BBA 45918

EFFECT OF AMMONIUM SALTS, AMINES AND ANTIBIOTICS ON PROTON UPTAKE AND PHOTOPHOSPHORYLATION IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

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(Received December 17th, 1969)

SUMMARY

1. NH_4Cl inhibits the light-induced proton uptake but not photophosphorylation in chromatophores prepared from *Rhodospirillum rubrum*. This selective inhibition of proton uptake was also obtained with various amines which are well-known uncouplers in chloroplasts.

2. Addition of ion-transporting antibiotics like valinomycin or nonactin resulted in the inhibition of photophosphorylation with NH_4Cl but not with the other amines. The synergistic inhibition with valinomycin and NH_4Cl was abolished in the presence of K^+.

3. Similar results were found also in subchloroplast particles. Both in chromatophores and in subchloroplast particles valinomycin was required to induce the inhibition of photophosphorylation with ammonium nitrate, bicarbonate and acetate as well as chloride.

4. The effect of the ion-transporting antibiotics can be attributed to an enhanced permeability of NH_4^+ in their presence. The resulting inhibition of photophosphorylation can be explained by the initiation of an energy-dependent cyclic cation transport involving an active influx of protons coupled with a passive efflux of NH_4^+ facilitated by the antibiotics. The results can also be explained according to the chemiosmotic theory, if it is assumed that all the anions tested, namely Cl^-, NO_3^-, HCO_3^- and acetate are impermeable in chromatophores as well as in subchloroplast particles.

INTRODUCTION

Ammonium salts and amines are well-known uncouplers of photophosphorylation in chloroplasts[1,2]. Like other uncouplers NH_4Cl was also found to inhibit the light-induced proton uptake at the same range of concentrations at which it uncouples[3]. In subchloroplast particles NH_4Cl was, however, reported to inhibit the proton uptake at concentrations which had little effect on photophosphorylation[4]. It was therefore suggested that the pH change observed on illumination was not the driving force for ATP formation in subchloroplast particles. SHAVIT *et al.*[5], working with

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PMS, *N*-methylphenazonium methosulfate.

chromatophores from *Rhodospirillum rubrum*, arrived at a similar conclusion while studying the effect of nigericin on proton uptake and photophosphorylation.

The effect of ammonium salts or amines in *R. rubrum* chromatophores has not been thoroughly studied. HORIO AND YAMASHITA[6] reported that phosphorylation in chromatophores was as resistant to $(NH_4)_2SO_4$ as to Na_2SO_4. Both salts caused complete inhibition at 0.4 M, probably due to a salt effect. The only other report dealing with the action of amines in chromatophores is that of COST AND FRENKEL[7]. They observed that methylamine caused the bromthymol blue absorbance change to proceed at a greatly enhanced rate, while other uncouplers strongly diminished this rate. Since, however, the use of bromthymol blue as an indicator of internal pH changes in chromatophores has been questioned recently[8,9], one cannot directly correlate the stimulation of bromthymol blue changes by amines with their possible effect on light-induced pH changes.

We, therefore, tested the effect of NH_4Cl as well as other ammonium salts and various amines on both ATP formation and the light-induced proton uptake in *R. rubrum* chromatophores. The studies described here indicate that all these compounds selectively inhibit the proton uptake without affecting the phosphorylation in the chromatophores as well as in subchloroplast particles. Addition of valinomycin or nonactin, which was found to induce the inhibition of ATP formation by nigericin in chromatophores[10,11], resulted in a pronounced block of ATP formation with the ammonium salts but not with amines. This synergistic effect was abolished by the presence of K^+. A preliminary report of these findings has appeared[12].

MATERIALS AND METHODS

R. rubrum cells were grown anaerobically in the medium of ORMEROD *et al.*[13], for 40 h at 33° and illuminated with 10000 lux of white light. Chromatophores were isolated as previously described[14], except that they were washed and finally suspended in 0.25 M sucrose instead of Tris–sucrose. Bacteriochlorophyll was determined using the extinction coefficient *in vivo* given by CLAYTON[15].

