BBA 46002

DIFFERENCES IN SENSITIVITY TO VALINOMYCIN AND NONACTIN OF VARIOUS PHOTOPHOSPHORYLATING AND PHOTOREDUCING SYSTEMS OF *RHODOSPIRILLUM RUBRUM* CHROMATPOHORES

Z. GROMET-ELHANAN

Department of Biochemistry, Weizmann Institute of Science, Rehovot (Israel)

(Received April 20th, 1970)

SUMMARY

1. The effect of valinomycin and nonactin on photophosphorylation and photoreduction by *Rhodospirillum rubrum* chromatophores was tested in the presence of various electron carriers and donors, which differed in their sensitivity to hydroxyquinoline *N*-oxide.

2. The phosphorylating system with *N*-methylphenazonium methosulphate was resistant to both antibiotics and to hydroxyquinoline *N*-oxide. All the other phosphorylating systems tested were inhibited far beyond the 50 % level by valinomycin and nonactin. No correlation was observed between the sensitivity to hydroxyquinoline *N*-oxide and to valinomycin or nonactin.

3. Both antibiotics inhibited NAD^+ photoreduction to the same extent as photophosphorylation. The addition of NH_4Cl resulted in an increased inhibition of both photoreactions.

4. The sensitivity, as well as the resistance to valinomycin and nonactin, was independent of the presence of transportable ions such as K^+.

5. According to these findings the inhibition by valinomycin and nonactin cannot be an inhibition of energy transfer or an uncoupling due to their ion-transporting activity. It is, however, impossible in these systems to resolve the question whether they are electron transport inhibitors or uncouplers in a way not related to their ion-transporting ability.

INTRODUCTION

BALTSCHEFFSKY AND ARWIDSSON[1] found that valinomycin at $3 \cdot 10^{-8}$ M inhibited ATP formation in the presence of succinate in *Rhodospirillum rubrum* chromatophores up to 50 %, and the titration curve then levelled off so that an increase in the valinomycin concentration of about 50-fold gave only a small additional effect. This system was also completely inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO)[2]. However, the HQNO-resistant *N*-methylphenazonium methosulphate (PMS)-catalysed phosphorylation was not inhibited at all by valinomycin

Abbreviations: DCIP, dichlorophenol indophenol; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; PMS, *N*-methylphenazonium methosulphate.

even at $2 \cdot 10^{-6}$ M. BALTSCHEFFSKY AND ARWIDSSON[1] therefore suggested that there are two sites of phosphorylation in the cyclic electron transport chain in these chromatophores: one site, which is sensitive to valinomycin and is located on that part of the electron transport chain that is inhibited by HQNO, and another site that is resistant to valinomycin and is located on the HQNO resistant part of the electron transport chain. In the PMS bypass only the site that is resistant to both valinomycin and HQNO participates in the photophosphorylation.

The resistance of the PMS system to valinomycin has been confirmed[3,4] and even extended to up to $1 \cdot 10^{-5}$M valinomycin[4]. With the succinate system however, controversial results have been reported[4,5]. Thus, THORE *et al.*[5] found no inhibition at $3 \cdot 10^{-8}$ M valinomycin, but at higher valinomycin concentrations they observed an increasing inhibition with no plateau, resulting in up to 82 % inhibition at $3 \cdot 10^{-5}$ M valinomycin. JACKSON AND CROFTS[4] on the other hand obtained a plateau at the high valinomycin concentrations, but at no more than 20 % inhibition.

A complete inhibition of photophosphorylation with either succinate[4,5] or PMS[4] was observed when nigericin (which by itself had no inhibitory effect[6]) was added together with valinomycin. In fact any combination of a nigericin type antibiotic with a valinomycin type antibiotic resulted in inhibition of phosphorylation[5]. This type of inhibition required the presence of K^+ and was interpreted as an uncoupling attributed to the ion-transporting activity of the two types of antibiotic[4,5]. We found a similar inhibition of PMS-catalysed phosphorylation when various ammonium salts were added together with valinomycin type antibiotics[7]. In these conditions K^+ was not required, but the inhibition could also be explained as an uncoupling due to the enhanced permeability of NH_4^+ in the presence of the antibiotics[7].

