Enzymes of mercapturic acid production in rat mammary gland

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1. The enzymes glutathione *S*-transferase, γ -glutamyl peptidyltransferase and dipeptidase, which participate in the detoxification pathway through mercapturic acid production, were measured in rat mammary gland during pregnancy and lactation. 2. Mammary-gland concentration of reduced glutathione showed, concomitantly with the enzyme activities, a significant increase during lactation. 3. The mammary-gland glutathione *S*-transferase exhibits characteristics quite similar to those described for the liver and kidney enzymes with respect to substrates, isoenzymes, molecular weight and probenecid and bilirubin inhibition. 4. In view of these similarities, mammary-gland glutathione *S*-transferase may play the same role as a cytoplasmic organic-anion receptor proposed for the hepatic enzyme. It may also represent a detoxification pathway for protecting the mammary tissue during the lactogenic cycle.

Among the several known physiological functions of GSH in higher animals are the detoxification reactions for a great variety of electrophilic compounds, many of them carcinogenic (Chasseaud, 1979; Tate, 1980). The detoxification pathway is initiated by glutathione *S*-transferase, which exists as a set of isoenzymes that not only catalyse the conjugation of GSH to those compounds but also bind a variety of organic anions (Boyland & Chasseaud, 1969; Habig *et al.*, 1974; Jakoby & Habig, 1980). The final detoxification product is mercapturic acid, which is eliminated.

Amino acid transport, as proposed by Meister (1973), is another important physiological process in which GSH takes part. In this case GSH is the acceptor of the entering amino acid in a reaction catalysed by the membrane enzyme γ -glutamyl peptidyltransferase, and the product is a γ -glutamyl-amino acid. GSH is regenerated through this γ -glutamyl cycle of six reactions. Some enzymes, such as γ -glutamyl peptidyltransferase and cysteinylglycine dipeptidase, participate both in the γ -glutamyl cycle and in the detoxification pathway to mercapturic acid.

The detoxification pathway has been described in liver, but glutathione *S*-transferase activities similar to that described in liver, with similar isoenzymes, substrate specificity, molecular weight etc., have also been identified in kidney and intestinal epithelium (Kaplowitz *et al.*, 1975, 1976).

We have previously described the presence of γ -glutamyl peptidyltransferase and the main enzymes of the γ -glutamyl cycle in mammary gland during the lactogenic cycle (Puente *et al.*, 1979, 1982). The present paper details properties of rat mammary-gland glutathione *S*-transferases, their relation to the detoxification pathway and the probable protective function in the tissue during the lactogenic cycle.

Experimental

Virgin and primiparous Sprague–Dawley rats (body wt. 150–200 g), 100–120 days old, at different stages of the lactogenic cycle were used; pregnancy was assayed by the observation of spermatozoa in the vaginal fluid. During lactation the rats were maintained with eight to ten pups. The animals were killed by decapitation after light ether anaesthesia.

The enzyme assays were performed in 20% (w/v) homogenates prepared in 10 mM-sodium phosphate buffer, pH 7.4, and centrifuged at 105 000 g for 1 h.

For determination of molecular weight, the 105 000 g supernatant was applied to a Sephadex G-200 column (1.5 cm $\times$ 50 cm) equilibrated with 0.1 M-phosphate buffer, pH 7.4. Five proteins of known molecular weights (cytochrome *c*, ovalbumin, bovine serum albumin, lactate dehydrogenase and pyruvate kinase) were used to calibrate the column.

Glutathione *S*-transferase activity was assayed in

Abbreviation used: GSH, reduced glutathione.

the 105 000 *g* supernatants and in the eluates from the Sephadex G-200 and CM-cellulose (see below) columns by using an assay medium with 1.0 mM 1-chloro-2,4-dinitrobenzene and 5.0 mM-GSH in 0.1 M-potassium phosphate buffer, pH 6.5, at 25°C in a double-beam spectrophotometer (Shimadzu-190) at 340 nm (Habig *et al.*, 1974). Enzyme activity is expressed as μmol of product formed/min at 25°C per g of tissue (units/g). The glutathione *S*-transferase activity was also assayed with benzyl chloride and methyl iodide as substrates and measurement of the disappearance of the free thiol groups from GSH with Ellman's reagent (Ellman, 1959). Results are expressed as indicated above.