Chloroplasts were prepared from lettuce leaves[16]. Subchloroplast particles were made according to MCCARTY[4], except that they were washed and finally suspended in a solution containing 0.4 M sucrose, 0.01 M choline chloride and 0.02 M Tricine (pH 7.5). Chlorophyll was assayed spectrophotometrically[17].

Proton uptake was measured as described by GROMET-ELHANAN AND BRILLER[9]. The samples were illuminated at room temperature using a 500-W slide projector (without its heat filter) through a combination of CS 2-59 and CS 7-69 corning filters and 9 cm of water. The reaction system used was the *N*-methylphenazonium methosulfate (PMS) *plus* 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) one, which was found to give a reversible and fully reproducible pH effect with the above far-red illumination setup in the presence of ascorbate[9]. The reaction mixture for proton uptake contained the following components in a total volume of 2.5 ml: KCl, 200 mM; PMS, 40 μM; sodium ascorbate, 60 μM; HQNO 1.2 μM and 50 μg of bacteriochlorophyll. The initial pH was 7.1. Any other additions or changes are stated in the text.

When ATP formation was followed simultaneously with proton uptake the reaction mixture contained also 1.6 mM potassium phosphate buffer and 1 mM $MgCl_2$ at the same initial pH of 7.1. After three light–dark cycles of the pH effect

were completed, 0.24 mM ADP was added and ATP formation was followed by the glass electrode method of NISHIMURA *et al.*[18]. The rate of ATP formation determined in this way was, however, low. Firstly, because the concentrations of ADP and P_i used were suboptimal in order to keep the buffer capacity of the system at a minimal level; and secondly, because the initial pH of 7.1 was a compromise between the lower optimal pH for proton uptake[19] and the higher optimal pH for ATP formation (Table I).

TABLE I

PHOTOPHOSPHORYLATION IN CHROMATOPHORES AS A FUNCTION OF pH

ATP formation was measured by the ^{32}P method. The reaction mixture contained also 200 mM choline chloride and the pH of the Tris–HCl buffer was as indicated.

pH	*ATP formation (μmoles/mg bacteriochlorophyll per h)*
7.1	447
7.4	484
7.7	538
8.1	639
8.4	623
8.8	476

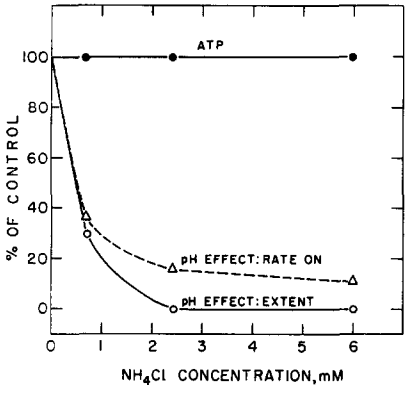
Phosphorylation was therefore also measured according to the method of AVRON[20]. The reactions were run for 3 min in test tubes inside a water bath at 25° and were illuminated by 50000 lux of white light. The reaction mixture contained the following components in a total volume of 3 ml: Tris–HCl buffer (pH 8.0), 27 mM; Tris phosphate, 3.3 mM (containing $4 \cdot 10^6$–$7 \cdot 10^6$ counts/min ^{32}P); ADP, 1.66 mM; $MgCl_2$, 2.66 mM; PMS, 33 μM; HQNO, 1 μM and 30–50 μg bacteriochlorophyll. No additional salt was required for ATP synthesis here and the presence of additional salts up to a concentration of 200 mM did not significantly affect the activity (see legends to Figs. 3 and 4).

HQNO was purchased from Sigma, valinomycin from CalBiochem and nonactin from Ciba. Nigericin was a gift from Dr. R.L. Harned (Commercial Indiana Solvents Corp.) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was generously provided by the Du Pont Co.

