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Pore formation by an outer membrane protein of the cyanobacterium *Anabaena variabilis*

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A cell-envelope fraction was isolated from cell walls of *Anabaena variabilis* after cell breakage and removal of thylakoid and plasmalemma membranes by repeated sodium dodecyl sulfate (SDS) washing. Following lysozyme and detergent treatment of the cell walls, a yellow to orange extract was obtained, which was subjected to gel filtration. Fractions with an apparent molecular weight between 40 000 and 80 000 showed a strong, pore-forming activity in lipid bilayer membranes from diphytanoylphosphatidylcholine/*n*-decane. The pores were not homogeneous in size presumably because of aggregate formation of the pore-forming protein. The single conductance unit had an average pore conductance of 3.5 nS in 1 M KCl. Further information was obtained by determining the single-channel conductance for various ions differing in size, and from zero-current membrane potential measurements. The pore was found to be permeable to large ions ($Tris^+$, $Hepes^-$), and a pore diameter of 1.6 nm could be estimated from the single-channel conductance. At neutral pH, the pore was about 2-times more permeable for potassium than for chloride ions, probably due to an excess of negative charges in or near the pore. Transmembrane potentials of 50 mV and larger caused a decrease of the pore conductance to substates which had about half the conductance of the pores at low voltages.

Introduction

The cell envelope of Gram-negative bacteria consists of three distinct layers: the outer membrane, the peptidoglycan layer and the inner membrane [1]. The inner membrane represents a real diffusion barrier. It contains the respiratory chain and a large number of transport systems for substrates. The outer membrane acts as a molecular filter with defined exclusion limits for hydrophilic substances [2]. These molecular-sieving properties result from integral outer membrane proteins, called porins [3], which form large water-filled channels through the outer membrane. The exclusion limit of these transmembrane channels is dependent on the type of Gram-negative bacteria and ranges between 600 for *Escherichia coli* [3] and 6000 for *Pseudomonas aeruginosa* [4]. Purified porins can be reconstituted in lipid vesicles [3,4] and artificial bilayer membranes [5]. In both systems, they form pores with diameters between 1.1 and 2.2 nm.

Whereas the structure of the cell wall of enteric bacteria like *E. coli* and *Salmonella typhimurium* is very well understood at present, very little is known about the structure and the permeability properties of the cell envelope of another important class of Gram-negative bacteria, the cyanobacteria. Cyanobacteria contain a photosynthetic system

Abbreviations: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

which is very similar to that of the chloroplasts [6] and they are very likely the ancestors of these photosynthetic cell organelles. On the other hand, some cyanobacteria like our *Anabaena variabilis* strain can also grow photoheterotrophically [7] which indicates that large molecules can pass the outer membrane. Recently, proteins of different molecular weights have been found in the outer membrane of the cyanobacterium *Anacystis nidulans* [8], but so far there does not exist any information on the function of these proteins.

In this paper, we show that proteins isolated from the cell wall of the cyanobacterium *A. variabilis* have pore-forming activity in lipid bilayer membranes. The pores have a single channel conductance of about 0.36 nS in 0.1 M KCl and they are moderately cation-selective. The pores of the outer membrane of *A. variabilis* show a strong similarity to pores of other Gram-negative bacteria. On the other hand, they appear to be voltage-dependent, which has not been observed in our experiments with porins from enteric bacteria like *E. coli* [9] and *S. typhimurium* [10], but has been found in reconstitution experiments with pore-forming protein from the outer membranes of chloroplasts and mitochondria [11,12].

Materials and Methods

Cultivation of algae. Anabaena variabilis Kütz (American Type Culture Collection 29413) was grown in an inorganic medium at 30°C as described [13]. After 48 h, the cyanobacteria were harvested at a chlorophyll concentration of approx. 20 μg/ml by centrifugation at 200 × *g* for 5 min. For one porin preparation we used algae equivalent to a total amount of 20–25 mg chlorophyll. The filaments were washed three times in 80 ml of a buffer (buffer A) consisting of 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH 7.8) by centrifugation at 200 × *g* and resuspension to remove possible bacterial contaminations. Thereafter, the pellet was suspended in an equal volume of buffer A frozen in liquid nitrogen and stored at −30°C.

