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TWOFOLD EFFECT OF VALINOMYCIN ON ISOLATED SPINACH CHLOROPLASTS: UNCOUPLING AND INHIBITION OF ELECTRON TRANSPORT

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SUMMARY

1. Treatment of isolated chloroplasts with valinomycin above 10^{-7} M inhibits both photophosphorylation and ms-delayed light emission, with maximum inhibition occurring at 10^{-5} M.

2. In the same concentration range it stimulates electron flow, when the pH of the medium is between 7 and 8, inhibits the extent of the light-induced pH change and reverses 2,3,5,6-tetramethyl-*p*-phenylenediamine-quenched chlorophyll *a* fluorescence in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

3. These effects of valinomycin occur in the absence of added K^+ although the presence of K^+ does facilitate its action.

4. It is suggested that high concentrations of valinomycin not only induce a change in the K^+ permeability but also in the H^+ permeability of the thylakoid membrane and in so doing dissipate the high-energy state, i.e. uncouple.

5. However, electron transport data do not clearly demonstrate the uncoupling action of valinomycin since it also inhibits electron flow.

6. Valinomycin at concentrations above 10^{-6} M inhibits electron flow and stimulates chlorophyll *a* fluorescence of chloroplasts uncoupled by nigericin or NH_4Cl.

7. The dual effect of valinomycin accounts both for the previous suggestion that this compound inhibits photophosphorylation by acting as an energy transfer inhibitor and also explains why valinomycin modifies both normal ms-delayed light and KCl-induced ms-delayed light transients.

INTRODUCTION

Valinomycin has been reported to increase the permeability of black lipid membranes to a number of monovalent cations: $H^+ > Rb^+ > K^+ > Cs^+ > Na^+$ in

Abbreviations: Diaminodurene, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DCIP, 2,6-dichlorophenol indophenol; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid; Tricine, *N*-tris-(hydroxymethyl)methyl glycine.

that order [1, 2]. However, it has usually been assumed that concentrations of valinomycin which increase the permeability of the coupling membrane of mitochondria, chloroplasts and chromatophores to K^+ do not affect the permeability of the membrane to H^+. Mitchell [3] argued that the H^+ permeability of mitochondrial membranes is not increased because of the low concentration of H^+ at physiological pH values compared to the usual concentration of monovalent cations present in the reaction medium.

Valinomycin alone is a very effective uncoupler of mitochondria [4] and will also uncouple chromatophores in conjunction with nigericin (neither being effective alone) [5]. At low concentration (i.e. a concentration that uncouples mitochondria) valinomycin has no effect on chloroplast photophosphorylation and electron transport measured under steady state illumination [6] while nigericin alone is a potent uncoupler. Low concentrations of valinomycin will, however, act synergistically with dinitrophenol or with a low concentration of nigericin (which do not uncouple on their own) to uncouple photophosphorylation [7, 8].

The various effects of valinomycin and other ionophorous antibiotics on mitochondria, chloroplasts and chromatophores have been regarded as strong support [9, 10] for Mitchell's proposal [11] that the major component of the proton motive force is an electrical potential ($\Delta\psi$) in mitochondria and chromatophores and a pH gradient (ΔpH) in chloroplasts.

Although increase in K^+ permeability of the chloroplast membrane by low concentrations of valinomycin usually has no effect on steady state photosynthetic activities there have been a number of confusing reports on the effect of valinomycin at higher concentrations. Initially Avron and Shavit [6] reported that at high concentration valinomycin acts as a weak uncoupler but Karlish and Avron [12] later concluded that it acts as an energy transfer inhibitor. Keister and Minton [13] found that ATP formation and coupled electron flow were inhibited by high concentrations of valinomycin in a K^+-independent reaction. However, they also showed that this inhibition was not completely reversed by uncouplers and that H^+ uptake and ATPase activity were affected by valinomycin in a manner more analogous to the effect of an uncoupler than an energy transfer inhibitor. They therefore concluded that valinomycin has several different actions at high concentration but they did not suggest a mechanism.