Since the inhibition by valinomycin alone in the succinate system was independent of transportable ions[4], it might be due to a different type of inhibition. In the light of this observation and the controversial results obtained with valinomycin alone[1,4,5], the effect of valinomycin type antibiotics was re-examined using various phosphorylating systems, which showed different sensitivities towards HQNO[8]. The action of these antibiotics on NAD^+ reduction with different electron donors was also tested as a means of checking their possible uncoupling activity, since uncouplers were found to inhibit NAD^+ reduction in *R. rubrum* chromatophores[8,9]. The effect of valinomycin type antibiotics in combination with ammonium salts on NAD^+ reduction was also examined.

MATERIALS AND METHODS

R. rubrum cells were grown as previously described[7]. Harvested cells were washed once in 0.1 M Tricine–0.25 M sucrose (pH 7.5) and broken under argon with the "Yeda Press" (Yeda Research and Development Co. Ltd., Rehovot) developed by SHNEYOUR AND AVRON[10]. Chromatophores were isolated from the broken cell suspension as described earlier[8], except that they were washed and finally suspended in 0.25 M sucrose instead of Tris–sucrose. In some experiments (see text) the chromatophores were washed and suspended in 0.125 M KCl or 0.125 M choline chloride instead of sucrose. Bacteriochlorophyll was determined using the extinction coefficient *in vivo* given by CLAYTON[11].

Experiments under air were carried out in test-tubes in a water-bath illuminated from both sides by 50 000 lux of white light at 25°. Experiments under argon were run in a Warburg bath as previously described[8].

The reaction mixture for photophosphorylation contained the following components in a total volume of 3 ml: Tricine–NaOH buffer (pH 8.0), 30 mM; $MgCl_2$, 2.66 mM; ADP, 1.66 mM; sodium phosphate, 3.33 mM (containing $2 \cdot 10^6$–$5 \cdot 10^6$ counts/min ^{32}P); chromatophores, containing 15–30 μg bacteriochlorophyll and, when indicated, PMS, 66 μM; succinate, 0.33 mM; ascorbate, 3.3 mM; and dichlorophenol indophenol (DCIP), 0.13 mM. Other additions are stated in the text. The reaction mixture for NAD^+ photoreduction contained in addition 0.33 mM NAD^+ and 1 mg of human serum albumin. When used as electron donor for NAD^+ reduction the concentration of succinate was 3.3 mM.

NAD^+ reduction was assayed as described by GROMET-ELHANAN[8], using the method developed by BEN-HAYYIM *et al.*[12] to preserve the reduced pyridine nucleotide. ATP formation was measured according to AVRON[13]. Diaminodurol was prepared fresh every day. It was dissolved in water by heating for 1 min at 60°, and then slowly cooled to room temperature.

HQNO was purchased from Sigma, valinomycin from CalBiochem and nonactin from CIBA. Diaminodurol was generously given by Dr. A. Trebst.

RESULTS

Inhibition by HQNO of various photophosphorylating systems

Different phosphorylating systems have been reported to differ in their sensitivity towards HQNO[2,8]. According to their sensitivity these systems could be arranged in three groups: (a) the resistant group, which includes only the PMS system (95% of the control at $2 \cdot 10^{-6}$ M HQNO); (b) the sensitive group, which includes the endogenous as well as the ascorbate and succinate systems (less than 10% of the control at $2 \cdot 10^{-6}$ M HQNO); and (c) the intermediate group of the ascorbate–DCIP system (60% of the control at $2 \cdot 10^{-6}$ M)[8]. As can be seen in Fig. 1, the ascorbate–diaminodurol system also belongs in the intermediate sensitivity group. This system was tested here since it was reported to serve as an electron donor to NAD^+ in *R. rubrum* chromatophores[14] and was even the best electron donor in these chromatophores[15].

Diaminodurol, unlike DCIP, was also found to catalyse phosphorylation when added in the absence of ascorbate both in chloroplasts[16] and in chromatophores (Fig. 2). In these conditions the rate of phosphorylation increased with increasing diaminodurol concentration and was almost similar under air or argon. At the highest diaminodurol concentration tested the rate of phosphorylation approached that obtained with the PMS system. In the presence of ascorbate, ATP formation was not dependent on diaminodurol concentration and was inhibited under argon owing to overreduction as was earlier reported with DCIP[17]. The phosphorylation system with various concentrations of diaminodurol by itself was as sensitive to HQNO as the ascorbate–diaminodurol system (Table I and Fig. 1).

