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Hyaluronidase in ram semen

Quantitative determination, and isolation of multiple forms

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A study was made of hyaluronidase in ram semen. The end-group assay conditions used to determine activity quantitatively were chosen to ensure reliability as well as sensitivity [Gacesa, Savitsky, Dodgson & Olavesen (1981) Anal. Biochem. **118**, 76–84]; they led to 1 W.H.O. Standard International Hyaluronidase Unit displaying 0.1263 EC munit (1 EC unit of activity releases 1 μmol equivalent of *N*-acetylglucosamine end groups/min at 37 °C). All the activity in the semen was shown to be sperm-derived, and intact spermatozoa were estimated to contain 1.23 EC units per 10^9 cells. In a low-ionic-strength medium, only some 20% of the hyaluronidase was extractable, although up to 80% of the activity could be extracted as the ionic strength was increased; further addition of detergent extracted the remainder. During purification of the enzyme, it was found that inclusion of poly(vinyl alcohol) in the media stabilized the activity; detergent inclusion also improved the yield, especially during early stages. As a consequence both of reliable quantitative determination and of stabilization, a number of forms of hyaluronidase could be isolated in high yield, by using anion-exchange chromatography, cation-exchange chromatography, affinity chromatography and gel filtration. The existence of all these forms was confirmed by electrophoresis and immunoblotting with the use of a monoclonal anti-(ram hyaluronidase) antibody, and their presence in very freshly prepared sperm extracts was demonstrated. The specific activity of the isolated major hyaluronidase form was 15.0 EC units/mg; this was equivalent to 119000 W.H.O. units/mg, higher than any other previously reported values.

INTRODUCTION

'Testicular' hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) is a sperm-specific enzyme that is first detectable at the round spermatid stage of sperm cell development. It is localized in the acrosomal region of the fully formed spermatozoon (Morton, 1976), and is believed to play an important role at fertilization, facilitating sperm passage through the cumulus mass surrounding the oocyte; this role, however, has not been confirmed (see Morton, 1976; Harrison, 1983; Talbot *et al.*, 1985).

Although hyaluronidase is generally presumed to be a soluble enzyme, bound activity has been detected in several sperm species (Stambaugh & Buckley, 1970; Brown, 1975). Although its intra-acrosomal localization (Gould & Bernstein, 1975; Sakai *et al.*, 1979) would imply that the enzyme is released from living spermatozoa only after the acrosome reaction (as observed by Shams-Borhan & Harrison, 1981), some investigators have reported release of hyaluronidase from capacitated spermatozoa before an observable acrosome reaction (Talbot & Franklin, 1974*a*,*b*; Zao *et al.*, 1985) and even rapid 'leakage' after ejaculation (Masaki & Hartree, 1962). A variety of M_r values have been ascribed to the enzyme (cf. Borders & Raftery, 1968; Zaneveld *et al.*, 1973; Nobuhara *et al.*, 1980), and a wide range of specific activity values have been reported for purified preparations, both within and between species (e.g. Morton, 1977; Srivastava & Farooqui, 1979; Gupta & Goldberg, 1981; Lyon & Phelps, 1981; Bansal *et al.*, 1982). In most cases, low recoveries of activity have been obtained after purification, leaving doubts as to whether the residual material was representative of the original enzyme population.

In the light of such uncertainties, a new investigation of hyaluronidase in ram spermatozoa has been undertaken, to try to establish reliable data as a basis for future work on the enzyme's putative role in fertilization. In the present work hyaluronidase activities in spermatozoa were measured, the extraction of the enzyme was optimized, and a procedure was established for the isolation of various hyaluronidase forms present in the sperm extracts.

METHODS

Reagents

PVA was 'type II, cold-water-soluble' (average M_r 10000), product P-8136 from Sigma Chemical Co. (Poole, Dorset, U.K.).

Hyaluronic acid was prepared from human umbilical cords by the method of Jeanloz (1965). It contained less than 2.5μg of protein/mg, and fewer than 0.13% of the estimated content of *N*-acetylglucosamine groups were free (i.e. the product was highly polymerized).

Mega 9 was prepared by the method of Hildreth

Abbreviations used: PVA, poly(vinyl alcohol); Mega 9, nonanoyl *N*-methylglucamide.

(1982), except that the crude product was not precipitated in diethyl ether but was recrystallized directly from the syrup left after rotary evaporation of the filtered reaction mixture.

Monoclonal mouse anti-(ram hyaluronidase) antibody was obtained as ascites fluid from tumours of the 1D6 monoclonal mouse cell line isolated by Harrison & Gaunt (1988). The ascites fluid was used as antibody without further purification.

Heparin–hydroxypropyl-Sepharose was prepared as follows. Sepharose CL-4B (Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K.) (5.4 g wet wt.) was activated with CNBr (680 μmol) in the presence of triethylamine in accordance with Kohn & Wilchek (1982). As a result, 21.4 μequiv. of cyanate esters was introduced per g wet wt. (Kohn & Wilchek, 1981). The activated Sepharose was then coupled overnight at 4 °C in 0.1 M-sodium bicarbonate buffer with 5.3 mmol of 1,3-diamino-2-hydroxypropane at pH 9.5. After treatment with 0.1 M-ethanolamine at pH 8.5 to block remaining cyanate ester groups, the 1-amino-2-hydroxypropyl-Sepharose beads were coupled overnight with 100 mg of heparin (H-3125 from Sigma Chemical Co.), with the use of 100 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide at pH 5.0 as coupling agent, according to the protocol given by Pharmacia Fine Chemicals (1974). Finally, remaining amino groups on the heparin–2-hydroxypropyl-Sepharose were acetylated by stepwise addition of acetic anhydride as described by Riordan & Vallee (1972).

Hyaluronidase assay

The assay was based on the method of Gacesa *et al.* (1981), and measured the rate of release of *N*-acetylglucosamine end groups from hyaluronic acid. The concentration of hyaluronic acid used (1 mg/ml) was chosen arbitrarily; the kinetic behaviour of hyaluronidase is complex (Gacesa *et al.*, 1981), and substrate saturation cannot be achieved under the pH and ionic-strength conditions that would yield maximal rates of catalysis. For the chosen substrate concentration, the following conditions were established as optimal by preliminary experimentation, and were subsequently confirmed by studies on purified hyaluronidase forms (Harrison, 1988). Measured activity was linear with time for at least 20 min.

