Free Brownian Motion of Individual Lipid Molecules in Biomembranes

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ABSTRACT The mobility of phospolipids in free-standing and supported membranes was investigated on the level of individual molecules. For the analysis of trajectories a new statistical treatment was developed that permitted us to clearly distinguish different types of diffusional motion. A freely diffusing subfraction of lipids within supported membranes was identified. Its mobility was characterized by a mean lateral diffusion constant of $D_{supp} = 4.6\ \mu m^2/s$. In comparison, the mobility of lipids embedded in "free-standing" planar membranes yielded an increase in the mean diffusion constant by a factor of 4.5, $D_{free} = 20.6\ \mu m^2/s$. This increase is attributed to the ultrathin ($\leq$1 nm) lubricating water layer between membranes and glass support.

INTRODUCTION

Advances in ultrasensitive optical microscopy have made possible the detection and characterization of single fluorescence-labeled molecules in biological systems with a positional accuracy down to 50 nm (Betzig and Chichester, 1993; Dunn et al., 1994; Funatsu et al., 1995; Sase et al., 1995; Schmidt et al., 1996; Ha et al., 1996; van Hulst et al., 1997; Jia et al., 1997). Further developments, in particular, on the imaging of individual fluorescence-labeled lipids in phospholipid membranes allowed for a detailed description of the mobility in these systems with a time resolution of milliseconds. It was found that diffusional processes exhibited deviations from free Brownian motion on length scales less than 100 nm. These deviations were interpreted as a consequence of defects in the lipid membrane due to membrane-support interactions (Schütz et al., 1997). We report here, for the first time, a detailed study of the mobility of individual fluorescence labeled lipids in a "free-standing" membrane showing unrestricted Brownian motion. Comparison of the results with those obtained on a freely diffusing subfraction of lipids within a glass-supported membrane allowed the direct measurement of the influence of the ultrathin lubrication water layer on the mobility of membrane components. This water layer might play an important role in studies of immobilized membrane proteins under physiological conditions on interaction-free membranes.

Unsupported phospholipid bilayers have been proved to be an important tool in the investigation of the electrophysiology of purified membrane proteins (White, 1992). In particular, this assembly is predominantly applied for the determination of the regulation mechanisms of ion channels located in internal cell membranes (e.g., of the endoplasmatic reticulum). While the electrophysiology of many membrane proteins is studied to quite an extent, information on the structural implications of functional changes is poor. With the advances of optical techniques as achieved in the current investigation, it seems possible to study simultaneously the structural parameters (e.g., association and reorientation) and functional parameters (e.g., conductance) of ion channels. Such combined studies will give a handle for directly identifying structure-function relationships in membrane proteins.

EXPERIMENTAL

A liquid cell was constructed for simultaneous electrical and optical measurements on "free-standing" membranes (see *inset* in Fig. 2 *A*). The *cis* and *trans* sides of the cell were electrically separated by a 12-μm-thick teflon sheet with a central hole of 80 μm diameter. The sheet was horizontally aligned 150 μm above a glass slide. Symmetrical buffer solutions (250 mM HEPES, 50 mM $Ca(OH)_2$, pH 7.40) were chosen. Planar, free-standing phospholipid bilayers were prepared across the hole by the Müller-Rudin technique (black lipid membranes, BLM) (Montal and Mueller, 1972) from a lipid mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (Avanti) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti) in a ratio of 7:3. This mixture was dissolved in decane to a concentration of 50 mg/ml. Membranes were formed spontaneously upon contact of an air bubble at the tip of a glass capillary with the hole (80 μm diameter). Bilayer formation was carefully controlled by capacitance measurements. The membranes thinned out spontaneously and exhibited a DC conductivity lower than 1 pS. They were free of solvent on visual inspection and as apparent from their specific capacitance of $0.56 \pm 0.10\ \mu F/cm^2$ (White, 1992), given the size of the undisturbed membrane $74 \pm 6\ \mu$m diameter. The lipid mixture contained small amounts of Cy5-labeled lipids (Cy5–1,2-dimiristoyl-*sn*-glycero-3-phosphoethanolamine, Cy5-DHPE, 5×10^{-10} mol/mol; Molecular Probes, Eugene, OR), equivalent to a surface density of fluorescent-labeled lipids of $<0.01\ \mu m^{-2}$.