The effect of valinomycin on photophosphorylating systems

Fig. 3 illustrates the effect of valinomycin on the various phosphorylating systems. The PMS system was resistant while all the other systems tested, namely,

succinate, ascorbate–DCIP, diaminodurol, ascorbate–diaminodurol, ascorbate by itself and the endogenous system (the last three not shown here) were sensitive with 50 % inhibition at $3 \cdot 10^{-6}$–$4 \cdot 10^{-6}$ M valinomycin. There was no indication of

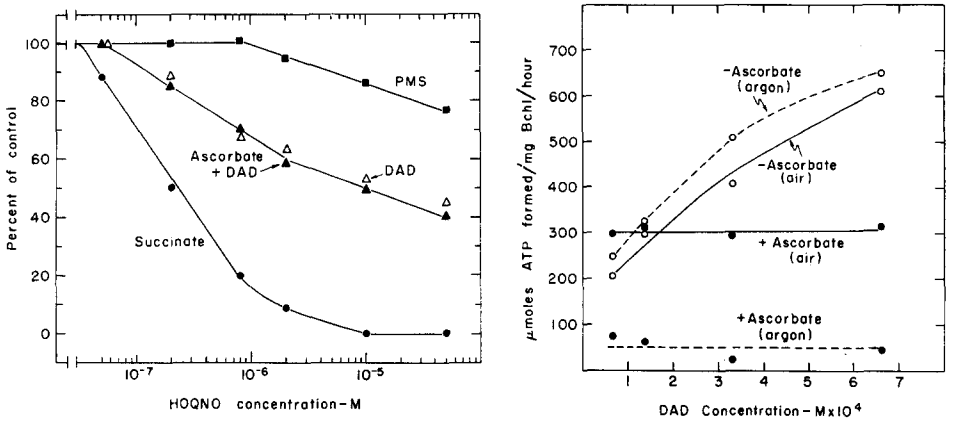


Fig. 1. Effect of HQNO on various photophosphorylating systems. All the reactions were run under air. The control values expressed in μmoles ATP/mg bacteriochlorophyll per h were as follows: ■ with PMS, 821; ▲ with ascorbate–diaminodurol (DAD) (0.13 mM), 272; △ with diaminodurol (DAD) (0.13 mM), 252; ● with succinate ,187.

Fig. 2. The dependence of photophosphorylation under air or argon on diaminodurol (DAD) concentration.

TABLE I

EFFECT OF HQNO AND VALINOMYCIN ON PHOTOPHOSPHORYLATION CATALYSED BY VARIOUS DIAMINODUROL CONCENTRATIONS

All the reactions were run under argon. When added, the concentration of HQNO was $2 \cdot 10^{-6}$ M, and that of valinomycin was $3 \cdot 10^{-6}$ M. Numbers in parentheses are per cent of the control in the absence of HQNO for the + HQNO series and in the presence of HQNO for the HQNO + valinomycin series.

Diaminodurol concn. (mM)	*Effect of added inhibitors on phosphorylation (μmoles ATP formed/mg bacteriochlorophyll per h)*		
	None	*HQNO*	*HQNO + valinomycin*
0.13	252	135 (54)	83 (61)
0.33	384	252 (66)	154 (61)
0.66	530	340 (64)	229 (67)

a plateau at any percentage inhibition and no clear cut correlation with the sensitivity to HQNO. Thus, the systems that have an intermediate sensitivity to HQNO were as sensitive to valinomycin as the HQNO-sensitive succinate system. Moreover, the effect of valinomycin on these systems was the same in the absence or presence of HQNO (*cf.* Fig. 3 and Table I). As was observed with HQNO, all the tested diaminodurol concentrations exhibited the same sensitivity to valinomycin (Table I).