Up to 2.5 EC munits of activity were incubated at 37 °C with hyaluronic acid (1 mg/ml) in the presence of 0.2 M-NaCl and 0.1 M-sodium citrate buffer, pH 4.5, in a final volume of 150 μl; the reaction was initiated by the addition of 60 μl of a stock hyaluronic acid/citrate buffer solution. After 15 min, the reaction was terminated by addition of 30 μl of 0.2 M-$K_2B_4O_7$, pH 9.1, plus 4 μl of 6 M-K_2CO_3; the final pH after this addition was 8.9. The mixture was heated for 8 min at 100 °C, and then cooled in wet ice. Finally, 0.9 ml of *p*-dimethylaminobenzaldehyde solution [1 % (w/v) in acetic acid containing 1.25 % (v/v) conc. HCl] was added and the mixture incubated at 37 °C for 10 min. The absorbance was read at 585 nm against acetic acid. Suitable 'blanks' and standard (15 nmol of *N*-acetylglucosamine) were included in each batch.

One EC unit of enzyme activity liberates 1 μmol equivalent of *N*-acetylglucosamine end groups/min at 37 °C. Under the above assay conditions, 1 W.H.O. International Hyaluronidase Unit (that activity exhibited by 0.1 mg of the W.H.O. International Hyaluronidase Standard; National Institute for Biological Standards and Control, now at Potters Bar, Herts., U.K.; Humphrey, 1957) was determined to be equivalent to 0.1263 ± 0.0132 EC munits (mean $\pm$ S.D., $n = 5$), i.e. 1 EC unit = 7918 ± 827 W.H.O. units.

Unless stated otherwise, hyaluronidase activity is expressed throughout as EC units.

Protein assays

Protein was assayed by the Coomassie Brilliant Blue dye-binding method as modified by Read & Northcote (1981), with crystalline bovine serum albumin (A-4378 from Sigma Chemical Co.) as standard and Serva Blue G (Uniscience, London, U.K.) as dye.

Sperm counts

Sperm concentrations were assessed by using a Double Neubauer haemocytometer. For each determination duplicate dilutions of the sperm suspension were made and single counts made on each dilution.

Spermatozoa

Semen was obtained from Suffolk rams in the Institute's colony, by means of an artificial vagina. Pooled ejaculates of normal motility were processed within 1 h of collection.

Purification of hyaluronidase

Step 1. Isolation of spermatozoa. Ram semen was diluted with 20 vol. of 0.25 M-sucrose/5 mM-glucose/20 mM-Mes buffered with 3 mM-KOH plus NaOH to pH 6.0, and centrifuged at 700 $g_{max.}$ for 20 min at 17 °C. The supernatant was drawn off by aspiration and discarded.

Step 2. Extraction. The loose sperm pellet was resuspended to approx. 1×10^9 cells/ml with aqueous solutions of NaCl, phosphate buffer, EDTA and Mega 9, such that the following final concentrations were achieved: 0.3 M-NaCl, 5 mM-EDTA, 5 mg of Mega 9/ml and 20 mM-sodium phosphate, pH 7.4. The cell suspension was stirred thoroughly at 0 °C for 15 min and then centrifuged at 38000 $g_{max.}$ for 20 min at 4 °C. The supernatant was removed and stored at −20 °C until required.

Step 3. Desalting. A maximum of 16 ml of sperm extract was applied to a column (2 $cm^2 \times 30$ cm) of Sephadex G-25 (superfine grade) (Pharmacia) that had been equilibrated with 10 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA, 1 mg of Mega 9/ml and 1 mg of PVA/ml. The column was eluted overnight at 4 °C at approx. 10 ml/h. The next morning, the high-M_r material, which contained the hyaluronidase activity, was pooled and subjected to f.p.l.c. without delay.

Step 4. Anion-exchange chromatography. With the use of a Pharmacia f.p.l.c. system, desalted sperm extract was applied to a Mono Q column (HR 5/5; Pharmacia) equilibrated in 10 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA, 1 mg of Mega 9/ml and 1 mg of PVA/ml; a maximum of 10 mg of protein (equivalent to 4 ml of original sperm extract) was applied at each run. After washing, the column was eluted at 1 ml/min with a gradient of 0–0.5 M-NaCl in 25 ml of equilibration medium at room temperature. Two peaks of hyaluron-

idase activity were detectable in the eluate. Each was collected and pooled at 0 °C, and was processed further without delay.

Step 5. Cation-exchange chromatography. Pooled fractions from anion-exchange chromatography were adjusted to pH 6.0 with acetic acid, and subjected to f.p.l.c. on a Mono S column (HR5/5; Pharmacia) equilibrated with 20 mM-Mes/NaOH buffer, pH 6.0, containing 1 mM-EDTA, 1 mg of Mega 9/ml and 1 mg PVA/ml; not more than 6 mg of protein was applied at each run. After washing, the column was eluted at 1 ml/min with a gradient of 0–0.3 M-NaCl in 40 ml of equilibration medium at room temperature. Hyaluronidase activity was detectable usually as a heterogeneous peak. All fractions comprising the peak were pooled together, and stored frozen at −20 °C.

Step 6. Affinity chromatography. Pooled fractions from cation-exchange chromatography were adjusted to pH 4.5 with acetic acid and applied at 10 ml/h to a column (0.8 $cm^2 \times 6.5$ cm) of heparin–hydroxypropyl-Sepharose (see above) equilibrated with 20 mM-sodium acetate buffer, pH 4.5, containing 1 mM-EDTA and 1 mg of Mega 9/ml. Not more than 40 units of enzyme activity were applied at each run. The column was eluted at 6 ml/h with a gradient of 0.1–0.7 M-NaCl in 100 ml of equilibration medium. Two peaks of hyaluronidase activity were detectable in the eluate. They were collected separately, concentrated to less than 4 ml with the use of an Amicon YM-10 ultrafiltration membrane (Amicon, Upper Mill, Gloucs., U.K.), and the pH adjusted to 6.0 with 1 M-NH_3. PVA was then added to a final concentration of 2 mg/ml, and the pool was stored frozen at −20 °C.

