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Combining isoflurane anesthesia with midazolam and butorphanol in rats

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Abstract: Representative inhalant anesthetic agent, isoflurane is commonly used during surgery in rats. However, isoflurane mediates relatively strong respiratory depression. In human and veterinary medicine, sedatives and analgesics are co-administered to complement the anesthetic action of inhalant anesthesia. The present study aimed to establish the novel balanced anesthesia that combines midazolam and butorphanol with isoflurane (MBI) in rats. Male Sprague Dawley rats were divided into 2 groups, and administered either isoflurane monoanesthesia or isoflurane with midazolam (2.5 mg/kg, ip) and butorphanol (2.0 mg/kg, ip). The minimum alveolar concentration (MAC) in each group was evaluated. Induction and recovery times were measured in each group. Adverse reactions during induction were also recorded. In each group, vital signs were assessed for 1 h under 1.5×MAC of isoflurane. Instability of vital signs was assessed under each anesthesia by calculating coefficient of variance. Compared with isoflurane monoanesthesia, MBI anesthesia caused 32% MAC reduction (isoflurane monoanesthesia: 1.30 ± 0.09%, MBI 0.87 ± 0.08%, *P*<0.05). MB premedication mediated smooth sedating action with low incidence of adverse reactions such as urination and defecation. Isoflurane monoanesthsesia remarkably decreased respiratory rate and saturation O_2 (SPO_2). In contrast, MBI anesthesia resulted in a relatively stable respiratory rate without decreases in SPO_2 during the anesthetic period. In summary, MB premedication is effective for attenuating respiratory depression induced by isoflurane, and achieving smooth induction. This anesthetic protocol serves as a novel option for appropriate anesthesia in rats.
Key words: inhalant anesthesia, preanesthesia, respiratory depression, rodents

Introduction

Achieving an appropriate anesthetic effect in animal experiments is essential for laboratory animal welfare. Owing to an increasing concern for laboratory animal welfare and third-party certification of experimental facilities, advances in rodent anesthesia is currently a topic of interest. Achieving appropriate anesthetic effect requires sufficient anesthetic depth and fewer adverse reactions. The administration of an anesthetic influences various physiological systems that include cardiorespiratory, neuronal, metabolic, and immune systems

[2, 9, 13, 15, 27]. Among them, cardiorespiratory depression is the main adverse reaction that is problematic during the operation. This may cause impairment of physiological function, and affect experimental data [12]. Therefore, an anesthetic protocol that mediates sufficient anesthetic depth with less cardiorespiratory influence would be valuable for laboratory animal welfare and reliability of experimental animal data.

Inhalant anesthesia enables regulation of anesthetic depth in real time and could available to both short and long durations of anesthesia [13]. Following the development of ready-to-use inhalant anesthesia devices for small rodents and advances in anesthetic techniques, the use of an inhalant anesthesia has increased in rodents [3, 7]. Isoflurane is a major volatile agent that has been used as rat inhalant anesthesia [12] and less influence on hepatic metabolism, making it suitable for pharmacological and metabolic experiments [10]. In addition, the induction and recovery with isoflurane is relatively rapid. However, it may cause some undesirable reactions. The main problem is its respiratory depression and clinical adverse reactions during induction [12, 25]. In addition, monoanesthesia with a volatile agent such as isoflurane may not provide a sufficient analgesic action when a highly invasive surgical procedure is performed [5, 11].

In human and veterinary medicine, a preanesthesic agent is administered to complement the anesthetic action of inhalant anesthesia. A preanesthetic agent is usually administered in combination with sedatives and analgesic agents [6, 20]. Among the combination of preanesthetics, midazolam and butorphanol (MB) are preferred in medicine since this combination has moderate sedative and analgesic actions and results in lesser cardiorespiratory influence [21]. This combination is also clinically used as a preanesthetic to complement of isoflurane's anesthetic action in dogs and cats [14, 21]. Following these studies, we recently reported that combining isoflurane anesthesia with midazolam and butorphanol (MBI) is effective for reducing the maintenance concentration of isoflurane that leads to the amelioration of respiratory depression induced by isoflurane in mice [24]. As to rats, the ideal combination of preanesthesia for isoflurane has not been fully evaluated. The aim of the present study was to apply MBI anesthesia in rats.

