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Sensitivity of *Aedes aegypti* adults (Diptera: Culicidae) to the vapors of *Eucalyptus* essential oils

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ABSTRACT

Vapors of essential oils extracted from various species of *Eucalyptus* (*E. gunnii*, *E. tereticornis*, *E. grandis*, *E. camaldulensis*, *E. dunnii*, *E. cinerea*, *E. saligna*, *E. sideroxylon*, *E. globulus* ssp. globulus, *E. globulus* ssp. maidenii, *E. viminalis* and the hybrids *E. grandis* × *E. tereticornis* and *E. grandis* × *E. camaldulensis*) and their major components were found to be toxic to *Aedes aegypti* adults, the yellow fever mosquito.

An aliquot of each oil was placed in a cylindrical test chamber and the number of knocked-down mosquitoes was recorded as function of time. Knockdown time 50% was then calculated. Results showed that *E. viminalis* had the fastest knockdown time at of 4.2 min, on the same order as dichlorvos, a standard knockdown agent. A correlation was observed between the content of 1,8-cineole in the *Eucalyptus* essential oils and the corresponding toxic effect.

The correlation between KT_{50} values and calculated vapor pressures of the essential oil components showed that the fumigant activity of simple organic compounds in insects is correlated with their volatility.

1. Introduction

Aedes aegypti (L.) is an urban mosquito that feeds almost exclusively on humans and has become adapted to use man-made containers for breeding (Christophers, 1960). During the continental *A. aegypti* eradication campaign in 1955, the area infested with this vector in Argentina was estimated to be 1,500,000 km. In 1963, *A. aegypti* was considered eradicated from Argentina (Bejarano, 1968). However, since 1992 the Argentine National Ministry of Health has reported its presence in almost every province of the country. The population of *A. aegypti*, which transmits the viruses responsible for dengue and yellow fever, has seen an unprecedented increase due to unplanned urbanization, industrialization, water storage practices, lack of piped water supply in rural areas and human migration (Tonn et al., 1982). Today public health experts are seriously concerned about a possible outbreak of yellow fever, especially after a few recent epizootic events reported in northern Argentina.

Local health authorities frequently use insecticide space treatments during epidemics and periods of high risk of dengue transmission in order to control adult mosquitoes. Ultra-low volume (ULV) application of adulticides is the recommended control method against *A. aegypti* adults in the Americas (PAHO, 1981). These sprays are usually delivered from portable (backpack or hand-held) or vehicle-mounted equipment, although helicopters and fixed wing aircraft have also been used. Their adulticidal effect is transitory with little or no effect on the aquatic stages of the vector. As *A. aegypti* prefers to rest inside houses, outdoor traditional insecticide spraying is ineffective (Chavasse and Yap, 1997; Mattews, 1996).

Emphasis has now shifted from natural pyrethrin and organophosphorus formulations to synthetic pyrethroids because they have good insecticidal activity at low application rates, short persistence in the environment and some even have a knockdown effect (PAHO, 1994). However, there is a great need to develop effective knockdown agents that can be combined with effective insecticides with larval and adulticidal activity to improve control strategies.

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) is an organophosphate compound with insecticidal and fumigant activity and a very high knockdown effect, used for controlling flies, mosquitos, gnats, cockroaches, fleas, and other insect pests (Tomlin, 1997). Dichlorvos formulations include pressurized liquids or aerosols, emulsifiable concentrates and slow-release impregnated materials such as resin strips. The US Environmental Protection Agency determined that adverse effects caused by dichlorvos, of primary concern to human health, are neurological effects

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related to the inhibition of cholinesterase activity. Therefore, in the near future dichlorvos will be seriously restricted, if not completely banned, due to it's serious risk to human health. Consequently, it is very important to find a compound with a high knockdown activity, high vapor pressure and high capacity of penetration that does not possess the adverse effects of dichlorvos, to be used for pest control. One alternative source may be plants containing bioactive chemicals (Miana et al., 1996; Sukumar et al., 1991). The genus *Eucalyptus*, native to Australia and other surrounding islands north of Australia, comprises more than 800 species of trees. This number continues to grow as new taxa are described. In their natural habitat *Eucalyptus* trees grow over a great variety of climatic and edaphic conditions. Therefore, the very large and varied gene pool that can be drawn upon for planting purposes is one of the reasons it has been so successfully introduced to so many different countries around the world (Coppen, 2002).