Preparation of the porin-containing extract. Whole filaments were mixed with an equal volume of glass beads (0.5 mm diameter) and shaken for 5 min at 4°C in a cell homogenizer (Vibrogen Zellmühle). The resulting cell homogenate was filtered through a glas suction filter to remove glass beads and centrifuged for 30 min at 48000 × *g*. The pellet was suspended in 80 ml buffer A and centrifuged again. This procedure was repeated. The pellet was then washed three times in 80 ml 50 mM Tris-HCl (pH 7.8) to remove phycobilisomes and other soluble protein components. The resulting green pellet was diluted to a chlorophyll concentration of 0.5 mg/ml by 50 mM Tris-HCl (pH 7.8) containing 0.1% SDS (final concentrations). The solution was incubated in the dark on ice for 10 min and centrifuged for 20 min at 48000 × *g*. The green supernatant containing solubilized thylakoid and cytoplasmic membranes was discarded, the pellet was resuspended in the same volume of the Tris-SDS buffer. The whole procedure was repeated four times. The resulting greyish pellet containing only cell walls was then suspended in 20 ml phosphate buffer (pH 7.0) containing 10 mM EDTA (buffer B). After centrifugation, the pellet was finally suspended in 2 ml buffer B containing lysozyme (1 mg/ml, grade VI, Sigma). The suspension was incubated under stirring at 30°C for 2 h. Then, Genapol X-80 was added to a final concentration of 1% and the sample was sonicated for 5 min in an ultrasonic cleaning bath (Sonorex PK 102, 240 W. Bandelin, Berlin). Genapol X-80 was a gift from Farbwerke Hoechst, Frankfurt, F.R.G. The mixture was centrifuged for 20 min at 50000 × *g* and the orange to yellow supernatant was frozen in liquid N_2 and stored at −30°C.

The pore-forming activity of the different supernatants obtained throughout the preparation of the protein extract was monitored in lipid bilayer experiments (see below). Pore-forming activity was only observed after lysozyme/EDTA treatment of the cell wall fraction. Subsequent mild sonification and the addition of detergent increased this activity.

HPLC. 200 μl of the yellow supernatant was applied to a TSK-G 3000 SW column (7.5 × 600 mm, LKB) equilibrated with a buffer (pH 7.2) containing in mM: Hepes/NaOH, 20; NaCl, 50; EDTA, 5; NaN_3, 3, and 1% Genapol X-80. 1-ml fractions were collected at a flow-rate of 0.1 ml/min. Colourless fractions with porin activity were eluted shortly after the exclusion volume of the column; they were pooled and subjected to the

measurements described below. Further biochemical analysis of these active fractions are currently being investigated.

Black lipid bilayer experiments. Optically black lipid bilayer membranes were formed as described previously [9]. The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole (area 0.1–2 mm^2). Membranes were formed across the hole by painting on a solution of 1–2% (w/v) diphytanoylphosphatidylcholine (Avanti Biochemicals, Birmingham, AL) dissolved in *n*-decane (Fluka, Buchs, Switzerland). The aqueous salt solutions (Merck, Darmstadt, F.R.G.) were unbuffered and had a pH between 5.5 and 6. The results were virtually pH-independent in the range of pH 5–7. The protein was added from a concentrated stock solution either to the aqueous phase bathing a membrane in the black state or prior to membrane formation. The temperature was kept at 25°C throughout.

Membrane conductance measurements were performed using a pair of Ag/AgCl electrodes switched in series with a voltage source and a current amplifier (Keithley 427). The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. Zero current measurements were performed using a pair of calomel electrodes with salt bridges as described earlier [14].

