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Published in:
Journal of Bacteriology

Publication date:
1988

JOURNAL OF BACTERIOLOGY, July 1988, p. 3194–3198
0021-9193/88/073194-05$02.00/0

Vol. 170, No. 7

Effect of Cholesterol on the Branched-Chain Amino Acid Transport System of *Streptococcus cremoris*

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Received 19 January 1988/Accepted 27 March 1988

The effect of cholesterol on the activity of the branched-chain amino acid transport system of *Streptococcus cremoris* was studied in membrane vesicles of *S. cremoris* fused with liposomes made of egg yolk phosphatidylcholine, soybean phosphatidylethanolamine, and various amounts of cholesterol. Cholesterol reduced both counterflow and proton motive force-driven leucine transport. Kinetic analysis of proton motive force-driven leucine uptake revealed that the V_{max} decreased with an increasing cholesterol/phospholipid ratio while the K_t remained unchanged. The leucine transport activity decreased with the membrane fluidity, as determined by steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated into the fused membranes, suggesting that the membrane fluidity controls the activity of the branched-chain amino acid carrier.

Studies of the relation between the physical properties of membranes and the activity of membrane-associated enzymes are of fundamental importance for understanding the organization of membrane structure. Three general types of protein-lipid interaction can be distinguished: (i) specific binding of lipids, which results in the modulation of enzyme activity by allosteric interactions; (ii) interactions in the hydrophobic core of the membrane, i.e., solvation effects by the lipid acyl chains providing a proper hydrophobic environment for optimum conformation and orientation of the protein; and (iii) interactions at the polar-apolar interface of the membrane, i.e., the polar phospholipid groups providing a suitable microenvironment between the hydrophilic and hydrophobic interface of the membrane for an optimum conformation of the protein. Changes in membrane protein activity caused by the in situ modification of bilayer features, such as the fluidity, are usually explained by one or more of these three types of interactions.

The role of membrane fluidity in the modulation of enzyme activity has received considerable attention (11, 18, 21, 27, 29). Bacterial membranes usually remain in the liquid-crystalline state under a variety of environmental conditions (21, 29). The proportion of saturated and long-chain fatty acids incorporated into phospholipids is increased when the cells are grown at elevated temperatures, such that the membrane viscosity ($\tilde{\eta}$) is maintained at a constant value independent of the growth temperature (21, 31). Several models have been proposed which describe the relation between membrane viscosity and solute transport activity (17, 32). However, studies of the role of membrane fluidity in bacterial transport in cells have not yielded evidence for any of these models (for a review, see reference 20). In a few systems, translocation rates indeed increase with increasing membrane fluidity. Usually this dependence is relatively small, whereas many transport systems appear to be almost insensitive to changes in membrane fluidity within the liquid-crystalline phase. Reconstitution of a transport system into liposomes with a defined lipid composition offers a very amendable experimental system for studies of the role of membrane fluidity. Such studies have been done with the eucaryotic transport system for glucose of erythrocytes (1, 3), which has been reconstituted into liposomes with a defined lipid acyl chain composition and cholesterol content. These studies have allowed a much clearer analysis of the effects of membrane composition on the functional properties of these proteins than was possible in in vitro experiments with altered native membranes. Recently, we have reported on the phospholipid polar group requirement of the branched-chain amino acid transport system of *Streptococcus cremoris* (9). A membrane fusion technique was used by which this transport protein could be studied in fused membranes with a defined phospholipid composition. Using a similar approach, we have studied the effect of cholesterol on both counterflow and proton motive force-driven uptake of leucine by fused membranes containing a mixture of soybean phosphatidylethanolamine (PE) and egg yolk phosphatidylcholine (PC) (3/1, mol/mol). Manipulation of the cholesterol content of the membrane has often been used to modulate the fluidity of the membrane (18, 20). Cholesterol increases the order of fatty acyl chains in the liquid-crystalline phase but decreases this order in the gel phase (5). It should be emphasized that none of these components (i.e., PE, PC, and cholesterol) are native components of the streptococcal membrane (9). Therefore, a physiological function of cholesterol seems to be unlikely. The effect of cholesterol on the membrane fluidity was estimated from the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). The possible significance of membrane fluidity with respect to leucine transport is discussed.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]leucine (12.4 TBq/mol) was purchased from Amersham Corp. (Buckinghamshire, England). Octadecyl rhodamine-β-chloride was obtained from Molecular Probes, Inc. (Junction City, Oreg.). DPH, egg yolk PC, and crude soybean PC (type II-S) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Soybean PE was isolated from crude soybean PC as described previously (16).

