Effects of Ionophorous Antibiotics on the Light-Induced Internal and External Hydrogen Ion Changes and Phosphorylation in Bacterial Chromatophores*

Mitsuo Nishimura and Berton C. Pressman†

ABSTRACT: The effects of ionophorous or transport-mediating antibiotics, valinomycin, nigericin, monensin, X-206, X-537A, and dianemycin on the light-induced external and internal H^+ changes of *Rhodospirillum rubrum* and *Chromatium* chromatophores were studied under phosphorylating and nonphosphorylating conditions. Valinomycin increased the rate and magnitude of the light-induced external pH rise in the presence of K^+, but inhibited the apparent light-induced internal acidification, as measured by bromothymol blue–spectrophotometric method. Nigericin, X-206, X-537A, and monensin decreased the light-induced external pH rise, but increased the light-induced bromothymol blue change. Dianemycin either increased or inhibited both the bromothymol blue and external H^+ changes, depending upon its concentration. Under phosphorylating conditions, these antibiotics had little effect on the phosphorylation-dependent external pH rise due chiefly to adenosine triphosphate synthesis in either the presence or absence of K^+ or Na^+. Combinations of valinomycin and nigericin, or valinomycin and uncouplers did not produce synergistic effects. Possible mechanisms for the observed effects of ionophorous antibiotics on the internal and external H^+ changes and the processes of energy transduction are presented. Differences between the actions of ionophorous antibiotics on bacterial chromatophores and chloroplasts are discussed.

Studies of the effects of the "ionophorous" (Pressman *et al.*, 1967) or transport-mediating antibiotics have been carried out extensively in biological and artificial lipid barrier systems and the mechanisms of antibiotic-mediated ion transport have been discussed (Moore and Pressman, 1964; Harris *et al.*, 1967; Mueller and Rudin, 1967). Two groups of antibiotics have been classified: the valinomycin type which forms charged lipophilic complexes with alkali ions, and the nigericin type which forms analogous charge-compensated zwitterion complexes (Pressman *et al.*, 1967).

The biological membrane systems which have been examined include suspensions of chloroplasts and chromatophores with respect to the effects of ionophorous antibiotics on the light-induced pH gradient formation. The acceleration of light-induced H^+ uptake in the presence of valinomycin and K^+ (or other cations), and the inhibition of the H^+ uptake by nigericin under similar conditions were the major effects reported (von Stedingk and Baltscheffsky, 1966; Packer, 1967; Shavit *et al.*, 1968a–c). The uncoupling of photophosphorylation by nigericin in the presence of K^+ was also observed in chloroplasts (Shavit and San Pietro, 1967; Shavit *et al.*, 1968a,b). However, photophosphorylation by chromatophores of *Rhodospirillum rubrum* was not affected by nigericin in the presence or absence of K^+ (Shavit *et al.*, 1968c).

This laboratory has reported the comparison of the kinetics of light-induced bromothymol blue and external H^+ changes of *R. rubrum* chromatophores (Chance *et al.*, 1966; Nishimura *et al.*, 1968) and reached the conclusion that the observed light-induced H^+ change does not precede phosphorylation and that it is not a necessary condition for phosphorylation. A similar view has also been expressed by Shavit *et al.* (1968c), although the hypothesis of a hydrogen ion gradient as the prerequisite, *i.e.*, the energized intermediate which drives photophosphorylation has been suggested by others (Mitchell, 1966; Jagendorf and Uribe, 1966a; Schwartz, 1968). In this paper the effects of several ionophorous antibiotics on the bromothymol blue and external H^+ changes under phosphorylating and nonphosphorylating conditions have been compared in *Chromatium* and *R. rubrum* chromatophores. Two general response patterns were observed as exemplified by the effects of nigericin and valinomycin; dianemycin had an effect which was intermediate between the two extreme response patterns. The results are interpreted as showing that hydrogen ion gradient formation and phosphorylation are parallel processes coupled *via* a common energy pool rather than that the light-induced hydrogen ion gradient behaves as an obligatory intermediate for phosphorylation.

Experimental Procedures

The photosynthetic nonsulfur purple bacterium, *R. rubrum*, van Niel strain 1, and sulfur purple bacterium, *Chromatium* sp. strain D, were cultured as

* From the Johnson Research Foundation, Department of Biophysics and Physical Biochemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received November 4, 1968.* This work was supported by a research grant from the National Institutes of Health, U. S. Public Health Service (Grant GM 12202).

† Recipient of a U. S. Public Health career development award (K3-GM-3626).

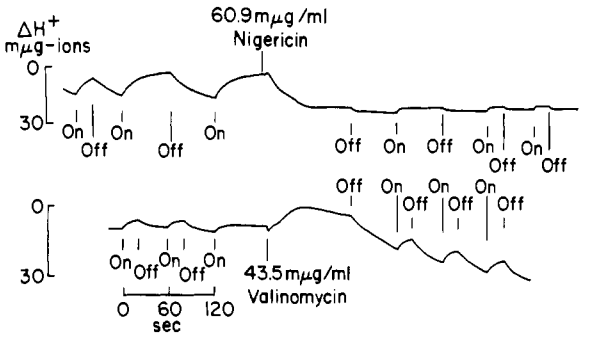

FIGURE 1: Effects of nigericin and valinomycin on the light-induced external pH shift in KCl. *R. rubrum* chromatophores, 97 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl (pH 6.5); temperature 23°. An upward deflection corresponds to external alkalinization.

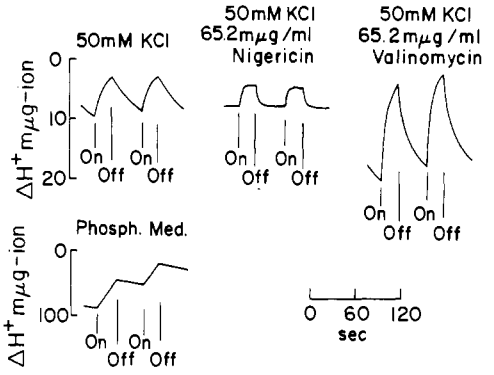

FIGURE 2: Effects of nigericin and valinomycin on the light-induced external pH shift in the KCl-nonphosphorylating medium, and photophosphorylation as measured by the pH shift due to ATP formation. *Chromatium* chromatophores, 25 mμmoles of bacteriochlorophyll/4.6 ml. Upper traces: 50 mM KCl, pH 6.5. Lower trace: phosphorylating medium, pH 7.8; temperature 28°. An upward deflection corresponds to external alkalinization.

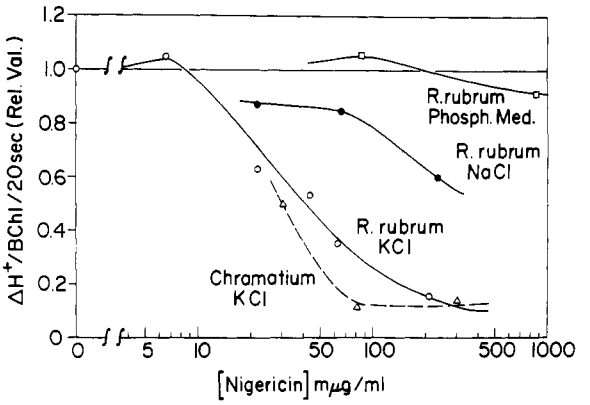

FIGURE 3: Concentration dependence of the effect of nigericin upon the light-induced external pH shift. *R. rubrum* chromatophores, 48 mμmoles of bacteriochlorophyll/4.6 ml; *Chromatium* chromatophores, 44 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl (pH 6.5) and 50 mM NaCl (pH 6.5); or phosphorylating medium, pH 7.8; temperature 23°. The rate of the pH shift in the absence of nigericin in each reaction mixture was taken as unity.

