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LIGHT-DEPENDENT REDUCTION OF PYRUVATE BY PEA CHLOROPLASTS IN THE PRESENCE OF GLUTAMATE DEHYDROGENASE AND C_5-DICARBOXYLIC ACIDS

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Key Word Index—*Pisum sativum* (cv Massey Gem); Leguminosae; peas; chloroplasts; α-oxoglutarate reduction; pyruvate reduction; glutamate oxidation; dicarboxylic C_5 acids; ammonia assimilation; glutamine metabolism; glutamate synthase; glutamine synthetase; glutamate dehydrogenase; light-dependent.

Abstract—Illuminated pea chloroplasts supported (glutamine plus α-oxoglutarate (α-OG)) and (NH_3 plus α-OG)-dependent O_2 evolution. The properties of these reactions were consistent with light-coupled glutamate synthase and glutamine synthetase activities. In the presence of a glutamate-oxidizing system (component C) comprised of NAD-specific glutamate dehydrogenase (NAD-GDH), lactate dehydrogenase (LDH), 4 mM pyruvate and 0.2 mM NAD, illuminated chloroplasts supported O_2 evolution in the presence of glutamine. The reaction did not proceed in the absence of any one of the constituents of component C and the properties of O_2 evolution were consistent with light-coupled glutamate synthase activity. In the presence of component C, chloroplasts also catalysed O_2 evolution in the presence of catalytic concentrations of glutamate. Studies of O_2 evolution and metabolism of [^{14}C]-glutamate in the presence of the inhibitors methionine sulphoximine (MSO) and azaserine suggest that O_2 evolution was dependent on the synthesis of glutamine from the products of glutamate oxidation. This was supported by polarographic studies using α-OG and NH_3 instead of glutamate. The results are consistent with a C_5-dicarboxylic acid shuttle mechanism for the export of reducing equivalents from illuminated chloroplasts (glutamate) and recycling of the oxidation products (α-OG and NH_3).

INTRODUCTION

The inner chloroplast membrane is impermeable to ferredoxin (Fd) and nicotinamide adeninine dinucleotides [1–3]. Thus Fd_{red} and NADPH formed within the chloroplast by light-dependent reduction of Fd_{ox} and NADP with H_2O as electron donor cannot serve directly as reductants in other metabolic compartments of the photosynthetic cell. Nevertheless, light-generated reducing equivalents are known to be transported from the chloroplast to other subcellular compartments and, under certain circumstances, vice versa [3–9]. It has been proposed that outward transport of reducing equivalents across the chloroplast envelope is mediated by shuttle mechanisms which involve: (i) light-coupled reduction of the oxidized form of a metabolite or carrier in the chloroplast; (ii) transport of both the oxidized and reduced forms of the metabolite across the envelope; (iii) oxidation of the reduced form in other metabolic compartments of the cell; and (iv) recycling of the oxidized form. Two shuttle mechanisms for the transport of reducing equivalents across the chloroplast envelope have been described; they are known as the phosphoglycerate (PGA)/dihydroxyacetone phosphate (DHAP) and C_4 shuttles and involve PGA/DHAP [3, 4, 7] and oxaloacetate (OAA)/malate [3, 6, 9] as the oxidized/reduced forms of the carrier respectively. Another shuttle involving glyoxylate/glycollate as carrier has been discounted [10].

Illuminated pea chloroplasts catalyse the reductive transamination of α-OG by glutamine to form 2 mol of glutamate with the concomitant evolution of 0.5 mol of O_2 [11–13]. This reaction is attributed to light-coupled glutamate synthase activity using H_2O as electron donor (Fig. 1, component A) [13]. Since the glutamate synthase of chloroplasts is Fd-specific [11, 12], light generated Fd_{red} is presumed to act as the electron donor. Glutamate synthase when coupled to glutamine synthetase forms the principle pathway for the incorporation of NH_3 into glutamate (Fig. 1, component B) [14, 15]. In theory this mechanism could serve as the light-dependent reductive component of a shuttle mechanism; glutamate could be transported out of the chloroplast by the dicarboxylate translocator of the inner membrane and oxidized to NH_3 and α-OG by GDH in other parts of the photosynthetic cell. Other oxidative mechanisms are also possible. The α-OG could return to the chloroplast by counter transport with glutamate via the dicarboxylate translocator (Fig. 1) [16]. This paper reports a study of the role of glutamate in the export of reducing equivalents from illuminated pea chloroplasts (Fig. 1) in the presence of catalytic amounts of C_5-dicarboxylic acids and a glutamate-oxidizing system external to the chloroplast with pyruvate as eventual electron acceptor (Fig. 1, component C).

