THE EFFECT OF NIGERICIN AND VALINOMYCIN ON CO_2 FIXATION ELECTRON TRANSPORT AND P518 IN INTACT SPINACH CHLOROPLASTS

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1. Introduction

Nigericin is a potent uncoupler of photophosphorylation in isolated chloroplasts [1, 2] and bacterial chromatophores [3] whereas valinomycin alone is ineffective [4, 5]. Nigericin is an ion transport antibiotic which brings about exchange of potassium ions and protons across natural and artificial membranes [3, 6, 7]. Valinomycin causes only the equilibration of potassium ions according to the electrochemical potential [6, 7].

In chromatophores [3] it has been shown that the effects of nigericin and valinomycin are consistent with the Mitchell hypothesis of phosphorylation in which a proton motive force, comprising essentially of a pH gradient and an electrial potential gradient, drives the phosphorylative process. Carotenoid absorption changes at 525 nm were also shown to be consistent with the hypothesis that these changes are an indicator of the membrane potential [8, 9]. In chloroplasts the evidence is less clear. The lack of uncoupling of photophosphorylation by valinomycin suggests that a membrane potential is not an appreciable component of the proposed proton motive foce. However, it is possible that in the type of chloroplasts [envelope-free and swollen] which were used for these measurements, the permeability properties of the thylakoid membranes were altered [10]. Witt et al. [11, 12] and Larkum and Bonner [10] have presented evidence correlating light-induced changes of P518 in chloroplasts with changes in membrane potential. Larkum and Bonner [10] showed that the P518 changes were much larger in intact chloroplasts than in swollen chloroplasts or fragments. It seemed possible, therefore, that the effects of nigericin and valinomycin on photophosphorylation would differ for intact and swollen chloroplasts. In the present work, the effects of these ion transport antibiotics were studied in relation to CO_2 fixation, electron transport and light induced P518 changes in intact chloroplasts with high rates of CO_2 fixation. The results suggest that an electrical potential component does not make a significant contribution to phosphorylation in chloroplasts.

2. Methods

Spinach plants were grown in nutrient culture [13]. Chloroplasts with high rates of CO_2 fixation [150–250 μmoles CO_2 (mg. Chl)$^{-1}$ hr^{-1}] were prepared by the technique of Jensen and Bassham [14] but the first pellet was washed in medium A. The second pellet was resuspended in 1 ml of medium C. Swollen chloroplasts were prepared by adding 0.1 ml of chloroplast suspension to 1.5 ml of distilled water and then adding 1.4 ml of double strength medium 'C'.

Oxygen exchange was recorded using a Clark-type electrode and temperature controlled cuvette (Rank Bros, Bottisham, Cambridge, U.K.). Saturating red light was provided from a quartz-iodine source and broad red filter. The temperature was 25°C ± 0.2°C.

P518 changes were recorded using an Aminco–Chance dual wavelength spectrophotometer with side illumination. Actinic illumination at the wavelengths 664 and 714 nm was obtained using interference filters (Balzer) with 95% transmission between ±5 nm. The light intensity at the cuvette was 3.4 mW/cm^2 at 664 nm and 1.8 mW/cm^2 at 714 nm.

Table 1
The effect of nigericin and valinomycin on HCO_3^--dependent O_2 evolution in intact spinach chloroplasts

Antibiotic	Concentration (μM)	O^2 evolution (% of control)
Nigericin	0.02	78
Nigericin	0.05	51
Nigericin	0.10	14.1
Nigericin	0.20	8.4
Nigericin	0.3	4.0
Nigericin	1.0	0.0
Valinomycin	0.3	75
Valinomycin	1.5	59
Valinomycin	1.5*	35
Valinomycin	5.0*	6.0

The reaction mixture contained Medium C of Jensen and Bassham [14] (1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.8 mM, KH_2PO_4, 10 mM NaCl, 50 mM HEPES buffer, pH 7.6 and 300 mM sorbitol) and chloroplasts equivalent to 63 μg/ml of chlorophyll in a total volume of 3 ml. The chloroplasts were illuminated with saturating red light at 25°C.

*) 30 min pre-incubation.

Chlorophyll was determined by the method of MacKinney [15].

