*Biochem. J.* (1981) **198**, 211–217
*Printed in Great Britain*



# The hypothalamic–hypophyseal–gonadal regulation of hepatic glutathione *S*-transferases in the rat

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(*Received 26 March 1981/Accepted 30 March 1981*)

Hepatic glutathione *S*-transferase activities were determined with the substrates 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene. Sexual differentiation of glutathione *S*-transferase activities is not evident during the prepubertal period, but glutathione conjugation with 1,2-dichloro-4-nitrobenzene is 2–3-fold greater in adult males than in females. Glutathione conjugation with 1-chloro-2,4-dinitrobenzene is slightly higher in adult males than adult females. No change in activity was observed after postpubertal gonadectomy of males or females. Neonatal castration of males results in a significant decrease in glutathione conjugation with 1,2-dichloro-4-nitrobenzene. Hypophysectomy, or hypophysectomy followed by gonadectomy did result in significantly higher glutathione *S*-transferase activities in both sexes. These increases can be reversed by implanting an adult male or female pituitary or four prepubertal pituitaries under the kidney capsule. Postpubertal sexual differentiation of glutathione *S*-transferase activities is neither dependent on pituitary sexual differentiation nor pituitary maturation. Prolactin concentrations are inversely related to glutathione *S*-transferase activities in hypophysectomized rats with or without ectopic pituitaries. Somatotropin exogenously administered to hypophysectomized rats results in decreased glutathione *S*-transferase activities, whereas prolactin has no effect. Adult male rats treated neonatally with monosodium L-glutamate to induce arcuate nucleus lesions of the hypothalamus have decreased glutathione *S*-transferase activities towards 1,2-dichloro-4-nitrobenzene and decreased somatotropin concentrations. Our experiments suggests that sexual differentiation of hepatic glutathione *S*-transferase is a result of a hypothalamic inhibiting factor in the male (absent in the female). This postpubertally expressed inhibiting factor acts on the pituitary to prevent secretion of a pituitary inhibiting factor (autonomously secreted by the female), resulting in higher glutathione *S*-transferase activities in the adult male than the adult female.

The glutathione *S*-transferases (EC 2.5.1.18) are a group of proteins that catalyse many reactions in which glutathione participates as a nucleophile (Jakoby *et al.*, 1976). These enzymes bind a large number of hydrophobic compounds and act as a storage facility before metabolism or excretion of the ligand (Jakoby & Keen, 1977) and undergo covalent-bond formation with reactive electrophilic carbon atoms (Keen & Jakoby, 1978). Some attention has been devoted to the postnatal developmental course of glutathione *S*-transferase (Hales & Neims, 1976*a*,*b*) and to its induction by xenobiotics (Darby & Grundy, 1972; Hales & Neims, 1977; Lamartiniere *et al.*, 1979*a*), but little to its regulation by endocrine factors (Reyes *et al.*, 1971). We have therefore initiated an in-depth study on the role that the hypothalamic–hypophyseal–gonadal axis plays in the sexual differentiation and regulation of hepatic glutathione *S*-transferases in the rat. We have investigated the 'activational effects' of sex steroids (direct hormone action in the presence of the effector) on adult male and female rats and the 'organizational effect' of hormones on postnatal enzyme development (McEwen, 1976*a*). Recent evidence suggests that the latter mechanism is latent and dependent on hypothalamic nerve endings being exposed to androgen during a critical period of early development, resulting in the programming

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for a male type of metabolism that is expressed postpubertally via the hypothalamic–hypophyseal–gonadal axis (Einarsson *et al.*, 1973; Gustafsson & Stenberg, 1976; McEwen, 1976*a*,*b*; Illsley & Lamartiniere, 1980).

Several glutathione *S*-transferases have been separated on the basis of differences in physical properties, and, although individual proteins have been found to catalyse reactions with more than one class of substrate, each protein displays preferred specificity (Habig *et al.*, 1974). Much of the work on this cytosolic drug-metabolizing enzyme system has utilized the substrates 1,2-dichloro-4-nitrobenzene and 1-chloro-2,4-dinitrobenzene. The present paper describes our findings concerning the endocrine control of glutathione *S*-transferases using these substrates.