In addition, Keister and Minton [13] showed that valinomycin, like well-established uncoupling agents, inhibited ms-delayed light emission and did not act like energy transfer inhibitors which increase the emission intensity. This sensitivity of ms-delayed light to the phosphorylating ability of chloroplasts was first demonstrated by Mayne and Clayton [14] and explained by Kraan et al. [15] and Wraight and Crofts [16] in terms of a model involving Mitchell's concepts of the high-energy state [11]. They suggested that ms-delayed light is sensitive to the pH gradient and the membrane potential. Wraight and Crofts [16] showed that at low concentrations of valinomycin only the induction kinetics of the ms emission process were altered and not the extent, in a K^+-dependent reaction, while at higher concentration both phosphorylation and the extent of ms-delayed light were inhibited, in a K^+-independent reaction.

In this paper we have attempted to clarify the mode of action of valinomycin in isolated chloroplasts with particular reference to its ability to inhibit phosphorylation and ms-delayed light emission.

MATERIALS AND METHODS

Chloroplasts were isolated essentially as described previously [17] from spinach purchased from the local market. Final suspension after a sucrose wash was either in 0.33 M sucrose and 5 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) brought to pH 7.0 with less than 2 mM KOH or NaOH or in 0.33 M sucrose and 20 mM *N*-tris(hydroxymethyl)-methyl glycine (Tricine) brought to pH 8.0 with KOH. Chlorophyll concentrations were determined by the method of Arnon [18]. Electron transport was measured as O_2 uptake in the presence of methyl viologen. Photophosphorylation was monitored by changes in pH of the reaction mixtures [19] and for these experiments the buffer concentration was lower than that routinely used. Low buffer concentration was also used for measurement of the extent of reversible light-induced pH changes. Simultaneous measurements of O_2 and pH changes were made in a Rank O_2 electrode cell fitted with a combined glass electrode (Pye Unicam EJ 702) through the lid of the reaction vessel. The reaction mixture was maintained at a temperature of 20 °C and was illuminated with $2 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} white light through a heat filter.

The intensity of 1 ms-delayed light was continuously measured with a rotating sector phosphoroscope as previously described [17]. Prompt fluorescence was measured simultaneously using a photomultiplier placed at right angles to the exciting light beam. The exciting light was transmitted by a Balzer Calflex C, 2-mm Schott BG38 and 4-mm Schott BG18 giving, an intensity of $2.5 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. The photomultipliers were shielded from the blue exciting light by appropriate red filters as described earlier [17]. Various additions to 3 ml of suspension contained in a 1-cm cuvette were often made during measurement of prompt and delayed fluorescence by rapidly injecting 100 μl of the appropriate stock using a syringe inserted through a light tight rubber diaphragm. This procedure gave reproducible signals with a mixing time in the region of 200 ms. These experiments were carried out at room temperature (approx. 25 °C).

Valinomycin and nigericin were obtained from the Lilly Laboratories, Indianapolis. Valinomycin was also purchased from Sigma Chemical Company, London. 2,3,5,6-Tetramethyl-*p*-phenylenediamine(diaminodurene) was purchased from Eastman Chemicals. Pure 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was recrystallized from technical grade DCMU obtained from Hickson and Welch Ltd. ADP and other biochemicals were obtained from Sigma Chemical Company, London.

RESULTS

The effect of valinomycin on ms-delayed light emission

Fig. 1 shows the effect of valinomycin concentrations on the intensity of delayed light emission from spinach chloroplasts and on the size of KCl-induced transients measured in the absence of an electron acceptor. In this experiment the intensity of ms-delayed light emission was recorded 1 min after the addition of valinomycin in the light before the injection of KCl.

The steady state level of ms-delayed light is not affected by low concentrations of valinomycin but is inhibited by concentrations above 10^{-8} M. In this experiment the reaction mixture contained a low concentration of K^+ (2 mM) in order that the

