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Photosynthesis by Isolated Protoplasts, Protoplast Extracts, and Chloroplasts of Wheat

INFLUENCE OF ORTHOPHOSPHATE, PYROPHOSPHATE, AND ADENYLATES

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ABSTRACT

Protoplasts, protoplast extracts (intact chloroplasts plus extrachloroplastic material), and chloroplasts isolated from protoplasts of wheat (*Triticum aestivum*) have rates of photosynthesis as measured by light-dependent O_2 evolution of about 100 to 150 micromoles of O_2 per milligram of chlorophyll per hour at 20 C and saturating bicarbonate. The assay conditions sufficient for this activity were 0.4 molar sorbitol, 50 millimolar N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid KOH (pH 7.6), and 10 millimolar $NaHCO_3$ with protoplast, plus a requirement of 1 to 10 millimolar ethylenediaminetetraacetate (EDTA) and 0.2 to 0.5 millimolar inorganic orthophosphate (Pi) with protoplast extracts and chloroplasts. Protoplast extracts evolved approximately 6 micromoles of O_2 per milligram of chlorophyll before photosynthesis became largely dependent on exogenous Pi while photosynthesis by chloroplasts had a much stronger dependence on exogenous Pi from the outset.

Photosynthesis by chloroplasts from 6-day-old wheat plants under optimum levels of Pi was similar to that with the addition of 5 millimolar inorganic pyrophosphate (PPi) plus 0.2 millimolar adenosine-5'-diphosphate (ADP). Either PPi or ADP added separately inhibited photosynthesis. When chloroplasts were incubated in the dark for 2 to 6 minutes, photosynthesis was strongly inhibited by 5 millimolar PPi and this inhibiting was relieved by including adenosine-5'-triphosphate (ATP) or ADP (0.2 to 0.6 millimolar). Chloroplasts from 9-day-old wheat leaves were slightly less sensitive to inhibition by PPi and showed little or no inhibition by ADP.

Chloroplasts isolated from protoplasts and assayed with 0.3 millimolar Pi added before illumination have an induction time from less than 1 minute up to 16 minutes depending on the time of the assay after isolation and the components of the medium. In order to obtain maximum rates of photosynthesis and minimum induction time, $NaHCO_3$ and chelating agents, EDTA or PPi (+ATP), are required in the chloroplast isolation, resuspension and assay medium. With these inclusions in the isolation and resuspension medium the induction time decreased rapidly during the first 20 to 30 minutes storage of chloroplasts on ice. Requirements for isolating intact and photosynthetically functional chloroplasts from wheat protoplasts are discussed.

Many attempts have been made in the past 20 years to achieve high rates of photosynthesis with chloroplasts isolated from a variety of species including peas and spinach. The problems involved fall into three categories. First, it is necessary, but by no means easy, to secure actively photosynthesizing tissue. Second, the chloroplasts must be isolated in a more or less undamaged state. Finally, they must be assayed in an environment which permits them to achieve their full photosynthetic potential.

With species such as peas and spinach active chloroplasts may be separated from the parent tissue by mechanical disruption and little or no improvement in chloroplast integrity has been achieved recently (26, 27). Chloroplasts isolated in this fashion did not, however, originally photosynthesize as well as the parent tissue because they were assayed under suboptimal conditions. Rates in excess of 100 μmol of CO_2 fixed mg^{-1} Chl hr^{-1} have been recorded (4, 14, 16) and were most readily attained in the presence of PPi. This relates to the need to provide chloroplasts with Pi within a very narrow range of concentrations. Isolated chloroplasts are Pi-consuming organelles as Pi is incorporated into sugar phosphates via the phosphoribulokinase reaction. Certainly in spinach the release of Pi in starch synthesis appears to be too slow to sustain maximal photosynthesis and the rate of CO_2-dependent O_2 evolution soon declines if exogenous Pi is not supplied. *In vivo,* Pi recycling probably results from sucrose synthesis which is now usually regarded as a cytoplasmic event. Thus, the phosphate translocator (12) permits the exchange of internal triose-P for external Pi and, in the cell, sucrose synthesis releases Pi so that this process may continue. *In vitro,* if the optimal (Pi) is exceeded this exchange may also inhibit photosynthesis by depleting the photosynthetic carbon cycle of its essential intermediates. For this reason PPi is particularly beneficial in experiments with isolated spinach chloroplasts because it not only releases Pi at an appropriate rate (as a result of enzymic hydrolysis of $Mg_2P_2O_7$) but also appears to alleviate Pi inhibition, possibly by interferring with the action of the phosphate translocator (30). In peas, however, PPi is inhibitory except when supplied together with ADP or ATP, when it leads to rates which are higher than those observed with Pi alone (24, 25).

Although mechanical disruption of tissue continues to be a convenient method for the isolation of chloroplasts from peas and spinach, enzymic digestion has been employed, to advantage, in other species. This study considers photosynthesis (at 20 C and saturating bicarbonate) of isolated protoplasts, protoplast extracts, and chloroplasts isolated from protoplasts of wheat. It includes a consideration of the requirements in the isolation and assay medium for high rates of photosynthesis and factors which influence the length of the induction period of photosynthesis.