γ -Glutamyl peptidyltransferase activity was determined in whole homogenates with L- γ -glutamate *p*-nitroanilide as the donor substrate for the γ -glutamyl group and glycylglycine as the acceptor (Tate & Meister, 1974). The dipeptidase activity was assayed in the 105 000 *g* supernatant with alanyl-glycine as the substrate and determination of the amino acids produced with the reagent ninhydrin. Both enzyme activities are expressed in units ($\mu\text{mol}/\text{min}$)/g.

Kinetics determinations were made with 1-chloro-2,4-dinitrobenzene as the substrate, and the Michaelis constant (K_m) was calculated from the abscissa intercept of plots of least-squares-regression equations.

Inhibition kinetics were studied with probenecid and bilirubin by the method of Dixon (1953). The inhibitory constants (K_i) were calculated from the least-squares-regression equations and represent the mean intersection of three lines.

Isoenzymes were studied by using the procedure of Habig *et al.* (1974), with 20 g of lactating gland (at 15–20 days of lactation). The isoenzyme pattern was obtained from a CM-cellulose column eluted with a linear gradient of KCl.

Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, and by absorbance measurements at 280 nm for column eluates.

The reagents 1-chloro-2,4-dinitrobenzene, probenecid, bilirubin, L- γ -glutamate *p*-nitroanilide, glycylglycine, alanylglycine, GSH, Sephadex G-200 and CM-cellulose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Benzyl chloride and methyl iodide were from Riedel-De Haen, Seelze-Hannover, West Germany.

Results

Fig. 1 shows glutathione *S*-transferase activity in mammary-gland tissue during the lactogenic cycle. It can be seen that increases in both the enzyme activity and the GSH concentration occur during

Fig. 1. Glutathione *S*-transferase activity and GSH concentration during the lactogenic cycle in rat mammary gland

The glutathione *S*-transferase activity (●) was assayed in a reaction mixture (1.0 ml) containing 0.1 M-potassium phosphate buffer, pH 6.5, 10.0 mM-GSH and 1.0 mM-1-chloro-2,4-dinitrobenzene; the reaction was initiated by the addition of 100 μl of mammary-gland 105 000 *g* supernatant obtained at different stages of the lactogenic cycle. GSH concentrations (○) were determined in 5% HClO_4 -treated homogenates (1:5, w/v). The reaction mixture (2.5 ml) contained 0.5 M-potassium phosphate buffer, pH 6.8, ascorbic acid (1 g/l), glyoxylic acid (0.5 g/l) and 0.5 ml of HClO_4 -treated homogenate. This mixture was incubated at 60°C for 5 min, and then 0.5 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (1.5 g/l) was added. The resultant colour was determined at 412 nm. Each value represents the mean for five animals $\pm$ S.E.M.

pregnancy, but near parturition the GSH concentration decreases, attaining a value similar to that of the mammary gland of virgin rats. At day 20 of lactation the GSH concentration has increased to about 3.5 mM and glutathione *S*-transferase activity to about 43 units/g with 1-chloro-2,4-dinitrobenzene as the substrate. The enzyme activity was 0.83 and 2.34 units/g respectively with benzyl chloride and methyl iodide as the substrates.

Table 1. Activities of enzymes of mercapturic acid formation in rat mammary gland during the lactogenic cycle. Assay conditions were as described in the text. Results are given as the mean values $\pm$ S.E.M.; each value represents the mean for five animals. All the activities during pregnancy and lactation were significantly larger than in virgin rats ($P < 0.05$).

Enzyme	Activity (units/g)		
	Virgin	Pregnancy	Lactation
Glutathione <i>S</i> -transferase	0.98 $\pm$ 0.09	7.73 $\pm$ 0.75	42.50 $\pm$ 3.12
γ -Glutamyl peptidyltransferase	0.24 $\pm$ 0.03	2.20 $\pm$ 0.16	16.40 $\pm$ 2.75
Dipeptidase	0.37 $\pm$ 0.07	0.74 $\pm$ 0.08	1.31 $\pm$ 0.15

