REGIONAL DIFFERENTIATION OF THE SPERM SURFACE AS STUDIED WITH ^{125}I-DIIODOFLUORESCEIN ISOTHIOCYANATE, AN IMPERMEANT REAGENT THAT ALLOWS ISOLATION OF THE LABELED COMPONENTS

CHRISTOPHER A. GABEL, E. M. EDDY, and BENNETT M. SHAPIRO

From the Departments of Biochemistry and Biological Structure, University of Washington, Seattle, Washington 98195

ABSTRACT

The regional differentiation of the sperm surface has been studied with the aid of a novel covalent labeling technique that permits concurrent cytological, biochemical, and immunological analyses. For these studies isothiocyanate derivatives of fluorescein (FITC) and diiodofluorescein (IFC) were employed: the latter can be prepared with radioiodine to high specific activity (^{125}IFC) and is an impermeant reagent for the erythrocyte surface. Sperm of sea urchin (*Strongylocentrotus purpuratus*), medaka (*Oryzias latipes*), and golden hamster bind the fluorescent chromophores with a nonuniform distribution, most of the fluorescence being associated with the midpiece. The radioactive derivative ^{125}IFC permits an analysis of the proteins that are responsible for most of the binding. Additionally, ^{125}IFC-labeled sperm are capable of fertilizing eggs, as assessed by autoradiography.

That IFC labels the surface of the sperm was inferred from the following: (*a*) the labeling of the surfaces of other cells by fluorescein isothiocyanate and its derivatives; (*b*) the agglutination of labeled sperm by antibodies directed against IFC; (*c*) the use of a peroxidase-dependent immunocytochemical reaction using anti-IFC antibodies, with analysis by electron microscopy; and (*d*) extraction of labeled sea urchin sperm with Triton X-100 under conditions that preferentially solubilize the plasma membrane. The antiserum directed against IFC was used to isolate the labeled surface components from Triton X-100 extracts of whole sperm, by immunoprecipitation, with *Staphylococcus*-A protein serving as a coprecipitant.

The results support previous data showing that the sperm surface is a heterogeneous mosaic of restricted domains, one notable zone being the midpiece, where common molecular properties may be shared by sperm with distinctly different morphologies. In addition, IFC-mediated covalent alteration of specific cell surface proteins may be used to label, to identify, and, with the use of anti-IFC antibodies, to isolate such proteins from other cellular constituents.

Many cells have regionally differentiated surfaces: for example, microvilli are present on the apical surface of gut epithelial cells, enzymes are restricted to the basal infoldings of kidney proximal tubule cells, neurons have specific synaptic regions, and liver cells form bile canaliculi between their lateral membranes. The sperm is a particularly good example of a cell with a regionally differentiated surface. Distinct regions have been identified by lectin binding (1, 4, 11, 17, 18, 30, 31), charge distribution (40), intramembrane particle arrangement (8, 9, 20), and surface antigen localization (6, 19, 21, 29). The patterns of local differentiation often correlate topographically with morphologically distinct portions of the sperm, suggesting that the sperm surface is a heterogeneous mosaic of restricted domains. This heterogeneity may reflect different functions of the sperm surface at different loci; the molecular basis of the regional differences have not been extensively explored.

Noncovalent probes that are used to determine the existence of surface heterogeneity do not permit identification of the molecular components involved. Some reagents, such as lectins and antibodies, additionally may be cytotoxic (23) or can cause redistribution of labeled components, due to reagent-induced crosslinking (32, 36). To allow identification of surface components responsible for topographic heterogeneity of the sperm, we have used a novel covalent labeling agent, ^{125}I-diiodofluorescein isothiocyanate (^{125}IFC). This reagent (10), like its congener fluorescein isothiocyanate (FITC), is fluorescent, allowing one to determine the location of labeled components by fluorescence microscopy, as well as to identify these components by biochemical fractionation of the radioactive material. In addition, the radioactive reagent ^{125}IFC, like FITC (28, 33), labels

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Abbreviations used in this paper are: IFC, diiodofluorescein isothiocyanate; FITC, fluorescein isothiocyanate; Tris, Tris(hydroxymethyl)aminomethane; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PBS, phosphate buffered saline; MSW, Millipore filtered seawater; SDS, sodium dodecyl sulfate; TX-100, Triton X-100; DAB, diaminobenzidine; TCA, trichloroacetic acid; HRP, horseradish peroxidase; BSA, bovine serum albumin; Staph A, Staphylococcus-A protein adsorbent; LCI, lactoperoxidase-catalyzed iodination.

mammalian sperm with no deleterious effect on morphology or motility. This paper shows that IFC labels a restricted set of proteins on the surface of the midpiece of spermatozoa. Labeled sperm are viable, as assessed by their ability to fertilize eggs. The IFC-labeled components may be isolated by immunoprecipitation using anti-IFC antibodies. The results expand our understanding of sperm surface differentiation and demonstrate the utility of ^{125}IFC as a covalent label for studying cell surfaces.

MATERIALS AND METHODS

Materials

IFC and its radioactive derivative, ^{125}IFC, were synthesized as previously described (10). FITC was obtained from Baltimore Biological Laboratories. Tris(hydroxymethyl)aminomethane (Tris), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), lactoperoxidase, and diaminobenzidine (DAB) were purchased from Sigma Chemical Co., St. Louis, Mo. Goat anti-rabbit IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP), were obtained from Miles-Yeda, as were the porcine-γ-globulins. Staphylococcus protein A-antibody adsorbent was purchased from Calbiochem, Behring Corp., American Hoechst Corp., San Diego, Calif.

Gametes

Sperm and eggs from the sea urchin, Strongylocentrotus purpuratus, were obtained as described (37). Sperm were washed twice before use, by suspending them in a 100-fold excess of Millipore-filtered sea water (MSW) and centrifuging at 3,000 g for 10 min. Golden hamster sperm were isolated from caudae epididymides in a small volume of Ringer's solution (25) containing 6 mM glucose (glucose-Ringer's) and were stored at 37°C in 5% CO_2 and 95% air.

Covalent Modification of Sperm

(a) Labeling sperm for fluoresence microscopy. Sea urchin sperm were labeled at a final concentration of 10^8–10^9 cells/ml in 10 mM Tris (pH 7.7) in MSW containing 30 μM FITC. After 30 min at 12°C, cells were diluted into a large volume of MSW, collected by centrifugation at 3,000 g for 10 min, and washed three times with MSW. Hamster sperm ($\sim 10^7$/ml) were incubated in glucose-Ringer's containing 13 μM FITC for 5 min at 37°C, then the suspension was layered onto 3% Ficoll in phosphate-buffered saline (PBS) and collected by centrifugation at 400 g for 10 min. The pellet was washed twice with PBS by centrifuging in a similar manner. Fluorescence was visualized with a Zeiss Universal epifluorescence microscope using a FITC exciter filter, a FL 500 beam splitter, and a No. 50 barrier filter. The

fluorescence micrographs shown in Fig. 1 are 3-min exposures taken with Kodak Tri-X film.