RESULTS

NH_4Cl was found to inhibit strongly the light-induced proton uptake in chromatophores from *R. rubrum*, without affecting the rate of ATP formation (Fig. 1). The type of inhibition exerted by NH_4Cl in chromatophores was thus similar to that observed in subchloroplast particles[4] and differed from the inhibition reported in chloroplasts of both proton uptake and phosphorylation[1,3]. This selective inhibition was not restricted to NH_4Cl. Table II summarizes the effect of several amines which were found to act as uncouplers in chloroplasts[2,21]. All the amines tested, except tetramethylammonium chloride, inhibited proton uptake while none by itself inhibited

phosphorylation (see Fig. 5). Tetramethylammonium chloride, which was inactive as an inhibitor of either of these reactions in chloroplasts[21], was also ineffective in chromatophores.



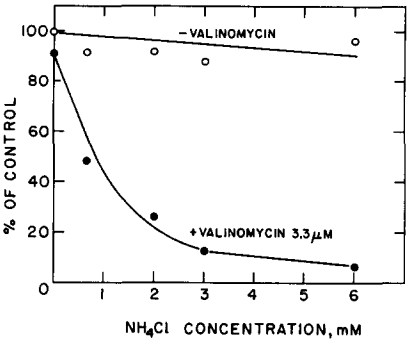


Fig. 1. Effect of NH_4Cl on proton uptake and photophosphorylation in chromatophores. Proton uptake and ATP formation were measured simultaneously as described under MATERIALS AND METHODS. The control values for ATP formation were 50 μmoles/mg bacteriochlorophyll per h, for the extent of proton uptake, 0.242 μequiv/mg bacteriochlorophyll and for the initial rate of proton uptake 0.720 μequiv/mg bacteriochlorophyll per min.

Fig. 2. Effect of NH_4Cl on photophosphorylation in chromatophores in the absence and presence of valinomycin. ATP formation was measured by the ^{32}P method. The reaction mixture also contained 200 mM choline chloride. The control value was 540 μmoles ATP formed per mg bacteriochlorophyll per h.

TABLE II

EFFECT OF AMINES ON THE LIGHT-INDUCED PROTON UPTAKE IN CHROMATOPHORES

The reaction mixture was as described under MATERIALS AND METHODS, except that 200 mM choline chloride was used instead of KCl.

Additions (mM)	*Proton uptake*			
	Extent		*Initial rate*	
	μequiv/mg bacteriochlorophyll	*% of control*	*μequiv/mg bacteriochlorophyll per min*	*% of control*
None	0.114	(100)	0.473	(100)
NH_4Cl, 2.4	0.019	17	0.170	36
Methylamine chloride, 2.4	0.086	75	0.458	97
Methylamine chloride, 12.0	0.038	33	0.278	59
Cyclohexylamine chloride, 2.4	0.016	14	0.236	50
N,N,N',N'-Tetramethylethylenediamine chloride, 2.4	0.050	44	0.222	47
Tetramethylammonium chloride, 2.4	0.121	106	0.736	156
Tetramethylammonium chloride, 12.0	0.116	102	0.720	153

A similar pattern of selective inhibition of proton uptake but not of ATP formation was observed with nigericin in chromatophores[5]. When, however, ion-transport-inducing antibiotics like valinomycin or nonactin were added together with nigericin, ATP formation was blocked and this synergistic inhibition was dependent upon the presence of K^+ or Rb^+ (refs. 10, 11). When valinomycin was added together with NH_4Cl in the presence of K^+, no significant synergistic inhibition of phosphorylation was observed (Table III, Expt. 1). Moreover, the stimulated rate and extent of proton uptake in the presence of valinomycin and K^+, although decreased by NH_4Cl, was closer to the control level than to the NH_4Cl-inhibited level. A pronounced synergistic block of phosphorylation was, however, obtained in the presence of Na^+ (Table III, Expt. 2). The proton uptake activity, which requires the presence of salts[19], was as active with NaCl as with KCl (Table III) or choline chloride (Table II) and was inhibited to the same extent by NH_4Cl in every case. But with NaCl, when valinomycin did not stimulate the proton uptake, its inhibition by NH_4Cl persisted also in the presence of valinomycin. These results indicate that the synergistic effect of NH_4Cl and valinomycin unlike that of nigericin and valinomycin is not only independent of K^+ but is inhibited by it.

