A Collagen-binding Protein on the Surface of Ejaculated Rabbit Spermatozoa

JAMES K. KOEHLER, E. D. NUDELMAN, and S. HAKOMORI
Department of Biological Structure, Division of Biochemical Oncology, Fred Hutchinson Cancer Research Center, Departments of Pathobiology and Microbiology and Immunology, University of Washington, School of Medicine, Seattle, Washington 98195

ABSTRACT A rabbit antibody to mouse 3T3 cell fibronectin was used in conjuction with a fluorescein-tagged second antibody to detect fibronectin-like activity on the surface of rabbit spermatozoa. Only ejaculated sperm displayed an intense and highly localized fluorescence over the acrosomal region. Cauda epididymal sperm of the rabbit as well as several other species did not exhibit any reaction. The fluorescent activity could be eliminated by trypsin treatment but was re-established by incubation in cell-free seminal fluid. Sperm recovered from females 10–12 h after mating showed a reduction or absence of antifibronectin fluorescence, suggesting that this component's loss could be a factor in sperm capacitation.

Because fibronectins show strong binding to collagen, mixtures of ejaculated sperm and collagen were examined in the light and electron microscope. Living sperm appear to have a strong affinity for collagen and quickly adhere to the filaments by their heads, while continuing vigorous flagellations.

Surface labeling of sperm with the galactose-oxidase-NaB[^{3}H]$_4$ technique, extraction with urea-detergent mixtures and affinity chromatography of extracts on gelatin-Sepharose revealed a single radioactive band of mot wt ~40,000 after SDS polyacrylamide gel electrophoresis and fluorography.

Fibronectin became an active focus of research when it was found that this cell surface protein decreased significantly in quantity after the malignant transformation of various cell lines (12, 18, 32, 40). This finding led to the use of the alternate term large, external, transformation-sensitive (LETS) protein for this substance. A similar, but probably not identical, molecule is found in serum and has been called cold insoluble globulin (CIG) or plasma fibronectin (25).

These glycoproteins are found on the surfaces of many cultured cells, in primitive mesenchyme, and in basement membranes (23, 35). They are also found in amniotic and cerebrospinal fluid in addition to blood plasma. The use of primary or secondary fluorescence techniques with antifibronectin antibody has provided striking evidence for the presence of this molecule on a large variety of cell surfaces. Typically, the material is arranged in a loose meshwork over the cell periphery (3, 13).

Many studies using both in vitro and in vivo systems have suggested that this molecule is involved in adhesion phenomena including cell-cell aggregation and cell-substrate attachment especially to collagen and basement membrane material (6, 31, 41). Plasma fibronectin may also be involved in opsonizing collagen and other debris for ingestion by macrophages (20).

Several studies have shown that fibronectin makes a rather late appearance in developing mouse embryos. Wartiovaara et al. (38) indicated no detectable fibronectin until the 7th or 8th d of development, whereas Zetter et al. (42) found expression of fibronectin 3 or 4 d postfertilization. Although not expressly stated, these studies also suggest that unfertilized eggs are devoid of surface fibronectin.

Spermatozoa possess a large number of cell membrane-associated proteins and glycoproteins. Esbenshade and Clegg (8) indicate at least a dozen protein species in a plasma membrane fraction from boar sperm, and Olson and Hamilton (29) found more than 25 Coomassie-staining bands of which five were periodic acid-Schiff positive. Herr et al. (15) used ^{125}I-lactoperoxidase labeling to identify six surface components on mouse spermatozoa after releasing 24 major polypeptide bands by detergent treatment. Nicolson et al. (27) used lactoperoxidase-catalyzed iodination to study the surface proteins of rabbit sperm. Among the eight surface-labeled bands, several were

THE JOURNAL OF CELL BIOLOGY · VOLUME 86 AUGUST 1980 529–536

enhanced in the cauda as compared to the caput epididymis, including a 39,000 component. Very few of these many proteins, however, have been correlated with a specific physiological phenomenon. Notable exceptions to this are the sperm-coating (decapacitation factor) proteins which seem to protect the sperm from undergoing a premature acrosome reaction (7, 17, 19, 39) and the forward motility factor of epididymal origin recently described by Hoskins et al. (16). No reports have appeared regarding the presence or absence of fibronectin or other collagen-binding proteins on male gametes. In a continuing effort to characterize the surface properties of mammalian spermatozoa and find suitable probes to monitor capacitation and the acrosome reaction, we present evidence to indicate the presence of a fibronectin-like, collagen-binding protein on the rabbit sperm surface.

MATERIALS AND METHODS

Animals and Sperm Acquisition

Three New Zealand White bucks of proven fertility were used to obtain ejaculated sperm with the aid of an artificial vagina. Immunofluorescence studies were carried out on samples of two pooled ejaculates, whereas biochemical determinations utilized 12–14 ejaculates obtained over a 24-h period. Cauda epididymal sperm were donated by normal New Zealand White male rabbits undergoing urelated surgical procedures. The cauda epididymides were dissected free of fat and testicular tissue and cut several times with a fresh razor blade, allowing sperm to disperse into Hanks' balanced salt solution (HBSS). Epididymal sperm from several other species (Swiss Webster mice, golden hamsters, voles, and guinea pigs) were also tested for antibody binding and were obtained in the same manner as described for the rabbit. In some cases the ejaculates were fractionated into a sperm fraction, granular fraction, and seminal fluid supernate by differential centrifugation.

Immunofluorescence

Antifibronectin was prepared by injecting two New Zealand White rabbits with purified samples of the released form of fibronectin made by 3T3 (BALB mouse) cells in culture. Antigen was purified on a gelatin Sepharose 4B affinity column (6). The rabbit immune serum was subject to ammonium sulfate precipitation and DEAE cellulose purification to obtain the IgG fraction. Immunodiffusion analysis of the 3T3 antifibronectin antibody showed precipitin bands between the antibody and purified 3T3 fibronectin as well as purified CIG from hamster plasma, whereas no bands formed with preimmune rabbit serum.