Eucalyptus leaves are well known for their aromatic oils. These are defined as steam-volatile components with a molecular weight of less than 250 amu that are terpenoid or aromatic in structure (Brophy and Southwell, 2002). The oil glands are located deeply within the leaves, well below the epidermal cuticle and other cells forming the surface layers of the foliage. These oils can be recovered by steam distillation and are thus referred to as essential oils (Denny, 2002). The facility with which essential oils are obtained from aromatic plants and their diverse chemical compositions makes them potential sources of natural pesticides – either by direct toxicity or repellency – and have attracted increasing attention among researchers (Singh and Upadhyay, 1993; Isman, 2000).

Several *Eucalyptus* oils are toxic to *A. aegypti* larvae (Lucia et al., 2008), suggesting that these oils deserve further attention as widely available, environmentally–friendly mosquito larvicides. Their volatility has potential benefits in terms of bringing the pesticide vapor into close contact with the pest while at the same time not leaving residues that might cause adverse affects to the protected objects, either crops, food products, or, in the case of aerosols to be used in urban environments, human beings. Hence, the purpose of this study is to evaluate the fumigant activity of several *Eucalyptus* essential oils and their major components against adult *A. aegypti*, contributing to the search of novel, safer products for mosquito control activities in urban areas.

2. Methods

2.1. Biological material

An insecticide-susceptible strain of *A. aegypti* (L.) (CIPEIN strain) was used, reared according to previous reports (Lucia et al., 2007). Third and fourth instar larvae were kept under the same conditions until the adults emerged. For the bioassays, we used batches of 13–15 adult mosquitoes, 1–3 days-old, fed only on raisins.

Essential oils were extracted from the following species of *Eucalyptus*: *E. saligna* Smith, *E. dunnii* Maiden, *E. globulus* ssp. maidenii F.v. Muell, *E. globulus* ssp. globulus Labill, *E. viminalis* Labill, *E. tereticornis* Smith, *E. camaldulensis* Dehnh, *E. cinerea* F.v. Muell. ex Benth, *E. sideroxylon* A. Cunn, *E. gunnii* Hook. *E. grandis* (Hill) ex Maiden (Lucia et al., 2008).

2.2. Chemicals

α-Terpineol (90%), *p*-cymene (99%), 1,8-cineole (99%), 4-terpineol (96%), γ-terpinene (97%) and α-pinene (97%) were purchased from Sigma–Aldrich (Buenos Aires, Argentina). Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) 96.7%, used as a positive control, was provided by Chemotecnica S.A (Buenos Aires, Argentina).

Essential oils were extracted on a laboratory scale for 70 min, using a hydrodistillation method in a modified Clevenger-type apparatus (Bandoni, 2002). The chemical composition of each oil was determined in previous studies in our laboratory (Lucia et al., 2008). Briefly, the main essential oil component of *E. cinerea*, *E. globulus* ssp. maidenii, *E. globulus* ssp. globulus, *E. sideroxylon* and *E. viminalis* is 1,8-cineole, whereas the main components of *E. grandis* × *E. tereticornis* and *E. grandis* × *E. camaldulensis* are 1,8-cineole and α-pinene. Essential oils of *E. tereticornis* and *E. camaldulensis* contain α-phellandrene, β-phellandrene and *p*-cymene as main components in addition to 1,8-cineole. The essential oils of *E. dunnii* and *E. gunnii* have a complex composition, with 1,8-cineole, γ-terpinene, *p*-cymene and spathulenol as main components (Lucia et al., 2008). Finally, the main component of *E. grandis* is α-pinene (Lucia et al., 2007).

2.3. Bioassay for fumigant activity

Fumigant tests developed in the lab were conducted in an enclosed specifically designed chamber that allowed concentration of the test vapors. Experimental units were composed of transparent acrylic tubes, 11.9 cm long, with an inner diameter of 4.4 cm and 164.5 ml capacity. One of the ends was covered by a neoprene disc and the other with a metal mesh (Bio Quip®, USA). A cover glass with 10 μl of essential oil was placed in a 5 cm Petri dish (Fig. 1).

