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The Properties of Rabbit Sperm Membranes in Contact with Electrode Surfaces

M. Blank, L. Soo and J. S. Britten

Department of Physiology, Columbia University College of Physicians and Surgeons, 630 W. 168 Street, New York, N.Y. 10032

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Summary. In dilute suspensions of sperm cells there is electrode noise due to the changes in the electrical double layer during contacts of the suspended cells with the electrode surface. The noise frequency, the number of current fluctuations above one nanoampere per unit time, depends upon the concentration of cells, the potential at the electrode surface and the presence of adsorbed material at the electrode. We have studied the electrode noise due to suspensions of rabbit sperm cells, and have obtained information about the properties of the cell membranes and their ability to adsorb or interact with substances in the environment. The observed differences in the noise frequency between ejaculated and epididymal sperm cells indicate that the ejaculated sperm cell does not adsorb material as readily. The titration of sperm cell suspensions with Zn^{++} ions shows a lower negative surface charge on the epididymal sperm cell, in agreement with published values on cell electrophoresis. The experiments reported here demonstrate that there are significant changes in the membranes of sperm cells at the later stages of maturation which are independent of membrane charge and affect the ability of the cells to adsorb material. Such adsorption processes could be the first stages of physiological processes (e.g. "sperm capacitation", "acrosome reaction") that are known to occur *in vivo*, and that play important roles in fertilization.

Mammalian sperm cells undergo continuous changes during their development, maturation and after contact with the uterine environment. The changes in the membrane properties of the cells are of particular interest, since the sperm cell membrane plays a crucial role in the process of fertilization.

Various techniques have been used to study the changes in the properties of the sperm cell membranes at different stages of development. The most frequently used physical technique has been cell electrophoresis. Such studies have given information about the isoelectric point of the sperm membrane (Bangham, 1961; Nevo, Michaeli & Schindler, 1961) and of the electrophoretic mobility of sperm cells of various species (Bey, 1967). It

has also been possible to show changes in the electrophoretic mobility of sperm cells on passage through the epididymis (Bedford, 1963) and after incubation in the uterus (Vaidya, Glass, Dandekar & Johnson, 1971). These results, together with morphological (Bedford, 1970) and physiological (Orgebin-Crist, 1969) studies, have yielded a better understanding of the changes in the sperm cell membrane related to the ability to fertilize.

As in the case of all physical techniques, there are limitations in the methods and in the conclusions that can be drawn from the measurements. For example, electrophoresis measures the surface charge at the plane of shear, which may be considerably different from the surface of interest. For this reason it is always useful to study the same properties with the aid of several techniques. One new approach that offers promise of obtaining new information about cell membranes is the study of polarography involving whole cells (Velazquez & Rosado, 1972). This technique is still new and there are questions about the quantitative aspects of the conclusions. For example, the measured value of 5 μEquiv of charge per 10^8 human sperm cells at neutral pH is equivalent to 1 charge/Å^2 (of a cell assumed to have an area of 200 μ^2). Similarly, the published data indicate 20 NH_2 groups/Å^2 of cell membrane. These values appear to be much too large and indicate the presence of unknown factors affecting the interpretation of the measurements.

We have attempted to develop new information about the cell membranes of sperm cells by analyzing the noise that the cells generate when in contact with an electrode surface (Blank & Britten, 1970). We have used the mercury/water interface as the electrode, since it is an almost ideal polarizable interface, i.e., an imposed potential will be maintained in the absence of substances that can be oxidized or reduced, with almost no current across the interface. However, when particles or cells are suspended in the medium there are random interfacial currents that vary with the concentration of the particles, and depend on the potential at the mercury/water interface. The mechanism of the random currents has been studied, and it is now possible to use the noise signals to obtain information about the surface properties of suspended cells (Blank, Soo & Britten, 1974). We have used this technique to study the properties of sperm cell membranes and, in particular, the differences between rabbit epididymal and ejaculated cells.

