Biochimica et Biophysica Acta 821 (1985) 56–62
Elsevier

BBA 72785

Urea reflection coefficient for the human red cell membrane

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(Received May 15th, 1985)

Key words: Reflection coefficient; Urea; Erythrocyte membrane; (Human)

The reflection coefficient, σ, is an irreversible thermodynamic parameter which measures the interaction between solute and solvent in passage across a membrane. The initial estimate of Goldstein and Solomon ((1960) J. Gen. Physiol. 44, 1–17) by the zero-time method gave $\sigma_{urea} = 0.6$ for the human red cell membrane and a more recent measurement by Levitt and Mlekoday ((1983) J. Gen. Physiol. 81, 239–253) using a different method gave $\sigma_{urea} = 0.95$. We have now developed a variant of the zero-time method which gives $\sigma_{urea} = 0.70 \pm 0.02$, which is significantly different from 1.0. There has been controversy as to whether urea permeates the human red cell by the same channel used by water or by a different route. The finding that σ_{urea} is significantly less than 1.0 (actually less than 0.95) makes it possible to discriminate between these two possibilities since completely independent transfer of urea and water mandates a value of $\sigma_{urea} = 0.95$. Values significantly lower than 0.95 can only be achieved if the transport of the solute, urea, is coupled to that of the solvent, water.

When the equivalent pore model of the human red cell was developed it was assumed implicitly that urea permeated the human red cell passively through the aqueous channel used by water (Paganelli and Solomon [1], Goldstein and Solomon [2]). The finding that phloretin inhibited urea transport, although it has little effect on water transport, caused Macey and Farmer [3] to suggest that water and urea did not share the same pathway. The experiment of Wieth et al. [4] indicated that urea transport in human red cells was saturated at high urea concentrations and could be inhibited competitively by thiourea. On this basis, they concluded that urea crossed the human red cell membrane by facilitated diffusion.

The reflection coefficient, σ, is a measure of the interaction between solvent and solute as they cross a permeability barrier. When the interaction is very large, as when ^2H_2O (radius = 1.9 Å) diffuses passively through a 23 Å radius pore in a dialysis tubing membrane in aqueous solution (Durbin [5]), σ approaches 0 ($\sigma = 0.002$). When the molecule is too large to permeate the channel, as for bovine serum albumin, $\sigma = 1$ which shows that there is no solute-solvent interaction in the membrane. In intermediate cases, as for sucrose, in which the ratio of the solute radius to the pore radius (a/r) is 0.23, $\sigma = 0.37$. In passive systems σ is less than unity as long as (a/r) is significantly less than 1.0.

If facilitated diffusion by a carrier molecule is the only urea permeation process, there should be no urea in the aqueous channel and hence no interaction between urea and water permeation so that σ_{urea} should be 1.0. In 1960, Goldstein and Solomon [2] developed the zero-time method to make the first measurement of σ in the human red cell which gave $\sigma_{urea} = 0.62$, consistent with their view that urea, whose hydrated radius (Durbin [5]) is 2.7 Å, permeated through the 4.2 Å radius red

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cell aqueous channel. Measurements of σ_{urea} by the zero-time and other methods [6–11] gave values ranging from 0.55 to 1.0. In view of the importance of σ_{urea} to understanding the basic mechanism of urea permeation in the human red cell, we have now developed a modified zero-time method to determine σ_{urea} and obtained a value of 0.70 ± 0.02.

Experimental methods

The time-course of red cell volume changes following exposure to a large osmotic pressure gradient was measured by 90° scattering of white light, using the stopped-flow apparatus described by Levin et al. [12]. Light intensity was measured over a 2–4-s time interval starting 20 ms after mixing of the cell suspension with the test solution. The scattered light intensity was digitized with a resolution of 2–4 ms and stored for further analysis.

