2′-(3α-Benzyloxy-24-norcholan-23-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazoline-N-oxyl as a potential spin probe for model membranes

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A new steroidal doxyl (4,4-dimethyloxazolidine-N-oxyl) nitroxide (SDN) viz. 2′-(3α-benzyloxy-24-norcholan-2′-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazoline-N-oxyl has been synthesized. This is expected to have higher mobility over other spin labels reported earlier. The localization of this spin probe in lipid bilayers has been determined using ^{1}H NMR and ^{31}P NMR techniques. The alterations induced by drugs in the membrane characteristics such as phase transition and permeability have been investigated using electron paramagnetic resonance (EPR) techniques. The results show the applicability of SDN as a potential spin probe in the study of biomembranes. (*Steroids* **59**:377–382, 1994)

Keywords: steroidal doxyl nitroxide; 2′-(3α-benzyloxy-24-norcholan-23-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazoline-N-oxyl; spin label; model membranes; localization; phase transition; permeability; mepivacaine; xylocaine; azelaic acid

Introduction

Several reports which describe syntheses and applications of steroid based nitroxyl spin labels are available in literature.[1,2] These spin labels contain rigidly attached doxyl nitroxide at the site of ketone moiety of steroid molecules and are widely used[1] for oriented multibilayer studies. Earlier we reported[3] the synthesis of steroidal doxyl nitroxide, 2′-(3α-benzyloxy-24-norcholan-23-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazoline-N-oxyl (SDN) with the nitroxide group in the side chain. Such nitroxides are expected to have higher mobility owing to the possibility of free rotation and are likely to exhibit greater versatility as spin probes in spin labeling studies. However, before SDN could be used as a spin probe, it was necessary to ascertain its incorporation and localization in membrane systems. This has been achieved with the help of electron paramagnetic resonance (EPR), ^{1}H NMR and ^{31}P NMR techniques. In the present study, we are reporting the potential applicability of the new nitroxide to study the phase transition and permeability properties of membranes made up of dipalmitoylphosphatidylcholine (DPPC) or egg phosphatidyl choline (EPC) dispersions. We also report the alterations induced by drugs such as the local anesthetics, mepivacaine[4] [N-(2,6-dimethylphenyl)-1-methyl-2-piperidinecarboxa-mide] and xylocaine[5] [2-(diethylamino)-N-(2,6-dimethylphenyl)-acetamide] and the antikeratinizing agent, azelaic acid[6] (1,7-heptanedicarboxylic acid), in the characteristics of model membranes.

Experimental

Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma Chemical Company, U.S.A. EPC was isolated and purified by the method of Keough[7]. Spin label SDN was synthesized in the laboratory (Scheme 1). Azelaic acid was obtained from Serva Fienobiochimica, (Heidelberg, Germany). Mepivacaine and xylocaine were used as their hydrochloride salts and were purchased from Astra Pharmaceutical Products, (Westborough, MA). Sodium ascorbate was obtained from Sisco Research Laboratories, India and other reagents used were of analytical grade.

Multilamellar dispersions of lipid used for the phase transition experiments were prepared following Hill's method.[8] Chloroform solutions of appropriate quantities of lipid (100 mM) and spin label (1 mM) were evaporated to dryness under a stream of nitrogen gas. The film was dried under vacuum for 3–4 h and then hydrated with appropriate amount of 10 mM phosphate buffer (pH 7.5) containing the desired

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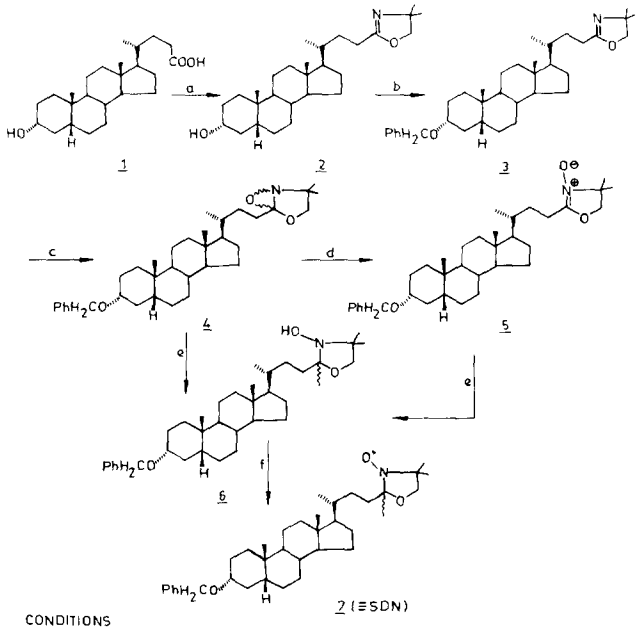