Materials and Methods

All sperm cells used in this study were obtained from New Zealand rabbits. Ejaculated sperm were collected with the aid of an artificial vagina and either kept in the

seminal fluid or centrifuged at $500 \times g$ for 2 min and the pellet diluted with a buffered isotonic solution containing 135 mM NaCl, 5 mM KCl and 1 ml (per liter) of 1 M Tris Cl at pH 7.5 (Solution I). (When experiments were done at pH's other than 7.5, the Tris Cl was replaced by the same amount of the buffer system involving carbonate (K_2), phosphate (K_2), phthallate and acetate. Very low pH's were obtained with HCl.) Epididymal sperm were obtained from the caudal region of the epididymis by placing the tissue in a watchglass and cutting it with fine scissors. The cells were extruded into about 1 ml of Solution I and collected with a Pasteur pipette. This process was repeated several times and the samples were pooled. The entire sample was then spun at $500 \times g$ for 2 min as in the case of ejaculated sperm. The sperm were transferred into the test solutions with lambda pipettes and the concentrations of the cell suspensions were determined by the standard cell count method using a Levy-Hausser Counting Chamber.

Electrode Noise Measurements

The cell suspensions were placed in contact with a rapidly dropping mercury electrode apparatus, the Metrohm Polarecord Model E 261, and the current across the mercury/water interface was measured. In the presence of suspended sperm cells (and also in suspensions of various other particles), there is a considerable amount of electrode noise that depends on the cell concentration, the electrode potential and the presence of adsorbed material at the electrode. We have studied the properties of the noise and have found ways of relating the measurements to the properties of the sperm cell membrane (Blank *et al.*, 1974).

The random currents in cell suspensions are due to changes in the electrical double layer at the mercury/water interface when a particle hits the electrode surface and causes changes in adsorption (Bach, Britten & Blank, 1973). On contact, the cell surface competes with the electrode surface for adsorbed material and this can lead to a transfer of adsorbate. In our experiments the cells adsorb material from the electrode, leading to desorption-adsorption cycles at the electrode and changes in the charging current. This process leads to a noise signal.

The quantitative aspects of the noise can be understood in terms of the charge on a surface $Q = CV$, where C = capacitance and V = potential relative to the point of zero surface charge (PZC). The current required to charge the surface at constant V,

$$i = \frac{dQ}{dt} = V\frac{dC}{dt}.$$

The sign of i depends on the value of the polarization and one can observe a change in direction of current at the PZC. If one counts all rapid current jumps in excess of an arbitrarily chosen threshold (10^{-9} amps) and divides by the time of observation, we obtain a frequency. The variation of the frequency and direction of the random currents with V follows from the variation of the sign and magnitude of Q on either side of the PZC. The noise disappears when outside the adsorption range for the electrode surface or when there is a strongly adsorbed substance that does not transfer to the particle. It has also been shown that the noise frequency varies with the square root of the number of cells in the suspension (Blank *et al.*, 1974). This enables us to compare measurements obtained with different cell concentrations.

We, therefore, have a measurement that is sensitive to the transfer of small amounts of adsorbate between electrode surface and cell surface, and we can learn about the properties of both surfaces from the results of competitive adsorption processes that arise upon contact. For example, we can compare the ability of the sperm membrane to

remove adsorbate from the electrode surface at various electrode potentials and at different stages of development, and determine if the differences are related to changes in the charge density at the membrane surface.

Ion Binding Studies

The ability of cations to bind with the anionic groups on the sperm cells was measured by two different methods. In one case, microliter quantities of 0.1 M or 0.01 M solutions of Cl^- or NO_3^- salts of the ions were added to the sperm cell suspensions, and the electrode noise measured after each addition. The concentration at which the noise frequency decreased to 50% of its original value was used as the measure of the binding.