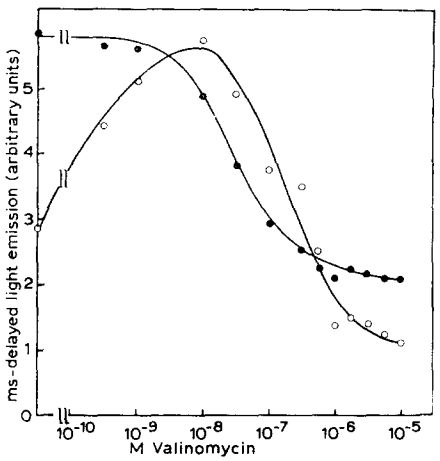

Fig. 1. Effect of valinomycin concentration on delayed light emission in the absence of an added electron acceptor and on KCl-induced delayed light transients stimulated under these conditions. Reaction mixtures contained in a final volume of 3 ml: 5 mM TES buffer (brought to pH 7.0 with KOH to give a final K^+ concentration of 2 mM), 0.33 M sucrose and chloroplasts equivalent to 34 μg of chlorophyll. Each reaction mixture was incubated in the dark for 1 min in the absence of valinomycin. Valinomycin was added after 30 s illumination. The level of delayed light emission (●–●) was measured after a total illumination period of 60 s. Salt-induced light emission was brought about by the rapid addition of 100 μl of 1.5 M KCl after 60 s of illumination to give a final concentration of 50 mM KCl. The initial height of the salt-induced light emission was measured (○–○). Other details as described in Materials and Methods.

sensitivity of KCl-induced transients to various concentrations of valinomycin could be studied [17]. However, inhibition of the steady state level of delayed light emission was brought about the by same concentration of valinomycin when the medium contained increased levels of KCl except that the inhibition by high concentrations of valinomycin was more pronounced.

In the presence of an electron acceptor the yield of delayed light was increased but again high concentrations of valinomycin inhibited the level, the degree of inhibition depending on the presence of K^+. We consistently found that in the presence of an electron acceptor a higher concentration of valinomycin was required (concentration for 50 % inhibition; 10^{-6} M in the presence of acceptor, $3 \cdot 10^{-8}$ M in the absence of acceptor). This inhibition of ms-delayed light emission by high concentrations of valinomycin has also been observed by Keister and Minton [13] and Wraight and Crofts [16] as well as by Barber [17].

Although low concentrations of valinomycin (less than 10^{-8} M) did not reduce the extent of ms-delayed light emission the K^+ permeability of the membrane was affected. Fig. 1 shows that the size of the KCl-induced ms-delayed light transients [17] were increased by low concentrations of valinomycin. In general it was found that the size of the KCl-induced transients increases with increasing valinomycin concentration as long as the steady state signal is not lowered. At approximately the concentration of valinomycin that begins to lower the steady state level of delayed light the KCl signals decrease in size.

The effect of valinomycin on electron transport, photophosphorylation and related processes

Table I shows the effect of a high concentration of valinomycin on electron transport uncoupled by nigericin. Electron flow from water to methyl viologen or ferricyanide is inhibited 60–70 % by 10^{-5} M valinomycin. In the presence of DCMU electron flow from reduced 2,6-dichlorophenol indophenol (DCIP) to methyl viologen is not affected by this concentration of valinomycin. Similar results are obtained if NH_4Cl is used in place of nigericin as uncoupler. Under these conditions electron flow is completely uncoupled from energy conserving processes and the inhibition of electron flow must result from a direct inhibition of the electron transport chain, before the site of donation of electrons by reduced DCIP. This effect of valinomycin is independent of K^+. The valinomycin inhibited rate of electron transport was found to have the same broad pH optimum as the nigericin uncoupled rate (pH 7.5–8.3).

TABLE I

INHIBITION OF NIGERICIN-UNCOUPLED ELECTRON TRANSPORT BY VALINOMYCIN

The reaction mixtures contained in a final volume of 3 ml: 20 mM Tricine buffer (pH 8.0), 0.33 M sucrose, 10 mM KCl, $9 \cdot 10^{-8}$ M nigericin and chloroplasts equivalent to 106 μg chlorophyll. In addition Expt (i) included 0.5 mM NaN_3 and 10 μM methyl viologen, Expt (ii) included 0.66 mM potassium ferricyanide and Expt (iii) included 3.3 μM DCMU, 33 μM DCIP and 1.66 mM sodium ascorbate. Other reaction conditions were as described in Materials and Methods.

