UPTAKE OF AMMONIUM ION BY CHLOROPLASTS, AND THE MECHANISM OF AMINE UNCOUPLING*

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Ammonium ions (Krogmann, Jagendorf and Avron, 1959) and substituted amines (Good, 1960) stimulate the ferricyanide Hill reaction, and uncouple electron transport from phosphorylation. NH_4Cl uncoupling is pH dependent, but if account is taken of the variation in concentration of NH_3, the Hill reaction stimulated by NH_4Cl is relatively independent of pH (Stiller, 1965), suggesting that NH_3 is the reactive species. Uncoupling by amines is associated with chloroplast swelling, which may be reversible (Izawa, 1965). Neumann and Jagendorf (1964) have demonstrated a reversible light-induced uptake of H^+ by chloroplasts, which is inhibited by NH_4Cl. Although charged ions equilibrate relatively slowly between chloroplasts and medium (Tolberg and Macey, 1965), Crofts, Deamer and Packer (1966) have concluded from the rapid swelling observed in solutions of the NH_4^+ salts of weak acids, that chloroplasts are freely permeable to NH_3.

A mechanism for NH_4Cl uncoupling can be deduced from these observations. It seems likely that following the uptake of H^+ on illumination of chloroplasts, the equilibration of NH_3 across the chloroplast membrane would be displaced so as to replace NH_3 lost by association with H^+, raising the concentration of NH_4^+ within the chloroplasts. Entry would continue until NH_4^+ diffused out at the same rate as H^+ were being pumped in. Such a mechanism would result in an inhibition of the pH change, as is

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observed, and would be accompanied by net uptake of NH_4^+. The consequent increase in osmolarity within the chloroplasts would lead to swelling. Chappell and Crofts (1965) have proposed a similar mechanism for NH_4^+-induced uncoupling of mitochondria, and Mitchell (1966) has suggested that NH_4^+-uncoupling in chloroplasts might result from ion movements.

It has now been found that on illumination, chloroplasts readily take up NH_4^+ from the medium, and that the kinetics of NH_4^+ uptake are consistent with the mechanism proposed above.

EXPERIMENTAL RESULTS

Nature and Extent of Cation Uptake. The effect of illumination on changes in H^+, cation concentration, and light-scattering, during PMS catalysed electron flow in a suspension of spinach chloroplasts in chloline chloride is shown in Figure 1. It can be seen that on illumination in the absence of NH_4Cl, the chloroplasts took up H^+ from the medium, and showed a parallel increase in light-scattering (Deamer, Crofts and Packer, 1966). A small uptake of cation was also observed, and these changes were reversible in the dark. On addition of a low concentration of NH_4Cl and subsequent illumination, the H^+ uptake and light-scattering change were partially inhibited, but the uptake of cation was much increased. The changes observed were reversible in the dark, and were reproduced on subsequent illumination cycles. Similar changes have been demonstrated in suspensions of chloroplasts in Tris Cl.

It seemed probable that the increased cation uptake observed reflected the movement of NH_4^+. The dependence of cation and H^+ uptake on cation concentration was therefore investigated, and these results are shown in Figure 2. The figure shows that cation uptake increased only slightly with increasing K^+ concentration and the H^+ uptake was unaffected. When K^+ was replaced

