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Observations on the Effect of the Retrosteroid Ro 4-8347 in the Female Rat

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Ro 4-8347 (6-chloro-9β,10α-pregna-1,4,6-triene-3,20-dione) is a potent, orally effective progestational agent with a close structural relationship to dydrogesterone. In clinical trials it could be shown that cyclic administration of this compound would induce ovulation in a certain number of anovulatory women (Stamm et al., 1968; Dapunt, 1968; Taubert und Jürgensen, 1968).

Ro 4-8347 was found to have a wide spectrum of progestational activity because it proved to be effective in the Clauberg-, Rubin-, and carbonic anhydrase test and also maintained pregnancy in the rat. Estrus and copulation were inhibited by daily administration of 2 mg/rat. There was no discernible estrogenic or anti-estrogenic effect in the vaginal opening test, and no myotrophic or androgenic effect could be shown.

Since little is known about the action mechanism of this compound, the effect of Ro 4-8347 administration upon some aspects of the reproductive function in the female rat was investigated.

Methods and materials

Female Wistar rats which had been kept under standard conditions with water and food 'ad libitum' were used for all experiments. Ro 4-8347 was supplied by Hoffmann-La Roche Ltd., Grenzach/Baden (Germany). Progesterone and estradiol-17β were obtained from commercial sources. – The following effects of Ro 4-8347 were studied:

A. Effect on uterine, ovarian, adrenal, and pituitary weight. – Female Wistar rats weighing 60–90 g were unilaterally oophorectomized by a dorso-lateral incision under light ether anesthesia. Each group contained 10 animals. The test substances were applied daily over a 42 day period by gastric lavage. Each dose was contained in 1.0 ml of a 5% gum arabic solution. At the time of sacrifice the body weight, and the wet weights of the remaining ovary, the uterus, the adrenal gland, and the pituitary were determined.

The following groups were studied: (1) Intact, untreated controls, (2) untreated oophorectomized controls, (3) controls treated with gum arabic solution, (4) 0.5 mg Ro 4-8347, (5) 1.0 mg Ro 4-8347/day, (6) 5 mg Ro 4-8347/day, (7) 10 mg Ro 4-8347/day.

B. Inhibition of exogenous gonadotropins. – Female rats of approximately 100 g body weight were used in groups of 6 animals per dose. The test substances were applied for 5 days. The animals were sacrificed on day 6, and the uterine and ovarian weights were determined. Ro 4-8347 was suspended in a dose of 5 mg/ml of a 5% gum arabic solution and applied by a gastric tube. HCG[1] and PMS[2] were dissolved in Ringer's solution and injected s.c.

The following groups were studied: (1) Untreated controls, (2) 5 mg Ro 4-8347/day from day 1 through 5, (3) 0.67 IU HCG + 6.7 IU PMS/day from day 3 through 5, (4) 5 mg Ro 4-8347/day from day 1 through 5, and 0.67 IU HCG + 6.7 IU/PMS/day from day 3 through 5.

C. Effect on LH content of the pituitary. – Ro 4-8347 and progesterone were administered in doses of 5 mg/day to groups of 6 intact female Wistar rats per dose for 14 days. The animals were sacrificed on day 15. The pituitaries were immediately removed and placed in acetone at —25° C until LH could be assayed.

Progesterone was injected as on oily solution s.c. Ro 4-8347 was administered by gastric tube in gum arabic solution as well as s.c. in a solution containing 60% arachis oil and 40% benzyl benzoate.

Techniques of LH assay. LH was measured by a modification of the method of Parlow (1958). Immature female rats weighing 40–50 g at the beginning of the experiment were used.

a) *Priming procedure:* Pseudopregnancy was induced by injection of 75 IU PMS on day 1 and 3. This was followed by injection of 75 IU HCG on day 5. The animals could be used for LH assay from day 11 through 14. Since assays performed on day 12 appeared to yield the best results, all experiments were done at this time.

b) *Method of assay.* 1 ml of pituitary suspension was injected into a tail vein of a pseudopregnant rat. Six animals were used per dose level. Each rat received 0.1 mg dried pituitary. They were sacrificed 2–3 h after injection. The time interval was standardized for each set of experiments. The ovaries were removed, cleaned of fat, and placed immediately in 2.5% metaphosphoric acid. The extracts obtained by homogenization were filtered, and subsequently assayed for their ascorbic acid content.

