Ion Translocation in Isolated Chloroplasts. Uncoupling of Photophosphorylation and Translocation of K^+ and H^+ Ions Induced by Nigericin*

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ABSTRACT: The effect of a novel type of uncoupling agent in chloroplasts is described. In the presence of K^+, nigericin uncoupled adenosine triphosphate (ATP) formation from electron transport, inhibited ATP hydrolysis, ATP–P_i exchange, and the light-induced proton uptake in spinach chloroplasts. Inhibition (50%) of these reactions was obtained at a concentration of about 5×10^{-8} M nigericin. The selectivity for K^+ among the alkali metal cations required for inhibition of these reactions was demonstrated. Nigericin induced, in the dark, a specific K^+–H^+-exchange reaction with chloroplasts suspended in the absence of external salts. The stoichiometry of this exchange was 1.2–1.5 K^+ effluxed/H^+ taken up. Under conditions that brought about uncoupling, nigericin was found to induce a light-dependent decrease in light-scattering intensity of chloroplast suspensions. This change in light-scattering intensity corresponds to swelling of the chloroplasts as corroborated by electron microscopy. The effect of nigericin, gramicidin, and valinomycin on H^+ uptake, K^+–H^+ exchange, and the light-induced absorbance changes were compared. From these results it is suggested that nigericin acts in chloroplasts at the level of the ion translocation mechanism in the membrane.

Nigericin is an antibiotic produced by a streptomycete isolated originally by Harned *et al.* (1951). The inhibition of the growth of a variety of microorganisms by this antibiotic is unique insofar as it is reversed by the addition of K^+ to the assay medium.

This antibiotic has been shown to exhibit biphasic properties in mitochondria (Graven *et al.*, 1967; Lardy *et al.*, 1967). At low concentrations (below 1 μg/ml) it inhibits the oxidation of many NAD-linked substrates concurrent with the loss of alkali metal cations accumulated previously in mitochondria. At higher concentrations and in the presence of K^+ it induces an ATPase activity, uncouples oxidative phosphorylation, and prevents the accumulation of K^+ and P_i.

The intent of this communication is to present evidence in accord with the suggestion that the action of nigericin in chloroplasts might be at the level of the ion translocation mechanism in the membrane. Nigericin may interact specifically with a component of the chloroplast membrane thereby promoting K^+–H^+ exchange which results in the dissipation of the high-energy intermediate or state derived from electron transport. A preliminary account of the action of this antibiotic on photoreactions in chloroplasts has been presented elsewhere (Shavit and San Pietro, 1967; Shavit *et al.*, 1967).

Methods and Materials

Chloroplasts were isolated from fresh market spinach leaves by standard procedures, except that the homogenizing medium contained 0.4 M sucrose and 0.01 M Tris-Cl (pH 7.8). ATP formation and ferricyanide reduction were assayed as described (Avron, 1960; Avron and Shavit, 1963). Reaction mixtures contained in a final volume of 3 ml the following components in micromoles: Tris-Cl (pH 7.8), 6; $MgCl_2$, 6; $^{32}P_i$–Tris, 2 (containing 1×10^6 cpm); ADP–Tris, 1; PMS,[1] 0.03; or ferricyanide, 1; and chloroplasts containing 60 μg of chlorophyll. Light intensity was 80,000 lux and the gas phase was air. Time of illumination was 30 sec. ATP hydrolysis, measured as $^{32}P_i$ released from [^{32}P]ATP, and ATP–$^{32}P_i$ exchange were assayed as described (Carmeli and Avron, 1966).

Hydrogen uptake by chloroplasts was measured as previously described (Dilley and Vernon, 1967). Potassium transport was measured either with a cation electrode (Electronic Instrument Ltd. Model GKN33) or by atomic absorption spectrophotometry. Chloroplast volume changes were measured turbidimetrically at 520 nm (Dilley and Vernon, 1964). Structural changes induced by nigericin were investigated by the freeze-etch technique (Mühlethaler *et al.*, 1965). ATP–Tris and ADP–Tris were purchased from Sigma Chemical Co. Nigericin was a gift from Dr. R. L. Harned, Commercial Solvents Corp., Ind.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PMS, phenazine methosulfate; DTT, dithiothreitol; TCA, trichloroacetic acid.

