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BINDING BY GLYCOPROTEINS OF SEMINAL PLASMA MEMBRANE VESICLES ACCELERATES DECAPACITATION IN RABBIT SPERMATOZOA

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Fertilizing capacity among uterine-capacitated rabbit sperm cells declined exponentially during incubation with membrane vesicles from seminal plasma. In suspensions containing an average of 0.42 mg vesicle protein/10^6 sperm, decapacitation occurred with a half-time of 23 min (k_i (native vesicles) = 1.78 ± 0.14 h^{-1}). Exposing these membrane vesicles to pronase retarded decapacitation, prolonging its half-time to 51 min (k_i (pronase-digested vesicles) = 0.81 ± 0.06 h^{-1}). Cholesterol-bearing liposomes suppressed sperm-fertilizing capacity at a comparable rate. In suspensions containing an average of 0.52 mg lipid/10^6 sperm, decapacitation had a half-time also of 51 min (k_i (liposomes) = 0.82 ± 0.14 h^{-1}). These lower inhibition rates accompanied diminished rates of vesicle uptake by spermatozoa. Membrane vesicles labeled with phosphatidyl[^{14}C]choline rapidly bound to epididymal sperm cells, displaying a half-time of 2.3 min (k_a (native vesicles) = 18.0 ± 0.35 h^{-1}). Following pronase treatment, this interval increased to 17 min (k_a (pronase-digested vesicles) = 2.48 ± 0.37 h^{-1}). Liposome binding data yielded a half-time of 28 min (k_a (liposomes) = 1.47 ± 0.17 h^{-1}). Postbinding decapacitation half-times for these vesicles, given by the difference between binding and decapacitation intervals, appear broadly alike: native vesicles, 21 min, pronase-digested vesicles, 34 min, and liposomes, 23 min. During this interval, a vesicle antifusigen (cholesterol) apparently transfers to the sperm plasma membrane inhibiting the acrosome reaction. The lipid bilayer in these membrane vesicles withstood proteolytic attack, as seen by electron microscopy. Pronase acted principally to hydrolyze vesicle glycoproteins, which evidently bind to the sperm surface during decapacitation.

Introduction

Membrane vesicles from seminal plasma reversibly inhibit fertilizing capacity among uterine-capacitated rabbit spermatozoa by a direct, non-species-specific action on the sperm cell [1–4]. Decapacitation assays with membrane vesicles modified through pronase digestion or lipid elution and synthetic phospholipid vesicles bearing cholesterol indicate that they act as sterol donors [5,6]. By elevating cholesterol levels in the phospholipid bilayer of the sperm membrane, these vesicles could block initiation of the acrosome reaction [4,7]. In this reaction, the plasma membrane undergoes a Ca^{2+} promoted fusion with the outer acrosomal membrane leading to exposure of hydrolytic enzymes that aid sperm penetration of the mammalian egg [8].

A careful study of decapacitation induced by membrane vesicles from rabbit seminal plasma revealed a decrease in effectiveness, approaching statistical significance, following pronase treatment [9]. Since pronase acts on these vesicles to hydrolyze surface glycoproteins quantitatively [5],

the possibility arises that they play a part in sperm decapacitation. If substantiated, this could involve facilitating transfer of an inhibitor (sterol) from vesicles to sperm plasma membrane. A number of previous investigators have suggested that glycoproteins may participate in decapacitation. Experiments with pronase-denuded vesicles seem to provide, therefore, an opportunity to obtain evidence supporting the proposal.

The present endeavor was undertaken primarily to clarify the effects of proteolysis by pronase on the decapacitation action of membrane vesicles from rabbit seminal plasma. In particular, evidence was sought for a protein facilitated inhibitor transfer process during decapacitation of uterine-capacitated rabbit sperm cells. For this purpose, the time course of decapacitation was established with native and pronase-digested membrane vesicles, and with liposomes prepared from phosphatidylcholine and cholesterol. Rate constants determined from these data on the kinetics of decapacitation were then compared with those found for sperm binding of radioactively labeled vesicles and liposomes.

