Our "two-charge" model shown in Figure 1a[10] suggests that it should be possible to titrate specific residues in bacteriorhodopsin and obtain large spectral shifts. The long-wavelength form of bacteriorhodopsin (λ_{max} 605 nm) observed at pH <2.8[1,2a] has been attributed to the titration of a charged amino acid near the Schiff base terminus.[11a,b] This presumably corresponds to the counterion. The 565-nm species formed at pH <0.8 could result from the titration of a second group[11a] which we have placed near the β-ionone ring. However, since bound anions have also been shown to induce wavelength shifts,[11b] the factor involved in the formation of the 565-nm species is still an open question.

It should be noted that the model carrying a negative charge in the vicinity of the β-ionone ring (Figure 1a) is in contrast to the model derived previously for bovine rhodopsin, where the important wavelength-determining interactions were located near the 11,12 double bond of the polyene side chain[7] (Figure 1b). We showed that simple synthetic compounds could simulate the spectral shifts observed in the binding site of bovine rhodopsin;[12] synthetic models, designed to simulate the bacteriorhodopsin binding site, are currently being prepared to check the external point-charge model for bacteriorhodopsin.

The influence of the opsin binding site on the absorption maximum of SBH^+, i.e., the opsin shift, is a central problem in the rhodopsin field since it accounts for the wide variance in λ_{max} encountered in the various pigments, including the visual cone pigments responsible for color perception. Thus numerous models have been proposed to account for the opsin shift. However, we point out that a model which rationalizes the opsin shifts of natural systems is far from satisfying the necessary conditions. The model should also account for the data derived from various synthetic retinals, e.g., data which led to the proposal of models 1a (Table I) and 1b.[13]

Acknowledgment. This work was supported by NIH Grant CA 11572 (K.N.) and NSF Grant PCM 80-10931 (B.H.).

(10) Honig, B.; Hudson, B.; Sykes, B. D.; Karplus, M. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1289–93.
(11) (a) Honig, B. In "Energetics and Structure of Halophilic Microorganisms"; Caplan, R., Ginzburg, M., Eds; Elsevier: New York, 1978; pp 99–121. (b) Fisher, U.; Oesterhelt, D. *Biophys. J.* **1979**, *28*, 211–30.
(12) Sheves, M.; Nakanishi, K.; Honig, B. *J. Am. Chem. Soc.* **1979**, *101*, 7086–8.
(13) Reference 6, Table I.

Koji Nakanishi,* Valeria Balogh-Nair
Maria Arnaboldi, Kazuo Tsujimoto
Department of Chemistry
Columbia University
New York, New York 10027

Barry Honig*
Department of Biophysics
University of Illinois
Urbana, Illinois 61801

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Opsin Shifts in Bovine Rhodopsin and Bacteriorhodopsin. Comparison of Two External Point-Charge Models

Sir:

We proposed the external point-charge model I (Figure 1)[1-3] to account for the variance in the absorption maxima of various visual pigments; more recently,[4] we proposed model II (Figure 1) to explain the purple color (λ_{max} 570 nm) of bacteriorhodopsin (bR), the pigment present in purple membrane.[5] Note that the two models have contrasting charge distributions; i.e., *the second charge is near C-12/C-14 in visual pigment I, whereas it is near the ring in bacteriorhodopsin II.*

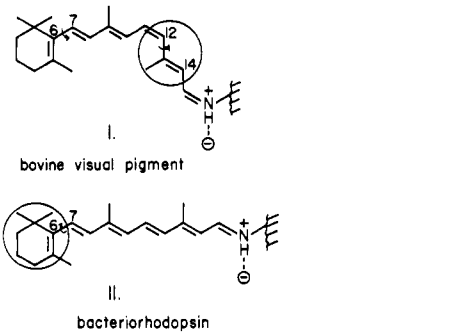

Figure 1. External point-charge models in the binding sites for bovine visual pigment (I) and bacteriorhodopsin (II). The existence of a counterion near the protonated nitrogen is assumed. The second negative charge shown by the circle could be a member of a charge pair in a salt bridge or one end of a neutral dipole. In I, it is located near C-12/C-14 whereas in II, it is near the ring. The small curved arrows indicate that the bonds are twisted.

