Effects of Antibiotics on Ion Transport and Photophosphorylation in *Rhodospirillum rubrum* Chromatophores*

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ABSTRACT: Nigericin, dianemycin, and monensin A inhibited the light-induced pH change in chromatophores prepared from *Rhodospirillum rubrum* but had little effect on photophosphorylation. This type of antibiotic stimulated the decay of the pH change but had little or no effect on the initial rate of formation. The ion transport inducing antibiotics, valinomycin and a mixture of nonactin and monactin, stimulated the rate of formation and extent of the pH change while inhibiting only slightly photophosphorylation. However, combinations of these antibiotics, *e.g.*, valinomycin and nigericin, strongly inhibited photophosphorylation. With nigericin or dianemycin, this synergistic inhibition was dependent upon the presence of K^+ or Rb^+ and was not observed in Na^+, Li^+, or Cs^+ with low antibiotic concentrations. Monensin A, in combination with valinomycin or nonactin, was active only in K^+. These results were interpreted to indicate that: (1) valinomycin type antibiotics stimulated an energy-linked ion transport, measured as the increase in pH of the medium, thereby promoting the formation of a gradient across the chromatophore membrane; and (2) the nigericin-type antibiotics dissipated this gradient, thus stimulating a cyclic ion transport which competed with adenosine triphosphate formation for a high-energy intermediate formed by light-induced electron transport.

In a recent publication we reported that nigericin strongly inhibited the light-dependent proton uptake in chromatophores from *Rhodospirillum rubrum* without affecting the rate of ATP formation (Shavit *et al.*, 1967). Based on these results we suggested that proton uptake in chromatophores represents a side reaction for the utilization of high-energy intermediates and was not on the direct pathway of ATP formation. Data presented in this paper is an extension of those results, using other antibiotics and combinations of antibiotics that have been shown to affect specifically the permeability of biological membranes for alkali metal cations.

The antibiotics have been divided into two groups (Lardy *et al.*, 1967; Pressman *et al.*, 1967). One group, represented by valinomycin, the gramicidins, the enniatins, monazomycin, and nonactin and its homologs, has the ability to induce active transport of alkali metal cations into mitochondria and intact cells. Valinomycin, which has been studied extensively, enhances specifically the permeability of many membranes for K^+ (Pressman, 1965; Chappell and Crofts, 1966; Mueller and Rudin, 1967; Lev and Buzhinsky, 1967). In mitochondria, valinomycin induces an energy-dependent K^+ uptake which is accompanied by a nearly stoichiometric efflux of H^+ provided no permeant anions are present (Cockrell *et al.*, 1966). This reaction can be described as an energy-dependent H^+–K^+ exchange. In chromatophores from *R. rubrum*, valinomycin has been demonstrated to stimulate light-induced proton uptake in the presence of K^+ but not of Na^+ (von Stedingk and Baltscheffsky, 1966).

The second group of antibiotics, represented by nigericin, dianemycin, and the monensins, is characterized in mitochondria by the ability to abolish the active uptake of alkali metal cations which was induced by antibiotics of the valinomycin class. This is observed as a rapid dissipation of the K^+ gradient formed in the presence of valinomycin (Graven *et al.*, 1966; Lardy *et al.*, 1967; Pressman *et al.*, 1967); the dissipation of the K^+ gradient is accompanied by an uptake of H^+. Another characteristic of this group of antibiotics is the ability to inhibit the oxidation of substrates by intact mitochondria (Lardy *et al.*, 1967). In chloroplasts (Shavit and San Pietro, 1967; Packer, 1967) and chromatophores (Shavit *et al.*, 1968), nigericin has been reported to inhibit the light-induced proton uptake. Nigericin also stimulated a passive efflux of K^+ from chloroplasts accompanied by an uptake of H^+ (Shavit *et al.*, 1967).

In respiring mitochondria the addition of both valinomycin and nigericin results in a strong stimulation of oxygen uptake (Pressman *et al.*, 1967). This stimulation is not observed with either antibiotic alone. The synergistic effect was ascribed to an uncoupling of respiration from phosphorylation as a result of an energy-requiring cyclic transport of K^+ across the mitochondrial membrane.

We have found that combinations of antibiotics from the two groups described above strongly inhibited photophosphorylation in *R. rubrum* chromatophores whereas

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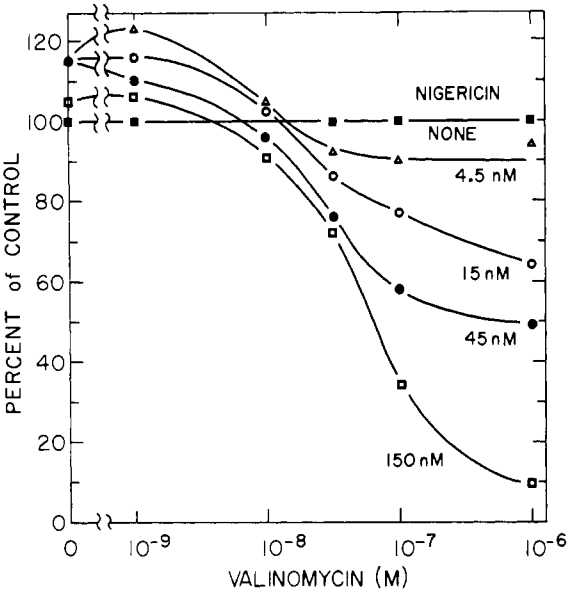

