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Large Amplitude Photo-Voltage Transients of Bilayer Lipid Membranes in the Presence of Chlorophyllin

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Abstract

Large amplitude electrical voltage transients result from the flash illumination of bilayer lipid membranes (BLM) in the presence of chlorophyllin, electron acceptors [$FeCl_3$ or $(NH_4)_2Ce(NO_3)_6$] and an electron donor ($FeCl_2$). The BLM were prepared from lecithin and oxidized cholesterol, or spinach chloroplast extracts. The photo-voltage waveforms observed may be resolved into three components, which have characteristic times of approximately the flash duration (8 μsec), 1 msec, and the BLM resistance-capacitance discharge time. These components are thus comparable to the Components A, B, and D previously reported for BLM and thin lipid membranes (TLM) of the spinach chloroplast extracts in the presence of electron acceptors. Component C of the chloroplast-BLM is extinguished by near trace quantities (1 μg/l) of chlorophyllin. Higher concentrations (1 to 20 mg/l) reduce the BLM resistance and stability but under some conditions the Component A response exceeds 200 mV. The inferred peak photo-current exceeds 10 mA/cm^2. Membrane resistance and stability data suggest that the chlorophyllin bonds within and disrupts the adjacent interface (monolayer), but that it does not permeate the BLM.

Introduction

Biophysical studies have revealed that the photosynthetic (thylakoid) membrane consists of an ultrathin layer of lipids together with sorbed

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proteins organized in a lamella about 100 Å thick [1]. Although the precise functions of the photosynthetic membrane is not known, it is believed that the membrane is the site of primary photophysical and photochemical processes. However, physical chemical characterization of the photosynthetic membrane has been difficult owing to the complexity of the membrane and lack of suitable technique for its isolation and study. One approach to mitigating these difficulties is to investigate a reconstituted membrane system [2]. We have prepared and investigated the so-called bimolecular or bilayer lipid membranes (BLM) of two molecules thick from the extract of chloroplast lamellae and used them as a model for the thylakoid membrane.

Recently, we have shown that the electrical voltage transients induced in BLM and thin lipid membranes (TLM) prepared from spinach chloroplast extracts by microsecond light flashes contain 3 photoactivated components and the resistance-capacitance discharge [3, 4]. The active pigments are contained in the membrane forming solution and are therefore incorporated into both monolayers which make up the BLM. The photo-responses may be induced by three types of membrane asymmetries: (i) a chemical difference, (ii) a pH difference, (iii) an applied voltage. Complex waveforms of light-elicited responses may also be obtained by combinations of these asymmetries. The limited amplitude of the fast component transients (less than 5 mV) presumably results from the limited solubility of the chloroplast pigments in the Chl-BLM (chloroplast bilayer lipid membrane) and TLM. Water soluble pigments and dyes are of interest since relative large quantities of the photoactive compounds may be available to the adjacent membrane interface, and in addition, membranes with pigment asymmetries may be studied [5]. We here report the results of recent experiments of the photo-effects which result from non-pigmented (lecithin and oxidized cholesterol) BLM and Chl-BLM in the presence of chlorophyllin, electron acceptors [$FeCl_3$ and $(NH_4)_2$ Ce $(NO_3)_6$], and an electron donor ($FeCl_2$).

Materials and Methods

The laboratory distilled water was re-distilled in an all-glass apparatus. The chemicals and solvents were reagent grade and were used as obtained. The chlorophyllin (water soluble chlorophyll) was obtained from the K and K Laboratories, Planview, New York. The $FeCl_3$ and $(NH_4)_2$ Ce $(NO_3)_6$ stock solutions were replaced with freshly mixed solutions periodically, so that none were over three days old when used. The $FeCl_2$ stock solution was prepared fresh daily. The spinach chloroplast extract membrane forming solutions were obtained from fresh spinach leaves, as previously described [6]. The lecithin and

oxidized cholesterol membrane forming solutions were prepared by the usual methods, described elsewhere [6].