The basic procedure for preparing cells for immunofluorescence is similar to that described by Schwarz and Koehler (34) in a lectin-labeling study. Ejaculates or cells were diluted two times with HBSS and passed through a small column (5-cm^3 disposable syringe) packed with fiber glass. Centrifugation was avoided at this step to minimize membrane damage or breakage. Cells were then fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) or 0.1 M cacodylate buffer. Live cells were also stained directly, but the results are less reproducible. If experimental procedures such as enzyme extractions were to be used, fixation was delayed. Samples that were trypsinized were incubated at 37°C for 20 min in 0.25% trypsin in HBSS (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.). Fixation was followed by a twofold wash in 0.2 M glycine to bind free aldehyde groups. Brief, slow-speed centrifugation was used (600 *g*, 3 min). Antibody (3T3 antifibronectin) was then added to aliquots of sperm (10–20 $\mu g/10^6$ cells) and incubated at 37°C for 20 min in a volume of 0.3 cm^3. Controls include sperm treated with normal rabbit IgG and sperm in HBSS. Antibody treatment was followed by a twofold wash in HBSS, and sperm were then treated with a diluted (10- to 50-fold) solution of goat anti-rabbit IgG tagged with fluorescein isothiocyanate (GARIg-FITC) (N. L. Cappel Laboratories, Cochranville, Pa.). Incubation was again carried out for 20 min at 37°C. An additional control included in this step consisted of untreated sperm incubated with GARIg-FITC lacking antibody. The labeled cells were loaded onto the top of a 3% bovine serum albumin solution in a 6-cm^3 Falcon tube and centrifuged for 5 min at slowly increasing speeds to 500 *g*. Fluorescence microscopy was carried out on a Zeiss model WL equipped with epifluorescence and a mercury HBO burner. Specimens were photographed with an Olympus camera (model PM-IDA, Olympus Corp. of America, New Hyde Park, N. Y.) using Tri-X film.

Sperm-Collagen Interactions

Type I collagen was obtained from human fetal skin, acetic acid, and pepsin extracted and centrifugally purified. The preparation was then precipitated with 1 M NaCl, redissolved in acetic acid, and extensively dialyzed against water before lyophilization. A small quantity of this collagen in PBS was placed on a microscope slide together with a drop of sperm suspension and sealed with a Vasoline-lined coverslip. Such specimens were viewed with a Zeiss phase-contrast microscope over periods of several minutes to several hours after preparation. Some samples were fixed with glutaraldehyde and osmium tetroxide, dehydrated in alcohols, and embedded in Epon 812 for thin-section preparation and viewed in a Philips 201 electron microscope.

Galactose Oxidase Surface Labeling

The method used was that described by Gahmberg and Hakomori (11). The cells or seminal particulates were washed gently two to three times with PBS and incubated with galactose oxidase (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 30 U/ml for 2 h at 37°C. The sample was then washed three times in PBS and pelleted at 800 *g* for 5–10 min. The pellet was then resuspended in buffer containing 2.5 mCi of $NaB[^3H]_4$ (New England Nuclear, Boston, Mass.). After 30 min of reduction, the label was chased with 1 mM cold $NaB[^1H]_4$ for 15 min and washed four times in PBS. Cell extracts obtained by the chromatographic methods described below were counted in a toluene-based scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.1% *p*-bis[2-(5-phenyloxazolyl)] benzene in a Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Six or 8 M urea alone or in combination with Triton X-100 (1–2%) was utilized for solubilization of the sperm cell membranes. The 8 M urea alone probably results in incomplete solubilization of the membrane. Protease inhibitors are routinely included in the solubilization medium (1.0 mM phenylmethylsulfonyl fluoride).

Gelatin (Collagen)-Sepharose Chromatography

Collagen-binding proteins such as cell surface fibronectin (LETS protein), the released form of fibronectin, plasma fibronectin (CIG) and associated substances such as polysaccharide ligands can be isolated from other surface components by virtue of their high affinity for collagen. Therefore, a gelatin Sepharose column was utilized for the isolation of these molecules after surface labeling and membrane solubilization. The procedure is based on the work of Engvall and Ruoslahti (6) in which gelatin (Sigma, type I) is coupled to BrCN-activated Sepharose 4B. Chromatography was carried out at room temperature on a 1.5 × 7.5 cm column of gelatin-Sepharose in PBS at pH 7.0. Adsorbed material was eluted with 4–8 M urea in 0.05 M Tris buffer at pH 7.5.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were prepared following the procedure of Laemmli (22). Samples were dissolved in buffer containing 2% SDS with or without 5% 2-mercaptoethanol (to reduce S-S bonds) and heated in a boiling water bath for 5 min. Slab gels were stained with Coomassie Blue. Standard proteins used in molecular weight estimations in the SDS polyacrylamide gel electrophoresis (PAGE) system include: myosin (200,000), bovine serum albumin (68,000), actin (45,000), and a ricin subunit (28,000). Fluorography of slab gels (containing surface-labeled components) followed the procedure of Bonner and Laskey (2). After gels were run containing ^3H-labeled molecules, the dried scintillator impregnated gels were placed in contact with x-ray film (Kodak X-Omat) and exposed at −70°C for periods of several days to 2 wk, depending on the specific activity, followed by routine photographic processing.