Scheme-I

amount of drug. The system was allowed to equilibrate for 30 min before vortexing.

For permeation experiments unilamellar vesicles were used. Unilamellar vesicles were prepared by sonicating multilamellar dispersions immersed in an ice bath using B-30 sonifier fitted with microtip (Branson Sonic Power Co.) at a duty cycle of 50%. Sonication was carried out until optical clarity was achieved. The preparations were centrifuged for 10 min at 20,000 × *g* (16,000 rpm) using Sorvall RC 5B centrifuge to separate out the titanium particles. Homogeneity of vesicle size of the phospholipid dispersions was analyzed by Sepharose 4B chromatography. Uniformity of vesicle size was checked on electron microscope. Vesicles of radii around 250 Å are formed under such conditions.[9] Electron microscopic studies were conducted using Jeol JEM 100 S electron microscope at a high voltage of 60 kV. The samples were prepared by placing a drop of suspension on a thin film of formvar-coated copper slot grid which was allowed to dry before placing under the microscope. Sodium ascorbate (5 mM) was added to the samples just before recording the EPR spectrum (t = 0).

Methods

EPR experiments were carried out on an X-band E-112 Varian Spectrometer with a 100 KHz field modulation and detection unit. Samples were taken in 50 μL glass capillaries sealed at one end and mounted in the variable temperature accessory of the Varian E-112 Spectrometer. Temperature could be controlled to an accuracy of ±1 C using a Varian V-4540 unit. The sample temperature was determined using a copper-constantan thermocouple kept in contact with the sample capillary.

^1H and ^{31}P NMR spectra of sonicated liposomes in D_2O were recorded using Varian VXR 300S NMR Spectrometer and Bruker AM-500 FT-NMR Spectrometer interfaced with an Aspect 3000 computer. The ^{31}P NMR spectra were obtained under proton decoupling and a line broadening of 20 Hz has been employed.

Results and Discussion

Synthesis of nitroxide (SDN) and its incorporation in liposomes

The SDN, 2′-(3α-benzyloxy-24-norcholan-23-yl)-2′,4′,4′-trimethyl-4′,5′-dihydrooxazoline-N-oxyl **7** was synthesized in an overall yield of 16.3% (Scheme 1) from lithocholic acid (3α-hydroxycholanoic acid) **1**. The nitroxide **7** (SDN) could also be obtained in an improved yield of 27.8% by a novel methodology involving a Grignard reaction of an oxaziridine **4**. The details of the synthesis have been reported elsewhere.[3]

The EPR spectrum of the nitroxide SDN in rapidly tumbling state, i.e., as dilute solution in chloroform (10^{-5} M), consists of a sharp triplet (Figure 1a). The observed isotropic hyperfine coupling constant is 14.75 G. The EPR spectrum of SDN in multilamellar dispersions of lipid (Figure 1b) exhibits a difference in the shape of the lines at the highest and the lowest field. This can be attributed to the anisotropy of the nuclear hyperfine coupling tensor T and the g tensor of the nitroxide radical in the membrane. The hyperfine coupling constant observed in this case is 14.10 G. An increase (by 28.5%) is observed in the line width of the lowest field line in the EPR spectrum of the nitroxide SDN incorporated in liposomes, as compared to the line width of the same line in the EPR spectrum of SDN in rapidly tumbling state. The changes in the line shapes, hyperfine coupling constant, and the line width of the EPR spectra of SDN in liposomes suggest the definite incorporation of the nitroxide in the model membranes.

