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Effect of Methionine Sulfoximine in nitrogenase activity by ammonia and glutamine in *Rhodopseudomonas sphaeroides*

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암모니아와 glutamine에 의한 *Rhodopseudomonas sphaeroides*의 질소 고정 효소 활성에 미치는 Methionine Sulfoximine의 영향

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ABSTRACT: The effect of ammonia and glutamine on nitrogenase activity of *Rhodopseudomonas sphaeroides* was examined. The nitrogenase activity of this strain was inhibited by ammonia and glutamine. When ammonia and glutamine were exhausted, nitrogenase activity promptly resumed at its original rate.

Methionine sulfoximine (MSX), irreversible glutamine synthetase (GS) inhibitor, is a structural analogue of glutamate. MSX was used in order to know whether the nitrogenase activity was inhibited by ammonia and glutamine directly or not. The ability of MSX to prevent nitrogenase switch-off by ammonia was found to be dependent upon the phase of culture. When the cells were sampled after 12 hour culture, 500 μM MSX would not prevent the nitrogenase switch-off by ammonia. Twenty one percents of GS activity was inhibited by 500 μM of MSX and concentration of released ammonia decreased. But nitrogenase activity was still inhibited by ammonia. However, nitrogenase switch-off after 20 hours would be prevented by 100 μM of MSX. On the other hand, GS activity was inhibited completely by 100 μM MSX and concentration of released ammonia somewhat increased. But nitrogenase activity was not inhibited. This data indicated that the inhibition of *in vivo* nitrogenase activity of *Rp. sphaeroides* by ammonia seemed to be mediated by products of ammonia assimilation rather than by ammonia itself.

KEY WORDS□*Rhodopseudomonas sphaeroides*, nitrogenase switch-off, methionine sufoximine, glutamine synthetase.

Nitrogenase activity of purple non-sulfur bacteria is regulated by the addition of certain nitrogenous compounds such as ammonia or glutamine. They inhibit nitrogenase activity rapidly and completely (Sweet and Burris, 1981). This inhibition is reversible and the activity of nitrogenase is fully restored on exhaustion of the nitrogen source (Arp and Zumft, 1983). This effect on nitrogenase has been termed a 'switch on/switch off' effect (Zumft and Castillo, 1978) and should be clearly distinguished from long term regulation at the genetic level. In most of the bacteria, except some photosynthetic bacteria, the nitrogenase remains active when measured in cell extracts. The nitrogenase of the nonsulfur photosynthetic bacteria appear to differ from those of the nonphotosynthetic heterotrophs in that their activity is subject to regulation.

Glutamine synthetase(GS) is a key enzyme in the assimilation of nitrogen and ammonia by various organisms (Tyler, 1978). GS has been implicated in the genetic regulation of nitrogenase

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synthesis from studies with glutamine auxotroph (Leonardo and Goldberg, 1980; Streicher *et al.*, 1974) and with the use of GS inhibitor such as DL-methionine-DL-sulfoximine (MSX) (Arp and Zumft, 1983) and α -N-oxalyl-L-α,β-diaminopropionic acid (ODAP) (Meyer and Vignais, 1979). However, detailed control mechanism of nitrogenase is unknown. Therefore, this report describes whether the nitrogenase activity is inhibited by ammonia and glutamine directly or not.

MATERIALS AND METHODS

Rhodopseudomonas sphaeroides isolated from mud flats of paddy field in Korea (Lee, 1986a) was used in this experiment. The cell growth and culture conditions were described in the previous report (Lee, 1986a). Glutamate-grown cells were cultured in the media containing 7 mM glutamate as a nitrogen source. N-limited-grown cells were cultured in nitrogen limiting media with various minerals (Lee, 1986a) and 30 mM succinate.

Nitrogenase activity in whole cells was assayed by manometric measurement (Hanus *et al.*, 1980) and amperometric method (Sweet *et al.*, 1980). The manometreic measurement was described previous report (Lee, 1986b). In amperometric method, a Clark type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) was used as the hydrogen probe. After 3 m*l* of culture broth were added in the glass chamber of oxygen monitor, the broth was bubbled with 100% O_2 free argon gas. The temperature of water jacket was 30 °C and light intensity was approximately 4,400 lux. Nitrogenase activity of *Rp. sphaeroides* was measured by amperometrically without mention separately.