RESULTS

Properties of (glutamine plus α-OG)-dependent O_2 evolution by isolated chloroplasts (component A)

The presence of light-coupled glutamate synthase activity, which incorporates light-generated reducing

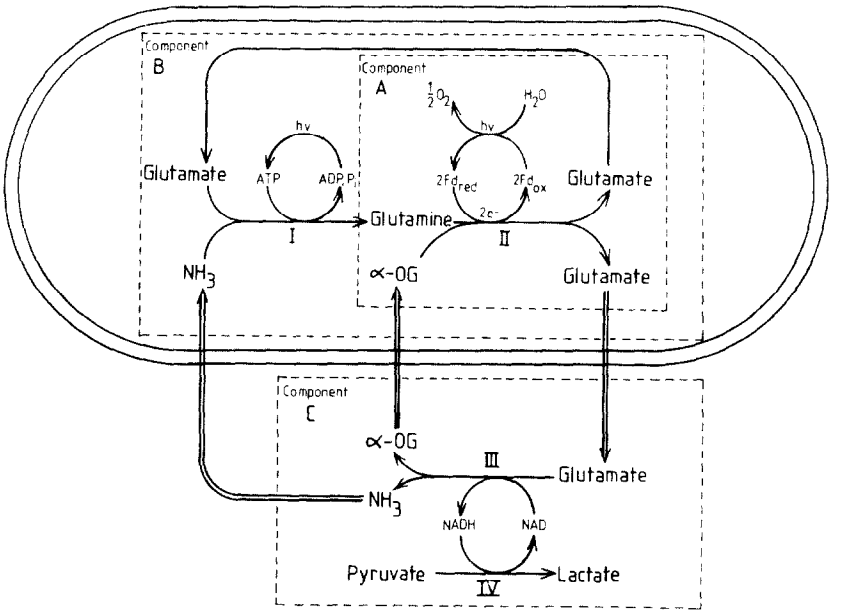

Fig. 1. Model for the export of reducing equivalents from illuminated chloroplasts involving a C_5-dicarboxylic acid shuttle. The area enclosed within the double lines represents a chloroplast. Reactions I and II involve light-coupled glutamine synthetase and glutamate synthase respectively. The theories of (glutamine plus α-OG)-dependent O_2 evolution [13] and (NH_3 plus α-OG)-dependent O_2 evolution [15] by illuminated chloroplasts are shown as components A and B respectively. Glutamate, synthesized from NH_3 and α-OG by reactions I and II, is exported from the chloroplast with the counter transport of α-OG by the dicarboxylate translocator [16] and oxidized to α-OG and NH_3 (component C). Glutamate oxidation by component C is shown as studied in this paper; it involves NAD-GDH, (reaction III), LDH (reaction IV) and catalytic amounts of NAD with pyruvate as eventual electron acceptor. Other models for component C involving a range of electron acceptors are also possible.

equivalents into the C_5 shuttle (Fig. 1, component A), was confirmed by a brief study of (glutamine plus α-OG)-dependent O_2 evolution by illuminated chloroplasts [13] in the presence of glyceraldehyde, an inhibitor of CO_2 assimilation [17]. The rate of O_2 evolution varied from 2.9 to 4.2 μmol/mg Chl per hr (mean 3.5, s.d. 0.8). O_2 evolution did not proceed in the dark or in the absence of either α-OG or glutamine. O_2 evolution was totally inhibited by 2 mM azaserine, an inhibitor of glutamate synthase [12, 13]. When chloroplasts were supplied with 5 mM glutamine, addition of limiting amounts of α-OG (e.g. 250 nmol/ml) initiated O_2 evolution but the rate gradually decreased and finally ceased. O_2 evolution recommenced when more α-OG was added and this sequence could be repeated two or three times before the chloroplasts became unresponsive to further additions. For the first addition of α-OG the ratio of O_2 evolved/α-OG supplied was 0.36–0.42 but the ratio decreased with each subsequent addition. When limiting amounts of glutamine (125 nmol/ml) were supplied in the presence of 2.5 mM α-OG, similar results were observed except that for the first addition of glutamine the O_2/glutamine ratio was 0.45–0.49.

