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STRUCTURAL PROPERTIES OF THE MEMBRANE OF INTACT HUMAN SPERMATOZOA

A STUDY WITH FLUORESCENT PROBES*

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SUMMARY

The properties of the membrane of intact, metabolically active, human spermatozoa have been studied by the use of 1-anilino-8-naphthalene sulfonate (ANS). By fluorescence microscopy it was found that at neutral pH ANS is bound exclusively to the membrane of the entire sperm with some preferential binding to the midpiece, while at low pH some preferential binding to the acrosome was observed. By spectrofluorimetry, fluorescence was found to be enhanced 48-fold on binding of ANS to the spermatozoal membrane, with a 50-nm shift in the emission spectrum of the bound dye. 2.47 ± 0.02 nmoles of ANS were bound per 10^6 spermatozoa ($K = 2.3 \cdot 10^{-5}$ M). Scatchard plots indicate that all the binding sites on the spermatozoal membrane have similar binding characteristics with a Z value of 84.8. Energy transfer with an efficiency of 7% was found for recently ejaculated spermatozoa. The fluorescence of bound ANS depends on the pH of the medium and possibly on the metabolic state of the cell, since addition of succinate or fructose produces an enhancement of fluorescence, while addition of glucose results in a decrease of this parameter. These changes are inhibited by the presence of cyanide.

INTRODUCTION

In the biology of reproduction, membranes play an important role in many processes such as sperm motility, capacitation, syngamy, *etc.*[1]. The study of the membrane of gametes and the changes in structure and/or function accompanying these processes is therefore of great importance.

A fluorimetric technique for measurement of sperm capacitation has been proposed by Ericsson[2,3]. This technique, based on the release of membrane-bound tetracycline, has been recently validated in the human (Hicks *et al.*[4]) by showing that incubation of [^{3}H]tetracycline·HCl-labelled sperm cells in the presence of some media known to capacitate hamster sperm *in vitro* induces the release of the bound probe.

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

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More recently, Edelman and Millete[5] have found by means of fluorescence microscopy that ANS binds to the sperm plasma membrane producing an immediate loss of motility.

We now report our studies of the binding of 1-anilino-8-naphthalene sulfonate (ANS) to the membrane of intact human sperm cells, of energy transfer from aromatic amino acid-rich membrane regions to the fluorescent probe, and of the changes induced in these parameters by variations in pH and substrate availability.

MATERIALS AND METHODS

Human spermatozoa were obtained from fresh ejaculates and were suspended and washed in 0.01 M Tris–HCl (pH 7.38). Freshly prepared suspensions of spermatozoa were adjusted to about 10^8 cells per ml, as determined with a hemocytometer, and the number was checked afterwards by absorbance measurement at 230 nm of an aliquot of the spermatozoal suspension[6].

Neuraminidase treatment

10^8 spermatozoa per ml were suspended in 0.25 M sucrose solution buffered to pH 6.5 with 0.01 M phosphate buffer. 50 μg of neuraminidase, chromatographically purified from *Clostridium perfringens* (Sigma type VI), were added at time zero, and the mixture was incubated at 37 °C for 60 min. Controls were run at the same time using heat-inactivated neuraminidase. After incubation, the sperm cells were recovered by centrifugation, washed once and finally resuspended in 0.01 M Tris–HCl (pH 7.38).

The magnesium salt of ANS was obtained from Eastman Organic Chemicals (Cat. No. 10990) and used without further purification. ANS concentration was calculated by weighing and checked using the value of $4.95 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ for the molar absorption coefficient at 350 nm (ref. 7). All other reagents used were of the best quality obtainable through Sigma Chemical Co.

Fluorescence spectroscopy

Fluorescence determinations were made in a Perkin–Elmer MPF-2A recording spectrophotofluorimeter at room temperature in the ratio mode. The slit widths of excitation and emission were 6 nm, unless stated otherwise. Unless indicated, the recorded readings were not corrected for the spectral dispersion of excitation energy, nor for the spectral characteristics of the detection system. All fluorescent measurements were carried out 30 s after the addition of the reagents to the cell. The observed fluorescence intensities were corrected for the emission of the spermatozoa and of free ANS.

Absorbance spectroscopy and fluorescence microscopy

Absorbance measurements were carried out at room temperature in a Unicam SP-800 double-beam recording spectrophotometer.

Since it was conceivable that ANS might bind to limited patches on cell membranes, we examined human spermatozoa with and without ANS by phase-fluorescence microscopy using a Reichet Zetopan Universal microscope equipped for fluorescent observation.

Calculation of quantum yield and number of binding sites

Quantum yield of the produced fluorescence was measured by the comparative method of Parker and Reese[8] using quinine hydrochloride as a reference. ANS bound

to bovine serum albumin was also used as a reference; its quantum yield has been determined to be 0.75 by Weber and Young[9]. Measurements on the reference materials gave results within 10% of each other.