FIGURE 1: Inhibition of photophosphorylation by valinomycin and nigericin.[4] The reaction mixture was as in Table I and contained 15.7 μg of Bchl/ml. The rate of ATP formation in the absence of antibiotics was 168 μmoles/mg of Bchl per hr. The 100% level was taken as the activity with valinomycin alone. This corresponds to 35, 14, 10, 3, and 0% inhibition at valinomycin concentrations of 10^{-6}, 10^{-7}, 3.3×10^{-8}, 10^{-8}, and 10^{-9} M, respectively.

either antibiotic alone has little effect. We believe that these results support the postulation that combinations of these antibiotics induce an energy-requiring cyclic transport of K^+ or H^+ (our results do not discriminate) across membranes (Pressman *et al.*, 1967).

Methods

R. rubrum, S 1, was grown and chromatophores prepared as described earlier (Keister and Yike, 1966) with the exception that chromatophores which were to be used for measurements of the light-induced pH changes were washed once in 0.1 M NaCl instead of 10% sucrose in 0.1 M Tris-Cl. Bchl[1] was determined from the *in vivo* absorption at 880 mμ (Clayton, 1963). pH changes were measured aerobically as described (Shavit *et al.*, 1968) at an incident red light intensity of 1.3×10^5 ergs cm^{-2} sec^{-1}. Before the start of each experiment the pH of the reaction mixture was adjusted to pH 6.45 $\pm$ 0.03 with NaOH or HCl. The temperature was 27°.

Reactions for the assay of ATP formation were terminated by addition of trichloroacetic acid to 5%. [^{32}P]ATP was assayed as previously described (Keister, 1965).

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Results

Synergistic Inhibition of Photophosphorylation by Combinations of Various Antibiotics. The effect on photophosphorylation of antibiotics of the valinomycin class (valinomycin and nonactin) and of the nigericin class (nigericin, dianemycin, and monesin A), singly and in combination, is presented in Table I. Combinations of antibiotics from each of the two classes, *e.g.*, valinomycin and nigericin, strongly inhibited photophosphorylation; in contrast, the antibiotics alone or in combination with other antibiotics of the same class, *e.g.*, valinomycin and nonactin, had little or no inhibitory effect at the concentrations used.[3] Although not shown in the table, little synergistic effect could be elicited with an antibiotic from either class in combination with uncoupling agents such as *m*-chlorocarbonyl cyanide phenylhydrazone or desaspidin.

A more detailed study of the inhibition obtained with varying concentrations of valinomycin and nigericin is presented in Figure 1.[4] For clarity the activity with valinomycin alone has been taken as 100%. At 10^{-7} and 10^{-6} M this assumption represents actually an inhibition of 14 and 35%, respectively. The synergistic inhibition was dependent upon both the valinomycin and nigericin concentrations. Nigericin, alone or at low concentrations of valinomycin, slightly stimulates photophosphorylation. This stimulation was also observed with dianemycin and monensin A. We do not have an explanation for this stimulatory effect.

Patterns of inhibition similar to those of Figure 1 were observed with valinomycin in combination with dianemycin or monensin A and with nonactin together with nigericin, dianemycin, or monensin A.

The light-driven energy-linked transhydrogenase (Keister and Yike, 1967) found in these chromatophores was inhibited by combinations of valinomycin and nigericin similar to the effects found on photophosphorylation.

Stimulation of the Light-Induced pH Change by Valinomycin-Type Antibiotics. Valinomycin has previously been demonstrated to stimulate the light-induced pH change in *R. rubrum* chromatophores (von Stedingk

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: Bchl, bacteriochlorophyll.

[2] This is a mixture of 72% nonactin and 28% monactin.

[3] Valinomycin has been reported to inhibit photophosphorylation by 50% maximally at concentrations of 3×10^{-8} M and higher (Baltscheffsky and Arwidsson, 1962). In our hands, valinomycin had little effect on photophosphorylation at 3×10^{-8} M; however, the per cent inhibition increased in a linear fashion when plotted against the logarithm of the concentration. In some experiments we obtained 82% inhibition at 3×10^{-6} M and 50% at 3×10^{-6} M. These concentrations were based on a molecular weight of 741. The actual molecular weight is probably 1111 (Brockman *et al.*, 1963).

[4] The valinomycin used for the experiments presented in Figure 1 and Table II was from a different source and was somewhat more inhibitory (on a molar basis) than that used in the other experiments.

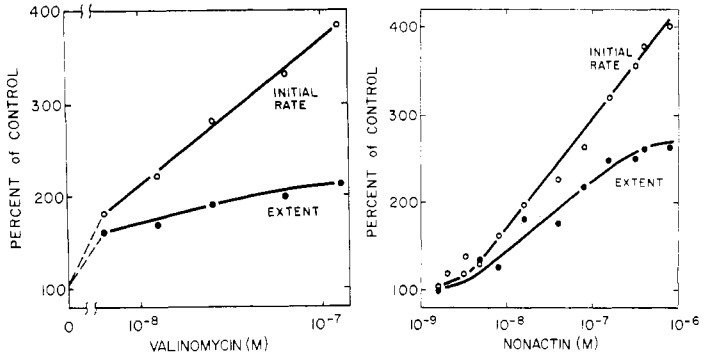

FIGURE 2: Stimulation of the light-induced pH change by (a) valinomycin and (b) nonactin. The reaction mixture contained 100 mM KCl, 0.02 mM succinate, and chromatophores corresponding to 7.4 μg of Bchl/ml in a volume of 8 ml. The initial rate of the pH change in the control (100%) was 199 μmoles of H^+/mg of Bchl per hr and the extent 0.25 μmole of H^+/mg of Bchl.