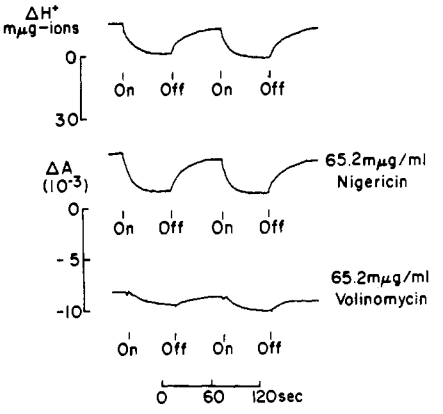

FIGURE 4: Effects of nigericin and valinomycin on the light-induced bromothymol blue change of *R. rubrum* chromatophores in KCl (double-beam spectrophotometric method). *R. rubrum* chromatophores, 52 mμmoles of bacteriochlorophyll/1.85 ml; 50 mM KCl (pH 7.3); bromothymol blue, 17 μM ; 5-mm path length; ΔA measured at 625–655 mμ; temperature 23°. The same ΔH^+ and ΔA calibrations apply to all three traces. A downward deflection corresponds to an apparent internal acidification.

described previously (Nishimura and Chance, 1963). Chromatophore preparations were obtained by disintegration of the bacteria in a Nossal shaker followed by differential centrifugation (Nishimura, 1962). Changes in pH in the reaction medium were recorded by coupling a Radiometer 22 pH meter to an Esterline-Angus Speed-Servo recorder and expressed in terms of equivalent ΔH^+ by calibrating the system with standard acid. Apparent changes in the internal $[H^+]$ were measured by the bromothymol blue–spectrophotometric method. Calibration of the bromothymol blue change as apparent ΔH^+ was carried out spectrophotometrically with an addition of standard acid. Details of the experimental procedures have been described in the preceding paper (Nishimura *et al.*, 1968).

The sample was illuminated through a Wratten 88A filter (>720 mμ) and a water layer (45 mm thickness) by a low-voltage lamp operated by direct current. Typical incident light intensity was 112 kerg/cm^2 per sec as measured by a Reedeer thermopile calibrated by a secondary standard lamp (National Bureau of Standards). We thank the Eli Lilly Co. for supplying the ionophorous antibiotics employed.

Results

Effect of Nigericin on Light-Induced External pH Changes. Typical traces showing the effect of nigericin on the light-induced external pH changes are shown in Figure 1 (*R. rubrum* chromatophores) and Figure 2 (*Chromatium* chromatophores). Nigericin induced a marked inhibition of the extent of light-induced pH

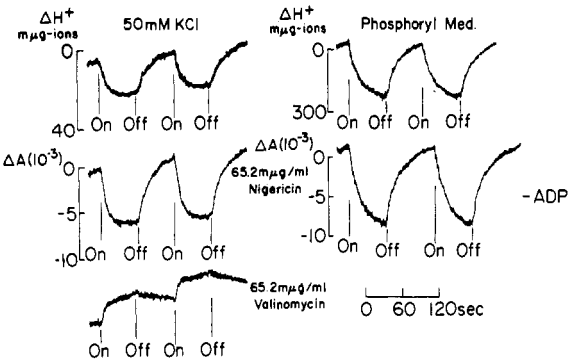

FIGURE 5: Effects of nigericin and valinomycin on the light-induced bromothymol blue change in KCl and effect of absence of ADP on the bromothymol blue change in the phosphorylating medium. KCl (50 mM, pH 7.3) or phosphorylating medium (pH 7.8). *Chromatium* chromatophores, 29 mμmoles of bacteriochlorophyll/1.85 ml; 5-mm path length; ΔA at 625–650 mμ; temperature 29°. The respective ΔH^+ and ΔA calibrations apply to each set of traces on the right and left, respectively. A downward deflection corresponds to an apparent internal acidification.

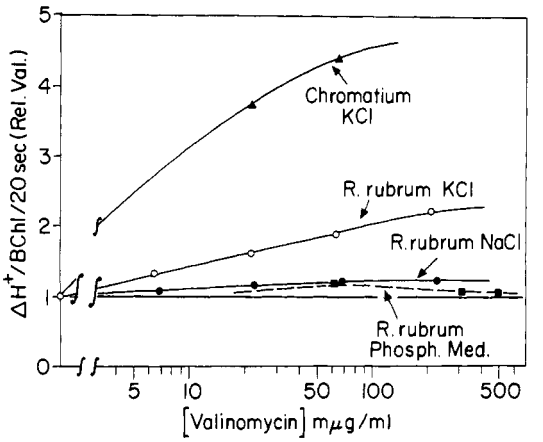

FIGURE 6: Concentration dependence of effect of valinomycin upon the light-induced external pH shifts. *R. rubrum* chromatophores, 48 mμmoles of bacteriochlorophyll/4.6 ml; *Chromatium* chromatophores, 83 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl, pH 6.5; 50 mM NaCl, pH 6.5; phosphorylating medium, pH 7.8; temperature 23°. The rate of the H^+ change in the absence of valinomycin in each reaction mixture was taken as unity.

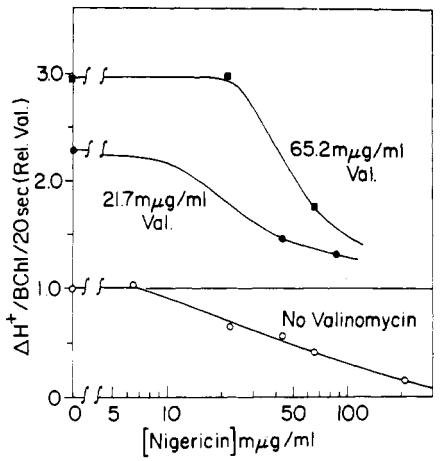

FIGURE 7: Effect of combination of nigericin and valinomycin on the light-induced external pH shift. *R. rubrum* chromatophores, 98 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl, pH 6.5; temperature 23°. The rate of the pH shift in the absence of nigericin and valinomycin was taken as unity.

changes both in *R. rubrum* and *Chromatium* chromatophores in the nonphosphorylating systems in the presence of K^+ or Na^+. When nigericin was added during illumination, a rapid drop in the light-induced pH change was observed. After the addition of nigericin, the light–dark pH cycles were much diminished as compared with those in the absence of nigericin. The effect of various concentrations of nigericin on the light-induced pH shift is shown in Figure 3. In the presence of 50 mM K^+, the responses to nigericin begin at 10 mμg/ml and are maximal at 100 mμg/ml. The finding by Shavit *et al.* (1968c) that K^+ is more effective than Na^+ in manifesting the effect of nigericin was confirmed and is consistent with the intrinsic ion selectivity of the antibiotic as measured in model systems (Pressman, 1968). The half-maximum inhibitory concentration of nigericin for *R. rubrum* chromatophores is ten times higher in NaCl than in KCl. The degree of inhibition by nigericin of the net pH change is larger than its effect on the initial rate of pH change, as can be seen in Figure 1 (*R. rubrum*) and Figure 2 (*Chromatium*).

