Correlation between Photosynthetic Activity and Membrane Integrity in Isolated Pea Chloroplasts

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Introduction

Although it is widely recognized that the photosynthetic activity of isolated chloroplasts may be modified by changes in structure during extraction it is by no means certain to what extent these effects can be attributed to the presence or absence of the chloroplast membrane. The difficulties experienced in defining structural modifications in isolated chloroplasts and relating these to changes in activity are great and call for considerable caution in interpretation. However, evidence is now available (8, 10) which supports the view that chloroplasts which appear opaque under the light microscope have retained their membranes whereas those which have lost their membranes appear granulated (9). By this criterion, chloroplasts isolated from peas (11) initially contain a large proportion of chloroplasts with intact membranes. This paper describes the use of these preparations in an attempt to investigate the relationships between photosynthetic activity and membrane integrity.

Materials and Methods

Plant Material. Peas (var. Laxton Superb and Feltham First) were grown for 13 to 16 days in moist vermiculite in a box which was illuminated for 11 hours out of each 24 by five 40-w fluorescent tubes (Ecko Daylight) at a distance of 30 inches. This gave a light intensity of approximately 150 ft-c. In the dark period the temperature was maintained at 15° ± 1 but in the light period it increased to between 20° and 25° according to the ambient temperature. Taken from the dark, the leaves contained little or no starch.

Reagents. Pyocyanine, prepared from phenazine methosulphate by the method of McIlwain and purified by repeated passage from chloroform to acid, was kindly donated by Dr. Geoffrey Hind. Trishydroxymethylglycine (Tricine) was prepared according to Good (6) and twice recrystallized from aqueous ethanol.

Inorganic Phosphate Estimation. Inorganic phosphate was determined by a modification (7) of Allen's method (1). The disappearance of Pi in the light is assumed to represent ATP formation from ADP. It has been shown that the Pi content of similar reaction mixtures was restored to the level prior to illumination by 7-minute hydrolysis in N HCl (7).

CO_2 Fixation. This was estimated by using C^{14}, as before (11). The method used to relate counts recorded to μmoles CO_2 fixed (11) was also checked against standard solutions in a scintillation counter and its validity confirmed.

Illumination. Reaction mixtures were illuminated in saturating light (> 4000 ft-c) provided by a circular bank of nineteen 150-w tungsten bulbs. The apparatus was essentially a more sophisticated version of one previously described (7). Temperature was maintained at 20° ± 0.25°.

Chloroplasts. Whole chloroplasts were prepared from 50 g of leaf material by a method similar to one previously described (11). In this method a Waring blendor (M.S.E. Atomix) is used to disrupt the cells but the leaf material is added to the grinding medium with the blades revolving at low speed and when the last leaf is drawn below the surface (after approx. 10 sec) the speed is increased to maximum for only 5 seconds. This is necessarily a relatively inefficient homogenization but a large proportion of chloroplasts survive in an apparently entire state. In preliminary experiments it was found that the concentrations of sorbitol used previously (11) interfered with the phosphate estimation and therefore in all the experiments described in this paper sucrose was substituted for sorbitol. Resuspending medium for whole chloroplasts contained 0.1 % NaCl, 0.1 % $MgCl_2$ 0.175 % sodium isoascorbate in 0.45 M sucrose. In all but one of the reported experiments chloroplasts without membranes were prepared by resuspending the pellet of whole chloroplasts in media from which only the sucrose was omitted. In most experiments additional sucrose was added to reaction mixtures which were to receive membrane-free chloroplasts so that the final concentration was the same as that in reaction mixtures containing whole chloroplasts. In the experiment illustrated in figure 5, whole chloroplasts were added directly to reaction mixtures from which the sucrose had been omitted.

Chloroplasts were always prepared immediately before use and added to each reaction mixture 20 seconds before illumination. Reactions were started and stopped sequentially at 1-minute or 30-second intervals. Where different periods of illumination were necessary the reactions which were to last longest were started first in order to minimize any

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effect of aging. Normally less than 45 minutes elapsed between cutting the leaves from the growing plants and terminating the last reaction.

Chlorophyll Determination. Duplicate samples were extracted in 80 % acetone in subdued light. After centrifugation in stainless steel tubes the chlorophyll content was determined by spectrophotometric measurement at 652 mμ (*2*).

Microscope. Chloroplasts were examined using a Gillet-Sibert photomicroscope equipped with phase contrast and lenses which gave a magnification of 2000 diameters.