## Experimental

Studies were performed with birth-dated Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.). The animals were maintained on synthetic diet [NIH (National Institutes of Health) Feed-31] and allowed free access to water. The animals were housed in a controlled environment (temperature 21°C; 12h light/12h dark cycle), weaned at 21–23 days of age and housed (four animals/cage) after weaning. Oestradiol benzoate, testosterone propionate and diethylstilboestrol were purchased from Steraloids, Wilton, NH, U.S.A., and dissolved in propylene glycol. To determine responsiveness of hepatic enzymes to steroid hormones, oestradiol benzoate (10$\mu$g/kg body wt.) or testosterone propionate (2mg/kg body wt.) or propylene glycol was injected subcutaneously into adult animals daily for 1 week before they were killed. Castration of neonatal (day 1) animals ('neonatal castration') was performed on rats hypothermally anaesthetized. Subcutaneous injections of pharmacological doses (1.45$\mu$mol) of testosterone propionate, diethylstilboestrol, or 0.02ml of propylene glycol, were given on day 2 after parturition. Animals in different treatment groups were housed separately. For pituitary-transplantation experiments, either one whole pituitary from age-matched adult animals or four whole pituitaries (equivalent weight) from prepubertal animals were transplanted under the kidney capsule after sodium pentabarbital-induced anaesthesia. Control animals were sham-operated. Hypophysectomized and gonadectomized rats were obtained from Charles River Breeding Laboratories.

Serum prolactin concentrations were determined by radioimmunoassay by Dr. A. F. Parlow, Harbour General Hospital, Torrance, CA, U.S.A., by using N.I.A.M.D.D. Rat PRL-I-3 standard. Serum somatotropin concentrations were determined by Dr. G. A. Mason, University of North Carolina, Chapel Hill, NC, U.S.A., by using N.I.A.M.D.D. Rat GH-RP-1 standard. Exogenously administered rat prolactin (N.I.A.M.D.D. Rat Prolactin-B-1, 7 units/mg) and bovine somatotropin (N.I.H.-GH-B-18, 0.83 unit/mg) to hypophysectomized rats were given by subcutaneous injections in saline (0.9% NaCl) at concentrations of 780$\mu$g/kg body weight and 5mg/kg body weight respectively, twice daily for 8 days, followed by a single injection 2h before the animals were killed. Lesions in the area of the arcuate nucleus of the hypothalamus were induced through intraperitoneal injections of 4mg of monosodium L-glutamate/g body weight on alternate days for the first 10 days of life (Nemeroff *et al.*, 1978). Controls received 10% NaCl (osmotic control) on the same schedule.

Animals were killed by decapitation, allowed to bleed, and their livers were rapidly removed and placed on ice. Liver homogenates (20%, w/v) in 10mM-Tris/HCl, pH7.2, containing 14mM-$MgCl_2$ and 0.6M-KCl, were prepared in a motor-driven glass Potter–Elvehjem homogenizer equipped with a Teflon pestle, and centrifuged at 105000*g* for 60min. These operations were performed at 4°C. This high-speed supernatant was used for enzyme assays. Glutathione *S*-transferase activities were measured as a function of reduced-glutathione conjugation towards the substrates 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as described by Habig *et al.* (1974), with modifications. Assays were conducted at 37°C in 0.1M-potassium phosphate buffer (for pH, see below) and monitored on a Gilford spectrophotometer (model 250). In the assay of transferase activity towards 1-chloro-2,4-dinitrobenzene, the pH was 6.5 and $\Delta A_{340}$ was monitored. In the assay of transferase activity towards 1,2-dichloro-4-nitrobenzene, the pH was 7.5 and $\Delta A_{345}$ was monitored. Electrophilic-substrate concentrations were 1mM, and the glutathione concentration was 5mM. One unit of enzyme activity is defined as the amount catabolizing 1$\mu$mol of substrate/min. Protein determinations were performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistical comparisons between groups were made using the Mann–Whitney U-tests.