Step 7. Gel filtration. Concentrated pools from affinity chromatography were applied to a column (2 $cm^2 \times$ 90 cm) of Ultrogel AcA 34 (LKB Instruments, South Croydon, Surrey, U.K.) previously equilibrated with 10 mM-Mes/NaOH buffer, pH 6.0, containing 0.3 M-NaCl, 1 mM-EDTA, 1 mg of Mega 9/ml and 0.5 mg of PVA/ml, and eluted at approx. 10 ml/h. Active fractions of the eluate were pooled as required, concentrated with the use of an Amicon YM-10 ultrafiltration membrane, and stored frozen at −20 °C.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out in polyacrylamide slabs 1.5 mm thick, with a 3.75% (w/v) stacking gel and either a 10% (w/v) homogeneous or a 5–17% (w/v) gradient separating gel [acrylamide/bisacrylamide ratio 36.5:1 (w/w) throughout]. The discontinuous buffer system, which contained 0.1% (w/v) SDS, was that of Laemmli (1970). Before electrophoresis, samples were mixed with 1% (w/v) SDS, stacking buffer (40% of that in the stacking gel), 10% (w/v) sucrose and 0.001% Bromophenol Blue (all final concentrations); the mixture was then heated at 65 °C for 20 min (Kowit & Maloney, 1982). In the work described in the present paper all samples were run in a non-reduced state.

Afterwards gels were either (*a*) fixed and stained for protein with 0.25% Coomassie Brilliant Blue R in methanol/acetic acid/water (4:1:5, by vol.) and then destained in methanol/acetic acid/water (5:2:13, by vol.), or (*b*) used for immunoblotting (see below).

Immunoblotting

This was carried out essentially according to the procedure of Burnette (1981). Transverse electrophoresis was carried out at about 3.5 V/cm for 15–17 h at 4 °C, and Schleicher and Schull 0.2 μm-pore-size nitrocellulose membrane filter (BA83; Anderman and Co., East Molesey, Surrey, U.K.) was used to bind the transferred proteins. Afterwards, the nitrocellulose membrane was rinsed in phosphate-buffered saline (0.125 M-NaCl/0.015 M-sodium phosphate buffer, pH 7.3).

Hyaluronidase bands were detected as follows: unfilled binding sites on the membrane were saturated by incubation for 1 h at room temperature on a rocker with 5% (w/v) bovine serum albumin (fraction V, product A-9647 from Sigma Chemical Co.) in phosphate-buffered saline. Next the paper was rocked for 3 h with a 1:2000 dilution of monoclonal mouse anti-(ram hyaluronidase) antibody in 1% (w/v) bovine serum albumin in phosphate-buffered saline. After a thorough washing in phosphate-buffered saline, the paper was rocked for 2 h with a 1:500 dilution of peroxidase-conjugated rabbit anti-[mouse IgG (H + L)] antibody (Miles Scientific, Stoke Poges, Bucks., U.K.) in 1% (w/v) bovine serum albumin in phosphate-buffered saline. Finally, after further thorough washing in phosphate-buffered saline, the bound peroxidase activity was detected by brief (approx. 1 min) incubation of the paper in a solution composed of 5 ml of methanol containing 15 mg of 4-chloro-1-naphthol, 25 ml of phosphate-buffered saline and 10 μl of 30% (w/v) H_2O_2 (Hawkes *et al.*, 1982).

Alternatively, total transferred proteins were detected by staining for 30–60 min with 0.001% Coomassie Brilliant Blue R in methanol/acetic acid/water (4:1:5, by vol.), followed by destaining with the same solvent.

RESULTS

Distribution of hyaluronidase in ram semen

Semen is composed of spermatozoa, cytoplasmic droplets (vesicles of superfluous cellular material discarded from spermatozoa during maturation), small numbers of moribund germ and somatic cells, and cell debris, all suspended in a fluid comprising secretions from the several regions of the male reproductive tract. The data in Table 1 show that the hyaluronidase in ram semen emanates solely from the spermatozoa.

(*a*) The values obtained for the hyaluronidase content of whole semen were closely correlated with the sperm densities of the different semen samples ($r = 0.919$, 4 degrees of freedom); moreover, it was estimated from the regression curve that the hyaluronidase content of semen lacking spermatozoa (i.e. zero sperm density) would not differ significantly from zero. Thus there was no hyaluronidase in the male-reproductive-tract fluid secretions, nor was the enzyme associated with cells other than spermatozoa.

(*b*) Cytoplasmic droplets are sperm-derived and the possibility of their containing hyaluronidase was not excluded by the argument in (*a*). However, droplets are sedimented with the spermatozoa during preparation of seminal plasma, but very largely removed from the spermatozoa during the washing procedure; any hyaluronidase in them would thus contribute to the

Table 1. Distribution of hyaluronidase activity in ram semen

Equal-sized portions from six samples of ram semen were diluted 20-fold in phosphate-buffered saline, pH 7.2, containing 10 mM-glucose. Part of each diluted sample was centrifuged for 2.5 min in a Beckman Microfuge B, and a sample of each supernatant removed for assay, to give 'seminal plasma' values. The rest of each diluted sample was sonicated for 3 × 10 s, and assayed, to give 'whole semen' values. From the same original semen samples, other equal-sized portions were diluted 5-fold with phosphate-buffered saline, pH 7.2, containing 10 mM-glucose and washed (see Harrison *et al.*, 1982) into 0.23 M-sucrose/10 mM-NaCl/5 mM-glucose/20 mM-Hepes buffered with 3 mM-KOH and NaOH to pH 7.0. The loosely pelleted spermatozoa were diluted to about 2×10^8 cells/ml, sonicated and assayed, to give 'measured sperm content' values. 'Percentage extracellular' values are defined as:

$$\frac{\text{Seminal plasma activity} \times 100}{\text{Whole semen activity}}$$

'Estimated sperm content' values are defined as:

$$\frac{\text{Whole semen activity} - \text{seminal plasma activity}}{\text{Sperm density in semen}}$$

Cell counts were performed on all samples containing spermatozoa. Hyaluronidase activity is expressed as EC units.

Sample	Sperm density in semen ($\times 10^9$/ml)	Whole semen (units/ml)	Seminal plasma (units/ml)	Percentage extracellular	Estimated sperm content (units/10^9 cells)	Measured sperm content (units/10^9 cells)
1	4.41	5.06	0.574	11.3	1.02	1.13
2	4.02	4.29	0.705	16.4	0.89	0.92
3	3.39	4.70	0.883	18.8	1.13	0.84
4	4.80	5.48	0.439	8.0	1.05	1.08
5	2.23	2.87	0.879	30.6	0.89	0.72
6	3.06	4.17	0.975	23.4	1.04	0.84
$\bar{x} \pm$ S.D.	3.65 ± 0.94	4.43 ± 0.91	0.742 ± 0.207	18.1 ± 8.2	1.00 ± 0.09	0.92 ± 0.16

'estimated sperm content' but not to the 'measured sperm content'. Because there was no significant difference between these two values, the droplets must have contained negligible amounts of hyaluronidase.

