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UPTAKE AND LIGHT ACTIVATED ESTERIFICATION OF ^{32}P BY ISOLATED CHLOROPLASTS

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SUMMARY

1. Esterification of $^{32}P_i$ by illuminated chloroplasts prepared on a sucrose gradient was examined to establish the optimal incubation conditions.

2. The evidence is consistent with phosphorylation being closely coupled to the sum of noncyclic and pseudocyclic electron flow and with the rate of electron flow responding to the availability of electron acceptors.

3. Apparent K_m values for ADP and Mg^{2+} were found to be 40 and 250 μM, respectively. The K_m value for Mg^{2+} was increased by the presence of Ca^{2+}. Two apparent values were observed for P_i at 0.2 and 1.1 mM. Chloroplast damage resulted in increased apparent K_m (P_i) values.

4. Acceleration of the esterification resulting from the addition of ADP and P_i to the medium indicated that these compounds were able to penetrate to the active site of esterification.

5. Ribose 5-phosphate (Rib-5-*P*) was shown to inhibit P_i esterification without affecting the apparent K_m for ADP or P_i. The evidence suggests that Rib-5-*P* interferes with the uptake of P_i, and possibly ADP.

INTRODUCTION

Most information available today on photophosphorylation is derived from experiments on chloroplast fragments or on preparations now believed to have been damaged structurally during the isolation procedure. Such preparations were suitable for establishing maximum rates of esterification and electron flow in the presence of redox mediators, *e.g.*, phenazine methosulfate (PMS). Recent reports that correlate maximal CO_2 fixation rates[1,2] with chloroplast integrity indirectly question the relevance of photophosphorylation data to the performance of the intact chloroplast.

SPENCER AND UNT[3], WALKER[1] and GEE *et al.*[4] prepared isolated intact chloroplasts and found that the photophosphorylation activities of their preparations were much lower than the published maximal rates. The object of this study was to establish rates that were representative of good chloroplast preparations; this required the selection of optimal conditions, however. Evidence of chloroplast integrity

Abbreviations: PMS, phenazine methosulphate; CMU, 1-(*p*-chlorophenyl)-3,3'-dimethylurea.

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and biochemical activity was given in a previous report[5]. Here rates of P_i esterification have been examined in the absence of any exogenous electron acceptor, and the influence of a number of factors has been investigated. The findings confirm and extend those of earlier workers[1,3,4,16]. A preliminary report has been published[6].

MATERIALS AND METHODS

Chloroplasts from 13–17-day-old seedling peas were isolated on a discontinuous sucrose gradient as previously described[5]. Chlorophyll was assayed using the method of ARNON[7].

Except when stated otherwise in the text, the reaction medium was 0.4 M sucrose, 5 mM $MgCl_2$, 2.5 mM ADP, 2.5 mM NaH_2PO_4–Na_2HPO_4 buffer containing 5–25 μC $^{32}P_i$, 27.5 mM Tris–HCl (pH 7.8) and chloroplasts containing 40 $\pm$ 10 μg chlorophyll in a total volume of 2.5 ml. Incubation vessels were pyrex tubes (10 mm $\times$ 75 mm) maintained at 22–25° which were placed 10 cm from a photoflood tungsten lamp delivering 90000 lux at the vessel surface.

Reactions were terminated by the addition of 0.5 ml of 5 M $HClO_4$; the tubes were ice-cooled and centrifuged at 2000 $\times$ g for 7 min. Esterified P_i was separated from 0.5-ml aliquots of the supernatant using the method of HAGIHARA AND LARDY[8]. Recovery of [^{32}P]ATP was 80–85 %, and $^{32}P_i$ breakthrough was negligible. To separate [^{32}P]ATP from labelled sugar phosphates 0.5 ml of the esterified P_i eluate from the column used by HAGIHARA AND LARDY[8] was mixed with 1 ml of Norit A charcoal suspension (10 g per 100 ml water) and after 1 h the separation procedure of HIND AND JAGENDORF[9] was followed. ^{32}P-labelled solutions emit light (Cherenkov radiation)[10] and no added scintillator is required[11]. Samples in aqueous solution were counted at 20% efficiency in the Nuclear Chicago Model 725 counter. Results are presented as the difference between illuminated samples and dark controls represented by foil-covered tubes.

