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SUGAR TRANSPORT IN BEEF ERYTHROCYTES

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SUMMARY

1. The entry of sugar into beef erythrocytes was found to be facilitated by a saturable, passive process with unusually high affinity for D-glucose and D-3-*O*-methylglucose, the Michaelis constants for these two sugars being 0.09 and 0.17 mM respectively. In other mammalian cells, the transport K_m is about the same for these two sugars, and ranges between 6 and 15 mM. The transport V was about 0.1–0.2 μmole/min per ml for both sugars, slow by comparison to most other mammalian cells.

2. The beef erythrocyte differed from the human cell but resembled the rabbit cell in that extracellular methyl glucose did not affect efflux, and intracellular methylglucose appeared not to affect influx. However, the inhibitory effects of glucose on methylglucose entry were stronger if the glucose was added some time prior to the addition of methylglucose. Since intracellular methyl glucose did not inhibit influx, the time dependent inhibition by glucose was likely due to glucose metabolism rather than intracellular glucose *per se*. Other kinetic criteria affirmed this conclusion.

INTRODUCTION

Since the pioneering work of LeFevre[1], Widdas[2], and of Wilbrandt *et al.*[3] a considerable body of evidence has accumulated leading to the view that glucose enters erythrocytes[1–7], muscle cells[8], adipocytes[9], hepatocytes[10] and the brain parenchyma[11] by combining with a mobile carrier in the respective limiting membranes. Nevertheless, from studies with human erythrocytes, certain authors[12–14] have challenged the carrier concept while others[15–20] have argued for carrier models more elaborate than the simplest envisioned by Widdas[21] and Rosenberg and Wilbrandt[22]. It has been suggested, for example, that the formation of the sugar–carrier complex and its breakdown may require the action of an enzyme[15] or that the carrier may have two or more interacting sugar-binding sites[16,20] or that free and loaded carriers have different mobilities[17,18].

The present report describes a series of studies of sugar transport in beef erythrocytes. The results indicate facilitation by a mobile carrier with unusually high affinity for glucose and almost as high affinity for methylglucose. Although glucose inhibited 3-*O*-methylglucose influx in a manner not predicted by the simple mobile carrier model, the results did not support any of the alternatives cited above.

METHODS

Beef blood was collected in 0.2 vol. of citrate buffer (13.2 g trisodium citrate, 4.4 g citric acid, 13.2 g dextrose per l) and kept at 0°. On the same day 300 ml of this was filtered through glass wool, centrifuged at 6000 × *g* for 15–20 min and the supernatant and white layer aspirated. The cells were washed 3 times in isotonic NaCl (room temperature) once in the incubation buffer, and then 90 ml of the loosely packed cells were suspended with 160 ml of the buffer. The latter was prepared by mixing the following isotonic solutions (0.3 osmole/l) in the indicated volumes: 228 ml NaCl, 4 ml KCl, 4 ml $CaCl_2$, 2 ml $MgSO_4$, 4 ml $NaPO_4$ buffer, pH 7.3, 8 ml potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer, pH 7.3 (Calbiochem). Bovine serum albumin (Fraction V, Nutritional Biochem. Corp.) was present at 0.2 %.

Portions of the suspension (20 or 25 ml) were shaken gently at 37° in loosely capped 50-ml erlenmeyer flasks. In the studies of sugar entry, various amounts of ^{14}C-labeled D-3-*O*-methylglucose (International Chemical and Nuclear Corp.), D-[3-^{3}H]allose (Amersham/Searle) and/or D-glucose were added in volumes of 50 or 100 μl at the times indicated in the figures and tables. In studies of sugar exit, ^{14}C-labeled 3-*O*-methylglucose was allowed to equilibrate as in entry experiments. Two 10-ml protions of this suspension were centrifuged, the medium was aspirated, and the cells resuspended in 26 ml of new medium.

