Biochem. J. (1981) **198**, 243–246
Printed in Great Britain

Glutathione utilization by lactating bovine mammary secretory tissue *in vitro*

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(*Received 2 March 1981/Accepted 28 April 1981*)

γ-Glutamyltransferase (D-glutamyl transpeptidase, EC 2.3.2.2) activity has been shown to be located predominantly on the extracellular surface of the plasma membrane of lactating bovine mammary cells. Radioactive label from both oxidized ([^{14}C]-γ-glutamyl) and reduced ([^{35}S]cysteinyl) glutathione was taken up and incorporated into acid-precipitable proteins of mammary tissue. Uptake was shown to involve the transport of free amino acids, and incorporation was shown to involve the action of γ-glutamyltransferase. These results indicate that lactating mammary tissue utilizes the constituent amino acids of glutathione for milk-protein synthesis.

GSH (γ-L-glutamyl-L-cysteinylglycine) is distributed widely in most animals, plants and microorganisms (Meister & Tate, 1976). Because GSH is usually the most abundant intracellular thiol and γ-glutamyl compound (with the possible exception of glutamine), there has been much interest as to the cellular function of this molecule (Meister & Tate, 1976). In erythrocytes, GSH functions by protecting the cell from oxidizing agents (Smith, 1974), and it is probable that it has this function in all cells. However, because GSH turnover is rapid in some tissues [$t_{\frac{1}{2}} = 30$ min in kidney (Sekura & Meister, 1974)], it appears to have additional functions. Meister (1973) originally proposed that GSH might function in amino acid transport via membrane-bound γ-glutamyltransferase (for a review, see Meister *et al.*, 1976). However, recent evidence on the cellular localization and mode of action of γ-glutamyltransferase has led to a new hypothesis involving the function of this enzyme.

γ-Glutamyltransferase is located primarily on the plasma membrane of cells, with its active site (for GSH) oriented toward the extracellular surface (Hahn *et al.*, 1978). Although γ-glutamyltransferase is also present on internal cell membranes of certain cells (Marathe *et al.*, 1978), it is unclear whether this represents functional enzyme or simply processing of newly synthesized enzyme.

GSH has been shown to be transported out of certain cells, and this translocation appears to be necessary for GSH degradation (Griffith & Meister, 1979*a*; Griffith *et al.*, 1979). Certain cells such as hepatocytes lack substantial γ-glutamyltransferase activity, but have been shown to translocate GSH. It has been suggested that the translocated GSH from liver is carried by the blood and utilized by tissues with high γ-glutamyltransferase activity (Griffith & Meister, 1979*b*). Kinetic evidence suggests that the reaction of GSH with γ-glutamyltransferase results in degradation of GSH without the occurrence of transpeptidation (Elce & Broxmeyer, 1976). Thus the role of plasma-membrane γ-glutamyltransferase in mammalian tissues appears to be the degradation of blood GSH, resulting in the release of its constituent amino acids (Curthoys & Hughey, 1979).

Mammary tissues of cow and rat contain high γ-glutamyltransferase activity compared with that in other tissues (Baumrucker & Pocius, 1978). Mammary γ-glutamyltransferase activity has been shown to increase at the onset of lactation in the rat, and this increase appears to be hormonally regulated (Puente *et al.*, 1979; Pocius *et al.*, 1980). Recently, Pocius *et al.* (1981) have reported that there is an arterio-venous difference in GSH concentration across the bovine mammary gland.

This potential uptake (disappearance) of GSH provided the first evidence of a sufficient cysteine source for milk-protein synthesis. Previous studies were never able to account for the amount of cysteine in milk (Clark *et al.*, 1978). However, because only whole blood, and not plasma, showed significant arterio-venous differences of GSH concentration across the gland (Pocius *et al.*, 1981),

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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0306-3283/81/070243-04$01.50/1

questions still remain as to the availability and use of GSH by mammary tissue. We report here experiments designed to study the uptake and utilization of GSH by bovine mammary tissue and cells *in vitro*. These studies suggest that GSH can be used by mammary tissue as a source of amino acids for milk-protein synthesis.

