

# Effect of peripheral nerve on the neurite growth from retinal explants in culture

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## ABSTRACT

The effect of peripheral nerve (PN) on neurite outgrowth from retinal explants of adult hamsters was examined. Cultures of retinal explants, and co-cultures of retinal explants and PN were performed using chick retinal basement membrane (BM) as substrate. The presence of PN increases the number and length of neurite outgrowth. In addition, a high proportion of neurites situated close to PN tend to grow towards it. Since there was no contact between retinal explants and PN, we suggest that PN might secrete diffusible substances to attract the neurites to grow towards it.

**Key words:** *retinal explants, peripheral nerve, tissue culture, neurite outgrowth, adult hamster.*

## INTRODUCTION

Recent experiments showed that damaged retinal ganglion cells of adult mammals can regenerate extensively into a segment of peripheral nerve grafted into the eye of rats[1] or hamsters[2, 3, 4]. In addition, the graft in retina seems to play an active role in attracting and/or guiding the regenerating axons to grow into it [2]. In this work, we co-cultured segments of PN with strips of adult hamster retina to see the effects of PN on the neurite growth from retinal explants. The results suggest that PN segments may release into the environment soluble factors which seem to enhance the number and length of neurites outgrowth and affect the direction of growing neurites from adult hamster retinal neurons.

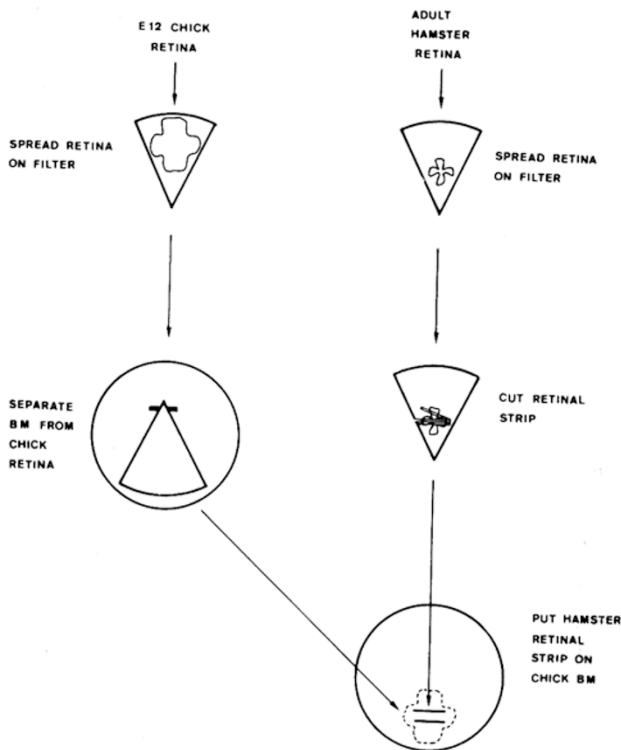
## MATERIALS AND METHODS

In order to select the appropriate substrate for neurite growth from adult hamster retinal

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neurons, we cultured retinal strips on different substrates in a set of preliminary experiments. The substrates tested included collagen, laminin, poly-L-lysine and basement membrane (BM) from retina of chick embryo of 6- and 12- day old (E6 and E12). The collagen solution was obtained from rat tail as described elsewhere[5]. A final collagen concentration of 0.4mg/ml was coated onto Petriperm (Heraeus) dishes. Laminin (1  $\mu$ g or 50  $\mu$ g per ml; Bethesda Res. Lab.) was coated onto glass cover slips which had been treated with poly-L-lysine (Sigma, MW 52,000, 100  $\mu$ g/ml).

Each Petriperm dish was incubated with two ml of poly-L-lysine (100  $\mu$ g/ml) for 30 min. After drying, the dish was put under UV light for 10 min and then calcium and magnesium free (CMF) Hank's solution was added into the dish. The Hank's solution should be removed before using the dish for preparation of BM. The BM was obtained from E6 or E12 chick retina[6] and Halfter, personal communication). The retina was dissected in CMF Hank's solution. The whole retina from chick was spread out on Sartorius black filter (SM 13006) with photoreceptor cell layer facing the filter (Fig. 1). The filter was then turned upside-down onto a poly-L-lysine coated dish prepared as above. The filter was removed 10 min later with retina still sticking to it while BM was left on the dish.



