EFFECTS OF SERUM ON MAMMARY EPITHELIAL PROLIFERATION IN VITRO DURING MAMMARY GLAND DEVELOPMENT

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ABSTRACT

The proliferative response of mammary gland epithelium from nonpregnant, pregnant, and lactating mice to mammary serum factor and insulin was studied in vitro. Mammary gland epithelium from nonpregnant and lactating animals has a delayed proliferative response to mammary serum factor and insulin when compared to the response of epithelium from pregnant animals. The results show that as the animals go through pregnancy into lactation the mammary gland epithelium becomes less responsive to mammary serum factor while it retains its responsiveness to insulin. The concentration of mammary serum factor in sera from animals at various physiological stages is constant. Sera from hypophysectomized rats, on the other hand, show a 50% drop in mammary serum factor activity. This loss of activity cannot be reversed by injecting prolactin, 17-β-estradiol, or growth hormone into the hypophysectomized animals. A hypothesis that the mammary gland is composed of two proliferative epithelial populations is developed, and the possible role of prolactin in stimulating DNA synthesis is discussed.

There have been described a number of tissue culture systems in which cellular proliferation is initiated by factors found in serum (1). Some of these studies have reported factors active in cell lines lacking particular differentiative functions, while others have reported factors that affect specific cell types or tissue types. That these factors may have some physiological role is suggested by the growing number of reports of peptide hormones such as somatomedin (2), erythropoietin (3), nerve growth factor (4), epidermal growth factor (5), and angiogenesis factor (6), which selectively promote cell proliferation in target tissues. Recently it has been reported that components of serum promote DNA synthesis in mammary epithelium in vitro (7–10). The effect is selective in that only the epithelial and not the stromal elements of the gland respond. This is due to serum components other than insulin, which is a known mitogen in this system (9). In this paper we describe further experiments on the biological response of mammary epithelium to mammary serum factor as the gland undergoes the changes of pregnancy and lactation, and we develop a hypothesis that the gland is composed of two proliferating epithelial cell populations.

MATERIALS AND METHODS

Animals and Supplies

C3H and BALB/C female mice at least 2 mo of age (Simonsen Laboratories, Gilroy, Calif.) were used in

these studies. Timed pregnancies were determined by the vaginal plug method. 2-mo old male hypophysectomized rats were purchased from Simonsen Laboratories. 2 wk after surgery, bovine growth hormone (200 μg/day), ovine prolactin (200 μg/day), or 17-β-estradiol (1 μg/day) was injected intraperitoneally into each animal for 3 consecutive days and the animals were bled on the 4th day.

Tissue Culture Techniques

The techniques used for organ culture of inguinal mammary glands of mice have been previously described (9). In brief, explants were cut from the inguinal mammary glands and placed on stainless steel screens floating in Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) for 48 h. At this time, the medium was removed and the test medium was added for an additional 22–24 h of incubation. In some experiments the test medium was added at the start of the experiment.

DNA Synthesis Determination

DNA synthesis was measured by the incorporation of [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., specific activity 17 Ci/mmol) into acid-insoluble materials during a 2-h pulse, 22–24 h after the addition of the test media. This technique has been described previously (9) and the amount of incorporation has been shown to correspond to the number of epithelial cells synthesizing DNA at the time. The incorporation was expressed as a percent stimulation over control values. The range of [^{3}H]thymidine incorporation was from 1,600 to 3,000 cpm per mg of stimulated tissue.

Previous studies have demonstrated that tritiated thymidine in organ cultures of mammary gland is principally incorporated into epithelial cells and that the incorporation correlates with the mitotic index and the labeling index (9, 11). Earlier work has shown that the incorporation of tritiated thymidine is dependent upon the amount of serum added to culture media (9). Where indicated, experiments were performed over a range of serum concentrations.

Hormones and Sera

The insulin solution (crystalline porcine insulin, 24.4 U/mg, which was a gift of Eli Lilly & Co., Indianapolis, Ind.) was prepared as previously described (9). Though rodent sera are most active in promoting growth in this system, it has been shown (9) that porcine sera are the most active of readily available sera, and therefore porcine serum was used in the experiments reported here. Sera were obtained from Grand Island Biological Co. or by bleeding laboratory animals.