TABLE I: Synergistic Inhibition of Photophosphorylation by Antibiotics.[a]

	Antibiotic (% inhibition)				
Additions (μM)	Valinomycin (0.1 μM)	Nonactin (0.42 μM)	Dianemycin (0.63 μM)	Nigericin (0.45 μM)	Monensin A (8.8 μM)
None	17	13	0	10	6
Valinomycin (0.1)		18	62	58	69
Nonactin (0.42)			71	74	79
Dianemycin (0.63)				7	13

[a] The reaction mixture contained in 3 ml the following: 10 mM Tris-Cl (pH 8), 3.3 mM $MgCl_2$, 1.67 mM ADP, 3.3 mM $^{32}P_i$, 100 mM KCl, and chromatophores corresponding to 15.5 μg of Bchl/ml. The reaction was illuminated for 3 min at a light intensity of 2.6×10^5 ergs cm^{-2} sec^{-1} and a temperature of 22°. The rate of ATP formation was 152 μmoles/mg of Bchl per hr.

and Baltscheffsky, 1966). In order to describe a mechanism for the synergistic inhibition of photophosphorylation by the above antibiotics, we have reinvestigated the effect and the results are shown in Figure 2a. Low concentrations of valinomycin stimulated both the extent and the initial rate of the light-induced pH change. These results differ somewhat with the results of the above authors who reported that valinomycin had only a slight effect on the extent of the reaction. However, it must be noted that the extent of the nonstimulated pH change in our preparations was 0.2–0.4 μmole of H^+/mg of Bchl; in comparison, the previous authors reported an extent of about 1 μmole of H^+/mg of Bchl. Similar results were obtained with nonactin (Figure 2b) except the extent was enhanced more than with valinomycin.

In these experiments the extent of the pH change apparently approached a saturation level whereas the initial rate continued to increase with antibiotic concentration. We would attribute this nonparallel effect on the extent, as contrasted with the initial rate, to a stimulation of the decay reaction at higher antibiotic concentrations, especially with valinomycin. Indeed, von Stedingk and Baltscheffsky (1966) reported about a twofold stimulation of the decay with 10^{-6} M valinomycin. The low concentration of valinomycin and nonactin that we have used for further studies in this paper had little effect on the decay of the pH change.

Inhibition of the Light-Induced pH Change by Nigericin-Type Antibiotics. In an extension of our previous studies on the effect of nigericin on the light-induced pH change (Shavit *et al.*, 1968) we have tested the effect of dianemycin and monensin A on this reaction. The open symbols of Figure 3 illustrate the effect of dianemycin on the extent of the proton uptake in the presence and in the absence of the ion transport inducing antibiotics, valinomycin and nonactin. Dianemycin was strongly inhibitory and, although not shown, similar results were obtained with nigericin and monensin A. The closed symbols denote the effects of dianemycin on photophosphorylation. In the absence of a valinomycin-type antibiotic, dianemycin had no inhibitory effect on photophosphorylation over a wide range of concentrations which strongly inhibited the pH change. However, in the presence of valinomycin or nonactin, dianemycin inhibited strongly. In view of the different experimental conditions required for measuring photophosphorylation as contrasted to the pH change, we consider the concentrations required to elicit the inhibition of the two reactions to be comparable.

Similar results were obtained with monensin A and nigericin and the concentrations required for 50% inhibition of the proton uptake and photophosphorylation, in the presence and absence of fixed amounts of valinomycin and nonactin, are presented in Table II. These figures are presented primarily for the purpose

TABLE II: Effect of Antibiotics on the Light-Induced pH Change and Photophosphorylation.[a,b]

Antibiotic	Concentration for 50% Inhibition (μM)					
	Extent of pH Change			ATP Formation		
	None	Valinomycin (0.1 μM)	Nonactin (0.42 μM)	None	Valinomycin (0.1 μM)	Nonactin (0.42 μM)
Nigericin	0.004	0.02	0.03	c	0.07	0.07
Dianemycin	0.02	0.07	0.09	c	0.12	0.18
Monensin A	0.3	0.8	0.8	c	2.00	2.00

[a] Experimental conditions were as in Table I. [b] See footnote 4. [c] Dianemycin had no effect at 1.5 μM. Nigericin and monensin A inhibited by 10% at 0.45 and 15 μM, respectively.

of comparing the relative effectiveness of the antibiotics, for a change in the concentration of valinomycin or nonactin would be reflected by a change in the level of the nigericin-type antibiotics required for 50% inhibition (see Figure 1).

