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BIOELECTRICAL RESPONSE OF THE *NITELLA FLEXILIS* CELL TO ILLUMINATION:

A NEW POSSIBLE STATE OF PLASMALEMMA IN A PLANT CELL

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SUMMARY

Transient hyperpolarization of the external cytoplasmatic membrane may be observed on rapid illumination of the *Nitella flexilis* cell. Several important properties of that response make the latter similar to a considerable degree to the excitation response.

The condition for transient hyperpolarization is the normal functioning of the electron transport chain conjugated with non-cyclic photophosphorylation.

The value of the membrane potential at the moment of hyperpolarization of the external cytoplasmic membrane, is determined by the difference in the electrochemical potential of HCO_3^- or H^+. This state of the plasmalemma supplements the two other known states: normal and depolarized (excited), when the main ions determining membrane potential are K^+ and Cl^-.

INTRODUCTION

Illumination of a plant cell causes a change in the electric potential difference not only on thylakoid membranes of chloroplasts[1], but on the external cytoplasmatic membrane of the cell, as well[2–9]. In the second case, considered by us, light illuminates only if it is absorbed by the cell chloroplasts[2,8,9]. Unfortunately, the data obtained by different authors on light-induced changes in the cell membrane potential are difficult to compare as the experiments were conducted after different intervals of time had elapsed following cell illumination (from a few minutes up to 24 h), in different seasons and on cells grown in different conditions. The latter could impart some individual peculiarities to the cells; up to now there has been no criterion for defining these peculiarities other than the cell response itself to the illumination. The latter is illustrated in Fig. 1A. Systematized data show the possible dynamics in time variations of the membrane potential in response to rapid illumination (dark–light response) of the *Nitella flexilis* cell. This dependence was obtained at different times in a single experiment for cells grown in different

Abbreviations: DCMU, 3-(3,4)-dichlorophenyl-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol.

conditions. It is to be noted that even in the course of one experiment with a dark–light alternation of the cell, other conditions being constant, along with the reproducible kind of relationship between the membrane potential and time (Fig. 2A), an evolution of this relationship with a disappearance of the phase of hyperpolarization of the membrane potential under subsequent cell illumination may also be observed, or the reverse. Both those cases are shown in Figs 2B and 2C. Here we have used the data obtained in experiments carried out during the spring and summer months. The cells we dealt with showed in more than 50% of all the cases studied a reproducible dark–light response with transient hyperpolarization of the membrane potential. Fig. 1B presents the full course of that response.

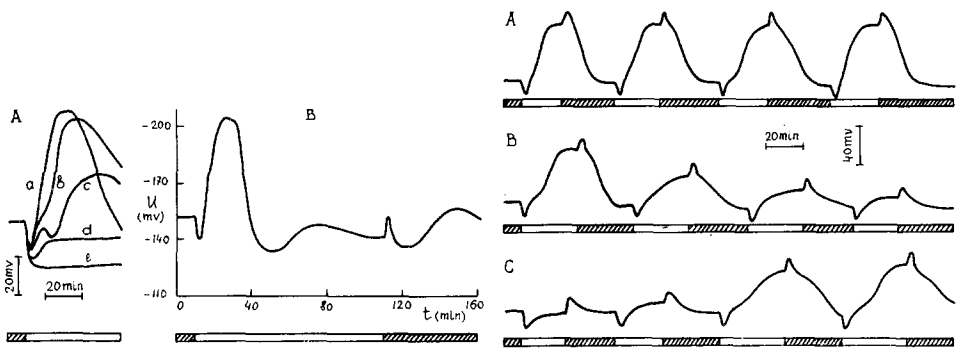

Fig. 1. Change in membrane potential with time in response to rapid illumination of the cell. (A) Possible ways of changes in membrane potential just after cell illumination. Various curves illustrate cases with a distinct hyperpolarization phase and those when that phase does not occur (see text). (B) Total course of changes in membrane potential in the presence of a hyperpolarization phase. Here too the change in the membrane is shown in response to the rapid darkening of the cell.
In this and the following figs, the vertical axis corresponds to hyperpolarization of membrane potential. The dashed band underneath the figure shows the period of dark storage of the cell; the light band, that in the light.

