A and negate the effect of the buffer anions on decreasing amounts of RNase A.

The phenomenon of anion binding to proteins is common[5-8]. RNase A contains 4 arginine residues and 11 lysine residues[9] and the positively charged side groups on these residues may interact with the phosphate groups in substrates. In addition, the binding of phosphate from buffers has been demonstrated by dialysis and gel filtration experiments[10]. The paper electrophoresis experiments of Crestfield and Allen[11] demonstrated a marked decrease in the isoelectric pH (from 7.8 to 5.9) as the ionic strength of phosphate buffer was increased. When the ionic strength was held constant, the isoelectric pH value in phosphate buffer was 6.4 whereas in ammonia-ammonium acetate buffer it was 9.1 These data demonstrated the differential effect of buffer ions on the mobility of RNase A. Electrophoretic experiments by a number of other authors[12-15] demonstrate the variation of isolelectric pH with changing ionic strength of various buffers. Comparison of the isoelectric pH values determined from RNase A mobility vs pH curves with the pI values determined by isoelectric focusing also suggest that the unusually low isoelectric pH values determined for RNase A are artifacts owing to interaction with buffer anions.

Our present results with RNase A provide a caveat in the use of electrophoretic mobility to discriminate proteins. We suggest that the phenomenon portrayed here may be particularly likely to occur with other RNase enzymes, and perhaps other cationic proteins, present at low concentrations in comparison to the buffer ions in electrophoresis. The use of equally sensitive zymogram techniques for other enzymes or improved methods of protein detection should allow a determination of whether this phenomenon is relatively common or is as rare as presently appreciated.

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2 A. Chrambach, R.A. Reisfeld, M. Wyckoff and J. Zaccari, Analyt. Biochem. *20*, 150 (1967).
3 E.S. Vessell, Nature *195*, 497 (1962).
4 N. Ressler, J.L. Schulz and R.R. Joseph, Nature *198*, 888 (1963).
5 R.A. Alberty, in: The Proteins, vol. 1, part A, p. 461. Academic Press, New York 1953.
6 I.M. Koltz, in: The Proteins, vol. 1, part B, p. 727. Academic Press, New York 1953.
7 J. Steinhardt and S. Beychok, in: The Proteins, vol. 2, p. 139. Academic Press, New York 1964.
8 J.F. Riordan, Molec. cell. Biochem. *26*, 71 (1979).
9 F.M. Richards and H.W. Wyckoff, in: The Enzymes, 3rd edn, vol. 4, p. 647. Academic Press, New York 1971.
10 J. Bartholeyns, D. Wang, P. Blackburn, G. Wilson, S. Moore and W.H. Stein, Int. J. Pept. Prot. Res. *10*, 172 (1977).
11 A.M. Crestfield and F.W. Allen, J. biol. Chem. *211*, 363 (1954).
12 L.B. Barnett and H.B. Bull, J. Am. chem. Soc. *81*, 5133 (1959).
13 A.A. Hakim, Biochim. biophys. Acta *20*, 581 (1956).
14 I.D. Raacke, Archs Biochem. Biophys. *62*, 184 (1956).
15 L.B. Barnett and H.B. Bull, Archs Biochem. Biophys. *89*, 167 (1960).
16 R.A. Reisfeld, J.J. Lewis and D.E. Williams, Nature *195*, 281 (1962).

Properties of γ-glutamyltranspeptidase, and glutathione levels in rat mammary gland

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Summary. γ-Glutamyltranspeptidase activity and glutathione levels were studied in rat mammary gland during the lactogenic cycle; both increased during mid-lactation. The enzyme's specific activity with several amino acids showed that glutamine and methionine were the best substrates. Maleate decreased the transpeptidation reaction and increased the hydrolytic activity. These results suggest that γ-glutamyltranspeptidase from the mammary gland is similar to the enzyme described in other tissues in relation to these properties and the physiological role proposed in amino acids transport.

γ-Glutamyltranspeptidase is a widely distributed enzyme which has been specially studied in kidney[1]. As it is a membrane-bound enzyme, an important role in the transport of amino acids into the cells has been atributed to it; this is achieved in a cycle of six enzymatic reactions known as the γ-glutamyl cycle[2]. The natural substrate postulated for the enzyme is reduced glutathione (GSH), a metabolite which is found in high concentrations in tissues like liver, kidneys, the intestinal mucous villi, erythrocytes, etc., which contain some or all of the cycle enzymes.