Results

When certain fractions obtained by gel filtration of a cell wall/outer membrane extract (see Materials and Methods) were added in small quantities to the aqueous solutions on both sides of a black lipid bilayer membrane, the membrane current started to increase in a stepwise fashion (Fig. 1). The occurrence of these conductance steps was specific to the fractions 20–25 which correspond to an apparent molecular weight of about 40 000 to 80 000. We observed many different conductance steps probably due to aggregate formation [15,16]. They were obviously multiples of a single conductance unit (Fig. 1) because we were not able to see intermediate steps within the time resolution of our instrumentation of 0.5 ms. The occurrence of these multiple steps is also seen in a histogram of conductance fluctuations observed in 1 M KCl solution (Fig. 2). Peaks were observed at 3.5, 7 and 10 nS. This result suggests the existence of dimers and trimers of the single conductance unit. Surprisingly, dimers of the single conduc-

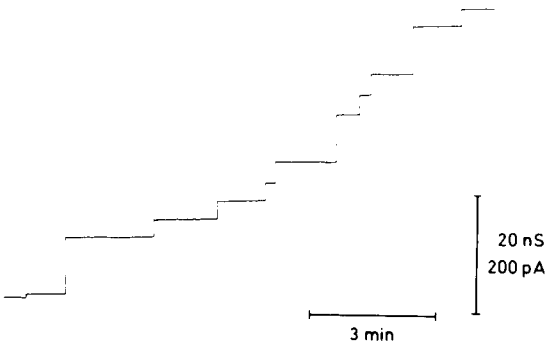

Fig. 1. Stepwise increase of the membrane current after the addition of fraction 22 (see Materials and Methods) to the aqueous phase bathing a black membrane from diphytanoylphosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl, 10 μg/ml Genapol X-80 and 5 μl fraction 22. The applied voltage was 10 mV.

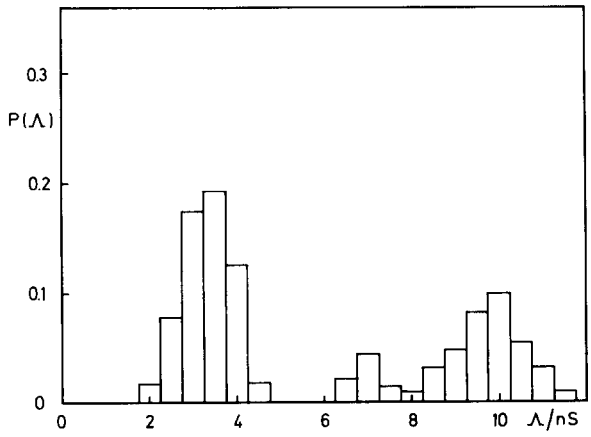

Fig. 2. Histogram of conductance fluctuation observed with fraction 22 of outer membrane of *A. variabilis*. The aqueous phase contained 1 M KCl, 10 μg/ml Genapol X-80 and 5–10 μl fraction 22. The applied voltage was 10 mV. Note the broad distribution of the histogram. The peak on the left side corresponds to an average single-channel conductance of 3.5 nS whereas the peak on the right side corresponds to that of about 9 nS. The total number of steps was 338.

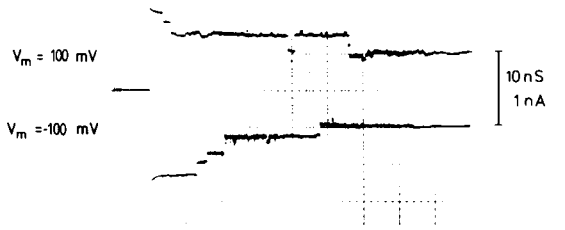

Fig. 3. Decrease of the membrane current after application of +100 mV and −100 mV (as referred to the side of the addition of fraction 22). The membrane was made of diphytanoylphosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl, 10 μg/ml Genapol X-80 and 10 μl HPLC-fraction 22; $T = 25°C$.

tance step were only rarely observed. On the other hand, it seems rather unlikely that the three maxima in Fig. 2 reflect different pores because of the uniform voltage dependence (see below). In this respect, we like to note that in some extracts a small pore with a single-channel conductance of about 200 pS in 1 M KCl was observed. This pore could not be investigated in detail because of the existence of the 10-times larger pore. The increase in membrane conductance was observed irrespective of whether the pore-forming protein was added to the aqueous solution on one side of the membrane only or to both sides. Most current fluctuations were directed upwards. Terminating events were only rarely observed at low transmembrane potentials on the order of 5 to 40 mV. From records extending over prolonged periods at these voltages, the average lifetime of the conductive unit was estimated to be at least several minutes. The lifetime of the pores was found to be independent of the salt and protein concentration in the aqueous phase.