RESULTS

Time-Course of [^{3}H]Thymidine Incorporation into Mammary Gland Explants at Various Physiological States

When mammary gland epithelium from mature mice is incubated in Medium 199 containing insulin or serum, DNA synthesis is initiated (8, 9, 11, 12). The nature of the response to insulin and serum differs both qualitatively and quantitatively depending upon the physiological state of the animal, that is, whether the animal is nonpregnant, pregnant, or lactating (8, 9, 13, 14). As is illustrated in Fig. 1, when epithelium from nonpreg-

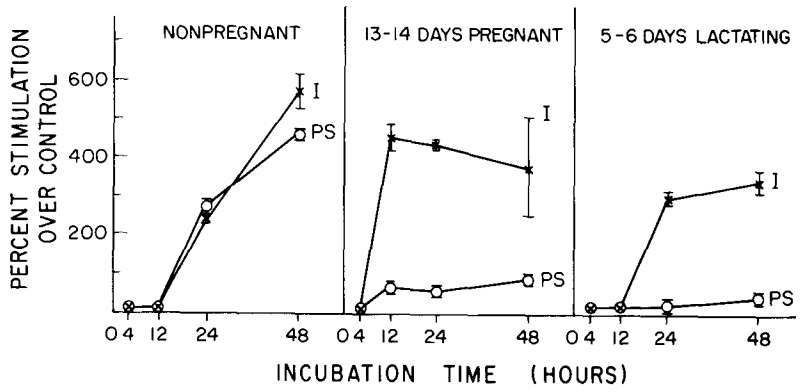

FIGURE 1 The effect of insulin (I) and porcine serum (PS) on DNA synthesis in explants of mammary gland from nonpregnant, midpregnant, and lactating animals. Explants were exposed to Medium 199 alone, Medium 199 plus insulin (5 μg/ml) or porcine serum (5%). At the indicated times, explants were pulsed with [^{3}H]thymidine (5 μCi/ml) for 1 h and the acid-insoluble counts were determined and expressed as percent stimulation of control. Nonpregnant control = 135 cpm/mg tissue; midpregnant control = 1,200 cpm/mg tissue; lactating control = 700 cpm/mg tissue.

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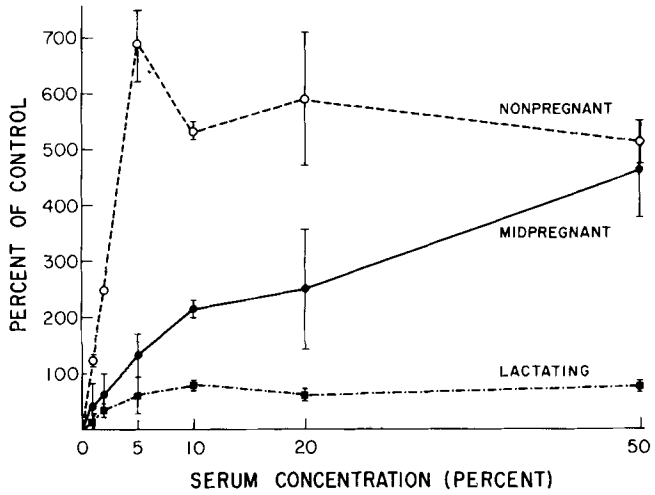

FIGURE 2 The effects of various concentrations of serum on DNA synthesis in explants of mammary gland from nonpregnant, midpregnant, and lactating animals. Control explants were incubated in Medium 199 alone; experimental tissues were incubated in Medium 199 plus porcine serum. Points are the mean of duplicate determinations of tissues exposed for 1 h to 2 μCi of [^{3}H]thymidine/ml. Nonpregnant control = 200 cpm/mg tissue; midpregnant control = 370 cpm/mg tissue; lactating control = 870 cpm/mg tissue.

nant animals is incubated in organ culture with insulin or serum, there is approximately a 20-h delay in augmented rates of DNA synthesis. On the other hand, such delay is not observed in mammary glands from pregnant animals.

