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Macromolecular Inhibitor of Fertilization in Rabbit Seminal Plasma

B. K. DAVIS

Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 01545

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ABSTRACT **A large (86S) glycoprotein isolated from rabbit seminal plasma acts on sperm cells to block fertilization. The proportion of fertilized eggs recovered from does 1 day after tubal insemination of sperm 5–6 hr before or 2–3 hr after ovulation was significantly depressed when uterine-capacitated spermatozoa were exposed to this substance at concentrations of 20–72 μg of protein/ml. Rabbit seminal plasma was estimated to have about 2 mg/ml of this factor. Another large glycoprotein (∽138S) is also present in rabbit seminal plasma; the function of this molecule awaits elucidation.**

Spermatozoa from several mammalian species have been known for some time to need a period of hours within the female reproductive tract before gaining the capacity for fertilization (1–3). The presence of an inhibitor of spermatozoan fertilizing capacity, of a high molecular weight, in rabbit seminal plasma, has been suspected since it was observed that ultracentrifugation removes within a few hours the inhibitory effect of seminal plasma upon fertilization (4, 5). Conceivably, this factor masks, under physiological conditions, the fertilizing capacity of sperm cells in rabbits and perhaps other mammalian species. If substantiated, it would be a novel molecular regulator of cellular function.

An attempt to isolate this putative inhibitor molecule was undertaken. Two high molecular weight fractions were recovered after ultracentrifugation of rabbit seminal plasma on relatively dense sucrose zones. The slower-sedimenting fraction inhibits fertilization in a manner similar to that of seminal plasma; evidence revealing it to be a glycoprotein was obtained.

MATERIALS AND METHODS

Seminal plasma

Samples of seminal plasma were collected with the aid of an artificial vagina from intact and vasectomized bucks. Seminal plasma from each source was treated separately, but the individual samples collected for a day were pooled. These samples were then centrifuged at 3000 rpm (1000 × g) for 5 min (seminal plasma from vasectomized bucks) or 30 min (seminal plasma from intact animals). The resulting supernatant was aspirated off and stored at −4°C. Samples obviously contaminated with urine were not included.

Fractionation

Initially, seminal plasma was concentrated about threefold in a collodion bag apparatus (Schleicher & Schuell) that retains molecules of molecular weight over 100,000. About 1.5–2.0 ml of concentrated seminal plasma was layered over a 5–25% (w/v) sucrose density gradient made up in a solution of 0.15 M KCl–0.01 M Tris, pH 7.4. Centrifugation was performed at 4°C in a Beckman L-2 ultracentrifuge with an SW 25.1 rotor at 25,000 rpm for 8.5–10 hr. The sucrose gradient preparation was removed through a hole pierced in the bottom of the cellulose nitrate centrifuge tube with a hollow needle (23 gauge). 0.8-ml fractions were collected at 4°C. The absorbance of the fractions at 278 or 280 nm was measured in a Beckman DB spectrophotometer. The gradient fractions in the vicinity of a peak were usually pooled and subsequently stored at −4°C.

Another procedure, which permitted reasonably large volumes of seminal plasma to be fractionated, was also employed. In this method, 6 ml of 20% sucrose was layered on 4 ml of 80% sucrose, and 17 ml of seminal plasma was pipetted on top. The sucrose solutions were made up in 0.15 M KCl–0.01 M Tris, pH 7.4, as before. Centrifugation was for 8.5 hr. 0.5-ml fractions were collected throughout the sucrose region of this preparation; the upper part, the original seminal plasma region, was aspirated off. The absorbance of these fractions at 270 nm was recorded, and the total UV-absorption spectrum was obtained with a Cary spectrophotometer operated at 0.5 nm/sec.

Material from some gradients was resedimented to furnish evidence of homogeneity or for further purification. In this operation, the fraction was first dialyzed against 1000 volumes of buffer (0.15 M KC1–0.01 M Tris, pH 7.4) for 12 hr in order to lower the concentration of sucrose. A collodion bag apparatus was then used to concentrate the sample. The concentrated fraction was resedimented on a linear sucrose density gradient (5–25%) as described in the legend to Fig. 1.