Blood was drawn from young healthy adults into heparin (10 USP units/ml blood), centrifuged and aspirated to remove components other than the packed red cells. The cells were washed twice and then suspended in an isosmolal buffered saline solution to form a 2% hematocrit suspension. The buffer composition is (mM): NaCl, 125; KCl, 4.4; $NaHCO_3$, 24.9; $CaCl_2$, 1.2; MgCl, 0.5; Na_2HPO_4, 4.2. The buffer was titrated to a pH of 7.4, and had a total osmolality of 294 ± 3 mosM. The hyperosmolal mixing solutions used to produce the gradients were made from this same isosmolal buffer, to which was added either urea, or, when an impermeant gradient was required, sucrose or NaCl. The solutions used in these measurements produced initial gradients which were as large as 300 mosM in the case of impermeant solutes and 600 mosM for urea. Measurements were also made on blood suspensions which had been allowed to stand for 1 h at room temperature after a 1 mM concentration of *p*-chloromercuribenzene sulfonate (pCMBS) (Sigma) had been added. pCMBS is a mercurial sulfhydryl reagent which effectively inhibits both water and urea permeation (Macey and Farmer [3]). All experiments were carried out at 20–23°C.

For each mixing solution, 10–15 runs and sometimes considerably more were averaged together in order to reduce random fluctuations in the data. The average cell volume/time records were corrected for scattered light intensity changes not associated with size changes by subtraction of control runs in which the blood suspensions were mixed with isomolal buffer solutions as described by Levin et al. [12]. For some of the urea experiments, additional control runs according to the procedure of Terwilliger and Solomon [13] were also obtained. For these controls the blood suspension and hyperosmolal mixing solution were mixed and allowed to come to equilibrium; the resulting solution was injected through both ports of the stop-flow device. When this additional control was used to correct the same experimental data, no significant difference was found from data corrected with a single control run. Hence, the second control run was omitted in the present experiments in which large osmotic gradients were customarily used. There is an additional factor for which no correction was made. As urea permeates into the cell, the refractive index of the red cell changes. Papanek [4] studied the effect of concentrated ethylene glycol solutions on scattered-light intensity in red cell stopped-flow experiments after equilibrium had been reached. His data indicate that the scattered light increased by 4.4% at an ethylene glycol concentration of 0.5 M. Our experimental data were taken at about 20 ms after mixing, when the urea concentration inside the cell was still relatively small; our urea concentrations ranged from 0.2 to 0.6 M. Consequently the error introduced by the refractive index change was small compared to 4.4%. The digitization, averaging and subsequent analysis were done with a PDP-11/34 computer (Digital Equipment Co., Maynard, MA), as described by Levin et al. [12] and Terwilliger and Solomon [13]. In order to determine the errors reported in Figs. 1 and 2, a visual estimate was made of the possible maximal and minimal values of dV/dt and these results are shown as the termini of the error bars.

Results and Discussion

Method for determination of σ

The volume change of a red cell following an osmotic gradient may be described by the Kedem

-Katchalsky equations,

$$dV/dt = -K_1[\Delta\pi_i + \sigma\Delta\pi_s] \quad (1a)$$

$$dS/dt = (1-\sigma)\bar{\pi}_s(dV/dt) + K_2\Delta\pi_s \quad (1b)$$

where (dV/dt) is the volume rate of change in units of cell volume. $\Delta\pi_i = (\pi_{i,out} - \pi_{i,in})$ is the impermeable solute osmotic pressure gradient, $\Delta\pi_s$ is the permeable solute osmotic gradient, and $\bar{\pi}_s \approx (\pi_{s,in} + \pi_{s,out})/2$ is the average osmolality of the permeant solute inside the membrane. All osmolarities are measured in units of $\pi_{iso} = 292$ mosM and cell volumes are normalized to V_{iso} (taken as 104 μm^3, Jay [15]). dS/dt is expressed in terms of the normalized number of osmoles of permeant solute in the cell, S, in units of $\pi_{iso}\ V_{iso}$. The constants are $K_1 = ARTL_pC_{iso} = 0.99\ L_p\ cm^{-2} \cdot s^{-1}$, $K_2 = \omega RT/V_{iso} = 0.33\ \omega\ cm^{-2} \cdot s^{-1}$ in which L_p is the red cell hydraulic conductivity in units of $cm^3 \cdot dyn^{-1} \cdot s^{-1}$, C_{iso} is 0.293 osmol/cm^3, ω is the solute permeability coefficient in units of $mol \cdot dyn^{-1} \cdot s^{-1}$ and A is cell area taken as 137 μm^2 (Jay [15]).

