Specific Binding of the Glycosaminoglycan ^{3}H-Heparin to Bull, Monkey, and Rabbit Spermatozoa *In Vitro*

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In Vitro **binding and some binding parameters of the glycosaminoglycan heparin to viable epididymal or ejaculated bull spermatozoa, ejaculated rabbit spermatozoa, and frozen-thawed rhesus monkey spermatozoa were investigated. Nonspecific binding was affected only by the concentration of ^{3}H-heparin, whereas specific binding was saturable, reversible, and dependent on the *p*H, temperature, and calcium concentration of the incubation medium. Magnesium concentration dependence was observed in the presence of calcium but could not be detected in the absence of calcium. Bound ^{3}H-heparin was displaced by several orders of magnitude greater concentrations of chondroitin sulfate. Scatchard plot analysis suggested multiple binding affinities for ^{3}H-heparin to spermatozoa. ^{3}H-heparin was shown to bind to sperm heads and flagella. Fluorescein-labeled heparin bound to acrosomal, postacrosomal, and flagellar membranes. It was concluded that the specific binding of heparin involved a proteinaceous component on, or intercalated with, spermatozoal membranes. Thus, glycosaminoglycans present in the female reproductive tract may contribute to sperm capacitation and enhance the likelihood of successful fertilization in mammals.**

Key words: sperm, acrosome reaction, glycosaminoglycans, heparin, binding.

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Mammalian spermatozoa obtained from either the epididymis or a fresh ejaculate are initially incapable of fertilizing ova of the same species. Before a spermatozoon can penetrate an ovum, it must become "capacitated" and lose its outer membranes via the "acrosome reaction" (for a detailed review see Bedford, 1983). "Capacitation" and the "acrosome reaction" are events that occur as the spermatozoa pass through the female reproductive tract; however, the actual mechanisms by which these events occur are not completely understood.

The acrosome reaction is calcium-dependent (Yanagamachi and Usui, 1974), and involves fusion at many points of the outer plasma and outer acrosomal membranes overlying the anterior portion of the sperm head, culminating in membrane vesiculation (Fawcett, 1975). Activation of acrosomal zymogens seems concomitant with the acrosome reaction. Evidence has been presented for regulation of the acrosome reaction by calmodulin (Jones et al, 1980, Lenz and Cormier, 1982).

Follicular fluid increased the number of spermatozoa having reacted acrosomes in many species (Barros and Austin, 1967; Bedford, 1969; Iwamatsu and Chang, 1969). Proteoglycan isolated from bovine follicular fluid promoted the acrosome reaction in bull spermatozoa (Lenz et al, 1982). The active component of that proteoglycan was shown to be the glycosaminoglycan (GAG) side-chains. An inhibitor of calmodulin activity reduced the ability of a chondroitin sulfate preparation to induce acrosome reactions.

Porcine uterine flushings stimulated the conversion of purified boar proacrosin to the active protease acrosin (Wincek et al, 1979). The active component was a glycosaminoglycan (GAG). Similar results were obtained when boar spermatozoa were incubated with commercially available GAGs

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(Parrish et al, 1980). No correlation could be found between the degree of sulfation on GAGs and their ability to catalyze the conversion of proacrosin to acrosin.

Conversely, comparisons of commercially available GAGs indicated that the most highly sulfated polysaccharide tested, heparin, was also the most effective for induction of an *in vitro* acrosome reaction in bull or rabbit spermatozoa. The increased sulfation of the amino sugar component of the disaccharides significantly enhanced their inductive quality for the acrosome reaction (Handrow et al, 1982). It was postulated that high doses of GAGs could contribute to the membrane stability of rabbit spermatozoa and consequently decrease the incidence of reacted acrosomes (Lenz et al, 1983).

^{3}H-heparin binds to the membranes of human spermatozoa (Delgado et al, 1982), and heparin induces the decondensation of human sperm nuclei (Delgado et al, 1980). The present study was designed to evaluate the ability of ^{3}H-heparin to bind *in vitro* to bull, rabbit, and rhesus monkey spermatozoa, as well as to assess the effects of varying calcium and magnesium concentrations, temperature, *p*H, time, and the presence of other glycosaminoglycans on that binding.

Materials and Methods

Spermatozoa

In order to make general comparisons, the binding of ^{3}H-heparin to epididymal or ejaculated bull spermatozoa, ejaculated rabbit spermatozoa, and frozen-thawed ejaculated rhesus monkey spermatozoa was examined.

Epididymal bull or ejaculated bull and rabbit spermatozoa suspensions were prepared with Tyrode's balanced salt system (TALP) (Leibfried and Bavister, 1981) supplemented with 0.6% bovine serum albumin, 21.6 mM lactate, 10 mM HEPES, and 0.2 mM pyruvate (final *p*H 7.35, except where otherwise noted). All incubations were performed with TALP or modified TALP. Calcium ion concentrations were modified by omission of $CaCl_2$ and the addition of 50 μM ethylene-glycol-bis-(β-aminoethyl ether) N_1N'-tetra-acetic acid (EGTA), or by the inclusion of additional $CaCl_2$ to a final concentration of 3.6 mM. Magnesium ion concentration was modified by the addition of $MgCl_2$-$6H_2O$ to final levels of 1, 4, and 8 mM. The proper osmolality (290 milliosmoles/Kg) was maintained by adjustment of the amount of NaCl added.

Bull spermatozoa from the cauda epididymis were collected as previously described (Lenz et al, 1982). Ejaculated rabbit spermatozoa were collected from New Zealand White bucks twice weekly for one month prior to and during the experiments, using an artificial vagina. Ejaculated bull spermatozoa were provided by American Breeders Service, DeForest, Wisconsin, and were transported in a sealed thermos container at ambient temperature. Rhesus monkey spermatozoa were collected by electro-ejaculation (Mastroianni and Manson, 1963) from eight adult rhesus males, 6 to 18 years of age and weighing from 5.80 to 9.35 kg. All samples were collected in the fall. Ejaculates were transported at ambient temperature in sealed centrifugation tubes.

Individual ejaculates or epididymal flushings were suspended in 10 ml of TALP, agitated gently, and centrifuged at 2000 rpm (200 xg) for 10 minutes. The supernatants were aspirated and the sperm pellets were resuspended in 2 ml TALP. Aliquots were evaluated for motility by light microscopy. Pools within each type of spermatozoa were made from those sperm suspensions having greater than 60% motile spermatozoa. An aliquot from each pool was incubated for 15 minutes at 37 C with an equal volume of 0.4% trypan blue. The percentage of living spermatozoa was estimated by evaluating 100 spermatozoa for dye exclusion by light microscopy. The percentage of viable spermatozoa exceeded 90% for all suspensions assayed. Sperm concentration was determined with a hematocytometer and light microscopy. An aliquot from the viable monkey sperm pool was evaluated in a displacement assay for comparison with results obtained with frozen-thawed monkey spermatozoa. Monkey sperm samples were frozen in 10% sucrose/90% TALP and stored at −70 C. Samples were thawed as needed and pelleted by centrifugation at 2000 xg. The supernatant was aspirated and the pellet resuspended by vortexing in 2 ml TALP. Binding equilibrium studies utilized 5×10^6 monkey spermatozoa incubated with 7.5×10^4 dpm of ^{3}H-heparin in a final volume of 1 ml, or 5×10^7 epididymal or ejaculated bull or rabbit spermatozoa in a final volume of 1 ml with 1.5×10^5 dpm (32.45 pmoles) of ^{3}H-heparin. Incubations of duplicate tubes for each treatment were conducted in 12 × 75 mm plastic capped tubes in a 37 C shaking water bath, except where noted otherwise. Varying doses of unlabeled heparin or other polysaccharides were added to obtain displacement data.

