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TEMPERATURE-SENSITIVE RNA SYNTHESIS DURING ADAPTIVE GROWTH ON TESTOSTERONE OF *PSEUDOMONAS TESTOSTERONI*

MAMORU WATANABE, KAM WOON IP and LUISA PO
Division of Endocrinology and Metabolism, University of Alberta, Edmonton, Alberta, and the Division of Medicine, University of Calgary, Calgary, Alberta, Canada

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SUMMARY

Pseudomonas testosteroni is able to grow on nutrients other than steroids at 37°C, but during adaptive growth on steroids demonstrates temperature sensitivity and grows at 30 but not at 37°C. Similarly, RNA synthesis, which precedes and is necessary for induction of degradative enzymes, steroid-binding proteins, and transport processes occurs only at 30°C and not at 37°C, whereas rate of RNA synthesis during growth on other nutrients is greater at 37°C. The temperature-sensitive step occurs early during the induction period and the critical period appears to be during 2 to $2\frac{1}{2}$ h of adaptive growth on testosterone. The critical step appears to be the initiation of specific RNA synthesis. Once RNA synthesis is initiated, synthesis continues at the non-permissive temperature. From these data we have postulated that the temperature-sensitive step involves initiation of RNA synthesis and may depend on a temperature-sensitive repressor molecule of the steroid operon.

INTRODUCTION

Pseudomonas testosteroni, an organism originally isolated from soil, is capable of adaptive growth on testosterone* and other C-19 and C-21 steroids [1–3]. Talalay and his co-workers [4] have indicated that during adaptive growth on steroids, several enzyme activities are induced which include 1-ene-dehydrogenase, 4-en-5α-dehydrogenase, 4-en-5β-dehydrogenase, and 3β and 17β-hydroxysteroid dehydrogenase. We have recently reported that there are also steroid-binding proteins induced during adaptive growth which are released from the periplasmic space by osmotic shock treatment [5–7]. In addition, a transport process for steroids is induced, whose characteristics and regulation can be studied using the membrane vesicle system prepared from osmotically-shocked preparations of spheroplasts [8–10].

We have previously reported that the optimum temperature for growth on testosterone of *P. testosteroni* was 30°C and very little growth occurred above this temperature [5]. In addition, induction of periplasmic steroid-binding activity occurred at 25 or 30°C but very little at 37°C and none at 45°C [5]. In addition, metabolism of [7-^{3}H]-testosterone to ^3H_2O occurred optimally at 30°C but not at 37 or 45°C [5]. Our studies of temperature dependence of transport of steroids in membrane vesicles indicates that transport is maximal around 37°C [8]. It would appear that the temperature sensitivity of growth of this organism on testosterone is not limited by steroid transport properties, but may relate to other metabolic requirements at the permissive or non-permissive temperature.

One of the other factors which we have examined with respect to temperature sensitivity relates to the stability of binding of steroids to induced bacterium. The steroid-binding activity of induced bacterium is irreversibly destroyed by incubation at 45°C [5]. On the other hand, binding is inhibited at 37°C but the inhibition is reversible [5].

Because *P. testosteroni* is able to grown on other carbon sources at 37°C, an investigation of the temperature-sensitive step required during growth on steroids was initiated. This report indicates that one of the temperature-sensitive steps may be at the level of specific RNA synthesis.

EXPERIMENTAL METHODS

Growth conditions. Media used for growth and conditions for induction of binding activity have been described [6]. Binding activity and 1-ene-dehydrogenase activity of bacterial cells were performed as previously indicated [5, 6].

Synthesis of RNA and protein. Synthesis of RNA and protein was determined by the incorporation of radioactive uracil and amino acids, respectively, into acid-insoluble material [11]. At various times after induction of stationary phase cultures with testosterone, or after initiation of growth, samples of bacteria (usually 0.2 ml) were removed and incubated at the temperature and times indicated in the presence of [^{3}H]-uracil or [^{3}H]-protein hydrolysate, usually added to a final concentration of 1 μCi/ml and

* Trivial and systematic nomenclature of steroids: Testosterone-17β-hydroxy-4-androsten-3-one.