(NH_3 plus α-OG)-dependent O_2 evolution by isolated chloroplasts (component B)

The chloroplasts used in this study also catalysed (NH_3 plus α-OG)-dependent O_2 evolution in the light in the presence of glyceraldehyde [15]. The rate of O_2 evolution varied from 3.0 to 4.0 μmol/mg Chl per hr (mean 3.4, s.d. 0.5). O_2 evolution did not occur in the absence of either α-OG or NH_4Cl. In the presence of 2.5 mM α-OG chloroplasts evolved *ca* 20 nmol O_2/ml for each addition of 40 nmol/ml of NH_4Cl; the mean ratio of O_2 evolved/NH_3 supplied was 0.49 (s.d. 0.11). (NH_3 plus α-OG)-dependent O_2 evolution was inhibited completely by 1 mM MSO and showed no requirement for exogenous ADP, PPi or $MgCl_2$ as reported previously [15].

Polarographic studies of the C_5-shuttle

All studies of the C_5-shuttle were conducted in the presence of a glutamate-oxidizing system (Fig. 1, component C) involving 4 mM pyruvate, 0.2 mM NAD, NAD-GDH and LDH. Three main types of approach were used in the polarographic studies. One of these entailed the addition of substrate amounts of glutamine (5 mM) in the absence of exogenous glutamate or α-OG. MSO, an inhibitor of glutamine synthetase [14, 15] was also added to prevent recycling of NH_3 (condition 1). In some experiments catalytic concentrations of α-OG (0.25 mM) were also added (condition 1a). The second involved the addition of catalytic concentrations of glutamate (<0.25 mM) in the absence of glutamine and MSO (condition 2). The third, involved the addition of 1 mM NH_3 (a precursor of glutamine [15, 18]) and catalytic concentrations of α-OG (condition 3). All experiments were conducted in the presence of glyceraldehyde to inhibit endogenous CO_2-dependent O_2 evolution [17].

For condition 1, illuminated chloroplasts evolved O_2 after a lag period of 3–6 min. The rate gradually increased during the ensuing 12 min and thereafter maintained a

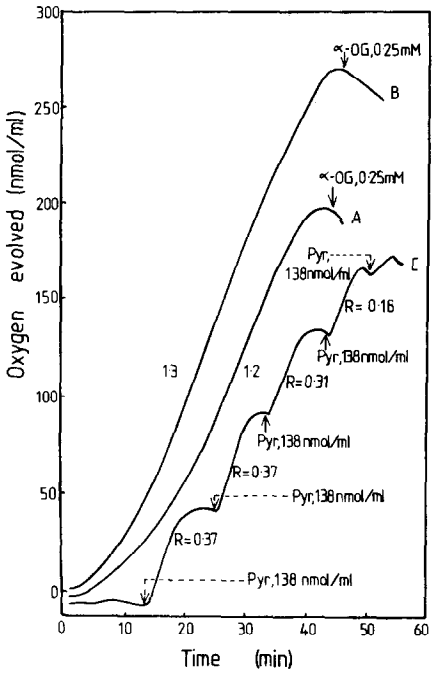

Fig. 2. Time course of O_2 evolution by illuminated chloroplasts in the presence of 5 mM glutamine, 0.5 mM MSO and component C. A, without further supplements (condition 1); B, with 0.25 mM α-OG (condition 1a); C, as for A but initially lacking pyruvate (Pyr). Further additions were made as indicated. The ratios for O_2 evolved/pyruvate supplied (R) for each addition of pyruvate in expt C are also shown. Chloroplast intactness: A, B, 89%; C, 87%.

