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Twenty Years of Study on Effects of Pineal Peptide Preparation: Epithalamin in Experimental Gerontology and Oncology

V. N. ANISIMOV, V. KH. KHAVINSON, AND V. G. MOROZOV

Laboratory of Experimental Tumors
N. N. Petrov Research Institute of Oncology
68, Leningradskaya Str., Pesochny-2
St. Petersburg 189646, Russia

INTRODUCTION

During the last decade a number of reports have appeared in the literature on a regulatory role of the pineal gland in aging and cancer development,[1-5] in which the modulating function of the pineal gland on neuroendocrine and immune system has been shown to be altered in aging[6,7] and tumor growth.[8-11] Pinealectomy of rats was followed by reduction of their life span[12] and by stimulation of tumor development.[9,13] The administration of the pineal hormone melatonin into old mice or the grafting of pineal glands from young donors into the thymuses of old mice prolongs their life span.[14] There is also evidence of an antitumor effect of melatonin.[8-10]

Most investigators have invoked melatonin as the primary mediator of the endocrine capabilities of the pineal gland. However, there is evidence that some of the effects of the pineal gland may be a result of pineal peptide secretion.[15] It has been shown that some crude peptide extracts or purified peptides isolated from the pineal gland have antigonadotropic, metabolic and antitumor activity.[10,11,16-19] In 1955 Parhon[20] reported on the life-span prolonging effect of a pineal extract in old rats. No details were given on the method of pineal extraction or on the experiment.

In 1973 the first evidence was published that administration of the low-molecular-weight pineal peptide extract (commercial form was named later as Epithalamin) was followed by restoration of the estrus cycle in old female rats with persistent estrus syndrome and by lowering of the threshold of sensitivity of the hypothalamo-pituitary complex to feedback inhibition by estrogens in old animals.[21] Since this work, the effect of epithalamin on the function of the reproductive, endocrine, neuroendocrine and immune systems was systematically studied in our own and our colleagues' experiments. There was shown a high biological activity of epithalamin. Long-term treatment with the preparation prolongs the life span of animals, slows down the aging of the reproductive system, improves the parameters of immune functions and inhibits the development of spontaneous tumors induced by some chemicals or X-irradiation and transplanted tumors.[1,9,22-34] In the present paper the results of twenty years of study of the biological effects of epithalamin, mainly in experimental gerontology and oncology, are summarized.

Epithalamin: A Low-Molecular Weight Pineal Peptide Preparation

The physiologically active substances were prepared from bovine pineal gland collected during all seasons of the year at the Leningrad Factory of Medical Preparation (p/o S. M. Kirov "Lenmyasokombinat") as described elsewhere.[24,33] The main steps of the preparation were as follows. The native tissues at −4°C were kept in acetone for 48 hours. After the acetone was poured off the tissues were homogenized and extraction was performed in 3% acetic acid at a ratio of 1 : 6 (v/v) in the presence of $ZnCl_2$ for 72 hours. After the final extraction, and centrifugation for 20 min at 3000 rpm, acetone was added to the supernatant (at −4°C; 1 : 8 v/v). After precipitate formation the acetone was poured off. The precipitate was treated with acetone and ether in the filter until a white powder was formed. The powder was dissolved, sterilized and lyophilized. When studying the prepared substances by ion-exchange chromatography on the carboxyl cationite Biocarb (USSR), gel-filtration on a Sephadex G-25 and electrophoresis on cellulose layers, it was found that the above substances are complexes of polypeptides. The three main fractions' ratio in the total preparation was 74 : 16 : 10%; the molecular weights were 250, 11000 and 1200, respectively, and the isoelectric points (pH units) were 3.0, 5.0 and 10.5, respectively.

The biological activity of epithalamin was estimated in a test with human chorionic gonadotropin (HCG) in infantile female mice.[34] More than 30 assays were performed during 20 years in our laboratory and the stability of the high antigonadotropic effect of every new series of epithalamin prepared was proved.

Epithalamin was gel-filtrated on a Sephadex-50 and the low- and high-molecular fractions were studied in the test with HCG.[35] It was shown that the antigonadotropic effect of the low-molecular fraction is higher than that of the high-molecular fraction. Subsequent purification of epithalamin, sequencing, synthesis and bioassay of a very low-molecular peptide are under way now.[36]

The commercial drug form of total pineal peptide preparation (Epithalamin) was permitted by the Pharmaceutical Committee of the USSR Ministry of Health for medical use and only the results of the experiments with this preparation are reported here.