The activity of glutamine synthetase and ammonia concentration were measured by according to the previous report (Lee, 1986b).

RESULTS AND DISCUSIONS

Many heterotrophic anaerobes can utilize and produce H_2 through the action of hydrogenase (Gray and Gest, 1965). However, in photosynthetic bacteria, the photoproduction of hydrogen is catalyzed by nitrogenase (Takakuwa *et al.*, 1983; Lee and Bae, 1983), whereas the utilization of hydrogen as a biosynthetic reductant is effected by another kind of (classical) hydrogenase (wall *et al.*, 1975). When *Rhodospirillum rubrum* allowed to grow photoheterotrophically in a medium containing as a carbon source either malate, fumarate, or pyruvate and either nitrogen gas, glutamate, or aspartate as a nitrogen source, the cells not only evolved carbon dioxide, but also exhibited a vigorous evolution of hydrogen gas (Gest and Kamen, 1949; Ormerod *et al.*, 1961). The photoevolution of hydrogen was completely inhibited by NH_4^+, high concentrations of yeast extract or peptone, or amino acids such as alanine and serine that are readily decomposed to release NH_4^+(Bregoff and Kamen,1952). The inhibitory effect of NH_4^+ on the photoevolution of H_2 (Gest *et al.*, 1950) suggested that it repressed the synthesis

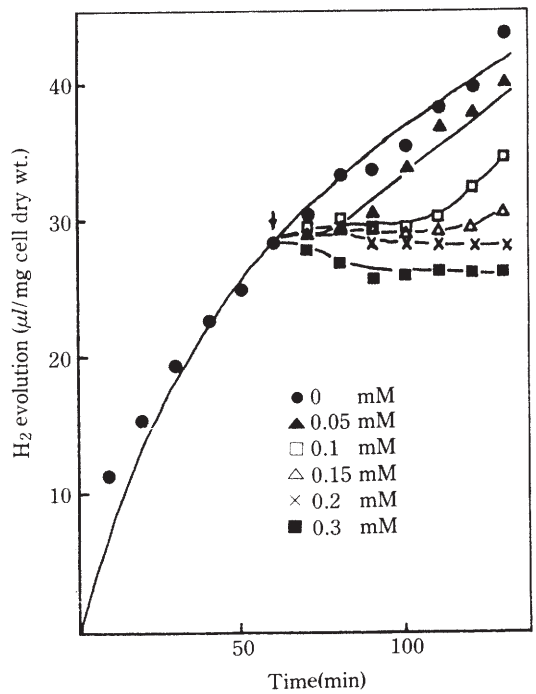

Fig. 1. *Inhibition of hydrogen evolution of Rp. sphaeroides by ammonia.*
The cells were cultured in N-limited medium for 20 hours. Arrow; addition of ammonium chloride.

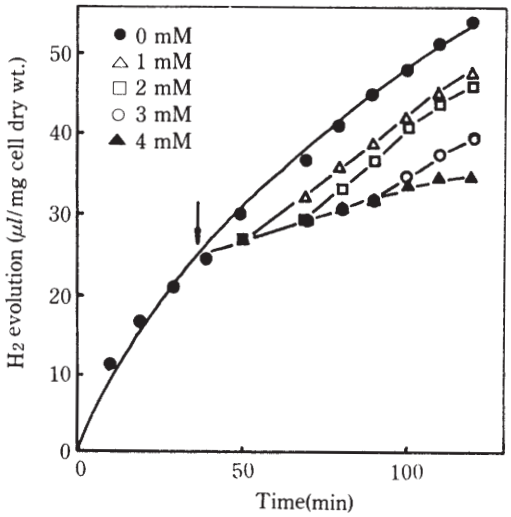

Fig. 2. *Inhibition of hydrogen evolution of Rp. sphaeroides by glutamine.*
The cells were cultured in N-limited medium for 20 hours. Arrow; addition of glutamine.

of the hydrogen-evolving system. Fig. 1,2 showed the effect of ammonium ion and glutamine on nitrogenase activity (measured by manometer) of *Rp. sphaeroides* grown for 20 hours in N-limited medium. Nitrogenase activity of this organism was inhibited by NH_4Cl and $(NH_4)_2SO_4$, but not by NaCl (Fig. 3). Therefore, nitrogenase activity of the cells was inhibited by ammonia. When ammonia and glutamine were exhausted, nitrogenase activity promptly resumed at its original rate. Therefore the inhibition of nitrogenase activity by ammonia and glutamine was reversible.