Materials and methods

Materials

γ-Glutamic acid *p*-nitroanilide, glycylglycine, buffers, amino acids, insulin and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase (Type III) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Medium 199 was from Grand Island Biological Co., Grand Island, NY, U.S.A. Radioactive compounds were from New England Nuclear Corp., Boston, MA, U.S.A. Radioactive compounds were GSSG ([^{14}C]glutamate), GSH ([^{35}S]cysteine) or [^{3}H]isoleucine. Filters and apparatus were from Millipore Corp., Bedford, MA, U.S.A.

Methods

Bovine mammary tissue was obtained at slaughter from lactating Holstein (Friesian) cows producing a minimum of 12kg of milk/day. Uptake of GSH was measured by using bovine mammary tissue slices as described previously (Pocius & Baumrucker, 1980). Tissue slices (0.2–0.4g) were pre-incubated at 37°C for 10min in a modified Krebs–Ringer bicarbonate (KRB) buffer (5ml) under an O_2/CO_2 (19:1) atmosphere. The KRB buffer, pH7.4, contained NaCl (118mM), KCl (4.74mM), $CaCl_2$ (2·54mM), $KHPO_4$ (1.18mM), $MgSO_4$ (1.18mM), $NaHCO_3$ (24.9mM), sodium acetate (10mM), glucose (10mM) and insulin (0.1i.u./ml). After preincubation, 100μl of KRB buffer containing GSH or -GSSG (0.02–0.05 μCi) was added to the medium so that each slice was exposed to 0.1mM-GSH or GSSG. Slices were incubated at 37°C for various times under an O_2/CO_2 (19:1) atmosphere. Methods for termination of incubation and calculation of uptake were as described previously (Pocius & Baumrucker, 1980). Uptake is expressed as μmol/ml of cell water, and was corrected for substrate present in the extracellular space.

Bovine mammary acini were prepared by a modification of the procedure of Katz *et al.* (1974), as follows. Finely diced mammary tissue was digested in Hank's balanced salt (HBS) solution containing 10mM-Hepes, pH7.4, and 400 units of Type III collagenase/ml. Digestion was for 2h at 37°C in a shaking incubator. The digest was filtered sequentially through a stocking nylon then a 140μm-pore-size nylon mesh. Acini were collected by centrifugation and suspended in HBS solution for incorporation studies. Amino-acid-incorporation experiments were conducted with 0.2–0.5mg of acini protein/ml ($\simeq 1\times10^4$–3×10^4 acini/ml) in Medium 199 (37°C) containing 10mM-Hepes, pH7.4, 50i.u. of benzylpenicillin sodium/ml, 0.25mg of streptomycin sulphate/ml plus the addition of radioactive substrates. GSH or GSSG (0.5μCi/ml) was added to make a final medium concentration of 0.8–1.0μg/ml. Isoleucine (0.3μCi/ml) was used as a control in amino-acid-incorporation experiments. Incorporation experiments were terminated by Millipore filtration (25mm × 0.45μm pore size), and acini were washed three times with 1ml of Medium 199 (37°C) under vacuum. The filters containing acini were washed twice with 1ml of cold 10% (w/v) $HClO_4$ (2min without vacuum, each), followed by vacuum filtration. Filters were dried and counted for radioactivity in 10ml of Tritosol (Fricke, 1975).

γ-Glutamyltransferase was assayed with the artificial substrate γ-glutamic acid *p*-nitroanilide as described previously (Baumrucker & Pocius, 1978). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results and discussion

Table 1 shows the specific activity of γ-glutamyltransferase for mammary-tissue total homogenate, intact acini and sonicated acini. The higher activity in intact acini compared with that in the homogenate was probably due to loss of non-alveolar protein. The enzyme activity of intact acini represents 75% of the total secretory-cell γ-glutamyltransferase activity (sonicated acini). Previous studies have shown that acini from lactating mammary tissue are viable (Park *et al.*, 1979*a*,*b*) and maintain intact tight junctions (O'Brien *et al.*, 1981). It has also been shown that the substrate, γ-glutamine *p*-nitroanilide, does not penetrate cell membranes (Inoue *et al.*, 1977). Thus the data suggest that 75% of the γ-glutamyltransferase activity is located on the exterior membrane surface of lactating mammary secretory cells. Because the plasma membranes of these cells represent only a small percentage of total cell membranes (Keenan *et al.*, 1974, 1978), the data imply that γ-glu-

Table 1. *γ-Glutamyltransferase activity of bovine mammary tissue and collagenase-isolated acini*

The specific activity is expressed as substrate (γ-glutamic acid *p*-nitroanilide) utilized/min per mg of protein (±S.E.M.). Data are from tissue and acini preparations from three separate animals.

