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Electrooptic study of the deionized form of bacteriorhodopsin

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Abstract. Electric field induced light scattering by suspensions of cation-depleted purple membranes, obtained by deionization of purple membrane (PM) suspensions on a cation exchange column or by electrodialysis at a pH around 6, shows a strong drop (more than 5 times) in the value of the permanent dipole moment relative to that of PM fragments. The membrane dipole moments were measured both at low dc and ac electric fields as well as by using electric field pulses with reversing polarity. Some slight changes in the dispersion of the electric polarizability were also observed.

Microelectrophoretic measurements showed that the electric charge of the membrane fragments is increased by 30% after deionization. The importance of these data for the understanding of the blue membrane properties and subsequently for the mechanism of proton pumping are discussed.

Key words: Bacteriorhodopsin, purple membrane, blue membrane, electric light scattering, permanent dipole moment, electric polarizability, electric charge

Introduction

The purple membrane protein, bacteriorhodopsin (bR) contains a retinal chromophore covalently bound to the ε-amino group of a Lys residue via a Schiff base (Oesterhelt and Stoeckenius 1971; Bridgen and Walker 1976). The free trans retinal has its absorption maximum at 380 nm, whereas the chromophore in the protein has a red shifted absorption maximum near 570 nm due to protonation of the Schiff base and interaction with charges in the vicinity of the cyclohexene ring (Nakanishi et al. 1980; Motto et al. 1980).

The reversible transition of purple to blue membrane (absorption maximum at 605 nm) can be induced by deionization of PM (Kimura et al. 1984b; Chang et al. 1985) or electrodialysis (Tsuji and Hess 1985). The blue form of PM can also be obtained by acidification ($pH < 3$) (see for example, Kimura et al. 1984b), by treatment with EDTA (Chang et al. 1985; Chen et al. 1984), by treatment with low concentrations of SDS (Padros et al. 1984), by reconstitution of bR with negative polar lipids (Lind et al. 1981), and by interaction with hydrophobic polar molecules (Drachev et al. 1981). Addition of cations (Kimura et al. 1984b; Dupuis et al. 1985) or increasing the pH above three restores the original state of native PM. Both absorption spectroscopy and circular dichroism experiments (Kimura et al. 1984b), as well as Resonance Raman spectroscopy (Smith and Mathies 1985) have shown that deionized and acid-induced species of PM are indistinguishable.

Several investigations on the electro-optical properties of PM suspensions have been performed during the past 6 years. The existence of a large permanent dipole moment in a direction perpendicular to the membrane plane has been demonstrated by electric dichroism (Keszthelyi 1980; Barabas et al. 1983; Kimura et al. 1981), electric light scattering (Todorov et al. 1980; Todorov et al. 1982, Stoylov et al. 1984) and electric birefringence (Stoylov et al. 1984; Kahn and Tu 1984; Todorov and Stoylov 1985). The effects of bR concentration, pH and electrolytes on the permanent dipole moment (Kimura et al. 1984a; Barabas et al. 1983), on the electric polarizability (Todorov et al. 1980, 1982; Kimura et al. 1984a; Petkanchin et al. 1985) and its dispersion (Stoylov et al. 1984) have been described and the angle of the chromophore with respect to the membrane normal has been found (Tsuji and Rosenheck 1979; Kimura et al. 1981, 1984a; Barabas et al. 1983).

High field electrooptic effects of PM in suspensions (Shinar et al. 1977; Hess 1978; Tsuji and Neu-

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mann 1981 a, b, 1983) and in dried films (Borisevitch et al. 1979; Lukashev et al. 1980; Chamorovsky et al. 1983) have been considered as an indication of conformational transitions of bR molecules connected with the orientation of both the retinal and tyrosine and/or tryptophan residues, saturated induced dipole moment, etc. Stoylov had drawn attention to some controversy in this field (Stoylov et al. 1984).

Studies of the electric properties of the blue form of PM is of great importance for elucidation of the molecular mechanism of the proton pump of bR, based on the fact that blue membranes do not pump protons (Mowery et al. 1979). It is supposed that there are one or two cation binding sites on the bR molecule (Kimura et al. 1984 b), probably tyrosine and the protonated Schiff base (Dupuis et al. 1985), which are responsible for the purple-blue transition and consequently for the proton pump functioning. Therefore, one might probe the changes in the permanent dipole moment after deionization in order to check the location of the binding sites.