RESULTS

The effect of the P_i concentration on the rate of esterification by illuminated chloroplasts is shown in Fig. 1a. In the range 4–400 μM P_i the v_{max} is 18 μmoles P_i esterified per mg chlorophyll per h, and the apparent K_m is 0.2 mM (see Fig. 1b). The second linear slope of Fig. 1a suggests a second low affinity site of P_i involvement in photophosphorylation. The enzyme characteristics of this second site can be calculated using the method of WALKER[36] to give v_{max} of approx. 47 μmoles P_i esterified per mg chlorophyll per h, and K_m of approx. 1.1 mM. In Table I these K_m values are compared with published data. Saturation was not achieved at P_i concentrations up to 0.01 M, whereas a maximum rate at 5 mM was reported by BLACK *et al.*[17] with inhibition at 10 mM P_i. The effect of sonication on the P_i requirement of chloroplast preparations is shown in Table II. The rise in apparent K_m (P_i) of the low affinity site (Table II) suggests that the active centre of this phosphorylation enzyme is intimately associated with the membrane structure and possibly that the P_i concentration at this site is maintained at a level above that represented by the medium. The response of the high affinity site to sonication is different and suggests that the active centre of this phosphorylation is not greatly modified by sonication.

The rate of photophosphorylation as a function of ADP concentration is shown in Fig. 2. The apparent K_m (ADP) was 40 μM which is compared with published values in Table I; the v_{max} was 25 μmoles P_i esterified per mg chlorophyll per h.

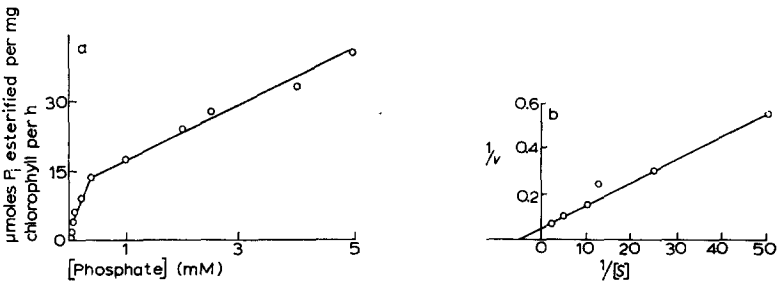


Fig. 1. a. Effect of P_i concentration on rate of P_i esterification. Medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 2.5 mM ADP; illumination period 3 min; K_m 0.2 mM; v_{max} 18 μmoles P_i esterified per mg chlorophyll per h; and $K_{m(2)}$ 1.1 mM; $v_{max(2)}$ 47 moles P_i esterified per mg chlorophyll per h. b. Data of a replotted as $1/v$ *vs.* $1/[S]$ for low $[S]$ values.

TABLE I

COMPARISON OF APPARENT K_m VALUES FOR ADP, P_i AND Mg^{2+}

	K_m (*mM*)		
	ADP	P_i	Mg^{2+}
Present work	0.04	(i) 0.2 (ii) 1.1	0.25
Ref. 12*	0.5	0.5	1.5
Ref. 13*	5.0	1.0	1.0
Ref. 4*	0.2	—	—
Ref. 14*	0.09	0.3	—
Ref. 15*	0.02	0.3	—
Ref. 16*	0.07	0.07	—

* Extrapolated from published results.

TABLE II

EFFECT OF CHLOROPLAST DAMAGE ON THE P_i AND ADP REQUIREMENT IN PHOTOPHOSPHORYLATION

Chloroplasts were sonicated in ice-cold 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8) using an MSE–Mullard 60-W ultrasonicator at 18–20 kcycles/sec. v_{max} presented as μmoles P_i esterified per mg chlorophyll per h.

	Condition	K_m (*mM*)	v_{max}
High P_i affinity site	Control	0.23	22.5
	18-sec sonicate	0.22	2.27
	180-sec sonicate	0.47	1.45
Low P_i affinity site	Control	1.5	45
	18-sec sonicate	3.6	18
	180-sec sonicate	10	18
ADP affinity site	Control	0.04	25
	Osmotic rupture	0.35	5.7
	18-sec sonicate	1	1.8

Inhibition of P_i esterification occurred at 0.2 mM ADP and was progressive to 2 mM ADP to give an esterification rate of 75% of the maximum. Increasing the ADP concentration beyond this range did not further increase the degree of inhibition.