Results

Microscopy. Using a phase contrast microscope with the condenser and iris diaphragm adjusted to give conditions approximating to dark ground illumination, 2 types of chloroplast could be readily distinguished. The majority were opaque and shiny with a halo which made them appear to stand out from the dark background. The remainder were dark and granulated, somewhat larger, flatter, and without the halo. The difference in appearance was pronounced and independent of the depth of focus. Chloroplasts prepared on sucrose density gradients by Dr. Rachel Leech, which have been shown by electron microscopy (10) to contain 90 % or more chloroplasts with intact membranes, were also examined in this way and again the same 2 categories were seen, the great majority opaque and haloed and the remainder dark and granulated. Following exposure to hypotonic media all the chloroplasts appeared dark and granulated. On this basis, and with regard to the results of Kahn and Von Wettstein (9) and Leech and Greenwood (10) the shiny opaque chloroplasts were identified as entire and the dark granulated chloroplasts were identified as ones which had lost their bounding membranes. This has now been confirmed by A. D. Greenwood in this department who has shown by electron microscopy that the entire chloroplasts retain their outer double membrane and their stroma. Routine examination of whole chloroplast preparations indicated that the proportion of chloroplasts without intact membranes was usually only about 20 % though in some experiments it fell to as little as 10 % and in others it increased to as much as 50 %. The source of this variation is not known but in earlier work peas which had been exposed to relatively high temperatures ($> 25°$) during growth invariably gave chloroplast preparations which fixed CO_2 at poor rates and our inadequately controlled growing conditions are therefore suspected.

Whole chloroplasts resuspended in media from which the sucrose was omitted lost their membranes within a matter of seconds and it was not normally possible to find any opaque chloroplasts by the time a sample could be placed under the microscope. Subsequent fragmentation occurred only slowly and the membrane-free chloroplasts remained relatively entire

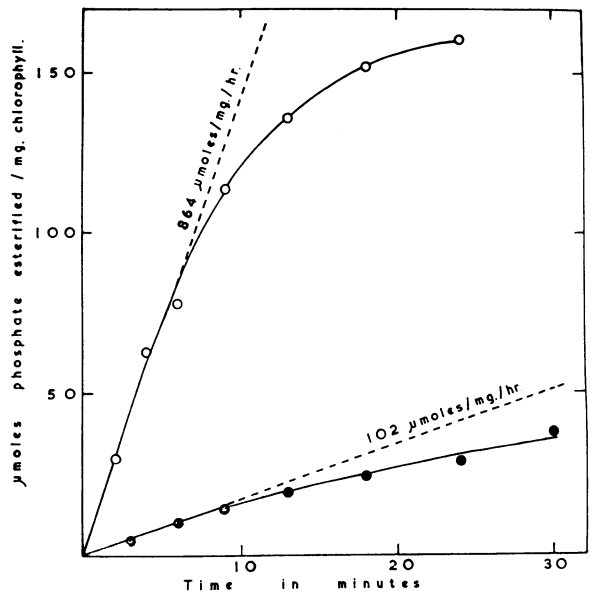

FIG. 1. Progress curve of cyclic photophosphorylation by whole and membrane-free chloroplasts. Reaction mixtures contained in a final volume of 0.3 ml, inorganic phosphate, 2 μmoles; ADP 2 μmoles (both at pH 7.5); pyocyanine, 0.006 μmole; Tris-HCl buffer, pH 7.5, 6.6 μmoles; 0.1 ml of chloroplast suspension containing whole (●) or membrane-free (○) chloroplasts. The respective chlorophyll content was 0.032 mg (whole) and 0.010 (membrane-free). Reaction mixtures containing membrane-free chloroplasts also contained additional sucrose to compensate for that omitted from the resuspending medium.

for half an hour or more though they showed some tendency to swell and also to coalesce with one another when they came into contact. After 10-minutes illumination at 20° in phosphorylating reaction mixtures the appearance of both whole and membrane-free chloroplast preparations (now in identical media) seemed much the same as it was immediately after isolation nor was there any readily detectable difference in the proportion of whole chloroplasts. It should be emphasized of course, that any visual estimate of the proportion of whole chloroplasts had of necessity to be based on a very small sample from a very large population.