Supported phospholipid membranes were deposited on glass substrates by the Langmuir-Blodgett technique (Schütz et al., 1997). Lipids were spread from chloroform at the air/buffer (100 mM NaCl, 10 mM NaH_2PO_4, pH 7.5) interface in a monolayer trough (Mayer Feintechnik, Göttingen, Germany). First a lipid monolayer was transferred vertically from the air/buffer interface onto a clean glass slide at a constant surface pressure of 32 mN/m. Subsequently, the monolayer-coated substrate was horizontally brought into contact with the second monolayer compressed to 32 mN/m, containing small amounts (10^{-9} mol/mol) of *N*-(6-tetramethylrhodamine-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine, triethylammonium salt (TRITC-DHPE) (T-1391; Molecular Probes) equivalent to a surface density less than $0.01\ \mu m^{-2}$. The lipid bilayer on the

Received for publication 11 May 1999 and in final form 6 August 1999.

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0006-3495/99/11/2638/05 $2.00

substrate was pushed through the interface and clamped underwater to an open quartz cell.

The detection system has been described elsewhere (Schmidt et al., 1995). In brief, samples were mounted on an epifluorescence microscope (Axiovert 135TV; Zeiss) and illuminated for 3 ms with 630-nm circular polarized light from a dye laser (SP375, rhodamine 6G dye; Spectra Physics) at an intensity of 3.4 kW/cm^2. Images were detected on a slow-scan liquid-nitrogen-cooled CCD camera (AT200, Photometrics; equipped with a Tek512B back-illuminated CCD chip, Tektronix), using a 60× water immersion objective (60×/1.2 W; Olympus). All experiments were performed at 23°C. A typical representation of the data is shown in Fig. 1 *A*. The fluorescence image of an individual fluorescence-labeled lipid molecule is shown. On average, 166 counts were detected from a single molecule, yielding a signal-to-background noise ratio of ~20 (background noise 8 counts RMS). The samples were consecutively observed, with the delay between observations set at 2, 4, and 7 ms. Image analysis was performed automatically, yielding the position of individual lipid molecules with an accuracy of 54 nm. Subsequently, the two-dimensional trajectories were reconstructed as exemplified in Fig. 1 *B* for the molecule seen in Fig. 1 *A*.

RESULTS AND DISCUSSION

Initially, the lipid trajectories were analyzed following a statistical approach published earlier (Schütz et al., 1997). Lateral diffusion of a Brownian particle in a medium characterized by a diffusion constant D is described by the distribution of distances, r, between two observations separated by the time lag, t:

$$p(r^2, t)\mathrm{d}r^2 = \frac{1}{r_0^2(t)} \exp\left(- \frac{r^2}{r_0^2(t)}\right)\mathrm{d}r^2 \qquad (1)$$

where $r_0^2(t) = 4Dt$ (Anderson et al., 1992; Almeida and Vaz, 1992). Integration of Eq. 1 yields the distribution function for the square displacements r^2:

$$P(r^2, t) = \int_0^{r^2} p(r_1^2, t)\mathrm{d}r_1^2 = 1 - \exp\left(\frac{r^2}{r_0^2(t)}\right) \qquad (2)$$

$P(r^2, t)$ is the probability that the Brownian particle stays within a circle of radius r during time t. To evaluate the trajectories, $P(r^2, t)$ was calculated by counting the number of square displacements with values less than or equal to r^2 normalized by the total number of data points for each value of t. In total, 104 trajectories in four different membranes were analyzed, exemplified for $t = 14$ ms in Fig. 2 *A*. The data were fitted according to Eq. 2, yielding the mean square displacement $r_0^2 = 1.43 \pm 0.01$ μm^2. For all time lags a monoexponential behavior was observed. r_0^2 increased linearly with the time lag (Fig. 4, *open circles*) characterized by a diffusion constant for the free-standing membrane of $D_{\mathrm{free}} = 20.6 \pm 0.9$ μm^2/s. This value is higher than that reported from fluorescence bleaching experiments (13.3 ± 0.8 μm^2/s) (Ladha et al., 1996). The difference might be attributed to a difference in length scales of the experiments (1.5 μm here and ~10 μm in Ladha et al., 1996). The latter becomes even more apparent (Vaz and Almeida, 1991) on comparison between nanometer diffusion constants by, e.g., nuclear magnetic resonance and micrometer diffusion constants as presented here.