MATERIALS AND METHODS

Plant Material and Protoplast Preparation. Wheat (*Triticum aestivum,* var. Sappo) was grown in Vermiculite in nutrient culture (21) in a greenhouse with sunlight supplemented by incandescent lamps. Plants were collected early each morning. Leaf segments 0.75 mm in width were prepared with a mechanical leaf cutter

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similar to that of Huber and Edwards (13). A double-edged razor was used as the cutting blade and a fresh cutting edge was used daily. A stream of air was passed through a 21-gauge hypodermic needle positioned just behind the cutting blade which swept the segments into a collecting tray underneath the instrument. Approximately 10 g of tissue was cut and placed in 40 ml of enzyme medium in a crystallizing dish 19 cm in diameter and 10 cm high. The segments were stirred into the enzyme medium with a glass rod. The enzyme medium contained 0.5 M sorbitol, 1 mM $CaCl_2$, 0.05% BSA, 2% cellulase (Onozuka 4S) and 0.3% pectinase (Macerozyme) adjusted to pH 5.5. The dish was placed in a 25 C water bath and illuminated with a 150-w incandescent bulb providing a quantum flux density of 5 nE/cm^2·sec between 400 and 700 nm at the surface of the leaf segments. A 5-cm layer of water was placed between the lamp and leaf segments to remove excess heat. After 3 hr the enzyme medium was removed with a 10-ml syringe and discarded because it contained little Chl. The segments were washed three times with 20-ml aliquots of 0.5 M sorbitol, 1 mM $CaCl_2$ by gentle manual shaking. After each washing the released protoplasts and chloroplasts were filtered through two nylon filters, the first a coarse mesh tea strainer (1-mm apertures), and the second of nylon cloth (195-μm apertures). The first filter retained leaf segments while the second filter retained released vascular strands. In subsequent procedures for purification and storage of protoplasts, preparations were maintained on ice or around 4 C. The filtrate was placed in six tubes (12 × 125 mm), centrifuged at 100*g* for 5 min, and the supernatant discarded. Protoplasts were purified by a modification of the procedure of Evans *et al.* (10). About 0.1 ml of 0.5 M sucrose, 1 mM $CaCl_2$ was added to each tube and the pellet resuspended by gentle shaking. Five ml of 0.5 M sucrose, 1 mM $CaCl_2$ was then added to each tube and thoroughly mixed by inversion. Two ml of a solution containing 0.4 M sucrose, 0.1 M sorbitol, 1 mM $CaCl_2$, 5 mM MES-KOH (pH 6) was then layered onto this followed by 1 ml of 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM MES-KOH (pH 6.0). After centrifugation at 200*g* for 5 min the protoplasts collected in a band between the layers of 0.5 M sorbitol, 1 mM $CaCl_2$ and 0.4 sucrose, 0.1 M sorbitol, 1 mM $CaCl_2$. The bands were removed with a disposable pipette and the purified protoplasts, 1 to 1.5 mg of Chl in 6 to 7 ml of medium, were stored on ice. Protoplasts were generally found to be stable with storage on ice up to 20 hr though they were usually used within 6 to 8 hr following isolation. Both preparation of the leaf segments for enzymic digestion and purification of the protoplasts after the 3-hr incubation require about 30 min.

Preparation of Protoplast Extracts and Chloroplasts from Protoplasts. Aliquots, usually 0.4 to 1 ml, containing about 20 μg of Chl/0.1 ml, were taken from the stock of protoplasts and to this were added 5 ml of 0.5 M sorbitol, 1 mM $CaCl_2$. After centrifugation at 250*g* for 2 min the protoplast pellet was suspended in 0.4 to 1 ml of 0.4 M sorbitol, 50 mM HEPES-KOH (pH 7.6), 10 mM $NaHCO_3$, and 1 mM EDTA unless otherwise indicated. For experiments with protoplasts, 0.1-ml aliquots were taken directly from this suspension and added to 0.5 ml of assay medium.

For protoplast extracts a 1-ml plastic disposable syringe was used. The tip of the syringe was cut off leaving an opening of about 2-mm diameter over which was attached a 20-μm nylon net (22). The protoplasts were broken by sucking into the syringe and ejecting three times. Denser protoplast suspensions and smaller pore areas may lead to blockage of the pores resulting in a strong resistance to pipetting and poor preparations. For each preparation a fresh 20-μm nylon net was used.

To prepare chloroplasts the protoplast extracts were centrifuged at 250*g* for 90 sec and the supernatant discarded. The chloroplast pellet was suspended by shaking in 0.1 ml of the same medium (50 mM HEPES-KOH [pH 7.6], 0.4 M sorbitol, 1 mM EDTA, and 10 mM $NaHCO_3$) in an ice bucket and the volume brought to that of the initial aliquot (0.4–1 ml). Protoplast extracts and chloroplasts were generally used within 1 hr after preparation. In one experiment to check stability, chloroplasts isolated and resuspended in media containing 10 mM EDTA and 10 mM $NaHCO_3$ had rates of photosynthesis (in the presence of 0.3 mM Pi) of 162, 162, and 155 μmol of O_2 mg^{-1} Chl hr^{-1} at 10, 30, and 75 min after isolation.

Assay of Photosynthesis. O_2 evolution was followed polarographically at 20 C using a twin Clark-type electrode system (8) purchased from Hansatech Ltd., Hardwick Industrial Estate, Kings Lynn, Norfolk. The assay medium of 0.6 ml was normally the same as the suspension medium: 50 mM HEPES-KOH (pH 7.6), 0.4 M sorbitol, 10 mM $NaHCO_3$, 1 or 10 mM EDTA, with other additives as indicated. With protoplast extracts and chloroplasts 110 units of catalase were routinely included as a protective agent against any peroxide which might be formed during photosynthesis. Chl content/reaction was generally 10 to 25 μg. In calculating rates of photosynthesis a correction was made for consumption of O_2 by the electrode which was significant due to the small reaction volume. Rates of dark respiration by the protoplasts at 20 C were about 5% of the rate of net photosynthesis. The light was provided by Rank-Aldis 105-w projectors with filters as previously described (8) giving a quantum flux density of 90 nE/cm^2·sec^{-1} between 400 to 700 nm and an irradiance of 300 w m^{-2} at the surface of the vessel. To prevent breakage of protoplasts the magnetic stirring speed was set at a minimum while still maintaining a stable response.

