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GLUTATHIONE TRANSFERASE ISOENZYMES FROM HUMAN TESTIS

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Abstract—By using affinity chromatography and isoelectric focusing techniques, several forms of glutathione transferase (GSTs) were resolved from human testis obtained from patients operated on for malignant diseases. Large interindividual variations in the expression of different isoenzymes resulted in the samples investigated. Five out of six samples analysed expressed GST-4.4 that resulted in being structurally and immunologically identical to GST-π (class Pi). All the cationic GSTs of human testis, except for GST-8.36, GST-9.1 and GST-10.1, are homodimers of 24,500 M_r subunit and cross reacted with antisera raised against class Alpha GST. Some of the forms isolated (GST-3.8, GST-8.36, GST-9.1 and GST-10.1) can not apparently be related to any of GSTs so far characterized in other human tissues. Upon SDS/polyacrylamide gel electrophoresis, GST-8.36 and GST-9.1 appeared to be heterodimers of 24,500 and 26,500 M_r subunits and were found only in the testis seminoma suggesting that they might be tumour specific isoenzymes. GST-3.8 appeared to be formed by heterodimers of 23,000 and 26,500 M_r subunits whereas, GST-10.1 was found to be dimers of 22,000 and 24,500 M_r subunits. In addition, the results of immunohistochemical studies with antisera raised against both class Pi and Alpha GSTs are reported.

Glutathione transferases (GSTs; EC. 2.5.1.18) are a family of multifunctional proteins that catalyse the conjugation of a wide number of electrophilic compounds with the thiol group of glutathione [1–4]. This reaction is considered to be the first step in the formation of mercapturic acids, a pathway through which hydrophobic substances are inactivated and eliminated from the body [1]. The GSTs are present in a large number of human tissues and in most instances, they occur in multiple forms [3–6]. The major human cytosolic GSTs have been described as dimeric proteins of at least three different subunits (ranging from 23,000 to 26,500 M_r), and have been grouped into three structurally and immunologically distinct classes, which includes acidic forms (pI 4.4–4.9; class Pi), near-neutral forms (pI 5.5–6.6; class Mu) and basic forms (pI > 7.5; class Alpha) [3, 4]. Genetic studies indicate that the GSTs of the three classes are the products of at least three distinct gene loci [7, 8]. Starch gel electrophoresis experiments, however, suggest that in human populations additional GST subunit loci might be present [9, 10]. The distribution of GSTs in different human tissues is not uniform. Certain forms that are the major components in one organ may be absent or present in trace amounts in other tissues. For example, the acid form (pI 4.6) GST-π which is the predominant GST in most tissues including placenta [3, 4] and erythrocytes [3, 4] is not present in significant amounts in human liver [3, 4]. GST-π is, however, overexpressed in a number of human tumor tissues [11–13]. On the other hand, the near-neutral form (pI 6.6) GST-μ in certain human tissues is expressed by only 50% of individuals [3, 4, 14]. The qualitative and quantitative differences in the occurrence of various GST in different organs as well as in the same organs of different individuals are of particular toxicological importance and might cause differential susceptibility of tissues to the toxic effect of particular xenobiotics. It is therefore important that the GSTs of other human tissues and organs are isolated and characterized in order to establish their relationships with the GSTs of the groups so far identified.

GSTs have been shown to be present in the human testis [15, 16], however, no definitive study of the isoenzyme forms present has been carried out. The present work describes the composition of GSTs in human testis. A major finding was that testis can express GSTs not yet identified in any of human tissues so far analysed.

MATERIALS AND METHODS

Testis procurement. The testis specimens (1.5–7.0 g) were obtained at the time of surgery from individuals operated on for prostatic carcinoma or seminoma. The histological examination of testis specimens obtained from patients operated on for prostatic carcinoma revealed a slight sclerotic hyalin degeneration of tubules with a thickening of basal membrane. A survey of the patients included in the present study are given in Table 1. The tissue was stored at −80° until used (storage time 3–5 weeks). No loss of enzymatic activity was noted during storage time.