(b) Labeling sperm with [125]IFC. [125]IFC (11 μM) was dissolved in 10 mM HEPES in MSW, pH 7.7 (HEPES-MSW), 3 × 10^{8} sea urchin sperm/ml were added, and after incubation for 30 min at 12°C, the reaction mixture was applied to a 1.5 × 10 cm Sephadex G-25 column, equilibrated in MSW. Labeled sea urchin sperm pass directly through the column, whereas free IFC is adsorbed to Sephadex. The eluted sperm were collected by centrifugation (10 min, 3,000 g) and resuspended in a small volume of MSW. Hamster sperm were labeled with [125]IFC in a protocol identical to that with FITC.

(c) Enzymatic iodination of sperm with ^{125}I and lactoperoxidase. Enzymatic iodination of sperm was carried out by a modification of a previously described procedure (35). Sea urchin sperm (2 × 10^{8} cells/ml) or hamster sperm (4 × 10^{7} cells/ml) in 10 mM HEPES-MSW or glucose-Ringer's (respectively) were incubated with 2.3 × 10^{-3} mM KI, 0.5 mCi of Na[125]I, and 50 μg/ml of lactoperoxidase. Aliquots of 18 μM H_2O_2 were added at 0, 7, and 14 min. At 20 min, cells were layered onto 3% Ficoll (in MSW or PBS for sea urchin or hamster sperm, respectively), collected by centrifugation as described above, and washed twice. When human erythrocytes were substituted for hamster sperm in the above reaction, ^{125}I was incorporated only into membrane proteins and hemoglobin was not labeled, indicating that under these conditions labeling is restricted to the surface of intact cells.

Fractionation of Labeled Sperm

Labeled sperm were suspended in 50 mM Tris, pH 6.8, containing 0.1% sodium dodecyl sulfate (SDS), 0.2 mM phenylmethyl sulfonylfluoride, 30% glycerol (disaggregation buffer) containing 10 μg/ml deoxyribonuclease (Type 1) for 5 min on ice. Samples were then made 1% in SDS and 1% in β-mercaptoethanol and heated for 3 min at 100°C. The protein concentration was determined after precipitation by addition of 9 vol of acetone; precipitated proteins were redissolved in 0.5 M NaOH and analyzed by a modified Lowry assay (13) with porcine-γ-globulin as standard. Chloroform/methanol extraction of [125]IFC-labeled sperm was carried out as described for bacteria (2). For electrophoresis, the extracted material was solubilized in disaggregation buffer containing 1% SDS and 1% β-mercaptoethanol. [125]IFC-labeled sea urchin sperm (1 × 10^{9} /ml) were extracted with 3.3% (vol/vol) Triton X-100 (TX-100) in MSW, using 35 pg of detergent/sperm. After 30 min at room temperature, the mixture was diluted eightfold with PBS and centrifuged at 27,000 g for 30 min to remove insoluble material. Before electrophoresis, proteins were precipitated with 9 vol of acetone and disaggregated as above. SDS polyacrylamide slab gel electrophoresis and autoradiography were done as previously described (22, 37).

Preparation of Rabbit Anti-IFC

Antiserum against IFC was prepared by a procedure described for the isolation of antibodies against FITC (24), using IFC coupled to porcine-γ-globulin. The derivatized protein contained 22 mol of IFC per mol of protein, determined from the absorbance at 511 nm (10) and the protein concentration (13). Anti-IFC antiserum was prepared by ammonium sulfate fractionation of the serum, obtained after cardiac puncture of immunized rabbits. Control serum, taken before immunization, was similarly fractionated. To isolate specific antibodies against IFC, anti-IFC was further fractionated by affinity chromatography on IFC-labeled bovine serum albumin coupled to CNBr activated Sepharose 4B (26). Anti-IFC was applied to the affinity column and, after extensive washing with 0.3 M NaCl in 50 mM phosphate, pH 8, the bound antibody was eluted with 0.1 M glycine, pH 3, and dialyzed against PBS. Specificities of the antisera were determined by double immunodiffusion on 1% agarose plates (12).

Immunocytochemical Electron Microscopy

The distribution of IFC binding sites on hamster sperm was demonstrated at the electron microscope level with an indirect immunocytochemical assay (39). IFC-labeled hamster sperm (2 × 10^{7}/ml), suspended in glucose-Ringer's containing a 36-fold dilution of anti-IFC, were incubated for 30 min at 37°C, washed twice by centrifugation in glucose-Ringer's containing 1 mg/ml BSA, and then suspended in 1 ml of glucose-Ringer's containing a 21-fold dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase for 30 min at 20°C. Sperm were washed as before, then with 50 mM Tris, pH 7.6, containing 0.15 M NaCl, and finally suspended in 1 ml of the same solution containing 0.5 mg/ml diaminobenzidine (DAB) and 0.01% H_2O_2 (15). After 7 min at 20°C, sperm were collected by centrifugation, washed twice with glucose-Ringer's, resuspended in 1 ml of glucose-Ringer's, and fixed in 0.2 ml of 4% OsO_4 for 30 min. Sperm were then washed twice with distilled water, dehydrated with three changes of 2,2-dimethoxypropane, and processed for electron microscopy. Hamster sperm were TX-100 extracted by suspending them in glucose-Ringer's containing 0.6 (low TX-100 extract) or 12 (high TX-100 extract) ng of detergent/sperm and incubating for 20 min at 20°C. Extracted cells were collected by centrifugation, washed twice with glucose-Ringer's, and treated as described above.

Immunoprecipitation

The affinity purified anti-IFC (4 mg/ml in PBS) was diluted 37-fold with the TX-100 extract of [125]IFC-labeled sea urchin sperm and incubated at 37°C for 60 min. To precipitate the antigen/antibody complexes, Staphylococcus-A protein (Staph A) was used as previously described (16): 0.3 ml of a 10% (wt/vol) solution in PBS,

was added to the affinity purified anti-IFC treated TX-100 extract, the mixture was incubated at 5°C for 20 min, and a pellet, containing the immunoprecipitate, was collected by centrifugation (1,000 g for 20 min), washed in PBS, and layered onto a shelf of 5% sucrose, 0.1% TX-100, in PBS. The precipitate was collected by centrifugation washed with PBS, suspended in disaggregation buffer containing 3% SDS and 3% β-mercaptoethanol, and treated for SDS gel electrophoresis as above. Recovery of radioactive antigen was determined on aliquots of the TX-100 extract, before and after the addition of

Staph A, and on the material eluted from the adsorbent with SDS in a Beckman Model 4000 Gamma-counter.