20 μCi/ml, respectively. At the end of the incubation period, 2 ml of cold 5% trichloroacetic acid (TCA) was added. Samples containing tritiated uracil were allowed to sit at 4°C for 30 min, while those containing tritiated protein hydrolysate were subjected to a boiling water bath for 30 min. The precipitate was then collected on Millipore HA filters and washed three times with 5% TCA. The filters were dried and the radioactivity was determined in a liquid scintillation spectrometer.

Materials. P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; unlabelled testosterone from Steraloids, Pawling, New York; [1,2-^{3}H]-testosterone from New England Nuclear Corporation, Boston, Massachusetts; [^{3}H]-uracil and ^{3}H-reconstituted protein hydrolysate from Schwarz–Mann, Orangeburg, New York; Millipore HA filters from Millipore Corp., Bedford, Massachusetts; and Rifampin from Calbiochem, Los Angeles, California.

RESULTS

Although the optimal temperature for growth on testosterone is 30°C, *P. testosteroni* was able to grow on other carbon sources at 37°C (Fig. 1). When this organism was grown on a medium containing yeast extract devoid of testosterone, growth was more rapid at 37°C than at 30°C. There was very little growth at 45°C. These studies indicated that the temperature sensitivity for growth was limited to growth on steroids and was not related to growth on other nutrients.

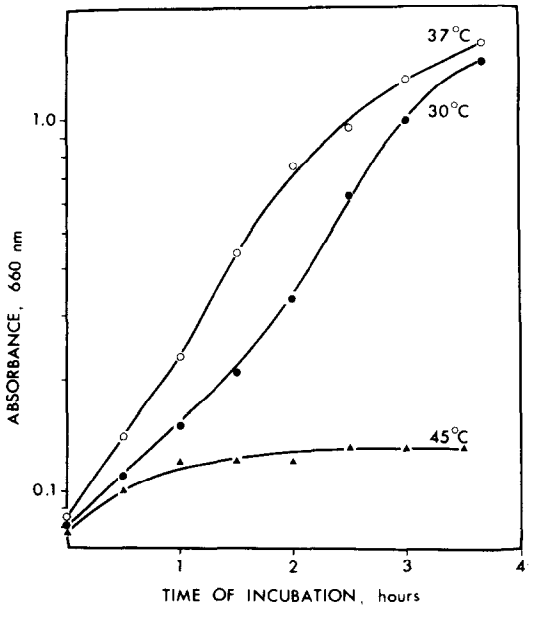

Fig. 1. Effect of temperature on growth of *P. testosteroni* in yeast extract. One ml of an overnight growth of *P. testosteroni* grown at 30°C was diluted 35-fold with fresh medium consisting of yeast extract not containing testosterone or other steroids and incubated at 30, 37 or 45°C. At 30 min intervals, samples were removed and absorbance at 660 nm determined in a spectrophotometer.

This finding suggested that there was not a generalized deleterious effect at 37°C, nor any generalized physiological requirements limiting growth of this organism to 30°C.

Before attempting to define the precise nature of the temperature-sensitive step during growth of this organism on testosterone, the sequence of RNA and protein synthesis during 72 h of growth at 30°C was determined. Rates of RNA and protein synthesis were examined by measuring the incorporation of [^{3}H]-uracil and [^{3}H]-amino acids, respectively, during 10 min of incubation at 30°C. The sequence of induction of binding and 1-ene-dehydrogenase activities was also examined in relation to RNA and protein synthesis. As previously reported [5], 1-ene-dehydrogenase activity appeared 8 to 9 h after induction, reaching a maximum at 15 h, whereas binding activity of steroids by whole cells reached a maximum at about 27 h (Fig. 2). Stimulation in the rate of RNA synthesis was first noted in induced cultures approx. 2 h after the addition of testosterone, with a maximum rate at 7–8 h (Fig. 2). Uninduced cultures demonstrated only basal levels of RNA synthesis without the stimulation observed with induced cultures. The stimulation in the rate of protein synthesis occurred concomitantly with that of RNA synthesis and preceded the appearance of enzyme or binding activities. It should be noted that maximum rates of both RNA and protein synthesis occurred at 7–8 h, while the 1-ene-dehydrogenase activity first became evident 8–9 h after induction.