The synergistic effect of NH_4Cl and valinomycin was also observed in the presence of choline chloride at pH 8.0 under optimal conditions for phosphorylation (Fig. 2). With 2 mM of NH_4Cl ATP synthesis was 75 % inhibited in the presence of valinomycin, while without valinomycin no inhibition was observed even with 6 mM NH_4Cl. In this system of phosphorylation the effect of increasing concentrations of KCl on the synergistic effect of NH_4Cl and valinomycin could be tested. As can be seen from Fig. 3, KCl did not inhibit the synergistic effect of NH_4Cl and valinomycin at concentration below 50 mM (a 20-fold excess over NH_4Cl), but at 130 mM KCl already eliminated 50 % of the effect and at 300 mM KCl completely abolished the synergistic effect. With choline chloride even at 350 mM only a slight effect was seen (Fig. 4).

TABLE III

EFFECT OF NH_4Cl AND VALINOMYCIN ON THE PROTON UPTAKE AND PHOTOPHOSPHORYLATION IN CHROMATOPHORES IN THE PRESENCE OF KCl OR NaCl

The reaction mixture was in Expt. 1 as described under MATERIALS AND METHODS; in Expt. 2 200 mM NaCl and 1.6 mM sodium phosphate were used instead of the corresponding K^+ salts. 2.4 mM NH_4Cl and 4.0 μM valinomycin were added when indicated. The control values in Expt. 1 were as follows: for the extent of proton uptake 0.080 μequiv H^+ per mg bacteriochlorophyll; for the initial rate 0.490 μequiv H^+ per mg bacteriochlorophyll per min and for ATP formation 39 μmoles/mg bacteriochlorophyll per h. The corresponding control values in Expt. 2 were: 0.102 μequiv H^+ per mg bacteriochlorophyll; 0.409 μequiv H^+ per mg bacteriochlorophyll per min and 35 μmoles ATP per mg bacteriochlorophyll/h.

Expt.	*Additions*	*Proton uptake (% of control)*		*ATP formation (% of control)*
		Extent	*Initial rate*	
1	NH_4Cl	14	24	116
	Valinomycin	295	244	80
	NH_4Cl + valinomycin	92	160	70
2	NH_4Cl	7	26	115
	Valinomycin	130	110	92
	NH_4Cl + valinomycin	10	30	15

The synergistic effect of valinomycin was rather specific and required the presence of NH_4^+, since no significant synergism was observed with any of the tested amines (Fig. 5), although they affected proton uptake and phosphorylation like NH_4Cl (Table II and Fig. 5). The anion on the other hand, was not an important factor for the expression of the synergistic effect (Table IV). Thus, all the tested