The observed inhibition of phosphorylation by valinomycin was independent

TABLE II

EFFECT OF K^+ CONCENTRATION ON THE INHIBITION BY VALINOMYCIN OF SUCCINATE-CATALYSED PHOTOPHOSPHORYLATION IN VARIOUS CHROMATOPHORE PREPARATIONS

All the reactions were run under air. When present, the concentration of valinomycin was $3 \cdot 10^{-6}$ M.

Additions	*Phosphorylation (μmoles ATP formed/mg bacteriochlorophyll per h) in chromatophores prepared in*		
	Sucrose	*KCl*	*Choline chloride*
15 mM KCl	156	168	170
15 mM KCl + valinomycin	75	89	85
250 mM KCl	129	119	123
250 mM KCl + valinomycin	63	58	62

TABLE III

EFFECT OF VALINOMYCIN ON SUCCINATE-LINKED PHOTOREACTIONS IN THE ABSENCE AND PRESENCE OF NH_4Cl

All the reactions were run under argon. The control values expressed in μmoles/mg bacteriochlorophyll per h were as follows: NAD^+ photoreduction (–ADP–P_i), 42; NAD^+ photoreduction (+ ADP + P_i), 22; photophosphorylation, 283.

Valinomycin concn. $\times 10^7$ *(M)*	*NH_4Cl concn. (mM)*	*Reaction measured (% of control)*		
		NAD^+ reduction (–ADP–P_i)	*NAD^+ reduction (+ ADP + P_i)*	*Phosphorylation*
1	–	104	105	105
10	–	75	86	89
30	–	50	64	58
100	–	27	36	34
–	6	109	112	101
10	6	46	48	54
30	6	20	24	22
100	6	4	6	3

of K^+, and did not change when assayed in the absence of K^+ in the reaction mixture (Fig. 3) or in the presence of 15 mM KCl or 250 mM KCl (Table II). The same effect of valinomycin was also obtained whether the chromatophores were prepared in sucrose, KCl or choline chloride (Table II).

The degree of inhibition by valinomycin alone is thus dependent only on the phosphorylating system. The PMS system was resistant under all the conditions tested, namely under air or argon, in the presence or absence of HQNO or KCl at concentrations between 0 and 250 mM. All the other systems tested were sensitive and showed the same sensitivity under all the above-mentioned conditions.

The effect of valinomycin alone and in combination with NH_4Cl on NAD^+ photoreduction

NAD^+ photoreduction in the absence or presence of the phosphorylating reagents as well as ATP formation in the system under argon were inhibited to about the same extent by valinomycin. An example with succinate as the electron donor is illustrated in Table III. Similar results were obtained also with ascorbate–diamino-

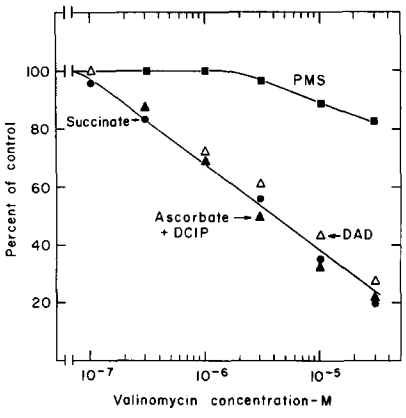


Fig. 3. Effect of valinomycin on various photophosphorylating systems. All the reactions were run under air. The control values were as follows: ■ with PMS, 703; ● with succinate, 170; ▲ with ascorbate–DCIP, 160; △ with 0.13 mM diaminodurol (DAD), 238.

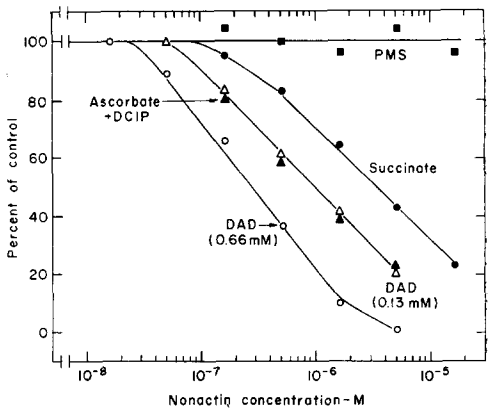


Fig. 4. Effect of nonactin on various photophosphorylating systems. All the reactions were run under air. The control values were as follows: ■ with PMS, 888; ● with succinate, 339; ▲ with ascorbate–DCIP, 232; △ with 0.13 mM diaminodurol (DAD), 289; O with 0.66 mM diaminodurol (DAD), 666.

durol as the electron donor. As in ATP formation in the absence of NAD^+, the inhibition was far beyond the 50% level, and addition of 100 mM KCl to the reaction mixture did not change the results.