Materials and Methods

In these experiments, rabbits of mixed strain, with proven fertility, were used. The isolation of membrane vesicles from freshly obtained seminal plasma was performed by ultracentrifugation on a discontinuous density gradient of sucrose, dissolved in 0.05 M KCl/0.01 M Tris (pH 7.4) at $90\,000 \times g$ for 4 h at 4°C [2]. Protein levels in the vesicle-containing fractions from the gradient were established colorimetrically [10]. Sucrose was removed by dialysis. Vesicle proteolysis occurred during a 4 h incubation at 37°C with 10% (w/w protein) pronase E (Sigma), assayed to contain 3–4 proteolytic units/mg protein. Postincubation vesicle recovery was performed by sedimentation on a sucrose layer (4–10%) at $110\,000 \times g$ for 4 h at 4°C. Sometimes this step was also performed by gel filtration on a Sephadex G-200 column (1 cm diameter, 25 cm length) using Tris-KCl buffer as eluant.

Liposome preparation involved prolonged ultrasonication, as previously described [6]. In five experiments, liposomes were used with a cholesterol/phospholipid mole ratio of 1.3 and for a remaining two the ratio was 0.6 and 0.4. Each liposome preparation inhibited fertilization and data from these seven experiments have been pooled.

Decapacitation by vesicle and liposome preparations was assayed using sperm cells flushed with 4 ml Hanks' solution (Difco) from the uterus of a donor 7–10 h after being bred to two or three bucks. The uterine sperm were incubated for specified intervals at 23°C while suspended in 1 ml of Ca^{2+} enriched Hanks' solution [3]. In test suspensions, native or pronase-modified membrane vesicles were present at an average of 0.42 mg vesicle protein/10^6 sperm. For the liposome experiments, there was an average of 0.52 mg lipid/10^6 sperm. These incubated sperm cells were then deposited into the oviduct of a recipient doe given human chorionic gonadotrophin (Squibb) to induce ovulation. Sperm fertilizing capacity was judged from the fertilization rate noted among eggs flushed from the oviducts of recipients 1 day post-insemination [3]. To establish control fertilization rates, sperm cells incubated in the absence of vesicles were routinely placed into the contralateral oviduct. In each experiment, sperm fertilizing capacity was assayed at all specified intervals. However, any interval represented by an aggregate of less than 10 eggs was omitted from the analysis of sperm decapacitation rate. The fertilization rates presented are based on results obtained in five to nine experiments.

The kinetics of association between membrane vesicles and spermatozoa were examined using cauda epididymal sperm cells and vesicles bearing phosphatidyl[^{14}C]choline. These vesicles achieved a specific activity of 0.07 μCi phosphatidyl [^{14}C]choline/mg protein following incubation with a phospholipid-Celite suspension [11]. Spermatozoa ($4 \cdot 10^6$ sperm/ml) from a freshly excised epididymis were suspended in 4.25 ml Krebs-Ringer bicarbonate medium (pH 7.0) and incubated in 12×75 mm plastic tubes (Falcon) at 23°C with 3600 cpm phosphatidyl[^{14}C]choline vesicles. After incubation, the suspension was rapidly chilled and the sperm cells sedimented at $1000 \times g$ for 30 min onto a 60% (w/v) layer of sucrose in Tris-KCl buffer. They were washed in 4 ml buffer, resedimented and then assayed for radioactivity in a liquid scintillation counter (Nuclear

Chicago) with Aquasol (New England Nuclear) as phosphor.

For electron microscopy, vesicle pellets formed by sedimentation at 168 000 × *g* for 2.5 h were fixed with OsO_4, embedded in Epon (Ladd), sectioned on a glass-blade microtome, and stained with uranyl acetate and lead citrate before viewing under a Zeiss (EM 9S2) electron microscope [2]. Polypeptide patterns of rabbit seminal plasma fractions were obtained by gel electrophoresis using Laemmli's method [12]. The gels were stained for protein with Coomassie Brilliant Blue (Mann) and for sugar with periodic acid-Schiff reagent (Fisher). A densitometer (Joyce-Loebl) provided quantitative scans of polypeptide bands.