c) *Reagents.* (1) 10 mg of 2,6-dichlorphenolindolphenol sodium were dissolved in 100 ml of distilled water at 85–95° C. The resulting solution was filtered and brought up to a total volume of 250 ml. (2) 11.3 g of sodium acetate ($CH_3COONa \cdot 3H_2O$) were dissolved in 250 ml H_2O. The pH was adjusted to 6.7 ± 0.2 by addition of acetic acid. (3) Xylene.

d) *Method of ascorbic acid assay.* Equal volumes of solutions (1) and (2) were mixed. 5 ml of the resulting mixture, 5 ml of xylene, and 2–3 ml of the ovarian extract were shaken in a separatory funnel for 30 sec. The color indicator was by this method quantitatively extracted into the xylene fraction. After separation and filtration the optical density was measured against a reagent blank at 500 mμ in a Zeiss spectrophotometer M 4 Q III. The LH assay was performed as a 3-point assay with 2.0 IU HCG and 0.9% NaCl as standards.

D. Induction of ovulation. – Daily vaginal smears were performed on female Wistar rats of approximately 200 g body weight. After the normalcy of the estrus cycle had been established, the animals received on the day of full estrus an electric shock of 7.5 V DC by means of a double vaginal electrode (day 0). This method had been shown in preliminary experiments to induce pseudopregnancy in more than 90% of the animals. At first experiments were conducted to establish the minimal effective dose of Ro 4-8347 which would prevent conception. This was achieved by administration of various test doses in volumes of 1 ml propylene glycol by gastric tubes for 12 days. The animals were placed with male rats of proven fertility from day 2 through 12. On day 20 they were sacrificed and examined for evidence of pregnancy.

[1] Predalon, Organon, Oss (Holland).

[2] Predalon S, Organon, Oss (Holland).

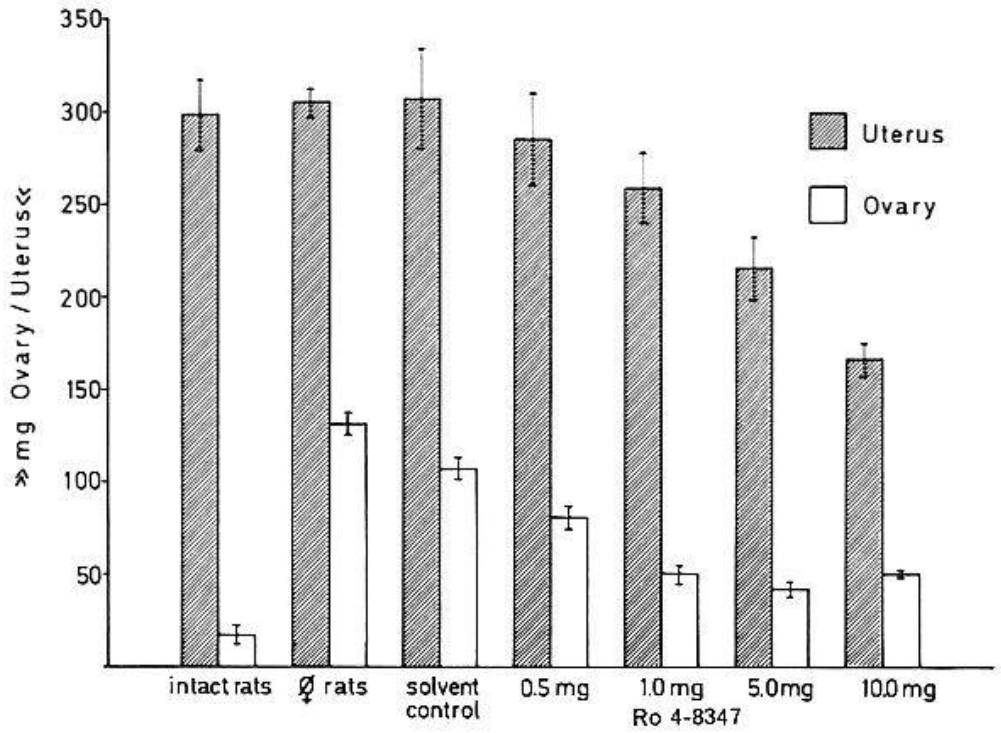

Fig. 1. Effect of Ro 4-8347 on ovarian and uterine weight of hemi-castrated female rats after 42 days of oral application. Each group contained 10 animals. Vertical bars delineate standard error of the mean.

