Acrosomal and Lysosomal Isoenzymes of β-Galactosidase and *N*-Acetyl-β-glucosaminidase in Rat Testis†

Gopal C. Majumder and Roger W. Turkington*

ABSTRACT: β-Galactosidase and *N*-acetyl-β-glucosaminidase were partially purified from epididymal spermatozoa and testes of mature rats. Two isoenzymes of β-galactosidase (I and II) were observed. Isoenzyme I was localized in the lysosomes of precursor germinal cells and isoenzyme II was derived from sperm acrosomes. The sedimentation constants of isoenzymes I and II were 9.9 and 6.4 S, respectively. β-Galactosidase I was eluted earlier than isoenzyme II during gel filtration through Sephadex G-200 and during chromatography on carboxymethylcellulose. The two isoenzymes also differed in relation to their relative sensitivities toward heat denaturation. Three isoenzymes of *N*-acetyl-β-glucosaminidase were observed in these tissues. Isoenzymes I and II were localized to the lysosomes of the testicular precursor cells and a third enzyme was derived from the acrosomes of the spermatozoa. The sedimentation constants of isoenzymes I and II and the sperm enzyme were 9.2, 8.1, and 6.4 S, respectively. Isoenzymes I and II were eluted earlier than the sperm enzyme during gel filtration through Sephadex G-200. Chromatography on carboxymethylcellulose resolved enzyme I from the other isoenzymes, which were eluted together later as a single peak. The isoenzyme derived from sperm also differed from the lysosomal isoenzymes I and II in relation to its pH optimum, K_m for the synthetic substrate, and sensitivity toward heat denaturation. These results demonstrate that during cell differentiation in testis unique molecular forms of these enzymes appear in sperm cell acrosomes.

Spermatogenesis is an orderly process of cell differentiation which leads to the formation of a highly specialized cell type, the spermatozoan. Among the few organelles retained in this motile cell is the acrosome, which has been regarded as a specialized lysosome which evolved to facilitate fertilization (Allison and Hartree, 1970; Mann, 1964; Stambaugh and Buckley, 1969). Although the acrosome contains acid hydrolases and thus resembles the lysosomes of precursor cell types (spermatogonia, spermatocytes), it is not clear what relationships the enzymes of the acrosome bear to specific lysosomal enzymes with similar substrate specificities. Recently it has been demonstrated that sperm acrosomal hyaluronidase (Zaneveld *et al.*, 1973; Bollet *et al.*, 1963; Vaes and Jacques, 1965; Aronson and Davidson, 1967) and proteinase (Zaneveld *et al.*, 1972) differ in certain biochemical and immunochemical properties from the hyaluronidase and proteinase enzymes prepared from lysosomes of organs other than the testis. It is generally agreed that acrosomal hydrolases disperse specific macromolecular substrates of the cumulus oophorus and zona pellucida of the ovum to permit penetration by the sperm cell (Allison and Hartree, 1970; Mann, 1964; Stambaugh and Buckley, 1969). Because the cumulus oophorus appears to contain large numbers of glycoproteins it was of interest to characterize two acrosomal enzymes which could participate in the hydrolysis of cumulus oligosaccharides, β-galactosidase and *N*-acetyl-β-glucosaminidase. The present studies compare certain properties of these enzymes with those of lysosomal enzymes derived from precursor cells of the rat testis. The results demonstrate that sperm cell acrosomes contain unique isoenzymes which differ significantly from the lysosomal enzymes of other testis cells.

Experimental Section

Animals. Male Sprague-Dawley rats (90–100 days old) were used in all experiments.

Chemicals. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide and *p*-nitrophenol were purchased from Calbiochem, San Diego. *p*-Nitrophenyl β-D-galactopyranoside, human serum albumin, and bovine serum albumin were products of Sigma, St. Louis. Carboxymethylcellulose was from Bio-Rad, Richmond, Calif. Sephadex G-200 was a product of Pharmacia, Sweden, and density gradient grade sucrose (ribonuclease free) was obtained from Mann Research, New York. Triton X-100 was obtained from Rohm and Haas Co., Philadelphia.

Isolation of Epididymal Spermatozoa. Rats were killed by cervical dislocation and epididymides were rapidly excised *via* the scrotal route. The tissues were chilled on ice and were maintained at 0–4° throughout the procedure. Each epididymis was trimmed of fat and connective tissue, bisected with a sharp razor blade, rinsed in 0.25 M sucrose, and finally suspended in 0.25 M sucrose (5 ml/epididymis) with occasional gentle stirring for the extraction of sperm cells. After 10 min, the suspension of spermatozoa in sucrose solution was removed by decantation and the extraction of the epididymides was repeated twice. The combined suspension of spermatozoa was filtered through eight layers of cheesecloth and the filtrate was centrifuged at 700*g* for 10 min to sediment spermatozoa. The pellet was washed three times with 0.25 M sucrose and the washed spermatozoa were suspended in 0.25 M sucrose. The preparation of sperm cells was observed to be highly pure by phase-contrast microscopy, and was contaminated by less than 2 epithelial cells/1000 sperm cells.

Isolation of Enzymes from Spermatozoa and Testis. Testes or epididymal spermatozoa were homogenized in 0.25 M sucrose containing 0.2% Triton X-100 with 120 passes in a glass homogenizer fitted with a motor-driven Teflon pestle. To the homogenate solid potassium chloride was added with stirring to bring its final concentration to 1.0 M. The suspension was homogenized again with 120 passes in the same homogenizer. After 30 min the suspension was centrifuged at 27,000*g* for 30 min and the pellet was discarded. The supernatant fluid was dialyzed against 50 volumes of 0.05 M sodium citrate buffer (pH 4.6) with three changes of the buffer. The dialyzed fraction

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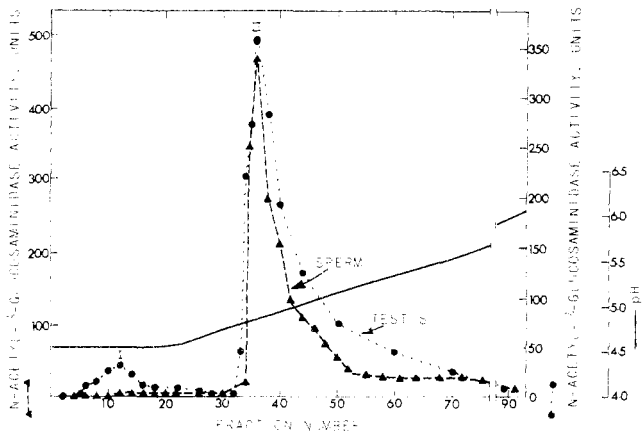