In this paper our results on some electrooptic parameters of the deionized form of PM are presented.

Materials and methods

Purple membranes from H. halobium ET 1000 were isolated according to a standard procedure (Oesterhelt and Stoeckenius 1974). bR concentration was determined on the basis of a molar extinction coefficient $\varepsilon_{568} = 63{,}000\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ (Oesterhelt and Hess 1973) for its light adapted state.

Blue species of PM ($\mathrm{bR}^{605}_{\mathrm{dei}}$) were prepared by deionization of PM suspensions according to Kimura et al. (1984 b) using a cation exchange column (AG-50 W, Fluka). The cation depleted PM's obtained by this procedure have an absorption maximum around 605 nm. Membrane suspension electrodialysis was carried out following Tsuji and Hess (1986). A molar extinction coefficient $\varepsilon_{605} = 60{,}000\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ (Kimura et al. 1984 b) was used to determine the concentration of $\mathrm{bR}^{605}_{\mathrm{dei}}$.

All measurements were performed on suspensions at a very low concentration (0,4 μM bR) at pH 5.7. The specific conductivity of the suspensions (PM, $\mathrm{bR}^{605}_{\mathrm{dei}}$) was measured at 1 kHz and was about $(1.8-2.4) \times 10^{-6}\ \Omega^{-1}\,\mathrm{cm}^{-1}$.

Electric light scattering

Changes in the intensity of light scattering were measured at 90° with the electric field perpendicular to the plane of observation using a device described by Todorov et al. (1982), the light source being a He-Ne laser (632.8 nm) in the case of PM and an incandescent lamp plus interference filter (430 nm) in the case of blue membranes.

The light scattering changes in an electric field are the result of the orientation of disperse particles, the driving force being the interaction of the external electric field with the membranes electric moments (permanent and induced). The electric light scattering effect α is defined as (Todorov et al. 1982):

$$\alpha = (I_0 - I_E)/I_0 \tag{1}$$

where I_E is the intensity of the scattered light when an electric field of strength E is applied to the suspension and I_0 is the intensity of the scattered light without field.

The electrooptic effect for disc shaped particles in the case of a low degree of orientation ($U \ll kT$, where U is the energy of orientation, k is the Boltzman constant and T the absolute temperature) is expressed by:

$$\alpha = \{A(KB)/I_0(KB)\}\,(\mu^2 + \delta)\,E^2 \tag{2}$$

where $K = (2\,\pi/\lambda')\sin(\theta'/2)$; $\mu = p_1/kT$; $\delta = (\gamma_1 - \gamma_2)/kT$ and B is the diameter of the particle; p_1 is the permanent dipole moment along the membrane symmetry axis; γ_1 and γ_2 are the electric polarizabilies along the symmetry and transverse particle axis, respectively; λ' is the wavelength of the incident light in the solution and θ' is the angle of observation; $A(KB)$ and $I_0(KB)$ are optical functions (see Todorov et al. 1982), depending on the particle size and experimental conditions.

Reversing pulse technique

O'Konski was the first to apply the reversing pulse technique in studying particle electric moments (O'Konski and Haltner 1957). The theoretical background of the optical phenomena occurring when the electric field is rapidly reversed in sign is described by Tinoko and Yamaoka (1959). They show that one can calculate, from the data of the electric birefringence at rapidly reversed electric field, the ratio ϱ which is defined by:

$$\varrho = \mu^2/2\,\delta \tag{3}$$

ϱ depends on the electric moments (permanent and induced) of the particle.

For the case of a low electric field birefringence ϱ can be calculated from the experimentally observed transient upon field reversal according to

$$\varrho = (1 - R_m)/(0.1547 + R_m); \quad D = \ln 3/4\,t_m, \tag{4}$$

where $R_m = \Delta n(t_m)/\Delta n(\infty)$; $\Delta n(t_m)$ is the electric birefringence at time t_m at which the extremum of the transient signal is reached after the field reversal and $\Delta n(\infty)$ is the steady-state birefringence; D is the rotational diffusion coefficient.