PMS: N-methyl phenazonium methosulfate

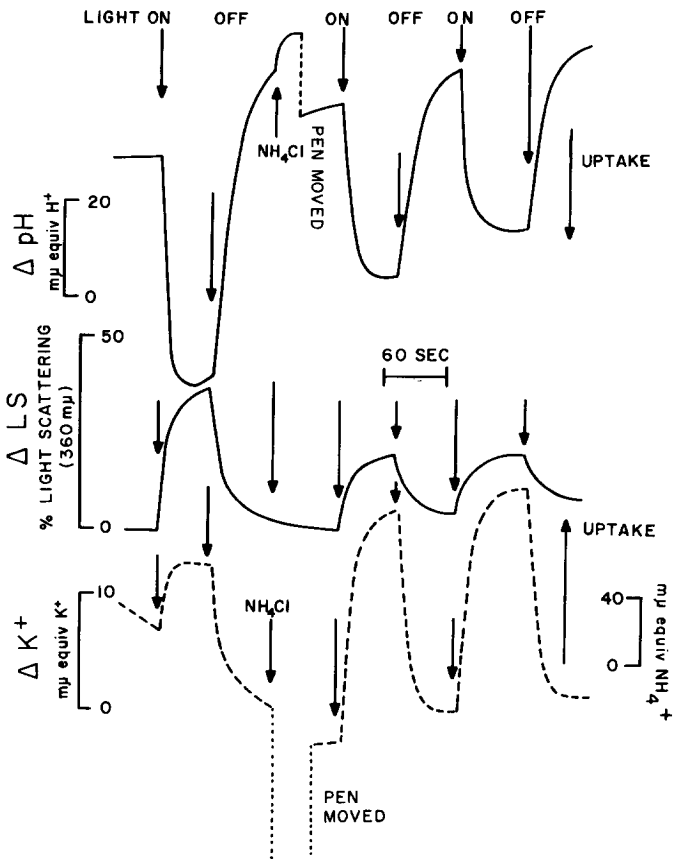

Figure 1. Light-dependent changes in H^+, cation concentration, and light-scattering in a chloroplast suspension. Preparation of chloroplasts, and measurements of changes in concentrations of cations in the reaction medium were as described by Crofts et al. (1966). The cation electrode used (Type GKN 33, Electronic Instruments Ltd., Richmond, Surrey, England) was 1.85 times as sensitive to K^+ as to NH_4^+, but insensitive to changes in pH of the magnitude involved in these experiments. Cation uptake was estimated by comparison with the deflection produced on addition of known ammounts of the ions. Chloroplasts (26 μg chlorophyll/ml) were suspended in 7.5 ml of 0.1 M choline chloride, 0.5 mM Tris-Cl, 20 μM PMS at pH 6.9 and 25°. Where indicated, NH_4Cl was added to 0.08 mM. Note the change in sensitivity of the electrode on addition of NH_4^+. K^+ (0.05 mM) from the chloroplasts was present initially.

by NH_4^+, the uptake of cation was much enhanced, and increased markedly with increasing NH_4Cl concentration, while H^+ uptake was progressively inhibited. It can be concluded that most of the cation disappearing when chloroplasts are illuminated in the presence of NH_4Cl is NH_4^+.

Light induced cation uptake in the presence of NH_4^+ during cyclic

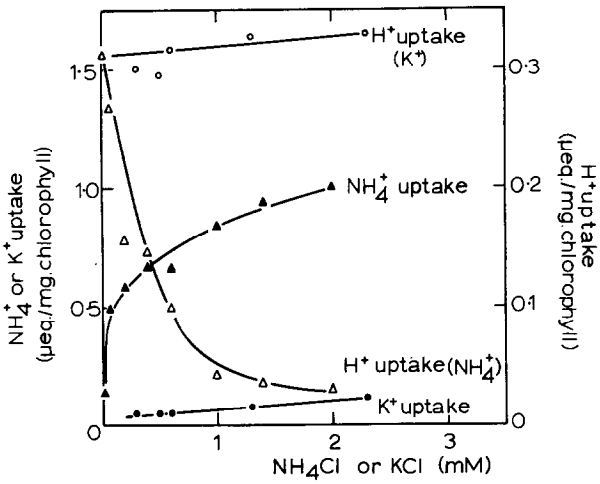

Figure 2. Relation between light dependent cation uptake by chloroplasts and concentration of K^+ or NH_4^+. Chloroplasts (57.5 μg chlorophyll/ml) were suspended in 5 ml 0.1 M choline chloride, 0.5 mM Tris-Cl, 20 μM PMS containing KCl or NH_4Cl at the concentrations indicated, at pH 7.1 and 25°. Cation equivalent to 0.3 mM K^+ was carried over with the chloroplasts. KCl (0.5 mM) was present in all NH_4Cl experiments, and allowance for K^+ uptake at this concentration has been made in plotting points, by subtraction.

electron flow in sonically disrupted chloroplasts which consist of isolated grana lamellae (Gross and Packer, 1965) is also observed, suggesting that the uptake in whole chloroplasts is into the grana compartment.

Kinetics of NH_4^+ Uptake. It would be expected from the ionic mechanism above, that the rate of NH_4^+ uptake into chloroplasts would be limited by the rate of uptake of H^+. H^+ uptake has been shown to be dependent on light-intensity, and to be reduced by inhibitors of electron flow (Neumann and Jagendorf, 1964) and it seems likely that it is closely coupled to electron flow. The relation between rates of ammonium uptake and electron flow was therefore investigated.