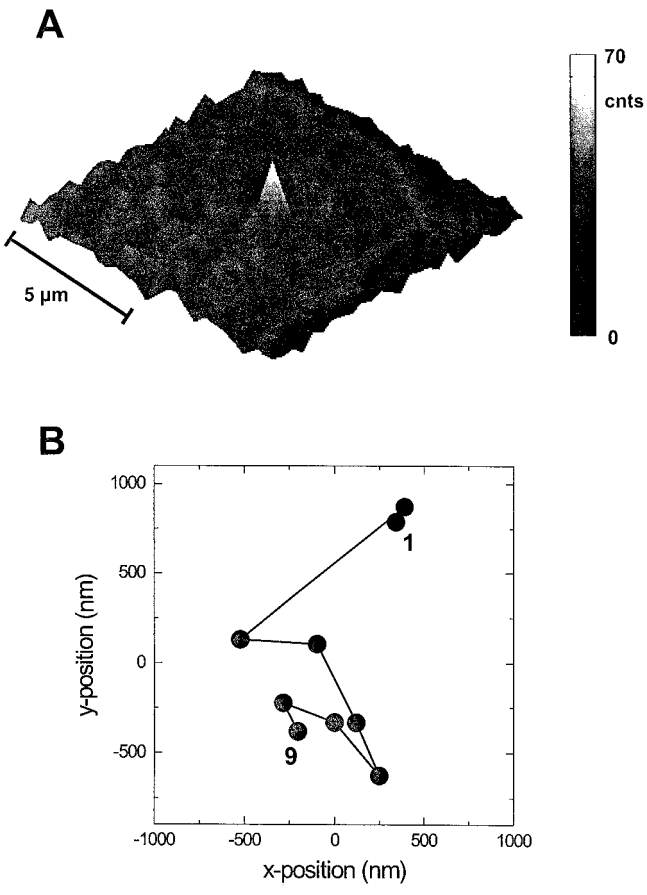

FIGURE 1 (*A*) Fluorescence image of an individual fluorescence-labeled lipid in a phospholipid membrane. The molecule was excited for 3 ms at an intensity of 3.4 kW/cm^2. (*B*) Trajectory of an individual lipid in a free-standing membrane. The time between observations was set at 5 ms.

Individual trajectories were analyzed in the same manner. Diffusion constants were determined for each combination of time steps, which yielded a mean diffusion constant and its variance for every trajectory. The distribution of diffusion constants obtained in such a manner is displayed in Fig. 2 *B*. As predicted, the distribution followed a gamma function (cf. Eq. 1) (Saxton, 1997) with a mean of $D_{\mathrm{free}} = 20.5$ μm^2/s and a variance of $\sigma_{\mathrm{free}}^2 = 153.9$ μm^4/s^2. The latter is given by the uncertainties for determination of diffusion constants from individual trajectories with limited length ($N = 10$ time steps minimum in analysis) (Saxton, 1997).

Equivalent experiments had been performed on supported lipid membranes that were deposited on glass substrates by the Langmuir-Blodgett technique (Schütz et al., 1997) (see *inset* of Fig. 3 *A*). In these samples it was found that the mobility of the lipids was readily characterized by two components. While the slow component presumably was due to lipids adherent to inhomogeneities in the membranes, the mobility of the fast component was suggested to characterize the mobility of freely diffusing molecules in such membranes. For further elucidation a clear distinction between molecules belonging to the fast and slow components