To measure the intracellular contents of the sugars, 5-ml samples were (at times indicated with figures and tables) mixed thoroughly with 35 ml ice-cold saline. Ice-cold isotonic saline containing 7 % bovine serum albumin was layered under the suspension. These samples were centrifuged 5 min at 6000 × *g* in a 3° cold room. Samples of supernatant near the top were saved for glucose analysis. The remaining supernatant was aspirated through half the albumin layer. The walls were washed with 15 ml cold saline and all the supernatant above the pellet aspirated. The cells were then lysed by addition of 8 ml distilled water. 1 ml of 5 M $HClO_4$ was added and the mixture shaken vigorously. After centrifugation, 5 ml of extract was taken with a cotton-tipped pipet and transferred to another test tube where 0.43 ml of 3 M K_2CO_3 was added. The above analytical steps were carried out in the cold. At this point the neutral extracts were mixed and allowed to warm at room temperature until further mixing caused no further CO_2 evolution. The extracts were then cooled in ice for 15 min, centrifuged and the supernatants analyzed for ^{3}H, ^{14}C and glucose. The two isotopes were measured in the Tri-Carb liquid scintillation spectrometer (1 ml extract in 10 ml Bray's solution). Glucose was measured by the hexokinase method[23] using a Gilford spectrophotometer adapted to 3-cm cuvets.

To measure the ^{3}H and ^{14}C in the whole suspension of cells, 1-ml samples were taken at any time after the isotope additions. These were treated with 8 ml distilled water and 1 ml 5 M $HClO_4$ and were further worked up as described above for the acid extracts of the cell pellets. A 1-ml portion of the cell suspension with no isotope additions was similarly worked up and used in the preparation of the ^{3}H and ^{14}C counting standards as well as the counting blank.

RESULTS

Most of the experiments reported here were repeated at least four times on separate days with separate collections of erythrocytes. Typical results are shown.

The theoretical basis for interpreting these experiments has been developed in previous communications[7,11].

In the figures and text, the following symbols will be used: S = D-3-*O*-methylglucose, A = D-allose, G = D-glucose, W = volume, t = time in min, U = rate of entry, influx or efflux, V = max. velocity, $K = K_m$, $F = V/K_m$, and k = apparent flux constant. The subscripts have the following meanings: $_o$ = outside, $_i$ = inside, $_j$ = total, $_s$ = methylglucose, $_a$ = allose, $_g$ = glucose, $_+$ = inward, $_-$ = outward. Thus, $[S_o]/U_{s+}$ = (concentration of methylglucose outside)/(methylglucose influx rate). Concentrations are in nmoles/ml and rates in nmoles/min per ml intracellular water (W_i judged from equilibrium intracellular methylglucose space.)

Concentration dependence of methylglucose entry

The kind of rate law governing methylglucose entry can be shown by a plot of initial $[S_o]/U_s$ *vs.* $[S_o]$. In such a plot, (1) simple diffusion gives a horizontal line, (2) classical Michaelis saturation gives a straight line with a positive slope, (3) a combination of the two (or two saturable processes in parallel with differing K_s or anticooperative interaction among sugar binding sites) gives a curved line, steepest near the ordinate with diminishing slope at high concentrations and (4) cooperativity gives a line with least positive slope (even a negative slope) near the ordinate and increasing slope at higher concentration. While in the latter three cases a simple and complete interpretation depends on measurements of initial rates (where the equations are simple), the general form of the curve may be preserved in measurements taken somewhat later.

Fig. 1 shows plots of $[S_o]/U_s +$ *vs* $[S_o]$ under several conditions. The three lines intercepting the ordinate near 1.5 min and formed by closed symbols show data obtained by adding various concentration of labeled methylglucose to cell suspension otherwise free of sugar. Each line is representative of a group (4–6) of experiments performed as a series, several months intervening between each series. The three plots (as well as others in this figure) are slightly curved as described under Case (3) above. Since several interpretations of the curvatures are possible, no sophisticated curve resolutions will be made (*cf.* ref. 7). The ordinate intercepts and the slopes near the intercepts are related almost purely to the high affinity, saturable process responsible for the majority of sugar entry. The poorly saturable process is of little consequence when sugar concentration are less than 100 nmoles/ml. The equation for this portion of the curve is: $[S_o]/U_{s+} = 1/F_s + [S_o] \cdot 1/V_{so}$ (see Eqn. 29 of ref. 7). The intercepts ($1/F_s$) place F_s near 0.66 min^{-1}. The initial slopes ($1/V_{so}$) place V_{so} near 110 nmoles/min per ml. Since $F_s = V_{so}/K_{so}$, then $K_{so} = V_{so}/F_s = 170$ nmoles/ml. This sugar carrier, therefore, binds methylglucose about 30 times more tightly than does that of the rabbit erythrocyte.[7] The other data of Fig. 1 will be presented later.

