Biochimica et Biophysica Acta, 314 (1973) 354–359

BBA 46595

THE MEASUREMENT OF CYCLIC PHOTOPHOSPHORYLATION IN ISOLATED CHLOROPLASTS BY DETERMINATION OF HYDROGEN ION CONSUMPTION.
AN EVALUATION OF THE METHOD USING TITRATION AT CONSTANT pH

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(Received April 2nd, 1973)

SUMMARY

1. An improved method for the continuous measurement of phosphorylation is reported.

2. This method, in which the consumption of H^+ during the reaction is followed by titration at constant pH using commercially available apparatus, has been evaluated for photophosphorylation by isolated chloroplasts.

3. By use of an independent assay for ATP it is shown that the method gives a valid and precise measurement of photophosphorylation.

INTRODUCTION

The measurement of phosphorylation from the uptake of H^+ during ATP formation was first investigated by Nishimura *et al.*[1]. These workers measured the pH change associated with phosphorylation by suspensions of mitochondria and chromatophores. The method has subsequently been widely used for the measurement of photophosphorylation by isolated chloroplasts[2–4], and it therefore seemed desirable to check its validity by an independent assay of the ATP formed.

A reaction that consumes protons may be followed either by measuring the pH change or by titration of acid at constant pH. The necessity to calibrate pH changes by subsequent back titration, which should be done in the same conditions of illumination[5], is avoided by pH-stat titration. A further advantage of measuring photophosphorylation at a constant pH value is that both the rate of ATP formation and the ratio of H^+ consumed to ATP formed vary with pH[1].

This paper reports an evaluation of the pH-stat method for the continuous measurement of cyclic photophosphorylation in isolated chloroplasts, using commercially available apparatus.

MATERIALS AND METHODS

Chloroplasts preparation

Envelope-free spinach chloroplasts were prepared in sorbitol–phosphate

medium by the method of Emmett and Walker[6]. Chlorophyll was determined by the method of Arnon[7].

Titration at constant pH

The twin reaction vessels and apparatus for illumination were similar to those described previously[8] for photosynthetic oxygen evolution measurements. Each reaction mixture was contained in a cylindrical glass vessel (15-mm diameter) surrounded by a water jacket through which water at 15 °C was circulated from a Haake model FJ thermostat. The reaction mixture was vigorously stirred by a magnetic stirrer. The reaction vessel was illuminated by the 150-W quartz–iodine lamp of a slide projector. The beam was passed through a 150-mm-diameter spherical water-filled flask, a red filter (I.C.I. Perspex No. 400) transmitting 80–90% of light at wavelengths above 625 nm, and an infrared filter (Balzer interference filter, Calflex-C, Tempax).

The pH of the reaction mixture was measured by miniature glass and reference electrodes (Radiometer type G222C and K4112 respectively), connected to a Radiometer PM 26 pH meter. The output of the meter was recorded on one channel of a Rikadenki B 24 recorder. The pH of the reaction mixture was controlled by a Radiometer titration assembly consisting of a TT 11 titration control, ABU 12 autoburette, and SBR 2 recorder. The titrant was standardised 10 mM HCl, delivered to the reaction vessel by polythene tubing with a finely drawn out tip. The entire apparatus described was duplicated to allow two experiments to be run simultaneously.

The reaction mixture contained, in a total volume of 2.0 ml 0.33 M glucose, 10 mM KCl, 2 mM $MgCl_2$, 1.25 mM sodium phosphate, 1.25 mM potassium phosphate, 30 μM pyocyanine and chloroplasts equivalent to 100 μg chlorophyll. The pH was adjusted with 0.1 M HCl or NaOH to 0.05 unit over the pH-stat value set on the titration apparatus. The reaction mixture was then illuminated and the titration assembly switched on. After titration to the selected pH-stat value, phosphorylation was initiated by addition of approximately 0.6 μmole ADP.

