Biochemical Pharmacology, Vol. 38, No. 23, pp. 4231–4236, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

SULFATION IN MALE REPRODUCTIVE ORGANS

BULL AND BOAR TESTIS PHENOL SULFOTRANSFERASES

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(*Received* 16 *March* 1989; *accepted* 23 *June* 1989)

Abstract—Phenol sulfotransferases (PST) from bull and boar testis were partially purified and characterized. A single form of PST adsorbed on DEAE–cellulose was found in the bull testis, whereas from boar testis two different peaks of PST activity were separated. The bull testis PST and both boar testis enzymes were active with *p*-nitrophenol and adrenalin. They all showed higher affinity to pNP than to adrenalin and were inhibited by these substrates at higher concentrations. Their optimal pH was at 8.5. Bull testis PST and boar PST II which were adsorbed on DEAE–cellulose were thermostable, whereas boar PST I was thermolabile. Those three PST forms differed in sensitivity to 2,6-dichloro-4-nitrophenol (DCNP), N-ethyl maleimide (NEM), iodoacetamide (IAA) and phenylglyoxal (PG). Bull and boar PST II were more rapidly inactivated in the presence of DCNP than boar PST I. In the presence of NEM, the —SH groups reagent, the bull phenol sulfotransferase and boar PST I lost their activity, whereas the activity of boar PST increased. Also iodoacetamide, another —SH group modificator, raised boar PST II activity and decreased boar PST I activity. DTT, which protects thiol groups, had an opposite effect on the enzymes studied than NEM. Phenylglyoxal, a reagent specific for arginine residues inhibited bull testis PST and both boar phenol sulfotransferases. Substrate protection experiments were also performed to determine the localization of reactive groups in bull and boar testis phenol sulfotransferases.

Phenol sulfotransferases (PST, EC 2.8.2.1) are a group of enzymes which play an important role in biotransformation of various biologically active exo- and endogenous compounds [1–3]. On the basis of substrate specificity and thermal stability phenol sulfotransferases have been divided into two groups: thermostable (TS) which are active with exogenous phenols, and thermolabile (TL) which are active with catecholamines [4, 5]. This classification is not very precise since the thermolabile forms catalyse also the sulfation of millimolar concentrations of phenols [4], and the thermostable form, e.g. of human liver, can use dopamine (at high concentrations) as a substrate [6].

In a previous works the properties of bovine lung and small intestine phenol sulfotransferase were studied [7, 8]. Two thermostable forms of PST, both active only with exogenous phenols, were found in bovine lung and only one thermostable form in bovine small intestine. In human lung [9] as well as in human ileum [10] there are two different forms of PST, thermostable and thermolabile. The differences observed between bovine and human phenol sulfotransferases led us to extend our studies to the enzyme present in bovine tissues.

Since phenol sulfotransferase is one of the most important enzymes in detoxification and until now no attempt has been made at checking the relationship of this enzyme's properties with spermatozoa fertility, we started a cycle of studies on sulfation in male reproductive system.

In this first work, we purified and characterized PSTs from bull and boar testis and isolated these enzymes from different parts of epididymides.

Studies on the boar testis PST seemed to be all the more interesting as it is believed that sulfation does not play an important role in pigs [11].

MATERIALS AND METHODS

Reagents. [^{35}S]PAPS (sp.act. 1.1–2.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and stored at −20°; DEAE–cellulose DE-11 was from Whatman Biochemicals (Maidstone, U.K.); acrylamide and N.N.N′.N′-tetramethylendiamide were from Fluka (Buchs, Switzerland), 2-naphthol was a product of Merck (Darmstadt, F.R.G.); *p*-nitrophenol (pNP), adrenalin, dopamine, phenylglyoxsal (PG), *N*-ethylmaleimide (NEM), dithiotreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma Chemical Co. (Poole, U.K.) 2,6-dichloro-4-nitrophenol (DCNP) was from Aldrich Chemical Co. (Gillingham, U.K.)

Tissues. Bull and boar testes and epididymides were obtained from a local slaughterhouse immediately after killing of the animals.