## Results

Glutathione conjugation with 1-chloro-2,4-dinitrobenzene is only slightly higher in adult male than in female rats, but with 1,2-dichloro-4-nitrobenzene it is 2–3-fold higher in adult males than in adult females ($P < 0.001$). Gonadectomy of postpubertal male and female rats followed by hormone replenishments was performed in order to gain insight into this sex difference. Castration

and ovariectomy results in no change in glutathione *S*-transferase activities in these adult animals (Fig. 1). Administration of exogenous androgen or oestrogen to the castrated males and oestrogen to the ovariectomized females had no significant effect on activities, but giving exogenous testosterone to adult ovariectomized females caused a significant increase in glutathione conjugation towards 1,2-dichloro-4-nitrobenzene.

We subsequently investigated the role of the pituitary on glutathione *S*-transferase regulation. Although hypophysectomy of male and female rats results in higher glutathione *S*-transferase activities, ectopic pituitaries of age-matched donors (male and female) transplanted under the kidney capsule of hypophysectomized rats were capable of reversing this effect (Figs. 2 and 3). In order to see if pituitary glands from prepubertal rats were capable of causing a similar effect, pituitaries from 21-day-old male rats were transplanted into adult hypophysectomized female rats. In was found that these prepubertal pituitaries were also capable of reversing the effect of hypophysectomy on enzyme activities (Fig. 4). Serum prolactin concentrations have been reported to be low in hypophysectomized animals, but significantly elevated in those hypophysectomized rats that received ectopic pituitaries (Lamartiniere *et al.*, 1979*b*; Illsley & Lamartiniere, 1980). Since prolactin concentrations are inversely related to glutathione *S*-transferase activities, and since prolactin and somatotropin are both regulated

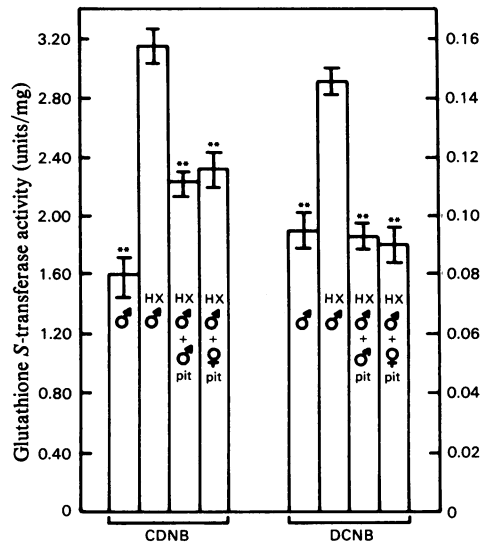


Fig. 2. *Effect of the pituitary gland on hepatic glutathione S-transferase activities in the adult male rat*

Hypophysectomy (HX) was performed on day 21 and transplantation of age-matched pituitaries (pit) on day 56. Animals were killed 8 days later. Glutathione *S*-transferase activities were measured by using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates. Values represent means ± S.E.M. ($n = 8$). $^{**}P < 0.01$ when compared with hypophysectomized males. ♂, Male; ♀, female.

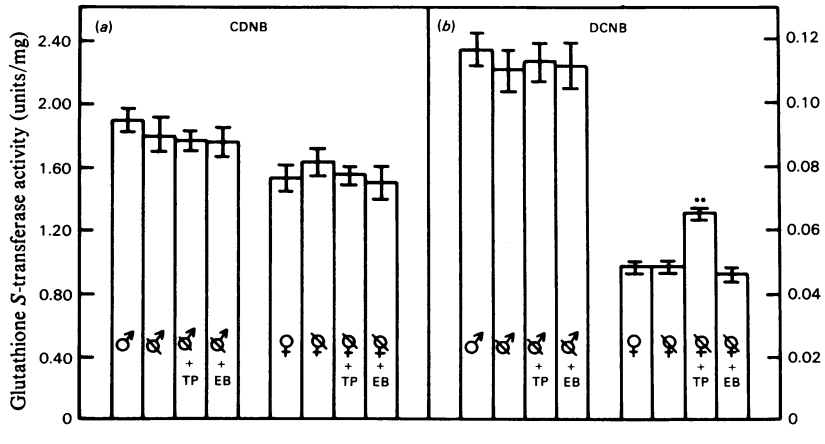


Fig. 1. *Effects of gonadectomy and steroid-hormone replacement on glutathione S-transferase activities*