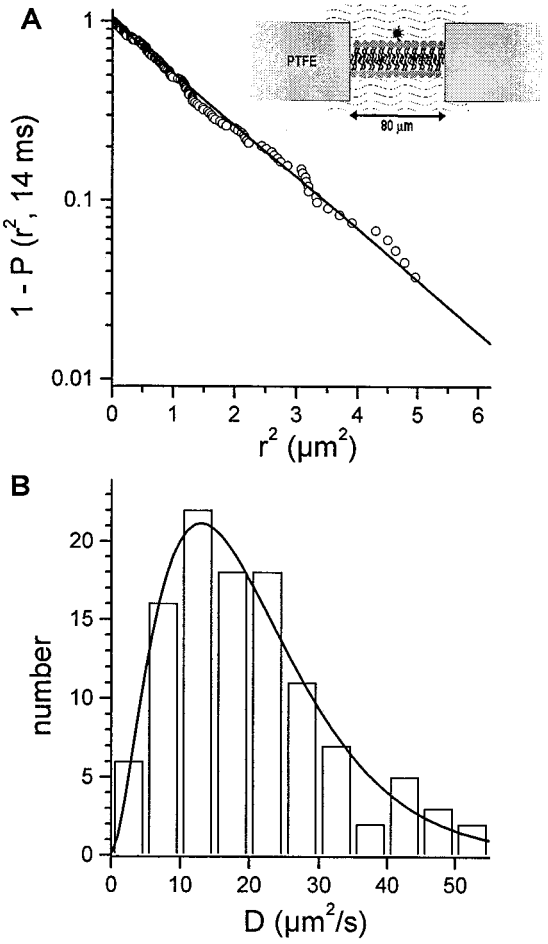

FIGURE 2 Diffusion in a free-standing membrane. (*A*) 104 trajectories for $t = 14$ ms were analyzed according to Eq. 2. The monoexponential behavior is characterized by the mean square displacement of $r_0^2 = 1.43 \pm 0.01$ μm^2. (*B*) Distribution of diffusion constants for individual trajectories. Tha data follow a gamma distribution characterized by a mean $D_{free} = 20.5$ μm^2/s and variance $\sigma^2_{free} = 153.9$ μm^4/s^2 (*solid line*).

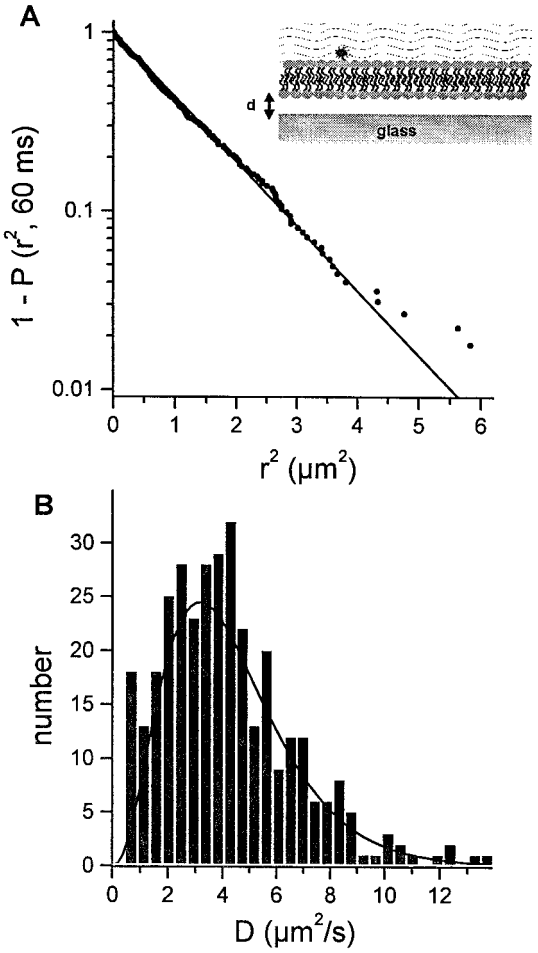

FIGURE 3 Diffusion in a supported membrane. (*A*) 223 trajectories for $t = 60$ ms were analyzed according to Eq. 2. The monoexponential behavior is characterized by the mean square displacement of $r_0^2 = 1.20 \pm 0.01$ μm^2. (*B*) Distribution of diffusion constants for individual trajectories. The data follow a gamma distribution characterized by a mean $D_{supp} = 4.40$ μm^2/s and variance $\sigma^2_{supp} = 5.41$ μm^4/s^2 (*solid line*).

and those that change their mobility during observation was needed. Such reliable criteria are developed in the following paragraph.

