Journal of Bioenergetics and Biomembranes (1977) **9**, 195–201

On the Estimation of Proton Gradient and Osmotic Volume in Chloroplast Membranes

E. De Benedetti and F. M. Garlaschi

Istituto di Scienze Botaniche dell'Università di Milano, via G. Colombo 60, 20133 Milano, Italy

Received 4 February 1977

Abstract

The dependence of thylakoid osmotic volume on NH_4Cl uncoupling and on phosphorylation substrates is determined by the centrifuge filtration method. The values obtained are used to evaluate the transmembrane proton gradient in conjunction with either the 9-aminoacridine fluorescence quenching method or the [^{14}C]methylamine uptake method. The ΔpH values obtained with the two methods are compared and a linear relationship is demonstrated in the ΔpH range from 1.4 to 2.7 ([^{14}C]methylamine values). Different linear relationships are obtained depending on the presence or absence of electron acceptor. We conclude that the 9-aminoacridine method can be used for ΔpH determination after calibration with other methods.

Introduction

The 9-aminoacridine method of Schuldiner et al. [1] for ΔpH determination in chloroplasts has been questioned by several authors on the basis of the fact that the 9-aminoacridine fluorescence quenching is dependent on probe and chlorophyll concentration and independent of the osmotic volume of the thylakoids. This would indicate amine binding and aggregation in the inner phase of thylakoids [2, 3]. Such dependencies are not in accordance with the model of Schuldiner et al., which therefore should be discarded. On the other hand, as Graber and Witt [4] recently

pointed out, as long as 9-aminoacridine fluorescence quenching is proportional to $[H^+]_{in}/[H^+]_{out}$, it can be used for relative ΔpH measurements, and absolute values may be obtained by calibration with other methods.

In this paper we present data showing the conclusion that 9-aminoacridine method is completely unreliable [2] is not warranted, and we indicate conditions under which it can be used meaningfully. We have performed a comparison between two different methods of ΔpH measurement: the 9-aminoacridine method and the [^{14}C]methylamine uptake method. It is shown that a linear relationship exists between the results obtained with the two methods. The estimation of ΔpH requires the accurate measurement of the thylakoid osmotic space. We have investigated the influence of our experimental conditions on the inner volume of thylakoids.

Materials and Methods

Intact spinach chloroplasts were prepared using a modification [5] of the procedure of Jensen and Bassham [6]. After resuspension in a solution containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, pH 7.6, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.33 M sorbitol (medium A), chloroplasts were sedimented at $1000 \times g$ for 1 min and finally resuspended in the same medium. All procedures were performed between 0 and 4°C and chloroplasts were stored in ice. Before each reaction chloroplasts were osmotically disrupted by 20-fold dilution in medium A modified by the omission of sorbitol and with 5 mM $MgCl_2$ (medium B). After 2 min of incubation in the dark, reactions were started with light.

Osmotic volume determination and [^{14}C]methylamine uptake were performed by centrifugation through a silicone oil layer [7] in a 152 Beckman Microfuge. White saturating light, filtered through 4 cm of running water, was provided by a 300 W lamp. Experiments were performed in an air-conditioned room at 20°C and fast cooling of lamp and centrifuge was obtained with an air blower. Control of temperature is critical since changes of density of the silicone oil, due to small increases of temperature, cause inversion of the layers; this inconvenience cannot be overcome by the use of denser and more viscous oil since we have observed (unpublished results) that this may lead to serious underestimation of thylakoid osmotic volume. We have found suitable a mixture of Wacker AR silicones in the following proportion: AR 50/AR 100, 10 : 1. Microfuge tubes were prepared by layering 70 µl of silicone oil mixture over 25 µl of 1 M sucrose. Aliquots of 200 µl of incubation mixture, containing 80 µg/ml of chlorophyll and all the other components, were layered over silicone during the 2-min dark period. Quadruplicate samples were run

