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Stage-dependent appearance of sulfhydryl oxidase during spermatogenesis in the testis of rat and hamster

An immunohistochemical study

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Summary. Sulfhydryl oxidase (SOx), an enzyme that catalyzes the oxidation of sulfhydryl compounds, appears in the spermatogenic cells of rat and hamster testes in a stage-dependent manner. It first appears in pachytene spermatocytes at stage I in both the animal species studied. SOx immunoreactivity is associated with mitochondria of these cells. The fate of such mitochondria is species-dependent. In rat, the immunoreactive mitochondria aggregate during maturation phase and are retained in the residual bodies. Spermatozoa free of SOx are released into the lumen. On the other hand, in hamster, the immunoreactive mitochondria arrange themselves around the midpiece of spermatozoa. In such a case, residual bodies lack SOx. The appearance of SOx coincides with the appearance of LDH-X in the spermatogenic cells. Like many other proteins such as LDH-X, RSA-1 and cytochrome c_t, SOx provides yet another example of differential gene activation associated with a developmental process of gametes.

Introduction

Sulfhydryl oxidase (SOx) or thiol oxidase catalyzes the oxidation of sulfhydryl compounds such as glutathione, cysteine, mercaptoethanol, dithiothreitol, thioglycerol etc., and utilizes molecular oxygen as an electron acceptor. The presence of this enzyme in the hamster epididymal fluid was first reported by Chang and Morton (1975). According to these authors, this enzyme is absent in the hamster epididymal sperms and is different from the thiol oxidase present in the bovine milk, in *Piricularia oryzae* and in *Myrothecium verrucaria*. Later, it was observed that large quantities of SOx are present in the seminal vesicle secretions of rat and hamster and lowest amount in the testis (Chang and Zirkin 1978).

Sulfhydryl oxidase has been purified from the seminal vesicle secretion of sexually active rats and well characterized (Ostrowski et al. 1979b; Ostrowski and Kistler 1980; Seitz et al. 1988). The functional significance of this enzyme is not yet known, though its role in 1) regulating disulfide bonding on spermatozoa surface during epididymal maturation and storage; 2) protecting spermatozoa against deleterious effects of low molecular weight thiols has been suggested (Chang and Morton 1975; Chang and Zirkin 1978). Ostrowski et al. (1979a) suggested that thiol oxidase may serve to generate disulfide bonds in one or more of the major seminal vesicle secretory proteins but they have no evidence for such an assumption. Wagner and Kistler (1987) reported the ability of SOx from seminal vesicles to link monomeric seminal vesicle secretory proteins namely SVS I, II and III to form homo- and hetero-oligomers in the presence of oxygen.

Using polyclonal antibodies raised against a purified preparation of SOx, we observed that SOx is specifically associated with the germ cells in the testis of rat and hamster (*Phodopus sungorus*) and that it appears in a stage-dependent manner. In this paper, we report the expression of this enzyme in the spermatogenic cells of rat and hamster testes using an immunohistochemical approach.

Materials and methods

A. Antigen isolation and antibody preparation. SOx was purified according to a method described elsewhere (Seitz and Aumüller 1989). A homogeneous preparation of the enzyme (molecular weight = 65 kDa based on SDS-PAGE; pI = 8.65) was used as an antigen for raising antibodies in rabbits. Antiserum was checked by immunoblotting. Briefly, the method was as follows: Testis were homogenized in *Tris*/HCl buffer (50 m*M*, pH 8.0) containing 150 m*M* NaCl and 5 mM EDTA and centrifuged at 100,000 *g* for 60 min. Supernatant was diluted with sample buffer and processed for SDS-PAGE. A purified preparation of SOx was directly suspended in sample buffer. Sample from liver mitochondria and pachytene spermatocytes was prepared according to methods described later (see: section C, D). Proteins were separated on SDS-PAGE according to the method of Laemmli (1970) and then transferred electrophoretically onto nitrocellulose according to the

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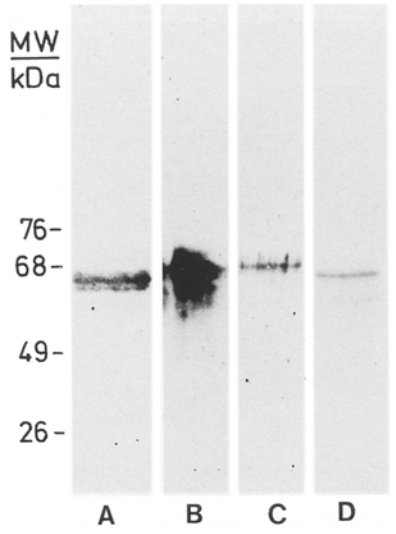