Effects of the Antibiotics on the Kinetics of the Light-Induced pH Change. Typical tracings of the light-induced pH change are presented in Figure 4. The effect of nonactin on the pH change is represented by curve A as compared with the control (curve B). The most striking feature of this curve is that nonactin stimulated the initial rate and extent of the H^+ uptake. This is to be contrasted with the effect of dianemycin (curve C) which inhibited the extent of the reaction but had little effect on the initial rate. The striking feature of this curve (curve C) is that the dark decay of the H^+ gradient was greatly accelerated even though the extent of the reaction was less than the control.

The decay of the pH gradient formed in both mitochondria and in chloroplasts has previously been demonstrated to be a first-order reaction with respect to the H^+ concentration. That the decay reaction is first order in chromatophores also is illustrated by the results of Figure 5. Curve A represents the initial rate of dark decay following various extents of the pH change. These were varied by increasing the extent of the reaction with nonactin (or valinomycin). Since curve A is a straight line, it means that the reaction is first order and is unaffected by nonactin or valinomycin. Curve A and the associated points were obtained from a single chromatophore preparation; a similar curve, but perhaps with a different slope, may be characteristic of another preparation.

In contrast, the slope of the curve (curve B) is greatly increased with monensin A but is still first order with

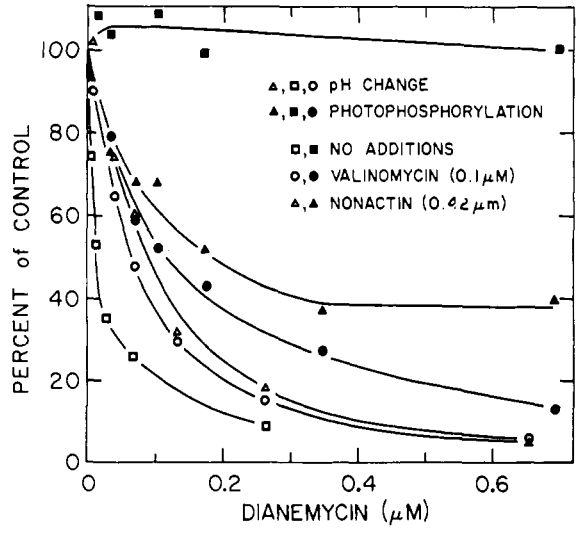

FIGURE 3: Effect of dianemycin on the light-induced pH change and photophosphorylation. Experimental conditions were similar to those of Figures 1 and 2. The initial rate of the pH change was 151 μmoles of H^+/mg of Bchl per hr and the extent was 0.31 μmole of H^+/mg of Bchl. This rate was stimulated 3.3- and 4.4-fold by valinomycin and nonactin, respectively, whereas the extent was stimulated 2.2- and 2.7-fold, respectively. The rate of ATP formation was 162 μmoles/mg of Bchl per hr and was inhibited 14 and 17%, respectively, by valinomycin and nonactin.

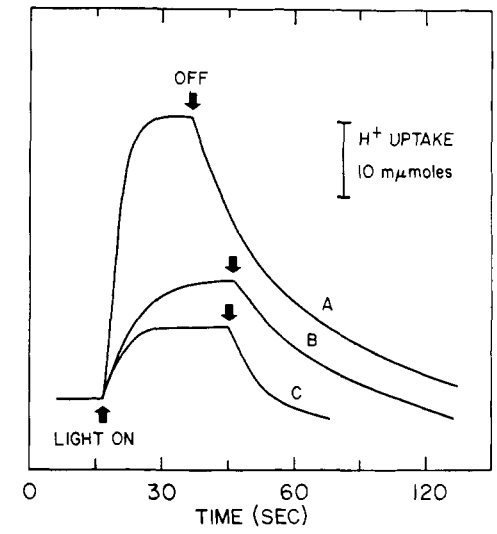

FIGURE 4: Kinetics of the light-induced pH change. Experimental conditions were as described for Figure 2 except the reaction mixture contained 7.4 μg of Bchl/ml. Curve A contained 1.6×10^{-7} M nonactin and curve C contained 6.5×10^{-9} M dianemycin. Curve B was the control. Control activity was as in Figure 2.

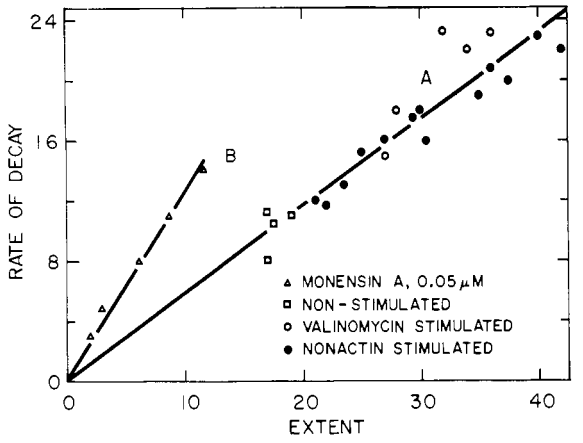

FIGURE 5: Decay of the light-induced pH change as a function of the extent. The rates of decay in curve A were determined from the experiments performed in Figure 2 and are presented in arbitrary units. The rates of decay in curve B were determined from a single curve containing monensin A.