In the low-to-medium concentration range of nigericin (up to 1 μg/ml), no effect of the antibiotic on the external alkalinization due to ATP formation in the phosphorylation medium (Nishimura *et al.*, 1962) occurred in absence or presence of 4–87 mM K^+. At higher concentrations of nigericin, a slight decrease of photophosphorylation was observed with *R. rubrum* chromatophores as reported by Shavit *et al.* (1968c).

Nigericin on Internal $[H^+]$ *Change.* When the apparent internal $[H^+]$ change was measured using the bromothymol blue–spectrophotometric method, a striking effect of nigericin was noticed. At the levels (10–100 mμg/ml) which markedly reduced the external pH change, the apparent internal light-induced acidification was increased (Figure 4, *R. rubrum* chromatophores; Figure 5, *Chromatium* chromatophores).

Effect of Valinomycin on Light-Induced External pH Changes in Chromatophores. Valinomycin induced a marked *increase* in the light-induced external alkalinization in the presence of K^+ or Na^+. Typical traces are shown in Figure 1 (*R. rubrum*) and Figure 2 (*Chromatium*). Addition of valinomycin during illumination caused a rapid rise in pH to a new steady-state level (Figure 1). The effect of valinomycin on the external pH change was qualitatively the same with both *R. rubrum* and *Chromatium* chromatophores. The increase in pH induced by valinomycin was plotted against its concentration (Figure 6). The action of low levels (10–100 mμg/ml) of valinomycin was quite large in

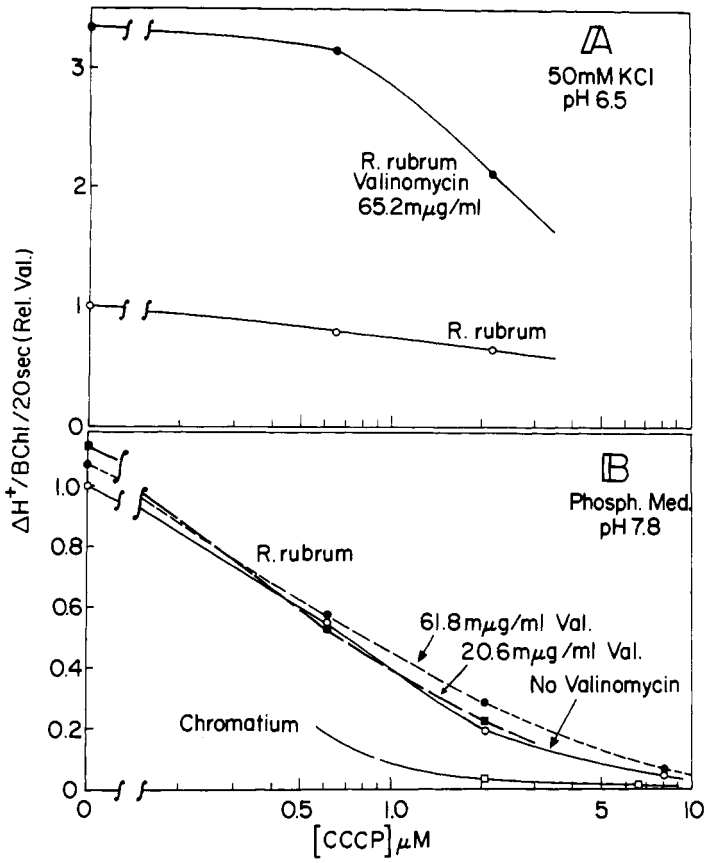

FIGURE 8: Effect of combination of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone on the light-induced external pH shift. (A) 50 mM KCl, pH 6.5; *R. rubrum* chromatophores, 78 mμmoles of bacteriochlorophyll/4.6 ml; temperature 23°. (B) Phosphorylating medium, pH 7.8; *R. rubrum* chromatophores, 64 mμmoles of bacteriochlorophyll/4.6 ml; *Chromatium* chromatophores, 67 mμmoles of bacteriochlorophyll/4.6 ml; temperature 28°. The rate of H^+ change in absence of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone in each reaction system was taken as unity.

KCl. In NaCl the effect of valinomycin was much smaller. In the presence of a phosphorylation medium valinomycin did not affect the larger pH change resulting from the reaction ADP + P_i → ATP + ΦOH^- other than to increase it slightly (Figure 6, see also Figure 8B).

Effect of Valinomycin on the Bromothymol Blue Change in Chromatophores. The effect of valinomycin on the apparent internal $[H^+]$ change with *R. rubrum* and *Chromatium* chromatophores as measured with bromothymol blue was also studied. Simultaneous measurement of the external pH shift was always conducted in parallel experiments. Valinomycin induced a *decreased* light-induced bromothymol blue response in *R. rubrum* chromatophores (Figure 4) which contrasted with its *increase* of the external light-induced alkalinization (Figure 1). In *Chromatium* chromatophores, the direction of the light-induced bromothymol blue change was actually reversed by valinomycin, resulting in a slight apparent alkalinization (Figure 5). These striking phenomena occur in the presence of valinomycin at concentrations at which augmentation of the light-induced external alkalinization can be clearly observed. The effect of valinomycin on the internal light-induced $[H^+]$ change in a weakly buffered system (*e.g.*, 5–50 mM glycylglycine, pH 7.3) was dependent upon the presence of either K^+ or Na^+.

Combined Effects of Valinomycin and Nigericin. In 50 mM KCl, the external pH shift was markedly increased by valinomycin and inhibited by nigericin. The combination of valinomycin and nigericin gave a simple intermediate pattern on the light-induced pH rise in the medium (Figure 7). The extent of the inhibition by nigericin was not affected by valinomycin if the rate observed at a given valinomycin concentration before nigericin addition is taken as the control value.

The light-induced external alkalinization caused by the phosphorylation of ADP was only slightly inhibited by nigericin and slightly activated by valinomycin (*cf.* Figures 3 and 6). Raising the KCl concentration to 100 mM increased the degree of inhibition or activation only slightly. A combination of nigericin and valinomycin (each 63 mμg/ml) introduced a modest inhibition of the pH increase accompanying phosphorylation with *R. rubrum* chromatophores (5–20% inhibition in the absence of KCl, and 22–37% inhibition in the presence of 83 mM KCl).

Combined Effects of Valinomycin and Phosphorylation Uncouplers. The external alkalinization by illumination in *R. rubrum* chromatophores was moderately inhibited by micromolar concentrations of carbonyl cyanide *m*-chlorophenylhydrazone in KCl. The addition of carbonyl cyanide *m*-chlorophenylhydrazone to the valinomycin-treated system induced a small inhibition of the light-induced external alkalinization. No synergistic effect was observed when valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone were added simultaneously (Figure 8A).

Under phosphorylating conditions, low concentrations of carbonyl cyanide *m*-chlorophenylhydrazone

TABLE I: Effects of Valinomycin and Nigericin on the Light-Induced Bromothymol Blue Changes of *R. rubrum* Chromatophores as Observed with the Double-Beam Spectrophotometer.[a]

Reaction System	pH	Antibiotic (mμg/ml)	Apparent Internal H^+ Change: Rate (ΔH^+/Bacteriochlorophyll per 20 sec)	Apparent Internal H^+ Change: Amount (ΔH^+/Bacteriochlorophyll)
Phosphorylation medium + 82 mM KCl	7.8		0.695	0.890
Phosphorylation medium + 82 mM KCl	7.8	Valinomycin (490)	0.556	0.776
Phosphorylation medium + 82 mM KCl	7.8	Nigericin (865)	0.785	1.059
50 mM KCl	7.3		0.061	0.108
50 mM KCl	7.3	Valinomycin (62.5)	−0.002	0.048
50 mM KCl	7.3	Nigericin (62.5)	0.089	0.168

[a] Bacteriochlorophyll 46 mμmoles/1.85 ml; bromothymol blue 17 μM; 5-mm path length; ΔA at 625–655 mμ; temperature 23°. Positive values correspond to apparent internal acidification.