The following methods were used to study the effectiveness of Ro 4-8347 as an inducer of ovulation. Various doses of the test substance were administered in a volume of 1 ml propylene glycol on the following days of pseudopregnancy: 2+3, 3+4, 4+5, and 6+7. The total dose was divided in three portions, one of which was given the first day, and the remaining two the second day.

On the second day of treatment the animals were caged with male rats. Three days following the last administration of Ro 4-8347 the right tube was removed and the number of ova counted. The animals were sacrificed 8 days later to examine implantation sites in the contralateral horn of the uterus.

In a second series Ro 4-8347 was administered daily from day 3 through 8. When sperm were identified in the vaginal smear the drug was discontinued. The animals were sacrificed on day 16 of pseudopregnancy, and the number of implantation sites was recorded.

Results

A. Effect on uterine, ovarian, adrenal, and pituitary weight. – Unilateral oophorectomy resulted in a significant increase in the mean weight of the remaining ovaries. There was no effect on uterine weight in solvent treated control animals. The daily application of 1.0 mg of Ro 4-8347 caused a decrease of uterine and ovarian weight, both of which were significant ($p < 0.05$). When the dose was raised to 5 and 10 mg/rat/day respectively a further significant reduction of uterine weight was noticed. When doses larger than 1 mg/rat/day were given, no noticeable changes of ovarian weight were seen ($p > 0.05$) (Fig. 1). An obvious effect on pituitary weight was not discernible, but there was a certain depression in adrenal weight

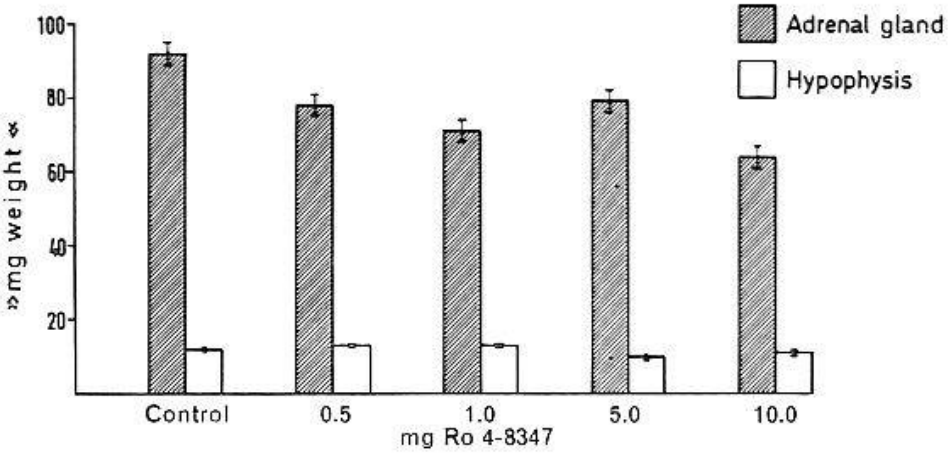

Fig. 2. Effect of Ro 4-8347 on adrenal and pituitary weight of hemi-castrated female rats. For details of experimental design refer to text and Fig. 1. Vertical bars indicate standard error of the mean.

