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Studies on the interaction of bacterial lipopolysaccharide with cationic dyes by absorbance and fluorescence spectroscopy

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Abstract

The interaction of cationic dyes, pinacyanol chloride, acridine orange and safranin O, with *Klebsiella* O3 lipopolysaccharide has been investigated by spectrophotometric and spectrofluorometric measurements. A strong metachromasy is induced by the acidic polysaccharide in pinacyanol chloride as revealed from the appearance of the blue-shifted absorption band at 495 nm from 600 nm. The stoichiometry of the polymer/dye in the metachromatic compound, the thermodynamic parameters of interaction and the effects of different cosolvents on the stability of complex have been studied. Quenching of fluorescence of acridine orange and safranin O by the lipopolysaccharide has also been studied. The chromotropic property of the biopolymer has been established by the spectral results. © 1997 Elsevier Science S.A.

Keywords: Dye-polymer interaction; Metachromasy bacterial lipopolysaccharide; Chromotropic property; Fluorescence quenching; Spectral properties

1. Introduction

The photophysical processes in absorbance and fluorescence properties of dye molecules play an important role in understanding the mechanism of many molecular complexes formed with dye molecules [1,2]. Interaction of cationic dyes with polyanions usually causes induction of metachromasy [3]. Formation of a metachromatic compound is characterized by the appearance of a blue-shift in the visible absorption spectrum of the dye [4]. The shape of the spectra, however, depends on the conformation of the polyanion as well as on the dye/polymer stoichiometry of the compound formed between them.

Lipopolysaccharides (LPS) are the integral constituents of the outer membrane of gram-negative bacteria [5]. These are amphiphilic in nature and posses endotoxic properties [6]. Structures of different *Enterobacteriaceae* have been extensively investigated during the last few decades. LPS consist of hydrophilic polysaccharide and lipophilic lipid moieties. The polysaccharide part comprises two distinct portions: core oligosaccharide and a polysaccharide chain of several repeating units, termed the O-specific chain. The lipid moiety, termed as lipid A, consists of diphosphoryiated glucosamine disaccharide, carrying a number of higher fatty acid chains. The presence of covalently bound phosphate groups, acidic sugars (KDO) and fatty acyl groups in the core and lipid A makes the LPS a highly anionic polyelectrolyte. As a whole, it forms a rigid highly charged surface. The isolated LPS has a wide range of immunobiological and pathophysiological activities. Such biological activities largely depend on the physical state of the LPS. The LPS is highly aggregated in aqueous medium and the variations in size and shape of these aggregates causing altered conformation may modify the biological activities. It is, therefore, of great importance to study the physical nature of the LPS which has yet to be adequately resolved. The amphiphilic nature as well as highly anionic polyelectrolytic behavior of the LPS offer potential sites for interaction with other molecules. Studies on binding of polycationic antibiotic and polyamines to LPS by ESR spectroscopy and electron microscopy are available in the literature [7–10]. Physical properties of LPS aggregates have been studied by complex formation of LPS with bovine serum albumin (BSA) and also by other methods [11–15].

Studies on binding of cationic dye to different synthetic and natural polyanions by absorbance, fluorescence and circular dichroism experiments during the last few decades yielded significant contributions in the field of dye–polymer interactions [16–18]. During the last few years, chromotropic properties of different bacterial capsular polysaccharides with respect to induction of metachromasy in different cationic dyes have been investigated in our laboratory [19–21]. But no work has yet been reported in the literature on the interaction of bacterial LPS with dye. It was, therefore,

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considered to be very important to also study the binding phenomenon of cationic dyes to the amphiphilic polyelectrolyte LPS. The results of such dye–LPS interactions are expected to yield additional informations on the properties of these biologically active LPS molecules in solution.

When an anionic polymer is added to a metachromatic dye, the monomeric dye cations bind to the anionic sites of the polyanion causing aggregation [22] and the blue-shifted band appears at the cost of the absorbance of the monomeric band. When a cationic fluorescent dye is added, the fluorescence is also quenched as a result of interaction with the polyanion. The present investigation deals with the interaction of the *Klebsiella* O3 lipopolysaccharide with pinacyanol chloride dye carried out by absorbance studies and also with acridine orange and safranin O carried out by fluorescence measurements. The spectral properties and thermodynamic parameters of the dye–LPS interaction are presented in this paper.