**Fig. 1** A schematic flow diagram demonstrating the preparation of E12 chick basement membrane (BM) and strips of retina from a adult hamster.

**Culture medium**

The culture medium consisted of Eagle's modified minimum essential medium complemented

with 2.4% NaHCO<sub>3</sub> and 5 mM Hepes, 10% fetal bovine serum, 2% chick serum, 1% chick embryo extract, 2 mM Glutamine, penicillin and streptomycin.

### ***Retinal strip cultures***

#### **1. Culturing on poly-L-lysine, laminin and collagen treated dishes.**

A whole hamster retina was spread out on a filter. Strips of 600  $\mu$ m wide were cut using a tissue chopper (McIlwain). They were then put onto the dishes with different culture substrates with ganglion cell layer facing downward: put up to 10 strips per dish. Culture medium was added and the cultures were placed in an incubator at 37°C with 4% CO<sub>2</sub>. Unlike the other dishes, the collagen treated dishes, were incubated for 2 hr before adding 2 ml culture medium. In all cases, retinal strips were cultured for 5 days, then fixed with 4% formalin.

#### **2. Culturing of chick basement membranes.**

After spreading a hamster retina on a filter, it was cut into 600  $\mu$ m wide strips (Fig. 1). Two strips were selected and put on E12 BM (prepared as described above). Only one retinal strip was put on E6 BM because of its small size. 2 ml of culture medium was added and the dishes placed in an incubator at 37°C with 4% CO<sub>2</sub>. Retinal strips were cultured for 3-5 days, then fixed with formalin.

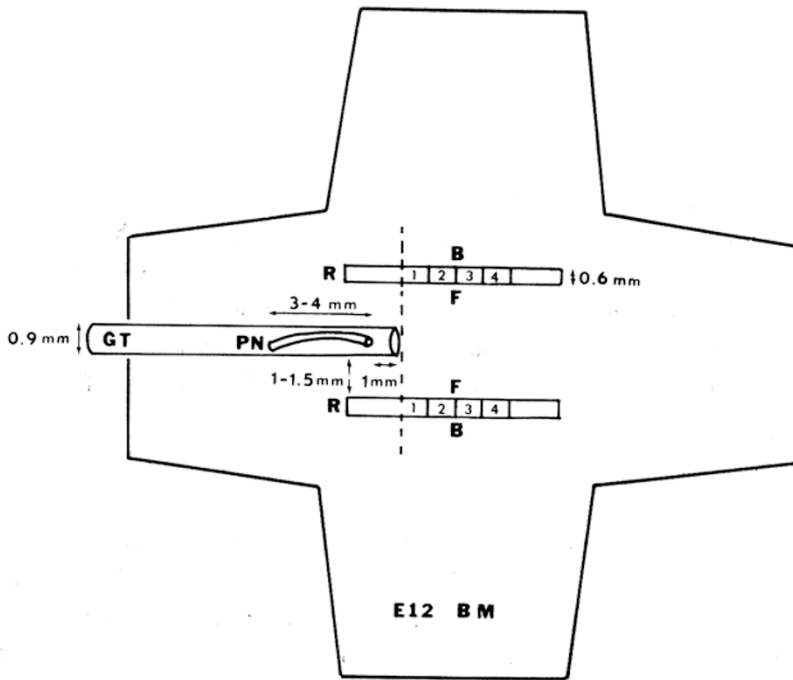
#### **3. Identification of retinal ganglion cells in cultures.**

After exposing the anterior portion of the superior colliculus in 3 adult hamsters by surgically removing their overlying cortex, a slit was made in the anterior tip of the superior colliculus and a small piece of gelform (Upjohn) soaked in horseradish peroxidase (HRP; Sigma, type VI, 50% solution in saline) was placed into the slit. The contralateral retina was removed two days later and was cultured on E12 chick BM for 4 days as described above. The retinal explants were then fixed and processed for HRP histochemistry with tetramethyl benzidine (TMB) as described before [1].