Results

Pronase-digested membrane vesicles

Native and pronase-digested membrane vesicles from rabbit seminal plasma form polydisperse populations comprising numerous small vesicles with cross-sectional diameters between 50 and 60 nm (Fig. 1). Enzymatic proteolysis has clearly not destroyed these vesicles or even noticeably altered their ultrastructure.

Protein recoveries for the modified vesicles given in Table I show that 29% of vesicle protein is removed during incubation with pronase. As vesicle structure withstands proteolytic attack by this enzyme (Fig. 1), hydrolyzed proteins necessarily

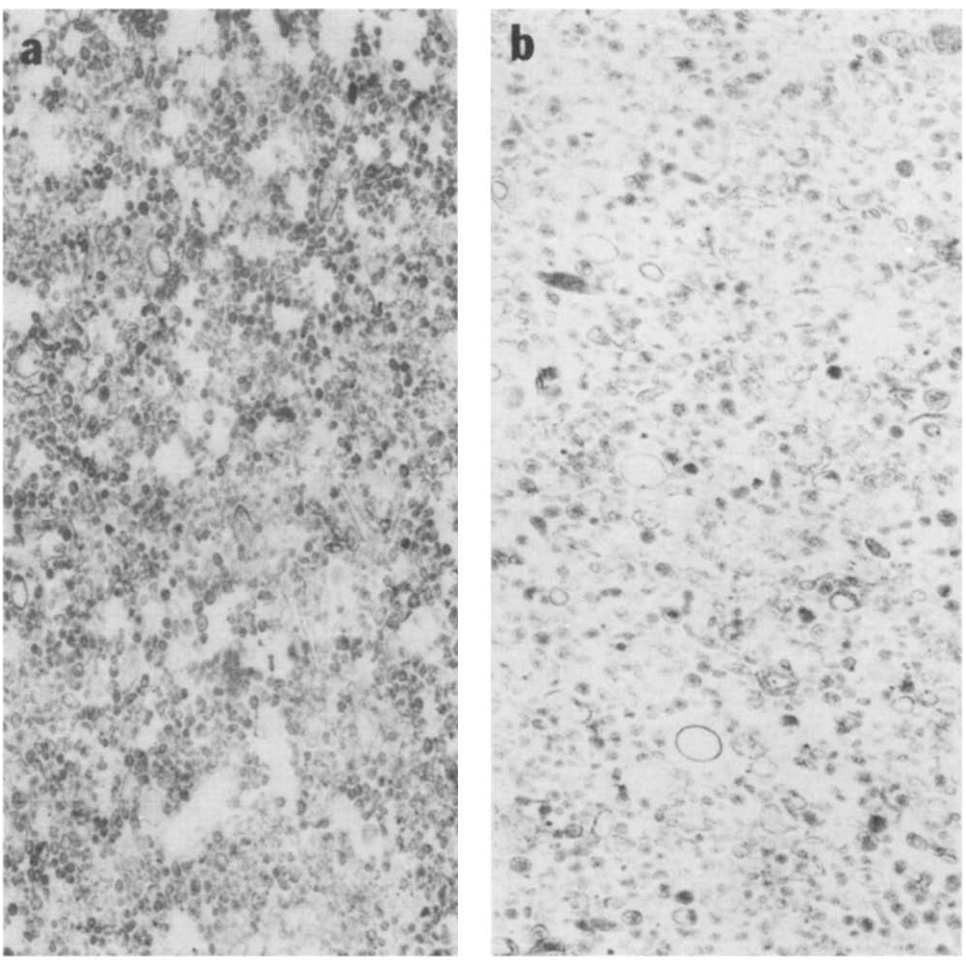

Fig. 1. Electron micrographs of membrane vesicles from rabbit seminal plasma. (a) Native vesicles, and (b) pronase-digested vesicles (magnification 35 000 ×).

TABLE I

EFFECT OF PRONASE ON PROTEIN LEVELS IN MEMBRANE VESICLES FROM RABBIT SEMINAL PLASMA

Following incubation for 4 h at 37°C in the presence and absence of 10% (w/w) pronase, vesicles and enzyme were separated (Materials and Methods), and the vesicle fraction then assayed for protein.