Chloroplast Intactness. This was determined by ferricyanide-dependent O_2 evolution before and after shock (18). Assay medium for intact chloroplasts contained 0.4 M sorbitol, 25 mM HEPES-KOH (pH 7.6), 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 10 mM D,L-glyceraldehyde, 3 mM ferricyanide, and 2.5 mM NH_4Cl. For osmotically shocked preparations, 0.1 ml of intact chloroplasts was added to 0.5 ml of assay medium, the latter without sorbitol, which resulted in a final sorbitol concentration of 0.067 M. Chloroplasts isolated from protoplasts were greater than 90% intact using this criterion for intactness.

Chl and Pi. Chl was determined in ethanol from *A* at 654 nm where μg of Chl ml^{-1} ethanol = A_{654}(1,000)/39.8 according to the extinction coefficients of Wintermans and De Mots (33). Pi determination was adapted from the method of Allen (1).

Material. Cellulase and Macerozyme were obtained from Yakult Biochemicals Co. Ltd., Nishinomiya, Japan. The 20- (HD 20, 14% open area) and 195-μm aperture nylon mesh was obtained from Henry Simon Ltd., P.O. Box 31, Stockport, England SK3 ORT.

RESULTS

Conditions for Photosynthesis by Wheat Protoplasts, Protoplast Extracts, and Chloroplasts. Figure 1 shows that protoplasts of wheat have reasonable rates of photosynthesis in the presence of buffer, sorbitol as an osmoticum, and bicarbonate with or without 1 mM EDTA. Addition of 0.3 mM and 5 mM Pi had little influence on rates of protoplast photosynthesis.

Photosynthesis in protoplast extracts of wheat (equivalent to that of protoplasts) requires 1 mM EDTA and in addition 0.3 mM Pi if rates are to be sustained (Fig. 2). With chloroplasts EDTA is required and photosynthesis just begins to rise out of the induction period before there is a requirement for Pi (Fig. 2). In contrast to protoplasts, high concentrations of Pi (5 mM) inhibit photosynthesis by protoplast extracts and chloroplasts.

Figure 3 shows that provided the cytosol fraction is added back to chloroplasts in equal proportion, the initial photosynthetic response typical of protoplast extracts is restored. This indicates that the difference between the response of protoplast extracts and chloroplasts is dependent on the cytosol fraction rather than some effect on the chloroplasts during centrifugation and resuspension. The Pi content of the cytosol fraction (after centrifugation at

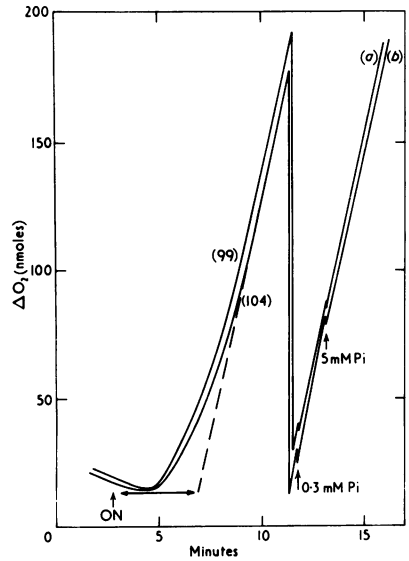

FIG. 1. Light-dependent O_2 evolution by wheat protoplasts from 8-day-old plants. (a): Reaction medium included 50 mM HEPES-KOH (pH 7.6), 0.4 M sorbitol, 10 mM $NaHCO_3$, 1 mM EDTA, and 24.5 μg of Chl. (b): Reaction medium same as above without EDTA. Numbers in parentheses are μmol of O_2 evolved mg^{-1} Chl hr^{-1}.

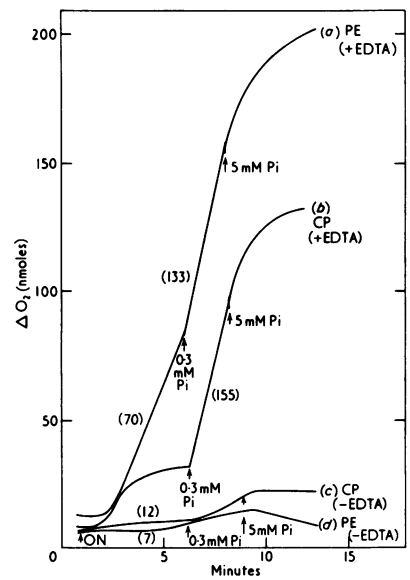

FIG. 2. Light-dependent O_2 evolution by protoplast extracts (PE) 17.2 μg of Chl added (a, d) and chloroplasts (CP) 14.2 μg of Chl added (b, c) from 7-day-old wheat plants. (a, b): Isolation and assay were in 50 mM HEPES-KOH (pH 7.5), 0.4 M sorbitol, 10 mM $NaHCO_3$, 1 mM EDTA. (c, d): Isolation and assay were same as above without 1 mM EDTA. Pi at 0.3 mM and 5 mM was added as indicated. Numbers in parentheses are μmol of O_2 evolved mg^{-1} Chl hr^{-1}.