In experiments to determine the location of binding sites, bull sperm pools were divided into three equal groups. Fifty million spermatozoa per milliliter from one group were incubated in displacement assays with ^{3}H-heparin and 25% sucrose for 2 hours, and the level of radioisotope bound was assessed. Spermatozoa from the second group were centrifuged through a sucrose density gradient of layered 65%, 25%, and 5% sucrose solutions at 750 rpm (75 xg) for 20 minutes, then washed as previously described. Fifty million spermatozoa per milliliter were then assayed in a normal binding displacement assay. Spermatozoa from the third group were sonicated at 1 amp for 1 minute to separate heads from tails. These spermatozoa were then centrifuged through a sucrose density gradient as above. Fractions containing predominantly heads (<1% tails) were aspirated from the bottom layer. Tail fragments were collected from the 65% sucrose layer. Head and tail fractions were washed as previously described and individually assayed in standard displacement assays.

The effects of the addition of sucrose to the culture

medium were assessed by incubating bull spermatozoa for varying periods of time with and without ^{3}H-heparin and/or sucrose. ^{3}H-heparin and/or sucrose were added to different incubations at different times.

Reagents

The standard GAGs employed in the assays were obtained from Sigma Chemical Co. The heparin was a sodium salt purified from porcine intestinal mucosa. Chondroitin-4-sulfate was a sodium salt isolated from whale cartilage, while the sodium salt of chondroitin-6-sulfate was purified from shark cartilage. The sodium salt of dermatan sulfate was purified from porcine skin. Hyaluronic acid (sodium salt) was isolated from bovine vitreous humor. Dextran sulfate, a sulfated polysaccharide containing monomers that differ from those of GAGs, was used as a control.

^{3}H-heparin with a specific activity of 0.14 mCi/mg was purchased from New England Nuclear. Heparin with fluorescein conjugated to the terminal sugar residues (end-labeled) was a gift from Drs. J. T. Holbrook and M. Luscombe of the University of Bristol Medical School, Bristol, England. All other chemicals used were of standard reagent quality.

Determination of Heparin Bound

At the end of the incubation period, tubes were centrifuged at 2800 rpm (280 xg) for 10 minutes at 4 C and the supernatant aspirated. Sperm pellets were resuspended in 1 ml ice cold Tyrode's buffer (*p*H 7.35, except where noted), centrifuged for 5 minutes as above, and the supernatant aspirated. This was repeated twice. The washed sperm pellets were resuspended in 1 ml of Tyrode's buffer, poured into scintillation vials, and 4 ml scintillation cocktail was added (RPI 3A70B). Radioactivity was monitored in a Beckman spectrophotometer for 1 minute. Machine efficiency averaged 35%.

Ejaculated bull or monkey sperm incubations were prepared for determination of radioactivity bound by filtration on a Millipore sampling manifold with glass filters (Whatman 934-AH) at a vacuum of 1 inch of mercury. Tubes and filters were rinsed twice with 1 ml ice cold Tyrode's buffer. Wet filters were put into scintillation vials with 400 µl of dH_2O and 4 ml of 3A70B scintillation cocktail. Radioactivity was then monitored in a Beckman spectrophotometer.

In all experiments, nonspecific binding was determined by the addition of 1 mg of unlabeled heparin to duplicate background tubes containing spermatozoa and ^{3}H-heparin.

Fluorescent Studies

Sperm preparations were smeared on slides and dried. Twenty µl of fluorescein end-labeled heparin solution (1.5 mg/ml in dH_2O) was placed on each slide, a coverslip was added, and the slide was incubated for 20 minutes in the dark. The incubated slide was then repeatedly dipped in Tyrode's buffered salt solution until the coverslip floated clear of the slide. Several drops of 5% n-propyl gallate in 80% glycerol/15% TALP (Giloh and Sedat, 1982) were put on the slide and a coverslip added. Prepared slides were examined on a Zeiss photomicroscope III under epifluorescence at an excitation wavelength of 493 nm with a magnification of 580 ×. Photomicrographs were made with Ilford HP-5 (ASA 400) black and white film exposed at ASA 1600. Exposures were developed in Kodak HS-70 developer and photo-enlarged.

Statistics

All data were corrected for nonspecific binding prior to final analysis on Hewlett-Packard desk-top computers (HP-35 or HP-85). Displacement curves were determined by the method of Rodbard (1976). Slopes of regression lines for the most steeply sloped linear portions of the daily displacement curves were analyzed for significance by F-tests, and were compared using *t* tests. Saturation data were analyzed by least-squares estimation. Scatchard data computations were modified from the suggestions of Rodbard (1973) and Feldman (1972). Estimates of binding affinities and the number of binding sites were calculated using a direct fit program to estimate the asymptotes of the polynomial regression curves (Cressie and Keightley, 1979).

Results

The results of a typical saturation kinetics experiment for ^{3}H-heparin binding to epididymal bull spermatozoa are shown in Fig. 1, Panel A. Panel B describes the results of similar incubations with rhesus monkey spermatozoa. Optimum conditions for ^{3}H-heparin binding to epididymal bull or rabbit spermatozoa were 1.5×10^5 dpm of ^{3}H-heparin per 5×10^7 spermatozoa. Binding equilibrium was reached at 2 hours of culture and was maintained for at least 8 hours of culture. Variability was observed in the saturation levels of ^{3}H-heparin binding to different sperm pools within each type of spermatozoa. The range of saturation levels for epididymal bull spermatozoa ranged from 6 to 16×10^3 dpm of ^{3}H-heparin, while bull spermatozoa had a range of 10 to 25×10^3 dpm of ^{3}H-heparin bound at saturation. There was no difference in the saturation level when 5×10^7 spermatozoa were incubated with 3×10^5 or 1.5×10^5 dpm of ^{3}H-heparin (data not shown). Optimum conditions for binding to monkey spermatozoa were 7.5×10^4 dpm of ^{3}H-heparin with 5×10^6 monkey spermatozoa. Binding equilibrium was reached at 2 hours of culture for rhesus monkey spermatozoa. Saturation levels for the rhesus monkey sperm pool ranged from 4 to 8×10^3 dpm of ^{3}H-heparin.

Binding of ^{3}H-heparin to bull and rhesus

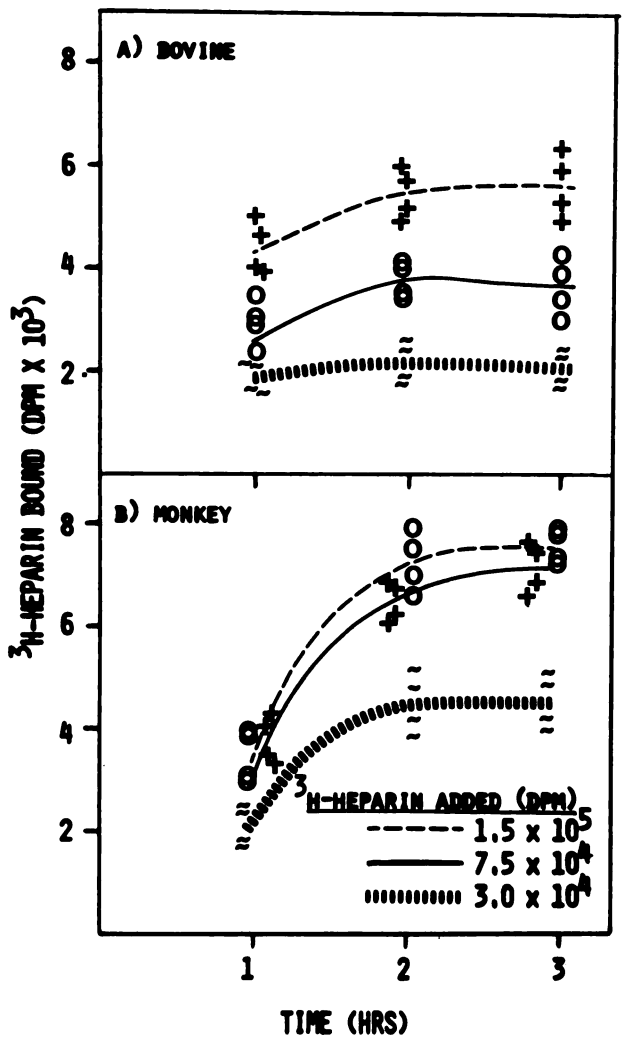

Fig. 1. Saturation kinetics for the specific binding of ^{3}H-heparin to bull epididymal spermatozoa (A) or ejaculated rhesus monkey spermatozoa (B) for three doses of ^{3}H-heparin.

