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II. EFFECTS OF IONOPHOROUS ANTIBIOTICS IN CHLOROPLASTS

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SUMMARY

The effects of the ionophorous or transport-inducing antibiotics, valinomycin, nigericin, monensin, dianemycin, X-206, X-464 and X-537A on the light-induced proton, K^+ and Na^+ uptake, electron transport and ATP formation were studied. Nigericin, monensin, dianemycin, X-206, X-464 and X-537A uncoupled photophosphorylation in the presence of a suitable alkali metal cation, inducing the uptake of the alkali metal cation while inhibiting the light-induced pH change. The selectivity for alkali-metal cations exhibited by these antibiotics was determined and found to be similar to that determined in mitochondrial and artificial membranes. The ratios of $K^+/\Delta H^+$ determined at a given concentration of antibiotics were in the order of 6–10, whereas the $Na^+/\Delta H^+$ ratios for a similar inhibition of the light-induced proton uptake were between 10–40. Valinomycin primarily enhanced permeability to K^+, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone enhanced permeability to H^+, and gramicidin that to both K^+ and H^+. With suboptimal concentrations of nigericin which only slightly uncoupled phosphorylation but induced a measurable K^+ uptake, the addition of valinomycin synergistically uncoupled photophosphorylation. These results suggest the participation of a membrane potential in ATP formation in chloroplasts and are discussed in comparison with similar effects described for subchloroplast and chromatophore particles.

INTRODUCTION

The relation between ion transport and energy transduction in mitochondrial and chloroplast systems has been considered in different hypotheses of energy coupling[1,2]. The chemical intermediate hypothesis regards ion transport as a process which competes with ATP formation. In the chemiosmotic hypothesis ion gradients are essential to the mechanism of energy transduction, and the total potential required for ATP formation is the sum of a transmembrane proton concentration gradient (ΔpH) and an electrical or membrane potential ($\Delta\psi$) (see ref. 1). The uncoupling of oxidation and photophosphorylation by a variety of agents was considered by proponents of both hypotheses. According to the chemiosmotic

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; diquat, 1,1'-ethylene-2,2'-dipyridylium dibromide; PMS, phenazine methosulfate.

hypothesis, uncouplers may act by dissipating transmembrane ion gradients. The ionophorous antibiotic nigericin was reported to uncouple photophosphorylation in chloroplasts and to enhance proton permeability in chloroplasts and chromatophores[3–6]. It was postulated that the dissipation of the proton gradient occurs *via* a K^+/H^+ exchange or antiport. A requirement for both nigericin and valinomycin for inhibition of photophosphorylation in chromatophores of *Rhodospirillum rubrum* was explained in terms of dissipation of the membrane potential[6,7]. In chloroplasts, where the greater part of the total potential was assumed to reside in the pH constituent, nigericin alone effectively uncoupled photophosphorylation. Several antibiotics of the nigericin type were found to increase the permeability of mitochondrial and artificial membranes to alkali–metal cations and to protons, by virtue of inducing a cation $\leftrightarrow$ H^+ exchange[8,9].

The data presented here give further results on the movements of cations, particularly K^+ and Na^+, induced by antibiotics of the nigericin type, and the effects of valinomycin, gramicidin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on these ion movements. These results are discussed in terms of ion permeability changes induced by the various compounds tested. The effects on cation permeability are related to the dissipation of the transmembrane proton gradient and the uncoupling of photophosphorylation.

METHODS AND MATERIALS

Chloroplasts were isolated from leaves of fresh market spinach or lettuce by standard procedures[3]. They were resuspended in 0.4 M sucrose, and ATP formation and $Fe(CN)_6^{3-}$ reduction were assayed as described[3]. pH changes were measured with a Radiometer G222C glass electrode; K^+ was measured with an Electronic Instrument Ltd. electrode, type GKN33B (BH115 glass); and Na^+ with a Beckman 39046 cationic electrode. A remote calomel reference electrode was used with a liquid junction to minimize effects of K^+ diffusion from the reference electrode into the cell with the cationic electrode. Calibrations were performed with standard solutions of HCl, KCl and NaCl. The sensitivity of the K^+-sensitive electrode to H^+ was negligible under the conditions used. The K^+/Na^+ selectivity was 10:1. The selectivity of the Na^+-sensitive electrode to Na^+ and H^+ was 3:1 and Na^+/K^+ was 6:1. In experiments where Na^+ uptake was determined, the actual pH change observed did not contribute more than 5% to the total amount of Na^+ uptake measured. Signals were recorded on a Rikadenki Model 341 recoder. Red light was provided by a 150-W quartz iodine lamp, through a heat filter and a Corning filter 2304. The intensity of illumination was about $1.8 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}.