Fig. 2. Glutathione *S*-transferase isoenzymes from rat mammary gland

A 20 g portion of lactating mammary gland was homogenized in 60 ml of distilled water and then centrifuged at 10000 *g* for 1.5 h. The supernatant was passed through glass-wool to remove the fat and then applied to a DEAE-cellulose column (40 cm $\times$ 3 cm) equilibrated with 10.0 mM-Tris/HCl buffer, pH 8.0. The eluate was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (660 g/l) and centrifuged at 10000 *g* for 30 min. The precipitate was dissolved in 10.0 ml of 10.0 mM-potassium phosphate buffer, pH 6.7 (buffer A), and dialysed for 24 h against the same buffer. The dialysed solution was applied to a CM-cellulose column (35 cm $\times$ 3 cm) equilibrated with buffer A and, after being washed with 300 ml of the same buffer, the column was eluted with a linear gradient of 0–75 mM-KCl, which allowed the separation of the isoenzymes. The glutathione *S*-transferases were assayed with 1-chloro-2,4-dinitrobenzene as substrate (●) as described in the legend to Fig. 1. ○. A_{280} .

Table 1 gives the activities of glutathione *S*-transferase and of enzymes of the γ -glutamyl cycle participating in detoxification by the mercapturic acid pathway. Dipeptidase activity increases about 4-fold during lactation, whereas glutathione *S*-transferase and γ -glutamyl peptidyltransferase activities increase 40–60-fold.

Gel-filtration chromatography of the 105000 *g* supernatant indicated a molecular weight of about 46000 for rat mammary-gland glutathione *S*-transferase.

Fig. 2 shows the isoenzyme pattern for mammary-gland glutathione *S*-transferase eluted from a CM-cellulose column. Six distinct peaks of activity were observed with 1-chloro-2,4-dinitrobenzene as the substrate.

The kinetics of glutathione *S*-transferase were studied with the supernatant of lactating mammary gland as the source of enzyme. A K_m value of 0.55 mM was obtained. Both probenecid and bilirubin were competitive inhibitors of the enzyme, the K_i values for these organic anions being 1.56 mM and 0.05 mM respectively.

Discussion

The glutathione *S*-transferases constitute a group of soluble enzymes whose function is to catalyse the detoxification of electrophilic drugs and some carcinogens by their conjugation with GSH. Also, some isoenzymes, such as ligandin (hepatic isoenzyme B), are capable of binding non-covalently a large number of compounds, including bilirubin, haem, dyes, certain steroids etc. (Chasseaud, 1979). These enzymes are widely distributed in liver, kidney, erythrocytes, intestinal epithelium etc., tissues that are also characterized by a high concentration of GSH (Boyland & Chasseaud, 1969; Beutler, 1971; Kaplowitz *et al.*, 1975).

We have demonstrated the presence in mammary gland of glutathione *S*-transferases that show a marked increase in activity during the lactogenic cycle paralleled by a significant increase in GSH concentration. At weaning both values reach the same range observed in the mammary gland of the virgin rat (results not shown). GSH concentration

shows a fall at about the time of parturition, for reasons currently unknown.

In liver, the reaction catalysed by glutathione *S*-transferase represents the initiation of a detoxification pathway that shares some steps with the γ -glutamyl cycle. Table 1 shows that the activities of glutathione *S*-transferase as well as of γ -glutamyl peptidyltransferase and dipeptidase increase during lactation, suggesting that this pathway is also present in mammary gland.

The substrates conjugated with GSH by the reaction catalysed by glutathione *S*-transferase are of varied nature. The mammary-gland enzyme has six isoenzymes, with aryl- and alkyl-transferase activities, a situation very much akin to that of liver. If we use the same system as that used for designation of the liver isoenzymes (Habig *et al.*, 1974), namely AA, A, B, C, D and E, in the reverse order of elution from the CM-cellulose column, then isoenzyme B is the major form in mammary gland and this corresponds to hepatic ligandin; isoenzyme E is present only in minute amounts. This suggestion is supported by the characteristics of binding of the organic anions bilirubin and probenecid, which are similar to those for the corresponding hepatic and renal glutathione *S*-transferases; in addition, the enzymes are competitively inhibited by these anions.

It is possible that the presence of these isoenzymes in mammary gland has something to do with a protective role, although there is no definite proof of this. Rats at the age of about 60 days are the most susceptible to tumour induction by hydrocarbons such as 9,10-dimethyl-1,2-benzanthracene and 3-methylcholanthrene (Dao, 1969). Although their concentration in the mammary gland is very low in such experiments, it is important to emphasize that the gland is refractory to their action during pregnancy and lactation (Dao *et al.*, 1960), periods

in which both GSH concentration and glutathione *S*-transferase activity are elevated.

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