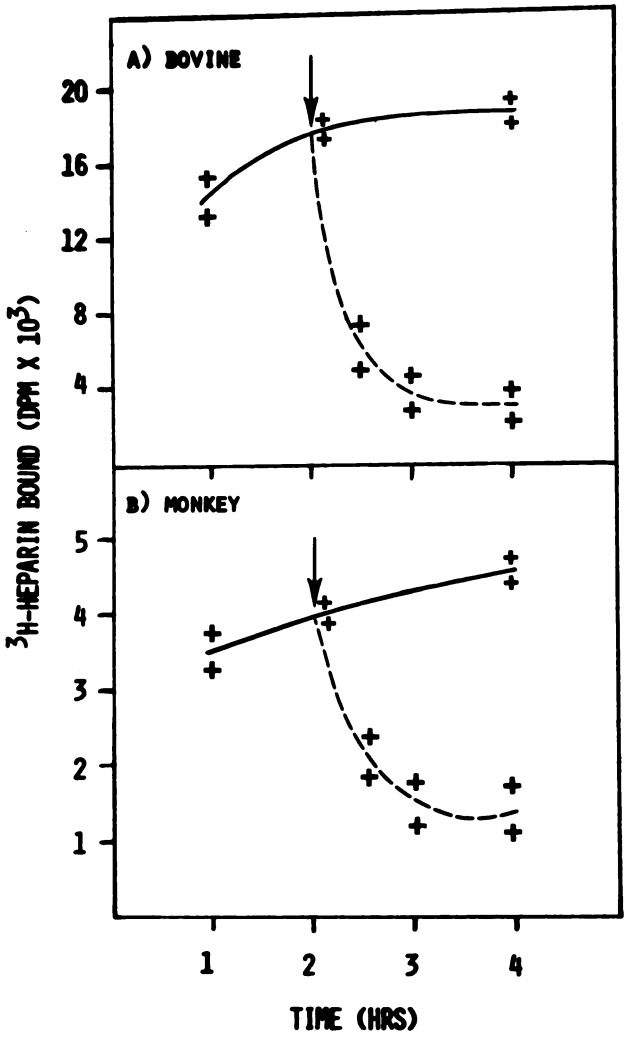

Fig. 2. Reversibility of ^{3}H-heparin specific binding to ejaculated bull spermatozoa (A) or rhesus monkey spermatozoa (B). Arrows mark time of addition of 100 μg (A) or 1 mg (B) of unlabeled heparin. (——) control tubes; (---) tubes to which unlabeled heparin was added.

monkey spermatozoa was reversible, as shown in Fig. 2. The addition of 100 μg/ml of unlabeled heparin to ejaculated bull spermatozoa preincubated for 2 hours with ^{3}H-heparin reduced the amount of specifically bound ^{3}H-heparin by 60% after 15 minutes, and an additional 4% after 30 minutes. In cultures of monkey spermatozoa preincubated for 2 hours with ^{3}H-heparin, the addition of 1 mg/ml of unlabeled heparin reduced by 60% the level of bound ^{3}H-heparin after 30 minutes. In both cases, control tubes incubated for the same total length of time had no reduction in binding. The binding of ^{3}H-heparin to sperm membranes was clearly reversible in a time-dependent manner.

Experiments to assess incubation effects on binding sites were performed as suggested by Ryan and Lee (1976). Ejaculated bull spermatozoa were incubated for 0 or 2 hours prior to the addition of ^{3}H-heparin. The tubes were then incubated for an additional 2 hours. The results shown in Table 1 suggest no apparent receptor damage. An increase in the amount of ^{3}H-heparin bound was noted. This is in contrast to saturation kinetics experiments that demonstrated saturable binding for spermatozoa incubated in the presence of ^{3}H-heparin for identical or longer incubation periods.

The results of displacement assays for epididymal or ejaculated bull, rabbit, or rhesus monkey

TABLE 1. Assessment of Incubation* Effects on Heparin Binding Sites on Ejaculated Bull Spermatozoa

Incubation Time Prior to Addition of ^{3}H-heparin† (Hours)	Heparin Bound‡
0	5846(539)
2	14214(1637)

* Incubations performed with Tyrode's balanced salt system supplemented by 0.6% BSA, 21.6 mM lactate, 10 mM HEPES, and 0.2 mM pyruvate, final *p*H 7.35.
† 32.45 picomoles.
‡ DPM ^{3}H-heparin bound to 5 × 10^7 spermatozoa expressed as Mean (SEM).

spermatozoa were used to compute individual displacement curves. These curves were similar or identical for all types of spermatozoa assayed. A typical displacement curve, for data from bull epididymal spermatozoa, is shown in Fig. 3. The variance of the experimental means, and deviations from the curve fit to those means, as shown in Fig. 3, are representative of all the subsequent displacement curves in this study. The steeply sloped linear portion of the curve occurs over an interval of three orders of magnitude of increasing heparin concentrations, which is characteristic of a limited number of binding sites and a physiological reaction. The slopes of regression lines calculated from daily results did not differ significantly. The slope factor of the displacement curves (the mathematical equivalent of the Hill coefficient) for epididymal or ejaculated bull, rabbit, or monkey spermatozoa was 0.8, with an effective dose for 50% displacement (ED 50) of 2.0 μg of heparin. The inter- and intra-assay coefficients of variance for rhesus monkey sperm assays were 18.1% and 6.3% respectively. The sensitivity was 0.54 ± 0.09 (Mean ± SEM, n = 8) μg/ml.

The results of incubations of ejaculated bull or monkey spermatozoa with ^{3}H-heparin in displacement assays at different temperatures indicated that heparin binding was temperature-dependent. Displacement curves obtained at 4, 20, and 37 C for ejaculated bull and monkey spermatozoa are shown in Fig. 4, Panels A and B, respectively. The kinetics of ^{3}H-heparin binding were similar for monkey or ejaculated bull spermatozoa. Binding equilibrium was reached after 8 hours of incubation at 4 C, 4 hours at 20 C, or 2 hours at 37 C. Total ^{3}H-heparin bound at 4 C was significantly lower ($P < 0.005$) than total ^{3}H-heparin bound at 37

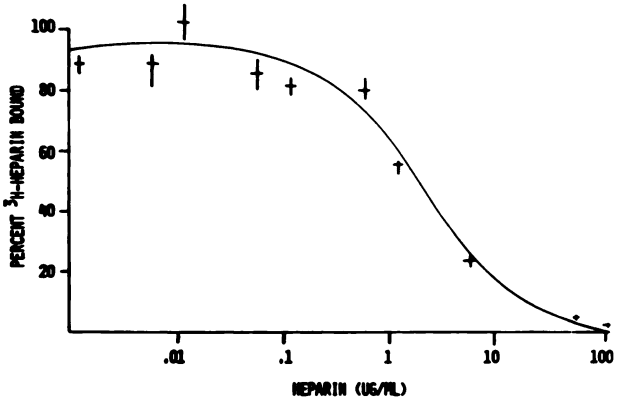

Fig. 3. Displacement curve for cultures of epididymal bull spermatozoa. The means of five replications are shown with standard errors of the mean.