The retinal chromophore is bound to the apoprotein lysine residue through a protonated Schiff base (SBH^+) in these pigments.[6] The charges shown by circles in Figure 1 do not exist in solutions of SBH^+ (formed from *n*-butylamine) and are present only in the binding sites. Hence the difference in the maxima between the *n*-butylamine SBH^+ and pigments can be regarded as a measure of the influence of the binding site and we have proposed to call it the "opsin shift".[4] The contrasting external point-charge distributions seen in models I and II should manifest themselves in the opsin shifts of pigments derived from appropriately tailored retinals; investigation of such data should in turn provide us with a means to check the validity of the two models. Namely, we may expect aromatic retinal **1** with an altered ring structure not to exert large effects on the λ_{max} of rhodopsin but instead affect that of bR; the opposite tendency is predicted for bromoretinals **4** and **5** having altered side chains. In the following we report the results of such observations.

Retinal Synthesis. Benzaldehyde and *p*-(dimethylamino)-benzaldehyde were converted into phenyl dienals **6** and **7** through conventional[7] Emmons reaction with ethyl phosphonosenecioate, Dibal reduction, MnO_2 oxidation, and flash chromatographic separations[8] of isomers. A second series of the same C-5 elongation process gave the all-trans, 13-cis, 9-cis, and 9,13-dicis isomers[9] of aromatic retinals exemplified by **1–3**.[10]

The synthesis of 13-bromoretinal **4** was carried out by condensation of Wittig salt **8**[11] with aldehyde **9** (Scheme I), which

(1) Nakanishi, K.; Balogh-Nair, V.; Gawinowicz, M. A.; Arnaboldi, M.; Motto, M.; Honig, B. *Photochem. Photobiol.* **1979**, *29*, 657.
(2) Honig, B.; Dinur, U.; Nakanishi, K.; Balogh-Nair, V.; Gawinowicz, M. A.; Arnaboldi, M.; Motto, M. G. *J. Am. Chem. Soc.* **1979**, *101*, 7084.
(3) See also: Arnaboldi, M.; Motto, M. G.; Tsujimoto, K.; Balogh-Nair, V.; Nakanishi, K. *J. Am. Chem. Soc.* **1979**, *101*, 7082. Sheves, M.; Nakanishi, K.; Honig, B. *Ibid.* **1979**, *101*, 7086.
(4) Nakanishi, K.; Balogh-Nair, V.; Arnaboldi, M.; Tsujimoto, K.; Honig, B. *J. Am. Chem. Soc.*, preceding paper.
(5) (a) Oesterhelt, D.; Stoeckenius, W. *Nature (London) New Biol.* **1971**, *233*, 149. (b) Henderson, R. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 87. (c) Stoeckenius, W.; Lozier, R. H.; Bogomolni, R. A. *Biochim. Biophys. Acta* **1978**, *505*, 215.
(6) The SBH^+ theory is the one generally accepted, especially on grounds of resonance Raman studies: Callender, R.; Honig, B. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 33. However, there is some controversy regarding the SBH^+ theory. For leading references see: Favrot, J.; Leclercq, J. M.; Roberge, R.; Sandorfy, C.; Vocelle, D. *Photochem. Photobiol.* **1979**, *29*, 99. See also ref 5b.
(7) Mayer, H.; Isler, O. In "Carotenoids"; Isler, O., Ed.; Birkhauser Verlag: Basel, 1971. Mayer, H. *Pure Appl. Chem.* **1979**, *51*, 535.
(8) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(9) For the sake of convenience, the conventional retinal numbering system is used.
(10) The purity of all retinals was ascertained by high-performance liquid chromatography before binding studies. The structures were fully corroborated by mass spectral, UV, ^1H NMR, and ^{13}C NMR data.
(11) Isler, O.; Gutmann, H.; Lindlar, H.; Montavon, M.; Rüegg, R.; Ryser, G.; Zeller, P. *Helv. Chim. Acta* **1956**, *39*, 463.