It has been shown that if nonpregnant animals are first primed with prolactin injections, the delay in DNA synthesis in vitro is abolished (10). This suggests that prolactin levels of the donor animal could be responsible for the differences in mammary epithelium sensitivity to serum and insulin in vitro. Since lactating animals are known to have a high level of endogenous prolactin, we compared the effects of serum and insulin on DNA synthesis of mammary epithelium from nonpregnant and pregnant animals with that found in lactating animals (Fig. 1). In spite of the high endogenous levels of prolactin, epithelium from lactating animals behaved like that from nonpregnant animals where endogenous prolactin levels are low. It should be noted that the insulin response with each type of tissue differs quantitatively from that of serum (see next section), but that it follows the same basic qualitative pattern, that is, there is initial unresponsiveness (for 12 h or more) in explant tissues from both nonpregnant and lactating animals whereas the epithelium from pregnant animals responds rapidly (13). Similar findings have been reported by Oka et al. (14).

Changes in Serum Sensitivity in Different Physiological States

Mammary epithelium from nonpregnant animals responds at maximal rates of DNA synthesis to serum concentrations as low as 5% and the response is linearly dependent upon serum to a concentration of 5% (9). Both Topper's (10) and Turkington's (7) groups suggest that serum concentrations as high as 50% may be necessary to maximally stimulate DNA synthesis in mammary gland epithelium from pregnant animals. We compared the responsiveness of mammary epithelium from nonpregnant, pregnant, and lactating animals to concentrations of serum of up to 50% (Fig. 2). These experiments demonstrate that mammary gland epithelium from nonpregnant animals is more responsive to 5% serum than epithelium from pregnant and lactating animals. With epithelium from pregnant animals a concentration of 50% is required to give effects comparable to those of 5% on mammary epithelium of nonpregnant animals. Like that of epithelium from nonpregnant animals, the response of epithelium from lactating animals is not further stimulated by serum concentrations higher than 5%. These observations suggest that the qualitative difference in the kinetics of response to serum and insulin between epithelium from pregnant

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animals, on the one hand, and nonpregnant and lactating animals on the other, cannot be explained simply by exposure to endogenously high levels of prolactin. These observations show that pregnancy and lactation are associated with decreased responsiveness to mammary serum factor. To substantiate that this decreased responsiveness correlates with progression through pregnancy, the effect of serum on DNA synthesis of epithelium from animals at various stages of pregnancy and lactation was determined. The results in Fig. 3 A show that as animals progress through pregnancy, mammary epithelium becomes less responsive to 5% serum, while the effect of 50% serum remains rather constant until late pregnancy. During lactation there is extreme unresponsiveness to serum over a wide range of concentrations.

To determine if during pregnancy there is a change in responsiveness of mammary epithelium to insulin as there is to serum, mammary epithelium from various stages of pregnancy was tested with low and high (1 or 5 μg/ml) concentrations of insulin (Fig. 3 B). The minimal concentration of insulin which gives maximal proliferation of mammary epithelium from nonpregnant animals is 1 μg/ml (9). As can be seen in Figs. 1 and 3 B, there is little change in responsiveness to insulin as the animal grows from pregnancy into lactation.

Serum Concentrations of Mammary Serum Factor in Various Physiological States

Progressive loss of epithelial responsiveness to mammary serum factor as pregnancy progresses