TABLE I: Effect of Nigericin on Chloroplast Photoreactions.[a]

Reaction	Nigericin Concentration (M)			
	None	2×10^{-8}	5×10^{-8}	5×10^{-7}
ATP formation with PMS	450	328	172	6.0
ATP formation with ferricyanide	44	19	6	0.5
Ferricyanide reduction	160	336	470	842
H^+ uptake	107	64	28	
Light-triggered ATP hydrolysis	117		77	21
Light-triggered ATP–$^{32}P_i$ exchange	25	14	9	0
Chloroplast coupling factor ATP hydrolysis	116		126	126

[a] ATP formation, ferricyanide reduction, ATP hydrolysis, ATP–$^{32}P_i$ exchange, and H^+ uptake were performed as indicated under Methods. All reactions mixtures contained 50 mM KCl except H^+-uptake reaction mixtures which contained 30 mM KCl. The final concentration of nigericin is given for the dark stages of the light-triggered ATPase and ATP–$^{32}P_i$ exchange. The latent Ca^{2+}-stimulated ATPase of chloroplast coupling factor was induced by incubation of a partially purified chloroplast coupling factor with 0.20 M DTT at pH 7.8 for 30 min at 22°. ATPase reaction mixtures contained in a final volume of 1 ml the following in micromoles: Tris-Cl (pH 8.0), 30; ATP, 2 (containing [^{32}P]ATP 3×10^4 cpm); $CaCl_2$, 3; KCl, 50; and chloroplast coupling factor, 25 μg of protein. The reaction mixtures were incubated for 10 min at 37°, then stopped by addition of TCA to a final concentration of 3%. The $^{32}P_i$ released was determined after extraction with isobutyl alcohol–benzene. Activities are given in micromoles per milligram of chlorophyll per hour except for the chloroplast coupling factor ATPase, which is given in micromoles of $^{32}P_i$ released per milligram of protein per hour. Chloroplast coupling factor was a gift from Drs. E. Racker and A. Bennun, Cornell University, Ithaca, N. Y.

Results

Effect of Nigericin on Chloroplast Photoreactions. Nigericin was recently shown to uncouple phosphorylation from electron transfer in the presence of K^+ (Shavit and San Pietro, 1967). The data presented in Table I illustrate the effect of nigericin, in the presence of K^+, on different chloroplast photoreactions. Ferricyanide reduction is stimulated but ATP formation concomitant with electron transfer is inhibited. Under these conditions, nigericin also inhibited the light-dependent ATP formation with PMS, H^+ uptake, light-triggered ATPase, and ATP–P_i exchange. Inhibition (50%) of these reactions was attained at a concentration of about 2–5 $\times 10^{-8}$ M nigericin. In contrast to the inhibition of the light-triggered ATPase, a tenfold higher concentration of nigericin did not inhibit the latent Ca^{2+}-stimulated ATPase activity exhibited by the chloroplast coupling factor (Vambutas and Racker, 1965), either in the presence or absence of KCl.

The stimulation of electron transport by nigericin as a function of KCl concentration is given in Figure 1. Maximal stimulation of ferricyanide reduction was observed at about 50 mM KCl. The apparent K_m for K^+ was close to 15 mM. In the absence of nigericin, the addition of KCl did not significantly affect the rate of ferricyanide reduction.

The uncoupling action of nigericin shows selectivity for K^+ among the alkali metal cations. As shown in Table II, the inhibition of ATP formation with PMS by nigericin is most effective in the presence of K^+. The requirement for a specific alkali metal cation is evident since sucrose did not have any effect nor could Na^+, Rb^+, Cs^+, Li^+, or Mg^{2+} replace K^+ efficiently. There was no difference in the degree of inhibition when KCl was replaced by KNO_3. From this it is concluded that the requirement is specifically for the alkali metal cation.

Lardy *et al.* (1967) have shown that phosphate and K^+ accumulation in mitochondria are inhibited by nigericin. Our experiments revealed identical inhibition of ATP formation rate at phosphate levels from 0.6 to 4.0 mM. It appears, therefore, that the inhibition of ATP formation by nigericin is not related to the availability of P_i for esterification.

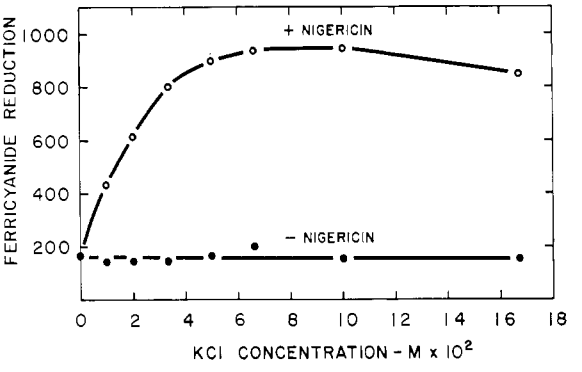

FIGURE 1: Stimulation of the rate of ferricyanide reduction by nigericin. Reaction mixtures and assay as described in Methods, except that ADP, P_i, and $MgCl_2$ were omitted. Nigericin concentration was 5×10^{-7} M. Rates of reduction are given in micromoles per milligram of chlorophyll per hour.