### ***Retinal strips and PN cultures***

Two retinal strips of 7-8 mm in length obtained from adult hamster were put on E12 chick retinal BM. In addition, a piece of predegenerated PN (3-4 mm long) was put between two retinal strips as in Fig. 2. In order to prevent cell migration from PN, it was put inside a glass tube (0.9 mm inner diameter, open at both ends). For any given culture, the retina and PN should be obtained from the same animal. Sciatic nerve, which was used as PN source, was crushed 4-10 days prior to culture experiment. The proximal end of the crushed nerve was tied with suture and the nerve segments were removed just before use.

After adding 2 ml culture medium, the cultures were placed in an incubator at 37°C with 4% CO<sub>2</sub>. The retinal strips were cultured for 5 days with no disturbance of dishes. Fixed with 4% formalin.



**Fig. 2** A schematic diagram showing two strips of adult hamster retinas(R) co-cultured with a segment of peripheral nerve (PN) situated inside a glass tube (GT). BM from an E12 chick embryo is used as substrate (E12 BM). Numerals 1, 2, 3, and 4 on retinal strips denote zones of 1 mm each, zone 1 starts from the mouth of glass tube. B, back-to-PN sides; F, face-to-PN sides.

## Analysis of cultures

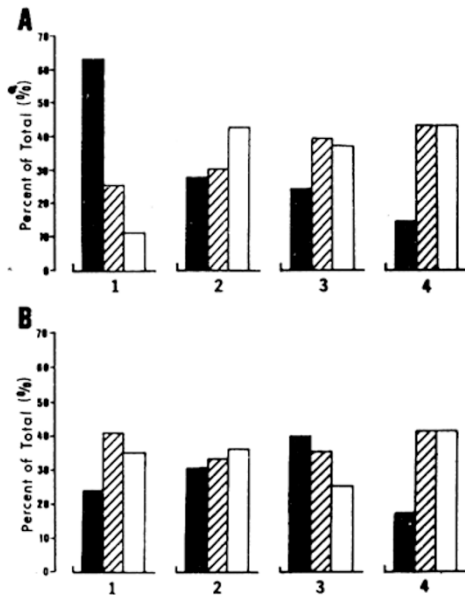
### 1. Retinal strip cultures.

After fixation, cultures were examined with an inverted microscope (Zeiss IM 35) and phase contrast optics. Ten longest neurites were measured from each retinal strip and their average length was used as an indicator of growth potential of each retinal strip cultured under different conditions.

### 2. Retinal strips and PN cultures.

The average length of neurite growth was obtained for each retinal strip as described above. In addition, the growth pattern of all neurites situated between the open end of glass tube and 4 mm of the retinal strips was analysed (Fig. 2). The 4 mm segment of retina was divided into 4 zones (Fig. 2, 3).

In order to quantify the effect of PN on growth pattern of neurites, the following method was designed to define their growth pattern. The exit point of a neurite from retinal strip was noted. Then, its growing tip was located and a perpendicular line was drawn from the tip of the neurite to intersect retinal strip at another point. If the distance between these two points was less than 250  $\mu\text{m}$ , the neurite was defined to grow in a straight mode. If this distance



**Fig. 3** Histograms demonstrating the percent of three neurite growth pattern of each zone in retinal strip co-cultured with PN. The experimental paradigm is illustrated in Fig. 2.

A, Neurite growth pattern on F side: B, Neurite growth pattern on B side. Black bars, positive curved neurites, strap bars, straight neurites. Open bars, negative curved neurites. Further details, see Tables 1 and 2.

was more than  $250 \mu\text{m}$  and the neurite was curving towards glass tube, it was defined as growing towards PN (positive curved neurite). If this distance was more than  $250 \mu\text{m}$  and the neurite was curving away from glass tube, it was defined as growing away from PN (negative curved neurite). The data from each mm zone of retinal strip (on both sides of the strips, Fig. 2) were summed up in Tables 1, and 2. The percentage of positive and negative curved and straight neurites, was calculated for each mm zone (Table 1, 2, and Fig. 3).

**Table 1.** Adult retinal strips co-cultured with a segment of predegenerated PN. Analysis of neurite growth pattern was performed on F side of retinal strips.