Fig. 2. Changes in membrane potential when darkness and illumination of the cell are alternating. (A) When the value of membrane potential hyperpolarization remains constant on reillumination of the cell. (B) Gradual decrease in membrane potential hyperpolarization on reillumination of the cell. (C) Gradual increase in membrane potential hyperpolarization on reillumination of the cell.

For convenience of investigation the dark–light response may be divided into several phases. The first phase is a depolarization of the membrane potential following just after the moment of cell illumination. This phase had been observed by some authors[3,5,8]. The second phase is a transient hyperpolarization of the membrane potential. This was observed in the experiments described in refs 2, 5. and 10. Then the membrane potential is reduced by a series of damping oscillations or in an asymptotic manner. The value of the membrane potential for the cell in that state may be equal to its value in the dark state[4], it may be higher[2] or lower[5,11] in the absolute value.

Obviously, the dark–light response with distinct manifestation of the second phase at the hyperpolarization of the membrane potential presents a more general

case and is of greater interest as compared to the dark–light response with no hyperpolarization of plasmalemma, *i.e.* when the second phase of the dark–light response is either "masked" due to the value of the membrane potential being at the initial or lower level in this phase, or does not exist at all.

The existence of the membrane potential level greatly exceeding the initial and final stable values is of some interest. As this becomes more evident, it gives rise to a hyperpolarized state of the plasmalemma, the latter possessing some valuable properties of principal importance.

METHODS

Single internodal cells of *N. flexilis* were the object under investigation.

The algae were cultivated in tap water in vessels containing peat and sand. The vessels were under natural illumination but protected from direct solar light. Usually, the fourth cell from the top was used in the experiment. On being separated the cell was stored in the dark for 2 h in the medium used for subsequent studies. Solutions of $NaHCO_3$ and KCl served as that medium. $NaHCO_3$ solutions were prepared just before use. In a number of experiments the medium contained some other ions, as well, which will be mentioned below.

The pH change of the medium was attained by adding the corresponding quantities of HCl or NaOH to the solution. The pH value was checked before and after the experiment. When studying the effect of various chemical agents on the cell, these were added to the initial solution used as a test medium. The cell under investigation was placed in a small bath, the flow of solution through it being continuous.

AgCl electrodes together with glass micropipettes containing 3 M KCl were used for recording the membrane potential values. The electric circuit for the membrane potential measurements was routine[5].

When the effect of light on some part of the cell was to be studied, the latter was placed in a special bath having 2 sections, one part of the cell being in the first section, the other in the second one. Electrical isolation between the sections was provided with lanoline. The construction of the bath allowed each section to be supplied with solution and illuminated independently. The basic circuit for measuring the corresponding membrane potentials is shown in Fig. 4A. Switching the outlets of electrometric amplifiers over to the recorder inlet (a multiple-pointed selfrecording potentiometer) was performed by means of a switch on the recorder. The temperature was 18–22 °C in all the experiments.

RESULTS

As was shown in the experiments, the first phase of the dark–light response being shown by a change in the membrane potential just after illumination of the cell, lasts for 5–15 min and may be rather complicated. For example, it may have two waves of depolarization as in the case of curve c (Fig. 1A). The "prehistory" of the response and the light regime before illumination, the chemical composition of the medium and the value of the cell illumination greatly account for the change in the membrane potential in that phase. The decrease of dark pretreatment

of the cell and the increase of intensity of the light flow used for illuminating the cell made the change of the membrane potential in this phase more simple, which was similar to the first phase for curves b and a (Fig. 1A). The first phase of the dark–light response is very stable, it is present even when the second phase is feeble or does not appear at all.

The change of the membrane potential in the course of the second phase of the dark–light response may attain a high value, namely: 60 mV and above this. The hyperpolarized state of the cell continues from several minutes up to much longer periods of time.

The dark–light response at the hyperpolarization of the membrane potential is of a threshold nature. When the illumination of the cell is lower than 100 lux there is no second phase of the dark–light response. With an increase in the applied illumination, the rate of hyperpolarization of the membrane potential rises, its value remains constant, however, other things being equal. Illumination of the cell by steps, when the given value of illumination was obtained in two stages, also only affects the rate of the membrane potential change. The data obtained are shown in Figs 3A and 3B. Finally, Fig. 3C indicates that the value of plasmalemma hyperpolarization does not depend on the value of the initial level of membrane potential either.