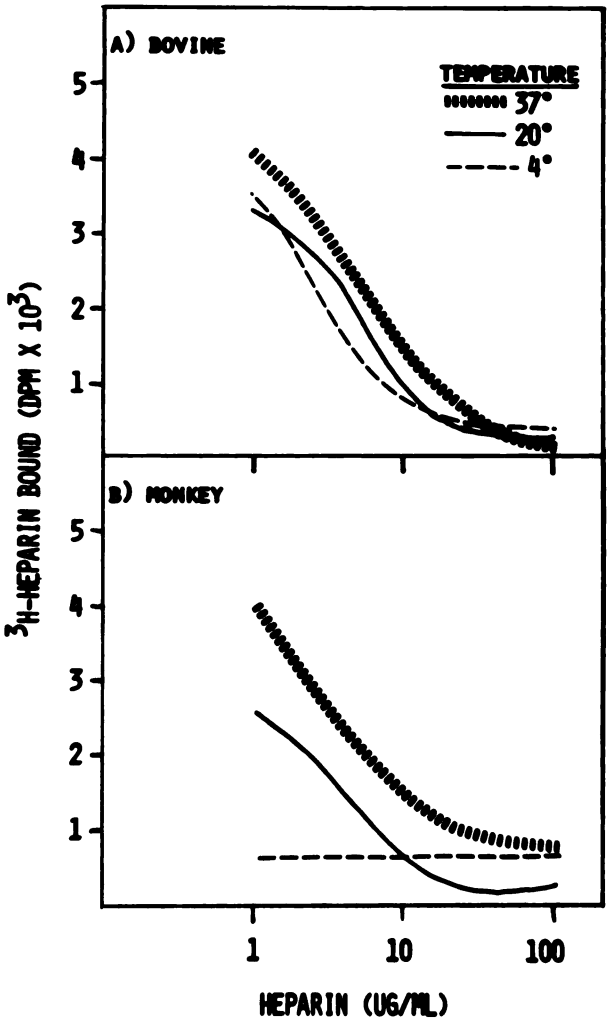

Fig. 4. The effects of temperature on the displacement of ^{3}H-heparin specific binding to ejaculated bull spermatozoa (A) or rhesus monkey spermatozoa (B).

TABLE 2. Van't Hoff Analysis of Temperature Effects on ^{3}H-heparin Binding to Ejaculated Bull and Rhesus Monkey Spermatozoa

Species of Spermatozoa	20 C	37 C
Bull	Kd* = 400 (90)	Kd = 700 (200)
ΔH‡ = − 7	ΔG† = −13	ΔG = −13
Rhesus Monkey	Kd = 24 (4)	Kd = 100 (20)
ΔH = −15	ΔG = −14	ΔG = −14

* Dissociation constants expressed in picomoles as Mean (SEM).

† Modified free energy for heparin binding expressed in Kcal/mole.

‡ Enthalpy change for heparin binding expressed as Kcal/mole.

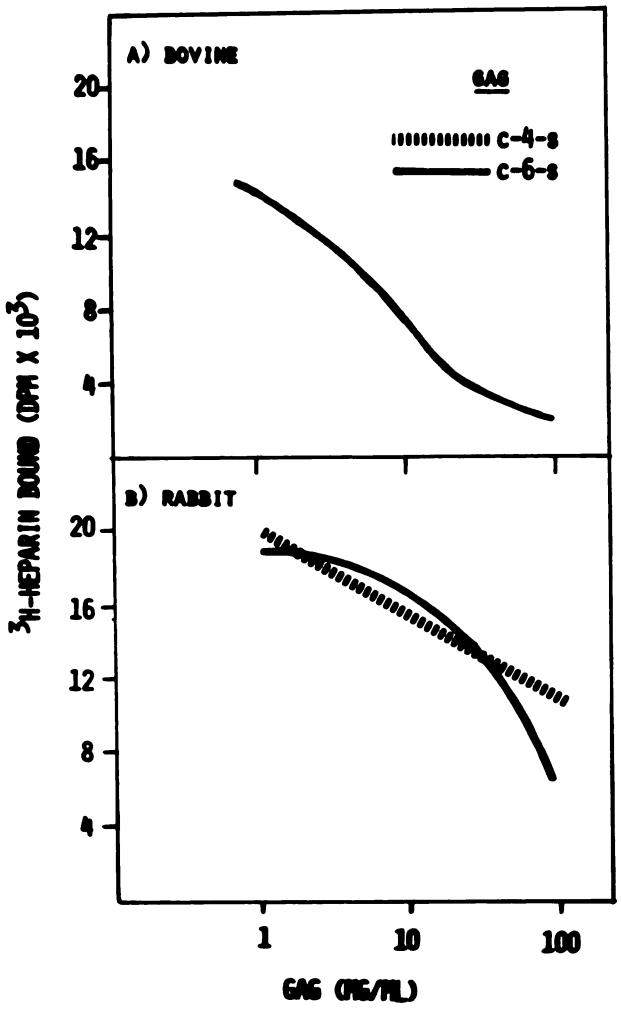

Fig. 5. Competitive displacement by chondroitin sulfates of ^{3}H-heparin specific binding to epididymal bull spermatozoa (A) or ejaculated rabbit spermatozoa (B). Normal displacement of ^{3}H-heparin by unlabeled heparin was observed in all cases.

C. At 20 C ^{3}H-heparin bound at a level intermediate to the results obtained at 4 and 37 C. Table 2 lists the equilibrium constants and results of Van't Hoff analysis of these data. The values obtained for the modified free energy change (ΔG') and enthalpy change (ΔH) indicate the binding reaction between bull ejaculated spermatozoa or monkey spermatozoa and ^{3}H-heparin was an exothermic, spontaneous process.

In order to assess the cross-reactivity of heparin binding sites with some other GAGs, attempts were made to competitively displace ^{3}H-heparin from bull epididymal spermatozoa with doses of 1 to 100 μg of chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, or hyaluronic acid (data not shown). These initial attempts were unsuccessful. However, doses of 1 to 100 mg of chondroitin-6-sulfate successfully displaced ^{3}H-heparin from bull epididymal or rabbit spermatozoal binding sites, as shown in Fig. 5, Panels A and B. The effective dose for 50% displacement (ED 50) of the labeled heparin by chondroitin-6-sulfate was 47 mg for both species. Attempts to displace ^{3}H-heparin with chondroitin-4-sulfate were unsuccessful for bull epididymal spermatozoa, whereas chondroitin-4-sulfate displaced ^{3}H-heparin from rabbit spermatozoa with an extrapolated ED 50 of 128 mg. These results indicate that heparin binding sites on rabbit or bull epididymal spermatozoa could also interact with chondroitin sulfates at high doses relative to heparin.

Attempts to displace ^{3}H-heparin from spermatozoa with dextran sulfate produced the results shown in Table 3. At doses of 100 μg per 5×10^7 spermatozoa, dextran sulfate displaced bound ^{3}H-heparin in a competitive manner equivalent to a dose of 1 mg of heparin in all species. Assuming that all molecules were bound, 100×10^{12} molecules of dextran sulfate would have been bound per spermatozoon. At slightly higher doses, dextran sulfate was capable of excluding both specific and nonspecific binding of ^{3}H-heparin to spermatozoa.

Scatchard analyses of displacement data were adjudged nonlinear for epididymal and ejaculated bull, rabbit, or monkey spermatozoa. A typical Scatchard plot, calculated from rabbit sperm displacement data, is shown in Fig. 6. The estimated first and second order binding affinities and the average number of binding sites per spermatozoon are listed in Table 4 for all types of spermatozoa

assayed. We have chosen to report only the principal binding affinities for the other experiments in this study, due to experimental considerations presented in the Discussion.

Changes in the *p*H of the incubation medium affected the displacement of ^{3}H-heparin bound to bull epididymal or monkey spermatozoa as shown in Fig. 7, Panels A and B. Corrected total binding for both species was greatest at *p*H 6.0 and lowest at *p*H 8.0. The principal binding affinities and average number of binding sites are listed in Table 5, together with results from *t* tests for significant differences between treatment means. These results suggest that a component of ^{3}H-heparin binding to spermatozoal membranes has zwitterionic character.