Experiment	Vesicle protein level (mg)		Recovery
	Native	Pronase-digested	
I	1.40	1.00	0.71
II	0.61	0.44	0.73
III	0.60	0.40	0.67
$\bar{X} \pm$ S.E.			0.71 ± 0.02

occupy exposed locations on the vesicle surface. To clarify this action, rabbit seminal plasma was fractionated into heavy (H) vesicles (density, 1.20 g/cm^3) and light (L) vesicles (density, 1.16 g/cm^3) by isopycnic sedimentation on a sucrose density gradient. Each vesicle fraction accounted for ap-

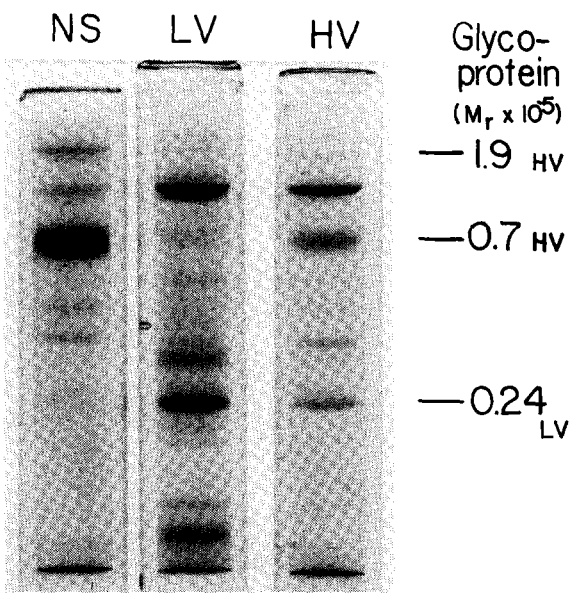

Fig. 2. Polypeptide bands characterizing rabbit seminal plasma fractions following electrophoresis on 7.5% polyacrylamide gels with glycine-Tris buffer (pH 8.5)/0.1% SDS. N.S., non-sedimenting fraction; L.V., L-vesicle (density, 1.16 g/cm^3) fraction; H.V., H-vesicle (density, 1.20 g/cm^3). Stain, Coomassie brilliant blue.

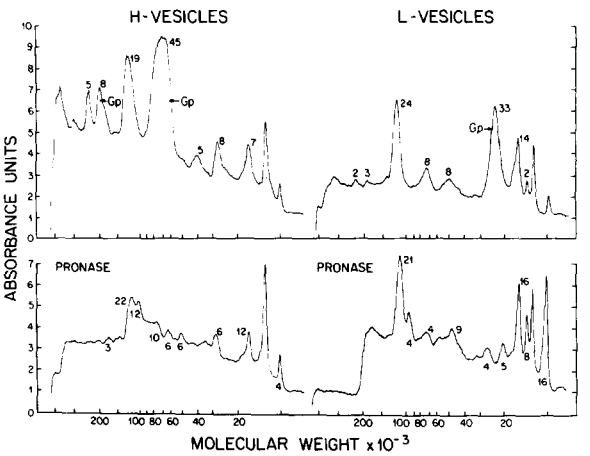

Fig. 3. Polypeptide profiles in native and pronase-digested membrane vesicles from rabbit seminal plasma after detergent-gel electrophoresis. Densitometer peak area, as percent ages, are shown; numbers appearing below the trace indicate bands apparent after vesicle exposure to pronase. Molecular weights were estimated from the mobility of serum albumin (68000), ovalbumin (44000), and lysozyme (14000). Gp denotes glycoprotein. Stain, Coomassie brilliant blue.