FIGURE 1: Carboxymethylcellulose chromatography of sperm (▲) and testicular (●) *N*-acetyl-β-glucosaminidases. An aliquot of 50 μl of each fraction was assayed for enzyme activity by the standard assay method.

was centrifuged at 240,000*g* for 60 min in a Spinco centrifuge with the SW50L rotor and the precipitate was discarded (step 1). The clear supernatant was applied to a carboxymethylcellulose column (0.9 × 24 cm) previously equilibrated with 0.05 M sodium citrate buffer (pH 4.6). The column was washed with 38 ml of the equilibrating buffer prior to further elution with a linear pH gradient of 0.05 M sodium citrate buffer (pH 4.6–7.0) in a total volume of 150 ml of the buffer. The flow rate was 9.0 ml/hr and the volume in each fraction was 1.8 ml (step 2). Active enzyme fractions were pooled together and concentrated by ultrafiltration through a UM-10 Millipore filter. The concentrated enzyme preparations (3.5 ml) were dialyzed against 0.05 M sodium citrate buffer (pH 4.6) and subjected to gel filtration on a column (2.8 × 38 cm) of Sephadex G-200 with 0.05 M sodium citrate buffer (pH 4.6) as the eluting buffer. The flow rate was 17.5 ml/hr and the volume in each fraction was 4.0 ml (step 3). All steps were carried out at 0–4°.

Assay of N-Acetyl-β-glucosaminidase. The activity of *N*-acetyl-β-glucosaminidase was measured by the method of Conchie (1954) with some modifications. The standard assay medium contained 20 μmol of sodium citrate buffer (pH 4.0), 300 μg of Triton X-100, 37 μmol of sucrose, 2.2 μmol of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, and the enzyme preparation in a total volume of 0.35 ml. The incubation was carried out at 37° for 30 min and the reaction was stopped by the addition of 2.5 ml of 0.05 *N* sodium hydroxide. The *p*-nitrophenol content of the mixture was measured by its absorbance at 400 nm in a Gilford spectrophotometer. A zero-min reaction tube served as the blank in all assays.

Assay of β-Galactosidase. The activity of β-galactosidase was measured by a slight modification of the method of Lederberg (1950). The standard assay mixture contained 20 μmol of sodium citrate buffer (pH 3.5), 300 μg of Triton X-100, 37 μmol of sucrose, 2.5 μmol of *p*-nitrophenyl β-D-galactoside, and the enzyme preparation in a total volume of 0.35 ml. The incubation was carried out at 37° for 30 min, and the reaction was stopped by the addition of 2.5 ml of 0.05 N sodium hydroxide. Samples were analyzed for the amount of *p*-nitrophenol released by the method described above. For each enzyme a unit of enzyme activity was defined as the amount of enzyme which catalyzed the liberation of 1 nmol of *p*-nitrophenol from the substrate during 30 min under the standard assay conditions.

Subcellular Fractionation. Cell fractionation procedures were performed by the method of DeDuve *et al.* (1955) as modified by Males and TTurkington (1970). Testes were homogenized in ice-cold 0.25 M sucrose containing 0.001 M sodium ethylenediaminetetraacetate (EDTA) with one passage of a motor-driven Teflon pestle. The homogenate was passed through four layers of cheesecloth before centrifugation at 1,000g_{av} for 10 min to sediment nuclei. Light and heavy mitochondrial fractions were sedimented by centrifugation at 17,000g_{av} for 30 min and the resulting supernatant was centrifuged at 105,000g_{av} for 60 min to isolate microsomes. The pellet at 17,000g_{av} was dispersed in 14.3% (w/v) sucrose and was fractionated by discontinuous sucrose gradient centrifugation through successive layers of 34.5 and 45% (w/v) sucrose at 56,000g_{av} for 2 hr at 4° in the SW50L rotor. Lysosomal (or acrosomal) and mitochondrial fractions were collected from the interface of 14.3 and 34.5% sucrose and of 34.5 and 45% sucrose, respectively. All the particulate fractions were washed with 0.25 M sucrose and finally dispersed in 0.25 M sucrose.

Subcellular fractions were isolated from spermatozoa by the same procedure except that spermatozoa were homogenized with five passes of the motor-driven pestle.

For electron microscopic examination each subcellular fraction was suspended in cold buffered saline and mixed with an equal volume of cold 4.5% glutaraldehyde in sodium cacodylate buffer (pH 7.0) containing 3 mM $CaCl_2$. After 1 hr at 4° the suspensions were centrifuged at 105,000*g* for 20 min, and the pellets were fixed in buffered osmium tetroxide for 2 hr at room temperature. The preparations were then dehydrated in 70% ethanol, embedded in Epon, sectioned at 0.05 μ, and stained with lead citrate.

Other Analytical Methods. Isokinetic sucrose density gradients (5–23.5%) were prepared according to McCarty *et al.* (1968) as previously described (Majumder and Turkington, 1971). Enzyme preparations (0.2 ml) were layered over 4.5 ml of the sucrose gradient and after centrifugation at 48,000 rpm for 13.5 hr at 5° in an SW50L rotor, the tube bottom was punctured and 10-drop fractions (about 0.15 ml) were collected and assayed for enzyme activity.

The protein concentration of the samples was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Results

Enzyme Purification. CARBOXYMETHYLCELLULOSE CHROMATOGRAPHY. The enzyme preparations from step 1 in purification were subjected to chromatography on carboxymethylcellulose. The elution profile shown in Figure 1 demonstrates the resolution of testicular *N*-acetyl-β-glucosaminidase into two distinct enzyme peaks (I and II). *N*-Acetyl-β-glucosaminidase II is the major enzyme peak which represented approximately 95% of the total testicular enzyme. Testicular enzyme I was not adsorbed by the column and was eluted with the first protein peak. Under identical conditions of carboxymethylcellulose chromatography, rat epididymal sperm enzyme was retained by the column and was eluted as a single peak following a linear pH gradient elution. The sperm enzyme showed a similar elution profile as the testicular enzyme peak II. Rechromatography of the testicular enzyme peaks I and II and the sperm enzyme did not alter significantly the elution profile of these enzymes, thereby confirming the presence of two isoenzymes (I and II) of *N*-acetyl-β-glucosaminidase in testis.