simultaneously. Illumination was for 2 min in the centrifuge, which was switched on after 1 min of light and run for a further 1 min. For osmotic volume determination tritiated water (30 μCi/ml) and 2 mM [^{14}C]sucrose (6 μCi/ml) were present during incubation; for methylamine uptake 10 μM [^{14}C]methylamine was present (0.5 μCi/ml). At the end of the centrifugation more than 95% of the chlorophyll was in the sucrose layer. Aliquots of supernatant were taken to determine the activity of $^{3}H_2O$ and [^{14}C]sucrose or [^{14}C]methylamine. The tubes were frozen to −80°C and then cut just above the sucrose layer. The bottoms of the tubes were placed in 5-ml centrifuge tubes with 0.5 ml of 5% perchloric acid, and the chloroplast pellet was resuspended with a glass rod. Denatured chloroplasts were sedimented and 0.25-ml aliquots of supernatant counted in polyethylene vials, with the addition of 10 ml of Instagel (Packard), in a liquid scintillation counter 3320 Packard. For the determination of osmotic volume of thylakoids it was assumed that water freely permeates thylakoid membranes [8] whereas sucrose does not [9]. The amount of incubation medium carried through with chloroplasts into the sucrose layer is very low, 0.5 to 0.7% of the suspending medium. ΔpH was determined according to Rottenberg et al. [10] from the [^{14}C]-methylamine distribution.

Measurement of the 9-aminoacridine fluorescence quenching was performed in a Perkin-Elmer MPF-3 spectrofluorimeter with 420 nm exciting light of 1000 ergs cm^{-2} sec^{-1} intensity. Emitted light was passed through the combination of a broadband Corning 4–96 filter and the monochromator of the instrument was set at 455 nm. Proton gradient was induced by actinic light filtered through a heat filter (Calflex C, Balzer) and a red Corning 2–58 filter. The actinic beam was directed on the sample by means of an Alflex-A mirror (Balzer) and light intensity was 200,000 ergs cm^{-2} sec^{-1}. 9-Aminoacridine was 1 μM chlorophyll 16 μg, in a final volume of 1 ml, unless otherwise stated. ΔpH was calculated according to Schuldiner et al. [1] using osmotic volumes determined, as described, under the same conditions and with every chloroplast preparation.

Ninety degree light scattering was determined under the same conditions of actinic illumination, with both monochromators set at 540 nm. Chlorophyll was 16 μg in a final volume of 1 ml.

Chlorophyll was determined by the method of Arnon [11].

Results and Discussion

Osmotic Volume

ΔpH was varied by treating chloroplasts with increasing concentration of ammonium or by phosphorylation substrates in the presence and absence

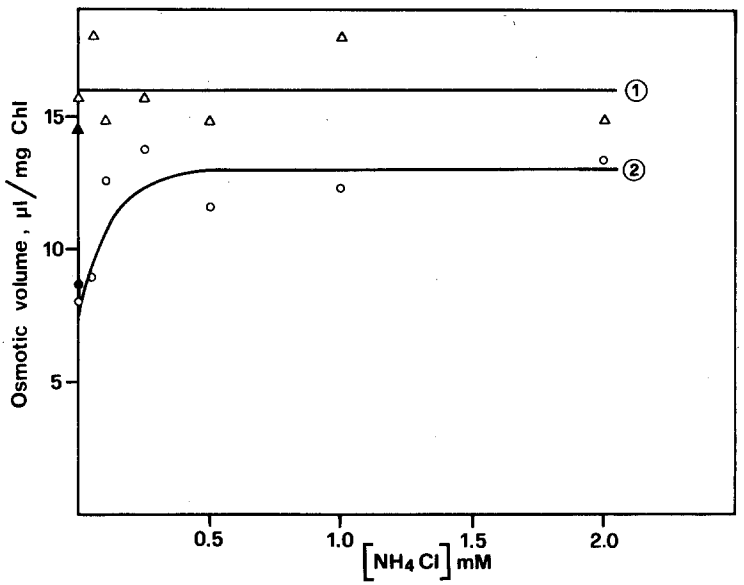

Figure 1. Osmotic volume as a function of NH_4Cl concentration. Conditions as in Methods. △, No electron acceptor; ○, 0.5 mM methyl viologen added. Filled symbols: 0.1 mM ADP plus 2 mM P_i added.

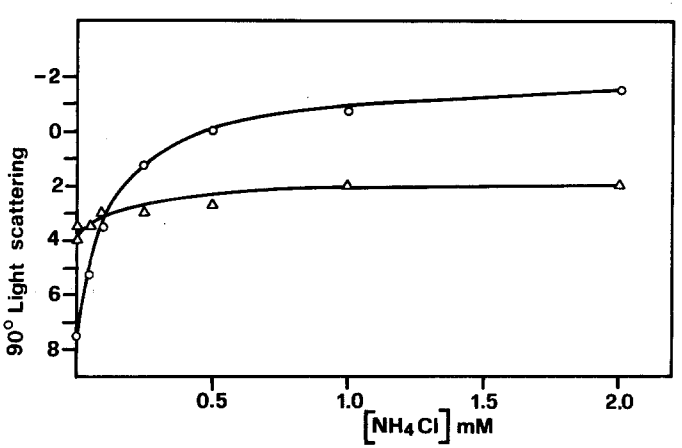

Figure 2. Light-dependent 90° light scattering as a function of NH_4Cl concentration. Arbitrary units. Ordinates represent the light scattering measured after 1 min of actinic light, minus that observed in the dark. Conditions as in Methods. △, No electron acceptor; ○, 0.5 mM methyl viologen added.