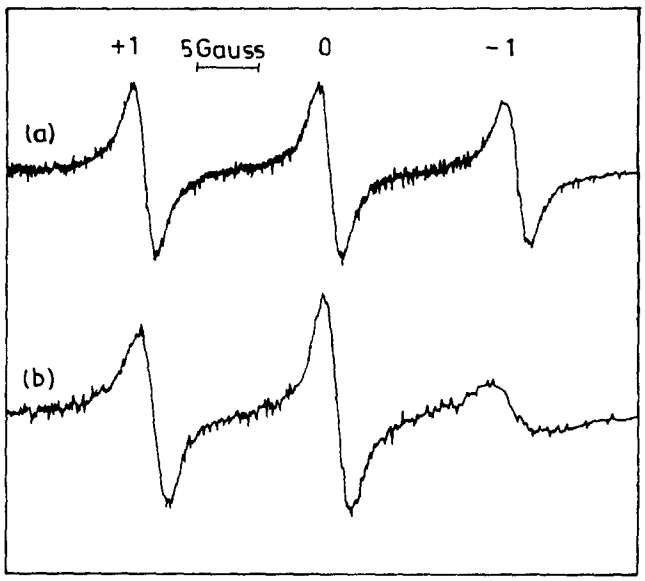

Figure 1 EPR Spectra of (a) spin label SDN in chloroform (10^{-5} M), (b) SDN incorporated into multilamellar vesicles of EPC. The concentrations of lipid and spin label are 100 mM and 1 mM, respectively.

The near isotropic EPR spectrum of the spin label SDN in liposomes can be attributed to two factors. Firstly, the doxyl nitroxide moiety is in the flexible side chain of the steroid and hence exhibits higher mobility than that of the rigidly attached 3-doxyl substituted steroids. Secondly, it can also be explained with the model proposed by Sackmann and Träuble.[10] As per this model, the spin label when incorporated into liposomes creates large pockets of free volume around its site of incorporation, particularly below the phase transition temperature (T_m) of the lipid. Even above T_m these pockets of free volume continue to exist. This allows the steroid nucleus to have high degree of motional freedom whereby the nitroxide undergoes fast tumbling. Owing to these two factors, the EPR spectrum of the spin label SDN in liposomes (Figure 1b) exhibits an intermediate situation of the two extreme cases viz. freely tumbling spin labels in organic solvents and restricted anisotropic motion.

Localization of spin label SDN in model membrane

Information regarding localization of the spin label has been obtained from NMR experiments. It is known that the NMR resonances arising from the nuclei (^1H, ^{31}P) located in the close proximity of the paramagnetic moiety (≤ 10 Å) experience line broadening due to dipole-dipole interactions[11]. We have recorded ^1H NMR and ^{31}P NMR spectra of EPC sonicated liposomes with and without incorporation of spin label at two different temperatures. The assignments of the lipid peaks have been made using the data reported in the literature.[12] It has been observed that on incorporating the spin label in liposomes, ^1H resonances of the different regions of the lipid molecule undergo line broadening to different extents (Table 1). The increase in the peak width observed for the N^+Me_3 group is the largest. This indicates that the site of residence of the doxyl nitroxide moiety is possibly close to the N^+Me_3 group. Such a mode of localization is in concurrence with the polarity profile of lipid bilayers and is also expected considering the substituents present in the molecule. The ^1H NMR signal for the terminal methyl group also shows line broadening, though to a smaller extent. This indicates that the doxyl group also spends a significant fraction of the time near the terminal methyl group.

The line broadening observed in the case of the terminal methyl group can be due to two factors. Firstly, the flexibility of the steroidal side chain keeps the nitroxide moiety in the neighborhood of the terminal methyl groups as well. Secondly, EPC is a mixture of lipid molecules having 16–22 carbon atoms[13] in the hydrocarbon chains. Moreover, there are double bonds in the chains at various positions leading to shorter effective chain lengths. Rotational isomerism about single bonds in the hydrocarbon chains is also possible.[14] As a result, the terminal methyl groups are located at various depths of the bilayer and experience the influence of the nitroxide group to different extents. Since the ^1H NMR signal observed for the terminal methyl is an average of the resonances of all the methyls, it is evident that the net effect is an increase in the line width of the terminal methyl group. Supporting evidence for the localization of SDN in lipid bilayers is also obtained from ^{31}P NMR spectrum. The full width at half maximum ($\Delta\nu_{1/2}$) for the ^{31}P NMR line of liposomes incorporated with SDN also revealed an increase by 6.6% (Table 1) compared to pure liposomes.