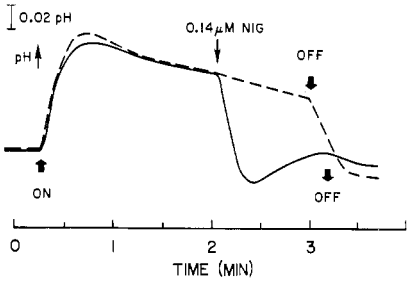

FIGURE 2: Effect of nigericin on light-dependent H^+ uptake. The reaction mixtures contained 0.1 M sucrose, either 30 mM KCl (——) or 30 mM LiCl (– – – –), 15 μM pyocyanine, and 20 μg of chlorophyll/ml as chloroplasts. Initial pH was 6.1. Red light (Corning filter 2304) was used for illumination at an incident intensity of 2×10^5 ergs cm^{-2} sec^{-1}.

Since nigericin is one of the most potent known uncouplers of photophosphorylation (50% inhibition of ATP formation being attained at between 2 and 5×10^{-8} M), it was of interest to determine whether or not nigericin is bound to the chloroplasts. The results of these experiments are presented in Table III. It appears that KCl enhances either the binding of nigericin to chloroplasts or the sedimentation of nigericin with chloroplasts since the chloroplast pellets obtained from suspensions of chloroplasts in the presence of nigericin and KCl were more inhibited (74% *vs.* 19% inhibition). Assays for the presence of nigericin in the supernatant solutions, conducted by adding aliquots to fresh chloroplasts, show that less nigericin was present in the supernatant solutions from chloroplasts treated with both nigericin and KCl than with nigericin alone. The inhibitory effect was completely reversed by washing the chloroplasts, indicating that nigericin was not tightly bound to the chloroplasts.

Effect of Nigericin on Light-Dependent H^+ Uptake. The interaction of nigericin and K^+ with the energy

TABLE II: Cation Specificity of the Uncoupling Action of Nigericin.[a]

Compounds	Added (mM)	Per Cent of Control
None		107
Sucrose	100	95
$MgCl_2$	50	111
LiCl	50	94
NaCl	50	92
CsCl	50	95
RbCl	50	87
KCl	50	48
KNO_3	50	41

[a] Reaction mixtures and assays as described under Methods. Control specific activities without nigericin varied with different compounds and were between 250 and 550 μmoles of ATP per mg of chlorophyll per hr. The nigericin concentration was 5×10^{-8} M.

TABLE III: Reversibility of Nigericin Uncoupling by Washing.[a]

Prepn	Conditions	ATP Formation (μmoles/mg of chlorophyll per hr)	% of Control
I	Chloroplast suspension	267	100
II	Chloroplast suspension + nigericin	60	22
III	Chloroplast suspension + KCl	274	100
IV	Chloroplast suspension + KCl + nigericin	62	23
	Resuspended pellets (first centrifugation)		
	From I	344	100
	From II	280	81
	From III	367	100
	From IV	93	26
	Supernatants (first centrifugation)		
	From I	286	100
	From II	81	28
	From III	286	100
	From IV	174	60
	Resuspended pellets after washing (second centrifugation)		
	From I	284	100
	From II	250	88
	From III	260	100
	From IV	249	96

[a] The initial suspensions of chloroplasts contained the following components in a total volume of 30 ml: I, 60 μg of chlorophyll/ml as chloroplasts; 5 mM Tris-Cl (pH 7.8); 150 mM sucrose; and 0.7% methanol; II, as I but with 9×10^{-7} M nigericin; III, as I but with 50 mM KCl; IV, as II but with 50 mM KCl. Aliquots of 1 ml were assayed for ATP formation with PMS as described in Methods. All assay reaction mixtures contained a final concentration of 50 mM of KCl, and, with preparations II and IV, nigericin at 3×10^{-7} M. The remainder of the suspensions were centrifuged at 10,000*g* for 10 min, and the pellets were resuspended in a volume of 0.4 M sucrose and 0.01 M Tris-Cl (pH 7.8) such to reproduce the original chlorophyll concentration. Aliquots (1 ml) of the supernatants and the resuspended pellets were taken for the phosphorylation assay. The remainder of the fractions were centrifuged and the pellets were resuspended as above and assayed for ATP formation.

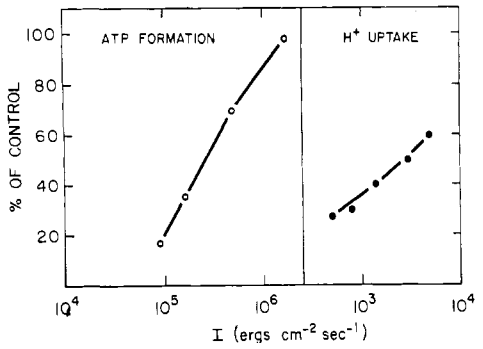

FIGURE 3: Light intensity dependence of the degree of inhibition of ATP formation and H^+ uptake by nigericin. ATP formation with PMS was assayed as described in Methods, in the presence of 50 mM KCl and 5×10^{-8} M nigericin. Control rates were: 12 and 425 μmoles of ATP formed per mg of chlorophyll per hr at the lowest and highest light intensities, respectively. H^+ uptake with pyocyanine was assayed as described in Methods, except that 50 mM KCl, 0.5 mM $MgCl_2$, and 1.4×10^{-9} M nigericin were present. Initial pH was 7.5. Control rates were 8 and 60 μmoles of H^+ per mg of chlorophyll per hr at the lowest and highest light intensities, respectively.