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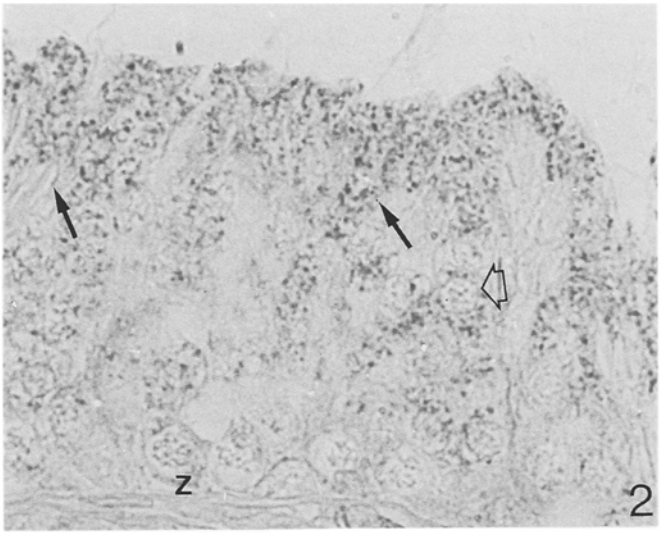

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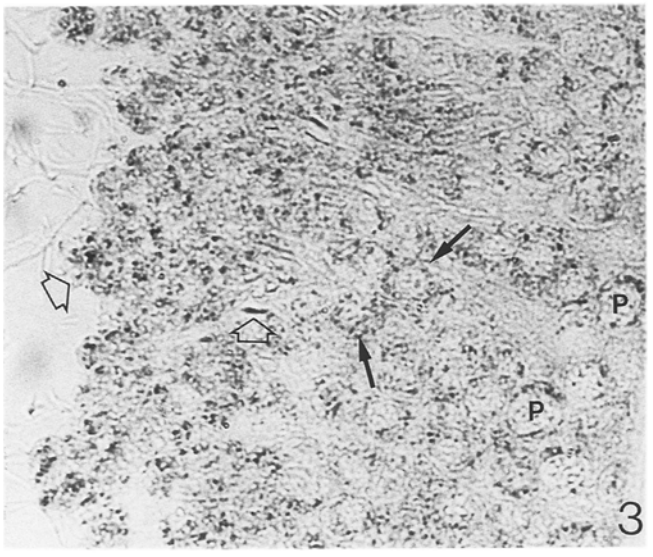

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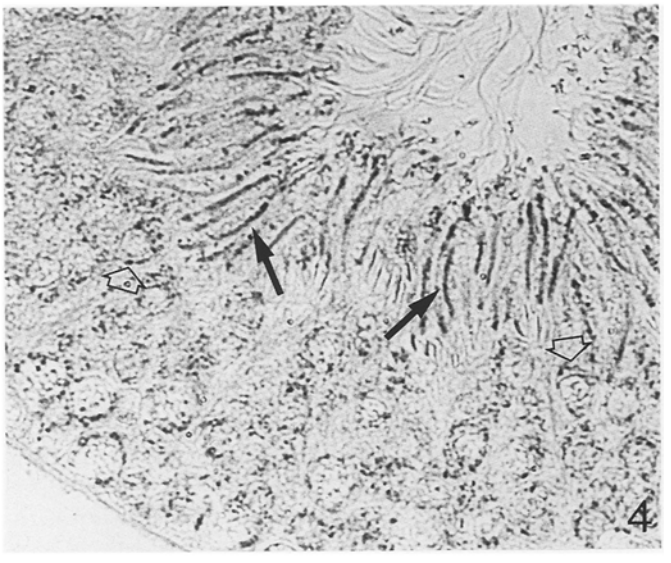

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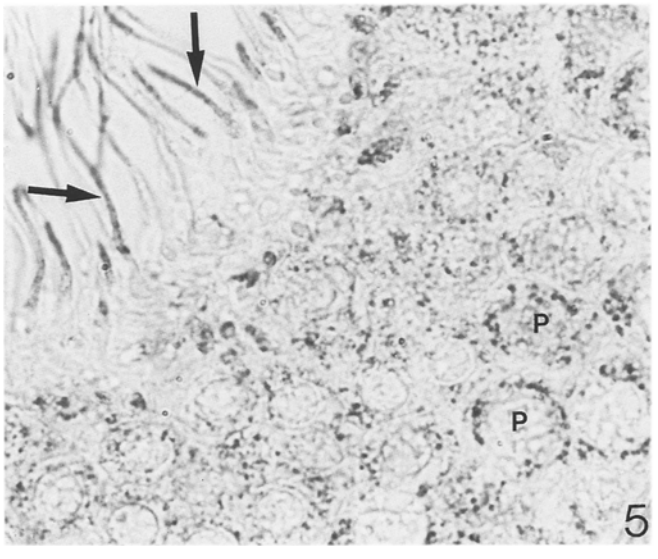