The effects of altered calcium concentrations on ^{3}H-heparin binding to bull epididymal spermatozoa are shown in Fig. 8, Panel A. Spermatozoa were collected, washed and resuspended in 50 μM EGTA TALP formulated without calcium. Bull epididymal spermatozoa were then incubated with ^{3}H-heparin in TALP formulated without calcium, with normal calcium concentration (1.8 mM) or with high calcium concentration (3.6 mM). There was a significant difference ($P < .05$) between the average specific binding of ^{3}H-heparin to spermatozoa incubated without calcium and to spermatozoa incubated with normal calcium concentration. There was a significant difference ($P < .05$) between the average specific binding of ^{3}H-heparin to spermatozoa incubated in the presence of normal as opposed to high calcium concentrations. Scatchard analysis of the displacement data indicated significant changes in the principal binding affinities and the number of corresponding

TABLE 3. Competitive Displacement of ^{3}H-heparin by Dextran Sulfate from Cultures of Epididymal Bull, Ejaculated Rabbit or Rhesus Monkey Spermatozoa

Species of Spermatozoa	Dextran Sulfate Added (μg/ml)	% Displacement*
Epididymal Bull	10	92(4)
	100	100(.5)
Ejaculated Rabbit	10	98(.5)
	100	100(.5)
Ejaculated Rhesus Monkey	10	91(4)
	100	100(.5)

* Percent displacement was calculated relative to controls containing spermatozoa and ^{3}H-heparin but no dextran sulfate, expressed as Mean (SEM).

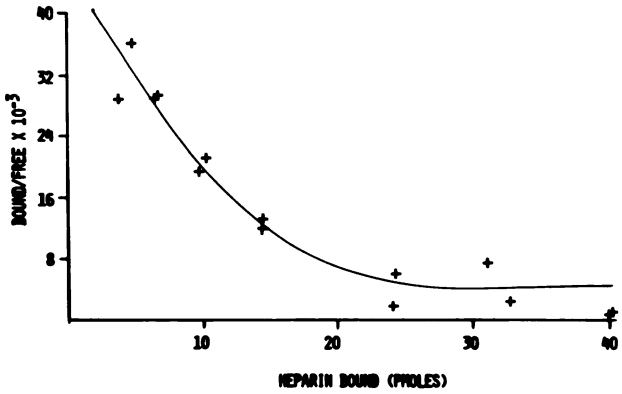

Fig. 6. Scatchard plot calculated from rabbit sperm displacement data. The plot was adjudged non-linear and the asymptotes estimated with direct fit lines.

binding sites for heparin on bull epididymal spermatozoa when the calcium concentration of the incubation medium was altered, as shown in Table 6. The specific binding of ^{3}H-heparin to ejaculated bull spermatozoa was unaffected by changing the concentration of calcium in the incubation medium (data not shown).

The effects of altered calcium concentration in the incubation medium on ^{3}H-heparin binding to rhesus monkey spermatozoa are shown in Fig. 8, Panel B. Monkey sperm pellets were resuspended in 150 mM EGTA TALP formulated without calcium. Incubations of 5×10^6 monkey spermatozoa were performed as with bull epididymal sperma-

TABLE 4. Estimated Dissociation Constants and the Average Number of Binding Sites per Spermatozoa for ^{3}H-heparin Specific Binding to Epididymal Bull, Rabbit, or Rhesus Monkey Spermatozoa*

Species of Spermatozoa	Kd†	Binding Sites‡
Epididymal Bull	200 (4)	3.7(.7)
	4000 (2000)	10(2)
Ejaculated Bull	700 (20)	4(2)
	10000 (400)	30(10)
Ejaculated Rabbit	100 (4)	6(.4)
	500 (200)	9(2)
Ejaculated Rhesus Monkey§	100 (20)	16(4)
	10000 (4500)	270(110)

* Doublets represent first and second order dissociation constants together with the respective number of binding sites.

† Dissociation constants expressed in picomoles as Mean (SEM).

‡ Average number of binding sites per spermatozoa expressed as Mean (SEM) ($\times 10^5$).

§ Obtained by electro-ejaculation.

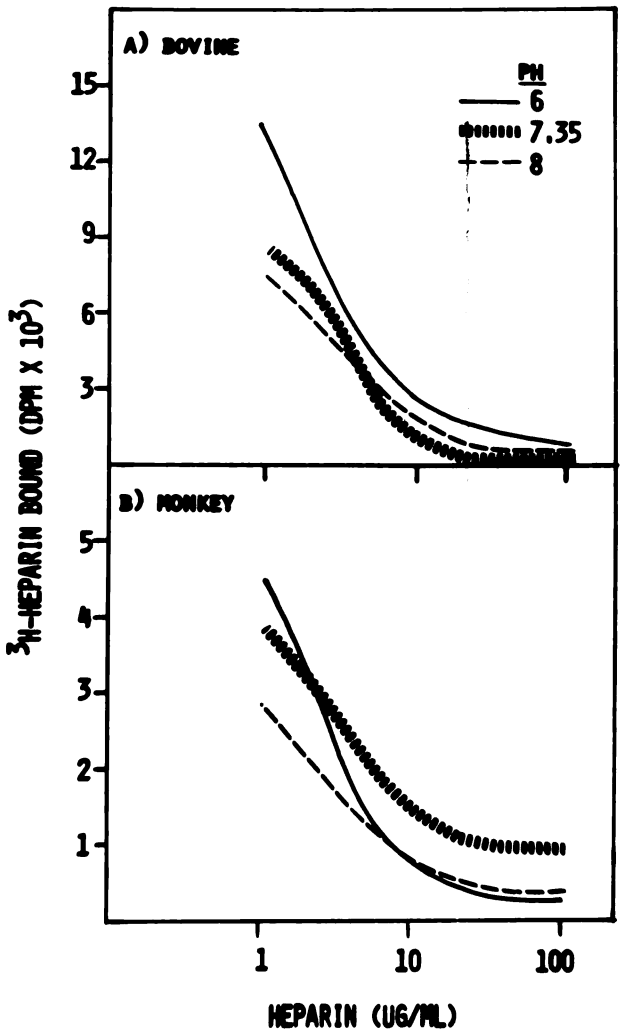

Fig. 7. The effects of varying *p*H of the incubation medium on the displacement of ^{3}H-heparin specific binding to epididymal bull spermatozoa (A) and ejaculated rhesus monkey spermatozoa (B).

tozoa. There was a significant difference ($P < 0.05$) between the average specific binding of ^{3}H-heparin to monkey spermatozoa without calcium and spermatozoa incubated with normal calcium concentration. Thus, the specific binding of ^{3}H-heparin to spermatozoal membranes was calcium-dependent. There was no significant difference between the average specific binding of ^{3}H-heparin to membranes incubated with normal or high calcium concentrations.

Increasing the magnesium concentration in the incubation medium had no effect on the specific binding of ^{3}H-heparin to bull epididymal spermatozoa that had been prepared in EGTA supplemented TALP formulated without calcium (data not shown). Results for epididymal spermatozoa prepared in TALP indicated a pronounced increase in total bound ^{3}H-heparin when sperm incubation included high levels of Mg^{+2} (data not shown). A four-fold increase was observed in total ^{3}H-heparin bound to spermatozoa when they were incubated with 8 mM Mg^{+2} concentration compared to normal Mg^{+2} concentration (.23 mM). High levels of Mg^{+2} produced a similar increase in total ^{3}H-heparin binding to ejaculated bull spermatozoa (data not shown). Both specific and nonspecific ^{3}H-heparin binding were augmented, with the greatest increase occurring to specific binding.

The results of experiments designed to discriminate between ^{3}H-heparin binding sites on the heads or tails of spermatozoa are presented in Table 7. The majority of binding sites were on the heads of ejaculated bull spermatozoa. Sperm tails bound 8% or less of the total amount bound by heads. The recovery rate of flagellar fragments in the sperm tail experiments could not be determined. Normal displacement curves were obtained with sperm head fractions and with whole spermatozoa centrifuged through a sucrose gradient without prior sonication (data not shown). These results indicate that ^{3}H-heparin was bound to the membranes of sperm heads and flagella, with the majority of the apparent binding sites on the sperm head. Whole spermatozoa or sperm heads centrifuged through a sucrose density gradient demonstrated a two to four-fold increase in ^{3}H-heparin bound over noncentrifuged spermatozoa incubated with or without sucrose (data not

TABLE 5. The Effects of Changing the *p*H of the Incubation Medium on the Estimated Parameters of ^{3}H-heparin Specific Binding to Epididymal Bull and Ejaculated Rhesus Monkey Spermatozoa

Species of Spermatozoa	Treatment*	Kd†	Binding Sites‡
Epididymal Bull	*p*H 6.00	90(20)^a	4.5(.3)^a
	*p*H 7.35	28(1)^b	1.4(.5)^b
	*p*H 8.00	50(10)^b	2(.5)^c
Ejaculated Rhesus Monkey	*p*H 6.00	140(30)^a	21(5)^a
	*p*H 7.35	100(20)^a	16(4)^a
	*p*H 8.00	270(60)^b	21(3)^a

* Spermatozoa were incubated and washed at the *p*H indicated.