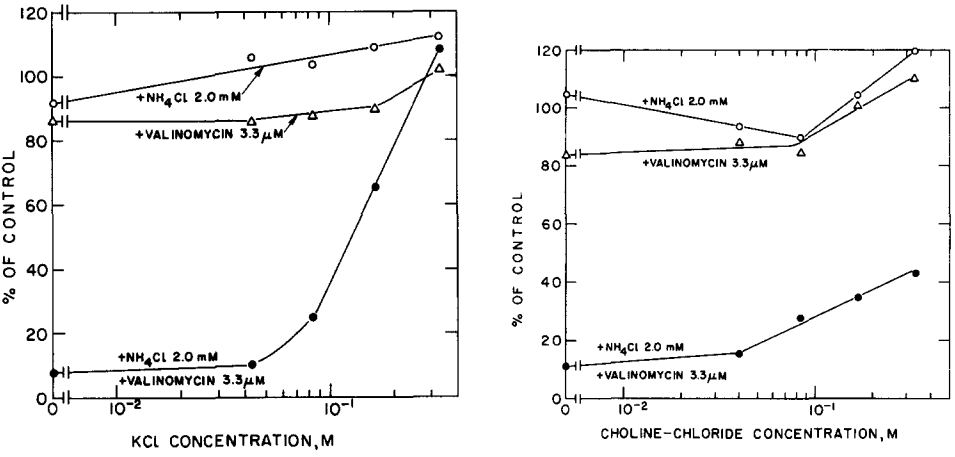


Fig. 3. Effect of KCl concentration on photophosphorylation in chromatophores in the presence of NH_4Cl and valinomycin. ATP formation was measured by the ^{32}P method. The control values in μmoles ATP formed per mg bacteriochlorophyll per h were as follows: in the absence of KCl, 471; with 42 mM KCl, 475; with 84 mM KCl, 456; with 167 mM KCl, 416 and with 330 mM KCl, 324.

Fig. 4. Effect of choline chloride concentration on photophosphorylation in chromatophores in the presence of NH_4Cl and valinomycin. ATP formation was measured by the ^{32}P method. The control values in μmoles ATP formed per mg bacteriochlorophyll per h were as follows: in the absence of choline chloride, 522; with 42 mM choline chloride, 582; with 84 mM, 617; with 167 mM, 509 and with 330 mM, 439.

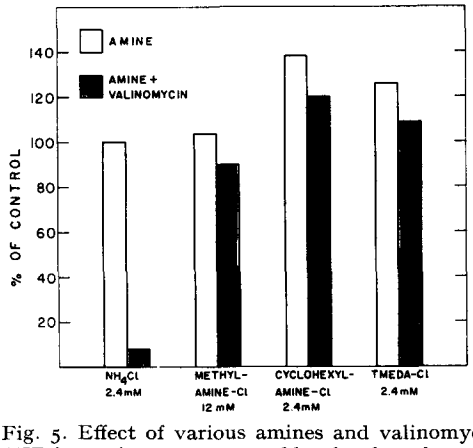


Fig. 5. Effect of various amines and valinomycin on photophosphorylation in chromatophores. ATP formation was measured by the glass electrode method. The reaction mixture was as described under MATERIALS AND METHODS, except that 200 mM choline chloride and 1.6 mM Tris phosphate were used instead of the corresponding potassium salts. 4 μM valinomycin was added where indicated. The control value was 78 μmoles ATP formed per mg bacteriochlorophyll per h. TMEDA-Cl, *N,N,N',N'*-tetramethylethylenediamine chloride.

ammonium salts did not by themselves inhibit the phosphorylation, and valinomycin induced a similar degree of inhibition in the presence of each of them.

An induction of the inhibition of phosphorylation with NH_4Cl was obtained also with ion-transporting antibiotics other than valinomycin. As can be seen from Fig. 6, nonactin caused even a more pronounced synergistic effect than valinomycin. No synergism was, however, observed with FCCP or with nigericin (Fig. 6).

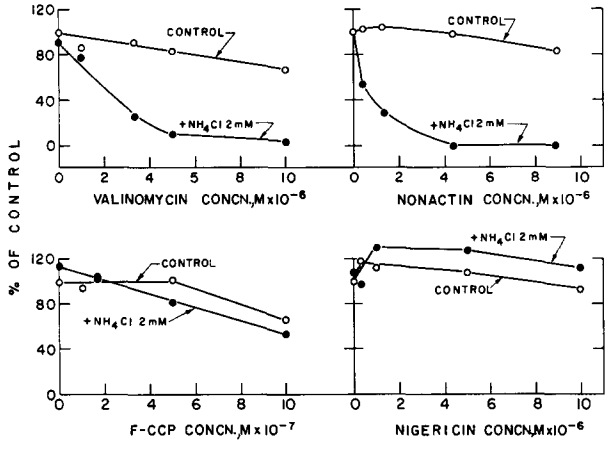


Fig. 6. Combined effect of NH_4Cl with valinomycin, nonactin, FCCP and nigericin on photophosphorylation in chromatophores. ATP formation was measured by the ^{32}P method. The reaction mixture also contained 200 mM choline chloride. The control value was 425 μmoles ATP formed per mg bacteriochlorophyll per h.