Organism, growth conditions, and isolation of membrane vesicles. *S. cremoris* Wg2 (Prt$^-$) was grown on MRS broth (4a) with 0.5% (wt/vol) lactose and converted into membrane vesicles by osmotic lysis (24).

Preparation of liposomes and fusion of liposomes with *S. cremoris* membrane vesicles. A mixture of soybean PE and

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egg yolk PC (3/1, mol/mol) with various amounts of cholesterol in chloroform was dried under a stream of N_2. A constant quantity of phospholipid and varying quantities of cholesterol were used. Trace amounts of solvent were removed under vacuum for 1 h, and the dried lipid film was suspended in 50 mM potassium phosphate (pH 6.0) to a final phospholipid concentration of 20 mg/ml. Liposomes were prepared by sonication with a probe sonicator (MSE Scientific Instruments, West Sussex, United Kingdom) at an intensity of 2 μm (peak to peak) for 5 min at 4°C under an N_2 atmosphere. Burst cycles of 15-s and 45-s rest were used. Liposomes were mixed with *S. cremoris* membrane vesicles at a protein-to-phospholipid ratio (wt/wt) of 1/10, and fusion was induced by rapidly freezing the suspension in liquid N_2 (9). Prior to use, the frozen membrane suspension was slowly thawed at room temperature and subsequently sonicated at 4°C for 8 s with the probe sonicator.

Fusion assay. The fusion efficiency was determined with the octadecyl rhodamine-β-chloride (R_{18}) fusion assay (15) essentially as described previously (9). Membrane vesicles of *S. cremoris* labeled with 4 mol% (total phospholipid) R_{18} were fused with nonlabeled liposomes, and the R_{18} fluorescence (excitation, 560 nm; emission, 590 nm) was determined before and after the addition of 1% (vol/vol) Triton X-100. The maximal level of probe dilution in the target membranes was determined as described previously (9).

Transport assays. Leucine counterflow and Δp-driven leucine transport were assayed as described previously (9). Uptake experiments were performed at 28°C.

Membrane fluidity assay. DPH polarization measurements were carried out essentially as described previously (30). For DPH labeling of the fused membranes, a small sample of DPH stock solution (4 mM in the solvent dimethyl sulfoxide) was diluted into 20 mM potassium phosphate (pH 6.0)–100 mM potassium acetate and vigorously vortexed. Samples were taken from this suspension and added to an equal volume of fused membrane suspension, yielding a DPH-to-lipid molar ratio of about 1/250. The mixture was slowly shaken at room temperature for 15 min, and incubation was continued for 1 h in the presence of 2 nmol of valinomycin per mg of protein. Labeled membranes were concentrated by centrifugation (9), and the steady-state DPH fluorescence polarization was measured with a Perkin-Elmer 204 spectrophotometer with the cuvette holder thermostated by circulating water at 28°C. The steady-state anisotropy (r_{ss}) was calculated according to the equation

$$r_{ss} = (I_{para} - I_{perp})/(I_{para} + 2I_{perp})$$

where I_{para} and I_{perp} are the corrected fluorescence intensities parallel and perpendicular to the emitted light measured at 430 nm with an excitation wavelength at 360 nm (28, 30). This relative index of microviscosity ($\bar{\eta}$) (28) was determined with the equation

$$\left(\frac{r_o}{r_{ss}} - 1\right)^{-1}$$

In this expression, r_o is the limiting value of the steady-state fluorescence anisotropy (r_o = 0.362) (28).

