Plant & Cell Physiol., **10**, 751–757 (1969)

Acid-base induced calcium uptake by spinach chloroplasts

KOJIRO NISHIDA and YOSHIYUKI HAYASHI

Botanical Institute, Faculty of Science, Kanazawa University, Kanazawa, Japan

(Received March 12, 1969)

Calcium uptake by spinach chloroplasts can be induced by the transition from an acidic to a basic medium. This reaction depends upon the pH difference. A difference by 4.3 to 4.5 pH units showed a maximum efficiency for calcium uptake. The acid-base induced calcium uptake is susceptible to various inhibitors in a way similar to that of light-induced calcium uptake. The same high energy intermediate (or state) produced in the chloroplast by this acid-base transition is inferred to be operating both in calcium uptake and ATP formation.

There have been several investigations on the uptake of anions and cations by chloroplasts under light (*1–5*). These investigations suggested that ion uptake is a manifestation of the operation of the energy conservation mechanism in the chloroplast. Recently, JAGENDORF and URIBE (*6*) discovered that chloroplasts, after a rapid transition from an acidic to a basic medium, synthesize small amounts of ATP in the dark. This finding was highly significant to the study of photophosphorylation. In addition, luminescence of chloroplasts was found to be induced by the acid-base transition (*7*) when a preillumination treatment had been applied to the chloroplasts (*8*), and that ATPase activity was induced in chloroplasts by the acid-base treatment (*9*, *10*).

Uptake of calcium by chloroplasts stimulated under light, was reported by NOBEL (*1*). Combining all these previous findings we posited that calcium uptake may be induced by the acid-base transition of the chloroplasts. This was experimentally proved to be the case. In this study isolated chloroplasts were transferred from an acidic to a basic medium (difference, about 4.5 pH units) and the resulting calcium uptake was examined.

Material and methods

Chloroplasts were prepared from market spinach by blending 150 g of deveined leaves in about 100 ml of grinding medium which consisted of 0.3 M sucrose. After blending for 3 min the brei was filtered through 12 layers of gauze and centrifuged for 7 min at $3,000 \times g$. The supernatant solution was again centrifuged for 5 min at $7,800 \times g$. Broken chloroplasts were suspended in a 20 mM NaCl solution and allowed to stand for 30 min in the dark before use.

The transition experiments were carried out as follows: 0.5 ml of chloroplast suspension, containing approximately 80 μg chlorophyll per milliliter, and 0.5 ml

Abbreviation: PMA, phenylmercuric acetate.

of an appropriate acid medium were taken up in a hypodermic syringe. Usually 10 mM succinic acid, which had been neutralized with an appropriate amount of NaOH to pH 3.8, was used as an acid medium. After 20 sec, the acidified chloroplasts were injected into 1.0 ml of buffer (0.1 M Tris-HCl, pH 8.5) containing $^{45}CaCl_2$ (4 μCi ^{45}Ca, final concentration of $CaCl_2$, about 10 μM). The final pH was directly checked in each experiment, and found to be about 8.3. After 50 sec of incubation in the base medium, chloroplasts were removed by a Millipore filter (25 mm diameter, 0.8 μ pore size). Chloroplasts on the filter were washed twice with 5 ml of a mixture of NaCl (10 mM) and succinate (5 mM) buffered with 0.05 M Tris-HCl, pH 8.3. Radioactivity of the chloroplasts was determined by a thin window G-M counter. The chlorophyll content was determined by the method of ARNON (*11*). To minimize the effect of light, experiments were carried out in dim light with intensities as low as possible. Moreover, 10^{-5} M DCMU was added in the acid medium.

All experiments were carried out at 3°C.

Results

1. *Calcium uptake induced by acid-base transition*

To determine the appropriate incubation time in the acid medium, chloroplasts were kept for 10, 20, 40 and 80 sec in an acid medium and subsequently injected into a base medium. As shown in Fig. 1, the amount of calcium uptake decreases with increasing incubation time. As the time required for the operation, 20 sec was chosen for acid incubation in the following experiments. Next, incubation time in a base medium was determined. Fig. 2 shows that the duration of incubation in the base medium has no significant effect on the uptake of calcium, almost the same amounts of uptake being observed for varied incubation times. Hence, 50 sec was chosen in the following experiments.