20,000*g* for 15 min to remove mitochondria and peroxisomes) was measured. This cytosol fraction had 3.2 μmol of Pi/mg of Chl while the respective value for O_2 evolution by protoplast extracts was 8.5 μmol/mg of Chl in the absence of exogenous Pi. An expected stoichiometry if all of the Pi were consumed would be 1 Pi/3 O_2 evolved since each Pi is thought to exchange 3 C atoms out of the chloroplasts in the form of triose-P on the phosphate transporter (31). Thus, there is sufficient Pi in the cytosol fraction to account for O_2 evolution by protoplast extracts. Passing the protoplast extract through a Sephadex G-10 column reduced the rate of photosynthesis to 5 μmol of O_2/mg Chl · hr and addition of 0.3 mM Pi increased the rate to 50 μmol/mg of Chl · hr. Since Sephadex G-10 would desalt the cytosol fraction of protoplast extracts but not remove cytosol enzymes this further suggests the initial high activity in protoplast extracts is due to Pi in the cytosol.

If low levels of Pi (0.05 mM) are added to wheat chloroplasts about 3 μmol of O_2 are evolved/Pi added before photosynthesis again falls to a low steady-state rate. In addition, low levels of PPi (0.025 mM) can serve as a Pi source provided Mg^{2+} is added in excess of EDTA. A stoichiometry of six O_2 evolved/PPi added is then observed. PPi at 5 mM, without exogenous addition of Mg^{2+}, induces some O_2 evolution which suggests that there is either slow uptake of PPi by the chloroplasts or slow external hydrolysis.

Influence of PPi, ADP, and ATP on Photosynthesis by Wheat Chloroplasts. High rates of photosynthesis with wheat chloroplasts were obtained without addition of Mg^{2+}, PPi, adenylates, or organic phosphates. PPi is of particular interest since it has been used to benefit in preparation and assay of photosynthesis in spinach chloroplasts (16, 19, 27). However, with chloroplasts from young pea leaves it inhibits photosynthesis and this inhibition is relieved by ADP or ATP (24, 25). Photosynthesis by wheat chloroplasts from 6-day-old plants is inhibited by 5 mM PPi and this is relieved by inclusion of 0.2 mM ADP (Fig. 4). If chloroplasts are added to the assay media in the light, near complete inhibition by 5 mM PPi occurs after 10 min. Preincubation of the chloroplasts in the dark with the assay medium containing 5 mM PPi for 2 or 6 min inhibited subsequent O_2 evolution in the light.

Photosynthesis by chloroplasts from 9-day-old wheat leaves (plants about 130 mm tall) was also inhibited by PPi but less so than those from 6-day plants. If chloroplasts from 9-day plants were added to the assay medium in the light there was only slight inhibition by PPi during the first few min of the assay. However, inhibition by PPi increased as the chloroplasts were preincubated in the dark for 2 to 6 min with severe inhibition after 6 min of dark preincubation. Inclusion of 0.6 mM ATP with PPi overcomes the inhibition by PPi and gave rates similar to that with 0.2 mM Pi added alone. Rates with 0.6 mM ATP + 0.3 mM Pi were similar to that with 0.3 mM Pi alone (data not shown).

With chloroplasts from 6-day-old wheat (plants about 75 mm tall) the optimum concentration of Pi for photosynthesis was 0.2 mM (Fig. 5A). With chloroplasts from 9-day-old plants the opti-

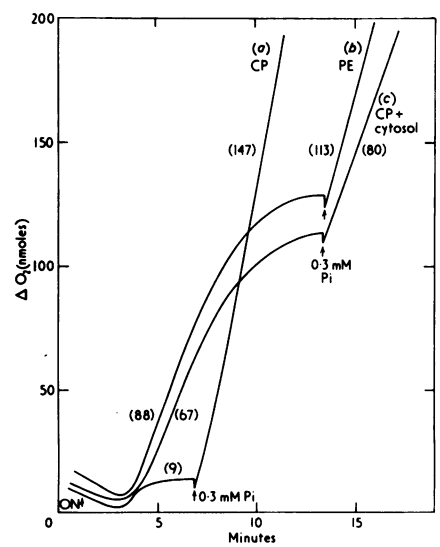

FIG. 3. Light-dependent O_2 evolution by chloroplasts (a), protoplast extracts (b), and chloroplasts + cytosol fraction (c) from 8-day-old wheat plants. For (c) chloroplast pellet from protoplast extract was resuspended and chloroplasts + an equivalent amount of supernatant added to the assay medium. Chl added was 18.9 μg in (b) and 20.5 μg in (a) and (c). Assay medium included 10 mM $NaHCO_2$ and 110 units of catalase. Numbers in parentheses are μmol of O_2 evolved mg^{-1} Chl hr^{-1}.

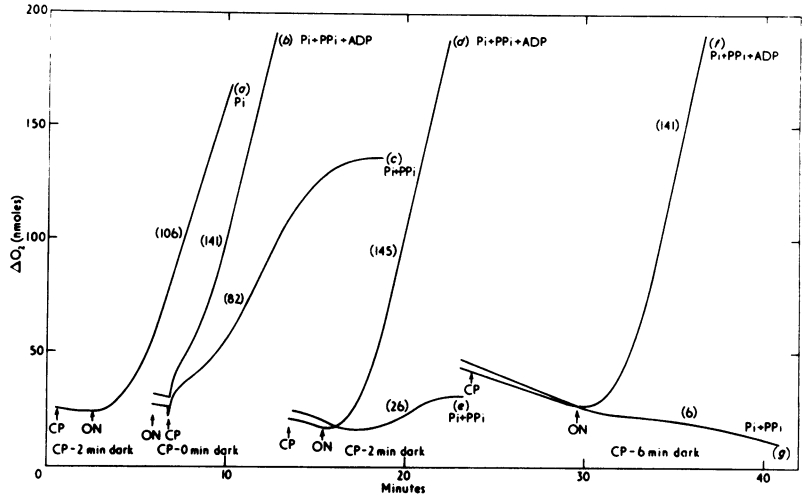

FIG. 4. Light-dependent O_2 evolution by chloroplasts isolated from protoplasts of 6-day-old wheat. Basic reaction medium was 50 mM HEPES-KOH (pH 7.6), 0.4 M sorbitol, 6 mM $NaHCO_3$, 1 mM EDTA, 0.2 mM Pi, 110 units of catalase, and 15.8 μg of Chl. (a): Chloroplasts in basic reaction medium in dark for 2 min. (b, d, f): Chloroplasts in basic reaction medium + 5 mM PPi, 0.2 mM ADP in the dark for 0, 2, and 6 min, respectively. (c, e, g): Chloroplasts in basic reaction medium + 5 mM PPi in the dark for 0, 2, and 6 min, respectively. Numbers in parentheses are μmol of O_2 evolved mg^{-1} Chl hr^{-1}.