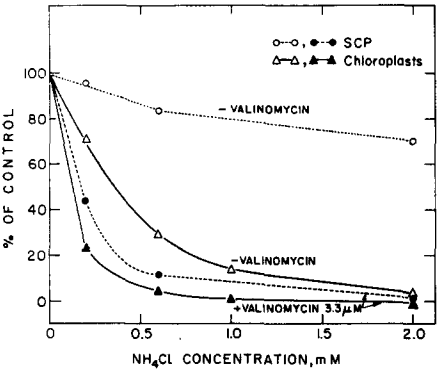


Fig. 7. Effect of NH_4Cl on photophosphorylation in chloroplasts and subchloroplast particles in the absence and presence of valinomycin. ATP formation was measured by the ^{32}P method. The reaction mixture also contained 200 mM choline chloride. With chloroplasts 42 μg chlorophyll were used and the control value was 677 μmoles ATP formed per mg chlorophyll per h. With subchloroplast particles 90 μg chlorophyll were used and the control value was 125 μmoles ATP formed per mg chlorophyll per h.

Since the effect of NH_4Cl on proton uptake and photophosphorylation observed here in chromatophores was similar to the effect of NH_4Cl reported in subchloroplast particles[4], we checked chloroplasts and subchloroplast particles for a synergistic effect of NH_4Cl and valinomycin (Fig. 7). While this manuscript was in preparation,

TABLE IV

EFFECT OF VARIOUS AMMONIUM SALTS AND VALINOMYCIN ON PHOTOPHOSPHORYLATION IN CHROMATOPHORES

ATP formation was measured by the ^{32}P method. The reaction mixture was as described under MATERIALS AND METHODS with no additional salt and with 10 mM Tris–HCl buffer and $MgSO_4$ instead of $MgCl_2$. Where indicated 2.0 mM ammonium salt and 3.3 μM valinomycin were added.

Additions	*ATP formation*			
	– valinomycin		*+ valinomycin*	
	μmoles/mg bacteriochlorophyll per h	*% of control*	*μmoles/mg bacteriochlorophyll per h*	*% of control*
None	488	(100)	472	96
NH_4Cl	484	99	157	32
NH_4HCO_3	456	93	134	27
NH_4NO_3	458	94	84	17
Ammonium acetate	468	96	146	30

TABLE V

EFFECT OF VARIOUS AMMONIUM SALTS AND VALINOMYCIN ON PHOTOPHOSPHORYLATION IN SUBCHLOROPLAST PARTICLES

ATP formation was measured by the ^{32}P method. The reaction mixture contained the following components in a total volume of 3 ml: Tricine (pH 8.0), 10 mM; $MgSO_4$, 3.3 mM; Tris–phosphate (containing $4 \cdot 10^6$ counts/min ^{32}P), 3.3 mM; ADP, 1.25 mM; sucrose, 83 mM and 40 μg of chlorophyll. Where indicated, 0.3 mM ammonium salt and 3.3 μM valinomycin were added.

Additions	*ATP formation*			
	–valinomycin		*+ valinomycin*	
	μmoles/mg chlorophyll per h	*% of control*	*μmoles/mg chlorophyll per h*	*% of control*
None	184	(100)	181	99
NH_4Cl	179	97	99	54
NH_4NO_3	163	89	106	58
NH_4HCO_3	186	101	102	56

McCARTY[22] reported on similar experiments in chloroplasts and subchloroplast particles with identical results. In subchloroplast particles (Table V) as well as in chromatophores (Table IV) the synergistic effect was independent of the anion, giving similar results with NH_4Cl, NH_4NO_3 or NH_4HCO_3.

DISCUSSION

Although a light-induced proton uptake has been observed in chromatophores[23] as well as in chloroplasts[3], these particles differ markedly in their sensitivity towards various compounds. Ammonium salts, or nigericin in the presence of K^+, were shown to inhibit both proton uptake and photophosphorylation in chloroplasts[1,3,24,25]. In chromatophores, however, the presence of either nigericin and K^+ (ref. 5) or ammonium salts (see Fig. 1 and Table III) resulted in the inhibition of proton uptake

without affecting the phosphorylation. A similar pattern of such selective inhibition was also observed in subchloroplast particles[4].

