The Isolation of Spinach Chloroplasts in Pyrophosphate Media

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Abstract. A simplified procedure (involving disruption in sorbitol-pyrophosphate mixtures) permits the separation of spinach chloroplasts which retain the ability to catalyze the photosynthetic assimilation of carbon dioxide and its associated oxygen evolution.

Photosynthetic carbon assimilation by isolated chloroplasts was first demonstrated by Arnon, Allen, and Whatley in 1954 (1). Subsequently many attempts have been made to bring about increases in rate. The respective contributions made by various groups have been recently discussed and need not be reiterated here (12,15). In retrospect, however, it seems clear that there were really 2 problems. The first was to isolate chloroplasts with intact envelopes, the second to devise reaction mixtures which would permit maximal rates and minimal loss of activity. There is now ample evidence that the ability of chloroplasts to catalyze extra-cellular photosynthesis is related to the integrity of the bounding membrane (14,15,17,18,20). Electron microscopy has shown that the most active chloroplasts retain an intact double envelope (14,18).

The isolation of chloroplasts with intact envelopes is facilitated by brief disruption, rapid separation and the use of a sugar or sugar alcohol (rather than NaCl) to maintain the osmotic pressure (16,18). The introduction of these variants in existing techniques brought about an immediate 10-fold increase in activity (16). Subsequent increases reported from Imperial College (5,6,7,9,10,17,18) culminating in rates of CO₂ fixation of just over 100 μmoles/mg chlorophyll/hr (9) involved no further improvements in separation, the technique remaining essentially unchanged for several years. They followed naturally upon the recognition of the initial lag or induction period (see e.g. 6,7,8,11,18) and upon certain changes in the conditions of assay. They were largely incidental to the main purpose of the investigation.

After the invaluable work of Good and his colleagues (13) on new buffers, an important contribution was made by Jensen and Bassham (14) when they used MES and inorganic pyrophosphate in their media in preference to orthophosphate (cf. 10).

Consequently, rates in excess of 100 were obtained for the first time (14) in reaction mixtures containing no added intermediates of the Benson-Calvin cycle. Such additives were necessary for the highest rates observed in the earlier work but following a reinvestigation it seems clear that sugar phosphates were not needed to overcome any intrinsic synthetic deficiency in the chloroplasts themselves but rather one which was imposed by the presence of orthophosphate in the grinding medium and the reaction mixtures (2,3,4,19).

Like other workers (see e.g. 15) we have also recently attempted to simplify existing procedures in the light of these findings. Our aim has not been to achieve the maximum possible rates but to devise the simplest possible procedure which might assist further investigations into cell-free photosynthesis.

Materials and Methods

Isolation of Chloroplasts. Chloroplasts were isolated from spinach leaves which were grown at the Chelsea Physic Garden or purchased at Covent Garden Market. Chilled laminae (50 g) were homogenized for 3 to 5 seconds in a domestic blender containing 200 ml of a semi-frozen solution containing sorbitol 0.33 M; MgCl₂, 5 mM; sodium isoascorbate, 2 mM and Na₄P₂O₇·10H₂O, 10 mM adjusted to pH 6.5 at 0° with HCl. This solution was prepared immediately before use from a stock solution of MgCl₂ and solid stock mixtures of sorbitol and pyrophosphate. Sodium isoascorbate was added after pH adjustment. The macerate was squeezed through 2 layers of muslin and filtered through 8 layers into 50 ml plastic tubes then centrifuged at 0° from rest to 4000g to rest, in approximately 90 seconds (other combinations of maximum centrifugal force and time may be necessary to suit the performance of particular centrifuges). The supernatant was decanted and the pellets were gently resuspended using a glass rod and a small piece of absorbent cotton wool in 1.0 ml of an ice cold...
solution containing sorbitol, 0.33 m; MgCl₂, 1.0 mm; MnCl₂, 1.0 mm; EDTA, 2.0 mm and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid). 50 mm adjusted to pH 7.6 at 20° with NaOH. At this point in the procedure the suspension was stored in an ice bath and the chlorophyll concentration measured so that assays could be performed with a standard amount of chlorophyll.

Measurement of Oxygen Evolution. Oxygen evolution by the chloroplasts was assayed in a solution identical to the resuspending solution described above except that it also contained NaHCO₃, 10 mm. The chlorophyll concentration in all assays was 100 μg/ml.

Evolution of oxygen was estimated polarographically (4) in a perspex, water-jacketed cell containing a basal Clark-type electrode separated from the magnetically-stirred assay mixture by a teflon membrane. The output of the cell was recorded electrically.

The difference between the output of the electrode system with air saturated water at 20° and water containing sodium dithionite was taken to represent 0.28 μmole of oxygen per ml. This value agreed with calibrations made using standardized H₂O₂ and catalase.