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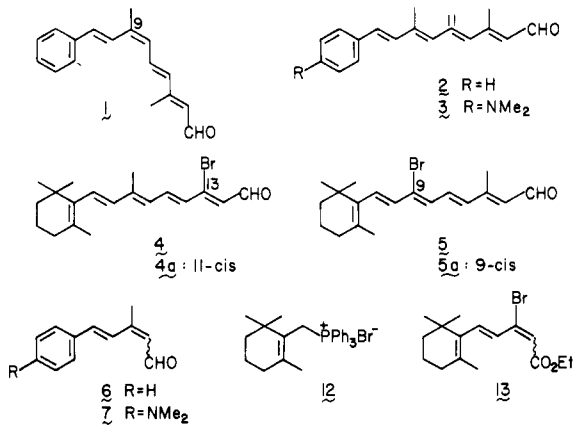


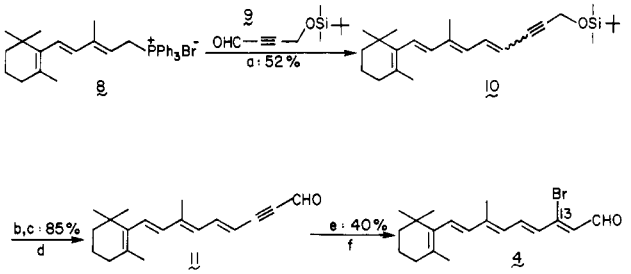
in turn was derived from 2-butyne-1,4-diol[12] by monosilylation (1.1 equiv of *tert*-butyldimethylsilyl chloride[13] in DMF, 0.5 h, 60% yield) and MnO_2 oxidation in CH_2Cl_2, 69% yield. Deprotected **10**, tetraenynal **11**,[14] and retinal **4** were all extremely unstable and decomposed even under argon in the dark at −70 °C after a few days; accordingly after deprotection of **10**, the subsequent reaction steps, high-pressure liquid chromatography (LC), and the binding were carried out as rapidly as possible.

9-Bromoretinal **5** was prepared as follows. Wittig salt **12**[15] was condensed with ethyl 3-bromo-4-oxocrotonate (prepared from 4-oxocrotonate[16] by bromination/dehydrobromination) to give bromo ester **13**, 63% yield, which, after Dibal reduction and MnO_2 oxidation, gave the aldehyde, quantitative yield. The usual sequence of Emmons reaction, Dibal reduction, and MnO_2 treatments[7] gave, in 70% yield, the trans and 13-cis mixture of 9-bromoretinals; the major trans isomer **5** was separated and purified by LC.

Binding Studies. I. Phenyl-9-*cis*-retinal 1 with Bovine Opsin. Bovine opsin was incubated with a 15-fold excess of chromophore **1**,[17] pH 7.0, 67 mM phosphate buffer, room temperature for 25 h; the suspension was then centrifuged, and the pigment pellet was washed several times with hexane at −20 °C and solubilized in 2% digitonin,[18] pH 7.0, phosphate buffer. Although the yield[19] is only ca. 10%, an aromatic rhodopsin is indeed formed.[20] The pigment, even in the absence of NH_2OH, is bleached in 40 s when irradiated with a 100-W tungsten lamp (Figure 2); the difference curve leads to λ_{max} 485 nm.

Although the natural chromophore is 11-*cis*-retinal, it is known that 9-*cis*-retinal also forms rhodopsins.[21] In the current studies we have worked with the 9-cis series due to accessibility. The maxima of 9-*cis*-rhodopsin **14** (2% digitonin) and the SBH^+ of 9-*cis*-retinal with *n*-butylamine **15** (MeOH)[22] are 485 and 440 nm, respectively; the red shift from 440 to 485 nm corresponds

Scheme I[a]



[a] (a) *n*-BuLi, THF, room temperature, 3 h; (b) Bu_4NF, THF; (c) MnO_2, CH_2Cl_2; (d) high-performance liquid chromatography, Lichrosorb, 3% Et_2O in hexane; (e) 40% aqueous HBr in C_6H_6, room temperature, 8 h; (f) flash chromatography[8] with 5% Et_2O in hexane, then high-performance liquid chromatography, 3% Et_2O in hexane.