Figure 2 shows the carboxymethylcellulose chromatography profiles of β-galactosidase enzymes from rat epididymal sperm and mature testes. Two enzyme peaks were observed in both the sperm and testicular enzyme preparations. The major sperm enzyme peak (II, approximately 90% of the total activity) was adsorbed by the column and was eluted with a linear

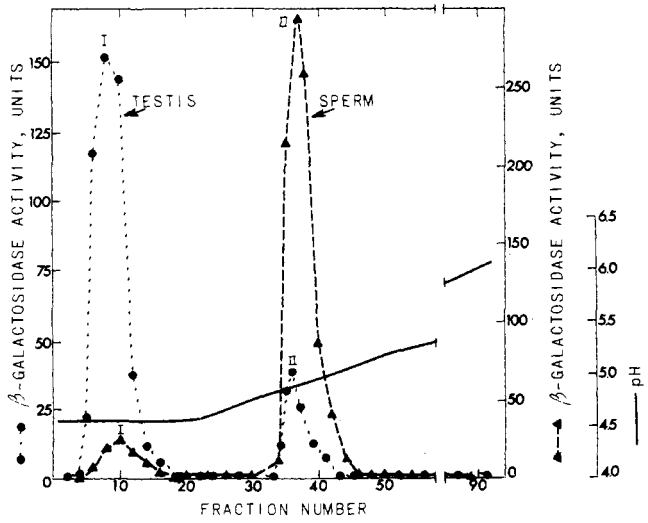

FIGURE 2: Fractionation of sperm (▲) and testicular (●) β-galactosidases by chromatography on carboxymethylcellulose. An aliquot of 75 μl of each fraction was assayed for enzyme activity by the standard assay method.

pH gradient, whereas the minor enzyme peak (I) was not retained by the column. In the case of the testicular enzymes, however, the major enzyme peak (II) which represented approximately 85% of the total activity was not adsorbed by the carboxymethylcellulose column. The minor testicular enzyme II (approximately 15% of total activity) had an elution profile similar to that of the major sperm enzyme II. Rechromatography on carboxymethylcellulose yielded a single peak of activity with each enzyme preparations indicating the presence of two isoenzymes (I and II) of β-galactosidase in sperm and mature testis.

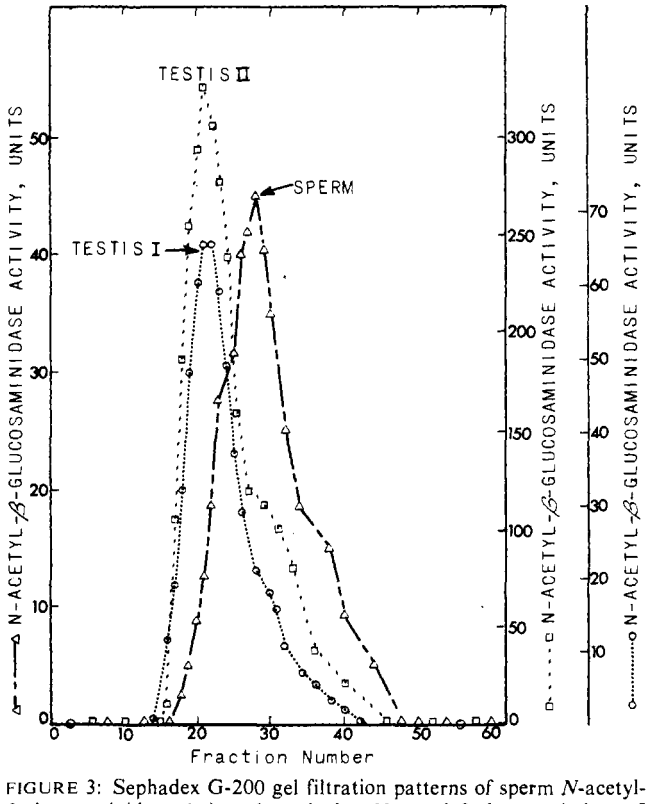

FIGURE 3: Sephadex G-200 gel filtration patterns of sperm *N*-acetyl-β-glucosaminidase (△) and testicular *N*-acetyl-β-glucosamindases I (○) and II (□). The following amounts of enzyme obtained by carboxymethylcellulose chromatography were applied to the column: sperm enzyme, 38,000 units; testicular enzyme I, 7,500 units; and testicular enzyme II, 115,000 units. An aliquot of each fraction (150 μl of fractions from sperm enzyme and testicular enzyme I and 50 μl of fractions from testicular enzyme II) was assayed for enzymatic activity by the standard assay method. Recovery of the enzyme activity by Sephadex gel filtration was approximately 80% for both testicular enzymes and 20% for the sperm enzyme.