Zone	Distance to glass tube ( $\times 100 \mu\text{m}$ )	Positive curved neurites	Straight neurites	Negative curved neurites	Total number of neurites
1	0 - 10	117(62.6%)	48(25.7%)	22(11.7%)	187
2	10 - 20	43(27.9%)	46(29.9%)	65(42.2%)	154
3	20 - 30	13(24.1%)	21(38.9%)	20(37%)	54
4	30 - 40	3(14.2%)	9(42.9%)	9(42.9%)	21

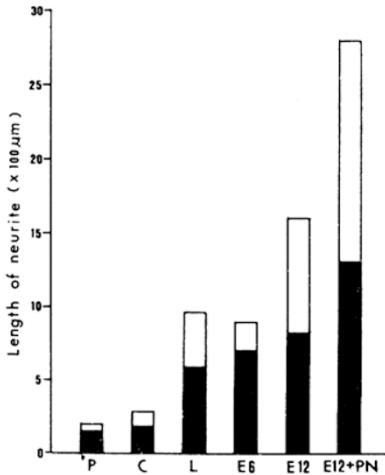
**Table 2.** Adult retinal strips co-cultured with a segment of predegenerated PN. Analysis of neurite growth pattern was performed on B side of retinal strips

Zone	Distance to glass tube ( $\times 100 \mu\text{m}$ )	Positive curved neurites	Straight neurites	Negative curved neurites	Total number of neurites
1	0 - 10	33(23.9%)	56(40.6%)	49(35.5%)	138
2	10 - 20	34(30.6%)	37(33.3%)	40(36.1%)	111
3	20 - 30	27(39.7%)	24(35.3%)	17(25%)	68
4	30 - 40	2(16.6%)	5(41.7%)	5(41.7%)	12

## RESULTS

### Effect of different substrates on neurite outgrowth for retinal explants

Number of neurite growing out from adult retinal explants was small, irrespective of the substrate used. However, length of neurite growth from adult hamster retina varied according to the substrate used (Fig. 4). An average neurite outgrowth length of 155  $\mu\text{m}$  was obtained when poly-L-lysine was used as substrate. The situation was similar for collagen which demonstrated an average neurite outgrowth length of 188  $\mu\text{m}$ . Neurites grew markedly better on laminin and reached an average outgrowth length of 582  $\mu\text{m}$ . However, neurite seemed to grow even better on retinal BM from E6 and E12 chicken. With average outgrowth length of 690 and 823  $\mu\text{m}$  respectively.



**Fig. 4** Histogram showing the length of neurite from retinal strips growing on different substrates. Shaded areas represent average neurite length obtained from all the experiments using a particular substrate. Enclosed white areas denote length of the longest neurite growing on each substrate. P, poly-L-lysine; C, collagen; L, laminin; E6 and E12, BM from E6 and E12 chick embryos; E12 + PN, BM from E12 chick embryo with PN in culture.

### *The origin of neurites in vitro*

After 4 days in cultures, many HRP labelled cell bodies were observed in the retinal strips obtained from animals in which HRP had been administered to the superior colliculus. These cell bodies were found in retinal ganglion cell layer. More importantly, HRP reaction products could be seen in the initial portion of some outgrowth neurites (not shown).

### *Effect of PN on neurite outgrowth*

Since E12 BM seemed to be the best substrate for neurite outgrowth from adult retinal explants, it was used for experiments on the effects of PN on neurite growth in cultures.

### *Length of neurite outgrowth.*

There seems to be difference in the length of neurite outgrowth in retinal strip

culture with or without PN after 5 days in cultures (Fig. 4). The average length of neurite in retinal strip cultures alone was 823  $\mu\text{m}$  whereas in retinal explants and PN co-cultures, the average neurite length was 1305  $\mu\text{m}$ . This increase of 58.6% suggests that the presence of PN enhanced the growth potential of neurites.

### **Number of neurites.**

In retinal explants and PN co-cultures, neurite number in different zones of retinal strip increased gradually as they approached the opening of glass tube (Table 1 and 2). This was true on both sides of the retinal strip suggesting an enhancement effect of PN on the number of growing neurites