steady rate (1.2 μmol/mg Chl per hr) for a further 20 min before O_2 evolution gradually declined and finally ceased (Fig. 2A). O_2 evolution generally ceased after 40–48 min and could not be re-initiated by addition of α-OG. Significant O_2 evolution was not detected in reaction mixtures lacking any one of glutamine, NAD, pyruvate, LDH or NAD-GDH but subsequent addition of the deleted reagent always resulted in the evolution of O_2. Addition of small amounts of pyruvate (138 nmol/ml) to reaction mixtures lacking this substrate initiated O_2 evolution but the rate soon decreased and finally ceased (Fig. 2C). O_2 evolution recommenced when more pyruvate was added and this procedure could be repeated several times. The ratio of O_2 evolved/pyruvate supplied was 0.37 for the first and second additions of pyruvate but thereafter decreased with each successive addition (Fig. 2C). Azaserine (2 mM), an inhibitor of light-coupled glutamate synthase [12, 13], completely inhibited O_2 evolution within *ca* 2 min but amino-oxyacetate (10–30 mM), an inhibitor of transaminases [19], was without effect on O_2 evolution for condition 1.

Relative to condition 1, addition of 0.25 mM α-OG (i.e. condition 1a) caused a small increase (*ca* 10%) in the rate of O_2 evolution. However α-OG virtually abolished the lag phase before O_2 was evolved and decreased the time taken to achieve a steady rate (Fig. 2B); α-OG also enhanced the total amount of O_2 evolved. With these exceptions the properties of O_2 evolution for condition 1a were essentially as described for condition 1. In the absence of MSO the rate of O_2 evolution for condition 1a was decreased by *ca* 30%. Accordingly MSO was routinely included in studies of conditions 1 and 1a.

Table 1. O_2 evolution by illuminated chloroplasts in the presence of component C and catalytic concentrations of glutamate (condition 2)

Expt	Chloroplast intactness (%)	Glutamate added (nmol/ml)	O_2 evolved* (nmol/ml) Without glutamate	O_2 evolved* (nmol/ml) With glutamate
1	75	204.5	22.7	135.3
2	75	204.5	22.7	145.5
3	87	210.0	nd†	308.6

*Determined after cessation of O_2 evolution (see text).
†nd: Not determined.

For condition 2 (i.e. 0.25 mM glutamate, without glutamine) illuminated chloroplasts catalysed O_2 evolution at rates of 0.6–0.9 μmol/mg Chl per hr. This rate was attained after a period of 20 min including a lag phase of 7 min. O_2 evolution ceased after approximately 50 min. In the absence of 0.25 mM glutamate, the maximum rate of O_2 evolution was 0.17 μmol/mg Chl per hr. Table 1 lists the total amount of O_2 evolved after cessation of O_2 evolution in experiments with and without glutamate. In all cases the ratio of O_2/glutamate exceeded 0.5. For condition 2, chloroplasts did not catalyse O_2 evolution in the absence of NAD-GDH but subsequent addition of this reagent caused immediate initiation of O_2 evolution which rapidly attained rates commensurate with those containing NAD-GDH initially. Azaserine (2 mM) completely inhibited O_2 evolution. MSO (0.5 mM) decreased the rate of O_2 evolution by 63% and O_2 evolution ceased completely after 16 min. Subsequent addition of 2 mM glutamine, however, initiated resumption of O_2 evolution at 1.1 μmol/mg Chl per hr.

For condition 3 (i.e. with 1 mM NH_3 and 0.305 mM α-OG), illuminated chloroplasts, after a lag phase of 14 min, evolved O_2 at rates of 0.6–0.9 μmol/mg Chl per hr. This rate was fairly constant for the ensuing 12 min but decreased and eventually ceased 40 min after initiation (Fig. 3A). Azaserine (2 mM) inhibited this reaction completely (Fig. 3C). MSO (0.5 mM) also inhibited the reaction completely but subsequent addition of 2 mM glutamine caused resumption of O_2 evolution at a rate of 1.4 μmol/mg Chl per hr. In the absence of NH_3 the rate of O_2 evolution for condition 3 was decreased by *ca* 45% (Fig. 3B). Reaction mixtures as for condition 3 but lacking NAD-GDH also catalysed O_2 evolution. After a lag period the rate attained 0.7 μmol/mg Chl per hr but ceased within 28 min. At this stage 146 nmol of O_2 were evolved; i.e. 0.47 mol O_2 per mol of α-OG supplied (Fig. 3D). Addition of NAD-GDH caused resumption of O_2 evolution at a rate of 1.1 μmol/mg Chl per hr (Fig. 3D).