PST assay. PST activity was measured using [^{35}S] 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the sulfate donor. The assay mixture consisted of 50 mM glycine buffer, pH 8.5, variable amounts of phenolic sulfate acceptor, 0.45 μCi [^{35}S]PAPS (0.29 μM), and the enzyme preparation in a total volume of 1 ml. The reaction mixtures were incubated for 30 min at 37°. Blanks were assayed as described above, except that the phenolic substrate was omitted. The amount of ^{35}S-labeled products

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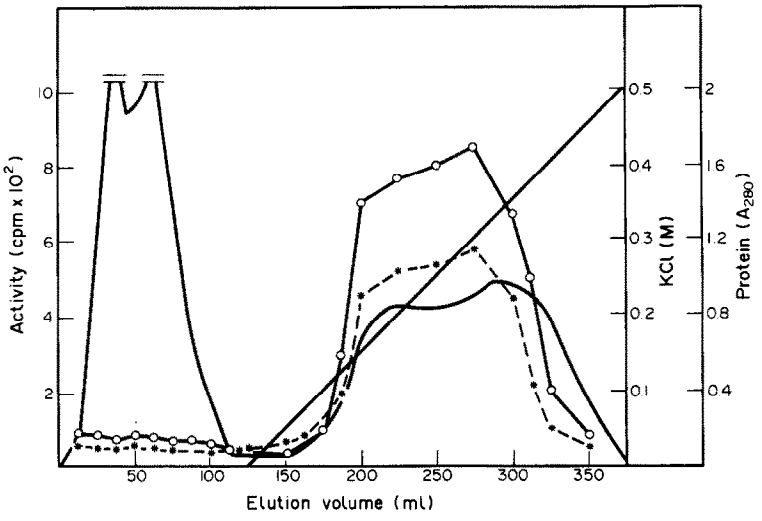

Fig. 1. DEAE–cellulose chromatography of bull testis PST. The enzyme obtained after ammonium sulfate precipitation (about 200 mg protein) was applied to a DEAE–cellulose column (20 × 1 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.4/3 mM 2-mercaptoethanol/0.25 M sucrose, and eluted with a KCl linear concentration gradient. Fractions of 5 ml were collected at a flow rate of 1–1.5 ml/min. PST activity was assayed using 50 μM *p*-nitrophenol (○), or 100 μM adrenalin (*) as substrate. Protein content (—).

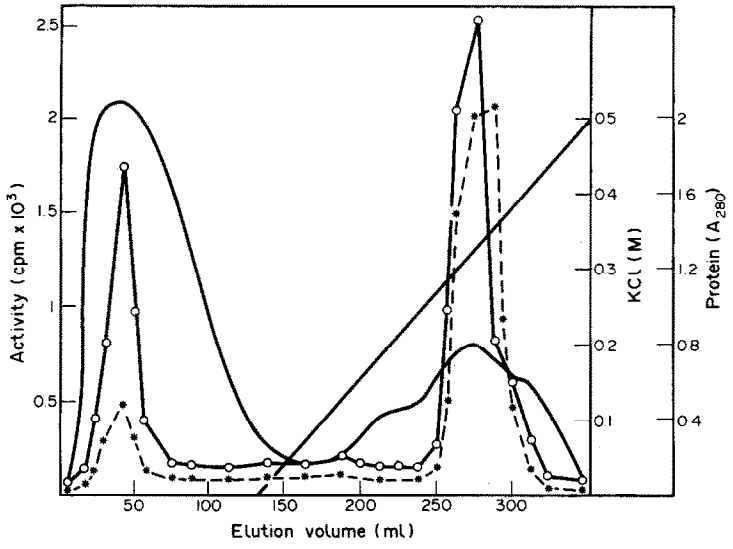

Fig. 2. DEAE-cellulose chromatography of boar testis PST. The enzyme obtained after ammonium sulfate precipitation (about 200 mg protein) was applied to a DEAE–cellulose column equilibrated as described for Fig. 1. PST activity was assayed using 50 μM *p*-nitrophenol (○), or 100 μM adrenalin (*) as substrate. Protein content (—).

was determined by the method of Foldes and Meek [12] as described previously [9].

Protein determination. Protein was determined according to Lowry *et al.* [13] or spectrophotometrically by the method of Warburg and Christian [14], with crystalline bovine serum albumin as a standard.

Thermal stability study. The enzymes after the DEAE–cellulose chromatography step were incubated in a shaker bath at various temperatures for 15 min. Then the samples were cooled to 4° and assayed for activity after adding buffer and substrates. Blanks without an accepting substrate were determined in parallel at each temperature tested.