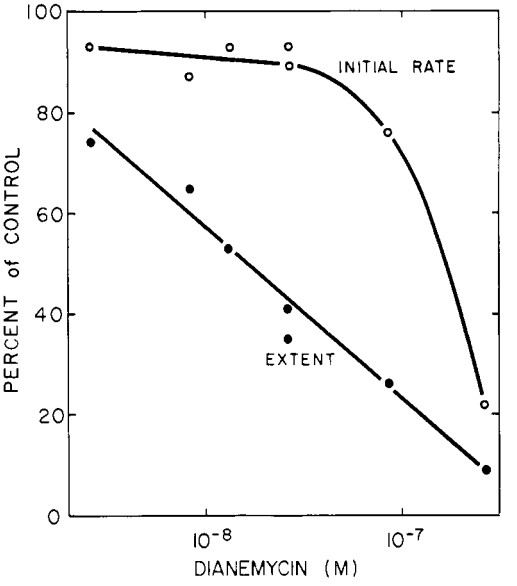

FIGURE 6: Effect of dianemycin on the initial rate and extent of the light-induced pH change. Experimental conditions were as in Figure 2.

respect to $[H^+]$. Similar results were obtained with nigericin and dianemycin.

The effect of dianemycin concentration is presented in more detail in Figure 6. Whereas the extent of the reaction was progressively inhibited by this antibiotic, the initial rate was unaffected until the extent was severely depressed. It seems probable that even this inhibition of the initial rate was due to the enhancement of the decay rate to a degree such that the initial rate of formation could not be accurately measured.

Salt Specificity for the Stimulation of the Light-Induced pH Change by Valinomycin and Nonactin. The relative effectiveness of fixed concentrations of valinomycin and nonactin for stimulation of the light-induced pH change in various alkali metal salts is presented in Table III. In this experiment valinomycin was equally effective in KCl, RbCl, and CsCl but has little or no stimulatory effect in NaCl and LiCl. The lack of stimulation in NaCl as contrasted to KCl has been reported previously by von Stedingk and Baltscheffsky (1966). In some experiments, CsCl was slightly less effective than KCl and RbCl. This specificity is similar to that found with valinomycin in mitochondria and red blood cells. Nonactin had a similar salt specificity except that little activity was observed in CsCl. This is quite different from the salt specificity shown by nonactin in mitochondria where it has been reported to induce the transport of all alkali metal cations except Li^+ (Lardy *et al.*, 1967).

TABLE III: Salt Specificity for Stimulation of the pH Change.[a]

	Per Cent Stimulation			
	Valinomycin, 2.5×10^{-8} M		Nonactin, 1.6×10^{-8} M	
Salt	Extent	Initial Rate	Extent	Initial Rate
KCl	126	167	76	86
RbCl	126	200	88	86
CsCl	132	210	10	6
NaCl	0	6	7	0
LiCl	7	24	0	2

[a] Experimental conditions were similar to Figure 3 except 50 mM salt and 6.5 μg of Bchl/ml were used. The 100% activity was essentially the same in all salts and corresponded to an extent of 0.21 μmole of H^+/mg of Bchl and an initial rate of 89 μmoles of H^+/mg of Bchl per hr.

Salt Specificity of the Synergistic Inhibition of Photophosphorylation. The effects observed in mitochondria, chloroplasts, and chromatophores due to these antibiotics have been shown to occur only in media containing specific alkali metal cations; generally K^+ elicits the greatest activity. The salt dependency for the inhibition of photophosphorylation (in this case, KCl) by three different combinations of antibiotics is presented in Figure 7. The figure shows that the inhibitory effect has an absolute dependency upon KCl and the effect is almost maximal at a concentration of about 50 mM KCl. The combinations of antibiotics not included in the figure show a similar dependency upon KCl for their inhibitory effect. Although not shown here, the salt dependency for inhibition of the proton pump was similar to that of Figure 7. The ion specificity for the synergistic inhibition is presented in Table IV. KCl was most effective in eliciting the inhibition with all combinations of antibiotics. Some inhibition was observed in RbCl with nigericin and dianemycin, but monensin A was ineffective in this salt. Essentially no effect was found in NaCl or LiCl and very little in CsCl.

Salt Specificity for the Inhibition of the pH Change by Nigericin-Type Antibiotics. If the inhibition of photo-

TABLE IV: Salt Specificity for the Synergistic Inhibition of Photophosphorylation by Antibiotics.[a]

Additions (μM)	Per Cent Inhibition				
	KCl	RbCl	CsCl	NaCl	LiCl
Valinomycin (0.1)	16	13	11	2	0
Valinomycin (0.1) + nigericin (0.45)	55	34	19	0	1
Valinomycin (0.1) + dianemycin (0.63)	58	34	15	9	4
Valinomycin (0.1) + monensin A (8.8)	48	6	4	2	4
Nonactin (0.42)	19	17	14	10	15
Nonactin (0.42) + nigericin (0.45)	77	35	10	10	4
Nonactin (0.42) + dianemycin (0.63)	70	42	20	19	3
Nonactin (0.42) + monensin A (8.8)	71	11	15	12	6

[a] Experimental conditions were as in Table I except 50 mM salts were used and 12.7 μg of Bchl/ml. The control rate of ATP formation was 230 μmoles /mg of Bchl per hr.

phosphorylation by combinations of nigericin-type and valinomycin-type antibiotics were due to the induction of a cyclic, energy-requiring ion transport which competes with ADP for a high-energy intermediate as has been suggested with mitochondria (Pressman *et al.*, 1967), then the ion specificity for the inhibition should be a reflection of the capacity of the valinomycin type agents to stimulate ion transport and of the nigericin-type compounds to dissipate the ion gradient formed in various salts. Salts of interest in this connection were KCl, RbCl, and CsCl since the stimulation of the light-induced pH change by valinomycin or nonactin occurred only in these salts (see Table III). Furthermore, the synergistic inhibition of photophosphorylation occurred only in KCl and RbCl with the antibiotic concentrations tested.