Antibiotics used were kindly given by Dr. R. L. Harned, Commercial Solvents Corp., Ind.; Dr. J. M. McGuire, Lilly Research Laboratories, Ind.; Dr. B. C. Pressman, University of Pennsylvania, Philadelphia, Pa.; Dr. J. Berger, The Hoffman-La Roche Co., N. J., and Dr. W. Simon, Eidg. Technische Hochschule, Zurich.

RESULTS

Uncoupling of photophosphorylation

Several antibiotics of the nigericin type were tested for their effect on ATP

formation and electron transport in chloroplasts (Fig.1). Dianemycin, monensin and Compounds X-206, X-537A and X-464 were found to stimulate electron transport and inhibit ATP formation in the presence of 50 mM KCl. Under these conditions, 50% inhibition of ATP formation was attained at concentrations of antibiotics below 0.1 μM, placing them among the most potent uncouplers of photophosphorylation. Monensin, although very similar to nigericin[10], was less inhibitory in a KCl medium. Replacing KCl by NaCl in the medium resulted in a greater degree of inhibition. Fig. 1 shows that 50% inhibition of ATP formation was attained at a concentration of about 0.5 μM of monensin in the presence of 50 mM NaCl.

The formation of ATP with phenazine methosulfate (PMS) as electron carrier was inhibited similarly by a given concentration of these antibiotics (see Fig. 3), except for Compound X-206, which at a given concentration in a KCl medium inhibited phosphorylation with $Fe(CN)_6^{3-}$ or 1,1-ethylene-2,2'-dipyridylium dibromide (diquat) to a larger extent than that with PMS (Fig. 2). Thus at a concentration

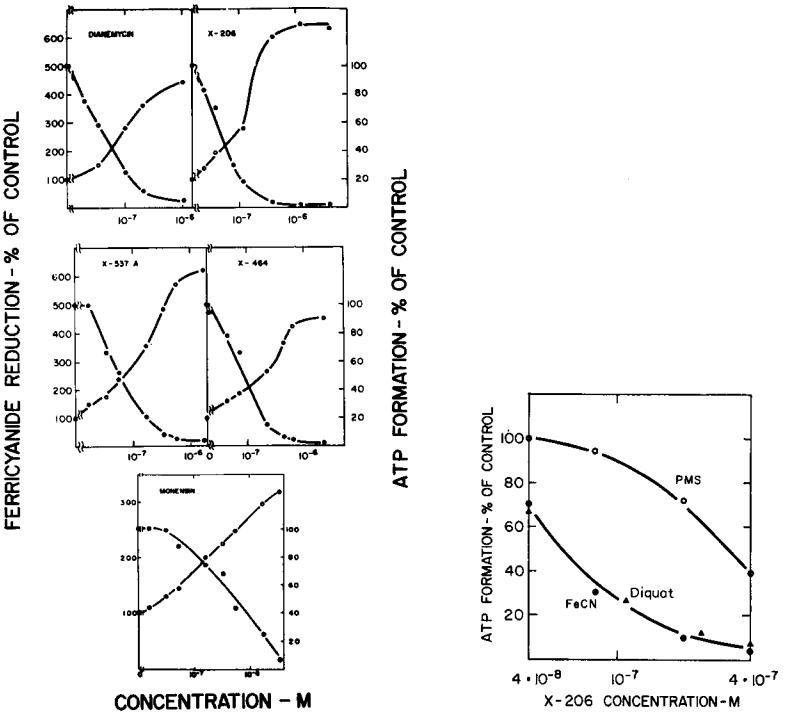


Fig. 1. Uncoupling of photophosphorylation by several antibiotics. Reaction mixtures contained in a final volume of 3 ml the following components: 2 mM Tris–HCl buffer (pH 7.8), 2 mM $MgCl_2$; 0.33 mM ADP; 0.66 mM $^{32}P_i$ (containing $1 \cdot 10^6$ counts/min). 0.33 mM Fe $(CN)_6^{3-}$; 50 mM KCl or NaCl; and chloroplasts containing 60 μg of chlorophyll. Assays were performed as described[3]. For monensin NaCl replaced KCl in the reaction medium. Control values for $Fe(CN)_6^{3-}$ reduction were 150–200 and for ATP formation 50–75, both expressed in μmoles per mg chlorophyll per h.