2. Experimental

The *Klebsiella* O3 (smooth) test strain was obtained from Dr. S. Schelecht of Max Planck Institute for Immunobiology, Freiburg, Germany. The dyes pinacyanol chloride, acridine orange and safranin O were purchased from Sigma Chemical Co., USA. The bacterial cells were grown in nutrient agar medium, harvested, dried and lipopolysaccharide was isolated and purified by phenol/chloroform/light petroleum according to the method of Galanos et al. [23]. Neutral sugars, GlcN, KDO, phosphate and fatty acids were analyzed as described by Kaca et al. [24].

Absorption spectra were recorded using a Milton Roy Spectronic-21D spectrophotometer. Concentrations of the aqueous solutions of dye and LPS were in the range of 10^{-6} to 10^{-4} M; one mole of LPS referred to the average mass of LPS containing one anionic charge group.

Fluorescence measurements were performed on a Shimadzu RF-5000 spectrofluorometer using acridine orange and safranin O dye solutions in the range of 10^{-6} to 10^{-5} M.

The equivalent weight of the LPS was determined by spectrophotometric and spectrofluorometric titration techniques [25,26]. Stoichiometry of the LPS–dye complex was determined by the isolation method of MacIntosh [27] and also by the centrifugation method [28]. Thermodynamic parameters of interaction were evaluated using Rose and Drago equation by measuring absorbance of the dye solution as well as that of the dye–polymer mixtures at different temperatures. General experimental details were the same as described earlier [29].

3. Results

Klebsiella O3 lipopolysaccharide is a heteropolymer consisting of O-specific polysaccharide, core oligosaccharide and lipid A. The complete primary structure of the LPS is not yet known. The O-specific chain is a homopolysaccharide consisting of *D*-mannose as the sole sugar unit [30]. The presence of KDO, phosphate group and higher fatty acid chains makes the LPS a unique polyelectrolyte offering potential anionic sites for interaction with dye cations. The equivalent weight of the LPS which is its mass containing one anionic group on the average, was determined by metachromatic titration [25,26]. The LPS induced strong metachromasy in the cationic dye pinacyanol chloride as a result of which a blue-shifted metachromatic band appeared at 495 nm at the cost of monomeric band at 600 nm (Fig. 2). Increasing amounts of the LPS solution (0.01–0.2 ml, 1.0×10^{-3} g ml^{-1}) were added to a fixed volume of pinacyanol chloride solution (1.0 ml, 10^{-4} M) in different sets of experiments and the total volume was made up to 10 ml by adding water in each case. The intensity was measured at 600 nm (α-band of the dye) which decreased progressively upon addition of increasing amounts of LPS until the point of equivalence for the dye cations and the polyanions was attained. Beyond this equivalence point the change in absorbance became less significant. The results are shown in Fig. 1. From the point of intersection of the two linear curves, the volume of the LPS solution required for equivalent consumption of the dye was calculated. Assuming 1:1 stoichiometry of the dye–polymer complex as revealed from the appearance of an isobestic point at 540 nm in the absorption spectra (Fig. 2), the equivalent weight of the LPS was found to be 820. One mole of LPS referred to this gram-equivalent mass in the present work.

The molar mass of the LPS as defined above was also determined by spectrofluorometric titration of the fluorescent dyes acridine orange and safranin O with the LPS in dilute solution. The quenching phenomenon was utilized for carrying out such fluorometric titration. The results are shown in Fig. 1 along with the spectrophotometric titration curves. The calculated molar mass of the LPS was found to be 880 with acridine orange and 790 when safranin O was used.

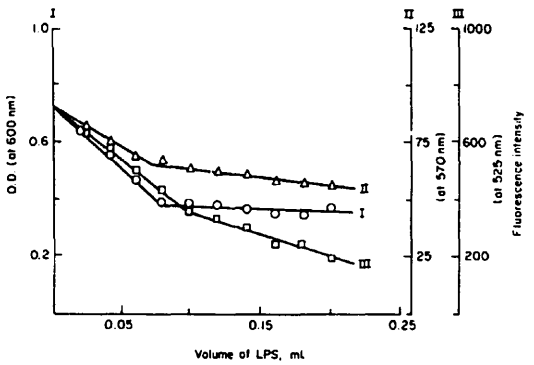

Fig. 1. Spectrophotometric titration of pinacyanol chloride (○) and spectrofluorometric titration of acridine orange (□) and safranin O (△) with *Klebsiella* O3 LPS at 298 K. Dye conc., 1.0×10^{-5} M; LPS conc., 1.0×10^{-3} gm cm^{-3}.

