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Separation of Non-Sperm Components from Seminal Preparations and Their Effect on the Analysis of Sperm Proteins (38744)

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Studies with rabbit sperm usually utilize "washed seminal particles", the low-speed pellet obtained from an ejaculate. We, as well as others (1–3), have observed that such "washed particle" preparations contain large numbers of particulate components which are not sperm. Benedict *et al.* (2) observed non-sperm particles in rabbit semen and suggested that they were of prostatic origin. Metz *et al.* (3) examined the ultrastructural and immunologic properties of these particles in ejaculates from vasectomized rabbits and characterized them by electron microscopy into three main classes: dense, vesicular and amorphous. In none of the above reports was any attempt made to assess the mass of these particles relative to the mass of the sperm in an ejaculate, or the extent to which the presence of these particles is "washed preparations" might interfere with biochemical studies of the sperm themselves. Nor, to our knowledge, have other investigators studying the biochemical composition or properties of sperm made any attempts to compensate for the presence of these non-sperm components (NSC).

We have devised a simple method for separating the NSC from sperm in seminal preparations and have measured the relative extent of their contribution to the mass of the whole "washed seminal particles", and of their contribution to protein content or lactic dehydrogenase (LDH) isozyme activity.

Methods. Preparation of sucrose gradient. A solution of sucrose in deuterium oxide (D_2O) of approximate density 1.343 was prepared by dissolving 6 g of sucrose and 73 mg of NaCl in 4 ml of D_2O to yield a solution which is 57.6% by weight sucrose (60% sucrose-D_2O-NaCl). This solution was diluted 1:1 with D_2O-NaCl (8.5 mg/ml) to yield 30% sucrose-D_2O-NaCl (approximate density = 1.219). Equal volumes of these solutions were used to pour a linear gradient of density 1.343–1.219 in cellulose nitrate centrifuge tubes (vol 5 ml).

Collection of seminal preparations. Semen and seminal plasma was collected respectively from intact and vasectomized male rabbits using an artificial vagina. Ejaculates from intact and vasectomized humans were collected by masturbation. Sperm were obtained from the vas deferens and epididymides of the male guinea pig and rabbit by flushing with physiologic saline. In the case of the male rabbit, the epididymis was removed and divided into three anatomical parts: head, body and tail. These parts were flushed using a blunt 30 gauge needle attached to a syringe filled with saline, and the flushings were collected in small watch glasses. Flushings from the body were not included in these determinations since no sperm or NSC could be detected. "Washed particles" were prepared by centrifuging the flushings or ejaculates at 10,000 rpm in a Sorvall Model RC2B centrifuge and resuspending the pellets in saline.

Separation of sperm and NSC. The components of "washed particles", whole ejaculates, and flushings from the epididymis or vas deferens were separated by sedimenting to equilibrium in the sucrose gradient. Samples of up to 0.2 ml were layered onto the sucrose gradient and centrifuged at 25,000 rpm (45,000 *g*) for 30 min at 20° in a Spinco SW 65 rotor. After centrifugation, the bands of sperm and NSC were removed by inserting a needle through the side of the tube in the area of the band and withdrawing the band with a syringe. The isolated components were immediately diluted with saline, pelleted at low speed and resuspended in a volume of saline equivalent to that of the sample applied to the gradient.

Coulter counter determinations. Determinations of particle counts were made with a Counter counter using a 50 μm aperture essentially as described by Fowler and Hellman (4). All counts were made immediately after resuspension of the isolated particles.

Electrophoresis and LDH isozyme activi-

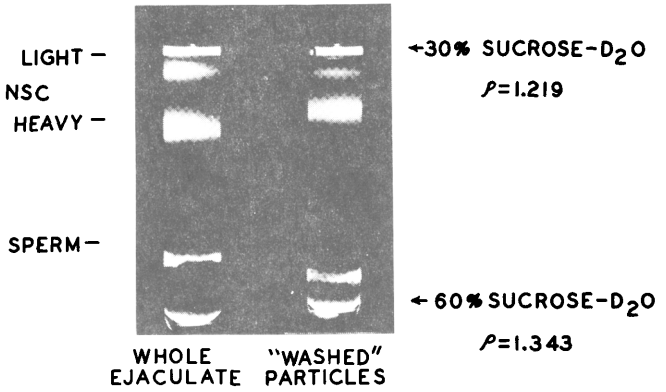

FIG. 1. Equilibrium sedimentation of rabbit semen. 0.2 ml of rabbit whole ejaculate or the equivalent amount of "washed particles" were layered onto the sucrose gradient and centrifuged as described in Methods.