The number of dye-binding sites per spermatozoon (n) and the dissociation constant for the dye–spermatozoon complex (K_c) were determined in two ways. First, the general method developed by Klotz[10] was applied. This method involves plotting the inverse of the number of moles of ANS bound per 10^6 spermatozoa against the inverse of the fraction of dye bound. The fraction, X, of dye bound can be obtained by the use of the formula proposed by Laurence[11] which requires knowledge of the fluorescent intensities of the all-bound and the all-free situations.

The fluorescent intensity produced when all the ANS is bound to spermatozoa was obtained by plotting the inverse of the results obtained by measuring the change in fluorescence produced by the addition of increasing amounts of spermatozoa to a fixed amount of ANS. The Y intercept of the line represents the inverse of the maximal fluorescence that could be obtained with the selected ANS concentration.

In the second method used, a fixed number of spermatozoa was titrated with increasing amounts of ANS up to a maximum concentration of 80 μM. The amount of dye bound at each concentration (and by subtraction, the amount free) was calculated by first determining the enhancement in fluorescence when 1 nmole of dye was completely bound. This value was obtained by extrapolation of plots of the reciprocal of fluorescence *versus* the reciprocal of protein concentration, in the manner suggested by Datta and Penefsky[12] (see above). This latter technique permits work with less turbid solutions. Addition of large amounts of sperm cells produces turbidity of the media and results in a tendency to agglutinate.

In all titrations the total dilution was maintained below 10% and was usually 5%. Unless stated otherwise, all experiments were performed in 0.020 M Tris buffer pH 7.38 prepared in 0.14 M NaCl. All reagents used, including ANS, were always prepared in the same buffer in which the titration was carried out.

RESULTS

Fluorescence microscopy

Incubation, even for 60 min, of twice-washed human spermatozoa with concentrations of ANS as high as 0.1 mM did not immobilize human spermatozoa. Motile spermatozoa can be observed as long as 24 h after addition of the fluorescent probe. Increasing ANS concentrations produced an increasing immobilization of spermatozoa. At 1 mM ANS, 80% of the spermatozoa were immobilized almost immediately[5] although 100% immobilization took at least 2 h. It is worth mentioning that immobilized spermatozoa under these conditions do not show a decrease in their oxygen uptake as measured in a Gilson oxygraph with a Clark type electrode. Microscopic observation shows the binding of the probe preferentially, if not exclusively, to the membrane of the entire sperm (Fig. 1) with some preferential binding to the midpiece. At pH 7.5, deficient binding of the probe to the acrosomal region was frequently observed. However, when the observation was carried out at lower pH values (around 4.5), the spermatozoa were labeled either uniformly or with a higher fluorescence in the acrosomal region (Fig. 1B).

