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THE EFFECT OF ANTIBIOTICS ON THE PHOTOCYCLE AND PROTONCYCLE OF PURPLE MEMBRANE SUSPENSIONS

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Summary

The interrelation was studied between the phototransient absorbing maximally at 412 nm (M_{412}) and light-induced proton release under steady-state conditions in aqueous suspensions of 'purple membrane' derived from *Halobacterium halobium*. The decay of M_{412} was slowed down by the simultaneous application of the ionophoric antibiotics valinomycin and beauvericin. The former had only slight activity alone and the latter was effective only in conjunction with valinomycin. The steady-state concentration of M_{412} which was formed on illumination was a direct function of the concentration of valinomycin. Maximum stabilization of M_{412} was obtained when the valinomycin was approximately equimolar with the bacteriorhodopsin. Addition of salts to the medium increased the number of protons released per molecule of M_{412} without affecting the level of M_{412} which was produced by continuous illumination. The effectiveness of the salts in this respect depended on the nature of the cation. Ca^{2+} and their antagonists La^{3+} and ruthenium red were found to have especially high affinity for the system. The extent of light-induced acidification could not be enhanced by increasing the pH of the medium from 6.5 to 7.8. The possible mechanism of action of the ionophores and of the cations on the photocycle and on the proton cycle is discussed.

Introduction

When *Halobacterium halobium* is grown at low oxygen concentrations in the light, it produces a purple coloured retinal · protein complex, bacteriorhodopsin. Bacteriorhodopsin, together with lipids, forms patches which are plugged across the cell membrane. Solar radiation absorbed by bacteriorho-

Abbreviations: BR_{570}, ground state of the chromatophore; M_{412}, phototransient with maximum absorption at 412 nm; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

dopsin creates a transmembrane proton gradient, which serves as an energy source for ATP synthesis [1,2]. The purple membrane patches can be isolated and studied in aqueous suspensions [3—8]. The absorption maximum of bacteriorhodopsin in the ground state is at 570 nm (BR_{570}). In an aqueous suspension of purple membrane a light flash induces a rapid sequence of spectral changes which are associated with cyclic proton release and uptake. The appearance of the proton in the bulk phase coincides with the rise of the transient designated as M_{412}, absorbing maximally at 412 nm [3]. Lewis et al. [4] have concluded on the basis of resonance Raman data that unexcited BR_{570} and M_{412} are respectively protonated and deprotonated at the Schiff-base linkage which connects the retinylidene prosthetic group and the free amino group of the lysine residue of the protein. Flash photometric studies led Lozier et al. [3,5] to suggest that the conformation changes which accompany the rise of M_{412} induce the release of the proton on one side of the purple membrane and subsequently a proton is taken up on the opposite side.

Actinic light of moderate intensity converts only a minor fraction of BR_{570} into M_{412} during continuous illumination. Therefore, in order to study the interrelation between the photocycle and the protoncycle in the steady-state it is necessary either to increase the rate of formation of M_{412} by using very high light intensities or to slow down the rate of its decay. Continuous illumination by high light intensities, however, may damage the system. Slowing down the rate of decay of M_{412} was achieved by Oesterhelt and Hess [6] by saturating with diethylether a suspension of purple membrane in 4 M NaCl. The presence of salt at high concentrations served to suppress a disturbing side effect of the organic solvent.

We intended to study the quantitative relation between light-induced net acidification and the level of M_{412} in the photosteady state under various environmental conditions. Therefore it was necessary to devise a method by which the steady-state concentration of M_{412} can be regulated. Since Sherman et al. [7] reported previously that valinomycin slows down the decay of the M_{412} transient, we tested the effect of various ionophores on the photocycle. In the present communication it is shown that the combined use of two ionophoric substances, beauvericin and valinomycin, caused a remarkable increment in the steady-state level of M_{412} and, as a corollary, in the light-induced acidification. Purple membrane suspensions stabilized by the ionophores were found to provide a convenient system for the study of the interrelation between the steady-state concentration of M_{412} and the extent of proton release. Some of these results have been reported briefly in a previous communication [8].