Purification of GSTs. Homogenates (25% w/v)

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Table 1. Clinical data of the patients

Patient	Age (years)	Diagnosis	Drug intake
I	70	Prostatic carcinoma	—
II	47	Seminoma	—
III	73	Prostatic carcinoma	Flutamide
IV	80	Prostatic carcinoma	Cyprosterone acetate
V	67	Prostatic carcinoma	—
VI	73	Prostatic carcinoma	—

Drugs were given in conventional doses for at least three months prior to surgery.

were separately prepared in 10 mM potassium phosphate buffer, pH 7.0 supplemented with 1 mM dithiothreitol (buffer A). The extract was centrifuged for 60 min at 105,000 *g* (4°) and the resulting supernatant was applied to a GSH-Sepharose affinity column [17] (1 cm × 10 cm) which was preequilibrated with buffer A. The column was exhaustively washed with buffer A supplemented with 50 mM KCl. The enzyme was eluted with 50 mM Tris/HCl buffer, pH 9.6 containing 5 mM GSH. The fractions showing GST activity were pooled, concentrated by ultrafiltration, dialysed against buffer A and subjected to isoelectric focusing on a column (110 ml; LKB produkter, Stockholm, Sweden) containing 1% ampholine, pH 3.5–10 plus 1% ampholine pH 9–11 in a 40% (w/v) sucrose density gradient. After focusing for 72 hr at a final voltage of 700 V (4°), the content of the column was eluted and collected in 1.2 ml fractions. The peaks of activity thus separated were concentrated by ultrafiltration, dialysed against buffer A and used for further characterization.

SDS/polyacrylamide slab gel electrophoresis. Subunit molecular weight of GSTs were determined by SDS/polyacrylamide slab gel electrophoresis as described by Laemmli [18]. SDS concentration was 0.1% (w/v), the spacer gel and the separating gel were 3% and 12.5%, respectively. Albumin (66,000 M_r), ovalbumin (45,000 M_r), glyceraldehyde-3-phosphate dehydrogenase (36,000 M_r), carbonic anhydrase (29,000 M_r), trypsinogen (24,000 M_r), trypsin inhibitor (20,100 M_r) and α-lactalbumin (14,200 M_r) were used as standards for characterization of the subunit's molecular size.

Immunological characterization. The immunological studies were carried out using antisera prepared against GST pI 8.5 of human skin (class Alpha) [5], GST V of human uterus (class Pi) [6] and GST III of human uterus (class Mu) [6]. These antisera recognize GST isoenzymes of the same class but do not recognize members of the other classes. Ouchterlony immunodiffusion experiments were performed at 4° in 1% agarose gels containing 50 mM potassium phosphate (pH 6.9) and 0.9% NaCl. The proteins fractionated in SDS/polyacryamide gel electrophoresis were transblotted on nitrocellulose paper according to Towbin *et al.* [19] and detected by an alkaline phosphatase-linked immunoassay.

Enzyme assay. GST activity with 1-chloro-2,4-dinitrobenzene was determined according to the method of Habig *et al.* [20]. Protein determination was performed by the method of Bradford [21] using γ-globulin as standard.

Immunohistochemical staining. An indirect immunoperoxidase sandwich (PAP) method was used to locate GSTs in formalin (10%) fixed, paraplast embedded human testis tissues. Antisera raised against both Pi and Alpha classes GSTs were diluted 1:250 in phosphate buffered saline solution and used as primary antibody and the enzyme–antibody complex was identified using a peroxidase/antiperoxidase method [22]. In negative control samples the primary antisera was omitted.

RESULTS

Purification of GSTs

We have purified the GST isoenzymes of human testis from six different samples to apparent homogenity by affinity chromatography followed by isoelectric focusing. The purification parameters for these samples are shown in Table 2. The testis GSTs represented about 1.1–2.6% of cytosolic proteins. The differences in the specific activity noted after affinity column may be indicative of differences in the isoenzymes distribution. Figure 1, in fact, shows that there were marked differences in activity profiles obtained after isoelectric focusing. The major peaks resolved were designed as testis GST suffixed by their isoelectric point. In two samples (testis V and testis VI) more than 90% of activity is expressed by GST-4.4. This peak is also present in testis II, testis III and testis IV where it constitutes about 25–35% of activity but it is absent in testis I. A more anionic peak of activity, GST-3.8 (5–7% of activity), is present in only one (testis I) out of six samples investigated. The cationic GSTs of both testis I and testis III resolved into three peaks of enzyme activity GST-7.7 (25–28% of activity), GST-9.54 (28–35% of activity), GST-10.1 (28–30% of activity) (testis I) and GST-8.44 (5–7% of activity), GST-8.71 (30–35% of activity), GST-8.92 (30–35% of activity) (testis III), respectively. The cationic GSTs of testis II resolved into two peaks of enzyme activity GST-8.36 (45–50% of activity) and GST-9.1 (8–12% of activity). Two cationic peaks of activity GST-8.25 (30–35% of activity) and GST-8.86 (35–40% of activity) were obtained from testis IV. None of the sample analysed expressed a significant peak of activity in the near-neutral region of pH gradient.