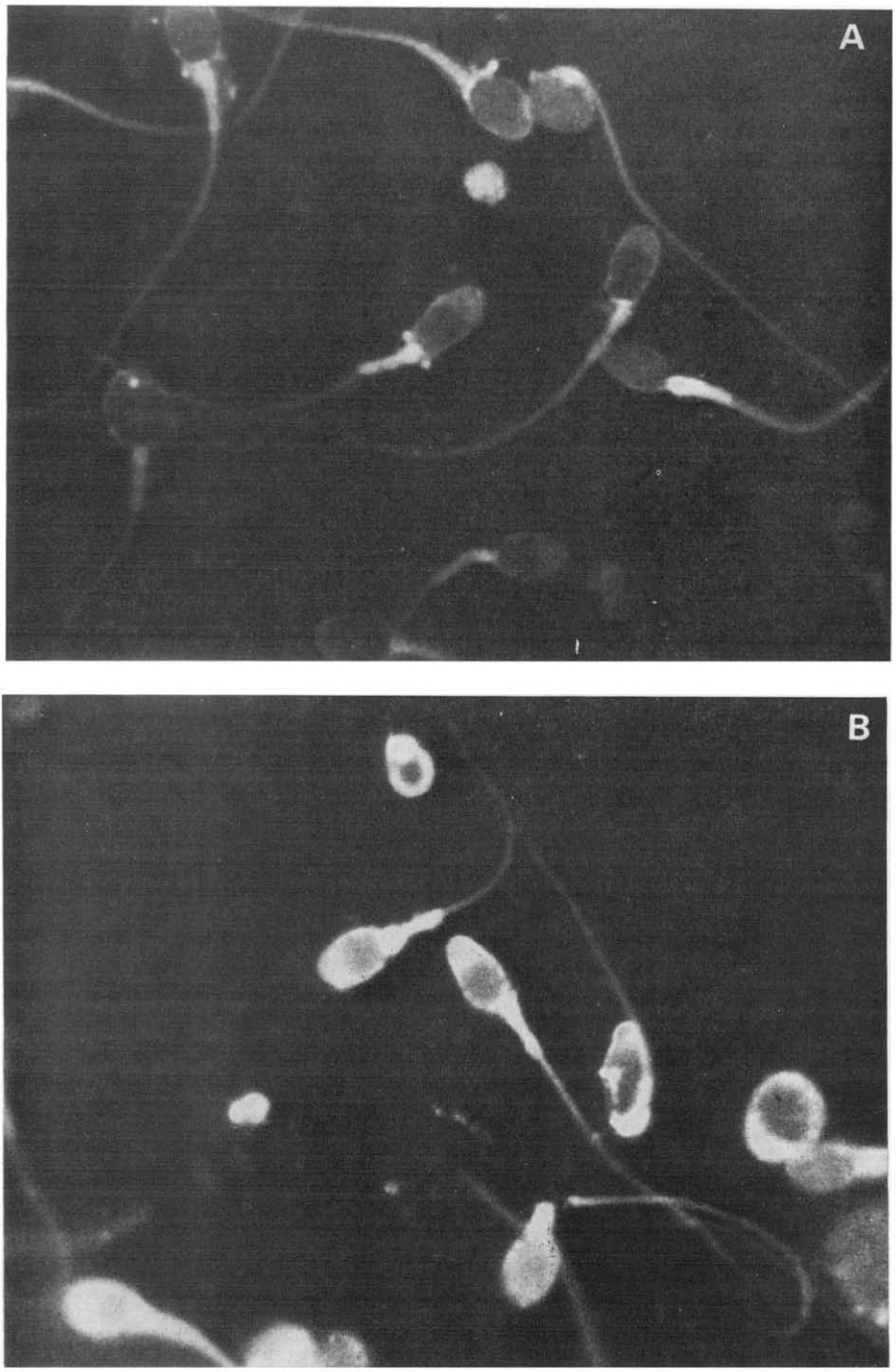


Fig. 1. Fluorescence microphotography of human spermatozoa labeled with ANS. Both photographs were taken under the same conditions with the only difference that spermatozoa in (A) were labeled and observed at pH 7.5 and those in (B) at pH 4.5.

Titration of spermatozoa with ANS

The binding of ANS to intact human spermatozoa results in a considerable enhancement of fluorescence, 48 times greater than the fluorescence of ANS alone, and in a blue shift of the fluorescent maximum (Fig. 2A). Fig. 2B shows the fluorescence increase resulting from the addition of increasing amounts of spermatozoa to

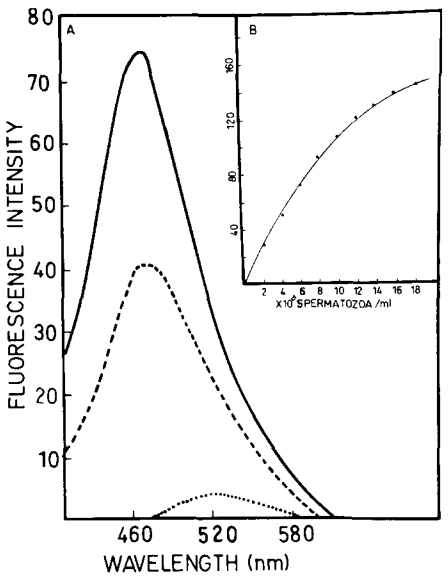


Fig. 2. ANS fluorescence in the presence and absence of intact human spermatozoa. Part A shows the uncorrected emission spectra of free and spermatozoa bound ANS. The dotted line represents the fluorescence of a 6 μM solution of ANS, while the continuous and the interrupted line represents the spectra observed by the addition of 6 and 3 millions of human spermatozoa, respectively. Fig. 2B represents the increase in the 470-nm fluorescence of a 6-μM solution of ANS when titrated with increasing amounts of human spermatozoa. Excitation was carried out at 370 nm in both cases.

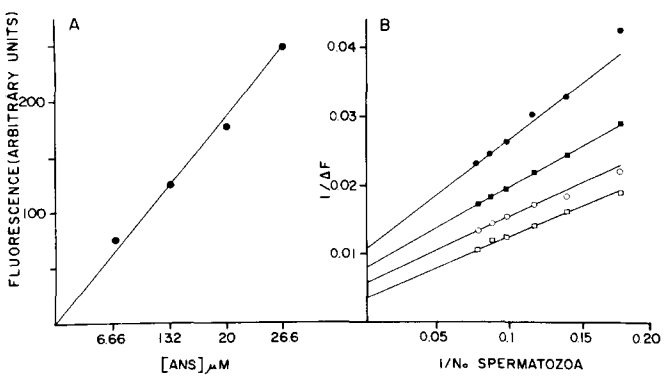


Fig. 3. Relative quantum yield of ANS fluorescence bound to intact human spermatozoa. Increasing amounts of human spermatozoa were added to 6.66 (●—●), 13.2 (■—■), 20 (○—○) and 26.6 (□—□) μM solutions of ANS, and the resulting fluorescence increase was plotted in reciprocal values against the reciprocal of the number of spermatozoa (in millions) added (B). The maximal increase in fluorescence obtained by the ordinate intercept in (B) for each ANS concentration were then plotted in (A) against the concentrations of ANS used.