Fig. 2. Differential effect of X-206 on photophosphorylation. Reaction mixtures and assay conditions were as described in Fig. 1. Where indicated, $Fe(CN)_6^{3-}$ was replaced by 0.01 mM PMS or 0.03 mM diquat. Control values for $Fe(CN)_6^{3-}$ or diquat were 100 μmoles ATP per mg chlorophyll per h and for PMS, 450 μmoles ATP per mg chlorophyll per h.

of about 0.1 μM, ATP formation with PMS was inhibited by 10 % while that with $Fe(CN)_6^{3-}$ or diquat, by 70 %. Although not shown here, it was found that Compound X-206 inhibited the pH change with diquat more than that in the presence of pyocyanine (R. A. DILLEY AND N. SHAVIT, unpublished results). Such a differential uncoupling of these two reactions is analogous to that observed with uncouplers like FCCP[11].

Uncoupling by nigericin and its related antibiotics required the presence of a suitable alkali-metal cation. The selectivity for alkali-metal cations of the various antibiotics is given in Fig. 3. At a given concentration of antibiotic and 50 mM of each of the alkali-metal cations, all compounds inhibited ATP formation with PMS in the presence of KCl; dianemycin was also effective in the presence of NaCl; X-537A in CsCl and RbCl; X-464 was more effective in KCl than in RbCl or NaCl; monensin was more inhibitory in the presence of NaCl than KCl. At these concentrations, none of the antibiotics was inhibitory in the absence of added alkali-metal cations.

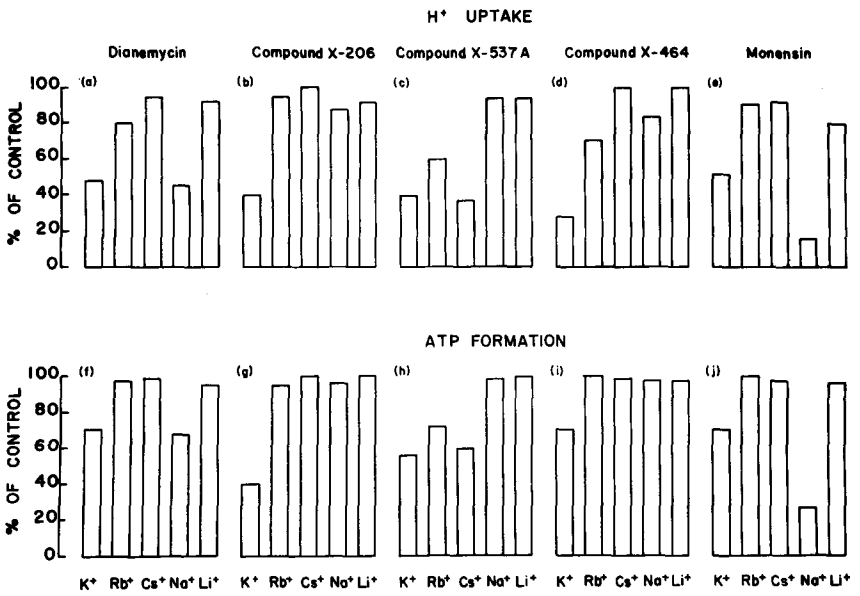
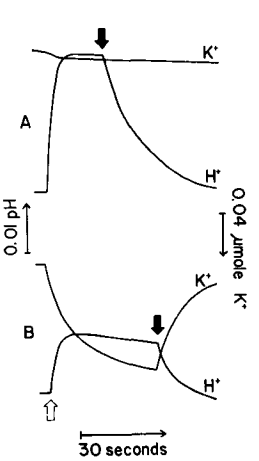


Fig. 3. Inhibition of H^+ uptake and ATP formation by several antibiotics in the presence of different alkali-metal cations. H^+ uptake was measured as described[3]. Initial pH 6.2. Control values were: 0.3–0.4 μmole H^+ per mg chlorophyll. ATP formation with PMS was assayed as described in Fig. 2. Control values were 400–600 μmoles ATP per mg chlorophyll per h. Antibiotic concentrations (μM) (a) 0.17 and (f) 0.035; (b) 0.1 and (g) 0.39; (c) 0.08 and (h) 0.17; (d) 0.11 and (i) 0.23; (e) 0.75 and (j) 1.5. Chloride salts of each of the alkali-metal cations were added to a final concentration of 50 mM.

Fig. 3 also shows that the light-induced pH change in chloroplasts was inhibited by agents of the nigericin type. The selectivity for alkali-metal cations at a given antibiotic concentration is similar to that observed for the uncoupling of photophosphorylation.