In all the free-living nitrogen-fixing bacteria examined thus far, synthesis of nitrogenase is repressed after addition of ammonia to the culture medium (Neilson and Nordlund, 1975). In most of the bacteria, except some photosynthetic bacteria, the nitrogenase remains active when measured in cell extracts.

In addition to this long-term effect of ammonia, some bacteria are capable of rapidly switching off whole-cell nitrogenase activity with added ammonia. Nitrogenase activity is inhibited within minutes after addition of NH_4^+ to whole cells. This so-called short-term effect is observed in *Azotobacter* (Eady, 1981), *Rhodospirillum* (Neilson and Nordlund, 1975) *Rhodopseudomonas* (Zumft and

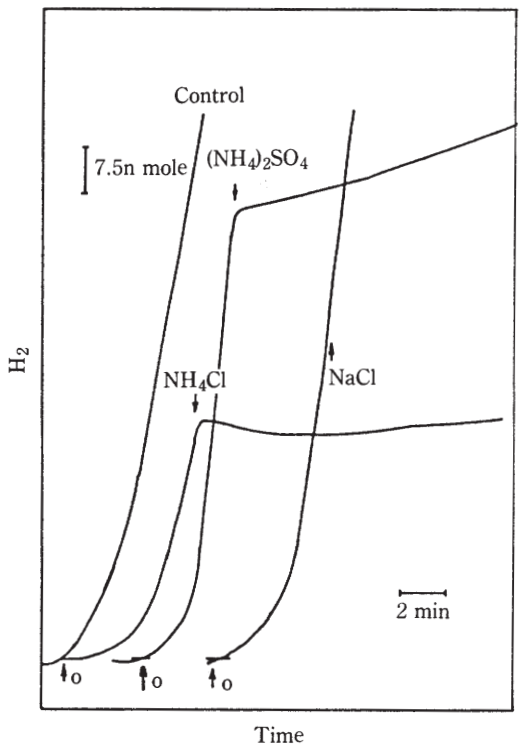

Fig. 3. *Inhibition of hydrogen evolution by ammonium ion.*
NH_4Cl, NaCl; 0.5 mM, $(NH_4)_2SO_4$; 0.25 mM, ↑; light on

Castillo, 1978), and *Anabaena* (Yoch and Gotto, 1982) species. For the phototrophic organisms, inhibition is dependent on the growth conditions (Alef *et al.*, 1981). In organisms that fix nitrogen only under fermentative conditions, like *Clostridium* and *Klebsiella* species, no short-term effect of ammonia is found (Yoch and Gotto, 1982).

The nitrogenases of the nonsulfur photosynthetic bacteria appear to differ from those of the nonphotosynthetic heterotrophs in that their activity is subject to regulation. Carithers *et al.*(1979) showed that the conditions under which nitrogenase of *Rs. rubrum* was depressed (i.e., growth on N-starvation, on N_2, or on glutamate) determined the regulatory state of the enzyme. That form of nitrogenase obtained from N-starved cells which is always fully active *in vivo* and *in vitro* and has characteristics common to the nitrogenase of other bacteria was called nitrogenase A. Nitrogenase from N_2-or glutamate-grown cells is