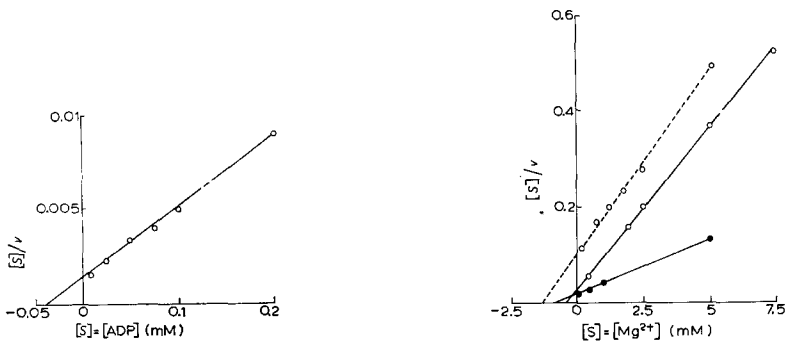


Fig. 2. Effect of ADP concentration ([S]) on photophosphorylation: [S]/v plotted against [S]. Apparent K_m (ADP) 40 μM; v_{max} 25 μmoles P_i esterified per mg chlorophyll per h. Medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 2.5 mM NaH_2PO_4–Na_2HPO_4 buffer, illumination period 3 min.

Fig. 3. Effect of Mg^{2+} concentration ([S]) on photophosphorylation: [S]/v plotted against [S]. O—O, in the absence of $CaCl_2$; ●—●, in the presence of 5 mM $CaCl_2$. Medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 2.5 mM ADP, 2.5 mM NaH_2PO_4–Na_2HPO_4 buffer. 3-min illumination. Apparent K_m (Mg^{2+}) 0.25 mM: in the presence of 5 mM $CaCl_2$ this becomes 0.75 mM Mg^{2+}. O---O, effect of Mg^{2+} concentration ([S]) on the $A_{540\ nm}$. This reading is generally regarded as an indication of the chloroplast volume. Plotted as [S]/v: the axis has been multiplied × 0.5 for this line.

After an 18-sec sonication, the apparent K_m (ADP) increased to 1 mM and v_{max} fell to 1.8 μmoles P_i esterified per mg chlorophyll per h (see Table II). Osmotic rupture of chloroplast preparations produced similar changes in these criteria, in agreement with the observations of GEE *et al.*[4].

In Fig. 3 the rate of esterification is shown as a function of an added concentration of Mg^{2+}. The apparent K_m (Mg^{2+}) was 0.25 mM and under these conditions v_{max} was 12.5 μmoles P_i esterified per mg chlorophyll per h. There was no evidence of inhibitory levels of Mg^{2+} below 12 mM.

Divalent cations have been shown to cause a rapid dark contraction of the chloroplast volume[18]. The work of IZAWA AND GOOD[19] suggested that the contraction is correlated with an enhanced photosynthetic electron transport. Thus Mg^{2+} could indirectly stimulate photophosphorylation by altering the chloroplast volume; however, the apparent K_m (Mg^{2+}) for contraction (Fig. 3) was found to be 1.25 mM, which is higher than the apparent K_m for photophosphorylation.

The effect of a 5-min dark incubation of chloroplasts in 5 mM $CaCl_2$ on the Mg^{2+} requirement for P_i esterification was also examined. Fig. 3 shows that in the presence of Ca^{2+} the apparent K_m (Mg^{2+}) was increased to approx. 0.75 mM and v_{max} was increased to 44.6 μmoles P_i esterified per mg chlorophyll per h. The $CaCl_2$ addition thus results in a noncompetitive stimulation of esterification. Dark contraction of chloroplasts does not discriminate between equimolar Mg^{2+}, Mn^{2+} or Ca^{2+} treatment, but the effect of Ca^{2+} on esterification is different from that of an equivalent increase in Mg^{2+} concentration. From this it appears that Ca^{2+} do not influence P_i esterification

indirectly through conformational changes but may possibly activate a second phosphorylation site. AVRON[20] and BENNUN AND AVRON[21] have reported a Ca^{2+}-stimulated ATPase activity in chloroplasts. The effect of various additions to a standard incubation on the esterification rate is given in Table III. From the data of SANTARIUS AND HEBER[22], the ATP concentrations in the chloroplast are 0.25–0.4 mM; thus the apparent product inhibition of esterification rates shown by 5.0 mM ATP in Table III may be of little significance to natural control mechanisms.