Materials and Methods

Animals and housing conditions

Thirty-four male-Sprague Dawley rats (slc: SD; Japan SLC Inc., Shizuoka, Japan) aged 8 weeks were used in the present study. The body weight of animals ranged from 250 to 270 g. Animals were housed in plastic cages with wood shavings, and were kept in a room equipped with a barrier at the Research Institute of Biosciences, Azabu University. The room temperature was $22 \pm 1°C$, with humidity of $50 \pm 5\%$. The room was lighted from 6:00 to 20:00. Animals were fed *ad libitum* with pelleted laboratory diet (Lab Diet, Japan SLC Inc.) and clean fresh water. The animals were acclimated to the facility for 1 week before the experiment, and all studies were performed when rats were 8 weeks of age. All experiments were performed between 13:00–17:00 to minimize influence from circadian rhythm. After the experiments, euthanasia was performed via the intraperitoneal administration of pentobarbital (Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) at 100 mg/kg, followed by cervical dislocation. This study was approved by the Animal Research Committee of Azabu University.

Anesthesia

Prior to the experiment, rats were randomly allocated to 2 groups. One group was administered isoflurane monoanesthesia (Isoflu, DS Pharma Animal Health Co., Ltd., Osaka, Japan), and the other received midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan) and butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) followed by isoflurane (MBI). In the MBI anesthetic group, anesthesia was induced by intraperitoneal administration of midazolam (2.5 mg/kg) and butorphanol (2.0 mg/kg). The dose setting of MB was preliminarily determined within the range of 2.0–4.0 mg/kg for midazolam and butorphanol in advance. In the isoflurane monoanesthetic group, anesthesia was induced with 5% isoflurane in an induction chamber. Following the loss of righting reflex, rats in both anesthetic groups were rapidly transferred to a nose cone mask, and maintained with isoflurane with room air. Isoflurane anesthesia was performed using a rodent inhalant anesthesia apparatus (SomnoSuite Small Animal Anesthesia System, Kent Scientific Corporation, Connecticut). The flow rate of isoflurane was determined using following formula; Flow rate (ml/min) = 0.65 × body weight (g).

Induction and recovery time

In each rat, induction and recovery times (sec) were recorded. Induction was completed when the righting reflex was lost after induction with 5% isoflurane or intraperitoneal MB administration. Sixty min after the induction, the supply of isoflurane was stopped and the recovery time was assessed. Recovery time was defined as the time between stopping gas supply and recovery of righting reflex.

Clinical adverse reactions

According to the previous reports on rodents [4, 24], the incidence of clinical adverse reactions during induction was recorded in each anesthetic group. The adverse reactions recorded in the present experiment included head shaking, urination, defecation, and apnea. We also evaluated the rate of rats that had at least 1 clinical adverse event in each group. The post-anesthetic abnormalities were clinically evaluated for 1 week.

Determination of minimum Alveolar concentration (MAC)

MAC measurement was performed using the blanketing method as previously described [18, 22]. In the assessment, the forelimbs, hind limbs, and tails of rats were stimulated under several isoflurane concentrations. All stimuli were induced by the same investigator by using forceps with a spacer between its arms. Motor activity was considered a positive response. After induction, rats were initially maintained with 1.4% isoflurane. Depending on the presence or absence of response, the concentration of isoflurane was increased or decreased in steps of 0.1–0.2%, respectively. In each test, a 30-min equilibration period was set. MAC was calculated as the mean of the lowest value for which a negative response was obtained and the highest value for which a positive response was obtained.

Vital sign recording

In the present study, rectal temperature, heart rate, respiratory rate, and SPO_2 were evaluated as vital signs. Vital signs were recorded in each individual under 1.5 ×MAC of isoflurane. We applied this concentration because appropriate anesthetic depth can be achieved in most surgical procedures [4, 8]. Initially, rats were introduced into animal holders and their baseline values were measured. For anesthesia, rats were located on a nylon pad to maintain a consistent surface temperature underneath. Vital signs were measured before anesthesia and every 5 min during the 60-min period. Rectal temperature was measured using a commercial rectal temperature sensor (Right Temp, Kent Scientific Corporation). Heart rate and SPO_2 were assessed using a rodent pulse oximeter and heart rate monitor (MouseSTAT, Kent Scientific Corporation). We adopted the maximum value during one min as the value of rectal temperature, heart rate, and SPO_2 at each time point. Respiratory rate was measured from the movement of the thorax wall per min. Time course of each vital signs in each anesthetic group was compared. Instability of vital signs was also compared by calculating the coefficient of variance (CV).