Studies with PG, NEM, IAA and DTT. Samples containing enzyme, 50 mM glycine buffer pH 8.5, and the compound studied at variable concentrations in a total volume of 0.9 ml, were preincubated at 37° for 10 min, then cooled to 4° and assayed for sulfotransferase activity. In the protection experiments pNP or/and PAPS were included during the preincubation period. Blanks without an acceptor substrate were determined for each concentration of the compound studied. In the control samples the compound studied was omitted.

Table 1. Purification of bull and boar testis PST

Step	Procedure	Bull PST Sp.act. (pmol/mg/min)	Bull PST Purification factor	Bull PST Yield (%)	Boar PST Sp.act. (pmol/mg/min)	Boar PST Purification factor	Boar PST Yield (%)
1.	9000 *g* supernatant	0.47	—	100	0.27	—	100
2.	100,000 *g* supernatant	0.75	1.6	95	0.41	1.5	96
3.	$(NH_4)_2SO_4$ fractionation	1.79	3.8	76	1.27	4.7	70
4.	DEAE–cellulose chromatography						
	Peak I	—	—	—	4.75	17.6	14
	Peak II	9.3	19.8	59	8.64	32.0	32

PST activity was assayed with 50 μM pNP as an acceptor substrate. Approximately 20 g of bull and boar testis was used for the purification of PST.

Table 2. PST activity in different parts of bull and boar reproductive organs

Tissue	Activity (nmol/mg/min × 10^{-4}) Bull PST	Boar PST
Testis	7.6 ± 0.45	4.0 ± 0.21
Epididymis		
Head	4.9 ± 0.21	2.25 ± 0.32
Corpus	7.1 ± 0.39	2.10 ± 0.07
Tail	0.5 ± 0.19	0.51 ± 0.23

The activity was measured using 50 μM pNP as an acceptor substance. Each value is the mean ± SE (N = 6).

RESULTS

PST preparation

The tissues were homogenized and extracted in 3 vol. of cold 10 mM sodium phosphate buffer, pH 7.4, containing 3 mM 2-mercaptoethanol and 0.25 M sucrose. The homogenates were centrifuged for 15 min at 9000 *g*. The pellets were discarded and supernatants were centrifuged at 100,000 *g* for 60 min, yielding the cytosolic fractions. The salt fractionation procedure at 55–75% and 35–55% ammonium sulfate concentration for the bull and boar testis PST, respectively, led to an increase in the enzyme specific activity. PST from bull testis was adsorbed on the DEAE–cellulose column (20 × 1 cm) and eluted with a linear gradient of KCl (0–0.5 M) as a single peak of activity (Fig. 1). Boar testis PST was divided on the DEAE–cellulose column into two peaks (Fig. 2), one was eluted with 10 mM sodium phosphate buffer pH 7.4 (PST I), and the other with a linear gradient of KCl (PST II). Activity was assayed with pNP and adrenalin (Figs. 1 and 2). The enzymes after DEAE–cellulose chromatography were used for all studies. The results of purification are summarized in Table 1.

PST activity

Activity of the bull testis PST assayed in cytosol in the presence of 50 μM pNP as an acceptor substrate is about twice as high as the activity of the boar testis enzyme (Table 2). Activity of the bull epididymis PST differs in various parts of the organ: it is the highest in the corpus and the lowest in the tail. The tail part of the boar epididymis also shows the lowest PST activity (Table 2).

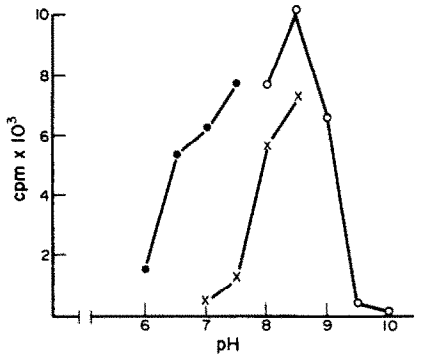

Fig. 3. Effect of pH on the activity of bull testis PST. Enzyme activity was determined in the presence of 10 mM sodium phosphate buffer (●), 50 mM TES (×) or 50 mM glycine buffer (○) using 50 μM pNP as substrate. Each value represents the mean of three determinations.

Optimal pH

The effect of pH on the bull and boar testes PST was studied in the presence of 50 μM pNP as an acceptor substrate. Three different buffer systems used were: 10 mM sodium phosphate, 50 mM TES and 50 mM glycine buffer, from pH 6 to 10. For all the enzymes studied optimal pH was 8.5 (Fig. 3).