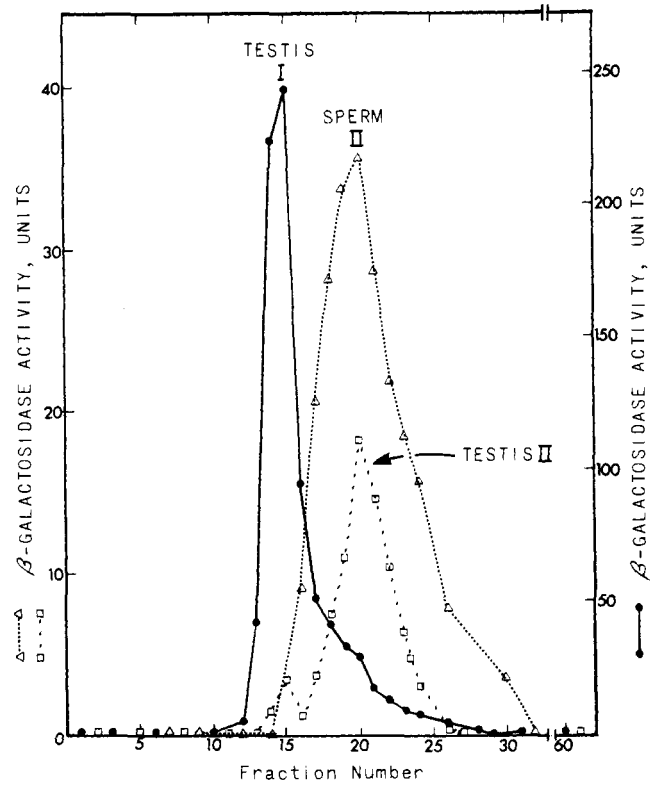

FIGURE 4: Sephadex G-200 gel filtration patterns of sperm β-galactosidase II (△) and testicular β-galactosidases I (●) and II (□). The following amounts of enzymes obtained by carboxymethylcellulose chromatography were applied to the column: sperm enzyme II, 10,000 units; testicular enzyme I, 18,000 units; and testicular enzyme II, 2500 units. An aliquot of 150 μl of each fraction was assayed for enzyme activity by the standard assay method. Recovery of enzyme activity by Sephadex gel filtration was approximately 80% for the testicular enzymes (I and II) and 50% for the sperm enzyme II.

SEPHADEX G-200 GEL FILTRATION. The enzymatic activities derived from sperm or testis and resolved by carboxymethylcellulose chromatography were analyzed further by molecular sieving on a column of Sephadex G-200. The testicular isoenzymes for *N*-acetyl-β-glucosaminidase activity showed nearly identical gel filtration patterns, indicating that these isoenzymes (I and II) are similar in molecular size (Figure 3). The sperm enzyme was eluted as a single peak of activity, and the filtration rate of this enzyme was distinctly slower than that for both the testicular isoenzymes I and II, indicating that the sperm enzyme has a smaller molecular size as compared to the testicular enzymes. Fractions containing testicular enzyme I (fractions 16–27) and enzyme II (fractions 17–27) were pooled together, concentrated by ultrafiltration, and dialyzed against 0.05 M sodium citrate buffer (pH 4.6). These enzyme preparations of testis were used for the studies reported below unless otherwised stated. The highly purified sperm enzyme following Sephadex filtration was found to be unstable. For the following studies on the properties of the sperm enzyme, the enzyme preparation as obtained by carboxymethylcellulose chromatography (fractions 34–52) was used.

The major sperm β-galactosidase enzyme II was also characterized further and its properties were compared with the testicular β-galactosidases I and II. Sephadex G-200 gel filtration patterns of these enzymes are shown in Figure 4. Each of these enzyme preparations were eluted as a single peak of activity

TABLE I: Summary of Partial Purification of β-Galactosidase from Rat Epididymal Spermatozoa and Testes.[a]

Tissue	Fraction	Act. (Units × 10^{-3})	Sp Act. (Units/μg of Protein)	Purifcn -fold	Recov, % Initial Act.
Spermatozoa	Homogenate	70	3.4	1	100
	Step 1	38	13.6	4	54
	Step 2, Peak II	33	270.0	79	46
Testis	Homogenate	54	0.3	1	100
	Step 1	27	2.9	9	50
	Step 2				
	Peak I	19	5.6	19	36
	Peak II	2.7	2.9	9	5
	Step 3				
	Peak I	16	37.2	124	30
	Peak II	2	6.5	22	4

[a] β-Galactosidases were isolated from 6 g of rat (90 days old) testes. Spermatozoa were obtained from epididymides of 12 rats (90–100 days old). At each step of purification the activities of the enzyme were measured by the standard assay method.

following molecular sieving on the Sephadex column. The major testicular β-galactosidase enzyme (I) exhibited a faster rate of filtration as compared to the testicular enzyme II and sperm enzyme II. However, the filtration pattern of the sperm enzyme was nearly identical with that of the testicular minor enzyme peak II. These results indicate that the molecular size of the sperm enzyme is clearly less than that of the major testicular enzyme (I), but it is similar to that of the minor testicular enzyme II. Fractions containing testicular β-galactosidases I (fractions 13–19) and II (fractions 17–25) were pooled together, concentrated by ultrafiltration, and dialyzed against 0.05 M sodium citrate buffer (pH 4.6). These preparations of testicular β-galactosidase were used for the studies described here unless otherwise indicated. The sperm β-galactosidase preparation after purification by Sephadex gel filtration was found to be unstable, unlike the testicular enzymes. The sperm β-galactosidase preparation as purified by carboxymethylcellulose chromatography was used for studies on the characterization of this enzyme.

Table I shows the results of a typical purification procedure for β-galactosidase. The specific activity of β-galactosidase in sperm was markedly higher (approximately 12-fold) than that in the mature testis. Approximately 50–55% of the total activity of these tissues was recovered in the high-speed supernatant at 240,000*g* following treatment of the tissue homogenates with 0.2% Triton X-100 and 1.0 M potassium chloride. The major β-galactosidase (II) of sperm was purified to 79-fold whereas the testicular enzymes I and II were purified to 124- and 22-fold, respectively.

As shown in Table II the specific activity of the *N*-acetyl-β-glucosaminidase in sperm was markedly higher (approximately 7-fold) as compared to the testes from adult rats. A combination of Triton X-100 (0.2%) and 1.0 M potassium chloride was very effective in solubilizing the particulate bound enzymes of testis and sperm. Testicular enzymes I and II were purified to 27- and 230-fold, respectively, whereas the sperm enzyme was purified to 97-fold.