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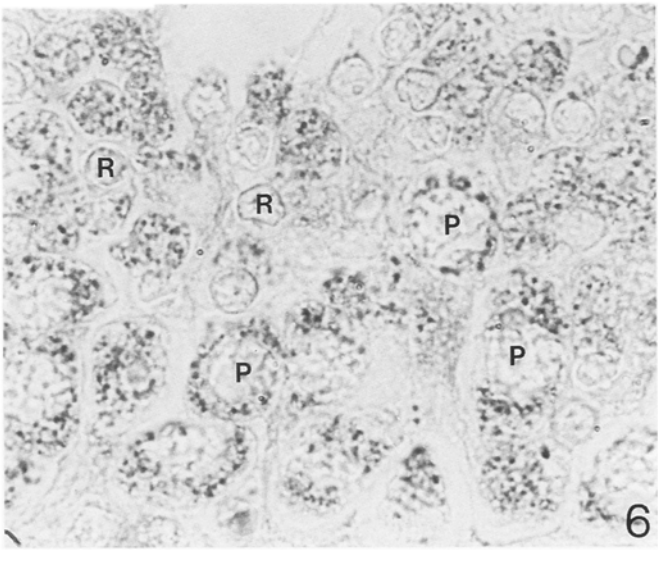

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method of Towbin et al. (1979) using a Biorad transblot cell. The blotted nitrocellulose sheets were blocked by incubation in saturation buffer (10 m*M* *Tris*/HCl, pH 7.5, containing 0.9% NaCl and 5% fat free milk powder (Instant Non-Fat Dry Milk) for 1 h (three times, 20 min) at 37° C. Individual strips were incubated with the first antibody (1:100) for 1 h at room temperature and then thoroughly washed (two times, 10 min) at 37° C in washing buffer (50 m*M* *Tris*/HCl, pH 7.5, containing 0.25 *M* NaCl, 0.003 M EDTA and 0.05% Tween 20). Incubation with pig anti-rabbit IgG (1:100) in saturation buffer (30 min, at room temperature) was followed by washing with washing buffer (2 times, 10 min) at 37° C. Strips were then incubated with rabbit anti-horseradish peroxidase (1:200) for 5 min at room temperature. After washing the strips in washing buffer (2 times, 10 min at 37° C), peroxidase activity was demonstrated with 3,3′-diaminobenzidine (35 mg/100 ml PBS, pH 7.0; 50 µl of H_2O_2 added just before use). The reaction was stopped by rinsing the strips with water.

B. Animals and immunocytochemistry. Testes from normal, sexually active adult rats ($n=15$) as well as hamsters ($n=15$) were fixed in Bouin's fixative, dehydrated through grades of alcohol, cleared in xylene, infiltrated and embedded in paraffin. Sections were cut at 5 µm and processed for the visualization of SOx according to the peroxidase-antiperoxidase complex (PAP) method of Sternberger et al. (1970).

Briefly, the method was as follows:-deparaffinized sections were treated with 3% H_2O_2 for 15 min, washed with phosphate buffered saline (PBS) (0.1 *M*, pH 7.3) and incubated with 10% bovine serum albumin for 30 min. Washed sections were first incubated with primary antibody (anti-SOx) (dilution = 1:300) for 1 h and then with pig anit-rabbit IgG (Dakopatts, Copenhagen, Denmark) for 30 min. Sections were thoroughly washed with PBS after each incubation. Antibody binding was visualized by incubating the sections with peroxidase-antiperoxidase complex (Dakopatts, Copenhagen, Denmark) for 5–15 min and with 3,3′-diaminobenzidine (DAB) in the presence of 3% hydrogen peroxide (Fluka, FRG). Dilution of commercial antibodies used was in the ratio of 1:200. Control sections were either incubated with PBS or normal rabbit serum instead of primary antibody. All the incubations were carried out at room temperature in a humid atmosphere. Sections were counterstained with PAS-hemalum and examined under Zeiss AXIOMAT. The stages of cycle of seminiferous epithelium in rat were identified according to the classification system of Leblond and Clermont (1952) and in hamster according to Frieling (1979).

C. Isolation of liver mitochondria. Mitochondria from adult rat liver were isolated according to a method described by Schnaitman and Greenawalt (1968) and were lysed in sample buffer for SDS-PAGE.

D. Isolation of pachytene spermatocytes. Pachytene spermatocytes (70% pure) were separated from 38-day-old rats by velocity sedimentation at unit gravity in albumin gradient according to a modified method of Lam et al. (1970). Briefly, a germ cell suspension was obtained by treating mechanically isolated seminiferous tubules with collagenase (0.5 mg/ml) in Eagle's minimal essential medium (MEM). A germ cell suspension containing 300–400 million cells were then allowed to settle in an albumin gradient (1–3% in MEM; total volume = 1 l) (size of the chamber = 16 cm diameter, 20 cm length) for three and half hours at room temperature. After elution, fractions (each 50 ml) were centrifuged to pellet down the cells. Cells were washed three times with MEM to get rid of albumin and were lysed directly in sample buffer for SDS-PAGE.

