The pyrano [2,3-a] carbazole derivatives (**VII**) were found to be active only against bacterial strains, more so against *S. aureus.* Among these isomers **VIIc** was found to be the most active.

Though the compounds **III a, b, c** and **IV a, b, c** showed antifungal and antibacterial activities respectively they do not compare very well with the standard antibiotics used in practice. However, from this lead further structural modifications should give promising antibiotics.

1 The authors are thankful to Dr B.N. Apte, Association of Microbiologists of India, Bombay Unit, for providing some of the organisms, to Prof. A.K. De, Indian Institute of Technology, for financial assistance, to Prof. G.D. Shah, Department of Chemistry, Indian Institute of Technology for guidance, encouragement and helpful discussions.

2 Present address: Department of Life Sciences, Ramnarain Ruia College, Matunga, Bombay-400 019 (India).

3 Address for reprint requests: Research Centre, Hoechst Pharmaceuticals Ltd, L.B.S. Marg, Mulund, Bombay-400 080 (India).

4 T.A. Pugsley and W. Lippmann, Experientia *33*, 568 (1975).
5 A.A. Asselin, L.G. Humber, T.A. Dobson, J. Komlossy and R.R. Martel, J. med. Chem. *19*, 787 (1976).
6 A. Shoeb, F. Anwar, R.S. Kapil, S.P. Popli, P. Dua and B.N. Dhawan, J. med. Chem. *16*, 425 (1973).
7 V.I. Shvedov, L.B. Altukhova and A.N. Grinev, Khim.-farm. Zh. *3*, 28 (1969); Chem. Abstr. *72*, 12476j (1970).
8 A. Mooradian, J. med. Chem. *20*, 487 (1977).
9 A. Mooradian, A.G. Hlavac, P.E. Dupont, M.R. Bell and A.A. Abousi, J. med. Chem. *18*, 640 (1975).
10 E.J. Alexander and A. Mooradian, U.S. Pat. 4,001,270 (1976); Chem. Abstr. *87*, 39275q (1977).
11 W.L. Albrecht and R.W. Fleming, U.S. Pat. 3,932,424 (1976); Chem. Abstr. *84*, 150498s (1976).
12 H. Biere, H. Ahrens, C. Rufer, E. Schroeder and H. Koch, Ger. Pat. 2,337,154 (1975); Chem. Abtr. *82*, 156068j (1975).
13 D.N. Chowdhury, S.K. Basak and B.P. Das, Curr. Sci. India *47*, 490 (1978).
14 D.P. Chakraborty, K.C. Das, B.P. Das and B.K. Chowdhury, Trans. Bose Res. Inst. *38*, 1 (1975).
15 K.C. Das, D.P. Chakraborty and P.K. Bose, Experientia *21*, 340 (1965).
16 G.D. Shah and B.P.J. Patel, Indian J. Chem. *18B*, 451 (1979).
17 F.M. Dean, in: The Total Synthesis of Natural Products, vol. 1, p. 467 and p. 534. Ed. J. Ap Simon. Wieley Interscience, New York 1973.

Glutathione and γ-glutamyl cycle enzymes in rat mammary gland*

J. Puente, E. Castellón and M. Sapag-Hagar

Departamento de Bioquímica, Facultad de Ciencias Básicas y Farmacéuticas, Universidad de Chile, Casilla 233, Santiago 1 (Chile), 31 August 1981

Summary. The main enzymes of the γ-glutamyl cycle during the lactogenic cycle in rat mammary gland were studied. A significant increase was found in all of them with the onset of lactogenesis. The effect of methionine sulfoximine on reduced glutathione concentration was studied in tissue slices of lactating mammary gland. The findings suggest that this compound affects glutathione synthesis by inhibiting γ-glutamylcysteine synthetase.

Reduced glutathione (GSH) is a tripeptide widely distributed in almost every living tissue at concentrations varying between 0.4 and 12.0 mM. Its intracellular level changes with growth, nutritional state and hormone levels in the organism[1,2]. In a tissue like mammary gland (in which its metabolism has been little studied), GSH concentration reaches 3.0 mM in the lactating gland[3].

Participation of GSH in different metabolic processes is determined by 2 of its most important structural characteristics, the presence of a γ-glutamyl bond and a SH group. The γ-glutamyl bond protects GSH against degradation by α-peptidases, and the only way to degrade it is by γ-glutamyltranspeptidase, the enzyme which initiates the γ-glutamyl cycle in a sequence of 6 reactions which allows GSH resynthesis and the transport of amino acids into the cell[4]. The presence of the SH group permits its participation in the protection of thiol groups in several detoxification reactions[5].