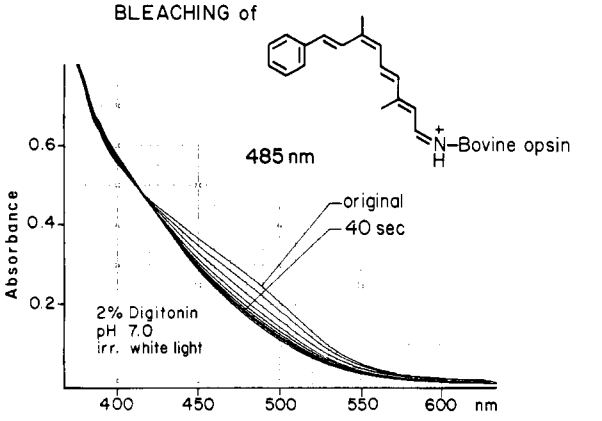


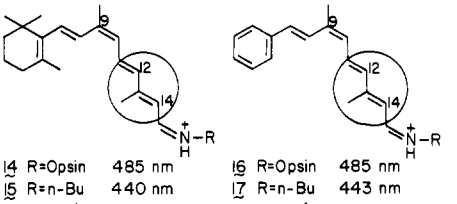
Figure 2. Bleaching of bovine rhodopsin from phenyl-9-*cis*-retinal. The pigment was irradiated with a 100-W tungsten lamp, >500 nm.

Table I. λ_{max} (nm) of Dark-Adapted Bacteriorhodopsins

retinal	aldehyde[a]	SBH^+ [b]	bR^{DA} [c]	Δ,[d] cm^{-1}
1. all-trans	381	440	560	4870
2. phenyl-2	373	455	480	1150
3. (dimethylamino)-phenyl-3	442	550	535	−510
4. 13-bromo-4	388	465	595	4700
5. 9-bromo-5	372	430	535	4560

[a] In MeOH. [b] Protonated Schiff base with *n*-$BuNH_2$, in MeOH. [c] In H_2O, pH 7.0. [d] Opsin shift, i.e., λ_{max} of SBH^+ (in cm^{-1}) minus λ_{max} of pigment (in cm^{-1}).

to an opsin shift of 2110 cm^{-1}. The opsin shift of the 9-cis isomers of the phenyl analogues **16** and **17** is 1960 cm^{-1}. Note that in



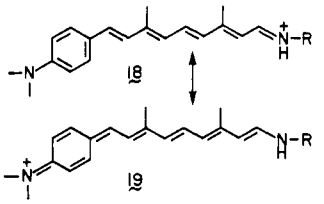
spite of the drastic change in ring structure and probably the dihedral angle around the 6,7 bond, the λ_{max} and opsin shifts are quite similar in the two series. This agrees with model I which has its point charge near the side chain rather than the ring.

II. Phenyl-*all-trans*-retinal 2 with Bacterioopsin. The opsin was incubated[17] with a 6-fold excess of the chromophore in distilled water or 67 mM phosphate buffer, pH 7.0, for 1 h in the dark. The dark-adapted pigment, thus formed, bR^{DA}, λ_{max} 480 nm (Table I), was not stable when treated with 0.1 M NH_2OH. The large shift of 4870 cm^{-1} between SBH^+ and bR^{DA} (Table I) was