a fixed amount of ANS. The graph indicates that in the presence of an excess of cells, all ANS molecules tend to be bound. The maximum increase in fluorescence produced by addition of different amounts of ANS, and obtained by a double-reciprocal plot, follows a linear pattern (Fig. 3) and indicates the average quantum yield, under normal conditions, of the spermatozoal membrane binding sites (Table I), as well as the fluorescence produced per nmole of dye bound.

TABLE I

DERIVED PARAMETERS FOR THE BINDING OF ANS TO HUMAN SPERMATOZOA

The data represent the means ±S.D. obtained from 4 to 5 determinations.

Method	*Fluorescence per nmoles ANS*	*n (nmoles per 10^6 spermatozoa)*	$\overline{K} \times 10^5$ *(M)*
Klotz	8.9 ± 1.2	2.47 ± 0.07	2.3 ± 0.18
Scatchard	—	2.58 ± 0.12	2.15 ± 0.23

This increase in fluorescence intensity is accompanied by a 50-nm shift in the emission spectrum of the bound dye. The emission peak of free ANS is at 520 nm (uncorrected), while the emission peak of the spermatozoa–ANS complex is 470 nm (uncorrected) (Fig. 2A). The relation of the blue shift with the number of spermatozoa is shown in Fig. 4B. Applying the procedures given by Turner and Brand[13] to our data, we found that the spermatozoa binding sites for ANS have, on the average, a Z value of 84.8.

Quick mixing of the sample into the cell compartment permits the time course of the increase in fluorescence (Fig. 4A) to be followed. The highest level of fluorescence is reached within 2 min, 85% of this increase is produced within the first 5 s after mixing. The increase is almost complete after 30 s.

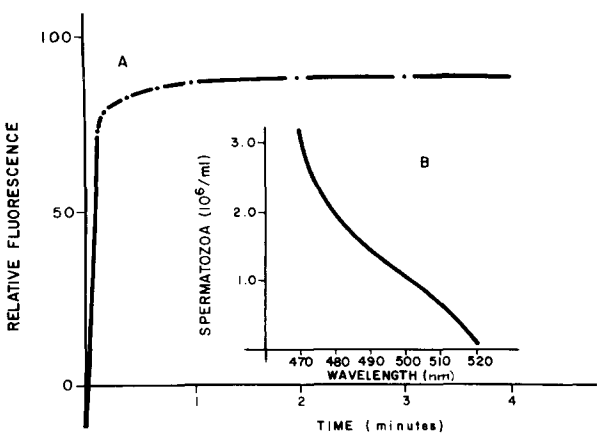
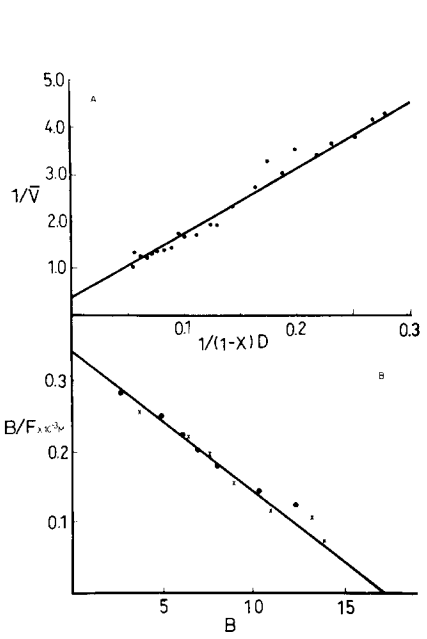


Fig. 4. (A) Time-related increase in the 470-nm fluorescence of ANS produced upon binding to human spermatozoa. At zero time $5 \cdot 10^6$ spermatozoa were added to a 6-μM solution of ANS and the increase in fluorescence was recorded. (B) Wavelength shift of the emission maxima of ANS produced by the addition of increasing amounts of human spermatozoa. Excitation wavelength was 370 nm in both cases.

ANS binding characteristics

An analysis of the type and number of ANS binding sites on the spermatozoal membrane was carried out. It appears from Fig. 5A that, when plotted in this manner, the data agree with the equation proposed by Klotz *et al*[10]. to describe the binding of dyes to proteins. The parameters derived for the equation are shown in Table I.

