Studies on Spermatogenesis in Rats

V. INCREASED THERMAL LABILITY OF LYOSOMES FROM TESTICULAR GERMINAL CELLS AND ITS POSSIBLE RELATIONSHIP TO IMPAIRMENTS IN SPERMATOGENESIS IN CRYPTOCHIDISM*

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SUMMARY

Lysosome-rich particles prepared from adult rat testes but not from liver released large amounts of various lysosomal enzymes into the supernatant medium when they were incubated at 37° at physiological pH. Evidence was presented that the thermally sensitive lysosomes were present in advanced germinal cells but not in spermatogonia. Lysosomal rich particles isolated from regressed testes, obtained from rats which previously had been hypophysectomized or made cryptorchid, were temperature-insensitive as those from liver. The hypothesis was advanced that the increased release of hydrolytic enzymes at 37° from lysosomes in certain germinal epithelial cells may be intimately associated with the cellular degeneration and cessation of spermatogenesis which occur in cryptorchid testes.

In many mammals, including rats and men, the testes within the scrotum are maintained at a cooler temperature than that prevailing within the body. In rats, this differential is approximately 5°, and the intratesticular temperature is around 32° (1). If the testicular temperature is elevated, spermatogenesis ceases (2). This can be made to occur either by increasing the environmental temperature, or by placing the testes into the abdominal cavity.

The mechanisms, by which a relatively slight elevation of temperature is able to induce damage to specific germinal epithelial cells (primarily spermatocytes and spermatids), remain unknown. Not all testicular cells degenerate in cryptorchid rats or in other cryptorchid mammals which normally have testes within the scrotum. Spermatogonia, Sertoli cells, and Leydig cells are reported to remain morphologically intact when the intratesticular temperature equals that of the body core temperature (2). One of the factors which renders certain germinal epithelial cells heat-sensitive may reside in the nature of the different lysosomes in various cell populations. In this communication, we report that lysosome-rich particles prepared from normal adult rat testes more readily release their hydrolytic enzymes when incubated at 37° than do lysosomes prepared from other cells. Ancillary data will be presented indicating that the heat-sensitive lysosomes are derived primarily from relatively advanced germinal cells of the testis, and that these lysosomes probably release their enzymic content into cells in vivo within 40 hours following the onset of experimental cryptorchidism.

EXPERIMENTAL PROCEDURE

Normal adult rats of the Wistar strain, weighing 280 to 300 g, were purchased from High Oak Company, Richmond Hill, Ontario. Normal adult rats of the Wistar strain, weighing 280 to 300 g, were purchased from High Oak Company, Richmond Hill, Ontario. Animals were maintained at 24° and had free access to rat chow (Rockland) and tap water. Adult hypophysectomized rats, purchased from the Charles River Breeding Laboratories, were allowed to regress for 30 to 60 days as previously described (3). Pigeons were obtained from Riemens Fur Company, Agatha, Ontario.

Cryptorchidism was produced by translocating both testes to the abdominal cavity. Testes were gently directed through the inguinal canal into the abdomen of rats anesthetized with ether, and testes were then attached to the inner portions of the abdominal wall by sowing a small piece of the testicular capsule (tunica albuginea) to abdominal musculature. Control operations, including ether anesthesia, incisions, and testes manipulation, were performed without suturing the testes into the abdominal cavity.

Preparation of Lysosome-rich Particles, and Testicular Cytosol Fractions—All animals were killed between 9 and 11 a.m. by cervical fracture. A known weight of liver or testis (with tunica albuginea removed) was minced with scissors and transferred to a glass homogenizing tube (Kontes 886000-0024). Ten volumes of ice cold 0.25 M sucrose, containing 10 mM Tris-HCl (pH 7.5), (Medium A) were added and the mixture was homogenized with a Teflon pestle driven by a low speed motor. The clearance between the Teflon pestle and tube was between 0.004 to 0.006 inch. Usually 10 strokes (45 s) were taken for homogenization, and all operations were carried out at 0-4°. The homogenate was centrifuged for 20 min at 800 × g. The pellet, containing nuclei, trapped mitochondria, and unbroken cells, was discarded. The supernatant fraction was centrifuged for 20 min at 40,000 × g at 0°. The pellet, containing mainly mitochondria and lysosomes, was then suspended in Medium A and recentrifuged as above. The washed residue was suspended in Medium A, and was used for the investigation of thermal effects on the release of enzymes from the lysosome rich particle preparation.