(12) Aldrich chemical, freshly distilled before use.
(13) Corey, E. J.; Venkateswarlu, A. *J. Am. Chem. Soc.* **1972**, *94*, 6190.
(14) 11-*cis*-**11** did not bind with bovine opsin, and *all-trans*-**11** did not bind with bacterioopsin.
(15) Analogous to the preparation of the corresponding α-ionyl compound: Barlett, L.; Klyne, W.; Mose, W. P.; Scopes, P. M.; Galsko, G.; Mallams, A. K.; Weedon, B. C. L.; Szabolcs, J.; Toth, G. *J. Chem. Soc. C* **1969**, *2527*.
(16) Rambaud, R.; Vessiere, M. *Bull. Soc. Chim. Fr.* **1961**, 1567.
(17) In all incubation studies, a solution of the chromophore in ethanol was added to the deoxygenated opsin suspension; the final volume of ethanol was 1.0–2.0% of the incubation mixture: Matsumoto, H.; Horiuchi, K.; Yoshizawa, T. *Biochim. Biophys. Acta* **1978**, *501*, 257.
(18) Bridges, C. D. B. *Vision Res.* **1977**, *17*, 301.
(19) For the sake of convenience the yield is expressed relative to that expected when the same amount of opsin was incubated with 9-*cis*-retinal.
(20) Kropf et al. have reported that an isomeric mixture of 9,13-demethyl analogue of **2** does not bind to bovine opsin: Kropf, A.; Whittenberger, B. P.; Goff, S. P.; Waggoner, A. S. *Exp. Eye Res.* **1973**, *17*, 591.
(21) Hubbard, R.; Wald, G. *J. Gen. Physiol.* **1952**, *36*, 269. Wald, G.; Brown, P. K.; Hubbard, R.; Oroshnik, W. *Proc. Natl. Acad. Sci. U.S.A.* **1958**, *41*, 438. Hubbard, R.; Kropf, A. *Ibid.* **1958**, *44*, 130.
(22) The λ_{max} values of SBH^+ are measured in MeOH for the sake of simplicity because it has been shown that in this solvent the maxima are not affected by the counteranion: Erickson, J. O.; Blatz, P. E. *Vision Res.* **1968**, *8*, 1367.

accounted for by the point charge near the ring (model II, Figure 1).[4] In the phenyl analogue this value is only 1150 cm^{-1} (Table I), thus implying that the influence of bR binding site greatly differs for the aromatic retinal. This contrasts with bovine rhodopsin (case I) and supports model II, which predicts that the difference in ring structure would affect the maximum of bR.

III. [*p*-(Dimethylamino)phenyl]retinal 3 with Bacterioopsin. The pigment was prepared (Table I) as for case II by incubating the opsin with a 15-fold excess of the retinal for 20 h in the dark at room temperature. Irradiation of bR^{DA}, λ_{max} 535 nm, with light >500 nm, for 15 min, gave the light-adapted species, bR^{LA}, λ_{max} 545 nm. The 535-nm maximum disappeared after 5.5 h in the presence of 0.1 M NH_2OH. This chromophore is unique since the SBH^+ form has two contributing forms **18** and **19** reminiscent



of cyanine dyes. Since the electronic and steric properties of the aromatic ring differ greatly from those of trimethylcyclohexene, the effect of the second point charge is also drastically altered and is now −510 cm^{-1}. Although the cause of this contrasting behavior is not clear, it is consistent with model II; i.e., a change in the ring structure alters the opsin shift.

IV. 13-Bromo-11-*cis*-retinal (4a) and 9-Bromo-9-*cis*-retinal (5a) with Bovine Opsin. Compounds **4a** and **5a** were prepared by photoisomerization of **4** and **5**, with LC and NMR characterization. The 11-*cis*-retinal **4a** was incubated immediately with opsin after it was eluted from the liquid chromatograph due to its extreme instability. Opsin was incubated with 3- and 5-fold excesses of **4a** and **5a**, respectively, pH 7.0, 67 mM phosphate buffer, room temperature for 23 h, and the pigments were processed as in case I. The pigments from **4a** and **5a** were bleached under conditions similar to those for case I in 110 s and 70 min, respectively. The spectral data are summarized in the following:

	4a (13-bromo-11-cis)	5a (9-bromo-9-cis)
aldehyde (MeOH) λ_{max}, nm	383	365
SBH^+ (MeOH) λ_{max}, nm	467	422
vis, nm	520	465
opsin shift (Δ), cm^{-1}	2180	2190
CD (see Figure 3 for unit)	520 (+6.0)	452 (+7.5)
	355 (+3.3)	322 (+6.7)

The opsin shift of the 13-bromo-11-*cis*-rhodopsin, 2180 cm^{-1}, should be compared to that of natural rhodopsin, 2700 cm^{-1}; as expected from model I, introduction of the 13-Br exerts a considerable change in the opsin shift because of its proximity to the site of the negative charge. The 2190-cm^{-1} value for the 9-bromo-9-*cis*-rhodopsin should be compared to the 2110-cm^{-1} value for the 9-*cis*-rhodopsin. The close similarity of these two values is again in agreement with model I; namely, position 9 is not sensitive to electronic and/or steric effects. Bovine pigments have been prepared from 9- and 13-demethylretinals[20,23] but since the SBH^+ values were not measured, the data cannot be compared with those discussed here.