When illuminating only one part of the cell placed in the bath with two sections, responses of two types as shown in Figs 4B and 4C may be observed. With a symmetrical arrangement of the cell (with respect to the section interface) the response of the first type was observed in 10 out of 15 cases. With a non-symmetrical arrangement, when a long portion of the cell was illuminated, the number of cells giving the first type response increased and on illumination of the short portion, the number of cells giving the second-type response increased.

The following series of experiments (Figs 5A–5F) show the effect of the light

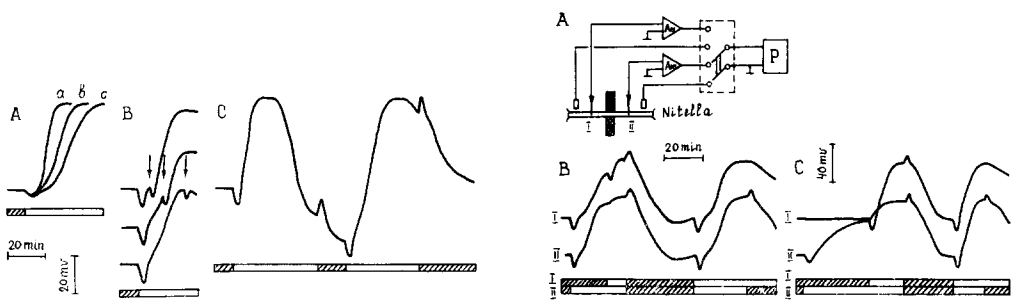

Fig. 3. The change of membrane potential in the dark–light response depending on the conditions used. (A) Dark–light response changes depending on illumination of the cell: 4000 lux (a), 800 lux (b), 400 lux (c). (B) The change in the dark–light response on cell illumination by 400-lux steps. The band underneath the figure shows the moment of initial illumination, the arrow indicates the moment of additional illumination. (C) Changes in the dark–light response depending on the value of the initial dark level for the membrane potential.

Fig. 4. (A) Basic circuit for measuring membrane potential of the cell parts located in different sections of the bath. A_m, electrometric amplifier; P, multiple-pointed selfrecording potentiometer. (B) and (C) Possible ways of membrane potential change on illumination or darkening of one part of the cell or both of them simultaneously. Nos I and II for the given dependences and bands refer to the corresponding bath section.

wavelength used for illuminating the cell and that of some inhibitors and cofactors of photosynthesis processes on the ability of the membrane potential to attain the hyperpolarization level. With red light illumination of the cell ($\lambda > 700$ nm), or when adding one of the chemical agents into the cell bathing solution, the second phase of the dark–light response appeared to be depressed. The following compounds were used as the agents: 3-(3,4)-dichlorophenyl-1,1-dimethylurea (DCMU), flavin mononucleotide (FMN), phenazine metasulphate and ferricyanide, at concentrations of 10^{-2}–10^{-4} mM. When DCMU was added to the solution after hyperpolarization of plasmalemma, the membrane potential decreased to the dark level, the manner of this decrease being similar to that of the membrane potential response to switching off the light[12].

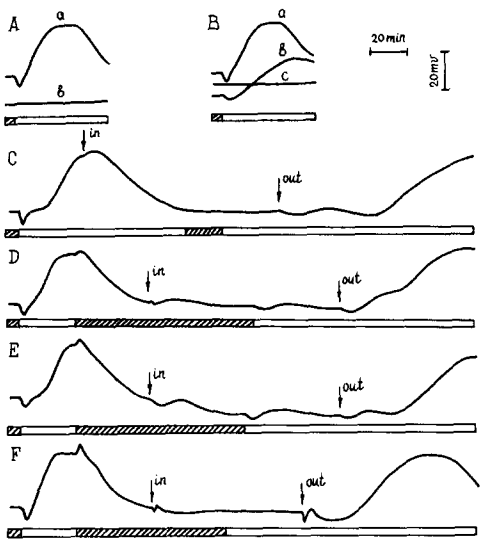

Fig. 5. Effect of light spectral composition and of some inhibitors and cofactors of photosynthetic processes on the change of membrane potential in the dark–light response. (A) Cell illumination by white light. (a); and red light with $\lambda > 700$ nm (b). (B) Check of the dark–light response (a); dark–light response in the presence of DCMU sodium ascorbate and DCIP in the medium (b); dark–light response in the presence of only DCMU in the medium (c). (C)–(F) Dark–light response in the presence of some chemical agent. Arrows "in" and "out" indicate the moments of introducing the chemical agent into the medium and its removal from the latter, respectively: DCMU (C); phenazine metasulphate (D); FMN (E) and ferricyanide (F).