† Dissociation constants expressed in picomoles as Mean(SEM). Numbers with different superscripts within each species differed significantly, $P < 0.05$.

‡ Average number of binding sites per spermatozoa expressed as Mean(SEM) ($\times 10^5$). Numbers with different superscripts within each species differed significantly, $P < 0.05$.

shown). These results indicated that an increase in the number of ^{3}H-heparin binding sites on ejaculated bull spermatozoa was a consequence of sucrose density gradient centrifugation rather than a result of a biochemical interaction with sucrose.

Evaluation by epifluorescent light microscopy of sperm smears incubated with fluorescein end-labeled heparin (Fl-heparin) indicated that ejaculated bull or monkey spermatozoa bound Fl-heparin over most parts of the spermatozoa, including the acrosome. No fluorescence was observed on the equatorial segment of spermatozoa with intact acrosomes. Figure 9 shows typical photomicrographs. When sperm smears prepared from ejaculated bull sperm suspensions, which previously had been incubated for 9.5 hours with 10 μg/ml of heparin, were examined, very few spermatozoa with fluorescent acrosomes were observed. An apparent greater intensity of fluorescence was observed on the equatorial segments of those samples.

Discussion

This study demonstrates the specific binding of ^{3}H-heparin to epididymal or ejaculated bull, rabbit, and rhesus monkey spermatozoa. Manipulation of ^{3}H-heparin levels in the incubation medium made possible the regulation of nonspecific binding at less than 10% of the total concentration of bound heparin. Total heparin concentration was the only variable that affected nonspecific binding. Specific, displaceable binding of ^{3}H-heparin *in vitro* to spermatozoa was reversible and affected by varying the *p*H, temperature, and divalent cation concentrations of the incubation medium. Displacement occurred over a range of heparin doses previously reported to induce an *in vitro* acrosome reaction in bull epididymal spermatozoa (Handrow et al, 1982) and ejaculated rabbit spermatozoa (Lenz et al, 1983). The principal binding affinity of Kd = 10^{-10} M is biologically significant and comparable to hormone-receptor binding affinities.

The ability of chondroitin sulfate to displace ^{3}H-heparin implies that heparin binding sites on spermatozoa were capable of interaction with chondroitin sulfate. The higher doses of chondroitin sulfate required for effective displacement of ^{3}H-heparin could suggest a high degree of specificity for heparin. Alternatively, this phenomenon may have been due to the lower degree of sulfation for chondroitin sulfates relative to heparin. The *p*H

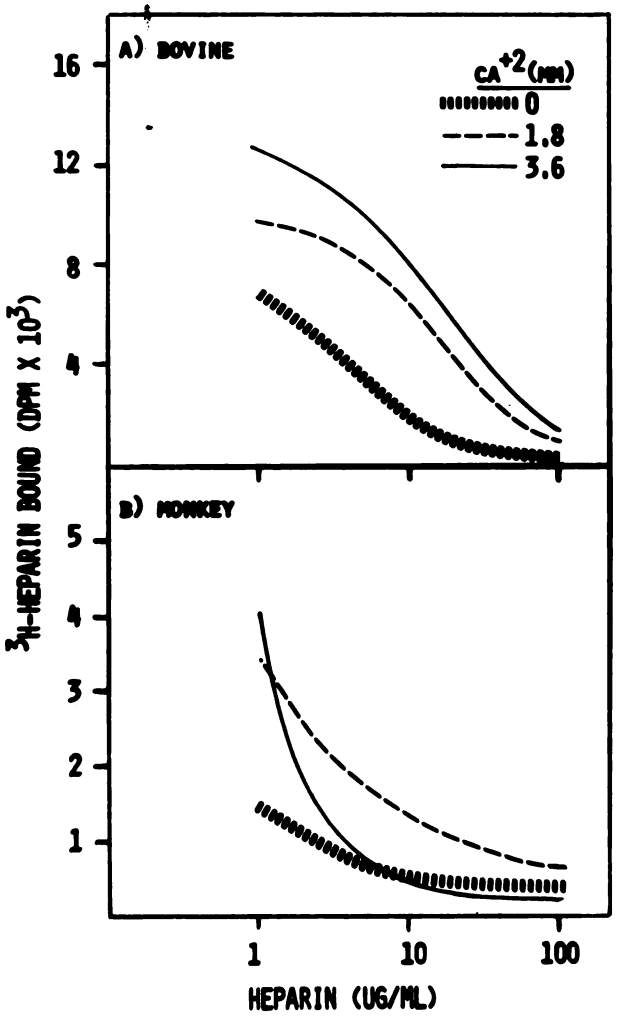

Fig. 8. The effects of altered calcium concentrations in the culture medium on ^{3}H-heparin specific binding to epididymal bull spermatozoa (A) or ejaculated rhesus monkey spermatozoa (B).

TABLE 6. The Effects of Altered [Ca^{+2}] in the Incubation Medium on Heparin Binding to Epididymal Bull Spermatozoa

Treatment*	Kd†	Binding Sites‡
OmM Ca^{+2} + EGTA	83(11)[a]	.8(.2)[a]
1.8 mM Ca^{+2}	148(7)[b]	9.5(2)[b]
3.6 mM Ca^{+2}	1166(90)[c]	20(1.6)[c]

* Spermatozoa were incubated and washed with the concentrations of Ca^{+2} indicated. The incubation medium employed Tyrodes Balanced Salt Solution plus 0.6% BSA, 10 mM HEPES, 0.2 mM pyruvate, 21.6 mM lactate, *p*H 7.35.

† Dissociation constant expressed in picomoles as Mean (SEM). Numbers with different superscripts differed significantly, $P < 0.05$.

‡ Average number of binding sites per spermatozoa expressed as Mean(SEM) ($\times 10^5$). Numbers with different superscripts differed significantly, $P < 0.05$.

TABLE 7. Comparison of Heparin Binding to Ejaculated Bull Sperm Heads and Tails

Treatment*	No. of Assays	Kd†	Binding Sites‡
Controls	2	1274(375)	10(2)[a]
Whole sperm	1	1445(300)	45(7)[b]
Sperm heads	2	837(240)	40(2)[b]
Sperm flagellae	1	N.A.§	3.3(.3)[c]

* *Controls:* spermatozoa incubated in standard displacement assay. *Whole spermatozoa:* intact spermatozoa centrifuged through sucrose gradients layered with 65%, 25%, and 5% sucrose solutions. *Sperm heads or tails:* fractions that were collected from the bottom or top, respectively, of a sucrose gradient. These spermatozoa were sonicated at 1 amp for 1 minute and centrifuged through a layered gradient of 65%, 25%, and 5% sucrose solutions.

† Dissociation constant expressed in picomoles as Mean(SEM). There were no significant differences between the various treatments.

‡ Average number of binding sites per spermatozoa expressed as Mean(SEM) ($\times 10^5$). Numbers with different superscripts differed significantly, $P < 0.05$.

§ N.A.: not assayed.

dependence and spontaneous, exothermic nature of heparin binding to spermatozoa suggest the involvement of ionic attractions. The ability of dextran sulfate to competitively displace ^{3}H-heparin indicates that heparin binding to sperm membranes is dependent in part on an interaction with sulfate groups. The inability of dextran sulfate to induce an acrosome reaction *in vitro* further suggests that other interactions are involved in GAG induction of the acrosome reaction. This is consistent with the previous suggestion that increased sulfation enhances the inductive ability of GAGs for the acrosome reaction (Handrow et al, 1982; Lenz et al, 1983).