Paradoxical inhibition of methylglucose entry by glucose

Fig. 2 shows that the inhibitory effects of glucose on methylglucose entry are dependent on the duration of exposure to glucose prior to addition of methylglucose. For this comparison, the half-closed circles are the controls for the open circles; the half closed squares are the controls for the open squares. The open triangles had no simultaneous controls, but agree with the other open symbols

reasonably well. The equation for competitive inhibition is: $[S_o]/U_{s+} = 1/F_s + [S_o]. 1/V_{so} + [G_o]\cdot 1/(F_s\cdot K_{go})$, (see Eqn. 33′ of ref. 11, dropping all terms with S_1 and G_1). When there was no prior exposure (half-closed symbols) glucose inhibited with an apparent K_{go} slightly less than 100 nmoles/ml. When glucose was present prior to methylglucose (open symbols), the inhibition was greater and the curves did not extrapolate to the zero glucose point. Since only minute amounts of methylglucose were used in this experiment, the ordinate intercept is $1/F_s$. Thus, prior exposure to glucose seems to have diminished F_s. A lower F_s means that either K_{so} was increased or V_{so} was diminished.

To determine which of these modulations occurred, several methylglucose concentrations were tested in the presence of glucose added simultaneously or present before methylglucose (Fig. 1). Comparing the half closed circles with the closed circles, we see that glucose, 200 nmoles/ml, added with methylglucose