The electrode cell was illuminated by a 150 watt Quartz-Iodine slide projector, the light from which passed through 14 cm of water and a Balzer (Calflex C Tempax) heat filter. The assay solution was maintained at 20° ± 1° by circulating temperature controlled water through the jacket of the cell.

Storage Properties of Pyrophosphate Isolated Chloroplasts. In experiments in which the storage properties of pyrophosphate isolated chloroplasts were investigated the suspension was stored in an ice bath and aliquots were removed at suitable intervals and brought up to 20° in the assay medium. Oxygen evolution was then measured as described above.

Comparison of Chloroplasts Isolated in Pyrophosphate or MES Buffered Media. A comparison was made between chloroplasts isolated in the media described above and those described by Jensen and Bassham (14). The isolating medium described by these workers contained: sorbitol, 0.33 m; NaNO₃, 2 mm; EDTA, 2 mm; sodium isoascorbate, 2 mm; MnCl₂, 1 mm; MgCl₂, 1 mm; KH₂PO₄, 0.5 mm; NaCl, 20 mm and MES [2-(N-morpholino) ethanesulfonic acid]. 50 mm adjusted to pH 6.1 with NaOH.

The resuspending medium (14) contained all the above constituents except MES. Instead it contained HEPES, 50 mm and was adjusted to pH 6.7 with NaOH. The assay solution was similar to the resuspending solution except that it did not contain NaCl contained Na₄P₂O₇, 10 H₂O, 5 mm; NaHCO₃, 10 mm and was adjusted to pH 7.6 with NaOH.

The experimental procedures were identical to those described above with reference to the preparation involving the use of pyrophosphate buffered media.

Results

Storage of Pyrophosphate Isolated Chloroplasts. During the first hour or so of storage at 0° it was found that the capacity of the chloroplasts to evolve oxygen increased, usually by about 25% of the initial rate (fig 1). Following this period of increase the rates of oxygen evolution obtained remained relatively constant for several hours after which a progressive loss of activity was observed.

Comparison of Chloroplasts Isolated in MES or Pyrophosphate Buffered Media. Figure 2 shows the course of oxygen evolution associated with CO₂ fixation by illuminated spinach chloroplasts isolated using pyrophosphate buffered media and also the solutions described by Jensen and Bassham. The respective preparations were made using leaf samples which were as far as possible identical. The experiment illustrated is representative of several, in all cases oxygen evolution commenced more rapidly and frequently attained a higher rate with chloroplasts isolated in the pyrophosphate buffered media.

Over a period of several months a considerable number of chloroplast preparations were made using either the pyrophosphate buffered media or the Jensen and Bassham media (14). Figure 3 shows the rates of oxygen evolution attained in these experiments and also the frequency of occurrence of each rate. The highest rate of all was obtained using the pyrophosphate preparation and generally the rates were higher with this method (54% of the pyrophosphate preparations attained rates in excess of 50 μmoles O₂ per mg chl per hr whereas only 17% of the preparations isolated in the other media exceeded this figure).
Discussion

Chloroplasts prepared in mixtures containing sugars such as sucrose, glucose, or fructose (16, 18) or sugar alcohols such as sorbitol (14, 15, 16, 18) or mannitol (15) are known to retain their outer double envelopes for relatively long periods and to show high rates of CO₂ fixation and its associated oxygen evolution. If orthophosphate is used to maintain the pH the best rates are realized only in the presence of added intermediates of the Benson-Calvin cycle. This effect may be avoided by the use of MES (13) or as shown in this paper by the use of inorganic pyrophosphate (cf. 14, 15). In some work the use of a buffer such as MES (13), which is not also a metabolite, may be obligatory. For other purposes there are obvious advantages attached to the use of standard laboratory reagents which are cheap and readily available. Sodium pyrophosphate comes into this category and in the present procedure it is used only in the grinding medium (so that it is discarded as a supernatant almost immediately) and is then replaced by another buffer such as HEPES (13) at a stage when the volumes involved are so small that the cost of reagents is of little consequence.

We believe that the present procedure may appeal to workers who have no past experience of chloroplast isolation but who may wish to employ chloroplasts for routine tests. There is little doubt, for example, that the combination of a readily obtainable cell-free photosynthetic system and an oxygen electrode would lend itself to work on herbicides or atmospheric pollutants. The point we wish to stress is that the isolation of entire chloroplasts is no longer the prerogative of laboratories with special interests in this field. If facilities are available which will allow the separation of chloroplasts capable of supporting the Hill reaction then the investigator should experience no additional difficulty in obtaining chloroplasts which will utilize CO₂ (as the precursor of the hydrogen acceptor) as readily and as rapidly as ferricyanide or other Hill reagents.

Literature Cited