RESULTS

Cultured mouse 3T3 cells treated with antifibronectin and GARIg-FITC show the characteristic, filamentous network staining associated with the cell surface (Fig. 1 *a* and *b*). Epididymal spermatozoa from several mammalian species did not demonstrate any significant fluorescence above that of normal rabbit IgG-treated cells. Animals in this group included mouse, vole, golden hamster, guinea pig, and New Zealand White rabbits. Ejaculated rabbit sperm, however, showed a brilliant and highly localized fluorescence over the acrosomal region of the sperm head (Fig. 2 *a* and *b*; Fig. 3 *a* and *b*). At low magnification (Fig. 2 *a* and *b*) it can be seen that essentially all of the sperm in a given field demonstrate the fluorescence. At higher magnification (Fig. 3 *a* and *b*), the fluorescence is shown to be highly localized to the acrosomal region of the spermatozoon. In controls treated with normal rabbit IgG little or no fluorescence can be detected (Fig. 4 *a* and *b*), although

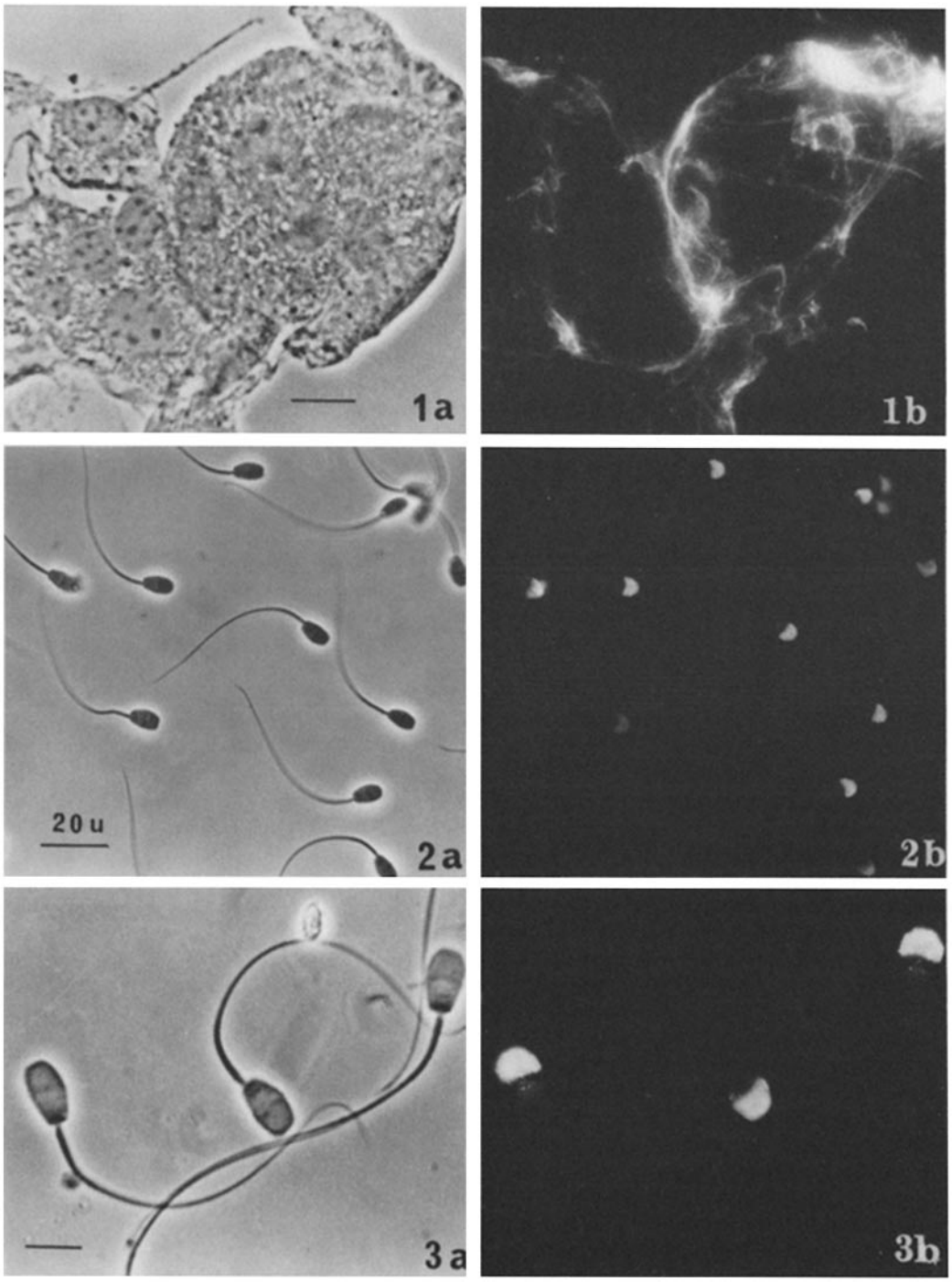


FIGURE 1 (*a*) Mouse 3T3 cells in culture. Phase-contrast image of fixed preparation. (*b*) Fluorescence image of same material after treatment with rabbit anti-3T3 fibronectin stained with fluorescein-tagged goat anti-rabbit IgG. The network of fibronectin filaments at the cells' periphery is clearly demonstrated. Magnification bar in this and subsequent micrographs equals 10 μm unless otherwise specified. × 1,000.

FIGURE 2 (*a*) Survey view of ejaculated rabbit sperm in phase contrast. (*b*) Fluorescence image of anti-3T3 fibronectin-stained sperm showing that essentially all the cells fluoresce in a highly localized region at the anterior of the sperm head. × 500.

FIGURE 3 (*a*) Higher magnification in phase contrast of several sperm in a fixed preparation. (*b*) The fluorescence after anti-3T3 fibronectin treatment and staining is uniformly distributed over the acrosomal surface. × 1,200.

sometimes a very faint, nonspecific reaction can be seen over the postacrosomal region. Cells treated just with GARIg-FITC were completely negative. Antifibronectin absorbed with 3T3 cells or cell-free seminal fluid also resulted in a negative reaction when used to label ejaculated sperm.