Photophosphorylation. Figure 1 shows a time course for cyclic photophosphorylation in which the 2 types of chloroplast suspensions were compared. This represents the largest difference observed in a series of experiments in which half of the chloroplast pellets prepared from a single batch of leaves were resuspended in normal media to give largely whole chloroplasts and the remainder were suspended in hypotonic media to give membrane-free chloroplasts. In a number of observations based on single time intervals as well as complete time course experiments the rates of phosphorylation by whole chloroplasts were always less than those catalyzed by membrane-free chloroplasts but the degree of dif-

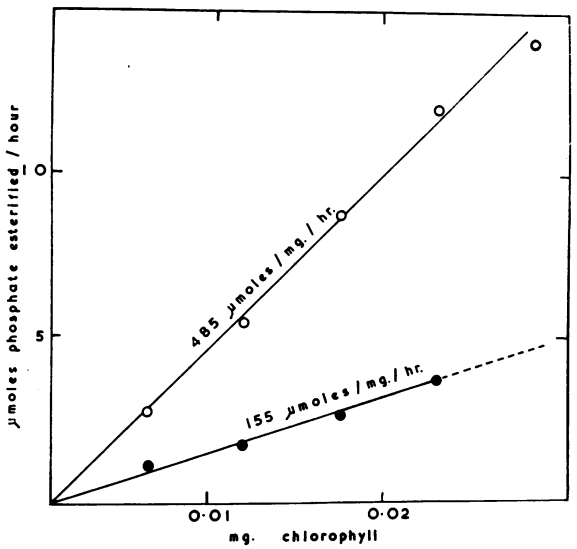

FIG. 2. Photophosphorylation by whole and membrane-free chloroplasts as a function of chlorophyll concentration. Reactants mixtures as for figure 1 except that the mixtures contained 0.02, 0.04, 0.06, 0.08 or 0.10 of whole (●) or membrane-free (○) chloroplast preparations and 0.08, 0.06, 0.04, 0.02 and zero of the appropriate resuspending medium. Chlorophyll content as indicated in the figure.

ference varied considerably from preparation to preparation. Expressed as a percentage of the rate achieved by membrane-free chloroplasts the phosphorylation by whole chloroplasts varied between 65 % and 12 %. As stated above, estimates of the percentage of membrane-free chloroplasts present in these whole chloroplast preparations varied between approximately the same limits.

Figure 2 shows that at low concentrations the relationship between chlorophyll concentration and rate of photophosphorylation was linear for both whole and broken chloroplasts so that at least within this range it would seem permissible to compare rates (on a chlorophyll basis) in reaction mixtures containing different amounts of chlorophyll (as in fig 1). At higher chlorophyll concentrations there was some experimental indication that departure from linearity might occur at a lower concentration in whole chloroplasts than it did in membrane-free chloroplasts. In the majority of experiments the chlorophyll content of whole and membrane-free preparations was adjusted so that it was the same (or approximately the same) in each.

Figure 3 shows a time course in an experiment in which the normal procedure was modified. Only whole chloroplasts were prepared and aliquots of these were then added to reaction mixtures containing either the normal sucrose concentration or to reaction mixtures from which the sucrose had been omitted. In a previous experiment it had been shown that the initial rates of photophosphorylation by membrane-free chloroplasts were identical in reaction mixtures with and without added sucrose so that it is probable that the difference in rate in figure 3 can be attributed entirely to membrane loss. In this experiment it can be seen that the rate achieved in the absence of sucrose was greatest at the outset and showed no tendency to increase with time. This would suggest that membrane-loss occurred very rapidly, and had gone to completion in the 20 seconds between the addition of the chloroplasts and the start of illumination. This is consistent with the visual observation (see above). In an additional experiment in which whole chloroplasts were preincubated in sucrose-free reaction mixtures for periods ranging between 30 seconds and 10 minutes there was no subsequent detectable difference in rate. It will be seen that in the lower curve in figure 3 (whole chloroplasts in reaction mixtures containing sucrose) there is a suggestion of a late increase in rate which

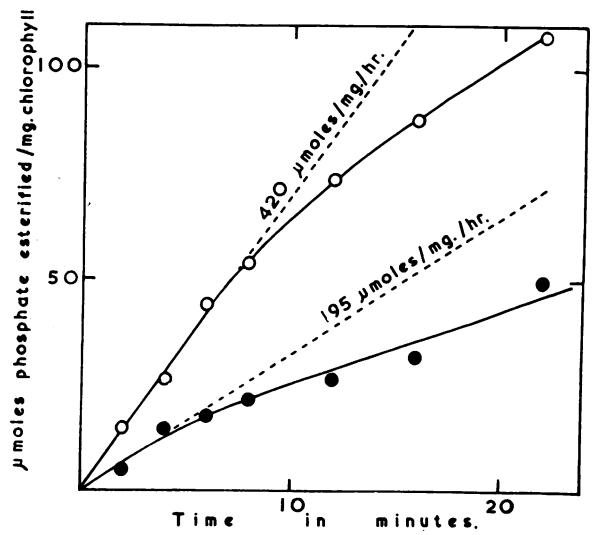

FIG. 3. Progress curves of cyclic phosphorylation in reaction mixtures of different tonicity. Reaction mixtures as for figure 1 except that 0.02 ml of whole chloroplasts were added to mixtures which initially contained 0.1 ml of 0.45 M sucrose (●) or no sucrose (○). Chlorophyll, 0.0108 mg.