ties. Polyacrylamide gel disc electrophoresis (PAGE) was performed as described by Davis (5). Samples of "washed particles" from rabbit ejaculates, or isolated components, were subjected to disruption by sonication in dilute solution or by homogenization in ground glass conical tissue grinders. There was no difference in the results obtained with either method, but neither method succeeded in disrupting the sperm heads. The insoluble material was removed by centrifugation at 10,000 rpm and the supernatants were dialyzed against 0.05 *M* Tris–HCl, pH 8.0 prior to being applied to the gel. Electrophoresis was conducted at 4 ma/gel until the tracking dye had migrated 6 cm. The gels were removed from the tubes and incubated in LDH-specific stain according to the method of Grell *et al.* (6). The gels were transferred to tubes and photographed or were scanned at 575 nm using a Gilford linear transport.

Results and Discussion. Separation of NSC and sperm from rabbit ejaculates. Centrifugation of either rabbit whole ejaculate or "washed particle" preparations to equilibrium in the sucrose gradient results in the separation of sperm and NSC shown in Fig. 1. The sperm occupy a region of higher density toward the bottom of the tube at a density of approximately 1.31 while the NSC separate into two bands, designated heavy and light, at a density of approximately 1.25. These two bands were pooled unless otherwise specified. The apparent bands at the top and the bottom of the tube are due to reflections of light off the meniscus and the tube bottom during photography. Each band can be removed from the tube, washed and further examined.

Photomicrographs of the particles and sperm are shown in Fig. 2. The "washed particles" from the ejaculate of a vasectomized rabbit (2a) show the NSC clearly without the interference caused by the sperm in the "washed particles" from the normal rabbit ejaculate (2b). After separation on the gradient, the lighter, pooled NSC bands (2c) can be seen to be free of sperm, although a sperm tail is occasionally found, and the sperm band from the gradient can be seen to be essentially free of any apparent NSC (2d).

Relative amounts of NSC and sperm. The relative distribution of the number of particles obtained after the gradient fractionation of several seminal preparations is presented in Table I. Approximately 80% of the total number of particles in rabbit ejaculates were NSC, while the NSC comprised 35% of the particles in human ejaculates. Nicander *et al.* (7) have recently reported studies on particle-secreting cells from rabbit prostate. We have found that flushings from the rabbit vas deferens and epididymides contained significant numbers of NSC, suggesting that the particles are not exclusively of prostatic origin (2). Flushings from guinea pig vas deferens and epididymis contained fewer, although easily detectable, numbers of NSC.

Since 80% of the total number of particles found in a rabbit ejaculate are *not* sperm,