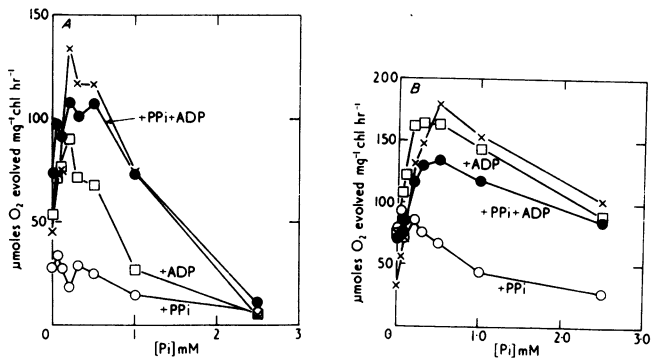

FIG. 5. A: light-dependent O_2 evolution by chloroplasts isolated from protoplasts of 6-day-old wheat. Chloroplasts isolated from protoplasts and assayed in 50 mM HEPES-KOH (pH 7.5), 0.4 M sorbitol, 1 mM EDTA, 10 mM $NaHCO_3$, 110 units of catalase, 19 μg of Chl, and the following additions: (×——×), varying concentrations of Pi; (●——●), varying concentrations of Pi + 5 mM PPi and 0.6 mM ADP; (□——□), varying concentrations of Pi and 0.2 mM ADP; and (○——○), varying concentrations of Pi and 5 mM PPi. Chloroplasts were preincubated with reaction media for 3 min in dark prior to illumination. Maximum rates of O_2 evolution were taken following 3 min of illumination. B: light-dependent O_2 evolution by chloroplasts isolated from protoplasts of 9-day-old wheat. Reaction medium included 22.7 μg of Chl. Other conditions as in A.

mum Pi concentration was 0.5 mM, and photosynthesis was much less sensitive to inhibition at high Pi levels (1–2.5 mM) in comparison to the 6-day-old plants (Fig. 5, A and B). Also, the chloroplasts from 9-day-old plants were less sensitive to inhibition by PPi. Again, inclusion of ADP with PPi tends to overcome the inhibition by PPi alone (Fig. 5B). Mg^{2+} was not added in these experiments in order to limit the production of Pi through pyrophosphatase (25). Under assay conditions similar to those in Figure 5B (plus 0.3 mM Pi) photosynthesis of chloroplasts isolated from the basal part (lower 5 cm of the leaf) of 9-day-old plants was inhibited 72% by 5 mM PPi while photosynthesis by chloroplasts isolated from the tips (upper 5 cm of leaf) was inhibited only 29% by 5 mM PPi. This further indicates that chloroplasts in the younger tissue are more sensitive to PPi inhibition of photosynthesis.

Variation in the Length of Induction of Photosynthesis with Wheat Chloroplasts. Protoplasts of wheat, with or without EDTA, had an induction period of 2 to 3 min (Fig. 1 and data not shown). Protoplasts isolated from leaf segments incubated in the dark during enzymic digestion had an induction period of about 5 min (data not shown). During this study (with chloroplasts in assays including 0.2–0.3 mM Pi prior to illumination) considerable variation in the induction time from about 2 to 10 min was observed. The influence of various components of the isolation, resuspension, and assay media on induction time and maximum rate of photosynthesis with chloroplasts (from several preparations of protoplasts) is recorded in Table I. The assay medium included 0.3 mM Pi added before illumination, and chloroplasts were added in the light. In all cases except isolations with buffer and sorbitol alone, the induction time decreased after 20- or 30-min storage of chloroplasts on ice. With certain mixtures, lags as small as 0.3 to 0.5 min were observed. With only HEPES buffer and sorbitol in the isolation and resuspension medium, induction time was 16 or 17 min (Table I, exp. V) and in some preparations no O_2 was evolved even after 25 min in light (data not shown). $NaHCO_3$, EDTA, or PPi + ATP decreased the length of the induction period and increased the maximum rate of photosynthesis. Ten mM EDTA was more effective than 1 or 2 mM (Table I, exp. III). PPi alone also shortened the lag (from 8.5 to 3 min in one experiment with freshly isolated chloroplasts) although PPi alone inhibited photosynthesis (not shown in Table I). The most effective medium for decreasing induction time and maximizing rates of photosynthesis was a combination of $NaHCO_3$ with EDTA or $NaHCO_3$ with PPi + ATP.

Chloroplasts isolated from protoplasts in a medium including 10 mM $NaHCO_3$ + 10 mM EDTA and stored on ice for 30 min had a short induction time of 0.3 min when added directly to the assay medium in the light (Table II). However, preincubation of chloroplasts in the dark in the assay medium (which contained 0.3 mM Pi) lengthened the induction period though the maximum rate of photosynthesis was unchanged.