Phase transition studies

Phase transition behavior of DPPC dispersions has been studied using SDN as the spin label. It is customary to use h_{+1}/h_0 (i.e., the ratio of the height of the low field line to that of the central line in the EPR spectrum of the nitroxide) as a parameter which relates to the changes in membrane fluidity.[15,16] Earlier investigations[17] have reported that this parameter contains contributions from both order as well as mobility. The values of the parameter h_{+1}/h_0 at various temperatures are plotted (Figure 2). The parameter shows initial gradual increase with increase in temperature which is followed by an abrupt, large change which levels off at higher temperatures. This change corresponds to the transition of lipid hydrocarbon chains from the gel to liquid crystalline phase.

Analyses of the phase transition plots reveal that the main transition for pure DPPC dispersions occurs at

Table 1 Effect of incorporation of spin label SDN on the ^1H NMR and ^{31}P NMR spectra of EPC liposomes

	Full width at half maximum ($\Delta\nu_{1/2}$ in Hz)				
	22 C N^+Me_3	(^1H NMR) terminal $-CH_3$	32 C N^+Me_3	(^1H NMR) terminal $-CH_3$	^{31}P NMR
Pure EPC $(\nu_{1/2})_L$	6.25	18.75	6.25	18.75	750
EPC + spin label $(\nu_{1/2})_{LS}$	15.00	31.25	12.50	25.00	800
$\frac{(\Delta\nu_{1/2})_{LS}}{(\Delta\nu_{1/2})_L}$	2.4	1.66	2.00	1.33	1.06

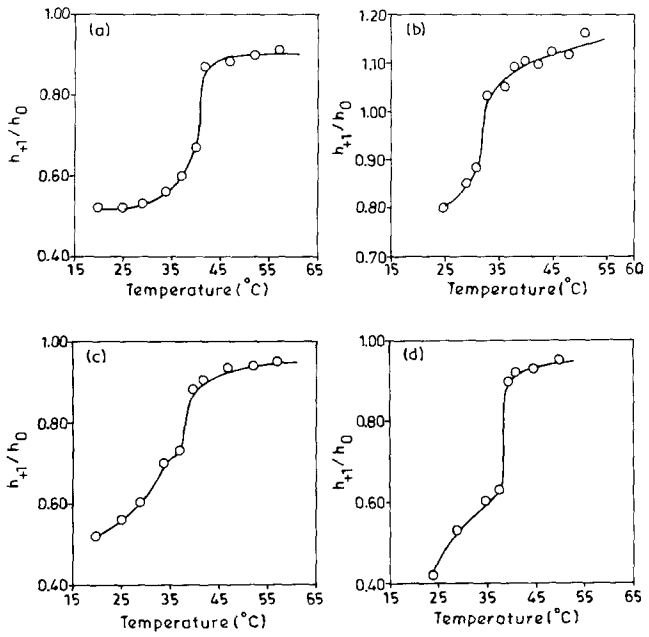

Figure 2 Spectral parameter h_{+1}/h_0, as a function of temperature, (a) pure DPPC (100 mM), (b) DPPC (100 mM) + mepivacaine (40 mM), (c) DPPC (100 mM) + xylocaine (40 mM), and (d) DPPC (100 mM) + azelaic acid (40 mM), using SDN (1 mM) as the spin label.

41 C and pretransition at 35.5 C. It is pertinent to note that these values are in agreement with those reported by other methods[6] as well as by using other spin labels.[18] This confirms that the newly synthesized spin label is suitable as an EPR sensitive probe for phase transition studies. In the presence of the drugs mepivacaine, xylocaine, and azelaic acid, we observe that the characteristic features of phase transition curves of DPPC dispersions are retained (Figure 2). The significant lowering of the main phase transition temperature (Table 2) to an extent of about 9 C in the presence of mepivacaine could be attributed to its affinity to bind to the hydrophobic phase of the lipid.[19] However, in the presence of xylocaine, the main phase transition temperature of DPPC vesicles was affected only marginally. It is pertinent to note that Hubbell et al[20] and Rosenberg et al[21] have reported similar findings in the case of xylocaine. On the other hand, we observe that in the presence of azelaic acid the main phase transition temperature remains nearly unaffected. This could possibly be explained by the fact that azelaic acid, having