The temporal sequence of appearance of RNA and protein synthesis, followed by the appearance of enzyme activities and steroid-binding properties indicated that the requirements for induction of this operon was similar to that reported for other inducible systems. In fact, RNA synthesis appeared to be essential for induction of both steroid receptor and dehydrogenase activities. Inhibition of RNA and protein synthesis by the addition of rifampicin to the culture medium at the time of testosterone addition prevented the induction of 1-ene-dehydrogenase activity and the appearance of the steroid-binding activity (Table 1).

Having established the requirement for RNA synthesis during growth on testosterone, synthesis of RNA was examined at the permissive (30°C) and non-permissive (37°C) temperatures during growth of *P. testosteroni* on testosterone or other carbon sources. When RNA synthesis was examined during growth of this organism on a carbon source other than testosterone, synthesis was greater at 37°C than at 30°C (Fig. 3), in keeping with the increased growth rate observed at this temperature (Fig. 1). Protein synthesis was also greater at 37°C than at 30°C. Neither RNA nor protein synthesis was increased above baseline levels at incubation temperatures of 45°C.

When RNA synthesis was examined during inductive growth on testosterone, however, the temperature profile differed from that observed with growth on

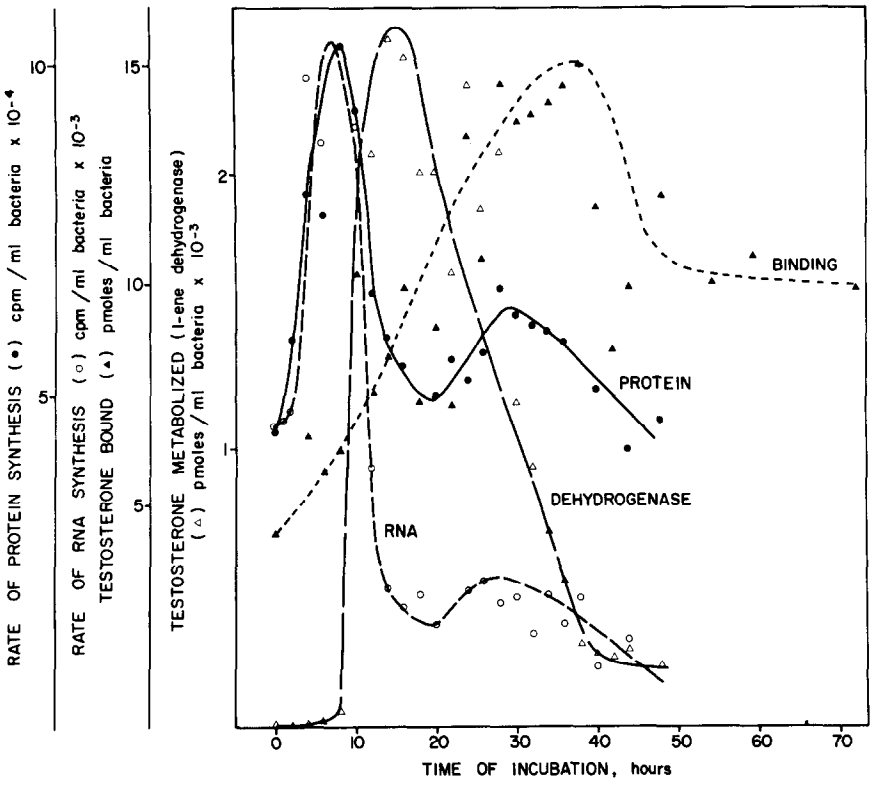

Fig. 2. Synthesis of RNA and protein during growth of *P. testosteroni* on testosterone. Stationary phase cultures of *P. testosteroni* were induced by the addition of testosterone to a final concentration of 0.5 g/l and incubated at 30°C for 72 h. At various times after induction, samples of bacteria were removed and rates of syntheses of RNA and protein were determined as described in Methods. 1-ene-Dehydrogenase activity was assayed at 30°C using limiting amounts of bacteria and [1,2-^{3}H]-testosterone as substrate [5]. Binding activity of whole bacteria was assayed on ethanol-washed cells as described [5, 6]. For determination of RNA and protein synthesis, 0.2 ml of bacteria was used and incubated at 30°C for 10 min in the presence of tritiated uracil or tritiated protein hydrolysate, respectively.

other carbon sources. RNA synthesis occurred at 30°C during growth on testosterone whereas there was little or no synthesis at 37 or 45°C (Fig. 4). Incubation of this organism for 6 h at 37 or 45°C in the presence of testosterone did not irreversibly damage the bacterium. Growth rate of such cells was restored to normal when subsequently incubated in fresh medium at 30°C.