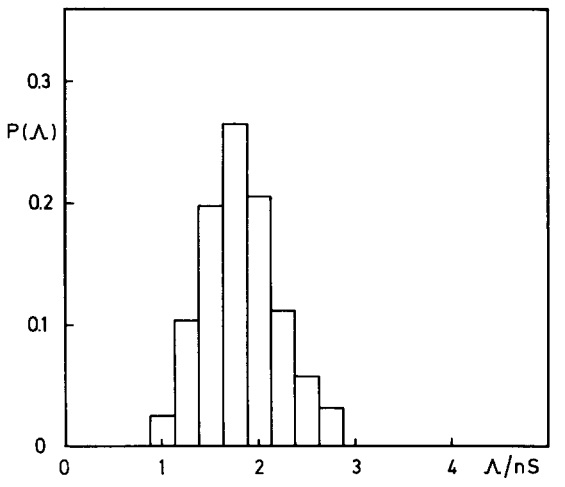

Fig. 4. Histogram of the terminating steps observed at 100 mV transmembrane potential. The membranes were made of diphytanoylphosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl, 10 μg/ml Genapol X-80 and 10 μl fraction 22; $T = 25°C$. The histogram was derived from 124 individual steps with an average single-channel conductance of 1.7 nS.

TABLE I

AVERAGE SINGLE-CHANNEL CONDUCTANCE

Average single-channel conductance $\bar{\Lambda}$ was measured on membranes from diphytanoylphosphatidylcholine/*n*-decane in the presence of the pore-forming protein from *A. variabilis* outer membrane. The pH of the aqueous salt solution was around 6 if not otherwise indicated; $T = 25°C$; $V_m = 10$ mV. $\bar{\Lambda}$ was determined by recording a large number *n* of conductance steps. Note that $\bar{\Lambda}$ corresponds always to the smaller peak in the histograms (compare Fig. 2).

Salt	Concn. (M)	$\bar{\Lambda}$ (nS)	$\bar{\Lambda}/\sigma$ (cm) ($\times 10^8$)	*n*
KCl	0.01	0.038	2.7	47
	0.1	0.36	2.8	83
	1.0	3.5	3.1	338
NaCl	1.0	1.8	2.1	93
LiCl	1.0	1.4	2.0	38
$CaCl_2$	0.5	1.3	1.7	57
Na^+ Hepes$^-$ (pH 8)	0.5	0.30	1.7	33
$Tris^+$ Cl^-	0.5	0.58	1.9	51
$Tris^+$ Hepes$^-$ (pH 8)	0.5	0.10	1.4	29

The increase of the transmembrane potential to values above 50 mV led to a decrease of the membrane conductance in discrete steps if several pores were incorporated into the membrane. Fig. 3 shows such an experiment. After incorporation of pores, the voltage was switched to 100 mV (upper trace) and to −100 mV (lower trace). In both cases, the membrane current was reduced in discrete steps in a more or less symmetric fashion. A histogram of these steps is given in Fig. 4 for a transmembrane potential of 100 mV. It is obvious that the average unit of these voltage-dependent terminating steps is smaller than that of the on-steps at low voltages (compare Fig. 2). There are two possible explanations for this. (i) The on-steps of smallest size could still be oligomeric pores,

where the single units switch off separately at larger voltages. (ii) The increased membrane potential could result in a reduction of the pore size to substates, which we consider to be more likely.