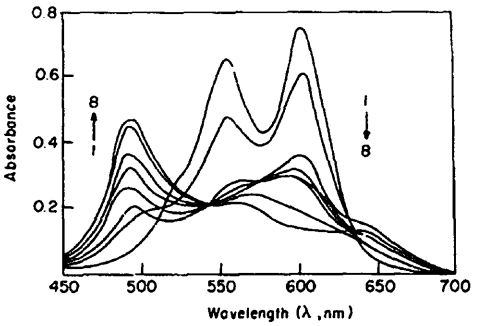

Fig. 2. Absorption spectra of pinacyanol chloride–*Klebsiella* O3 LPS at different LPS/dye (P/D) molar ratios at 298 K. Dye conc., 1.0×10^{-5} M. P/D: (1) 0.0; (2) 0.21; (3) 0.42; (4) 0.83; (5) 1.66; (6) 2.49; (7) 4.15; (8) 8.30.

From these three results average equivalent weight of the LPS was taken as 830 for further calculations.

Absorption spectra of the aqueous solution of pinacyanol chloride (1.0×10^{-5} M) upon addition of the LPS at different LPS/dye molar ratios (P/D = 0.0 ~ 10.0) are shown in Fig. 2. The pure dye solution showed two peaks at 600 nm and 550 nm corresponding to monomeric (α-band) and dimeric (β-band) forms respectively. On addition of the LPS, intensities of both the peaks decreased and a new blue-shifted metachromatic band (μ-band) appeared at 495 nm. At higher P/D value the spectral character changed significantly; a strong μ-band appeared at 495 nm with almost complete removal of the α-band and β-band. The absorption spectra also showed an isobestic point at 540 nm.

Stoichiometry of the LPS–dye complex was determined according to the method of MacIntosh [27]. Increasing amounts of the LPS solution (0.1–1.0 ml, 10^{-4} M) were added to 10 ml portions of pinacyanol chloride dye solution (1.0×10^{-5} M). The metachromatic compound was separated out from the aqueous phase by extraction with petroleum ether and the uncomplexed dye concentration was measured spectrophotometrically. The values of the complexed dye concentrations were plotted against the concentration of added LPS. The results are shown in Fig. 3. From the point of intersection of the two linear curves the stoichiometry of the LPS and dye in the LPS–dye compound was determined. Stoichiometry was also determined by the centrifugation method [28] in which the metachromatic LPS–dye compound was removed by centrifuging the solution; the supernatant containing free dye was analyzed colorimetrically. The results are shown in Fig. 3 along with the results obtained by MacIntosh method. Both the methods yielded identical results of stoichiometry (1:1).

The effects of different alcohols methanol, ethanol, n-propanol, n-butanol and other cosolvents, DMSO, DMF and urea, on the stability of LPS–dye complex have been studied by measuring absorbance of the solution at the α-band and also at the μ-band. Absorbance at 600 nm (α-band) increased with the increase in concentration of the added cosolvent and finally reached the constant value corresponding to that of the pure dye solution. Absorbance at 495 nm (μ-band), however, decreased simultaneously and reached a constant minimum value. The results are shown in Figs. 4 and 5. At a certain concentration of added cosolvent, disappearance of the μ-band indicated complete reversal of metachromasy. Amongst the alcohols, the ability of breaking the LPS–dye complex followed the order: methanol < ethanol < n-propanol < n-butanol. DMSO, DMF and urea were also found to be quite effective in causing reversal of metachromasy.