Uptake of alkali-metal cations

The ability of nigericin to induce a $K^+ \longleftrightarrow H^+$ exchange in natural and artificial membrane systems has been demonstrated and a similar exchange of cations was reported for chloroplasts[3,4]. Fig. 4 illustrates a simultaneous recording of the change in pH and K^+ concentration with illumination. Fig. 4A illustrates the change in pH and K^+ upon illumination of chloroplasts in a choline chloride medium in the absence of nigericin. No significant change in K^+ concentration was observed. The efflux of K^+ observed in chloroplasts[12] was not obtained here, probably because of the presence of 50 mM choline chloride in the medium[13]. When nigericin was added, the extent of the pH change was inhibited with a concomitant uptake of K^+ from the medium (Fig. 4B). The uptake of K^+ was slower than the uptake of protons and approached 1.6 μmoles/mg chlorophyll. Generally, the uptake of K^+ did not reach a steady state as was obtained for the pH change. The uptake of K^+ as a function of nigericin concentration is illustrated in Fig. 5. At a concentration that inhibited the light-induced pH change by about 50 %, uptake of K^+ was about 1.3 μmoles per mg chlorophyll. Fig. 6 indicates that this uptake was also dependent on the external KCl concentration, reaching saturation at about 4–5 mM KCl. No measurements were performed at concentrations of KCl higher than 10 mM.



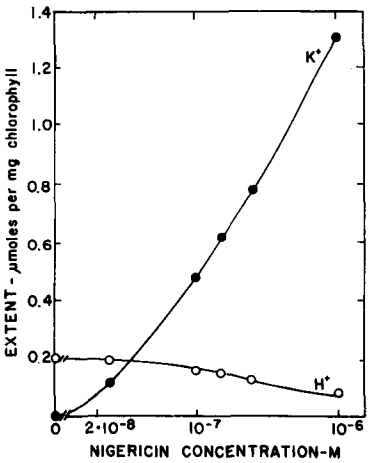
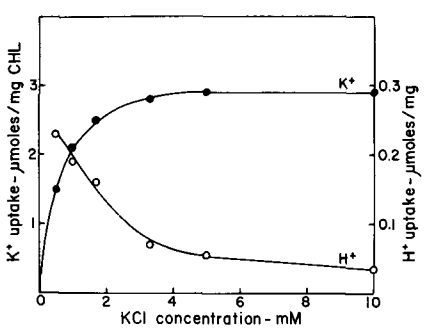


Fig. 4. Light-induced H^+ and K^+ uptake in the presence of nigericin. H^+ and K^+ changes were measured as described in METHODS AND MATERIALS. ↑, light on; ↓, light off. The reaction mixture contained in a final volume of 6 ml as follows: 100 mM choline chloride; 1 mM KCl; 7 μM pyocyanine and chloroplasts containing 130 μg chlorophyll. Initial pH, 6.3. A. Without nigericin, extent of proton uptake was 0.45 μmoles H^+ per mg chlorophyll. B. With 0.7 μM nigericin; extent of K^+ uptake and H^+ uptake was 1.6 and 0.27 μmoles/mg chlorophyll, respectively.

Fig. 5. Light-induced H^+ and K^+ uptake with nigericin as a function of nigericin concentration. Reaction mixtures and assay were as described in Fig. 4, except for the presence of 0.5 mM KCl.

Nigericin was shown to inhibit ATP formation and H^+ uptake more severely at low than at high light intensities[3]. As shown in Fig. 7, the K^+ uptake induced by nigericin was strongly dependent upon the incident light intensity. This dependence was more severe than that of H^+ uptake, with or without nigericin. The H^+ uptake



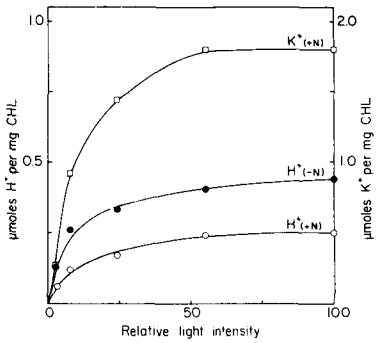


Fig. 6. Light-induced H^+ and K^+ uptake with nigericin as a function of KCl concentration. Reaction mixtures and assay as described in Fig. 4, except that the concentration of KCl varied as indicated. K^+ and H^+ uptake are given as extent reached after 1 min of illumination. The extent of H^+ uptake without nigericin was 0.42 μmoles H^+ per mg chlorophyll.

Fig. 7. The dependence upon incident light intensity of the H^+ and K^+ uptake. Reaction mixtures and assays as described in METHODS AND MATERIALS and Fig. 4. Light intensity (100 %) corresponds to $1.8 \cdot 10^5$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. Chlorophyll concn.: 21.3 μg/ml. Nigericin concn.: 0.7 μM. Initial pH, 6.3.

reached saturation at a lower light intensity than the uptake of K^+. Thus, the $K^+/\Delta H^+$ ratios determined varied between 10 to 4 at higher and lower light intensities, respectively.

