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Genetics

Genetic alterations in potassium transport in L cells

(furosemide-sensitive K^+ transport/K^+ efflux/intracellular Na^+/cell volume/mutation)

J. J. GARGUS, I. L. MILLER, C. W. SLAYMAN, AND E. A. ADELBERG

Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **Starting with mutagenized cultures of the mouse fibroblastic cell line LM(TK^-), we have selected mutant clones by their ability to grow at 0.2 mM K^+, a concentration unable to support the growth of the parent cell. The mutants fall into two classes on the basis of their potassium transport properties. Both classes maintain a high intracellular K^+ concentration when growing in low-potassium medium, and both are unaltered in the ouabain-sensitive Na/K pump. One class shows an increased activity of a ouabain-resistant, furosemide-sensitive K^+ transport system; the other class shows a decreased activity of a specific component of K^+ efflux.**

Mammalian cells maintain a high internal potassium concentration and a low internal sodium concentration relative to their environment; the resulting cation gradients play a central role in cell physiology, being responsible for such diverse functions as volume regulation (1), nutrient transport (2), and membrane excitability (3). Mechanistically, the potassium and sodium gradients reflect the steady-state concentrations at which all passive ion movements (leaks) are balanced exactly by all active ion movements (pumps) (1). Among the active pathways, the ouabain-sensitive Na^+,K^+-ATPase is the best characterized (4), but additional ouabain-insensitive active cation movements have been detected (for example, see refs. 5–7). There are also several modes of passive K^+ and Na^+ movement that are determined by the specific membrane permeabilities to the ions and are thought to occur through ion-selective channels (3). It is the ability of a class of these channels to alter permeability in response to membrane potential or receptor-bound neurohormone that confers excitability upon a membrane (8). These complex and interrelated pathways are difficult to sort out unambiguously either by kinetic measurements or with the use of inhibitors.

In view of the successful application of genetics to the analysis of complex transport processes in microorganisms (9), we have undertaken a genetic study of the mechanisms for cation movement in mammalian cells. One class of mutants, resistant to the cardiac glycoside ouabain, has already been isolated in a number of laboratories (for example, see refs. 10 and 11). In this paper we introduce two new classes of mutants, both selected for their ability to grow at a potassium concentration below that minimally required for the growth of parent cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The parental cell line used for the isolation of mutants was LM(TK^-), a thymidine kinase-deficient derivative of the mouse fibroblastic L cell line (12). All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere in flasks containing α medium (13) with horse serum added to a final concentration of 10% (vol/vol). For growth and flux experiments, suspension cultures were grown in spinner bottles containing SPF/C medium, an α medium formulation lacking nucleosides and calcium, and modified to contain the desired potassium concentration by isosmotic replacement of sodium for potassium. Fetal bovine serum or dialyzed fetal bovine serum was added to a final concentration of 5% (vol/vol), and the final potassium concentration of the complete growth medium was determined by flame photometry.

Mutagenesis. Logarithmic phase suspension cultures of LM(TK^-) grown at 5.4 mM K^+ were treated for 16 hr with ethyl methanesulfonate at 300–500 μg/ml. Survival varied in different experiments between 2% and 50% of the treated cells. The cells were washed and resuspended in 5.4 mM K^+ medium and grown for 8–12 days to allow for fixation and phenotypic expression of the induced mutations. The cells were then washed in medium lacking K^+ and plated in SPF/C medium containing 1.8 mM $CaCl_2$ and 3% horse serum, providing a final K^+ concentration of 0.2 mM. The medium was changed at 5-day intervals until the flasks were largely cleared of cells. The final number of primary clones able to grow at 0.2 mM K^+ was 1–10 per 10^7 cells plated. Individual clones were picked and grown in 0.2 mM K^+ medium and frozen at −70°C for preservation. Only one clone was chosen from each mutagenized cell suspension; thus each represents a unique mutational event. All mutants have remained stable in nonselective medium for more than 6 months.