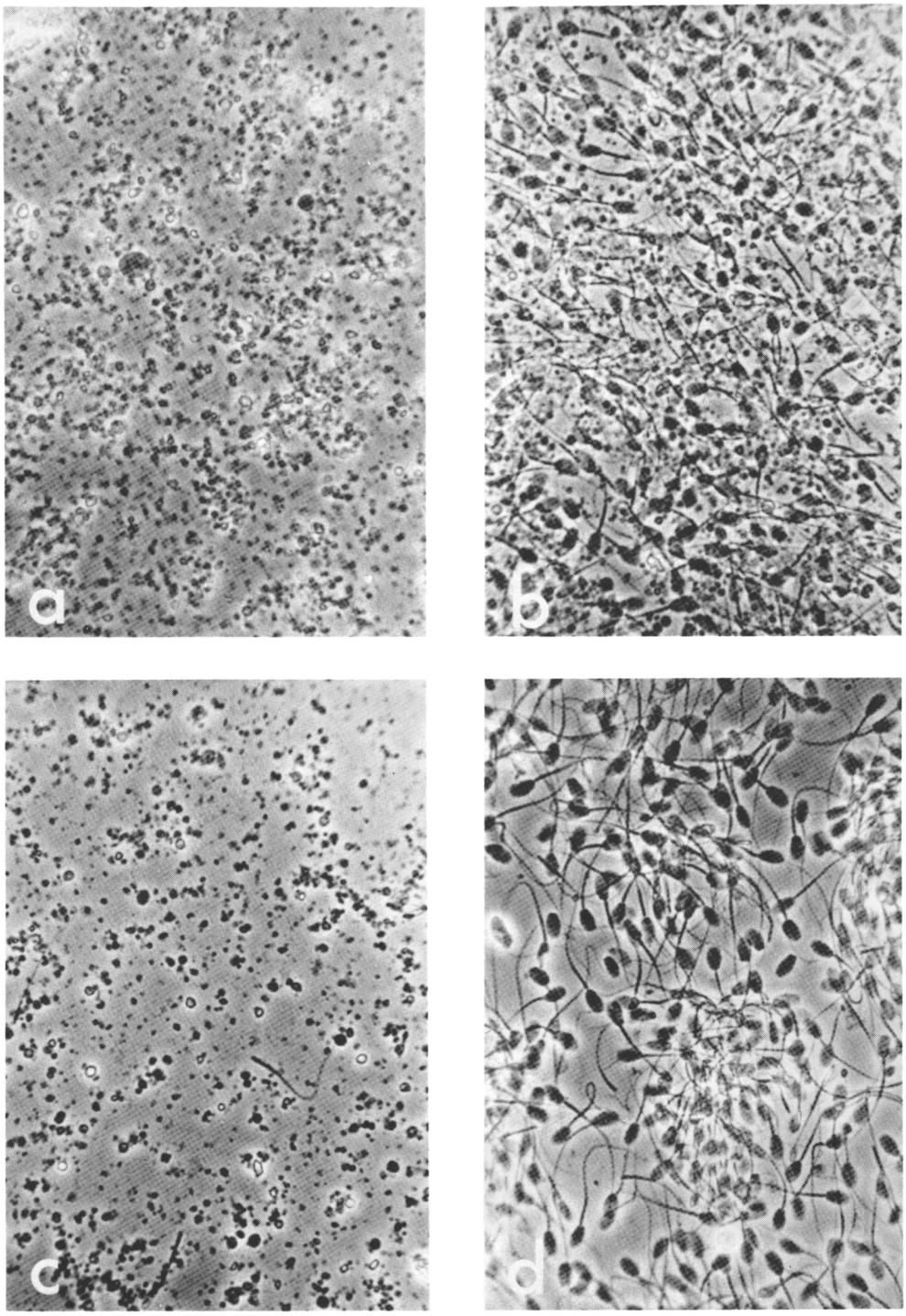

FIG. 2. Microscopic examination of rabbit seminal preparations. Samples were placed on a glass slide and examined under a Zeiss phase-contrast microscope (magnification 250 ×) and photographed. (a) "washed particle" preparation from vasectomized rabbit; (b) "washed particle" preparation from normal rabbit ejaculate; (c) pooled NSC bands from normal "washed particle" preparation after separation on sucrose gradient; (d) sperm band from same separation.

TABLE I. DISTRIBUTION OF TOTAL NUMBER OF PARTICLES[a] RECOVERED FROM GRADIENT.

Sample applied to gradient	% NSC	% Spermatozoa
Rabbit ejaculate[d]		
Whole	82[b]	18
Washed	81[c]	19
Human ejaculate[d]		
Washed	35	65
Rabbit flushings		
Vas deferens	10	90
Epididymis	33	67
Head	21	79
Tail	30	70
Guinea pig flushings		
Vas deferens	6	94
Epididymis	13	87

[a] Coulter Counter determinations.
[b] 11% Light, 71% heavy.
[c] 9% Light, 72% heavy.
[d] Ejaculates from normal males and vasectomized males contain approximately equal numbers of NSC.

TABLE II. DRY WEIGHT[a] OF PARTICULATE COMPONENTS OF RABBIT EJACULATE.

	mg/ml ejaculate equivalent[b]	% Total	% Recovery
NSC	9.3 ± 0.2	52	70
Spermatozoa	8.7 ± 0.9	48	
Whole washed ejaculate[c]	25.6 ± 0.3		

[a] By lyophilization.
[b] SD based on three determinations.
[c] Suspended in 60% sucrose-D_2O for 30 min.

the mass of the particles relative to that of sperm was determined. Table II shows the dry weight of the NSC and the sperm fractions after separation, as compared to that of the whole washed ejaculate. The distribution by weight in the gradient was approximately equally divided between the NSC and the sperm. A similar distribution was found in the fractions were homogenized and assayed for protein content. The recovery of total mass from the gradient was only 70%. This loss, which primarily reflects the inability to completely remove each band from the tube, is probably equally divided between the NSC and sperm fractions.