After trypsinization (15–20 min, 37°C), ejaculated sperm retain motility but fluorescence is either extinguished completely or, more commonly, restricted to a faint rim at the anterior margin of the acrosome (Fig. 5 *a* and *b*). Reincubation of similarly trypsinized sperm in cell-free seminal fluid for 30 min at 37°C fully re-established the intensity and acrosomal localization of the fluorescence (Fig. 6 *a* and *b*). Trypsinized

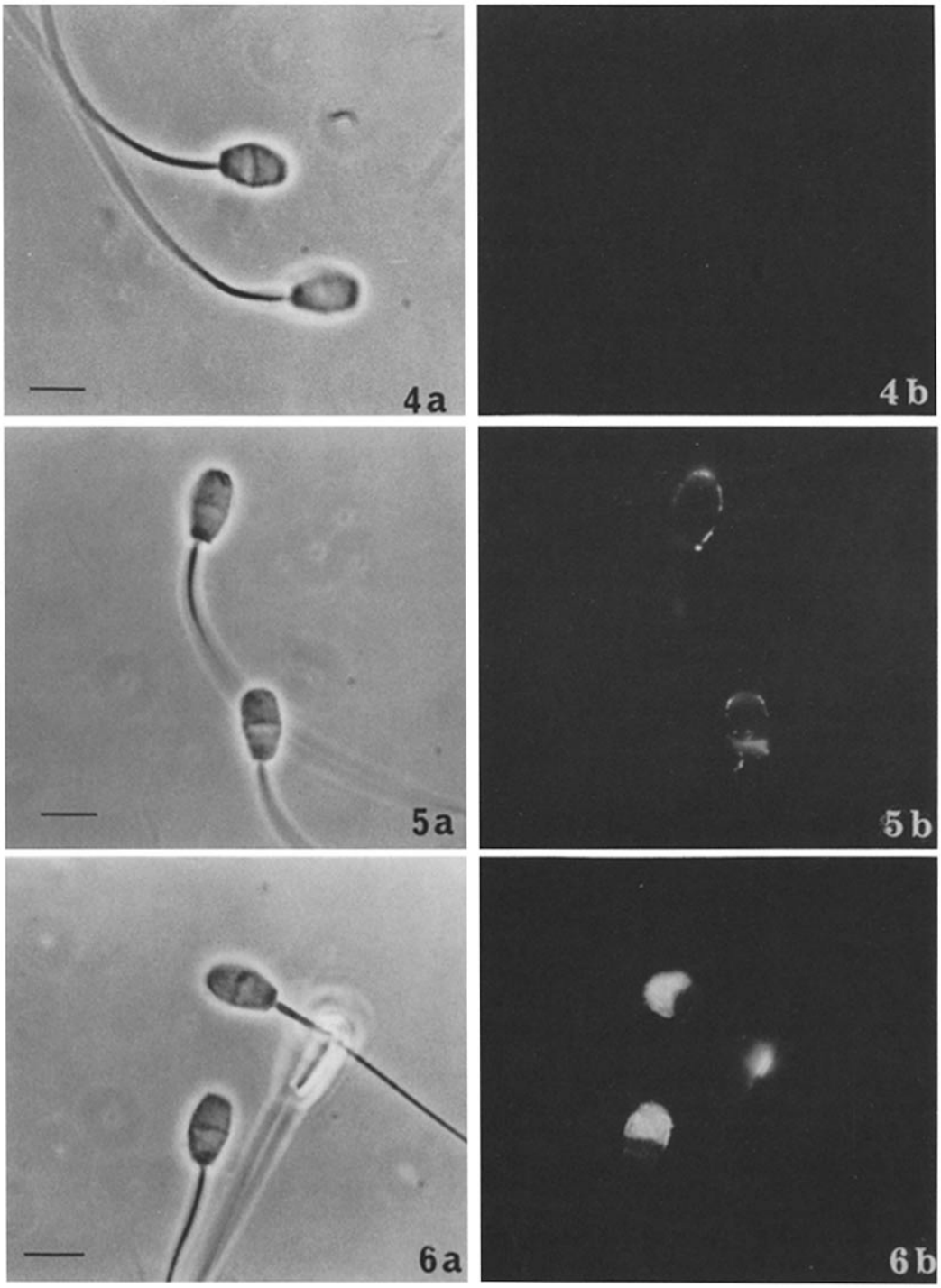


FIGURE 4 (*a*) Phase-contrast micrograph of rabbit sperm treated with normal rabbit IgG and GARIgG-FITC. (*b*) No fluorescence is detectable in this control preparation. × 1,200.

FIGURE 5 (*a*) Phase-contrast micrograph of rabbit sperm subjected to trypsin treatment (0.25%, 20 min at 37°C) before fixation. (*b*) After anti-3T3 fibronectin staining, the fluorescence is limited to a thin band at the anterior margin of the sperm head. Some trypsin-treated samples show no fluorescence. × 1,200.