Expt No.	Electron transport System	Additions	Electron transport rate (μequiv $e^-{}_2$/mg chlorophyll per h)
(i)	$H_2O \rightarrow$ methyl viologen	None	214
		10^{-5} M valinomycin	60
(ii)	$H_2O \rightarrow$ potassium ferricyanide	None	187
		10^{-5} M valinomycin	71
(iii)	$DCIPH_2 \rightarrow$ methyl viologen	None	160
		10^{-5} M valinomycin	160

There have been several reports that high concentrations of valinomycin inhibit photophosphorylation [7, 12, 13, 22] in a more or less K^+-independent reaction. We found that for 50 % inhibition of ATP formation approx. 10^{-6} M valinomycin was required. This was a similar concentration to that reported by Keister and Minton [13] but a slightly greater concentration than that reported by Wraight and Crofts [22]. Karlish and Avron [12] found an inhibition at much lower concentrations but this may be related to the low rate of phosphorylation in their experiments. They measured a P/2e^- ratio of only 0.35 while in our experiments the P/2e^- ratio was approx. 1, the normal ratio found with broken washed spinach chloroplasts. Inhibition of ATP formation could be caused by uncoupling, inhibition of electron transport or by energy transfer inhibition.

We therefore investigated the effect of a high concentration of valinomycin (10^{-5} M) on the basal rate of electron flow. Fig. 2 shows the effect of increasing valinomycin concentration on the basal rate of electron flow and on the extent of the light-induced pH change measured at pH 8.0. Above $3 \cdot 10^{-6}$ M valinomycin O_2

uptake in the presence of methyl viologen is stimulated while H^+ uptake is inhibited such that at 10^{-5} M valinomycin the rate of electron transport is doubled while H^+ uptake is 80 % inhibited.

Fig. 3 shows the pH dependence of the effect of 10^{-5} M valinomycin on the

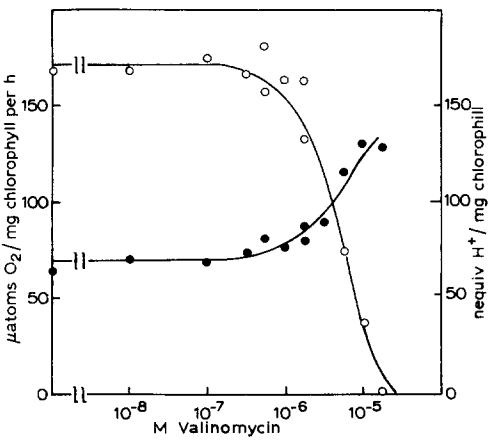

Fig. 2. Effect of various valinomycin concentrations on the basal rate of electron transport (●–●) and the extent of H^+ uptake (○–○). Reaction mixtures contained in a final volume of 3 ml: 1 mM Tricine buffer, 0.33 M sucrose, 100 mM KCl, 0.5 mM NaN_3, 10 μM methyl viologen and chloroplasts equivalent to 246 μg chlorophyll. Reaction mixtures were brought initially to pH 8.0 with KOH. Other reaction conditions were as described in Materials and Methods.

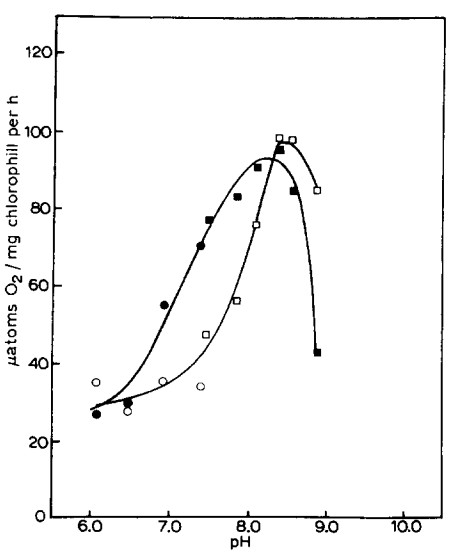

Fig. 3. pH dependence of the effect of valinomycin on the basal rate of electron transport. Reaction mixtures contained in a final volume of 3 ml: 10 mM Tricine buffer (□, ■) or 10 mM TES buffer (○, ●), 0.33 M sucrose, 0.5 mM NaN_3, 50 mM KCl, 10 μM methyl viologen and chloroplasts equivalent to 130 μg chlorophyll. □ and ○, no additions, ■ and ●, plus 10^{-5} M valinomycin. Other reaction conditions were as described in Materials and Methods.