To check the possible influence of the above agents directly on the properties of plasmalemma, test experiments were carried out. Table I shows the alteration of the membrane potential with a 10-times increase (up to 1 mM) in KCl concentration in the medium where the concentration of the agent remained constant.

If the given agent is removed from the solution bathing the light-treated cell, a transient hyperpolarization of plasmalemma to the level approaching that during the second phase of the dark–light response is observed. After washing the cell in the checking solution, the normal course of the dark–light response was restored[13].

Hyperpolarization of the membrane potential during cell illumination could

TABLE I

ALTERATION OF MEMBRANE POTENTIAL AT KCl CONCENTRATION INCREASE FROM 0.1 TO 1 mM

Composition of solution	*Membrane potential alteration (mV)*
KCl	41.8 ± 5.4
KCl + 10^{-5} M phenazine metasulphate	43.7 ± 3.5
KCl + 10^{-7} M FMN	40.2 ± 1.2
KCl + 10^{-7} M ferricyanide	56.0 ± 1.3
KCl + 10^{-6} M DCMU	36.2 ± 1.0

be obtained in the presence of DCMU in the medium, if, in addition, 10^{-2} mM sodium ascorbate and 2,6-dichlorophenolindophenol (DCIP) were also present.

Special attention was devoted to the influence of HCO_3^- and H^+ on membrane potential.

A rapid increase of bicarbonate concentration in the medium (from 0.1 mM to 1.0 mM) caused, in the case of an illuminated cell, transient hyperpolarization of the membrane potential, as shown in Fig. 6A.

When the pH of the medium varied (in the range of pH values corresponding to those of the bicarbonate containing solutions) at the expense of adding the correct quantities of HCl and NaOH, no transient hyperpolarization of plasmalemma, as in the case of the increase in HCO_3^- concentration could be observed.

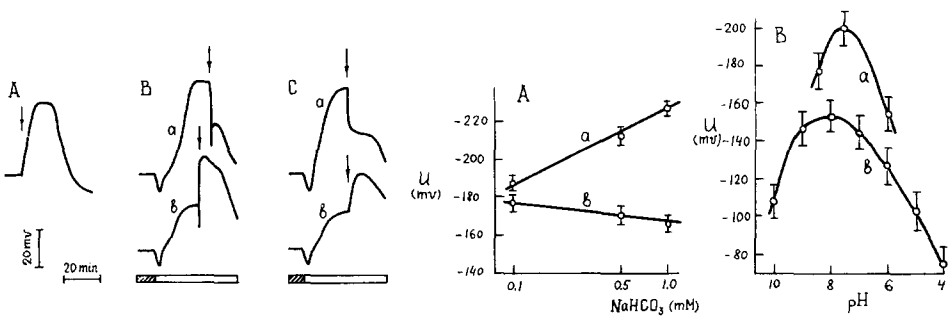

Fig. 6. (A) Membrane potential changes with an increase of HCO_3^- concentration in the medium from 0.1 to 1.0 mM. (B) Effect of a HCO_3^- concentration decrease in the medium from 1.0 to 0.1 mM (a); or its back increase (b) on the value of plasmalemma hyperpolarization in the second phase of the dark–light response. (C) Effect of pH increase in the medium from 7.5 to 8.5 (a); or its back decrease (b) on the value of plasmalemma hyperpolarization in the second phase of the dark–light response. The time of changes in HCO_3^- concentration or in pH are indicated with the arrows.

Fig. 7. (A) Relationship between membrane potential and $NaHCO_3$ concentration: at the moment of hyperpolarization in the second phase of the dark–light response (a); and in the dark-adapted cell (b). 0.08 mM KCl was continuously present in the medium. (B) Relationship between membrane potential and pH value in the medium: at the moment of hyperpolarization in the second phase of the dark–light response (a); and in the dark-adapted cell (b).