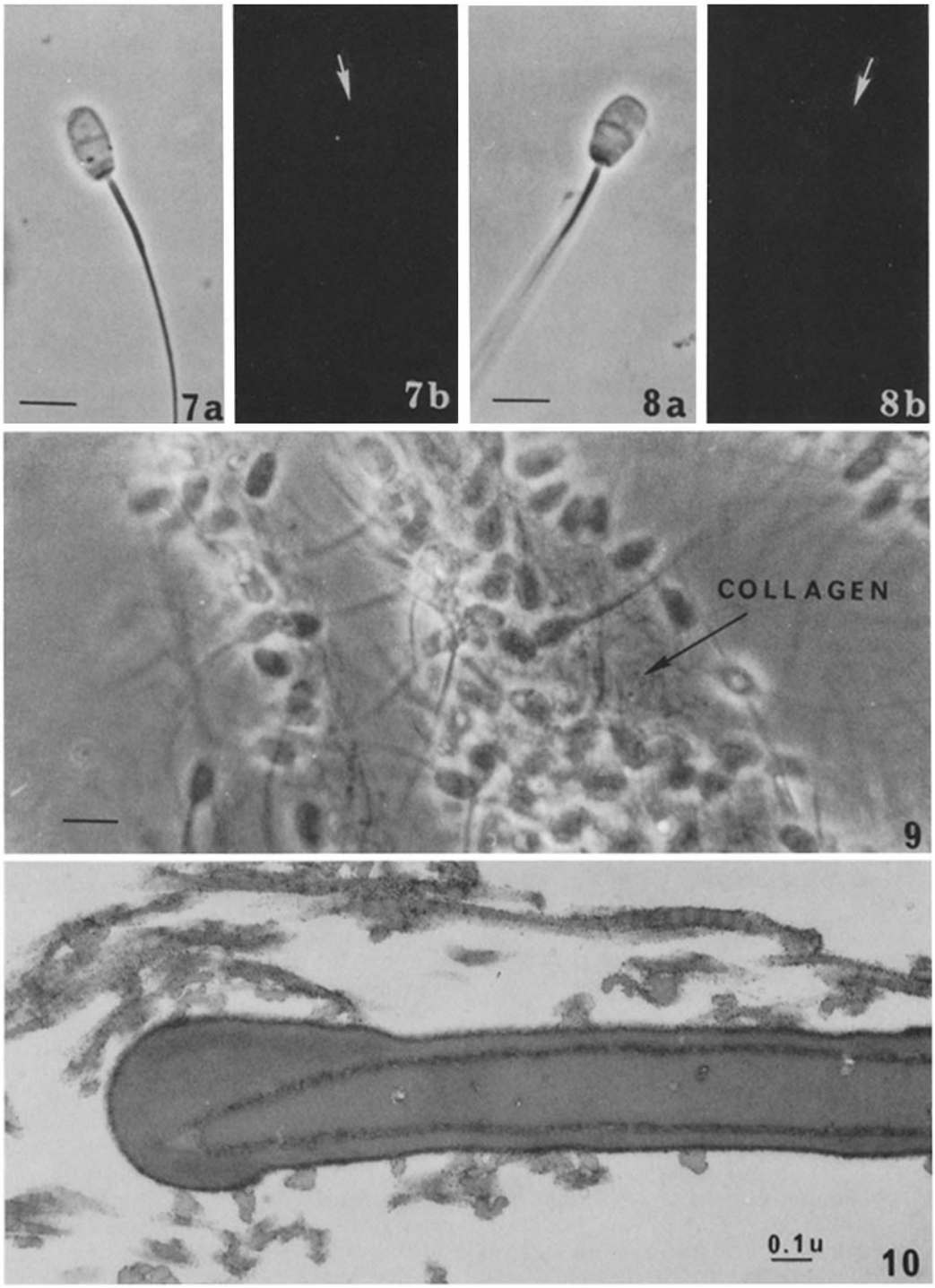
FIGURE 6 (*a*) Cells reincubated in cell-free seminal fluid (30 min at 37°C) after trypsin treatment. Phase contrast. (*b*) The fluorescence of the anterior region of the sperm head is completely re-established as shown by anti-3T3 fibronectin staining. × 1,200.

cells reincubated only in HBSS remained negative when tested with antifibronectin.

To determine the presence or absence of this material on sperm which had been in transit in the female tract, the uteri of three rabbits were flushed 10–12 h after natural mating, and recovered sperm were stained for antifibronectin. Although these cells show considerable variability in staining, most cells no longer show the acrosomal fluorescence of control ejaculated sperm (Fig. 7 *a* and *b*; Fig. 8 *a* and *b*).

Collagen-Sperm Interaction

When freshly ejaculated rabbit sperm are brought into con-



FIGURES 7 and 8 Sperm isolated from the female tract 10–12 h after natural mating. Although the morphology of the sperm appears normal, they no longer stain after treatment with anti-3T3 fibronectin. The arrows indicate the anterior tip of the acrosomes in the fluorescence figures (7 *b* and 8 *b*). × 1,200.

FIGURE 9 Preparation of collagen and ejaculated rabbit spermatozoa as seen in phase contrast ~15 min after mixing. The meshwork of collagen fibers can be seen (arrow), and many of the sperm appear attached to this matrix by the anterior region of the head. × 1,000.

FIGURE 10 Ejaculated rabbit spermatozoon incubated with collagen and prepared for electron microscopy. The collagen fibrils can be seen to be attached to the plasma membrane at multiple sites. Longitudinal sections of the collagen fibrils show the characteristic periodicity of this material. × 57,000.

tact with collagen on a slide preparation, the sperm appear to stick quickly and tenaciously to the collagen fibrils. The sticking takes place primarily at the anterior portion of the sperm head (i.e., acrosomal region) as seen in Fig. 9. The interaction takes place rapidly, the collagen matrix being essentially covered with attached sperm after 5–10 min of incubation. The appearance under the light microscope is similar to that of a living ciliated epithelium, the sperm vigorously flagellating.

Electron microscope preparations of collagen-sperm complexes reveal a very close association of collagen fibrils with the anterior portion of the plasma membrane of the sperm head (Fig. 10). Trypsin treatment of sperm greatly reduces the interaction with collagen. Incubation of sperm with "inert" fibrils such as cellulose or fiber glass does not elicit any significant association, nor is there any indication of attachment of sperm to Sepharose beads.

Surface-Labeling Studies

Ejaculated sperm and the particulate fraction were subjected to surface labeling using the galactose oxidase-NaB[^{3}H]$_4$ technique (11). This procedure places tritium in galactosyl and *N*-acetylgalactosaminosyl residues on exposed cell surface glycoconjugates. The membranes were solubilized, run on a collagen-Sepharose affinity column, and SDS PAGE determinations were made of bound components. Fig. 11 shows a fluorogram of such a gel pattern of sperm extracts indicating four major radioactive (i.e., surface-labeled) bands with approximate mol wt between 60,000 and 30,000 (lanes *b* and *c*). The supernate from the 6-M urea sperm extraction is seen to contain a single faint band at mol wt 40,000 (lane *d*), and this same component is strongly bound to the collagen column (lane *f*, arrow). Fig. 12 shows Coomassie Blue-stained SDS PAGE preparations and accompanying fluorogram (Fig. 13) of galactose oxidase-labeled, ejaculated granule fraction extracts. Four prominent bands again appear to be surface-associated glycoconjugates, including the 40,000 mol wt protein seen in the sperm preparations.