The parameter ϱ and the permanent dipole moment p could be approximately estimated from the data obtained by electric light scattering (Petkanchin and Bruckner 1976) in reversing fields, using Eqs. (3) and (4). The values of p is obtained from the ac field dependence of the electrooptic effect. The reversing pulse technique is not very sensitive for $\varrho > 10$, i.e. if the value of the permanent dipole moment is high or the electric polarizability is low (Greve and Blok 1973).

Microelectrophoretic measurements

The electrophoretic mobility of purple and blue membranes was measured using Rank Brothers Apparatus (Blume et al. 1978) with a flat cell, at 25 °C in dark field illumination, $\lambda = 545$ nm. The electrophoretic mobility was measured at two stationary levels where at least 20 particles were timed at different electric field polarity. The values when the difference at the two stationary levels was more than 5% were not included.

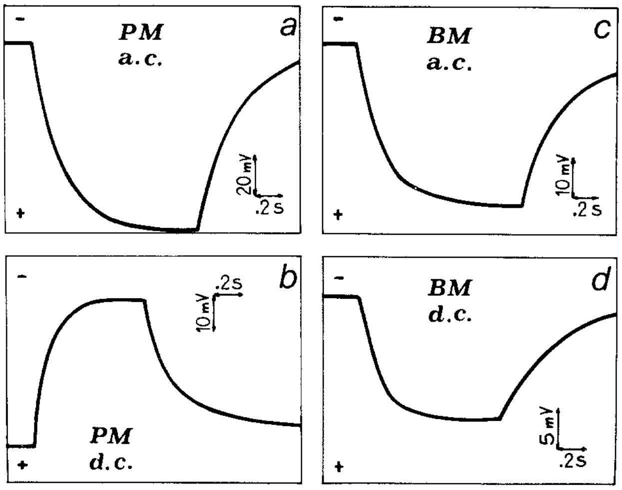


Fig. 1a–d. Time dependence of electric light scattering of purple **(a and b)** and blue **(c and d)** membranes; **b and d** for the case of dc orienting field of intensity 3×10^3 V/m; **a and c.** when ac field of 6×10^3 V/m and frequency 1 kHz is applied to the suspension. (Drawings from the oscilloscope screen)

The total net particle charge (Q) was calculated from the electrophoretic mobility using the following equation (Shaw 1969):

$$Q = 6\,\pi\,\eta\,a\,u_e(1 + \varkappa\,a)/f(\varkappa\,a)\,, \tag{5}$$

where u_e is the PM electrophoretic mobility, a – the particle radius, $1/\varkappa$ – the diffuse double layer thickness, $f(\varkappa a)$ – Henry function (varying between 1.0 for small $\varkappa a$ and 1.5 for large $\varkappa a$), which accounts for the influence of an insulating particle on the imposed electric field (Henry 1931).

Results

The typical time course of the scattered light intensity of purple and cation-depleted membranes upon applying dc and ac electric fields is presented in Fig. 1. At low dc electric field strengths purple membranes show a negative electrooptic effect (which passes through minimum when increasing the strength of the electric field and becomes positive at high dc field strengths; see Todorov et al. 1982) whereas blue membranes show a positive effect even at the lowest dc fields. In the case of the ac orienting field the effect is positive in the whole range of field strengths both for purple and blue membranes.

The dependence of the electrooptic effect on the square of the dc electric field strength for purple (dotted line) and blue (full line) membranes is shown in Fig. 2. The dependences, $\alpha(E^2)$, for purple and blue membranes are qualitatively different. A