Fig. 1. Western blots of SOx (*A*: purified preparation of SOx from seminal vesicles; *B*: testis; *C*: liver mitochondria; *D*: pachytene spermatocytes) are shown following protein separation by SDS-PAGE. After transfer to nitrocellulose, protein bands were probed with antiserum for SOx. Immunostaining for SOx from seminal vesicle shows two bands at $M_r = 65000$ and 64000. Band at $M_r = 64000$ is a degradation product of band at $M_r = 65000$. Single band at $M_r = 65000$ in extracts of testis and liver mitochondria corresponds to SOx band in Lane A while in pachytene spermatocytes, SOx band is at $M_r = 64000$

Figs. 2 to 12. Salient spermatogenic stages in hamster are shown in Figs. 2 to 6 and in rat from Figs. 7 to 12. Paraffin sections were labeled for SOx by PAP method. For more details, see the text

Fig. 2. Hamster (stage VIII): SOx is uniformly distributed in secondary spermatocytes (*empty arrow*) and spermatids at step 8 (*solid arrows*). Zytogene spermatocytes (*Z*) lack SOx immunoreactivity. ×136

Fig. 3. Hamster (stage I): Pachytene spermatocytes (*P*) display minimum level of immunoreaction for SOx. In round spermatids (*solid arrows*), SOx is localized around the nucleus but more or less uniformly distributed in spermatids at step 9 (*empty arrows*) ×136

Fig. 4. Hamster (stage II): Immunoreactive material is seen to be arranged in a specific manner in spermatids at step 10 (*solid arrows*). Staining pattern of round spermatids (*empty arrows*) is same as in stage I. ×136

Fig. 5. Hamster (stage III): Unlike rat, SOx is localized in the mid piece of spermatozoa (*solid arrows*) in hamster. Pachytene spermatocytes (*P*) at this stage display maximum immunoreaction. ×214

Fig. 6. Hamster (stage IV): Pachytene spermatocytes (*P*) and spermatids (*R*) display a strong immunoreaction for SOx. SOx immunoreactivity is homogeneously distributed in these cell types. ×214

Results

Antibody specificity

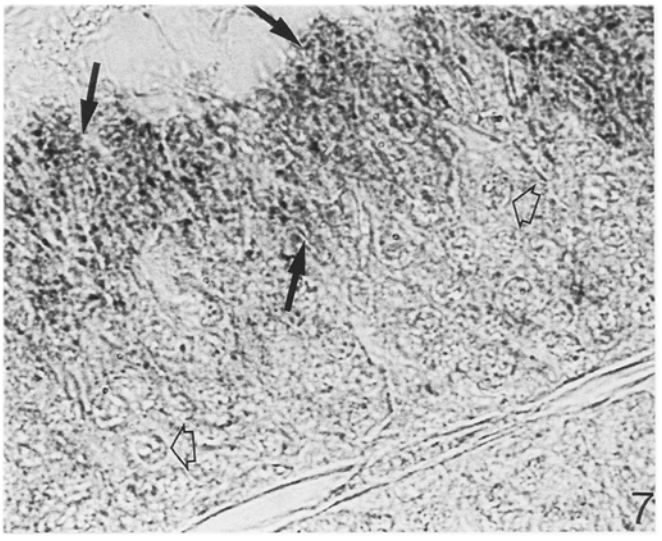
Immunoblot analysis of our antibody using SDS-gel electrophoresis followed by Western blotting, resulted in an immunoreaction of the antibody with two bands at $M_r = 65000$ and $M_r = 64000$ when purified preparation of SOx is used (Fig. 1A). Low molecular weight band ($M_r = 64000$) is a degradation product of a bigger molecule ($M_r = 65000$). A clear cut reaction was observed with a protein in testis and liver mitochondria ($M_r = 65000$) and in isolated pachytene spermatocytes ($M_r = 64000$).

Immunohistochemistry of sulfhydryl oxidase

Sulfhydryl oxidase immunoreactivity is associated with germ cells in a specific manner in the testis of both the species studied. The pattern of SOx distribution in these cells seems to be stage-dependent.

In *hamster*, SOx first appears in pachytene spermatocytes at stage I and continues to be present in spermatids. Immunoreactive SOx is uniformly distributed in spermatids from steps 1 to 8 (Figs. 2–6). During maturation phase, it is seen to be associated with the middle piece of the spermatozoa (Figs. 4 and 5). During spermiation, spermatozoa loaded with SOx immunoreactivity are released into lumen while residual bodies devoid of SOx are retained (Fig. 6).