The experimental arrangement is described in more detail elsewhere [4]. Briefly, the transmembrane voltage was measured with miniature calomel electrodes with salt bridges and a high input impedance buffer amplifier, and were displayed on an oscilloscope screen and photographed. Various precision resistors were used to shunt the membrane in a circuit which also allowed a calibrated voltage source to be applied in series with this shunt resistor and the membrane. Upon application of the voltage source, the resulting voltage division between the shunt and membrane resistance allowed the membrane resistance to be determined. Upon application or removal of the voltage source, the time variation of the membrane voltage allowed the membrane capacitance to be estimated. A Strobotac 1538-A (General Radio Company) with a type 1538-P4 capacitor was used for flash illumination. The high intensity xenon flash illumination was of approximately 8 μsec duration, and was modified by the use of colored or grey filters where noted. A pyrex glass cup and filters prevented ultraviolet light from reaching the BLM.

The BLM's were observed to thin in dim green light in symmetric 0.1 M acetate solutions. A 10-15 min dark period was then allowed before other chemicals were added. The chlorophyllin was added to the solution on the opposite side of the membrane from which the light was incident. This prevented the colored solution from reducing the light intensity at the membrane. The solution pH values and the value of the shunt resistor employed will be specified for each membrane. All measurements were made at room temperature, approximately 23° C. For pH values above 6, phosphate buffer was added to one solution just prior to adding the chlorophyllin, to stabilize the pH at the desired value. The pH of the opposite solution was allowed to vary upon the addition of the electron acceptor. Hydrolysis rendered the electron acceptor ineffective if the electron acceptor solution pH was maintained above about 6.3. In addition, other chemicals which are more effective buffers than acetate in this pH range, such as phosphate, lactate, and malonate, also rendered the electron acceptors ineffective.

The absorption of the filters and various solutions of interest were determined on a dual beam spectro-photometer (Model 14, Cary Instruments). The solution absorption measurements were made using a 1 cm quartz cell with an identical solvent filled reference cell. The spinach chloroplast extract solution was diluted directly from the petroleum ether phase in the extraction procedure. The amount of extract in solution was determined by weighing the residue after solvent evaporation. A sub-micron particle suspension of the spinach chloroplast extract was prepared by ultrasonically dispersing the residue, then filtering it through a 0.45 micron grid membrane (The Nalge Co.,

Rochester, New York). A cell disruptor microtip (Model W140D, Heat Systems-Ultrasonics) with a cooling water bath was used to disperse the residue. The amount of the extract in suspension was determined by ultrasonically mixing petroleum ether with the aqueous suspension, centrifugally collecting, then evaporating the petroleum ether. This process was repeated 6 times, although the residue mass ceased increasing, and the aqueous phase appeared clear, after the third such extraction.

Results

The absorption spectra of the red, green, blue, 12% and 50% grey filters are shown in Fig. 1. Also shown are the absorption spectra of dilute spinach chloroplast extract in petroleum ether, a sub-micron suspension of the spinach chloroplast extract in 0.1 M acetate pH 6.5 solution, and chlorophyllin in 0.1 M acetate pH 6.5 solution.

The photo-responses of a Chl-BLM in the presence of an applied voltage, an electron acceptor asymmetry, and dilute chlorophyllin, are illustrated in Fig. 2. The small amplitude of the Component C response at pH 7.1 (Fig. 2a) may be compared to the previously reported 1.6 mV response at pH 5.0 (Fig. 1c, Ref. 3). The larger response at pH 5.0 occurs in spite of the approximately order-of-magnitude larger illumination intensity-duration product used in the present study. The presence of a 3 mM $(NH_4)_2Ce(NO_3)_6$ asymmetry is seen in Fig. 2b to result in a Component A and B, and an enhanced Component C. The polarity of Components A and B are such that the electron acceptor side of the BLM becomes negative. Figure 2d illustrates that a 10^{-5} g/l concentration of chlorophyllin quenches Component C. The loss of Component C was complete in 3 min after the chlorophyllin addition. Approximately 15 min were required for Component C to be quenched with a 1 μg/l concentration of chlorophyllin. That the Chl-BLM remained in the black state was confirmed visually and by capacitance measurements, which showed that the capacitance varied less than 10% from the initial value of about 4000 pF/mm^2.

Figure 2c and e illustrate the responses obtained with the flash illumination modified by inserting the colored filters in the light path. The analysis of present data shows that each component of the Chl-BLM is activated in near accordance with the chloroplast extract absorption spectra (Fig. 2c), and that chlorophyllin enhances the blue response preferentially, which is in accordance with the chlorophyllin absorption spectra. Comparable results were also obtained using millimolar concentrations of $FeCl_3$ as the electron acceptor.