### **Direction of neurite outgrowth.**

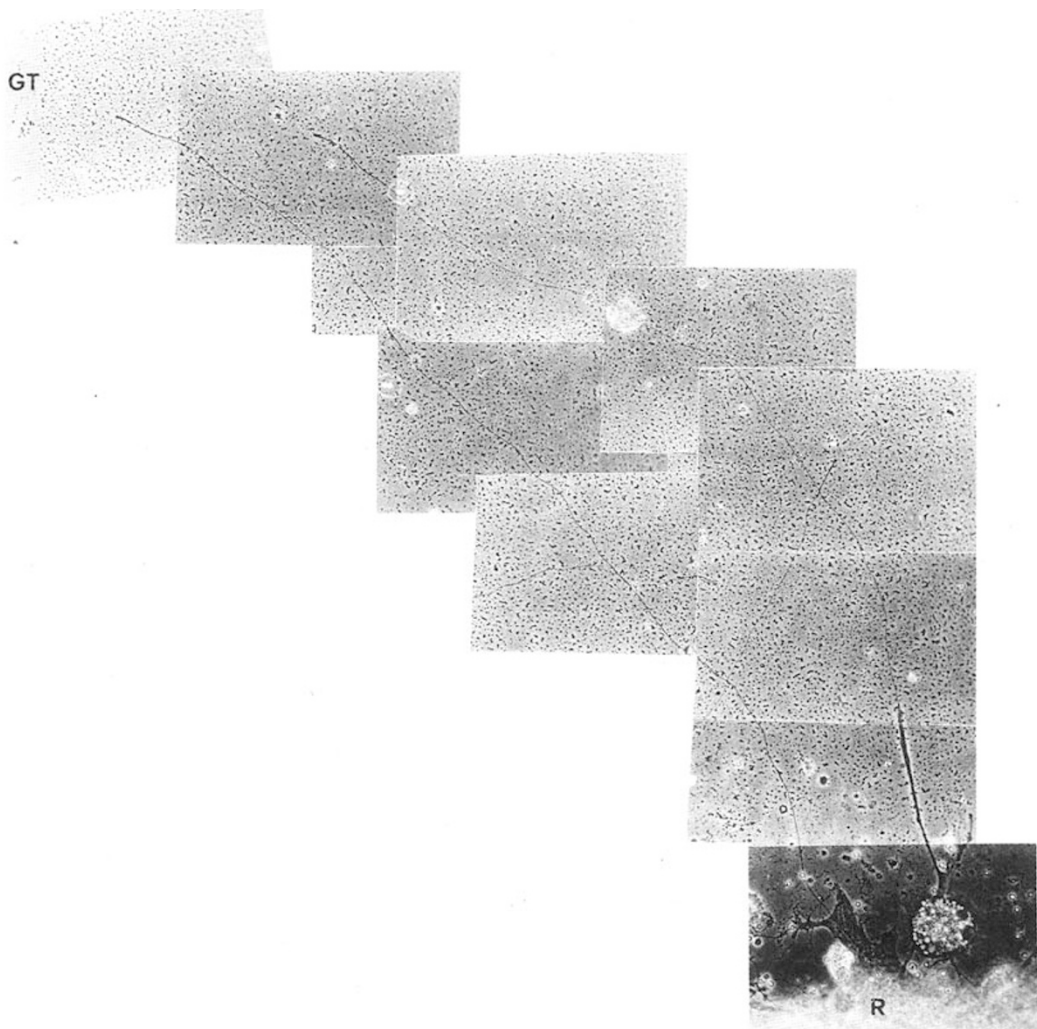
40 retinal strips were cultured on 20 E12 BM. 416 neurites on the F side and 329 on the B side were classified as being straight, positive or negative curved neurites as defined in Materials and Methods' section. Results indicated that the percentage of positive curved neurites was substantially higher in zone 1 on F side, but not on B side (Table 1 and 2, Fig. 4). Two neurites growing towards the glass tubes (positive curved neurites) are illustrated in Fig. 5. Out of 187 axons analysed in zone 1 on experimental F side, 117 of them curved towards PN, 48 were straight, and only 22 curved away from PN (Table 1). There was no obvious difference in the direction of neurite growth on B side, or in the remaining zones on F side. The maximum distance between PN and neurites as they emerged from zone 1 on F side, was about 2.5-2.8mm, thus 2.8 mm seems to be the limiting distance beyond which the direction of neurite growth was not affected by PN. No neurite has been shown to grow into the glass tube and to contact PN.

In most cultures, no cell was observed to migrate from PN, via glass tube, onto BM. But a few cells were seen in a few cultures. However, these cells were situated immediately adjacent to the mouth of glass tube and they did not in contact with any neurites.

