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Update on Metabolism

Efficiency of Nitrogen Utilization in C_3 and C_4 Cereals

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Addition of nitrogen leads to increased dry matter accumulation in vegetative plant parts and to increased final yields in cereal crops (Hageman and Lambert, 1988). The efficiency with which nitrogen is used varies with plant species and with environmental conditions. For example, plants that possess a C_4 pattern of photosynthesis have, in addition to a superior method for trapping CO_2 from the atmosphere, a greater nitrogen use efficiency (g dry matter gain per mg nitrogen utilized) than do C_3 plants (Brown, 1978). Although there are many differences in the metabolism of C_3 and C_4 plants, the major difference between these two patterns of photosynthesis is the contribution of photorespiration to both carbon and nitrogen metabolism. When photorespiration is reduced in C_3 plants either by increasing ambient levels of CO_2 or reducing levels of O_2, both the yield (vegetative dry matter) and nitrogen use efficiency are enhanced (Evans, 1989). As indicated in Table I, this effect is apparent in wheat, a C_3 cereal, but not in maize, a C_4 cereal (Hocking and Meyer, 1991).

Factors that could be altered by reducing the contribution of photorespiration are the carbon supply necessary to drive the net increase in carbohydrate and protein and the availability of reductant and ATP. In this article I demonstrate that high levels of NO_3^- seen in barley and wheat relative to maize and sorghum (Martin et al., 1983) are related to a carbon deficiency caused by the inhibition of the mitochondrial PDC by monovalent cations, in particular by the NH_4^+ produced by photorespiration in C_3 plants (Schuller and Randall, 1989; Gemel and Randall, 1992). NH_4^+ production is lower in C_4 than in C_3 cereals (Martin et al., 1983). In addition, since NH_4^+ production is localized in bundle sheath cells in C_4 plants, whereas NO_3^- assimilation is found in mesophyll cells (Edwards, 1986; Becker et al., 1993), its impact on the carbon flow required for NO_3^- assimilation should be negligible in C_4 plants.

THE PRODUCTION OF NH_4^+

The NO_3^- Assimilation Pathway

A NO_3^--uptake system, NR, and NiR are required for the uptake of NO_3^- and its conversion to NH_4^+ (Fig. 1A). Both energy and reductant in the form of NADH or NAD(P)H and reduced Fd are required to drive these processes (Sechley et al., 1992). A major source of cytosolic NADH for the reduction of NO_3^- is a malate/oxaloacetate shuttle that operates in green leaves between the chloroplast and cytosol (House and Anderson, 1980; Heldt and Flügge, 1992). In this shuttle, NADPH produced by PSII in the chloroplast mediates the reduction of OAA (OAA + NADPH → malate + NADP) and is transferred via malate, the product of that reduction, to the cytosol. In the cytosol, malate oxidation to OAA is coupled to the reduction of NAD (malate + NAD → OAA + NADH). The OAA is cycled back to the chloroplast and the NADH produced can be used in the reduction of NO_3^-. A malate/oxaloacetate shuttle from mitochondria is an alternative source of NADH for reductions localized in the cytosol (Naik and Nicholas, 1986). The availability of this source of reductant would depend mainly on the oxidation of Gly in C_3 plants in the light (see Gemel and Randall, 1992; Heldt and Flügge, 1992; Hänning and Heldt, 1993). Reduced Fd is supplied directly to NiR within the chloroplast, as is the ATP, which is required for the synthesis of Gln (Fig. 1A). Reductant [Fd or NAD(P)H] is required for the synthesis of glutamate and both reductant and ATP are required for the synthesis of other amino acids within the chloroplast and, subsequently, for the incorporation of those amino acids into protein (Fig. 1B) (Sechley et al., 1992). Carbon supplied by the Calvin cycle is required for the net synthesis of carbohydrate (Suc or starch) and of glutamate, Gln, and protein. A sophisticated carbon shuttle between chloroplast, mitochondria, peroxisome, and cytosol has evolved to maintain the supply of carbon, reductant, and ATP necessary to support photosynthesis, photorespiration, NO_3^- reduction, and the biosynthetic reactions (Ogren, 1984; Naik and Nicholas, 1986; Heldt and Flügge, 1992).