The interaction constant (K_C) between the LPS and pinacyanol chloride was determined by measuring absorbance of the LPS–dye mixture (A) at the metachromatic band (495 nm) at four different temperatures taking different sets of solutions containing varying amounts of LPS (C_S) in a fixed volume of the dye solution; the absorbance (A_O) of the initial

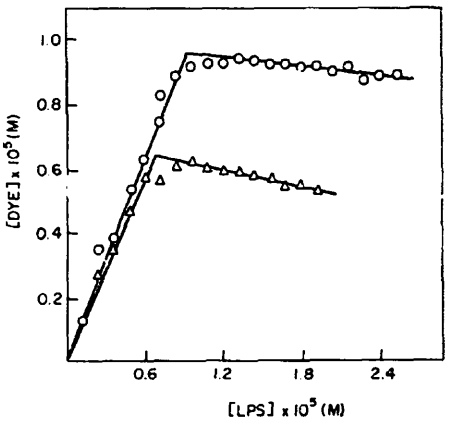

Fig. 3. Stoichiometry of *Klebsiella* O3 LPS/pinacyanol chloride at 298 K. MacIntosh method (△); centrifugation method (○).

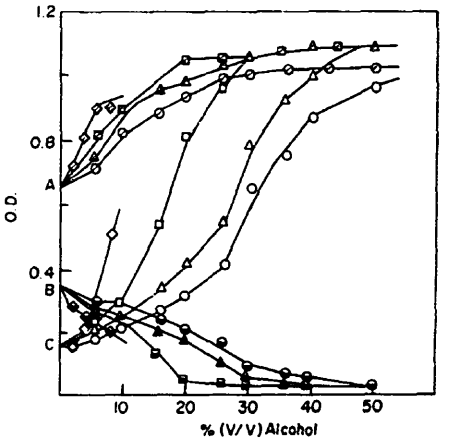

Fig. 4. Reversal of metachromasy in *Klebsiella* O3 LPS/pinacyanol chloride complex (dye conc., 1.0×10^{-5} M; P/D, 5.0) by the addition of methanol (○); ethanol (△); n-propanol (□) and n-butanol (◇) at 298 K. (a) Absorbance of the pure dye at 600 nm, (b) absorbance of the dye–LPS mixture at 495 nm and (c) absorbance of the dye–LPS mixture at 600 nm.

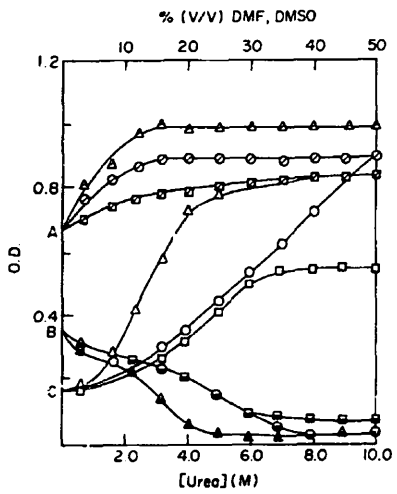

Fig. 5. Reversal of metachromasy in *Klebsiella* O3 LPS/pinacyanol chloride complex (dye conc., 1.0×10^{-5} M; P/D, 5.0) by the addition of DMSO (○); DMF (△) and urea (□) at 298 K. (a) Absorbance of the pure dye at 600 nm, (b) absorbance of the dye–LPS mixture at 495 nm and (c) absorbance of the dye–LPS mixture at 600 nm.

concentration of the dye (C_D) was also measured at 495 nm. Absorbance results were treated using the Rose and Drago equation [31]:

$$\frac{C_D \cdot C_S}{(A-A_0)} = \frac{1}{K_C \cdot L(\epsilon_{DS}-\epsilon_D)} + \frac{C_S}{L(\epsilon_{DS}-\epsilon_D)} \qquad (1)$$

From the slope and intercept of the linear line obtained by plotting $C_D C_S/(A-A_0)$ against C_S, the value of K_C was evaluated in each case (Fig. 6). The thermodynamic parameters of interaction such as enthalpy of complex formation (ΔH), free energy change (ΔG) and entropy change (ΔS) were also calculated. The results are given in Table 1.

Fluorescence studies on interaction of the LPS with acridine orange and safranin O dye were carried out by adding increasing amounts of the LPS to a fixed volume of the dye in separate experiments. The intensity of fluorescence of the dye was quenched in both cases. Acridine orange solution was excited at 440 nm (λ_{ex}) and fluorescence intensity was recorded at 520 nm (λ_{em}). When safranin O was used the solutions were excited at 526 nm (λ_{ex}) and fluorescence was measured at 570 nm (λ_{em}). The fluorescence spectra are shown in Fig. 7.