always inactive *in vitro* (due to inactivation of the Fe protein), but it can be reactivated by a Mn^{++} (ATP)-dependent activating protein (Zumft and Nordlund, 1981). This regulatory form of nitrogenase A has different enzymatic and physical characteristics. The physiological significance of the Mn^{++}-dependent nitrogenase R-activating system was suggested by the observation that the growth of *Rs. rubrum* and *Rp. capsulata* in nitrogen gas (a condition that produce nitrogenase R.) required Mn^{++} in the culture medium, but growth in NH_4^+ or glutamate did not. Carithers *et al.*(1979) reported that extracts from nitrogen starved cells contained fully active nitrogenase, whereas the enzyme from cells grown in glutamate or nitrogen required *in vitro* activation. They called the active form that required activation nitrogenase R; they reported that the addition of NH_4^+or glutamate to a culture of nitrogen starved cells caused on *in vivo* conversion of form A to form R. Yoch and Cantu (1980) have extended these model to include both on active and inactive form of nitrogenase R. Changes in the nitrogen environment of *Rs. rubrum* drastically influences the concentration of nitrogenase A and nitrogenase R in these cells, and this is accompanied by changes in whole-cell activity. This phenomenon is most dramatic after adding NH_4^+ to an N-starved culture; here the nitrogenase A of the cell is rapidly converted to nitrogenase R. These observation suggested that the NH_4^+-induced inhibition of nitrogenase activity commonly observed in the purple nonsulfur bacteria involved the conversion of nitrogenase A to R (Yoch and Cantu, 1980).

But Sweet and Burris(1981) reported that this model is not correct. As mentioned above, differences in culture and assay conditions may account for these discrepancies. Instead of postulating the existence of two types of active nitrogenase in the cells, Sweet and Burris (1981) reported that enzyme could be simply active or inactive, and that the patterns of inhibition could be interpreted as arising from the regulation of an inhibitory mechanism. This regulation seems to be accomplished by products of ammonia assimilation; unless there has been a recent flux of NH_4^+through the assimilatory system, the inhibitory mechanism does not respond rapidly to sudden additions of NH_4^+. Other events, such as changes in the balance of energy supplies in the cell, periods of total darkness, and exposure to traces of oxygen could cause changes in nitrogenase activity by different mechanisms than the one triggered by NH_4^+.

Methionine sulfoximine (MSX), irreversible glutamine synthetase (GS) inhibitor, is a structural analogue of glutamate. Meister(1974) showed that strong inhibition of GS activity by MSX required ATP in cell-free extracts. This is consistent with the mechanism of inhibition of this enzyme from other sources, where phosphorylation of MSX by ATP at the active site resulted in a tight-binding and irreversible inactivation.

Alef *et al.*(1981) reported that nitrogenase of N_2-grown cells of *Rp. palustris* responded to ammonia with a rapid and complete inactivation, which was prevented by addition of MSX to cells prior to ammonia. When MSX was added to an ammonia-inactivated cells, nitrogenase activity was restored (Fig. 4). When *Rp. sphaeroides* was

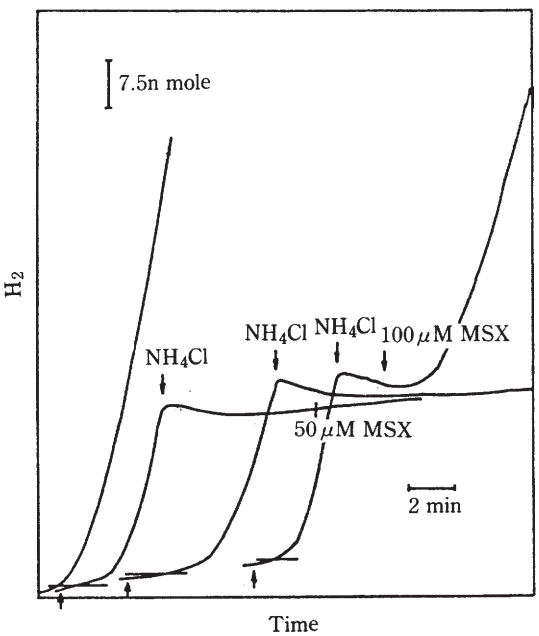

Fig. 4. *Effect of MSX on the nitrogenase switch-off by ammonium ion.*
Rp. sphaeroides was grown in glutamate medium. 0.5 mM NH_4Cl was aded. ↑ ; light on