(0.2–2 μM) showed a strong inhibition of phosphorylation as evident from the decreased external alkalinization (Figure 8B). The addition of valinomycin to the phosphorylating reaction system produced only a slight increase of external alkalinization (Figure 8B, see also Figure 6). The addition of valinomycin after carbonyl cyanide *m*-chlorophenylhydrazone produced no increased inhibition of the external alkalinization indicating no additional inhibition of phosphorylation.

2,4-Dinitrophenol, at 206 μM, did not inhibit the photophosphorylation by *R. rubrum* chromatophores, even in the presence of valinomycin (62 mμg/ml). In *Chromatium* chromatophores, 2,4-dinitrophenol was slightly inhibitory to phosphorylation (14–37% inhibition at 620 μM) in the presence of 83 mM KCl. Valinomycin (29 mμg/ml) increased the light-induced pH rise slightly in the presence of KCl, ADP, P_i, and Mg^{2+}. An addition of 620 μM 2,4-dinitrophenol to the valinomycin-activated system (in the presence of 83 mM KCl) reduced the pH rise somewhat to the same extent as it did in the absence of valinomycin. This contrasts with the synergistic effect of 2,4-dinitrophenol and valinomycin on the photophosphorylation capacity of chloroplasts, where each of these reagents alone failed to uncouple, but in combination completely uncoupled phosphorylation (Karlish and Avron, 1968). Similar synergistic effects between uncouplers and valinomycin on inducing ion movements have been described for mitochondria (Pressman *et al.*, 1967) and erythrocytes (Harris and Pressman, 1967).

Effects of Nigericin and Valinomycin on the Bromothymol Blue Change under Phosphorylating Conditions. With a complete phosphorylating system, valinomycin and nigericin produced little effect on the internal bromothymol blue changes in the absence of K^+ or Na^+. In the presence of these alkali ions (50–100 mM), the light-induced apparent internal acidification was increased by nigericin and decreased by valinomycin. These effects were minimal and high concentrations of the antibiotics were needed to observe appreciable effects. This contrasts with the observations that in dilute KCl or NaCl, very low concentrations of valinomycin or nigericin were sufficient to induce strong inhibition and accentuation of the bromothymol blue response, respectively (Table I, compare also Figures 4 and 5).

Effects of Dianemycin on External and Internal $[H^+]$ *Changes.* With *R. rubrum* chromatophores, low concentration of dianemycin (2–20 mμg/ml) induced a small increase of the extent of the light-induced external alkalinization in KCl or NaCl. At higher concentrations, the light-induced external pH rise was inhibited by dianemycin (Figure 9). In the case of *Chromatium* chromatophores, the increased pH rise at low concentrations of dianemycin was not markedly apparent. Alkalinization due to phosphorylation of ADP with *R. rubrum* chromatophores was not inhibited by dianemycin in the concentration range where activation or inhibition of the external pH shift of the nonphosphorylating system was observed in NaCl or KCl. The presence of KCl did not affect the action of dianemycin on the phosphorylating system (Figure 9).

Dianemycin induced a moderate increase of the light-induced internal acidification at concentrations slightly higher than those necessary for activation of the external pH shift of the nonphosphorylating system (Figure 10). At higher concentrations of dianemycin, the extent of the light-induced bromothymol blue response was inhibited as shown in Figures 10 and 11.

Effects of Monensin, X-206, and X-537A on External and Internal $[H^+]$ *Changes.* In *Chromatium* chromatophores, the presence of monensin, X-206, or X-537A induced a decrease of the external pH shift in KCl (Figure 12). The half-inhibition concentrations were in the order of X-206 < X-537A < monensin. X-206 was approximately as effective as nigericin, but the other

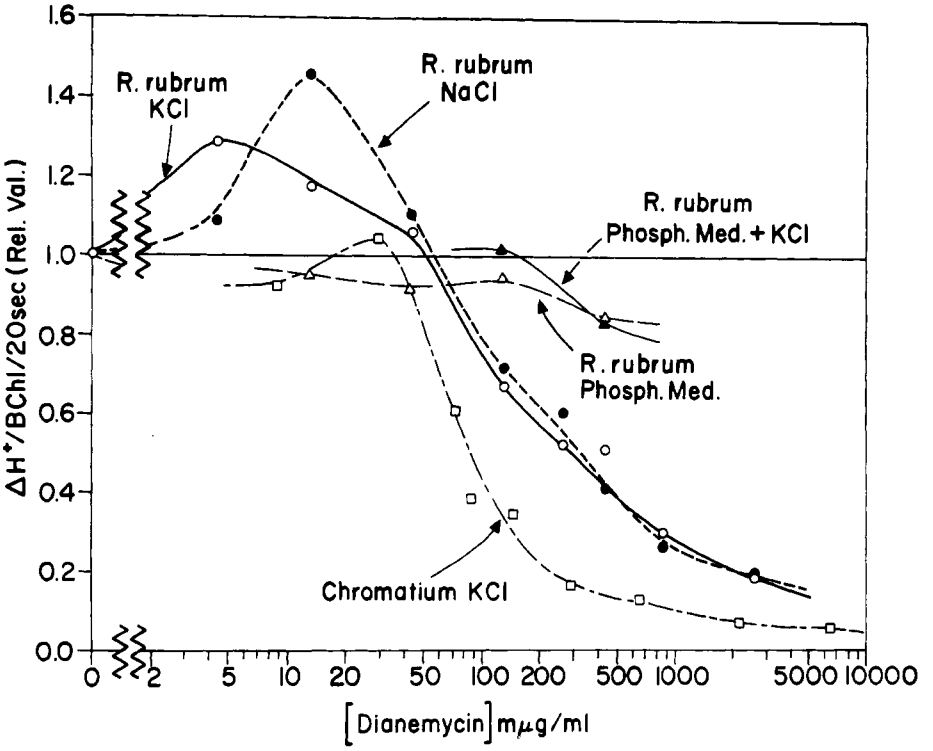

FIGURE 9: Concentration dependence of effect of dianemycin upon the light-induced external pH shift. The nonphosphorylating system contained: *R. rubrum* chromatophores, 129 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl, pH 6.5 or 50 mM NaCl, pH 6.5; or *Chromatium* chromatophores, 44 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl, pH 7.3; temperature 23°. The phosphorylating system contained: *R. rubrum* chromatophores, 26 mμmoles of bacteriochlorophyll/4.6 ml; phosphorylating medium, pH 7.8 or phosphorylating medium plus 85 mM KCl, pH 7.8; temperature 23°. The rate of the pH shift in the absence of dianemycin in each reaction mixture was taken as unity.