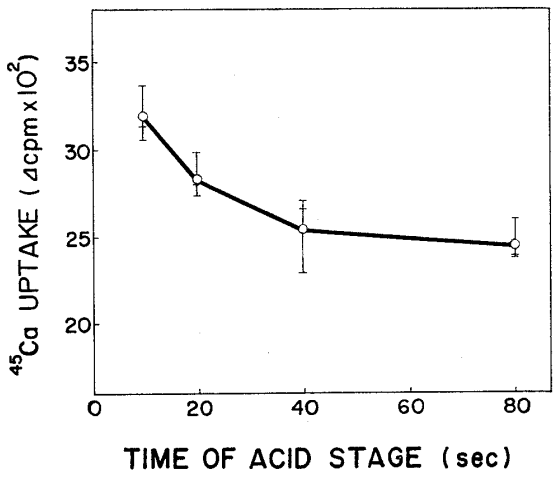

Fig. 1. Amount of acid-base induced uptake of calcium as a function of incubation time in an acid medium. Incubation time in the base medium was 50 sec. Acid medium, pH 3.8; base medium, pH 8.5.

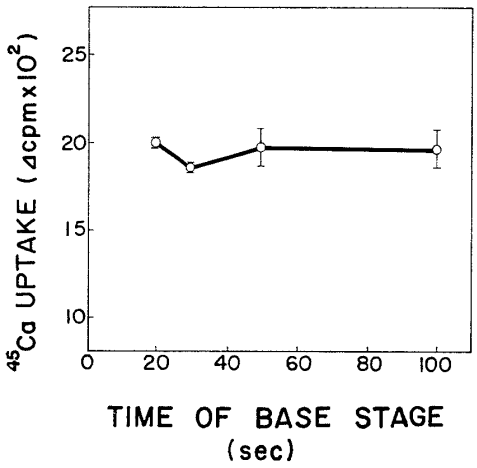

Fig. 2. Time course of acid-base induced calcium uptake in a base medium. Chloroplasts were incubated in the acid medium for 20 sec. Other conditions as in Fig. 1.

Table 1 shows typical data for acid-base induced calcium uptake of chloroplasts, following 20 sec of incubation in the acid medium and a subsequent 50 sec of incubation in the base medium. About a 30–35% increase in uptake of calcium was obtained as compared with that in the control sample incubated at pH's 7.2 and 8.3. The pH difference of 1.1 in the control runs was shown to be ineffective in inducing any uptake (see Fig. 3); a finding which is in harmony with experiments by JAGENDORF and URIBE on ATP formation.

2. Calcium uptake as a function of pH in the base stage

According to JAGENDORF and URIBE, yields of ATP induced by an acid-base transition were affected by both acid and base stage pH values. Hence, in our experiments, calcium uptake was examined as a function of pH in the base stage. As shown in Fig. 3, uptake was most pronounced with the highest pH in the base stage, namely, 8.3 in experiment I and 8.1 in experiment II, which correspond to pH differences of 4.5 and 4.3 units, respectively. Uptake was markedly diminished by decreasing the pH difference and at pH 7.2 (pH difference, 3.4) the calcium uptake was reduced to about 1/4 of the highest values obtained.

Table 1

Acid-base induced calcium uptake in spinach chloroplasts

Treatment	Exp. I			Exp. II		
	cpm	Δcpm	% increase	cpm	Δcpm	% increase
Control	4014			3414		
		1281	31		1235	36
Acid-base treated[a]	5295			4649		

Exp. I and Exp. II exemplify 2 parallel runs under the same experimental conditions.

[a] Acid medium, pH 3.8 for 20 sec; base medium, pH 8.5 for 50 sec.

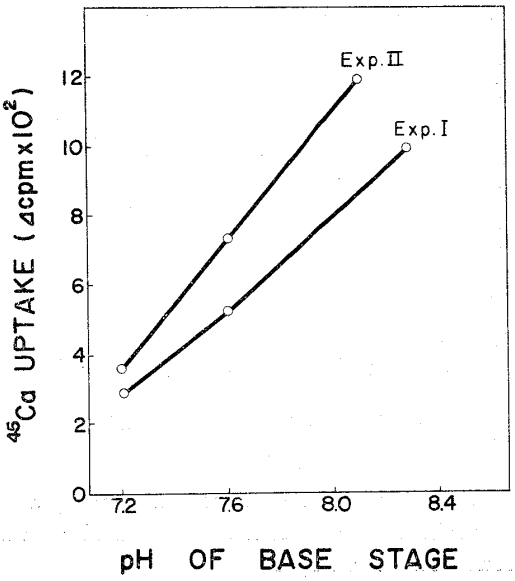

Fig. 3. Effect of pH difference on acid-base induced calcium uptake. Exp. I and Exp. II exemplify 2 parallel runs under the same experimental conditions. pH of the acid stage, 3.8.