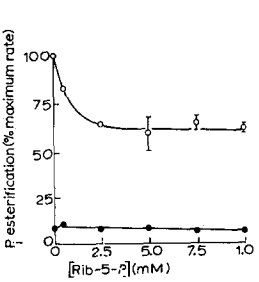
TABLE III

EFFECT OF ADDED PHOSPHORYLATED COMPOUNDS ON THE RATE OF ESTERIFICATION

Standard medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 2.5 mM ADP, 5 mM $MgCl_2$, 2.5 mM NaH_2PO_4–Na_2HPO_4, volume 2.5 ml, containing chloroplasts equivalent to 40 ± 10 μg chlorophyll. Illumination period 3 min. Results are expressed as μmoles P_i esterified per mg chlorophyll per h.

Addition	*Rate of esterification*		
	0.1 mM	*0.7 mM*	*5.0 mM*
ATP	22.9	22.8	11.7
AMP	23.3	22.8	21.3
$Na_4P_2O_7$	24.2	21.9	18.6
Fru-1,6-P_2	21.0	22.6	23.6
Glc-6-*P*	24.4	25.6	26.8
Rib-5-*P*	22.6	19.0	13.6
Control (standard medium)		23.3	

The apparent inhibition due to ribose 5-phosphate (Rib-5-*P*) shown in Table III was examined more closely. WALKER[23], and BUCKE *et al.*[24] reported enhanced CO_2 fixation by isolated chloroplasts on adding Rib-5-*P*, but KALBERER *et al.*[25] and JENSEN AND BASSHAM[2,26] were unable to confirm this. The effect of the concentration of Rib-5-*P* is shown in Fig. 4. The apparent K_m (ADP) and apparent K_m (P_i) (Fig. 5) were unaltered by the addition of 5 mM Rib-5-*P*. The percentage distribution of ^{32}P between the sugar phosphate fraction and ATP after separation on charcoal was also



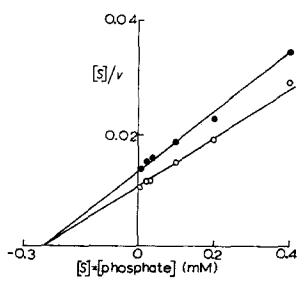


Fig. 4. Effect of Rib-5-*P* concentration on P_i esterification as percent of maximal rate. Medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 2.5 mM NaH_2PO_4–Na_2HPO_4 buffer. O—O, 2.5 mM ADP; ●—●, no ADP. 3-min illumination. Results are expressed as the mean of three determinations ± S.E.

Fig. 5. Effect of 5 mM Rib-5-*P* on the apparent K_m (P_i). Medium as Fig. 4, with 2.5 mM ADP. O—O, 5 mM Rib-5-*P*; ●—●, no Rib-5-*P*.

unaffected. A 3–30-min dark preincubation of chloroplasts in a P_i-deficient standard medium supplemented with 5 mM Rib-5-*P* revealed a time-dependent inhibition of esterification amounting to 68% inhibition at 30 min during subsequent illumination in the presence of P_i. Inhibition was less (40% at 30 min) if P_i was present in the preincubation medium and if ADP was omitted until the illumination period. These results suggest that Rib-5-*P* interfered with the availability of P_i but not by competition at the active site of esterification. The results of JOHNSON AND BRUFF[29] suggest that the chloroplast is not readily penetrated by Rib-5-*P*, and a possible interpretation of our observations is that Rib-5-*P* competes with P_i and perhaps with ADP in the mechanism of entry into the chloroplast. The addition of Rib-5-*P* made no detectable difference to the low rate of P_i esterification (2.5 μmoles per mg chlorophyll per h) dependent on the endogenous ADP content (see Fig. 4), which is consistent with the above interpretation.