Structural properties

To study the subunits composition of the GSTs obtained from the isoelectric focusing run, the protein recovered from each peak was subjected to SDS/polyacrylamide slab gel electrophoresis (PAGE) [18]. The results obtained are summarized in Table 2. GST-4.4 showed a single band on the gel of 23,000 M_r as compared with the marker proteins used. The most anionic isoenzyme GST-3.8 gave two bands of 23,000 M_r and 26,500 M_r, respectively. The cationic isoenzymes GST-7.7, GST-8.25, GST-8.44,

Table 2. Purification parameters for glutathione transferases from human testis specimens

Sample and step	GST activity			
	Protein (mg)	Activity (μmol/min/mg)	Yield (%)	Purification (fold)
I cytosol	150	0.22	—	—
affinity column	1.7	14.2	73	64
II cytosol	177	0.43	—	—
affinity column	3.2	21.1	88	49
III cytosol	135	0.39	—	—
affinity column	1.8	24.6	84	63
IV cytosol	153	0.15	—	—
affinity column	1.8	11.7	91	78
V cytosol	100	0.10	—	—
affinity column	1.6	6.1	97	61
VI cytosol	78	0.13	—	—
affinity column	1.2	3.7	44	30

GST-8.71, GST-8.86, GST-8.92 and GST-9.54 gave essentially a single polypeptide band of 24,500 M_r. Testis GST-8.36 and GST-9.1, both expressed by testis IV, was found to be formed by two subunits of 24,500 and 26,500 M_r. The most cationic testis isoenzyme GST-10.1 is also composed by two subunits of 23,000 and 24,500 M_r. It has to be noted that SDS/polyacrylamide slab gel electrophoresis of testis I revealed that the affinity purified fraction contained four different bands corresponding to apparent molecular weight values of 22,000, 23,000, 24,500 and 26,500 M_r.

Immunological characterization

The immunological characterization of GSTs resolved from human testis was performed using the antisera raised against the three major classes of human GSTs Alpha, Mu and Pi and were the same as those used in our previous works [6, 12, 13]. In immunodiffusion experiments, testis GST-7.7, GST-8.25, GST-8.44, GST-8.71, GST-8.86, GST-8.92 and GST-9.54 all cross-reacted with antisera raised against class Alpha GST but did not with antisera against classes Mu and Pi (Table 3). GST-4.4 cross-reacted with class Pi antisera but not with Mu and Alpha antisera (Table 3). GST-8.36 cross-reacted with both antisera raised against class Alpha and class Mu GST, whereas GST-9.1 cross-reacted with only antisera raised against class Alpha GST (Table 3). This result indicates that the 26,500 M_r subunit of GST-8.36 is immunologically distinct from the 26,500 M_r subunit of GST-9.1. The most cationic enzyme GST-10.1 cross-reacted with both classes Alpha and Pi GST antisera (Table 3). The results of Western blot analysis in which nitrocellulose membrane was incubated using Pi class antisera followed by Alpha class antisera revealed that the faster subunit of GST-10.1 is immunologically identical to class Pi GST whereas the other is immunologically identical to class Alpha GST (Table 3). In immunodiffusion experiment GST-3.8 cross-reacted with only class Pi GST antisera (Table 3) and Western blot analysis revealed that the positive reaction was due to the faster subunit (Table 3).

Immunohistochemical detection

The results of immunohistochemical staining for both Alpha and Pi classes GST are shown in Fig. 2. A widespread positive reaction with anti Pi and anti Alpha antisera resulted in an interstitial area and a strong positive staining with anti Pi antisera was found in the basal area of seminiferous epithelium.