ATP analysis

Firefly luciferin and luciferase were extracted from desiccated *Photinus pyralis* tails (Sigma) and partially purified by the methods of Nielsen and Rasmussen[9]. Light emission by assay mixtures was measured in a Nuclear-Chicago Unilux 2 scintillation counter by the method of Stanley and Williams[10]. Each series of assays was calibrated with freshly prepared ATP standard solutions and a linear relation was observed between count rate and amount of ATP within the range 1–10 pmoles ATP for assay. Assay specificity checks showed that the count rate per pmole ADP was less than 2% of that for ATP.

ATP was extracted from reaction mixtures as follows: 10 μl of chloroplast suspension was injected into 1.0 ml of ice-cold ethanol–5 mM EDTA (4:1, v/v). This was placed in a boiling water bath until the volume reduced to 0.1 ml (approx. 5 min), removed and placed on ice, and the volume made up to 1.0 ml with water. After centrifugation at $1000 \times g$ for 2 min, and 0.2 ml sample was taken and mixed with 50 μl 20 mM $MgCl_2$, 0.1 M potassium phosphate (pH 7.3). The ATP concentration of this solution was determined as described. Each series of assay was accom-

panied by triplicated control extractions from chloroplast suspensions containing a known amount of ATP standard. For all experiments reported the mean recovery of ATP in the control experiments was ±5% of the ATP added to the chloroplast suspensions.

Preparation of ATP and ADP solutions

ATP and ADP (sodium salts) were of the highest grade obtainable from the Sigma Chemical Co. The concentration of standard solutions was checked by absorbance measurements, and the solutions stored at −15 °C. The pH of ADP solutions was adjusted with 0.1 M NaOH to the same value as that to be maintained during the pH-stat titration. Standard ADP solutions were prepared immediately on receipt of ADP from the manufacturer, and were used within a few days.

RESULTS AND DISCUSSION

Simultaneous recorder traces of the pH of the reaction mixture and of the volume of titrant acid added are shown in Fig. 1. The pH of the reaction mixture during photophosphorylation is controlled by the apparatus to within 0.01 unit, apart from the initial transient excursion of pH on the addition of ADP. The rate of titration of acid remained linear until the reaction was about 90% complete, suggesting that the addition of titrant to the chloroplast suspension was without

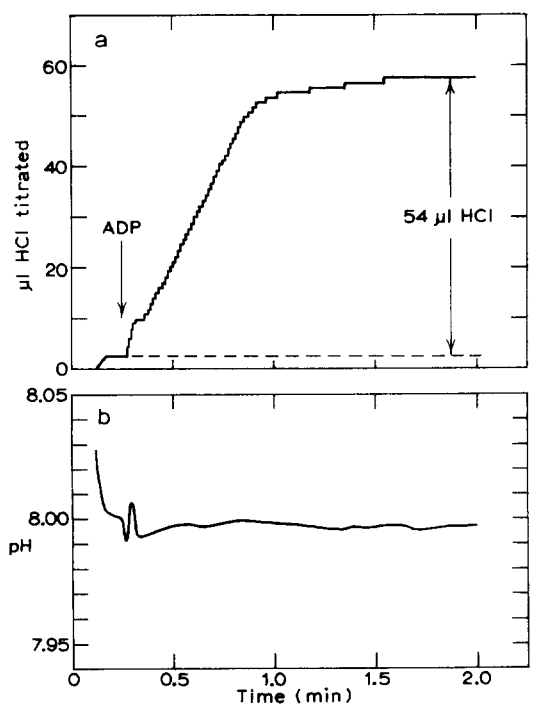

Fig. 1. Simultaneous recorder traces of (a) the volume of 0.0107 M HCl titrated and (b) the pH of the chloroplast suspension during photophosphorylation of 0.6 µmole ADP. Reaction conditions as described in Methods.