Several compounds of the nigericin-type-induced K^+ uptake with a concomitant inhibition of the pH change, as indicated in Table I. In the presence of 1 mM KCl and at concentrations of antibiotics that gave about 50 % inhibition of the proton gradient, a considerable uptake of K^+ was observed. The ratio of $K^+/\Delta H^+$ calculated for all the antibiotics tested except monensin was about 6–8. With monensin, the ratio observed was about 2. These compounds also induced uptake of Na^+, as indicated in Fig. 8 and Table II. The uptake of Na^+ induced by monensin is presented in Fig. 8B. Upon illumination in the absence of monensin, the sodium electrode showed a response which may reflect both pH and cation movement, since the electrode is somewhat

TABLE I

K^+ UPTAKE INDUCED BY COMPOUNDS OF THE NIGERICIN TYPE

H^+ and K^+ uptake were measured as described in METHODS AND MATERIALS and in Fig. 4. KCl concn., 1 mM; chlorophyll., concn., 28 μg/ml and the initial pH, 6.2–6.5. ΔH^+ is the difference between control and inhibited states. Extent of both K^+ and H^+ were taken after 1 min.

Compound	*Concn. (μM)*	*% Inhibition of H^+ uptake*	*K^+ uptake (μmoles/mg chlorophyll)*	*ΔH^+ (μmoles/mg chlorophyll)*	*Ratio $K^+/\Delta H^+$*
Nigericin	0.7	50	1.34	0.17	7.9
Dianemycin	1.0	50	1.05	0.17	6.2
X-464	1.2	55	1.66	0.21	7.9
X-537A	5.6	47	0.97	0.16	6.1
X-206	6.0	59	1.62	0.20	8.1
Monensin	12.0	41	0.31	0.14	2.2

sensitive to changes in pH (Fig. 8A). Upon illumination in the presence of monensin extensive Na^+ uptake took place together with inhibition of the pH change (Fig. 8B). In Table II the extent of Na^+ uptake by several compounds is given at concentrations that cause about 50% inhibition of the pH change. The uptake of Na^+ observed with monensin and other compounds in relation to the inhibition of the H^+ uptake is given by the $Na^+/\Delta H^+$ ratios calculated, which were about 20–40. These ratios are significantly higher than those observed for K^+ uptake with concentrations of antibiotics that cause similar inhibition of the H^+ uptake.

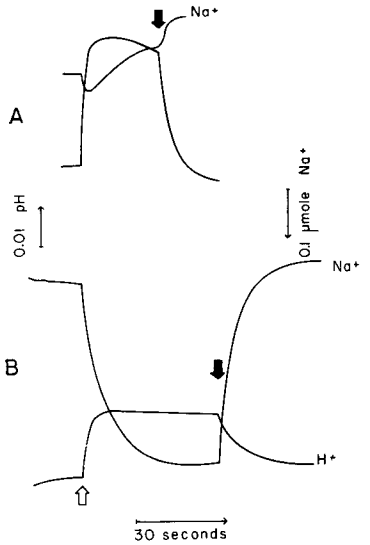


Fig. 8. Light-induced H^+ and Na^+ uptake in the presence of monensin. H^+ and Na^+ uptakes were measured as described in METHODS AND MATERIALS. ⇑, light on; ↓, light off. The reaction mixture contained in a final volume of 3 ml as follows: 100 mM choline chloride; 1 mM NaCl; 1.3 mM Tris–HCl buffer; 7 μM pyocyanine and chloroplasts containing 133 μg chlorophyll. Initial pH, 6.7. A. Without monensin; extent of proton uptake was 0.3 μmoles H^+ per mg chlorophyll. B. With monensin, 5 μM; extent of Na^+ uptake was 3.5 μmoles/mg chlorophyll.

The effect of valinomycin, gramicidin and FCCP upon the K^+ uptake induced by nigericin is shown in Table III. Valinomycin is known to enhance permeability to K^+, and gramicidin to all alkali metal cations and protons, whereas FCCP enhances permeability primarily to protons. Uptake of K^+ induced by nigericin was completely abolished by low concentrations of valinomycin, which alone did not affect proton uptake. At these concentrations of valinomycin we did not observe any effect on pH change, as was observed by PACKER *et al.*[14]. Gramicidin inhibited both K^+ and proton uptake, having a more pronounced effect on K^+ than on proton uptake. At a concentration at which gramicidin alone inhibited the proton uptake by about 50%, it inhibited the uptake of K^+ in the presence of nigericin by about 90%, without any appreciably greater effect on the H^+ uptake. FCCP, at a concentration that inhibited the proton uptake to about 30%, had a slight effect on the K^+ uptake induced by nigericin (10% inhibition). This is also evident from the calculated $K^+/\Delta H^+$ ratios (Column 3).