Neither neuraminidase treatment nor reducing conditions (7% mercaptoethanol, boiled for 5 min) appeared to significantly shift the electrophoretic mobility of this 40,000 mol wt collagen-binding material. This suggests that sialic acid is not a major component of this glycoprotein and that interchain S-S bonds are not involved in its structure.

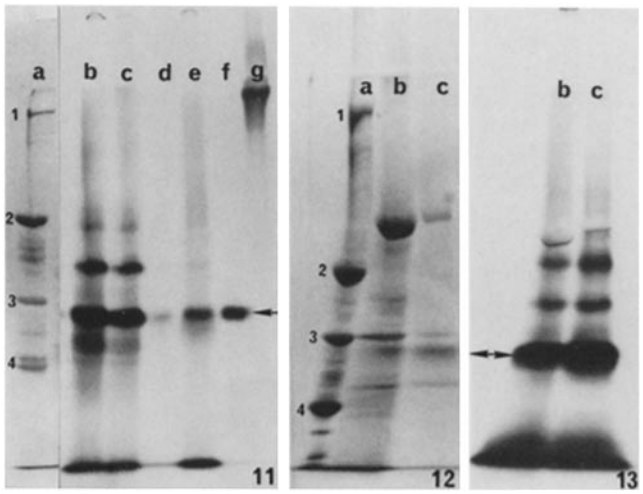


FIGURE 11 SDS polyacrylamide gel (5–14% gradient) patterns of galactose oxidase-NaB[^{3}H]$_4$-labeled ejaculated rabbit sperm extracts. (*a*) Coomassie Blue-stained molecular weight standards: (*1*) myosin, 200,000 mol wt, (*2*) bovine serum albumin, 68,000 mol wt, (*3*) actin, 45,000 mol wt, and (*4*) a ricin subunit, 28,000 mol wt. Lanes *b*–*g* are fluorograms showing radioactive bands. (*b*) SDS extraction of sperm cells. (*c*) Sperm pellet after 6 M urea extraction. (*d*) Supernate from 6 M urea extraction. (*e*) Pass through from collagen column. (*f*) Collagen-binding material from sperm extract. (*g*) 220,000 mol wt standard of [^{3}H]proline CIG. Of prime interest is the ~40,000-dalton labeled protein that binds to collagen (arrow, *f*) and is present in all other sperm extracts.

FIGURES 12 and 13 Coomassie Blue-stained (Fig. 12) and fluorograms (Fig. 13) of SDS PAGE patterns of galactose oxidase-labeled ejaculate particle extracts. (*a*) Molecular weight standards (same as Fig. 11), (*b*) Triton-treated particle suspension, (*c*) Triton extract supernate. Several prominent bands appear to be surface associated, including the ~40,000 mol wt protein (arrows).

DISCUSSION

The fluorescence studies indicate the presence on the ejaculated sperm surface of a molecule having antigenic determinants similar or identical to those of fibronectin. The activity is highly localized to the surface over the acrosomal region, can be removed by trypsin, and is rapidly reestablished by incubation of trypsinized cells in seminal fluid. Immunofluorescent localization of a fibronectin-like activity is also demonstrated on the seminal granules found in the ejaculate. Surprisingly, analysis of SDS polyacrylamide gels of galactose oxidase surface-labeled cells did not reveal a high molecular weight (~220,000) component similar to fibronectin, but rather a 40,000-dalton species that bound to a collagen-Sepharose affinity column. The collagen-binding region within the fibronectin molecule has been isolated from proteolytic products by several investigators using gelatin-Sepharose columns. Balian et al. (1) have isolated a 40,000-dalton fragment from cathepsin-D hydrolysates. 70,000- and 40,000-dalton fragments were found in trypsin hydrolysates (33) and a 40,000-dalton moiety was isolated from chymotrypsin hydrolysates (14). A highly glycosylated collagen-binding fragment with a mol wt of 70,000 has been isolated from hamster fibroblast culture media (10). Because addition of protease inhibitors decreased the quantity of this material, it may represent a degradation product of fibronectin (Sekiguchi and Hakomori, unpublished observations). Naturally occurring collagen-binding proteins with molecular weighrs in these ranges have not been described, but it is plausible that the sperm collagen-binding protein may be related to such a smaller moiety rather than high molecular weight native fibronectin.

A 40,000-dalton activity could also be detected in gels of seminal fluid and in fluorograms of surface-labeled isolated seminal granules in the present work. Mayol and Longenecker (24) have shown that these granules can constitute a large mass of the ejaculated material (approximately equivalent to the spermatozoa themselves). These granules are membrane bounded as visualized in thin-section preparations and have been described by Nicander et al. (26) as having a prostatic origin. Using freeze fracture, we have also observed that the granule membrane faces display intramembrane particles.

Although numerous studies have been carried out on mammalian sperm surface membrane components, few of these have focused specifically on molecules that show changes in quantity or distribution during development or fertilization. Eng and Oliphant (7) have isolated a large glycoprotein (~121,000 mol wt) from rabbit seminal fluid that inhibits the acrosome reaction and fertilization in vivo. They have termed this substance acrosome-stabilizing factor. More recently, the same group has found a similar material on the rabbit sperm surface using iodination techniques (28). This material has the approximate subunit molecular weight of fibronectin and it is possible that a connection exists between this substance and the fluorescent activity that we have detected over the acrosomal region. Hoskins et al. (16) have described a 37,000 mol wt

glycoprotein produced in the epididymis of several mammalian species which apparently binds to sperm during transit and promotes the development of progressive forward motility via a cyclic nucleotide mechanism. Olson and Hamilton (29) using galactose oxidase -NaB[^{3}H]$_4$ labeling found a 37,000 mol wt glycoprotein associated with cauda, but not caput, epididymal sperm of the rat, suggesting that it may play a developmental role in sperm maturation or capacitation. Nicolson et al. (27) have studied the changes in surface proteins of rabbit sperm progressing from the caput to the cauda epididymis using lactoperoxidase-catalyzed iodination. They found seven radioactive peaks using SDS gels ranging in mol wt from ~35,000 to 100,000. Four of these bands increased significantly in cauda epididymal preparations including a 39,000 mol wt component. This is once again similar to the 40,000 mol wt species reported here. However, because the lactoperoxidase method does not discriminate between proteins and glycoproteins, it is difficult to directly compare these substances solely on an SDS gel weight determination. The data presented by Nicolson et al. (27) furthermore provide no information on the localization of this activity on the sperm surface. The present study again identifies a molecule of apparent similar molecular weight (~40,000) found on the surface of mammalian sperm using galactose oxidase labeling. In contrast to that in the study of Nicolson et al. (27), this substance makes its appearance or becomes activated only upon ejaculation rather than in the epididymis, and preliminary data indicate that it is lost (or masked) during the capacitation process. The data thus far suggest the working hypothesis that the molecule responsible for the antifibronectin staining is related or identical to the collagen-binding component extracted from the cell surface.