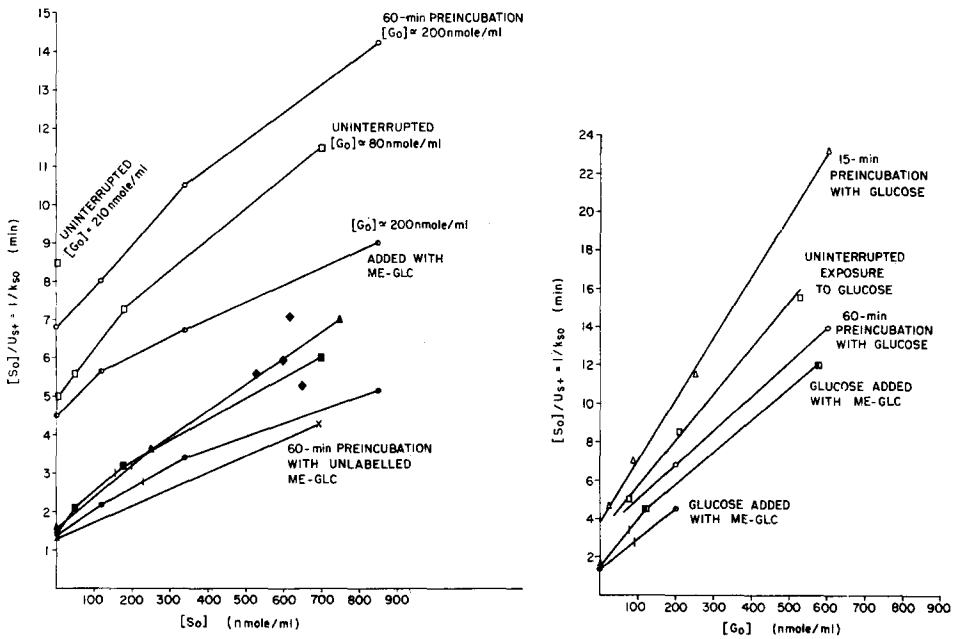

Fig. 1. Concentration dependence of methyl glucose entry in absence and presence of glucose. Samples for measurement of entry were taken 1 min after the addition of radioactive methylglucose to the cell suspensions. Samples were also taken after 60 min to determine the equilibrium content. $[S_o]/U_{s+}$ was taken to be the reciprocal of the apparent influx constant, k_{so} (see Eqn. *a* of ref. 11) obtained in the equation: $k_{so} = (-\ln(1-(S_1/S_1)/(S_{1\infty}/S_1)))\cdot(S_{1\infty}/S_1)\cdot(W_o/W_1)/t$. This calculation is very precise when $[S_o]$ is below k_{so}, but tends to overestimate $[S_o]/U_{s+}$ when $[S_o]$ is high. Since the curves were concave downward, the error of this calculation was such as to make the curves less curved than they actually should have been. Three sets of experiments are shown: (1) ▲; (2) ■, □, ◆; and (3) ●, ◐, ○, ×. The experimental conditions were as follows: entry into cells without glucose (▲, ■, ●); entry into the cells metabolizing glucose in steady state (□, ○); entry into cells receiving glucose simultaneous with methylglucose (◐); influx into cells equilibrated with methylglucose; efflux from cells equilibrated with methylglucose (◆) as obtained in Fig. 3.

Fig. 2. Effect of glucose on methylglucose entry. Sampling and calculations were as described under Fig. 1. Three sets of experiments are shown: (1) △; (2) ◧, □; (3) ◐, ○. The half-closed symbols, ◧ and ◐, show effects of glucose added with methylglucose. The open symbols, △, □ and ○, show effects of glucose added prior to methylglucose.

increased $[S_o]/U_{s+}$ about the same at each $[S_o]$, a typical competitive inhibitory effect. However, about the same concentration of glucose remaining after one hour preincubation (open circles, initial $[G_o] = 300$ nmoles/ml) was a much more potent inhibitor at each $[S_o]$ and increased $[S_o]/U_{s+}$ more at higher $[S_o]$. Since slope is $1/V_{so}$, the data indicate that preincubation with glucose lowered V_{so}, *i.e.* exerted a non-competitive inhibition on methylglucose entry. The open squares are data obtained in experiments where glucose was never completely removed from the cells. Comparing those data with the closed squares, where no glucose was present, one sees that 80 nmoles/ml glucose was a very potent inhibitor and again increased $[S_o]/U_s$ more when $[S_o]$ was high.

The non-competitive inhibition could have been due to glucose inside the cell, if for some reason the loaded carrier were much less able to move from inside to outside than the empty carrier, while the movements of free and loaded carrier towards the inside were about equally facile. This circumstance would have another effect on the kinetics, making the efflux V much less than the influx V, *i.e.* $V_{gi} << V_{go}$, also $K_{gi} << K_{go}$ since $V_{gi}/K_{gi} = V_{go}/K_{go} = F_g$ (see Eqns. 21 and 30 of ref. 7. Make the following assignments in keeping with earlier findings and the modifications suggested above: $k_4 = k_{-4} = k_2 = 0.2$, $k_{-2} = 0.02$, $k_{-1} = 20$, $k_3 = 2$, $k_1 = k_{-3} = 0.1$. This will show by analogy how a low k_{-6} and low k_7 would affect K_{gi} and K_{go}.).

Alternatively, the non-competitive inhibition could have been due to the metabolism of glucose. For example, a participant of the phosphate cycle, a glycolytic intermediate, or lactic acid might bind the carrier in a non-competitive manner and affect its mobility.

Methylglucose efflux

It is difficult to study glucose efflux kinetics in order to test whether $V_{gi} << V_{go}$ and $K_{gi} << K_{go}$. However, if glucose-loaded carrier has great difficulty moving outward, the same should be true of methylglucose-loaded carrier. Fig. 3 shows methylglucose efflux from pre-loaded cells. The four tests were carried out on four separate days. In each experiment, efflux of labelled sugar into sugar-free medium was compared to efflux into a medium containing unlabelled sugar at the concentration inside the cells. It is seen that the external sugar did not affect efflux. The curves diverge only after the inside concentration fell in the cells incubating in sugar-free medium. Thus, the rate constants, k_{si}, obtained for the efflux into sugar-containing medium may be taken as the initial $U_{s-}/[S_i]$ in either flask. The reciprocals are displayed in Fig. 1 as the diamond symbols. They are seen to agree reasonably well with the line formed by the closed squares, which represents the entry experiments carried out with the efflux experiments. If $V_{si} << V_{so}$, then the diamonds would have been much higher than the closed squares. The evidence, therefore, favors the view that pre-incubation with glucose lowers V_{so} by a metabolic effect.

