Biochemical Pharmacology, Vol. 52, pp. 801–808, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI S0006-2952(96)00341-3

Regulation of Rat Olfactory Glutathione S-Transferase Expression

INVESTIGATION OF SEX DIFFERENCES, INDUCTION, AND ONTOGENESIS

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ABSTRACT. The glutathione S-transferases (GSTs) of rat olfactory epithelium have been characterised with regard to sex differences, induction, and developmental regulation, and compared to those of the liver. Olfactory cytosolic GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was similar in both male and female animals, and there were no differences in subunit profile. Administration of *trans*-stilbene oxide and β-naphthoflavone had no effect on olfactory GST activity with CDNB, although phenobarbitone treatment resulted in a small, but significant, increase in activity (130% compared to controls). HPLC analysis of subunit profiles indicated that all three agents induced olfactory subunit 1b and decreased subunit 6. The effect of age (3 to 84 days) on both cytosolic and microsomal CDNB activity was examined. In the liver, cytosolic activity was low at 3 days and climbed steadily to reach maximal levels around 28 days, but microsomal activity was relatively constant at all ages. Olfactory cytosolic activity was similar at all ages; microsomal activity was low until 21 days and then increased to reach a maximum at 56 days. Changes in individual cytosolic subunits were assessed by SDS-PAGE followed by immunoblotting. The significance of these results with regard to putative physiological roles for olfactory GSTs is discussed. BIOCHEM PHARMACOL 52;5:801–808, 1996.

KEY WORDS. glutathione S-transferases; olfactory epithelium; nasal cavity; induction; sex differences; ontogenesis

The GSTs† (EC 2.5.1.18) are a group of enzymes that catalyse the conjugation of glutathione with a variety of electrophilic compounds; both cytosolic and microsomal forms have been described [1, 2]. The cytosolic GSTs are dimers formed by combinations of subunits belonging to four multigene families, namely Alpha, Mu, Pi, and Theta. The alpha family comprises subunits 1, 2, 8, and 10; the Mu family subunits 3, 4, 6, 9, and 11; subunit 7 is the only member of the Pi family and subunits 5, 12, and 13 are members of the Theta family [1–5]. Within each family, subunits form homo- or heterodimers that exhibit broad and overlapping substrate specificity and immunological cross-reactivity with antisera. The microsomal GST is a trimer formed of 3 identical subunits that are distinct from any of the cytosolic subunits [6].

Sex- and age-related expression of cytosolic GSTs has been studied in some detail in the rat liver. Differences between male and female hepatic GST activity with some substrates has been reported, with up to 3-fold greater activity in male compared to female animals [7–11]. Analysis of specific subunits has, however, shown that subunit 2 is expressed at significantly higher levels in female compared to male rat liver [12–14]. GST activity in livers of rats of both sexes is low at birth and then increases steadily to reach adult levels at around 40 days postpartum [15–17].

The hepatic cytosolic GSTs are inducible by a variety of xenobiotics, both naturally occurring and synthetic. Barbiturates, polycyclic aromatic hydrocarbons, arylmethanes, dithiolethiones, antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole, the substrate *trans*-stilbene oxide, and natural dietary products all induce hepatic GST activity [18 and references therein, 19–24]. These inducing agents tend to be selective for subunits 1 and 3 [20, 24–30].

GSTs are also present in many extrahepatic tissues, and we have recently reported a detailed characterisation of the GSTs of adult rat olfactory epithelium [31]. GST activity using 1-chloro-2,4-dinitrobenzene as substrate is significantly lower in olfactory cytosol compared to hepatic, but the microsomal GST activities of the two tissues are comparable. The GST subunit profile of olfactory cytosol is unique, with subunits 3 and 4 predominant and an absence

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BNF, β-naphthoflavone; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione S-transferase; NBT, Nitro Blue Tetrazolium; PB, phenobarbitone, TFA, trifluoroacetic acid; TSO, *trans*-stilbene oxide.

† Received 29 November 1995; accepted 25 April 1996.

of subunit 1. The physiological role of nasal GSTs is unclear, but it has been suggested that they may provide a first line of defence for the lungs or have a role in olfaction, rapidly clearing odorant molecules so that acuity of olfaction is maintained [32]. They may also provide a defence mechanism for the brain by limiting retrograde axonal transport from the olfactory sensory cells, which are directly exposed to the environment, to the olfactory bulb [33–35]. Understanding the regulation of expression of nasal GSTs may cast some light on the question of their physiological significance and, thus, the aim of this study was to characterise olfactory GSTs with regard to sex differences, age-related changes, and response to classical hepatic-inducing agents.