The metabolism of this tripeptide, the functions of which are the protection of −SH groups, detoxification, the provision of a reservoir of cysteine, etc.[3,4], has not been studied in the mammary gland. In a previous paper we described the hormonal dependence of γ-glutamyltranspeptidase of rat mammary gland[5]; now we present a study of the activity of the partially purified enzyme with some L-amino acids as substrates; the effect of maleate on enzyme activity, and the GSH levels in the lactogenic cycle.

Materials and methods. L-γ-Glutamyl-p-nitroanilide, GSH and glycylglycine were obtained from Sigma Chemical Co.; L-U-^{14}C-glutamic acid from the Radiochemical Centre, Amersham.

Primiparous Sprague-Dawley rats (150–200 g b.wt) were taken at different stages of the lactogenic cycle. During lactation the rats were maintained with 8–10 pups.

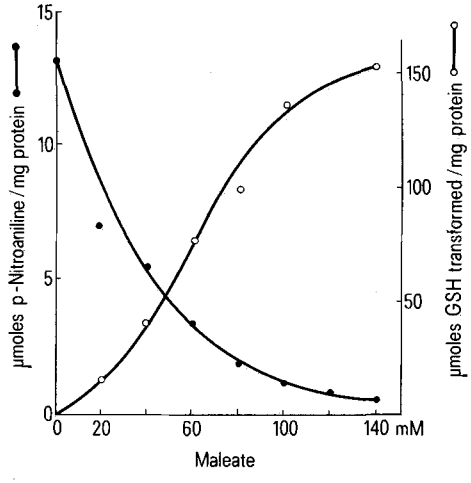
The enzyme assay was carried out using L-γ-glutamyl-p-nitroanilide as donor and glycylglycine as acceptor[6]. The enzyme activity was also assayed with GSH as the γ-glutamyl moiety donor, and glycylglycine and several amino acids as acceptor substrates; when L-U-^{14}C-glutamic acid was used as the acceptor, the transpeptidation product was isolated by paper electrophoresis[6] and counted in a Searle Delta 300 scientillation counter. GSH was assayed as described by Ball[7] and proteins were determined by the Lowry method[8]. Enzymatic activity for all methods is expressed as μmoles of product formed (or γ-glutamyl donor utilized) per min at 37 °C (units); specific activity is expressed as units per mg of protein.

The enzyme was purified from lactating mammary gland using the method already described for the kidney enzyme[9]. For the kinetic studies the double reciprocal plot of

Table 1. Activity of mammary gland γ-glutamyltranspeptidase toward various amino acid acceptors, and kinetic parameters for both γ-glutamyl donors studied

Acceptor		Specific activity (U/mg protein) γ-glutamyl donor L-γ-glutamyl-p-nitroanilide (2.5 mM)	GSH (5.0 mM)
None (control)		1.12	6.80
L-methione	20 mM	1.86	11.13
L-glutamine	20 mM	1.77	27.93
L-alanine	20 mM	1.23	15.67
L-glutamate	20 mM	1.46	6.90
L-arginine	20 mM	1.31	5.50
L-phenylalanine	20 mM	1.26	6.30
L-valine	20 mM	1.19	6.67
L-aspartate	20 mM	1.43	15.83
L-serine	20 mM	1.48	13.20
K_m GSH[a]	1.0 mM	V_{max} 90.9 µmoles/mg	
K_m L-γ-glutamyl-p-nitroanilide[a]	0.6 mM	V_{max} 11.1 µmoles/mg	

[a] Both with glycyglycine as acceptor.



The effects of increasing concentrations of maleate on the hydrolysis (-○-○-) and transpeptidation (-●-●-) reactions of γ-glutamyltranspeptidase of rat mammary gland.

Lineweaver and Burk was used and the product never exceeded 15% of the substrate concentration.

Results. The γ-glutamyltranspeptidase of rat mammary gland was purified 130 times with a sp. act. of 8.67 units/mg protein when L-γ-glutamyl-p-nitroanilide was used as the substrate. The enzyme proved to be quite stable for long periods (6 months). Table 1 shows kinetic parameters, K_m and V_m, for L-γ-glutamyl-p-nitroanilide and GSH as well as the substrate specificity for the amino acids studied. This specificity is coincident with that observed in mammary gland extracts during the lactogenic cycle (not shown) and with transpeptidases from other sources, i.e. glutamine and methionine are the best acceptor amino acids when the synthetic substrate is used, and glutamine, aspartic acid and alanine when GSH is the donor[6,10]. The effects of different concentrations of maleate on the hydrolytic and transpeptidation reactions are shown in the figure.