Electrophoresis

Gelatinized cellulose acetate strips (Colab), 2.5 × 18 cm' were used in electrophoresis. The electrophoresis was actually conducted in a Gelman tray containing barbitone buffer, pH 8.6, $\Gamma/2 = 0.05$ (6). During the run, a Canalco power-pack (model 200) delivered a dc current of 1 mA/strip and the run was allowed to proceed for 4 hr. In this time, bromophenol (Canalco), which was spotted at the origin on either side of a 1-cm sample band, migrated approximately 8 cm toward the anode. The sample, 10 or 15 μl, was applied with a microcapillary pipette (Drummond). On completion of electrophoresis, the cellulose acetate strips were stained by treatment with Ponceau S in 3% trichloroacetic acid or by periodic acid–Schiff (7).

Sedimentation in the analytical ultracentrifuge

An An-D rotor and a 4° sector cell (standard and wedge) in a Spinco model E ultracentrifuge, operated at 33,450 rpm, furnished sedimentation velocities for the present work. Throughout the centrifugal run, photographs were taken of the Schlieren patterns that developed. The run was usually completed after 20 min of centrifugation at the set speed. The distance of

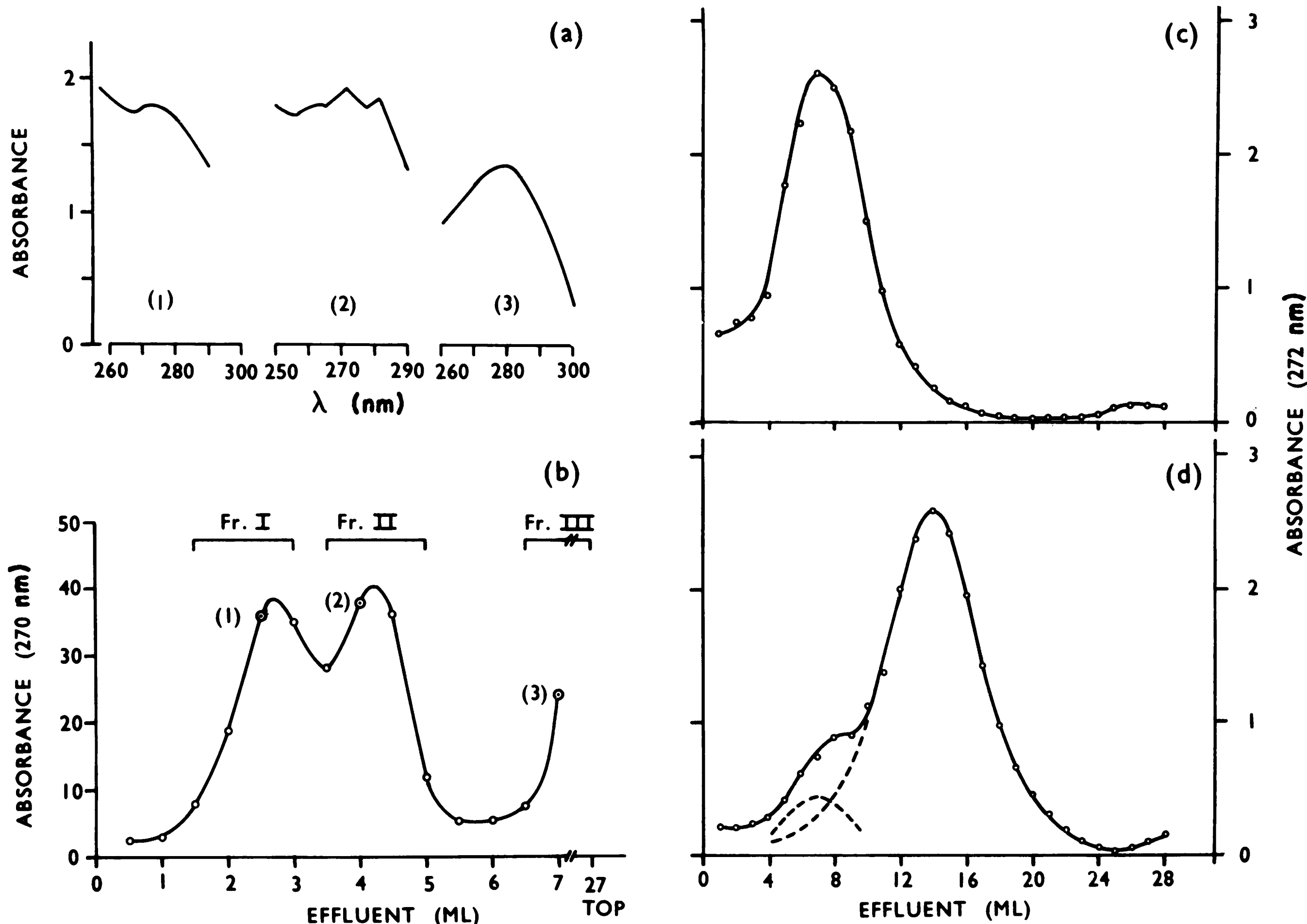

FIG. 1. Sedimentation pattern and UV spectral properties of seminal plasma obtained from intact bucks. (*a*) UV absorption spectra of three fractions are given from the sucrose zonal preparation shown in (*b*), where they are indicated as circled and numbered fractions. (*b*) Seminal plasma (17 ml) was centrifuged at 25,000 rpm for 8.5 hr at 5°C on a sucrose zonal preparation with 6 ml of 20% sucrose (w/v) on 4 ml of 80% sucrose. 0.5-ml fractions were collected in the 'sucrose region' of the preparation and their absorbance at 270 nm is given. Pooling the effluent under the bars gave fractions I, II, and III. (*c*) Result of resedimenting fraction I after dialysis against buffer to lower the concentration of sucrose followed by concentration with a collodion bag apparatus. Sedimentation on a linear gradient of 5–25% sucrose at 25,000 rpm for 7 hr. The absorbance at 272 nm of 1-ml fractions obtained from the gradient are given in the figure. (*d*) Result of resedimenting fraction II in the same way.