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In other experiments designed to estimate the amount of lysosomal enzymes in the cytoplasm of unbroken cells from testes of normal and cryptorchid rats, the cytosol fractions were prepared as above, with the following modifications. The testicular mince was washed with 10 volumes of Medium A prior to homogenization to remove contamination by possible lysosomal enzymes in the extracellular compartments. The washed mince was otherwise treated as above, with the exception that the 800 x g supernatant fraction was centrifuged at 40,000 x g for 30 min. The resulting supernatant fraction was called the cytosol fraction, and it was analyzed for the presence of lysosomal enzymes in testes obtained from rats experimentally made cryptorchid for varying time intervals.

Investigations of Effect of Temperature on Release of Lysosomal Enzymes from Lysosome-rich Particles—Lysosome-rich particles suspended in Medium A (2 mg of protein per ml), were incubated for 1 hour at various temperatures. The suspensions were then centrifuged at 40,000 x g for 1 hour at 0°C. The supernatant fraction was carefully separated from the pellet, and the residual fraction was suspended in the original volume of Medium A. The pellet fraction was subjected to ultrasonication (three times, 20 s each, at 21 kc per s, using the standard probe of a 300 W Biosonic BP-III sonicator from Bronwll Scientific Co., Rochester, N. Y.). Lysosomal enzyme activities were assayed in both the supernatant and sonicated pellet fractions. The sum of the enzyme activities in these fractions was made equivalent to 100% of the enzyme activities in the original lysosomal-rich particles.

Enzyme Assays—Arylsulfatase (type II) (arylsulfate sulphohydrolase A + B) (4), EC 3.1.6.1) was measured at pH 4.9, with N-nitroacetoclsulfate as substrate, using the incubation conditions of Kaback and Howell (5). The reaction was stopped after incubation for 30 min at 25°C by addition of 2 ml of 0.2 M NaOH, and the change in absorbance at 515 nm was measured. N-Nitroacetocls was used as standard.

β-N-Acetylhexosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetimidodeoxyglucosidase) (EC 3.2.1.30) was measured by using 2 μmoles of β-nitrophenyl-N-acetyl-β-D-glucosaminidase (6) in citrate-NaOH buffer (0.04 M, pH 4.5) in a final volume of 1.0 ml. The reaction was initiated by addition of the enzyme preparation, and the reaction was incubated at 25°C for 30 min. The reaction was stopped by addition of 2.0 ml of 0.2 M NaOH, and the change in absorbance at 400 nm was measured. β-Nitrophenol was used as standard.

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed using two different substrates. Acid phosphatase-1 was measured using 5 μmoles of β-nitrophenylphosphate (7) in citrate-NaOH buffer (0.04 M, pH 4.5) in a final volume of 1.5 ml. The reaction was initiated by addition of the enzyme preparations, and the reaction was incubated at 25°C for 30 min. The reaction was stopped by addition of 1.5 ml of 0.2 M NaOH, and the change in absorbance at 490 nm was measured. β-Nitrophenol was used as standard. Acid phosphatase-2 was measured using 50 μmoles of β-glucocerebroside (8) in acetyl-NaOH buffer (0.04 M, pH 4.5) in a final volume of 2.5 ml. After addition of the enzyme preparation to initiate the reaction, and incubation at 25°C for 30 min, 2.0 ml of 10% trichloroacetic acid were added to stop the reaction. Inorganic phosphate was determined according to the method of Lindberg and Ernster (9). Acid phosphatase-2 activity was exclusively localized in the lysosomal fractions of rat testes. In contrast, 20 to 30% of acid phosphatase-1 activity was observed to be present in the cytosol fractions, while the remainder was found in lysosomal fractions.

Freshly obtained samples of testicular tissue from normal and cryptorchid rats were fixed in Bouin’s solution (10), embedded in paraffin by conventional techniques, and 5 μm transverse sections were prepared and stained with periodic acid-Schiff-hematoxylin for subsequent histological examination.

RESULTS AND DISCUSSION

The release of acid phosphatase-1 and β-N-acetylhexosaminidase from rat testicular and liver preparations of lysosomes incubated at various temperatures is shown in Figs. 1 and 2. When lysosome-rich particles from rat testes and rat liver were incubated in 0.25 M sucrose solution at physiological pH 7.4 for 1 hour, there was little but little release of enzymes at 4°C. The amount of enzyme activity released was slightly increased in liver lysosome-rich particles as the incubation temperatures were increased to 37°C (Figs. 1 and 2). In contrast, there was a relatively large amount of enzymes released from testicular lysosome-rich particles incubated at 37°C (Figs. 1 and 2). Comparable results were obtained when the release of other hydrolytic enzymes from lysosome-rich particles of rat testes and rat liver was measured. The release of acid phosphatase-2 activity and arylsulfatase activity from lysosome-rich particles of testes was much greater at 37°C than the corresponding release of these enzymes from liver lysosome-rich particles incubated under identical conditions (Table I, Part A).