Flux Experiments. Influx experiments were carried out in buffer A, which contains 140 mM NaCl, 5.5 mM glucose, 25 mM Hepes at pH 7.00, and phenol red; the osmolarity of the buffer was 295 mosM. For measurement of the initial rate of K^+ influx, cells were depleted of potassium by incubation in buffer A at 0°C for 2 hr and then kept at 0°C in a dense suspension above the Millipore filter of a Nalgene filter unit (Nalge Co., Rochester, NY) in ionic equilibrium with excess buffer A below the filter. At intervals, cells from the suspension were diluted into buffer A at 20°C and equilibrated in a shaking water bath for 10 min. The flux was started by the addition of $^{42}K^+$ (and carrier potassium, if needed), and samples were taken at 2-min intervals for 8 min. Internal ion contents were determined by centrifuging cells in a microcentrifuge tube through a layer of silicone oil (GE F50 Versilube, R. H. Carlson Co., Greenwich, CT) into a layer of 12% perchloric acid (14). Cation content was measured by flame photometry or atomic absorption spectrophotometry, and intracellular $^{42}K^+$ was counted in an auto-gamma counter. Correction was made for trapped extracellular water by using [^{3}H]inulin as a marker. Cell water, as determined by wet weight/dry weight (15), accounted for 77% of the cell volume determined using a Coulter Counter Channelyzer, and ion concentration was expressed as mmol/liter of cells. The influx of potassium was linear for the first 10 min at all potassium concentrations used; the initial rate of influx was determined from a regression line through the points and expressed as μmol/10^9 cells per min.

For determination of unidirectional potassium efflux, cells grown in suspension culture at 5.4 mM K^+ were washed once

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Table 1. Properties of parental and mutant cells

Property	LM(TK⁻)	LTK-1	LTK-5
Growth in 5.4 mM K⁺			
Growth rate, generations/day	1.00	0.73	0.55
Cell volume, μm^3	1546 ± 185	1358 ± 149	2684 ± 240
Intracellular K^+, mmol/liter cells	111 ± 10	117 ± 16	98 ± 12
Intracellular Na^+, mmol/liter cells	15 ± 4	16 ± 4	23 ± 1
DNA content,* $\mu g/10^8$ cells	856	807	925
Growth in 0.2 mM K⁺			
Growth rate, generations/day	—†	0.30	0.22
Cell volume, μm^3	1600 ± 100†	1269 ± 47	2904 ± 104
Intracellular K^+, mmol/liter cells	28 ± 3†	77 ± 4	43 ± 5
Intracellular Na^+, mmol/liter cells	95 ± 15†	53 ± 5	75 ± 10

Data with uncertainties are given as mean ± SD.
* Determined by the method of Burton (16).
† LM(TK⁻) is not capable of growth at 0.2 mM K^+. Determinations of cell volume, K^+, and Na^+ were made after 4 hr at 37°C in this medium.

and suspended in potassium-free SPF/C medium to which 5% dialyzed fetal bovine serum was added (giving a total K^+ concentration of less than 0.03 mM). The cell suspension was transferred to a spinner bottle and returned to a 37°C CO_2 incubator. At intervals, samples were removed from the spinner bottle, centrifuged, and suspended in isotonic choline chloride. Aliquots were rapidly taken from the choline chloride suspension for determination of internal ion contents as in influx experiments.