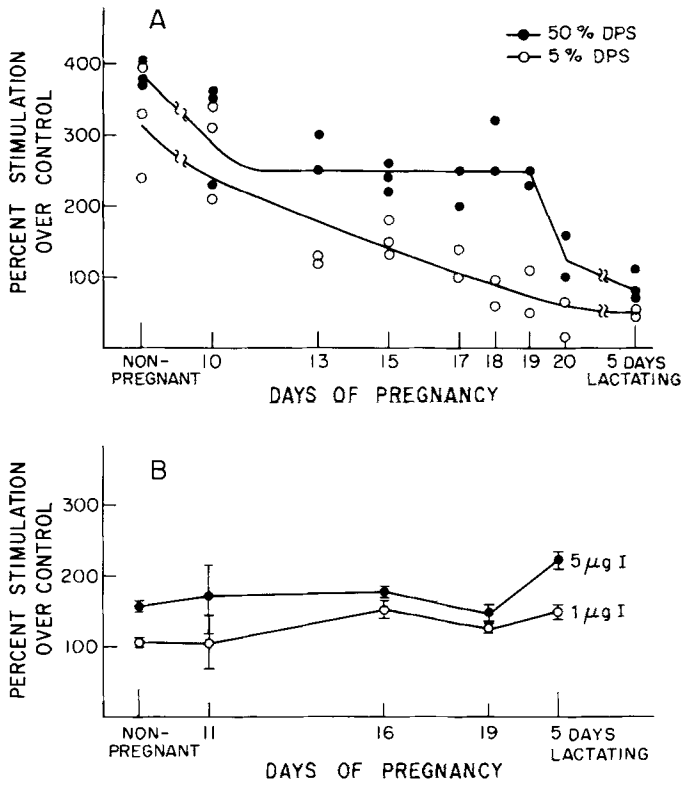

FIGURE 3 The effect of serum and insulin on DNA synthesis in explants of mammary gland from mice at different physiological stages. (A) Explants were exposed to Medium 199 or Medium 199 plus either 5% or 50% dialyzed porcine serum (DPS). Explants from nonpregnant and lactating mice were pulsed with [^{3}H]thymidine as in Fig. 1 after 40 h in the medium, and the explants from pregnant mice were pulsed after 24 h in the medium. Acid-insoluble counts were determined and expressed as percent stimulation of control. (B) Explants were exposed to Medium 199 or Medium 199 supplemented with 1 μg/ml or 5 μg/ml of insulin (I). Experimental procedures were the same as in Fig. 3 A.

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TABLE I

Effect of Various Sera on DNA Synthesis

Source of Serum	Porcine Serum Activity
	%
Porcine serum	100
Nonpregnant	99
11-day pregnant	116
16-day pregnant	120
19-day pregnant	96
Lactating	97

Effects of various sera on [^{3}H]thymidine incorporation into mammary gland explants from nonpregnant mice. Explants were preincubated in Medium 199 for 48 h, and then Medium 199 plus 5% serum was added. The explants were pulsed from the 20th to the 21st h with 5 μCi of [^{3}H]thymidine/ml, and acid-insoluble counts were determined and expressed as percent stimulation over control. There were 3,100 cpm/mg tissue incorporated in response to porcine serum. Sera were tested over a concentration range of 0.01-10%.

could be due to saturation of the epithelial cell by exposure to high endogenous levels of mammary serum factor during pregnancy. To test this possibility, sera from nonpregnant, pregnant, and lactating animals were obtained and tested (Table I). Though the levels of mammary serum factor increase slightly at midpregnancy, this amount of change over the control is within the variability found within this system. These observations suggest that there is probably very little, if any, change in the concentration of mammary serum factor in serum during pregnancy.

To determine if the factor responsible could be somatomedin or could be mediated by the pituitary gland, young rats were hypophysectomized, bled 2 wk later, and the sera were tested in this system. Some hypophysectomized animals were injected with bovine growth hormone, ovine prolactin, or 17-β-estradiol before exsanguination. Table II shows that hypophysectomy decreases mammary serum factor concentrations by about 50% and that injection of growth hormones or other hormones does not restore the lost activity. The failure of bovine growth hormone to restore activity, as it does for somatomedin, suggests that the factor responsible for the serum effect on growth is not somatomedin.

The results of hypophysectomy on mammary serum factor levels and the known effects of prolactin on proliferation of the mammary gland when administered to nonpregnant animals suggest that prolactin could play a role in mammary serum effects. However, the failure of prolactin injections to restore mammary serum factor activity, and the lack of a significant effect of prolactin on mammary epithelial proliferation in organ culture (Table III), suggests that prolactin alone may not be sufficient to promote growth in this gland. It is possible that prolactin-primed tissue becomes responsive to mammary serum factor or

TABLE II

Effect of Hypophysectomy on Serum Levels of Mammary Serum Factor

Source of Serum	Stimulation	Activity
	%	%
No additions	0	
Porcine	242	
Normal rat	354	100
Hypox rat	208	42
Hypox rat injected with:		
Bovine growth hormone (200 μg/day $\times$ 3 days)	178	50
Ovine prolactin (200 μg/day $\times$ 3 days)	162	54
17-β-estradiol (1 μg/day $\times$ 3 days)	163	54

Effects of various sera on [^{3}H]thymidine incorporation into mammary gland explants from nonpregnant mice. Explants were preincubated in Medium 199 for 48 h, and then Medium 199 plus 5% serum was added. Explants were pulsed for 2 h with 2 μCi [^{3}H]thymidine/ml 22 h after addition of the test medium. Sera were tested over a concentration range of 0.01-10%. No addition control = 1,200 cpm/mg tissue.