DISCUSSION

With the use of standard techniques, i.e. affinity chromatography and isoelectric focusing, different isoenzymes of GST were resolved from several samples of human testis. The different forms present in this human tissue constitute 1–2.5% of the total cytosolic proteins. This value is quite similar to the value of 2–3% found for GSTs of adult human liver, which is one of the human tissues that is most rich in GST [3]. The present study also provides evidence that there are considerable interindividual variations in the expression of different isoenzymes of GST in human testis. This occurrence was also seen in other human tissues [3, 10, 23]. On the basis of subunit molecular mass, isoelectric point value and immunological characteristic testis GST-4.4 is very similar if not identical to the anionic isoenzyme GST-π first described in human placenta [24, 25] but also present in the majority of human tissues so far analysed (3, 6, 26–28). The immunohistochemical studies seem to indicate that this enzymes is predominantly located in the seminiferous epithelium cells. Thus,

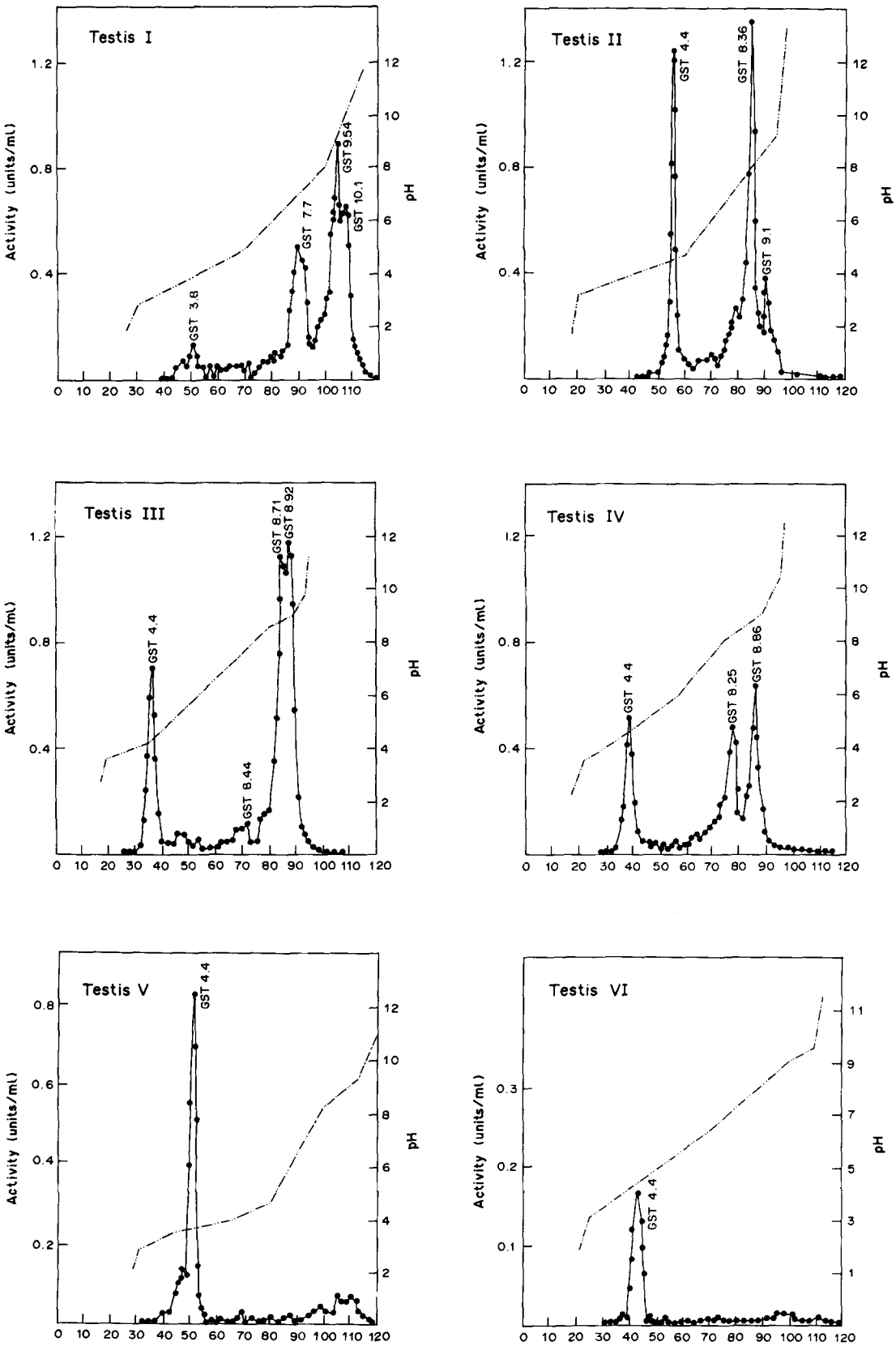
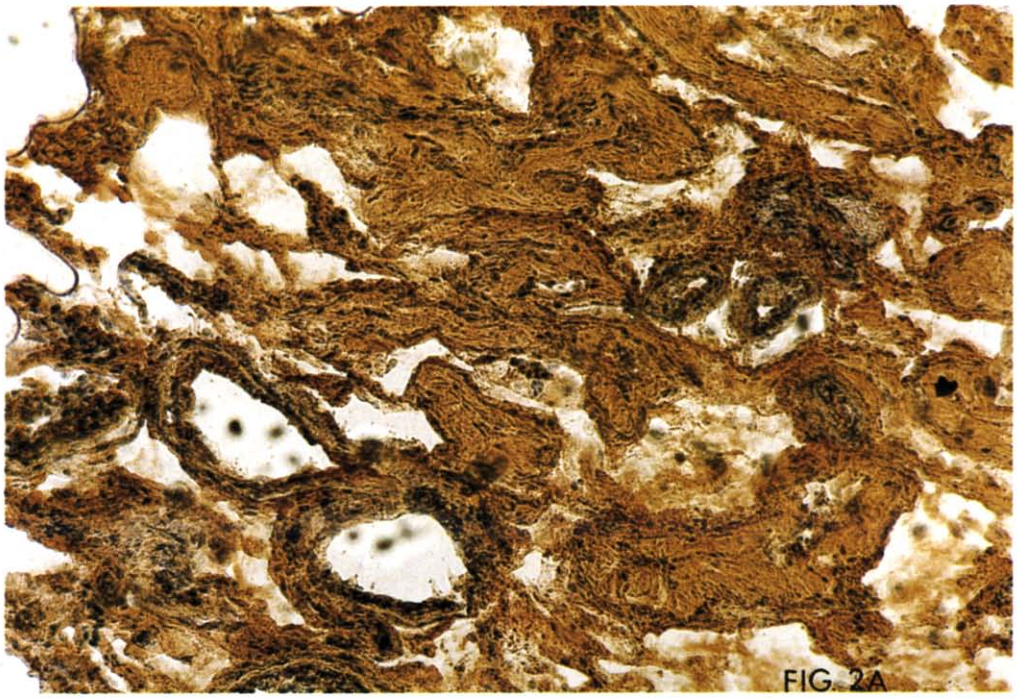