a given peak from the upper reference line was measured on a shadowgraph projector (Nikon).

Bioassay of fertilization capacity

The capacity of sperm for fertilization was judged from the proportion of fertilized ova recovered from does 1 day after insemination (2). Usually about 20,000 sperm were instilled into the oviduct in 0.01–0.02 ml of Hank's medium or Medium 199 (Difco). The sperm were obtained by flushing, with 4–8 ml of medium, the uteri of does bred thrice in a short interval 11–12 hr before autopsy. Washings from the uterus were subdivided, usually 1 ml per 12-ml centrifuge tube; the test solution was then added and the sperm were concentrated by sedimentation at 3000 rpm for 15 min and resuspension, by gentle agitation, in 0.2 ml of supernatant. In order to minimize the effect of animal variation, control (buffer only) sperm were usually instilled into the oviduct contralateral to that in which treated sperm were deposited. Upon completion of a series of inseminations, frequently involving four does, the cell count and motility of sperm samples were determined. Motility was obvious at this time in all the semen samples used. During insemination a recipient doe was subjected to anesthesia with an intravenous injection of sodium pentobarbital (Diamond), 30 mg/kg body weight. Ovulation was induced by an injection into the ear vein of 90 IU of chorionic gonadotropin (Squibb), either 4–5 or 12–13 hr before insemination. One day after insemination, the eggs were recovered under a dissecting microscope after the oviducts had been flushed with isotonic saline onto a watch-glass. At the time of recovery, fertilized eggs were nearly all 8-cell and, consequently, conspicuous. The recovered eggs were, however, fixed in acetic acid–alcohol 1:3 and subsequently stained with lacmoid (8) for cytological inspection.