Enzymatic Characteristics of the Isoenzymes. The amount of *p*-nitrophenol liberated from the synthetic substrates by the enzyme preparations from testis and sperm increased linearly with time for at least 40 min under the standard assay conditions. Proportional increases in enzyme activities were observed with as much as 300 units of β-galactosidase or *N*-acetyl-β-glucosaminidase in the reaction mixture of 0.35 ml. The divalent metal ions Mg^{2+}, Co^{2+}, and Ca^{2+} (10 mM as the chloride salts) had no effect on the activity of enzymes derived from either testis or sperm. The activities of these enzymes were not affected by EDTA or dithiothreitol (5 mM). However, *p*-chlo-

TABLE II: Summary of Partial Purification of *N*-Acetyl-β-glucosaminidase from Rat Epididymal Spermatozoa and Testes.[a]

Tissue	Fraction	Act. (Units × 10^{-3})	Sp Act. (Units/μg of Protein	Purifcn -fold	Recov, % Initial Act.
Spermatozoa	Homogenate	205	10	1	100
	Step 1	146	53	5	71
	Step 2	116	970	97	57
Testis	Homogenate	248	1.4	1	100
	Step 1	151	16.2	12	61
	Step 2				
	Peak I	7.6	5.4	4	3
	Peak II	116	129	92	47
	Step 3				
	Peak I	6.2	38.4	27	2.5
	Peak II	96	322	230	39

[a] *N*-Acetyl-β-glucosaminidases were isolated from 6 g of rat (90 days old) testes. Spermatozoa were obtained from epididymides of 12 rats (90–100 days of age). At each step of the purification the enzyme activity was measured by the standard assay method.

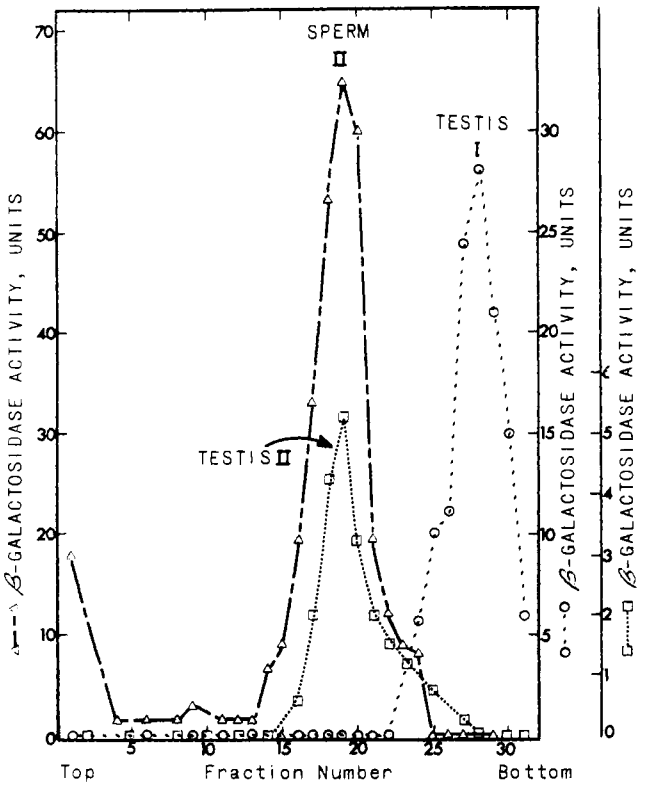

FIGURE 5: Sedimentation profiles of sperm β-galactosidase II (△) and testicular β-galactosidases I (○) and II (□) in isokinetic sucrose density gradients. The following amounts of the enzyme were used for the sedimentation analysis: sperm enzyme II, 1200 units; testicular enzyme I, 450 units; and testicular enzyme II, 80 units. An aliquot of 50 μl of each fraction was assayed for the activity of the enzyme by the standard assay method. Human serum albumin (4.6 S) was used as the standard protein for the determination of the sedimentation constants of the isoenzymes of β-galactosidase.

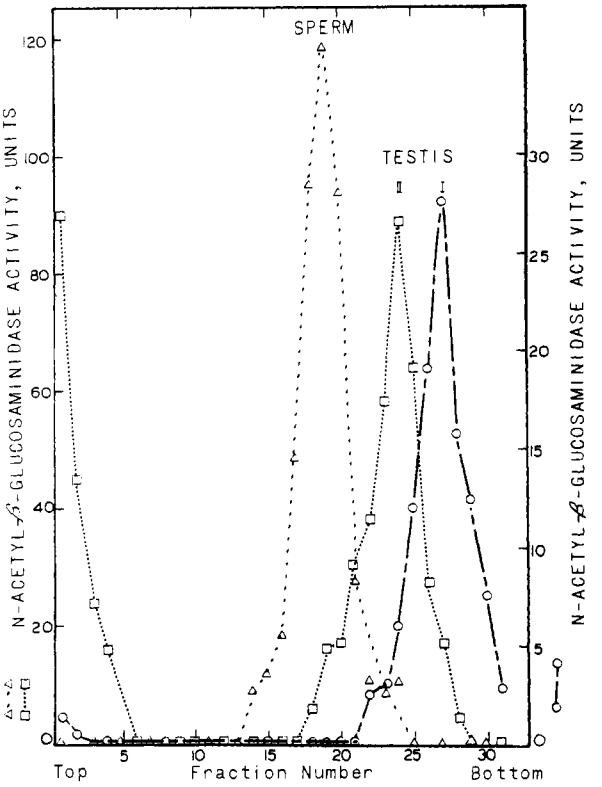

FIGURE 6: Sedimentation profiles of sperm *N*-acetyl-β-glucosaminidase (△) and testicular *N*-acetyl-β-glucosaminidases I (○) and II (□) in isokinetic sucrose density gradients. The following amounts of the enzyme were applied for the sedimentation analysis: sperm enzyme, 1500 units; testicular enzyme I, 380 units; and testicular enzyme II, 1800 units. An aliquot of 50 μl of each fraction was assayed for the activity of the enzyme by the standard assay method. Human serum albumin (4.6 S) was used as the standard protein for the determination of the sedimentation constants of the isoenzymes of *N*-acetyl-β-glucosaminidase.

romercuribenzoate (1.25 mM) strongly inhibited the activities of all the isoenzymes from sperm and testis, indicating that thiol groups of the isoenzymes are essential for enzymatic activity.

pH OPTIMA. The testicular and sperm β-galactosidases exhibited similar pH-activity curves, and maximal enzyme activity was observed in the pH range of 3.25–3.50. The optimal pH range for both the testicular *N*-acetyl-β-glucosaminidase isoenzymes (I and II) was between 4.0 and 4.25, whereas the sperm enzyme had highest activity in the pH range of 3.75–4.25. Striking differences among the *N*-acetyl-β-glucosaminidases were observed in the degree of activation over the pH range of 3.0–3.5. At 3.0 the degree of activation was highest with the sperm enzyme and least with the testicular enzyme II. The ratios of enzymatic activities at pH 4.00 and 3.00 for the sperm and testicular enzymes I and II were 1.2, 2.0, and 12.0, respectively.