The binding constant between the LPS and the dye was determined by treating the fluorescence data with the Stern–Volmer equation [32]. The ratio of the fluorescence intensity of the dye solution (F_0) to the intensity of the LPS–dye mixture (F) was plotted against the concentration of the added LPS which acted as the quencher (Q). The plots are shown in Fig. 8. The fluorescence data satisfied the Stern–Volmer equation $F_0/F = 1 + K_{SV}[Q]$. The value of the binding constant (K_{SV}) was determined from the linear plot.

4. Discussion

Induction of metachromasy in cationic dye pinacyanol chloride by the *Klebsiella* O3 lipopolysaccharide was clearly observed in the visible absorption spectra (Fig. 2). A metachromatic blue shift of about 105 nm indicated induction of strong metachromasy. The spectral changes were studied during interaction of the dye cations with the polyanions at different LPS/dye molar ratios. The equivalent weight of the LPS, referred as its mass containing one anionic group on average, was successfully determined by metachromatic titrations (Fig. 1). In the interaction between anionic polymer and cationic dye, the monomeric dye cations bind at the anionic sites of the polymer causing aggregation which is

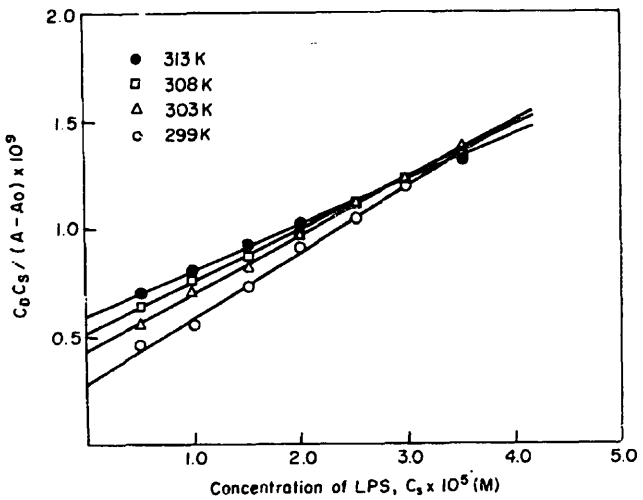

Fig. 6. Plot of $C_D C_S/(A-A_0)$ vs. C_S in *Klebsiella* O3 LPS/pinacyanol chloride dye interaction at different temperatures. Dye conc., 1.0×10^{-5} M. Temperatures (K): 299 (○); 303 (△); 308 (□); and 313 (●).

Table 1
Thermodynamic parameters for the interaction of *Klebsiella* O3 lipopolysaccharide and pinacyanol chloride

Temperature (K)	$K_c \times 10^{-4}$ (dm^3 mol^{-1}) [a]	ΔG (kcal mol^{-1}) [b]	ΔH (kcal mol^{-1}) [c]	ΔS (cal mol^{-1} deg^{-1}) [c]
299	11.02	−4.17		
303	6.07	−3.99		
308	4.40	−3.95	−5.83	−9.30
313	3.40	−3.91		

[a] Calculated from Fig. 6 according to equation of Rose and Drago [31].
[b] Calculated from thermodynamic equation: $\Delta G = -RT\ln k_c$.
[c] Calculated graphically by plotting ΔG against T according to the thermodynamic equation: $\Delta G = \Delta H - T\Delta S$.

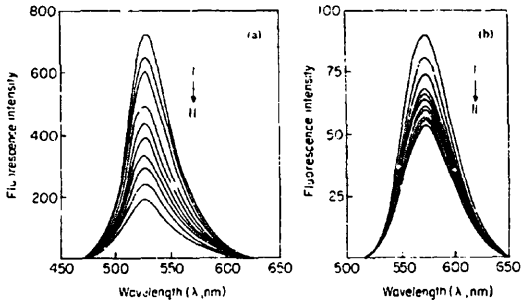

Fig. 7. Emission spectra of (a) acridine orange and (b) safranin O in the presence of different *Klebsiella* O3 LPS/dye (P/D) molar ratios at 298 K. Dye conc., 1.0×10^{-5} M. Excitation wavelength (λ_{ex}): acridine orange, 440 nm; safranin O, 526 nm. P/D: (1) 0.0; (2) 0.24; (3) 0.48; (4) 0.72; (5) 0.96; (6) 1.20; (7) 1.45; (8) 1.69; (9) 1.93; (10) 2.17; (11) 2.41.