MATERIALS AND METHODS

Materials

Reduced GSH, CDNB, acrylamide/bisacrylamide (40% solution), BCIP, NBT, alkaline phosphatase-conjugated anti-sheep IgG, S-hexyl-glutathione and GSH-agarose were all purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Antibodies raised against rat hepatic GSTs 1-1, 2-2, 3-3, 4-4, 7-7, and 8-8 were supplied by Biotrin International (Dublin, Ireland). Nitrocellulose, trifluoroacetic acid, and acetonitrile were from Pharmacia (Uppsala, Sweden), Applied Biosystems Ltd. (Warrington, Cheshire, U.K.) and Romil Chemicals (Cambridge, U.K.), respectively. All other chemicals were of the highest quality available commercially.

Animals

Wistar rats (males of varying ages and adult females) used in these studies were allowed food and water *ad lib*. For experiments using inducing agents, adult male rats were given either three i.p. injections of β-naphthoflavone (40 mg/kg/5 mL corn oil), or four i.p. injections of *trans*-stilbene oxide (400 mg/kg/5 mL corn oil) or corn oil (5 mL/kg) at 24-hr intervals and killed 24 hrs after the last injection. Phenobarbitone was administered as 0.1% (w/v) of the drinking water for 7 days. Animals were killed by exposure to a rising concentration of CO_2. Liver and nasal S9, cytosolic, and microsomal fractions were prepared by differential ultracentrifugation of tissues homogenised in 0.25 M sucrose and 0.1 M Tris-HCl, pH 7.4, containing 1.15% KCl, respectively [31].

Assays

GST activity with CDNB as substrate was determined according to the method of Habig *et al.* [36] using final concentrations of 1 mM CDNB for both cytosolic and microsomal fractions, and 1 mM GSH and 5 mM GSH for cytosolic and microsomal enzymes, respectively. Protein concentrations were determined by the method of Lowry *et al.* [37] using bovine serum albumin as standard.

SDS-PAGE and Immunoblotting

SDS-PAGE of liver and nasal S9 fractions was carried out using a Bio-Rad Minielectrophoresis system with a 12% (w/v) resolving gel as described by Laemmli [38]. The same amount of protein (30 μg) was loaded per lane, regardless of tissue or age of animal. It had previously been ascertained, using different loadings of protein, that 30 μg was within the linear range for both tissues.

Proteins were transferred to nitrocellulose using the method of Towbin *et al.* [39], and the blots were probed with antibodies against specific rat hepatic GSTs, followed by an alkaline phosphatase-conjugated second antibody. BCIP was used in conjunction with NBT for the detection of the alkaline phosphatase-conjugated complexes, as described by Blake *et al.* [40].

Densitometry of the immunoblots was carried out on an OmniMedia XRS scanner and the areas of the peaks integrated using the Millipore BioImage analysis software. For each blot, the area of the peak at 3 days was assigned an arbitrary value of 1, and the areas of peaks at other time points are expressed as factors of the peak area at 3 days.

Separation of GST Subunits by HPLC

Reverse phase HPLC was carried out according to the method of Ostlund-Farrants *et al.* [41] using a Dynamax C_{18} column (4.6 mm × 250 mm) (Rainin Instrument Co., Woburn, MA, U.S.A.). The solvents were 0.06% trifluoroacetic acid (TFA) in water (solvent A) and 0.04% TFA in acetonitrile (solvent B). Samples (1 mL), affinity purified on GSH-agarose, were injected at 35% solvent B and a gradient was run from 35 to 65% solvent B over 60 min with a flow rate of 1 mL/min. Absorbances at 214 nm were measured and areas integrated using a Shimadzu C-R5A computing integrator.

Statistical Analysis

The probability, P, of the significance of the difference between 2 sets of results was calculated using the Student's t-test. Values of $P < 0.01$ or $P < 0.05$ were considered to indicate that 2 sets of results were statistically significantly different.