Evidence for the coupling of esterification to electron flow

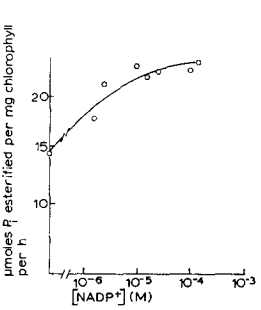
Photophosphorylation was more than 95% inhibited by 0.1 mM 1-(*p*-chlorophenyl)-3,3'-dimethylurea (CMU) and 50% inhibited by 4 μM CMU in the standard incubation conditions. Using current interpretations this indicates approx. 100% noncyclic (including pseudocyclic) photophosphorylation coupled to the flow of electrons from water photolysis.

In Fig. 6 the effect of added $NADP^+$ on the rate of P_i esterification is shown. Concentrations up to 0.1 mM $NADP^+$ gave enhanced rates; at higher concentrations the flow rates of extracts on the columns used by HAGIHARA AND LARDY[8] were sometimes arbitrarily extended by the formation of a precipitate. Runs which are free of this artifact suggest that the graph of Fig. 6 can probably be extended up to 1 mM $NADP^+$ concentration.

The effect of passing a stream of N_2 gas through the incubation for a 6-min illumination period and a 3-min preillumination period also was examined. A linear esterification rate was obtained that was 20% of the controls under atmospheric conditions, the conditions were possibly not fully anaerobic, but this result indicates that some 80% of the photophosphorylation normally observed is dependent on autoxidation of the endogenous electron acceptor(s), *i.e.*, is pseudocyclic photophosphorylation. Also, consistent with the results of Fig. 6, the rate of esterification is shown to be conditioned by the availability of electron acceptors. Similarly the addition of 90 μM PMS to a sucrose–Tris medium containing 1 mM $MgCl_2$, 1 mM ADP and 1 mM P_i increased the esterification rate from 15 to 180 μmoles P_i esterified per mg chlorophyll per h, presumably by increasing the availability of electron acceptors.

A time-course of P_i esterification by broken and intact chloroplasts is shown in Fig. 7. Chloroplasts were broken by osmotic shock in 27.5 mM Tris–HCl buffer (pH 7.8) (sucrose approx. 0.075 M) and then were incubated under the same phosphorylating conditions as the intact preparation. The asymptotic time-course was also observed by GEE *et al.*[4] and suggests that some limitation is imposed on the esterification. In the presence of 3 μM PMS the esterification rate in a 3-min period was substantially (20 ×) increased, and the incorporation in a 15-min period, instead of being asymptotic, was an approximately linear function of time. The addition of PMS has obviously by-passed the natural limitation which is thus unlikely to be

a physical limitation such as product inhibition (unless PMS-mediated phosphorylation is very different from basal phosphorylation), membrane permeability (unless PMS-mediated ion uptake is invoked) or a chloroplast volume imposing limits on an endogenous phosphate cycle.



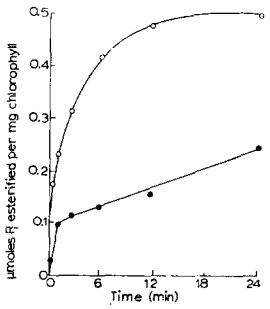


Fig. 6. Effect of $NADP^+$ concentration on the rate of $^{32}P_i$ esterification. Medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 2.5 mM ADP, 1 mM NaH_2PO_4–Na_2HPO_4 buffer. Illumination time 3 min.

Fig. 7. Time-course of ^{32}P esterification. O—O, intact chloroplasts; ●—●, chloroplasts broken by osmotic shock. Incubation medium 0.4 M sucrose, 27.5 mM Tris–HCl buffer (pH 7.8), 1 mM $MgCl_2$, 1 mM ADP, 1 mM NaH_2PO_4–Na_2HPO_4 buffer.

DISCUSSION

The rates of P_i esterification found here are comparable with published values for preparations having a high content of intact chloroplasts, *e.g.* ref. 16. The evidence of response to $NADP^+$ addition, N_2 atmosphere and CMU treatments suggests that photophosphorylation in intact chloroplast is closely linked to the sum of non-cyclic and pseudocyclic electron flow with a negligible contribution from cyclic photophosphorylation. The relatively low rates of P_i esterification in intact chloroplasts are thus attributable to the dependence on endogenous electron mediators, *i.e.*, to the absence of PMS, and suggest that a measure of control over electron flow and coupled photophosphorylation occurs.