Addition of NH_4Cl in the presence of valinomycin resulted in a synergistic inhibition of NAD^+ reduction as well as phosphorylation (Table III). However, owing to the sensitivity of this system to valinomycin alone, the synergism was less pronounced than in the phosphorylating system with PMS, where valinomycin by itself was much less effective[7].

The effect of nonactin alone and in combination with NH_4Cl on various photophosphorylating systems

Nonactin, which is an ion-transporting antibiotic of the valinomycin type[18,19], induced in the presence of NH_4Cl a synergistic inhibition of the PMS-catalysed phosphorylation which was even more pronounced than with valinomycin[7]. Therefore nonactin was tested by itself, as well as in the presence of NH_4Cl, on the various phosphorylating systems. As can be seen from Fig. 4, the PMS system was completely resistant to nonactin by itself, while all the other systems tested were sensitive. However, unlike the effect with valinomycin, the systems that were more resistant to HQNO than the succinate system (Fig. 1) were rather more sensitive to nonactin (Fig. 4 and Table IV). Furthermore, the sensitivity to nonactin of the phosphorylating system with diaminodurol increased with increasing diaminodurol concentrations. As was observed with valinomycin, the same degree of inhibition by nonactin was obtained in the absence or presence of HQNO, and K^+ was not required (Table IV).

Addition of NH_4Cl in the presence of nonactin resulted in a pronounced synergistic inhibition of all the phosphorylating systems tested including the PMS one (Fig. 5). In some experiments when diaminodurol was rapidly cooled and used

within less than 1 h after preparation it proved, when used by itself, to be rather resistant (like PMS) both to nonactin and to valinomycin. In these experiments the sensitivity to the antibiotics reappeared when the diaminodurol was used about 2 h after it had been prepared. The rate of phosphorylation was similar in both experiments. In the presence of ascorbate the system with diaminodurol was always sensitive.

The effect of nonactin on NAD$^+$ photoreduction

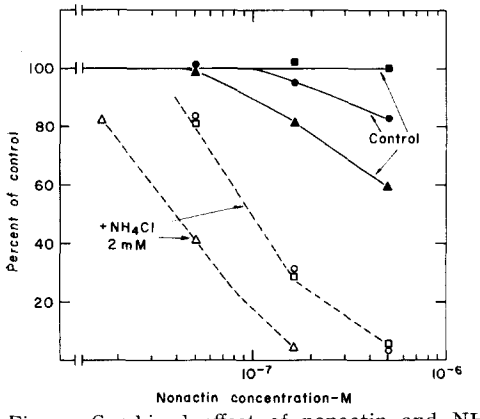
Nonactin, like valinomycin, inhibited NAD$^+$ reduction to the same extent as ATP formation (Fig. 6). The differences in sensitivity to nonactin of the various

TABLE IV

EFFECT OF K^+ CONCENTRATION AND HQNO ON THE INHIBITION BY NONACTIN OF VARIOUS PHOTOPHOSPHORYLATING SYSTEMS

All the reactions were run under air. The ascorbate–diaminodurol system contained 0.13 mM diaminodurol and no KCl. When present, the concentration of KCl was 100 mM and that of HQNO $2 \cdot 10^{-6}$ M. Control values were as follows: with succinate 356 and 334 without and with KCl, respectively; with ascorbate–diaminodurol, 292 and 180 without and with HQNO, respectively.