RESULTS

Fluorescence Localization

Hamster or sea urchin sperm treated with FITC (Fig. 1) or IFC show identical patterns of labeling, but IFC is less fluorescent. Labeling is nonuniform for both species, with most of the fluorescence
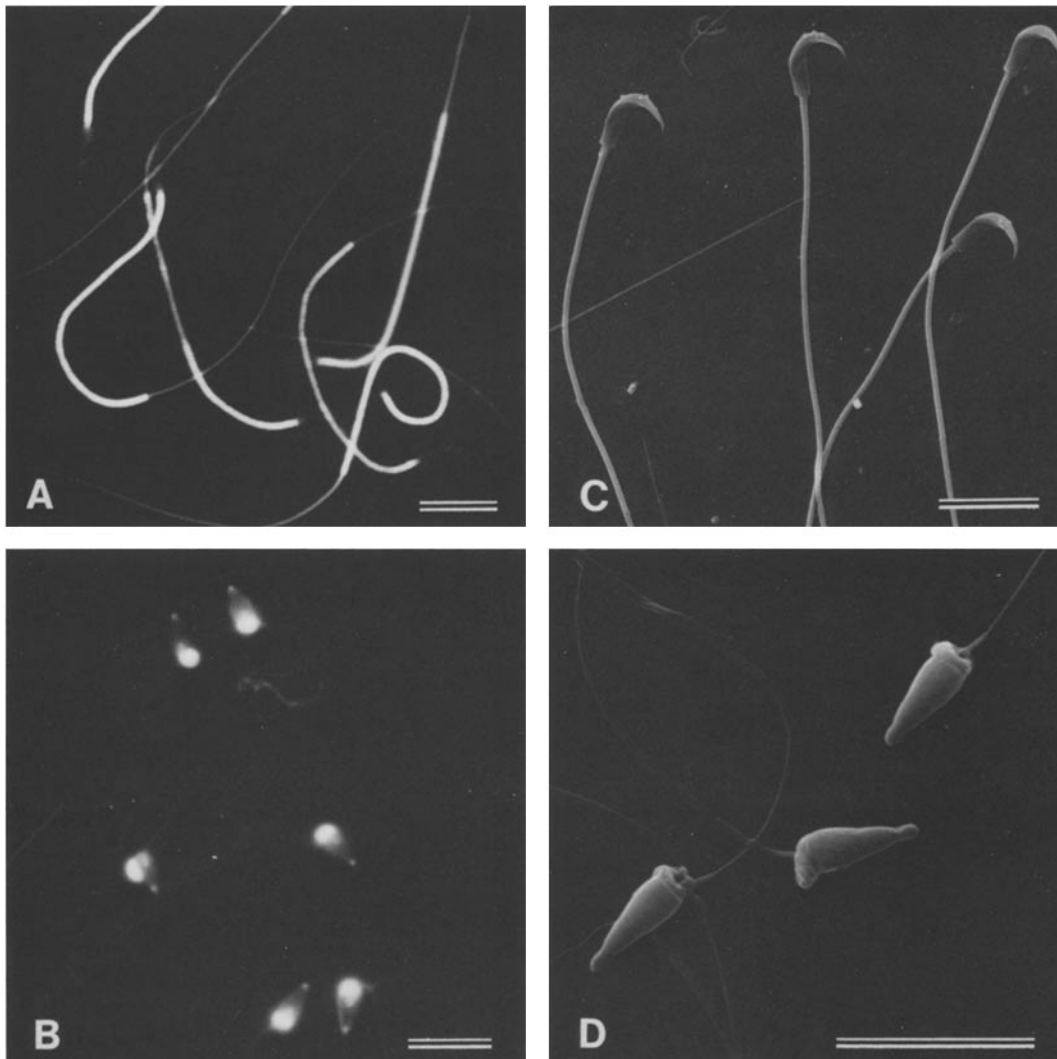

FIGURE 1   Fluorescence micrographs of hamster (A) and sea urchin (B) sperm after treatment with FITC. When these are compared to the corresponding scanning electron micrographs, in C and D, the majority of the fluorescence is found localized to the midpiece of both cell types; in sea urchin sperm, some labeling occurs in the acrosomal region as well. (A) Bar, 10 μM, × 1,000; (B) Bar, 5 μM, × 2,200; (C) Bar, 10 μM, × 1,300; (D) Bar, 5 μM, × 5,300.

occurring over the midpiece (Fig. 1). There is some fluorescence over the acrosome of sea urchin sperm but little on the remainder of the head or tail. In addition, sperm from a teleost fish (*Oryzias latipes*), bull, mouse, and guinea pig also show preferential binding of FITC over the midpiece. The regional differentiation of covalent labeling probably reflects the presence of an abundance of accessible amino groups over the sperm midpiece. Isothiocyanates react principally with amino groups to produce covalent thiourea derivatives. Sulfhydryl groups also can react with isothiocyanates (3), but pretreatment of sea urchin sperm with 20 mM 5-5'-dithio-bis(2-nitrobenzoic acid), a sulfhydryl group blocking reagent (27), for 15 min at 12°C and pH 7.6, did not alter the pattern of fluorescence, while treatment with 20 mM acetic anhydride, an amino group blocking reagent (27), for 15 min at 12°C and pH 7.6, prevented binding of FITC to the midpiece. In addition, pretreatment of sea urchin sperm with 2.5 mM dinitrophenol for 5 min at 12°C reduced their motility but did not change the pattern of labeling.

Characterization of the Covalently Labeled Sperm

Labeled sperm retained viability, as assessed by their ability to fertilize. When sea urchin sperm were labeled with [125]IFC, they incorporated radioactivity (Fig. 2) without losing viability, when limiting quantities of sperm were tested in a fertilization assay. To see whether labeled sperm were responsible for the fertilization seen in Fig. 2, rather than a population of unlabeled, but highly fertile sperm, we examined sections of fertilized eggs by autoradiography (Fig. 3). Silver grains were present over the midpiece of sperm, and at the site of sperm-egg fusion. The eggs used in this experiment were treated to allow them to become polyspermic, and thus facilitate observation of sperm-egg fusion. All the sperm in a population were labeled, as determined by autoradiography of sperm, in agreement with the fluorescence data. [125]IFC-labeled mouse sperm also retained the ability to fertilize mouse eggs after artificial insemination (Gabel and Eddy, unpublished data).

Sea urchin sperm labeled with [125]IFC were analyzed by polyacrylamide gel electrophoresis, to determine the number and size of the labeled components. Although several major sperm proteins (Fig. 4 A) were labeled by [125]IFC (Fig. 4 B), the majority of the polypeptide bands of sperm
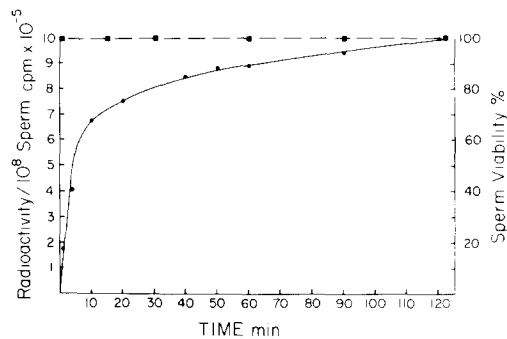

FIGURE 2 The kinetics of the reaction between sea urchin sperm and [125]IFC. Sperm were incubated with [125]IFC as described in Materials and Methods and, at various times after the addition of sperm, aliquots of the reaction mixture were removed, spotted onto glass fiber filter discs, and dropped into 10% TCA (0°C). The filters were washed twice with cold 5% TCA, then once each with 5% TCA, 95% ethanol, acetone, and ether at 20°C, then air dried and radioactivity (-●-●-●-) determined in a liquid scintillation counter, using Liquifluor scintillant. The viability of the sperm was assessed with a limiting concentration of sperm by removing aliquots of the reaction mixture containing just enough sperm to give 100% fertilization in untreated controls and adding them to a 5% egg suspension (vol/vol, as determined by the egg volume after settling at unit gravity for 30 min). The percent fertilization (-■-■-) was determined from the number of eggs with fertilization membranes.

were not radioactive. Sea urchin sperm were labeled by lactoperoxidase catalyzed iodination (LCI) (35) and analyzed in the same manner as [125]IFC-labeled sperm (Fig. 4 C). Several polypeptides of the same size were labeled by [125]IFC and LCI, while each technique also labeled some unique species. LCI labeled many more high molecular weight components than [125]IFC.