When increasing concentrations of dye were added to a fixed concentration of spermatozoa, the observed fluorescence increased to a limiting value. That is, it appeared to be possible to saturate all the binding sites on the spermatozoal surface with excess dye. Scatchard plots[14] constructed from titrations of this type (Fig. 5B) yielded data similar to those obtained by the Klotz method. From the linear character of the Scatchard plot it is possible to suggest that all the binding sites on the spermatozoal membrane have similar binding characteristics.



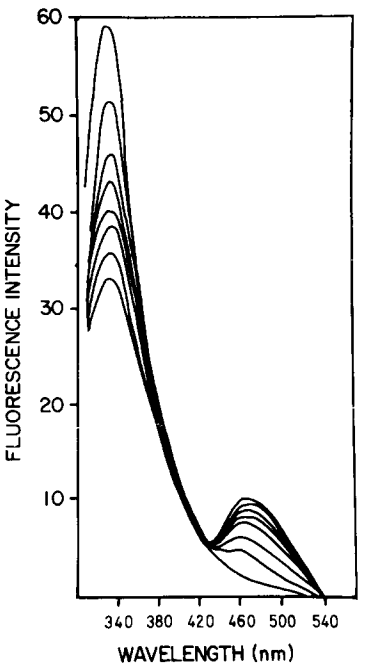


Fig. 5. Klotz (A) and Scatchard (B) plotts of ANS binding to intact human spermatozoa. The parameters obtained are indicated in Table I. In the Klotz plot (A) the ordinates indicate the reciprocal of the number of nmoles bound per 10^6 spermatozoa and the abscissas the reciprocal of the concentration of free dye. In the Scatchard plot (B) the ratio of bound over free ANS (B/F) is plotted against the nmoles of bound dye. In this case $8 \cdot 10^6$ spermatozoa were titrated by increasing amount of ANS following the method proposed by Datta and Penefsky[15].

Fig. 6. Uncorrected emission spectra of spermatozoa and of the ANS–spermatozoon complex when excited at 290 nm to study the presence of energy transfer. Additions of 6.6 nmoles of ANS induce a progressive quenching of the fluorescence at 335 nm with a progressive increase of that at 470 nm.

Energy transfer

Fig. 6 shows the emission spectra of spermatozoa and of the ANS–spermatozoon complex when excited at 290 nm. In the absence of ANS, the fluorescence at 338 nm arises from the excitation of the tryptophan residues on the spermatozoal

membrane. When ANS is added to a spermatozoal suspension, the tryptophan fluorescence at 338 nm is seen to be progressively quenched, while a second peak appears in the 470 nm region, which represents ANS fluorescence. This phenomenon has been repeatedly explained as being due to the transfer of excitation energy from protein chromophores to the bound dye[15]. A second manifestation of energy transfer is seen in the corrected excitation spectrum (Fig. 7). In the absence of energy transfer, the excitation spectrum of ANS fluorescence would be expected to coincide with the absorption spectrum of bound ANS. This is the case for the region above 300 nm. Below this wavelength the observed fluorescence is at least double that of the absorption spectra. The additional fluorescence results from a transfer of the excitation energy in the 280-nm region from tryptophan residues to the bound ANS. Using the equations proposed by Cheung and Morales[16] for the estimation of efficiency of transfer, a value of about 7% was found for recently ejaculated, washed human spermatozoa.