These coupled differential equations may be solved numerically for any choice of L_p, ω and σ (Milgram and Solomon [16], Levin, private communication). Such solutions are of apparent potential utility in comparing experiment with theory, and it is possible to obtain the set of permeability parameters giving the best fit to the data, as has been done by Levitt and Mlekoday [11]. In an extensive series of experiments (Levin, Levin, and Solomon, private communication), we have found that this procedure is not particularly sensitive to σ, and generally seems too insensitive to simultaneous changes in the three parameters for us to have confidence in data obtained by this procedure. We have decided instead to use the Kedem-Katchalsky equations to extract σ from specific features of the cell volume versus time curve which are sensitive to this parameter.

Measurement of σ

When the osmotic pressure gradient is produced by an impermeant solute the initial rate of red cell volume change in Eqn. 1 becomes

$$(dV/dt)_i = -K_1\Delta\pi_i \quad (\Delta\pi_s = 0) \quad (2a)$$

If the cells are instead subjected to an osmotic pressure gradient produced by a permeant solute only,

$$(dV/dt)_s = -K_1\sigma\Delta\pi_s \quad (\Delta\pi_i = 0) \quad (2b)$$

In the zero-time method of Goldstein and Solomon [2], σ was obtained from the ratio of the initial slopes of the cell volume-time curve in paired experiments under these specific conditions. However, the determination is difficult because the first 50–100 ms of the average cell volume-time record are noisy, even when 10–15 individual experiments are averaged. In addition the zero-time point is not directly accessible for analysis because of unstirred layer effects. Consequently dV/dt was measured at 20 ms and a correction procedure was devised to determine its value at zero-time. The slope at 20 ms of each averaged curve was determined by drawing a smooth curve through the region between approx. 20 and approx. 200 ms, constructing the tangent at 20 ms, measuring its slope, and subsequently applying the zero-time correction discussed below. For some runs this procedure was checked by taking the average slope between 20 ms and an upper time which varied between 30 and 200 ms. The average slope was plotted as a function of Δt, the time difference from time zero, and the resulting linear curve extrapolated to $\Delta t = 0$.

The zero-time correction was obtained by solving Eqns. 1 numerically for dV/dt as a function of time with reasonable choices of L_p, ω and σ. We found that, if the slopes at 20 ms are used to calculate σ, there is an underestimate of approx. 10%. It is also possible to solve Eqns. 1 analytically for very small values of t to obtain the time dependence of dV/dt in both the permeant and impermeant cases, as shown in the appendix. Both methods show that reflection coefficients obtained by comparing slopes at time $t > 0$ should be multiplied by a correction factor

$$f = 1 + \lambda t \quad (3)$$

where

$$\lambda = 0.47\omega - 1.8L_p$$

ω is given in units of 10^{-15} $mol \cdot dyn^{-1} \cdot s^{-1}$ and

L_p in units of 10^{-11} cm$^3 \cdot$ dyn$^{-1} \cdot$ s^{-1}. For $t_i = 20$ ms, $\omega = 14 \cdot 10^{-15}$ moles $\cdot$ dyn$^{-1} \cdot$ s^{-1} (this value is the same as that determined by Sha'afi et al. [17]), $L_p = 1.3 \cdot 10^{-11}$ cm$^3 \cdot$ dyn$^{-1} \cdot$ s^{-1} and f (20 ms) $\cong$ 1.1. However, this correction is an overestimate because the actual zero time is determined by a calibration procedure based on absorption changes in a homogeneous chemical reaction. Such a procedure is not sensitive to the problem of an unstirred layer at the cell membrane which effectively delays the time at which cell permeation can begin. Terwilliger and Solomon [13] estimate this delay to be approx. 10 ms, based on the measurements of Sha'afi et al. [17]. It is therefore reasonable to assign a time $t = 10 \pm 5$ ms to our nominal 20 ms measurement point, and to adopt a correction factor $f = 1.05 \pm 0.03$.