The expected increased variance in the diffusion constant within a trajectory for molecules that jump between the fast and slow components appears to be a good figure for such a sorting criterion. However, because of the broad distributions of diffusion constants (see, e.g., Fig. 2 *B*), a simple threshold criterion would fail. By defining $\rho = \ln(r^2/r_0^2)$, the distribution function in Eq. 1 transforms to $p(\rho)d\rho = \exp(\rho - \exp(\rho))d\rho$. The latter is characterized by a standard deviation $\bar{\sigma}_\rho = \pi/\sqrt{6}$, which is independent of D. This unitary value was used for the identification of molecules that stayed within a single mobility component. For each trajectory ρ was calculated for all possible time lags, yielding the mean, $\bar{\rho}$, and standard deviation, $\bar{\sigma}_\rho$. Trajectories with $\bar{\sigma}_\rho < 1.5$ were viewed as representatives of one mobility class. In addition, we selected trajectories that were characterized by an apparent diffusion constant larger than 0.45 μm^2/s. Both criteria reliably ensured that for further analysis only molecules diffusing freely in the membrane were taken into account.

Trajectories with a minimum of $N = 6$ observations were preselected. Sixty-six percent of all trajectories (i.e., 343) were assigned according to the above criteria. Collective analysis of the selected trajectories according to the statistical approach of Eq. 2 yielded distribution functions of the square displacements as shown in Fig. 3 *A* for $t = 60$ ms. A clear monoexponential increase according to Eq. 2, characterized by $r_0^2 = 1.13 \pm 0.08$ μm^2, was observed. The monoexponential behavior, which has been observed for all time lags measured, corroborates the reliability of the selection criteria. Without the selection criteria at least a biexponential increase has to be considered to model the data (Schütz et al., 1997), and the distribution of diffusion constants is much broader. Furthermore, our finding strongly supports the existence of freely diffusing lipids in a supported bilayer on a subsecond time scale as suggested

earlier. The distribution of diffusion constants is shown in Fig. 3 *B*. The histogram follows a gamma distribution characterized by a mean of $D_{supp} = 4.40\ \mu m^2/s$ and a variance of $\sigma^2_{supp} = 5.41\ \mu m^4/s^2$. From the linear increase in r_0^2 with time lag (Fig. 4, *closed circles*) the diffusion constant of freely diffusing molecules in a supported membrane $D_{supp} = 4.6 \pm 0.1\ \mu m^2/s$ was determined in registry with that reported earlier ($4.4 \pm 0.1\ \mu m^2/s$) (Schütz et al., 1997). It is noteworthy that the lateral diffusion constant as determined by photobleaching experiments on supported membranes (Tamm and McConnell, 1985; Kalb et al., 1992) is smaller ($D = 3.5\ \mu m^2/s$) than those reported here and by Schütz et al. (1997). Again, this difference is probably due to the difference in length scales of the experiments (Vaz and Almeida, 1991).

Our results show that the mobility of lipids is a factor of 4.5 slower for freely diffusing molecules in supported lipid membranes compared to those in free-standing membranes (Fig. 4, *open and closed circles*, respectively). The decrease in mobility is attributed to the interaction between the bilayer and the glass support in close proximity. This interaction is probably mediated by the viscosity of a minute hydration layer of thickness d between the support and the membrane (Kühner et al., 1994; Ladha et al., 1996). Its viscosity increases from the value of bulk water, $\eta_W = 1$ cP, with decreasing distance to the support. In turn, the increasing frictional forces on the lipids in the membrane opposing the support decrease their mobility. Because the mobility of lipids was found to be independent on the side of the supported membrane (Tamm and McConnell, 1985), which was due to the strong coupling between the two layers (Evans and Sackmann, 1988), our findings can be qualitatively understood. However, the thickness of this water layer is not exactly known, and values range from 2–8 Å (Koenig et al., 1996) to 20–30 Å (Bayerl and Bloom, 1990; Johnson et al., 1991), as determined by neutron scattering, and between 1 and 50 Å, as determined by optical microscopy (Johnson et al., 1991). Following the description of lipid diffusion in membranes (Saffmann and Dellbrück, 1975) and its extension for supported membranes (Evans and Sackmann, 1988), the diffusion constant is given by (Kühner et al., 1994)