Chloroplasts isolated in media including 10 mM $NaHCO_3$, 10 mM EDTA had high rates of O_2 evolution over the pH range assayed from 7.2 to 8.4, with the highest rate at pH 7.6 (Fig. 6). Rates were relatively low over this pH range with chloroplasts prepared without EDTA and bicarbonate or with only bicarbonate in the isolation and suspension medium. In all three isolation media the length of induction was greater at lower pH, although the shortest induction time in each medium (pH 8.4) does not provide the highest rate of photosynthesis.

The relatively long induction time with chloroplasts isolated in sorbitol-HEPES with 10 mM $NaHCO_3$ could be reduced by in-

Table I Influence of isolation and suspension medium and chloroplast storage on induction time and maximum rate of photosynthesis by wheat chloroplasts.

Protoplasts were stored in 0.5 M sucrose-sorbitol mixture, 1 mM $CaCl_2$, 5 mM MES-KOH pH 6.0 (see Methods). Protoplasts were resuspended in chloroplast isolation medium, the protoplasts broken, and chloroplast pellets obtained following centrifugation were suspended in the isolation medium and stored on ice. The basic isolation and suspension medium included 50 mM HEPES-KOH pH 7.6, 0.4 M sorbitol and other additive as indicated. Assay medium was same as isolation medium + 0.3 mM Pi, 110 units catalase and 10 mM $NaHCO_3$. Assay time indicates time after storage of chloroplasts on ice (0 time is 7 to 10 min after protoplast breakage). The method of evaluating the induction time is illustrated in Fig 1. All experiments were with chloroplasts from 8 or 9 day old wheat plants.

Exp.	Additives to chloroplast isolation and suspension medium	Assay time	Induction time	Rate
		Min	Min	μmoles mg^{-1} Chl hr^{-1}
I[a]	1 mM EDTA	0	16.5	37
		45	8.2	47
	1 mM EDTA, 10 mM $NaHCO_3$	0	9.5	85
		45	3.0	60
	1 mM EDTA, 10 mM $NaHCO_3$, 5 mM PPi, 0.2 mM ATP	0	5.0	132
		45	1.4	99
II[b]	1 mM EDTA, 10 mM $NaHCO_3$ (27 μg Chl)	0	8.5	100
		20	2.8	102
		40	1.2	89
	(13.5 μg Chl)	0	13.5	98
		20	4.0	93
	2 mM EDTA, 10 mM $NaHCO_3$ (27 μg Chl)	0	8.0	109
		20	2.1	129
	(13.5 μg Chl)	0	11.7	111
		20	3.7	124
	10 mM EDTA, 10 mM $NaHCO_3$ (27 μg Chl)	0	5.0	136
		20	1.8	138
	(13.5 μg Chl)	0	7.2	80
		20	2.9	151
III[c]	1 mM EDTA, 10 mM $NaHCO_3$	0	7.0	152
		30	2.1	138
	1 mM EDTA, 5 mM PPi, 0.2 mM ATP	0	8.5	130
		30	5.0	118
	1 mM EDTA, 5 mM PPi, 0.2 mM ATP, 10 mM $NaHCO_3$	0	4.8	166
		30	0.3	137
IV[d]	none	0	>12.0	0
		20	>12.0	0
	10 mM EDTA	0	7.5	110
		20	4.7	78
	10 mM EDTA, 10 mM $NaHCO_3$	0	4.8	181
		20	1.0	170
	10 mM PPi, 0.4 mM ATP	0	3.5	73
		20	1.8	104
	10 mM PPi, 0.4 mM ATP, 10 mM $NaHCO_3$	0	4.9	123
		20	0.4	147
V[e]	none	0	16.8	32
		20	16.1	15
	10 mM $NaHCO_3$	0	12.5	27
		20	7.7	42
	10 mM EDTA	0	7.7	77
		20	2.9	92
	10 mM EDTA, 10 mM $NaHCO_3$	0	4.7	91
		20	1.4	133

[a]17 μg Chl/assay; [b]Chl/assay as indicated; [c]15 μg Chl/assay; [d]17 μg Chl/assay [e]36 μg Chl/assay.

Table II Influence of dark preincubation of wheat chloroplasts in assay medium on induction and maximum rates of photosynthesis.

Chloroplasts were isolated from protoplasts obtained from 9-day old wheat. Isolation, resuspension, and assay medium contained 50 mM HEPES-KOH pH 7.6, 0.4 M sucrose, 10 mM EDTA and 10 mM $NaHCO_3$. In addition the assay medium contained 0.3 mM Pi and 110 units catalase. Chloroplasts were stored on ice for 30 min after isolation from protoplasts.

Time of dark preincubation	Chl/assay	Induction time	Rates
Min	μg	Min	μmoles O_2 mg^{-1} Chl hr^{-1}
0	16.1	0.3	142
0.5	14.9	1.9	145
1.0	16.1	1.7	145
4.0	14.9	3.1	149

cluding low levels of dihydroxyacetone-P (50–400 μM) or fructose bis-P (0.5 mM) in the assay medium. However, rates with these organic phosphates added were only about half that of chloroplasts isolated with EDTA and $NaHCO_3$.

DISCUSSION

Conditions for Photosynthesis by Wheat Protoplasts, Protoplast Extracts, and Chloroplasts. Average rates of leaf photosynthesis by C3 plants under nonstressed conditions are expected to be in the order of 100 μmol of CO_2 fixed mg^{-1} Chl hr^{-1} at 20 C while CO_2-saturated rates may be 1.5- to 2-fold higher. It is of interest to see if protoplasts may provide a means of isolating functional chloroplasts from species which may prove difficult by mechanical procedures. Some evidence for this potential was given by Rathnam and Edwards when chloroplasts isolated from protoplasts of several C3 species had rates of photosynthesis greater than 100 μmol of mg^{-1} Chl hr^{-1} (22). However, in that study the arbitrary use of 37 C is abnormally high for C3 photosynthesis and inclusion of 2 mM Pi in the assay medium may be inhibitory. In other studies protoplasts of wheat and barley were reported to have rates of photosynthesis of 100 to 150 μmol of CO_2 fixed mg^{-1} Chl hr^{-1} at 28 or 30 C and saturating bicarbonate (9, 13).