prox. 5% of fluid protein. The remaining 90% of protein forms a non-rapidly sedimenting fraction. Fig. 2 shows polypeptide profiles for these seminal plasma fractions obtained with detergent-gel electrophoresis. These seminal plasma fractions can be seen to display distinct, complex polypeptide patterns. Differences between H- and L-vesicle proteins reflect the fact that they have distinct sites of origin within the male reproductive tract [9]. Glycoproteins in H-vesicles have M_r $1.9 \cdot 10^5$ and $0.7 \cdot 10^5$ and in L-vesicles there is a single glycoprotein of $0.24 \cdot 10^5$ (Fig. 2). The glycoproteins are quantitatively the most significant proteins in both classes of vesicle (Fig. 3). Fig. 3 shows that they are completely hydrolyzed by pronase. In contrast, non-sugar-bearing proteins reveal mainly slight or moderate change with proteolysis. Pronase promoted increases in some small bands presumably reflect migration by hydrolysis products. No Coomassie blue staining bands were visible following electrophoresis of these vesicle preparations on 5% (w/v) per cent polyacrylamide gels using 0.188 M glycine/0.188 M Tris buffer (pH 8.5) and omitting detergent. This indicates they were free from detectable protein contaminants.

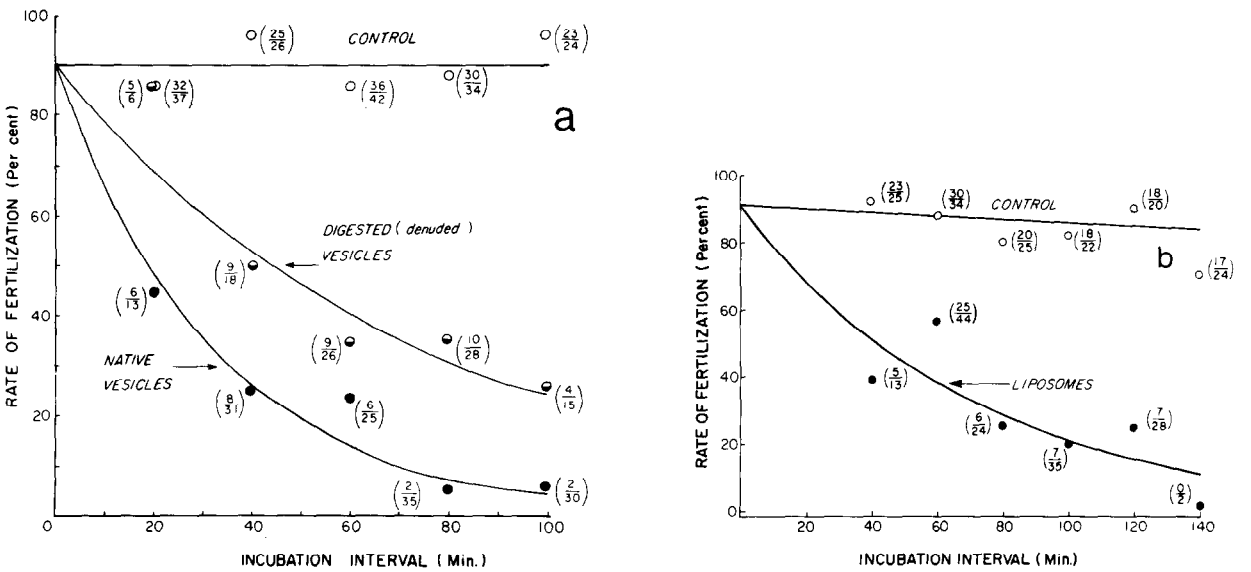

Fig. 4. Decrease in fertilization rate achieved by uterine-capacitated rabbit spermatozoa after incubation with membrane vesicles and liposomes. (a) Native and pronase-digested (denuded) membrane vesicles from rabbit seminal plasma, and (b) phosphatidylcholine liposomes bearing cholesterol. Given in parenthesis are the fertilized and total number of eggs. The curve is specified by: $F_t/F_0 = \exp[-\bar{k}t]$, F_0 and F_t represent fertilization rates corresponding to incubation intervals of 0 and t h, and $\bar{k}$ is an average rate constant (Table II).