Some comparative values for the kinetics of NH_4^+ and H^+ uptake under different conditions of electron flow are shown in Table I. At low concentrations of NH_4Cl the rate of uptake accompanying PMS catalysed electron flow is less than the rate of H^+ uptake in the absence of inhibitor. However, above 0.2 mM, the rate of NH_4^+ uptake is greater, and may be more than double

TABLE I

Kinetics of light dependent NH_4^+ uptake, H^+ uptake and electron flow in chloroplasts.

Experimental Parameter	Uncoupler	Km (mM)	Qmax. (μeq/mg/sec)or(μeq/mg)
Experiment 1			
Rate of ferricyanide reduction	NH_4Cl	0.40	0.12
Rate of NH_4^+ uptake (PMS)	NH_4Cl	0.41	0.31
Experiment 2			
Rate of NH_4^+ uptake (PMS)	NH_4Cl	0.50	0.27
Rate of NH_4^+ uptake (PMS)	NH_4Cl+m-ClCCP	1.40	0.23
Extent of NH_4^+ uptake (PMS)	NH_4Cl	0.45	2.25
Extent of NH_4^+ uptake (PMS)	NH_4Cl+ m-ClCCP	0.91	1.92
			Rate (μeq/mg/sec)
Experiment 1			
Ferricyanide reduction	none		0.019
Ferricyanide reduction	0.8 mM NH_4Cl		0.085
H^+ uptake (ferricyanide)	none		0.108
NH_4^+ uptake (ferricyanide)	0.8 mM NH_4Cl		0.160
H^+ uptake (PMS)	none		0.124
Experiment 2			
H^+ uptake (PMS)	none		0.122

Experiment 1

Chloroplasts (43 μg chlorophyll/ml) were suspended in 5 ml 0.1 M choline chloride, with 5 mM Tris-Cl and either 0.4 mM $K_3Fe(CN)_6$ or 20 μM PMS plus 1.6 mM KCl, at pH 7.2 and 25°. For ferricyanide reduction, $1/(V_{NH_4}-V_{no\ NH_4})$ was plotted against $1/[NH_4Cl]$. The value in the table is $(V_{max}+V_{no\ NH_4})$. Other values are derived from double reciprocal plots. Rates of NH_4^+ uptake were corrected for a K^+ uptake of 0.01 μeq/mg/sec. Above 0.8 mM NH_4Cl the rate of NH_4^+ uptake accompanying ferricyanide reduction declined; the value given is maximal.

Experiment 2

Chloroplasts (28 μg chlorophyll/ml) were suspended in 5 ml of 0.1 M choline chloride, with 2 mM Tris-Cl, 20 μM PMS plus 0.4 mM KCl at pH 6.9 and 25°. Where indicated, m-ClCCP was present at 2×10^{-6} M, at which concentration H^+ uptake was inhibited 49%. Rates of NH_4^+ uptake were corrected for a K^+ uptake of 0.01 μeq/mg/sec.

that of the uninhibited rate of H^+ uptake (see Table I). A similar uptake of NH_4^+ accompanies light-induced electron flow during NH_4^+ uncoupling of the ferricyanide Hill reaction. NH_4^+ uptake is reversed in the dark or on exhaustion of ferricyanide. Below 0.8 mM, both extent and rate of uptake increase with NH_4Cl concentration, but above this level, NH_4^+ uptake falls off slightly, though the rate of electron flow continues to increase. This observation is being further investigated.

In Table I it is shown that the concentration on NH_4Cl required for half-maximal stimulation of NH_4^+ uptake coupled to cyclic electron flow is similar to that for uncoupling of ferricyanide reduction. The fact that uptake coupled to ferricyanide reduction falls off at higher concentrations of NH_4Cl makes it impossible to obtain values for Km and maximal rate comparable to those shown for the PMS system. However as is the case when NH_4 uptake is coupled to the PMS system the rate of uptake can exceed that of H^+ uptake in the absence of NH_4Cl (see Table). In the case of ferricyanide coupled NH_4^+ uptake it is clear that the increased rate is associated with an increase in the rate of electron flow. It seems probable from these observations that NH_4Cl is able to stimulate the rate of electron flow in both the Hill reaction and the PMS system, and that the high rates of NH_4^+ uptake observed are a reflection of H^+ uptake coupled to an increased electron transport.