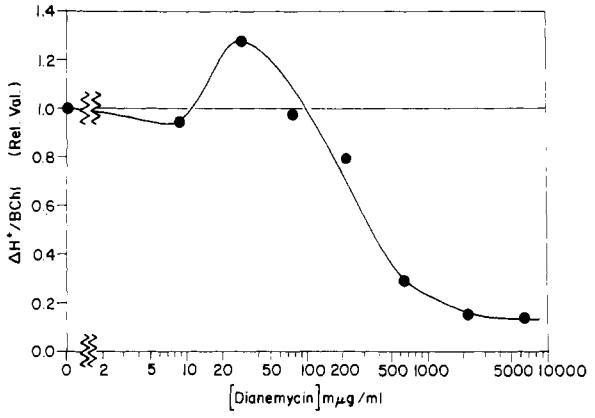

FIGURE 10: Concentration dependence of the effect of dianemycin upon the extent of light-induced bromothymol blue change. *Chromatium* chromatophores, 17 mμmoles of bacteriochlorophyll/1.85 ml; 5-mm path length; ΔA was measured at 625–650 mμ; 50 mM KCl, pH 7.3; bromothymol blue 17 μM; temperature 26°. The extent of $[H^+]$ change in the absence of dianemycin was taken as unity.

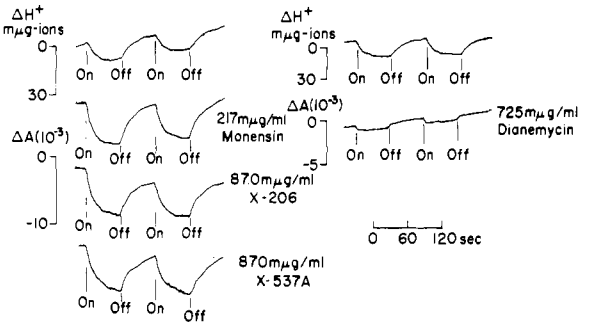

FIGURE 11: Effects of monensin, X-206, X-537A, and dianemycin on light-induced bromothymol blue change. *Chromatium* chromatophores; 50 mM KCl, pH 7.3. Left-side traces: 26 mμmoles of bacteriochlorophyll/1.85 ml; bromothymol blue 17 μM; temperature 26°. Right-side trace: 17 mμmoles of bacteriochlorophyll/1.825 ml; bromothymol blue 8.8 μM; temperature 28°; 5-mm path length; ΔA at 625–650 mμ. The ΔH^+ and ΔA calibrations apply to all traces in each of right-side and left-side sets. A downward deflection corresponds to an apparent internal acidification.

antibiotics were less inhibitory to the light-induced external alkalinization under nonphosphorylating conditions. In the case of the phosphorylation-associated external alkalinization, monensin and X-537A had no effect, while X-206 was only slightly inhibitory (Figure 12). The lack of inhibition of the phosphorylating system is similar to the results obtained with nigericin and valinomycin.

Examples of the traces showing the actions of monensin, X-206, and X-537A on the light-induced apparent internal acidification in KCl are presented in Figure 11. This response was increased by all these antibiotics.

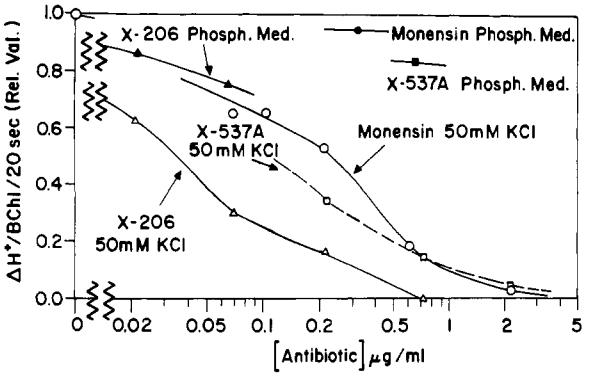

FIGURE 12: Concentration dependence of effects of monensin, X-206, and X-537A on light-induced external pH changes. Nonphosphorylating system: *Chromatium* chromatophores, 66 mμmoles of bacteriochlorophyll/4.6 ml; 50 mM KCl, pH 7.3; temperature 26°. Phosphorylating system: *Chromatium* chromatophores, 45 mμmoles of bacteriochlorophyll/4.6 ml; phosphorylating medium, pH 7.8; temperature 27°. The rate of $[H^+]$ change in the absence of antibiotic in each reaction system was taken as unity.

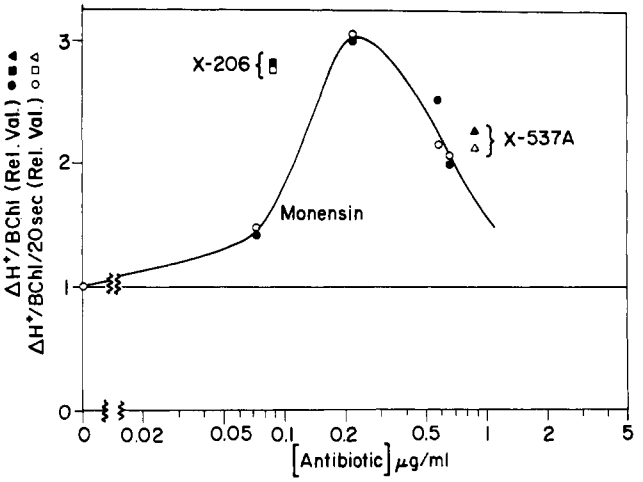

FIGURE 13: Concentration dependence of effects of monensin, X-206, and X-537A upon the light-induced bromothymol blue changes. *Chromatium* chromatophores, 26 mμmoles of bacteriochlorophyll/1.85 ml; 50 mM KCl, pH 7.3; bromothymol blue 17 μM; 5-mm path length; ΔA at 625–650 mμ; temperature 26°. The rates of light-induced apparent internal acidification are indicated by the open symbols; the extent of light-induced bromothymol blue changes are indicated by the closed symbols. The rate or amount of bromothymol blue change in the absence of antibiotic was taken as unity.

The concentration dependence of this effect was studied most extensively with monensin. The concentration range where the acceleration of the bromothymol blue response was observed was rather narrow (0.1–0.5 μg/ml) (Figure 13). X-206 and X-537A produced similar effects in the same concentration range, a much higher one than that required in the case of nigericin.

These inhibitors have been collectively referred to as the nigericin-type ionophorous agents since they all possess ionizable carboxyls and form lipid-soluble zwitterionic lipid-soluble complexes with alkali ions (Pressman, 1968). In the experiments reported here they all exert similar effects on the internal and external $[H^+]$ changes, differing only quantitatively, but not qualitatively.

Effects of Ionophorous Antibiotics on the Light-Induced Absorbance Changes in Chromatophores. Neither valinomycin nor nigericin affected the light-induced absorbance changes (434 and 603 mμ) of *R. rubrum* chromatophores in terms of steady-state absorbance change and half-decay time of the "light-off" reactions. Rather high concentrations of antibiotics (310 mμg of valinomycin/ml or 913 mμg of nigericin/ml) had no influence on the absorbance changes under various reaction conditions (phosphorylating medium, pH 7.8; phosphorylating medium plus 87 mM KCl, pH 7.8; 50 mM KCl, pH 6.5). However, with *Chromatium* chromatophores, the addition of ionophorous antibiotics induced a marked change in the kinetics of light-induced cytochrome reactions (changes in the steady-state level and rate of the "light-off" reaction). Detailed analyses of the effects of antibiotics on the electron transfer will appear elsewhere.

Discussion

If the transmembrane electrochemical H^+ gradient of photosynthetic particles is linked to their energy reserves, provided permeability is not limiting, perturbation by light would produce a translocation of H^+. The light-induced extraparticulate alkalinization sensed by the glass electrode has been interpreted in this fashion by several investigators (*cf.* review of Jagendorf and Uribe, 1966a).