The second method for studying binding was to measure the concentration of the added ion polarographically as the diffusion current associated with the half wave potential (e.g., -0.8 V in the case of Zn^{++}). Microliter quantities of the cation containing solutions were added to the sperm cell suspensions, and the polarographic current measured after each addition. The bound ions do not contribute to the polarographic current and they can be detected as the difference between the expected and observed currents. The current associated with the bound ions is corrected for the ions that engage in other equilibria (e.g. complex ion formation or binding with buffer anions, which accounts for 16% of the initial addition of Zn^{++} to solutions at pH 7.5).

Results

Electrode Noise in Ejaculate and Epididymal Sperm Suspensions

Electrode noise due to sperm cell suspensions is similar to that observed in the other particulate systems studied with regard to the dependence of the noise frequency on electrode potential (Blank & Britten, 1970). There is no observable noise at the PZC, which is at about -500 mV, and the noise signals have opposite directions at potentials on the two sides of this point. The noise frequency for the positively charged surface is greatest at -200 mV and for the negatively charged surface at -800 mV. If we consider the maximum noise frequency at a positively charged electrode surface, the variation with cell concentration, shown in Fig. 1, is virtually identical with results obtained using suspensions of "membrane particles". (These were obtained from homogenates of rabbit kidney, sedimented at $1{,}500 \times g$ for one-half hour, and had approximately the same average particle volume as the sperm cells.) However, when we consider the maximum noise frequency at the negatively charged electrode surface we see differences. In the case of epididymal sperm we obtain a pattern similar to Fig. 1, where the sperm cells indicate approximately the same signal frequency with the negative electrode surfaces, while the ejaculated sperm suspensions show a much lower frequency with negative surfaces. Fig. 2 presents the results of one experiment with ejaculated sperm suspensions. We can summarize

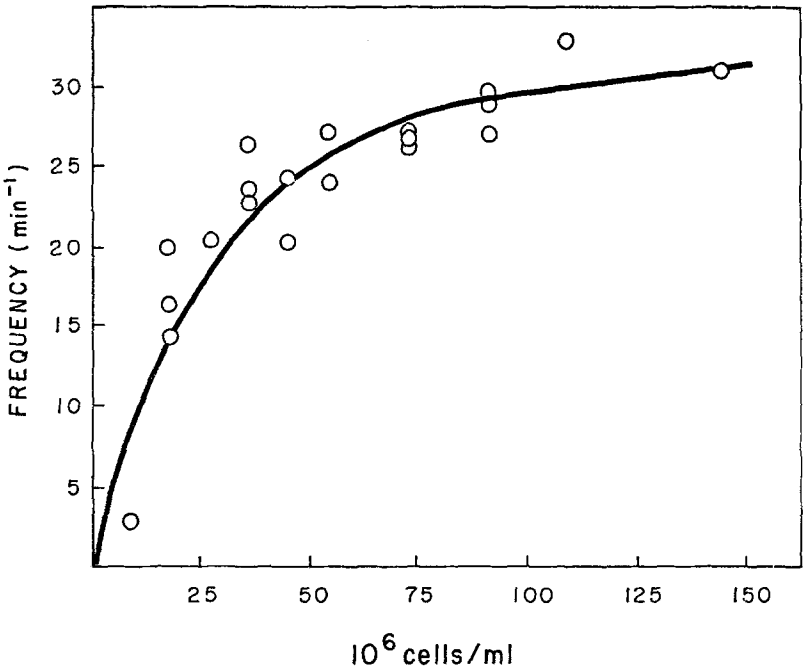

Fig. 1. The noise frequency (the number of current pulses $>10^{-9}$ amps divided by the time in minutes) *vs.* the concentration of rabbit sperm cells suspended in solution I. This is the maximum frequency obtained for a positively charged electrode and it occurs at -200 mV polarization