Fig. 1. Isoelectric focusing profiles of affinity purified GSTs from human testis. Experimental details are given in the text. pH gradient (··—··—··) and GST activity (—●—) determined with 1-chloro-2,4-dinitrobenzene.



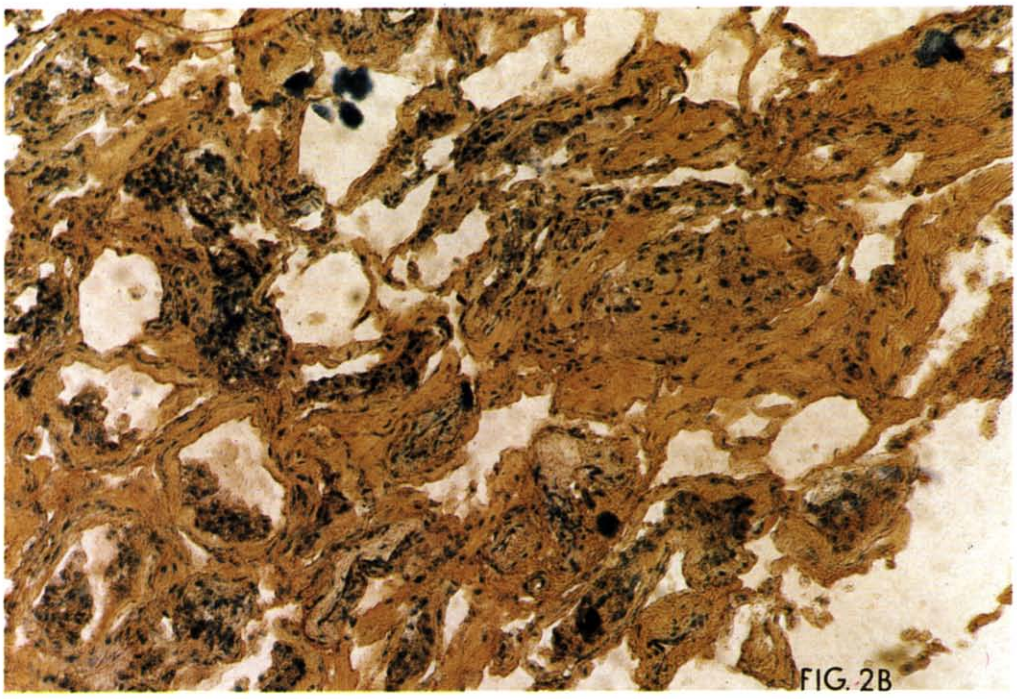


Fig. 2. Immunohistochemical staining for Pi class GST (A) and Alpha class GST (B) in human testis (magnification × 100).