Methods and Materials

Methods

For growth of *H. halobium* and isolation of purple membrane the method of Oesterhelt and Stoeckenius [9] was used with slight modifications. Light-induced proton release was assayed at 22°C in a water jacketed cell with temperature control as described by Racker and Stoeckenius [10]. A slide projector equipped with a 500 W tungsten lamp was used for illumination of the cell. The intensity of the light was $6.0 \cdot 10^5$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. A slide pro-

jector with a 150 W lamp was used as a source for actinic light to induce spectral changes by continuous illumination. The intensity of the light beams, after passing a Corning Nr. 3486 filter, was $1.5 \cdot 10^5$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. The optical measurements were carried out at ambient room temperature (20—22°C) in a Cary, model 118, spectrophotometer. The guard filters, Corning 9782 and 3966, were placed in front of the photomultiplier tube to protect against scattered light.

In the 'basic assay medium' the purple membrane concentration was 4 μM with respect to bacteriorhodopsin, assuming an extinction coefficient of ϵ^{570}: 63 mM^{-1} $\cdot$ cm^{-1} [3]. Unless stated otherwise membrane fragments were suspended in water and brought to pH 6.5 by adding a minute amount of NaOH.

Definition of concepts and units

ΔH_L^+: Increment in the proton concentration of the assay medium (g equiv. H^+/mol bacteriorhodopsin) elicited by continuous illumation. Calibration of the increment was made with 0.1 mM HCl. ΔA_L^{412nm}: Increment in the absorbance of bacteriorhodopsin (A cm^{-1}/μmol bacteriorhodopsin/ml) elicited by continuous illumination at 412 nm. The concentration of M_{412} phototransient was calculated from ΔA_L^{412nm} assuming an extinction coefficient of $\Delta\epsilon^{412}$: 23 mM^{-1} $\cdot$ cm^{-1} [3]. For the determination of the proton yield the experimentally obtained value of M_{412} was extrapolated for the higher light intensity used in ΔH_H^+ measurements. For the extrapolation the equation $M_{412} = BR_{570}/(1 + k_2/k_1 I)$ was used [11], where k_1 and k_2 are the rate constants for the formation and the decay of M_{412} respectively, and I the light intensity.

Materials

Valinomycin and gramicidin S were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The following substances were received as gifts: beauvericin and A23187 from Eli Lilly and Co., Indianapolis, Ind., U.S.A.; monensin, dianemycin and A-204 from Dr. H.A. Lardy, Institute for Enzyme Research, Madison, Wisc., U.S.A.; nigericin from Dr. N. Shavit, Ben-Gurion University of the Negev, Beer-Sheva, Israel; gramicidin A from Dr. S.R. Caplan, Weizmann Institute of Science, Rehovot, Israel.

Results

Screen-test for compounds which stabilize M_{412}

Light-induced acidification in aqueous suspensions of purple membrane is known to be correlated with the steady-state concentration of M_{412} [6]. Therefore, we searched for compounds which cause an increase in the absorbancy at 412 nm when the purple membrane suspension is illuminated by actinic light. Substances with known (cf. refs. 12, 13, 14) ionophoric and/or protonophoric capacity were tested. Since ionophores may require the presence of specific cations for their action, a mixture of salts containing Na^+, K^+ and Ca^{2+} was added to the incubation mixture. No stabilization of M_{412} was noted with the following substances: the channel forming non-cyclic depsipeptide, gramicidin A; the negatively charged ionophores nigericin, monensin, dianemycin, A-204

TABLE I

EFFECT OF ANTIBIOTICS ON LIGHT-INDUCED ABSORBANCE CHANGE AT 412 nm

The basic assay medium was incubated for 60 min at 22°C with 20 μg/ml of one of the substances listed in this table and ΔA_L^{412nm} was measured. A salt mixture (NaCl, KCl, $CaCl_2$, 10 mM with respect to each) was added subsequently to the assay medium and after readjustment of the pH to 6.5, and further 60 min of incubation ΔA_L^{412nm} was redetermined.