Determinations of the urea reflection coefficient were carried out on five donors. In three cases (Expts. 1, 3 and 5), one of which is shown in Fig. 1, initial (nominal 20 ms) slopes were studied as a function of $\Delta\pi$ for both permeable and impermeable solutes. σ_{urea} was determined by measuring the slopes of the two lines as in Fig. 1, applying the zero-time correction given above and determining σ_{urea} from the ratio of the slopes. For the other two determinations of σ_{urea} it was decided to take all data at single values of $\Delta\pi_s$ and $\Delta\pi_i$ and determine σ_{urea} from the ratio of the two volume rates of change. These experiments used 25 (Expt. 2) and 40 (Expt. 4) individual runs for each data at single values of $\Delta\pi_i$ and $\Delta\pi_s$ and Eqns. 2 were used directly. The corrected results for each of our five experiments are given in Table I. The average value of the reflection coefficient is $\sigma_{urea} = 0.70 \pm 0.02$, which is significantly different from 1.0 ($P < 0.001$, t-test) *. This value is higher than those of 0.62 ± 0.02 and 0.55 ± 0.02 previously obtained in this laboratory by Goldstein and Solomon [2] and Sha'afi et al. [6], but is slightly lower than the figure of 0.79 ± 0.02 obtained by Owen and Eyring [7] in experiments in which an uncorrected zero-time method calculation was applied to experiments in which the 'zero time' was taken as 20 to 130 ms after mixing (see also Owen [10]). The data of Sha'afi et al. [6] have been analyzed extensively, first by Macey and Wadzinski [8] and subsequently by Owen and Galey [9] who have concluded that these experimental data could be fit by computer programs to yield either $\sigma_{urea} = 1.0$ (Macey and Wadzinsky [8]) or $\sigma_{urea} = 0.8$ (Owen and Galey [9]). Levitt and Mlekoday [11] have developed a computational technique for simultaneous determination of L_p, ω and σ in stopped-flow experiments. The values of the coefficients are obtained from a computer fit which provides

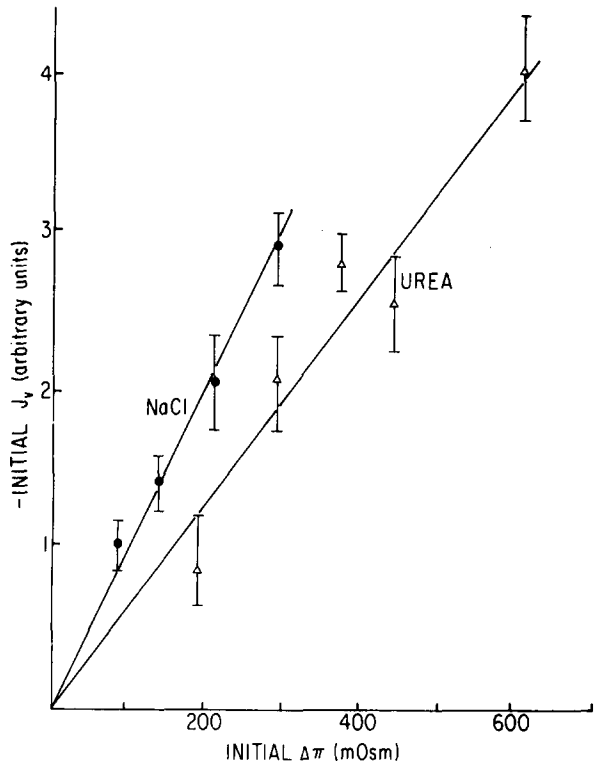

Fig. 1. Dependence of initial rate of red cell volume change ($J_v = (dV/dt)/A$) on initial gradient of osmotic pressure, in one of the experiments from Table I. Error bars are computed as described under Experimental Methods and the lines have been drawn by least squares. ●, solute, NaCl; △, solute, urea.

* Strictly speaking, the criterion for interaction between solute and solvent in a membrane pore is

$$\sigma < 1 - (\omega \bar{V}_s / L_p)$$

The second term on the right is ordinarily very small for non-lipophilic solutes in biological membranes. For urea in the red cell membrane, the correction term is 0.048, based on the values for L_p and ω given above and taking 45 cm^3/mol as the partial molar volume of urea ($\bar{V}_s$). Thus the corrected criterion becomes $\sigma < 0.95$.