Increased concentrations of chlorophyllin increased the amplitude of the photo-effects. However, if the chlorophyllin and the electron

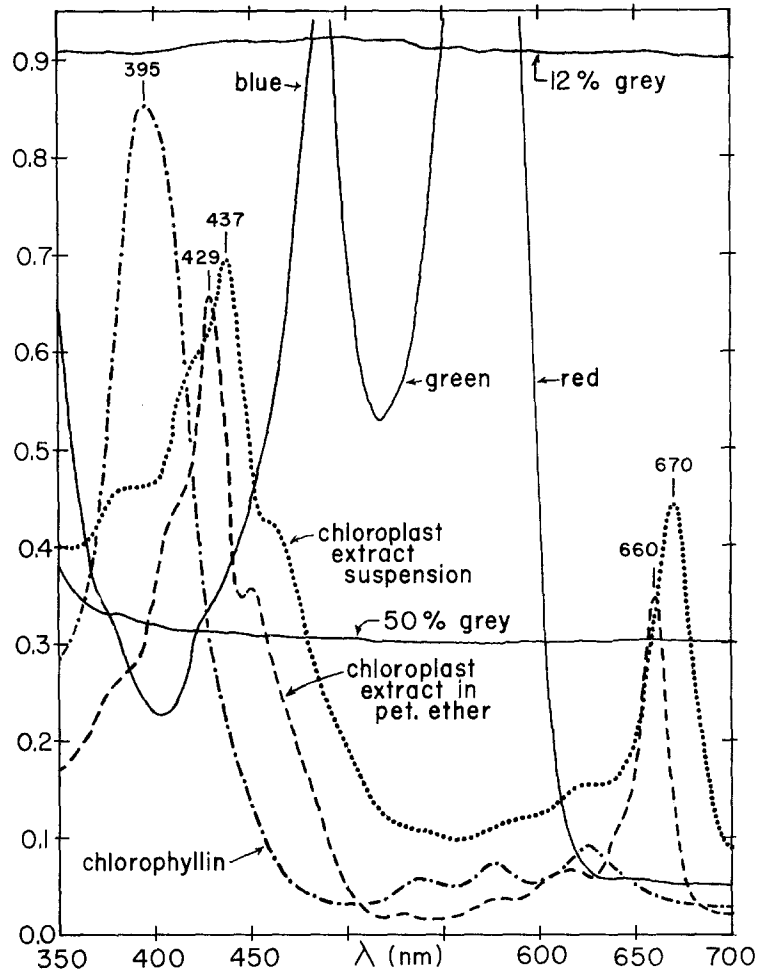

Figure 1. The spectroscopic absorption of the red, green, blue, 12% and 50% grey filters, and a 1 cm cell containing 30 g spinach chloroplast extract per liter petroleum ether, a submicron particle size suspension of 0.19 g spinach chloroplast extract per liter 0.1 M acetate pH 7 solution, and 0.50 g chlorophyllin per liter 0.1 M acetate pH 7 solution.

acceptor were added to the same side of the bathing solution, the chlorophyllin would flocculate and precipitate. If chlorophyllin was added to the solutions on both sides of a BLM, the membrane resistance would decrease and the membrane would rupture. The Chl-BLM would typically rupture in less than one minute after 5 mg chlorophyllin per liter was added to both solutions, in about one minute if 0.5 mg/l were