The asymptotic time-course of P_i esterification in Fig. 7 also suggests that a control mechanism exists. In the work of GEE *et al.*[4] the esterification rates are such that at the lower levels of ADP, exhaustion of the P_i acceptor could explain the time-course, as the authors suggest. In the present work the esterification shown in Fig. 7 consumed 0.5 μmole ADP per mg chlorophyll in 20 min amounting to the removal of 0.025 μmole ADP from an original content of 2.5 μmole ADP per incubation, so that exhaustion of the medium cannot apply here. The response of photophosphorylation to an exogenous supply of ADP is so well defined (Fig. 2) and consistent with other reports (*e.g.*, refs. 4, 16, 32, 33) that there is no need to question the penetrating ability of ADP.

Using pea Class 1 chloroplast preparations reinforced with substrate level concentrations of electron acceptors, WEST AND WISKICH[32] have demonstrated the controlled coupling of phosphorylation to electron flow by methods analogous to those used to demonstrate the respiratory control in mitochondria. If it is assumed that

our preparations behaved similarly, the possibility arises that phosphorylation is controlled by the availability of electron acceptors. The stimulated P_i esterification in the presence of added $NADP^+$ (Fig. 6) is consistent with the report of FORTI[34] and with the observations of ROBINSON AND STOCKING[33] that O_2 release, taken as an indicator of photosynthetic activity, is enhanced on adding $NADP^+$, although they tentatively ascribe this response[33] to activities of the 20–30% of swollen chloroplasts in their preparation. It is also consistent with our earlier report[5] of exogenous $NADP^+$ reduction that led to the conclusion that $NADP^+$ can penetrate into the chloroplast or that a trans-membrane NADPH (internal)/$NADP^+$ (external) transhydrogenation exists. The evidence suggests that in the absence of added electron acceptors when the endogenous pool(s) is totally reduced and the auto-oxidation of NADPH and ferredoxin is prevented (*e.g.*, under N_2), the flow of electrons through the phosphorylating site ceases. This situation might arise directly by saturation of the electron acceptor pools within the chloroplast, but it may alternatively include the conversion of compound Q (refs. 28, 29) into the reduced form incapable of quenching Photosystem II fluorescence, by a feed-back reaction as suggested by WITT *et al.*[35].

Only the portion of $^{32}P_i$ converted into phosphate ester has been considered here, another portion may coexist in the chloroplast as $^{32}P_i$, but to study this involves the risk of post-illumination changes in label distribution during the necessary washing procedures. Although it may be desirable to distinguish $^{32}P_i$ uptake from the uptake coupled to photophosphorylation, the two must be closely interrelated *in vivo*, for illumination and consequent esterification will almost certainly alter the chemical potential of a P_i gradient and thus impose a new set of (illuminated) conditions on P_i translocation.

It is questionable whether the uptake of P_i into the chloroplast is a process of simple diffusion, thus the evidence of DEAMER *et al.*[30] suggests that ions or charged molecules are less easily taken up than are uncharged molecules. The preliminary report of SANTARIUS *et al.*[31] on the compartmentation of $^{32}P_i$ in the intact leaf also suggests that chloroplast phosphate in the light is derived by phosphatase (including ATPase) activity rather than from penetration of cytoplasmic P_i. In the present work the high affinities for ADP and P_i in the intact chloroplast were lowered by disruptive treatments which might indicate the partial destruction of specific ADP and P_i translocation mechanisms. The inhibitory effect of Rib-5-*P* on the overall esterification rates has been interpreted as possibly acting on a translocation mechanism since it had neither a detectable effect on endogenous ADP-limited esterification nor on the apparent K_m for ADP or P_i. The crux of this matter is whether P_i penetrates into the chloroplast in the free form or is combined, complexed or undergoes reaction as a necessary preliminary step to translocation. If the latter is the true state, the evidence of two P_i affinity sites and the cooperating effect of Ca^{2+} and Mg^{2+} on overall esterification are readily interpreted. With the intention of clarifying some of the issues raised here, ^{32}P pulse labelling experiments have been initiated in which the pulse is terminated by a substantial dilution (*e.g.*, 20×–100×) with unlabelled P_i. The outcome of these experiments will be published elsewhere at a later date, but preliminary findings support the view that P_i from the medium does not penetrate by a simple diffusion.

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