NH_4^+ uptake is inhibited by uncoupling agents, but the inhibition observed is more nearly competitive than non-competitive. This can be seen from the data for rates and extent of NH_4^+ uptake in the presence and absence of m-ClCCP in Table I.

DISCUSSION

The effects of uncoupling agents and ammonium ions in stimulating electron flow and inhibiting phosphorylation have suggested a common site for their interaction with the energy coupling reactions. Theories of energy

m-ClCCP: m-chlorocarbonylcyanide phenylhydrazone.

coupling have been proposed, involving either energy-rich intermediates or a chemi-osmotic mechanism directly coupled to electron flow (Mitchell, 1961), as energetic states leading to ion movements or phosphorylation. The mechanism of amine uncoupling proposed above, and the evidence presented in support of it, may seem compatible with either of these hypotheses, but because of the following considerations it seems more difficult to explain them in terms of interaction with intermediates.

a) H^+ and NH_4^+ may be competing for a high energy intermediate. If this were so the uptake of other monovalent cations in comparable quantities would be expected. This is not seen for K^+ (Figure 2), and Good (1960) has shown that only monovalent amines without other charged groups are effective in uncoupling.

b) H^+ and NH_3 may be competing for a high energy intermediate. This is not likely, since no net production of H^+ is observed as a result of NH_4^+ dissociation, as would be expected on loss of NH_3 from the medium.

c) An ionic mechanism operates, but H^+ uptake may be driven by high-energy intermediates. This is plausible, but leads to two possible explanations of the effects of uncoupling agents. 1) It is possible that m-ClCCP inhibits NH_4^+ uptake by uncoupling high-energy intermediates from H^+ uptake. This is improbable, since such a mechanism would be expected to give non-competitive inhibition of NH_4^+ uptake, and competitive inhibition is observed (Table I). 2) NH_3 and m-ClCCP compete for H^+, but H^+ uptake is driven by high-energy intermediates. Although this is plausible, the high energy intermediates invoked here are superfluous to an explanation of the effects of uncoupling agents.

The observations reported here seem more compatible with a chemi-osmotic mechanism of energy coupling than one involving intermediates, and they may afford a means of distinguishing experimentally between these hypotheses.

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COENZYME CONTENT OF PURIFIED ALANINE RACEMASE FROM PSEUDOMONAS

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Alanine racemase, induced in Pseudomonas putida by growth on DL-alanine, was purified about 1000-fold to homogeneity. The purified enzyme contains approximately one molar equivalent of pyridoxal phosphate by absorbance, fluorescence, and microbiological assay. No evidence for the presence of a flavin coenzyme was found.

Alanine racemase was the first amino acid racemase to be studied enzymatically (Wood and Gunsalus, 1951). Racemases for other common amino acids have subsequently been detected in various bacteria (for references before 1964, see Adams and Norton, 1964; also Markovetz et al, 1966; Soda et al, 1967). In certain of these enzymes, including the alanine racemase first studied (Wood and Gunsalus, 1951), pyridoxal phosphate has been implicated as a coenzyme by inhibition or resolution studies. No direct evidence, however, has been reported for pyridoxal phosphate as a component of racemases for primary amino acids; such observations would require high absolute purity of the racemase studied. The question of coenzyme content is made more cogent by the finding that active preparations of racemases for secondary amino acids, hydroxyproline-2-epimerase (Adams and Norton, 1964) and proline racemase (Cardinale, 1965), lack pyridoxal phosphate, and also by the conclusion that partly-purified glutamic racemase of Lactobacillus fermenti (Tanaka et al, 1961) and alanine racemase of Bacillus subtilis (Diven et al, 1964) require a flavin coenzyme for activity.

The present communication describes the purification to apparent homogeneity of an inducible alanine racemase from Pseudomonas putida. From both absorption spectrophotometry, fluorescence measurements, and microbiological assays, the purified enzyme contains approximately one mole of pyridoxal phosphate per mole of enzyme. In contrast, no stoichiometrically-significant quantity of flavin could be detected spectrally or fluorometrically in the purified enzyme.

Growth of Cells, Enzyme Assay and Purification - A strain of P. putida (ATCC 15070), earlier used for studies of hydroxyproline