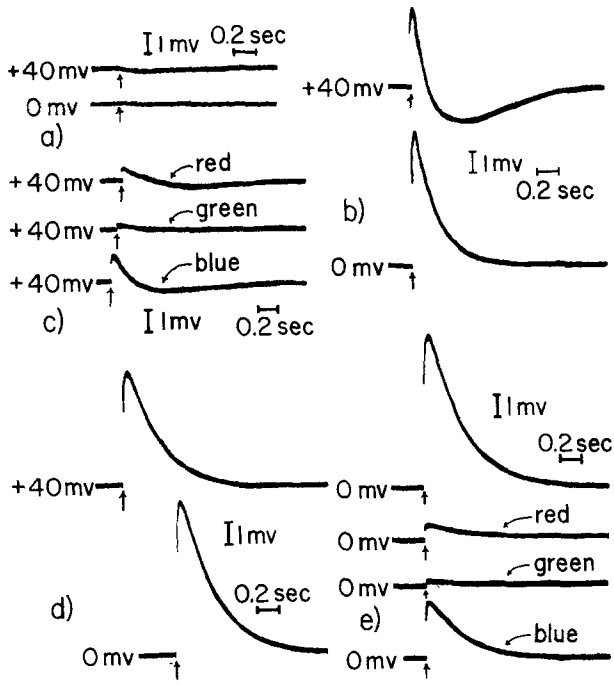

Figure 2. Waveforms from a single Chl-BLM prepared in pH 7.1 acetate buffer solution with a 10^8 ohm shunt resistor. The traces illustrate the photo-effects which result from, (a) no asymmetry (the 0 mV trace), and with +40 mV applied. The small vertical arrow marks the time of the flash illumination. The polarity of Component C response, which occurs on the +40 mV trace, is to reduce the voltage across the membrane. (b) a 3 mM $(NH_4)_2Ce(NO_3)_6$ gradient at 0 mV and +40 mV. The polarity of Components A and B is to make the Ce^{+4} side of the membrane negative. (c) a 3 mM $(NH_4)_2Ce(NO_3)_6$ gradient and +40 mV, with the light intensity reduced by the red, green, and blue filters. (d) 3 mM $(NH_4)_2Ce(NO_3)_6$ and 10^{-5} g/l chlorophyllin gradients, at 0 mV and +40 mV. The $(NH_4)_2Ce(NO_3)_6$ and chlorophyllin were added to opposite solutions. (e) 3 mM $(NH_4)_2Ce(NO_3)_6$ and 10^{-5} g/l chlorophyllin at 0 mV, with the light intensity reduced by the red, green and blue filters. The membrane resistance was initially 2×10^8 ohms, but fell to 2×10^7 after the chlorophyllin was added.

added, and in about 7 min if 25 μg/l were added. These results are also typical of oxidized cholesterol-lecithin BLM. Small photo-effects could be achieved from the oxidized cholesterol-lecithin BLM by adding chlorophyllin to both solutions, and after the resistance has decreased, but before the BLM ruptures, adding an electron acceptor. These membranes were generally unstable, however.

The amplitude of the photo-effects induced by chlorophyllin were found to increase with the solution pH to about 7.0 or 7.5. Since the

electron acceptors used were ineffective at this pH, and phosphate buffer rendered the electron acceptors ineffective, large amplitude photo-responses required a pH/buffer asymmetry. Since Component C is quenched by chlorophyllin, Component C did not appear under these conditions. Figure 3a and b illustrate large amplitude photo-responses obtained from oxidized cholesterol-lecithin BLM in the presence of chlorophyllin and an electron acceptor. No photo-voltages were observed from these non-pigmented BLM in the presence of chlorophyllin alone (i.e., in the absence of an electron acceptor, but with a chlorophyllin asymmetry, applied voltages, and/or pH gradients). Similarly, no photo-responses were observed from these BLM in the presence of electron acceptors alone (i.e., in the absence of chlorophyllin). The polarity of the chlorophyllin/electron acceptor photo-response was such that the chlorophyllin became electrically positive, with the electron acceptor becoming negative. The dark BLM resistance was always substantially reduced with the concomitant development of the BLM's capacity to produce a large amplitude voltage transient upon flash illumination. For the oxidized cholesterol-lecithin BLM used to obtain the traces shown in Fig. 3a, the transmembrane resistance before the chemical addition was $\gtrsim 10^9$ ohms, and dropped to $\sim 10^7$ ohms in about 30 min after the phosphate, chlorophyllin and $(NH_4)_2Ce(NO_3)_6$ addition. The 65 to 80 mV responses were observed over a period extending from 30 to 90 min after the chemical additions. The pH values of the two solutions were subsequently determined to be 7.1 for the chlorophyllin side, and 6.2 for the $(NH_4)_2Ce(NO_3)_6$ side. The rapid rise in the membrane voltage during the period of illumination (approximately the width of the arrow in the 10 μsec/cm trace) is directly comparable to Component A, as previously described [3, 4]. The 1 msec/cm trace shows the discharge of the photo-voltage after flash excitation (solid line) and the membrane voltage discharge after disconnecting an applied voltage (dashed line). The faster initial discharge of the flash induced voltage is attributed to Component B, which in this case has a polarity opposed to that of Component A. The characteristic time of Component B may be seen to be about one millisecond.