In *rat*, the initial pattern of labeling is same as in the case of hamster (Figs. 7, 8, 9, 12). Immunoreactive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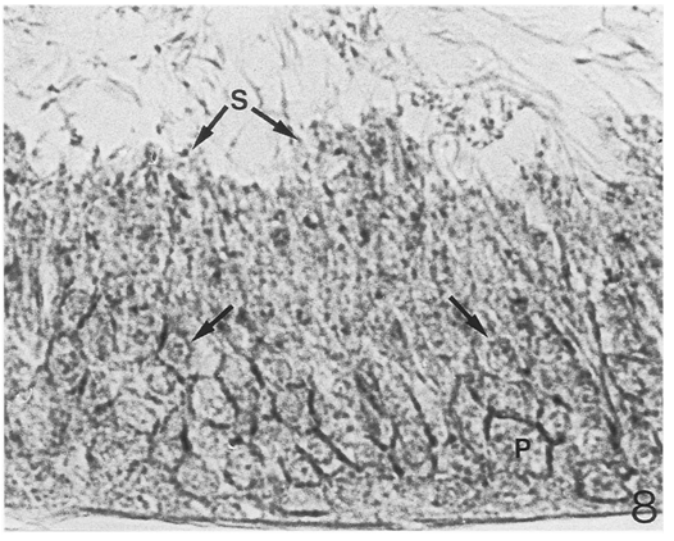
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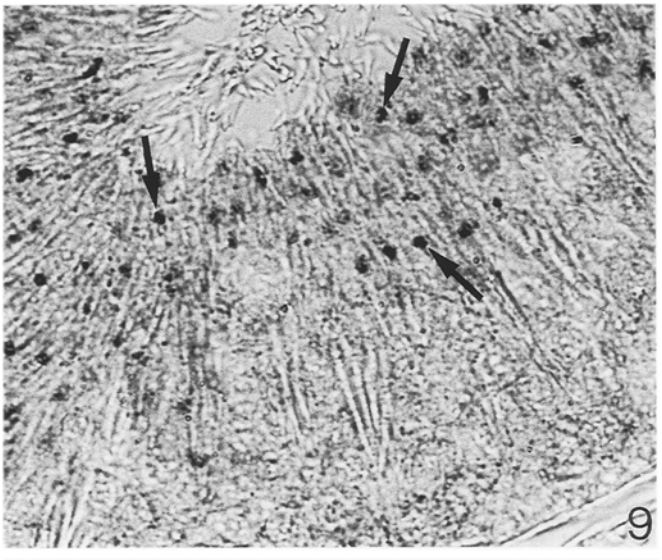
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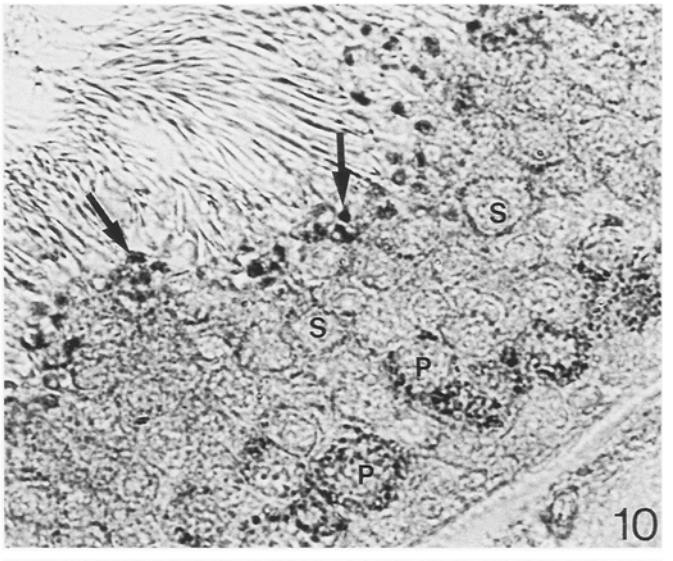
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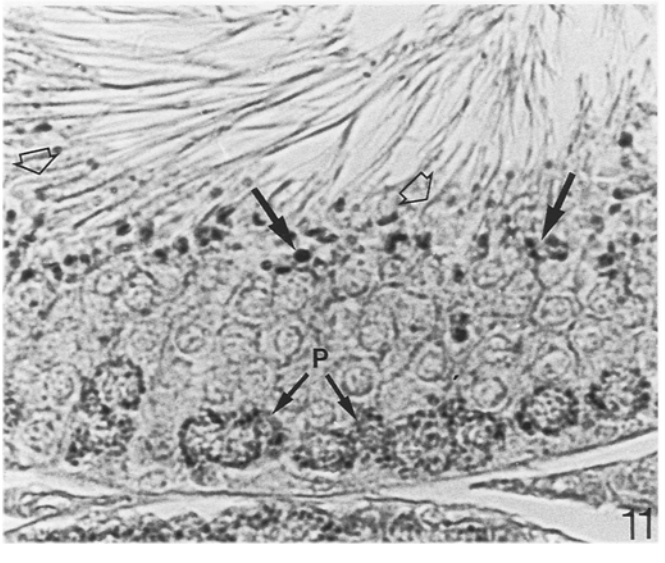
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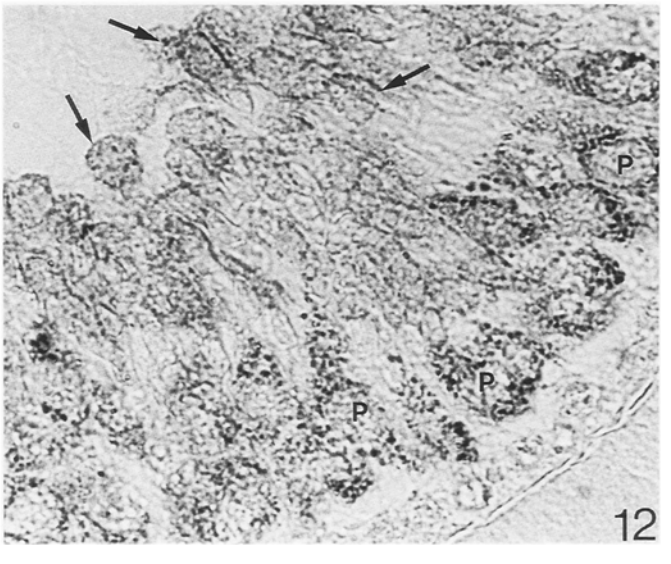
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SOx is present along the plasma membrane in round spermatids (Figs. 8–11) and is more or less uniformly distributed in spermatids in acrosome phase (Figs. 7 and 12). During early part of maturation phase, SOx starts aggregating and later gets concentrated into a small ball-like structure (Figs. 10 and 11). During spermiation, SOx immunoreactivity is seen to be retained in the residual bodies and spermatozoa free of SOx are released into the lumen (Figs. 11 and 12).