The results from the *p*H, cation, and temperature dependence experiments indicate that heparin interacts with a proteinaceous component or components on or intercalated with the membranes of bull, rabbit, and rhesus monkey sper-

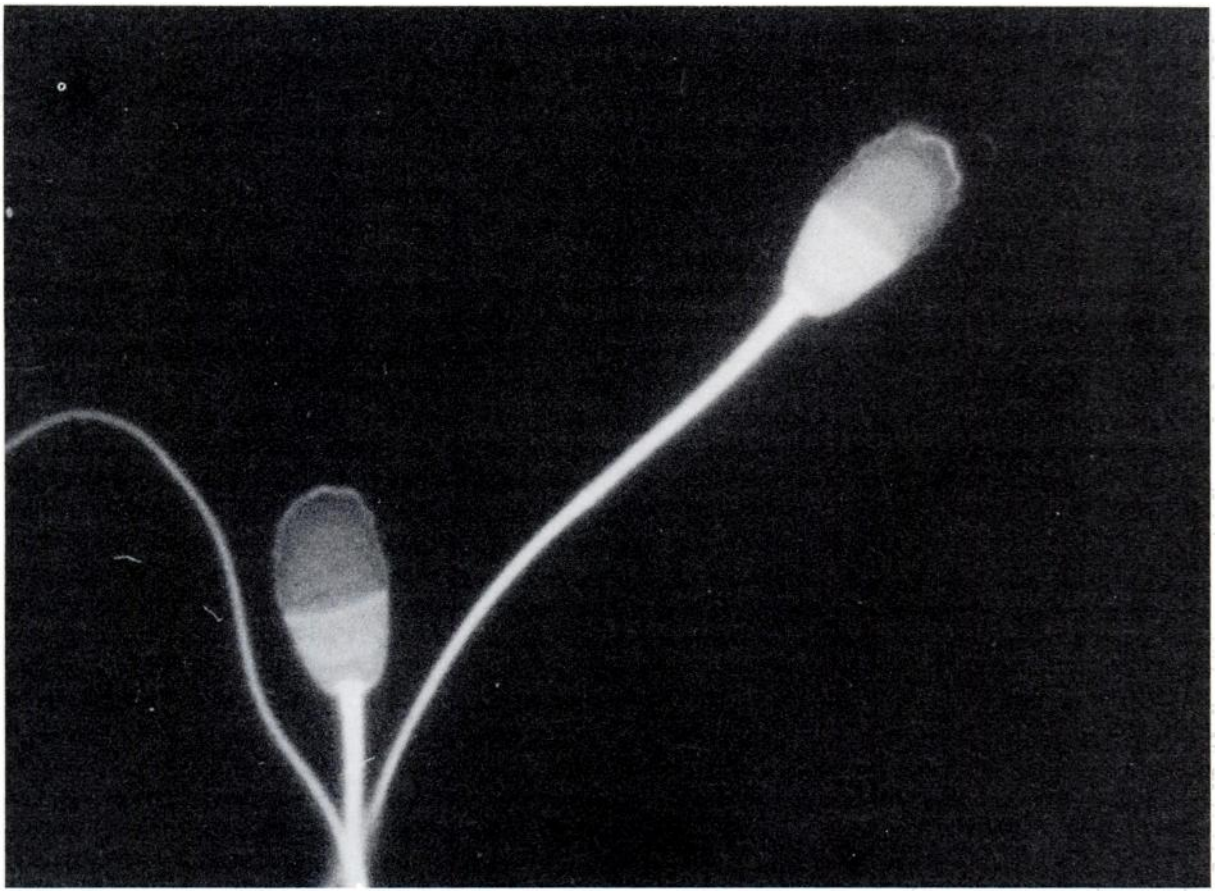

Fig. 9. Photomicrographs of epididymal bull spermatozoa labeled with fluoresceinated heparin and exposed with epifluorescence. Original magnification ×580.

matozoa. The spermatozoal binding affinities and numbers of binding sites for heparin were enhanced by increased calcium concentrations in the incubation medium. In the presence of normal calcium concentrations (1.8 mM), magnesium added to the incubation medium also augmented heparin binding to spermatozoa. Trifluoroperazine, a calmodulin antagonist, inhibits GAG induction of the acrosome reaction *in vitro* (Lenz et al, 1982), suggesting the involvement of calmodulin. Heparin and other GAGs have a biologically significant affinity for calcium (Boyd and Williamson, 1980) and to a lesser degree magnesium (Stivala, 1977). In the presence of divalent cations, GAGs are capable of forming ternary complexes with proteins. The results presented here indicate that further research is needed to evaluate a possible correlation between the reported GAG sperm capacitation (Lee et al, 1983), GAG binding to sperm membranes, and a possible regulation by GAGs of spermatozoal membrane proteins having an affinity for calcium and/or magnesium.

The increase in ^{3}H-heparin binding observed following incubation of ejaculated bull spermatozoa without GAGs or sucrose density gradient ultracentrifugation would be consistent with removal of membrane components that mask GAG binding sites. The principal heparin binding affinity for whole spermatozoa that had been centrifuged through a sucrose density gradient was similar to the heparin binding affinity for whole spermatozoa that had not undergone sucrose density gradient centrifugation. Thus, the primary effect of centrifugation through sucrose was to increase (unmask?) the number of binding sites for heparin. Further experiments are needed to determine whether specific binding of ^{3}H-heparin to spermatozoa increases as a result of such a removal of membrane components. The lack of an increase in specific binding of ^{3}H-heparin at equilibrium in saturation studies suggests that heparin may stabilize some membrane components *in vitro*. A similar GAG stabilization of acrosomal membranes has been postulated for rabbit spermatozoa (Lenz et al, 1983).

The temperature dependence of ^{3}H-heparin binding to spermatozoa implies an endocytotic process. Simple macromolecular adsorption to cell surfaces is temperature independent, whereas membrane binding followed by absorption is temperature dependent (Ryser, 1968). The internalization of heparin could account for the anomalous results from membrane damage experiments. Cultured mammalian cells were reported to absorb ^{14}C-hyaluronic acid and ^{35}S-proteoglycan (Truppe et al, 1977). Binding to surface membranes preceded internalization. The absorption of ^{35}S-chondroitin sulfate by cultured mammalian cells was shown to be temperature dependent (Saito and Uzman, 1971). If the characteristics of heparin binding to nonviable monkey spermatozoa are truly indicative of an endocytotic process, it may be surmised that internalization may not be dependent on active metabolism. Endocytosis of heparin by spermatozoa may utilize preformed metabolites or could result from a conformational change of membrane components induced by heparin itself. Further research is needed to clarify the processes involved in heparin endocytosis.

The preliminary results of studies employing fluoresceinated heparin provide visual evidence of heparin binding to the acrosomal membranes on ejaculated bull and monkey spermatozoa. These results are in agreement with the data obtained with ^{3}H-heparin. The addition of 1 mg of heparin competitively displaced bound ^{3}H-heparin to background levels or bound Fl-heparin to dark-field levels. Both types of labeled heparin were bound to the heads and flagella of bull and monkey spermatozoa. Most of the bound ^{3}H-heparin was on the sperm head, whereas fluorescence studies suggested a greater level of fluorescence on the tail and midpiece of spermatozoa. It is not known if the greater fluorescence on the flagella of spermatozoa was due to an increased concentration of binding sites, a greater binding affinity, or other factors.