(*c*) The proportion of the total hyaluronidase activity found to be extracellular in the different semen samples ('percentage extracellular') was inversely correlated ($r = -0.937$, 4 degrees of freedom) with the hyaluronidase content of the washed sperm populations ('measured sperm content'); this suggests strongly that the presence of hyaluronidase in the seminal plasma was due to leakage of hyaluronidase from the spermatozoa.

Moribund spermatozoa are always present to some degree in naturally occurring sperm populations, and these individuals will almost certainly have lost their hyaluronidase. The data for the washed sperm populations, therefore, do not truly reflect the hyaluronidase content of intact spermatozoa, but rather represent normal preparative expectations. However, since all the hyaluronidase in the semen apparently originated from the spermatozoa, an estimate of the normal hyaluronidase content of an intact ram spermatozoon could be made by dividing the total hyaluronidase activity in the semen samples by the sperm density of those samples: all the spermatozoa had been at one time intact and had contained all the hyaluronidase. A value of 1.23 ± 0.13 EC units (or 9750 ± 1040 W.H.O. units) per 10^9 spermatozoa was thereby obtained (mean values ± S.D., $n = 6$).

Table 2. Extraction of hyaluronidase from ram spermatozoa

Ejaculates from two rams were pooled, diluted with 5 vol. of 0.15 M-NaCl, and washed (Harrison *et al.*, 1982) into 0.25 M-sucrose/5 mM-glucose/20 mM-Hepes buffered with 3 mM-KOH and NaOH to pH 7.0. The washed sperm suspensions were then sonicated for 3 × 10 s. Equal-sized portions were diluted with 1 vol. of extraction medium and incubated for 15 min at 0 °C. After centrifugation in a Microfuge B for 2.5 min, the supernatants were assayed, and the values compared with the total hyaluronidase activity of the homogenate. Three replicate experiments were performed; values given are means ± S.D. Total measurable activity was not affected by the additives.

Extraction medium (final concns.)	Percentage of total in supernatant
Water	21.8 ± 3.4
NaCl (0.1 M)	74.2 ± 1.7
NaCl (0.2 M)	79.7 ± 3.3
NaCl (0.4 M)	80.9 ± 2.1
Mega 9 (1.25 mg/ml)	27.7 ± 7.4
Mega 9 (2.5 mg/ml)	29.0 ± 7.2
Mega 9 (5 mg/ml)	32.1 ± 8.4
NaCl (0.3 M) + Mega 9 (1.25 mg/ml)	90.6 ± 2.8
NaCl (0.3 M) + Mega 9 (2.5 mg/ml)	94.7 ± 4.5
NaCl (0.3 M) + Mega 9 (5 mg/ml)	98.4 ± 2.6

Extraction of hyaluronidase from spermatozoa

Only about 20% of the total hyaluronidase activity was released from disrupted spermatozoa at low ionic strength (Table 2); a further 20% resisted extraction by saline. Detergent treatment alone did not greatly assist extraction, but in combination with saline enabled complete recovery of the enzyme in a soluble form, without affecting its activity.

In a subsidiary experiment (results not shown), it was found that similar complete extraction of hyaluronidase

Table 3. Purification of ram sperm hyaluronidase

See the Methods section for details of procedures; values are means ± S.D. ($n = 4$).

Purification stage	Resultant fraction	Specific activity (units/mg of protein)	Mean overall purification factor	Relative proportions of fractions from given stages (%)	Mean overall yield (%)
Desalting	DS	0.586±0.022	(1)		(100)
Anion exchange					
of: DS	Q− and	1.35±0.10	2.3	72.3±4.1	64.6 } 89.3
	Q+	1.21±0.17	2.1	27.7±4.1	24.7 }
Cation exchange					
of: Q−	S/Q−	5.91±0.82	10.1		61.4 } 83.3
Q+	S/Q+	4.06±0.79	6.9		21.9 }
Affinity chromatography					
of: S/Q−	H_I/S/Q− and	12.68±0.82	21.7	57.2±6.3	30.5 }
	H_{II}/S/Q−	13.79±0.72	23.5	42.8±6.3	22.8 } 73.2
S/Q+	H_I/S/Q+ and	1.77±0.46	3.0	3.6±0.9	0.7 }
	H_{II}/S/Q+	14.32±1.02	24.4	96.4±0.9	19.2 }
Gel filtration					
of: H_I/S/Q−	A/H_I/S/Q−	14.70±1.15	25.1		27.5 }
H_{II}/S/Q−	A/H_{II}/S/Q−	15.91±0.97	27.2		19.9 } 63.6
H_{II}/S/Q+	A/H_{II}/S/Q+	14.69±0.91	25.1		16.2 }

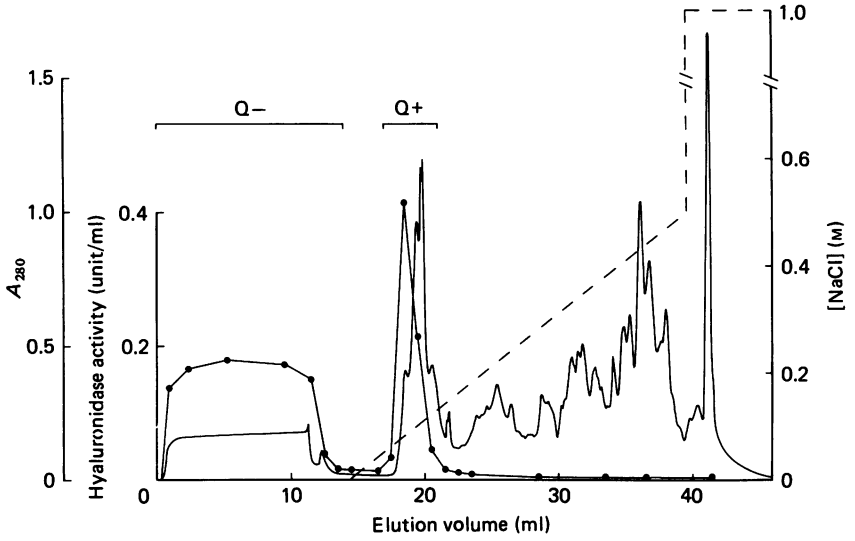

Fig. 1. Anion-exchange chromatography of hyaluronidase in desalted extracts of ram spermatozoa

A sample containing 8.8 mg of protein was applied to a Mono Q HR5/5 column and eluted with an NaCl gradient at pH 7.0, as described in the Methods section. The hyaluronidase activity profile (●—●) was obtained by assay of individual fractions (1 ml); the A_{280} profile (——) was obtained by continuous monitoring of the effluent; the NaCl gradient profile shown (----) was the pump activity trace, and refers essentially to the composition of the medium being applied to the column at the time the effluent was being monitored.

could be achieved by simply stirring the washed cells with 0.3 M-NaCl and 5 mg of Mega 9/ml.