The most intense immunoreaction is observed in pachytene spermatocytes at stages VIII–IX in rat (Figs. 11 and 12) and at stages III–IV in hamster (Figs. 5 and 6). The overall distribution pattern of SOx in the testes of rat and hamster is shown in Figs. 13 and 14 respectively.

The Sertoli cells and peritubular cells are completely devoid of SOx immunoreactivity, whereas Leydig cells and vascular smooth muscle cells are positive though the intensity of immunoreaction is far less than that of germ cells. Control sections incubated with either PBS or normal rabbit serum showed no reaction product.

Discussion

In a survey of distribution of SOx activity in the male reproductive tract of rat and hamster, Chang and Zirkin (1978) observed that rat as well as hamster testis contained 800 and 100 times respectively less activity than that of the seminal vesicles.

Using an immunohistochemical approach, we observed that germ cells from pachytene spermatocytes stage onwards have this enzyme. Initially, the SOx immunoreactivity is low in pachytene spermatocytes at stage I, but slowly increases and is found to be maximum in pachytene spermatocytes at stages VIII–IX in rat (Figs. 7 and 8) and at stages III–IV in hamster (Figs. 12 and 13) and remains same upto diplotene/diakinesis stage. Thereafter, it seems to be less in spermatids though uniformly distributed in the cells immediately below the plasma membrane (Figs. 4, 5, 10, 11).

The appearance of immunoreactive SOx in germ cells seems to be directly related to the appearance of "condensed" mitochondria in germ cells (De Martino et al. 1979). The earliest sign of formation of "condensed" mitochondria are observed in early spermatocytes, but only few mitochondria are involved in this process. In zygotene and early pachytene spermatocytes, this biological process is seen in numerous mitochondria. The observed 1) increase in SOx in meitoic cells, 2) cytoplasmic distribution pattern in spermatocytes and spermatids (steps 1 to 8), 3) aggregation of SOx immunoreactivity into a small ball-like structure in maturing spermatids (steps 15 to 18) and , 4) retention of SOx in the residual bodies are in agreement with the observation of De Martino et al. (1979) on the fate of "condensed" mitochondria in spermatocytes and spermatids in rat. It is interesting to note here that SOx has been shown to be present in the mitchondrial matrix in the glandular cells of rat seminal vesicles (Seitz et al. 1988).

The distribution pattern of SOx in rat testis is strikingly similar to that of LDH-X which has also been shown to be located within the mitochondrial matrix (Blanco et al. 1975). Machado de Domenech et al. (1972) reported its association with a special type of mitochondria. Using a histochemical approach, Hintz and Goldberg (1977) reported the presence of LDH-X only at mid pachytene stage onwards. With more refined and better techniques, Li et al. (1989) were able to detect LDH-X synthesis at an earlier stage (preleptotene spermatocytes) though at a much lower level. LDH-X activity like SOx increases substantially at mid pachytene spermatocyte stage. LDH-X is suggested to mediate the transfer of reducing equivalents between the cytoplasm and mitochondria via a 2-oxoacid-2 hydroxyacid redox couple (Goldberg 1972). SOx may also be involved in that shuttle mechanism by regulating the number of sulfhydryl groups and disulfide bonds.