SUBSTRATE CONCENTRATION. The apparent K_m values of the sperm and testicular β-galactosidases I and II for the synthetic substrate were similar, being 0.34, 0.35, and 0.34 mM, respectively. The apparent K_m of the testicular *N*-acetyl-β-glucosaminidase II (1.19 mM) was significantly higher than those of the testicular enzyme I (0.73 mM) and the sperm enzyme (0.68 mM).

HEAT STABILITY. The activities of all the enzyme preparations were stable after heating at 50° for 15 min and were destroyed completely when heated at 70° for 15 min. The sperm enzymes exhibited greater stability toward heat inactivation as compared to the testicular isoenzymes.

SUCROSE DENSITY GRADIENT FRACTIONATION. Figure 5 shows the sucrose density gradient sedimentation patterns of the sperm and testicular β-galactosidases. Each of the enzyme preparations sedimented as a single activity peak in the sucrose gradient. The sperm enzyme II showed a sedimentation profile which was nearly identical with that of testicular enzyme II. Both sperm enzyme II (6.4 S) and testicular enzyme II (6.4 S) sedimented as lighter particles as compared with the testicular β-galactosidase I (9.9 S). The sedimentation properties of the sperm and testicular *N*-acetyl-β-glucosaminidases I and II in isokinetic sucrose gradients were also determined (Figure 6). Only one activity peak could be detected in each enzyme preparation. The sperm enzyme (6.4 S) sedimented as a lighter particle as compared to the testicular enzymes I (9.2 S) and II (8.1 S). These results further demonstrate that the sperm *N*-acetyl-β-glucosaminidase is physically different from the testicular isoenzymes I and II.

SUBCELLULAR DISTRIBUTION. To determine the subcellular localization of these enzyme activities in mature rat testis and sperm, the tissue homogenates were fractionated by differential centrifugation techniques. Figures 7 and 8 show the distribution of the recovered enzyme activities in the nuclear (N), mitochondrial (M), lysosomal (L), acrosomal (A), microsomal (R), and cytosol (S) fractions. The results are expressed as the percentage of total recovered activity/percentage of total recovered protein for each enzyme activity. The protein content of each fraction is represented by the scale on the abscissa. In both tissues the enzymes were primarily particulate and the specific activity of the enzymes was highest in the lysosomes of

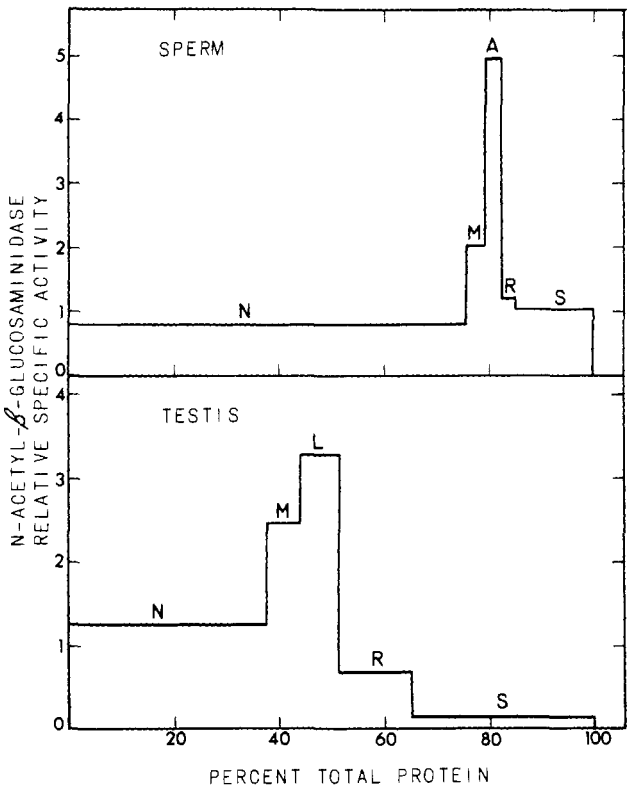

FIGURE 7: Subcellular distribution patterns of *N*-acetyl-β-glucosaminidase in rat epididymal spermatozoa and testis. The activities of the enzyme in all the fractions were measured by the standard assay method: N, nuclear; M, mitochondrial; L, lysosomal; A, acrosomal; R, microsomal; S, supernatant. Electron microscopic examination of the "lysosomal" fraction revealed it to be not less than 95% pure lysosomes. The acrosome fractions were 95–98% pure, being contaminated by occasional mitochondria and nonspecific debris.

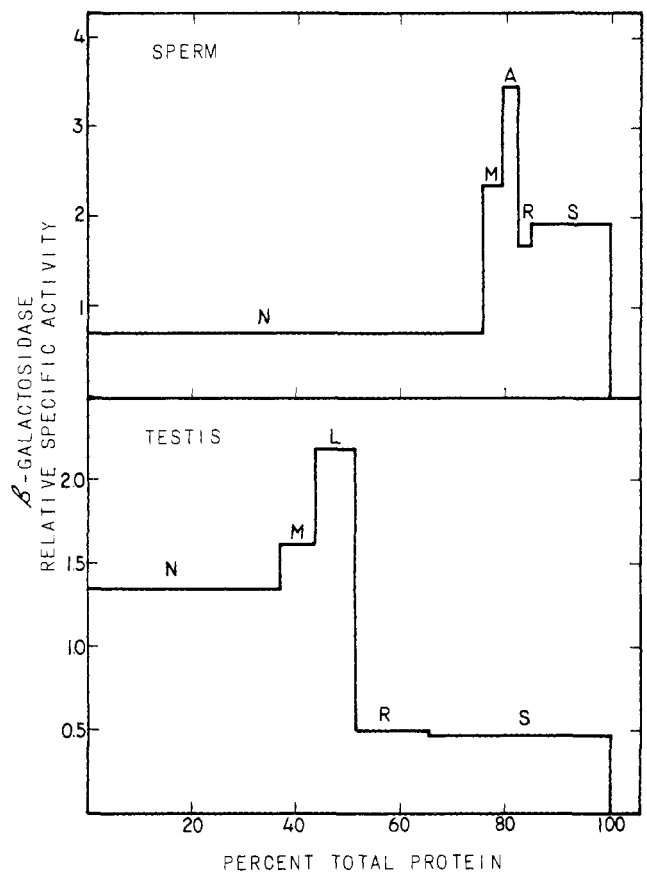

FIGURE 8: Subcellular distribution patterns of β-galactosidase in rat epididymal spermatozoa and testis. The activities of the enzymes in all the fractions were measured by the standard assay method: N, nuclear; M, mitochondrial; L, lysosomal; A, acrosomal; R, microsomal; S, supernatant.

testis and acrosomes of spermatozoa. The nuclear pellet from spermatozoa contains a significant amount of unbroken cells which possibly contributed to the large amount of enzyme activity in the crude nuclear fraction.

