Some Effects of Sugars and Sugar Phosphates on Carbon Dioxide Fixation by Isolated Chloroplasts

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(Received 9 May 1966)

1. Carbon dioxide fixation by isolated pea chloroplasts was stimulated by the addition of intermediates of the Calvin photosynthesis cycle and by some related compounds. 2. Ribose 5-phosphate and fructose 1,6-diphosphate consistently produced the largest effects; free sugars such as erythrose and sedoheptulose and acids such as glycollate and glyoxylate were largely ineffective or even inhibitory. 3. Small effects were produced by fructose and ribose but not by their isomers, glucose and xylose. 4. Maximal rates in the presence of ribose 5-phosphate varied between 10 and 50 μ moles of carbon dioxide fixed/mg. of chlorophyll/hr.

Chloroplasts isolated from spinach or from peas, in saline (e.g. Arnon, Allen & Whatley, 1954; Allen, Arnon, Capindale, Whatley & Durham, 1955; Whatley, Allen, Rosenberg, Capindale & Arnon, 1956; Gibbs & Calo, 1959; Havir & Gibbs, 1963) or in sugar media (e.g. Walker, 1964), retain the ability to carry out light-induced carbon dioxide fixation. In the absence of added substrate, fixation is non-linear with respect to time, showing an initial lag or induction period (Gibbs & Bamberger, 1962; Bamberger & Gibbs, 1963; Walker, 1965a). In the presence of certain sugar phosphates the lag period is shortened or eliminated (Bamberger & Gibbs, 1965; Walker, 1965b), so that in the first 10–15 min. the total fixation may be increased as much as 40-fold. The present paper is not concerned with the kinetics of the induction period but is intended to record the relative effectiveness of the various sugar phosphates that increase total fixation and to show that free fructose and ribose may act similarly, though to a smaller degree.

MATERIALS AND METHODS

Plant material. Peas (*Pisum sativum*, mostly var. Laxton Superb, occasionally var. Feltham First and var. Progress no. 9) were grown for 13–16 days in moist vermiculite (heat-expanded mica) in a temperature-controlled box ($18 \pm 0.5^\circ$) that was illuminated for 11 hr. out of each 24 hr. by five 40w fluorescent tubes and two 25w tungsten lamps (giving a light-intensity of approx. 150ft.-candles at the level of the leaves). Under these conditions the plants showed no signs of etiolation but they were essentially starch-free even when taken directly out of the light. Spinach (*Spinacia oleracea*) was bought from Covent Garden Market, London. Other material was collected from the field.

Chloroplasts. Chloroplasts were isolated (cf. Walker,

1964, 1965a,b) at pH 6.8 in 0.33M-sorbitol (or sucrose) containing $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer (0.1M), NaCl (0.1%), MgCl_2 (0.1%) and sodium isascorbate (20mM). Leaves (50g.) were homogenized for 5 sec. in a Waring Blendor in 200 ml. of solution (which had been chilled to the point of freezing) and the brei was squeezed through two layers of muslin and then filtered through eight layers. The filtrate was centrifuged at 0° in four 50 ml. centrifuge tubes by allowing the centrifuge to accelerate to 7000 rev./min. (approx. 4500g) and then stopping as quickly as possible (total time 100 sec.). This procedure was preferred to centrifugation at lower speeds, which keeps the chloroplasts in contact with the other cell contents for a correspondingly longer period. The supernatant was discarded. Each pellet was washed with 40–50 ml. of cold 0.45M-sorbitol (or sucrose) containing NaCl (0.1%), MgCl_2 (0.1%) and sodium isascorbate (20mM) and finally resuspended in 5 ml. (or occasionally less) of the same solution.