Some radioactivity associated with [125]IFC-labeled sea urchin sperm migrated as low molecular weight material not stained by Coomassie blue (Fig. 4 B). Extraction of [125]IFC-labeled sea urchin sperm with chloroform/methanol solubilized 25% of the radioactivity. The material extracted in this manner, when analyzed on SDS gels, migrated at the front and did not show Coomassie brilliant blue staining, but did show radioactivity (Fig. 4 D), indicating that it was probably lipid; it was not characterized further.

[125]IFC-labeled hamster sperm analyzed by SDS gel electrophoresis also showed many radioactive polypeptide bands (Fig. 5 B) that were a restricted class of the total sperm proteins (Fig. 5 A). When
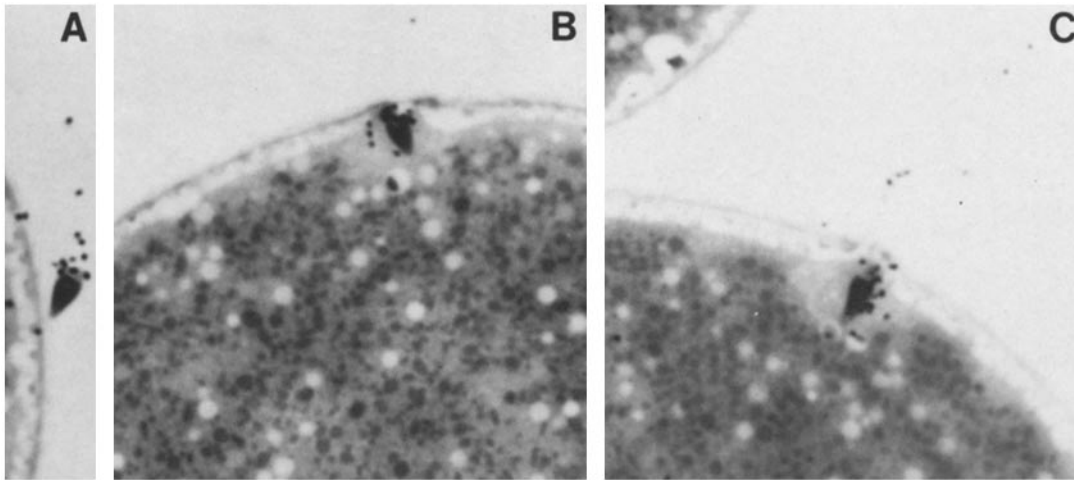
FIGURE 3 Autoradiography of [125]IFC-labeled sea urchin sperm and of eggs fertilized with these sperm. To induce polyspermy, eggs were pretreated for 2 min with 1 M glycerol, 0.1 mM $CaCl_2$ (adjusted to pH 9 with NH_4OH) then resuspended in MSW and inseminated with [125]IFC-labeled sperm. After 3 min, the eggs were added to 1% glutaraldehyde, in Ca^{+2} and Mg^+ free artificial seawater, and fixed for 20 min at 12°C. The fixed eggs were osmicated, dehydrated with dimethyoxypropane, and embedded in Epon. Thick sections were coated with Kodak NTB2 emulsion and, after developing, were stained with Toluidine blue. (A) Section of an [125]IFC-labeled sperm; note silver grains in the midpiece region and along the tail. (B and C) Sections of fertilized eggs with the penetrating sperm and associated silver grains visible in the egg cortex (× 1,600).

this pattern of radioactivity is compared to one obtained for LCI-labeled hamster sperm (Fig. 5 C), polypeptides of similar size were labeled by both techniques, but as with sea urchin sperm, there were substantial differences in the patterns. Chloroform/methanol extraction of [125]IFC-labeled hamster sperm solubilized only 0.2% of the bound radioactivity, in contrast to the case with the sea urchin.

Immunological Analyses of Binding Loci

The antiserum to IFC-conjugated porcine-γ-globulin crossreacted with porcine-γ-globulin and IFC-BSA in a double immunodiffusion test (Fig. 6A), but not with BSA or unbound IFC. When affinity purified anti-IFC was used in place of crude antiserum, precipitates formed only with the IFC-coupled proteins, and not with the proteins themselves (Fig. 6 B). Control preimmune sera did not react with any of the antigens. Thus, the antisera had the requisite specificity for the following analyses.

To demonstrate the site of IFC binding, we labeled hamster sperm with IFC, and then sequentially exposed them to anti-IFC and goat anti-rabbit IgG conjugated to HRP, as described in