Other analytical procedures. The internal volume of the fused membranes was estimated from the amount of entrapped calcein (8, 9, 23). Membranes were fused in the presence of 100 μM calcein, and the calcein fluorescence (excitation, 480 nm; emission, 520 nm) was determined before and after the addition of 100 μM $CoCl_2$. The residual fluorescence after the addition of 1% (vol/vol) Triton X-100 was subtracted from these recordings. Filter entrapment efficiency was estimated from recovery of the trapped amount of calcein by the fused membranes after filtration. The extent to which the fused membranes were retained by the 0.45-μm-pore-size cellulose nitrate filters (Millipore Corp., Bedford, Mass.) used in the transport assay was calculated from the total volume of calcein, the volume trapped by the fused membranes, and the volume that went through the filter. The filter entrapment efficiency is defined as $(V_b - V_a)/V_b$, where V_b and V_a denote the internal volume of the fused membrane suspension before and after filtration, respectively. Calcein fluorescence measurements were performed as described above. The electrical potential across the membrane (Δψ, interior negative) was determined from the distribution across the membrane of the lipophilic cation tetraphenylphosphonium by using a tetraphenylphosphonium-selective electrode as described previously (6). Protein was assayed by the method of Lowry in the presence of 0.5% (wt/vol) sodium dodecyl sulfate (10). Bovine serum albumin was used as a standard.

TABLE 1. Effect of cholesterol on the fusion efficiency and general properties of membrane vesicles of *S. cremoris* fused with PE-PC (3/1, mol/mol) liposomes

Cholesterol/PE-PC molar ratio[a]	Protein recovery (%)	Internal vol (μl/mg of protein)	Filter entrapment efficiency (%)	Fusion efficiency (%)	ΔΨ (mV)
0	66.7	14.2	83.6	82.9	−110
0.05	70.6	13.2	87.8	84.5	−113
0.1	68.7	13.5	85.0	74.4	−106
0.2	63.7	14.0	83.9	81.1	−106

[a] PE/PC ratio of 3/1 (mol/mol).

RESULTS

Effect of cholesterol on the fusion efficiency and characteristics of the fused membranes. The effect of the cholesterol content of the liposomes containing a mixture of soybean PE and egg yolk PC (3/1, mol/mol) on the fusion efficiency was studied with the R_{18} fusion assay (9, 15). For this purpose, *S. cremoris* membrane vesicles were labeled with 4 mol% (total phospholipid) R_{18} and fused with nonlabeled liposomes. The enhancement of R_{18} fluorescence as a result of probe dilution was recorded. At all cholesterol concentrations, a high extent of membrane fusion was observed (Table 1).

The fused membranes were further examined with respect to a number of general characteristics of these membranes. Approximately 65 to 70% of the *S. cremoris* membrane vesicle protein was recovered in the pellet after centrifugation of the fused membranes, irrespective of the cholesterol content of the liposomes (Table 1). The internal volume of the fused membranes was estimated from the entrapped amount of the fluorophore calcein. Virtually identical values were found with the different preparations, indicating that cholesterol had no effect on the internal volume. The extent to which the fused membranes were retained by the 0.45-μm-pore-size cellulose nitrate filters (Millipore) used in the transport assay was calculated from the total volume of calcein, the volume trapped by the fused membranes, and the volume that went through the filter. With all fused membrane preparations, a filter entrapment efficiency of approximately 85% was found (Table 1). To exclude the possibility that the effects of cholesterol are due to an alteration in the ion permeability of the membrane, the