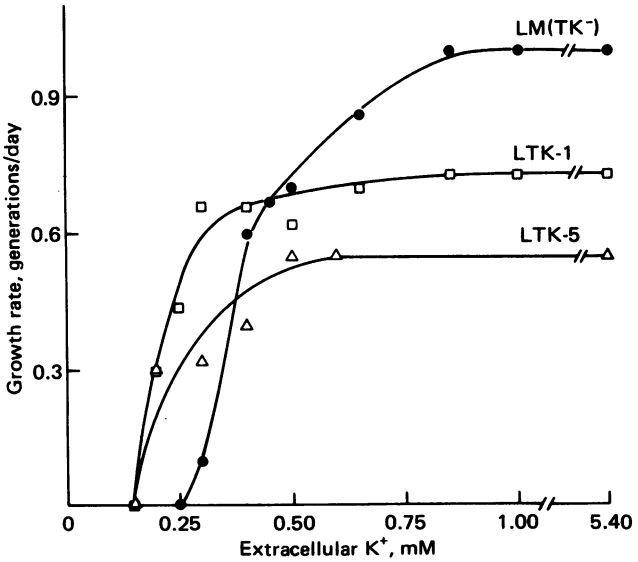

FIG. 1. Growth rate of LM(TK⁻) (●), LTK-1 (□), and LTK-5 (△) as a function of K^+ concentration. Cells from suspension cultures grown at 5.4 mM K^+ were washed, suspended in SPF/C medium containing 5% dialyzed fetal bovine serum and 0.15–5.4 mM K^+, and maintained at 37°C in a CO_2 incubator for 5 days with daily determinations of cell number. Growth rates are expressed as the reciprocal of generation time. Data were pooled from three experiments for LM(TK⁻) and LTK-1 and from two experiments for LTK-5.

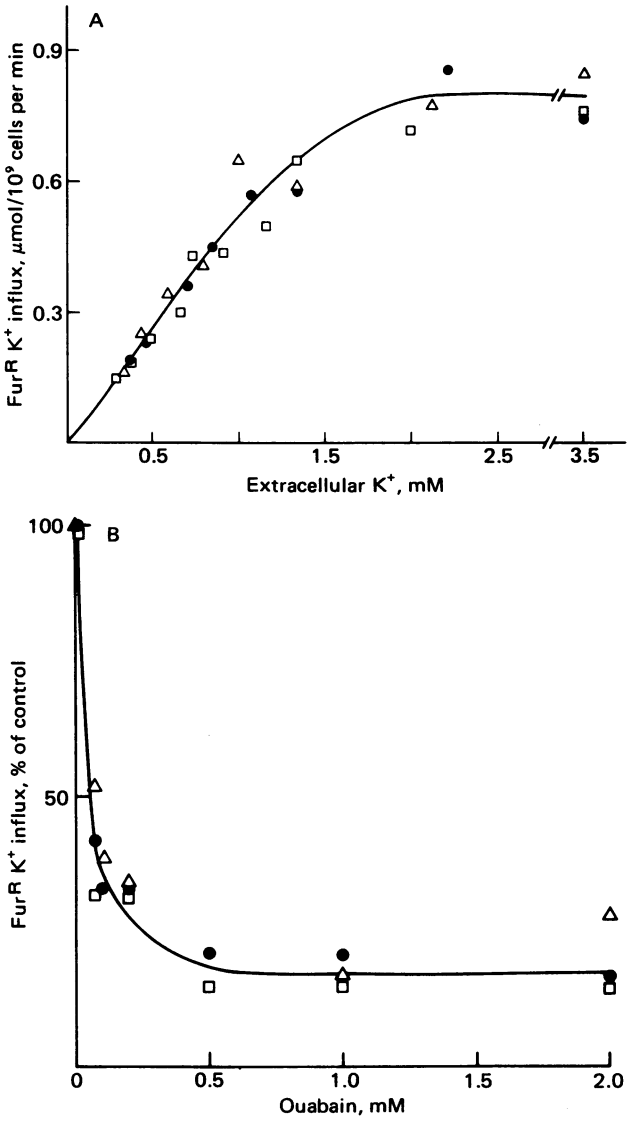

FIG. 2. (*A*) Furosemide-resistant (Fur^R) K^+ influx in LM(TK⁻) (●), LTK-1 (□), and LTK-5 (△). The initial rate of $^{42}K^+$ influx was determined in the presence of 1 mM furosemide. Data were averaged from three experiments. The maximal rate of furosemide-resistant K^+ influx is 0.8 μmol/10^9 cells per min and the $K_{1/2}$, the concentration at half-maximal influx, is 0.75 mM in all three cell lines. (*B*) Inhibition of furosemide-resistant K^+ influx by ouabain in LM(TK⁻) (●), LTK-1 (□), and LTK-5 (△). The initial rate of $^{42}K^+$ influx was determined in the presence of 1 mM furosemide and 0–2 mM ouabain, in buffer containing 0.4 mM K^+ [LM(TK⁻) and LTK-5] or 0.2 mM K^+ (LTK-1). Results are expressed as percent of the control rate in the presence of furosemide but in the absence of ouabain. Half-maximal inhibition was observed at 50 μM ouabain in all three cell lines.