RESULTS

Sedimentation on sucrose of seminal plasma collected from intact bucks revealed the presence of two fast-sedimenting fractions (Fig. 1*b*). These two fractions and the upper part of the preparation, corresponding to the original sample region, were recovered upon fractionation. From Fig. 1*b* it is apparent

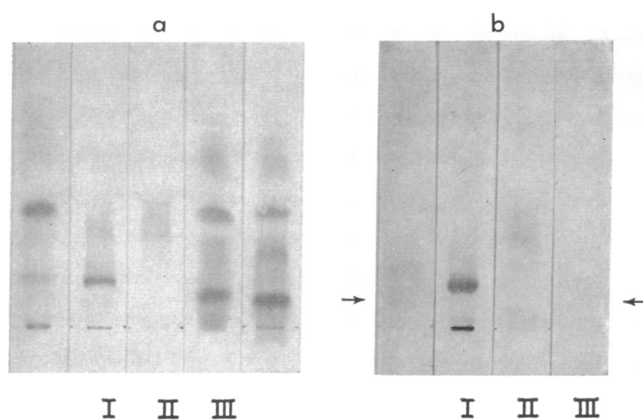

FIG. 2. Cellulose acetate electrophoresis of fractions of seminal plasma collected from intact bucks and of unfractionated seminal plasma from intact rabbits (*last lane of a* and *first lane of b*) and vasectomized rabbits (*first lane of a*) rabbits. Fractions I, II, and III designated here correspond to those in Fig. 1*b*. The electrophoresis, in barbitone buffer, was for 4 hr at 1 mA/strip. Staining with (*a*) Ponceau S, (*b*) periodic acid–Schiff. The arrows denote the location of faded bands in fraction III and in unfractionated seminal plasma.

that fractions I and II were only partially resolved. Resedimentation of fraction (I on a 5–25% (w/v) sucrose gradient showed it to be fairly homogeneous (Fig. 1*c*). Fraction II, which included the fraction tube with material from between the peaks (Fig. 1*b*), obviously contained some of fraction I and this appeared as a small leading peak upon resedimentation (Fig. 1*d*); this material was excluded from fraction II. When a small volume (1.5–2.0 ml) of concentrated seminal plasma was sedimented on a linear density gradient of 5–25% sucrose at a speed of 25,000 rpm for 8.5–10 hr as in the initial method of fractionation, fraction II alone was recovered and it was found to be homogeneous on resedimentation; fraction I sedimented to the bottom of the centrifuge tube in this procedure. Sedimentation causes seminal plasma to lose its cloudy opalescence, which is then associated with the fraction I and II regions in the centrifuge tube. These fast-sedimenting fractions are apparently large molecules that cause appreciable light scattering. Fig. 1*a* shows that the three fractions have distinct UV-absorption spectra: for fraction I, λ(max) ≌ 270 nm; II had λ (max) = 272 nm, with lesser peaks discernible at 264 and 281 nm; III had λ(max) = 280 nm. Absorption in the 280-nm region could be due to tyrosine present in protein. Biuret-positive material was present in each fraction. About two-thirds of seminal plasma protein (30 mg/ml) was estimated to remain during sedimentation in fraction III (21 mg/ml). Fractions I and II occurred in approximately equal amounts, 2 mg/ml seminal plasma. A sample of seminal plasma from vasectomized rabbits was estimated to have 19 mg protein/ml.

Electrophoresis was performed with strips of cellulose acetate and barbitone buffer. Seminal plasma from intact bucks had more bands sensitive to Ponceau S than that of vasectomized animals (Fig. 2*a*), and this is consistent with its higher protein content. Fraction III displayed several bands (Fig. 2*a*), which were closely homologous with those of unfractionated seminal plasma from intact rabbits. On the other hand, fractions I and II were rather homogeneous; fraction I had only a single strong band, which migrated slowly, and

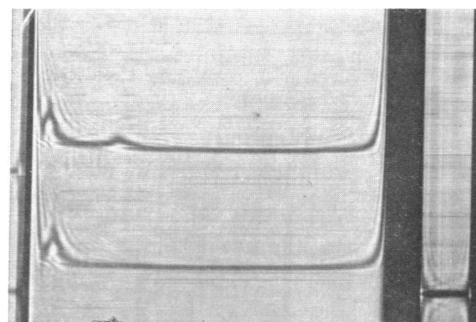

FIG. 3. Schlieren patterns of native seminal plasma from vasectomized rabbits (*below*; standard cell) and intact rabbits (wedge cell) in an analytical ultracentrifuge; 33,450 rpm for 3 min at 20°C; bar angle, 65°.

fraction II showed a broad band that migrated to the center of the strip. Both bands reacted positively with periodic acid–Schiff stain (Fig. 2b), which indicates that these species of molecules have sugar moieties. There were two Schiff-reactive bands in seminal plasma and only one, which faded, in fraction III.