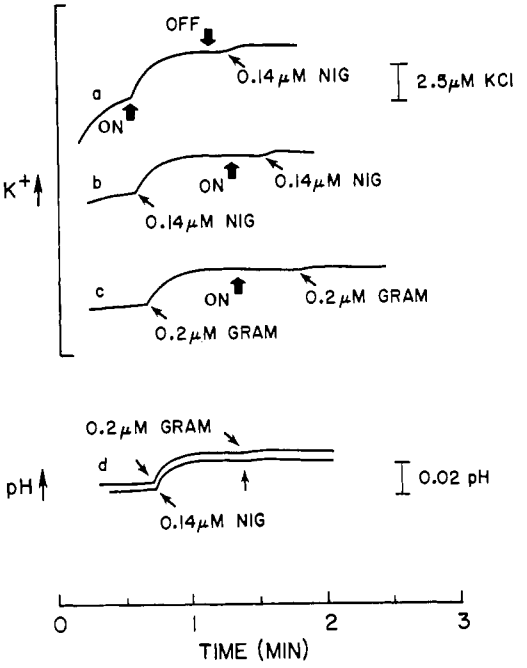

FIGURE 4: Nigericin- and gramicidin-induced K^+–H^+ dark exchange in low salt medium. Potassium efflux was measured in chloroplast suspensions with a cation electrode as described in Methods. The reaction mixtures for K^+ measurement contained the following: 0.1 M Tris-acetate (pH 6.0), 155 μg of chlorophyll as chloroplasts, and 15 μM pyocyanine in a total volume of 10 ml. An increase in the K^+ concentration in the suspension is given by an upward deflection of the trace. (⬆, ⬇) Light on, light off. (↘) Addition of an antibiotic. (a) Light-induced K^+ efflux; (b and c) K^+ efflux caused by addition of 0.14 μM nigericin or 0.2 μM gramicidin, respectively, in the dark. H^+ ion uptake was measured in chloroplast suspensions containing the following: 0.2 M sucrose, 155 μg of chlorophyll as chloroplasts, and 15 μM pyocyanine in a 10-ml total volume. The pH was adjusted to 6.0 with KOH. An increase in the pH of the suspension is given by an upward deflection. (d) H^+ uptake caused by addition of 0.14 μM nigericin or 0.2 μM gramicidin (signified by the arrows) in the dark.

transfer process is demonstrated further by the data presented in Figure 2. The light-dependent H^+ gradient established in a medium containing KCl was collapsed by the addition of nigericin in the light; in contrast, the H^+ gradient established in a medium containing LiCl was not affected by the addition of nigericin. The same cation selectivity was observed for the inhibition of the light-dependent H^+ uptake by nigericin as that given above for ATP formation. Addition of nigericin prior to illumination inhibited the generation of the H^+ gradient in KCl but not in media containing either CsCl, LiCl, or NaCl. The results are consistent with an uncoupling effect of nigericin in the presence of K^+ and the effect of most known uncouplers on H^+ uptake (Jagendorf and Neumann, 1965).

Effect of Light Intensity on Inhibition of ATP Formation and H^+ Uptake by Nigericin. The relationship between the Hill reaction velocity and light intensity developed by Rieske *et al.* (1959) has been applied to the effect of uncouplers and inhibitors on photophosphorylation (Avron and Shavit, 1963) and on H^+ uptake (Dilley, 1967) in order to establish whether the uncoupler acts by affecting light or dark reactions. As shown in Figure 3, nigericin inhibits ATP formation and H^+ uptake more severely at lower light intensities. A logical explanation of these results is that nigericin affects step(s) closely related to a light reaction.