of the electron transport cofactor methyl viologen. The effect of these treatments on thylakoid osmotic volume is shown in Fig. 1. Curves 1 and 2 show osmotic volume as a function of NH_4Cl concentration and of ADP plus P_i in the absence of methyl viologen and in its presence, respectively. As can be seen, in the absence of electron acceptor no effect of NH_4Cl is observed, even though there was a considerable effect of the uncoupler on methylamine uptake measured in the same experiment. This rules out the possibility that NH_4Cl was not taken up under these conditions. In the presence of methyl viologen considerable shrinking of thylakoids occurs, up to 50% of their volume measured in the absence of electron acceptor. Here NH_4Cl induced swelling, its effect being saturated at around 250 μM. ADP plus P_i was without effect in both cases (Fig. 1, filled symbols). These observations are supported by independent 90° light scattering measurements. Light-dependent 90° light scattering changes have been related to thylakoid volume [9, 12]. Figure 2 shows that in the presence of methyl viologen NH_4Cl induced a decrease in the scattering of chloroplast suspension, and the concentration dependence of this effect is very similar to that of osmotic volume. In the absence of methyl viologen this effect, though discernible, is much less pronounced. The apparent discrepancy here between light scattering and direct osmotic volume determination may be due to the inexactness of the osmotic volume determination, where large errors [13] make it impossible to detect small variations.

Our results differ substantially from the findings of Rottenberg et al. [10] on the osmotic volume dependence upon NH_4Cl concentration in lettuce chloroplasts, but the difference may be attributed to the different preparation of the chloroplasts and to the tonicity of the incubation medium.

9-Aminoacridine Fluorescence Quenching and ΔpH

Figure 3 shows a plot of the values of ΔpH obtained by the 9-aminoacridine method versus those obtained by the [^{14}C]methylamine method in the same experiment. ΔpH was varied by increasing NH_4Cl concentration. Curves 1 and 2 refer, respectively, to an experiment performed in the absence of electron transport cofactor and to one performed with 0.5 mM methyl viologen present. From these data it is clear that, under our experimental conditions, a linear relationship exists between the two measurements in the ΔpH range from 1.4 to 2.4 (methylamine) without electron acceptor and from 1.4 to 2.7 (methylamine) with methyl viologen. The noncoincidence of curves 1 and 2 indicates that ΔpH is not the only factor affecting 9-aminoacridine fluorescence quenching, as suggested by Fiolet et al. [2] and Buchholz et al.

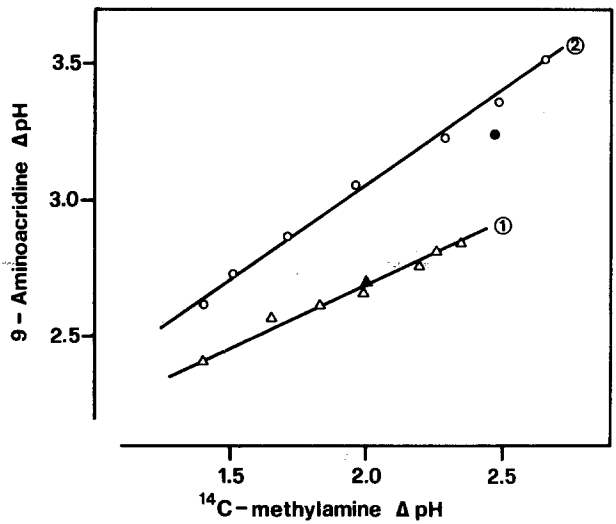

Figure 3. 9-aminoacridine ΔpH versus [^{14}C]methylamine ΔpH. Conditions as in Methods. △, No electron acceptor; ○, 0.5 mM methyl viologen added. NH_4Cl concentrations used: 0, 50, 100, 250, 500, 1000, 2000 μM. Filled symbols: 0.1 mM ADP plus 2 mM P_i added.