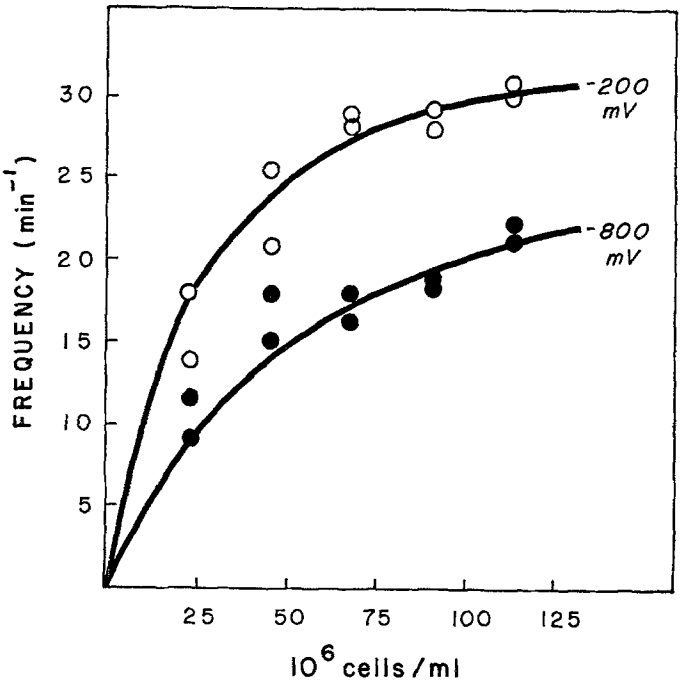

Fig. 2. The noise frequency *vs.* the concentration of (one sample) ejaculated rabbit sperm cells suspended in solution I. Values are given for positively (○) and negatively (●) charged electrodes

Table 1. Noise frequency ratio $\left(\frac{\text{pos}}{\text{neg}}\right)$

Suspensions	No. of Exps.	Mean $\pm\sigma$	Asymmetry (%)
Membrane particles	25	1.11 ± 0.23	~10
Epididymal sperm	5	1.23 ± 0.12	
	5	1.35 ± 0.57	~35
	19	1.47 ± 0.17	
Ejaculated sperm	10	1.81 ± 0.31	
	5	1.85 ± 0.11	~80
	9	1.77 ± 0.24	

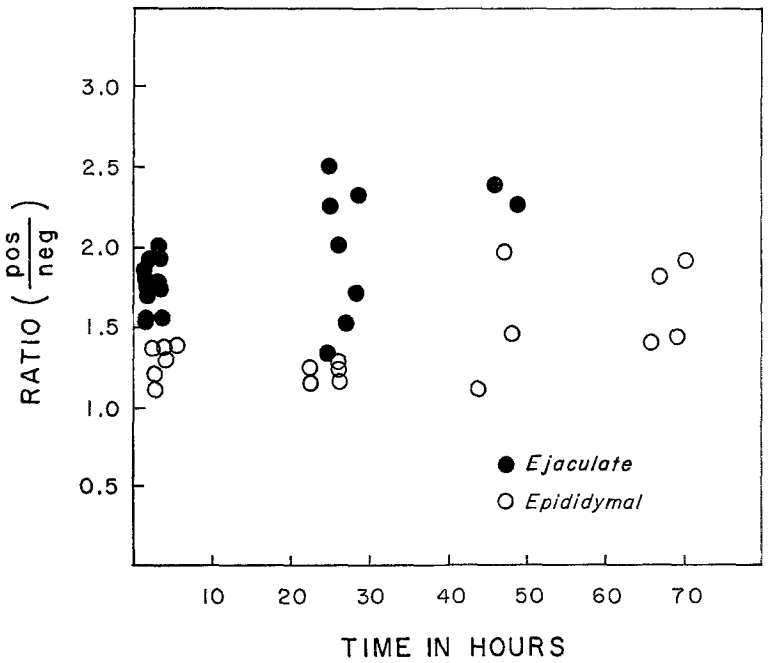

Fig. 3. The ratio of the electrode noise frequency at the positive electrode divided by the frequency at the negative electrode as a function of the age of the sperm sample (stored at ~10 °C). The ratios are given for ejaculate (●) and epididymal (○) samples, and the individual points represent averages of at least three values

the results of many experiments by calculating a ratio of the maximum frequency at a positive surface divided by the maximum frequency at a negative surface. The ratios for newly obtained cells are given in Table 1 for three different types of suspensions, and are shown in Fig. 3 as a function of time after collection for ejaculated and epididymal sperm cells.