Labelling studies of the C_5-acids in the C_5-shuttle

Metabolism of [^{14}C]-glutamate (0.25 mM) was examined under condition 2 as described for the polarographic studies. Duplicate incubations containing unlabelled glutamate were monitored for O_2 evolution by polarography. Table 2 shows that glutamate was readily oxidized to α-OG by the glutamate-oxidizing system (Fig.

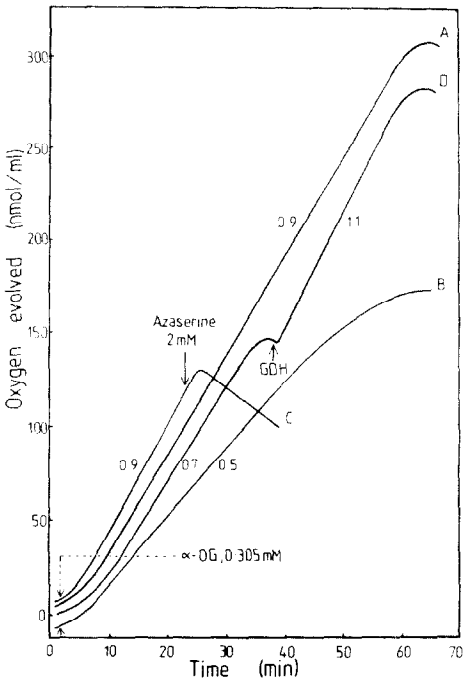

Fig. 3. Some properties of O_2 evolution by illuminated chloroplasts in the presence of 1 mM NH_3 and 0.305 mM α-OG and component C (condition 3). Experiments B and D initially lacked NH_3 and NAD-GDH respectively. Further additions were made as shown. Chloroplast intactness: A–D, 86%.

1, component C) in the absence of chloroplasts. Some ^{14}C-label was also associated with an unknown compound, referred to as X. Since the labelling of X was proportional to the labelling of α-OG (Table 2), it was assumed that X was a degradation or metabolic product derived from α-OG. When chloroplasts were incubated in the dark most of the label was again associated with α-OG and X; essentially no label was present in glutamine and only 6% of the label was recovered as glutamate. Relatively less label was associated with α-OG and X in the light; significantly more of the label was recovered as glutamate and *ca* 22% was present in glutamine. Illuminated reaction mixtures containing MSO (0.5 mM) or azaserine (2 mM) also contained less ^{14}C-label in α-OG and X than the dark control but relatively more than a complete illuminated reaction mixture. Incorporation of label into glutamine was inhibited by MSO but not by azaserine. In the absence of NAD-GDH a large proportion of the label (*ca* 64%) was recovered as glutamate although *ca* 16% was incorporated into glutamine; the remainder was recovered in α-OG.

Metabolism of [^{14}C]-glutamine (0.25 mM) was examined in the presence of 0.25 mM glutamate (Table 3). Under these conditions illuminated chloroplasts metabolized *ca* 40% of the glutamine to glutamate, α-OG and X. Much less net metabolism of glutamine occurred in the dark (*ca* 14%) or in the absence of chloroplasts (*ca* 9%) or NAD-GDH (*ca* 18%). In the presence of azaserine only *ca* 11% of the glutamine was metabolized but MSO promoted net glutamine metabolism (*ca* 62%). In the absence of NAD-GDH most of the metabolized glutamine was recovered as glutamate but in most other treatments the amounts of ^{14}C-label recovered in glutamate and α-OG were less disparate.