Effect of Epithalamin on the Life Span of Mice and Rats

The effect of epithalamin on the life span of C3H/Sn and SHR female mice was studied in two sets of experiments.[24,27] Chronic treatment of female C3H/Sn mice with epithalamin in a single dose 0.5 mg per mouse, started at the age of 3.5 months, prolonged their mean life span by 40% and increased their maximum life span by 3.5 months.[24] The survival curves of mice exposed to epithalamin were significantly shifted to the right. Calculation of parameters of the Gompertz equation have shown that the aging rate (α) of mice treated with epithalamin was significantly decreased. Long-term injections of epithalamin into SHR mice in a single dose of 0.1 mg per mouse slightly increased their mean life span and failed to change their maximum life span.[27] It was observed also that exposure to epithalamin was followed by the significant increase of life span of tumor-free mice of both strains (by 41 and 26%, respectively).

The comparative effect of long-term treatment with epithalamin started at the age of 3.5 and 12 months on survival of female SHR mice was evaluated.[29] It was shown that epithalamin increased the mean life span by 14% and 17% in young and middle-aged groups, respectively, in comparison to groups treated with saline

controls. Treatment with epithalamin markedly shifted survival curves of both age groups to the right compared to corresponding controls. The aging rate calculation in young and middle-aged groups treated with epithalamin revealed the slow down of aging in the young group in comparison with the middle-aged group. Also, it was observed that administration of epithalamin was followed by increased life span of tumor-free young (by 20%) and middle-aged (by 36%) mice.

Epithalamin was also administered to female outbred rats starting from the age of 3.5 months for 20 months in daily doses of 0.1 or 0.5 mg per animal.[37] The mean life span of rats exposed to epithalamin at these doses was increased by 25 and 45%, respectively, and the maximum life span was increased by 2 months only in rats exposed to major doses of the preparation (TABLE 1). The analysis of the slope of survival curves of rats treated with epithalamin suggests the slow-down of the aging rate under the influence of the pineal peptides. It is worthwhile to note that the age-related dynamics of body weight in control rats and those exposed to epithalamin were similar.

In other experiments female rats were injected chronically with epithalamin in a single dose 0.5 mg per rat starting at the age of 15 months.[32,38] It was shown that this treatment insignificantly increased their life span: by 6.2% when calculated from birth and by 18% when calculated form the start of experiment ($p > 0.05$). At the same time, 23% of rats treated with epithalamin survived longer than the longest survived rat in control group. The maximum life span in the group exposed to epithalamin was increased by 3 months compared to controls. Calculation of the parameters of the Gompertz equation has shown that epithalamin treatment slows down the aging rate in comparison to controls.

Thus, both in mice and in rats we observed the capacity of epithalamin to increase their mean and, sometimes, their maximum life span and to slow down the aging rate of exposed populations. These effects were less expressed when the treatment with epithalamin was started after the age of 1 year in mice and 15 months in rats.

Effect of Epithalamin on Function of the Reproductive System in Young and Old Rats

Females

It has been shown that the threshold of sensitivity of the hypothalamo-pituitary complex to feedback suppression by estrogens increases with the age in female rats.[39] This mechanism was suggested as the leading one in age-related switching-off of the reproductive function in the rat.[39] Subcutaneous injections of epithalamin in old rats increased sensitivity of the hypothalamo-pituitary complex to estrogen suppression and restored cyclic estrus activity in persistent estrus rats.[21,40] Injection of epithalamin into the third brain ventricle was followed by the same effect. On the other hand, pinealectomy or exposure to a constant light regime was followed by an increase in the threshold of hypothalamic sensitivity to the feedback inhibition by estrogen.[39]

In the study of the estrus function of female rats it was shown that at the age of 16–18 months, 38% of control animals had persistent estrus, anestrus or repeated pseudopregnancies, while among rats exposed to epithalamin from the age of 3.5 months estrus cycle disturbances had been observed in 7% of the animals.[37]