It is possible that the collagen-binding properties of this protein are purely coincidental. It may be yet another of the several sperm coating antigens or decapacitation factors described in recent years (17, 19, 39). These substances are added to the sperm during their transit through the tract, possibly to protect them from undergoing a premature acrosome reaction. Under normal circumstances sperm are restricted to compartments lined by epithelium and should not come into contact with collagen. There are, however, several lines of evidence which allow one to construct a hypothetical scenario which might account for the collagen-binding properties of sperm. For some time a collagenase-like enzyme has been implicated in the process of follicle rupture during ovulation (9). More recently, it has been shown that a plasminogen activator is produced by presumptive follicular cells in response to gonadotrophin, thereby producing plasmin (fibrinolysin) which attacks collagen and weakens the follicle wall to allow ovulation (36, 37). This process may allow some collagenous products and other debris to escape the ovarian surface during follicle rupture and, along with the egg mass, be swept into the oviduct. These collagenous elements could serve as a focus for sperm attachment as demonstrated in this report. Once localized to the general vicinity of the egg, the fibrinolysin remaining in the oviduct could aid in the removal of the collagen-binding protein from the sperm surface. Fibronectin is a known substrate for fibrinolysin (41). Furthermore, a collagenase-like peptidase has also been described as being present in sperm (21) and could also aid in detachment of the cells from the collagen fragments. Capacitation, therefore, could involve the removal of this collagen-binding surface material, allowing the acrosome reaction to proceed, leading in turn to the penetration of the sperm through the granulosa cells and zona pellucida of the ovum. That this is not an obligatory sequence of events is demonstrated by the successful fertilization of rabbits inseminated with epididymal spermatozoa (30).

Although in vivo interactions between sperm and collagen have not been described, it has been shown that an intravaginal sponge composed of cross-linked collagen acts as an efficient contraceptive device in humans (4, 5). These authors suggest that the contraceptive action of the sponge is probably the result of simple mechanical trapping of the ejaculate in the interstices of the sponge, but the possibility now exists that a more direct sperm-collagen association may be involved.

Human fetal skin collagen was kindly supplied by Dr. A. S. Narayanan (Department of Pathology) and cultures of mouse 3T3 cells were made available by Dr. T. Norwood (Department of Pathology). Secretarial and editorial work was provided by Ms. Doris Ringer.

This research was supported by National Science Foundation Grant PCM 77-01138 and by U. S. Public Health Service grants HD-03752 (J. K. Koehler) and CA-23907 (S. Hakomori) from the National Institutes of Health.

Received for publication 14 January 1980, and in revised form 25 April 1980.

REFERENCES

1. Balian, G., E. M. Glick, E. Crouch, J. M. Davidson, and P. Bornstein. 1979. Isolation of a collagen-binding fragment from fibronectin and cold insoluble globulin. *J. Biol. Chem.* 254:1429–1432.
2. Bonner, W. M. T., and R. A. Laskey. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *J. Biochem.* 46:83–88.
3. Chen, L. B., K. Burridge, A. Murray, M. L. Walsh, C. D. Copple, A. Bushness, J. K. McDougall, and P. H. Gallimore. 1978. Modulation of cell surface glycocalyx: studies on LETS protein. *Ann. N. Y. Acad. Sci.* 312:366–381.
4. Chvapil, M. 1976. An intravaginal contraceptive diaphragm made of collagen sponge: new old principle. *Fertil. Steril.* 27:1387–1397.
5. Chvapil, M., T. Chvapil, S. Jacobs, and J. S. Owen. 1977. Laboratory and clinical testing of an intravaginal contraceptive collagen sponge. *Contraception* 15:693–703.
6. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin to collagen. *Int. J. Cancer.* 20:1–5.
7. Eng, L. A., and G. Oliphant. 1978. Rabbit sperm reversible decapacitation by membrane stabilization with a highly purified glycoprotein from seminal plasma. *Biol. Reprod.* 19: 1083–1094.
8. Esbenshade, K. L., and E. D. Clegg. 1976. Electrophoretic characterization of protein in the plasma membrane of porcine spermatozoa. *J. Reprod. Fertil.* 47:333–337.
9. Espey, L. L., and P. Rondell. 1968. Collagenolytic activity in the rabbit and sow Graafian follicle during ovulation. *Am. J. Physiol.* 214:326–329.
10. Fukuda, M., and S. Hakomori. 1979. Proteolytic and chemical fragmentation of galactoprotein a, a major transformation sensitive glycoprotein released from hamster embryo fibroblasts. *J. Biol. Chem.* 254:5442–5450.
11. Gahmberg, C. G., and S. Hakomori. 1973. External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. *J. Biol. Chem.* 248: 4311–4317.
12. Gahmberg, C. G., and S. Hakomori. 1973. Altered growth behavior of malignant cells associated with changes in externally labeled glycoproteins and glycolipids. *Proc. Natl. Acad. Sci. U. S. A.* 70:3329–3333.
13. Hayman, E. G., and E. Ruoslahti. 1979. Distribution of fetal bovine serum fibronectin and endogenous rat cell fibronectin in extracellular matrix. *J. Cell Biol.* 83:255–259.
14. Hahn, L.-H. E., and K. M. Yamada. 1979. Identification and isolation of a collagen-binding fragment of the adhesive glycoprotein fibronectin. *Proc. Natl. Acad. Sci. U. S. A.* 76:1160–1163.
15. Herr, J. C., A. C. Hahnel, and E. M. Eddy. 1979. Mouse sperm surface antigens identified with heterologous antiserum and immunoabsorption. *J. Cell Biol.* 83(2, Pt. 2):228 *a* (Abstr.).
16. Hoskins, D. D., D. Johnson, H. Brandt, and T. S. Acott. 1979. Evidence for a role for a forward motility protein in the epididymal development of sperm motility. *In* The Spermatozoon, Maturation, Motility, Surface Properties and Comparative Aspects. D. W. Fawcett and J. M. Bedford, editors. Urban and Schwarzenberg, Munich. 43–56.
17. Hunter, A. G., and H. O. Nornes. 1969. Characterization and isolation of a sperm coating antigen from rabbit seminal plasma with capacity to block fertilization. *J. Reprod. Fertil.* 20:419–427.
18. Hynes, R. O. 1973. Alteration of cell surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. U. S. A.* 70:3170–3174.
19. Johnson, W. L., and A. G. Hunter. 1972. Seminal antigens: their alterations in the genital tract of female rabbits and during partial *in vitro* capacitation with β-amylase and β-glucuronidase. *Biol. Reprod.* 7:332–340.
20. Kaplan, J. E., and T. M. Saba. 1976. Humoral deficiency and reticuloendothelial depression after traumatic shock. *Am. J. Physiol.* 230:7–14.
21. Koren, E., and S. Milkovic. 1973. Collagenase-like peptidase in human, rat and bull spermatozoa. *J. Reprod. Fertil.* 32:349–356.
22. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond).* 227:680–685.
23. Linder, E., E. Vaheri, E. Ruoslahti, and J. Wartiovaara. 1975. Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med.* 142:41–49.
24. Mayol, R. F., and D. E. Longenecker. 1975. Separation of non-sperm components from