basal rate of electron transport. The basal rate of electron transport shows a pH dependence similar to that reported by others [20, 21] with a pH optimum at pH 8.5. The effect of valinomycin is such that the pH optimum is decreased to around pH 8.0 more like that of the uncoupled rate of electron flow. It can be seen that the stimulation of the basal rate by valinomycin occurs only between pH 6.5 and pH 8.3. Around pH 8.4 there is no significant effect of 10^{-5} M valinomycin on the basal rate of electron transport and above this pH electron transport is inhibited. In view of the change of the pH profile of electron transport to one more like that of the uncoupled rate and to the fact that this concentration of valinomycin inhibits electron transport uncoupled by nigericin (see Table I) it seems likely that valinomycin acts as an uncoupler at high concentrations. However its uncoupling action cannot be observed in the classic way (as a strong stimulation of the basal rate of electron flow) because it has a second inhibitory effect directly on electron transport. For example in the experiment of Fig. 3 addition of nigericin after 10^{-5} M valinomycin caused no further change in the rate of electron flow.

Table II shows that for maximal effect of valinomycin on electron transport and the extent of H^+ uptake, K^+ were required as was also seen in the case of delayed light emission.

TABLE II

THE EFFECT OF VALINOMYCIN ON ELECTRON TRANSPORT AND PROTON UPTAKE IN THE PRESENCE OF DIFFERENT CATIONS

The reaction mixtures contained in a final volume of 3 ml: 0.33 M sucrose, 0.5 mM NaN_3 and 20 μM methyl viologen. Chloroplasts equivalent to 150 μg chlorophyll were present in Expt (i) and 230 μg in Expt (ii). The reaction mixtures were brought initially to approx. pH 8.0 with NaOH. Reaction conditions were as described in Materials and Methods.

Expt No.	Salt	Additions	Electron transport rate (μequiv e^-_2/mg chlorophyll per h)	H^+ uptake (μequiv H^+/mg chlorophyll)
(i)	100 mM KCl	None	57	124
		10^{-5} M valinomycin	120	15
	100 mM choline chloride	None	70	148
		10^{-5} M valinomycin	75	67
(ii)	50 mM KCl	None	46	242
		10^{-5} M valinomycin	75	11
	50 mM NaCl	None	48	188
		10^{-5} M valinomycin	50	83

Both inhibition of electron transport and uncoupling by valinomycin may be observed by studying prompt chlorophyll *a* fluorescence.

Quenching of the fluorescence of broken chloroplasts occurs on addition of an electron acceptor. It is thought that this is due not only to the redox state of the primary acceptor, Q, but also to the establishment of a high energy state [22, 23]. We found that quenching of fluorescence brought about by potassium ferricyanide in the presence of the uncoupler, nigericin, and therefore due only to the redox state of Q is partially reversed (approx. 50 %) by 10^{-5} M valinomycin. We also found

that quenching of fluorescence brought about by diaminodurene in the presence of DCMU and therefore due only to the high energy state is reversed by 10^{-5} M valinomycin. Uncouplers which also reverse quenching in the presence of diaminodurene cause no further reversal when added after 10^{-5} M valinomycin.

DISCUSSION

The results presented above suggest that the effect of valinomycin on photosynthetic electron transport and energy conservation may be interpreted as a combination of two independent actions: uncoupling of ATP formation from electron flow and direct inhibition of electron transport.

The complete inhibition of ATP formation, H^+ uptake and ms-delayed light emission are in agreement with the suggestion that valinomycin acts as an uncoupler. We have also shown that uncoupled electron transport is inhibited by a high concentration of valinomycin and that the site of inhibition is before the site of donation of electrons by reduced DCIP. This is in agreement with reports that electron transport coupled to ATP formation is inhibited by valinomycin [7, 13] and that this inhibition is only partially reversed by addition of well-known uncouplers [13].