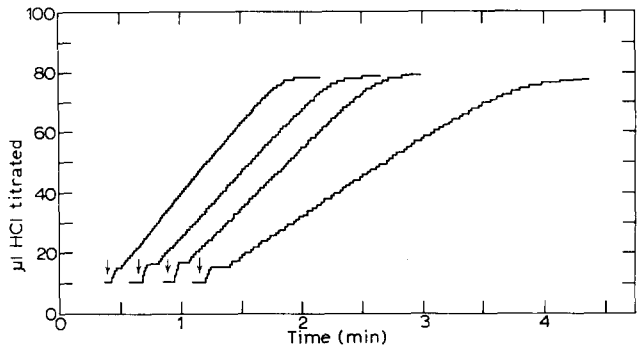

Fig. 2. Consecutive recorder traces of the volume of acid titrated showing the effects of chloroplast preparation ageing on the rate of H^+ consumption during photophosphorylation of equal amounts of ADP. The time interval between each measurement was 30 min, and the amount of ADP added, indicated by the vertical arrow, was approx. 0.8 μmole. Other conditions as described in Methods.

effect on the reaction (total dilution of reaction mixture $<5\%$). The rate of H^+ consumption was determined from the linear section of the titrant volume recording.

The consecutive traces in Fig. 2 have been chosen to illustrate the behaviour of a preparation of chloroplasts showing unusually marked ageing effects. Although the rate of H^+ consumption during photophosphorylation decreased as the chloroplasts aged, the total amount of H^+ consumed during phosphorylation of equal amounts of ADP was not affected. The decrease in rate with time was normally rather less than that exhibited by the particular chloroplast suspension used for this experiment. Ageing effects were compensated for in routine measurements by conducting control and test experiments simultaneously on the duplicated apparatus.

In Fig. 3, the relation between the rate of H^+ consumption during photophosphorylation and the amount of chloroplasts in the reaction mixture is shown. The rate of H^+ consumption during photophosphorylation measured by the appara-

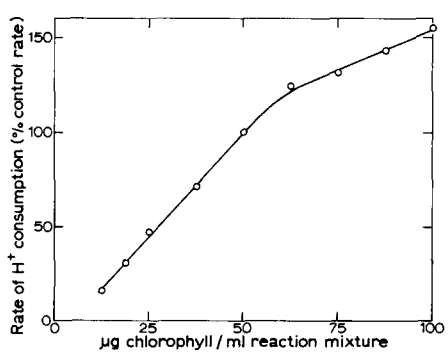

Fig. 3. The relation between the amount of chlorophyll present and the rate of H^+ consumption during photophosphorylation by spinach chloroplasts. Conditions, other than variation of chlorophyll content, as described in Methods. The control reaction mixture contained 50 μg chlorophyll/ml. Average control rate of H^+ consumption, 547 μmoles $H^+ \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$.

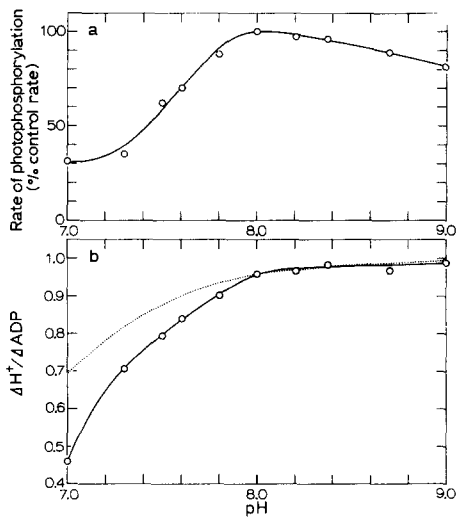

Fig. 4. Effect of pH on the rate of photophosphorylation and $\Delta H^+/\Delta ADP$. Conditions other than pH as described in Methods. (a) Rate of photophosphorylation determined from the rate of H^+ consumption and the $\Delta H^+/\Delta ADP$ value shown in (b). Average control rate, 332 μmoles ADP·mg^{-1} chlorophyll·h^{-1} at pH 8.00±0.01. (b) Effect of pH on $\Delta H^+/\Delta ADP$ determined from the total HCl titrated during photophosphorylation of 0.612 μmole ADP. The dotted line represents a theoretical curve calculated[1] for the magnesium salts of ATP, ADP and P_i.

tus was linearly related to the amount of chloroplasts in the suspension up to a chlorophyll concentration of 60 μg/ml. The slight departure from direct proportionality with decreasing chlorophyll concentrations is typical for photophosphorylation[11] and other chloroplast reactions[12], while the departure from linearity at high concentrations is caused by light limitation. Photophosphorylation with pyocyanine as cofactor requires extremely high light intensities for saturation[13]. The theoretical maximum rate of addition of titrant by the apparatus is 5 times greater than the highest rates observed here, and there was never any indication from the pH recordings of inadequate pH control.