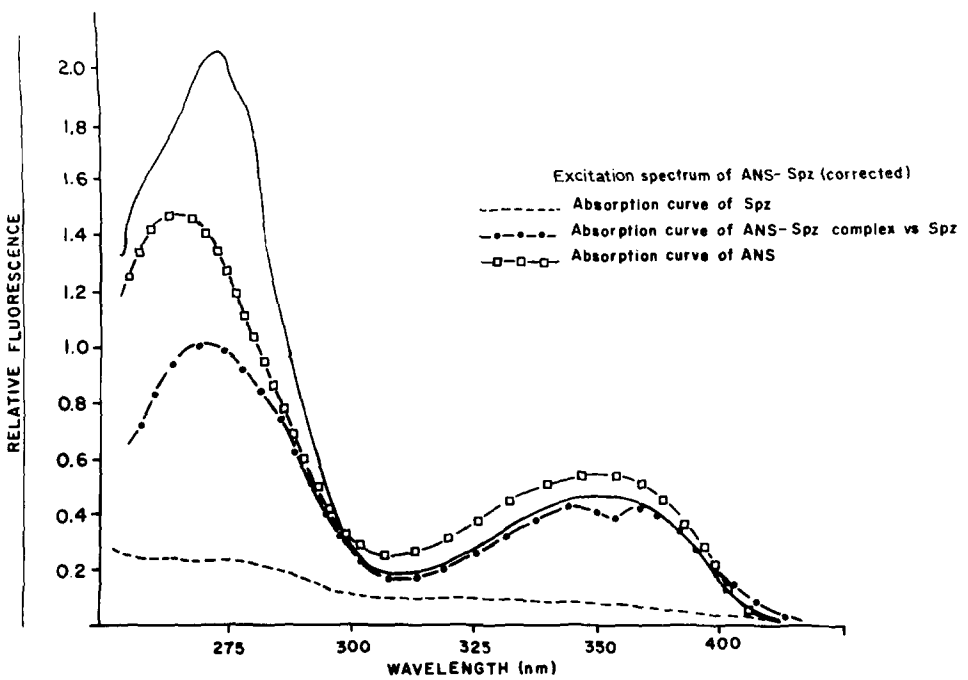


Fig. 7. Absorption and excitation (corrected) spectra of ANS bound to spermatozoa. The absorption spectrum of ANS complexed to sperm cells was obtained as a difference spectrum (ANS–spermatozoon complex *versus* spermatozoa) in a double-beam spectrophotometer.

Effect of substrate availability on ANS fluorescence

A significant increase in fluorescence was observed when succinate or fructose was added to a mixture containing spermatozoa almost saturated with dye (Fig. 8 and Table II). The increase produced by fructose was less marked but highly reproducible. These enhancements of fluorescence were not accompanied by an increase in the blue shift. When fixed amounts of spermatozoa resuspended in Tris–HCl (pH 7.4) were titrated with increasing amounts of ANS in the presence of 10 mM

TABLE II

EFFECT OF SUBSTRATE AVAILABILITY ON ANS FLUORESCENCE IN THE PRESENCE OF HUMAN SPERMATOZOA

The data represent the means ±S.D. obtained from 4 to 5 determinations.

Additions	*% maximal fluorescence per 10^6 spermatozoa*	*n (nmoles per 10^6 spermatozoa)*	$\overline{K} \times 10^5$ *(M)*
None	100	2.58 ± 0.12	2.15 ± 0.13
Succinate (10 mM)	142*	2.45 ± 0.09	1.45 ± 0.18*
Succinate *plus* NaCN	98	2.69 ± 0.11	2.00 ± 0.13
Fructose (10 mM)	128*	2.53 ± 0.13	1.60 ± 0.11*
Fructose *plus* NaCN	96	2.65 ± 0.14	2.05 ± 0.16
Glucose (10 mM)	88*	2.44 ± 0.09	2.33 ± 0.12
Glucose *plus* NaCN	96	2.55 ± 0.12	2.19 ± 0.09

* $P < 0.01$ when compared with the group with no additions.

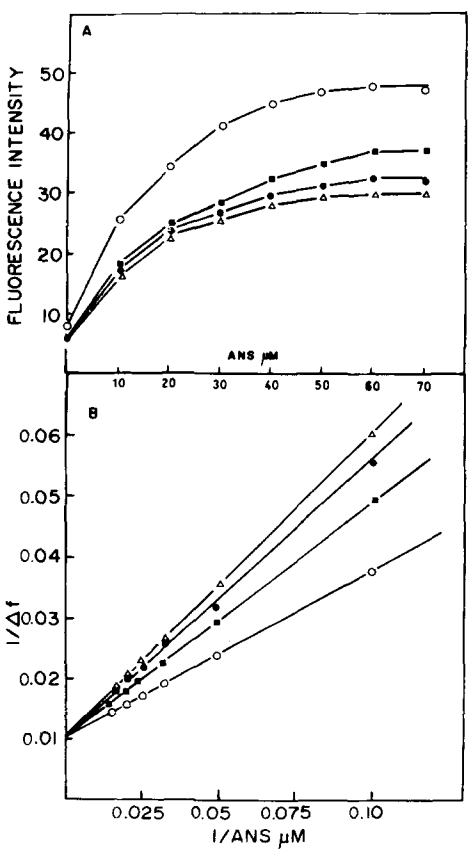


Fig. 8. (A) Effect of the presence of succinate (○—○), fructose (■—■) and glucose (△—△) on the fluorescence produced by adding ANS to a suspension of intact human spermatozoa, as compared with the fluorescence produced with no added substrate (●—●). (B) Double-reciprocal plot of the data presented in (A). The final concentrations of the substrates used was 10 mM in all cases.

succinate, or 10 mM fructose, the results obtained, plotted as the reciprocals of the obtained values (Fig. 8B) and then analyzed by Scatchard's method, indicate that the action of these substrates upon the spermatozoal membrane produces an increase in the quantum yield of the bound ANS and a modification of the apparent dissociation constant without any change in the number of binding sites (Table II). Of special interest is the fact that glucose, in a concentration similar to that of fructose, either does not produce any change in the pattern of ANS binding to spermatozoa or, more frequently, induces a small but significant decrease in the ANS-induced fluorescence (Table II). Also, substrate availability produces changes only with recently ejaculated spermatozoa and addition of sodium cyanide decreases the ANS fluorescence to the value prior to substrate addition (Table II).