Associated with the absence of a scrotum in birds, avian species have testes within the abdominal cavity. In pigeons, for example, the testicular temperature within the abdomen is normally maintained at 40°C or higher (11). We compared the

![Fig. 1. Release of acid phosphatase-1 (AP1) from lysosome-rich particles prepared from rat testes and rat liver. Lysosomal preparations were incubated at various temperatures shown for 1 hour. Results, expressed as the percentages of total enzyme activity, are given as means ± S.E.M., and the number of separate experiments is recorded within parentheses. The actual specific activities are shown in Table I. For other details of methods, see “Experimental Procedure.”](http://www.jbc.org/doi/abs/10.1074/jbc.270.20.7957)
properties of lysosomes prepared from pigeon testes with those prepared from rat testes. As shown in Table I, Part A, the release of enzymes from lysosome-rich particles of pigeon testes incubated at various temperatures was no greater than that released from lysosome-rich particles of pigeon liver. When both sets of lysosome-rich particles were incubated at 37° for 1 hour, the lysosomal enzyme activities released into the supernatant fractions from pigeon testes preparations were considerably lower than those obtained from rat testes (Table I, Part A). Lysosomes from pigeon testes did not show any greater temperature sensitivity with respect to enzyme release than did lysosomes prepared from livers obtained from rats or pigeons (Table I, Part A).

The specific activities of lysosomal enzymes in lysosome-rich particles isolated from rat liver, pigeon liver, and pigeon testes were as high as those observed in corresponding fractions from testes of normal adult rat (Table I, Part B), indicating that the observed differences in enzymes released into supernatant fractions did not result from an absence of enzymes in lysosomal-rich particles isolated from the former tissues.

In regressed cryptorchid rats (2) and in regressed hypophysectomized rats (3, 12, 13), the cessation of spermatogenesis is associated with a relative failure of germinal cells to develop beyond the early primary spermatocyte stage. The composition of the testes from these regressed animals consists primarily of peritubular cells, Leydig cells, Sertoli cells, spermatogonia, and preleptotene spermatocytes (2, 12), but advanced spermatids and spermatozoa are virtually absent, and there is a great reduction in the numbers of late stage spermatocytes and early spermatids (12). Results shown in Table II, Part A demon-

![Graph showing release of p-N-acetylhexosaminidase (NAG) from lysosome-rich particles prepared from rat testes and rat liver.](http://www.jbc.org/)

**Fig. 2.** Release of $\beta$-N-acetylhexosaminidase (NAG) from lysosome-rich particles prepared from rat testes and rat liver. Lysosomal preparations were incubated at various temperatures shown for 1 hour. Results, expressed as the percentages of total enzyme activity, are given as means $\pm$ S.E.M., and the number of separate experiments is recorded within parentheses. The actual specific activities are shown in Table I. For other details of methods, see “Experimental Procedure.”

### Table I

**A. Release of various lysosomal enzymes from lysosome-rich particles incubated at 4° versus 37°**

<table>
<thead>
<tr>
<th>Source of lysosome-rich particles</th>
<th>Incubation temperature</th>
<th>Percentage of enzyme released per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AP$_1$</td>
</tr>
<tr>
<td></td>
<td>$°C$</td>
<td></td>
</tr>
<tr>
<td>Rat testes</td>
<td>4</td>
<td>2.0 ± 0.3 (10)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>11.8 ± 1.3 (10)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>4</td>
<td>2.0 ± 0.3 (5)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4.1 ± 0.1 (5)</td>
</tr>
<tr>
<td>Pigeon testes</td>
<td>4</td>
<td>0.7 ± 0.2 (3)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>2.4 ± 1.0 (3)</td>
</tr>
<tr>
<td>Pigeon liver</td>
<td>4</td>
<td>1.0 ± 0.3 (3)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>2.7 ± 1.1 (3)</td>
</tr>
</tbody>
</table>