The resistance of the oxidized cholesterol-lecithin BLM used to produce the photo-effects in Fig. 3b was reduced to 7×10^6 ohms in 20 min after the addition of chlorophyllin to 10 mg/l to one solution, and $(NH_4)_2Ce(NO_3)_6$ to 1.5 mM to the other. The amplitude of the photo-response may be seen to increase nearly linearly with the light intensity from 3% to 12%, (from 5.5 mV to 21 mV), but the increase is less than linear from 12% to 50% (64 mV), and from 50% to 100% (100 mV). Repeated illumination (of up to 100 flashes) did not significantly alter the amplitude of the photo-responses, if a dark period

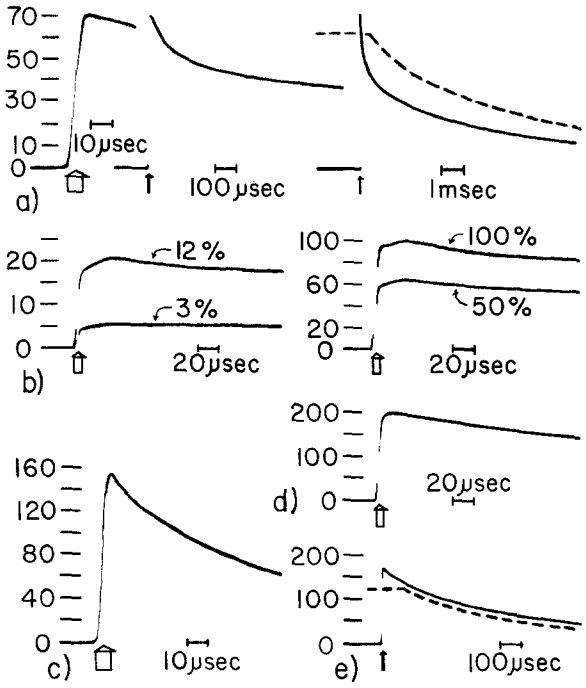

Figure 3. Waveforms (in mV) illustrating large amplitude photo-voltage transients resulting from the flash illumination of BLM in the presence of chlorophyllin and other chemicals. (a) Successive traces at progressively slower sweep speeds of an oxidized cholesterol-lecithin BLM prepared in 0.1 M acetate pH 6.0 solution with chlorophyllin and $(NH_4)_2Ce(NO_3)_6$ subsequently added to 1 mg/l and 1.5 mM strength respectively in the opposite solutions. K_2HPO_4 at pH 7.5 was also added to the chlorophyllin solution side of the BLM to 0.01 M strength. The traces were recorded about one hour after these chemicals were added. The initial transmembrane resistance of $\gtrsim 5 \times 10^9$ ohms fell to 1×10^7 ohms during this period. The dashed line illustrates the variation in the membrane voltage when an applied voltage was removed. A 10^8 ohm shunt resistor was used. (b) The photo-responses of another oxidized cholesterol-lecithin BLM prepared under identical conditions, except for the absence of K_2HPO_4 in this case, with the flash illumination intensity reduced by the 3%, 12%, and 50% grey filters, and with the full light intensity. The transmembrane resistance in this case fell to 0.7×10^7 ohms. (c) The photo-voltages obtained from a Chl-BLM prepared in 0.1 M acetate pH 6.4 solution about 80 min after the addition of chlorophyllin to 10 mg/l and K_2HPO_4 to 0.01 M at pH 7.0 to one solution, and $(NH_4)_2Ce(NO_3)_6$ to 2 mM to the other solutions. The initial transmembrane resistance of $\sim 10^8$ ohms fell to $\sim 10^5$ ohms in 4 min after the chemical additions. A 10^7 ohm shunt resistor was used. (d) The photo-voltage response obtained from the Chl-BLM used in part c, two minutes after the addition of $FeCl_2$ to 1 mM to the chlorophyllin solution. (e) The photo-voltage response obtained from the same Chl-BLM used in parts c and d, 10 min after the $FeCl_2$ addition. The dashed line shows the membrane voltage variation upon removal of an applied voltage. The membrane resistance by this time had increased to 3.5×10^5 ohms.

of 30 to 60 seconds was allowed between the flashes. This time period was required for the flash apparatus to be fully recharged.