RESULTS

Comparison of GST activity using CDNB as substrate in liver and olfactory epithelium of adult male and female rats indicated that there were no sex differences in activity in either tissue. Activities (nmol/min/mg protein; N = 3) were 968.3 ± 146.0 and 921.5 ± 40.2 in the liver, and 594.1 ± 50.6 and 610.4 ± 80.4 in olfactory epithelium for male and female rats, respectively. However, a more detailed analysis of the GST profile by HPLC showed that, in the liver of female rats, there was an increase in subunit 2 and a small decrease in the levels of the majority of the other subunits

compared to the male profile (Fig. 1a). No major differences were observed in the profiles of olfactory GSTs of male and female rats (Fig. 1b).

Treatment of adult male rats with classic inducing agents of hepatic GSTs (PB, TSO, and BNF) resulted in significant increases in hepatic GST activity with CDNB as substrate (Table 1). In the olfactory epithelium, TSO and BNF had no effect; PB administration caused a small, but significant, increase in GST activity (Table 1).

The effects on individual subunits were determined by HPLC and the results are shown in Fig. 2a and b. In the olfactory epithelium, the most striking observations were the appearance of a peak at a retention time corresponding to that of subunit 1b and the decrease in levels of subunit 6 to below the limits of detection, following treatment with all 3 chemicals. No peak corresponding to subunit 1b was detected in either water or corn oil controls, treatment with PB, BNF, or TSO resulted in the appearance of peaks with areas of 0.75 ± 0.21, 0.71 ± 0.30 and 0.70 ± 0.21, respectively. The only other significant effects were a decrease in the levels of subunits 4 and 11 following BNF treatment, and an increase in subunit 3 and a decrease in 11 following TSO induction. In the liver, PB administration significantly increased expression of subunits 1a, 1b, and 3, and decreased that of subunit 2. BNF induced subunits 1b and 3 and decreased levels of subunit 1a. TSO increased expression of subunits 1b, 3, and 7 and decreased that of subunits 2, 4, and 6.

The effect of age (3 to 84 days) of male rats on both cytosolic and microsomal GST activity using CDNB as substrate was studied. Olfactory cytosolic GST activity was similar at all ages studied (Fig. 3a), whereas the olfactory microsomal activity was low until 3 weeks, when it started to climb, reaching its highest level at 56 days (Fig. 3b). In contrast, in the liver, cytosolic activity was approximately 20% of "adult" levels at 3 days and climbed steadily before beginning to level off at 28 days (Fig. 3a), whereas hepatic microsomal GST activity was relatively constant at all ages (Fig. 3b).

SDS PAGE followed by immunoblotting was performed on some of the samples to probe for age-related changes in individual cytosolic GST subunits. In both tissues, levels of subunits 2, 3, and 4 were low at 3 days and increased steadily to reach maximum levels between 28 and 35 days (Fig. 4), this increase was particularly marked for olfactory subunit 2. In the olfactory epithelium, levels of subunits 7 and 8 were similar at all ages; in the liver, levels of subunit 7 declined steadily and subunit 8 showed a trend similar to subunits 2, 3, and 4 (Fig. 4). Subunit 1 appeared to be expressed in the liver at a constant level at all ages (Fig. 4).

DISCUSSION

The olfactory epithelium of a number of species has been shown to be rich in drug-metabolising enzymes, including the GSTs [for a review see 42]. The physiological role of