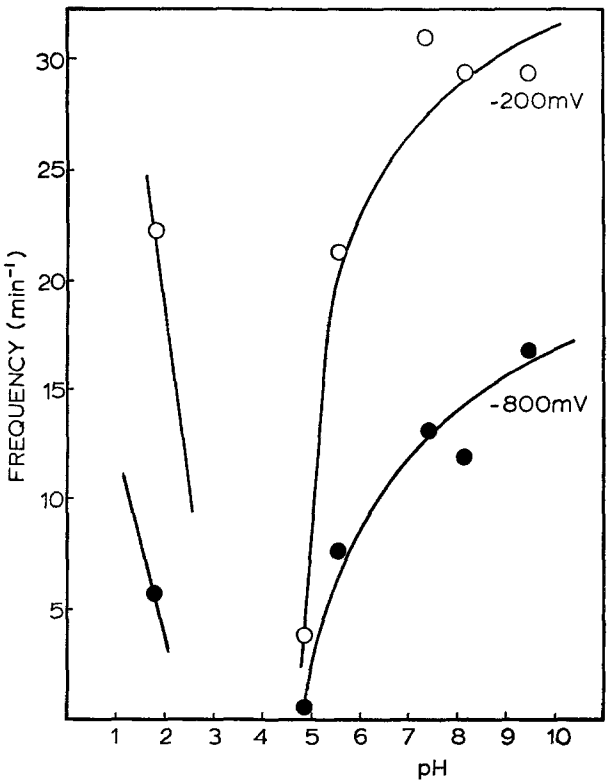

Fig. 4. The average noise frequency (in min^{-1}) *vs.* the pH of the solution for 10^8 cells/ml of ejaculated sperm at −200 mV (○) and at −800 mV (●) electrode polarization

The ratio obtained for "membrane particles" is not different from unity, while the epididymal sperm are about 35% higher and the ejaculated sperm are 80% higher. The differences between the two populations of sperm studied are independent of the degree of motility and persist over many hours (although there appears to be a tendency for ratios of both populations to increase upon storage). The differences are also independent of the solutions in which they are suspended. Thus, if epididymal sperm are mixed with seminal fluid obtained from an ejaculate sample, or ejaculated sperm are suspended in the saline solution used, they retain the characteristics of the sperm. The differences are therefore due to changes that arise during the sperm maturation process, and not to the solutions used for collection or storage.

Since the isoelectric point of rabbit sperm cells is about pH 3, it was of interest to study the variation of electrode noise over a wide pH range. Fig. 4 presents the average noise frequency (for at least three samples of

ejaculated cells at each pH) as a function of pH for both positive and negative electrode frequencies. It should be noted that the ratio agrees with those listed in Table 1 and that the values obtained for epididymal sperm, which are not shown, are also as expected. (The -200 mV values for epididymal sperm are about the same as for the ejaculated sperm cells, and the -800 mV values lie between the two curves.)

The disappearance of the noise signals around pH 3 to 4, the isoelectric region of the sperm membrane (Bangham, 1961; Nevo *et al.*, 1961), indicates that the noise requires the presence of a charge on the cell membrane. (In this region the cells also tend to coagulate, as expected.) The reappearance of the noise at low pH in approximately the same ratio of positive-to-negative electrode surfaces as in Table 1, even though the charge on the membrane has reversed sign, indicates that the mechanism giving rise to the noise signal is not simply related to the charge.