Specific K^+–H^+ Exchange Induced by Nigericin. Studies with mitochondria have revealed that a large number of antibiotic substances act as uncouplers of oxidative phosphorylation and increase mitochondrial permeability to monovalent cations; the specificity for K^+ varied with the uncoupler (Pressman, 1965; Chappell and Crofts, 1965). The effect of nigericin and K^+ in chloroplasts suggested that this antibiotic acts in a manner analogous to the transport-inducing antibiotics in mitochondria (Moore and Pressman, 1964; Lardy *et al.*, 1967). Some typical potassium and hydrogen electrode responses of chloroplast suspensions, when nigericin or gramicidin was added, are shown in Figure 4. Traces a, b, and c are K^+ electrode signals due to (a) light-dependent K^+ efflux in the presence of pyocyanine, (b) nigericin-induced K^+ efflux in the dark, and (c) gramicidin-induced K^+ efflux in the dark, respectively. These signals were obtained in a medium containing 0.1 M Tris-acetate in order to assure that the electrode responses obtained were not a consequence of a change of the pH of the medium. Under these conditions it is apparent that both nigericin and gramicidin cause a K^+ efflux in the dark which is equivalent to the light-dependent K^+ efflux (Dilley and Vernon, 1965). However, this dark K^+ efflux is not unique to nigericin since gramicidin (Figure 4, trace c) or valinomycin, Triton X-100, and chlorpromazine gave similar results (unpublished experiments). When the K^+ efflux response reached its maximal value either by illumination or in the dark by addition of nigericin or gramicidin, no further transport of K^+ could be observed by any combined addition of light or antibiotics. These observations suggest that both the dark and the light

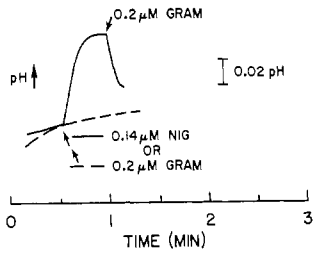

FIGURE 5: Nigericin-induced H^+ uptake and gramicidin-induced H^+ efflux in the dark and in high salt. The reaction mixture contained the following: 0.1 M LiCl, 0.2 M sucrose, and 200 μg of chlorophyll as chloroplasts in a total volume of 10 ml at pH 6.0. The pH of the suspension was measured continuously as the antibiotics were added. (——) 0.14 μM nigericin added at bottom arrow and 0.2 μM gramicidin added at top arrow; (– – – –) 0.2 μM gramicidin added at bottom arrow.

efflux originate from the same pool of internal K^+. The initial efflux of K^+ induced by nigericin and other uncouplers probably results from the existent concentration gradient, since these experiments were performed in the absence of externally added K^+ and the internal K^+ concentration was probably in the range of 10–40 mM (Dilley and Vernon, 1965).

The response observed with the hydrogen electrode is given in Figure 4 (trace d). It is evident that the addition of nigericin or gramicidin to a chloroplast suspension, in the absence of externally added salts, causes an uptake of H^+ in the dark. This dark H^+ uptake appears to have kinetics similar to the dark K^+ efflux observed under similar conditions (Figure 4, traces b and c). These results demonstrate the existence of a dark K^+–H^+ exchange induced by nigericin and gramicidin. The K^+ efflux induced by nigericin was verified using atomic absorption spectrophotometry. The stoichiometry of this exchange reaction was in the range of 1.2–1.5 K^+ effluxed/H^+ taken up.

As mentioned above, the K^+–H^+-exchange reaction, observed in the absence of externally added salts, is not unique to nigericin since it is induced by other compounds as well. It was of interest, therefore, to study the nigericin- and gramicidin-induced H^+ uptake in the presence of various salts added externally. As shown in Figure 5, when the external salt present was LiCl, addition of nigericin induced an H^+ uptake (solid line). This H^+ uptake in the dark was partially reversed by the subsequent addition of gramicidin. Moreover, similar H^+ gradients could be induced by nigericin in either CsCl or NaCl but not in KCl. Gramicidin added initially did not cause an uptake of H^+ in 0.1 M LiCl (Figure 6, dashed line), or in CsCl, NaCl, and KCl. This is as expected since Pressman (1965) has shown that in mitochondria, gramicidin induces greater permeability to all the alkali metal cations, *i.e.*, external Li^+, Cs^+, Na^+, or K^+ rather than H^+ could exchange for internal K^+. These results are in accord with the postulate that nigericin increases the permeability of the chloroplast membrane specifically to K^+, whereas gramicidin-induced permeability is not cation specific. Valinomycin gave a response similar to nigericin in LiCl or NaCl media, including the gramicidin-induced