Single channel measurements were performed with a variety of different electrolytes and different concentrations at a membrane potential of 10 mV. From records similar to those given in Fig. 1, the average single channel conductance $\overline{\Lambda}$ was obtained by measuring a large number of individual events. The results are summarized in Table I which contains also the ratio between the average single channel conductance, $\overline{\Lambda}$, and the specific conductance, σ, of the corresponding aqueous salt solution. The values for $\overline{\Lambda}$ correspond to the smaller peak in the histogram. $\overline{\Lambda}/\sigma$ varies for salts containing small cations or anions only between $1.7 \cdot 10^{-8}$ cm and $3.1 \cdot 10^{-8}$ cm by less than a factor of 2 despite variations of $\overline{\Lambda}$ (and of σ) by a factor of 80. A comparison of the single channel conductances for KCl, NaCl and LiCl with the limiting molar conductivities of K^+, Na^+ and Li^+ (73.5, 50.1 and 38.7 Scm^2/mol, respectively [26]) shows that the single channel conductance follows the mobility sequence of the cations in the aqueous phase. This indicates that the pore formed by the protein of the outer membrane of *A. variabilis* could be cation-selective. On the other hand, it is also evident from the data given in Table I that large organic ions such as $Tris^+$ or $Hepes^-$ permeate through the pore showing little or no interaction with the pore interior because $\overline{\Lambda}/\sigma$ is only slightly smaller for $Tris^+$ or $Hepes^-$ than for KCl or NaCl. This finding clearly argues against the possibility that the pore of the outer membrane of *A. variabilis* consists of a bundle of small pores.

Zero-current membrane potentials

The conclusions drawn from the single channel experiments are supported by measurements of zero-current membrane potentials V_m in the presence of a salt gradient across the membranes. In these experiments, a certain amount of the active fractions was added to a 10 mM solution of either KCl or LiCl. The amount was adjusted in such a way that the conductance of membranes from diphytanoylphosphatidylcholine/*n*-decane increased within 20 min about 100-fold above the specific conductance of undoped membranes (10 nS/cm^2). Then the salt concentration on one side of the membranes was raised by adding small amounts of concentrated salt solution while stirring. The diluted side (10 mM) became positive in all experiments with KCl due to preferential movement of the potassium ion. It became negative in the presence of LiCl due to the preferential movement of the anion. The potential reached its final value 5–10 min after the salt gradient was established.

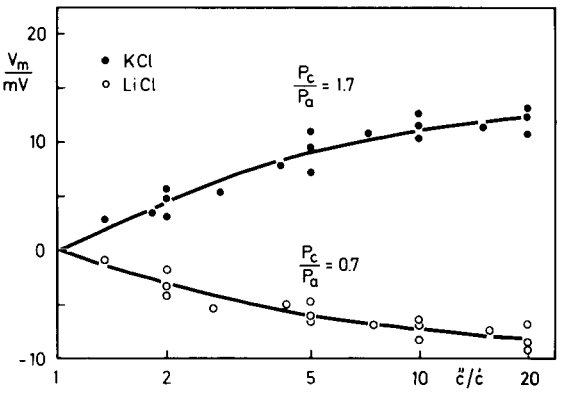

Fig. 5. Zero current membrane potentials V_m as a function of the ratio c''/c' of the salt concentrations on both sides of the membrane. c' was fixed at 10^{-2} M KCl, or LiCl and c'' was varied between 10^{-2} M and 0.2 M. In addition, the aqueous phase contained 20 μg/ml Genapol X-80 and 20 μl HPLC-fraction 22; $T = 25°C$. The membranes were made of diphytanoylphosphatidylcholine/*n*-decane. V_m was positive on the dilute side for KCl and negative on the same side for LiCl. The lines were drawn according to the Goldman-Hodgkin-Katz equation [14] with $P_{K^+}/P_{Cl^-} = 1.7$ and $P_{Li^+}/P_{Cl^+} = 0.7$.

The observed values of the zero current membrane potentials for KCl and LiCl are given in Fig. 5. The experimental results were fitted to the Goldman-Hodgkin-Katz equation [14], which gave a reasonable fit of the experimental data assuming a ratio of the permeability P_c (cation) and P_a (anion) of 1.7 for KCl and 0.7 for LiCl. The monovalent cation K^+ has obviously a somewhat higher permeability through the pore than the counterion chloride. The inverse situation for the less mobile Li^+ shows on the other hand that ions have in the pore a similar mobility as in the aqueous phase. The slight cation selectivity in the presence of KCl (where cation and anion have approximately the same mobility in the aqueous

phase) may result from an excess of negatively charged groups in or near the pore. In this respect, it is interesting to note that most porins from Gram-negative bacteria from also slightly cation selective pores in lipid bilayer membranes [17]. Chemical modification of carboxyl groups in the case of the OmpF-porin has clearly shown that the cation selectivity is in this case caused by negatively charged groups in or near the pore [18].