Rats were gonadectomized on day 35; testosterone propionate (TP), oestradiol benzoate (EB) or propylene glycol was administered subcutaneously daily for 1 week before the animals were killed at day 63. Glutathione *S*-transferase activities were measured by using (*a*) 1-chloro-2,4-dinitrobenzene (CDNB) or (*b*) 1,2-dichloro-4-nitrobenzene (DCNB) as substrates. All values represent means ± S.E.M. ($n = 8$). $^{**}P < 0.01$ when compared with ovariectomized females. ♂ and ♀, Sham-operated males and females; ⚲ and ⚲, gonadectomized males and females.

by hypothalamic inhibiting factors (prolactin inhibiting factor and somatostatin respectively), their actions on glutathione *S*-transferase activities in hypophysectomized rats were investigated (Table 1). Prolactin was not capable of reversing the effect of hypophysectomy. Somatotropin exogenously administered to hypophysectomized rats resulted in decreasing glutathione *S*-transferase activities to values that were similar to those in sham-operated control males.

In an attempt to gain further insight into the sexual differentiation of glutathione *S*-transferases, we investigated effects that neonatal castration of male rats had on adult enzyme activity. Neonatal castration resulted in a significant decrease in glutathione conjugation with 1,2-dichloro-4-nitrobenzene, but not with 1-chloro-2,4-dinitrobenzene

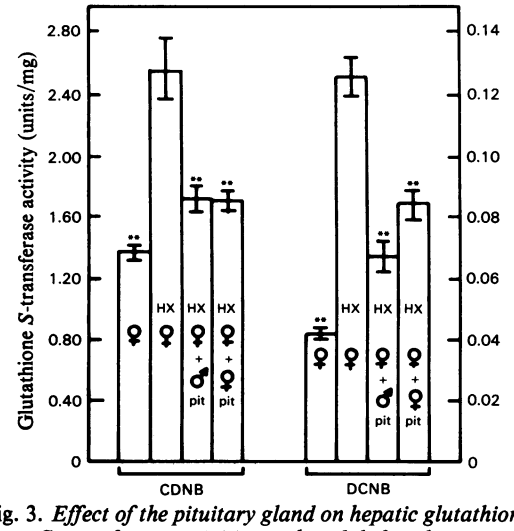


Fig. 3. *Effect of the pituitary gland on hepatic glutathione S-transferase activities in the adult female rat*
Hypophysectomy (HX) was performed on day 21 and transplantation of age-matched pituitaries (pit) on day 56. Animals were killed 8 days later. Glutathione *S*-transferase activities were measured with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates. Values represent means ± S.E.M. ($n = 8$). $^{**}P < 0.01$ when compared with hypophysectomized females. ♂, Male; ♀, female.

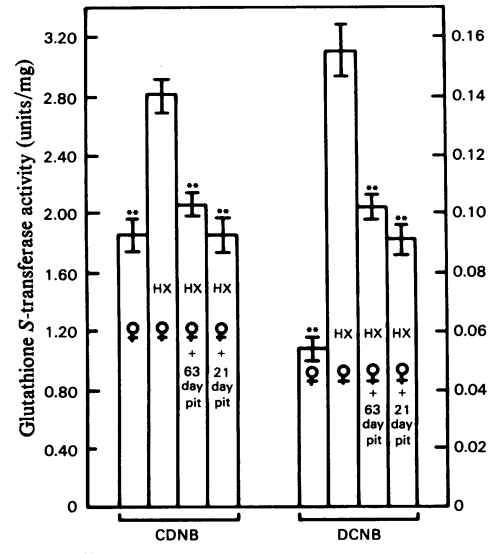


Fig. 4. *Effect of prepubertal pituitary gland on glutathione S-transferase activities in the hypophysectomized female rat*
Hypophysectomy (HX) was performed on day 21 and pituitaries (pit) transplanted on day 63. Either one pituitary from a 63-day-old male rat or four pituitaries from 21-day-old male rats were transplanted per hypophysectomized female (♀) rat. Glutathione *S*-transferase activities were measured with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates. Values represent means ± S.E.M. ($n = 8$). $^{**}P < 0.01$ when compared with hypophysectomized females.