If we were to consider the data obtained at pH 7.5, which is well above the membrane isoelectric point, we would reason as follows. Since the current pulses arise when the cells remove adsorbate from the electrode surface, the higher ratio for the ejaculated sperm indicates that it cannot compete for adsorbate as effectively against a negatively charged electrode surface. This might occur if the sperm membrane lost negative charges toward the end of the process of maturation. However, below the isoelectric point, where the sperm membrane is positive, the same effect occurs. It appears then that the ejaculated sperm cell cannot compete for adsorbate as effectively against a negatively charged electrode surface, regardless of the charge on the membrane. We can probably generalize this observation to adsorption from all negatively charged surfaces, including other cells. It therefore appears that the ejaculated sperm cells tend to adsorb less or more slowly than the epididymal sperm.

Titration of the Membrane Anionic Groups

If the negative charges on the membranes are involved in the adsorption process, i.e., compete for adsorbate with the negative charge on the electrode, we should be able to titrate them by adding cations that bind strongly to the membrane. When we add salt solutions to the suspension we find a gradual decrease in the noise, and Fig. 5 shows the variation of the maximum frequency with the concentration of added cadmium ion in the form of $CdCl_2$. The 50% inhibition concentration is a measure of the effect of the ion, and Table 2 gives the concentrations of various ions in suspensions of ejaculated and epididymal sperm cells that reduce the noise frequency at -200 mV.

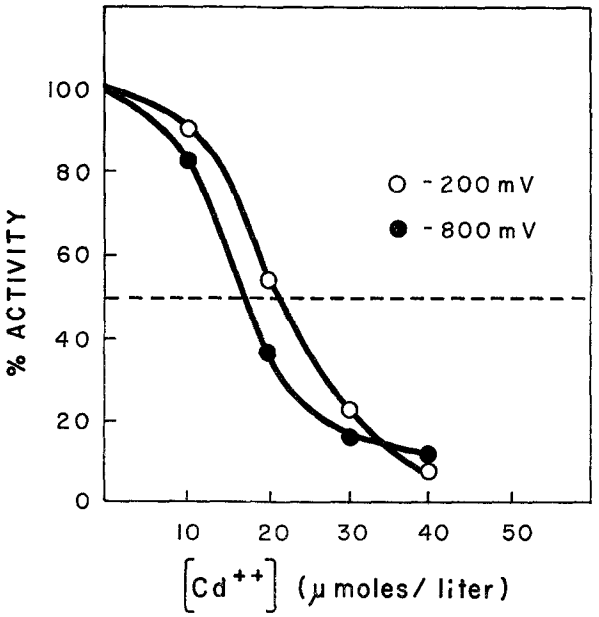

Fig. 5. The decrease in the electrode noise frequency, plotted as per cent activity due to the presence of Cd^{++} ion which has been added to solution I. Values are given for -200 mV (○) and -800 mV (●) electrode surfaces

Table 2. Inactivating concentrations of ions

Added ion	Cell suspensions (6 to 8 × 10^6 cells/ml)	
	Ejaculated cells (Ion conc., μM)	Epididymal cells (Ion conc., μM)
La^{+3}	1.8	
Zn^{+2}	5.5	53
Ni^{+2}	12	
Cd^{+2}	15	
Ca^{+2}	44	500
Mg^{+2}	94	1,600
Mn^{+2}	125	

Average deviations of these values are about 30%.

The results indicate that an order of magnitude lower concentration is required to affect the electrode noise in ejaculated cell suspensions. We can conclude that adsorption on ejaculated cells is more easily stopped.