TABLE 1. The Biological Effects of Epithalamin

Target	Effect
Life span	Increases the mean life span and slows down the aging rate in mice and rats;
Reproductive system	Inhibits compensatory ovarian hypertropy in hemiovariectomized rats;
	Inhibits the HCG-induced increase of uterine weight in infantile mice and rats;
	Decreases serum LH level in adult male rats;
	Decreases the threshold of sensitivity of the hypothalamo-pituitary complex to feedback inhibition by estrogens in old rats;
	Restores estrus cycles and fertility in old persistent-estrus rats;
	Delays age-related switching-off of estrus function in female rats;
Nervous and neuroendocrine system	Increases the learning capacity of rats in the labyrinth test;
	Influences circadian locomotor activity in rats;
	Sedative effect in dogs;
	Activates neurosecretion in n. paraventricularis, n. supraopticus and neurohypophysis;
Pineal gland	Stimulates the activity of pinealocytes;
	Increases the night synthesis of pineal serotonin, N-acetylserotonin and melatonin in adult and old rats;
	Increases the synthesis of melatonin in the gland *in vitro*;
	Increases the night level of serum melatonin in old rats;
Hormones and metabolism	Increases T3 and decreases T4 levels in adult rat serum;
	Decreases the serum level of corticosterone in mice;
	Increases the sensitivity of hypothalamo-pituitary complex to feedback inhibition by glucocorticoids in old rats;
	Decreases serum levels of insulin and triglycerides in rabbits;
	Increases the tolerance to glucose loading in rabbits;
	Does not influence the body weight of rats;
	Increases diuresis;
Immune system	Stimulates T- and B-mediated immunity in adult and old mice;
	Increases the serum level of thymic serum factor and serum titer of CTLA in old mice;
Cancer	Decreases the incidence of spontaneous tumors and increases their latency in mice and rats;
	Inhibits the development of DMBA and X-irradiation-induced mammary carcinogenesis in rats;
	Inhibits the realization of NEU-induced transplacental carcinogenesis in rats;
	Inhibits the growth of some transplantable tumors and their metastasizing;
	Increases the sensitivity of tumors to cytostatic and laser therapy

However, among 16 old female rats which remained sterile after mating with adult males, four became pregnant and gave birth to 5–9 fetuses per litter after a two-week course of epithalamin administration.[37]

Daily cytological study of vaginal smears in rats treated with epithalamin from the age of 15 months revealed the slowdown of age-related switching-off of estrus function in them as compared with controls. Thus, while at the age of 18 months the number of rats with disturbances of estrus function (persistent estrus and anestrus) was equal in both groups, at the age of 27 months these disturbances

were observed in 50% of control and 30% of females exposed to the pineal preparation ($p < 0.05$).[32,38]

Thus, we observed the slowdown of age-related switching-off of estrus function in female rats exposed to epithalamin. Also shown was the capacity of epithalamin to recover estrus function, ovulation and fertility in persistent estrus rats. Epithalamin also restored the regular estrus cycles in young and old rats with persistent estrus syndrome induced by exposure to constant illumination.[41] These effects of epithalamin, as suggested, are dependent on the capacity of the preparation to prevent the age-related increase of the threshold of sensitivity of the hypothalamo-pituitary complex to feedback regulation by estrogen and on its decrease in old female rats.

Males

A single morning administration of epithalamin was followed by the decrease in serum luteinizing hormone (LH) level and failed to influence the serum testosterone level in 4–5-month-old males, but increased both hormones 30 min after injection into 16-month-old ones. A daily treatment in the morning (between 10:00 and 11:00) with epithalamin for 5 days failed to influence the serum LH level in both young and old male rats, but was followed by the significant decrease of serum testosterone level in young rats at 12:00 and 17:00, and failed to influence serum testosterone in older males at any time of day (Anisimov *et al.*, unpublished data).