Discussion. The analysis of table 1 shows that the activity of γ-glutamyltranspeptidase with GSH as the substrate is from 4 to 16 times higher than the activity with the synthetic substrate using glycylglycine or amino acids as acceptors.

Table 2. γ-glutamyltranspeptidase activity and GSH levels through the lactogenic cycle

Condition		γ-Glutamyl-transpeptidase (mU/mg)	GSH ($\frac{\text{µmoles}}{\text{g fresh tissue}}$)
Virgin (control)	(7)	6.7± 0.7[a]	0.35±0.03
15 days pregnancy	(5)	18.3± 3.0[b]	0.88±0.08
20 days pregnancy	(5)	32.3±11.3[b]	0.62±0.11
1 day lactation	(4)	80.0±14.7[b]	0.34±0.09
10–15 days lactation	(3)	143.7± 6.3[b]	2.40±0.12[c]
20 days lactation	(4)	106.7±13.0	1.13±0.25[c]

The number of determinations is given in parentheses. [a] SEM; [b] Significantly larger than control; $p<0.05$. [c] Significantly larger than control; $p<0.05$.

The kidney enzyme activity with GSH is only 50% of that obtained with the synthetic substrate. This has a special significance considering that GSH is the natural substrate of the enzyme.

It has been proposed that the effect of maleic acid on the reactivity of γ-glutamyltranspeptidase, i.e. enhancement of the hydrolytic activity and decrease of the transpeptidase activity, is achieved when it is bound at the acceptor site of the enzyme[11]. With the mammary γ-glutamyltranspeptidase (figure) the enzyme behaviour can be explained in a similar way: maleate enables the hydrolysis of γ-glutamyl enzyme and prevents binding of the acceptor amino acid or peptide. It has also been shown that maleate makes the kidney enzyme susceptible to inactivation by serine modifier reagents[12] but we found no change in activity with diisopropylfluorophosphate at the maleate concentration which produces the maximum effect (0.1 M).

The K_m value for GSH is roughly equal to GSH concentration in the mammary gland during lactogenesis, a common feature for many metabolites. As shown in table 2, there is an increase, both in enzyme activity and GSH concentration during lactation, which would support the proposed role for γ-glutamyltranspeptidase in amino acid transport in the mammary gland, a distintive feature of tissues in which there is evidence of this function[1,2].

The increase of GSH can be explained by an active resynthesis or by blockade of its reoxidation, as glutathione reductase does not change during the lactogenic cycle[13]. An additional physiological meaning of this increase may be provided by the $-SH$ group protection of enzymes and other proteins which are induced during the lactogenic cycle, and also in the detoxification functions which take place in such a short and important period as lactogenesis.

1 A. Meister and S. Tate, A. Rev. Biochem. *45*, 559 (1976).
2 A. Meister, Science *180*, 33 (1973).
3 A. Meister, in: Metabolism of Sulfur Compounds, p. 148. Ed. D. M. Greenberg. Academic Press, New York 1975.
4 N. Tateishi, T. Higashi, A. Naruse, K. Nakashima, H. Shiozaki and Y. Sakamoto, J. Nutr. *107*, 51 (1977).
5 J. Puente, M.A. Varas, G. Beckhaus and M. Sapag-Hagar, FEBS Lett. *99*, 215 (1979).
6 A. Meister and S. Tate, J. biol. Chem. *249*, 7593 (1974).
7 C.R. Ball, Biochem. Pharmac. *15*, 809 (1966).
8 O. Lowry, N. Rosebrough, A. Farr and R. Randall, J. biol. Chem. *193*, 265 (1951).
9 S. Tate and A. Meister, J. biol. Chem. *250*, 4619 (1975).
10 C. DeLap, S. Tate and A. Meister, Life Sci. *16*, 691 (1975).
11 G.A. Thompson and A. Meister, J. biol. Chem. *254*, 2956 (1979).
12 M. Inoue, S. Horiushi and Y. Morino, Biochem. biophys. Res. Commun. *82*, 1183 (1978).
13 R. Hilf, R. Ickowicz, J.C. Bartley and S. Abraham, Cancer Res. *35*, 2109 (1975).