LDH isozyme activity in NSC and sperm. In an attempt to evaluate the possible extent of interference of the NSC on the analysis of sperm proteins, we examined the electrophoretic distribution of lactic dehydrogenase (LDH) isoenzymes (8–11) from the NSC and sperm of rabbit ejaculates after separation of "washed particles" on gradients. The separated sperm and NSC were diluted, centrifuged at low speed and resuspended in equal volumes of saline and were subjected to sonication and electrophoresis, followed by staining of the gel in LDH stain (Methods). The results (Fig. 3a) indicate that the majority of LDH isozyme activity is associated with the NSC. Analysis of the "washed particle" extracts prior to centrifugation shows a profile which is a combination of these two gels, as would be expected. There is a large amount of enzyme activity at the interface of the running and stacking gels in the sperm profile and a heterogenous diffusion of activity just below the interface. The fast-moving bands are the tracking dye. These gels were incubated in the LDH stain for only 30 min to ensure that the staining intensity was proportional to enzyme activity. The gels were then scanned in a Gilford spectrophotometer in an attempt to quantitatively assess the enzyme activity present (Fig. 4). Five LDH isozyme bands were observed in the NSC, while the sperm extract showed only two minor bands and a large, diffuse area between 0.5 and 1.5 cm. When the gels were incubated in the stain for a longer time period, more bands were apparent in the sperm profile (Fig. 3b), with the area between 0.5 and 1.5 cm still being predominant. We are unsure at this time as to whether this is the "band x" isozyme (8–11) but if so, the NSC also contains "band x", although in lesser amounts.

These data show that while rabbit spermatozoa contain LDH isozyme activity, the contribution of the NSC to the total LDH activity of a preparation of "washed particles" is indeed sizeable. Therefore "washed seminal particles" from rabbit whole ejacu-

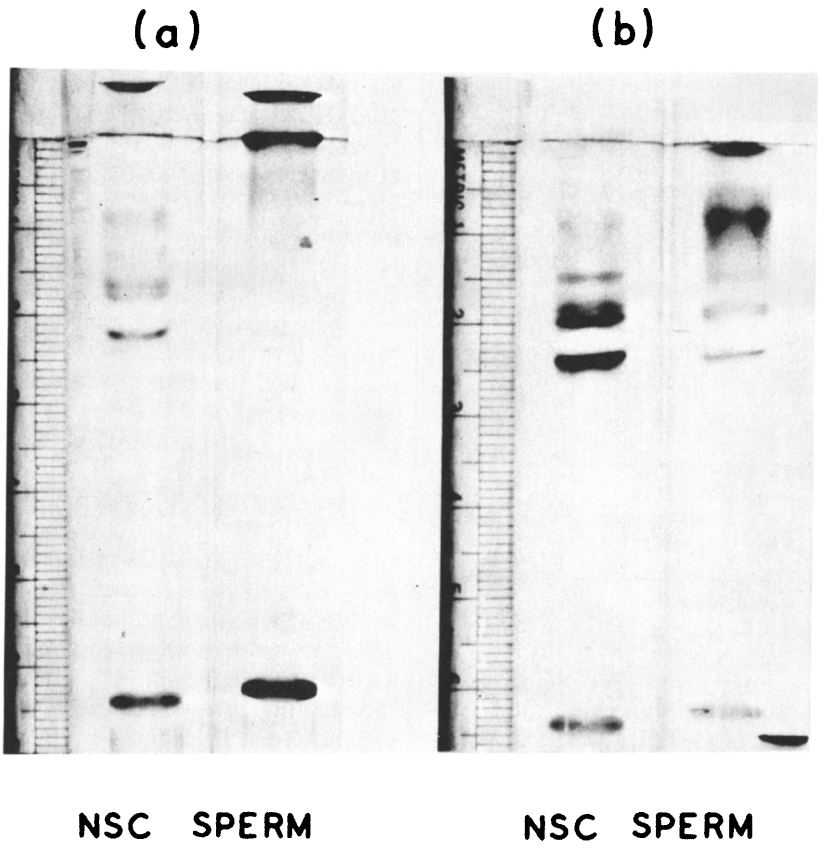

FIG. 3. LDH isozyme activity in separated NSC and sperm. Rabbit "washed particles" were separated into NSC and sperm on sucrose gradients and the individual bands were removed from the tube, diluted in saline and centrifuged at low speed. The pellets were immediately suspended in equal volumes of saline, sonicated and the extracts dialyzed overnight against 0.05 *M* Tris–HCl pH 8.0. Aliquots of the dialyzed preparation were subjected to electrophoresis and staining for LDH isozyme activity. (a) incubated in stain for 30 minutes; (b) incubated in stain for 1 hr. (a) and (b) represent different experimental samples from ejaculates of different rabbits.

lates are not equivalent to spermatozoa, and the "contaminant" or non-spermatozoan particles must first be separated from the sperm for studies of sperm proteins or other components to be meaningful. NSC were also found in ejaculates from both intact and vasectomized humans as well as in flushings from rabbit, and to a lesser extent in guinea pig, epididymides and vas deferens.