Reagent added	Structure	Selectivity for cations	ΔA_L^{412nm}	
			No salt	With salt
None	—	—	0.02	0.07
Valinomycin	Neutral, cyclic dodecadepsipeptide	$K^+ >> Na^+$	0.75	1.00
Beauvericin	Neutral, cyclic hexadepsipeptide	Ba^{2+}, $Ca^{2+} >> Na^+$, K^+	0.06	0.10
Valinomycin plus beauvericin	—	—	5.00	6.50
Gramicidin S	Neutral, cyclic decapeptide	Protonophore	0.60	0.60
Gramicidin S plus valinomycin	—	—	1.40	1.60
Gramicidin S plus beauvericin	—	—	0.70	0.70

and A-23187. Also the uncoupling agents 2,4-dinitrophenol and FCCP and the inhibitors of energy transfer oligomycin and *N'*,*N'*-dicyclohexylcarbodiimide were found to be ineffective in this respect. On the other hand, as shown in Table I, significant stabilization of M_{412} resulted in the presence of the neutral, cyclic depsipeptides valinomycin and gramicidin S. The neutral cyclodepsipeptide, beauvericin, had little effect when added alone but acted as a powerful synergist in combination with valinomycin. It is also apparent that the salt mixture moderately enhanced ΔA_L^{412} when valinomycin was present either alone or together with beauvericin. From experiments which were not included in Table I, it became apparent that the salt-effect was entirely due to the KCl added. There was some variation in the extent of K^+-dependence, probably because of differences in the residual amount of K^+ associated with various purple membrane preparations. The maximum increment obtained by KCl was 20—40% and the KCl concentration, which was necessary to attain maximum enhancement was 1—3 mM. KCl had no effect on ΔA_L^{412} up to a concentration of 50 mM in the absence of valinomycin.

The time-course of the ionophore effect

In the experiment shown in Fig. 1 valinomycin was added to an aqueous suspension of purple membrane, which was preincubated with beauvericin. ΔA_L^{412nm} and ΔH_L^+ were assayed at the time intervals indicated. As seen from Fig. 1 the interaction between valinomycin and purple membrane was a time-dependent process. The stabilization of M_{412} and the correlated light-induced acidification reached their maxima after approximately 5 min. The ratio between ΔA_L^{412nm} and ΔH_L^+ remained constant throughout the experiment, indicating a direct correlation between these two parameters.

The stoichiometry of interaction between valinomycin and bacteriorhodopsin

In the experiments shown in Fig. 2 increasing amounts of valinomycin were added to an aqueous purple membrane suspension with or without preincuba-