Table 1. Effect of rifampicin on induction process in *Pseudomonas testosteroni*

Measurement	Growth Conditions	
	No Addition	Rifampicin Added
Absorbance (λ_{660}) (induced/uninduced after 12 h)	1.33	1.04
Increase in rate of RNA Synthesis (cpm/ml of bacteria)	7,220	0
Increase in rate of protein Synthesis (cpm/ml of bacteria)	35,080	0
1-ene-Dehydrogenase Activity (pmoles/ml of bacteria)	3,243	42
Binding Activity (pmoles/ml of bacteria)	8.1	0.9

Note. Stationary phase cultures of *P. testosteroni* grown at 30°C were induced by the addition of testosterone to a final concentration of 0.5 g/l. Rifampicin was added to one-half of the culture to a final concentration of 62.5 μg/ml and the culture was shielded from light during the experimental period. Rates of syntheses of RNA and protein were determined 5 h after induction as described in Methods and the incubation period was for 10 min at 30°C. 1-ene-Dehydrogenase activity [5] was assayed 12 h after induction using limiting amounts of bacteria and [1,2-^{3}H]-testosterone as substrate. Binding activity of whole bacteria was assayed on ethanol-washed cells [5, 6] 24 h after induction.

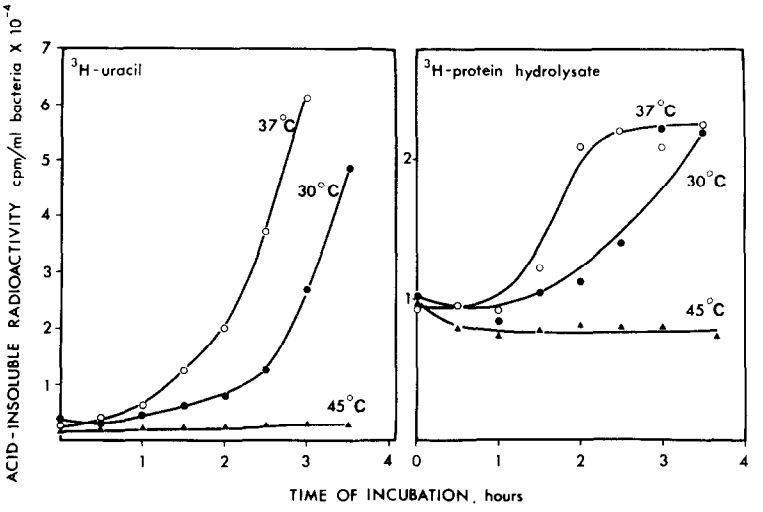

Fig. 3. RNA and protein synthesis during growth of *P. testosteroni* on yeast extract. Bacteria were grown in yeast extract lacking testosterone at 30, 37 and 45°C as described in Fig. 1. At 30 min intervals, 0.1 ml of bacteria was removed from these cultures and added to tubes containing either 0.1 μCi of ^{3}H-uracil or 4 μCi of ^{3}H-protein hydrolysate and incubated for 10 min at the temperature of growth. Acid-insoluble radioactivity was determined as described.

These studies indicated that 37°C was not a non-permissive temperature for *P. testosteroni* and RNA could be synthesized at 37°C except when testosterone served as the carbon source. In the latter situation, 30°C was the permissive temperature for growth and RNA synthesis. In order to determine the critical period of temperature sensitivity, bacterial cultures induced by testosterone at the permissive temperature were shifted to the non-permissive temperature at various times after induction, and the ability to synthesize RNA was examined.