The acrylic cylinder was placed over the Petri dish with the end covered by the metallic mesh facing downwards and thermostabilized for 10 min at 26–28 °C. Batches of 13–15 adult mosquitoes were collected with a mouth aspirator and introduced to the tube through the neoprene disc located on the upper side. This way the insects were exposed to the essential oil vapors but had no direct contact with the source. The tube was closed and carefully examined to ensure that the transferred mosquitoes were in good condition.

The number of knocked-down mosquitoes was recorded every minute for approximately 20–30 min. Knockdown was considered

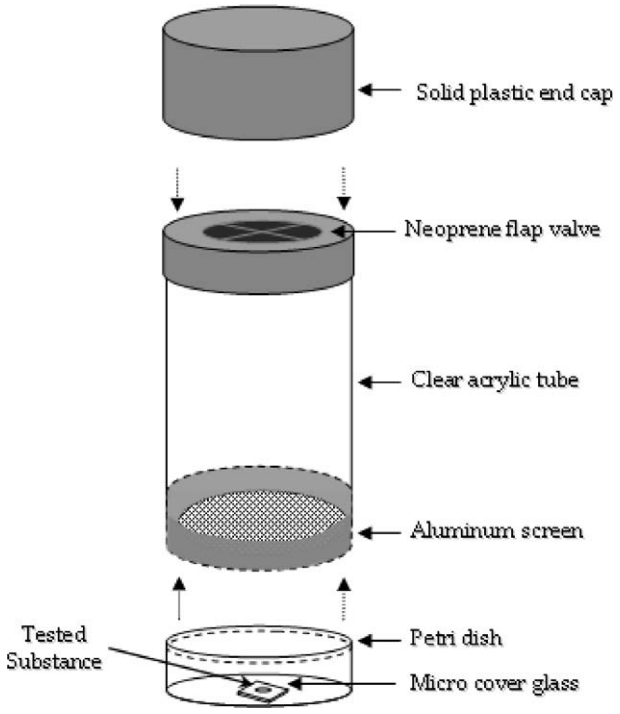

Fig. 1. Test device used to evaluate the fumigant activity of essential oils against *Aedes aegypti*.

as the inability of adult mosquitoes to fly. Four replicates were made for each tested substance. Dichlorvos was used as a positive control. The total number of mosquitoes in each tube was counted after each test.

2.4. Determination of vapor pressures of essential oils components (Vp)

Our objective was to calculate the vapor pressures of components using chromatographic data as experimental inputs, as introduced by Van Roon et al. (2002) and refined by Hoskovec et al. (2005).

According to these authors, the vapor pressure Px can be written as a function of the Kovats index Ix of the component X and some easy accessible non-chromatographic, physicochemical quantities (Eq. (1))

$$\ln Px = \ln Pz + (\ln X_Z^g + \ln X_X^g) + \frac{(100z - Ix)\left[\ln\left(\frac{Pz}{Pz+1}\right) + (\ln X_z^g - \ln X_{z+1}^g)\right]}{100} \quad (1)$$

where subscripts z and $z+1$ identify the reference n-alkanes with z and $z+1$ carbon atoms whose retention times encompass that of solute X.

Pz and $Pz+1$ can be calculated at temperature T (Weast, 1983) by means of Eq. (2).

$$Pz = \exp[C_1 + (C_2/T) + C_3 \ln T + C_4 T \exp^{C_5}] \quad (2)$$

As proposed by Yalkowsky et al. (1994), the difference in logarithms of ideal gas solubility, X^g, could be derived from the Van't Hoff equation (Eq. (3))

$$\ln X_i^g - \ln X_j^g \cong \frac{\Delta S_{b,i}^v (T_b^i - T)}{RT} - \frac{\Delta S_{b,i}^v (T_b^i - T)}{RT} \quad (3)$$

where ΔS_b^v is the entropy of vaporization at the normal boiling point T_b.