Rate of decapacitation

Fertilizing capacity among uterine-capacitated rabbit sperm cells decreased exponentially with time in the presence of membrane vesicles isolated from homologous seminal plasma (Fig. 4a). Fig. 4 also shows that proteolytic digestion retarded the rate of vesicle-induced decapacitation. It is apparent, nevertheless, these modified vesicles are still potent inhibitors of fertilization. Thus, spermatozoa incubated for 100 min with 0.42 mg pronase-digested vesicle protein/10^6 sperm (around $30 \cdot 10^6$ vesicles/sperm) fertilized 27% (4/15) of eggs flushed from the oviducts of does 1 day post-insemination. At this interval, control sperm can be seen to have achieved a fertilization rate of 96% (23/24). By comparison, native vesicles lowered fertilization to 6% (2/32). Sperm-fertilizing capacity declined exponentially also during incubation in a suspension of liposomes containing cholesterol (Fig. 4b). The rate of decapacitation in the liposome suspension (0.52 mg lipid/10^6 sperm) broadly compares with that for pronase-digested membrane vesicles. For example, at the 100 min-interval, liposome-treated sperm fertilized 20% (7/35) of eggs vs. 27% for those exposed to pronase-digested vesicles.

Sperm binding of phosphatidyl[^{14}C]choline vesicles

Native membrane vesicles labeled with phosphatidyl[^{14}C]choline bound rapidly to cauda epididymal spermatozoa during incubation at 23°C

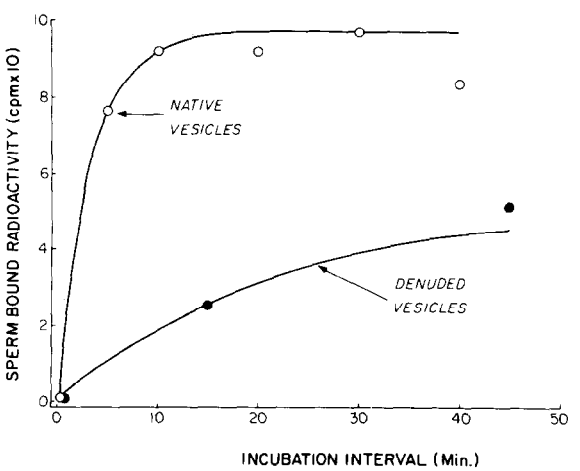

Fig. 5. Effect of pronase on the uptake of phosphatidyl [^{14}C]choline bearing membrane vesicles from rabbit seminal plasma by epididymal sperm cells. Vesicle suspensions lacking spermatozoa were also included to establish the level of non-sperm bound radioactivity. The results are based on duplicate observations. Each curve is based on an average first-order rate constant (Table II).

TABLE II

RATE CONSTANTS FOR BINDING AND DECAPACITATION WITH NATIVE AND PRONASE-DIGESTED MEMBRANE VESICLES FROM SEMINAL PLASMA AND LIPOSOMES CONTAINING CHOLESTEROL DURING INCUBATION OF RABBIT SPERMATOZOA

Average rate constants ($\bar{X} \pm$ S.E.) for binding (k_a) and decapacitation (k_i) were determined from data in Figs. 5 and 4, respectively. A first order relationship was used: $k = -(\ln(x/x_0)/t)$, x_0 and x are the fractions, at zero time and t h of available binding sites and fertilizing spermatozoa.

Vesicles	k_a (h^{-1})	k_i (h^{-1})
Native	18.1 ± 0.35	1.78 ± 0.14
Pronase-digested	2.5 ± 0.37	0.81 ± 0.06
Liposomes	1.5 ± 0.17	0.82 ± 0.14

(Fig. 5). At 50 μg vesicle protein/$17 \cdot 10^6$ sperm (approximately $5 \cdot 10^6$ vesicles/sperm), uptake was virtually complete in 10 min and amounted to nearly 3% of suspended vesicles. Pronase-modified vesicles displayed diminished affinity for the sperm surface. After incubation for 50 min, only about 1.5% of these vesicles bound to sperm cells. When spermatozoa incubated with radioactively labeled H-vesicles were subjected to brief sonication, it could be demonstrated that the sperm-bound radioactivity largely retained the bouyant density of these vesicles during floatation centrifugation in a sucrose density gradient. This finding indicates the membrane vesicles bind to the sperm surface when they interact with spermatozoa.