TABLE III

Effect of Prolactin on DNA Synthesis

	Tissue	Stimulation
	cpm/mg	%
No additions	1,130	0
Porcine serum(5%)	3,270	198
Prolactin(20 μg)	1,242	9
Prolactin(5 μg)	1,200	6
Prolactin(1 μg)	1,220	8
Prolactin(0.1 μg)	1,450	28
Prolactin(0.01 μg)	1,570	30

Effects of prolactin on [^{3}H]thymidine incorporation into mammary gland explants from nonpregnant mice. Explants were preincubated in Medium 199 for 48 h, and then Medium 199 plus various concentrations of prolactin were added. Explants were pulsed as in Table II.

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that mammary serum factor interacts with prolactin to promote the growth effect. To test these possibilities, mammary gland tissue from nonpregnant animals was preincubated for 48 h in various concentrations of prolactin before exposure to serum, or various concentrations of prolactin and serum were added simultaneously to explants. Preincubation in prolactin does not alter the response to serum (Table IV). However, when prolactin is added simultaneously with serum there is an alteration in serum-promoted growth (Table V). Over a large range of concentrations (0.01–1 μg/ml), prolactin has no effect on mammary serum factor-mediated DNA synthesis. At higher concentrations (5–20 μg/ml), prolactin appears to block the mammary serum factor-mediated DNA synthesis. When prolactin is added simultaneously with insulin there is no altered effect on DNA synthesis (Table VI). It appears that prolactin selectively interferes with the serum-mediated DNA synthesis and not the insulin-mediated DNA synthesis.

TABLE IV

Effect of Previous Exposure to Prolactin on Serum Effects

	Tissue	Stimulation
	cpm/mg	%
No additions	1,350	—
Porcine serum(5%)	2,800	110
48 h preincubation in:		
Prolactin(20 μg)	3,620	168
Prolactin(5 μg)	3,060	126
Prolactin(1 μg)	2,750	104
Prolactin(0.1 μg)	3,205	137
Prolactin(0.01 μg0	3,075	127

Mammary gland explants from nonpregnant mice were preincubated in Medium 199 plus various concentrations of prolactin for 48 h, at which point Medium 199 plus 5% serum was added. Explants were pulsed as in Table II.

TABLE V

Effects of Prolactin Plus Serum on DNA Synthesis

	Tissue	Stimulation
	cpm/mg	%
No addition	710	0
PS*(5%)	2,365	233
PS + prolactin(20 μg)	1,263	78
PS + prolactin(5 μg)	1,550	118
PS + prolactin(1 μg)	2,044	188
PS + prolactin(0.1 μg)	2,335	229
PS + prolactin(0.01 μg)	2,054	189

Mammary gland explants from nonpregnant mice were preincubated in Medium 199 alone for 48 h and then Medium 199 plus serum (5%) and various concentrations of prolactin for an additional 20 h. Explants were pulsed as in Table II.

* PS, porcine serum.

TABLE VI

Effects of Prolactin Plus Insulin on DNA Synthesis

	Tissue	Stimulation
	cpm/mg	%
No additions	1,380	0
I*(5 μg/ml)	4,050	194
I + prolactin(20 μg)	4,040	193
I + prolactin(5 μg)	3,150	126
I + prolactin(1 μg)	3,950	186
I + prolactin(0.01 μg)	3,995	189

Effects of insulin and prolactin on DNA synthesis in mammary gland explants from nonpregnant mice. Explants were preincubated in Medium 199 alone for 48 h and then Medium 199 plus insulin (5 μg/ml) plus various concentrations of prolactin. Explants were pulsed as in Table II.