DISCUSSION

Studies of components A and B

The properties of (glutamine plus α-OG)-dependent O_2 evolution are consistent with light-coupled glutamate synthase activity (Fig. 1, component A). The specificity of the reaction for glutamine and α-OG, its sensitivity to azaserine but not MSO and the O_2/α-OG ratio are in general agreement with the previous study [13]. However, unlike the low O_2/glutamine ratio (*ca* 0.2) reported for pea cv Feltham First [13], the ratios for cv Massey Gem (0.45–0.49) are in general agreement with the theoretical stoichiometry (0.5) suggesting that virtually all of the exogenous glutamine was metabolized by light-coupled glutamate synthase in this cultivar. The mean rate of (glutamine plus α-OG)-dependent O_2 evolution (3.5 μmol/mg Chl per hr) was *ca* 30% of the rate for cv

Table 2. Metabolism of 0.25 mM [^{14}C]-glutamate by illuminated chloroplasts in the presence of component C (condition 2)

Treatment	Time sampled (min)	^{14}C-Label in metabolite (% of total label)			
		Glutamate	Glutamine	α-OG	X
Without Chl	0.6	65.7	2.1	27.4	4.8
	30.0	3.4	0.7	73.7	22.2
Without light	0.6	73.4	1.4	22.8	2.4
	30.0	6.1	1.0	70.1	22.8
Complete	0.6	65.7	2.1	27.4	4.8
	31.2*	22.4	22.4	44.9	10.3
Complete + 2 mM azaserine	32.0	10.2	19.8	51.3	18.7
Complete + 0.5 mM MSO	28.6	19.3	0.4	57.9	22.4
Without NAD-GDH	30.0	64.4	15.8	19.8	0

*Incubation mixtures evolved 83.5 nmol O_2/ml. O_2 evolution was not associated with any other treatment or sample.

Table 3. Metabolism of 0.25 mM [^{14}C]-glutamine by illuminated chloroplasts in the presence of 0.25 mM glutamate and component C

Treatment	Time sampled (min)	^{14}C-Label in metabolite (% of total label)				O_2 evolved (nmol/ml)
		Glutamate	Glutamine	α-OG	X	
Without Chl	0.5	1.2	97.4	1.2	0.2	0
	25.0	6.6	91.5	1.1	0	0
Without light	25.0*	8.9	86.0	4.0	0.9	0
Complete	25.2*	13.7	60.2	16.1	9.8	111.3
Complete + 2 mM azaserine	24.0*	6.7	88.7	4.4	0	0
Complete + 0.5 mM MSO	24.4*	27.0	38.5	25.0	9.5	106.2
Without NAD-GDH	25.0*	14.1	82.2	3.5	0	0

*Samples removed at 0.5 min were indistinguishable from those taken at 0.5 min from incubation mixtures lacking Chl.

Feltham First but this is consistent with the lower rates of other light-coupled reactions in this cultivar [20, 21].

The properties of (NH_3 plus α-OG)-dependent O_2 evolution are consistent with the light-coupled pathway of NH_3 assimilation shown in Fig. 1, component B; the sensitivity of the reaction to MSO (which was alleviated by glutamine) implies that glutamine synthetase and glutamate synthase are involved [14, 15]. However unlike the previous study of this reaction [15], ADP, PPi and high concentrations of Mg^{2+} were not required. We presume that the lower concentration of Mg^{2+} in the extracting medium used in the current experiments [21] is relevant here.

Studies in the presence of component C

Two basic experimental approaches were used to study the proposed C_5-shuttle. The first involved the use of substrate concentrations of glutamine and MSO (i.e. conditions 1 and 1a) to restrict the study to cycling of glutamate and α-OG in the absence of NH_3 assimilation. Studies with catalytic concentrations of α-OG or glutamate in the absence of MSO and glutamine (conditions 2 and 3) were directed towards cycling of NH_3 in addition to the C_5-dicarboxylic acids.