Nigericin was a gift of Eli Lilley Co. Valinomycin was obtained from Calbiochem. FCCP and DCMU were gifts of Dr. P.G. Heytler, Dupont Nemours Co. and DBMIB was a gift of Dr. A. Trebst.

3. Results

Nigericin at concentrations above 0.05 μM was found to be a potent inhibitor of CO_2 fixation in intact chloroplasts, as measured by oxygen evolution with 1 mM $KHCO_3$ as substrate. The results of one such experiment are shown in table 1 in which the rate of O_2 evolution of the control was 185 μmoles/mg Chl/hr.

Nigericin, over a similar range of concentrations, had little effect on light-induced P518 changes (fig. 1). On some occasions the P518 response was stimulated with a steady-state level in the light 30% greater than the control.

The effect of valinomycin on P518 was similar to that reported previously [10]. The fast response of the normal P518 change was replaced by a very slow response and a depressed steady state. A typical trace is shown in fig. 2. Only in the presence of nigericin plus valinomycin was the P518 response completely abolished.

Valinomycin had little effect on CO_2 fixation in intact chloroplasts up to a concentration of 0.5 μM (table 1). Above this concentration the initial rate was reduced below 70% of the control rate. However, a preincubation time of 20 to 30 min was necessary at concentrations of 1.5 μM or above to reduce the rate below 50% of the control; presumably, potassium is slowly released from the thylakoids during the prein-

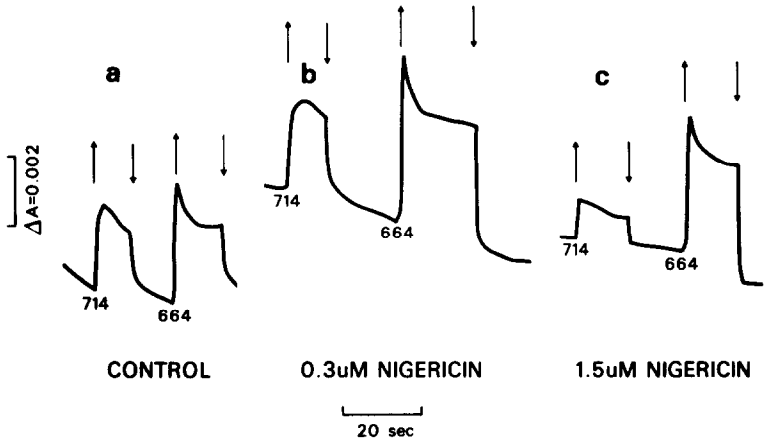

Fig. 1. Effect of nigericin on light-induced absorbance changes of intact chloroplasts at 518 nm. Reference wavelength: 540 nm. The reaction mixture contained medium C of Jensen and Bassham [14] and chloroplasts containing 189 μg chlorophyll in a total volume of 3 ml. Light intensity: 714 nm, 1.8 mW.cm^{-2}; 664 nm, 3.4 mWcm^{-2}. Temperature: 25°C. Upward arrow indicates light on and downward arrow light off. (a) Control; (b) 0.3 μM nigericin; (c) 1.5 μM nigericin.

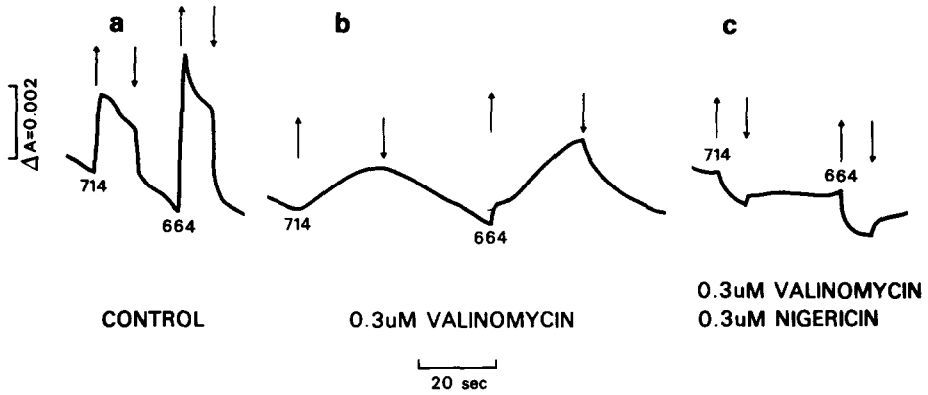

Fig. 2. Effect of valinomycin and nigericin on light induced absorbance changes of intact chloroplasts at 518 nm. Reference wavelength: 540 nm. Reaction mixture and illumination conditions as for fig. 1. (a) control; (b) 0.3 μM valinomycin; (c) 0.3 μM valinomycin plus 0.3 μM nigericin.

cubation time and this has an effect on energy-storage mechanisms.