Effect of Epithalamin on the Nervous and Endocrine Systems in Young and Old Animals

Administration of epithalamin to adult rats during 5 consecutive days was followed by increased activity of succinate-, alpha-ketoglutarate- and pyruvate-dehydrogenases in rat brain and by a 39% increase of learning capacity in a labyrinth test and some other behavior tests.[42] Arushanian *et al.*[43] observed the increase in the amplitude and shifts in the acrophase of circadian locomotor activity to late hours in rats exposed to long-term administration of epithalamin. Epithalamin treatment was also followed by a change in the time-course of forced swimming and by a decrease of the rhythmic index of depression. Intravenous treatment with epithalamin was followed by sedative effects in dogs, and animals were sleepy for 2–3 hours.[44]

A ten-day-long administration of epithalamin to adult male rats was followed by a significant activation of neurosecretory elements in the hypothalamic nucleus paraventricularis, and a slighter one in the nucleus supraopticus, and by the increase of the content of neurosecretory substances in the neurohypophysis.[45] The ultramicroscopic study showed that a single morning injection of epithalamin to adult rats was followed by the signs of activation of pinealocyte function.[46]

A single or 2-week-long administration of epithalamin failed to change the level of dopamine, norepinephrine, serotonin or 5-hydroxyindolyl acetic acid in the hypothalamus of adult male rats (Ostroumoa & Anisimov, unpublished data). In our other studies the effect of epithalamin on serotonin metabolism in the pineal gland of young and old male rats was studied.[30–32,47,48] It was shown that in the morning a single treatment or a 5-day course of epithalamin in 4–5-month-old rats was followed by an increase of the night level of serotonin. N-acetylserotonin and

melatonin in the pineal gland. In 18–20-month-old rats a similar treatment was followed only by a tendency to increase the pineal melatonin level. At the same time, an increase of the serum melatonin level in old rats exposed to epithalamin was observed. In young as well as in old rats the exposure to epithalamin failed to influence the reaction of direct O-methylation of serotonin in 5-methyltryptamine and oxidative deamination and subsequent O-methylation into 5-HIAA and 5-MIAA. These data suggest the existence of an ultra-short loop between pineal peptides and indoles. Pineal peptides may influence the metabolism of tryptophan into serotonin and its subsequent transformation into melatonin. As age advances, the intensity of these reactions is decreased. It is noteworthy that exposure to epithalamin at the 18:00 hours was followed by a decrease in the night peaks of serotonin, N-acetylserotonin and melatonin in the rat pineal gland.

The induction of the synthesis of melatonin by epithalamin was studied in the rat pineal gland *in vitro*. Pineals were collected from rats killed in the light period (09:00) and incubated for 3 hours with either isoproterenol (1 μM), epithalamin (25 μg/ml) or isoproterenol and epithalamin at the same concentrations. Additional pineals were incubated in the absence of these drugs. Epithalamin as well as isoproterenol significantly increased melatonin levels in the rat pineal gland, whereas epithalamin did not potentiate the effect of isoproterenol in increasing the melatonin content in the pineal gland (R. J. Reiter, personal communication).

We also observed the stimulating effect of a 5-day-long treatment with epithalamin on the serum triiodothyronine (T3) level and the inhibitory effect of this treatment on the serum thyroxine (T4) level in 4–5-month-old male rats. These data suggest the influence of the preparation on the metabolism of T3 into T4. In the future, a direct measurement of the activity of 5'-deiodinase in the thyroid gland of rats exposed to epithalamin will help to elucidate these results. In 16-month-old male rats the course administration of epithalamin was followed by a decrease of both T3 and T4 levels in animal serum (Anisimov *et al.*, unpublished data).

One hour after the administration of epithalamin at the dose of 0.5 mg/kg into adult male rats the serum level of corticosterone and aldosterone was increased, whereas the serum level of ACTH was decreased.[49] Five-day-long exposure of 2-month-old mice to epithalamin was followed by a decrease in the serum corticosterone level by 3.2 times in comparison to the control level.[50]

The value of the suppression of the serum corticosterone level by prednisone or dexamethasone in rats was shown to be less pronounced with their advance in age.[51,52] The administration of epithalamin to rats in the dose which failed to change the basal serum corticosterone level was followed by the restoration of the sensitivity of the hypothalamo-pituitary axis to the feedback suppression by exogenous corticosteroid.[51,52]

The treatment of rabbits with epithalamin was followed by an increase of glucose tolerance, but the tolerance to insulin loading was unaffected.[53] The authors also observed that a 3-week-long exposure to epithalamin led to the decrease of both the serum insulin and triglycerides level. It was suggested that epithalamin increases the amount of the insulin fraction which is active in relation to the utilization of glucose in a muscle tissue.