* I, insulin.

DISCUSSION

These experiments demonstrate that there is a factor(s) in sera, mammary serum factor, which promotes the proliferation of mammary explant epithelium and that the epithelium of the gland becomes increasingly less responsive to the factor(s) during pregnancy and lactation. Epithelium from nonpregnant animals is most responsive to mammary serum factor, but late in pregnancy increasing concentrations are required to give maximal stimulation. During lactation, even concentrations as high as 50% give only minimal responses. Responsiveness to insulin, another epithelial proliferation-promotion agent, on the other hand, is retained through pregnancy and on into lactation. The qualitative nature of the response to serum and insulin also differs in the various physiological states. There is approximately a 20-h delay in augmented rates of DNA synthesis in explants from both nonpregnant and lactating animals (11, 14). Explants from pregnant animals respond with increased rates of DNA synthesis within a few hours of culture in the presence of insulin or serum. The difference in response between tissues from nonpregnant and pregnant

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animals has been attributed to prolactin, in that Oka and Topper (10) have shown that explants from animals previously injected with prolactin do not have a delay in their response to insulin or serum. However, the results reported here on lactating animals suggest that factors other than exposure to prolactin alone must alter responsiveness to insulin and mammary serum factor. At times of high endogenous levels of prolactin there is the same delay in response to both agents that is found in tissues from nonpregnant animals where there are low endogenous levels of prolactin.

Differences in response to mammary serum factor and insulin may be more related to shifts in the composition of the epithelial cell population comprising the gland rather than to exposure to prolactin per se. We know that the growth-promoting effect of serum and insulin is exclusively on the epithelial cells of the mammary gland (9). These agents appear to affect different groups of cells in that if each agent alone is added to organ culture at maximally effective concentration, a limited percentage of epithelial cells will initiate DNA synthesis. When the two agents are combined there are additive effects on the numbers of cells synthesizing DNA (9). These observations, coupled with the demonstration of the retention of insulin responsiveness with changes in responsiveness to mammary serum factor as the gland progresses from pregnancy into lactation, suggest that there are at least two populations of mammary gland epithelial cells. These populations are operationally defined by their response to insulin and serum. One population is present at all stages of development and is responsive to insulin; the other, a serum-responsive population, decreases as pregnancy progresses. There are probably a number of schemes which could explain the two cell populations and their changes, for example: (*a*) a stem-cell scheme in which the insulin population continues to produce insulin-responsive cells whereas the stem-cell population responsive to mammary serum factor can only go through a limited number of cell cycles; (*b*) a scheme whereby there is competition between one or more peptide hormones and mammary serum factor for active site in or on the epithelial cell population; or (*c*) a scheme whereby cells which undergo proliferation and respond to the hormones of pregnancy produce daughter cells lacking mammary serum factor-responsive sites. It may be possible to test these latter possibilities. The in vitro competition experiments reported here lend some credence to the second possibility, even though the concentration of prolactin required to see this effect was extremely high.

To better understand the biological function of mammary serum factor and how it interacts with the hormones of pregnancy requires better characterization of the factor(s). Preliminary studies indicate that the mammary serum factor is heat stable, and at neutral pH is associated with high molecular weight material. It is present in highest concentrations in rodent serum when the test system used is rodent mammary gland (9). Preliminary investigations on partially purified materials indicate that it is an acidic protein.[1] The origin of the factor is not known. Serum from animals previously hypophysectomized loses 50% of its activity for promoting DNA synthesis in mammary gland epithelium. Injections in vivo of growth hormone, prolactin, and estrogen do not restore the activity to the serum. These observations suggest that the pituitary may be involved in the serum effect, as has been suggested by the work of Gospodarowicz (15) where brain and pituitary extracts promote the growth of 3T3 cells. The fact that growth hormone does not restore activity suggests that the factor under study is probably not somatomedin. The failure of prolactin to restore activity suggests that prolactin itself is probably not the operative component. This observation plus the demonstration that in vitro prolactin alone does not promote mammary gland proliferation suggests that, to be operative, prolactin may have to be presented to the tissue in the presence of other factors, or at a time in development in which a particular cell population has been produced.

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