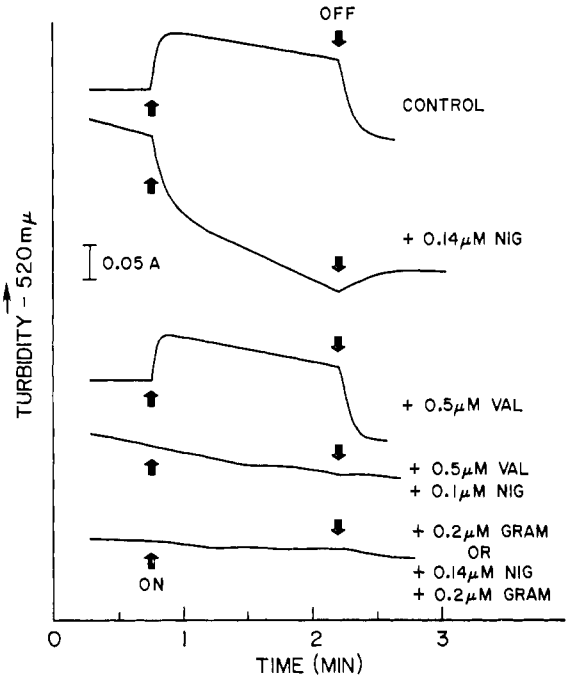

FIGURE 6: Effect of nigericin, gramicidin, and valinomycin on light-induced absorbance changes of chloroplast suspensions. The reaction mixtures contained 0.1 M KCl, 20 μg of chlorophyll as chloroplasts, and 15 μM pyocyanine in a volume of 1 ml at a pH between 6.0 and 6.2. Various additions were made as indicated. The change in absorbance at 520 nm was followed in a Beckman Model DB spectrophotometer as described (Dilley and Vernon, 1964).

collapse of the preestablished H^+ gradient. However, with valinomycin the requirement for the cation is less specific than with nigericin, since Cs^+ can replace K^+. No H^+ uptake induced by valinomycin was observed in media containing either CsCl or KCl (unpublished results), probably because valinomycin allows both external Cs^+ and K^+ to traverse rapidly the membrane in exchange for internal K^+.

Light-Scatter Changes Induced by Nigericin upon Illumination. As shown in the uppermost trace of Figure 6, chloroplasts suspended in KCl show, upon illumination, an increase in absorbance. In contrast, the presence of nigericin plus KCl reverses the direction of the light-induced absorbance change (Figure 6, second trace). In the presence of CsCl, NaCl, or LiCl, nigericin did not reverse the direction or affect the magnitude of the increase in light scattering upon illumination. The decrease in absorbance observed with nigericin is rapid and dependent upon the concentration of nigericin and KCl in the assay medium. At 100 mM KCl and 0.15 μM nigericin the rate and extent of the light-induced absorbance decrease was about threefold greater than at 20 mM KCl. Similarly, at 100 mM KCl, the rate and extent of the absorbance decrease at 1.4 μM nigericin was about threefold greater than at 0.014 μM nigericin. An increase in absorbance was also observed in the dark, when the chloroplasts were suspended in the presence of increasing concentrations of KCl, corresponding to shrinkage as reported earlier (Shavit and Avron, 1967). That the decrease in absorbance