Table 1. *Effects of somatotropin and prolactin on glutathione S-transferase activities in hypophysectomized male rats*
Surgery was performed at 56 days of age. Pituitary-hormone injections were initiated 7 days later and were performed as described in the Experimental section. Values represent means ± S.E.M. ($n = 8$). $^{**}P < 0.01$ when compared with sham-operated and with hypophysectomized + somatotropin.

| | | Glutathione *S*-transferase activity (munits/mg) | |
|---|---|---|---|
| Treatment | Substrate ... | 1-Chloro-2,4-dinitrobenzene | 1,2-Dichloro-4-nitrobenzene |
| Sham-operated | | 1962 ± 157 | 148 ± 10 |
| Hypophysectomized | | | |
| +0.9% NaCl | | 3017 ± 105** | 186 ± 8** |
| +Somatotropin | | 2310 ± 80 | 149 ± 6 |
| +0.9% NaCl | | 3072 ± 84 | 162 ± 8 |
| +Prolactin | | 3288 ± 188 | 176 ± 4 |

(Table 2). Testosterone propionate or diethylstilboesterol administered on day 2 was not capable of reversing the effect of neonatal castration. Prolactin concentrations in these adult rats that were neonatally castrated were significantly decreased. Testosterone propionate (2 mg/kg body wt.) administered in adulthood to those neonatally castrated animals primed on day 1 with testosterone or diethylstilboestrol was also incapable of reconstituting the system (results not shown). These findings demonstrate that, in the rat, the hormonal environment during the neonatal period is critical for the eventual sexual expression of glutathione S-transferase activities in adult animals and that regulation of this differentiation must involve more than the gonads.

In an effort to elucidate the role of the hypothalamus in the pituitary regulation of glutathione S-transferase activities, arcuate-nucleus lesions of the hypothalamus were induced by injections of monosodium L-glutamate into neonatal animals (Nemeroff *et al.*, 1978). Light-microscopic examination of Nissl-stained sections of the medial basal hypothalamus of neonatal rats shortly after monosodium L-glutamate injections revealed considerable damage in the region of the arcuate nucleus. Enzyme measurements in the resulting adult male animals revealed decreased glutathione conjugation with 1,2-dichloro-4-nitrobenzene, but no significant changes in activities with 1-chloro-2,4-dinitrobenzene. Glutathione S-transferase activities in females were not significantly altered. Serum somatotropin concentrations in monosodium L-glutamate-treated male and female rats were drastically decreased (Table 3).

## Discussion

Gonadectomy of postpubertal male and female rats does not result in changes in glutathione S-transferase activities in adult animals, and testosterone administered exogenously to adult castrated males and oestrogen to adult ovariectomized females did not cause significant changes in enzyme activities. These results are similar to those reported by Reyes *et al.* (1971). However, testosterone propionate administered exogenously to postpubertally ovariectomized females results in significantly higher glutathione conjugation with 1,2-dichloro-4-nitrobenzene, not only pointing to a selectivity in enzyme modulation, but suggesting that this may be a result of hepatic target-organ response to steroids due to

Table 2. *Glutathione S-transferase activities and prolactin concentrations in adult male rats after neonatal castration and hormone treatment*

Castrations were performed on day 1, hormone (1.45 μmol) or vehicle was given on day 2 and the animals were killed on day 63. Values represent means ± S.E.M. ($n = 8$). **$P < 0.01$ when compared with sham-operated males.