Analysis of data

All statistical analyses were performed using Stat Mate IV (ATMS. Co., Ltd., Tokyo). Student's *t* test was used for comparison of induction time, recovery time, MAC, and CV of vital signs in each group. The incidence of adverse reactions during induction was analyzed using Fisher's exact test. Repeated measures analysis of variance (ANOVA) was used to analyze differences in vital sign measurements. When a significant difference was detected using ANOVA, a Dunnett's *t* test was performed to assess differences between baseline and subsequent time points. Following one-way ANOVA, the post hoc analysis with Bonferroni test was performed to identify significant differences between groups at each time point. Data in the present study were expressed as mean ± SD. Statistical significance was defined as *P*<0.05.

Results

Induction and recovery time

Induction and recovery times (sec) are shown in Table 1. Both anesthetic groups achieved rapid induction (isoflurane monoanesthesia: 240 ± 37, MBI: 223 ± 89). Intraperitoneal administration of MB mediated moderate sedative action sufficient to immobilize the rats until maintenance with isoflurane. In addition, both anesthetic groups showed rapid recovery from anesthesia (isoflurane monoanesthesia: 180 ± 83, MBI anesthesia: 288 ± 208). There were no significant differences in induction and recovery times between the 2 anesthetic groups.

Adverse reactions during induction

The incidence of adverse reactions observed during

Table 1. Induction and recovery times in each anesthetic group

	Isoflurane alone	MBI	P value
Induction time (sec)	240 ± 37	223 ± 89	0.17
Recovery time (sec)	180 ± 83	288 ± 208	0.15

Table 2. Incidence of clinical adverse reactions during induction

	Isoflurane alone	MBI
Head shaking	4	0
Urination*	45	14
Defecation*	40	0
Apnea	0	0
Either adverse events*	71	14

*P<0.05.
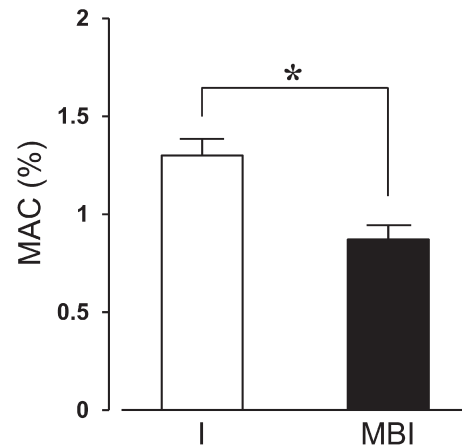
Fig. 1. Comparison of MACs in groups treated with isoflurane alone (I) and a combination of midazolam, butorphanol, and isoflurane (MBI). Results are represented as mean ± SD of 7 rats. *P<0.05.
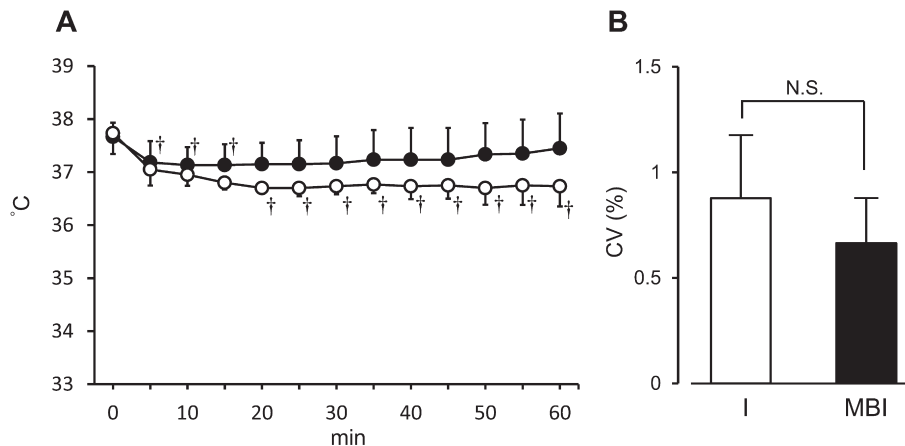
Fig. 2. Measured rectal temperature in each anesthetic group. (A): rectal temperature over time in each group. (○): isoflurane-treated group. (●): group treated with a combination of midazolam, butorphanol, and isoflurane. (B): instability in rectal temperature in each group represented as coefficient variance (CV). Results are expressed as mean ± SD in 6 rats. †Significant difference from baseline (P<0.05). N.S.: not significant.