The results of Fig. 5 show that the competition of the sperm cell for adsorbate is affected by the divalent cation for both positively and negatively charged electrodes. This is true for all the ions studied, and in all

cases, the interaction with the negatively charged electrode is affected to a somewhat greater extent. The decrease in electrode noise for positive and negative surfaces is probably due to the interaction of the divalent cations with the negatively charged groups on the sperm membrane as well as on the adsorbed film at the electrode, since both reactions would reduce the electrostatic forces leading to desorption from the electrode. Control experiments in which epididymal sperm were suspended (and in some cases collected as well) in seminal fluid obtained from ejaculate samples, again indicated that the observed differences were due to the properties of the cell membranes.

The order of effectiveness of the ions listed in Table 2 provides a clue as to the identity of the types of groups that are interacting with the cations. In general, the order follows the pattern of the interaction of cations with carboxyl colloids in charge reversal studies in electrophoresis (Booij & Bungenberg de Jong, 1956). In particular, the effective concentrations of Mn^{++} and Ni^{++} compared to Ca^{++}, and the relative effectiveness of La^{+3} suggest the presence of carboxyl groups.

The Stoichiometry of Zn^{++} Binding

Since Zn^{++} ions were the most effectively bound divalent cations studied and since Zn^{++} is also easily detected at the mercury/water electrode, we measured the amount of this ion bound to ejaculated sperm cells. The Zn^{++} was measured polarographically in sperm suspensions to which aliquots of $ZnCl_2$ solution were added (*see* Fig. 6). The number of Zn^{++} ions that disappeared from solution was 0.052 ± 0.011 μEquiv per 10^8 ejaculated sperm cells. Assuming an area of $200\ \mu^2$ per cell, there are 1.56 ± 0.32 ions bound per $100\ Å^2$ of membrane in ejaculated cells. The concentration at which the Zn^{++} binding is complete is definitely lower than the 50% inhibition concentration listed in Table 2. Binding is apparently complete after the addition of an amount of Zn^{++} that would be 10^{-5} M, were it all in solution. In Table 2, we see that 50% of the electrode noise remains at over five times this concentration. Therefore, desorption from the electrode continues even after all of the Zn^{++} has been bound by the sperm cells.

On measuring the binding of Zn^{++} ions to epididymal sperm in the same way as above, we obtain a smaller number of ions bound to the cell membrane, 0.036 ± 0.010 μEquiv per 10^8 cells, or 1.09 ± 0.30 ions per $100\ Å^2$ of membrane. This indicates that there are definitely fewer Zn^{++} binding sites on the epididymal cell membranes as compared to ejaculated cells.

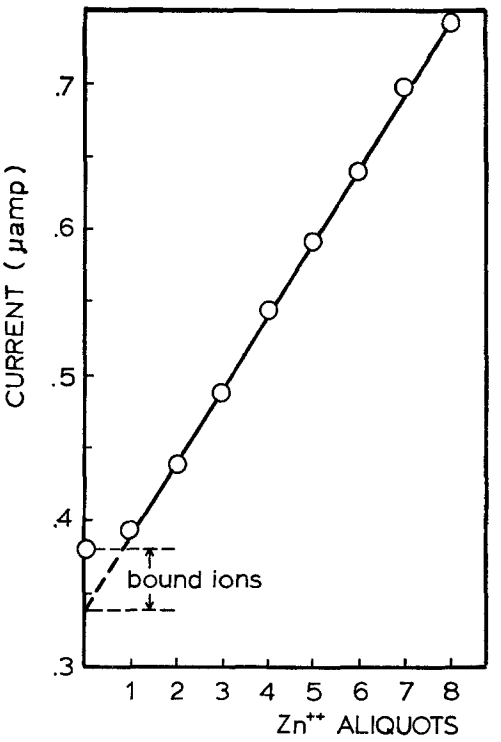

Fig. 6. The polarographic current (in μamps) *vs.* the number of 10 λ aliquots of 0.01 M Zn^{++} added to the solution. The difference between the initial current and the extrapolated current at 0 aliquots represents the amount of Zn^{++} ions bound

This fact must be viewed in conjunction with the data of Table 2, which indicate that higher concentrations of divalent ions are needed in epididymal sperm suspensions (even in those that have been in contact with seminal fluid of ejaculate samples) to eliminate electrode noise and the process of desorption from the electrode. The ability to adsorb and the magnitude of the surface charge are apparently not simply related.