Isolation of various forms of hyaluronidase

Data relating to the isolation of hyaluronidase forms are presented in Table 3. Mean recoveries in excess of 85% were obtained in a highly repeatable fashion at each stage of the process, (*a*) by including Mega 9 and PVA wherever practical and (*b*) by completing the stages during which the enzyme was in low-ionic-strength environments (i.e. purification steps 3–5; see the Methods section) as rapidly as possible (always within a 24 h period).

Anion-exchange chromatography. Hyaluronidase activity could always be separated into two fractions by anion-exchange chromatography (Fig. 1). The major portion was not adsorbed and was therefore presumed to be non-anionic (designated Q−), but some activity was always adsorbed, albeit weakly, and was therefore presumed to be anionic (designated Q+). The ratio of non-anionic to anionic activity in crude extracts varied somewhat but was usually about 3:1 (see Table 3).

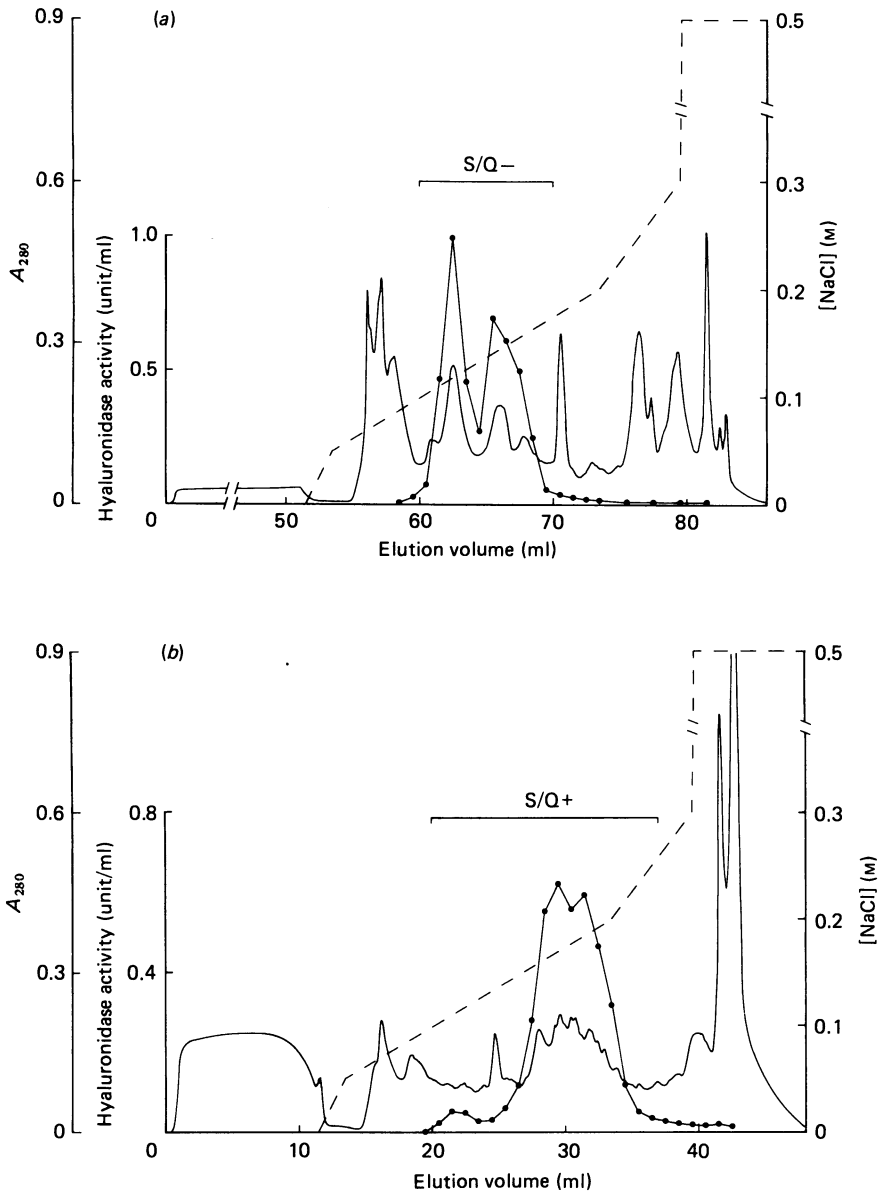

Fig. 2. Cation-exchange chromatography of (*a*) non-anionic (fraction Q−) and (*b*) anionic (fraction Q+) populations of ram sperm hyaluronidase

In (*a*), 4.7 mg of protein was applied to a Mono S HR5/5 column; in (*b*), 4.4 mg of protein was applied. Elution was carried out with an NaCl gradient at pH 6.0, as described in the Methods section. The profiles were derived as described in Fig. 1 legend.

Cation-exchange chromatography. Both non-anionic (Q−) and anionic (Q+) hyaluronidase displayed heterogeneity on cation-exchange chromatography; the activity elution patterns of the two types differed (compare Fig. 2*a* with Fig. 2*b*), confirming that the Q− population was intrinsically different from the Q+ population. Because the constituent subpopulations could not be clearly resolved, all the eluted activity was pooled together in each case to yield fraction S/Q− and fraction S/Q+ respectively.

Affinity chromatography. Two fractions (H_I and H_{II}) could be clearly separated on heparin–hydroxypropyl-Sepharose, both from fraction S/Q− (Fig. 3*a*) and from fraction S/Q+ (Fig. 3*b*). It was found in preliminary experiments that the nature of the 'spacer' molecule used to link the heparin to the Sepharose affected this separation, as well as the degree of purification obtained; 1,3-diaminopropan-2-ol (O'Carra *et al.*, 1974) gave better results than 1,6-diaminohexane (Cuatrecasas, 1970) or adipic acid dihydrazide (Nishikawa & Bailon, 1975). Heparin, being a highly sulphated polysaccharide, is a polyanion, and some degree of cation-exchange chromatography undoubtedly took place, yet the hyaluronidase separations achieved with heparin–Sepharose were much more effective than those achieved with Mono S under the same conditions. This suggests that some form of affinity chromatography was occurring on the heparin column.