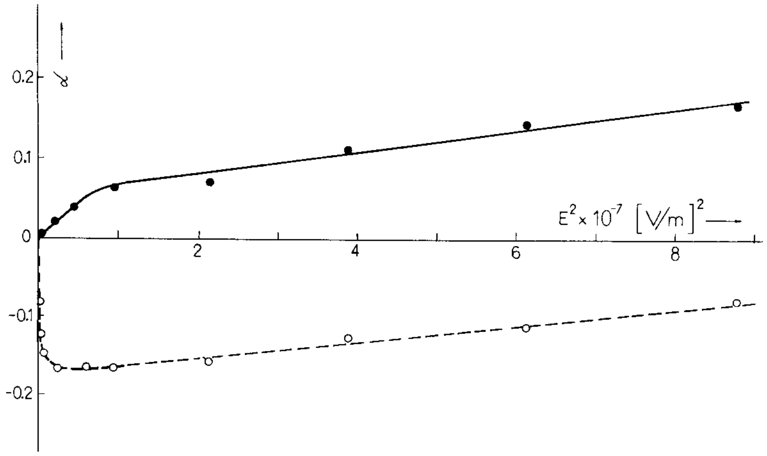
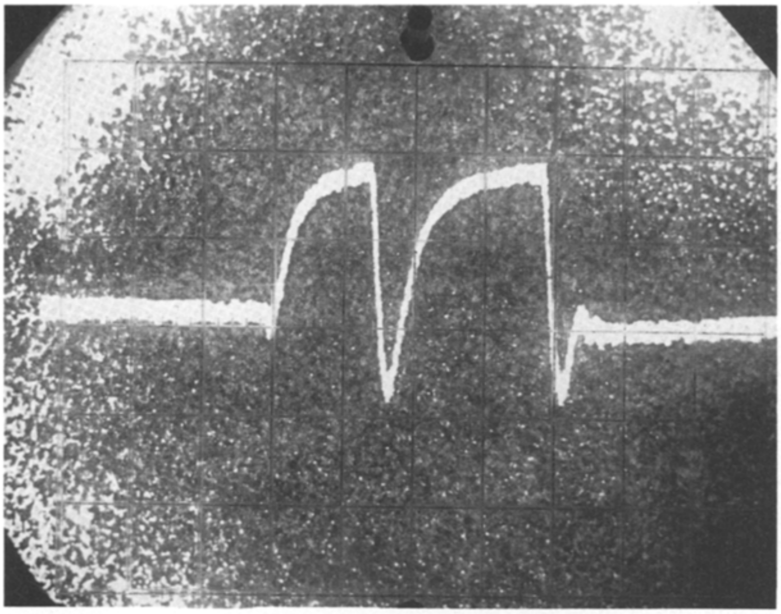
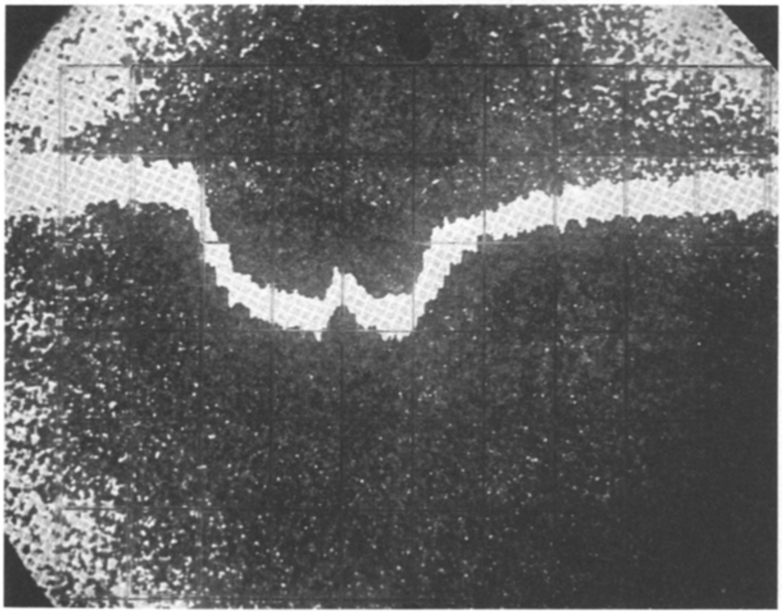


Fig. 2. Dependence of the electric light scattering on the square of the dc electric field in purple *(dotted line)* and blue *(full line)* membranes



a



b

Fig. 3a and b. Time course of the electric light scattering – orienting electric pulses of reversing polarity, $\nu = 2$ Hz; $E = 3 \times 10^3$ V/m; (**a.** PM: 20 mV/div: 0.2 s/div; **b.** BM: 10 mV/div; 0.2 s/div)

negative effect connected with the large transverse permanent dipole moment of PM is observed. In contrast, there is no negative part in the case of the blue membranes.