Quantitatively, the molar free energy in calories, ΔF, required to move H^+ against an electrochemical gradient is given by the expression

$$\Delta F = 2.3RT\left(\Delta\text{pH} + \frac{\Delta E}{59}\right) \qquad (1)$$

where R is the gas constant, T the absolute temperature, ΔpH the transmembrane pH gradient, and ΔE the transmembrane potential in millivolts, both the latter assumed opposing H^+ translocation. Thus the electrochemical H^+ gradient has two independently variable components, a chemical concentration term, ΔpH, and an electropotential term, ΔE. The light-induced H^+ translocation would progress until the combined ΔpH and ΔE terms attain the limit permitted by the light-induced driving force, ΔF.

Accentuation by valinomycin of the external alkalinization of photosynthetic particles can be related to the known ability of this agent to carry cations across membranes as charged complexes (Pressman *et al.*, 1967). Valinomycin and related compounds thus permit appropriate cations such as K^+ to move across the membrane electrophoretically thereby discharging the membrane potential. If the ΔF term remained constant, reduction of the ΔE term would permit the ΔpH term to rise thereby permitting an additional translocation of H^+.

A priori it is conceivable that H^+ translocation is electrophoretically driven by a membrane potential created by a more primary light-induced reaction. If

this were true, however, discharge of the membrane potential by valinomycin plus K^+ would be expected to reduce H^+ movement, hence the observed data support the electrogenic rather than the electrophoretic character of H^+ translocation.

The requirement for external K^+ for accentuation by valinomycin of the light-induced H^+ translocation (Figure 6) also deserves comment. In order to facilitate H^+ uptake, the movement of K^+ must be directed outward from the particles. It thus appears that the role of the extraparticulate K^+ is to diffuse into the particles and raise the internal K^+ level spontaneously during the equilibration period prior to illumination. The relatively large concentrations of K^+ (*ca.* 10^{-2} M) preclude its translocation of the magnitudes of the changes in H^+ gradient (*ca.* 10^{-8} M) from producing any correspondingly significant alteration of the K^+ gradient.

In a previous report from this laboratory (Nishimura *et al.*, 1968) it was shown that the spectral changes in intraparticulate bromothymol blue correlate with the expected kinetics and extent of formation of a high-energy intermediate. The bromothymol blue changes were assumed, as in the case of mitochondria (Chance and Mela, 1966) to arise from intraparticulate pH changes. If the light-induced reaction is truly a translocation of H^+ (or OH^-) between the intra- and extraparticulate phases of a closed system, then any ΔH^+ in one phase must generate a ΔH^+ in the opposite direction in the second phase. Difficulty in estimating the effective buffer capacity of the intraparticulate phase precludes a rigorous evaluation of the precise ΔpH expected for internal phase corresponding to a given external alkalinization.

In the present report, however, a condition has been found in which the internal bromothymol blue changes in *Chromatium* chromatophores, as calibrated by the addition of external acid or alkali, are in the same direction as the extraparticulate H^+ shift as sensed by the glass electrode (*cf.* Figure 2, top right trace, and Figure 5, bottom left trace). Since calibration of the glass electrode measurements is more direct, these measurements are regarded as more reliable than the bromothymol blue data regarding ΔpH. Accordingly, the dominant factor determining the bromothymol blue response of valinomycin-treated *Chromatium* chromatophores is probably *not* a simple function of the intraparticulate pH. The indicator may be in equilibrium with the pH of a subcompartment or membrane phase of the particle, but may alternatively be responding to some other physical parameter such as the membrane potential as suggested by previous investigators (Cost and Frankel, 1967, 1968; Mitchell *et al.*, 1968).

The effects of nigericin and related ionophores on the light-induced alkalinization and intraparticulate bromothymol blue changes are opposite to those produced by valinomycin; extraparticulate alkalinization is reduced and the bromothymol blue changes are accentuated. This class of ionophores carry H^+ across membranes as undissociated carboxylic acids, and alkali ions as zwitterions devoid of net charge (Pressman *et al.*, 1967). Since the transported forms of H^+ and cations are both electrically neutral, their passage across membranes is neither influenced by a potential, nor are they capable of discharging any existent membrane potential. The relationship governing the ion equilibrations catalyzed by nigericin-type ionophores is (Pressman, 1968)

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{[M^+]_{in}}{[M^+]_{out}} \qquad (2)$$

When relatively large and equivalent concentrations of a cation M^+, such as K^+, are present on both sides of the membrane and equilibrate with the transmembrane H^+ gradient, the H^+ gradient would be dissipated removing the ΔpH term of eq 1. This is consistent with the observed reduction of the external alkalinization reaction by nigericin (*cf.* Figures 1 and 2). Again a paradox arises as the reduction of H^+ translocation reflected by extraparticulate alkalinization is accompanied by an *increased* bromothymol blue response (*cf.* Figures 4 and 5). This can be resolved if a significant component of the bromothymol blue response is due to the membrane potential as suggested above. If the light-induced driving force, ΔF, is sustained, reduction of the pH term by nigericin *via* eq 2 would permit the ΔE term of eq 1 to rise to the limits set by the light-induced driving force, ΔF. This would lead to a corresponding shift of the bromothymol blue spectrum. Thus valinomycin and nigericin added separately act to shift the dominant component of the electrochemical H^+ gradient to the chemical concentration term, ΔpH, or the electropotential term, ΔE, respectively.

Although bromothymol blue may not be in strict equilibrium with the pH of the bulk of the intraparticulate space and may partially reflect alterations in membrane potential, intraparticulate compartmentation or other physical properties of the system, it does appear to reflect the energetic state of the particles. The ability of bromothymol blue measurements to respond to rapid kinetics and their usual but not universal opposing correlation with the extraparticulate ΔH^+, render this indicator an interesting dynamic probe of photosynthetic processes.

Nigericin, but not valinomycin has been reported to inhibit photophosphorylation by chloroplasts (Shavit *et al.*, 1968a,b; Karlish and Avron, 1968). Within the context of the above discussion it appears likely that the dominant component of the transmembrane electrochemical H^+ gradient in chloroplasts is the chemical concentration or pH term which is susceptible to dissipation by nigericin. By the same token, the uncoupling action of valinomycin plus K^+ on mitochondria (Moore and Pressman, 1964) suggests that in these organelles the electropotential term predominates. The inhibitory action of nigericin on mitochondria is one of restricting substrate permeation rather than an energy-dissipating effect (Graven *et al.*, 1966; Pressman *et al.*, 1967).

The effects of valinomycin, nigericin, and other ionophorous antibiotics on the light-induced internal $[H^+]$ changes of *Chromatium* chromatophores are more marked than those of *R. rubrum* chromatophores. This is due in part to the fact that light-induced change in bacteriochlorophyll, which interferes in the spectral region used to monitor bromothymol blue, is more

extensive in *R. rubrum* chromatophores than in those of *Chromatium*. The actual reversal of direction by valinomycin of the internal bromothymol blue change in *Chromatium* chromatophores upon illumination is especially dramatic.