Whereas fraction S/Q− divided at this stage about equally into fraction H_I/S/Q− and H_{II}/S/Q− (Fig. 3*a*

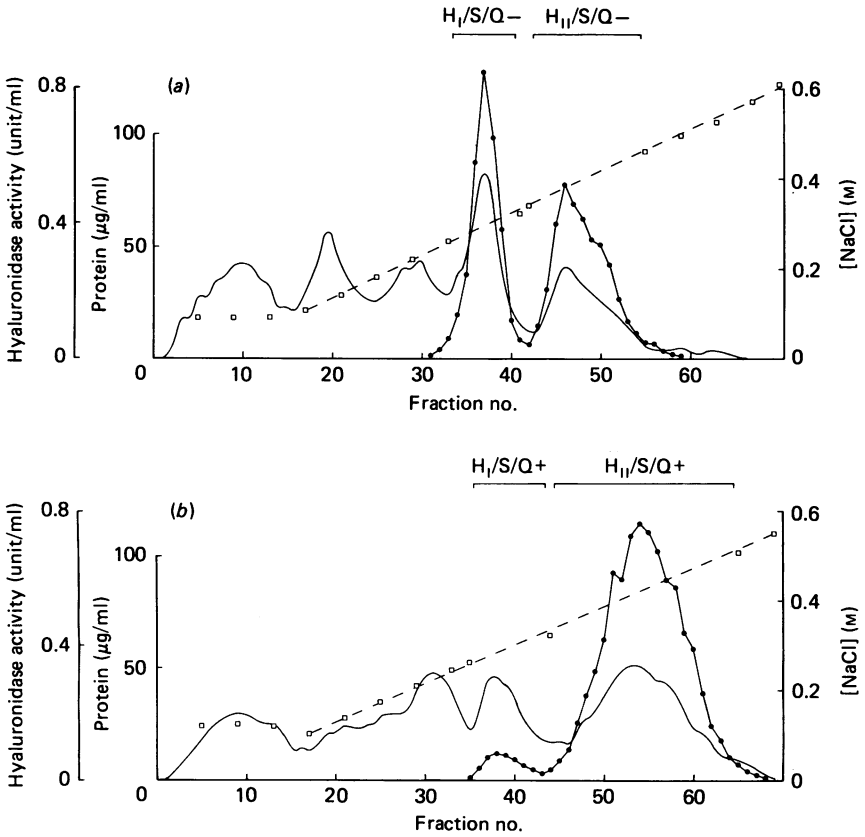

Fig. 3. Affinity chromatography of partially purified (*a*) non-anionic (fraction S/Q−) and (*b*) anionic (fraction S/Q+) populations of ram sperm hyaluronidase

In (*a*), 2.4 mg of protein was applied to a heparin–hydroxypropyl-Sepharose column (0.8 cm^2 × 6.5 cm); in (*b*), 2.6 mg of protein was applied. Elution was carried out with an NaCl gradient at pH 4.5, as described in the Methods section. The hyaluronidase activity profile (●—●) was obtained by assay of individual fractions (1 ml); the protein (——) was obtained by assaying each fraction by the Coomassie Brilliant Blue dye-binding method (see the Methods section); the salt gradient profile (□--□) was estimated by measuring the conductivity of individual fractions and comparing the values obtained with those of standard eluent solutions.

and Table 3), fraction S/Q+ yielded almost entirely fraction H$_{II}$/S/Q+, only a very little activity being detected as the first (H$_I$) peak (Fig. 3*b* and Table 3). Recovery from the heparin column was always high, and thus the absence of the H$_I$ peak was not due to specific losses or instability: immunoblotting (see below) confirmed the virtual absence from the S/Q+ fraction of the hyaluronidase component responsible for the H$_I$ peak (see Fig. 5).

Gel filtration. Small increases in specific activity were achieved by subjecting the three major H/S/Q fractions to gel filtration (Table 3); the resultant preparations were prefixed A (e.g. A/H$_I$/S/Q−). Fraction H$_I$/S/Q− ran as a monodispersed single component, whereas fractions H$_{II}$/S/Q− and H$_{II}$/S/Q+ ran as multidispersed components of much higher M_r than the H$_I$ material. [Gel-filtration profiles are presented in the accompanying paper (Harrison, 1988).]

Electrophoresis of fraction A/H$_I$/S/Q− revealed essentially a single band of protein, whereas multiple bands, of higher M_r, were seen with fractions A/H$_{II}$/S/Q− and A/H$_{II}$/S/Q+ (see Harrison, 1988). However, monoclonal anti-(ram hyaluronidase) antibody reacted with all these bands after immunoblotting, showing that all three pools from gel filtration contained pure hyaluronidase, in differing but related molecular forms.

Verification of the existence of multiple hyaluronidase forms

With the use of monoclonal antibody to ram hyaluronidase for immunoblotting, a series of antigenic bands could be demonstrated in electrophoretograms of freshly prepared sperm extracts; moreover, an identical pattern of bands was seen in sperm extracts that had been stored frozen for more than 14 months (Fig. 4). Blotting of the different fractions from cation-exchange and affinity chromatography revealed a specific partition of the different bands between the various fractions (Fig. 5). In accord with the gel-filtration findings, the low-M_r band was seen almost exclusively in the H$_I$ fractions whereas the higher-M_r bands were seen in the H$_{II}$ fractions; very-high-M_r bands predominated in fraction H$_{II}$/S/Q+. Thus the presence of multiple forms of hyaluronidase in ram sperm extracts could be demonstrated both by SDS/polyacrylamide-gel electrophoresis and by various kinds of column chromatography, and the same population of forms was present both in extracts analysed as soon as

