests that either the free ^{125}IFC in sperm is not available for labeling or that if labeling does occur it is below our detection limits. Finally, even when ^{125}IFC -labeled polypeptides were recovered from embryos with high transfer ratios, the resulting pattern of ^{125}IFC -labeled polypeptides was identical to that seen when the transfer ratio is 1. The reason for the high transfer ratios is still unknown, although Gabel et al. (13), found that it was dependent on live sperm and suggested that ^{125}IFC -labeled material might be transferred to the egg by transient sperm-egg interactions. Thus, although ^{125}IFC -labeled sperm have a small amount of free ^{125}IFC , this free ^{125}IFC does not appear to label egg proteins during fertilization or react with exogenously added proteins.

The apparent proteolysis of many ^{125}IFC -labeled sperm proteins after fertilization is interesting from the standpoint of insertion of the sperm membrane during fertilization.

Several protease activities are released from eggs during fertilization in sea urchins (30); these have been shown to cleave egg surface proteins labeled with ^{125}I (32). We suspected that these proteases might be responsible for some of the apparent alterations of ^{125}IFC -labeled sperm proteins observed after fertilization. However, when ^{125}IFC -labeled polypeptides were recovered from supernumerary sperm after fertilization and analyzed by SDS PAGE, no alterations in the labeled sperm polypeptides were observed. Thus, it is likely that the modifications in the ^{125}IFC -labeled sperm proteins occur during or subsequent to gamete membrane fusion. At least some of the FITC- (or ^{125}IFC -)labeled sperm material is internalized during fertilization (15), and it may be this process that results in the changes seen in the ^{125}IFC -labeled sperm proteins after fertilization. The apparent proteolysis of many of the ^{125}IFC -labeled sperm proteins after fertilization and during development appears paradoxical in that a derivative of FITC-labeled sperm, the "fluorescent patch," remains intact throughout early development (Fig. 1; also see reference 11). However, it is unknown what quantity of the originally labeled sperm material remains in the patch, or whether the patch contains only unmodified, labeled proteins. In fact, in using antisera specific for the labeled sperm proteins, we have recently found that some sperm proteins reside at a locus distinct from the patch (Gundersen, G. G., and B. M. Shapiro, manuscript in preparation). We do not know the quantitative contribution of the labeled low-molecular-weight components (see Results) to the patch.

Several general conclusions can be reached from the observation that labeled sperm proteins undergo distinct fates after fertilization. The first is that persistence of IFC- (and, by analogy, FITC-) labeled material after fertilization is not due to its being protected from the cellular degradative pathways, because most of the labeled proteins are disposed of rapidly. The second conclusion is that at least partial cellular identity of the minority partner (by size) in this cell fusion is preserved long after the fusion event, in that several sperm proteins remain undegraded, or with specific limited proteolytic modifications, for days after fertilization. Whether this is a general property of other physiological cell fusions (e.g., in myogenesis) or is specific to embryogenesis, remains to be determined. The persistence and specific cleavage of individual sperm proteins makes them candidates for a potential role in embryogenesis, an intriguing possibility supported by recent findings in other systems. A mutation that leads to embryonic death and exhibits paternal inheritance has been characterized in *Caenorhabditis elegans* (39). In the mouse, Hoppe and Illmensee (17) recently showed that the failure of parthenogenetic embryos to develop could not be ascribed to nuclear factors and suggested that the sperm may be contributing extranuclear components necessary for successful development. The sperm proteins that we have found to persist in sea urchin embryos are potential candidates for such a developmental role.

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