Finally, the structure of *Y. pseudotuberculosis* acyloxy acid was confirmed by ^{13}C NMR spectroscopy. For interpretation of the spectroscopic data, D,L-3-hydroxy-(I)[6], D,L-3-acetoxy-(II)[7], D,L-3-dodecanoyl-(III), and D,L-3-tetradecanoyltetradecanoic-(IV) acids were synthesized and ^{13}C NMR spectra of these compounds and their methyl esters (Ia–IVa) were studied. To prepare D,L-3-tetradecanoyltetradecanoic acid (IV), tetradecanoyl chloride (10 µmoles) was added to a solution of the compound I (5.5 µmoles) and p-dimethylpyridine (0.5 µmoles) in dry chloroform (40 ml) and dry pyridine (10 ml) at −10 °C. After 20 h at room temperature the reaction mixture was poured into ice-water, the organic layer was washed with 1 N HCl and evaporated. The residue was separated by sephadex LH-20 column chromatography with chloroform as eluent to yield the compound IV (melting point 38.5 °C, 54%). The compound III was obtained in an analogous way (oil, 52%). The fatty acids methyl esters were prepared by treatment with diazomethane.

Compounds: I, D,L-3-hydroxy-; II, D,L-acetoxy-; III, D,L-3-dodecanoyl-; IV, D,L-3-tetradecanoyltetradecanoic acids; Ia–IVa, methyl esters of the aforementioned acids; lip A, acyloxy fatty acid from lipid A of *Y. pseudotuberculosis*. C′-Carbon atoms of acid residue acylating C-3 atom of hydroxytetradecanoic acid. The chemical shift values of other carbon atoms: C-5–C-11 = 29.7; C-12 = 32.0; C-13 = 22.7; C-14 = 14.1; OCH_3 = 51.7 ppm.

An analysis of the data in the table shows that acylation of the hydroxyl group on the C-3 atom of 3-hydroxytetradecanioc acid shifts the signals of C-2 (up field), C-3 (down field), and C-4 (up field) atoms. It seems possible to use these finding of the structure investigation of the native lipid A as well. Thus, the results of ^{13}C NMR spectroscopy, chromato-mass-spectrometry, and alkaline degradation point to the presence of dodecanoyltetradecanoic acid in the lipid A of *Y. pseudotuberculosis*. Synthesis of glucosamine derivatives with hydroxy- and acyloxy fatty acids may help to elucidate the role of the latter in the biological activity of lipid A.

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0014-4754/84/070709-02$1.50 + 0.20/0

Effect of hormones and cyclic AMP on γ-glutamyltranspeptidase activity of rat mammary gland explants[1]

M.P. Valdivieso, J.F. Puente and M. Sapag-Hagar

Departamento de Bioquímica, Facultad de Ciencias Básicas y Farmacéuticas, Universidad de Chile, Casilla 233, Santiago 1 (Chile), 13 June 1983

Summary. γ-Glutamyl transpeptidase activity was assayed in midpregnant rat mammary gland explants at 14, 22 and 38 h, in the presence and absence of insulin, prolactin and corticosterone. With these 3 hormones the explants attained the characteristics of a secretory gland after 22 h of tissue culture, at which time the enzyme exhibited its maximal activity. The addition of dibutyryl cyclic AMP in the presence of the 3 hormones produced a significant increase in enzyme activity, which was maximal with a 1 mM concentration of the cyclic nucleotide. A similar effect was observed when theophylline or theophylline plus dibutyryl cyclic AMP were added to the culture medium.