Table 2 Phase transition temperatures of DPPC dispersions in presence of and in absence of drugs using SDN as the spin label

Type of vesicles	Pretransition temperature (C)	Main transition temperature (C)
Pure DPPC	35.5	41.0
DPPC + mepivacaine	Not observable	32.0
DPPC + xylocaine	32.0	38.0
DPPC + azelaic acid	32.0	39.0

The molar ratios of DPPC : spin label : drug are 100 : 1 : 40.

two carboxylic acid groups, remains bound to the head group of the lipid and does not alter much the hydrophobic interior. A decrease of 3.5 C in the pretransition temperature which corresponds to tilting of head group, supports the binding of the drug to the head group. Our inferences in the case of azelaic acid are in agreement with the results obtained by Bossi et al.[6]

Permeability studies

Permeability of model membranes has been studied by monitoring the reduction of the spin label SDN by ascorbate ions added externally to the water phase.[22] The rate of reduction of the bilayer-fixed spin label SDN has been determined as the ascorbate ions diffuse through the bilayer. The reduction of the spin label has been monitored by observing the changes in the height of the low-field line in the EPR spectrum as a function of time. The EPR signal heights decrease as shown in Figure 3. It is pertinent to note that though Kornberg and McConnell[23] observed that the ascorbate ions do not penetrate the EPC bilayer at 0 C, the present permeation experiments have been carried out at 30 C. This temperature is conducive to the permeation of ascorbate ions through the bilayer, as is well documented in the literature.[22]

Let the rate constants for the reduction of the spin labels present in the outer and the inner monolayer be k_o and k_i, respectively. The number of spin labels in the outer and inner monolayers of the vesicle is directly related to the respective surface areas, $4\pi r_o^2$ and $4\pi r_i^2$, where r_o and r_i are the radii of the respective monolayers. In the plots depicted in Figure 3 the points represent experimental points through which theoretical curves are drawn by fitting the data to equation 1.

$$S(t) = S_o(0)e^{-k_o t} + S_i(0)e^{-k_i t} \quad (1)$$

where S(t) is the signal height due to total spin label present at time t, $S_o(0)$ and $S_i(0)$ are signal heights due to initial concentration of spin labels present in the outer and the inner monolayer respectively. The values of k_o and k_i have been obtained by using equations 2 and 3.

$$S(0) = S_o(0) + S_i(0) \quad (2)$$

$$S_o(0)/S_i(0) = r_o^2/r_i^2 \quad (3)$$

It has been reported[9] that the outer radius of the sonicated vesicles is around 250 Å and that the thickness of bilayers is around 50 Å. Taking into account these facts, the value of $S_o(0)/S_i(0)$ can be determined. The values of k_o and k_i are obtained by least square fitting of the data utilizing equation (1).[18] The half-life times for the reduction of spin labels deduced from k_o and k_i are listed in Table 3. It is interesting to note that the time-scales of our permeation experiments are much shorter than that of flip-flop experiments of Kornberg and McConnell.[23] Since these authors have shown that the asymmetry in the distribution of spin labels between the bilayers decays with a half-life of about 6.5 h at 30 C, the flip-flop motion from the inner monolayer to the outer monolayer is clearly a very slow process. Hence, the flip-flop motion is highly unlikely in

the present case and has not been considered in the interpretation of our results.

For pure EPC, the half-lives for the reduction of spin labels present in the outer and the inner monolayers show a very large difference. This is attributed to the fact that the ascorbate ions, when introduced on the outer side of the EPC vesicles, diffuse through the bilayer and reduce the spin labels. The spin labels residing in the outer monolayer are easily accessible and therefore undergo reduction at a faster rate as compared to spin labels in the inner monolayer. One observes that the half-life times for reduction of spin labels in the outer and inner monolayers of EPC incorporated with mepivacaine are of the same order as observed in the case of EPC dispersions. This indicates that incorporation of mepivacaine does not alter permeability profile of the lipid matrix. The observed higher value of half-life time for reduction of spin labels residing in the outer monolayer in the presence of xylocaine can be attributed to binding of the drug to the head group of the lipid which, in turn, obstructs permeation. However, a decrease in the half-life time for reduction of spin labels in the inner monolayer indicates enhanced accessibility of the interior of the lipid matrix to ascorbate ions.