RESULTS

Isolation and Growth of Mutants. In medium containing 5.4 mM K^+, LM(TK⁻) grows with a doubling time of 24 hr; it shows progressively slower growth as K^+ is reduced below 1 mM, and fails to grow at 0.25 mM K^+ or below. When LM(TK⁻) cells were treated with ethyl methanesulfonate and plated at 0.2 mM K^+, five independent mutant clones were isolated by their ability to grow at the low K^+ concentration. Initial screening of cation fluxes showed the mutant clones to fall into two classes (see below), and a representative of each class (LTK-1 and LTK-5, respectively) was chosen for further study.

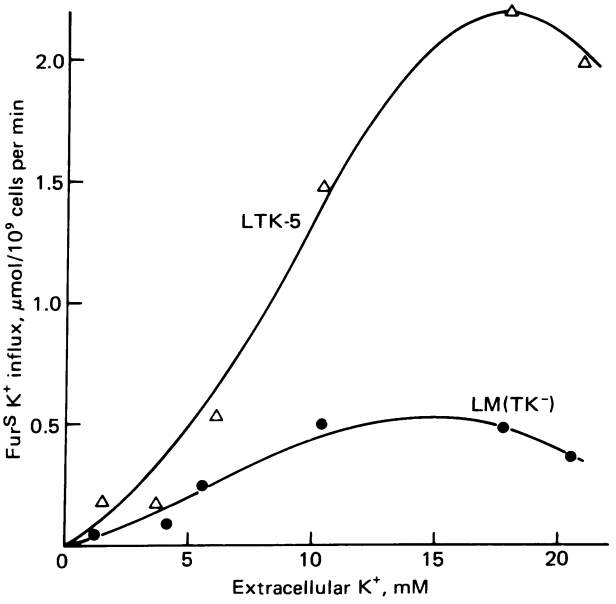

FIG. 3. Furosemide-sensitive (Fur^S) K^+ influx in LM(TK$^-$) (●) and LTK-5 (△). The initial rates of $^{42}K^+$ influx were determined at each K^+ concentration (from 1 to 20 mM) in the presence and absence of 1 mM furosemide, and the furosemide-sensitive influx was obtained by subtraction. The maximal rates of furosemide-sensitive influx were 0.5 μmol/10^9 cells per min in LM(TK$^-$) and 2.2 μmol/10^9 cells per min in LTK-5.

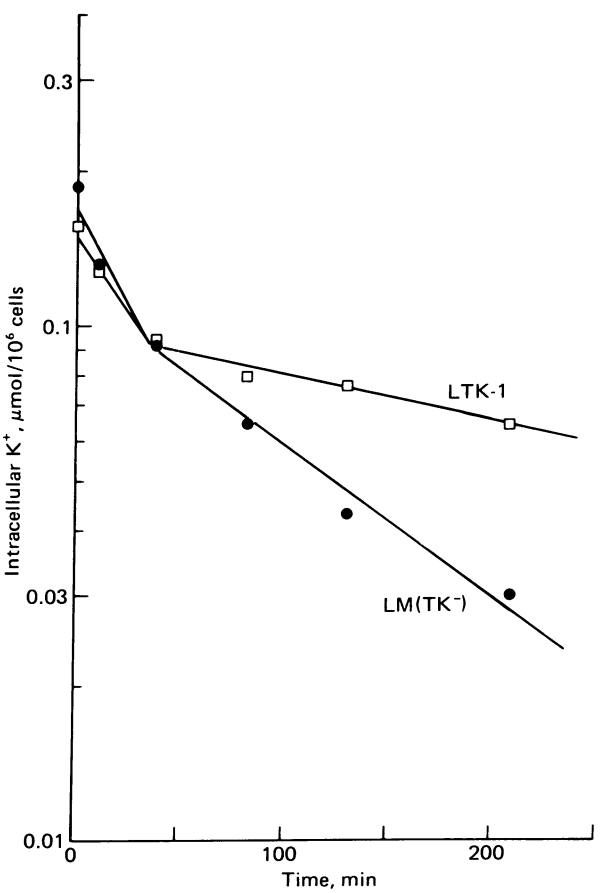

FIG. 4. Semilogarithmic plot of potassium efflux from LM(TK$^-$) (●) and LTK-1 (□) into K^+-free SPF/C medium at 37°C. The data points plotted are the average of results from two experiments. The fast component of efflux amounts to 35% of cellular K^+ and has a half-time of 10 min in both cell lines. The slow component amounts to 65% of cellular K^+ in both cell lines, and has a half-time of 105 min in LM(TK$^-$) and 330 min in LTK-1.