The significant influence of epithalamin on salt and water metabolism was observed: the exposure of dogs or rats to the preparation was followed by an increase in urine excretion, hyposodiumuria, hyperpotassium-, calcium- and magnesiumuria.[44]

Effect of Epithalamin on Immune Function in Young and Old Rodents

Epithalamin proved to increase the number of antibody-forming cells generated in the spleen and the level of serum hemagglutinins in response to immunization with sheep red cells.[54,55] Treatment with epithalamin increased the survival of CC57Br/Mv mice infected with 1.5×10^9 bacterium *S. typhimurium* and the survival of skin allografts in mice and rats; it also stimulated both the reaction of delayed type hypersensitivity in guinea pigs and also the phagocytic activity of blood neutrophils.[24,55] It was shown also that epithalamin restored the level of proliferation of granulocytes and macrophages (CFU-GM) in pinealectomized rats up to the intact level (D. Gupta, personal communication).

The exposure of both young (2-month-old) and 2-year-old mice with intact thymus to epithalamin was followed by an increase in the level of thymic serum factor (TSF), of compounds with thymosin-like activity (CTLA) and the thymic index, and by an increase in spleen cellularity in old animals.[50] Thus, in these experiments it was shown that exposure to epithalamin was followed by an increase in the titers of serum TSF and CTLA, and by morphological features of thymic and spleen function stimulation. The activation of the proliferation and maturation of thymic cortical thymocytes, hyperplasia and medullar differentiation of epithelial cells in the thymus and epithelioid cells in the spleen of AKR mice exposed to long-term treatment with epithalamin in comparison to controls.[56] In C3H/Sn mice exposed to epithalamin starting at the age of 3.5 months we observed a delay in the age-related decrease of the value of the phytohemagglutinin-induced reaction of blast transformation of T-lymphocytes, in comparison to controls.[26]

Epithalamin and Tumor Growth

Spontaneous Tumor Development in Rats and Mice Exposed to Epithalamin

In the first of our experiments female outbred rats were injected s.c. with 0.2 ml of saline or dissolved in this volume 0.1 or 0.5 mg of epithalamin 5 times per week for 20 months, starting at age of 3.5 months. Autopsies were performed on all animals and tumors discovered were studied histologically. It was shown that any dose of epithalamin failed to change both the total and malignant tumor incidence in female rats while the tumor latency was significantly increased.[57]

In another experiment female rats were exposed to long-term injections with saline or 0.5 mg epithalamin starting at age of 15 months. In this case total tumor incidence in the epithalamin-treated group was decreased by 1.6 times in comparison to the control group ($p < 0.004$), while the incidence of malignant tumors decreased by 2.7 times ($p < 0.04$).[32,38]

Female C3H/Sn mice from the age of 3.5 months were treated s.c. with 0.2 ml of saline (control group) or with 0.5 mg of epithalamin dissolved in 0.2 ml saline for five consecutive days once a month up to their natural death.[24,26] It was shown that the total tumor incidence in epithalamin-treated female C3H/Sn decreases by 2.1 times in comparison to saline-treated control animals. The antitumor effect of epithalamin was most pronounced in relation to mammary adenocarcinomas. Their incidence decreased by 2.9 times, the multiplicity of mammary adenocarcinomas (number of tumors per mouse) also significantly decreased.

Outbred female Swiss-derived SHR mice from the age of 3.5 or 12 months were treated s.c. with saline or 0.1 mg of epithalamin dissolved in saline for five consecutive days monthly.[29] Administration of epithalamin to young mice was

followed by a significant reduction in spontaneous tumor incidence, first of all because by the 2.6-fold decrease of mammary adenocarcinomas incidence, $p < 0.025$. The exposure to epithalamin was also followed by the shift of the curves of tumor-free survival to the right in young mice, whereas in the middle-aged group the treatment with the drug exerted similar, but feebly marked effect. The incidence of spontaneous thymic lymphomas was 39% in 12-month-old intact AKR mice and only 14% in mice exposed to long-term administration of epithalamin.[56] In treated mice the serum level of TSF was increased as compared with controls and morphological features of the activation of cellular elements of thymus and spleen were observed.