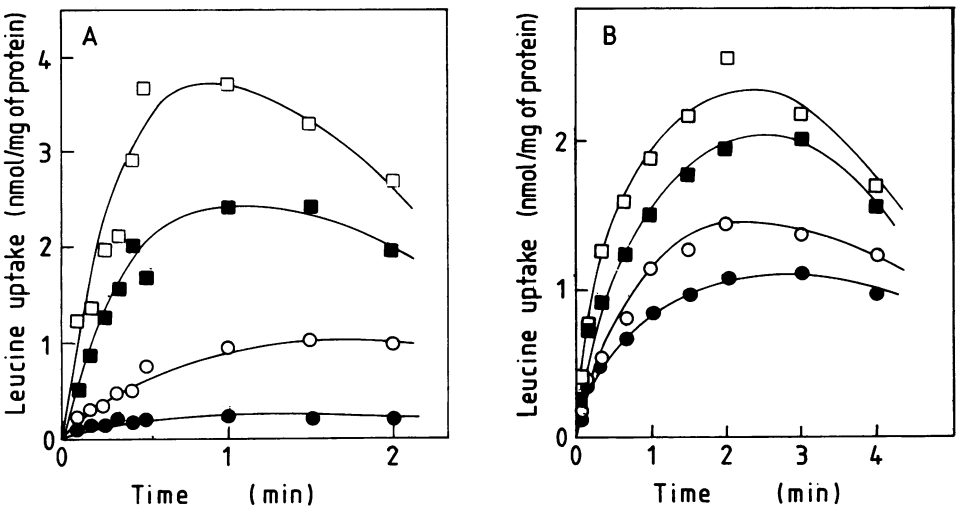

FIG. 1. Δp-driven leucine transport (A) and counterflow (B) by membrane vesicles of *S. cremoris* fused with PE-PC (3/1, mol/mol) liposomes containing various amounts of cholesterol. Fused membranes had a cholesterol/PE-PC molar ratio of 0 (□); 0.05 (■); 0.1 (○); and 0.2 (●). A fixed amount of phospholipid was used, and the amount of cholesterol was varied. The final [^{14}C]leucine concentrations during Δp-driven leucine transport and counterflow were 1.5 and 20 μM, respectively.

ability of the fused membranes to maintain an imposed electrical membrane potential (Δψ) was determined. A Δψ with a theoretical value of −120 mV was imposed by the use of an outwardly directed potassium diffusion gradient in the presence of valinomycin, and the magnitude of the generated Δψ was estimated from the distribution across the membrane of the lipophilic cation tetraphenylphosphonium ion as measured with an ion-selective electrode (6). Hardly any effect of cholesterol on the Δψ was observed (Table 1). Cholesterol clearly has little effect on any of these parameters. Furthermore, with all the different lipid mixtures, closed membrane structures were obtained upon fusion.

Effect of cholesterol on leucine uptake. The effect of the cholesterol on the activity of the branched-chain amino acid transport system was investigated by assaying counterflow and Δp-driven uptake of leucine. In contrast to Δp-driven leucine transport, the translocation cycle of counterflow does not involve a deprotonation and protonation step (7). Under those conditions, the carrier merely catalyzes an exchange reaction between internal and external leucine. A progressive decrease in both Δp-driven uptake (Fig. 1A) and counterflow (Fig. 1B) of leucine was observed when membrane vesicles were fused with PE-PC (3/1, mol/mol) liposomes containing increasing amounts of cholesterol. For kinetic analysis of Δp-driven leucine uptake, initial rates were determined from the uptake values obtained after a 10-s incubation. A concentration range of 0.75 to 20 μM leucine was used, and results were analyzed by Eady-Hofstee plots. As shown in Table 2, with increasing cholesterol content of the membrane, a reduction of the V_{max} of leucine transport is observed whereas the K_t remains unaffected (Table 2).

To test the possibility that cholesterol either excludes the leucine carrier during the fusion step or irreversibly inactivates the transport system, fused membranes containing 20 mol% cholesterol were re-fused with liposomes containing no cholesterol. This treatment reduced the cholesterol/PE-PC molar ratio from 0.2 to 0.1 and resulted in a reactivation of leucine transport (Fig. 2A). As a control, raising the cholesterol/PE-PC molar ratio from 0 to 0.1, by re-fusing fused membranes containing no cholesterol with liposomes containing 20 mol% cholesterol, led in a similar way to depression of leucine transport activity (Fig. 2B). These results demonstrate that the activity of the leucine carrier is reversibly influenced by the cholesterol content of a membrane with a fixed phospholipid composition.

Effect of cholesterol on membrane fluidity. The effect of cholesterol on membrane fluidity was assessed by the steady-state fluorescence polarization of DPH incorporated in the membrane. In agreement with previous observations (2), an increase in DPH anisotropy was observed with increasing cholesterol content (Table 2), indicating that cholesterol effectively reduced the motional flexibility and increased the order of fatty acyl chain. Consequently, a reduction of the membrane fluidity at the temperature at which the transport assays was performed was obtained by increasing the cholesterol content in the membrane.