Reaction mixtures. In a final volume of 0.3 ml. the mixtures contained 0.05 ml. of a solution of reduced glutathione (1 μ mole), sorbitol (or sucrose) (30 μ moles), MnCl_2 (0.5 μ mole), MgCl_2 (0.25 μ mole), EDTA (0.5 μ mole), KH_2PO_4 (0.5 μ mole) and tricine (Good, 1962) (7.5 μ moles), adjusted to pH 7.5 with NaOH, and in addition 0.10 ml. of chloroplast suspension and sugar phosphates, sugars and related compounds as indicated in the text. Reactions were carried out in round-ended Pyrex-glass centrifuge tubes (1.5 cm. $\times$ 10 cm.) sealed with Suba-Seal stoppers or in similar short tubes (1.5 cm. $\times$ 4 cm.) for time-course experiments. Reaction mixtures were completed by the addition of Na_2CO_3 solution, immediately followed by chloroplasts, to a darkened tube. Illumination followed within 15–30 sec. and reactions were started at 30 sec., 40 sec. or 1 min. intervals. The Na_2CO_3 (2.95 μ moles except where indicated) contained a proportion of ^{14}C (20 μC , or 50 μC for time-course experiments). Reactions were stopped by the addition of 0.5 ml. of 0.3N-HCl, or (in time-course experiments) 10 μl . samples were withdrawn with a Hamilton micro-syringe and injected into 40 μl . of 0.3N-HCl.

Measurement of radioactivity. Samples (20 μl .) were dried on lens-tissue disks (diam. 1.7 cm.) on aluminium planchets

and their radioactivity was determined in a Nuclear-Chicago gas-flow detector (model D47). The relationship between radioactivity measured and CO_2 fixed was calculated from values obtained when known quantities of labelled sucrose were added to reaction mixtures (containing no carbonate) that were then acidified, sampled and counted as before. The validity of this procedure, which is accurate to within approx. $\pm 5\%$, was also checked by counting selected samples, radioactive carbonate and known standards (in solution) in a Nuclear-Chicago scintillation counter.

Illumination. Tungsten or quartz iodine lamps gave 8000–10000 ft.-candles at the surface of the reaction vessels. In most experiments a red Perspex filter, transmitting 90% of the incident light above $595\text{ m}\mu$, was inserted between the light-source and the reaction vessels. Except where indicated the temperature was maintained at 20° .

RESULTS

Ideally, it would be desirable to plot complete time-courses or progress curves (Figs. 1, 2 and 3) for a large number of additives to a single chloroplast preparation. In practice the rate of decay of the isolated chloroplast makes it mandatory to start and stop, within a fairly short period, all reactions that are to be directly compared with one another. This in turn limits the total number of determinations that can be made. Nevertheless, the extent of carbon dioxide fixation within a single time-interval can provide an indication of the relative effective-

ness of various substrates, and if necessary small differences can then be verified in separate experiments (cf. Table 1 and Figs. 1, 2 and 3). Table 1 is representative of results from a larger number of experiments from which three consistent features emerge:

- (1) In 'substrate' quantities ($2\text{ }\mu\text{moles}$ in 0.3 ml.),

Fig. 2. Progress curves of $^{14}\text{CO}_2$ fixation by isolated pea chloroplasts prepared and resuspended in saline. Additions were: $2.0\text{ }\mu\text{moles}$ of fructose (○); $2.0\text{ }\mu\text{moles}$ of glucose (Δ). The chlorophyll concentration was $61\text{ }\mu\text{g./reaction mixture}$.

Fig. 1. Progress curves of $^{14}\text{CO}_2$ fixation by isolated pea chloroplasts prepared and resuspended in a medium containing sorbitol. Additions were: $2.0\text{ }\mu\text{moles}$ of fructose (○); $2.0\text{ }\mu\text{moles}$ of glucose (Δ). The chlorophyll concentration was $70\text{ }\mu\text{g./reaction mixture}$ (see the Materials and Methods section). In single time-interval experiments (see Table 1) the fixation in the presence of glucose was similar to that in the absence of any added sugar (other than the sorbitol used to maintain the osmotic pressure).

Fig. 3. Progress curves of $^{14}\text{CO}_2$ fixation by isolated pea chloroplasts prepared and resuspended in a medium containing sorbitol. Additions were: $2.0\text{ }\mu\text{moles}$ of ribose (□); $2.0\text{ }\mu\text{moles}$ of xylose (Δ); none (○). The chlorophyll concentration was $27\text{ }\mu\text{g./reaction mixture}$.

ribose 5-phosphate was the most effective of the additives tested. Fructose 1,6-diphosphate stimulated carbon dioxide fixation to a slightly smaller extent but was superior to hexose monophosphates.