TABLE II

Na^+ UPTAKE INDUCED BY COMPOUNDS OF THE NIGERICIN TYPE

H^+ and Na^+ uptake were measured as described in METHODS AND MATERIALS and in Fig. 8. NaCl concn., 1 mM; chlorophyll., 22 μg/ml and the initial pH,6.5. ΔH^+ is the difference between control and inhibited states. Extent of both Na^+ and H^+ were taken after 1 min.

Compound	*Concn. (μM)*	*% Inhibition of H^+ uptake*	*Na^+ uptake (μmoles/mg chlorophyll)*	*ΔH^+ (μmoles/mg chlorophyll)*	*Ratio $Na^+/\Delta H^+$*
Monensin	1.5	39	2.7	0.09	30
	5.0	48	4.0	0.11	36
Dianemycin	1.0	35	3.3	0.09	37
	2.1	45	4.3	0.12	36
Nigericin	2.8	26	1.3	0.06	22
	6.4	40	2.4	0.09	27
X-206	14.0	41	2.0	0.09	22
X-537A	28.0	39	1.0	0.08	12

TABLE III

EFFECT OF VARIOUS ANTIBIOTICS ON H^+ AND K^+ UPTAKE

H^+ and K^+ uptake were measured as described in METHODS AND MATERIALS and in Fig. 4. KCl concn., 1 mM and the initial pH, 6.2–6.5. chlorophyll concn. was: Expt. I, 17 μg/ml and Expt. II, 22 μg/ml; nigericin concn. was: Expt. I, 1.2 μM and Expt. II, 0.7 μM; concn. of gramidicin, valinomycin and FCCP were, respectively: 0.05, 0.45 and 5.0. μM. ΔH^+ is the difference between control and inhibited states. Extent of both $K^+$and H^+ were taken after 1 min.

Expt. No.	*Compound*	*H^+ uptake (μmoles/mg chlorophyll)*	*K^+ uptake (μmoles/mg chlorophyll)*	*Ratio $K^+/\Delta H^+$*
I	None	0.39	0	
	Nigericin	0.14	1.75	7.0
	Valinomycin	0.40	0	
	Nigericin + valinomycin	0.12	0.09	0.3
II	None	0.42	0	
	Nigericin	0.19	1.54	8.1
	Gramicidin	0.23	0	
	FCCP	0.28	0	
	Nigericin + gramicidin	0.18	0.15	0.6
	Nigericin + FCCP	0.16	1.39	5.3

Synergistic effect of nigericin and valinomycin

Since the nigericin type of antibiotics induced a $K^+ \leftrightarrow H^+$ exchange with a ratio of K^+/H^+ higher than one, we considered the possibility that under these conditions there would be a build-up of an electrochemical gradient of K^+ that might contribute some of its energy to the formation of ATP. If this were so, the addition of valinomycin (which, as shown, had no effect on ΔpH but did abolish the K^+ gradient; Table III) should uncouple photophosphorylation. Table IV shows that a combination of nigericin and valinomycin acts synergistically to produce uncoupling. It should be noted that relatively high concentrations of the antibiotics were used in order to observe the stimulation of electron transport[11]. In the absence

TABLE IV

EFFECT OF NIGERICIN AND VALINOMYCIN ON $Fe(CN)_6^{3-}$ REDUCTION

The reaction mixture contained in a volume of 3.0 ml: 50 mM choline chloride; 3 mM Tris–HCl buffer (pH 7.8); 1 mM $K_3Fe(CN)_6$ and chloroplasts containing 60 μg chlorophyll. Light intensity 160 000 lux of white light for 30 sec. $Fe(CN)_6^{3-}$ reduction was assayed as described in METHODS AND MATERIALS. $V + N$, rate with valinomycin and nigericin; N, rate with nigericin.

Additions	*Concn.* (μM)	*$Fe(CN)_6^{3-}$ reduced* (*μmoles/h/mg chlorophyll*)	*Ratio* $\frac{V + N \text{ rate}}{N \text{ rate}}$
None		91	
Nigericin	0.046	138	
	0.46	171	
	1.4	257	
Valinomycin	0.3	102	
+ nigericin	0.046	196	1.4
+ nigericin	0.46	246	1.4
+ nigericin	1.4	396	1.5

of phosphorylating reagents, valinomycin itself did not affect electron transport, and nigericin alone uncoupled only partially. The addition of both ionophorous agents enhanced the electron transport rate above the rate observed with nigericin alone. In the presence of phosphorylating reagents similar results were obtained, but valinomycin itself, at these relatively high concentrations, inhibited both electron transport and phosphorylation without causing uncoupling[15].