## **DISCUSSION**

Neurite outgrowth was observed on various substrates, although there were differences in growth potential of neurites growing on different substrates. Neurite outgrowth on collagen was slightly better than that on poly-L-lysine, but laminin turned out to be a much better substrate than collagen or poly-L-lysine. The fact that laminin is an effective substrate for enhancing neurite outgrowth from retina has been shown in retinal cultures from fetal mice[7] or adult rat[8]. Laminin is an important component in BM[9] and its presence was demonstrated on E6 and E12 (Liu and So, unpublished results) and on E5 to E8[6] chick retinal BM with immunocytochemical method. This might explain why chick retinal BM was best substrate for neurite outgrowth from adult hamster retinas. That BM is a better





**Fig. 5** A photomontage showing two neurites growing towards the mouth of glass tube  
Phase contrast microscopy. Scale: 100  $\mu$ m.  
GT, glass tube; R, retinal strip.

substrate than laminin might be due to the fact that in addition to laminin, other neurite promoting substances such as heparan sulfate, proteoglycan might present in chick retinal BM. These neurite promoting substances on BM, in contrast to laminin which was plated on a culture dish, are more likely to be in their normal biological configuration, thus providing a more favorable environment for neurite growth.

Our results suggest that the growth pattern of neurites from adult hamster retinal explants can be modified by soluble factors secreted by the segments of pre-degenerated sciatic nerve cultured in the same dish. The presence of PN seems



also increase the length and number of neurite outgrowth, however, the effect decreases with their distance from PN. Thus, more outgrowing neurites were observed in areas (of retinal explant) close to PN. In addition, the neurites within about 2.8 mm from PN were attracted to grow towards the PN while those situated outside this zone did not seem to be affected.

Although we conclude that PN secretes diffusible substances *in vitro* to attract regenerating axons, we have to consider the possibility that in *in vivo* situation Schwann or other cell types might migrate out of PN onto retina to guide damaged axons to grow towards the nerve. In fact, in a couple of experiments, in which retinal strip were co-cultured with PN which was not put into a glass tube, we could observe a lot of cells migrating out of the nerve to intermingle with neurites growing out from retinal explants. Nevertheless, since no contact between neurites and cellular components from PN was observed in our experiments with the nerve cultured inside a glass tube, we suggest that the diffusible substances secreted by PN seem to be sufficient to alter the growth of neurites from adult hamster retinal neurons.

Various cellular components in injured PN could be sources of trophic factors. Schwann cells are likely to be an important contributor since they multiply after PN is injured[10], and it has been shown recently that conditioned medium from Schwann cells in culture seems to stimulate neurite outgrowth[11]. Other trophic factors have also been shown to be released from PN in *in vitro* experiments [12, 13]. These factors seem to enhance neurite outgrowth of dorsal root ganglion neurons from embryonic rats [13]. In addition, Varon and his colleagues have also illustrated neurite-promoting activity in fluid collected from chambers surrounding regenerating rat sciatic nerve[14]. Results from our experiments suggest that the trophic factors released from segments of PNs are also able to enhance the number and length and to influence the direction of neurite outgrowth from retinal explants of adult hamsters. Although we did not positively identify the source of all these neurites, we suppose that they are from retinal ganglion cells. Since the retinal ganglion cell layer of retinal explants in culture was in direct contact with substrate, the axotomized retinal ganglion cells, in comparison to the other retinal neurons, should have a much higher chance to grow neurites on the substrate. Indeed, by applying HRP to the superior colliculus in 3 animals, we have been able to show that many retinal ganglion cells survive in our culture system and HRP reaction products have also been observed in initial portion in some of the growing neurites. It has been shown from other peripheral nerve grafting experiments that diffusible factors have a 'rescue' effect in the retinal ganglion cell layer[15] and reduce the effects of axotomy on damaged retinal ganglion cells[16] in adult rats. We do not know what these trophic factors are at the moment. They can be nerve growth factor-like substances or other growth related substances[12, 13, 17, 18, 19, 20]. Of particular interest is the recent work showing that fibroblast growth

factors (FGF) seems to promote the survival of axotomized retinal ganglion cells of adult rat[21]. Thus, it will be of interest to determine if FGF is also being released by injured peripheral nerve.

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