We have studied conditions affecting photosynthetic O_2 evolution of protoplasts, protoplast extracts, and chloroplasts of wheat at 20 C and saturating bicarbonate. A relatively simple isolation, resuspension, and assay medium with pH 7.6 throughout was found to give reasonable rates of photosynthesis. Sorbitol as an osmoticum, HEPES-KOH buffer (pH 7.6) and bicarbonate were sufficient with protoplasts. With protoplast extracts and chloroplasts there was a marked requirement for a chelating agent, EDTA or PPi, and optimum levels of Pi. Protective agents such as sodium ascorbate, DTT, and BSA were not required, although other additives may eventually prove beneficial.

In the absence of Pi, photosynthesis by wheat chloroplasts quickly reached a low rate at most of 6 to 8 μmol of O_2 evolved mg^{-1} Chl hr^{-1}. With chloroplasts of spinach and peas, photosynthesis typically starts off at a relatively high rate and proceeds for several min before exogenous Pi is required (4, 5, 6, 24, 29). During the induction phase of photosynthesis the Pi level in the chloroplasts would be reduced as pools of organic phosphates of the Benson-Calvin pathway increase. Starch synthesis would release Pi and allow photosynthesis to continue but recycling of this nature may well be too slow to permit maximal rates of photosynthesis (31). Pi uptake and triose-P release from chloroplasts, catalyzed by the phosphate transporter, is thought to be required for sucrose synthesis (31). In wheat chloroplasts, at least under the present conditions, both the level of Pi and rate of

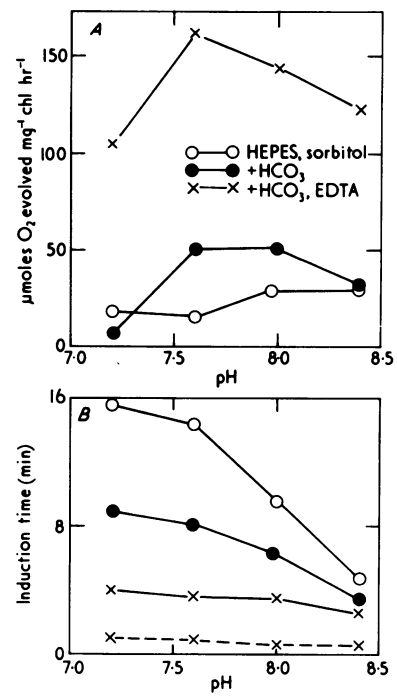

FIG. 6. Influence of pH of isolation, resuspension, and assay medium on maximum rate of photosynthesis and length of induction of photosynthesis of wheat chloroplasts. Each set of data on induction and rate of photosynthesis represents a separate isolation of chloroplasts from protoplasts. Protoplasts were isolated from 8-day-old wheat. Standard components of isolation, resuspension, and assay medium were 0.4 M sorbitol and 50 mM HEPES-KOH. Other additions to isolation, resuspension, and assay medium were: (×——×), 10 mM $NaHCO_3$, 10 mM EDTA; (●——●): 10 mM $NaHCO_3$; (○——○), none. Chloroplasts were assayed 10 min after isolation from protoplasts; (×- - -×) indicates induction time 30 min after isolating chloroplasts from protoplasts in medium including 10 mM EDTA + 10 mM $NaHCO_3$. All assays included 10 mM $NaHCO_3$, 0.3 mM Pi, 110 units of catalase, and 19 μg of Chl.

starch synthesis in the chloroplast in the absence of exogenous Pi must be low. Under the growth conditions used the leaves were found to contain no starch (A. Herold, personal communication) and the capacity of the chloroplasts to synthesize starch *in vivo* is uncertain.

The protoplast extract had an initially higher rate of photosynthesis than chloroplasts in the absence of exogenous Pi which could be accounted for from the Pi contained in the cytosol of the protoplast extract. The fact that photosynthesis was not sustained by the protoplast extracts without addition of Pi suggests that under these conditions organic phosphates released from chloroplasts are not being metabolized to sugars with release of Pi.

The stoichiometry of about 3 μmol O_2 evolved/Pi added with wheat chloroplasts is consistent with an obligate uptake of Pi in exchange for triose-P on the phosphate transporter and supports similar results with spinach (17, 25). Also, low levels of PPi can apparently serve as a Pi source if Mg^{2+} is present although PPi is of no benefit at optimum levels of Pi. Inhibition of photosynthesis by high levels of Pi with both chloroplasts and protoplast extracts is consistent with previous results with spinach and pea chloroplasts on the basis that high Pi would severely deplete the chloroplasts of triose-P which are needed to sustain the Benson-Calvin cycle (19, 24, 29, 30). The optimum concentration of Pi for photosynthesis by wheat chloroplasts of 0.2 to 0.5 mM (Fig. 5) is also in the range of that of pea and spinach chloroplasts (19, 24), although it is apparent with wheat chloroplasts that the Pi optimum can change with leaf age.

Lack of a requirement for exogenous Pi for photosynthesis by wheat protoplasts suggests that the protoplasts have maintained their Pi in the chloroplasts and cytosol and synthesis of sucrose and or starch would continue to recycle Pi for photosynthesis. Lack of inhibition of photosynthesis by 5 mM Pi indicates that protoplasts are relatively impermeable to Pi, and this can be used as a biochemical criterion for protoplast intactness.