Following the formation of ATP by the change in pH of the suspension[16], as shown in Fig. 9, permitted a clear demonstration of the synergistic effect on ATP synthesis, with lower concentrations of ionophorous antibiotics. Addition of either nigericin or valinomycin inhibited ATP formation very slightly. Considerably more inhibition of ATP synthesis was observed when illumination in the presence of nigericin induced uptake of K^+, and the subsequent adddition of valinomycin, in the light, collapsed the K^+ gradient established. The rate of ATP synthesis was inhibited (Fig. 9C) concomitantly with the efflux of K^+.

DISCUSSION

The alkali-metal cation selectivity for the uncoupling of photophosphorylation exhibited by the antibiotics of the nigericin type and the known size of the hydrated alkali-metal cations indicate, as already discussed[9], that the mode of action of these agents cannot be explained by the creation of pores in the membranes, which permit the passage of cations with a small hydration shell. The structures of the metal complexes of monensin and nigericin were recently determined[10,17]. The antibiotic molecule surrounds the alkali-metal cation conferring lipid solubility to the complex. The bound cation is assumed to be nonhydrated and the complex electrically neutral. Thus nigericin would be more lipid soluble when its carboxylic acid group is neutralized by either a proton or a cation[8].

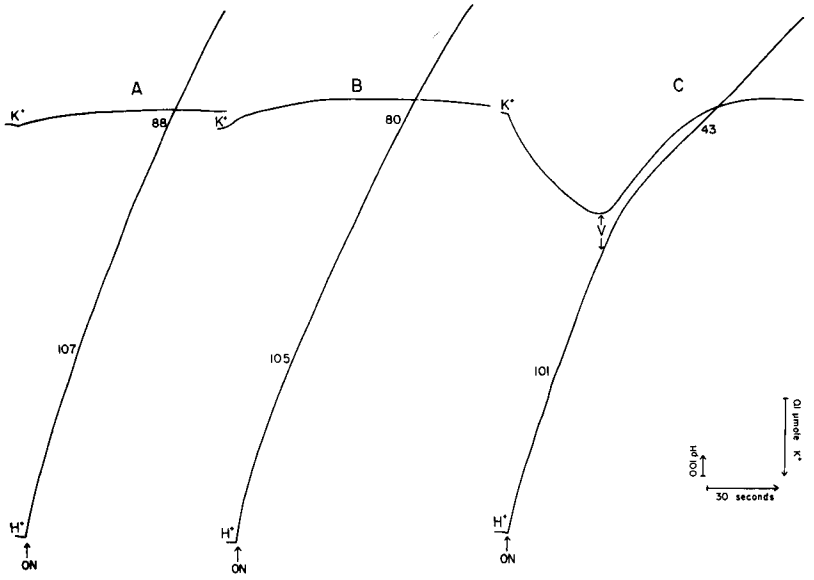


Fig. 9. Photophosphorylation and K^+ movements in the presence of nigericin and valinomycin. ATP synthesis and K^+ concentration were measured as described[16,7]. The reaction mixture contained in a volume of 6.0 ml: 50 mM choline chloride; 1 mM KCl; 7 μM pyocyanine; 0.2 mM ADP; 0.2 mM P_i–Tris buffer (pH 7.8); 0.5 mM $MgCl_2$ and chloroplasts containing 150–200 μg chlorophyll Initial pH, 7.8. 0.045 μM valinomycin (V) and 0.092 μM nigericin (N) were present where indicated. A, Control; B, + valinomycin; C, nigericin prior to illumination and valinomycin where indicated. The numbers inserted indicate rates of ATP formation in μmoles/h per mg chlorophyll.

The cation $\leftrightarrow$ H^+ exchange catalyzed by nigericin and other antibiotics of this group would therefore have to be 1:1 for the transport to be electrically neutral. Under the conditions employed in our experiments, in a medium containing choline chloride, we have determined a ratio of $K^+/\Delta H^+$ of about 6–10. This rather high ratio was also observed with several concentrations of the antibiotic or KCl (Figs. 5 and 6 and Table I). Similar high ratios of $K^+/\Delta H^+$ were observed with other antibiotics of the nigericin type; only with monensin, which is selective for Na^+, the ratio observed was about 2. We also calculated the $K^+/\Delta H^+$ ratio at different intervals of the K^+ and H^+ uptake curve and compared them with the ratio usually determined when the H^+ uptake reached the steady-state condition under illumination. At all stages the ratio obtained was high, varying between 6–10, and similar to that determined during the steady-state condition. Although the ΔH^+'s obtained during the initial stages of cation uptake were small and the ratios calculated less accurate than in the steady-state condition, the agreement is quite good.