Figure 3 presents the time dependence of the electric light scattering effect when sign reversing pulses are applied to suspensions of purple and of blue membranes. Again purple membranes show a typical curve for disperse particles with a large transverse permanent dipole moment (Tinoko and Yamaoka 1959) while for blue membranes the change of the electrooptic effect on field reversal is hardly observed.

The dispersion curves for purple (dotted line) and blue (full line) membranes at sufficiently low electric field strength ($E = 3.2 \times 10^3$ V/m) corresponding to the linear part of the $\alpha(E^2)$ dependence are presented in Fig. 4. There is again a qualitative difference in the dispersion dependence for purple and blue membranes. The PM effect reaches a negative value at the lowest frequencies used, whereas although the electrooptic effect for blue membranes decreases with frequency in the same low frequency region it remains positive and the plateau region shortens and shifts slightly to higher frequencies. The slope of the curve at low frequencies is smaller for blue membranes, obviously indicating a decrease in the blue membrane permanent dipole moment.

The dependence of the electrooptic effect on the square of the ac electric field strength was measured for a frequency 1 kHz (corresponding to the plateau of the dispersion curves (Fig. 4) where fragment orientation is believed to be due only to electric polarizability) and is shown in Fig. 5. The magnitude of the effect is smaller for blue membranes as compared to PM within the whole range of the applied electric field. It can be seen from Fig. 5 that the initial slope $(d\alpha/dE^2)_{E\to 0}$ for the purple membranes is up to ten times bigger than that for the blue membranes. This ratio of slopes is presented here as a ratio of electric polarizabilities (Table 1) with the assumption that the optical functions of the membrane fragments are not different.

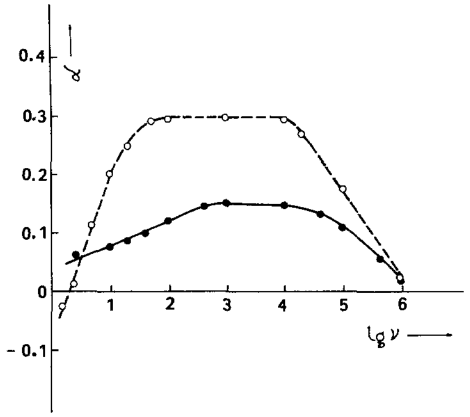


Fig. 4. Dispersion dependence of the relative effect on the applied electric field (3×10^3 V/m); (*dotted line* – purple membranes; *full line* – blue membranes)

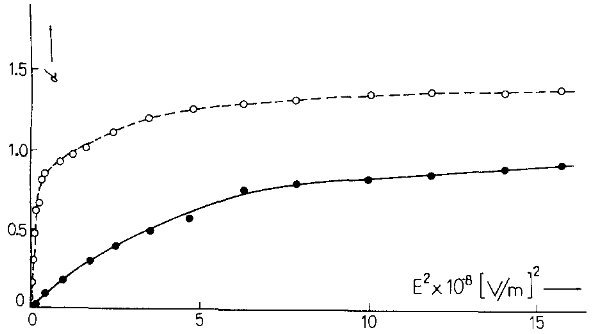


Fig. 5. Field dependence of the electric light scattering – ac field 1 kHz: *dotted line* – purple membranes and *full line* – blue membranes

The saturation values of the electrooptic effect (corresponding to a complete particle orientation) were obtained by plotting the light scattering effect versus the inverse value of the square of the electric field; these values are slightly different for blue and purple membranes though within the limits of the experimental error particle relaxation times (decay curves) are identical.

The calculated values for the electric polarizability, permanent dipole moment and total net charge are summarized in Table 1. The electric polarizability and permanent dipole moment were determined from the field dependence of the electrooptic effect and by the reversing pulse technique using Eqs. (3) and (4) respectively.

Q_e is the total net charge calculated from the electrophoretic mobility by means of Eq. (5) assuming that the particle surface is $2 \times 10^{-9}\,\mathrm{cm}^2$ ($R = 0.25 \times 10^{-4}$ cm, $\varkappa a = 5$ and $f(\varkappa a) = 0.22$; $c_{el} = 10^{-5}$ M/l).