cultured in glutamate-media for 20 hours, switch-off of nitrogenase by ammonia was prevented by 100μM of MSX, but not by 50 μM MSX (Fig. 4). Fig. 5 showed the effect of MSX on the switch-off of nitrogenase by ammonia depending on the culture phase in the strain grown in glutamate-medium. Switch-off of nitrogenase by ammonia could not be prevented in exponential phase (12 hour old) even in the presence of MSX at the concentrations as high as 500μM. When the strain was sampled after stationary phase (20 hour old), however, 100μM of MSX would prevent the nitrogenase switch-off by ammonia. The ability of MSX to prevent nitrogenase switch-off by ammonia was found to be dependent upon the phase of culture. Addition of 150 mM NH_4Cl to 20 hour-old glutamate-grown cells caused inhibition of nitrogenase activity. But addition of 30 mM MSX, which caused rapid inhibition of GS, to the cells before NH_4Cl prevented nitrogenase switch-off (Fig. 6). Fig. 7, 8 showed the interactions of ammonia, MSX, nitrogenase and GS of the cells grown in glutamate-medium for 12 hours and for 20 hours. When the cells were sampled after 12 hour culture, 500μM MSX would not prevent the nitrogenase switch-off by ammonia. The twenty one percents of GS activity was inhibited by 500μM of MSX and concentration of released ammonia decreased. But nitroge-

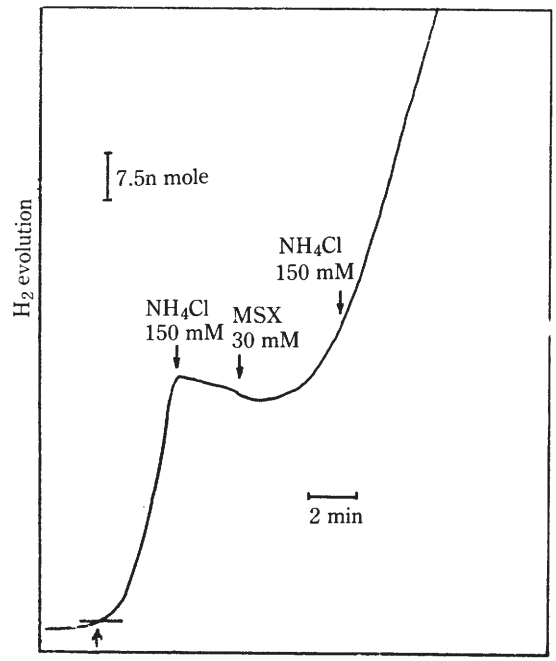

Fig. 6. *Interactions of NH_4Cl, MSX, and nitrogenase in Rp. sphaeroides cultured in glutamate-medium for 20 hours.*

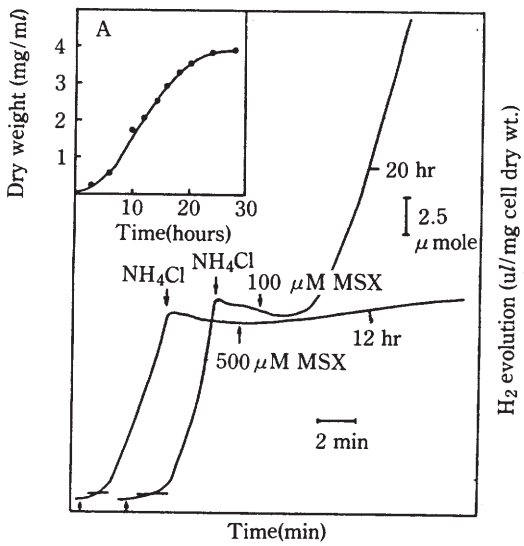

Fig. 5. *Effect of MSX on the nitrogenase switch-off by ammonium ion according to culture phase.*
0.5 mM NH_4Cl was added. A: Growth curve of *Rp. sphaeroides* cultured in glutamate-medium.

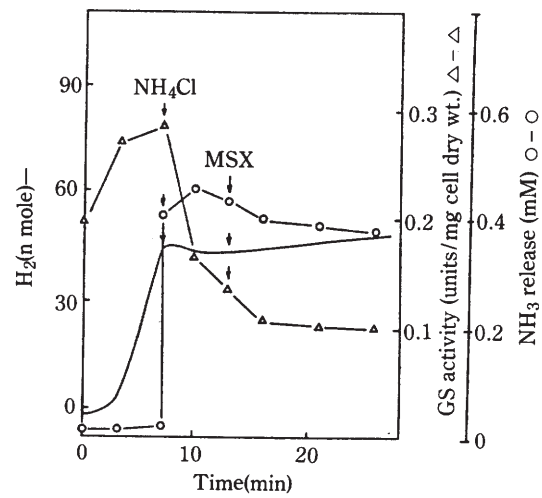

Fig. 7. *Interactions of ammonia, MSX, nitrogenase, and glutamine synthetase in Rp. sphaeroides grown in glutamate medium for 12 hours.*
First arrow; addition of 0.5 mM NH_4Cl, Second arrow; addition of 0.5 mM MSX