induction is shown in Table 2. In the isoflurane-treated group, head shaking (4%), urination (45%), and defecation (40%) were observed. In contrast, the rat treated with MBI only experienced urination (14%). Compared to isoflurane monoanesthesia, the incidence of urination and defecation was significantly lower in the MBI anesthetic group. Furthermore, in the MBI anesthetic group, the number of rats with either adverse reaction was significantly lower than that in the isoflurane induction group (isoflurane monoanesthesia: 71%, MBI anesthesia: 14%). No prominent post-anesthetic abnormalities were observed in any rat.

Determination of MAC

MAC results in both the anesthetic groups are shown in Fig. 1. Mean ± SD of MAC for the isoflurane monoanesthesia and MBI groups were 1.30 ± 0.09% and 0.87 ± 0.08%, respectively. MB premedication resulted in 32% MAC decrease in rat.

Vital signs recording

Finally, we compared the influence of each anesthetic on vital signs. In either parameter, baseline values between groups were not statistically significant. In the isoflurane-treated group, rectal temperature significant-
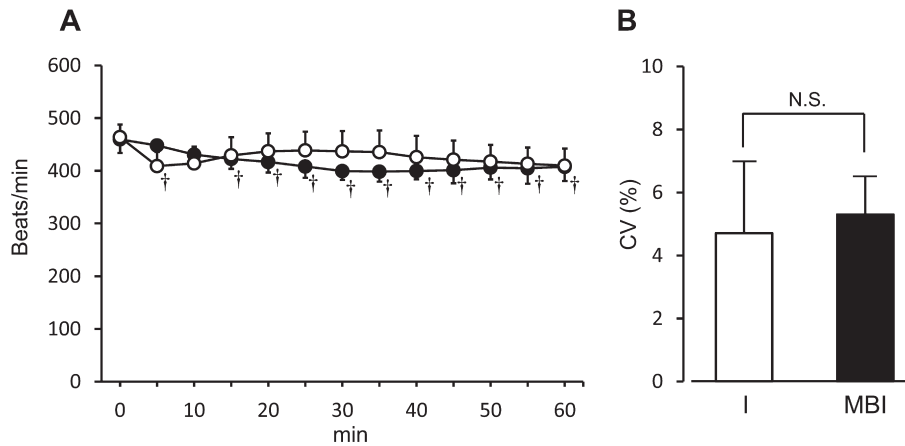
Fig. 3. Measured heart rate in each anesthetic group. (A): heart rate over time in each group. (○): isoflurane-treated group. (●): group treated with a combination of midazolam, butorphanol, and isoflurane. (B): instability in heart rate in each group represented as coefficient variance (CV). Results are expressed as mean ± SD in 6 rats. †Significant difference from baseline ($P<0.05$). N.S.: not significant.
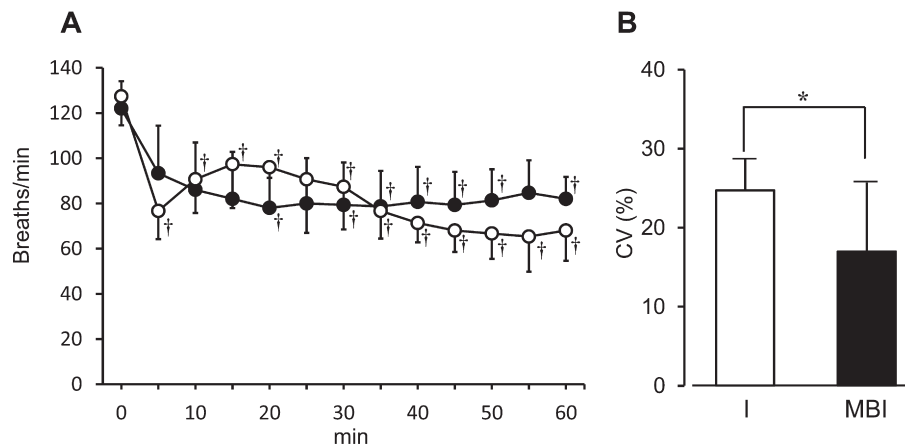
Fig. 4. Measured respiratory rate in each anesthetic group. (A): respiratory rate over time in each group. (○): isoflurane-treated group. (●): group treated with a combination of midazolam, butorphanol, and isoflurane. (B): instability in respiratory rate over time represented by coefficient variance (CV). Results are expressed as mean ± SD in 6 rats. *Significant difference between groups ($P<0.05$). †Significant difference from baseline ($P<0.05$).