Cell surface markers such as galactosyltransferase and rabbit sperm autoantigen-1 (RSA-1) are other examples whose distribution pattern is similar to SOx (O'Rand et al. 1984; Scully et al. 1987).

The fate of SOx in maturing spermatids seems to be species dependent. For instance, in rat, immunoreactive material is thrown out of the sperm cell so that sperms free of SOx are released into the lumen (Fig. 7). On the other hand, in hamster, the SOx immunoreactivity is seen to be incorporated into the sperm and is localized in the mid piece (Fig. 12). This observation in hamster is contrary to the report of Chang and Morton (1975) who observed that sperms from cauda epididymidis are devoid of SOx activity. This difference may be due to: 1) shedding of SOx by sperms during their pas-

Fig. 7. Rat (stage XIV): SOx is uniformly distributed in spermatids at step 14 (*solid arrows*) as well as in dividing secondary spermatocytes (*empty arrows*). ×136

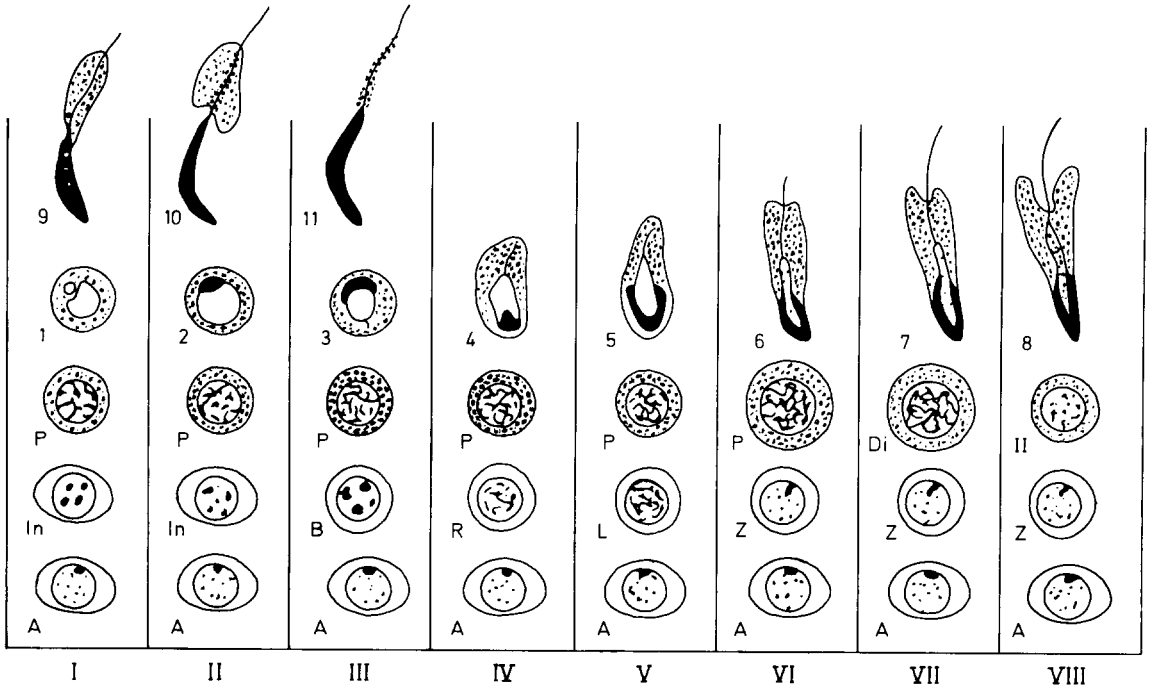
Fig. 8. Rat (stage I): Pachytene spermatocytes (*P*) at this stage display very little immunoreaction. Compare with pachytene spermatocytes at stages VII/IX (Figs. 11, 12). In round spermatids (*solid arrows*), SOx is present very close to the plasma membrane. SOx in spermatids at step 15 (*S*) show signs of aggregation. ×1162

Fig. 9. Rat (stage II): Aggregation of SOx (*arrows*) in spermatids at step 16 is almost complete. The pattern of distribution of SOx in pachytene spermatocytes and round spermatids is same as in Fig. 8. ×136

Fig. 10. Rat (stage VII): Aggregated SOx condenses in spermatids from steps 16 to 18 and display a very strong immunoreaction (*solid arrows*). Note the increase in SOx immunoreactivity in pachytene spermatocytes (*P*). Compare with stages I and II (Figs. 8, 9). Distribution of SOx in round spermatids (*S*) remains the same as in stage I. ×136

Fig. 11. Rat (stage VIII): Condensed immunoreactive material (*solid arrow*) is retained in the residual bodies and sperms (*empty arrows*) free of SOx are released into the lumen. Pachytene spermatocytes (*P*) display strong immunoreaction. ×162

Fig. 12. Rat (stage IX): Homogeneous distribution of SOx is seen in spermatids (*solid arrows*) at this stage. Pachytene spermatocytes (*P*) continue to display strong immunoreaction. ×136