EFFECT OF TREATMENTS WITH DETERGENTS. Table III shows the effect of treatments with sodium deoxycholate, Triton X-100, and cetyltrimethylammonium bromide on the release of the enzymes from the particulate fractions of the cells. There was a marked increase in the activities of both enzymes in the extracts (30,000*g* supernatant) of testis and sperm following treatment with these detergents. A variable effect was observed on the extraction of enzymes from the particulate fractions depending upon the nature and source of these enzymes. The effects of these detergents on the activities of the enzymes in crude tissue homogenates are also shown in Table III. Treatments with detergents, particularly with cetyltri-

TABLE III: Effect of Detergents on the Release and Activation of β-Galactosidase and *N*-Acetyl-β-glucosaminidase in Homogenates of Testis and Spermatozoa.[a]

Enzyme	Tissue	Expt 1: Enzyme Activity in Homogenate Treatments (Units/μg of Protein)				Expt 2: Enzyme Activity in High-Speed Supernatant Treatments (Units/μg of Protein)			
		Nil	Triton X-100	DOC	CTAB	Nil	Triton X-100	DOC	CTAB
β-Galactosidase	Testis	0.08	0.09	0.08	0.12	0.02	0.28	0.27	0.00
	Spermatozoa	0.93	1.10	0.93	1.69	0.36	1.54	4.04	5.40
N-Acetyl-β-glucosaminidase	Testis	0.44	0.67	0.57	1.00	0.05	0.87	1.02	0.82
	Spermatozoa	3.43	4.43	4.00	4.09	3.50	6.0	18.0	39.0

[a] Testes and epididymal spermatozoa were homogenized in ice-cold 0.25 M sucrose with 1 and 5 passes, respectively, in a glass homogenizer fitted with a motor-driven Teflon pestle. The homogenates were incubated without or with detergents at 0–4° for 30 min. They were then assayed at 37° for 10 min (expt 1). In a separate experiment (expt 2) the incubated homogenates were subjected to centrifugation at 30,000*g* for 15 min, and the high-speed supernatant fluids were assayed by the standard assay method. The results are expressed as units of enzyme activity per μg of protein of the homogenates. Final concentrations of detergents in the homogenates: expt 1, 0.16%; expt 2, 0.24%. DOC, sodium deoxycholate; CTAB, cetyltrimethylammonium bromide.

methylammonium bromide, effectively increased the total enzymatic activity of testicular and sperm β-galactosidase and *N*-acetyl-β-glucosaminidase, indicating the "latent" nature of these enzymes in crude tissue homogenates.

ADMIXTURES OF ENZYME PREPARATIONS. In other experiments preparations of lysosomal isoenzymes of β-galactosidase and *N*-acetyl-β-glucosaminidase were mixed in various proportions with crude sperm homogenate. In each admixture the enzymatic activities were additive amounts of the individual preparations. This result indicates that the total or nearly total absence of these lysosomal enzymes from mature spermatozoa cannot be explained by the presence of an inhibitor or the absence of an activator in the sperm cells. Partially purified lysosomal β-galactosidase I of testes was also incubated with crude sperm homogenate in 0.25 M sucrose (pH 7.0) at 30° for 30 min. Lysosomal β-galactosidase I was also added to one of two aliquots of a sperm suspension, and sperm β-galactosidase was isolated. Based upon the carboxymethylcellulose chromatography profiles, no conversion of the lysosomal to the acrosomal isoenzyme was observed. Prolonged incubations of crude testis homogenates (up to 3 hr at 37°) did not alter the ratios of lysosomal to acrosomal forms of β-galactosidase. These results provide no support for the proposition that the larger lysosomal isoenzyme could bear a precursor–product relationship to the smaller acrosomal enzyme.

COMPARISONS WITH EPIDIDYMAL ENZYMES. In order to determine whether the isoenzymes derived from sperm could represent contaminant molecules originating from the cells of the epididymis several additional observations were made. *N*-Acetyl-β-glucosaminidase and β-galactosidase were extracted from rat epididymis at 46 days of age. At this age the epididymis is mature (Reid and Cleland, 1957) but spermatozoa have not yet appeared in the seminiferous tubules of the testis (Clermont, 1962). The morphological maturity and sperm-free state of the epididymis were confirmed by light microscopy of fixed tissue sections. Extracted enzymes were characterized after filtration through Sephadex G-200 or chromatography on carboxymethylcellulose. Although large amounts of both enzyme activities were recovered, the isoenzyme activities characteristic of epididymal spermatozoa were both undetectable.

Discussion

The acrosome is regarded as a specialized lysosome which has evolved to facilitate fertilization in multicellular organisms (Allison and Hartree, 1970). The acrosome is uniquely formed in spermatids and spermatozoa during spermatogenesis. The enzymes β-galactosidase and *N*-acetyl-β-glucosaminidase derived from testis and sperm appear to be typical of lysosomal enzymes by a number of criteria (Maggi, 1973). They are acid hydrolases which sediment in the "light" mitochondrial or lysosomal fraction and have the same subcellular distribution as two other testicular lysosomal enzymes, acid phosphatase and β-glucuronidase (Males and Turkington, 1971). The "latent" nature of these particulate enzymes in crude tissue homogenates has been demonstrated by the releasing and activating effects of detergents.