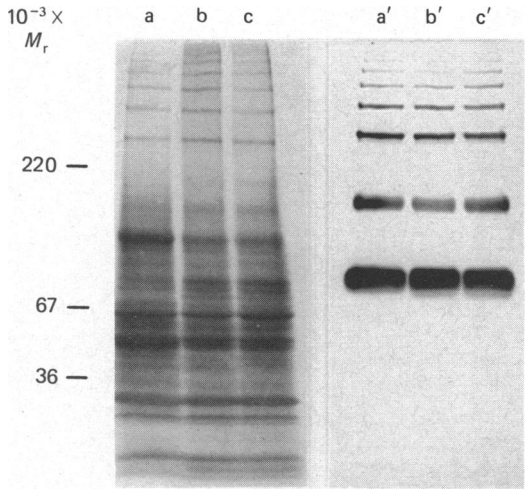

Fig. 4. Hyaluronidase forms in fresh and stored extracts

Washed spermatozoa were extracted for 15 min with NaCl and Mega 9 as described in the Methods section, and immediately centrifuged for 4 min in a Beckman Microfuge B. Samples of the supernatant were immediately prepared non-reduced for SDS/polyacrylamide-gel electrophoresis. Together with samples of similar extracts that had been stored at −20 °C for 2 or 14 months, these were subjected to electrophoresis on a 5–17% gradient gel and then blotted. Lanes a and a′, freshly prepared extract; lanes b and b′, extract stored for 2 months; lanes c and c′, extract stored for 14 months. Lanes a–c, stained for protein; lanes a′–c′, probed for hyaluronidase with the monoclonal antibody 1D6.

possible and in extracts stored for long periods. It was concluded that the multiple forms of hyaluronidase observed were not an artifact of purification.

Specific activity of purified hyaluronidase

The predominant hyaluronidase form was that in the fraction $H_I/S/Q-$, which represented some 42% of the total activity in the spermatozoa (assuming that losses were evenly distributed between forms). Evidence is presented in the accompanying paper (Harrison, 1988) that this form was the monomer. Gel filtration of fraction $H_I/S/Q-$ yielded essentially pure monomeric hyaluronidase (fraction $A/H_I/S/Q-$), electrophoresis revealing only traces of other material (see Harrison, 1988); quantitative analyses were performed on three such preparations.

The mean specific activity was 15.0 (13.9–16.2) EC units/mg or 119000 (110000–128000) W.H.O. units/mg. When protein was measured in these preparations with the Folin reagent [method of Peterson (1977), with the linear-transform procedure of Coakley & James (1978)], the resultant specific activity was 11.4 (6.9–13.9) EC units/mg or 90300 (54600–110000) W.H.O. units/mg.

Contaminating β-*N*-acetylhexosaminidase activity, measured essentially by the method of Parkes *et al.* (1984), was found to be between 0.93 and 1.67 units/mg; as the specific activities of purified hexosaminidases A and B from boar epididymal extracts have been reported by these authors as 115 and 380 units/mg respectively, this contamination represented at worst 1.45% on a protein basis.

Acrosin activity in these preparations was undetectable, extrapolating to a contamination limit for this enzyme of 0.48 unit/mg; as the specific activity of ram acrosin has been reported to be 142 units/mg at 25 °C (Brown & Hartree, 1978), this represented at worst an acrosin contamination of 0.17% on a protein basis [assuming acrosin's activity at 37 °C to be no more than double that at 25 °C (see Dixon & Webb, 1964)].

DISCUSSION

Isolation of multiple hyaluronidase forms

Two particular problems have contributed greatly to previous uncertainties and discrepancies relating to sperm ('testicular') hyaluronidase: quantitative determination of enzyme activity, and loss of enzyme activity during purification. Sperm hyaluronidase is stimulated both by protein and by high salt, though the effect of salt supersedes the effect of protein (Gacesa *et al.*, 1981; Harrison, 1988); however, for various reasons, rapid catalysis is maintained over only a relatively short period. It is therefore essential to employ assay conditions under which there is both a constant degree of stimulation regardless of the source of the enzyme sample (i.e. purified or crude, in salt solution or water) and also a linear rate of activity throughout the period of incubation. These criteria have rarely been satisfied. In consequence, most of the previously published quantitative data on hyaluronidase activity have been of doubtful validity, quite apart from any uncertainties arising from the comparison of data from widely different assay types (e.g. 'turbidometric' as compared with 'end-group analysis'). In the present study an assay protocol was used that allowed reliable quantitative determination at all stages of enzyme purification, and thus enzyme losses could be readily detected and, where possible, prevented.

Sperm hyaluronidase has a long-standing reputation for instability and for low yields during purification (Rasmussen, 1954; Brunish & Högberg, 1960; Borders & Raftery, 1968; Rhodes *et al.*, 1971; Yang & Srivastava, 1975; Gupta & Goldberg, 1981). My discovery that inclusion of poly(vinyl alcohol) in the purification media decreased hyaluronidase losses considerably, probably by preventing adsorption on surfaces (Harrison, 1988), was of crucial importance, especially during the later stages of isolation when purity was high and protein concentrations low; in addition, inclusion of the non-ionic detergent Mega 9 improved recoveries considerably during the early stages of isolation, possibly by decreasing interactions between the hyaluronidase and other extracted components.

As a result of the attention paid to the quantitative determination and recovery of activity, multiple forms of ram sperm hyaluronidase could be isolated in very high yield, essentially free of extraneous proteins. The characteristics of these different forms are described and discussed in detail in the accompanying paper (Harrison, 1988), but two points may be made here. Firstly, the multiplicity of enzyme forms does not seem to be an artifact of isolation. All forms are detectable in freshly prepared extracts analysed as early as possible, and they do not appear to change during 14 months of storage. It seems likely, therefore, that the multiple forms are present within the intact spermatozoon. Secondly, the specific-activity values of 90000–119000 W.H.O. units/