γ-Glutamyl transpeptidase is a membrane enzyme which has been described in several tissues, specially those which have a secretory function[2]. The metabolic turnover of reduced glutathione (GSH) is related to transpeptidase activity since this enzyme removes the γ-glutamyl moiety of the tripeptide, catalysing one of the first steps of a detoxification pathway which leads to mercapturic acid formation[3]. Another important function attributed to γ-glutamyl transpeptidase is related to the incorporation of amino acids into the cell through six enzymatic reactions known as the γ-glutamyl cycle[4]. In a previous publication we described the transpeptidase activity in the mammary gland of the rat during the lactogenic cycle, where we found a maximum of activity at the tenth day of lactation[5]. We have also reported some properties of this enzyme from rat mammary gland[6], as well as the presence of three enzymes of the γ-glutamyl cycle whose activities increased during lactation[7]. Here, we present a study of γ-glutamyl transpeptidase activity in rat mammary explants and the effect of insulin, prolactin and corticosterone and of cyclic adenosine monophosphate (cAMP) on this enzyme activity.

Material and methods. L-γ-glutamyl-p-nitroanilide, glycylglycine, prolactin, corticosterone, theophylline, dibutyryl cyclic AMP and cycloheximide were obtained from Sigma Chemical Co.; insulin from Lilly Laboratories and Eagle's Medium Modified by Dulbecco (MEMD), penicillin and streptomycin from Flow Laboratories. Mammary gland explants (2–3 mg each) from Sprague-Dawley midpregnant rats (12–13 days of pregnancy) were incubated in MEMD with glutamine (0.584 mg/ml), sodium bicarbonate (1.25 mg/ml) and penicillin and streptomycin (50 U/ml each). The hormones used in this study and their concentrations in the culture media were as follows: ovine prolactin (33 IU/mg) 10 µg/ml; bovine pancreatic insulin (23 IU/mg) 5 µg/ml and corticosterone 1 µg/ml[8].

The explants were removed and homogenized in 4 vol. 0.15 M Tris (pH 8.0)/0.01 M KCl in an Ultra-Turrax blendor (Janke and Kunkel, Staufen). The enzymatic assay was carried out using whole homogenates with L-γ-glutamyl-p-nitroanilide as donor and glycylglycine as acceptor[9]. Enzymatic activity is expressed as µmoles of product formed per minute at 37 °C (units) per mg of protein. Lactose was assayed enzymatically with β-galactosidase and β-galactose dehydrogenase[10]. Proteins were determined by Lowry's method[11].

Results. The time course of γ-glutamyl transpeptidase activity in mammary gland explants incubated with insulin, prolactin

and corticosterone (I + P + C) and dibutyryl cAMP (1.0 mM) is shown in the table. The enzyme activity showed a significant increase in those explants incubated with the 3 hormones only after 22 h of culture, while dibutyryl cAMP caused a significant increase, maximal at 14 h of incubation. The effects of theophylline and theophylline plus dibutyryl cAMP were also studied at this optimal time (14 h). The dibutyryl cAMP concentration which produced the maximum rise in the activity proved to be 1.0 mM (fig.). It can be observed (figures within brackets) that lactose production increases, and that after 22 h of tissue culture the gland reaches the characteristics condition of a secretory gland. In experiments carried out at short incubation times it was confirmed that the increase in enzymatic activity produced by dibutyryl cAMP could already be observed at the first hour of culture (not shown).

Discussion. Insulin, prolactin and corticosterone are well known lactogenic hormones which stimulate pregnant mammary explants to attain a lactogenic state[12]. There are no reports of the analysis of γ-glutamyl transpeptidase in mammary explant cultures nor of the best conditions for culture to obtain maximal activity. In the present study we have used the procedure of Topper et al.[8] for attaining lactating characteristics in the midpregnant gland after 24 h of tissue culture. The increase in enzyme activity produced by the hormones is significant with respect to the control only after 22 h of culture. The enzyme did not show any change when the explants were exposed to each hormone alone.