In suspension culture, both LTK-1 and LTK-5 are clearly distinguishable from the LM(TK$^-$) parent cells. At 5.4 mM K^+, both mutants grow at reduced rates (LTK-1 reduced by 30%; LTK-5 by 45%), and LTK-5 has an increased cell volume [2684 ± 240 μm^3 compared with 1546 ± 185 μm^3 in LM(TK$^-$)]. LTK-1 is normal in cell volume, and both mutants are normal in internal ion concentrations and DNA content (Table 1). A more striking difference is seen in growth at low potassium concentrations. Both mutants are able to grow in suspension culture at K^+ concentrations that do not support the growth of LM(TK$^-$) (Fig. 1). Furthermore, at these low K^+ concentrations, LTK-1 and LTK-5 maintain high internal K^+ concentrations, and therefore must have either increased K^+ influx or decreased K^+ efflux compared with the parent cells (Table 1).

Potassium Influx. Direct investigation of K^+ influx in LM(TK$^-$) cells revealed two major pathways: a ouabain-sensitive Na/K pump, and a furosemide-sensitive potassium transport system (17–21).

Neither LTK-1 nor LTK-5 is altered in the ouabain-sensitive Na/K pump. Fig. 2*A* illustrates the dependence of K^+ influx on the extracellular K^+ concentration, measured in the presence of 1 mM furosemide to eliminate the contribution of the furosemide-sensitive system. Under these conditions, all three cell lines show identical concentration dependence of potassium transport, with a $K_{1/2}$ of 0.75 mM extracellular potassium and a V_{max} of 0.8 μmol/10^9 cells per min. Fig. 2*B* illustrates the inhibition of potassium influx by ouabain, again in the presence of furosemide. Because rodent cells are relatively insensitive to ouabain (22) and because there is a pseudocompetitive inhibition between external K^+ and cardiac glycosides (4), this experiment was carried out at low external K^+. Ouabain gave essentially complete inhibition of furosemide-resistant K^+ influx in all three cell lines, with a half-maximal effect at 50 μM.

LTK-5, in contrast, shows a substantial increase in K^+ influx via the furosemide-sensitive pathway. Fig. 3 illustrates the results of an experiment in which K^+ influx was measured as a function of the extracellular K^+ concentration in the presence and absence of 1 mM furosemide. The furosemide-sensitive component of influx is plotted in Fig. 3. The V_{max} of this system in LTK-5 (2.2 μmol/10^9 cells per min) is 4 times that of the LM(TK$^-$) parent (0.5 μmol/10^9 cells per min); the $K_{1/2}$ is between 7 and 8 mM in both cell lines. By contrast with LTK-5, LTK-1 is unaltered in furosemide-sensitive K^+ transport (data not shown).

Potassium Efflux. Studies of unidirectional K^+ efflux from LM(TK$^-$) showed K^+ loss to follow two-compartment kinetics (23). One compartment, containing 35% of the internal potassium, empties with a half-time of 10 min; the other, containing 65% of the internal potassium, empties with a half-time of 105 min (Fig. 4).

LTK-1, while unaltered in the fast component of efflux, shows a reduction to 1/3 in the rate of K^+ loss from the slowly emptying pool, with the half-time increased from 105 to 330 min (Fig. 4). By contrast with LTK-1, LTK-5 exhibits a slightly increased rate of efflux from the slowly emptying K^+ pool (data not shown).

DISCUSSION

The importance of the Na/K pump to cation metabolism in mammalian cells has been well documented (4). The present results demonstrate that it alone is not responsible for all physiologically important cation movements in L cells, however, because mutations affecting two other cation transport

pathways can allow cells to survive the stress of low external potassium.