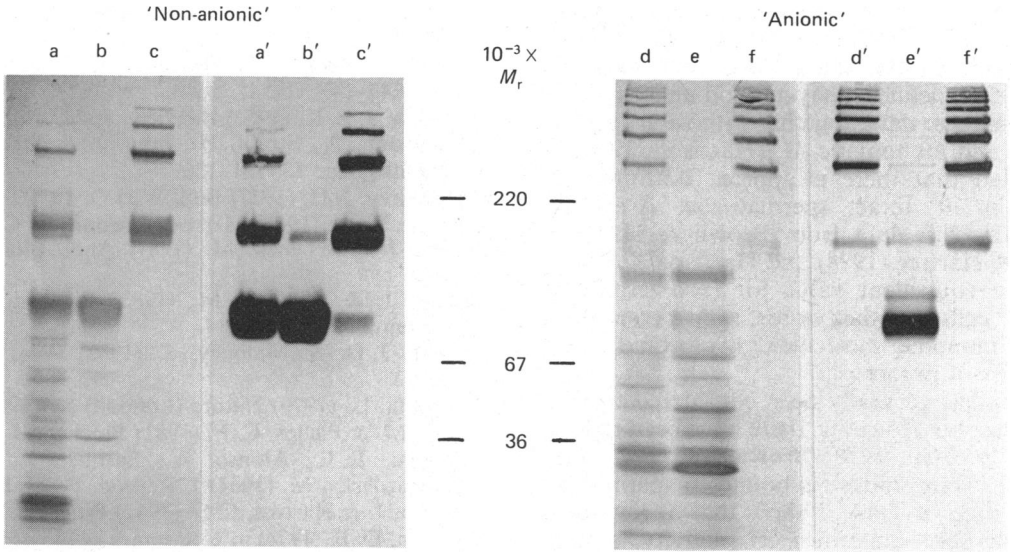

Fig. 5. Distribution of hyaluronidase forms in various chromatographic fractions

Duplicate samples, each containing 30 munits of hyaluronidase activity, were taken from various chromatographic fractions at intermediate stages during the isolation procedure; they were prepared non-reduced for SDS/polyacrylamide-gel electrophoresis, run on a 5–17% gradient gel, and blotted. Lanes a and a', S/Q−; fraction lanes b and b', fraction H_I/S/Q−; lanes c and c', fraction H_{II}/S/Q−; lanes d and d', fraction S/Q+; lanes e and e', fraction H_I/S/Q+; lanes f and f', fraction H_{II}/S/Q+. Lanes a–f, stained for protein; lanes a'–f', probed for hyaluronidase with the monoclonal antibody 1D6. Note that, in order to examine each fraction, equal quantities of activity were run in each track; in fact, b and c represent about 57% and 43% of a respectively, and e and f represent about 4% and 96% of d respectively. This should be taken into account when comparing H/S/Q fractions with the S/Q fractions from which they were obtained.

mg obtained for the ram hyaluronidase monomer preparations are the highest so far reported that have been based on reliable methodology. Although a similar figure of 104000 W.H.O. units/mg was given by Srivastava & Farooqui (1979) for bull (seminal-plasma) hyaluronidase, there is some doubt as to the validity of the assay protocol used. More easily comparable are the values for bull hyaluronidase of 42000 W.H.O. units/mg (Gacesa *et al.*, 1981) and 60800 W.H.O. units/mg (calculated from the data of Lyon & Phelps, 1981); those activity values can be related to mine directly via the International Hyaluronidase Standard, and the Coomassie Blue dye-binding assay procedure I used to measure protein was developed (Read & Northcote, 1981) to obviate variability of response to different proteins (see comments of Lyon & Phelps, 1981). There is evidence (R. A. P. Harrison, unpublished work) that bull hyaluronidase, like ram hyaluronidase (Harrison, 1988), exists as two monomeric species, and thus the Lyon & Phelps (1981) preparation, which contained two enzymatically active components, was almost certainly essentially pure. Its lower specific activity in comparison with my ram preparation may prove to be simply a species difference. On the other hand, the data of Lyon & Phelps (1981) indicated substantial losses of activity during their purification procedure.

Amounts, distribution and state of hyaluronidase in ram semen

It has long been recognized that testicular hyaluronidase is of germ-cell origin and that the activity in seminal plasma is sperm-derived (Swyer, 1947*a*). The data in Table 1 are further quantitative proof of this. However, they also indicate that very little hyaluronidase is associated with the cytoplasmic droplet. This is at variance with data presented in Morton (1977), but is what would be expected if the hyaluronidase is, as is believed, an acrosomal enzyme (Mancini *et al.*, 1964; Morton, 1976; Sakai *et al.*, 1979; Triana *et al.*, 1980; Shams-Borhan & Harrison, 1981); there is little likelihood that residual acrosomal components would remain in the spermatid cytoplasm throughout spermiogenesis, to be discarded later in the cytoplasmic droplet.

All samples of semen contain dead or dying spermatozoa. These may be expected to have lost some or all of their soluble enzyme contents into the surrounding milieu. It was therefore no surprise to find that some 18% of the hyaluronidase in semen was extracellular (i.e. in the seminal plasma). Indeed, hyaluronidase activities in seminal plasma have been used as an indicator of semen quality (Foulkes & Watson, 1975; Holtzmann *et al.*, 1978). However, there have also been reports that hyaluronidase 'leaches' out rapidly from live spermatozoa into the suspending medium, especially if the latter was originally hyaluronidase-free (Swyer, 1947*b*; Masaki & Hartree, 1962), though a subsequent study failed to observe such rapid leakage (Foulkes & Watson, 1975). The data in Table 1 show that no rapid leakage occurred during isolation of the spermatozoa, even though the cells were being washed through a hyaluronidase-free medium; the 'estimated sperm content' was not significantly different from the 'measured sperm content'. It seems possible that earlier observations of hyaluronidase leakage may have resulted from sperm damage caused by the experimental protocols employed.

No clearly interpretable data on hyaluronidase activities in spermatozoa have been published since those of Swyer (1947*a*); although some estimations of content have been made, they have not been related to cell numbers. The values of 0.92 EC unit or 7300 W.H.O.

units per 10^9 cells for washed sperm populations and of 1.23 EC units or 9750 W.H.O. units per 10^9 cells for intact spermatozoa are the first to have been obtained with a biochemically defined assay method under reliable conditions. From these data, together with values for the specific activity and monomeric M_r (Harrison, 1988), it can be calculated that there is approx. 0.92 nmol of hyaluronidase in 10^9 intact spermatozoa. A similar calculation made with data from Brown & Harrison (1978), Brown & Hartree (1978) and Harrison & Brown (1979) gives the equivalent value for (pro)acrosin as 4.6 nmol per 10^9 cells. In other words, ram spermatozoa contain about 1 monomer molecule of hyaluronidase for every 5 molecules of proacrosin.

Hyaluronidase has generally been considered to be a soluble sperm enzyme. However, there have been reports (Stambaugh & Buckley, 1970; Brown, 1975; Morton, 1976) that some hyaluronidase is bound to sperm head structures. The data of Table 2 show that at low ionic strength considerable quantities of activity remain bound to disrupted ram spermatozoa. Although most can be extracted by increasing the ionic strength, an appreciable proportion is only solubilized by detergent. This finding has considerable implications with respect to the role of hyaluronidase in fertilization; much of the activity is released at the start of the acrosome reaction (Shams-Borhan & Harrison, 1981), but it now appears that some may be retained to act at a later stage.

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