Figure 3c illustrates the photo-response obtained from a Chl-BLM with phosphate and chlorophyllin added to one solution, and $(NH_4)_2Ce(NO_3)_6$ added to the other solution. The 155 mV response occured in about 30 min. The initial Chl-BLM resistance was about 10^8 ohms, and fell to about 10^5 ohms after the chemical additions. The low transmembrane resistance produced a rapid discharge (Component D) of the photo-voltage; still a Component B with a polarity opposite to that of Component A was apparent. This 150 to 160 mV response was observed on this particular membrane every few minutes for a period extending over 80 min.

The largest amplitude photo-responses observed were obtained under the transient conditions created when $FeCl_2$ to 1 mM strength was added to the chlorophyllin solution of the Chl-BLM in the configuration described just above. Within 2 min of the $FeCl_2$ addition, the photo-response often exceeded 200 mV. Subsequently, the response decreased so that after 10 to 15 min, the response amplitude had decreased to the value obtained prior to the $FeCl_2$ addition, and after about 60 mins, the response had decreased to about 50% of the initial value. Figure 3d and e, respectively, show the photo-response of the Chl-BLM used to obtain the traces shown in Fig. 3c, two and ten minutes after the $FeCl_2$ addition. An increased amplitude of Component A, and the absence of Component B may be observed. In addition, an increased membrane resistance, which causes a longer discharge time, was observed. The presence of $FeCl_2$ also causes the chlorophyllin in solution to precipitate. $FeCl_2$ additions also produced temporary enhancement of the Component A amplitudes in oxidized cholesterol-lecithin BLM in the presence of chlorophyllin and $(NH_4)_2Ce(NO_3)_6$, although the maximum Component A amplitude values were from 25% to 50% less than those obtained with Chl-BLM.

Discussion

We have found conditions under which photo-voltages can be generated across BLM in 8 μsec that are of sufficient magnitude, if maintained, to rupture most BLM from dielectric breakdown. The conditions require an intense light flash, chlorophyllin and $FeCl_2$ on one side of the BLM, and $FeCl_3$ or $(NH_4)_2Ce(NO_3)_6$ on the other side. The concomitant appearance of the photo-voltage upon illumination with the resistance reduction suggests an interaction of chlorophyllin and $(NH_4)_2Ce(NO_3)_6$ with the BLM surfaces is essential for the efficient transport of the photo-induced charges. Since the BLM rupture with near trace quantities

of chlorophyllin present on both sides, we infer that the chlorophyllin interacts with the adjacent monolayer of the BLM in such a manner as to disrupt its structure and cohesion. The fact that BLM may survive several hours with high concentrations of chlorophyllin on one side only (with no electron acceptor present on the other side) suggests that the chlorophyllin may not permeate the BLM at an appreciable rate.

The quenching of Component B with the $FeCl_2$ addition suggests that Component B results from the reduction of the photo-oxidized pigment molecules. With the pigment present on only one side of the membrane, which we infer to be the case here, reduction of the photo-oxidized pigment should draw electrons from both sides of the membrane, which may be able to cross the membrane as holes. This charge transport will reduce the membrane voltage, i.e., it is a negative Component B, and its amplitude must be less than that of Component A. The addition of $FeCl_2$ to the pigment side of the membrane makes it much more effective in reducing the photo-oxidized pigment, eliminating the charge being transported across the membrane to reduce the pigment, thus quenching Component B. The enhanced amplitude of Component A upon the $FeCl_2$ addition may result from a more complete reduction of the pigment to flash excitation, or from the pigment being oxidized more than once during the 8 μsec flash period. The subsequent reduction of the Component A amplitude and the increase of the BLM resistance appear to be a direct consequence of the chlorophyllin being precipitated by the $FeCl_2$, with the pigment de-sorbing from the BLM interface.

Acknowledgements

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