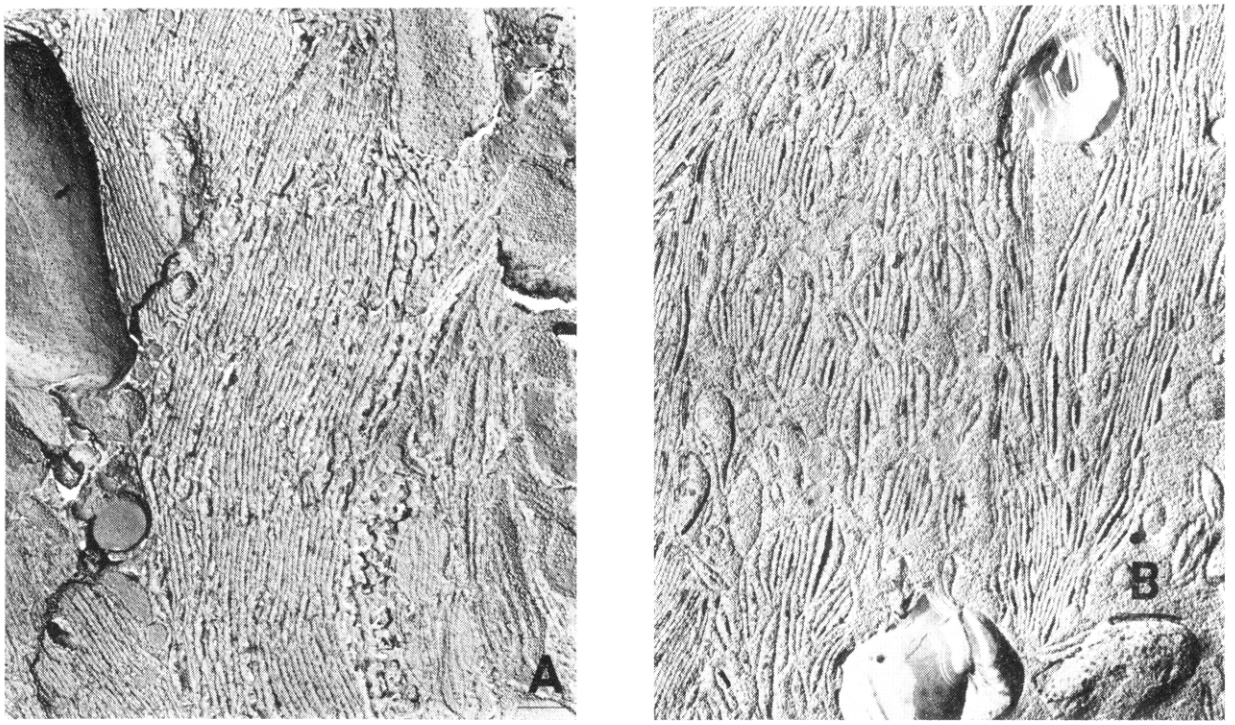

FIGURE 7: Effect of nigericin on chloroplast structure. Reaction mixtures contained in a total volume of 1 ml the following: 0.1 M KCl, 0.03 M Tris-acetate (pH 7.8), 0.1 M sucrose, 5×10^{-5} M pyocyanine, 15% glycerol, 0.3% methanol, 0.3 mg of chlorophyll, and where indicated 4.5×10^{-7} M nigericin. The suspensions were centrifuged in the dark at 1000*g* for 10 min and aliquots from the pellets were illuminated in white light, frozen, and freeze-etched as described (Dilley *et al.*, 1967). Electron micrographs were taken with a Phillips Model 200 electron microscope. (A) Control; (B) control + nigericin. Magnification 22,600×.

corresponds to swelling of the chloroplasts was corroborated by coulter counter measurements and by electron microscopy. Using the freeze-etch technique (Mühlethaler *et al.*, 1965), it is apparent that illumination of chloroplasts in the presence of nigericin and KCl has brought about a swelling of the thylakoids (Figure 7).

The effect of nigericin, gramicidin, and valinomycin on the changes in the absorbance of chloroplast suspensions is illustrated further in Figure 6. The light-induced increase in absorbance in the control experiments was found to be inhibited by gramicidin but not by valinomycin (Figure 6, third and fifth traces). Interestingly, this light-dependent swelling (decrease in absorbance) induced by nigericin in a medium containing KCl was inhibited either by gramicidin, an uncoupler of photophosphorylation, or by valinomycin, (Figure 6, fourth and fifth traces) which, at these concentrations did not affect the light-dependent H^+ uptake, either in the presence or absence of KCl, nor photophosphorylation (unpublished experiments).

Discussion

The results presented above extend our prior observations (Shavit *et al.*, 1967) concerning the effect of nigericin on the energy transfer steps in photophosphorylation. Nigericin is the most potent uncoupler of photophosphorylation known to date, and is representative of a new type of uncoupling agent of photophosphorylation, that share in common the selective requirement for an alkali metal cation for uncoupling to occur (N. Shavit, in preparation). In the presence of K^+, nigericin inhibited ATP formation, light-dependent H^+ uptake, ATP hydrolysis, ATP–P_i exchange but not the latent ATPase activity of chloroplast-coupling factor (Vambutas and Racker, 1965). The inhibition of ATP–P_i exchange in mitochondria has been rationalized on the basis of the inhibition of P_i accumulation (Lardy *et al.*, 1967). This is apparently not the case in chloroplasts, since the degree of inhibition of ATP formation (Table III) was unaffected by the concentration of P_i. The reactions affected by nigericin are light triggered or depend upon illumination, *i.e.*, conditions under which a high-energy intermediate or state is assumed to occur. Our data strongly suggest that nigericin acts on this high-energy intermediate or state, and below we shall consider the possible ways such interactions may occur.

Proposed mechanisms of uncoupling of photophosphorylation have assumed that either the uncoupler acts by catalyzing the hydrolysis of a high-energy intermediate (see Slater, 1967) or that the uncoupler dissipates the high-energy state (Mitchell, 1966). This high-energy intermediate or state has been proposed to be a H^+ gradient across the thylakoid membrane, a necessary step in the synthesis of ATP (Mitchell, 1966; Neumann and Jagendorf, 1964). In considering the mechanism of action of nigericin the question arises whether the uncoupling of ATP formation is the result of the utilization of a high-energy intermediate for ion transport (*i.e.*, K^+) or that an ion-exchange reaction (*i.e.*,

H^+–K^+) catalyzed by nigericin results in the dissipation of the H^+ gradient.

Three antibiotics known to affect ion transport in mitochondria were compared with respect to their effect on light-dependent reactions and the induction of ion-exchange reactions in the dark. Gramicidin, valinomycin, and nigericin were equally effective in causing an exchange *in the dark* of external H^+ for internal K^+ when the chloroplasts were suspended in a medium without added salts (Figure 4). The fact that the stoichiometry of the exchange was close to unity supports the claim that we are dealing with an exchange reaction resulting probably from the collapse of the K^+ permeability barrier. There is diffusion of K^+ outward driven by the chemical potential counterbalanced by the inward flow of H^+. These agents appear to increase the permeability of the membrane toward alkali metal cations, without appreciably affecting the permeability to anions; otherwise, an anion could accompany the cation and exclude the cation-exchange phenomena observed. Moreover, the effect of these three agents on the H^+–cation exchange when the concentration gradient of the cations is reversed (high salt, Figure 5) indicates that gramicidin is indeed capable of exchanging H^+ for Li^+, Cs^+, K^+, or Na^+; valinomycin exchanges K^+ or Cs^+ for H^+; and nigericin exchanges only K^+ for H^+. These data agree with the known effects of the three agents on mitochondrial ion movements (Pressman, 1965; Chappell and Crofts, 1965; Lardy *et al.*, 1967).