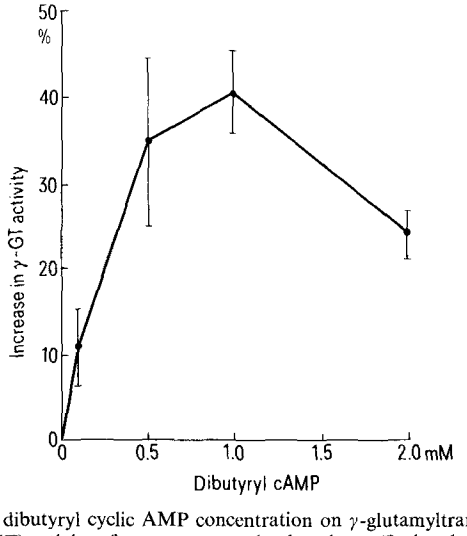
Cyclic AMP has been described as a strong negative controlling factor for lactogenesis in vitro, judged by its inhibitory effect on lipogenic enzymes and by its depressing action on DNA synthesis and specific milk constituents[13]; for these reasons, a decrease in γ-glutamyl transpeptidase activity was anticipated upon the addition of the cyclic nucleotide analog, as it is known that this enzyme exhibits a remarkable increase during lactation[5]. Nevertheless this cyclic nucleotide produced an increase in enzyme activity greater than that obtained with the combination of the 3 hormones, insulin, prolactin and corticosterone. The effect was maximal after a 14 h culture period, at an optimal concentration of 1 mM.

As mammary explants start showing the characteristics of a secretory tissue after 24–30 h of culture, the effect of cAMP at 14 h may well be ascribed to the fact that the gland still retains the properties of the organ during pregnancy. On the other hand a time course study showed no increase in enzyme activity with the 3 lactogenic hormones. Also the incubations of mammary explants with cycloheximide, an inhibitor of protein biosynthesis, did not supress the effect produced by dibutyryl cAMP (results not shown). In order to eliminate any non-specific effect of the dibutyryl derivative on the enzyme, mammary explants were incubated with theophylline which increases the endogenous levels of cAMP. Under these conditions and also at submaximal levels of cAMP and theophylline, significant increases of enzyme activity were achieved. When a mammary gland extract or a partially purified γ-glutamyl transpeptidase preparation were incubated with cAMP, the activity of the enzyme remained unaffected in both cases, suggesting an indirect effect of the cyclic nucleotide on the enzyme. It may be assumed that the tissue must be intact in order to observe the increase in the transpeptidase activity produced by cAMP. Apparently the cell integrity is a requisite for this phenomenon to occur.

γ-Glutamyltranspeptidase activity (1 U/mg protein) in rat mammary gland explants at different tissue culture times

	14 h	22 h	38 h
a MEMD (Control)	0.77 ± 0.09 (1.0)	0.40 ± 0.03 (1.1)	0.62 ± 0.06 (0.8)
b MEMD + I + P + C	0.87 ± 0.08 (1.3)	0.53 ± 0.03[1] (3.2)	0.57 ± 0.08 (2.2)
c MEMD + I + P + C + dib-cAMP (1.0 mM)	1.25 ± 0.06[2]	0.69 ± 0.06[2]	0.73 ± 0.04
d MEMD + Theophylline (1.0 mM)	1.54 ± 0.03[3]		
e MEMD + dib-cAMP (0.5 mM) + Theophylline (0.5 mM)	1.48 ± 0.05[3]		

γ-Glutamyltranspeptidase activity was assayed at the indicated times. Each value represents the mean ± SEM of 6 separate determinations. Numbers shown in parentheses represent lactose concentration (μmoles/μg DNA) in duplicate. [1] Significantly larger than control, $p < 0.05$; [2] significantly larger than b, $p < 0.05$; [3] significantly larger than b, $p < 0.05$.



Effect of dibutyryl cyclic AMP concentration on γ-glutamyltranspeptidase (γ-GT) activity of rat mammary gland explants. Each value represents the mean ± SEM of 3 determinations and are expressed as percent increase with respect to control values (without cyclic AMP). The MEMD incubation medium contained insulin (23 IU/mg), prolactin (33 IU/mg) and corticosterone (1 μg/mg). Explants were cultured for 14 h.

1 This work was supported by grant B1138–8333 from the Departamento de Desarrollo de la Investigación, Universidad de Chile.

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