In the culture grown continuously at 30°C, a significant increase in the rate of RNA synthesis was first

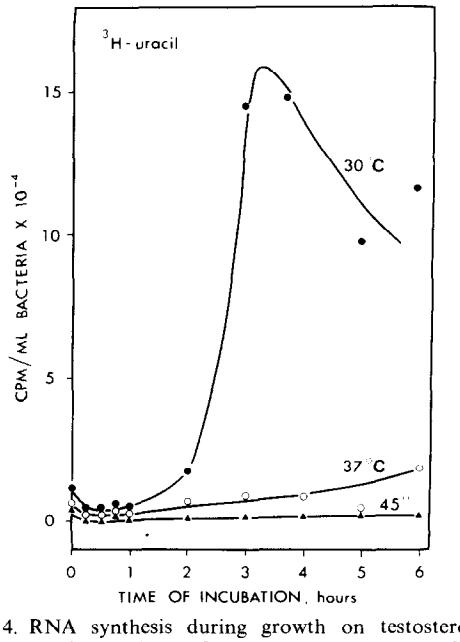

Fig. 4. RNA synthesis during growth on testosterone. Stationary phase culture of *P. testosteroni* grown at 30°C was induced by the addition of testosterone to a final concentration of 0.5 g/l. The induced cultures were incubated at 30, 37 or 45°C for 6 h. At time intervals indicated, rate of RNA synthesis was determined by adding 0.2 ml of bacteria to tubes containing 0.2 μCi of ^{3}H-uracil. After 10 min of incubation at the temperatures of growth, 2 ml of 5% TCA were added and acid-insoluble radioactivity determined as described.

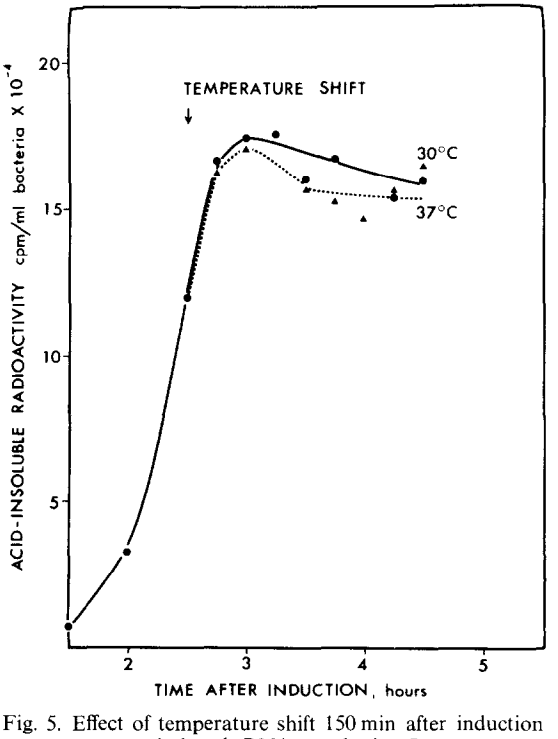

Fig. 5. Effect of temperature shift 150 min after induction on testosterone-induced RNA synthesis. *P. testosteroni* grown to stationary phase, induced at 30°C by addition of testosterone to a final concentration of 0.5 g/l, was shifted to 37°C 150 min after induction. Rate of RNA synthesis was determined by incubating 0.1 ml bacteria with 0.1 μCi of ^{3}H-uracil at 30°C for 5 min.

Table 2. Effect of temperature shift on RNA synthesis induced by growth on testosterone

Experiment	Time of Temperature Shift to 37°C	Rate of RNA Synthesis		
		At Time of Shift	4h After Induction	3h After Restoring to 30°C
	min after induction		cpm/ml of bacteria	
I	No shift	-	210,940	129,135
	15	4,590	6,120	200,245
	30	4,740	9,690	131,185
	45	5,655	5,370	163,950
	60	6,005	6,770	190,750
	90	14,025	6,700	177,320
	120	29,735	11,880	54,405
II	No shift	-	144,960	
	150	61,820	144,950	
	180	149,040	142,340	

Note 1. Stationary phase cultures of *P. testosteroni* grown at 30°C were induced by the addition of testosterone to a final concentration of 0.5 g/l and incubated at 30°C in a gyrotory shaker. At various times after induction, samples of the culture were raised to 37°C and incubated at the non-permissive temperature for a total of 4 h from the time of induction. At the end of 4 h the cultures were restored to an incubation temperature of 30°C and incubated for a further 3 h. At various intervals in Experiment I, 0.1 ml samples of bacteria were removed from each of the cultures and RNA synthesis was assayed at 37°C for 10 mins in the presence of 0.1 μCi of ^{3}H-uracil. There was no significant difference in the rate of synthesis when the incubation temperature during the assay for RNA synthesis was 30°C. In this experiment, we have reported the rate of RNA synthesis at the time of the shift, 4 h after induction, regardless of the time of the shift, and again 3 h after restoration to the permissive temperature.