While T_b data for many compounds are available from standard sources, ΔS_b^v estimates can be easily deduced from structural information such us the torsional bond number (i.e. an estimate of molecular flexibility) and number of polar groups capable of hydrogen bonding. Myrdal et al. (1996) described a procedure to obtain ΔS_b^v values by means of simple modifications of Trouton's Rule (Eq. (4)):

$$\Delta S_b = 86 + 0.4\tau + 1421\ \mathrm{HBN} \quad (4)$$

These authors also developed an empirical parameter called the hydrogen bond number, HBN, defined as

$$\mathrm{HBN} = \frac{\sqrt{\mathrm{OH} + \mathrm{COOH}} + 0.33\sqrt{\mathrm{NH_2}}}{\mathrm{MW}} \quad (5)$$

where OH, COOH and NH_2 represent the number of alcohols, carboxylic acids or primary amines, respectively, and MW is the molecular weight of the compound.

On the other side τ, the effective number of torsional bonds, has been defined by Myrdal et al. (1996) by

$$\tau = \sum(\mathrm{SP3} + 0.5\mathrm{SP2} + 0.5\mathrm{RING}) - 1 \quad (6)$$

where SP3 and SP2 are the number of nonring, nonterminal sp^3 (including NH, N, O, and S) and sp^2 atoms, respectively. RING indicates the number of independent ring systems in the compound. Note that t is set equal to zero if this equation gives a negative value.

2.4.1. Gas chromatography

The retention indexes of the examined compounds were determined by gas chromatography coupled to mass spectrometry using a GCMS-QP 5050 *A* (Shimadzu, Japan) instrument. Gas chromatography conditions were the following: injection of a 0.4 μl of a hexane solution (1 mg/ml); capillary column HP-1 (Crosslinked Methyl Silicone Gum) (50 m × 0.32 mm × 0.52 μm); helium as carrier gas (1.3 ml/min). Analytical conditions: injector and interface temperatures of 250 °C and 280 °C, respectively, split ratio of 13:1, initial isothermic temperature of 50 °C for 10 min, programmed temperature of 50–68 °C (1 °C/min), programmed temperature of 68–75 °C (0.5 °C/min), programmed temperature of 75 °C to 250 °C (20 °C/min), final isothermic temperature of 280 °C for 10 min; electron impact 70 eV.

2.5. Statistical analysis

Knockdown data for each essential oil or component and for dichlorvos were subjected to probit analysis (Litchfield and Wilcoxon, 1949) and KT_{50} values were obtained using POLO PC 2.0 software (LeOra Software 2002). KT_{50} values were considered significantly different when the 95% confident limits (CL) did not overlap.

The relationship between the fumigant activity (KT_{50}) of the essential oil components and their respective vapor pressures (Vp) was analysed using Statistical Graphics SGWIN® software (Statgraphics Plus 4.0; Statistical Graphics Corporation, 1994–1999, Herndon, VA, USA).

3. Results and discussion

The results of the fumigant activity tests for the 13 essential oils are shown in Table 1. The fumigant activity measured as KT_{50} represents the time needed to observe a 50% knockdown effect in the mosquito population exposed to the experimental substance. A

Table 1
Fumigant activity of the essential oil vapors from different species of *Eucalyptus* against *Aedes aegypti*.

Essential oils	Fumigant activity			
	KT_{50} (min)[a]	Statistics[b]		
	(CI)	Slope ± SE	χ^2	df
E. gunnii	12.03 (11.63–12.43)	8.27 ± 0.55	9.20	12
E. tereticornis	10.55 (10.22–10.87)	11.69 ± 0.94	4.23	8
E. grandis	10.31 (9.91–10.71)	8.74 ± 0.70	7.71	9
E. camaldulensis	10,35 (9.95–10.75)	8.61 ± 0.66	3.79	10
E. dunnii	6,99 (6.39–7.56)	4.631 ± 0.30	18.79	13
E. cinerea	6.33 (6.04–6.60)	9.28 ± 0.92	0.99	4
E. saligna	5.66 (5.40–5.92)	10.07 ± 0.92	0.66	6
E. grandis × *E. tereticornis*	5.21 (4.98–5.43)	10.62 ± 1.03	2.98	4
E. grandis × *E. camaldulensis*	5.07 (4.85–5.29)	11.89 ± 1.23	3.35	4
E. globulus ssp. globulus	4.81 (4.44–5.05)	8.74 ± 0.82	1.50	5
E. sideroxylon	4.77 (4.38–5.15)	10.35 ± 1.07	4.98	4
E. globulus ssp. maidenii	4.28 (4.03–4.52)	8.44 ± 0.82	1.60	5
E. viminalis	4.19 (3.95–4.42)	9.13 ± 1.01	1.87	3

[a] Time to 50% knockdown with a 95% confidence interval (CI). KT_{50} values are the means of four replicates using 13–15 adults (total 54–60 adults) for each essential oil, determined at 1-min intervals until 100% of the adults were knocked-down.
[b] Statistics of the probit analysis of knockdown times.