seminal preparations and their effect on the analysis of sperm proteins. *Proc. Soc. Exp. Biol. Med.* 149:64–69.
25. Mosesson, M. W. 1978. Structure of human plasma cold insoluble globulin and the mechanism of its precipitation in the cold with heparin or fibrin-fibrinogen complexes. *Ann. N. Y. Acad. Sci.* 312:11–30.
26. Nicander, L., L. Plöen, and M. Larsson. 1974. Specific apocrine secretion in the anterior lobe of the prostate gland of rabbits. *Cell Tissue Res.* 151:69–77.
27. Nicolson, G. L., A. B. Brodginski, G. Beattie, and R. Yanagimachi. 1979. Cell surface changes in the proteins of rabbit spermatozoa during epididymal passage. *Gamete Res.* 2: 153–162.
28. Oliphant, G., and C. A. Singhal. 1979. Iodination of rabbit sperm plasma membrane: relationship of specific surface proteins to epididymal function and sperm capacitation. *Biol. Reprod.* 21:937–944.
29. Olson, G. E., and D. W. Hamilton. 1978. Characterization of the surface glycoproteins of rat spermatozoa. *Biol. Reprod.* 19:26–35.
30. Orgebin-Crist, M. C. 1967. Maturation of spermatozoa in the rabbit epididymis: fertilizing ability and embryonic mortality in does inseminated with epididymal spermatozoa. *Ann. Biol. Anim. Biochim. Biophys.* 7:373–389.
31. Pearlstein, E. 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature (Lond).* 262:497–499.
32. Ruoslahti, E. and A. Vaheri. 1974. Disappearance of a major cell-type specific surface glycoprotein antigen (SF) after transformation of fibroblasts by Rous Sarcoma virus. *Int. J. Cancer.* 13:579–586.
33. Ruoslahti, E., E. G. Hayman, J. E. Kumsela, J. E. Shirely, and E. Engvall. 1979. Isolation of a tryptic fragment containing the collagen-binding site of plasma fibronectin. *J. Biol. Chem.* 254:6054–6059.
34. Schwarz, M., and J. K. Koehler. 1979. Alterations in lectin binding to guinea pig spermatozoa accompanying *in vitro* capacitation and the acrosome reaction. *Biol. Reprod.* 21:1295–1307.
35. Stenman, S., and A. Vaheri. 1978. Distribution of new major connective tissue protein, fibronectin, in normal tissue. *J. Exp. Med.* 7:1054–1064.
36. Strickland, C., and W. H. Beers. 1976. Studies on the role of plasminogen activator in ovulation. *J. Biol. Chem.* 251:5694–5702.
37. Strickland, C., and W. H. Beers. 1979. Studies of the enzymatic basis and hormonal control of ovulation. *In* Ovarian Follicular Development and Function. A. R. Midgley and W. A. Sadler, editor. Raven Press, New York. 143–153.
38. Wartiovaara, J., I. Leivo, I. Virtanen, and A. Vaheri. 1978. Cell surface and extracellular matrix glycoprotein, fibronectin: expression in embryogenesis and teratocarcinoma differentiation. *Ann. N. Y. Acad. Sci.* 312:132–141.
39. Williams, W. L., R. T. Robertson, and W. R. Dukelow. 1979. Decapacitation factor and capacitation. *In* Advances in Bioscience, Vol. 4. G. Raspe, editor. Pergamon Press, New York. 61–72.
40. Yamada, K. M., and J. A. Weston. 1974. Isolation of a major cell surface glycoprotein from fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 71:3493–3496.
41. Yamada, K. J., and K. Olden. 1978. Fibronectins-adhesive glycoproteins of cell surface and blood. *Nature (Lond).* 275:179–184.
42. Zetter, B. R., G. R. Martin, C. R. Birdwell, and D. Gospodarouwicz. 1978. Role of high molecular weight glycoprotein in cellular morphology adhesion and differentiation. *Ann. N. Y. Acad. Sci.* 312:299–316.