O_2 evolution under condition 1 did not occur in the absence of any one of NAD, pyruvate, LDH or NAD-GDH (i.e. component C inactive) implying that endogenous glutamate served as a source of α-OG for the glutamate synthase reaction. Assuming that endogenous glutamate is present in relatively small amounts [22], Fig. 1 predicts that, under condition 1, the concentration of α-OG and glutamate will constantly increase and O_2 evolution will therefore show autocatalysis before attaining a steady rate. In this event exogenous α-OG would cause more rapid attainment of maximum rate. The results (Figs. 2A and 2B) are consistent with this proposal although we presume that inward transport of α-OG or glutamine was initially rate-limiting (Fig. 2B) since condition 1 reaction mixtures which were pre-incubated in the dark for 8–15 min showed no lag upon illumination. The ratio of O_2 evolved/pyruvate supplied (Fig. 2C) for condition 1 is in fair agreement with the theoretical value (0.5) predicted by Fig. 1 and implies that pyruvate outside the chloroplast served as the eventual electron acceptor.

The properties of O_2 evolution for condition 2 imply that glutamate serves as a source of both glutamine and α-OG. In theory exogenous glutamate could serve either directly and/or indirectly in glutamine synthesis as glutamate and/or NH_3 respectively, the latter being derived from glutamate oxidation by component C. However, the absence of a glutamate requirement for (NH_3 plus α-OG)-dependent O_2 evolution (i.e. component B) and the enhanced rate of O_2 evolution by NH_3 in shuttle experiments with α-OG as the C_5-dicarboxylic acid (condition 3) suggest that exogenous glutamate serves as a source of NH_3 for glutamine synthesis for condition 2. In this event labelling of glutamine from exogenous [^{14}C]-glutamate would involve successive labelling of glutamate → α-OG → glutamate → glutamine entailing components C and A and glutamine synthetase, respectively. Recovery of relatively large amounts of label in α-OG under conditions which do not support component A or glutamine synthetase are consistent with this possibility. Exogenous glutamate appears to act as an important direct precursor of glutamine only if light-dependent glutamate synthase is blocked (e.g. with azaserine or in the absence of NAD-GDH).

Other aspects of the labelling data under condition 2 (Table 2) are consistent with the proposed C_5-shuttle. Most significant is the higher net retention of ^{14}C-label as glutamate and the small amount of label associated with α-OG in the light relative to the dark. This implies that glutamate was subject to recycling in a light-dependent reaction. The O_2/glutamate ratios from polarographic studies of condition 2 (Table 1) are also consistent with cycling of the C_5-dicarboxylic acids; after correcting for endogenous O_2 evolution the ratios exceeded the theoretical value for a single turnover (0.5). The labelling experiments with [^{14}C]-glutamine (Table 3) are also in agreement with the C_5-shuttle. Most notable is the large net loss of label from glutamine in the presence of MSO implying that the C_5-skeleton of glutamine in the absence of MSO is also subject to cycling.

For condition 3, α-OG and NH_3, which represent glutamate oxidation products produced by component C, were supplied instead of glutamate. The results (Fig. 3) imply that NH_3 is assimilated into glutamine and that this

reaction forms an integral part of the shuttle in the absence of exogenous glutamine.

The data we report imply that a C_5-shuttle could in theory operate in the photosynthetic cell. The rate for condition 1a represents the oxidation of 2.2 μmol NAD/mg Chl per hr. In contrast, rates of 15.2 and 10 μmol/mg Chl per hr and 40–50 μmol/mg Chl per hr have been reported for the C_4 [3, 6] and PGA/DHAP [7] shuttles respectively. On these grounds the C_5 shuttle, if operative *in vivo*, would make a relatively small contribution to the transfer of light generated reducing equivalents from the chloroplast to other subcellular compartments. We presume that the low affinity of the dicarboxylate translocator for α-OG and glutamate [16] and the inherently low rate of glutamate synthase of the chloroplasts used in this study are the most likely causes of the low rates of the C_5-shuttle. Further, since components A and B are also common to the N-assimilation pathway [14], the C_5 shuttle would confound this process. On the other hand the C_5-shuttle is consistent with the proposed photorespiratory N cycle [23]. In this regard the rates we report are sufficient to account for the photorespiratory NH_3 flux from glycine by isolated pea leaf protoplasts (0.77 μmol/mg Chl per hr) [23] though both values are much less than the flux predicted from partioning 15–30% of assimilated C through the photorespiratory pathway in C_3 plants.

EXPERIMENTAL

Plant material. Pea seedlings (*Pisum sativum* cv Massey Gem) were grown as before [20] and harvested 13–15 days after imbibition.