Table 2
Fumigant activity of the individual oil components from different species of *Eucalyptus* against *Aedes aegypti*.

Oil components	Fumigant activity			
	KT_{50} (min)[a]	Statistics[b]		
	(CI)	Slope ± SE	χ^2	df
α-Terpineol	26.38 (25.55–27.29)	10.83 ± 0.68	31.39	18
γ-Terpinene	9.31 (8.94–9.67)	8.73 ± 0.59	11.42	10
4-Terpineol	9.27 (8.86–9.68)	7.99 ± 0.54	9.06	11
p-Cymene	5.82 (5.52–6.10)	7.16 ± 0.56	4.73	8
α-Pinene	5.36 (4.95–5.73)	10.18 ± 1.01	4.89	4
1,8-Cineole	3.90 (3.56–4.20)	8.26 ± 0.75	5.80	5
Dichlorvos	2.40 (2.14–2.67)	9.25 ± 1.05	4.34	6

[a] Time to 50% knockdown with a 95% confidence interval (CI). KT_{50} values are the means of four replicates using 13–15 adults (total 54–60 adults) for each essential oil, determined at 1-min intervals until 100% of the adults were knocked-down.
[b] Statistics of the probit analysis of knockdown times. Dichlorvos was used as a positive control.

Table 3
Vapor pressures of *n*-alkanes calculated at 25 °C derived from Eq. (2).

Alkane	Formula	$P_{Vap.}$ (mmHg)	ln P
n-Octane	C_8H_{18}	14.03	2.64
n-Nonane	C_9H_{20}	4.35	1.47
n-Decane	$C_{10}H_{22}$	1.36	0.31
n-Undecane	$C_{11}H_{24}$	0.42	−0.87
n-Dodecane	$C_{12}H_{26}$	0.13	−2.01

Table 4
Parameters and vapor pressures of components from different species of *Eucalyptus* derived from Eqs. (1), (3), (4), (5), (6).

Compound	*Ix*	τ	HBN	ΔS (J $K^{-1}mol^{-1}$)	T_b (K)	$P_{vap.}$ (mmHg)
1,8-Cineole	1027	0.0	0.00	86.00	449.65	1.34
γ-Terpinene	1059	0.5	0.00	86.20	456.15	0.93
α-Pinene	935	0.0	0.00	86.00	429.35	3.66
α-Terpineol	1183	0.5	0.01	95.41	490.65	0.09
4-Terpineol	1172	0.5	0.01	95.41	482.15	0.13
p-Cymene	1017	0.5	0.00	86.20	450.15	1.35

Ix retention index, τ effective number of torsional bonds, HBN hydrogen bond number, ΔS_b^v entropy of vaporization at T_b, T_b boiling temperature, P_{vap} vapor pressure.

lower knockdown time represents a more active compound. The KT_{50} values of the essential oils from different *Eucalyptus* plants vary according to the chemical composition of the oil. The most active compound was the essential oil of *E. viminalis*, with a KT_{50} of 4.2 min. However, it was not significantly different from the essential oils of *E. globulus* ssp. *maidenii* and *E. sideroxylon* (KT_{50} = 4.3 and 4.8 min, respectively). A previous study showed that the oils of these species contained large quantities of 1,8-cineole (85.0%, 77.9% and 91.3%, respectively; Lucia et al., 2008). Therefore, the fumigant activity of these essential oils could be due to the presence of 1,8-cineole.

The results of the fumigant activity tests for the individual pure oil components are shown in Table 2. The following order of activity was obtained by comparing the KT_{50} values of each oil component: 1,8-cineole > α-pinene = *p*-cymene > 4-terpineol = γ-terpinene > α-terpineol. Tables 3 and 4 show respectively the calculated vapor pressures of *n*-alkanes and the calculated vapor pressures of components based on experimental data for different species of *Eucalyptus*.