ly decreased within 20–60 min, while in the MBI anesthesia groups, it decreased within 5–15 min (Fig. 2). The rectal temperature in the late phase of anesthesia was relatively lower in the isoflurane monoanesthesia group. Heart rate was not significantly different between groups (Fig. 3). The respiratory rates over time in both the anesthetic groups are shown in Fig. 4A. When comparing values at each time point, there was no significant difference between groups. However, CV in MBI anesthesia is significantly lower than that with isoflurane monoanesthesia, indicating stable values during the

anesthetic period (Fig. 4B). Time course of SPO_2 in both the anesthetic groups are shown in Fig. 5A. Isoflurane monoanesthesia resulted in a significant decrease in SPO_2 during the entire anesthetic period. In contrast, MBI anesthesia did not show a decrease in SPO_2 at any time point. As a result, CV in MBI anesthesia is lower than that of isoflurane monoanesthesia (Fig. 5B).

Discussion

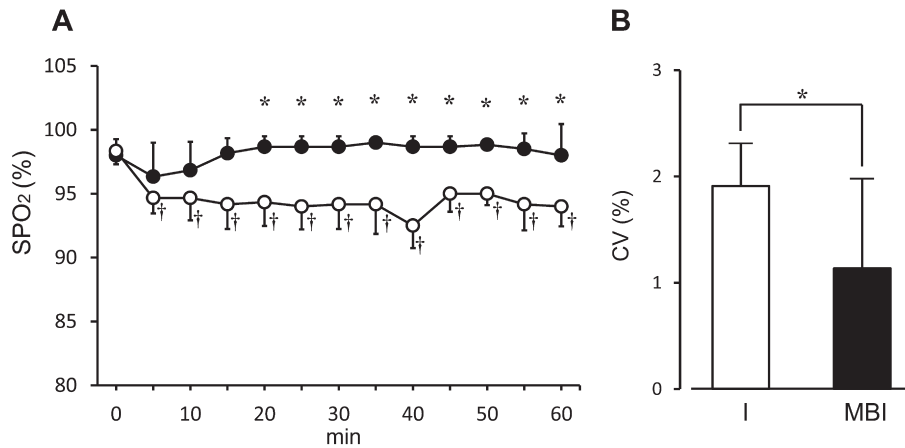The present study aimed to investigate the efficacy of

A

B

Fig. 5. Measured SPO_2 in each anesthetic group. (A): SPO_2 over time in each group. (○): isoflurane-treated group. (●): group treated with a combination of midazolam, butorphanol, and isoflurane. (B): instability in SPO_2 over time represented by coefficient variance (CV). Results are expressed as mean ± SD of 6 rats. *Significant difference between groups ($P<0.05$). †Significant difference from baseline ($P<0.05$).

MBI anesthesia in rats. We used midazolam and butorphanol as preanesthetics in rats. We selected these agents since they have a rapid onset of action with less cardiorespiratory influence, and these were reported in other species [17, 19, 23, 24]. As a result, premedication with MB showed a moderate sedative action sufficient to achieve rapid induction. In addition, preanesthesia with MB resulted in a lower incidence of adverse reactions during induction, compared with isoflurane induction. Notably, the incidence of urination and defecation reduced in MBI anesthesia, possibly because of less hypnotic action. MBI anesthesia did not show delayed awakening from anesthesia, compared to isoflurane monoanesthesia. This result suggests that sedative and hypnotic properties of midazolam diminished within 1 h. The combination of midazolam and butorphanol is effective for the induction of isoflurane in rats.