The γ-glutamyl cycle has been fully described in rat kidney, liver and brain and partially in erythrocytes and lens[6,7]; in general these tissues also have a high level of GSH. The cycle is regulated by GSH which in certain concentrations inhibits the enzyme γ-glutamylcysteine synthetase[8]. This enzyme is also inhibited by the convulsivant methionine sulfoximine[9].

We have already studied in our laboratory the hormonal dependence and some properties of γ-glutamyltranspeptidase from rat mammary gland[3,10]. Now we are studying the main enzymes of the γ-glutamyl cycle (γ-glutamyltranspeptidase, 5-oxoprolinase and γ-glutamylcysteine synthetase) in order to obtain information on its physiological role during the lactogenic cycle. We have also looked at the effect of methionine sulfoximine in GSH levels in tissue slices of mammary gland in lactogenesis.

Material and methods. Virgin and primiparous Sprague-Dawley rats (180–200 g) were taken at different stages of pregnancy and lactation. During lactation they were always kept with up to 8–10 pups.

Tissue slices were obtained from 12 to 15 days lactating mammary gland by using a Stadie-Riggs tissue slicer. Slices (100 mg) were incubated in Krebs-Ringer bicarbonate buffer pH 7.4, gassed with 95% O_2-5% CO_2 and containing 5 mM of each of the constitutive amino acids of glutathione (glycine, cysteine and glutamate) or the 3 amino acids plus 5 mM methionine sulfoximine. The slices were incubated with continuous shaking, up to 1 h. The γ-glutamyltranspeptidase activity was assayed using L-γ-glutamyl-p-nitroanilide as the donor substrate of the γ-glutamyl group and glycylglycine as the acceptor[11], 5-oxoprolinase was evaluated by quantification with glutamate dehydrogenase of the glutamate produced[12], and γ-glutamylcysteine synthetase was assayed by precipitation of the product L-[U-^{14}C] glutamylcysteine as cadmium mercaptide and subsequent counting by liquid scintillation[13]. The activities were expressed in units/g tissue, 1 unit being defined as the amount of enzyme that catalyzes the formation of 1 μmole product/min/g tissue.

GSH was determined by the method of Ball[16]. L-[U-^{14}C]glutamate was obtained from the Radiochemical Centre, Amersham (285 mCi/mmole). L-γ-glutamyl-p-nitroa-

nilide, dithio-bis-nitrobenzoic acid and glycylglycine were from Sigma Chemical Co. All other reagents were from Merck, Darmstadt.

Results. The γ-glutamyl cycle enzymes increase during pregnancy (fig. 1); this increase becomes quite significant in proportion to the extent of lactogenesis, with the higher activity at the 20th day of lactation. 5-Oxoprolinase level is quantitatively lower (2 orders of magnitude) than for the other enzymes.

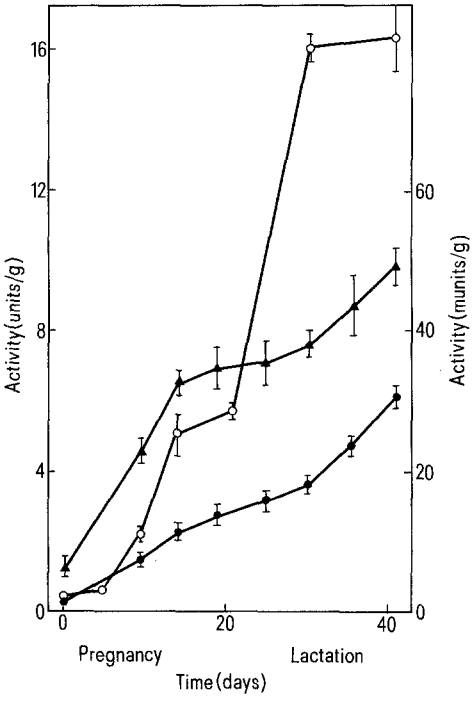

Figure 1. γ-Glutamyl cycle enzyme activities in rat mammary gland during pregnancy and lactation. Results are given as the mean from 5 animals. γ-Glutamultranspeptidase (units/g) -O-O-, 5-oxo-prolinase (munits/g) -▲-▲- and γ-glutamylcysteine synthetase (units/g) -●-●-.