Regression analysis evidenced a statistically significant relationship between fumigant activity (KT_{50}) and the inverse function of 1,8-cineole concentration (1/1,8-cineole (%); $p < 0.01$), according to the following equation:

$$KT_{50} = 2.94 + 143.66 \times 1/1,8\text{-cineole } (\%) \quad (7)$$

with a coefficient of determination of 91.70% and a standard deviation of residuals of 0.96 (Fig. 2). *Eucalyptus* species with a high content of 1,8-cineole in their essential oils have a greater fumigant activity against *A. aegypti*. The knockdown activity of dichlorvos was of the same order of magnitude as 1,8-cineole (Table 2). Reversion of the knockdown effect after 24 h was observed in 77.5 ± 33% of the individuals in the assays with the most active component, 1,8-cineole.

The fast knockdown effect produced by 1,8-cineole together with results obtained in head lice (Picollo et al., 2008), suggest that this monoterpene probably has a neurotoxic effect. The toxic action of monoterpenoids and the essential oils containing them may be mediated by pathways such as GABA receptors (Priestley et al., 2003) and octopamine receptors (Enan, 2001; Kostyukovsky et al., 2002).

Regression analysis also showed a statistically significant relationship between KT_{50} values and the inverse function of vapor pressures (1/*Vp*) of the different essential oil components (*p* value < 0.05), based on the following equation:

$$KT_{50} = 4.38 + 1.52 \times (1/Vp) \quad (8)$$

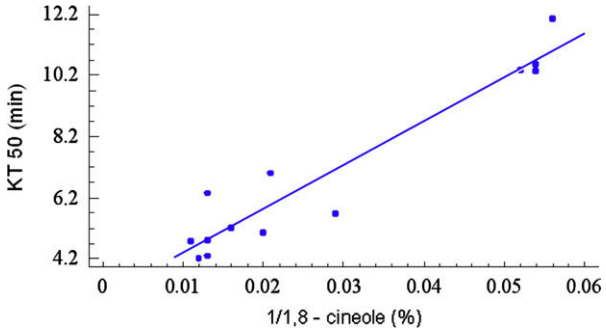

Fig. 2. Relationship between fumigant activity (KT_{50}) and the percentage of 1,8-cineole in essential oils from different species of *Eucalyptus*. Each point represents the KT_{50} value for each species of *Eucalyptus* and its corresponding concentration of 1,8-cineole, expressed as the inverse function of 1,8-cineole concentration (1/1,8-cineole (%)).

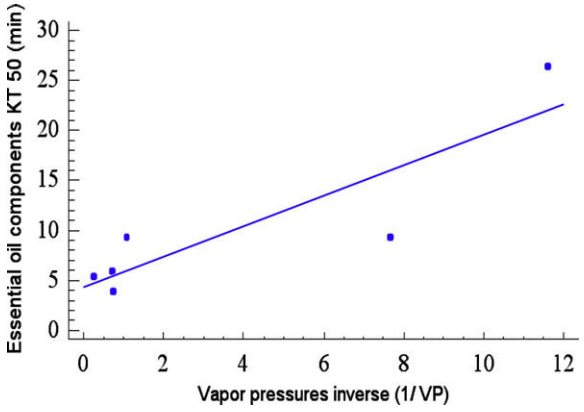

Fig. 3. Relationship between fumigant activity (KT_{50}) and the vapor pressure of the essential oil components. Each point represents the KT_{50} value of each essential oil component and its corresponding vapor pressure in mmHg at 25 °C, expressed as the inverse function of vapor pressures (1/*Vp*).

The coefficient of determination was 77.18% and the standard deviation of residuals was 4.44 (Fig. 3).

The correlation between KT_{50} values and vapor pressures of the essential oil components was in accordance with previous studies showing that the fumigant activity of simple organic compounds in insects is correlated with their volatilities (Hansch, 1971). 1,8-cineole and *Eucalyptus* essential oils with an elevated concentration of this monoterpene present a high knockdown activity against *A. aegypti*. These compounds are a promising alternative with a low environmental impact for controlling the yellow fever mosquito.

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