Table 3. Characteristic features of glutathione transferases isolated from human testis

Isoenzyme	Subunits molecular weight (M_r)	Cross-reactivity		
		Anti-Pi	Anti-Mu	Anti-Alpha
GST-3.8	23,000	+	−	−
	26,500	−	−	−
GST-4.4	23,000	+	−	−
GST-7.7	24,500	−	−	+
GST-8.25	24,500	−	−	+
GST-8.36	24,500	−	−	+
	26,500	−	+	−
GST-8.44	24,500	−	−	+
GST-8.71	24,500	−	−	+
GST-8.86	24,500	−	−	+
GST-8.92	24,500	−	−	+
GST-9.1	24,500	−	−	+
	26,500	−	−	−
GST-9.54	24,500	−	−	+
GST-10.1	22,000	+	−	−
	24,500	−	−	+

from the structural and immunological data obtained it is reasonable to include the testis GST-4.4 in the Pi family. On the other hand, the basic isoenzymes of human testis namely GST-7.7, GST-8.25, GST-8.44, GST-8.71; GST-8.86, GST-8.92 and GST-9.54 can be referred to class Alpha by being structurally and immunologically identical to the cationic GSTs isolated from liver (GST-α-ε) [3, 11, 29, 30], skin [5] and kidney [12, 31, 32]. The enzyme we have identified as GST-3.8 has several features which distinguish it from all human GSTs so far identified. It is the most acidic isoenzyme from human tissues, appears to consist of two different subunits (23,000/26,500 M_r) the smaller of which cross reacted with anti-Pi GST antisera, whereas the larger did not cross react with any of the antisera raised against the three major classes of human GST. A GST isoenzyme more acidic than GST-π and composed of two different subunits (pI 4.25; 26,950/27,500 M_r), named GST6, has been previously identified by Suzuki *et al.* [10]. GST6, however, failed to cross react with antisera raised against any of the three major classes of GST [10] and appears to be immunologically different from testis GST-3.8. Testis I also expressed GST-10.1 which has not been previously described. This enzyme is composed of two subunits, one of which migrates faster than the testis GST-4.4 subunit on SDS/polyacrylamide slab gel electrophoresis but had an antigenic determinant in common with it. The other subunit of this enzyme is structurally and immunologically identical to the subunit forming Alpha class GSTs. Thus, testis I contained two subunits with different electrophoretic mobility, immunologically identical to Pi class GST. This occurrence has never been described for other human tissues. The cationic isoenzymes of testis II, GST-8.36 and GST 9.1, also seems to be novel GSTs. They have an apparently similar subunit composition, both being dimers of 24,500 M_r and 26,500 M_r. Although the 24,500 M_r subunit of the two enzymes appears to be immunologically identical, the 26,500 M_r subunit of testis GST-9.1 resulted in being different from that of testis GST-8.36. The 26,500 M_r subunit of this latter form shows a common antigenicity with the subunit of Mu class GST. Previous work from this laboratory [5] has shown that in human skin a GST is contained with a pI value of 9.9 composed by a dimer of 26,500 M_r subunit which cross-reacted with antisera raised against rat GST 2.2 rather than against class anti-Mu GST. Unfortunately we had insufficient quantity of testis GSTs to establish whether the 26,500 M_r subunits of testis and skin GSTs are immunologically related. It has to be noted that testis II was obtained from a patient operated on for metastatic seminoma and the possibility that the two cationic isoenzymes (GST 8.36 and GST 9.1) isolated from this sample are tumor specific can not be ruled out. Tumor specific GST isoenzymes have been recently identified in the human kidney [33].

In conclusion, the present work shows that in human testis tissues, in addition to classes Pi and Alpha enzymes, also present are GSTs not yet described in any of the human tissues so far analysed.

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