β-Galactosidase, which participates in the clevage of the glycosyl side chain of glycoproteins, has been found in a wide variety of mammalian tissues (Dott, 1969; Conchie *et al.*, 1959; Caygill *et al.*, 1966). The present studies for the first time demonstrate the presence of β-galactosidase in spermatozoa. This enzymatic activity is located predominantly in the lysosomes of mature testis and in the acrosomes of epididymal spermatozoa. Both spermatozoa and mature testis contain two isoenzymes (I and II) of β-galactosidase, as demonstrated by carboxymethylcellulose chromatography. In the sperm cells β-galactosidase II is the principal form (approximately 90% of total activity) of the enzyme in contrast to testis, where isoenzyme I represents approximately 85% of the total activity. That the two isoenzymes of testis are different enzyme molecules is indicated by their separate elution profiles on rechromatography on carboxymethylcellulose, different rates of gel filtration through a column of Sephadex G-200, and differential rates of sedimentation through sucrose density gradients. The physical and heat inactivation properties of the major sperm enzyme (II) were clearly different from those of the major testicular enzyme (I), but were nearly identical with those of the testicular minor enzyme II, suggesting that testicular β-galactosidase II is probably of acrosomal origin. Subsequent studies on the isoenzyme profiles of β-galactosidase during testicular development have shown that the specific activity of isoenzyme I is high while isoenzyme II is undetectable during spermatogenesis up to the formation of spermatocytes. Isoenzyme II is first detectable with the appearance of acrosome-containing spermatids in the developing testis (Majumder *et al.*, 1974). Results of studies on the hormonal regulation of testicular β-galactosidases demonstrated that β-galactosidase II is specifically induced in testis in response to stimulation with luteinizing hormone and follicle-stimulating hormone or with testosterone (Majumder *et al.*, 1974), hormones required for the formation of acrosome-containing spermatids. These findings are compatible with the view that testicular β-galactosidase II is of acrosomal origin and isoenzyme I is of lysosmal origin. The data of this study demonstrate that the acrosomal enzyme β-galactosidase differs significantly in many of its molecular properties from the lysosomal enzyme of testis.

The presence of *N*-acetyl-β-glucosaminidase has been demonstrated previously in spermatozoa (Conchie and Mann, 1957). This is the first report describing certain physical and enzymatic characteristics of the enzyme partially purified from spermatozoa. The testicular enzyme preparation consists of two isoenzymes (I and II), isoenzyme II being the dominant form of the enzyme (approximately 94% of total activity) in mature testis. Rechromatography of these enzymes on carboxymethylcellulose and sedimentation and enzymatic analyses established further the separate identities of the testicular isoenzymes. In spermatozoa only one enzyme peak was detected by carboxymethylcellulose chromatography. The observation that the molecular size (gel filtration) and sedimentation constant of the sperm enzyme were less than those of both the testicular isoenzymes (I and II) is an indication that the sperm *N*-acetyl-β-glucosaminidase differs significantly in its physicochemical properties from the testicular isoenzymes. Enzymatic characteristics of the sperm enzyme were also clearly different from the testicular isoenzymes in relation to their pH optima, K_m values for the synthetic substrate, and heat stabilities. Studies on the isoenzymes of *N*-acetyl-β-glucosaminidase during testicular development have shown that the specific activities of isoenzymes I and II are maximal in rat testis at approximately 25 days of age when all the various cell types in testis contain lysosomes, and are decreased by dilution during the subsequent formation of acrosome-containing spermatids (Majumder *et al.*, 1974).

Several lines of evidence support the concept that the isoenzyme species recovered from epididymal sperm represent acrosomal enzyme. Their highest specific activities were recovered from subcellular fractions which represented highly purified acrosomes. The low level of contamination of the isolated spermatozoa by epithelial cells would appear to be an insignificant source of contamination by epididymal lysosomes, and the re-

peated washings of spermatozoa would serve to remove nonsedimentable enzymes derived from epididymal secretions. Finally, no isoenzyme activities identical with those derived from sperm could be detected in sperm-free epididymides of 46-day-old rats.

These results suggest that the more specialized enzymatic functions of the highly differentiated cells (spermatozoa) require an altered form of the enzyme molecule. The smaller acrosomal enzymes may represent an advantage for binding to acrosomal sites, for tight packaging within the motile sperm cells, or for interaction with specific substrate sites in the ovum. These findings are also consistent with the view that during the hormone dependent differentiation of spermatocytes to spermatids in testis the expression of genes for the lysosomal β-galactosidase and N-acetyl-β-glucosaminidase is repressed and new genes in spermatids are uniquely expressed for the formation of the acrosome-specific enzymes, β-galactosidase and N-acetyl-β-glucosaminidase. Similar observations have been reported in the case of hyaluronidase, which is specifically induced in the acrosomes of spermatids and spermatozoa during cell differentiation in the germinal epithelium of the testis (Males and Turkington, 1970).

Sperm acrosomal hyaluronidase and proteinase, whose characteristics are also different from the corresponding lysosomal enzymes of other tissues (Zaneveld *et al.*, 1972, 1973; Bollet *et al.*, 1963; Vaes and Jacques, 1965; Aronson *et al.*, 1967), may be released during fertilization to facilitate the penetration of the sperm into the ovum (Allison and Hartree, 1970; Stambaugh and Buckley, 1969; Zaneveld *et al.*, 1970). Specific induction of the unique enzymatic species of glycosyl side-chain cleavage enzymes, β-galactosidase and N-acetyl-β-glucosaminidase, in spermatids which are subsequently packaged into acrosomes of spermatozoa strongly suggests that these enzymes may also participate in the process of fertilization. The side chains of glycoproteins of the outer surface of the plasma membranes of cells have been shown to be important determinants of specificity in cell–cell interactions and other information recognition systems (Cook, 1968; Roseman, 1970; Oseroff *et al.*, 1973). It is possible that during fertilization the rupture of the acrosome releases specific glycosyl side-chain cleavage enzymes which can alter glycoproteins of the ovum and thereby potentially facilitate the process of fertilization. There exists the possibility that the concerted action of the acrosomal endoenzyme hyaluronidase and the glycosyl side-chain cleavage exoenzymes may be effective in the degradation of mucopolysaccharides or glycoproteins of the cumulus oophorus and corona radiata of the ovum. The functional significance of the evolution of unique enzyme molecules of β-galactosidase and N-acetyl-β-glucosaminidase in the acrosomes of spermatozoa is currently under investigation in this laboratory.

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