Relation between membrane fluidity and leucine uptake. To analyze whether the activity of the branched-chain amino acid carrier depended on the viscosity of the membrane, the V_{max} of Δp-driven leucine transport and the reciprocal of the relative index of microviscosity of the membrane were compared (Fig. 3). The results suggest that the initial rate of Δp-driven leucine uptake is a function of the microviscosity of the membrane.

DISCUSSION

The results presented in this paper suggest that the activity of the branched-chain amino acid transport system is mod-

TABLE 2. Kinetic parameters of Δp-driven leucine transport and the anisotropy of DPH fluorescence by membrane vesicles of *S. cremoris* fused with PE-PC liposomes containing various amounts of cholesterol

Cholesterol/PE-PC molar ratio[a]	Δp-driven leucine transport		DPH anisotropy [r_{ss}] (10^2)
	V_{max} (nmol/min × mg of protein)	K_t (μM)	
0	40.8	4.7	10.33
0.05	28.5	4.4	11.40
0.1	19.2	4.5	12.43
0.2	10.8	4.2	13.97

[a] PE/PC ratio of 3/1 (mol/mol).

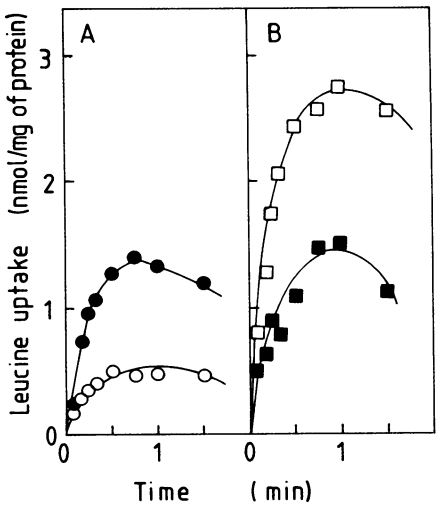

FIG. 2. Reversible activation and inactivation of Δp-driven leucine transport in fused membranes composed of mixtures of PE, PC, and cholesterol. (A) Reactivation of leucine transport by re-fusing fused membranes containing cholesterol/PE-PC at a molar ratio of 0.2 (○) with an equivalent amount of PE/PC liposomes, yielding a final cholesterol/PE-PC molar ratio of 0.1 (●). (B) Same as in A, except that membranes previously fused with PE/PC liposomes (□) were re-fused with an equivalent amount of cholesterol/PE-PC (molar ratio, 0.2) liposomes, yielding a final cholesterol/PE-PC molar ratio of 0.1 (■). A constant PE/PC ratio of 3:1 (mol/mol) was used.

ulated in a reversible manner by the fluidity of the membrane. Kotyk and Janacek (17) proposed that the rate of carrier-mediated solute transport across the membrane is proportional to the (rotational and/or lateral) diffusion constant of the protein in the membrane and, thus, inversely proportional to $\bar{\eta}$. They assumed that mechanical movement of solute translocation across the membrane is rate limited by the fluidity of the membrane. The results presented in Fig. 3 are consistent with this model. Since Δp-driven leucine transport and counterflow are both affected (Fig. 1A and B), it is likely that at least the reorientation step of the carrier-H^+-leucine complex (7) is sensitive to the fluidity of the membrane. The introduction of cholesterol, however, has a more pronounced effect on Δp-driven leucine transport than on leucine counterflow, suggesting that the membrane fluidity acts primarily on H^+-coupled leucine translocation. It should be noted that DPH fluorescence polarization measurements can be used only as a relative index of fluidity. For the estimation of the absolute values of $\bar{\eta}$, more information is required about the motional parameters of DPH, information which might be gained by the use of time-resolved techniques (19, 26).