**B. Specific activities of enzymes from lysosome-rich particles from various tissues**

<table>
<thead>
<tr>
<th>Specific activities</th>
<th>AP$_1$</th>
<th>AP$_2$</th>
<th>ARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat testes</td>
<td>0.788 ± 0.036 (10)</td>
<td>1.81 ± 0.087 (8)</td>
<td>0.178 ± 0.041 (10)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>1.55 ± 0.141 (5)</td>
<td>3.12 ± 0.292 (5)</td>
<td>0.141 ± 0.014 (6)</td>
</tr>
<tr>
<td>Pigeon testes</td>
<td>0.649 ± 0.059 (3)</td>
<td>1.11 ± 0.101 (3)</td>
<td>0.166 ± 0.014 (6)</td>
</tr>
<tr>
<td>Pigeon liver</td>
<td>0.903 ± 0.014 (3)</td>
<td>1.55 ± 0.341 (3)</td>
<td>0.03 ± 0.002 (3)</td>
</tr>
</tbody>
</table>

*AP$_1$ represents acid phosphatase activity assayed against p-nitrophenylphosphate; AP$_2$ represents acid phosphatase activity assayed against $\beta$-glycerophosphate; ARS represents arylsulfatase activity assayed against p-nitrocatecholsulfate; and NAG represents $\beta$-N-acetylhexosaminidase activity, assayed against p-nitrophenyl-$\beta$-N-acetyl-$\beta$-glucosaminide as substrate. All activities are expressed as the percentage of total activities obtained in sonicated lysosome-rich particles plus that released during incubation for 1 hour. The results are given as means $\pm$ S.E.M. with the number of determinations within parentheses.
TABLE II

A. Release of various lysosomal enzymes from lysosome-rich particles prepared from testes of normal, cryptorchid,
and hypophysectomized rats

<table>
<thead>
<tr>
<th>Source of testicular lysosome-rich particles</th>
<th>Incubation temperature</th>
<th>Percentage of enzyme released per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>AP₁</td>
</tr>
<tr>
<td>Normal adult rats</td>
<td>4</td>
<td>2.0 ± 0.3 (10)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>11.8 ± 1.3 (10)</td>
</tr>
<tr>
<td>Cryptorchid rats (20 days postoperative)</td>
<td>4</td>
<td>2.6 ± 0.3 (3)</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4.0 ± 0.6 (3)</td>
</tr>
<tr>
<td>Hypophysectomized rats (35 days postoperative)</td>
<td>4</td>
<td>0.9 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4.8 ± 0.2</td>
</tr>
</tbody>
</table>

B. Specific activities of enzymes from lysosome-rich particles from various groups of rats

<table>
<thead>
<tr>
<th>Specific activities</th>
<th>AP₁</th>
<th>AP₂</th>
<th>ARS</th>
<th>NAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adult rats</td>
<td>0.788 ± 0.036 (10)</td>
<td>1.51 ± 0.08 (8)</td>
<td>0.175 ± 0.041 (10)</td>
<td>0.66 ± 0.036 (10)</td>
</tr>
<tr>
<td>Cryptorchid rats</td>
<td>0.852 ± 0.03 (3)</td>
<td>1.82 (2)</td>
<td>0.166 ± 0.08 (3)</td>
<td>1.42 ± 0.03 (3)</td>
</tr>
<tr>
<td>Hypophysectomized rats</td>
<td>0.639 (2)</td>
<td>1.27 (2)</td>
<td>0.113 (2)</td>
<td>0.655 (2)</td>
</tr>
</tbody>
</table>

* The same symbols described in the legend to Table I are employed, and data are expressed in the same manner.

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Fig. 3. Amount of β-N-acetylhexosaminidase (NAG) activity in cytosol fractions from testicular cells obtained from both testes of cryptorchid rats at varying times after operation. For details of methods, see “Experimental Procedure.”

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The results are given for both testes as means ± S.E.M., with the number of determinations within parentheses.

The same symbols described in the legend to Table I are employed, and data are expressed as units of total lysosomal enzyme activity in sonicated homogenates from both testes obtained from cryptorchid rats at varying times after operation. The results are given as means ± S.E.M. with the number of determinations within parentheses.

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The specific activities of lysosomal enzymes in lysosome-rich particles isolated from testes of regressed hypophysectomized or cryptorchid rats were as high as those ob-
served in corresponding fractions from normal adult rats (Table II, Part B), indicating that the failure to observe an increased enzyme release into supernatant fractions did not result from an absence of available lysosomal enzymes in testicular cells remaining after the advanced germinal cells had been destroyed.