Substrate specificity and affinity

Bull testis PST and the two boar testis phenol sulfotransferases (PST I, PST II) are active with exogenous phenols (pNP, phenol, 2-naphthol) and catecholamines (adrenaline, dopamine). Bull enzyme as well as the two boar sulfotransferases show higher affinity towards pNP than towards adrenaline. K_m values, calculated by the method of Eisenthal and Cornish-Bowden [15], for the studied enzymes assayed with pNP as a variable substrate were as follows: bull testis PST, 1.67 ± 0.14 μM; boar testis PST I, 11.8 ± 0.7 μM; PST II, 4.42 ± 0.58 μM; and with adrenalin: 50.0 ± 1.8 μM for bull testis PST; 500 ± 8.1 μM and 125 ± 12.5 μM for boar testis PST I and PST II, respectively. The Lineweaver–Burk double reciprocal plots in all cases did not give biphasic curves. A marked substrate inhibition was observed at pNP concentrations exceeding 300 μM.

Thermal stability

Bull testis phenol sulfotransferase as well as boar

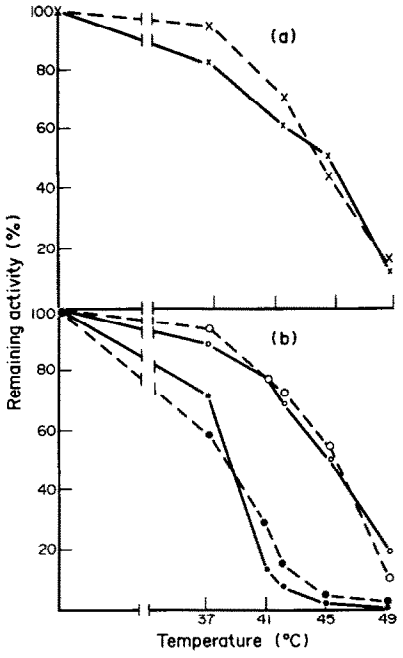

Fig. 4. Thermalstability of bull (A) and boar (B) testis phenol sulfotransferase. The activity was measured after 15 min preincubation at the indicated temperature with 50 μM pNP (×) or 200 μM adrenalin (×) as substrate for the bull testis enzyme; 100 μM pNP (●) or 500 μM adrenalin (●) as substrate for the boar testis PST II, and 50 μM pNP (○) or 250 μM adrenalin (○) as substrate for the boar testis PST II. Each point represents the mean of three determinations.

testis PST II show very high thermal stability, whereas boar testis PST I is thermolabile (Fig. 4). After 15 min preincubation at 45° the thermostable enzymes retained about 50% of their initial activity when assayed with pNP or adrenalin, whereas boar testis PST I lost about 90% of its initial activity after preincubation at 42° (Fig. 4B). No differences in thermal stability were observed with pNP or adrenalin as the acceptor substrates for all the enzymes studied (Fig. 4A and B).

2,6-*Dichloro-4-nitrophenol (DCNP) inhibition*

The thermostable form of boar testis phenol sulfotransferase, PST II, is more sensitive to the inhibitory effect of DCNP, a selective inhibitor of sulfation [16] than is the thermolabile PST I. After 10 min preincubation at 37° with 50 μM DCNP, PST II lost 60% of its initial activity, whereas PST I only about 20% (Fig. 5). The thermostable bull testis PST preincubated under the same conditions lost about 90% of its initial activity (Fig. 5).

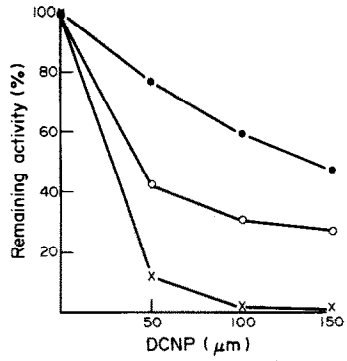

Fig. 5. Effect of 2,6-dichloro-4-nitrophenol (DCNP) on the bull and boar testis PSTs. The enzymes were preincubated for 10 min at 37° in the absence or presence of different concentrations of DCNP as described under Materials and Methods. The activity of the bull testis enzyme (×) was measured with 50 μM pNP as substrate, and of boar PST I (●) and boar PST II (○) with 100 μM pNP. Each point represents the mean of three determinations.