The LTK-5 mutant appears unaltered in the ouabain-sensitive Na/K pump, but possesses an increased activity of a furosemide-sensitive K^+ transport system. Ouabain-insensitive, diuretic-sensitive cation transport of this kind has been described by Hoffman and Kregenow (5) in human erythrocytes and has also been reported in several lines of cultured cells [for example, Ehrlich cells (20, 24, 25); L-cells (26); 3T3 (21, 27)]. It has been best studied in duck erythrocytes, in which Kregenow demonstrated its independence from the Na^+,K^+-ATPase and implicated it in the control of cell volume (7, 28). Thus, cells that have been allowed to shrink in hyperosmotic medium regain their original volume by the transport of K^+ into the cell against its electrochemical gradient along with associated anions and osmotically obligated water. The system can also be activated by the binding of norepinephrine at a β-adrenergic receptor on the cell surface (29). More recently, Schmidt and McManus (17–19), continuing the studies on duck erythrocytes, have shown the system to be inhibited by furosemide, and have defined the mechanism as Na–K cotransport. The present results demonstrate that the furosemide-sensitive K^+ transport system of LM(TK^-) is similar to that of duck erythrocytes in its requirement for high external K^+. [As expected from the fact that L cells lack β-adrenergic receptors (30), it is not influenced by norepinephrine (J. J. Gargus, unpublished experiments)]. Whether the LM(TK^-) system operates by means of Na–K cotransport, and whether it functions in volume regulation, are not yet known. It also remains to be determined whether the 4-fold increase in the furosemide-sensitive system accounts for the increased ability of LTK-5 to survive at low external K^+ concentrations.

The second mutant, LTK-1, is also unaltered in ouabain-sensitive Na/K transport, but shows a decreased rate of K^+ efflux from the major cellular pool. A possible candidate for this efflux pathway emerges from two very different kinds of experiments. (*i*) Electrophysiological studies by Nelson and Peacock (31, 32) and by Okada and coworkers (33–35) have indicated that L cells may possess a regulated passive potassium permeability (P_K). Specifically, membrane hyperpolarizations interpretable in terms of an increased P_K can be induced by acetylcholine binding to muscarinic receptors on the cell membrane, by mechanical and electrical stimulation, and by a still unidentified mechanism of cell–cell communication (31); under some experimental conditions, the hyperpolarizations also occur spontaneously and rhythmically (33). Unlike the classical Hodgkin–Huxley potassium "channel" (36), the P_K of L cells is not activated by membrane depolarization (33). Whether it behaves like the calcium-activated potassium "channel" seen in a variety of cells (37) has yet to be determined. (*ii*) An independent line of experiments on volume regulation has also pointed to a regulated passive potassium permeability in L cells. Again the process is similar to one seen in duck erythrocytes, which not only regulate their volume by swelling in hyperosmotic medium (as described above) but also are capable of shrinking back to their original volume when swollen in hyposmotic medium (38, 39). This shrinking is achieved by the loss of K^+ as the passive permeability of the membrane to K^+ is dramatically increased in the swollen cell (38, 39). The same mechanism of volume regulation has been demonstrated in cultured rodent cells by Roti Roti and Rothstein (40) and confirmed by us in LM(TK^-) (unpublished data). Such a change in potassium permeability would be expected to move the membrane potential in a hyperpolarizing direction, towards the Nernst potential for potassium (41), and may therefore be equivalent to the hyperpolarizing response measured in the electrophysiological experiment described above.

Thus, L cells appear to possess at least two pathways in addition to the Na/K pump by which potassium can move across the cell membrane: (*i*) a furosemide-sensitive K^+ transport system and (*ii*) a regulated potassium "channel." Furthermore, mutations causing either an increase in transport through the first pathway or a decrease in that through the second can confer upon the cell the ability to grow in low-potassium medium. More work is needed to clarify the physiological role of the two pathways under normal growth conditions—a role suggested by the facts that, in standard growth medium containing 5.4 mM K^+, the LTK-5 mutant has an abnormally large cell volume and both mutants have reduced growth rates. There has been considerable speculation that cellular cations are important not only in volume regulation and nutrient cotransport but also in processes such as cell cycle regulation (24, 27, 42, 43), transformation (21, 42), and differentiation (44). The study of transport mutants should help to establish whether the furosemide-sensitive K^+ transport system and the regulated potassium "channel" are involved in any of these processes.

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