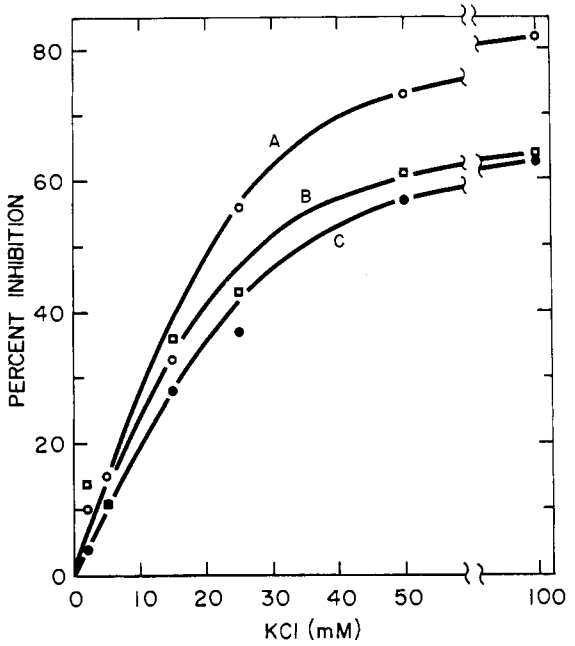

FIGURE 7: Effect of KCl on the inhibition of photophosphorylation by combinations of antibiotics. Experimental conditions were as in Table I. Control activities were 125 μmoles of ATP/mg of Bchl per hr for curves A and C and 152 μmoles of ATP/mg of Bchl per hr for curve B. Curve A contained nonactin (0.42 μM) plus monensin A (0.96 μM); curve B contained nonactin (0.42 μM) plus nigericin (0.45 μM); curve C contained valinomycin (0.10 μM) plus monensin A (0.88 μM).

Table V shows the relative degrees of inhibition obtained with the nigericin-type antibiotics in KCl, RbCl, and CsCl when the pH change was stimulated by valinomycin or nonactin. At these concentrations, nigericin and dianemycin had an absolute requirement for K^+ when the proton uptake was stimulated by valinomycin whereas dianemycin inhibited somewhat in RbCl when the reaction was stimulated by nonactin. Monensin A, while most effective in KCl, inhibited also in RbCl and CsCl. Low concentrations of antibiotic were chosen to illustrate the salt specificity. Higher concentrations of dianemycin and nigericin inhibited somewhat in RbCl, as would be expected from the inhibition of photophosphorylation observed in this salt (Table IV). A comparison of Tables IV and V reveals apparent discrepancies between the salt specificity for the synergistic inhibition of photophosphorylation and that of the valinomycin- and nigericin-type antibiotics for stimulation and inhibition of the pH change. The inhibition of the pH change by monensin A in RbCl and CsCl was not reflected by inhibition of photophosphorylation in these salts. Moreover, while nigericin and dianemycin inhibited photophosphorylation in RbCl they did not inhibit the light-induced pH change in this salt. These discrepancies led us to examine the effect of nigericin-type antibiotics on the dark decay of the pH change.

Effect of Nigericin-Type Antibiotics on the Dark Decay of the pH Change. Figure 8 illustrates the effect of nigericin-type antibiotics on the rate of dark decay of the pH change in various salts. In KCl all of the antibiotics strongly inhibited the extent of the reaction and stimulated the rate of decay by severalfold. In RbCl, the same concentrations of nigericin and dianemycin had little effect on the extent of the reaction. This is reflected in the lower effectiveness of these antibiotics in

TABLE V: Inhibition of the Extent of the pH Change in Various Salt.[a]

Salt	Per Cent Inhibition					
	Valinomycin, 2.5×10^{-8} M			Nonactin, 3.2×10^{-8} M		
	Nigericin, 5.1×10^{-7} M	Dianemycin, 6.5×10^{-7} M	Monensin A, 1.8×10^{-6} M	Nigericin, 5.1×10^{-7} M	Dianemycin, 6.5×10^{-7} M	Monensin A, 1.8×10^{-6} M
KCl	72	60	67	81	71	74
RbCl	0[b]	3[b]	30	0	34	34
CsCl	0[b]	0[b]	30			

[a] Experimental conditions were as in Table IV. The numbers are averages of three experiments. The extent and initial rate were stimulated two- to threefold by valinomycin and nonactin. [b] Stimulated slightly in some experiments.

inhibiting photophosphorylation in this salt as compared with KCl. Higher concentrations increasingly inhibited the extent. These antibiotics stimulated the decay reaction in RbCl but to a lesser degree than in KCl. In contrast, monensin A inhibited the extent but has no effect on the rate of decay. In CsCl, none of the antibiotics stimulated the decay. Monensin A, however, markedly inhibited the extent of the pH change in this salt.