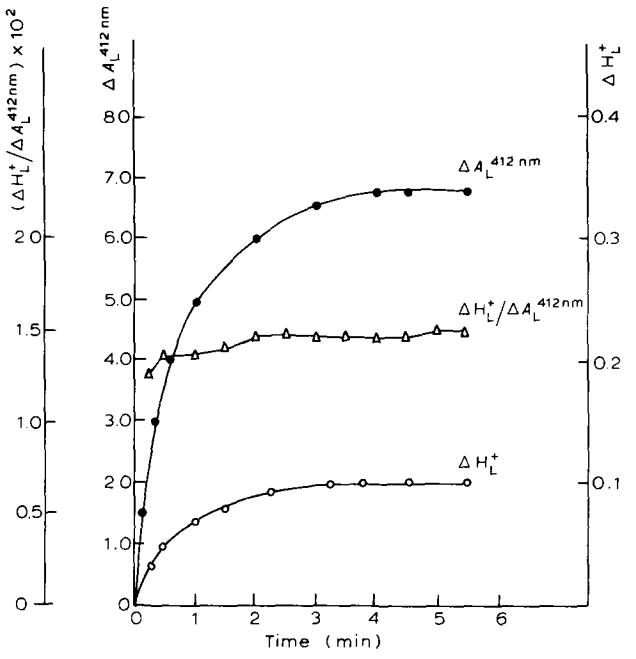

Fig. 1. The time-course of the valinomycin effect on ΔH_L^+ and ΔA_L^{412nm}. The basic assay medium also containing 3 mM KCl was incubated with 4 μg of beauvericin/ml for 60 min at 22°C. After adjusting the pH to 6.5 by NaOH the reaction was started by the addition of 10 μg of valinomycin/ml. The rise in the light-induced spectral change (ΔA_L^{412nm}) and proton release (ΔH_L^+) was followed in separate experiments as described under Materials and Methods.

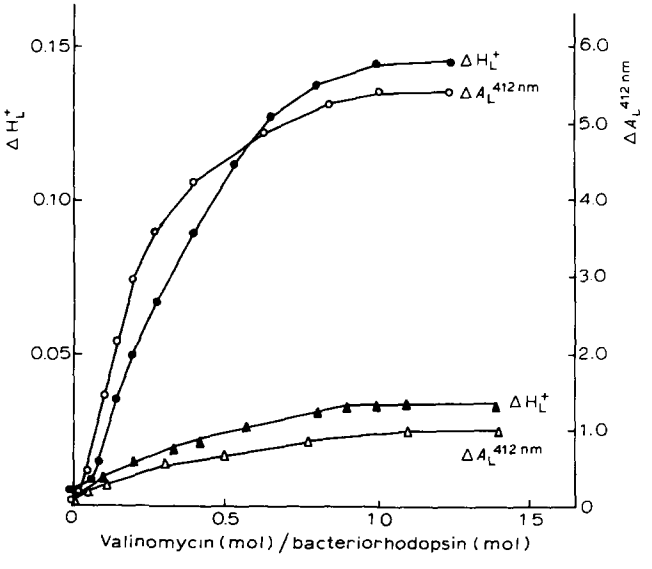

Fig. 2. The stoichiometry of the interaction between BR and valinomycin. The basic assay medium containing 3.0 mM KCl was titrated with valinomycin as indicated. Between each addition of valinomycin there was an interval of 15 min. All incubations were at 22°C. △, ▲, no beauvericin added; ○, ●, preincubation with 4 μg/ml of beauvericin for 60 min.

tion of the latter with beauvericin. After each addition of valinomycin ΔA_L^{412nm} and ΔH_L^+ were measured. The data presented in Fig. 2 show that both in the presence and in the absence of beauvericin approximately one molecule of valinomycin was necessary per molecule of bacteriorhodopsin to produce maximum ΔA_L^{412nm} and as a corollary also maximum light-induced acidification. When the concentration of purple membrane was lowered the amount of valinomycin to produce maximum effect was also reduced correspondingly (not shown).

Effect of salts on ΔH_L^+

When purple membrane was preincubated with the ionophores in the presence of a few millimolar KCl, addition of salts was observed to cause a significant increase in ΔH_L^+ without a corresponding rise in ΔH_L^{412nm}. Some typical results are shown in Fig. 3 in which ΔH_L^+ is presented as a function of the concentration of NaCl, $CaCl_2$ or $La(NO_3)_3$ respectively. The maximum enhancement in ΔH_L^+ induced by the above three salts is of similar magnitude (200—300%), however there is a large difference in the apparent K_m values.