We therefore conclude that the newly synthesized nitroxide SDN can be easily incorporated in the membranes and can be conveniently used as a potential spin probe for studies such as phase transition, permeability, etc., of membranes.

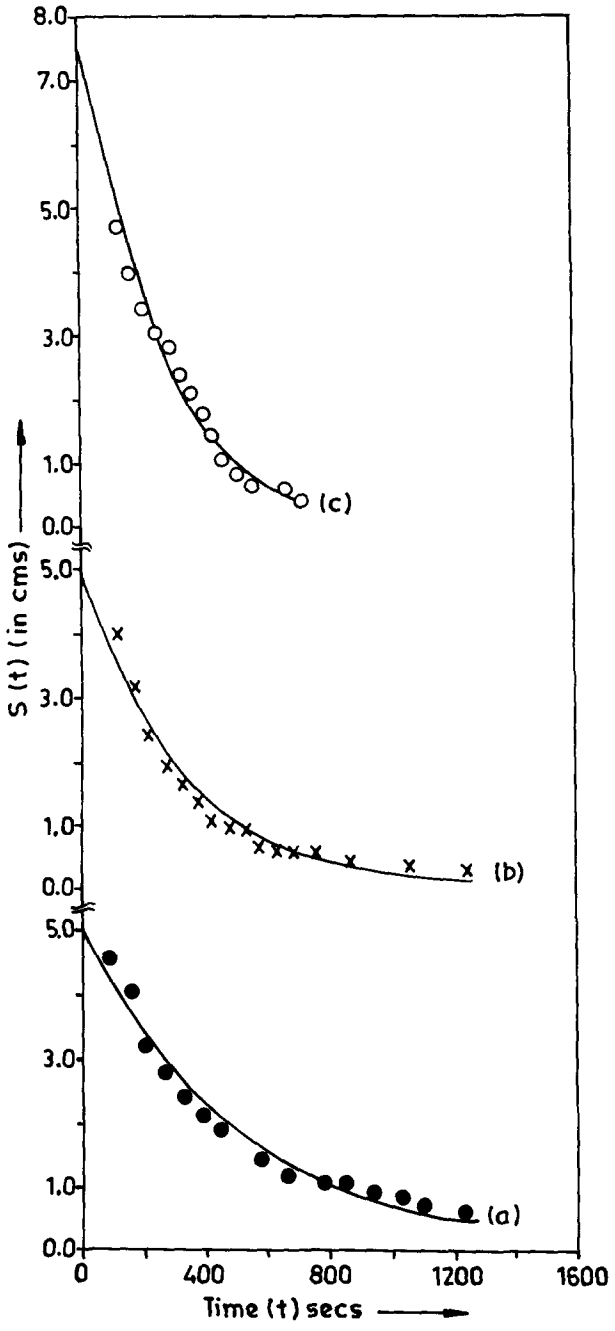

Figure 3 Signal height S(t) of the EPR spectral line of spin label SDN (1.0 mM) versus time. The points shown are experimental points through which theoretical curves have been drawn after fitting the data to $S(t) = S_o(0)e^{-k_o t} + S_i(0)e^{-k_i t}$, where $S_o(0)$ and $S_i(0)$ are respective initial signal heights due to the spin label in the outer and inner monolayers of sonicated vesicles. (a) (●), pure EPC (100 mM); (b) (×) EPC (100 mM) + mepivacaine (40 mM), and (c) (○) EPC (100 mM) + xylocaine (40 mM).

Table 3 Half-life times (min) for the reduction of spin label SDN incorporated in EPC bilayers in presence of different drugs at 30 C

	Pure EPC	EPC + mepivacaine	EPC + xylocaine
Outer monolayer	0.07	0.08	2.05
Inner monolayer	5.83	3.55	3.26

Permeating agent used was sodium ascorbate.

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