Other effects of cholesterol on the transport system, effects unrelated to membrane fluidity, should be considered as well. Cholesterol can occupy substitutional sites for phospholipids at the lipid-protein interface (19) and might, therefore, affect the conformation of the protein and, thus, the transport activity. Streptococci completely lack cholesterol or any other steroid (9) (G. In 't Veld and A. J. M. Driessen, unpublished results), which makes such a specific interaction unlikely. Cholesterol might also reduce the effective surface charge density of the phospholipid bilayer by increasing the distance between the charged phospholipid polar groups (27). In some systems, cholesterol also increases the hydration of the phospholipid bilayer surface (27). Cholesterol does not affect the binding of the surface probe 1-anilinonaphthalene-8-sulphonate (ANS) to fused membranes containing a fixed ratio of PE to PC (T. Zheng, unpublished results). Although the interpretation of results obtained with ANS as a probe of membrane polarity has been the subject of some controversy (26), it appears that the effect of cholesterol cannot be attributed to an alteration in the surface properties of the phospholipid bilayer. On the other hand, cholesterol promotes the bilayer to hexagonal (H_{II})-phase transition of lipid mixtures containing unsaturated PE, such as soybean PE (4, 12). Without calcium-chelating or -precipitating agents present in the intraliposomal space, the sarcoplasmic Ca^{2+}-ATPase demonstrates a strict requirement for lipids with a cone-shaped polar group, e.g., unsaturated PEs and monogalactosyl diglyceride for Ca^{2+} transport (13, 14, 22). Cholesterol promotes the Ca^{2+} transport activity of the Ca^{2+}-ATPase when reconstituted in liposomes of a phospholipid composition similar to that used in this study, e.g., mixtures of egg yolk PC with soybean PE, containing high levels of unsaturated phospholipid acyl chains (2). This effect was correlated with a bilayer to H_{II}-phase transition of the lipid. However, for the branched-chain amino acid transport system of *S. cremoris*, there is no absolute requirement for cone-shaped lipids (9). High rates of leucine transport were observed with fused membranes containing either phosphatidylserine, PE, or various glycolipids, including mono- and digalactosyl diglycerides. Although the incorporation of cholesterol in soybean PE-containing membranes may give rise to formation of H_{II} phases, activity is clearly not related to this ability of cholesterol.

In this paper, we have shown that cholesterol efficiently reduces leucine transport activity, an effect which is paralleled by a relative increase in membrane viscosity. We have

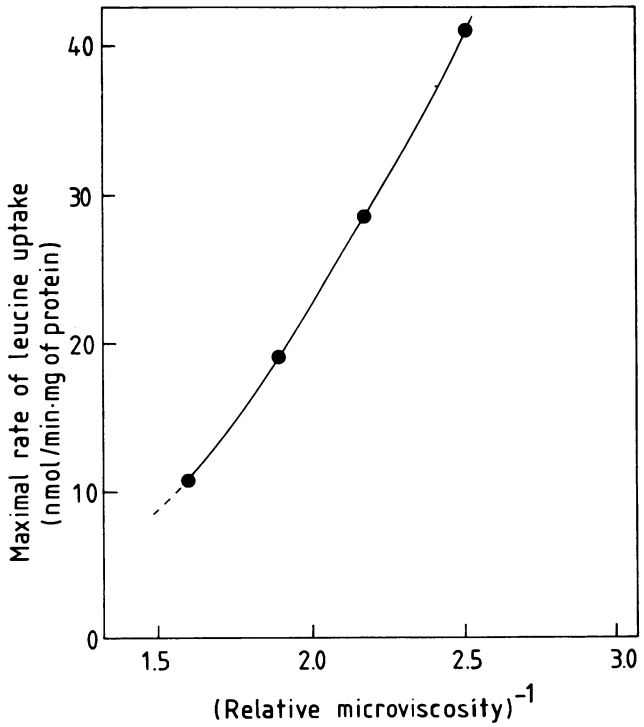

FIG. 3. Relation between the maximal rate of leucine transport (V_{max}) and the reciprocal of the relative index of lipid microviscosity calculated from the steady-state fluorescence polarization of DPH.

used cholesterol as a modulator of membrane fluidity in membrane vesicles of *S. cremoris* fused with liposomes of soybean PE and egg yolk PC. For further analysis of the role of membrane fluidity in branched-chain amino acid transport, studies should be performed with fused membranes containing phospholipids with various acyl chain lengths and degrees of saturation.

ACKNOWLEDGMENTS

This study has been made possible by the Stichting voor Biofysica with financial support from the Netherlands Organization for Advancement of Pure Scientific Research. One of us (T.Z.) is a postdoctoral fellow supported by the Dutch Government in the context of a scientific collaboration with the People's Republic of China.

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