The present study demonstrated that midazolam and butorphanol premedication decreased MACs of isoflurane to 32%. To our knowledge, there has been no report that investigated the combination of sedatives and analgesics on MACs of isoflurane in rats. A previous report demonstrated that total intravenous infusion of urethane decreased the MACs of isoflurane [1]. However, this anesthetic technique requires placement of an intravenous catheter, and cannot be allowed to recover after being anesthetized since it include urethane. Another report investigated that tramadol, an opioid analgesic, alone decreases MACs of isoflurane to 15% [26]. Compared with previous protocols, the combination of midazolam and butorphanol has advantage in MAC reduction of isoflurane in practical use.

Following MAC determination, vital signs were recorded in each anesthesia group to compare cardiorespiratory influences. Although there was no significant difference, MBI anesthesia showed a higher rectal temperature, compared with isoflurane monoanesthesia, particularly during the late phase of the anesthetic period. This result is consistent with that of a previous report in mice [24], and this may be associated with a decrease of isoflurane concentration. In both anesthesia groups, heart rate varied slightly during the anesthetic period. A previous study indicated that isoflurane monoanesthesia resulted in lower cardiac influence than injectable anesthesia such as pentobarbital and ketamine [16, 25]. In addition, the decrease in heart rate in rats observed in the present study was relatively mild compared with those reported in previous mice studies [24]. Although blood pressure measurement may be additionally required, cardiovascular influence induced by both anesthetic protocols can be physiologically tolerable in rats.

Isoflurane is known to have a relatively strong respiratory depression in various species [12, 17, 25]. Therefore, the main purpose of MB preanesthesia was to attenuate the respiratory depression by decreasing the MAC of isoflurane. For the assessment of respiratory function, we measured respiratory rate and SPO_2. As to

respiratory rate, isoflurane monoanesthesia mediated a marked decrease in respiratory rate and SPO$_2$ in rats. When compared to MBI anesthesia, the instability of respiratory rates during the anesthetic period was prominent in isoflurane monoanesthesia. The unstable respiratory rate shift observed in the isoflurane monoanesthesia group can be associated with an exposure to a higher concentration during induction. In addition, MBI anesthesia showed no significant decrease in SPO$_2$ during the entire study period, while isoflurane alone mediates a continuous SPO$_2$ decrease. Although there was no significant difference of respiratory rate in both anesthetic groups, remarkable increase of SPO$_2$ was observed in MBI anesthesia. The stabilization of SPO$_2$ in MBI anesthesia can be associated with reduction of isoflurane concentration, leading to high tidal volume during anesthesia. Taken together, premedication with MB attenuates respiratory depression induced by isoflurane in rats.

In the present study, the appropriate dose of MB in rats was preliminary investigated. The criteria for dose setting were that marked reduction of MAC can be achieved with attenuation of adverse events, particularly respiratory depression. As a result, preanesthesia with midazolam (2.5 mg/kg) and butorphanol (2 mg/kg) resulted in ideal gas-saving action with attenuation of respiratory depression. The required dose of MB in rats was relatively lower than that in mice [24], indicating a higher chemosensitivity to MB. Notably, MB at the dose achieved moderate sedative action and there was no need for exposure to a high concentration of isoflurane. Therefore, the dose of MB used in the present study was appropriate as a preanesthetic of isoflurane in rats.

In the present study, we established a novel isoflurane-based balanced anesthesia in rats. As MB premedication is attributed to the reduction of isoflurane concentration, it will be suitable for the prevention of accumulative toxicity of isoflurane, particularly for long durations of anesthesia. It is also effective in cases of highly invasive surgical treatment because MB can complement the analgesic and sadative action of isoflurane. The combination and dose of preanesthetics proposed in the present study can be attributed to the safe use of isoflurane anesthesia in various conditions. In summary, the efficacy of MB preanesthesia in rats was described. This anesthetic protocol serves as a novel option for anesthesia in rats, leading to laboratory animal welfare-based experiments.

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