Fig. 4 shows the effect of pH on the $\Delta H^+/\Delta ADP$ ratio and on the rate of photophosphorylation. The maximum rate was observed at pH 8.0. The measured values of $\Delta H^+/\Delta ADP$ were close to those calculated from the pK_a' values for the magnesium salts of ATP, ADP and P_i by Nishimura *et al.*[1] at pH values of 8.0 and above. However, at lower pH a progressive departure was observed. The values measured here are also lower than the $\Delta H^+/\Delta P_i$ ratio of 0.882±0.048 at pH 7.4 determined by these authors from the pH change during photophosphorylation by *Rhodospirillum rubrum* chromatophores. The reason for this disparity is not known, although a possible cause might be the influence of divalent cations other than magnesium in the chloroplasts on the pK_a' values for ATP, ADP and P_i. For practical purposes it is obviously desirable to determine the $\Delta H^+/\Delta ADP$ ratio for individual conditions.

The validity of pH-stat titration as a measure of photophosphorylation was tested by comparing the $\Delta H^+/\Delta ADP$ ratio measured during phosphorylation of a standard amount of ADP with $\Delta H^+/\Delta ATP$ ratios determined from the direct assay for ATP by firefly luciferase, and the results are shown in Table I. The $\Delta H^+/\Delta ATP$

TABLE I

STOICHIOMETRY OF H^+ CONSUMPTION DURING PHOTOPHOSPHORYLATION

pH 8.00 ± 0.01, reaction conditions described in Methods. Values are expressed as means ± S.E. (number of determinations).

$\Delta H^+/\Delta ATP$ (phosphorylation of approx. 50% added ADP)	0.967 ± 0.022 (8)
$\Delta H^+/\Delta ATP$ (phosphorylation to completion)	0.976 ± 0.020 (8)
$\Delta H^+/\Delta ADP$ (phosphorylation to completion)	0.959 ± 0.004 (14)

ratios were calculated from the amount of acid titrated after addition of approx. 0.8 μmole ADP to the illuminated chloroplasts, and the amount of ATP formed as determined by the firefly luciferase assay procedure. The reaction was terminated either by switching off the light after phosphorylation of about 50% of the added ADP in the first set of experiments, or by allowing the reaction to continue in the light until titration of acid ceased due to phosphorylation of all the ADP, as in the second set of experiments. The $\Delta H^+/\Delta ADP$ ratio was determined from the total amount of acid titrated during photophosphorylation of a standard amount of ADP. Similar mean values for $\Delta H^+/\Delta ATP$ were obtained whether phosphorylation was allowed to proceed to completion in the light, or terminated at 50% completion. The volume of acid titrated by the apparatus should not be affected by any net H^+ efflux from the chloroplast thylakoids on darkening, if phosphorylation in the dark is negligible. The $\Delta H^+/\Delta ATP$ values were not significantly different from the mean $\Delta H^+/\Delta ADP$ ratio. This gives considerable confidence that under these conditions of cyclic photophosphorylation, the reaction mixture is free from any variable side reactions involving hydrogen ions, and from any other reactions involving ADP or ATP.

It may be concluded from these results that the continuous recording of H^+ consumption by titration at constant pH in the apparatus described can be used as a convenient and precise method for the continuous measurement of photophosphorylation.

ACKNOWLEDGEMENTS

We wish to thank Mrs K. Holborow and Mrs C. Case for their technical assistance. This work was supported by a grant from the S.R.C. (U.K.).

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