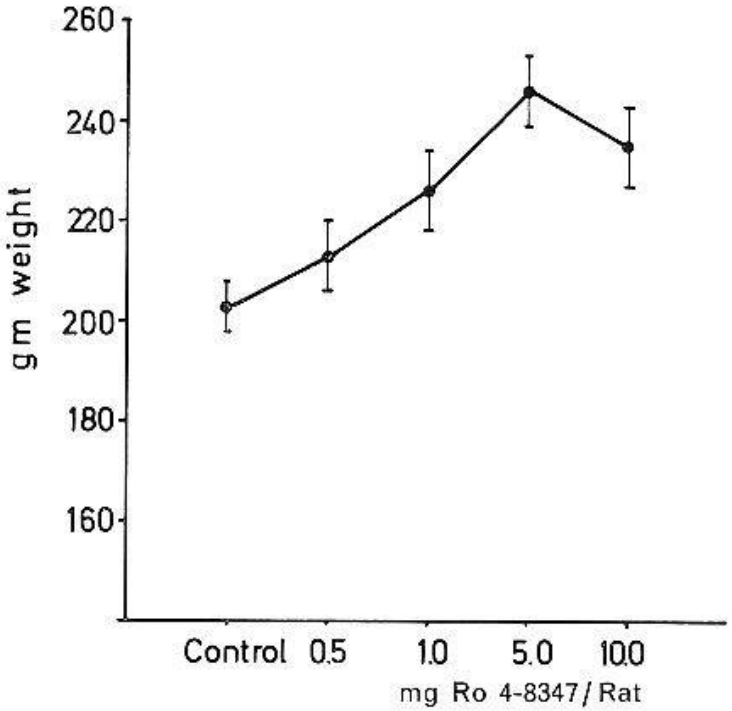

Fig. 3. Effect of Ro 4-8347 on body weight of unilaterally oophorectomized rats. The substance was applied for 42 days. Each point represents the mean weight of 10 animals. Vertical bars delineate the standard error of the mean.

(Fig. 2). It was found, however, in a separate experiment that the suppressive effect of 5 mg of Ro 4-8347 was much less marked than that observed by the application of equal doses of progesterone (progesterone 24.4 ± 1.2 mg, Ro 4-8347 43 ± 1.9 mg, $p < 0.01$).

Previous experiments had indicated that the daily dose of 2 mg of Ro 4-8347 to female rats over a 7 week period would induce an increase in body weight over that of untreated controls. It was found that animals treated with 1 mg/rat/day for 42 days gained on the average 20 g, and