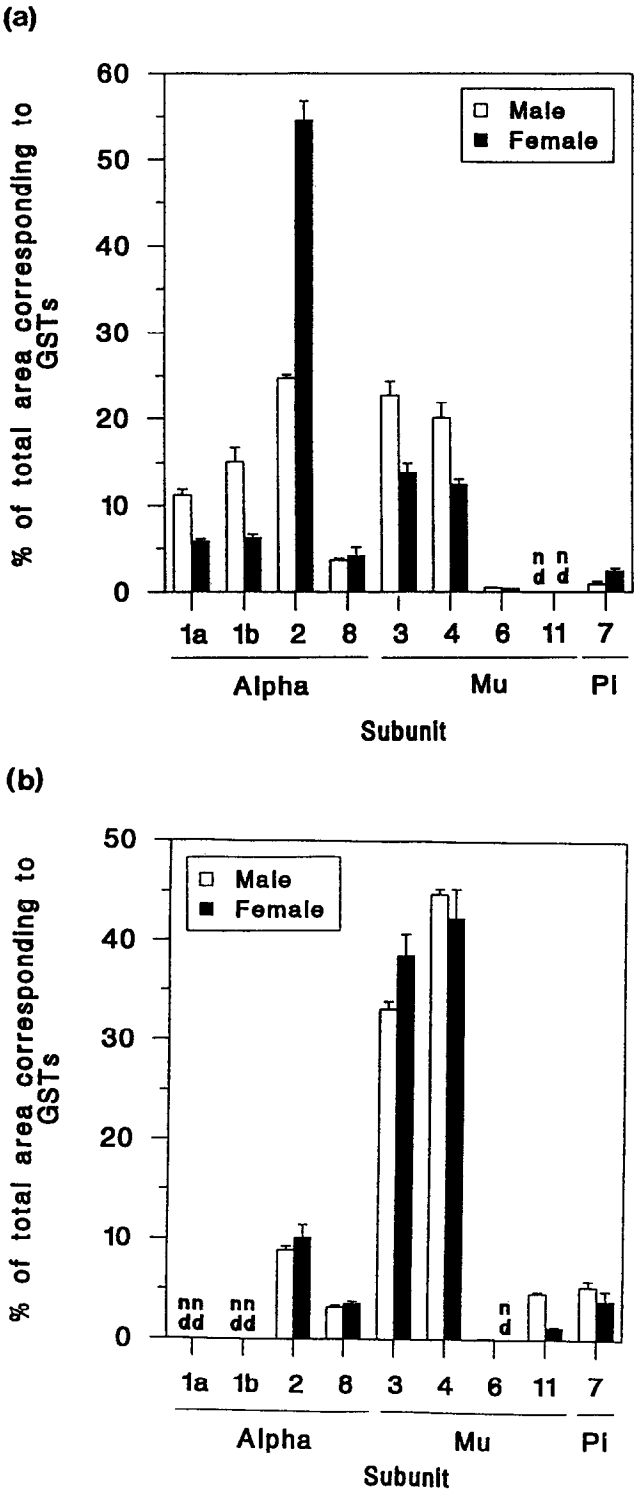

FIG. 1. Relative contribution of specific subunits to the total GST content of (a) liver and (b) olfactory epithelium from adult male and female rats. Reverse phase HPLC was performed as described in Materials and Methods. Results are means ± SD of 3 HPLC separations, each carried out on a separate cytosolic preparation. Absolute areas corresponding to GST peaks were: male liver, 11.6 × 10^6; female liver, 8.4 × 10^6; male olfactory epithelium, 9.6 × 10^6, female olfactory epithelium, 8.1 × 10^6. nd, below the limits of detection.

TABLE 1. GST activity in liver and olfactory epithelium of adult male rats treated with inducing agents

Treatment	Liver		Olfactory epithelium	
	Specific activity*	% of control	Specific activity*	% of control
Water	924.7 ± 73.3	–	453.5 ± 90.2	–
Corn oil	1111.8 ± 58.5	–	572.5 ± 64.6	–
PB	2587.3 ± 268.9†	280	591.2 ± 31.6†	130
BNF	1382.4 ± 62.1†	125	483.9 ± 38.3	85
TSO	2189.8 ± 256.3†	200	506.9 ± 79.5	88

Induction of GSTs and the GST assay using CDNB as substrate were performed as described in Materials and Methods. Results are means ± SD of 6 determinations, each carried out with tissue pooled from 3 animals. * nmol/min/mg protein; † $P < 0.05$, when compared to the appropriate control cytosol.

these enzymes is unclear. They are certainly responsible for the *in situ* activation of a variety of protoxins, resulting in nasal toxicity/carcinogenicity [42, 43], and it has also been suggested that they may provide a first line of defense for the lungs and/or the brain, or have a role in olfaction, rapidly clearing odorant molecules so that acuity of olfaction is maintained [32]. Detailed characterisation of nasal xenobiotic transformation and the regulation of expression of the enzymes involved may cast some light on the question of their physiological significance. In this study, we have confined our investigations to GSTs belonging to the Alpha, Mu, and Pi classes, and have not considered Theta class enzymes, because: 1. Earlier work failed to detect any activity with dichloromethane, a Theta class substrate, in olfactory epithelium (Banger *et al.*, unpublished results); 2. The affinity chromatography and HPLC methodology used does not detect Theta class GSTs; and 3. Antibodies to GSTs 5-5 and 12-12 are not readily available.