Influence of PPi, ADP, and ATP on Photosynthesis of Wheat Chloroplasts. Photosynthesis by wheat chloroplasts was inhibited by PPi and this inhibition was reversed by ADP or ATP (Fig. 4). The effects of PPi and adenine nucleotides on wheat chloroplasts are similar to those reported for pea chloroplasts (23–25). Robinson and Wiskich (24) showed that PPi entered pea chloroplasts via the adenine nucleotide transporter in exchange for endogenous nucleotides. The resulting depletion of the chloroplasts of ATP inhibited CO_2 fixation while the addition of exogenous ADP or ATP prevented this loss by competing with PPi for uptake on the adenine nucleotide transporter. This inhibitory action of PPi normally is not observed with spinach chloroplasts (25) and the inhibition is most pronounced with chloroplasts isolated from young pea shoots (24). This apparently reflects a higher activity of the adenine nucleotide transporter in chloroplasts from young pea shoots. Similar differences were observed with chloroplasts isolated from wheat plants of differing ages. Oxygen evolution by chloroplasts from 6-day-old wheat plants was inhibited by PPi more rapidly and to a greater extent than with chloroplasts from 9-day-old wheat. In addition, chloroplasts isolated from the physiologically younger basal regions of wheat leaves were more sensitive to PPi than those isolated from the tip regions. These differences are consistent with the notion that chloroplasts from developing tissues have higher rates of adenine nucleotide transport than chloroplasts from mature tissues (24).

With wheat chloroplasts, the inhibition of photosynthesis by PPi was most severe after preincubation in the dark for 2 to 6 min. This probably reflects the time taken for PPi to exchange for the chloroplast adenine nucleotides. Since dark preincubation of chloroplasts prior to illumination is a common practice, it is obvious that different times of dark preincubation in some cases will give subsequently very different rates of photosynthesis. With chloroplasts from young pea leaves, PPi + ADP (or ATP) tends to give higher rates of photosynthesis than with the optimum level of Pi alone (24, 25). In the present study with wheat chloroplasts inclusion of PPi (with or without adenylates) in isolation or assay media is not necessary or of benefit when adequate levels of EDTA are provided.

Variation in Length of Induction of Photosynthesis with Wheat Chloroplasts. In both leaves and isolated chloroplasts several min are required following illumination for photosynthesis to reach its maximum rate. It has been suggested that this period may be required for the chloroplast to build up its pool of metabolites of the Benson-Calvin pathway and for activation of certain catalysts of the cycle (for review see 11, 28, 29). When protoplasts are isolated from leaf segments which have been preilluminated during enzymic digestion, there is a lag period of 2 or 3 min before maximum rates of photosynthesis are attained. With wheat chloroplasts we found a long induction period with 0.3 mM Pi and low rates of photosynthesis if $NaHCO_3$, EDTA or PPi were not included in the chloroplast isolation and resuspension medium (Table I). Inclusion of $NaHCO_3$ in the isolation and chloroplast suspension medium reduced the induction period particularly after the chloroplasts had been stored on ice for 15 to 20 min (Table I). When chloroplasts are isolated and stored on ice for 15 to 30 min in a medium including $NaHCO_3$ + 10 mM EDTA, or $NaHCO_3$ + 5 mM PPi + 0.2 mM ATP, photosynthesis subsequently begins immediately and maximum rates are reached within 1 min if chloroplasts are added to the assay medium in the light (Tables I and II). However, preincubation of the chloroplasts in the assay medium in the dark increased the length of the induction (Table II). This suggests that the chloroplasts have maintained a pool of triose-P during isolation. Dark preincubation with 0.3 mM Pi would exchange these out of the chloroplasts on the phosphate transporter and thus lengthen the subsequent induction period in the light.

PPi was originally used by Jensen and Bassham (16) in attaining high rates of photosynthesis by spinach chloroplasts. Cockburn *et al.* (7) found it generally superior as a constituent of isolation media. As in the present work with wheat it also shortened induction when used in isolation (7) procedures. Together with Mg it serves as a 'Pi stat' in assay because of pyrophosphatase action (19, 27).

It seems likely that both PPi and EDTA may act beneficially by chelating inhibitory cations but whether these become attached to the envelope during isolation or play some natural role in metabolite transfer remains to be determined. Divalent cations such as Mg^{2+} and Ca^{2+} are relatively impermeable to impact chloroplasts (20). PPi has been found to protect against Pi inhibition with spinach chloroplasts (19), and PPi and citrate inhibit the phosphate transporter (17). These compounds along with EDTA might in part reduce phosphate transport through chelation of certain divalent cations which bind to the phosphate transporter and influence its activity.

Avron and Gibbs (2) found that 4 mM $MgCl_2$ inhibited photosynthesis in spinach chloroplasts if 2 mM EDTA was not included in the assay medium, although Mg^{2+} inhibition may not be pronounced in all circumstances (19; and S. Huber, personal communication). Avron and Gibbs (2) noted that Mg^{2+} and chelating agents could be eliminated without influencing activity, however EDTA was included in the isolation and resuspension medium at a concentration equivalent to divalent cations (and at least some EDTA would be carried over from the chloroplasts resuspension medium to the assay medium).

High pH shortened the induction time with the wheat chloroplasts (Fig. 6). A higher pH outside chloroplasts can also increase the pH in the chloroplast stroma (32) favoring activation of enzymes such as RBP-carboxylase and fructose bis-P (3, 14).

Although the basis for the influence of bicarbonate is uncertain. it might shorten the induction period when included in the isolation and resuspension medium by activating RBP-carboxylase as reported by Jensen *et al.* (15).

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