(2) There was a small response to free fructose and free ribose (see also Figs. 1, 2 and 3).

(3) In general, any component of the Calvin photosynthesis cycle (e.g. see Calvin & Bassham, 1962) enhanced fixation. Small responses were also brought about by some substances that could be fairly readily converted into Calvin-cycle intermediates by known metabolic routes. Other

Table 1. *Effect of additives on photosynthetic carbon dioxide fixation by isolated chloroplasts.*

These values are representative of a larger number of experiments (over 60). They show that, under a variety of conditions (see the text), the responses to different additives were broadly similar. Replicates in experiments with the same chloroplast preparation normally varied by less than 5%. In Expts. 7-9 the additional sugar used to maintain the osmotic pressure was sucrose; in the other experiments it was the same as that in the resuspending medium. The values underlined are those obtained in the absence of any added metabolite other than the sucrose or sorbitol used to maintain the osmotic pressure. Except where indicated the other reaction mixtures contained 2 μ moles of additive in a total volume of 0.3 ml. (see the Materials and Methods section).

	CO ₂ fixed (μ moles)								
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 8	Expt. 9
Resuspending medium	Sorbitol	Sorbitol	Sorbitol	Sorbitol	Sorbitol	Sucrose	Sorbitol	Sorbitol	Sorbitol
Time	20 min.	21 min.	15 min.	15 min.	10 min.	15 min.	18 min.	18 min.	18 min.
Temperature	20°	20°	20°	20°	20°	20°	20°	20°	20°
Chlorophyll	34 μ g.	118 μ g.	62 μ g.	58 μ g.	73 μ g.	40 μ g.	53 μ g.	59 μ g.	34 μ g.
Material	Peas	Spinach	Peas	Peas	Peas	Peas	Peas	Peas	Peas
Sugar phosphates and related compounds that increased fixation									
Ribose 5-phosphate	7.4	10.1	4.3	2.60	4.0*	2.5	4.45	5.3	6.6
Fructose 1,6-diphosphate	7.3	9.0	2.6	1.14	2.5*	—	—	4.0	5.5
Glucose 6-phosphate	1.6	2.6	—	0.25	—	—	—	1.2	1.6
Fructose 6-phosphate	1.1	3.2	—	0.29	—	—	—	0.85	1.1
Dihydroxyacetone phosphate	2.2	—	—	—	—	—	—	—	—
3-Phosphoglycerate	1.6	8.4	—	—	—	—	—	—	—
2-Phosphoglycerate	—	—	—	—	1.0*	—	—	—	—
Sugars that increased fixation									
Ribose	1.3	2.5	0.23	0.12	0.13†	0.69	0.71	—	1.05
Fructose	2.7	3.7	0.25	0.12	0.75†	0.27	0.55	—	0.85
Sugar phosphates, sugars and related compounds that caused little or no increase in fixation									
Glucose 1-phosphate	0.8	2.3	0.35	0.18	0.8*	—	—	0.60	0.9
6-Phosphogluconate	0.7	2.8	—	—	—	—	—	—	0.75
Phosphoenolpyruvate	0.3	2.8	—	—	—	—	—	—	0.35
Xylose	0.3	2.1	—	0.07	—	0.27	0.30	—	—
Deoxyribose	0.3	1.7	—	—	—	0.27	0.25	—	—
Arabinose	0.3	1.7	—	—	—	0.27	0.25	—	—
Glucose	0.4	1.7	0.11	0.05	0.13	0.27	0.30	—	0.50
Galactose	0.6	1.4	—	—	0.07	0.27	0.30	—	—
Erythrose	0.1	1.4	—	—	—	—	—	0.08	—
Glyceraldehyde	0.04	0.1	—	—	—	—	—	0.025	—
Glycerol	0.4	1.3	0.09	—	—	—	—	0.30	—
Glycollate	0.3	0.8	0.08	—	—	—	—	0.20	—
Glyoxylate	0.1	0.3	—	—	—	—	—	0.04	—
Sedoheptulose	0.4	1.6	0.09	—	—	0.27	0.30	—	—
Sucrose	0.4	1.6	0.11	—	—	0.27	0.25	0.20	0.50
Maltose	0.4	1.8	—	—	—	0.27	0.25	—	—
Sorbitol	0.3	1.7	—	0.07	0.20	—	0.25	—	—
Isocitrate	0.6	2.4	—	—	—	—	—	0.35	—
Glutamate	0.3	2.1	—	—	—	—	—	—	—

* 4 μ moles added.