The induction of a K^+–H^+ exchange in the dark by these agents does not seem to us sufficient to explain their activity on chloroplast photoreactions. Thus, nigericin uncouples photophosphorylation and inhibits the generation of a H^+ gradient and other processes (Table I) in the presence of about 50 mM KCl, gramicidin affects them in the same manner in the presence of any one of the alkali metal cations, and valinomycin is without effect on these reactions under similar conditions. The exchange reactions induced by these antibiotics *in the dark* clearly demonstrate their capacity to enhance the membrane permeability to cations (with significant differences in their specificity for alkali metal cations). However, the relationship between these dark exchanges and the effect of the antibiotics on light-induced chloroplast reactions is not obvious.

It appears that nigericin interacts with the ion translocation system of the chloroplast membrane. As mentioned above, this interaction could cause the dissipation of the proton gradient by affecting the exchange diffusion system, presumably by transport of K^+ inward in exchange for protons. We propose as a working hypothesis, that the following series of exchange reactions may occur subsequent to the light-driven H^+ uptake. In the presence of nigericin, an exchange of internal H^+ for external K^+ occurs, resulting in the uptake of K^+ which is neutralized by the uptake of an anion (and water). The observed swelling of chloroplasts in the light induced by nigericin and KCl (Figures 6 and 7) supports such a mechanism; we confirmed the uptake of K^+ reported by Packer (1967) at low concentrations of KCl and a high concentration of nigericin ($\sim 10^{-6}$ M). It should be noted, however, that these conditions are different from those under which the uncoupling and the swelling of chloroplasts were measured. Studies with the potassium electrode did not demonstrate a change in K^+ concentration of the suspending medium under conditions which gave the nigericin-dependent swelling. It may be that an equivalent amount of water and K^+ was accumulated by the chloroplasts with no change in K^+ concentration in the suspension. This point remains uncertain, however.

Additional evidence that salt uptake is responsible for the observed swelling is found in the fact that when K_2SO_4 replaced KCl, the rate of nigericin and light-induced swelling was greatly decreased. This would be expected since SO_4^{2-} is a less permeant anion than Cl^-.

Valinomycin does not affect either the light-induced shrinkage or the H^+ gradient nor does it induce a swelling as does nigericin (Figure 6). It appears, therefore, that the mechanism of action of valinomycin is different from that of nigericin. The inhibition by valinomycin of the light-induced swelling observed with nigericin (Figure 6) is consistent with the view that valinomycin causes an increase in K^+ permeability. Hence, no K^+ accumulation would occur because of the rapid equilibration of external and internal K^+. In the absence of any accumulation of K^+ there could be no uptake of anions (and water) and, therefore, no swelling would be observed. However, the valinomycin effects are very complex, and the above model is at best a working hypothesis on which to base further experiments.

For the case of gramicidin the mechanism of action must be different from that of nigericin and valinomycin, since gramidicin inhibits the H^+ uptake in the absence of any added alkali metal cation and does not induce swelling upon illumination of chloroplasts in the presence of salts. If gramicidin enhances the permeability of the membrane to alkali metal cations and H^+ its uncoupling action may involve the exchange reactions postulated to participate in the case of nigericin. Whether nigericin induces the utilization of a high-energy intermediate for K^+ uptake or the dissipation of a H^+ gradient by H^+–K^+ exchange is not distinguished in these experiments.

The effect of nigericin on the light-induced H^+ uptake and photophosphorylation in *R. rubrum* chromatophores was reported recently (Shavit *et al.*, 1968) and is relevant to the mechanism of uncoupling by nigericin. In chromatophores, the inhibition of the light-induced H^+ uptake by nigericin does not affect the capacity to form ATP. If nigericin does induce a H^+–K^+ exchange in chromatophores, this exchange does not result in the inhibition of ATP formation. It would appear that the light-induced H^+ uptake in chloroplasts is more intimately related to ATP formation than in the case of chromatophores. A less plausible explanation would be that uncoupling of ATP formation and the induction of a K^+–H^+ exchange are two separate effects of nigericin; thus, collapse of the H^+ gradient occurs both in chromatophores and chloroplasts but the inhibition of ATP formation only in chloroplasts. These possibilities and the light-induced K^+ uptake by nigericin in chloroplasts are currently under investigation.

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