From titration curves the following apparent K_m values were obtained for different salts: NaCl: 15 mM; KCl: 10 mM; $MgCl_2$, $MnCl_2$ and $BaCl_2$: 2 mM; $CaCl_2$: 0.5 mM; $La(NO_3)_3$: 5 μM and for the cationic dye, ruthenium red: 5 μM. It is noteworthy that the K_m for Ca^{2+} is considerably lower than for the other divalent cations and that La^{3+} and ruthenium red, which are known [15] to

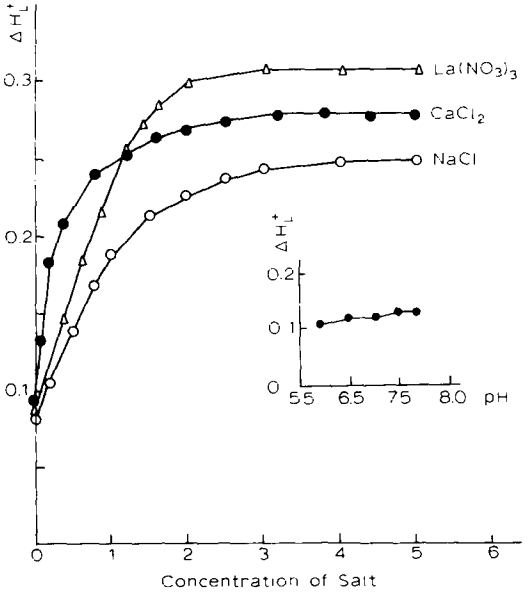

Fig. 3. Effect of salts on ΔH_L^+. The basic assay medium containing 3.0 mM KCl was incubated with 4 μg/ml of beauvericin and 10 μg/ml of valinomycin at 22°C for 60 min and titrated with the respective salt. After each salt addition the pH was readjusted by NaOH to 6.5. One unit on the abscissa represents the following concentrations of salt: NaCl, 20 mM; $CaCl_2$, 2.5 mM; $La(NO_3)_3$, 5 μM. The conditions of the experiment shown as an insert to Fig. 3 were as above. No salts added. The pH was adjusted, as indicated, by NaOH.

TABLE II

EFFECT OF SALTS ON THE PROTON-YIELD

To the basic assay medium also containing 3.0 mM KCl and 4 μg/ml of beauvericin the indicated concentrations of valinomycin were added. After incubation at 22°C for 60 min light-induced pH and absorbancy changes were measured. The measurements were then repeated after adding, in Expt. I, 20 mM $CaCl_2$ and in Expt. II, 20 μM $La(NO_3)_3$ to the medium. M_{412} was calculated from the absorbance changes at 412 nm as described under Materials and Methods.

Valinomycin (μg/ml)	M_{412} * (nmol/ml)	nequiv. H^+/ml		H^+/M_{412} **	
		No salt	With salt	No salt	With salt
Expt. I					
0.5	0.51	0.10	0.42	0.20	0.82
1.0	0.85	0.17	0.75	0.20	0.71
2.0	1.37	0.30	1.08	0.22	0.79
4.0	1.70	0.50	1.42	0.30	0.83
Expt. II					
0.4	0.40	0.09	0.20	0.22	0.50
1.0	0.75	0.14	0.40	0.19	0.53
2.0	0.99	0.24	0.66	0.25	0.67
5.0	1.90	0.59	1.45	0.31	0.76

* Extrapolated for the light intensity of $6.0 \cdot 10^5$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} by which the proton release was induced.

** Number of protons released per molcule of M_{412} present in the steady-state.

bind in various systems to the same site as Ca^+, are effective already in the micromolar concentration range.

Since in the range of concentration in which they enhanced the acidification, salts had practically no effect on ΔA_L^{412}, the question could be raised whether the increment in proton release is connected at all with the formation of M_{412}. The experiment presented in Table II was performed in order to assess whether, also in the presence of salts, the proton release is proportional with the extent of changes in the absorbancy at 412 nm. M_{412} was produced at various steady-state levels by variation of the valinomycin concentration. For each level of M_{412} the light-induced acidification was determined on the one hand in the absence of added salt and on the other in presence of optimum concentrations of Ca^{2+} or La^{3+}. As seen from Table II, a near proportionality was found to be maintained between M_{412} and proton release both in the absence and in the presence of salts, although in the latter case the number of protons produced per molecule of M_{412} was considerably increased.