† 33 μ moles added.

metabolites such as sucrose, glycollate and isocitrate were largely without effect or gave stimulations less than those of free fructose or ribose.

(4) A small number of additives decreased fixation. Of these, the most inhibitory was glyceraldehyde.

These features were seen in results obtained over a period of 3 years with three different varieties of peas and in occasional experiments (Table 1) with other species such as spinach (*Spinacia oleracea*), spinach beet (*Beta vulgaris*) and New Zealand spinach (*Tetragonia expansa*). Much of the work was designed to promote improvements in the rates of fixation and accordingly involved differences in pretreatment of the leaf material, differences in grinding medium, differences in the composition of the reaction mixtures (such as carbon dioxide concentration) and differences in light-intensity, light-quality and temperature during incubation. In general, though changes in conditions were accompanied by quantitative changes in the response to additives, the qualitative picture remained unchanged.

Effect of mixtures. Table 2 shows the effect on carbon dioxide fixation of two triose phosphates and some related compounds alone and in mixtures. Most of these were added in smaller quantities than the 2.0 μ moles used in most of the experiments in Table 1. The effects produced were more variable, and, for example, in one experiment the two trioses together were clearly more effective than either alone, whereas in another this response was less marked. The consistent features that did emerge from experiments of this type were: (a) that the same compounds gave the largest responses (as in Table 1) but that the differences were less, and that the decreasing order of effectiveness was now fructose 1,6-diphosphate, ribose 5-phosphate, dihydroxyacetone phosphate or 3-phosphogly-

cerate, glyceraldehyde 3-phosphate, hexose monophosphates; (b) that, in general, mixtures of these compounds gave increases in fixation that were little different to those brought about by increased quantities of single compounds; (c) that no mixtures produced effects much greater than that brought about by 2 μ moles of ribose 5-phosphate alone.

Effect of fructose and ribose. In preliminary experiments, chloroplasts prepared in media containing fructose consistently gave higher carbon dioxide-fixation values than those prepared in otherwise similar mixtures containing glucose. When sorbitol was used to maintain the osmotic pressure of the isolating medium, added fructose again produced a small stimulation that could not be induced by equimolar glucose or galactose (Table 1). This effect is shown more clearly in Fig. 1, which is a time-course for carbon dioxide fixation by chloroplasts prepared and resuspended in sorbitol. The reaction mixtures giving the shorter induction period contained 2.0 μ moles of fructose; that giving the longer lag contained 2.0 μ moles of glucose. Fig. 2 shows a similar effect with chloroplasts prepared and resuspended in saline (Arnon *et al.* 1954). Fig. 3 compares time-courses for carbon dioxide fixation by chloroplasts prepared in sorbitol and incubated in mixtures containing 2 μ moles of ribose (upper curve) or ones (lower curve) in which the ribose was either omitted or replaced by equimolar xylose.

Maximal rates. Although the best rates of fixation were obtained in the presence of substrate concentrations of ribose 5-phosphate (Table 1) the actual values were variable. Fully controlled growing conditions were introduced when it became clear that the poorest rates (in the presence or absence of substrate) were obtained with preparations from pea seedlings that had been exposed to high temperatures (25° or above) during growth. The

Table 3. Rates of $^{14}\text{CO}_2$ fixation by isolated chloroplasts from a series of experiments at 20°

For details of the grinding medium see the Materials and Methods section.