Nonactin concn. $\times 10^7$ (*M*)	*Phosphorylation (% of control) with*			
	Succinate		*Ascorbate–diaminodurol*	
	−KCl	*+KCl*	*−HQNO*	*+HQNO*
5	—	—	62	57
16	72	64	38	31
50	55	32	18	20
160	20	27	—	—



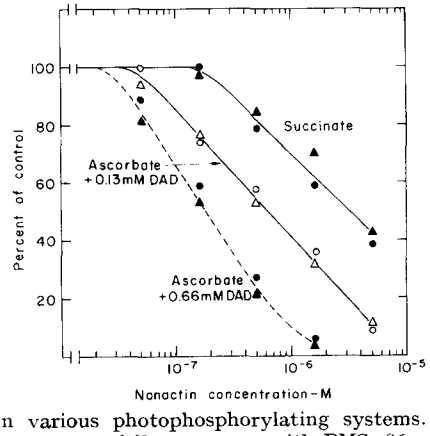


Fig. 5. Combined effect of nonactin and NH_4Cl on various photophosphorylating systems. All the reactions were run under air. The control values were as follows: ■, □ with PMS, 869; ●, ○ with succinate, 321; ▲, △ with ascorbate–diaminodurol (0.13 mM), 273.

Fig. 6. Effect of nonactin on NAD$^+$ photoreduction and ATP formation with various electron donors. All the reactions were run under argon. Circles designate NAD$^+$ reduction, and triangles ATP formation. The control values expressed in μmoles/mg bacteriochlorophyll per h were as follows: ●—●, ▲—▲ with succinate, 25 and 303, respectively; ○—○, △—△ with ascorbate–diaminodurol (DAD) (0.13 mM), 55 and 247, respectively; ●---●, ▲---▲ with ascorbate–diaminodurol (DAD) (0.66 mM), 58 and 240, respectively.

electron donors were obesrved here too. Succinate was less sensitive than ascorbate–diaminodurol, and the sensitivity of the last donor couple increased with increasing diaminodurol concentrations. The rate of NAD^+ reduction as well as of ATP formation (see also Fig. 2) in the presence of ascorbate and diaminodurol was, however, similar with all the diaminodurol concentrations tested.

Addition of NH_4Cl in the presence of nonactin resulted in a synergistic inhibition of NAD^+ reduction as well as phosphorylation.

DISCUSSION

Valinomycin and nonactin inhibited ATP formation by *R. rubrum* chromatophores when tested in the endogenous system as well as in the presence of succinate, ascorbate, ascorbate–DCIP, ascorbate–diaminodurol and diaminodurol. In the presence of PMS, ATP formation was resistant to both antibiotics.

The inhibition of phosphorylation by valinomycin and nonactin is different from the synergistic inhibition of phosphorylation which was obtained with these antibiotics in the presence of nigericin and KCl[4,5] or NH_4Cl[7] in two respects. (a) It was ineffective with PMS (Figs. 3 and 4), while the synergistic inhibition was as effective with PMS as with succinate (see refs. 5 and 7 and Fig. 5). (b) It was independent of the presence of transportable ions. The PMS system was resistant, and all the other systems were sensitive, whether KCl was absent or present in the chromatophore preparation or in the reaction mixture or in both (Tables II and IV). It is therefore concluded that the inhibition of phosphorylation by valinomycin and nonactin alone cannot be interpreted as an uncoupling due to their ion transporting activity.

Although the resistance of the PMS system to valinomycin, which was first reported by BALTSCHEFFSKY AND ARWIDSSON[1] was confirmed and extended also to nonactin, the results reported in this paper do not corroborate their suggestion that valinomycin inhibits only one out of two sites of phosphorylation in the cyclic electron transport in *R. rubrum* chromatophores. In contrast with their results with succinate and valinomycin[1], ATP formation in all the sensitive systems tested here was inhibited by valinomycin as well as by nonactin far beyond the 50% level with no indication of a plateau near 50% inhibition (Figs. 3 and 4 and Table IV). Furthermore, the sensitivity to valinomycin and nonactin could not be correlated with the sensitivity to HQNO. Thus, the systems with ascorbate–DCIP, ascorbate–diaminodurol and diaminodurol alone, which were relatively resistant to HQNO, were as sensitive to valinomycin as the succinate system (Fig. 3) and even more sensitive to nonactin (Fig. 4).