It is this dual action of valinomycin which probably accounts for why the uncoupling action of this compound has not been reported previously. Classical detection of uncoupling by stimulation of electron transport can only clearly been seen at pH values in the region of 7.3. At higher pH values the basal rate was either not affected by valinomycin as reported by Keister and Minton [13] or was inhibited. The data on chlorophyll *a* fluorescence also suggests that valinomycin acts both as an uncoupler and as an inhibitor and agrees with the report of Wraight and Crofts [22] that valinomycin reverses diaminodurene-quenched fluorescence as do well-known uncouplers such as nigericin and dianemycin.

In terms of Mitchell's concepts [11] uncouplers act on chloroplasts by directly or indirectly increasing the permeability of the thylakoid membrane to H^+ and thus allowing the high-energy state to be dissipated in a non-energetically useful manner. Mitochondria are uncoupled by low concentrations of valinomycin (approx. $5 \cdot 10^{-8}$ M) in a K^+-dependent reaction [4]. Uncoupling is accompanied by K^+ uptake and swelling. Mitchell [11] suggested that its action on mitochondria could be explained entirely in terms of an increase in K^+ permeability, arguing that the net K^+ influx was electrogenic and dissipated the membrane potential component of the proton motive force.

Uncoupling of chloroplasts by valinomycin only occurs at high concentrations ($> 10^{-6}$ M) and will occur in the absence of added K^+ although its presence does enhance the uncoupling effect. Isolated chloroplasts unlike mitochondria exhibit a large pH difference across the coupling membrane and probably maintain a relatively small membrane potential. It has been suggested that this is because the thylakoid membrane is generally more permeable to ions than the mitochondrial membrane. In the case of chloroplasts for example, it seems that nigericin uncouples by allowing H^+ to efflux down its concentration gradient via an electrically neutral exchange with K^+.

Although valinomycin has been reported to increase the permeability of synthetic membranes to H^+, as well as K^+ [1], it has been suggested that this increase is

not significant at physiological pH values [3]. However, Andreoli et al. [1] showed that the fall in electrical resistance of black lipid membranes was directly proportional not only to K^+ and H^+ levels in the media but also to the valinomycin concentration. Thus one might expect even at physiological pH values to see an increase in the H^+ permeability of membranes in the presence of high valinomycin concentrations. As chloroplasts are uncoupled by valinomycin under these conditions and assuming that uncoupling occurs when H^+ permeability is increased, it seems likely that valinomycin can increase the rate of H^+ conduction across the thylakoid membrane.

This effect may be amplified because, unlike mitochondria and bacterial chromatophores, illuminated chloroplasts can maintain a substantial pH gradient across the thylakoid membrane. Rottenberg et al. [24], among others have demonstrated that the internal pH during illumination is in the order of three pH units below that of the external pH. The optimum internal pH for photophosphorylation is around pH 5.5. The increase in internal H^+ concentration may also contribute to the uncoupling action of high concentrations of valinomycin.

From ms-delayed light measurements we were able to detect that a low concentration of valinomycin ($< 10^{-8}$ M) which did not reduce the extent of the steady state emission or inhibit ATP synthesis did affect K^+ permeability. This was seen as a stimulation in the extent of KCl-induced transients and is consistent with the effect of low concentrations of valinomycin on the decay of the flash-induced 515-nm absorbance signal [25] and the inhibition of the fast component of ms-delayed light induction kinetics [16], both of which are K^+-dependent processes. At high concentrations the steady state level is inhibited and this is accompanied by a reduction in the size of the KCl transients (see Fig. 1). This has already been explained in earlier papers [17, 26] by assuming an exponential relationship between the signal observed and the membrane potentials created by KCl diffusion across the thylakoids and by light-induced processes. However, in the earlier analyses of the KCl transients it was assumed that K^+ and Cl^- were the main diffusing ions. From the work presented in this paper it now seems likely that there is an increase in the H^+ permeability of the thylakoid membranes when high concentrations of valinomycin are used. Although this probably makes very little difference to the concepts and quantitative analyses presented in the previous papers [17, 26] it does clarify several observations. It seems reasonable to assume that the decrease in the steady state level of ms-delayed light is due to H^+ leakage causing a reduction of electrical and pH gradients which constitute the high-energy state. Moreover, the increase in H^+ permeability when valinomycin is present would account for the deviation from a simple Nernst relationship between the KCl-induced signals and the K^+ gradients used, a possibility which was suggested previously [17].

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