Source	Specific activity
Mammary-tissue homogenate	0.165 ± 0.037
Intact acini	0.182 ± 0.031
Sonicated acini	0.242 ± 0.028

tamyltransferase is concentrated on the extracellular surface of mammary cells. This is also true of other cells, such as intestinal epithelial cells (Marathe *et al.*, 1979).

The presence of some internal enzyme activity (Table 1; activity in sonicated acini represents a 25% increase over that in intact acini) may explain the occurrence of this enzyme in milk membranes (milk-fat-globule and skim membranes) as previously reported (Baumrucker, 1979). Milk membranes originate from apical plasma membrane and other intracellular membranes (i.e. secretory vesicles and Golgi apparatus) (Keenan *et al.*, 1974). In other cells it was speculated that the occurrence of intracellular activity was the result of synthesis and replacement of plasma-membrane enzyme, little or no functional significance being attributed to intracellular enzyme activity. With this concept in mind, it is surprising that such high γ-glutamyltransferase activities are secreted in milk (Baumrucker, 1979).

The extracellular occurrence of γ-glutamyltransferase activity (Table 1) suggests that mammary secretory cells have the potential to hydrolyse blood GSH. By using bovine mammary-tissue, we tested the ability of mammary slices to take up GSH or its constituent amino acids. Fig. 1 shows that the radioactive labels from both GSSG and GSH are concentrated in the cell water of mammary slices after 30 min. Recoveries of the isotope in the soluble fractions (Pocius & Baumrucker, 1980) were 78% for GSSG and 96% for GSH after 3 h incubation. This suggests that under these experimental conditions, radioactive label was not being significantly removed by protein synthesis.

After 1 h of incubation, the cellular concentration of GSSG was 2–3-fold greater than GSH (Fig. 1). After 3 h of incubation, this ratio was approx. 1.5. This may reflect the preference of γ-glutamyltransferase for oxidized substrate, as reported with other tissues (Griffith & Meister, 1979*a*,*b*). However, because GSSG provides 2 mol of free glutamate/mol of GSSG, the higher uptake of glutamate compared with cysteine may also be due to a concentration effect.

Previous studies have suggested that the main physiological function of γ-glutamyltransferase is the hydrolysis of GSH in the extracellular space (Griffith & Meister, 1979*b*; Curthoys & Hughey, 1979). Fig. 1 shows that concentrative uptake of radioactive label from both GSSG and GSH was prevented when the incubation media contained excess free unlabelled glutamate and cysteine respectively. This suggests that the unlabelled excess free amino acids saturated amino-acid-transport systems, preventing the concentrative uptake of the labelled amino acids from the GSH source and that the free amino acids from GSH are taken up after extracellular GSH hydrolysis by γ-glutamyltransferase.