This conclusion is also supported by a measurement of methylglucose influx into cells equilibrated with unlabeled methylglucose (the X symbols in Fig. 1). This experiment was simultaneous with the closed-circle series and agrees rather well. Had internal methylglucose inhibited influx, the X symbol would have fallen above the closed circle line.

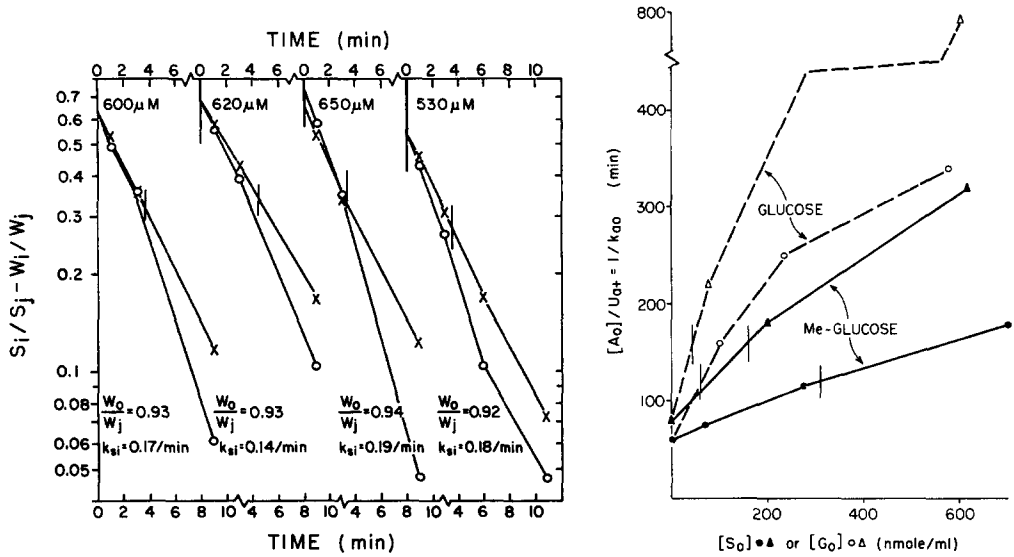

Fig. 3. Efflux of [^{14}C]methylglucose from cells incubating in sugar-free medium (O) and incubating in medium containing methylglucose at the inside concentration (×). The initial cellular methylglucose concentrations are shown at the top (530–650 nmoles/ml), for each of the four experiments. The data are given as the difference between S_i/S_j (the fraction of total labeled sugar remaining inside) and W_i/W_j (the fraction of total water which is inside), since it is this difference which falls exponentially towards zero (*i.e.* S_i/S_j approaches W_i/W_j). If all the label were inside, S_i/S_j would be 1; and $S_i/S_j - W_i/W = W_o/W_j$ is about 0.93. Thus, at the beginning of these experiments, about 70% (0.65/0.93) of the labeled sugar was inside; and the concentration of inside label was 220 times that outside (70%/0.07 ÷ 30%/0.93). The efflux constants (k_{si}, see Eqn. *a* of ref. 11) were obtained by the equation: $k_{si} = 0.693 \cdot (W_o/W_j)/t_{1/2}$. It is strictly applicable only when $[S_o] = [S_i]$, or when $[S_i] << K_{si}$, or when measurements are made early enough that influx is unimportant.

Fig. 4. Effects of methylglucose and glucose on allose influx. Methylglucose (▲, ●) was allowed to equilibrate for 60 min prior to addition of allose. Glucose was allowed 15 min (△) or 60 min (○) to achieve a steady state prior to addition of allose. Samples were taken 20 min after allose addition. Two series of experiments are shown: (1) ▲, △; (2) ●, ○. $[A_o]/U_{a+}$ was taken to be the reciprocal of the influx constant, k_{ao}, obtained in the equation: $k_{ao} = (-\ln(1-(A_i/A_j)/(S_{i\infty}/S_j))) \cdot (S_{i\infty}/S_j) \cdot (W_o/W_i)/t$. Under the conditions of the experiment, allose entry was approximately exponential.