Materials and Methods. The DAB reaction product formed a uniform dense layer over the sperm midpiece but was patchy and faint over the principal piece and head (Fig. 7 A-C). Hamster sperm that were not labeled with IFC, but treated with all the antisera and reagents, in a control experiment, did not have reaction product over the midpiece (Fig. 7 D and E). Occasional small patches of reaction product presumably represented nonspecific binding of the antiserum. Since the DAB reaction product was over the midpiece, as was the fluorescence (Fig. 1), the nonuniform distribution of label seen by fluorescence could be accounted for by a nonuniform distribution of label on the sperm surface, for antisera are impermeant reagents. To further substantiate the surface localization of the label, IFC-labeled hamster sperm were extracted with TX-100 and then sequentially exposed to the immunocytochemical reagents, as described above. Sperm extracted with a low concentration of TX-100 had reaction product over the sperm midpiece (Fig. 8A), but no cytoplasmic staining. However, labeled sperm extracted at a higher TX-100 concentration had no reaction product over the midpiece or other regions of the cell (Fig. 8 B and C). The plasma
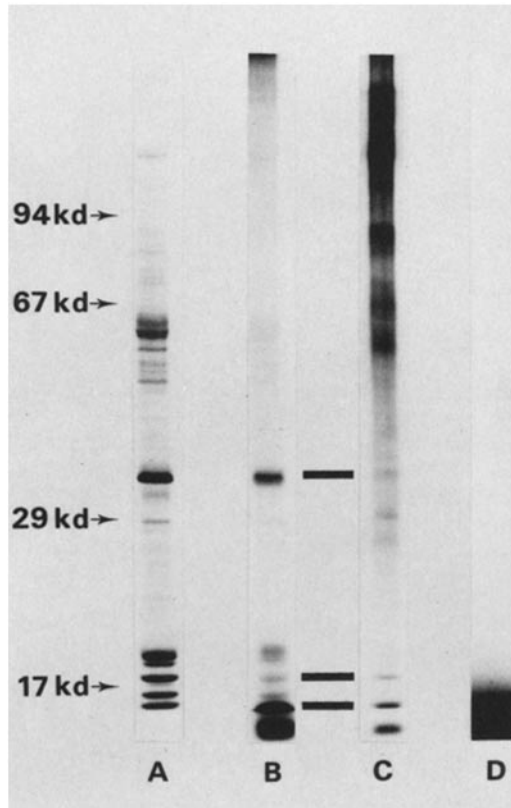
FIGURE 4 Fractionation of labeled sea urchin sperm by SDS gel electrophoresis. Channel A shows the polypeptides detected after Coomassie brilliant blue staining of the gel, while channels B and C show the radioautograms obtained from gels of sea urchin sperm labeled with [125]IFC and by LCI respectively. The radioautogram in channel D was obtained from a gel of the chloroform/methanol extract of [125]IFC-labeled sea urchin sperm. The numbers on the left indicate approximate molecular weights in kilodaltons (Kd). The gel was composed of a linear acrylamide gradient (12% at the bottom and 7.5% at the top) 25 cm in length. The gels were stained with 0.1% Coomassie brilliant blue R 250 in 25% TCA for 30 min, destained in 8% acetic acid overnight, dried, and analyzed by autoradiography as described (37). The lines between channels B and C indicate bands of similar molecular weight.
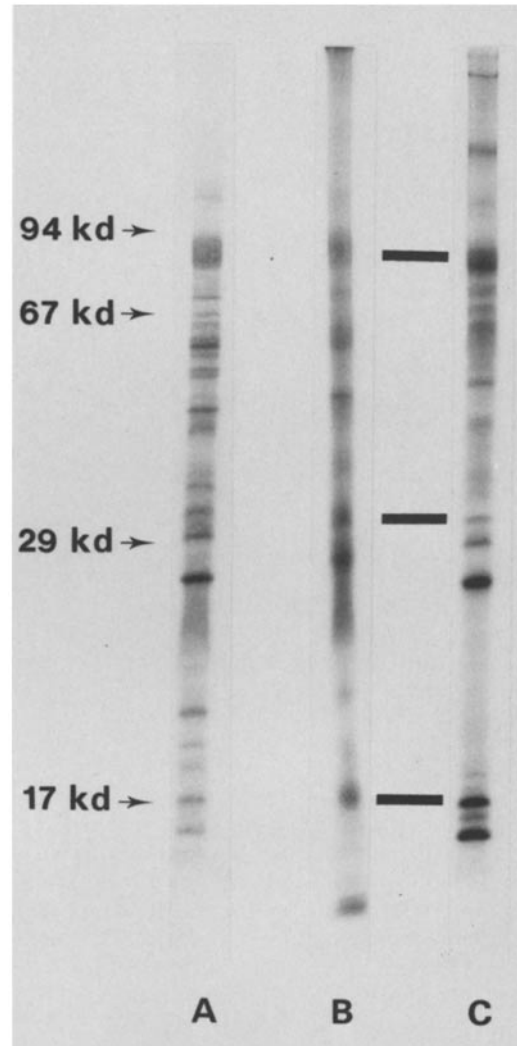
FIGURE 5 Fractionation of labeled hamster sperm by SDS gel electrophoresis. The polypeptides detected after Coomassie brilliant blue staining of the gel are shown in channel A, while the radioautograms of hamster sperm labeled by [125]IFC and LCI are shown in channels B and C respectively. The gel composition was as in Fig. 4. The lines between channels B and C indicate bands of similar molecular weight.

membrane was not detected at either TX-100 concentration. [125]IFC-labeled hamster sperm extracted with TX-100 under the same conditions released 30% of the total bound radioactivity at the lower TX-100 concentration, and 60% at the higher. There was presumably additional loss of labeled components from sperm during the proc-

essing for immunocytochemistry and electron microscopy. Sperm were also extracted with TX-100 and then exposed to IFC and the immunocytochemical reagents. This resulted in a uniform distribution of reaction product over the entire sperm (Fig. 8 D and E). Unlabeled extracted cells were similar to unlabeled intact cells in that no appre-
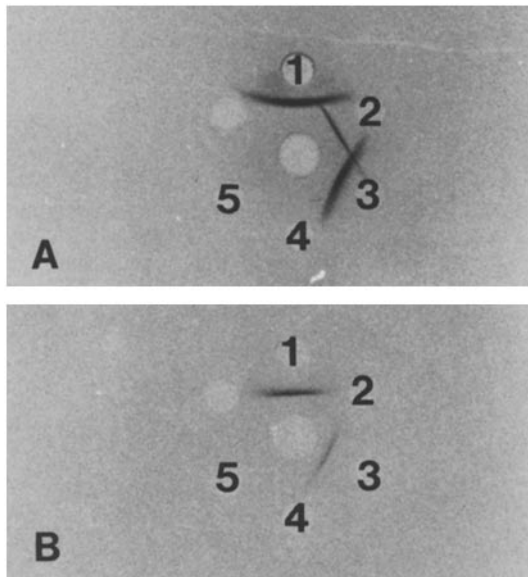
FIGURE 6 Immunodiffusion plate showing the specificities of crude anti-IFC (*A*) and the affinity purified anti-IFC (*B*). The wells in both plates received 10 μl of the following solutions: (*1*) IFC-γ-globulin; (*2*) γ-globulin; (*3*) IFC-BSA; (*4*) BSA (all proteins at 1 mg/ml); or (*5*) 10 μg/ml IFC. 15 μl of the appropriate antisera were in the center wells.

ciable reaction product was visible after exposure to immunocytochemical reagents. These observations indicate that there are cytoplasmic sites capable of reacting with IFC, but that an intact sperm plasma membrane presents a permeability barrier to IFC.

Sea urchin sperm treated with IFC and then with affinity purified anti-IFC were agglutinated by goat anti-rabbit IgG, whereas unlabeled cells treated with antisera in a similar fashion were not agglutinated. This indicates that at least a portion of the IFC is bound to the surface of sea urchin sperm, where it is accessible to antibodies. Further evidence that surface components are involved was obtained by extracting ^{125}IFC-labeled sea urchin sperm with 3.3% TX-100. Such extraction conditions demembranate sea urchin spermatozoa without disrupting the nucleus or tail structure (14). Extraction with 3.3% TX-100 in MSW solubilized 96% of the total radioactivity. SDS gel electrophoresis of material released by TX-100 indicated that many of the radioactive components of the sperm (Fig. 9*A*) were found in the TX-100 extract (Fig. 9*C*). Additionally, the TX-100 extraction enriched for a group of proteins that were

not detectable after disaggregation of whole sperm. The TX-100 insoluble material still contained some labeled proteins (Fig. 9*B*), but they accounted for <5% of the total radioactivity.