The surface charge density (σ_0) data are quoted in the last column of Table 1 for purple (Ehrenberg and Berezin 1984) and for blue membrane (Kimura et al. 1984b).

Discussion

The calculated value of the permanent dipole moment per bR molecule for PM using the reversing pulse technique data is around 70 D which agrees well with the data of other studies (Kimura et al. 1981; Todorov et al. 1982; Barabas et al. 1983; Kimura et al. 1984a). An estimation of the blue membranes permanent dipole moment gives a value less then 16 D.

This great decrease in the magnitude of the permanent dipole moment of blue membranes could be connected with counterions removal from the extracellular side of the membrane decreasing the charge asymmetry distribution. This is valid if the permanent dipole moment of the PM fragments is directed from the intracellular toward the extracellular side of the membrane as accepted by Kimura on the basis of proteolytic treatment data (Kimura

Table 1. Electric polarizability, permanent dipole moment, total net charge density Q_e, and surface charge density (* Ehrenberg and Berezin, 1984; ** Kimura et al. 1984b)

Sample	$[\gamma_1 - \gamma_2$, F · m$^2]$	[p, C · m]	$[Q_e, \mu$ C/cm$^2]$	$[\sigma_0, \mu$ C/cm$^2]$
Purple membranes	5.8×10^{-28}	4×10^{-24}	0.22	0.85*
Blue membranes	0.61×10^{-28}	0.9×10^{-24}	0.29	14.0**

et al. 1984a) and other studies (Tsuji and Hess 1986; Keszthelyi and Ormos 1980). A decrease of the permanent dipole moment is observed by Kimura when increasing the ionic strength (Kimura et al. 1984a). A strong screening effect occurs, diminishing the membrane asymmetry and consequently diminishing the value of the permanent dipole moment. All these observations support the idea for a surface origin of the permanent dipole moment (Dukhin and Stoylov 1968).

Alternatively, exchange of cations with protons (decrease of the surface pH value) might lead to a change of the protonation state of certain groups of the polypeptide moiety as shown for acid-induced blue membranes. On the other hand the possible effect of negatively charged lipids must not be neglected.

It should be kept in mind that all this is reasonable when conformational contributions are negligible (Kimura et al. 1984b; Dupuis et al. 1985; Tsuji and Hess 1986).

Finally it should be stressed that the values given are only rough estimates. The value of the permanent dipole moment needs improvement since the reversing pulse technique theory applied to electric light scattering is better the smaller is the ratio of particle dimension to the wavelength of the light at the angle of observation (Stoylov 1971; Koopmans et al. 1979). Furthermore, this method is not very sensitive for a high value of the permanent dipole moment. To a certain extent this problem occurs with other electrooptic methods such as electric birefringence and electric dichroism (Stoimenova and Stoylov 1980).

Considering that divalent ions bind to negatively charged sites at the membrane surface it is quite reasonable that the total negative charge obtained electrophoretically is higher for deionized than for purple membranes. This trend is supported by literature data (Kimura et al. 1984b) and could be the basis for interpretating the drastic decrease in the permanent dipole moment. The great difference between the electrophoretically determined total charge and the surface charge (σ_0) could be sought in the methods used for σ_0 determination (Resonance Raman spectroscopy or fluorescence quenching). The adsorption of the probes would depend on the ionic strength. Secondly, a gel-like layer (Stern layer) could be responsible for this great difference as in the case of an oxide surface. Thirdly, the influence of the aggregation state on the electrophoretic mobility is not clear. All these questions need further attention.

At the present state of this electrooptic study it is too early to discuss the electric polarizability changes, its dispersion and its relation to microelectrophoretically determined total net charge before sufficient data are available for the optical anisotropy of the purple and blue membranes. But it is evident that the change of the total net charge is in the opposite direction to those of the electric moments.

Conclusions

The drastic drop of the permanent dipole moment upon deionisation of PM substantiates the vital role of cations in the functioning of the proton pump of bR. The sharp decrease of the permanent dipole moment favours the interfacial origin interpretation. Before reaching more decisive conclusions about the location of cation binding sites in bR further studies of the optical anisotropy, the addition of different cations, experiments on model particles, spectral, photokinetic and other studies on blue membranes are necessary for a better understanding of the mechanism of the proton pump.

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