Plant material	Grinding medium	Chlorophyll ($\mu\text{g.}$)	Time-interval or max. rate	Rate ($\mu\text{moles of CO}_2/\text{mg. of chlorophyll/hr.}$)
Pea seedlings (Laxton Superb, 14 days)	Sorbitol	28	Max. rate	48.8
Pea seedlings (Laxton Superb, 14 days)	Sucrose	27	6-18 min.	23.2
Pea seedlings (Laxton Superb, 14 days)	Sucrose	33	Max. rate	55.3
Pea seedlings (Laxton Superb, 14 days)	Sucrose	27	6-18 min.	44.4
Spinach	Sucrose	55	8-18 min.	16.4
Spinach	Sucrose	55	6-18 min.	24.7
Pea seedlings (Laxton Superb, 14 days)	Sucrose	46	Max. rate	18.8
Pea seedlings (Laxton Superb, 14 days, previous year's seed)	Sucrose	48	Max. rate	29.0
Pea seedlings (Laxton Superb, 14 days, previous year's seed)	Sucrose	40	Max. rate	26.2
Pea seedlings (Laxton Superb, 15 days)	Sucrose	53	6-18 min.	30.0

peas were grown under low light-intensity to preclude starch formation (see the Materials and Methods section), and the combination of low light-intensity and high temperature induces a degree of etiolation that is apparently unfavourable. With the temperature fully controlled at 18° the plants were healthy in appearance and, though the variability in preparations persisted and is still unaccounted for, the maximum rates improved (Table 3).

In similar experiments, over the same period, ten determinations of maximum rate in the absence of added substrate (other than carbonate) gave values that fell between 4.8 and 19.8 with a mean of 11.3. It should be noted that in all of the experiments reported in the present paper (except for that illustrated in Fig. 2) the chloroplasts were suspended in a relatively high concentration of sucrose or sorbitol for purposes of maintaining the osmotic pressure. Carbon dioxide fixation does occur, however, in saline alone (i.e. in the complete absence of any added compound, other than carbon dioxide, which could be used as a metabolite), and it seems clear that the general pattern in saline, sucrose or sorbitol is the same (cf. Figs. 1 and 2; also Bamberger & Gibbs, 1965; Walker, 1965*b*; Baldry, Walker & Bucke, 1966).

The rates obtained with chloroplasts prepared from market spinach (see the Materials and Methods section) were generally similar to those from peas (see e.g. Table 3). More recently, higher rates (65–105 μ moles/mg. of chlorophyll/hr., in the presence of substrate concentrations of ribose 5-phosphate) have been obtained with chloroplasts from freshly gathered field-grown spinach.

DISCUSSION

The results show that phosphoglycerate, and several sugar phosphates that are known intermediates of the Calvin photosynthetic carbon cycle, stimulate light-dependent carbon dioxide fixation by isolated chloroplasts. This ability is shared by certain other metabolites (e.g. glucose 1-phosphate) from which the accepted Calvin-cycle intermediates could easily be derived by known metabolic routes. Taken in the context of other work (e.g. Arnon *et al.* 1954; Havir & Gibbs, 1963) the results support the studies *in vivo* on which the Calvin cycle is based (e.g. see Calvin & Bassham, 1962) and the view that isolated chloroplasts retain, at least to some extent, the ability to carry out the characteristic photosynthetic processes of whole leaves.

The smaller effects produced by fructose and ribose, but not by their isomers glucose and xylose, suggest the presence of specific kinases in the chloroplast preparations. The occurrence of a fructokinase in peas has been reported by Medina & Sols (1956).

Our results are also consistent with the view that the chloroplast envelope is permeable, or partly permeable, to several intermediates of the Calvin cycle. It is possible, but perhaps unlikely (because of the degree of dilution involved), that enzymes that had escaped from damaged chloroplasts could convert any additives that could not penetrate intact envelopes into those that might enter more readily. The possibility that the stimulatory effects could be attributed entirely to the presence of chloroplasts that have lost their envelopes is not supported by the fact that carbon dioxide fixation falls to less than 10% in pea chloroplasts that have been exposed to osmotic shock and in which the outer envelope is lost (Whatley *et al.* 1956; Walker, 1965*a*).

This work was supported by the Science Research Council and the University of London Central Research Fund. Special apparatus was provided by the Royal Society. We thank Professor C. P. Whittingham for his advice and criticism.

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