The TX-100 extract of labeled sea urchin sperm was treated with affinity purified anti-IFC and Staph A (16) to precipitate labeled proteins. Less than 2% of the radioactivity was precipitated by Staph A alone, but 50% was precipitated when anti-IFC was present during the incubation. SDS gel electrophoresis of the precipitated material confirmed that all of the labeled sperm components present in the TX-100 extract were precipitated by this technique (Fig. 9*D*). Some ^{125}IFC-labeled components present as minor species in the TX-100 extract were enriched by Staph A precipitation.

DISCUSSION

The studies reported above cast additional light on the nature of the regional differentiation of the sperm surface and demonstrate the utility of ^{125}IFC as a reagent for studying molecular components of cell surfaces. An important feature of this labeling technique is that it is a gentle one, in which sperm viability is preserved. We have tried repeatedly to label sea urchin sperm by lactoperoxidase catalyzed iodination, while preserving fertilizing ability, without success (B. M. Shapiro, unpublished data). In every attempt, radioactivity was incorporated along with a loss of viability of the sperm population; we never could obtain direct evidence, as in Fig. 3, that radiolabeled sperm could fertilize. This observation was in keeping with others suggesting that sperm may be killed by peroxidase-mediated halogenation (38), but of course such a negative result does not imply that all attempts at labeling sperm with lactoperoxidase need fail, for other conditions may be more gentle. Since the sperm is sensitive to manipulation, and yet can be labeled with ^{125}IFC, other cells are likely to be labeled with similar success. Thus, with the ability to label surface components of a living cell, one can follow the fate of specific labeled macromolecular components in that cell's future experience. In our case, we are using the technique to follow the fate of sperm surface components in developing sea urchin and mouse embryos (Gabel, Eddy, and Shapiro, manuscript in preparation).

The evidence that ^{125}IFC labels the sperm surface is several-fold. In the first place, both ^{125}IFC and its congener FITC (10, 34) label membrane
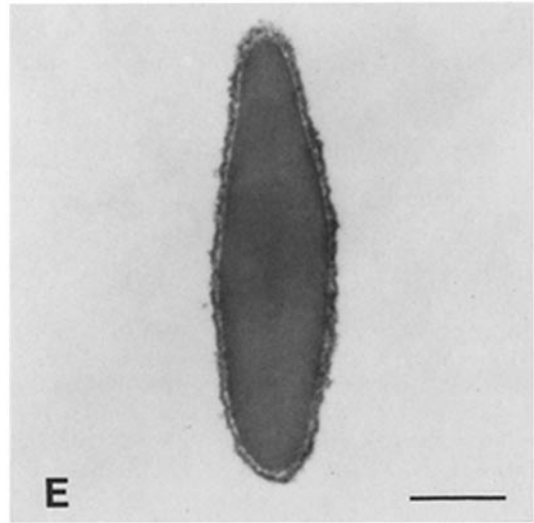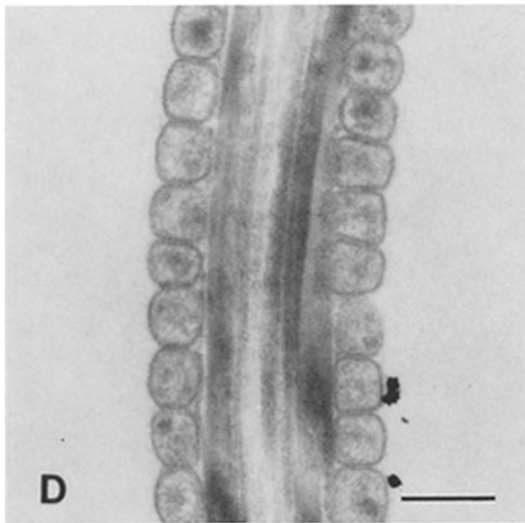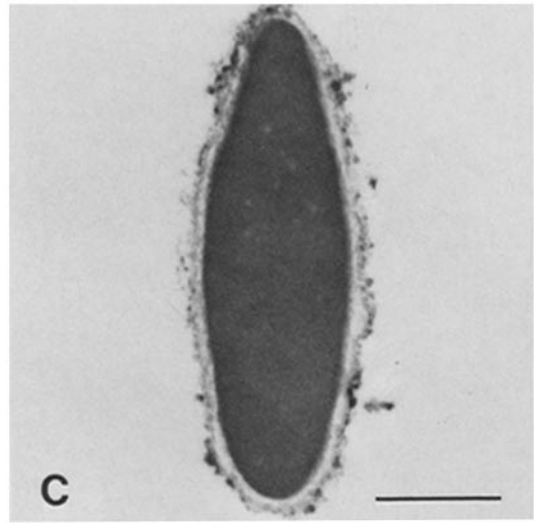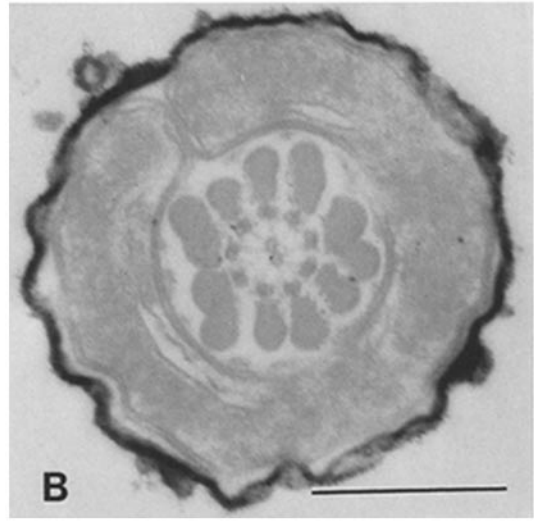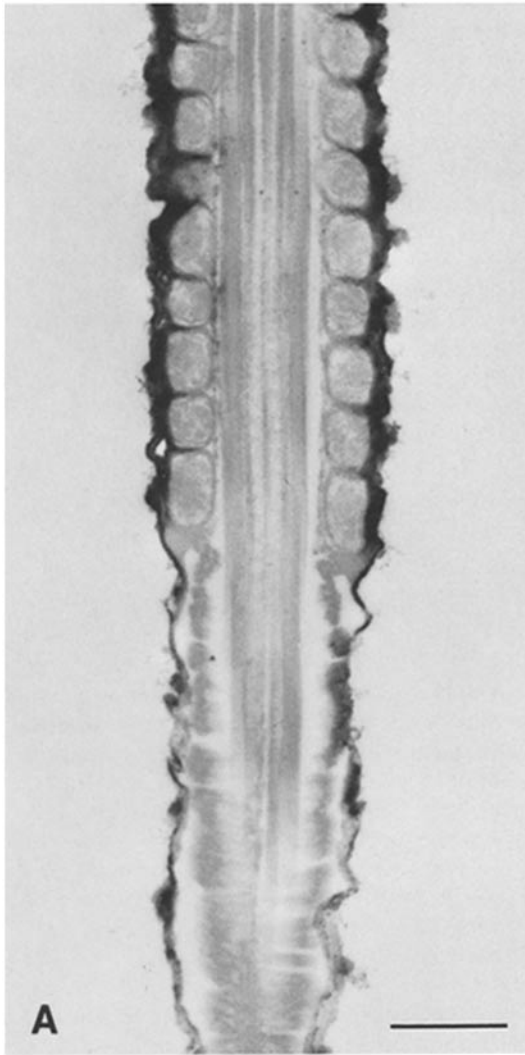
proteins of the erythrocyte without labeling hemoglobin, and FITC-labeling is restricted to the surface of viable muscle cells (5). In the second place, the regional labeling of the sperm surface seen by fluorescence microscopy of both IFC and FITC-labeled cells is also seen when IFC is detected with an immunocytochemical technique (Fig. 7). Since such a test relies on the properties of antibodies to be excluded from the cell by the plasma membrane, we conclude that the regionally differentiated fluorescence pattern reflects the distribution of components on the sperm surface. An alternate interpretation of the fluorescence distribution would have been that the labels penetrate the plasma membrane to label mitochondrial elements, as was seen with anilinonaphthalene sulfonate (7) which labels the midpiece region of dead sperm. However, the sperm in our study are viable (Figs. 2 and 3) and surface labeling can account for the regional heterogeneity (Fig. 7). When the permeability barrier of the labeled sperm was removed (Fig. 8A-C), no additional cryptic labeled sites were exposed, either in the mitochondria or elsewhere.