TABLE I

DETERMINATION OF σ_{urea} IN HUMAN RED CELL

Expt.	σ_{urea}	$\sigma_{urea,pCMBS}$
1	0.64 ± 0.08	0.87 ± 0.1
2	0.74 ± 0.06	0.97 ± 0.1
3	0.75 ± 0.09	0.93 ± 0.08
4	0.66 ± 0.09	–
5	0.67 ± 0.08	–
Av. *	0.70 ± 0.02	0.93 ± 0.02

* Error weighted average ± S.E.

the best values for all three parameters from a single shrink-swell curve. Levitt and Mlekoday conclude that their data are completely consistent with $\sigma_{urea} = 0.95$, but they also point out that the fit for $\sigma_{urea} = 0.75$ is also acceptable. This insensitivity of the simultaneous solution method to σ is consistent with our findings, as discussed above.

Since Macey and Farmer [3] had shown that pCMBS inhibited osmotic water transport by 80%, σ_{urea} was also determined in blood suspensions in which water transport had been inhibited by 1 mM pCMBS. Fig. 2 shows a representative plot of the initial rate of volume change against $\Delta\pi$ for pCMBS-treated blood. It is clear that pCMBS has brought σ very close to 1. An average of all three experiments yields $\sigma_{urea,pCMBS} = 0.93 \pm 0.02$ as shown in Table I. This observation is entirely consistent with the conclusion that pCMBS exercises its inhibition of urea transport by closing the aqueous channel, but it does not specify the mechanism.

There are a number of observations in the literature that have been interpreted as evidence that urea and water cross the human red cell membrane by separate pathways, but in each case there are alternative explanations that may also be put forward, based on the model of the red cell aqueous channel given by Solomon et al. [18]. According to this model there is a single class of aqueous channels across the red cell membrane with a radius of approx. 4.5 Å, which is responsible for the transport of water, nonelectrolytes and anion exchange. Although the channel provides the route of transport, the flux of each solute is modulated by specific interaction with the walls of the channel. Thus Cl^- flux is modulated by exchange of its water of hydration with hydrogen bonds on the pore wall. The one-for-one exchange characteristic of Cl^- flux is regulated by movement of a negatively charged residue attached to the pore wall, as suggested by Knauf [19] and others. Passive loss of the cation content of the cell does not take place because there is a positively charged region in the pore. Urea has the ability to form many hydrogen bonds and is therefore highly hydrated in aqueous solution, so that urea transport also requires exchange of the hydrated shell

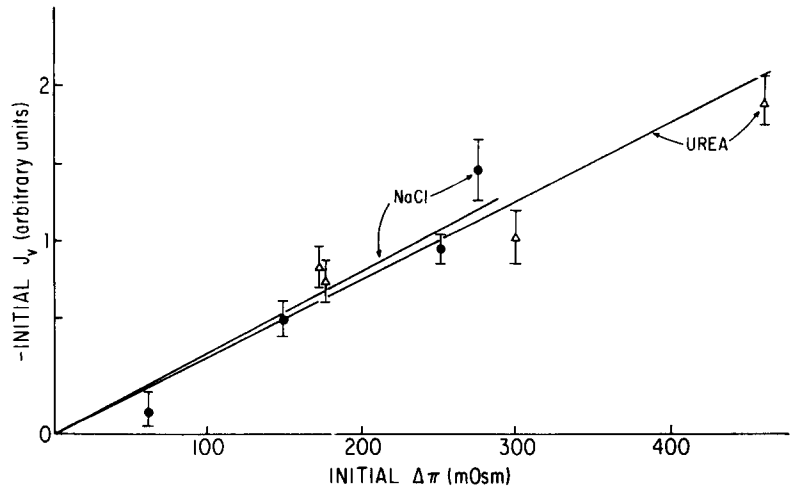

Fig. 2. Effect of pCMBS (1 mM) on the initial rate of red cell volume change in one experiment.

for hydrogen bonds formed with polar groups on the wall. Each of these solutes shares a common pathway with the solvent, but the passage of each is carefully modulated by specific chemical interactions which recognize the shape and the physical properties characteristic of each molecule.