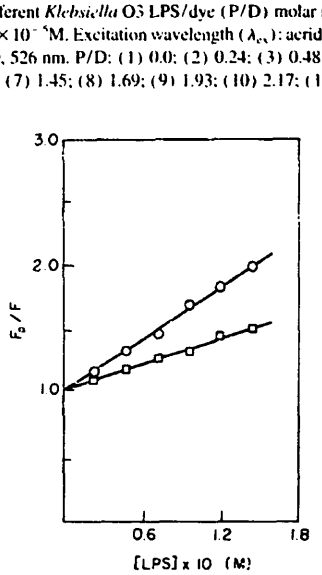

Fig. 8. Stern–Volmer plots for the interaction of acridine orange (○) and safranin O (□) with *Klebsiella* O3 LPS at 298 K. Dye conc., 1.0×10^{-5} M.

exhibited by the appearence of a blue-shifted band at the cost of the monomeric band. Usually, overcrowding of the dye on the chromotrope results in broad and multiple banded metachromasy. The present observations suggested that the anionic sites of the LPS were not very closely packed [33]. Moreover, formation of a broad multiple-banded spectrum suggests random coil structure of the polymer, whereas the polymer of helical form produces single-banded spectrum [34]. The absorption spectra shown in Fig. 2 indicated that the LPS might have random coil structure in aqueous solution of the metachromatic compound at lower LPS/dye molar ratios (P/D), but at higher P/D values (P/D>8) the random coil conformation changed to a helical one. At the intermediate P/D values, the spectra demonstrated a transition in conformation.

The absorption spectra of the interaction of the LPS with pinacyanol chloride showed an isobestic point at 540 nm (Fig. 2) during formation of the metachromatic band, suggesting a charge-transfer type complex formation with 1:1 stoichiometry. The 1:1 stoichiometry of the LPS and dye molecule in the metachromatic compound was also supported by the results obtained according to the method of MacIntosh and also by the centrifugation method (Fig. 3).

The reversal of metachromasy by addition of different cosolvents was studied by absorbance measurements both at the α-band and μ-band. The results depicted in Figs. 4 and 5 indicated that the efficiency of the added alcohols in disrupting metachromasy was of the order methanol < ethanol < n-propanol < n-butanol, which was in good agreement with the earlier reports [21,29]. Urea, DMSO and DMF were also found to be quite effective in causing reversal of metachromasy. Progressive destruction of the metachromatic compound by the co-solvents suggested involvement of hydrophobic bonds in aggregation of dyes leading to dimerization as well as metachromatic compound formation. The results of reversal of metachromasy can be usefully utilized to determine the stability of the metachromatic compound as well as the nature of the binding.

The value of the interaction constant (K_c) between LPS and pinacyanol chloride dye decreased with a rise in temperature suggesting that the interaction was exothermic in nature. The negative value of ΔH also supported it. The negative value of ΔG was within the range of a reversible biological process and such a low value of ΔG suggested non-chemical type of interaction. The negative value of entropy change indicated a more ordered state of the ions due to aggregation. Thus, all these thermodynamic parameters (Table 1) suggested the interaction between the anionic sites of the polyanions and the dye counterions resulting in aggregation and induction of metachromasy.

The Stern–Volmer type of quenching phenomenon was observed in both the cationic fluorescent dyes acridine orange and safranin O due to the interaction between the dye and LPS. The value of the Stern–Volmer constant (K_{SV}) in the case of acridine orange and safranin O were found to be 5.1×10^4 dm^3 mol^{-1} and 2.6×10^4 dm^3 mol^{-1} respectively. The linearity of the plots indicated static quenching.

From the spectral and thermodynamic properties discussed above, the chromotropic character of the *Klebsiella* O3 lipopolysaccharide was established, and it indicated the involve-

ment of electrostatic as well as hydrophobic bonds in interaction between the LPS polyanions and dye cations.

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