Effect of the pH of the solution on ANS fluorescence

Because of its negative charge, ANS should be repelled by anionic sites in the membrane at neutral pH. Human spermatozoa possess an excess of negative charges at neutral pH[17]. If anionic groups of spermatozoa are neutralized by reducing the pH, an increased binding of ANS may result. As observed in Fig. 9, the pH of the reaction

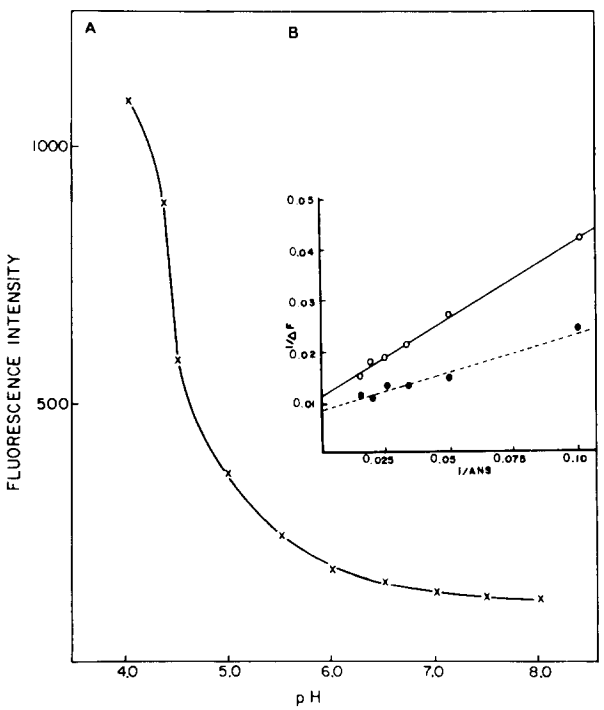


Fig. 9. (A) pH profile of the spermatozoon–ANS complex. At each pH $4.5 \cdot 10^6$ spermatozoa were titrated to the saturation point with increasing amounts of ANS. The data obtained were double-reciprocal plotted and the maximal fluorescence, obtained by extrapolation to the ordinate, is related in the figure to the pH. pH maintained with 20 mM Tris–acetate buffer was measured before and after titration was completed. In (B) the double-reciprocal plot of the ANS titration of neuraminidase-treated spermatozoa is shown (●---●), compared with the data obtained in the spermatozoa treated under the same conditions with heat-inactivated enzyme (○—○).

TABLE III

pH EFFECT ON THE DERIVED PARAMETERS FOR THE BINDING OF ANS TO HUMAN SPERMATOZOA

The data represent the means ±S.D. obtained from 4 to 5 determinations.

pH	*% maximal fluorescence per 10^6 spermatozoa*	*n (nmoles per 10^6 spermatozoa)*	$\overline{K} \times 10^5$ *(M)*
4.0	1000	8.13 ± 0.37	0.48 ± 0.05
5.0	300	4.73 ± 0.28	1.06 ± 0.09
6.0	140	2.98 ± 0.11	1.72 ± 0.09
7.0	100	2.47 ± 0.07	2.15 ± 0.23
8.0	95	2.49 ± 0.09	2.23 ± 0.18

mixture has a profound effect upon the enhancement of fluorescence which accompanied the addition of dye to human spermatozoa. A 2-fold increase occurred over the pH range 5.5 to 8.0, the normal biological range of pH variation to which the spermatozoa may be exposed. However, further lowering of the pH of the spermatozoal suspension produces a drastic increase in fluorescence of bound ANS (Fig. 9A) and indicates a pK of 4.4 for the ANS-responsive fixed anionic group. When the data obtained are analyzed by the methods described, it is observed that, although the increase in fluorescence is mainly due to an increase in the quantum yield of bound ANS, it is also partially explained by the appearance of new binding sites (Table III). This last effect may be produced by the refolding of some membrane peptide chains into B-structures, a process which occurs at low pH[18].

Effect of neuraminidase treatment

Incubation of human spermatozoa with chromatographically purified neuraminidase for 60 min produced a notable increment in the ANS-induced fluorescence of human spermatozoa. As can be seen in the Fig. 9B, when the data obtained are double reciprocal plotted it is found that the increase in fluorescence is only partially explained by the increase in the number of binding sites and that it is mainly due to an increase in the quantum yield of bound ANS. This is a behavior similar to that observed by the change in the pH of the medium and may be related to the same mechanism of action.