Allose entry

Fig. 4 shows the effects of incubation with methylglucose (at equilibrium) or glucose (at steady state)on the entry of allose. Despite considerable discrepancy between the two series of experiments, the following comments seem warranted. The ordinate intercepts show allose to be a much poorer permeant than methylglucose ($F_a << F_g$). The inhibitory effects of methylglucose are about as expected from a K_g in the neighborhood of 200 nmoles/ml (this constant is strictly B_g as defined earlier[7]). Had internal methylglucose inhibited influx, the apparent K_g in this experiment would have been less than that obtained in the methylglucose entry experiments. This result, therefore, supports the conclusion of the previous section that internal sugar *per se* does not interfere with influx. The inhibitory effects of glucose on the other hand suggest a K_g of about 50 nmoles/ml, somewhat less than expected from the effects of glucose added simultaneously with

methylglucose in Fig. 2. Thus, glucose but not methylglucose is an exceptionally potent inhibitor when added prior to allose.

DISCUSSION

Judging from the above data, the entry of glucose into the beef erythrocyte is facilitated by a carrier of low activity (V about 1/2 that of the rabbit erythrocyte[7] and 1/2000 that of the human erythrocyte[2,5,18,19]). The beef erythrocyte transport is distinguished by its unusually high affinity for D-glucose (K_g is about 0.09 mM). This suggests an interesting problem of comparative biochemistry. It would be interesting to know whether other beef tissues show such a low transport K_m and, if so, the survival significance of this peculiarity. In the beef erythrocyte the affinity for methylglucose is only 1/2 that for glucose whereas in other mammalian cells the two sugars show similar transport kinetics[7,24]. Entry seems to occur also by a much less readily saturable process such as simple diffusion or by a mediated transport of low affinity. The latter is compatible with anticooperative interaction but is no argument for it. The data are definitely incompatible with cooperatively interacting sites as suggested by Wilbrandt and Kotyk[16] for human erythrocytes. The inability of sugar on one side of the membrane to influence the amount of free carrier on the other side (flux from the other side) indicates that the loaded and free carrier move with similar facility. We observed the same relations in the rabbit erythrocyte[7]. These relations are precisely the assumptions of the simple model of Widdas[2,21] and of Rosenberg and Wilbrandt[4,22]. By contrast, in the human erythrocyte external glucose enhances efflux, suggesting more facile movement of the loaded carrier[17–19]. However, Miller[19] suggested an alternate interpretation for the enhancement of efflux by outside sugar, namely that a diffusion barrier or gradient exists between the point of sugar–carrier dissociation and the bulk of the external medium. According to that interpretation, an effluxing sugar after dissociation at the outside interface, stands a good chance of reassociating owing to difficulty in diffusing away from the external interface. External glucose facilitates efflux by competing for that reassociation thus increasing the probability of diffusion of the effluxing sugar into the bulk external medium. This interpretation seems reasonable in view of the fact that no such anomalies occur in rabbit[7] and beef erythrocytes where carrier transport is slow but only in human erythrocytes where carrier transport is extremely fast. Presumably diffusion from the site of dissociation is no obstacle in the slowly transporting cells. To determine the kinetic significance of diffusion to and from the membrane in human erythrocytes, kinetic studies of the kind reported here and earlier[7,17–19] should be repeated with human erythrocytes in which glucose transport has been inhibited by a non-competitive, irreversible inhibitor such as *N*-ethylmaleimide. Naftalin[25] has recently studied this question by observing the effects of agitation rate, temperature and external sugars on the efflux of labeled sugar from human erythrocytes. He concluded that restricted diffusion away from the external surface is responsible for the acceleration of efflux by external sugar. However, it is not clear to what extent his noncarrier model of membrane penetration influences this conclusion.

The ability for glucose metabolism to diminish the transport V is reminiscent of a similar finding in rabbit erythrocytes[7], where the V for glucose transport seemed

to be less than that for methylglucose. It is tempting to speculate that this effect may be related to the Pasteur effect in muscle, wherein, respiration inhibits glucose transport[26]; for, glucose metabolism is to the erythrocyte what respiration is to the other cells. If so, then some use may be made of this phenomenon in working toward an understanding of transport regulation in more complex cells.

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Dr. Joseph Katz suggested the possible usefulness of D-[3-^{3}H]allose as a readily available, non-metabolized glucose analogue. He kindly provided a preparation and we have been addicted ever since[24].

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