Wieth et al. [4] found that urea inhibits thiourea transport and Solomon and Chasan [20] have shown that thiourea inhibition is specific to amides, but has no effect on hydrophilic alcohol flux. Mayrand and Levitt [21] and Brahm [22] have shown that urea transport is saturable at high urea concentrations and that the K_d differs on the two faces of the membrane. Although these are characteristics typical of saturable enzyme-substrate reactions, they are also characteristic of surface absorption sites which could be located at the entrance to the channel. Thus, saturation cannot be used to discriminate between these two alternative methods of transport. Similarly, Macey and Farmer's [3] original observation that phloretin inhibited urea transport while it had no effect on water transport does not necessarily mean that two separate channels are involved. It can be interpreted just as well as a specific interdiction by phloretin of the hydrogen bond exchange which is necessary for urea permeation. The observation that chicken red cells have an effective anion exchange system, but transport urea and water slowly, with no evidence of any urea saturation (Brahm and Wieth, [23]) need have no relevance to the situation in the human red cell. Chicken red cells are nucleated; human red cells are not.

Our observation that $\sigma_{urea} = 0.70 \pm 0.02$ provides thermodynamic proof that urea and water transport share a common pathway. It does not prove that 'all' the urea and 'all' the water share the same channel, but it does show that there is a significant coupling between the two flows. It is not possible to accomodate this observation by any mechanism in which the urea pathway and the water pathway are entirely separate.

Appendix

We define an 'apparent' reflection coefficient

$$\sigma_{app} = R\frac{(\pi_{i,out} - 1)}{\pi_{s,out}} \tag{A-1}$$

where

$$R = (dV/dt)_s/(dV/dt)_i$$

It it were possible to measure R at $t = 0$ then Eqn. A-1 would yield the true σ. Actually R must be measured at a later time, but the time dependence of R may be calculated, and $R(0)$ obtained from a measurement of $R(t)$. This calculation is presented here for times short compared to the time scales on which significant volume and solute concentration changes occur.

For an initially 'permeant' solute gradient the term in $(dV/dt)_i$ in Eqn. 1b is small, so

$$\frac{dS}{dt} = K_2\Delta\pi_s = K_2\left(\pi_{s,out} - \frac{S}{V-a}\right) \tag{A-2}$$

where a is the fraction of cell volume not occupied by aqueous solution. At early times $V - a \cong 1 - a$, and the solution to Eqn. A-2 is

$$S = (1-a)\pi_{s,out}(1 - e^{-K_2 t/(1-a)}) \tag{A-3}$$

Therefore Eqn. 1a reduces to

$$(dV/dt)_s = -K_1\sigma\left(\pi_{s,out} - \frac{S}{V-a}\right) = -K_1\sigma\pi_{s,out}e^{-K_2 t/(1-a)} \tag{A-4}$$

For an initially 'impermeant' solute gradient, Eqn. 1a at early times becomes

$$\left(\frac{dV}{dt}\right)_i = -K_1\left(\pi_{i,out} - \frac{1-b}{V-b}\right) \tag{A-5}$$

where we have used the well known phenomenological description of the red cell as an imperfect osmometer (Savitz et al. [24]),

$$\pi_{i,in} = \frac{1-b}{V-b}$$

With $V = 1 + \Delta V$, Eqn. A-5 may be rewritten to first order in ΔV as

$$\left(\frac{dV}{dt}\right)_i = \frac{d}{dt}(\Delta V) = \frac{-K_1}{1-b}[(\pi_{i,out} - 1)(1-b) + \Delta V]$$

solving for ΔV and differentiating,

$$(dV/dt)_i = -K_1(\pi_{i,out} - 1)\, e^{-K_1 t/(1-b)} \tag{A-6}$$

and therefore

$$R(t) = R(0)\, e^{-\lambda t} \qquad \text{(A-7)}$$

where

$$\lambda = \frac{K_2}{1-a} - \frac{K_1}{1-b} = 0.47\omega - 1.8L_p$$

In evaluating λ we have used the values for K_1 and K_2 given in the text and the values (see Ref. 24), $a = 0.28$ and $b = 0.43$.

Eqn. A-7 has no physical meaning for $\lambda t > 1$, since $1/\lambda$ represents the approximate time scale for substantial changes in the cell, so higher terms in the expansion may be dropped. It is therefore appropriate to write

$$R(0) = R(t)(1 + \lambda t)$$

and

$$\sigma = \sigma_{app}(1 + \lambda t)$$

as in Eqn. 3 in the text.

Acknowledgements

We should like to express our thanks to Mr. Bernard Corrow for his help in construction and maintenance of the stopped-flow apparatus. This study has been supported in part by NIH grants GM 00782 and HL 14820.

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