| | | Glutathione S-transferase activity (munits/mg) | | |
|---|---|---|---|---|
| | Substrate ... | 1-Chloro-2-4-dinitrobenzene | 1,2-Dichloro-4-nitrobenzene | Prolactin (ng/ml) |
| Sex | Treatment | | | |
| Male | Sham-operated | 1697 ± 102 | 123 ± 5 | 50 ± 6 |
| | Castrated | | | |
| | +Propylene glycol | 1652 ± 99 | 86 ± 6** | 26 ± 4** |
| | +Testosterone propionate | 1570 ± 132 | 87 ± 6** | 16 ± 2** |
| | +Diethylstilboestrol | 1801 ± 144 | 85 ± 6** | |
| Female | | 1544 ± 79 | 49 ± 2** | |

Table 3. *Adult glutathione S-transferase activities and somatotropin concentrations in rats treated neonatally with monosodium L-glutamate*

Rats were injected intraperitoneally with 4 mg of monosodium L-glutamate/g body weight or saline (10% NaCl) on alternate days for the first 10 days of life. Animals were killed at 63 days of age. All values represent means ± S.E.M. ($n = 8$). *$P < 0.01$ when compared with saline-treated males; **$P < 0.01$ when compared with saline-treated females.

| | | Glutathione S-transferase activity (munits/mg) | | |
|---|---|---|---|---|
| | Substrate ... | 1-Chloro-2-4-dinitrobenzene | 1,2-Dichloro-4-nitrobenzene | Somatotropin (ng/ml) |
| Sex | Treatment | | | |
| Male | Saline | 2118 ± 88 | 118 ± 3 | 56.4 ± 11.8 |
| | Monosodium L-glutamate | 1894 ± 80 | 58 ± 4* | 4.0 ± 0.2* |
| Female | Saline | 1825 ± 164 | 47 ± 4 | 53.6 ± 6.2 |
| | Monosodium L-glutamate | 1690 ± 43 | 38 ± 3 | 4.0 ± 0.1** |

neonatal programming. We therefore investigated the effect of neonatal androgen deprivation on the resulting adult male rat and found that neonatal castration resulted in lowered glutathione conjugation with 1,2-dichloro-4-nitrobenzene (feminization). This is contrary to results obtained in the adult-castration experiments. Androgen deprivation did not, however, abolish enzyme sexual differentiation, and exogenously administered testoterone propionate or diethylstilboestrol on day 2 with or without androgen replacement for a week before animals were killed did not reverse the effect of day-1 castration. Obviously these gonadectomized animals are without the appropriate cellular secretions throughout their life span, and the hormone-dependent functions of the hypothalamic–hypophyseal–liver axis are therefore affected. Nevertheless, Gustafsson & Stenberg (1974, 1976) have demonstrated that testosterone administered to the neonatal castrated animals can program for a male-type steroid metabolism, and Illsley & Lamartiniere (1980) showed similar results for hepatic monoamine oxidase by using testosterone and diethylstilbesterol.