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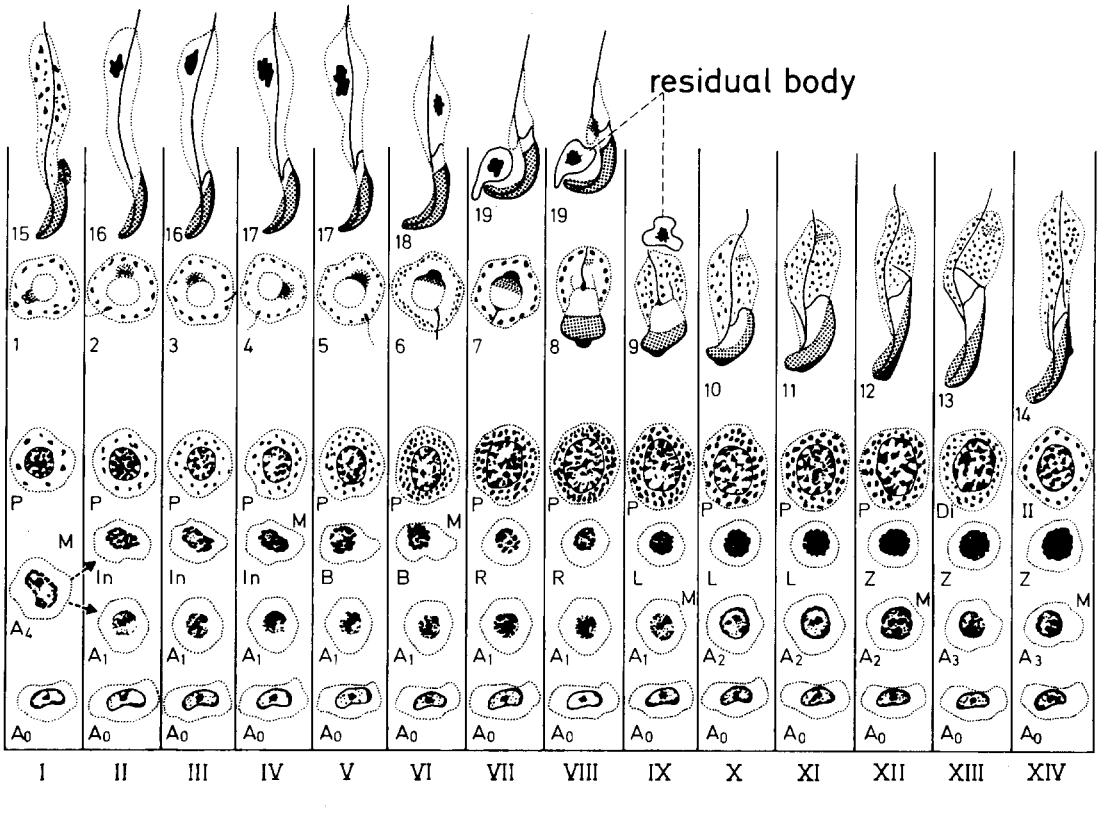

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Figs. 13 and 14. Overall distribution pattern of SOx in germ cells during various stages of cycle of seminiferous epithelium. **Fig. 13** Hamster; **Fig. 14** Rat

sage to cauda epididiymidis, and/or, 2) SOx localized immunologically is functionally an inactive enzyme. The first possibility is ruled out by the observation of Seitz et al. (1988) who reported the presence of SOx within the mitochondria of seminal vesicle epithelial cells.

The second possibility that SOx becomes functionally inactive by the time they reach cauda epididymidis, seems plausible. Majority of sulfhydryl groups on caput sperms are oxidized to disulfide bonds as spermatozoa migrate through epididymis (Calvin and Bedford 1971).

The important role played by SOx in the formation of disulfide bonds on sperm surface is further evident by the observation that disulfide bonding within nucleus and tail structures can occur independently of epididymal environment (Haugaard 1968). Haugaard (1968) presumed a spontaneous oxidation of protein thiol groups to disulfide bonds in in vitro studies. We are reporting for the first time the presence of SOx in testicular sperms in hamster. Probably this enzyme was involved in the spontaneous oxidation of protein thiol groups as observed by Haugaard (1968).

Our observations suggest that SOx like LDH-X, RSA-1 and cytochrome c_t is another example of differential gene activation associated with a developmental process in gametes (O'Rand et al. 1984; Scully et al. 1987; Wheat et al. 1977). The significance of appearance of SOx during early development of sperm is not yet known. It has been reported that mitochondrial precursor protein(s) unfold to a protease-sensitive conformation at the surface of mitochondria before being translocated into the mitochondria (Eilers et al. 1988). We suggest that mitochondrial SOx may help these translocated proteins to stabilize their native tertiary structure. Further experiments are underway to reveal the functional aspects of this enzyme.

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