The maximum fluxes of energy in chromatophores for light-induced nonphosphorylating ion transport and phosphorylation differ in magnitude, the latter being much larger, as judged by the maximum amount of equivalent H^+ change under the respective reaction conditions (*cf.* Figure 1 of Nishimura *et al.*, 1968). If the nonphosphorylating pH shift is measured at the optimal pH used in the phosphorylation system, the discrepancy between their respective rates is even greater. This would explain why the energy-dissipating fluxes set up by nigericin and K^+ do not inhibit phosphorylation extensively. The relative maximum fluxes of energy which can be diverted into energy-dissipating ion fluxes may be greater in chloroplasts than in chromatophores. In this case the larger energy loss caused by the collapse of the pH gradient by nigericin-induced ion flux could compete more effectively with phosphorylation for a common energized intermediate. This would also account for the inability of valinomycin and K^+ plus 2,4-dinitrophenol to uncouple photophosphorylation with chromatophores. In the case of chloroplasts, the drainage of high-energy intermediates through the work of cyclic ion translocation (Moore and Pressman, 1964) accelerated by 2,4-dinitrophenol was suggested (Karlish and Avron 1968). But in the chromatophores, the maximum energy dissipation through ion transport is much smaller than the rate of energy production utilizable for phosphorylation as indicated by the present and previous papers (Shavit *et al.*, 1968c; Nishimura *et al.*, 1968).

Although the capacity for H^+ translocation is associated with several phosphorylating systems, chromatophores, chloroplasts, and mitochondria, this does not establish that a transport process is the *serial* precursor of phosphorylation. In the case of chromatophores, from the comparison of the rates and time ranges of the consecutive reactions in energy transduction (Nishimura *et al.*, 1968) the differential effects of nigericin on ion transport and phosphorylation (Shavit *et al.*, 1968c, and present work), and effects of ionophorous antibiotics on the bromothymol blue changes, it appears that the observed H^+ changes are energetically parallel to phosphorylation and do not represent an energized intermediate obligatory for driving phosphorylation.

The demonstration of "alkali bath" phosphorylation in chloroplasts (Jagendorf and Uribe, 1966b) also supports a close linkage between ion translocation and phosphorylation. But the interaction of the two processes most probably takes place through a common pool of energized intermediate. In this regard it is relevant to point out that electroneutrality requires counterions to accompany any net H^+ or OH^- translocation which occurs during the "alkali bath" synthesis of ATP by chloroplasts. Thus the driving force for ATP formation might be the movement of ions other than H^+ or OH^- and hence the latter experiments may be more analogous to the formation of ATP in mitochondria at a fixed pH by a cation gradient (Cockrell *et al.*, 1967) than has been considered. A thermodynamic analysis of the conditions of the "alkali bath" synthesis of ATP also indicates a requirement for the translocation of more than two H^+ for each ATP synthesized (Cockrell *et al.*, 1966). This is inconsistent with the most widely considered mechanism for serial linkage of H^+ transport and ATP synthesis, namely, the chemiosmotic hypothesis of Mitchell (1966).

The apparent response of bromothymol blue to the particulate membrane is reminiscent of the explanation offered by Junge and Witt (1968) for the light-induced absorbance changes in chloroplasts. The decay of this effect, measured at 515 mμ, is accelerated by the valinomycin-type ionophore, gramicidin D, which is consistent with the present finding that valinomycin itself reduces the bromothymol blue changes. If the predicted effects of valinomycin-type ionophores on the membrane potential developed in the present paper are valid, it also explains the Junge and Witt observation in terms of a gramicidin-accentuated collapse of the membrane potential. It should be pointed out that the time course of the 515-mμ absorbance shift (rise time <20 μsec) is considerably faster than the bromothymol blue changes (rise time 0.1–1 sec after pulse), and hence even if both responses arise from a common origin, *i.e.*, the membrane potential, they are probably not mediated by the same physical process. The membrane potential may operate on the bromothymol blue by causing it to migrate from one locus to another and the corresponding changes in its environment shifting its p*K*. Natural chromophores such as carotenoids would be expected to be integral components of membranes and not subject to such relatively slow processes as intracompartmental migration. No simple explanation can be offered at present for the precise nature of the rapid 515-mμ changes.

Dilley and Shavit (1968) studied the relationship between light-induced ATP formation and the dark decay of the pH gradient in spinach chloroplasts. They found that rapid ATP synthesis decreased the rate and extent of the dark H^+ efflux, but not the initial rate of H^+ uptake. This was interpreted as indicating that the esterification of ADP to ATP (with a stoichiometric consumption of H^+) occurs in a compartment in contact with that acidified by the light-driven H^+ uptake, and that the proton pump is an *obligatory* part of the ATP-forming mechanism rather than that H^+ transport is an energy utilization *alternative* to photophosphorylation. The present data obtained with *R. rubrum* and *Chromatium* chromatophores are consistent with the first point (*cf.* Nishimura *et al.*, 1962, 1968), but the competition between H^+ transport and phosphorylation is clearly shown in the kinetics and extent of the internal and external $[H^+]$ changes and the effects of ionophorous antibiotics on these processes. It appears easier to demonstrate unequivocally the parallel energy utilization of a common intermediate by phosphorylation and transport in bacterial chromatophores than in chloroplasts. Not only are the ionophorous agents in-

capable of uncoupling photophosphorylation in chromatophores, but also their charge separation rates, as indicated by ion movements, cannot account for the observed phosphorylation rates according to the chemiosmotic hypothesis. It is of interest to note that the converse obtains in mitochondria where the ATP-supported valinomycin-induced K^+ transport is greater than can be accounted for by the chemiosmotic hypothesis (Cockrell *et al.*, 1966).

In a recent paper, McCarty (1968) showed that in subchloroplast particles NH_4Cl abolished the light-induced pH rise at concentrations which had little effect on ATP synthesis. He concluded that the pH change observed on illumination is not the driving force for ATP synthesis in subchloroplast particles. The difference of the maximum energy fluxes used for ion transport in chloroplasts and chromatophores has been discussed already. Current experiments on relaxation processes in electron and ion transport in our laboratory using flash illumination (*cf.* Geller, 1967), and application of these methods to record rapid H^+ changes (Izawa and Hind, 1967; Schwartz, 1968) should prove useful for the further clarification of the mechanism of energy transfer in photosynthetic systems.

The control of electron flow in the oxidative pathways of mitochondria by phosphorylation has been extensively studied (Lardy and Wellman, 1952). The rate of electron flow in the dark phase of photosynthesis is also controlled, in part, by the energetic level of the chromatophore membrane systems. Energy-driven NAD reduction and energy-linked transhydrogenase have been demonstrated in *R. rubrum* (Keister and Yike, 1966, 1967) and the dependence of electron transfer upon the coupling to phosphorylation or ion transport has also been shown (Nishimura *et al.*, 1968; Geller, 1967). Experiments in progress in this laboratory indicate that the rate of cytochrome reduction following illumination can be dependent upon the coupling of electron transfer to ion transport. Further studies on the nature of energetic coupling of electron transfer, ion transport, and phosphorylation are being carried out. The effects of ionophorous antibiotics on the utilization of energy by nonphosphorylating and phosphorylating photosynthetic systems under flash illumination will also be described in a subsequent communication.

Acknowledgment

The authors thank Dr. Britton Chance for his stimulating discussion. Technical assistance of Miss Kiyoko Kadota and Miss Reiko Fugono is gratefully acknowledged.

Addendum

Following the submission of this manuscript, a paper covering related work on *R. rubrum* chromatophores came to our attention (Jackson *et al.*, 1968; Thore *et al.*, 1968). A most significant discrepancy between the papers is our consistent inability to observe appreciable synergistic effects of valinomycin plus nigericin, or valinomycin plus uncoupler on chromatophore preparations comparable with those found with chloroplasts and mitochondria.