Decapacitation and binding rate constants

First-order rate constants derived from present findings on the kinetics of vesicle-sperm association (k_a) and inhibition of fertilization (k_i) are listed in Table II. The liposome k_a given by Table II was determined from previously reported uptake data for dipalmitoylphosphatidylcholine liposomes containing either [^{3}H]cholesterol or phosphatidyl[^{14}C]choline [13]. These rate constants obviously reflect the rapidity of binding relative to decapacitation. Retardation of both processes following proteolysis of membrane vesicles is also apparent. In fact, it is evident that pronase reduced membrane-vesicle affinity and decapacitation activity to roughly liposome levels.

Discussion

Membrane vesicles with decapacitation activity, from seminal plasma, were demonstrated to bind rapidly with rabbit spermatozoa and inhibit their fertilizing capacity, following uterine-capacitation, at an exponential rate. Rate constants determined under present conditions for these processes indicate $t_{1/2}$ (binding) = 2.3 min and $t_{1/2}$ (decapacitation) = 23 min. Their difference yields an interval of almost 21 min that corresponds to a post-binding period for vesicle-induced decapacitation. During this stage, an inhibitor, such as cholesterol, could transfer by exchange diffusion from membrane vesicle to sperm plasma membrane.

Decapacitation was retarded among pronase-treated vesicles. Their binding affinity also diminished. As the enzyme acts principally to hydrolyze glycoproteins, they evidently occupy exposed sites on the vesicle surface and participate in vesicle-sperm binding interactions. Interestingly, binding and decapacitation by pronase-denuded vesicles and cholesterol-bearing liposomes occurred at comparable rates. For these preparations, the post-binding stage of decapacitation can be estimated to be 34 and 22 min, respectively. It may be noted that this stage has a similar duration with native membrane vesicles. Bound native and glycoprotein-free vesicles, therefore, possess somewhat analogous decapacitation activities. In view of the proposed sterol-transfer mechanism, it is relevant that, additionally, they behave in a liposome-like manner. These results can be viewed as suggesting that vesicle glycoproteins enhance decapacitation activity, not by exerting a direct inhibitory action on sperm fertilizing capacity, but through an acceleration of vesicle uptake by spermatozoa.

Retarded decapacitation apparently explains an early report [14] that the rapidly sedimenting decapacitation factor in rabbit seminal plasma is inactivated by pronase. In the report, spermatozoa were exposed to the inhibitor for an unspecified interval, and since the conclusion that inactivation occurred rests upon the fertilization rate observed among a small number of eggs, the exposure period was possibly too brief for decapacitation.

Bedford and Chang [15] suggested that a glycoprotein might cause decapacitation, on the basis of

indirect evidence. They drew this inference largely from the ability of cold ethanol to precipitate the macromolecular principle in rabbit seminal plasma responsible for decapacitation. However, it is not surprising that membrane vesicles, which possess a glycoprotein coat, precipitate from this fluid under identical conditions (unpublished observation). These investigators were also unaware that seminal plasma decapacitation activity can withstand proteolytic attack by pronase, making a protein inhibitor highly unlikely. Notwithstanding this finding, some investigators attribute decapacitation activity to specific seminal plasma glycoproteins [16]. Estimates of their molecular weight range from $1 \cdot 10^5$ to $3 \cdot 10^5$. Decapacitation factor in rabbit seminal plasma sediments at 55 s and above [1,9], and this insures it of a hydrodynamic molecular weight at least an order of magnitude higher. Moreover, these reports involve unwarranted assumptions in calculating activity or, alternatively, they rely upon only indirect evidence of decapacitation.

Although the present endeavor establishes a role for glycoproteins in decapacitation, much remains to be elucidated about the location and identity of vesicle interaction sites on the sperm plasma membrane. Electron micrographs showing liposome-sperm interactions have been obtained by Friend [17]. He has also found membrane vesicle-sperm association (personal communication). Immunochemical evidence of a macromolecular seminal plasma coat on the mammalian sperm has been presented by Weil [18]. The coat-forming substance displayed a molecular weight around $4 \cdot 10^6$, and this could correspond to a small (hydrated diameter, 20 to 30 nm) membrane vesicle from seminal plasma.

Acknowledgments

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