Reyes *et al.* (1971) demonstrated that hypophysectomy of male rats resulted in an increase of Y protein (glutathione *S*-transferase B or ligandin), whereas Hales & Neims (1976*a*) reported that glutathione conjugation with 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene was increased after hypophysectomy in female rats but not in male rats. Our results demonstrate that hypophysectomy of male and female rats results in increased glutathione conjugation with both substrates. Our investigations furthermore demonstrate significant feminization of hepatic glutathione *S*-transferase activities in hypophysectomized rats by a pituitary transplant placed under the kidney capsule. This suggests that secretion of an inhibiting factor from the pituitary that results in lower glutathione *S*-transferase activities. This is unlike the steroid-metabolizing enzyme system (Gustafsson & Stenberg, 1974; Dieringer *et al.*, 1979), where the pituitary is a positive modulator of some enzyme activities. Since the ectopic pituitary is capable of reversing the effect of hypophysectomy, we conclude that this pituitary negative modulator is not dependent on a hypothalamic releasing factor, at least in a classical sense. The existence of higher glutathione *S*-transferase activities in the adult male suggest that the release of this pituitary factor may be prevented by a hypothalamic inhibiting factor in the male that acts postpubertally on the pituitary to modulate the secretion of this pituitary effector. Our conjecture concerning the action of the hypothalamus and hypophysis is supported by our experiments where feminization of glutathione conjugation with 1,2-dichloro-4-nitrobenzene is observed in male rats treated with monosodium L-glutamate to induce arcuate-nucleus lesions of the hypothalamus. By destroying the neurons of the arcuate nucleus, we might have destroyed the ability to produce a hypothalamic inhibiting factor in the male. The pituitary inhibiting factor is then allowed to be autonomously secreted, the end result being lowered glutathione *S*-transferase activities (Fig. 5). This hypothalamic inhibiting factor may or may not be analogous to the one proposed by Gustafsson & Stenberg (1976). Adult male and female pituitary and prepubertal pituitaries were capable of eliciting the same effect after transplantation into hypophysectomized rats. This suggests that sexual differentiation of glutathione *S*-transferase activities is not dependent on pituitary sexual differentiation or pituitary maturation. The latter finding is contrary to those found for the steroid-metabolizing system (Einarsson *et al.*, 1973; Gustafsson & Stenberg, 1976) and to the pituitary regulation of hepatic monoamine oxidase (Illsley & Lamartiniere, 1980). In these systems, ectopic prepubertal pituitaries are not capable of reversing the effect of hypophysectomy.

Although Gustafsson & Stenberg (1976) have proposed the existence of a novel pituitary factor ('feminotropin') to be involved in the regulation of hepatic steroid metabolism, prolactin and somatotropin have been implicated to be the feminizing factor (Stenberg *et al.*, 1977; Rumbaugh & Colby, 1980). Our results show that exogenously administered prolactin did not reverse the effect of hypophysectomy on glutathione *S*-transferase activities. Coupling this with the fact that low prolactin concentrations were found with low

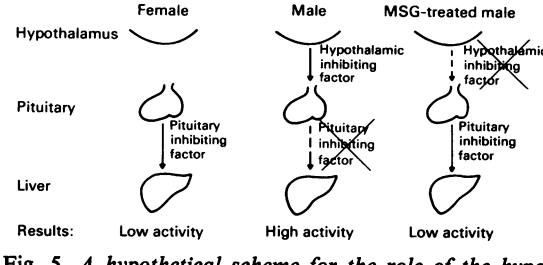


Fig. 5. *A hypothetical scheme for the role of the hypothalamus and pituitary on the regulation of hepatic glutathione S-transferases in the rat*

Left: in the adult female, a pituitary factor modules glutathione *S*-transferases. Centre: in the adult male, a neonatally programmed hypothalamic inhibiting factor modulates the pituitary inhibiting factor. Right: monosodium L-glutamate (MSG)-induced hypothalamic lesions prevent the production of the inhibiting factor, the end result being low glutathione *S*-transferase activities.

glutathione *S*-transferase activities in adult rats that were neonatally castrated, and the inverse relationship between prolactin concentrations and glutathione *S*-transferase activities in hypophysectomized rats with or without ectopic pituitaries, we rule out prolactin as being responsible for the feminization of glutathione *S*-transferase activities. In contrast, exogenously administered somatotropin is capable of reversing the effect of hypophysectomy on the glutathione *S*-transferases (decreased activities); however, decreased glutathione *S*-transferase activities in adult male rats treated neonatally with monosodium L-glutamate are paralleled by significantly decreased somatotropin concentrations, therefore suggesting that somatotropin is also not the feminizing factor. The possibilities exist, however, that prolactin or somatotropin or a novel pituitary hormone may play a role in the hormonal regulation of glutathione *S*-transferases by concerted action with other peripheral organs such as the thyroid or adrenals, or act directly on the liver to mediate specific protein synthesis of hepatic peptides such as somatomedin (Van Wyk & Underwood, 1975) that in turn can regulate glutathione *S*-transferases.

I thank Dr. Etsuko Kita, Mr. Carl Lowe, Mr. Mike Luther and Ms. Brenda Smith for excellent technical assistance, and Dr. G. W. Lucier for advice during this project.

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