The exception was when additional amounts of 10 mM $CaCl_2$ were present in the solution.

Figs 6B and 6C illustrate the direct influence of variations in HCO_3^- concentration and of pH of the medium on the value of membrane potential in the second phase of the dark–light response.

Finally, the relationships between the membrane potential and $NaHCO_3$ concentration and pH of the medium are presented in Figs 7A and 7B. In dark-adapted cells, the membrane potential alteration is mainly defined by the change in Na^+ concentration but at the moment of plasmalemma hyperpolarization however, by that of HCO_3^- concentration. The shift in pH of the medium from 7.5–8 to the region of acid or alkaline enough values, caused a decrease in the membrane potential value for the dark-adapted cell, and on illumination of the latter, the pH change depressed the appearance of the second phase in the dark–light response.

DISCUSSION

Transient hyperpolarization of the membrane potential, which is observed in the second phase of the dark–light response, may be caused by one of the following factors[12]: (a) The existence of electrogenic active transport. (b) Increase of plasmalemma permeability to some ion.

Using inhibitors and cofactors of photosynthetic processes, we could influence the functioning of certain links in the photosynthetic chain of electron transfer. As it is seen from Table I, all these chemical agents probably do not significantly effect the properties of the cell plasmalemma in a direct way.

When the cell was affected by DCMU, water photolysis was inhibited[17], *i.e.* the second photochemical system was switched off[16]. The energy thus obtained, at the expense of cyclic photophosphorylation, may be used to provide active transfer of HCO_3^-, for example[7], or to intensify the work of the Na^+ pump.

When sodium ascorbate and DCIP, being a donor and electron carrier, respectively[18], were present in the solution, along with DCMU, the normal functioning of the Calvin cycle was implemented by the products of electron transfer chain action, *i.e.* by NADPH and ATP.

Ferricyanide, as well as phenazine metasulphate and FMN, may replace NADP as an electron acceptor, though in essence their action is completely different. In the presence of ferricyanide, the Hill reaction[18] will also proceed together with the process of photophosphorylation, *i.e.* conditions will exist for stimulating active transport and particularly for immediate conjugation of Cl^- transfer with the action of the electron transfer chain[14,15]. Phenazine metasulphate is a cofactor of cyclic photophosphorylation[19] and FMN, depending if the regime of the second photochemical system is a cofactor of cyclic[20] or pseudocyclic photophosphorylation[21]. That is why the action of the above agents is in the end similar to the action of DCMU or ferricyanide.

Red light (700 nm) applied for cell illumination the latter inhibiting the functioning of the second photochemical system[16], also has a similar action to DCMU.

It was stated above that of all the variants of the experiments, the hyperpolarization of the membrane potential was observed only if sodium ascorbate and DCIP were present in the medium together with DCMU. Taking into account the possible transient hyperpolarization of plasmalemma of the illuminated cell, in response to the removal of the chemical agents mentioned from the medium, which is similar in its sense to reillumination of the cell, we may draw the following important conclusion: the onset of functioning of the electron transfer chain conjugated with non-cyclic photophosphorylation[12] is the condition for causing hyperpolarization of the external cytoplasmatic cell membrane in the process of the dark–light response.

It should be emphasized that each single variant of the above experiments may probably arouse certain objections, due to which the arguments in favour of that conclusion will not be serious enough. However, one cannot but take into consideration the total data obtained.

The given data do not include an involvement in active transport of any ions, including H^+, as discussed in Spanswick's work[34]. at least the ways considered by MacRobbie for light-dependent K^+ and Cl^- flows [14,15].

The connection between hyperpolarization of plasmalemma in the second phase of the dark–light response and commencement of the electron transport chain, and thus the working of the Calvin cycle, can be carried out first of all through a change in intracellular concentration of HCO_3^- or H^+. Besides, transient hyperpolarization can be attained by a rapid increase of HCO_3^- concentration in the medium[22] or, according to Spanswick[23], by the corresponding pH increase. The above stated allows the following postulation: the mechanism inducing the response studied presents a rapid change in the gradient of the chemical potential of HCO_3^- or H^+ (ref. 13) and the response itself is the result of a change in membrane permeability to one of these ions.