V. Bromoretinals 4 and 5 with Bacterioopsin. The bR's were prepared by incubation of 1:1 mixtures of the opsin and chromophores **4** and **5** for 20 and 6 h, respectively (Figure 3, Table I). When a suspension of 13-Br-bR^{LA} (595 nm) was treated with *all-trans*-retinal in the dark, room temperature, pH 7.0, the 13-bromo chromophore was slowly displaced; after 120 h the λ_{max} was shifted to 570 nm, i.e., the λ_{max} of natural bR (isosbestic point at 575 nm). In contrast, the 9-bromo chromophore was not displaced by *all-trans*-retinal. Both the 13- and 9-bromo analogues

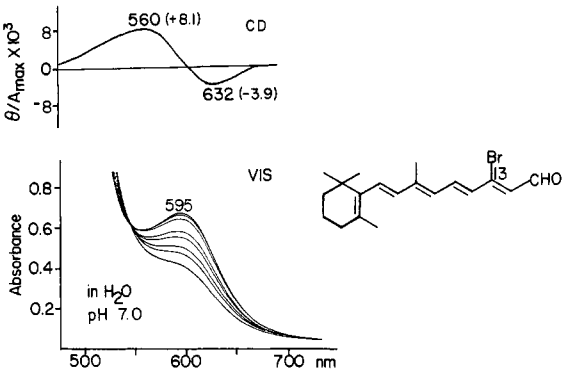


Figure 3. Absorption and CD curves of 13-bromo-bR^{DA}.

of bR underwent the following transformations at room temperature between the dark- and light-adapted species:

(a) 13-bromo-bR^{DA} $\underset{\text{30 h, in the dark}}{\overset{\text{30 min, 1000 W, } \lambda > 500 \text{ nm}}{\rightleftharpoons}}$ 13-bromo-bR^{LA}

vis: 595 nm	vis: ca. 15% reduction in intensity
CD: 632 nm (−3.9) 560 nm (+8.1) (see Figure 3)	CD: ca. 15% reduction in intensity

(b) 9-bromo-bR^{DA} $\underset{\text{50 h, in the dark}}{\overset{\text{15 min, 1000 W, } \lambda > 500 \text{ nm}}{\rightleftharpoons}}$ 9-bromo-bR^{LA}

vis: 535 nm	vis: 545 nm
CD: 585 nm (−1.6) 505 nm (+7.2)	CD: 590 nm (−1.5) 512 nm (+7.8)

Note that in case a, the differences between the species are only in a reduction in intensities of maxima and no shifts in wavelength; this is unlike typical cases where ca. 10-nm shifts are encountered. This unusual behavior may be related to the fact that in bR it is the 13-ene which undergoes isomerization during the light cycle;[5] hence it is plausible that the 13-bromine is affecting the light cycle and the proton pumping of this bR species.

In accordance with the red and blue shifts in the λ_{max} of the 13-bromo and the 9-bromo analogues, respectively, the extremes of the split CD also undergo similar shifts. This suggests that the CD of bacteriorhodopsin is a consequence of a coupled oscillator type of interaction among the three retinal moieties present in the three bR molecules of the crystalline lattice.[5b] A comparison of entries 1, 4, and 5 (Table I) shows that the opsin shifts are all similar; i.e., the 13- and 9-bromo atoms do not alter the binding site influence. This again agrees with the bR model which has no point charge in the vicinity of the side chain.

To summarize, the shifts encountered in the absorption maxima of artifical pigments support the point-charge models of rhodopsin and bacteriorhodopsin (Figure 1).

Acknowledgment. We thank John D. Carriker for technical assistance and the National Institutes of Health (Grant CA 11572) for financial support.

(23) Nelson, R.; DeRiel, J. K.; Kropf, A. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *66*, 531.

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Michael G. Motto, Mordechai Sheves,[24] Kazuo Tsujimoto Valeria Balogh-Nair, Koji Nakanishi*

Department of Chemistry
Columbia University
New York, New York 10027

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