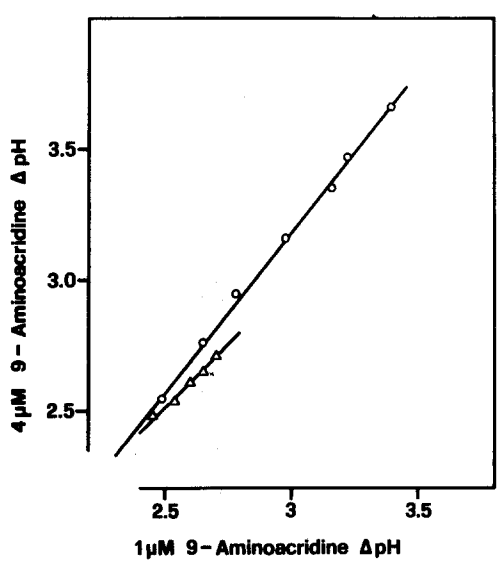

Figure 4. ΔpH measured with 9-aminoacridine 4 μM versus ΔpH measured with 9-aminoacridine 1 μM. Conditions as in Methods. ○, 0.5 mM methyl viologen added; ΔpH varied with NH_4Cl in the following concentrations: 0, 50, 100, 250, 500, 1000, 2000 μM. △, No methyl viologen; ΔpH varied with NH_4Cl in the following concentrations: 0, 50, 100, 250, 500 μM.

[3]. Indeed, according to their hypothesis, it seems conceivable that the different electron transport pathways involved in experiments 1 and 2 affect 9-aminoacridine fluorescence quenching differently (possibly via different effects on the charge state of the membrane).

Filled symbols in Fig. 3 refer to samples where ΔpH was varied by addition of the phosphorylation substrates ADP and P_i [14]. Whereas in the absence of methyl viologen no deviation from linearity is observed, in its presence the 9-aminoacridine method indicates a greater ΔpH decrease than the methylamine method.

Since it has been demonstrated [2] that 9-aminoacridine fluorescence quenching is dependent on probe concentration, we determined ΔpH at two probe concentrations, 1 and 4 μM, with and without electron acceptor. The results show (Fig. 4) a linear relationship between the two measurements. From this experiment and from the data in Fig. 3 we conclude that a linear relationship exists between the two methods of estimation of ΔpH if 9-aminoacridine concentration is also changed from 1 to 4 μM. Thus we conclude that the 9-aminoacridine method for ΔpH determination can be used in a certain ΔpH range and under defined conditions to provide relative ΔpH values. When absolute values are required it should be calibrated with other methods.

Acknowledgments

This work was supported by the Consiglio Nazionale delle Ricerche of Italy.

References

1. S. Schuldiner, H. Rottenberg, and M. Avron, *Eur. J. Biochem.*, 25 (1972) 64–70.
2. J. W. T. Fiolet, E. P. Bakker, and K. Van Dam, *Biochim. Biophys. Acta,* 368 (1974) 432–445.
3. J. Buchholz, Th. Heinze, and B. Rumberg, *5th International Biophysics Congress, Copenhagen 1975*. Villadsen and Christensen, Copenhagen (1975), p. 334.
4. P. Graber and H. T. Witt, *Biochim. Biophys. Acta,* 423 (1976) 141–163.
5. R. C. Jennings, F. M. Garlaschi, and G. Forti, *Biochim. Biophys. Acta,* 423 (1976) 264–274.
6. R. G. Jensen and J. A. Bassham, *Proc. Natl. Acad. Sci. U.S.A.,* 56 (1966) 1095–1101.
7. M. Klingenberg and E. Pfaff, in *Methods in Enzymology,* Vol. x, S. P. Colowik and N. O. Kaplan, eds., Academic Press, New York (1967), pp. 680–684.
8. H. W. Heldt and L. Rapley, *FEBS Lett.,* 7 (1970) 139–142.
9. R. A. Dilley and A. Rathstein, *Biochim. Biophys. Acta,* 135 (1967) 427–443.
10. H. Rottenberg, T. Grunwald, and M. Avron, *Eur. J. Biochem.,* 25 (1972) 54–63.
11. D. Arnon, *Plant Physiol.,* 24 (1949) 1–15.
12. L. Packer and S. Murakami, in *Methods in Enzymology,* Vol. xxiv, S. P. Colowik and N. O. Kaplan, eds., Academic Press, New York (1972), pp. 181–205.
13. A. R. Portis and R. E. McCarty, *J. Biol. Chem.,* 251 (1976) 1610–1617.
14. U. Pick, H. Rottenberg, and M. Avron, *FEBS Lett.,* 32 (1973) 91–94.