References

Chance, B., and Mela, L. (1966), *J. Biol. Chem. 241*, 4588.

Chance, B., Nishimura, M., Avron, M., and Baltscheffsky, M. (1966), *Arch. Biochem. Biophys. 117*, 158.

Cockrell, R. S., Harris, E. J., and Pressman, B. C. (1966), *Biochemistry 5*, 2326.

Cockrell, R. S., Harris, E. J., and Pressman, B. C. (1967), *Nature 215*, 1487.

Cost, K., and Frankel, A. W. (1967), *Biochemistry 6*, 663.

Cost, K., and Frankel, A. W. (1968), *in* Comparative Biochemistry and Biophysics of Photosynthesis, Shibata, K., Takamiya, A., Jagendorf, A. T., and Fuller, R. C., Ed., Tokyo, University of Tokyo, p 266.

Dilley, R. A., and Shavit, N. (1968), *Biochim. Biophys. Acta 162*, 86.

Geller, D. M. (1967), *J. Biol. Chem. 242*, 40.

Graven, S. N., Estrada-O., S., and Lardy, H. A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 654.

Harris, E. J., Catlin, G., and Pressman, B. C. (1967), *Biochemistry 6*, 1360.

Harris, E. J., and Pressman, B. C. (1967), *Nature 216*, 918.

Izawa, S., and Hind, G. (1967), *Biochim. Biophys. Acta 143*, 377.

Jackson, J. B., Crofts, A. R., and von Stedingk, L.-V. (1968), *European J. Biochem. 6*, 41.

Jagendorf, A. T., and Uribe, E. (1966a), *Brookhaven Symp. Biol. 19*, 215.

Jagendorf, A. T., and Uribe, E. (1966b), *Proc. Natl. Acad. Sci. U. S. 55*, 170.

Junge, W., and Witt, H. T. (1968), *Z. Naturforsch. 23b*, 244.

Karlish, S. J. D., and Avron, M. (1968), *FEBS Letters 1*, 21.

Keister, D. L., and Yike, N. J. (1966), *Biochem. Biophys. Res. Commun. 24*, 519.

Keister, D. L., and Yike, N. J. (1967), *Arch. Biochem. Biophys. 121*, 415.

Lardy, H. A., and Wellman, H. (1952), *J. Biol. Chem. 195*, 215.

McCarty, R. E. (1968), *Biochem. Biophys. Res. Commun. 32*, 37.

Mitchell, P. (1966), Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Cornwall, England, Glynn Research Ltd.

Mitchell, P., Moyle, J., and Smith, L. (1968), *European J. Biochem. 4*, 9.

Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.

Mueller, P., and Rudin, D. O. (1967), *Biochem. Biophys. Res. Commun. 26*, 398.

Nishimura, M. (1962), *Biochim. Biophys. Acta 57*, 88.

Nishimura, M., and Chance, B. (1963), *Biochim. Biophys. Acta 66*, 1.

Nishimura, M., Ito, T., and Chance, B. (1962), *Biochim. Biophys. Acta 59*, 177.
Nishimura, M., Kadota, K., and Chance, B. (1968), *Arch. Biochem. Biophys. 125*, 308.
Packer, L. (1967), *Biochem. Biophys. Res. Commun. 28*, 1022.
Pressman, B. C. (1968), *Federation Proc. 27*, 1283.
Pressman, B. C., Harris, E. J., Jagger, W. S., and Johnson, J. H. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1949.
Schwartz, M. (1968), *Nature 219*, 915.
Shavit, N., Dilley, R. A., and San Pietro, A. (1968a), *in* Comparative Biochemistry and Biophysics of Photosynthesis, Shibata, K., Takamiya, A., Jagendorf, A. T., and Fuller, R. C., Ed., Tokyo, University of Tokyo, p 253.
Shavit, N., Dilley, R. A., and San Pietro, A. (1968b), *Biochemistry 7*, 2356.
Shavit, N., and San Pietro, A. (1967), *Biochem. Biophys. Res. Commun. 28*, 277.
Shavit, N., Thore, A., Keister, D. L., and San Pietro, A. (1968c), *Proc. Natl. Acad. Sci. U. S. 59*, 917.
Thore, A., Keister, D. L., Shavit, N., and San Pietro, A. (1968), *Biochemistry 7*, 3499.
von Stedingk, L.-V., and Baltscheffsky, H. (1966), *Arch. Biochem. Biophys. 117*, 400.

A Naturally Occurring Indolylpteridine*

Walter S. McNutt and Isao Takeda

ABSTRACT: A mutant strain of *Achromobacter petrophilum* produces a green-yellow fluorescent compound when grown in a medium containing an excess of guanine. The efficient incorporation of ^{14}C from [2-^{14}C]guanine (but not from [8-^{14}C]guanine) into the unknown substance suggested that it might be a pteridine. The substance, crystallized from 4 N HCl, gave dark red needles with a neutralization equivalent of 465 and the analysis $C_{19}H_{21}N_5O_7 \cdot 3H_2O$. It consumed 3 moles of periodate/mole of compound and was converted upon reduction with Zn and HCl into a blue fluorescent compound and indole. The blue fluorescent compound was characterized as 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine. The structure of the green-yellow fluorescent compound was established as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine by chemical synthesis. The substance differs from naturally occurring pteridines hitherto known in having an indolyl group on the 6 position of the pteridine ring.

Takeda and Hayakawa (1968) obtained a purine-requiring mutant of *Achromobacter petrophilum*, which produces a green-yellow fluorescent compound when cultured in the presence of excess guanine.

In this article evidence is presented to show that Takeda's green-yellow fluorescent compound is another in the class of naturally occurring ribityllumazines (Masuda, 1956; Masuda *et al.*, 1958; McNutt, 1960). It differs from the naturally occurring pteridines previously known in having a 3-indolyl group at position 6 of the pteridine. Its structure has been established as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (I).

Results

The ^{14}C of [2-^{14}C]guanine, but not [8-^{14}C]guanine, was effectively incorporated into the green-yellow fluorescent compound by cultures of the organism (Figure 1), suggesting that the compound was probably a pteridine.

The neutralization equivalent (465; see Experimental Section) showed the presence of a large group or groups on the pteridine ring, and the periodate consumption; 3 moles of periodate/mole of compound (see Experimental Section) was consistent with a ribityl group.

The substance analyzed, $C_{19}H_{21}N_5O_7 \cdot 3H_2O$, and as hitherto known ribitylpteridines are lumazines it seemed likely that one of the nitrogen atoms was in a side chain. Also, the ultraviolet absorption spectrum showed a marked bathochromic shift in 4 N HCl (Figure 2, B *vs.* C) unlike that expected of a 2-amino-4-hydroxypteridine, and this indicated a weakly basic nitrogen atom in the side chain in conjugation with the pteridine ring.

The substance was readily reduced by Zn and HCl, giving rise to a blue fluorescent compound having ultraviolet absorption spectra (Figure 3) similar to those of 6-methyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (III) (McNutt, 1960). The structure of the blue fluorescent compound was established as 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (II) by

* From the Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111. *Received December 9, 1968.* We are indebted to the National Science Foundation (Grant GB-4961) for financial support of this work and for Dr. Takeda's salary while on leave from the Asahi Chemical Co., Tokyo, Japan.