This study found no sex differences in the GST activity or subunit profile of rat olfactory epithelium. In the liver, there was no difference between males and females in rates of CDNB conjugation, as has been reported previously for rats of a similar age [44, 45]. However, in agreement with the results of Carrillo *et al.* [12], Rogiers *et al.* [13] and Srivastava and Waxman [14], there was a marked increase in the level of hepatic expression of subunit 2 in females compared to that in males. The lack of a sex difference in olfactory GSTs is not unexpected because the putative physiological roles for nasal xenobiotic biotransformation [32] are not gender-specific.

Numerous studies have demonstrated that nasal cytochromes P450 show little or no induction following treatment of animals with classical hepatic inducing agents [42 and references therein]. We now report that a similar situation exists for olfactory GSTs. No induction of olfactory CDNB activity was detected following administration of TSO or BNF, and the increase due to PB treatment was far less than that seen in the liver (Table 1). It is not known whether nasal xenobiotic biotransformation enzymes are 1. inherently noninducible, 2. respond to agents other than those tested (for instance odorants), or 3. exist in a fully induced state in the adult. The rapid increases reported for some olfactory cytochrome P450 levels and activities after birth [46, 47] are compatible with explanations 2. and 3. and also with a role in olfaction.

HPLC analysis of subunit profiles confirmed previous studies that showed that these inducing agents increase the relative amounts of subunits 1 and 3 in liver [20, 24–30], and demonstrated in olfactory cytosol the appearance of a peak with a retention time corresponding to subunit 1b and a decrease in levels of subunit 6 to below the limits of detection (Fig. 2a and b). These results emphasise the need to analyse effects of inducing agents/inhibitors on individual drug-metabolising enzymes, rather than using general, nonspecific substrates. Alpha class GSTs exhibit high activity with the products of lipid peroxidation of biological membranes [48, 49] and, thus, the relative lack of this class of GST within olfactory epithelium [31] may render this tissue susceptible to peroxidative damage. The results of this study suggest that subunit 1b is present in olfactory epithelium, but at levels below limits of detection by immunoblotting, FPLC, or HPLC until after induction. If this subunit can be induced by xenobiotics administered systemically, it is reasonable to assume that inhaled compounds may have a similar effect and, thus, protection against lipid peroxidation may be afforded by induction of subunit 1b.

In olfactory cytosol, there were no significant changes in CDNB activity between days 3 and 84 (Fig. 3a). However, analysis of olfactory subunit profiles indicated that levels of the majority of subunits increased gradually with age, reaching maximal levels around day 30 (Fig. 4). The reason for the discrepancy in the 2 data sets is unclear, but could be explained by the presence of catalytically inactive subunits in adult, but not young, rat olfactory epithelium. We have previously reported that GST subunits constitute approximately the same percentage of cytosolic protein in both liver and olfactory epithelium of adult male rats, despite the low olfactory conjugation of CDNB [31], and it can be seen in the legend to Fig. 1 that the total areas of GST peaks are similar in the two tissues (11.6×10^6 and 9.6×10^6 for male liver and olfactory epithelium, respectively). Olfactory GSTs could be catalytically inactive due to the presence of an endogenous inhibitor; *in vivo* hepatic GST activity may be modulated by endogenous metabolites including bilirubin, haematin, bile acids, leukotriene C4, and unsaturated