A comparison of the ability of the nigericin-type antibiotics to inhibit photophosphorylation (Table IV) with their effect on stimulating the decay of the pH change (Figure 8) reveals a correlation between these activities. This correlation explains the apparent discrepancies between the results shown in Tables IV and V. With monensin A, the inhibition of the extent was not accompanied by a stimulation of the decay in Rb^+ or Cs^+. This indicates that the mechanism may be different in these salts than in K^+, but we have not further investigated these effects with monensin A.

Discussion

Currently there are several postulations for the mechanism of action of valinomycin- and nigericin-type antibiotics on ion transport. Cockrell *et al.* (1966) have proposed a model in which a carrier (C) is energized to C* by using the energy available as a high-energy intermediate. Metal ions (M^+) then combine with C* and are transported across the membrane. Valinomycin was proposed to stimulate M^+ uptake by interacting with C*. Lardy and his collaborators (1967) have proposed a varient of this mechanism in which the alkali metal ions are transported across the membrane as a complex, $C–M^+$. The energy available as a high-energy intermediate converts C into C* (a form which has less affinity for cations than C) at the inner surface of the inner mitochondrial membrane. This facilitates the formation of a gradient across the membrane. Valinomycin-type antibiotics were postulated to act by facilitating the conversion of C into C* or alternatively by delivering the cations to the activated carrier. Nigericin-type antibiotics which inhibit ion uptake were suggested to act by combining with C or with the $C–M^+$ complex, thereby preventing the activation of the carrier by the high-energy intermediate.

Pressman and coworkers (1967) have proposed a different mechanism of action for the nigericin-type antibiotics. Whereas valinomycin was proposed to act only at the loci of the mitochondrial ion pump, nigericin was proposed to confer ion permeability at random loci in the membrane and to increase H^+ permeability as well as alkali metal cation permeability. The result would be a passive $H^+–M^+$ exchange which would prevent the formation of a proton gradient or dissipate any gradient already formed. This mechanism was supported by the observation that the addition of valinomycin plus nigericin to a suspension of mitochondria resulted in a strong stimulation of respiration. This uncoupling would be due to an energy-requiring uptake of alkali metal ions induced by valinomycin and a concomitant increase in permeability induced by nigericin. This results in a cyclic energy utilizing transport of ions.

Chappell and Crofts (1966) have proposed a mechanism which differs from those outlined above by en-

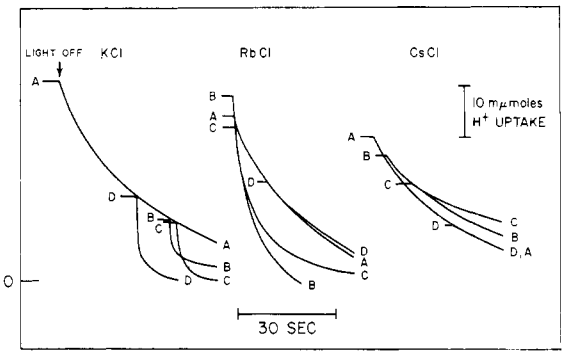

FIGURE 8: Effect of nigericin-type antibiotics on the decay of the pH change in various salts. Conditions were as described in Figure 2 except 50 mM salts were used. All reaction mixtures contained 2.5×10^{-8} M valinomycin and the initial rate and extent was 248 μmoles of H^+/mg of Bchl per hr and 0.68 μmole of H^+/mg of Bchl, respectively, in KCl and RbCl. The rate and extent was about 35% lower in CsCl. (A) Control; (B) nigericin, 5.1×10^{-7} M; (C) dianemycin, 6.5×10^{-7} M; (D) monensin A, 1.8×10^{-6} M. The light off points of curves B–D were drawn at the appropriate extent on curve A for ease of comparing the decay rate with the control.

visioning an energy-linked transport of protons rather than alkali metal cations. In this model the increased ion transport due to valinomycin-type antibiotics can be explained by the ability of these compounds to increase specifically the permeability of biological membranes for alkali metal cations, which would allow these ions to penetrate the mitochondrial membrane in exchange for protons. Similarly, nigericin-type antibiotics were postulated to increase the permeability of membranes for both protons and alkali metal cations (Henderson and Chappell, 1967).

In this investigation we have used the light-induced proton uptake in chromatophores as a convenient measure of ion transport. Our results, therefore, do not indicate whether the actively transported ions are protons or alkali metal cations. In some preliminary experiments we have been unable to observe any light-induced movements of alkali metal cations. However, the ion requirement of the proton pump and the ion-specific effects of the antibiotics suggest that an H^+–M^+ exchange must occur in this system. Thus, we ascribe our inability to observe alkali metal cation movements to unsuitable experimental conditions.

We propose that valinomycin-type antibiotics stimulate proton uptake in chromatophores by facilitating the energy-requiring transport of ions across the membrane. This is reflected in a stimulation of the initial rate and extent of the pH change (Figure 2). Low concentrations of these antibiotics had little effect on the decay of the gradient (Figure 5). It is likely that higher concentrations would stimulate the decay, and indeed this has been noted by von Stedingk and Baltscheffsky (1966) who reported that 10^{-6} M valinomycin stimulated the decay by about twofold. It seems reasonable that the nonenergized carrier would retain some affinity for cations and thus valinomycin would also facilitate the decay of the gradient. Alternatively, valinomycin may have a secondary effect on membrane permeability in addition to the effect on the energy-linked ion transport. This would be compatible with the observations of Mueller and Rudin (1967) and Chappell and Haarhoff (1967) who demonstrated that valinomycin enhanced the permeability of lipid membranes for K^+.