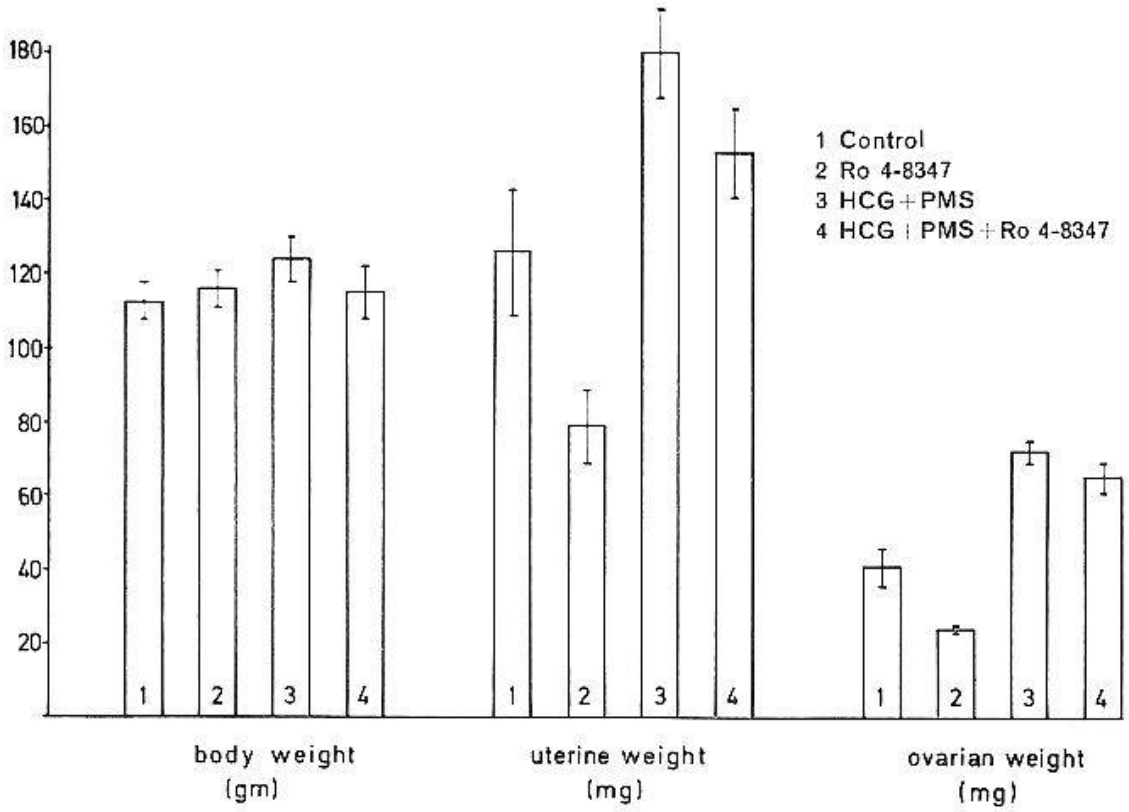

Fig. 4. Failure of Ro 4-8347 to inhibit the increase of ovarian and uterine weight induced by PMS and HCG. Each group contained 6 animals. Vertical bars stand for standard error of the mean.

animals treated with 5 mg gained on the average 40 g more than untreated controls. These differences were significant at the 1% level. When the dose was raised to 10 mg/rat/day the average body weight tended to be somewhat lower, but the difference as compared to the 5 mg dose level was of no statistical significance ($p>0.05$) (Fig. 3).

B. Inhibition of exogenous gonadotropins. – Experiments were conducted to determine if Ro 4-8347 would interfere with the action of exogenous gonadotropins on the ovary. The results of these experiments are depicted in Fig. 4. The administration of 5 mg Ro 4-8347/day resulted within 5 days in a significant reduction in ovarian and uterine weight. When applied together with PMS and HCG the gonadotropin induced stimulation of organ weights was not affected. This indicates that Ro 4-8347 acts upon the ovary through the mediation of the pituitary.

C. Effect on LH content of the pituitary. – An experiment was carried out to determine whether or not Ro 4-8347 would affect pituitary LH content.

The results of this experiment were shown in Table I. The injection of 5 mg progesterone resulted in a slight increase of the pituitary LH content. Contrary to this there was no effect in when the same amount of Ro 4-8347 was given orally. Subcutaneous injection of 5 mg Ro 4-8347 resulted, however, in a significant increase in the pituitary LH content (Table I).

Table I

LH content of rat pituitaries after 14 days application of Ro 4-8347 and progesterone. LH assay was assayed by the OAAD-test (PARLOW, 1958). The results were expressed as percentage of extinction of the reagent blank after injection of 0.1 mg dried pituitary per rat. OD_{500} of 2 IU was 0.091. For further details of experimental design refer to text

Treatment	OD_{500} (mean ± S.E.M.)	p
None	0.159 ± 0.010	–
Progesterone, 5 mg/day s.c.	0.127 ± 0.012	<0.05
Ro 4-8347, 5 mg/day p.o. .	0.164 ± 0.012	>0.05
Ro 4-8347, 5 mg/day s.c. ..	0.070 ± 0.002	<0.01

Table II

Determination of the minimal ovulation inhibiting dose of Ro 4-8347 in the rat. Ro 4-8347 was applied in 1 ml propylene glycol orally from day 1 to 12 of the experiment. The animals were caged with male rats from day 2 to 12, sacrificed on day 20, and the number of implantation sites was recorded

Ro 4-8347 mg/kg rat per day	N	Animals with sperm	Average number of implantation sites	Estrus vaginal smear
25.0	12	0	0	–
12.5	12	0	0	–
6.25	12	0	0	–
1.0	12	0	0	–
0.5	12	3	0	atypical
0.25	12	6	4.4	atypical
0.1	12	10	6.8	atypical

D. Induction of ovulation. – It could be shown that the administration of 0.5 mg Ro 4-8347 per kg rat over a 12-day period was just sufficient to block ovulation in the majority of animals (Table II). Attempts to induce ovulation in the pseudopregnant rat were therefore made with doses ranging from a total dose of 1.5 mg/kg/rat down to as little as 0.03 mg. The results summarized in Table III indicated that the administration of these doses for various 2-day periods of pseudopregnancy was largely ineffectual. A noticeable number of ovulations was obtained only in the group of animals receiving 0.05 mg/kg irrespective of the time of administration. There were, however, no pregnancies. The continuous application of the same dose from day 3 through 8 of pseudopregnancy was found to result in 8 out of 12 animals conceiving. The number of implantation sites was within normal range. When the dosage was decreased to 0.03 mg/kg the results did not differ significantly from those obtained with the placebo (Table IV).