When low TX-100 concentrations were used to disrupt the sperm surface, the plasma membrane was removed from other areas of the sperm, but labeling persisted over the midpiece (Fig. 8A). We interpret this to mean that an additional feature of the regional differentiation of the midpiece is a tight association between the plasma membrane and underlying components. This may be one of the reasons why the midpiece regions from sperm of diverse phyla react with the fluorescein isothiocyanate derivatives, and may reflect the presence of unusual structural components in that area (see below). In any event, the mitochondrial and plasma membranes are tightly apposed in this region, a distinct array of intramembrane particles is found there (9), and this particle array is disrupted by inhibitors of mitochondrial activity and

by displacement of the plasma membrane away from the mitochondria. Thus, the midpiece surface has unusual properties that might reflect a functional association between the plasma and mitochondrial membranes (8). Other evidence that supports the surface localization of [125]IFC is the finding that sea urchin sperm labeled with the reagent agglutinate when treated with anti-IFC, and that most of the radioactivity may be extracted under conditions previously shown to remove the plasma membrane. Although 96% of the radioactivity of [125]IFC-labeled sea urchin sperm was extracted with TX-100, the mitochondria were disrupted, so that any association between surface components and mitochondria would have been destroyed.

A comparison of the proteins labeled by [125]IFC (Figs. 4 and 5) and lactoperoxidase catalyzed iodination (LCI) shows important similarities and differences. In both sea urchin and hamster sperm, some proteins of the same size are labeled. However, especially in the case of sea urchin sperm, there are substantial differences; few proteins >50,000 daltons are labeled by [125]IFC in sea urchin sperm (Fig. 4), whereas most of the LCI labeling is found in much larger components. Some differences are to be expected, since the two techniques modify different amino acid residues, and, under the vectorial labeling conditions we employed (see above and Materials and Methods), labeling should be restricted to exposed tyrosyl and amine residues, for LCI and [125]IFC, respectively. The differences may also relate to the regional distribution of IFC and could indicate that certain proteins (e.g., the 35,000 dalton band of Fig. 4B) are localized on the midpiece surface. However, since no information exists on the topographic localization of LCI-dependent iodination sites on the sperm surface, this point will need to be pursued further. The experiments reported above also raise the question of whether components labeled by [125]IFC exist in higher concentration in the