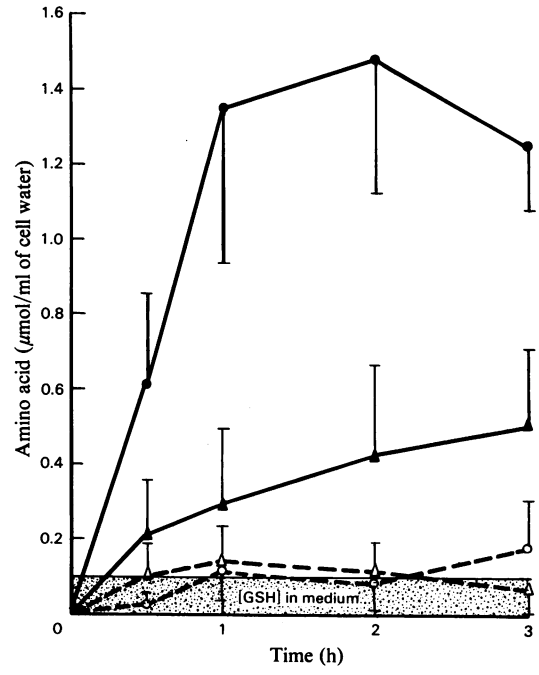

Fig. 1. *Uptake of glutathione by bovine mammary slices*
Media was modified Krebs–Ringer bicarbonate (see under 'Methods') containing radioactive label (*) in GSSG or GSH (see under 'Materials'); ●, 0.1 mM-*GSSG*; ○, 0.1 mM-*GSSG* and 20 mM-glutamate; ●, 0.1 mM-GS*H; and △, 0.1 mM-GS*H and 20 mM-cysteine. The results are means ± S.D. for at least nine slices from two separate animals.

Although tissue slices showed concentrative uptake of GSH, these experiments were conducted with relatively high concentrations of GSH (0.1 mM) when compared with those in blood plasma (≃0.001 mM). Fig. 2 shows that acini preparations incorporate the ^{35}S label from GSH into acid-precipitable proteins when substrates are provided to promote protein synthesis. Similar results were obtained with GSSG as a substrate (results not shown). For incorporation experiments the concentration of GSH was that reported for bovine intercellular fluid (1.0 μg/ml; Pocius *et al.*, 1981). Fig. 2 also shows that the inclusion of serine plus borate, an established inhibitor of γ-glutamyltransferase activity (Marczewska & Szewczuk, 1971), decreases the incorporation of amino acids from glutathione while not inhibiting isoleucine incorporation. Serine or borate alone had no effect on isoleucine or GSH-amino-acid incorporation (results not shown). The large difference in incorporation between isoleucine and cysteine (from glutathione) may be explained by the relatively lower amount of cysteine and cystine compared with isoleucine in milk proteins (Gordon & Kalan, 1978).

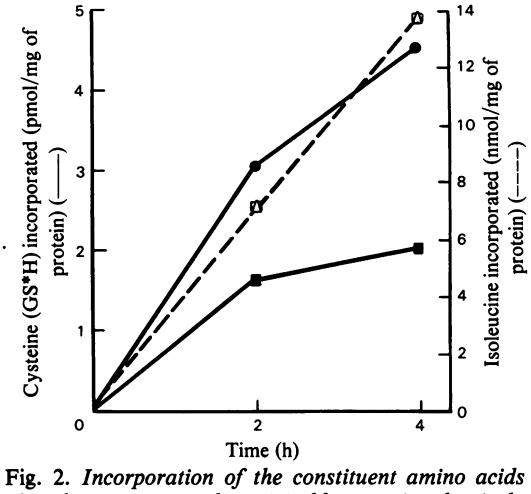

Fig. 2. *Incorporation of the constituent amino acids of glutathione into acid-precipitable proteins by isolated mammary acini*

Collagenase-isolated acini were incubated in Medium 199 plus the addition of [*cysteine*-^{35}S]-GSH (GS*H; 0.8–1.0 μg/ml). [^{3}H]Isoleucine was added to label the isoleucine pool in Medium 199 without altering the concentration significantly. The results are amounts of amino acids (pmol of cysteine or nmol of isoleucine) occurring in acid-precipitable proteins per mg of acini protein. ●, GS*H; ■, GS*H plus 20mM-serine and -borate; △, isoleucine; and □, isoleucine plus 20mM-serine and -borate as a control.

The timing and the magnitude of changes in γ-GTPase activity during lactogenesis suggest that this enzyme plays an important role in milk synthesis (Pocius *et al.*, 1980). The present study demonstrates that lactating bovine mammary cells have γ-glutamyltransferase situated on their extracellular surface and therefore have the potential to hydrolyse blood glutathione. This evidence is consistent with our previous findings of the disappearance of glutathione from blood passing the mammary gland (Pocius *et al.*, 1981). The incorporation of the constituent amino acids of GSH into mammary-cell proteins suggests that they may be utilized as a source for secretory protein synthesis.

This research was supported by a University of Illinois Agriculture Experiment Station project and National Institutes of Health grant no. HEW PHS HD 10942.

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