Since NO_3^- accumulates in C_3 relative to C_4 cereals (Martin et al., 1983), it could be that reactions involved in its uptake or in its reduction are important in controlling the efficient flow of NO_3^--N to protein. It could be, for example, that the permease mediating the uptake of NO_3^- is more active or more abundant in C_3 than in C_4 cereals, or that NR is less active or less abundant. To examine these possibilities, Oaks et al. (1990) grew barley and maize seedlings with three levels of NO_3^- (1, 5, and 20 mM), and measured the loss of NO_3^- from the medium, its accumulation in leaf tissue, NR

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Abbreviations: AAN, amino acetonitrile; GDC, Gly decarboxylase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS-1, cytosolic Gln synthetase; GS-2, chloroplastic Gln synthetase; HPMS, α-hydroxypyridine methane sulfonate; INH, isonicotinyl hydrazide; NiR, nitrite reductase; NR, nitrate reductase; OAA, oxaloacetic acid; PDC, pyruvate dehydrogenase complex; PEPcase, PEP carboxylase; PGA, phosphoglyceric acid; SPS, Suc phosphate synthase; TCA, tricarboxylic acid.

Table I. *Effect of nitrogen supply and CO^2 enrichment on nitrogen use efficiency in C_3 (wheat) and C_4 (maize) cereals*

Control (C; 340 $cm^3\ m^{-3}\ CO_2$) and CO_2-enriched (E; 1500 $cm^3\ m^{-3}\ CO_2$) plants were grown for 52 d in plastic pots (23 cm in diameter) under natural light in duplicate greenhouses. Nitrogen use efficiency is defined as weight of biomass produced per unit of N in the plant expressed as a percentage (g dry matter mg^{-1} N × 100). (Adapted from Hocking and Meyer [1991] with permission.)

N Supply	Nitrogen Use Efficiency		
	Wheat		Maize (C or E)
	Control (C)	Enriched CO_2 (E)	
mM			
0.5	5.8	7.4	7.4
2.5	4.1	6.8	6.6
6.0	3.6	5.8	5.6
12.0	3.1	5.0	5.1
25.0	2.8	4.4	4.9

activity, and the levels of total soluble protein (Table II). From those data it is clear that the uptake of NO_3^-, tissue NO_3^- levels, and NR are higher in barley than in maize. At the same time, levels of soluble protein are low relative to levels found in maize. Thus, the fate of tissue NO_3^- clearly represents a crossover point where the flow of NO_3^--N to protein-N is restricted in barley leaves; however, this restriction is not related to levels of extractable NR (Table II). This is good evidence that the flow of NO_3^--N is controlled not by mass flow but by a regulated NR. Thus, although there is ample extractable NR in barley leaves, that NR may, in fact, be in an inactive form in situ.

When wheat plants were grown in elevated levels of CO_2, i.e. when photorespiration was inhibited, the flow of NO_3^--N to protein was enhanced (Table I) and levels of both tissue NO_3^- and NR activity (Table III) were lower than levels found in control plants (Hocking and Meyer, 1991). The accumulation of tissue NO_3^-, which is reduced when photorespiration is inhibited, suggests that the true in vivo reduction of NO_3^- is inhibited either directly or indirectly by a product or products resulting from photorespiration, and that the NR activity as measured by the usual in vitro and in vivo methods reflects levels of tissue NO_3^- and not necessarily the true in vivo capacity for the reduction of NO_3^-. In other words, the total level of NR protein induced by NO_3^- is not necessarily expressed as NR activity in situ. According to current research, variable amounts of that protein are in an active form at any one time (see Lillo, 1994). This activation potential of NR could be one reason for the poor correlations found between yield and nitrogen additions in Hageman's experiments (Hageman and Lambert, 1988).