3. *Decay of the energetic state at the base stage as measured by calcium uptake*

Decay of the energetic state produced in chloroplasts by the acid-base treatment was followed using the method devised by JAGENDORF and URIBE in their study of ATP formation. Calcium was introduced at various periods after the transfer of chloroplasts into the base medium. The uptake of calcium was measured as usual. As shown in Fig. 4, the capacity for calcium uptake was highest immediately after chloroplasts were transferred to the base medium, and it sharply diminished thereafter. After 30 sec, the effect of the acid-base treatment was not detectable.

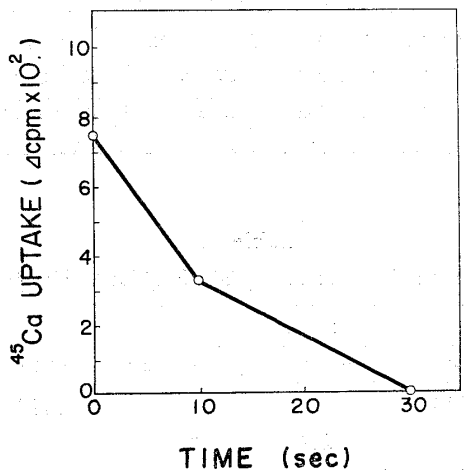

Fig. 4. Decay of acid-base induced capacity for calcium uptake in the base stage. ^{45}Ca was added 0, 10 and 30 sec after chloroplasts had been transferred to the base medium. Other conditions as in Fig. 1.

4. Effects of reagents

Table 2 shows that calcium uptake under investigation is inhibited by the uncouplers of photophosphorylation, including NH_4Cl, PMA and atebrin. Among the uncouplers tested, atebrin was the most effective in inhibiting calcium uptake. When ADP with 2 mM of $MgCl_2$ and phosphate was added to the base medium, calcium uptake was reduced by 40–60% (Table 3). These facts indicate that there is a competition between calcium uptake and ATP formation concerning the consumption of the high energy intermediate (or state) produced by the acid-base treatment.

5. Effects of organic acids

URIBE and JAGENDORF (*12*) showed various effects of various kinds of organic acids as the acid medium for the induction of ATP formation. In this experiment

Table 2

Effects of photophosphorylation uncouplers on acid-base induced calcium uptake

Uncouplers added	Exp. I			Exp. II		
	Conc (mM)	Δ cpm	% of control	Conc (mM)	Δ cpm	% of control
None	—	591	100	—	721	100
NH_4Cl	2	290	49	—	—	—
PMA	0.05	502	85	—	—	—
Atebrin	—	—	—	0.5	51	8

Acidified chloroplasts were transferred into base media (pH 8.5) containing each uncoupler.

Table 3

Effects of the presence and absence of ADP on acid-base induced calcium uptake

Exp.	Control (Δ cpm)	+ADP (Δ cpm)	% of control
1	1427	902	63.1
2	1064	1120	105.3
3	596	274	45.9
4	590	280	47.5

Acidified chloroplasts were transferred into a base medium (pH 8.5) containing ADP (4 mM), $MgCl_2$ (2 mM) and Na_2HPO_4 (2 mM).
ADP was excluded in the control.

Table 4

Effects of various dicarboxylic acids on acid-base induced calcium uptake

Acid	Conc (mM)	^{45}Ca uptake (Δ cpm)
Succinic	2.5	789
Malic	2.5	440
Glutamic	2.5	289

Acid medium pH 3.8; base medium pH 8.5.

the effects of organic acids including succinic acid, malic and glutamic acids on calcium uptake were examined. Table 4 shows that uptake was highest when succinic acid was used. Malic acid was less effective and glutamic least. The order of effectiveness of these organic acids is similar to that observed by URIBE and JAGENDORF in their experiments of ATP formation.

Discussion

That calcium ion is absorbed by the chloroplasts was first suggested by NISHIDA and KOSHII (*13*) from the results of their measurement of the optical density changes in chloroplasts suspended in $CaCl_2$ solution in the dark. DILLEY and VERNON (*4*), NOBEL and PACKER (*5*) and NOBEL (*1*) have shown that the uptake of calcium is accelerated by light, also that uptake is inhibited by uncouplers and energy transfer inhibitors. Thus, we inferred that the uptake of calcium has an intimate relation to the high energy intermediate (or state) produced in the chloroplasts.