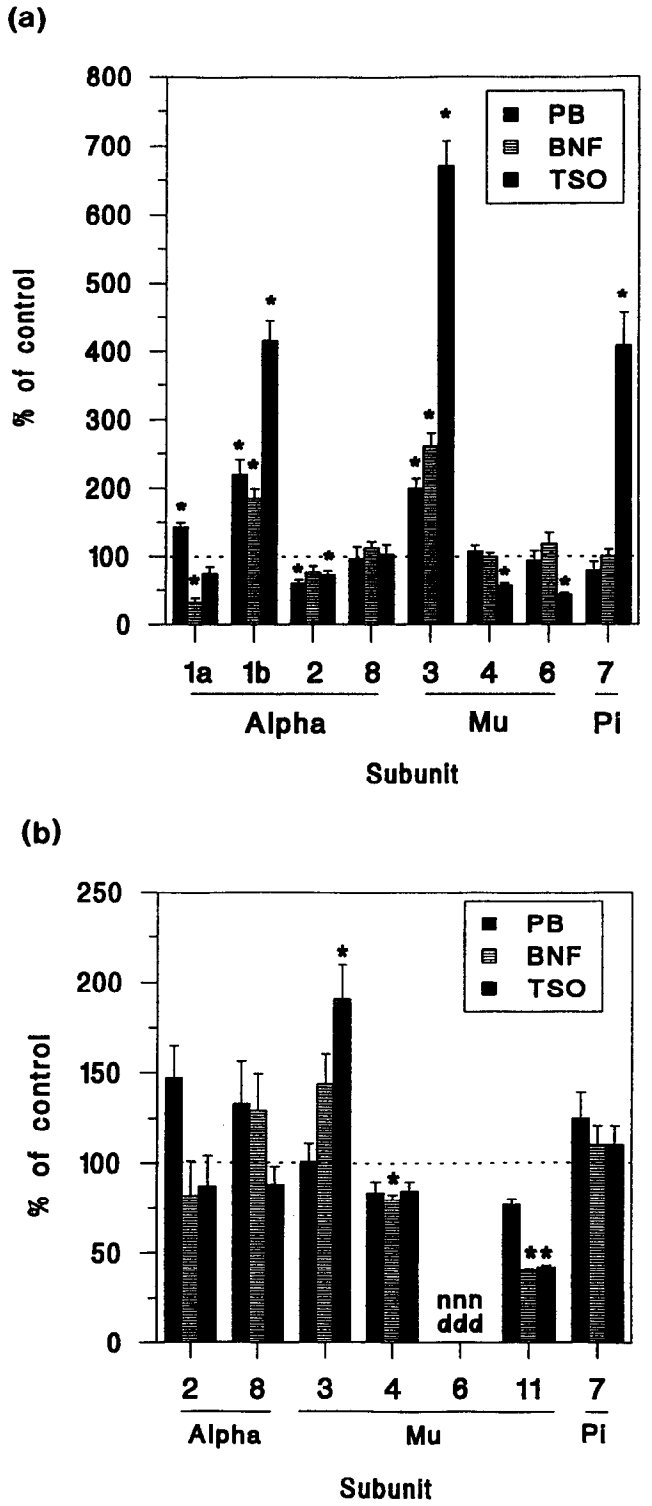

FIG. 2. Effect of inducing agents on specific subunits of (a) liver and (b) olfactory epithelium from adult male rats. Reverse phase HPLC was performed as described in Materials and Methods. Results are means ± SD of 3 HPLC separations, each carried out on a separate cytosolic preparation. Absolute areas of hepatic GST peaks in water and corn oil controls, respectively, were: 1a, 3.67 and 5.80; 1b, 1.10 and 1.31; 2, 3.70 and 4.82; 8, 0.66 and 0.86; 3, 4.01 and 1.72; 4, 3.62 and 4.72; 6, 0.33 and 0.33; 7, 0.05 and 0.12. Absolute areas of olfactory GST peaks in water and corn oil controls, respectively, were: 2, 0.96 and 1.03: 8, 0.15 and 0.17; 3, 2.41 and 1.33; 4, 4.31 and 4.52; 6, 0.13 and 0.21; 11, 0.35 and 0.43; 7, 0.20 and 0.21. nd, below the limits of detection; *$P < 0.05$.