Since there were only two fast-sedimenting peaks visible with Schlieren optics when native seminal plasma from intact bucks was subjected to ultracentrifugation in a Spinco model E centrifuge (Fig. 3), their direct identification with fractions I and II is possible. In seminal plasma, which is a relatively dense medium, their sedimentation coefficients were 89 S and 55 S. In buffer (0.15 M KC1–0.01 M Tris, pH 7.4), fraction II had a sedimentation coefficient of 86 S. The sedimentation coefficient of fraction I in buffer was not determined, but it may be estimated from the foregoing results to be around 138 S. It may be observed that the ratio of S values for fractions I and II and the ratio of their extent of sedimentation (Fig. 1*c* and 1*d*) are nearly equal (1.6 *v* 1.5) in these experiments; agreement between these ratios is expected (9) with the present peak identifications. A preliminary estimate of the molecular weight of fraction II, obtained from the application of Archibald's method (10) to the boundaries present on 70-min centrifugation at 1974 rpm, yielded values in the neighborhood of 4×10^6. By 90 min at this speed, fraction II sedimented from the miniscus. This fact seems to assure it of a molecular weight (*M*) that is greater than 0.5×10^6, judging from the equilibrium conditions for rotor speed in the analytical ultracentrifuge and molecular weight [$\text{rpm} = [5 \times 10^{11}/M(1 - \bar{v}\rho)]^{1/2}$; typically (11), $\bar{v}$ (partial specific volume) = 0.73 cm^3/g and ρ (solution density) = 1.02 g/cm^3]. It can be seen from Fig. 3 that seminal plasma obtained from bucks that had been vasectomized displayed only one peak in the Schlieren pattern. The sedimentation coefficient of this peak was determined to be 56 S, which is in good agreement with the value of 55 S derived for fraction II in seminal plasma. Since the seminal plasma from both intact and vasectomized rabbit inhibit fertilization, a deficiency of fraction I in the vasectomized rabbits argues against it being the principle sought.

The results obtained in bioassay of the three fractions and of unfractionated seminal plasma are given in Table 1. Control sperm (buffer only) and sperm treated with fraction I or III were associated with fertilization rates of 63.9, 53.8, and 43.5%, respectively, among day-1 eggs recovered. The control and fraction III rates had the larger difference, but it was non-

TABLE 1. *Fertilizing capacity of uterine-capacitated sperm cells after treatment with whole and fractionated seminal plasma*

Additive	Amount per ml (ml)	Time of insemn. before (−) or after (+) ovuln. (hr)	No. of rabbits (oviducts)	Eggs Total	Eggs Fert.	Fertiln. rate (%)
None	...	+(2-3)	9	44	28	63.9
Seminal plasma	0.1–0.125	+(2-3)	5	15	1	6.7
	0.1–0.125	−(5-6)	7	17	1	5.9
	(μg protein)					
Fraction I	48–160	+(2-3)	7	26	14	53.8
Fraction II	20–72	+(2-3)	20	46	3	6.5
	15–72	−(5-6)	15	62	5	8.0
Fraction III	160–1300	+(2-3)	8	23	10	43.5

Anesthetized does were tubally inseminated at the times indicated, with respect to the ovulation at 10 hr after the intravenous administration of 90 IU of chorionic gonadotropin. Usually, 20,000 sperm cells (range 5,000–39,000), recovered from the uterus of a doe bred 11–12 hr previously, were instilled into an oviduct in 10–20 μl. Spermatozoa recovered from the uterus, suspended in 1 or 2 ml of Hank's medium or Medium 199, were mixed at room temperature with the respective additives briefly before sedimentation and subsequent re-suspension in 0.2 ml of supernatant. The eggs were recovered 1 day after insemination.