has been ignored in drawing the curve. A similar tendency has been observed in 2 other experiments but enough data is not at present available to rule out coincidental experimental error. A late increase of this sort would be expected if slow membrane loss in sucrose containing reaction mixtures was not offset by thermal denaturation and other factors.

CO_2 Fixation. Figure 4 shows the progress curves for CO_2 fixation by whole and membrane-free chloroplasts. This was the converse of the photophosphorylation results in that the higher rate was now supported by the whole chloroplasts. The initial lag or induction period has been observed in a number of time course experiments with whole chloroplasts and has varied between 1 and 6 minutes.

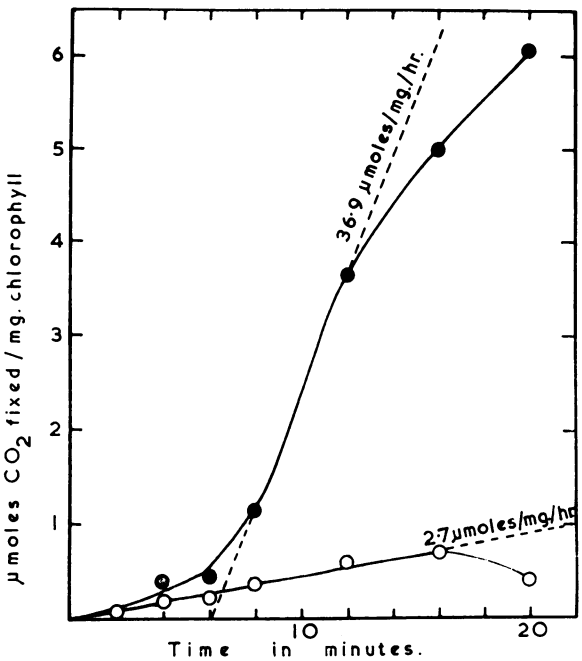

FIG. 4. Progress curves of CO_2 fixation by whole and membrane-free chloroplasts. Reaction mixtures contained in a final volume of 0.3 ml Pi, 0.25 μmole; $MgCl_2$, 0.25 μmole; $MnCl_2$, 0.25 μmole; EDTA, 0.25 μmole; Na isoascorbate, 0.50 μmole; tricine-NaOH, pH 7.5, 7.5 μmoles; GSH, 1 μmole; ribose-5-phosphate, 2 μmoles. Whole (●) or membrane-free chloroplasts (○) in 0.1 ml suspension containing 0.028 mg chlorophyll. Reaction mixtures containing membrane-free chloroplasts also contained additional sucrose to compensate for that omitted from the resuspending medium.

This seems to be in accord with a previous observation by Gibbs and Bamberger (3, 5) of a lag period of about 5 minutes which could be abolished by the addition of certain sugar phosphates. Under the present experimental conditions ribose-5-phosphate stimulated the maximum rate of CO_2 fixation and also diminished the length of the even longer lag period which was observed in its absence. It remains a possibility therefore that the induction period in figure 4 might be attributable in part to the time taken for ribose-5-phosphate to penetrate the intact membranes. A decrease in the ability of whole chloroplasts to fix CO_2 in the light following exposure to hypotonic media is not in any sense a new observation (see e.g. Whatley et al. 12). It is included here for completeness and also because it supports the interpretation that the visual changes represent membrane loss followed by loss of stroma.

Discussion

Preparations from peas containing a mixture of whole and membrane-free chloroplasts supported slower rates of photosynthetic phosphorylation than those in which all the chloroplasts had lost their membranes (cf. Whatley et al. 12). The difference between the rates was sufficiently large to suggest that most, if not all, of the photophosphorylation may have been mediated by membrane-free chloroplasts. It might be inferred that the intact membrane restricts or excludes the entry of an exogenous reactant such as ADP or a cofactor such as pyocyanine or alternatively that it prevents the dilution of an active endogenous adenosine triphosphatase. There are of course many other possible explanations but in view of the relative rate of the respective in vitro systems it seems impossible that the utilization of ATP in CO_2 fixation could account for the observed differences. In the absence of added sugar phosphate, fixation could be expected to fall below 5 μmoles/mg chlorophyll hour (11) and CO_2 fixation by whole chloroplasts is also strongly depressed by pyocyanine at the concentration used.