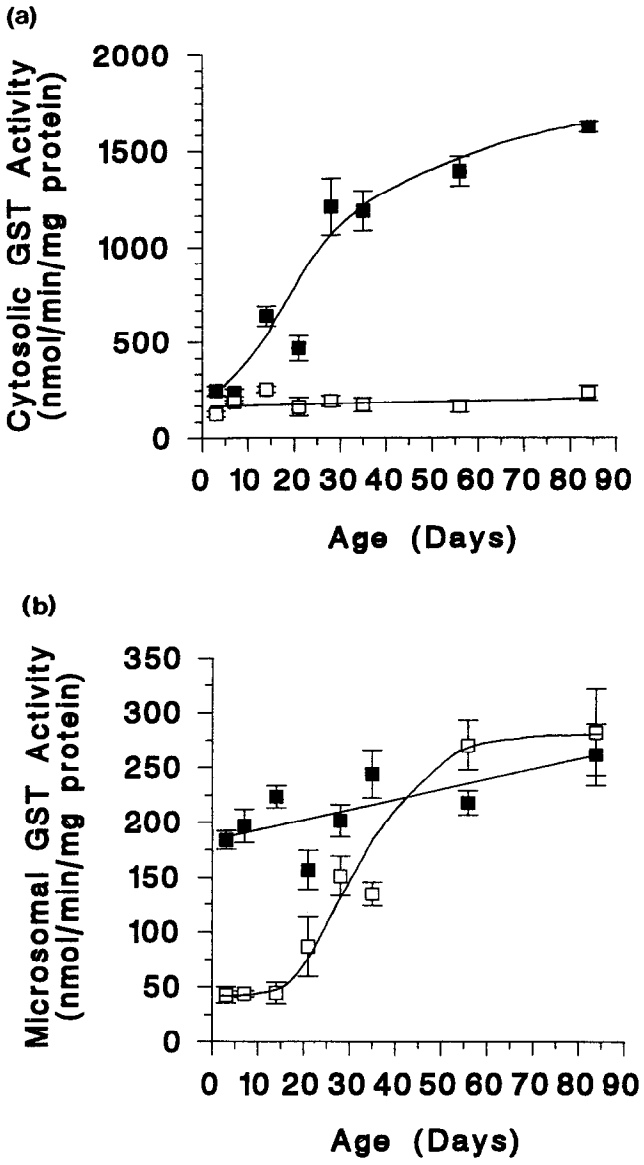

FIG. 3. Age-related changes in (a) cytosolic and (b) microsomal GST activity in liver and olfactory epithelium of male rats. GST activity with CDNB as substrate was measured as described in Materials and Methods. Results are means ± SD of 3 determinations, each carried out on tissue pooled from 1–2 animals. ■, hepatic; □, olfactory.

fatty acids [18]. Alternatively, it is possible that the GST proteins of liver and olfactory epithelium differ in the extent of some posttranslational modification (phosphorylation, methylation, or glycosylation [50–54]) resulting in a lower specific activity for olfactory GSTs, particularly those of older animals. However, an irreversible, covalently-bound inhibitor would almost certainly affect the HPLC profile of the olfactory GSTs, as would posttranslational modification. The effect of a reversible inhibitor would be diminished by dilution of the cytosol, and we have no evidence for this occurring. Thus, the reasons for the apparent discrepancy between the activity and the amount of GSTs in olfactory epithelium are unclear.

Cytochromes P450 1A2, 2G1 (NMb, Olf1), and a member of the 2A family have all been shown to be expressed