Discussion

The results of these measurements indicate clear differences between ejaculated and caudal epididymal sperm cells of rabbit, with regard to the ability to adsorb surface active material in competition with a charged electrode surface and the ability to bind Zn^{++} ions. Ejaculated sperm cells are less able to adsorb surface active material in comparison to the epididymal cells, and the adsorption is more easily stopped by divalent cations.

Titration experiments with divalent Zn^{++} show that ejaculated cells have a much greater cation binding ability. In all cases, control experiments interchanging cells and suspending media, e.g. using epididymal cells in seminal fluid, indicate that the measured differences relate to the cell membranes and not the suspending media.

Previous measurements of electrophoretic mobility, which are related to the surface charge, agree that the electrophoretic mobility of the mammalian (and in particular rabbit) ejaculated cell is about -1.0×10^{-4} cm^2 sec^{-1} volt^{-1} (Bangham, 1961; Nevo *et al.*, 1961; Bey, 1967). The epididymal cell of rabbit has about one-half the mobility, approximately -0.5×10^{-4} in the same units (Bedford, 1963), showing that there is a large increase in negative mobility (and presumably surface charge) on going from epididymal to ejaculated sperm cells. The results of our Zn^{++} binding experiments support these conclusions, and indicate that there are about 1.43 times as many cation binding sites on ejaculated cells. However, because of the double valence of the ion it is not possible to draw any conclusion about the ratio of membrane charge.

The ion binding values reported for ejaculated rabbit cells can be compared to the published values for human cells (Velazquez & Rosado, 1972). The Ca^{++} binding of human sperm is reported to be about 10 μEquiv per 10^8 sperm from cell polarography, while our Zn^{++} binding is 0.052 μEquiv per 10^8 sperm. The charge on the human sperm is somewhat higher (Bey, 1967), but the difference in binding is greater than an order of magnitude. This difference is probably due to the difference between the Zn^{++} and the Ca^{++} ions as probes of the surface charge. The affinity of the two ions for the same sites may differ by over an order of magnitude, as already noted in connection with the results of the electrode noise experiments reported in Table 2. The two ions may also be binding at different sites, including ion exchange reactions at sites that do not normally contribute to the surface charge. Therefore, it is possible that our results are consistent with those of Velazquez and Rosado (1972) regarding ion binding. However, we feel that our results give a better measure of the number of free anionic groups that normally exist on the available membrane area.

The value that we reported for the anionic binding sites on the membrane is probably still too high as a measure of the membrane charge, since analysis of the normal charge bearing groups on a membrane, the N-acetylneuraminic acid residues, shows that they are present in smaller quantitites on the membranes of sperm cells (Bey, 1967). Bull sperm cells, which have a somewhat greater electrophoretic mobility than rabbit cells, have neur-

aminic acid that is equivalent to about 0.6 charges per 100 $Å^2$ of membrane. This is approximately one-third of the value we determined on the basis of Zn^{++} binding.

Returning to the properties of the sperm cells at electrodes, we see that the electrode noise measurement, which results from adsorption processes, relates only indirectly to the membrane surface charge. It appears that despite the higher negative charge on the ejaculated sperm cell it does not adsorb substances from a negatively charged surface as well as an epididymal cell with about half the charge. Since cells usually are negatively charged, ejaculated sperm would not adsorb material from surrounding cells as readily. The ability of a sperm cell to adsorb substances in the environment is probably quite important in terms of its role in fertilization. The membrane changes in capacitation and the acrosome reaction may be initiated by adsorption or desorption processes. However, more information about the properties of sperm cells under different conditions is needed to establish the role of adsorption.

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