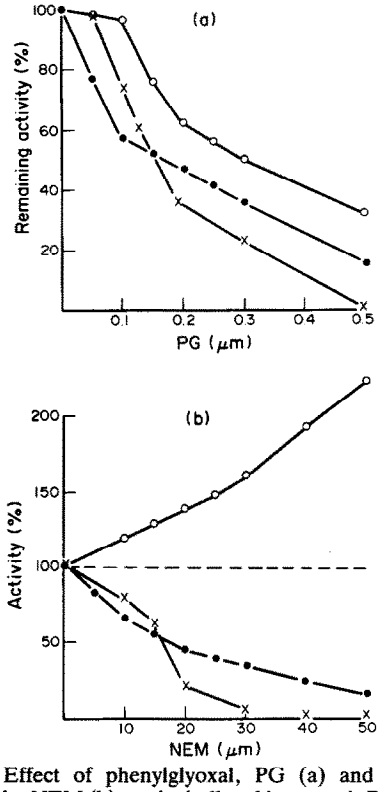

Fig. 6. Effect of phenylglyoxal, PG (a) and *N*-ethylmaleimide, NEM (b) on the bull and boar testis PSTs. The enzymes were preincubated in the absence or presence of different concentrations of the searching compounds. Conditions and measurements, as well as the symbols used, are the same as for Fig. 5.

Effect of group-specific reagents

Phenylglyoxal (PG). Phenylglyoxal, a reagent specific for arginine residues [17], inhibits all the enzymes studied (Fig. 6a).

To define the location of the arginine residues on the bull and boar testis phenol sulfotransferases, substrate protection experiments were carried out. Inclusion of the sulfate acceptor, pNP, into the preincubation mixture does not protect the enzymes from inactivation (Table 3). Inclusion of PAPS, a sulfate donor, in the preincubation mixture protects the bull testis PST and boar testis PST I, but has no effect on the boar testis PST II activity. The effect of inclusion of both substrates in the preincubation mixture has a similar effect as the inclusion of PAPS alone (Table 3).

Table 3. Substrate protection of the bull and boar testis phenol sulfotransferases from inactivation by phenylglyoxal (PG)

Substrate	Activity (%)		
	Bull PST	Boar PST I	Boar PST II
None	61.7 ± 6.8	49.5 ± 4.5	58.0 ± 2.8
pNP	67.2 ± 3.4	52.4 ± 2.6	59.8 ± 1.1
PAPS	96.5 ± 5.2	73.2 ± 3.3	58.3 ± 2.0
pNP, PAPS	96.7 ± 4.8	76.1 ± 1.8	55.3 ± 4.1

The enzymes were preincubated as described under Material and Methods in the absence or presence of 125 μM PG (bull testis PST), 200 μM PG (boar testis PST I), or 250 μM PG (boar testis PST II). Substrates at concentrations of: 50 and 100 μM pNP for the bull and boar testis enzymes, respectively, and 0.29 μM PAPS were included or not during the preincubation period.

The activity after preincubation for 10 min at 37° in the absence of NEM was taken as 100.

Each value is the mean ± SE (N = 4).

Table 4. Effect of various substrates on the activity of bull and boar testis phenol sulfotransferases in the presence of *N*-ethylmaleimide (NEM)

Substrate	Activity (%)		
	Bull PST	Boar PST I	Boar PST II
None	24.0 ± 3.2	25.5 ± 2.5	148.0 ± 6.2
pNP	65.4 ± 4.5	45.5 ± 1.8	264.0 ± 8.4
PAPS	64.5 ± 5.3	54.0 ± 2.7	101.8 ± 3.6
pNP, PAPS	98.7 ± 2.3	70.5 ± 3.1	96.5 ± 4.2

The enzymes were preincubated as described under Material and Methods in the absence or presence of 20 μM NEM (bull testis PST), 100 μM NEM (boar testis PST I), or 25 μM NEM (boar testis PST II). Conditions and activity measurements are the same as for Table 3.

N-Ethylmaleimide (NEM) and iodoacetamide (IAA). NEM, a sulfhydryl reagent, inhibits bull testis PST and boar testis PST I but activates boar testis PST II (Fig. 6b).