The experiments reported here show that the uptake of calcium by chloroplasts in the dark can be accelerated by the acid-base transition. The conditions necessary to induce the uptake of calcium are similar to those required for acid-base induced ATP synthesis. These start out with the absolute requirement for a transition from an acidic (pH 3.8) to a basic (pH 8.3) environment. The responses of the 2 reactions toward various substances also bear similarities. An addition of NH_4Cl, PMA and atebrin inhibits both the uptake of calcium and ATP formation. Moreover, grades of effectiveness of various organic acids used as the acid medium are also similar in both cases. That the phosphorylating system, *i.e.*, ADP with Mg and phosphate, affects calcium uptake is highly interesting. This suggests that the uptake of calcium induced by the acid-base transition competes with phosphorylation for available energy.

The amount of acid-base induced calcium uptake obtained in the present experiments was around 0.8 mμmoles/mg chlorophyll under the most favorable conditions. This value is rather low as compared with that for light-induced calcium uptake as investigated by NOBEL (*1*). He obtained values mostly higher than 2 mμmoles/mg chlorophyll and even values as high as 16 mμmoles/mg chlorophyll when sulfhydryl reagents were added to the reaction mixture. JAGENDORF and URIBE obtained ATP formation around 100–200 mμmoles/mg chlorophyll by the acid-base treatment. This is also much higher in efficiency than the calcium uptake obtained in this study using the same treatment of chloroplasts.

Thanks are due to Messrs. TAMAI and HOSHINA for many helpful discussions.

References

(*1*) NOBEL, P. S.: Calcium uptake, ATPase and photophosphorylation by chloroplasts *in vitro*. *Nature,* **214,** 875–877 (1967).

(*2*) NOBEL, P. S.: Relation of swelling and photophosphorylation to light-induced ion uptake by chloroplasts *in vitro*. *Biochim. Biophys. Acta,* **131,** 127–140 (1967).

(*3*) PACKER, L. and P. A. SIEGENTHALER: Light-dependent volume changes and reactions in chloroplasts II. Action of anions. *Plant Physiol.,* **40,** 1080–1085 (1965).

(*4*) DILLEY, R. A. and L. P. VERNON: Ion and water transport process related to the light-dependent shrinkage of spinach chloroplasts. *Arch. Biochem. Biophys.,* **111,** 365–375 (1965).

(*5*) Nobel, P. S. and L. Packer: Light-dependent ion translocation in spinach chloroplasts. *Plant Physiol.*, **40,** 633–640 (1965).
(*6*) Jagendorf, A. T. and E. G. Uribe: ATP formation caused by acid-base transition of spinach chloroplasts. *Proc. Natl. Acad. Sci. U.S.*, **55,** 170–177 (1966).
(*7*) Mayne, B. C. and R. K. Clayton: Luminescence of chlorophyll in spinach chloroplasts induced by acid-base transition. *ibid.*, **55,** 494–497 (1966).
(*8*) Mayne, B. C.: The light requirement of acid-base transition induced luminescence of chloroplasts. *Photochem. Photobiol.*, **8,** 107–113 (1968).
(*9*) Kaplan, J. H., E. G. Uribe and A. T. Jagendorf: ATP hydrolysis caused by acid-base transition of spinach chloroplasts. *Arch. Biochem. Biophys.*, **120,** 365–370 (1967).
(*10*) Kaplan, J. H. and A. T. Jagendorf: Further studies on chloroplast adenosine triphosphatase activation by acid-base transition. *J. Biol. Chem.*, **243,** 972–979 (1968).
(*11*) Arnon, K. I.: Copper enzymes in isolated chloroplasts. Polyphenolase in *Beta vulgaris*. *Plant Physiol.*, **24,** 1–15 (1949).
(*12*) Uribe, E. G. and A. T. Jagendorf: Organic acid specificity for acid-induced ATP synthesis by isolated chloroplasts. *ibid.*, **42,** 706–711 (1967).
(*13*) Nishida, K. and K. Koshii: On the volume changes of isolated spinach chloroplasts caused by electrolytes and sugars. *Physiol. Plant.*, **17,** 846–854 (1964).