The inhibition of the H^+ uptake with several of the antibiotics was also obtained in the presence of Na^+, with a concomitant uptake of Na^+. The $Na^+/\Delta H^+$ ratios (Fig. 8 and Table II) were about 10–40. Monensin and dianemycin, which are selective for Na^+, gave a higher ratio of $Na^+/\Delta H^+$ than nigericin and Compounds X-206 and X-537A. These ratios reinforce the argument against a simple cation $\leftrightarrow H^+$ exchange of 1:1 under these experimental conditions.

In chromatophores, JACKSON *et al.*[6] have reported data for nigericin showing

a ratio of about 1 for $K^+/\Delta H^+$. PACKER *et al.*[14] have observed a disparity between the K^+ and H^+ uptake at low and high concentrations of nigericin. The cation transport-inducing capability of nigericin was also dependent upon the pH of the medium[7,14].

The deviation from a 1:1 stoichiometry of the cation to proton movement induced by the ionophorous antibiotics may be apparent. Assuming that the introduction of an extrinsic permeability to K^+ and H^+ results in an enhancement of the light-dependent proton uptake itself, and taking into consideration that we determined net changes in the bulk phase and not fluxes, the enhancement of the proton uptake could mask a real ratio of K^+/H^+ of unity. In such a case, one might expect an enhancement of the initial rate of proton uptake in the presence of the antibiotics. However, from the data presented an inhibition rather than stimulation of the initial rates of H^+ uptake was observed under the conditions at which the cation/proton ratios were determined. Similar high $K^+/\Delta H^+$ ratios were obtained when the antibiotic was added under continuous illumination, after the proton uptake reached its steady-state condition. (H. DEGANI AND N. SHAVIT, unpublished results).

The high ratios obtained in chloroplasts could also be explained by the fact that these antibiotics do not act as simple exchange carriers, which cross the membrane only in the associated and noncharged form, but can cross in both the dissociated and associated forms. This interpretation is supported by the findings of MUELLER AND RUDIN[18], namely, that nigericin increases the conductance of artificial membranes, thus indicating that the dissociated complex is also slightly permeable. Another indication is probably the different light-intensity dependence of the K^+ and H^+ uptake (Fig. 7), which is not to be expected for a simple exchange carrier of K^+.

We thus suggest that the mode of action of the antibiotics of the nigericin type in chloroplasts is the induction of a cation transport which is rather "loosely" coupled to that of protons and which is not electrically neutral. Since the permeability of Cl^-, the major anion in the medium, is relatively low (swelling of chloroplasts in the presence of nigericin[3] is a much slower process than is K^+ uptake), the movement of K^+ (electrogenic) would result in the build-up of the membrane potential ($\Delta\psi$). The experiments with low nigericin concentration, which partially inhibited ΔpH, induced K^+ uptake (Table III) and had almost no effect on the rate of ATP formation (Fig. 9), suggest that a membrane potential established by the electrogenic movement of K^+ may contribute to the total potential required for ATP synthesis. Introduction of valinomycin collapses $\Delta\psi$ by enhancing K^+ permeability, producing considerable inhibition of ATP synthesis. It is this cooperative effect between nigericin and valinomycin that strongly suggests the participation of a membrane potential in chloroplast ATP formation.

Uncoupling in the presence of NH_4Cl and valinomycin was observed in subchloroplast particles[19], submitochondrial particles[20,21] and chromatophores[22], or with nigericin and valinomycin in chromatophores[6,23] and submitochondrial particles[24]. In chloroplasts, however, no such cooperative effect with these two antibiotics was observed[15]. NISHIMURA AND PRESSMAN[25] also reported that with *R. rubrum* chromatophores they could not observe any appreciable synergistic effect of valinomycin *plus* nigericin or valinomycin and uncoupler. These effects were interpreted in terms of the chemiosmotic hypothesis, namely, consideration of the relative contribution of ΔpH and $\Delta\psi$ to the total available energy for ATP formation. An alternative interpretation was the postulation that the rapid cyclic ion transport

induced in such a way occurred at the expense of a high-energy intermediate other than a transmembrane H^+ gradient. Although our experiments do not distinguish between these two possible mechanisms, they suggest that the mechanism of coupling may be the same in chloroplasts, subchloroplasts and chromatophores. The differences observed in different particles result from the experimental conditions and the consequent membrane permeability to ions, determining the relative contributions of ΔpH and $\Delta\psi$, as proposed in the chemiosmotic hypothesis. More definite experiments on other ion movements (anions) and on the movement of the ionophorous agents across the membrane are required to test these interpretations more rigorously.

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