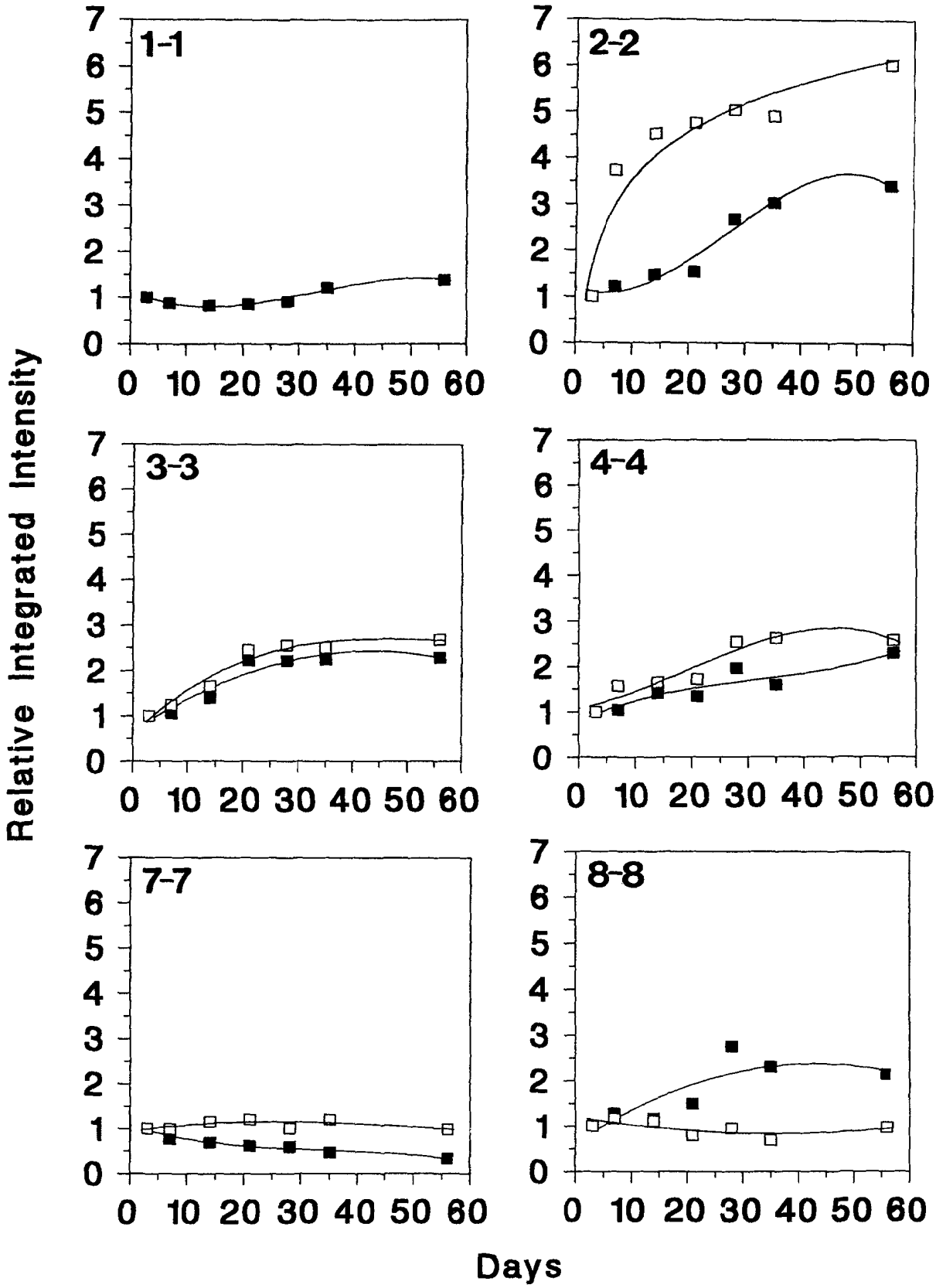

FIG. 4. Age-related changes in expression of individual GST subunits in liver and olfactory epithelium of male rats. SDS-PAGE of hepatic and olfactory S9 samples, immunoblotting with antibodies against hepatic GSTs 1-1, 2-2, 3-3, 4-4, 7-7, and 8-8 followed by densitometry of the immunoblots were carried out as described in Materials and Methods. For each subunit, the integrated peak area at 3 days was assigned an arbitrary value of 1 and peak areas at other time points are expressed as factors of this value. ■, hepatic; □, olfactory.

within the olfactory epithelium, but not the liver, of experimental animals at birth, and to reach "adult" levels around 21 days postpartum [46, 47]. The synchrony between the development of cytochromes P450 within olfactory epithelium with the ontogenesis of the sense of smell suggests a possible involvement of these enzymes in olfaction. Because it is enzyme activity, rather than concentration, that is critical in this respect, our present results with olfactory GSTs are consistent with their also having a role in olfaction.

In the liver, cytosolic GST activity has been shown to be low at birth and then increase steadily to reach adult levels around 40 days postpartum [15–17], and our results are in general agreement with this finding. We have also analysed the subunit profiles at each time and found that the majority of subunits mirror the changes in CDNB activity, but that levels of subunit 1 remain constant for up to 60 days and those of subunit 7 decrease. In adult rats, subunit 7 is a marker for preneoplastic foci in hepatocarcinogenesis, and its concentration in adult liver has been shown to be lower than that of foetal liver [55].

In contrast to the cytosolic results, olfactory microsomal GST activity was low 3 days postpartum and increased steadily to reach adult levels around 60 days; that of the liver was relatively constant at all ages (Fig. 3b). Microsomal GSTs afford protection against lipid peroxidation [56, 57] and can be oxidatively activated by reactive oxygen species [58]. We have reported that the microsomal GST of adult rat olfactory epithelium can be activated by incubation with the sulphydryl agent N-ethylmaleimide to a similar extent as the hepatic enzyme [31], suggesting that the increase of olfactory microsomal activity seen with age is unlikely to be due to *in vivo* oxidative activation. The low levels of microsomal GST activity in olfactory epithelium postpartum, combined with the relative absence of Alpha class cytosolic GSTs, may predispose the neonate to oxidative damage within the nasal cavity.

In conclusion, although direct evidence is still unavailable, our results with the GSTs are compatible with nasal xenobiotic biotransformation enzymes having a role in olfaction.

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