Table III

Induction of ovulation in the pseudopregnant rat with Ro 4-8347. The test substance was administered in 1 ml of propylene glycol once during the 1st, and twice during the 2nd day. For experimental detail refer to text

Ro 4-8347 dose/48 h	Days of treatment	N	Sperm	Ovulation	Pregnancy	χ^2
1.5 mg/kg	2 and 3	6	0	0	0	–
	3 and 4	6	0	1	0	<2.706
	4 and 5	6	0	1	0	<2.706
	6 and 7	6	0	0	0	–
0.3 mg/kg	2 and 3	6	0	0	0	–
	3 and 4	6	0	0	0	–
	4 and 5	6	0	2	2	>2.706
	6 and 7	6	2	2	1	>2.706
0.15 mg/kg	2 and 3	12	0	5	0	>5.412
	3 and 4	12	1	4	0	<2.706
	4 and 5	12	0	3	0	>2.706
	6 and 7	12	0	5	0	>2.706
0.03 mg/kg	2 and 3	12	0	0	0	–
	3 and 4	12	0	0	0	–
	4 and 5	12	0	0	0	–
	6 and 7	12	0	0	0	–
Placebo	2 and 3	6	0	0	0	–
	3 and 4	6	0	1	0	–
	4 and 5	6	0	0	0	–
	6 and 7	6	0	1	0	–

Table IV

Induction of ovulation in the pseudopregnant rat by p.o. administration of Ro 4-8347 from day 3 to 8 of pseudopregnancy. For details of experimental design refer to text

Ro 4-8347 dose	Days of treatment	N	Number of rats with sperm	Mean number of implantations	χ^2
0.05 mg/kg/day	3 to 8	12	8	6.7	>5.412
0.01 mg/kg/day	3 to 8	12	2	5.8	<2.706
Placebo	3 to 8	12	1	0	–

Comment

The results of these experiments showed that Ro 4-8347 is a retrosteroid with a relatively strong central effect. When applied over several weeks to unilaterally oophorectomized rats, a dose-dependent decrease in ovarian and uterine weight was found. It could also be shown that this effect could

be demonstrated after only 5 instead of 42 days of treatment. There was no interference with the effect of exogenously administered gonadotropins upon the ovary. This suggests a direct effect of this substance on the hypothalamic-pituitary axis. Preliminary experiments not reported here had shown that the efficacy of Ro 4-8347 in suppressing ovarian and uterine weight was in the same order of magnitude as that of equal doses of progesterone.

Progesterone has been used with success in the induction of premature ovulation when given to proestrous rats (ZEILMAKER, 1966), for the facilitation of ovulation in PMS treated, immature rats (MCCORMACK, 1964), and for the synchronization of estrus in animal husbandry (CARRICK and SHELTON, 1967; FOHNING et al., 1966).

In contrast to a previous report, it was found that even a daily dose of as little as 0.5 mg/kg rat would suffice to inhibit ovulation effectively. It was not without interest to note that the application of 0.05 mg/kg rat/day, i.e. $^1/_{10}$ of the ovulation inhibiting dose, from day 3 through 8 of pseudopregnancy would result in 8 out of 12 animals ovulating and conceiving. This dose proved to be much less effective when applied at various 48-hour periods during pseudopregnancy. Higher and also lower doses proved to be singularly ineffective. This seems to indicate that there is, at least in the rat, a definite correlation between the dose and effect of this compound regarding ovulation induction.

When the pituitary LH contents of Ro 4-8347 treated rats and progesterone treated rats were compared it was found that the pituitaries of the former contained significantly higher amounts of LH than the latter. As these results were obtained with the much higher dose of 5 mg/rat, it remains a matter of speculation whether or not this finding bears any relationship to the ovulation inducing effect of this compound in the rat and in the human.

Summary

1. The effect of the retrosteroid Ro 4-8347 upon some aspects of the reproductive function of the female rat was studied.

2. It was found to suppress uterine and ovarian weight in doses of 0.5 to 10 mg/rat/day.

3. The effect of exogenously administered PMS and HCG on the ovary was not interfered with.

4. The pituitary LH content of rats treated with Ro 4-8347 was significantly higher than that of progesterone treated rats.

5. Ovulation induction was achieved by the administration of 0.05 mg/day per rat from day 3 to 8 of pseudopregnancy.

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