Transient hyperpolarization of plasmalemma in the second phase of the dark–light response suggests a transient increase in the permeability of the potential-determining ion, *i.e.* a process similar to inactivation in the excitation phenomenon should exist[24].

If on hyperpolarization of plasmalemma the potential-determining ion is HCO_3^-, its permeability should increase up to approx. $1 \cdot 10^{-3}$–$1 \cdot 10^{-4}$ cm·s^{-1} (compare with approx. $1 \cdot 10^{-7}$–$1 \cdot 10^{-8}$ cm·s^{-1} for a cell in the dark). We could estimate these values from the data presented in Fig. 7A using the formula of Goldman for the membrane potential and assuming intracellular concentration of K^+ and Na^+ equal to 114 and 14 mM, respectively[25], the permeability for K^+ being assumed as $1 \cdot 10^{-5}$–$1 \cdot 10^{-6}$ cm·s^{-1} (ref. 7). Raven[26] gives similar values for HCO_3^- permeability calculated by the formula of Goldman for ion flow with respect to *Hydroctyon africanum* and Smith[27] also for *Chara Austr.*, *Nitella tr.*, *Nitellopsis obtusa* and *Tolypella intricata*.

Raven[26] and Smith[27] suggest that anion membrane permeability can hardly assume such a high value. Moreover, with reference to Hope and Findlay[28,29] Raven stated that the likely increase of another anion, Cl^-, was not obtained even during cell excitation. The above stated is an argument in favour of electrogenic active transport. However, according to the latest work by Findlay and Hope[30], permeability for chlorine on excitation is about $1 \cdot 10^{-3}$–$1 \cdot 10^{-4}$ cm·s^{-1} (compare

with approx. $1 \cdot 10^{-8}$ cm·s^{-1} in the normal state[26,31]). Thus, the similar mechanism with respect to HCO_3^-, suggested by us, appears to be quite possible.

Hydrogen, as a potential-determining ion on hyperpolarization, had been considered by Hope[7]. As a matter of fact, plasmalemma permeability to H^+ may be great enough[31,32]. According to Hope's estimation[7], in this case the value of intracellular pH on plasmalemma hyperpolarization should decrease to 3.2 (the pH of the medium being 7), which is doubtful. Comparison of the data presented in Fig. 7 also shows that cell plasmalemma in the hyperpolarized state acquires properties rather close to those of a "bicarbonate electrode".

Only special investigations can give a final solution to the problem as to the kind of ion determining the potential. In our particular case, however, what is most important, is not the kind of ion but the fact that the process of hyperpolarization of plasmalemma is in many respects similar to the process of its depolarization when the cell is excited.

The response discussed has a threshold relative to the force of the irritant (the value of sudden illumination of the cell). When the threshold is exceeded, the level of membrane potential hyperpolarization does not depend on the intensity of illumination. It is defined by the value of the electrochemical potential difference of its main potential defining ion. The response may be observed both over the whole cell and in single areas. Finally, the response is connected with an inactivation mechanism.

It should be noted that contrary to normal and excited states of the external cytoplasmatic membrane, when the principal potential-defining ions are K^+ and Cl^-, respectively[33], the intracellular concentration of HCO_3^- in the state investigated, can vary depending on many conditions and primarily on the functioning intensity of the photosynthetic apparatus of the cell. Due to this fact it is very likely, that there was a scattering in the values of hyperpolarization for different cells and on reillumination sometimes even for one cell as, can be seen from Figs 2B and 2C. Depending on the ratio between the values of electrochemical potential differences for HCO_3^- and K^+, in some cases hyperpolarization of the cell plasmalemma will not be observed and its depolarization will occur. Responses of the above kind are presented by the Curves g and e in Fig. 1A. High hyperpolarization of the membrane potential in the second phase of the dark–light response is most probably a particular case for which the investigated state of plasmalemma appears most clearly. The latter makes that particular case very convenient for conducting the study.

Thus, when defining the state of the external cytoplasmatic membrane of the cell by the kind of principal potential-defining ion, into the two already known states, *i.e.* potassic (normal dark-adapted and light-adapted)and chlorous (excited) ones we have to add a new state: either a HCO_3^- or a H^+ one.

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