FIGURE 7 Thin sections of hamster sperm after treatment with antisera and DAB to localize IFC binding sites on intact sperm. Sections A-C are from IFC-labeled sperm and D and E are from unlabeled cells. (A) Longitudinal section through the midpiece and anterior portion of the principal piece. Note that the DAB reaction product is dense and uniform over the midpiece but becomes patchy and random posterior to the annulus. (B) Cross section through a sperm midpiece. (C) Cross section through the head region of a labeled sperm. Longitudinal section through the midpiece (D) and cross section through the head (E) of unlabeled sperm; all the antisera were used as in A-C, but there was no initial IFC labeling. Note the absence of the DAB reaction product. Bars, 0.5 μM. (A) × 31,000; (B) × 51,600; (C) × 33,250; (D) × 25,800; (E) × 25,300.
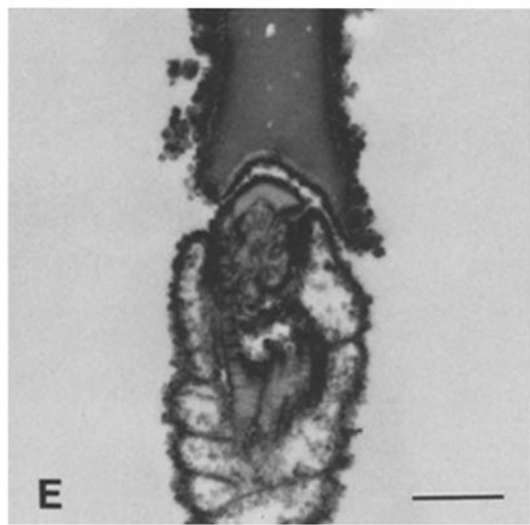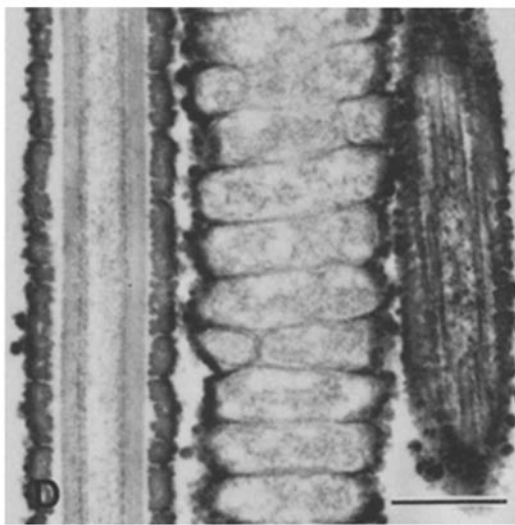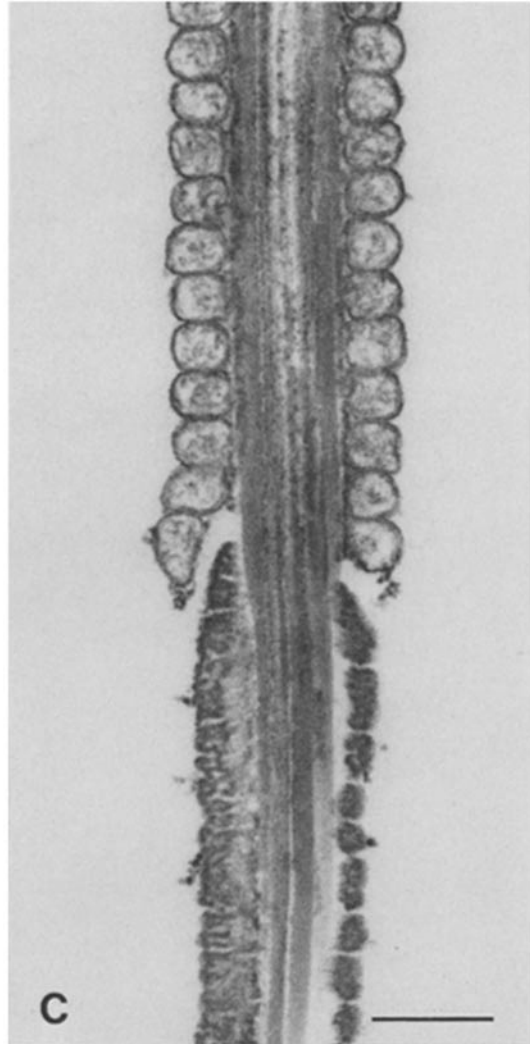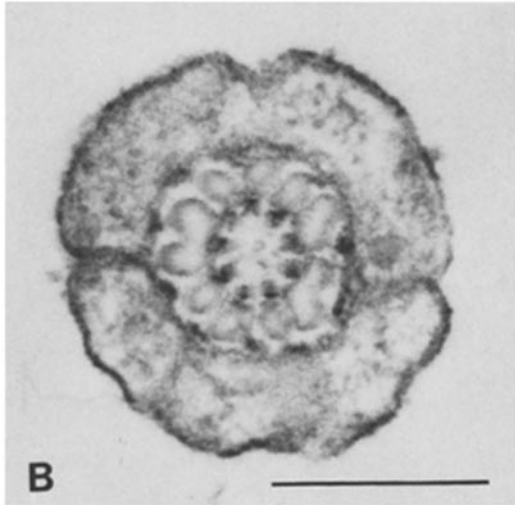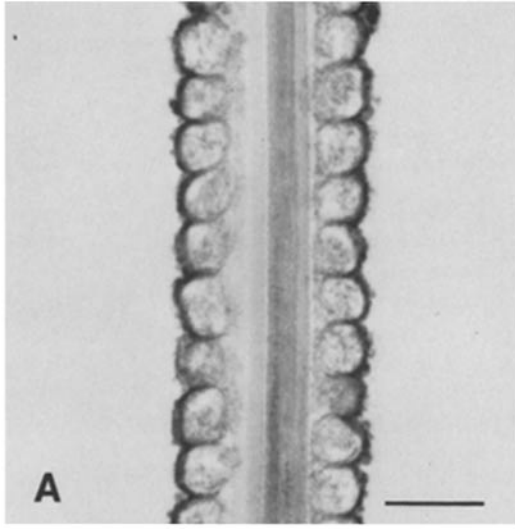
midpiece region of sperm, or whether they are homogeneously distributed but only exposed to the labeling reagent at that site. The resolution of
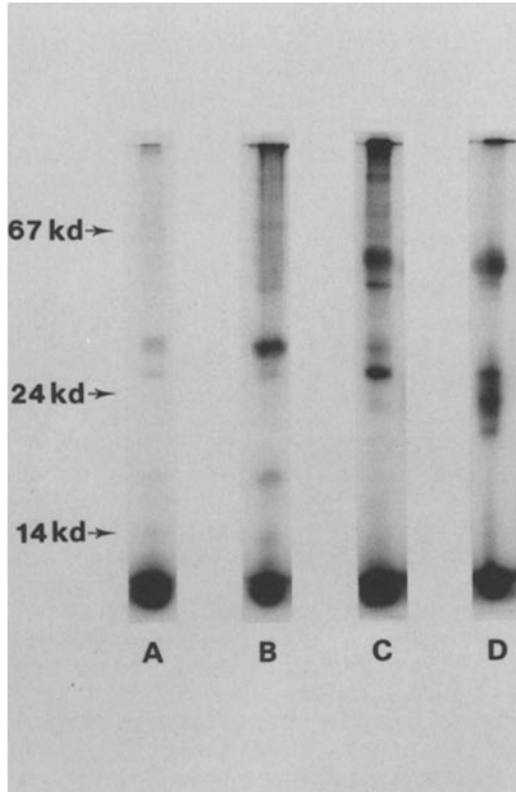

FIGURE 9   Analysis of the Triton extract from [125]IFC-labeled sea urchin sperm and the Staph A immunoprecipitate of this extract by SDS gel electrophoresis. The channels show the radioautograms from 12 cm, 12.5% acrylamide gels after separation of whole [125]IFC-labeled sperm, (A); the Triton insoluble material, (B); the components solubilized by Triton, (C); and the components precipitated by Staph A adsorbent after treatment of the TX-100 extract with affinity purified anti-IFC, (D). The same amount of radioactivity (2×10^5 cpm) were applied for each sample.

such questions will be facilitated by the ability to perform specific immunoprecipitation of IFC-labeled components with affinity purified anti-IFC antiserum, as shown in Fig. 9. We are presently extending this immunoprecipitation technique, that enriches for IFC-labeled surface components (Fig. 9), to develop it into a general scheme for the purification of labeled cell surface proteins. Thus, by using IFC-labeling and antibody precipitation of solubilized cell preparations, one should be able to purify surface proteins without first purifying the plasma membrane, which has not been accomplished in sperm.

The studies in this paper have shown that IFC binds to the surface of the midpiece of sperm, perhaps because of a high density of exposed amino residues. The nonuniform distribution of labeling occurs in sperm from diverse phyla, suggesting that the property may have general significance for sperm function. Additionally, [125]IFC-labeling of surface components is a useful technique, not only because it complements the more commonly employed lactoperoxidase-catalyzed iodination, but also because it expands the experimental possibilities for subsequent analysis. The technique is gentle, so that living cells may be studied after labeling. Labeled components can be localized by fluorescence microscopy or electron microscope immunocytochemistry. Finally, the labeled components can be isolated by using specific immunoreagents directed against IFC. Thus, [125]IFC surface labeling should provide a valuable tool for studying the biology of diverse cell types.

FIGURE 8   Triton X-100 extraction of hamster sperm affects the distribution of IFC. IFC-labeled cells were first treated with detergent, then exposed to the antisera and DAB. (A) Longitudinal section through the midpiece of a sperm extracted at the lower TX-100 concentration (0.6 ng/sperm). The DAB reaction product is still present. Cross sectional (B) and longitudinal (C) sections through the midpiece of labeled sperm after extraction at the higher TX-100 concentration (12 ng/sperm). There is no DAB reaction product associated with these cells. (D and E) Sections from sperm which were first extracted with TX-100 (either the high or low concentration) then labeled with IFC and treated with the antisera. The DAB reaction product forms a uniform layer over all regions of the cell. Bars, 0.5 μM. (A) $\times$ 26,000; (B) $\times$ 57,000; (C) $\times$ 32,300; (D) $\times$ 30,000; (E) $\times$ 24,300.

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