If the increased temperature sensitivity of lysosomes in specific germinal cells were responsible for the damage and destruction of these testicular cells during cryptorchidism in vivo, it would be anticipated that the amounts of nonparticulate lysosomal enzymes should increase within testicular cells shortly after cryptorchidism was experimentally produced. The activities in the testicular cytosol fractions of $\beta$-N-acetylhexasaminidase (Fig. 3) and arylsulfatase (Fig. 4) increased within 40 hours after the operation, and these elevated activities were sustained during the time intervals measured. In contrast, the decreases in testicular weight and protein content, which follow the production of cryptorchidism (2), were not clearly evident until 120 and 140 hours postoperatively (Table III). Histological and morphological changes in the testis which occur following cryptorchidism were not detectable 40 hours postoperatively. In agreement with observations of previous workers (3), degenerative changes were observed in testes obtained from rats rendered cryptorchid for 3 or more days, but not in testes from rats rendered cryptorchid for 40 hours (Fig. 5).

The increases in lysosomal enzyme activities in cytosol fractions of testes obtained from rats at varying periods following the onset of cryptorchidism (Figs. 3 and 4) were not simply a reflection of increased total testicular lysosomal enzymes in the cryptorchid state. As shown in Table III, the total lysosomal $\beta$-N-acetylhexasaminidase and arylsulfatase activities remained at the same levels during all time intervals investigated up to 5 days after the production of cryptorchidism.

![Arylsulfatase (ARS) activities in cytosol fractions from testicular cells obtained from both testes of cryptorchid rats at varying times after operation. For details of methods, see "Experimental Procedure."](image)

![Microphotographs of sections of testes from normal adult rats (A) and from rats rendered cryptorchid for 40 hours (B), 80 hours (C), or 120 hours (D). Note abnormal tubules in C and D, containing degenerating spermatids and spermatocytes. Spermatogonia and Leydig cells appear normal in all cases. Stained with periodic acid-Schiff-hematoxylin; $\times$ 400](image)
Combined data are compatible with the interpretation that certain germinal cells of rat testes contain lysosomes which are more temperature-sensitive than are lysosomes from liver, and that these lysosomes probably release their enzymes into the cytosol fraction within 40 hours after the onset of cryptorchidism. The possible effects of the released lysosomal enzymes on cell structure and function remain debatable, but it appears reasonable to suggest that they may well be intimately associated with the subsequent destruction of affected germinal cells and the subsequent cessation of spermatogenesis. It is generally accepted that lysosomes contain a host of hydrolytic enzymes capable of catalyzing degradative reactions, using intracellular components as substrates. These processes may play an important role in the response of cellular injury (14, 15).

Dingle (16) reported that lysosomes from rat liver did not readily release hydrolytic enzymes when incubated in 0.25% sucrose for 45 min at 37° at neutral pH, and our data confirm these observations. Dingle noted, however, that incubation of liver lysosomal preparations at pH 5 or lower resulted in enzyme release (15). We repeated these experiments, and showed that the differences in behavior between rat testes and liver lysosomal preparations incubated at 37° were abolished when the pH of the medium was lowered to 4.5 (data not shown). The additive effects of increased hydrogen ion concentrations and temperature on functions of lysosomes isolated from various tissues remain to be investigated.

Blackshaw and Hamilton (17) recently reported histochemical observations on dye chromophilia, and on changing patterns of testicular lysosomal acid phosphatases and aminopeptidases following immersion of rat testes into a water bath at either 42° for 30 min, or at 43° for 60 min. The histologic appearance of pachytene spermatocytes was observed to be altered qualitatively within 2 to 4 hours after exposure of testes to these elevated temperatures (17). Increases in the percentages of free acid phosphatases (from 53% base-line to 66% at 4 hours and 61% at 24 hours after immersion at 43° for 1 hour) and free acid proteinases (from 6% base-line to 13% at 4 hours and 20% at 24 hours after heat treatment) were also reported (17). These observations, coupled with a loss of DNA in affected tubules, suggested to the authors that there was a lysosomal effect obtained by heating the testis which may be a consequence of injury to various cellular membranes at critical stages of development (17).

We provisionally interpret our results to signify that membranes of lysosomes in temperature-sensitive germinal cells may be more readily altered by exposure to elevated temperature than are membranes of lysosomes from cells normally adapted to a temperature of 37°. Investigations to test this hypothesis are in progress, and the properties of lysosomes prepared from various classes of germinal cells are being examined.

Results presented are compatible with the concept that the testicular damage leading to cessation of spermatogenesis which occurs during cryptorchidism may be a consequence of lysosomal enzyme release from lysosomes ruptured by exposure to higher temperature. However, it remains equally plausible that these various abnormalities are simply reflections of associated events which occur in response to a more fundamental common cause.

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