Preincubation of boar testis phenol sulfotransferases with iodoacetamide, another sulfhydryl reagent, enhances the PST II activity, but lowers the activity of PST I. After 10 min preincubation at 37° with 5 mM IAA, PST II activity was more than doubled, whereas PST I was inhibited and the enzyme retained less than 40% of its initial activity.

Substrate protection experiments were also carried out to define the location of sulfhydryl groups on the enzymes studied. Inclusion of pNP or PAPS in the preincubation mixture partially protects bull phenol sulfotransferase and boar PST I from NEM inactivation, and inclusion of both substrates almost totally protects the activity of those enzymes (Table 4). In the case of boar testis PST II, inclusion of pNP raises the enzyme activity, whereas inclusion of PAPS lowers the activity (Table 4). When both substrates were added to the preincubation mixture the effect was the same as with PAPS alone.

Dithiothreitol (DTT). Dithiothreitol added to the preincubation mixture containing enzyme and buffer activates strongly boar testis PST I assayed with pNP or adrenaline but inhibits boar testis PST II. After 10 min preincubation at 37° with 3 mM DTT, the activity of PST I assayed with 100 μM pNP was more than 12 times higher than the activity assayed without DTT, whereas the activity of PST II did not exceed 45%. Inclusion of PAPS in the preincubation mixture containing PST II and DTT did not increase the activity.

DISCUSSION

Two different forms of phenol sulfotransferase were separated by DEAE–cellulose chromatography from boar testis. The forms differ in their electric charge, affinity to substrates, sensitivity to inhibitors and thermal stability. PST I which is not adsorbed on the DEAE–cellulose column is thermolabile and PST II which is adsorbed is thermostable. Either form is active both with exogenous phenols and adrenalin.

Only one form of PST was isolated on the DEAE–cellulose column from bull testsis. This form is adsorbed on the cellulose and, like boar testis phenol sulfotransferases, is active both with pNP and adrenalin. The enzyme like boar PST II, bovine lung [7] and bovine small intestine [8] enzymes, is very stable during preincubation at high temperatures. Bull testis PST, as well as the thermostable boar PST II, shows higher affinity to pNP and adrenaline than the thermolabile boar PST I. The affinity of all studied enzymes to pNP is much higher than to adrenalin. No correlation between the substrate specificity and thermal stability was observed as it has been demonstrated for human tissue phenol sulfotransferases [4–6].

The testis thermostable sulfotransferases (bull and boar PST II) like the human platelet, brain, and liver [5, 6, 18, 19] enzymes, are more sensitive to DCNP, a specific inhibitor of sulfation [16], than the thermostable boar PST I. Phenylglyoxal, an arginine residue specific reagent, inhibits the activity of bull testis PST and boar testis phenol sulfotransferases. PAPS but not pNP protects the bull PST and boar PST I activity from inactivation but has no influence on boar PST II. These data suggest that the bull testis enzyme and boar PST I, like rat liver sulfotransferase [20], contain arginine residues which are essential for their catalytic activity and play the role as anion recognition sites for binding of the sulfate donor.

When the enzymes studied were preincubated with sulfhydryl group modifiers (NEM or IAA) a marked inhibition of bull testis PST and boar PST I activity was observed, whereas boar PST II activity increased. Dithiothreitol, a reducing agent used for stabilization of phenol sulfotransferases during their isolation and purification [6, 21], has an effect opposite to that of NEM. Binding of PAPS or pNP partially protects bull and boar testis PST I from inactivation by NEM, and binding of both substrates totally protects the activity of those enzymes. The data suggest that sulfhydryl groups are present both at the sulfate donor and sulfate acceptor sites of those enzymes or at least necessary for the correct configuration of the enzyme required for substrate binding. On the other hand, inclusion of pNP into the preincubation mixture caused an additional increase of boar testis PST II activity, and inclusion of PAPS abolished the activating effect of NEM. It

seems that boar testis PST II is more active when the —SH groups are in the oxidized form and that sulfhydryl groups (as disulfides) may play an important role in the proper conformation of the enzyme.

The three studied enzymes seem to be different proteins responsible for inactivation of exo- and endogenous phenols. Further studies are needed to determine their immunological properties, and regulation, and to answer the question of whether species dependent differences in sulfation are in anyway correlated with spermatozoa fertility.

Acknowledgement—This work was supported by a grant from the Polish Government, Project C.P.B.P., No. 05.06.1.1.2.

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