Discussion

The experiments described here show that certain fractions derived from the cell envelope of *A. variabilis* are able to form pores in lipid bilayer experiments. These pores show a striking similarity to the pores of the outer membrane of *E. coli* [9], *S. typhimurium* [10] and *P. aeruginosa* [19]. The cyanobacterial porin is able to increase the membrane conductance by many orders of magnitude in discrete steps. This result indicates that the single conductance unit is a channel. The average single channel conductance $\overline{\Lambda}$ of a porin pore of *A. variabilis* outer membrane is about 3.5 nS in 1 M KCl. If we assume that the pore is a hollow cylinder with spherical cross-sections and filled with an aqueous solution of the same specific conductance, σ, than the bulk aqueous phase then the average pore diameter $d\ (=2r)$ may be calculated for a certain length l of the pore according to:

$$\overline{\Lambda} = \sigma\pi r^2/l \qquad (1)$$

Assuming a pore length l of 7.5 nm (corresponding to the average thickness of an outer membrane [2,20]) the pore diameter is calculated to be about 1.7 nm ($\sigma = 112\ \text{mS} \cdot \text{cm}^{-1}$). This value for the diameter of the pore is a little larger than found for the porin pores of *E. coli* [9] and *S. typhimurium* [10]. The exclusion limit for the diffusion of hydrophilic substrates through these pores is between 600 (*E. coli* [15]) and 800 (*S. typhimurium* [3]). Considering this and the dependence of molecular weight of globular molecules on the third power of the radii of the molecules [21], we would expect an exclusion limit around M_r 2000 for the outer membrane of *A. variabilis*.

The single-channel conductance of the porin pore from *A. variabilis* is closely related to the bulk aqueous conductance of the salt solutions (compare Table I and the selectivity measurements). This result is consistent with the notion that this porin forms wide water-filled channels in the outer membrane, which has also been observed for most but not all porins from Gram-negative bacteria [17]. Such channels should have a poor cation-to-anion discrimination and a low selectivity for small ions. In agreement with this, the pore of *A. variabilis* has a small cation selectivity for KCl as a substrate. Because of the same aqueous mobility of both ions, this may be caused by an excess of negatively charged groups in or near the pore. Furthermore, the current vs. voltage character of a wide channel with a limited selectivity should be ohmic as was in fact observed.

The lifetime of the single channels was found to be voltage-dependent. The pore switches to substates if the transmembrane potential exceeds values of about 50 mV. This can be concluded from the difference of the conductance levels of the opening of the pores at low voltage and the off-steps at high voltages. On the other hand, we cannot exclude that the on-steps at low voltages reflect the opening of a bundle of pores which inactivate separately at high voltages. So far it is not clear if the closing of the pores at large transmembrane potential has any physiological meaning. The conductance of the pores is extremely large and their selectivity is so poor that ionic gradients across the outer membrane of *A. variabilis* cannot exist for a long time [10]. On the other hand, gram-negative bacteria are presumably the ancestors of mitochondria and chloroplasts [27]. The outer membrane of both cell organelles contains voltage-dependent pores, which have most probably to do with their metabolic regulation [11,12]. The observation that the porin pore of a cyanobacteria is voltage-dependent may support this relationship although its physiological meaning remains obscure.

The structure of porin pores from enteric bacteria has been studied in great detail in the past [22,23]. the active unit consists of trimers of identical subunits and contains most probably only one pore with three openings faced to the outside [23]. There exists only very limited information on the structure and the composition of the cell wall of

cyanobacteria. To our knowledge, this is the first information that their outer membranes contain porin-like proteins. The aggregation state of pore-forming protein in the conductive units is completely unknown. It could also be arranged as trimers. The results of the single channel measurements (compare Fig. 2) would suggest in this case, however, that a monomer contains in contrast to the situation in *E. coli* a complete pore, i.e., a trimer would contain three independent pores. It is interesting to note that such an arrangement has recently been reported for porin pores from *Rhodopseudomonase sphaeroides* [28].

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