In contrast, the nigericin-type antibiotics had little effect on the initial rate of the pH change until the extent was severely inhibited (Figures 4 and 6). The decay of the light-induced pH change, however, was markedly stimulated even at low concentrations of antibiotic (Figures 4, 6, and 8). Thus, we propose that this type of antibiotic acts by conferring ion permeability to the membrane at sites not associated with the ion pump mechanism.

If we assume that the initial rate of dark decay reflects the turnover of protons during the steady state in the light, then an estimate can be made of the magnitude of the energy requirement. In the absence of antibiotics this rate is typically 20–30 μmoles of H^+/mg of Bchl per hr. The addition of 2.5×10^{-8} M valinomycin results in an increase of this rate to 50–60 μmoles/mg of Bchl per hr due to the stimulation of the extent. Nigericin at concentrations sufficient to inhibit the pH change by about 50% stimulated the initial rate of decay to 150–200 μmoles/mg of Bchl per hr. If we assume a requirement of one high-energy bond for four H^+ transported (Carafoli *et al.*, 1965; Cockrell *et al.*, 1966), then 40–50 μmole equiv of ATP would be required and this would be reflected as an inhibition of photophosphorylation. In a separate experiment, the combination of valinomycin and nigericin mentioned above inhibited photophosphorylation by 35%, and this corresponded to a decrease in ATP of 50 μmoles/mg of Bchl per hr. Valinomycin alone at low concentrations had little effect on photophosphorylation since the rate of decay of the gradient was limiting the turnover. With nigericin alone, although the rate of decay was markedly increased, the rate of H^+ uptake was low and not of sufficient magnitude to inhibit significantly phosphorylation.

Thus, we would ascribe the synergistic inhibition of photophosphorylation by nigericin- and valinomycin-type antibiotics to the induction of a cyclic, energy-requiring ion transport which is of sufficient magnitude to compete with ADP and inorganic phosphate for the energy of a high-energy intermediate of phosphorylation.

Another observation which supports this hypothesis is that the combination of valinomycin and nigericin exhibits a progressively greater inhibitory effect as the light intensity is decreased. This condition mimics the effect of uncouplers on photophosphorylation (Keister and Yike, 1967) and supports the idea that active ion transport competes with ADP and inorganic phosphate for energized intermediates of phosphorylation. This mechanism is similar to that proposed by Pressman *et al.* (1967) to account for their observations with mitochondria.

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Effect of Alkyldinitrophenols on Photophosphorylation in Chloroplasts*

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ABSTRACT: Both 2,4- and 2,6-dinitrophenol are known to exhibit uncoupling effects at lower pH, and inhibition of electron transport at higher pH. Alkylated dinitrophenols are much more effective both as uncouplers and inhibitors of photophosphorylation. Lipid solubility as well as dissociation were found to be key factors associated with the effect of alkyldinitrophenols on photophosphorylation in chloroplasts. The rate of adenosine triphosphate formation was essentially proportional to electron transport if the nonphosphorylating rate is substracted from the rate obtained in phosphorylating systems regardless of the over-all rate of reaction. This is interpreted as meaning that the phosphorylation reaction is exclusively associated with that part of electron transport stimulated by the presence of phosphorylating reagents. The stoichiometry (P/2e ratio) of the phosphorylation process therefore is probably 2.0. Since the inhibition of electron transport and its associated adenosine triphosphate formation by dinitrophenols is more severe as the light intensity is lowered, the site of inhibition by these compounds is assumed to be at or very close to the photoinduced reaction step.

2,4-Dinitrophenol is undoubtedly one of the best known uncouplers of oxidative phosphorylation although much stronger uncouplers are now available. In contrast, photophosphorylation was first shown to be relatively insensitive to this compound (Arnon *et al.*, 1954; Avron, 1960; Krogmann *et al.*, 1959; Whatley *et al.*, 1959) and it was at one time claimed that it was not an uncoupler but only an inhibitor of electron transport for chloroplasts. This led to the earlier speculations that the mechanisms for the electron transport coupled energy transfer in oxidative phosphorylation and photophosphorylation were basically different (Jagendorf, 1959). The observation of a true uncoupling effect of 2,4-dinitrophenol in photophosphorylation at lower pH values (Neumann and Jagendorf, 1964) and of a similar effect of 4-isooctyl-2,6-dinitrophenol (Baltscheffsky, 1965) seems to indicate that, at least to some extent, other factors may be involved in the action of dinitrophenols on chloroplast systems. In some earlier experiments in our laboratory involving carrot disks, a rather distinctly different action pattern was observed for alkylated 2,4- and 2,6-dinitrophenols. These observations prompted a more detailed investigation on the effect of some alkylated 2,4- and 2,6-dinitrophenols on photophosphorylation.

Experimental Section

Chemicals. The compounds used in this investigation were as follows: 2,4-dinitrophenol, 6-(1,2-dimethylbutyl)-2,4-dinitrophenol, 6-(1-methylpentyl)-2,4-dinitrophenol, 2,6-dinitrophenol, 4-(1,2-dimethylbutyl)-2,6-

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