Recently, Gee et al. (4) have established that it is possible, with membrane-free chloroplasts, to achieve labelling patterns resembling those in the intact leaf and their comprehensive study also serves to underline the complexities of the various factors involved and the importance of intra-organelle integrity. Quantitatively, however, it seems probable that the decreased ability of chloroplasts to fix CO_2 when they have lost their membranes is at least partly attributable to the loss of soluble enzymes (12). Conversely the ability of whole chloroplasts from peas to fix CO_2 at relatively fast rates must in some part be related to the large proportion which have intact membranes. This in turn may reflect the toughness of the pea chloroplast membrane rather than the superiority of the isolation procedure. Applied to chickweed (*Stellaria media*) the normal technique yielded chloroplasts (in sucrose) which were almost entirely devoid of membranes. Work by James, Leech and Greenwood (8, 10) on which the present criteria for whole and membrane-free chloroplasts is largely based, also established that it is possible to isolate almost completely pure membrane-free chloroplasts from media of relatively high osmotic pressure. It seems more than likely therefore, that many chloroplast preparations described in the literature as whole may have contained a large proportion of otherwise entire chloroplasts which had lost their membranes and the truly intact chloroplast may therefore be less permeable than may have been imagined.

D. Spence and H. Unt (Aust. J. Biol. Sci., 1965, 18: 197–210) have now reported decreased rates of photophosphorylation in spinach chloroplasts after resuspension in dilute Mg^{++}-free media; uncoupling may be assumed to have occurred. It should be emphasized that in the present work chloroplasts were made membrane-free by exposure to hypotonic media containing $MgCl_2$ (see text).

Summary

Pea chloroplasts, isolated in sucrose media were examined under the phase contrast microscope. The

proportion of otherwise entire chloroplasts which showed the granular appearance characteristic of membrane loss varied between 10 % and 50 % in individual preparations.

Exposure to hypotonic media was followed by immediate membrane loss. Membrane-free chloroplasts showed an enhanced ability to catalyse cyclic photophosphorylation and a reduced ability to catalyse carbon dioxide fixation. Expressed as a fraction of the rate achieved by membrane-free chloroplasts, photophosphorylation by chloroplasts with intact membranes varied between 12 % and 65 %.

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Literature Cited

1. Allen, R. J. L. 1940. The estimation of phosphorus. Biochem. 34: 858–65.
2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
3. Bamberger, E. S. and M. Gibbs. 1963. Studies on photosynthesis carbon dioxide fixation by whole spinach chloroplasts. Plant Physiol. 38: x–xi.
4. Gee, R., G. Joshi, R. F. Bils, and P. Saltman. 1965. Light and dark $C^{14}O_2$ fixation by spinach leaf systems. Plant Physiol. 40: 89–96.
5. Gibbs, M. and E. S. Bamberger. 1962. Studies on photosynthetic carbon dioxide fixation by whole spinach chloroplasts. Plant Physiol. 37: lxiii.
6. Good, N. E. 1962. Uncoupling of the Hill reaction in isolated chloroplasts. Arch. Biochem. Biophys. 96: 653–61.
7. Hill, R. and D. A. Walker. 1959. Pyocyanine and phosphorylation with chloroplasts. Plant Physiol. 34: 240–45.
8. James, W. O. and R. M. Leech. 1964. The cytochromes of isolated chloroplasts. Proc. Roy. Soc. B. 160: 13–24.
9. Kahn, A. and D. Von Wettstein. 1961. Macromolecular physiology of plastids. II. Structure of isolated spinach chloroplasts. J. Ultrastruct. Res. 5: 557–74.
10. Leech, R. M. 1964. The isolation of structurally intact chloroplasts. Biochim. Biophys. Acta 79: 637–39.
11. Walker, D. A. 1964. Improved rates of carbon dioxide fixation by illuminated chloroplasts. Biochem. J. 92: 22c–23c.
12. Whatley, F. R., M. B. Allen, L. L. Rosenburg, J. B. Capindale, and D. I. Arnon. 1956. Photosynthesis by isolated chloroplasts. V. Phosphorylation and carbon dioxide fixation by broken chloroplasts. Biochim. Biophys. Acta 20: 462–68.