Note 2. In Experiment II the conditions were identical except that the temperature shift occurred at 150 and 180 min after induction. In these experiments, the incubation period during measurement of RNA synthesis was 5 min instead of 10 min and the incubation temperature was 30°C. In these experiments the cultures were not restored to 30°C for a further incubation period.

noted at 90 min with considerable RNA synthesis 4 h after induction (Table 2). Cultures maintained at 30°C for up to 120 min were unable to attain the increased rate of RNA synthesis when subsequently switched to 37°C. It should be noted, however, that when cultures were shifted after significant RNA synthesis had begun, i.e., at 150 and 180 min, a shift to the non-permissive temperature did not affect the subsequent rate of RNA synthesis. In the culture shifted at 150 min, rate of RNA synthesis was examined every 15 min after the shift. The pattern of RNA synthesis after the shift was identical at 37 and 30°C (Fig. 5).

It should be noted that in the case of the 90 and 120 min cultures, there was a decrease in the rate of RNA synthesis when assayed 4 hr after induction, or at least 2 hr after the temperature shift. The difference in incorporation did not appear to be related to the incubation temperatures during RNA synthesis since control cultures showed identical rates of RNA synthesis at equivalent stages of induction at incubation temperatures of 30 and 37°C. It would appear that unless RNA synthesis is well on its way prior to the shift in temperatures, there is no further synthesis of RNA at 37°C since these cultures were allowed to incubate at this temperature for at least a further 2 h. These studies indicated that the most critical period of temperature sensitivity was during 2 to $2\frac{1}{2}$ h after induction.

It should be noted that bacterial cultures shifted to 37°C were not destroyed by the temperature shift. When these cultures were subsequently restored to 30°C and RNA synthesis during a further 3 h period was determined, the stimulation of RNA synthesis in all of these cultures was quantitatively and temporally similar to the pattern of stimulation observed with the cultures induced and maintained at 30°C (Table 2).

DISCUSSION

It would appear from the studies reported here that the temperature-sensitive step regulating growth on testosterone may be at the level of specific RNA synthesis. The critical period appeared to be early in the induction process, between 2 and $2\frac{1}{2}$ h after addition of testosterone to the bacterial culture. Cultures exposed to testosterone for 2 h at 30°C were unable to synthesize RNA when shifted to 37°C. However, cultures shifted $2\frac{1}{2}$ h after induction were able to synthesize RNA at 37°C at a rate equivalent to the permissive temperature.

Several mechanisms could explain the deleterious effect of the temperature during 2–$2\frac{1}{2}$ h after induction. Transcription could be the primary target site of temperature sensitivity, although this seemed unlikely in view of the inability to detect differences in rate of RNA synthesis when cultures induced at 30°C were

assayed for RNA synthesis at both 30 and 37°C. A second piece of evidence against this possibility was that RNA synthesis in cultures shifted after RNA synthesis had already been initiated continued as well at 37°C as at 30°C and at a rate equivalent to the permissive temperature. However, before RNA synthesis is initiated, a temperature shift to 37°C was deleterious to further RNA synthesis. It is most likely, therefore, that the temperature-sensitive step is at the level of initiation of RNA synthesis and may be due to a temperature-sensitive repressor of the "steroid operon". We hope to further elucidate the temperature-sensitive step by isolating a mutant which is stable at 37°C for growth on testosterone.

It should be noted that our investigations have indicated more than one possible site for temperature-sensitivity for growth on testosterone. We have previously indicated that binding of steroids to induced bacteria is inhibited at 37°C, although the damage is reversible [5]. On the other hand, it may be possible that steroid-binding activity is linked in some way to the repression of the "steroid operon" and the temperature-sensitive step may represent a single defect. It is important to emphasize that the effect on RNA synthesis may not be the primary target of the non-permissive temperature, but may represent a secondary phenomenon of a more basic temperature-sensitive process, whose integrity and function plays a vital role in the initiation of specific RNA synthesis.

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