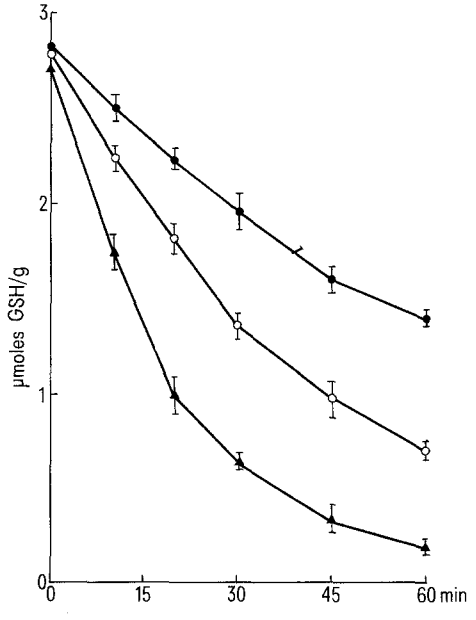

Figure 2. Depleting effect of methionine sulfoximine on GSH levels of tissue slices from lactating mammary gland. All values represent the mean of 4 different experiments ± SEM. -O-O- Krebs Ringer bicarbonate (KRB), control; -●-●- KRB plus glutamate, cysteine and glycine, 5 mM each; -▲-▲- KRB plus the same 3 amino acids plus 5 mM methionine sulfoximine.

Fig. 2 shows the depleting effect of methionine sulfoximine on GSH levels in tissue slices from lactating mammary gland. The initial GSH concentration is 3.0 mM.

Discussion. The enzymes of the γ-glutamyl cycle studied in this work were chosen for the following reasons: γ-glutamyltranspeptidase initiates the degradation of glutathione and the cycle reactions, 5-oxoprolinase is probably the rate-limiting enzyme in other tissues[12] and γ-glutamylcysteine synthetase is the only one under regulation.

All these enzymes show a remarkable increase through pregnancy and lactation, while their activities are very low in the virgin mammary gland. γ-Glutamyltranspeptidase is the one which presents the greatest increase and is also the predominant enzyme of the γ-glutamyl cycle in kidney. 5-Oxoprolinase is one of the key enzymes as it links degradation with its biosynthesis and because its lower activity in mammary gland is also in agreement with its rate-limiting role on the cycle in this tissue. It has to be emphasized that this is precisely the enzyme which has not been found in those tissues in which it has been possible to demonstrate the existence of the full cycle[7]. γ-Glutamylcysteine synthetase, the 1st enzyme in the biosynthesis of GSH, is very active during lactation and susceptible of inhibition by methionine sulfoximine. The remarkable decrease in GSH observed in tissue slices of lactating mammary gland incubated with this compound is most likely due to inhibition of the enzyme, an effect which also has been observed in liver and kidney[17], suggesting that methionine sulfoximine may prove to be useful as an inhibitor of GSH synthesis in various experimental systems. Nevertheless, in the particular case of mammary gland, this effect has to be studied 'in vitro' since the i.p. injection of methionine sulfoximine could upset lactogenesis.

We conclude that, in general, the γ-glutamyl cycle increases its activity throughout lactogenesis in mammary gland, in agreement with the proposed function for the cycle as a mechanism of transport of amino acids into the cell, a situation which is highly demanding in mammary gland, due to the increase in protein synthesis taking place for gland growth and for the protein components of milk.

* This work was supported by grant B1138-8223 from the Servicio de Desarrollo Científico, Universidad de Chile.

1 P.C. Jocelyn, in: Biochem. Soc. Symp. No. 17, p. 43. Ed. E.M. Crooke. Cambridge University Press, London and New York 1959.
2 J. Isaacs and F. Binkley, Biochim. biophys. Acta *497*, 192 (1977).
3 J. Puente, A.M. Martínez, G. Beckhaus and M. Sapag-Hagar, Experientia *37*, 459 (1981).
4 A. Meister, Ann. Int. Med. *81*, 247 (1974).
5 L.F. Chasseaud, Adv. Cancer Res. *29*, 175 (1979).
6 M. Orlowsky and S. Wilk, Eur. J. Biochem. *53*, 581 (1975).
7 A. Meister and S. Tate, A. Rev. Biochem. *45*, 559 (1976).
8 P. Richman and A. Meister, J. biol. Chem. *250*, 1422 (1975).
9 P. Richman, M. Orlowsky and A. Meister, J. biol. Chem. *248*, 6648 (1973).
10 J. Puente, M. Varas, G. Beckhaus and M. Sapag-Hagar, FEBS Lett. *99*, 215 (1979).
11 S. Tate and A. Meister, J. biol. Chem. *249*, 7593 (1974).
12 A. Wendel and I. Flugge, Hoppe-Seyler's Z. Physiol. Chem. *356*, 873 (1975).
13 H. Heinle, A. Wendel and U. Schmidt, FEBS Lett. *73*, 220 (1977).
14 M. Orlowsky and A. Meister, Biochemistry *10*, 372 (1971).
15 C. Fiske and Y. Subbarow, J. biol. Chem. *66*, 375 (1925).
16 C.R. Ball, Biochem. Pharmac. *15*, 809 (1966).
17 A. Palekar, S. Tate and A. Meister, Biochem. biophys. Res. Commun. *62*, 651 (1975).