A mechanism for the inhibition of proton uptake in chloroplasts by ammonium salts and amines has been proposed by CROFTS[26]. According to this mechanism the chloroplast membrane is freely permeable to NH_3. When light-induced proton uptake occurs, the NH_3 combines with H^+ to form NH_4^+, more NH_3 enters to replace that lost by association with H^+, leading to an accumulation of NH_4^+ and a dissipation of the proton gradient. This mechanism can also explain the inhibition of proton uptake in chromatophores, where NH_4^+ uptake similar in extent to the proton uptake has been observed (Z. GROMET-ELHANAN, unpublished observations). However, since there is no concomitant inhibition of phosphorylation, it follows that phosphorylation in chromatophores is not dependent upon a proton gradient.

An inhibition of photophosphorylation in the presence of ammonium salts in chromatophores could be obtained by the addition of ion-transporting antibiotics like valinomycin or nonactin (see Figs. 2 and 6 and Table IV). This synergistic effect was specific to NH_4^+, since it did not occur with amines (see Fig. 5), although they inhibited the proton uptake like NH_4Cl (see Table II). The presence of K^+, which was required for the synergistic inhibition of phosphorylation with nigericin and valinomycin[10,11], resulted in the abolition of the synergistic effect with NH_4Cl and valinomycin (see Fig. 3 and Table III). The specificity to NH_4^+ and the inhibition by K^+, which is transported by valinomycin preferentially to NH_4^+ (ref. 27), indicate that the synergistic effect of valinomycin and NH_4^+ might be associated with an enhanced permeability of NH_4^+ in the presence of valinomycin. The inhibition of phosphorylation can thus be explained by an energy-dependent cyclic cation transport[11,28,29], which is facilitated by the spontaneous penetration of NH_3 into the chromatophores where it combines with H^+ to form NH_4^+ which is effluxed in the presence of valinomycin.

These observations may also be interpreted in terms of the chemiosmotic mechanism[30], as was proposed by JACKSON *et al.*[10] for nigericin and valinomycin in chromatophores and by MCCARTY[22] for NH_4Cl and valinomycin in subchloroplast particles. They suggested that the difference between chloroplasts and the other particles is due to a difference in their permeability to Cl^-. In chloroplasts Cl^- was assumed to cross the membrane by passive diffusion[26], thus eliminating the electrochemical gradient created by H^+ (or NH_4^+) uptake and leaving the pH gradient as the main driving force for ATP formation. On the other hand chromatophores[10] and subchloroplast particles[22] were assumed to be impermeable to Cl^- and therefore a membrane potential rather than a pH gradient would be the important factor for photophosphorylation. Elimination of the pH gradient by NH_4Cl would thus result in inhibition of phosphorylation in chloroplasts but not necessarily in chromatophores or subchloroplast particles. Phosphorylation would be inhibited in the last two particles in the presence of NH_4Cl and valinomycin when both the pH and the electrochemical gradients are dissipated.

Since Cl^- (or anion) permeability has not been studied in chromatophores or subchloroplast particles, there is at yet no experimental evidence for the assumed impermeability of these particles. In submitochondrial particles, where the loss of respiratory control by NH_4Cl was also found to be dependent on the presence of valinomycin[31], indirect evidence for the impermeability of Cl^- has recently been repor-

ted by MONTAL *et al.*[32]. They observed that when NH_4Cl was replaced by NH_4NO_3 the respiratory control was abolished even in the absence of valinomycin. They therefore concluded that in submitochondrial particles NO_3^- (but not Cl^-) is a permeant anion. Such a permeant anion has as yet not been found in chromatophores or subchloroplast particles, where valinomycin was required for the inhibition of phosphorylation with ammonium nitrate, bicarbonate and acetate as well as chloride (see Tables IV and V).

A careful study of the permeability of chromatophores and subchloroplast particles, as compared to chloroplasts, to various anions is, therefore, required before any convincing conclusions regarding the differences between these particles can be drawn.

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