NAD^+ reduction was inhibited by valinomycin and nonactin to the same extent as phosphorylation when either succinate or ascorbate–diaminodurol served as the electron donor (Table III and Fig. 6). This parallel inhibition of the reduction and phosphorylation is different from the effect of the energy transfer inhibitor oligomycin, which was reported to stimulate NAD^+ reduction while inhibiting ATP formation in chromatophores[8,9]. It is, however, impossible with these assays to resolve the question whether the inhibition by valinomycin and nonactin is due to inhibition of the electron transport between the donors and NAD^+ or to uncoupling,

since uncouplers were also found to block NAD^+ reduction as well as phosphorylation in these chromatophores[8,9].

MCCARTY[20] has recently reported that valinomycin inhibited both ferricyanide reduction and the coupled phosphorylation in chloroplasts and subchloroplast particles. In these systems, however, this type of inhibition rather rules out an uncoupling effect[21]. Since the addition of NH_4Cl relieved the inhibition of the electron transport but further increased the inhibition of the phosphorylation[20], it seems that valinomycin by itself acts as an energy transfer inhibitor in these particles[21]. Thus, although the effect of valinomycin on electron transport and phosphorylation was similar in chloroplasts and chromatophores, this similarity does not indicate an analogous type of inhibition. Indeed, the addition of NH_4Cl in the presence of valinomycin resulted in opposite effetcs on the electron transport in the different particles.

A possible way of resolving the type of inhibition exerted by valinomycin and nonactin in the chromatophores, which is now under investigation, is the examination of their effect on the oxidation–reduction state of components of the cyclic electron transport.

ACKNOWLEDGEMENT

The capable technical assistance of Mrs. O. Kraus is gratefully acknowledged.

REFERENCES

1 H. BALTSCHEFFSKY AND B. ARWIDSSON, *Biochim. Biophys. Acta*, 65 (1962) 425.
2 H. BALTSCHEFFSKY AND M. BALTSCHEFFSKY, *Acta Chem. Scand.*, 12 (1958) 1333.
3 H. SATO, K. TAKAHASHI AND G. KIKUCHI, *Biochim. Biophys. Acta*, 112 (1966) 8.
4 J. B. JACKSON AND A. R. CROFTS, *European J. Biochem.*, 6 (1968) 41.
5 A. THORE, D. L. KEISTER, N. SHAVIT AND A. SAN PIETRO, *Biochemistry*, 7 (1968) 3499.
6 N. SHAVIT, A. THORE, D. L. KEISTER AND A. SAN PIETRO, *Proc. Natl. Acad. Sci. U. S.*, 59 (1968) 917.
7 S. BRILLER AND Z. GROMET-ELHANAN, *Biochim. Biophys. Acta*, 205 (1970) 263.
8 Z. GROMET-ELHANAN, *Arch. Biochem. Biophys.*, 131 (1969) 299.
9 D. L. KEISTER AND N. J. YIKE, *Arch. Biochem. Biophys.* 121 (1967) 415.
10 A. SHNEYOUR AND M. AVRON, *FEBS Letters*, 8 (1970) 164.
11 R. K. CLAYTON, in H. GEST, A. SAN PIETRO AND L. P. VERNON, *Bacterial Photosynthesis*, Antioch Press, Yellow Springs, Ohio, 1963, p. 495.
12 G. BEN-HAYYIM, Z. GROMET-ELHANAN AND M. AVRON, *Anal. Biochem.*, 28 (1969) 6.
13 M. AVRON, *Biochim. Biophys. Acta*, 40 (1960) 257.
14 A. TREBST, E. PISTORIUS AND H. BALTSCHEFFSKY, *Biochim. Biophys. Acta*, 143 (1967) 257.
15 D. L. KEISTER AND N. J. MINTON, *Biochemistry*, 8 (1969) 167.
16 Z. GROMET-ELHANAN AND N. RADLICH, in preparation.
17 S. K. BOSE AND H. GEST, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 337.
18 H. A. LARDY, S. N. GRAVEN AND S. ESTRADA-O, *Federation Proc.*, 26 (1967) 1355.
19 B. C. PRESSMAN, E. J. HARRIS, W. S. JAGGER AND J. H. JOHNSON, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1949.
20 R. E. MCCARTY, *J. Biol. Chem.*, 244 (1969) 4292.
21 N. E. GOOD, S. IZAWA AND G. HIND, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol.1, Academic Press, New York, 1966, p. 75.