Summary. The presence of non-sperm components (NSC) in "washed" rabbit epididymal and ejaculated collections and their interference with the analysis of sperm proteins have been demonstrated. NSC were also found in ejaculates from normal and vasectomized humans and from vasectomized rabbits, and in epididymal preparations from guinea pigs. They comprise about 80% of the total number of particles in rabbit ejaculates and 35% in human ejaculates. The dry weight is approximately equally distributed in rabbit ejaculates between NSC and sperm. The majority of the LDH isozyme activity of the "washed particles" in an ejaculate were associated with the NSC and this study shows that "washed particle" preparations are not equivalent to sperm and that NSC must first be separated from the sperm themselves for studies of sperm proteins to be meaningful. A method for the separation of the NSC from the sperm by equilibrium sedimentation in gradients of sucrose-D_2O is described.

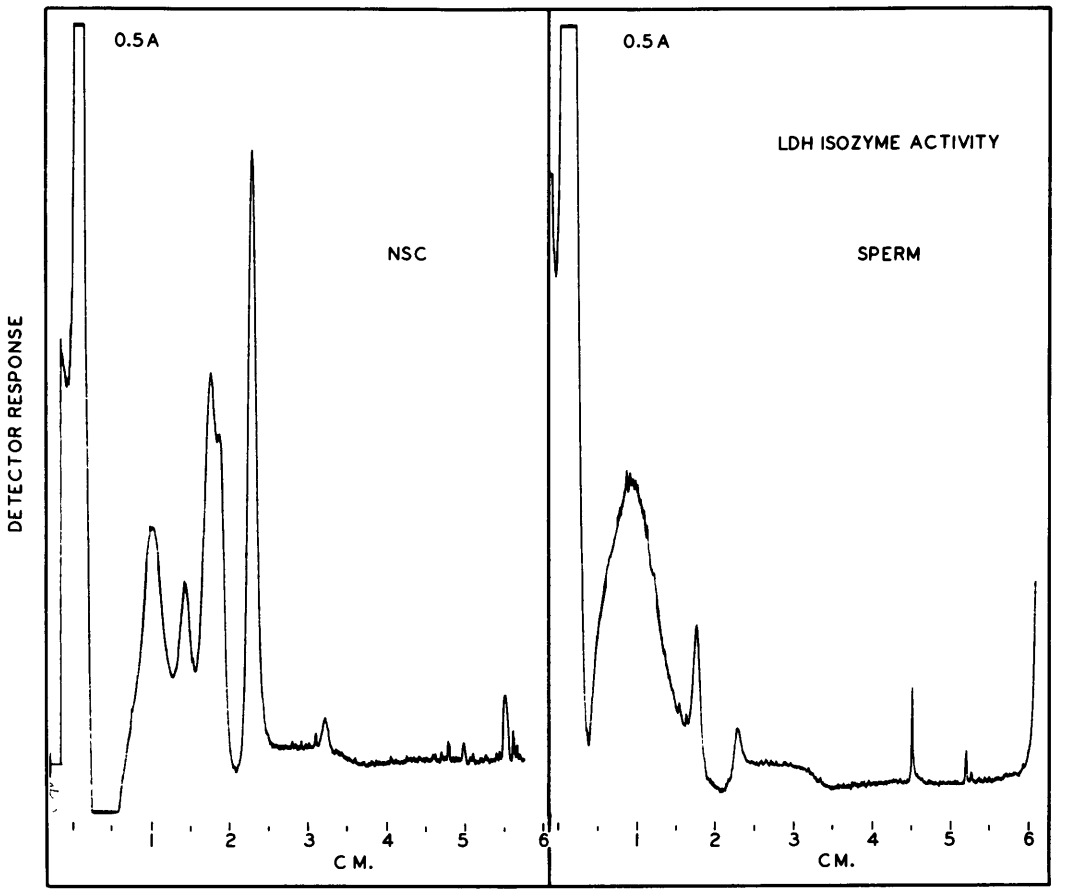

FIG. 4. Gel scan of LDH isozyme activity. The gels from Fig. 3a were scanned at 575 nm with a Gilford linear transport. Maximum pen deflection corresponds to 0.5A.

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