The results of this study are analogous to previously reported properties of GAGs. Radiolabeled GAGs bound to rat hepatocytes with two apparent binding affinities (Kjellen et al, 1977) and were displaced by dextran sulfate. Additional studies indicated competitive displacement of ^{3}H-heparan sulfate by dermatan sulfate and suggested the possibility of high and low binding affinities (Kjellen et al, 1980). High affinity ($K_d = 10^{-7} - 10^{-11}$ M) binding sites for radiolabeled GAGs have been reported for several other types of mammalian cell lines (Glimelius et al, 1978; Underhill and Toole, 1979, 1981; Winterbourne, 1982). The latter study also reported that altered sulfation of GAGs from transformed cells resulted in a lowered affinity for

cell-surface receptors. Membrane binding sites for ^{3}H-heparin were demonstrated on human spermatozoa (Delgado et al, 1982). They reported competitive displacement of ^{3}H-heparin by dextran sulfate but not by "some other sulfated GAGs" tested. Treatment of human spermatozoa with large doses of heparin had been reported to induce the decondensation of sperm heads and nuclei (Delgado et al, 1980). Rabbit, ram, and bull sperm nuclei remained highly condensed under similar experimental conditions. The heparin dose ranges employed in those studies with human spermatozoa were several orders of magnitude greater than the range of heparin concentrations used in the present study. No report was made of the amount of ^{3}H-heparin bound that was due to reversible, displaceable binding to human spermatozoa. Consequently, it is impossible to ascribe the substantial difference in heparin binding affinities between human spermatozoa and the species examined in the present study to either procedural or specific differences. It should be noted, however, that the lower range of heparin concentrations utilized in this study afforded increased assay sensitivity while approximating the levels reported for 1) heparin-like activity in human uterine fluid (Foley et al, 1978), 2) heparin-like material recovered from flushings of female bovine reproductive tracts obtained at slaughter (Lee and Ax, 1983), and 3) heparin-like material purified from bull seminal plasma (Lee et al, 1983).

Several statistical factors in this study suggest negative cooperativity or a multiplicity of binding affinities for heparin. These include a Hill coefficient of .8, upward concavity of the Scatchard plot, interdecile range greater than 81, and competitive displacement over a range of three orders of magnitude of increasing ligand concentration. Commercially available heparin preparations such as were employed in this study are heterogenous mixtures of varying molecular weight. Statistical analysis of the results of this study relied in part on an application of the law of mass action, with its inherent assumptions, to describe the parameters of heparin binding to spermatozoa. It was not within the scope of this study to determine if all the assumptions of the law of mass action were justified. Therefore, further research is needed to evaluate the number and types of GAG binding sites on spermatozoa, the functions of those binding sites, and the mechanisms through which those functions are affected. This report of GAG binding sites on mammalian spermatozoa contributes to the growing evidence that glycosaminoglycans may serve as capacitating agents in the female reproductive tract and regulate eutherian fertilization.

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References

Barros C, Austin CR. *In vitro* fertilization and the sperm acrosome reaction in the hamster. J Exp Zool 1967; 166:317–324.

Bedford JM. Morphological aspects of sperm capacitation in mammals. Adv Biosci 1969; 4:35–50.

Bedford, JM. Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biol Reprod 1983; 28:108–120.

Boyd J, Williamson FB. A physico-chemical study of heparin: evidence for a calcium-induced co-operative conformational transition. J Mol Biol 1980; 137:175–190.

Cressie NAC, Keightley DD. The underlying structure of the direct linear plot with application to the analysis of hormone-receptor interactions. J Steroid Biochem 1979; 11:1173–1180.

Delgado NM, Huacuja L, Merchant H, Reyes R, Rosado A. Species specific decondensation of human spermatozoa nuclei by heparin. Arch Androl 1980; 4:305–313.

Delgado NM, Reyes R, Huacuja L, Merchant H, Rosado A. Heparin binding sites in the human spermatozoa membrane. Arch Androl 1982; 8:87–95.

Fawcett DW. The mammalian spermatozoon. Dev Biol 1975; 44:394–436.

Feldman HA. Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. Anal Biochem 1972; 48:317–338.

Foley ME, Griffin BD, Zuzel M, Aparicio SR, Bradbury K, Bird CC, Clayton JK, Jenkins DM, Scott JS, Rajah SM, McNichol GP. Heparin-like activity in uterine fluid. Br Med J 1978; 2:322–324.

Giloh H, Sedat JW. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Science 1982; 217:1252–1255.

Glimelius B, Busch C, Hook M. Binding of heparin on the surface of cultured human endothelial cells. Thromb Res 1978; 12:773–782.

Handrow RR, Lenz RW, Ax RL. Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. Biochem Biophys Res Commun 1982; 107:1326–1332.

Iwamatsu T, Chang MC. *In vitro* fertilization of mouse eggs in the presence of bovine follicular fluid. Nature 1969; 224:919–920.

Jones HP, Lenz RW, Palevitz BA, Cormier RJ. Calmodulin localization in mammalian spermatozoa. Proc Natl Acad Sci USA 1980; 77:2772–2776.

Kjellen L, Oldberg A, Rubin K, Hook M. Binding of heparin and heparan sulfate to rat liver cells. Biochem Biophys Res Commun 1977; 74:126–133.

jellen L, Oldberg A, Hook M. Cell-surface heparan sulfate: mechanisms of proteoglycan-cell association. J Biol Chem 1980; 255:10407–10413.
ee CN, Ax RL. Concentrations of glycosaminoglycans in the female bovine reproductive tract. Ann Mtng Am Dairy Sci Assoc, 1983 (abstract).
ee CN, Lenz RW, Ax RL. Bovine sperm undergo capacitation when exposed to glycosaminoglycans *in vitro*. Ann Mtg Am Soc Anim Sci 1983 (abstract).
iebfried ML, Bavister BD. The effects of taurine and hypotaurine on *in vitro* fertilization in the golden hamster. Gamete Res 1981; 4:57–63.
enz RW, Cormier JM. Effects of calmodulin-binding drugs on the guinea pig spermatozoon acrosome reaction and the use of these drugs as vaginal contraceptive agents in rabbits. Ann NY Acad Sci 1982; 383:85–97.
enz RW, Ax RL, Grimek HJ, First NL, Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. Biochem Biophys Res Commun 1982; 106:1092–1098.
enz RW, Bellin ME, Ax RL. Rabbit spermatozoa undergo an acrosome reaction in the presence of glycosaminoglycans. Gamete Res 1983; 8:11–19.
lastroianni L Jr, Manson, WA Jr. Collection of monkey semen by electro-ejaculation. Proc Soc Exp Biol Med 1963; 112:1025–1027.
arrish RF, Wincek TJ, Polakoski KL. Glycosaminoglycan stimulation of the *in vitro* conversion of boar proacrosin into acrosin. J Androl 1980; 1:89–95.
odbard D. Estimation of molecular weight by gel filtration and gel electrophoresis. II. Statistical and computational considerations. In: Catsimpoolas N, ed. Methods of protein separation. New York: Plenum Press, 1976; 181–217.
Rodbard D. Mathematics of hormone-receptor interaction. Adv Exp Med Biol 1973; 36:289–326.
Ryan RJ, Lee CY. The role of membrane bound receptors. Biol Reprod 1976; 14:16–29.
Ryser HJ-P. Uptake of protein by mammalian cells: an underdeveloped area. Science 1968; 169:390–396.
Saito H, Uzman BG. Uptake of chondroitin sulfate by mammalian cells in culture. Exp Cell Res 1971; 66:90–96.
Stivala SS. Physicochemical properties of heparin, and its interaction with Cu(II) and calcium in relation to anticoagulation. Fed Proc 1977; 36:83–88.
Truppe W, Basner R, Von Figura K, Kresse H. Uptake of hyaluronate by cultured cells. Biochem Biophys Res Commun 1977; 78:713–719.
Underhill CB, Toole BP. Binding of hyaluronate to the surface of cultured cells. J Cell Biol 1979; 82:475–484.
Underhill CB, Toole BP. Receptors for hyaluronate on the surface of parent and virus-transformed cell lines. Exp Cell Res 1981; 131:419–423.
Wincek TJ, Parrish RF, Polakoski KL. Fertilization: a uterine glycosaminoglycan stimulates the conversion of sperm proacrosin to acrosin. Science 1979; 203:553–554.
Winterbourne DJ. Binding of heparan sulfate and heparin to control and virus-transformed cells. Biosci Rep 1982; 2:1009–1015.
Yanagamachi R, Usui N. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp Cell Res 1974; 89:161–174.

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