The strong inhibition of CO_2 fixation by nigericin and the lack of effect on the P518 response raises important questions about the linkage between energy conversion in the thylakoid and the nature of the P518 response. However, it is necessary to establish that nigericin does not affect a critical transport step at the chloroplast outer envelope. This possibility was ruled out by using 3-phosphoglycerate (PGA) or oxaloacetate (OAA) as the electron acceptor for the photosynthetic electron transport chain. Both PGA and OAA readily penetrate CO_2-fixing chloroplasts as indicated by their stimulation of oxygen evolution. Neither acceptor was found to stimulate O_2 evolution in swollen chloroplasts, since the reduction of PGA or OAA requires chloroplast stromal enzymes. The reduction of PGA requires in addition a coupled phosphorylation system.

Table 2 shows the effect of nigericin on O_2 evolution by intact spinach chloroplasts with $K_3Fe(CN)_6$, PGA or OAA as electron acceptor. Ferricyanide does not penetrate intact chloroplasts [16] and the low rate of ferricyanide-dependent O_2 evolution is ascribed to broken chloroplasts. Microscopic examination indicated that 10 to 20% of the chloroplasts in the preparation used to obtain the data of table 2 were broken. O_2 evolution was completely inhibited by 0.3 μM nigericin when PGA was the electron acceptor. In contrast, nigericin stimulated O_2 evolution with OAA as acceptor. If it is assumed that the O_2 evolution supported by OAA or PGA is catalysed by intact chloroplasts, as seems highly likely, then it must be concluded that nigericin uncouples phosphorylation from electron transport. The stimulation by nigericin of O_2 evolution by broken chloroplasts with ferricyanide as acceptor is consistent with this conclusion. Nevertheless, a normal P518 response was observed under all these conditions and it could only be completed suppressed by the addition of nigericin plus valinomycin.

Table 2
The effect of nigericin on O_2 evolution by intact spinach chloroplasts

Electron acceptor	O_2 evolution [μmoles (mg Chl^{-1} hr^{-1}]
HCO_3^- (1 mM)	217
$K_3Fe(CN)_6$ (1 mM)	10
$K_3Fe(CN)_6$ (1 mM) + Nigericin (0.3 μM)	32
PGA (1 mM)	123
PGA + Nigericin (0.3 μM)	0
OAA (1 mM)	40
OAA + Nigericin (0.3 μM)	116

The reaction mixture contained Medium C of Jensen and Bassham [14] chloroplasts containing 174 μg chlorophyll in a total volume of 3 ml.

4. Discussion

Nigericin catalyses an exchange of K^+ for H^+ across both natural and artificial membranes [3, 6, 7] and in the present experiments it is assumed to act on the thylakoid membrane. Its strong uncoupling action indicates that a proton gradient is associated with photophosphorylation. Since the exchange of K^+ for H^+ can be assumed to be nearly electrically neutral [3, 7], nigericin should have no effect on any electrical potential difference across the thylakoid. According to Mitchell [17] (see also Larkum and Bonner [10]) phosphorylation is driven by both a ΔpH component and an electrical potential component (ΔE). The evidence here suggests that in the presence of nigericin any ΔE component does not contribute to the phosphorylation mechanism.

The relative ineffectiveness of valinomycin (alone), which catalyses K^+ exchange and would therefore tend to abolish any electrical potential gradient, further indicates that any ΔE component does not contribute to the phosphorylation mechanism. However, the effect of valinomycin after long preincubation times indicates that the K^+ status of the thylakoid is possibly important in the phosphorylation mechanism.

The present data do not rule out the existence of electrical potential differences across the thylakoid membrane. The electrochromic hypothesis for P518 seems still to be the best explanation for the effects of nigericin and valinomycin and for other observations [18]. However, the present results indicate that a ΔE component does not contribute to the phosphorylative process in the presence of nigericin, and the effect of valinomycin indicates that ΔE may not contribute under any circumstances.

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