DISCUSSION

The object of this study was to determine whether some of the physicochemical properties of the membrane structures of whole, intact, mammalian cells (in this case human spermatozoa) could be established by current methods. Mammalian spermatozoa were chosen as models because they can be easily obtained as free cells and because important membrane changes accompany the maturation and the physiological behavior of these cells[19].

It can be roughly calculated that $6 \cdot 10^8$ molecules of the fluorescent probe can

be bound by a single spermatozoon in 20 mM Tris–HCl buffer (pH 7.4), which is 20 times higher than the value reported by Gitler and Rubalcava for hemoglobin-free erythrocytes[20]. This high hydrophobicity could be related to the low electrophoretic mobility of spermatozoa[21] when compared with that of erythrocytes[22], and to the susceptibility of human spermatozoal membranes to detergents[23]. Unfortunately no data exist that allow us to relate the binding of ANS to the composition of the spermatozoal membrane.

The low transfer efficiency (7%) found by us (Figs 6 and 7) may be explained if it is assumed either that only a small proportion of the tryptophan residues on the spermatozoal coat protein are in appropriate non-polar regions or that the proportion of this amino acid in the membrane proteins of human spermatozoa is exceptionally low.

Transfer efficiency is sensitive to small perturbations of protein conformation[24], and therefore, as expected, the transfer efficiency of the spermatozoal membrane changes with the ionic environment. Thus the increase in fluorescence of the spermatozoon–ANS complex at acid pH when excited at 288 nm cannot be explained only by the increase in fluorescence of this complex when excited at 370 nm. An important increase in the transfer efficiency in this pH region is also necessary. Since pH modifications comprise both an increase in the quantum yield of the fixed ANS and an increase in the number of binding sites, it may be postulated that changes in environmental pH alter the structure of the coat protein of human spermatozoa in such a way that more of the ANS-unexposed tryptophan regions may be available to ANS binding. This is similar to the effect of pH on the binding of ANS to isolated erythrocyte membranes[20].

Since, particularly in the experiments of Gompertz and Stock[25], treatment of microsomes with neuraminidase does not produce any change in the pH titration curve, the changes in fluorescence produced by changes in the pH of the ANS–membrane complex have been related to the presence of acidic side-chains of protein amino acid[20]. We may remark, however, that although sialic acid may not be important in the structure of intracellular membranes, it has been reported to account for an important part of the negative surface charge of cell membranes[26,27]. In particular, the net negative surface charge of human spermatozoa seems to be related to the presence of membrane sialic acid as has been shown by cell polarography[17] and by modifications in the electrophoretic mobility of spermatozoa[21]. A further datum supporting the hypothesis that sialic acid represents the fixed anionic group of pK approx. 4.4 is that treatment of human spermatozoa with chromatographically pure neuraminidase produces a significant decrease in its electrophoretic mobility[21] and also a large increase in the fluorescence induced by ANS binding (Fig. 9B). Furthermore, microscopic observations of fluorescence have shown that the acrosome region of human spermatozoa fluoresces more at acid pH when labelled with ANS, than when the labeling is carried out at a pH higher than 7.0. It is tempting, then, to speculate that sialic acid is randomly distributed through the spermatozoa membrane, but with a higher density in the acrosomal region.

In the experiments reported here, addition of metabolizable substrates to metabolically active spermatozoa produced a modification of the fluorescence observed in the absence of the substrate (Table II).

Previous work has shown that human spermatozoal oxygen uptake is stimulated

by the addition of fructose or succinate, the latter being much more active[28]. On the other hand, glucose addition induces the production of a Crabtree effect[28] and inhibits oxygen uptake. These facts point to the existence of different mechanisms in the utilization of the two hexoses. It is difficult to compare these results with those presented here, but the production of an increase of fluorescence by fructose, compared with the slight, but reproducible, decrease induced by glucose, may suggest differential mechanisms of transport and/or utilization of these carbohydrates at the membrane level.

Although some alternative interpretations of the data reported here should be considered, it is important that the modifications in fluorescence induced by succinate and fructose are observable only in recently ejaculated spermatozoa and that they decrease and ultimately disappear in aged spermatozoa. Moreover, the presence, or addition, of 3 mM NaCN inhibits the occurrence or reverses the changes in fluorescence induced by these substrates (Table II).

It is clear that the experimental strategy adopted here to study the behavior of the cell membrane of normal, untreated, spermatozoa is, in principle, applicable to other situations where the state of this membrane may have been modified by exposure to some experimental or *in vivo* conditions similar to those of the physiological environment of the cell. It is possible, then, to postulate that fluorescent probes are useful in studying the changes in surface characteristics that accompany spermatozoal maturation and/or capacitation and that changes in membrane architecture produced during active transport, metabolic activity, hormone action, *etc.* in intact cells may be studied by this procedure.

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