significant: $\chi^2_{(1)} = 1.75$ ($\chi^2_{(0.05,\ 1)} = 3.84$). As a corollary, the smaller difference, between control and fraction I rates, is also not statistically significant (neglecting theoretical requirements for statistical orthogonality). Sperm treated with fraction II or seminal plasma, inseminated 5–6 hr before or 2–3 hr after the presumed time of ovulation, fertilized between 5.9 and 8.0% of recovered eggs. While these four groups (2 treatments × 2 times) were comparable ($\chi^2_{(3)} = 2.08$; NS), they had very significantly lower fertilization rates than in the other groups: Control, fraction I, and fraction II $\chi^2_{(1)} = 65.5$; $P < 0.005$. Clearly, fraction II is an effective inhibitor of fertilization and, apparently, it is the factor responsible for the deleterious action of rabbit seminal plasma on fertilization. Table 2 indicates that under these assay conditions, fraction II, at 18–72 μg protein/ml, had an untoward effect on fertilization. At the lower concentrations employed, it was without apparent effect.

DISCUSSION

These results suggest that the deleterious influence of rabbit seminal plasma on fertilization can be attributed to a large glycoprotein. This finding confirms a previous view (5) that a rapidly-sedimenting protein-like substance in seminal plasma blocks fertilization. It was found that 20–72 μg protein/ml of the inhibitor suppressed fertilization as effectively as seminal plasma, in the presence of approximately 200,000 uterine-capacitated sperm cells (Table 1); concentrations much below this range were ineffective (Table 2). There are about 15–54 $\times 10^6$ inhibitor molecules per sperm cell under these conditions, on the basis of a molecular weight of 4×10^6. Seminal plasma is 0.2% with respect to this substance and, as rabbit semen has about 160×10^6 spermatozoa per ml, there are evidently 2×10^6 free molecules of inhibitor per sperm cell in semen. The excess of inhibitor molecules may indicate that spermatozoa are coated by them. Removal of this coat could occur during capacitation before fertilization. However, this is a matter of conjecture; the present picture regarding the mode of action of the inhibitor is far from complete.

TABLE 2. *Fertilizing capacity of uterine-capacitated sperm cells after treatment with fraction II at various concentrations*

Concn. of fraction II (μg protein/ml)	No. of rabbits (oviducts)	Eggs Total	Eggs Fert.	Fertiln. rate (%)
0.14–0.80	6	19	12	63.2
3.2–7.2	3	12	8	66.7
18–72	21	50	3	6.0

The results obtained are in reasonable accord with previous observations (4) showing that fertilization is depressed by 2–5% (v/v) rabbit seminal plasma, although insemination of sperm treated with seminal plasma or inhibitor fraction II (Table 1) 5–6 hr before ovulation did not enhance the rate of fertilization as expected (4). Concerning the results with seminal plasma, where a direct comparison is possible, the present practice of using fewer sperm and the use of a higher mean concentration of seminal plasma may have contributed to the different outcome. Recapacitation of spermatozoa under present conditions might be achieved with longer sperm incubation in the oviduct, which is a poor site for capacitation of spermatozoa (12).

Isolation of the inhibitor of fertilization in rabbit seminal plasma has recently been reported by other workers (13). These authors failed, however, to give data directly concerning inhibitory activity in the purified fraction; moreover, they attribute to it a molecular weight of 170,000. It is improbable a molecule of this size could sediment in 4 hr when centrifuged at 105,000 × *g* in a Spinco 40 rotor (14) as originally reported (5) and as their (13) work demonstrated. Another group of workers (15), who have not actually obtained the inhibitor in a purified state, claim that its activity is present in a small fragment isolated from a pronase digest of the pellet formed on sedimentation of seminal plasma. This fragment, termed "DF" (decapitation factor), has a molecular weight of 300–500 and is reported to inhibit the fertilizing capacity of 100,000 spermatozoa at a concentration of 50 μg/ml; evidently around 750×10^9 DF molecules per sperm cell are needed to block fertilization. The DF activity on a

molar basis is clearly 50,000 times lower than that obtained with fraction II.

That a specific macromolecule should control a cellular function such as fertilization has intriguing implications. More information regarding the synthesis and mode of action of the fraction II glycoprotein is needed before the mechanism of this control over cellular function can be understood. The presence of a second macromolecular glycoprotein fraction (fraction I) in rabbit seminal plasma, at least in the seminal plasma obtained from intact rabbits, is also curious and merits further study.

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