$$D = \frac{k_B T}{4\pi\eta_M} \frac{1}{\epsilon/4 + \epsilon K_1(\epsilon)/K_0(\epsilon)} \qquad (3)$$

$$\epsilon = a\sqrt{\frac{\eta_W}{\eta_M}\left(\frac{1}{d} + \frac{2\eta_W}{\eta_M}\right)}$$

with modified Bessel functions K_0 and K_1 of first and second order, respectively, lipid radius $a = 4.6$ Å (calculated from the mean area per lipid of 60 Å^2), and viscosity of the membrane η_M. We determined the last from the diffusion constant in the free-standing membrane, $D = 20.6 \pm 0.9\ \mu m^2/s$, yielding $\eta_M = (7 \pm 1)\ 10^{-8}$ Pcm. Comparison with the diffusion constant of freely diffusing molecules in the supported membranes, $D = 4.6\ \mu m^2/s$, allows an estimate of the thickness of the lubricating water layer of ≤ 1 nm, which supports the low values obtained by neutron scattering (Koenig et al., 1996). It should be noted, however, that the description of such small layers by a hydrodynamic approach is inappropriate. Thus the theory by Sackmann et al. is used here to determine an upper limit of the layer thickness.

In conclusion, we succeeded in detecting and following the motion of individual fluorescence-labeled molecules in a free-standing phospholipid membrane. The ultrathin water layer between a phospholipid membrane and a solid support was found to be on the order of ≤ 1 nm. This figure clearly demonstrates the necessity of biocompatible layers for the

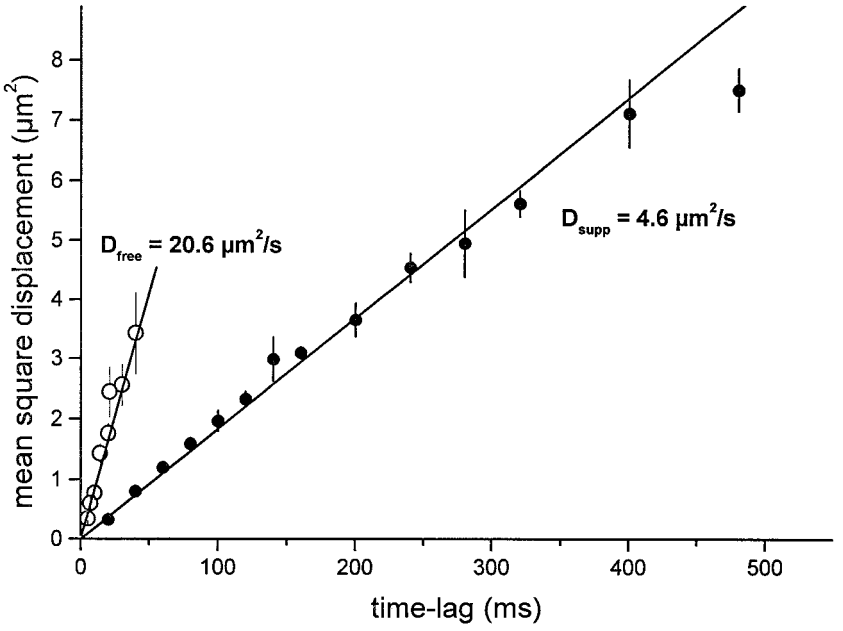

FIGURE 4 Comparison of the diffusion in a free-standing (○) and supported membrane (●). For both, the mean square displacement increases linearly with time lag, yielding diffusion constants of $D_{free} = 20.6 \pm 0.9$ and $D_{supp} = 4.6 \pm 0.1$, respectively.

study of membrane proteins on solid supports. On the other side, it should be noted that "free-standing" membranes, as investigated here, are proven testbeds for the study of membrane proteins in a close-to-native environment. The presented methodology opens up the possibility for simultaneous structural (by optical means) and functional measurements of membrane proteins embedded in "free-standing" membranes. Such experiments are currently under way in our laboratory.

We acknowledge H. Schindler for discussions on mobility in phospholipid membranes and M. Saxton for insights into the statistics of diffusion analysis.

This work was supported by the Austria Research Funds, projects S6610-MED and P12097-PHY.

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