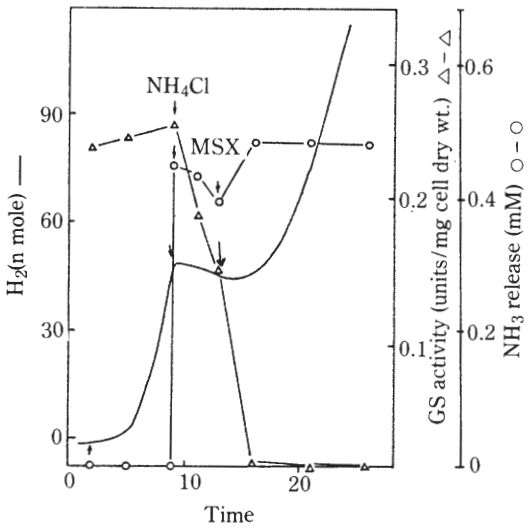

Fig. 8. *Interactions of ammonia, MSX, nitrogenase, and glutamine synthetase in Rp. sphaeroides grown in glutamate medium for 20 hours.*
First arrow; 0.5 mM NH_4Cl, Second arrow; 0.1 mM MSX

nase activity was still inhibited by ammonia (Fig. 7). However, nitrogenase switch-off after 20 hours would be prevented by 100μM of MSX (Fig. 8). On the other hand, GS activity was inhibited completely by 100μM MSX and concentration of released ammonia somewhat increased. But nitrogenase activity was not inhibited. From these observations, one can reason that the effect of MSX is related with the residual level of GS in such a way that the remaining GS activity in exponentially growing cells caused ammonia related inhibition of nitrogenase. Therefore, it is reasonable to assume that GS is the target enzyme for the action of MSX in nitrogenase inactivation. These data (Fig. 6,7,8) indicated that the inhibition of *in vivo* nitrogenase activity of *Rp. sphaeroides* by ammonia seemed to be mediated by products of ammonia assimilation rather than by ammonia itself.

적 요

광합성 세균인 *Rhodopsedomonas sphaeroides*의 질소 고정 효소 활성에 미치는 암모니아와 glutamine의 영향을 조사하였다. 그 결과 *Rp. sphaeroides*의 질소 고정 효소 활성은 암모니아와 glutamine에 의해 저해를 받았으며, 이들의 저해는 암모니아와 glutamine이 다 사용된 후에는 질소 고정 효소의 활성이 되살아나는 가역적인 반응이었다. glutamine synthetase의 활성을 비가역적으로 저해하는 methionine sulfoximine(MSX)을 사용하여 암모니아와 glutamine이 직접 질소 고정 효소의 활성을 저해하는지를 알아보았다. 암모니아에 의한 질소 고정 효소의 switch-off에 미치는 MSX의 영향은 균의 배양 시기에 의존함을 알 수 있었다. 12시간 배양한 경우, 500 μM NH_4Cl에 의해 질소 고정 효소는 저해를 받았으며, 500 μM의 MSX를 추가로 처리하였을 경우, GS는 21% 저해를 받았으며, 이때 유리된 암모니아의 량은 감소하였고, 질소 고정 효소의 활성은 회복되지 않았다. 그러나 20시간 배양한 경우, 500 μM NH_4Cl을 처리한 후 100 μM MSX를 첨가하면, GS의 활성은 완전히 저해되고, 유리된 암모니아의 량은 약간 증가하였으나 질소 고정 효소의 활성의 저해는 MSX에 의해 회복되었다.

따라서 *Rp. sphaeroides*의 경우, *in vivo* 상태에서 암모니아에 의한 질소 고정 효소의 활성 저해는 암모니아 자체에 의한 것이 아니라 암모니아 동화산물에 의한 것임을 알 수 있었다.

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