One possible interpretation of the above results could be, that the salts increased the extent of dissociation of a group on the surface of the membrane which is directly connected with the release of the proton into the bulk phase. Therefore, we tried to bring about the same effect by increasing the pH of the medium. However, as seen from the curve which is inserted in Fig. 3, the light-induced acidification was not significantly changed between pH 6.5 and 7.8.

Discussion

The simultaneous addition of the two ionophores, valinomycin and beauvericin, to an aqueous suspension of purple membrane sheets was found to offer

a convenient method for the study of the interrelation between the proton-cycle and the photocycle elicited by the illumination of bacteriorhodopsin. With the aid of the ionophores the steady-state level of M_{412} can be changed in a controlled manner and the proton yield for each level of M_{412} can be determined. Variations in the steady-state concentration of M_{412} between 8 and 38% of the total bacteriorhodopsin caused a correspondingly large change in the light-induced acidification.

Sherman et al. [7] suggested that the active species which perturbs the photocycle is the positively charged ionophore · cation complex. In agreement with their view, among the large number of ionophores tested, those which were found to stabilize M_{412} in the photo-steady state form positively charged complexes with cations or proton [12]. However, it also became clear that only a few of the ionophores belonging to this class are active. The most interesting case is that of beauvericin. This neutral hexadepsicyclopeptide is ineffective but acts as a powerful synergist when applied in conjunction with the neutral dodecadepsicyclopeptide, valinomycin. The rather stringent structural requirements for the action and the finding that valinomycin is most effective when added in a molecular ratio of one to one in relation to bacteriorhodopsin, point to the possibility that the charged hydrophobic complex interacts specifically with the bacteriorhodopsin protein.

In the light of the work of Lozier et al. [5], of several other authors and most recently of Drachev et al. [16] the major events which take place when BR_{570} is exposed to a light flash are as follows: (a) Dissociation of a proton from the Schiff base gives rise to the M_{412} transient and the proton is translocated during the 570 nm → 412 nm transition to the external medium; (b) Coincidentally with the 412 nm → 570 nm regenerative transition, M_{412} is reprotonated by a proton which originated from the water of the external medium; (c) Proton extrusion and proton uptake occur on opposite surfaces of the assymetrical purple membrane sheet. In a suspension of purple membrane sheets, in which both surfaces are in contact with the same medium, net acidification is expected to be the greater the slower is the proton uptake on one surface relative to the proton release from the other surface. Moreover, if the rate of the reprotonation of the Schiff base is controlled by the rate at which protons can diffuse through the peripherial regions of the bacteriorhodopsin molecule (cf. ref. 17) then inhibition of the proton uptake will be reflected by a corresponding rise in the steady state level of M_{412}. It is, therefore, conceivable that the hydrophobic, positively charged, antibiotic-cation complex penetrates the membrane, binds to the protein and by electrostatic charge screening (cf. ref. 16) impedes the movement of the proton from the medium to the Schiff base. The observed constancy of the proton yield (H^+/M_{412}) at various valinomycin concentrations is in accordance with such assumption. Alternative mechanisms which are worthy of consideration are: (a) Change in the state of aggregation of the lipid moiety of the membrane, similar to valinomycin-induced aggregation in lecithin liposomes [18]; (b) perturbation of the water structure near the active site. (Dehydration of purple membrane films has been shown to increase the stability of M_{412} [19].)

The enhancement of the light-induced acidification in the photosteady state by cations has quite different characteristics than that mediated by the anti-

biotics. The former, like the latter, are connected with 'stoichiometric' protons [20], i.e. there is a stoichiometric relation between the extent of cation-induced acidification and the level of M_{412} in photosteady state. However, the cations at their effective concentrations do not increase the steady state level of M_{412} but only the number of protons released into the medium per molecule of M_{412} present (i.e. increase H^+/M_{412}). This pattern of action suggests that the cations by binding on the surface reduce the internal buffer capacity of the membrane sheets. Thus, a larger fraction of the photodissociated protons will reach the external medium and will be sensed by the glass electrode.

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