Chemicals. GDH (in 50% glycerol) was obtained from Calbiochem (La Jolla, CA., U.S.A.) and LDH (in 50% glycerol) from Sigma (St Louis, MO, U.S.A.); LDH was dialysed as in [6]. All acidic substrates were adjusted to pH 7.5 with KOH. The concentration of solns of α-OG and pyruvate were ascertained immediately after use by oxidation of NADH at 340 nm in the presence of NAD-GDH [24] and LDH [25] respectively; glutamate was determined by reduction of NAD in the presence of NAD-GDH [26]. [U-^{14}C]-L-Glutamate and [U-^{14}C]-L-glutamine were obtained from The Radiochemical Centre (Amersham, Bucks, U.K.).

Chloroplasts were prepared as in ref. [21]. Chl was measured in EtOH, O_2 evolution by polarography and chloroplast intactness by the ratio of the uncoupled rates of O_2 evolution for osmotically shocked and intact chloroplasts using $Fe(CN)_6^{3-}$ as electron acceptor [21].

Reaction mixtures for polarographic studies of components A and B and the C_5 shuttle. All incubations were conducted in the light at 25° in medium containing 0.33 M sorbitol, 50 mM HEPES, 0.1% BSA, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and adjusted to pH 7.6 with KOH [21]. These conditions were maintained by addition of double strength incubating medium where necessary. In addition all incubations were conducted in the presence of 10 mM DL-glyceraldehyde to inhibit CO_2 assimilation [17]. For (glutamine plus α-OG)-dependent O_2 evolution (component A), incubations contained 200 μg Chl/ml, 5 mM glutamine and reactions were initiated with 2.5 mM α-OG. For (NH_3 plus α-OG)-dependent O_2 evolution (component B), incubations contained 200 μg Chl/ml, 2.5 mM α-OG and reactions were initiated with 2 mM NH_4Cl.

All studies of the C_5-shuttle were conducted at 400 μg Chl/ml in the presence of 10 mM DL-glyceraldehyde and a glutamate-oxidizing system (component C) comprised of 4 mM pyruvate, 0.2 mM NAD, NAD-GDH (48 units/ml) and LDH (28 units/ml). For condition 1, reaction mixtures contained 0.5 mM MSO and 5 mM L-glutamine and reactions were initiated by addition of chloroplasts. For condition 1a, incubations contained 5 mM glutamine, 0.5 mM MSO and reactions were initiated with 0.25 mM α-OG. For condition 2, reactions were initiated with 0.25 mM L-glutamate (no other additions were made). For condition 3, reaction mixtures contained 1 mM NH_4Cl and O_2 evolution was initiated with 0.25–0.35 mM α-OG.

Metabolism of [^{14}C]-glutamate and [^{14}C]-glutamine by the C_5-shuttle. Metabolism of [^{14}C]-glutamate (0.25 mM, 10 Ci/mol) was examined in reaction mixtures otherwise as described for condition 2 for the polarographic study of the C_5-shuttle. Metabolism of [^{14}C]-glutamine was also studied in reaction mixtures as described for condition 2 but contained [^{14}C]-glutamine (0.25 mM, 19.6 Ci/mol) in addition to unlabelled glutamate (0.25 mM). These reactions were monitored for O_2 evolution in duplicate reaction mixtures using unlabelled substrates. Samples (50 μl) from ^{14}C-labelled incubation mixtures were treated with 100 μl of satd 2,4-dinitrophenylhydrazine (DNP) in 80% EtOH containing 1 M HCl and 17 μl of 3 M trichloroacetic acid. (Sufficient DNP was added to ensure that the large amount of glyceraldehyde present in the samples did not interfere with the formation of the hydrazones of α-OG and pyruvate.) After centrifugation, samples were subjected to PC on Whatman 3 MM paper in *n*-BuOH–EtOH–0.5 M NH_3 (7:1:2) for 24 hr [26]. Labelled compounds were detected with a gas-flow radiochromatogram scanner. Marker amino acids were detected with 0.1% ninhydrin in *n*-BuOH and oxo acids as their DNP-hydrazones.

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