Effect of Epithalamin on Carcinogenesis Induced by Chemicals or X-Irradiation

The administration of epithalamin to female rats significantly inhibited mammary carcinogenesis induced by 7,12-dimethylbenz-(a) anthracene (DMBA).[57,58] Mammary adenocarcinomas developed in 81% of rats exposed to DMBA and saline and in 26% of rats treated with DMBA and epithalamin. Epithalamin also inhibited carcinogenesis induced by a single total-body X-ray irradiation (4 Gy) in female rats.[25] The incidence of the total or only malignant tumors under the influence of epithalamin was decreased by 1.3 and 2.7 times, respectively, as compared with controls. The exposure to epithalamin was followed by a significant decrease in the incidence of mammary adenocarcinomas, tumors of the thyroid, uterus, ovaries and the hematopoietic system.

The effect of epithalamin on transplacental carcinogenesis was studied in rats exposed at the 21st day of pregnancy to 75 mg/kg of N-nitrosoethylurea (NEU).[59] The progeny was subdivided into two groups and starting at the age of 2 months was treated with saline or epithalamin for a year. The exposure to epithalamin was followed by a decrease of total tumor incidence of 10.3%, as compared to controls, and by a decrease of spinal cord tumor incidence (of 28%); kidney tumor incidence (of 25%), peripheral nerve tumors (of 15%), and by the increase of their latency.

Effect of Epithalamin on the Growth of Transplantable Tumors

Our studies of the anti-tumor activity of epithalamin was carried out on the following transplantable tumors: hepatoma-22a, squamous cell cervical carcinoma SCC, melanoma B16, Harding-Passey melanoma, microalveolar mammary carcinoma RSM, Ehrlich carcinoma, carcinoma NK/Ly, Lewis lung carcinoma, lympholeukemia LIO-1 and leukemia L-1210.[1,23,33,34,57,60] It was observed that epithalamin expressed a pronounced inhibitory effect on RSM and SCC tumors, a weak influence on hepatoma-22a, and had no effect on Harding-Passey melanoma and Lewis lung carcinoma. In addition, epithalamin increased the survival time of SHR mice inoculated with transplantable lympholeukemia LIO-1 but had no effect on the survival time of mice with leukemia L-1210. The administration of epithalamin also was followed by the enhancement of the anti-tumor effect of cyclophosphamine against SSC tumor in mice.[60]

Moskalik[61] s.c. transplanted melanoma B16 or Lewis lung carcinoma to C57BL mice and irradiated them with the neodium laser GOS-1001 (USSR), wave length 1060 nm, impulse length 1 μsec., energy power 350–400 J/sq. cm. During 5 days

before the start of the laser irradiation and 5 days after mice were injected with 1 mg of epithalamin or saline. Animals were killed in 24–27 days after the laser irradiation and the number of lung metastases was estimated. It was showed that epithalamin increased the antimetastatic effect of the laser irradiation. The treatment with epithalamin of rats with transplanted Pliss lymphosarcoma was followed by a decrease in the incidence of the tumor metastasizing induced by the surgical laparotomy.[62] Similar data were received by these authors in the experiment with Lewis lung carcinoma.

Thus, treatment with epithalamin was followed by an inhibition of spontaneous carcinogenesis and that induced by chemicals or X-irradiation, by an inhibition of the growth of some transplanted tumors, by a decrease of their metastatic potential and by potentiation of the therapeutic effects of some anti-tumor chemicals and laser irradiation.

CONCLUSION

Thus, during the last two decades a wide spectrum of the biological activity of the low-molecular-weight pineal preparation epithalamin was observed (TABLE 1). Administration of epithalamin to old animals led to the normalization of some parameters in the endocrine, reproductive, and immune systems and some other functions in the organism, to the inhibition of tumor growth and metastasizing. Long-term exposure to epithalamin was followed by an increase in the life span of mice and rats, by a slowdown of the age-related switching-off of reproductive function and immune function decline, and by the inhibition of spontaneous carcinogenesis and that induced by chemicals or ionizing radiation. The mechanism of the biological effects of epithalamin includes the enhancement of the night peak of melatonin in the pineal gland and serum, the lowering the hypothalamic threshold of sensitivity to homeostatic feedback stimuli, the modulation of some T- and B-mediated immune functions. These observations provide evidence supporting a concept of the possibilities of practical application of epithalamin in clinics for prevention and treatment of age-related pathology, including cancer. The results of clinical trials of epithalamin were reported elsewhere.[55,63]

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