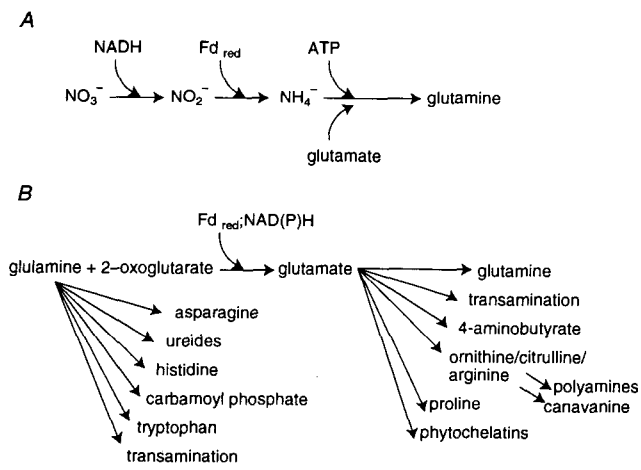

Figure 1. Primary reactions in the assimilation of NO_3^-. A, $NO_3^- \rightarrow$ Gln; B, potential fates of Gln and glutamate. In some of the reactions listed in B, NH_4^+ may substitute for Gln, e.g. in the synthesis of Asn, carbamoylphosphate, or glutamate. In these alternative reactions distinct gene products are also required.

The Photorespiratory Supply of NH_4^+

The photorespiratory nitrogen cycle first proposed by Keys et al. (1978) has been proven to be basically correct by mutant studies of Sommerville and Ogren and the inhibitor studies of Zelitch (reviewed by Ogren, 1984; Sechley et al., 1992). Although not involved in the primary net assimilation of nitrogen, the NH_4^+ handled by the photorespiratory-N cycle in a particular photorespiring C_3 leaf can be up to 20 times that handled by the reduction of NO_3^- (Canvin, 1990); in fact, in the light when photosynthesis and photorespiration are active in green leaves, it is the major player in the nitrogen economy of that leaf.

The products of photorespiration, as illustrated in Figure 2, are:

1. Ser, which is converted to hydroxypyruvate via a transaminase reaction in the peroxisome. This is followed by a reduction requiring NADH to yield peroxisomal glycerate. The glycerate is transferred to the chloroplast, where it is phosphorylated. The resultant 3-PGA is reduced via an NADP-requiring glyceraldehyde phosphate dehydrogenase. The NADH required for the production of glycerate is supplied either by the conversion of Gly to Ser in the mitochondria cycle or by reductant produced in the chloroplast. In either case, a malate/OAA cycle would be involved in the transfer of reducing equivalents to the peroxisome. Where

there are other reactions competing for this reductant, the rates of either those other reactions or the photorespiratory-N cycle would be reduced. Hence, reductant supply required for reduction of NO_3^- is in direct competition with the reductant supply necessary to drive photorespiration.

2. CO_2 is reassimilated via Rubisco or PEPcase or is lost to the atmosphere.

3. NH_4^+, judging from the effect of GS- or GOGAT-minus mutants in *Arabidopsis* and *Hordeum* (see Sechley et al., 1992), is either reassimilated via GS or is lost from the system.

4. NADH can be used in the reduction of oxaloacetate to yield malate, or in the electron transport system of the mitochondria to yield ATP. The malate produced in the mitochondria can then be used to supply the reductant necessary for the production of glycerate or NO_2^-.

If the photorespiratory system were truly cyclic, as was suggested initially (Keys et al., 1978), the stoichiometry of the products would be 1:1:1:1; Ser would be converted back to phosphoglycerate and in the process would use the NADH generated by the conversion of Gly to Ser (Canvin, 1990). Such stoichiometries do not take into account competition for the products of photorespiration, for example, competition for the available glutamate or NADH or the export of intermediates such as Ser or Gly to other parts of the plant (see Sechley et al., 1992, for a more detailed discussion of this point and for the pertinent primary references).

The levels of phosphoglycerate generated by Rubisco can be modified by altering the CO_2 or O_2 concentration of the atmosphere (Fig. 2), with the carboxylation reaction being favored by elevated CO_2 concentrations (Ogren, 1984). Such modifications of the atmosphere as well as the use of inhibitors of photorespiration have proven useful in determining the contribution of photorespiration to metabolism in different plant species and under different environmental conditions (Evans, 1989). Using such techniques, Martin et al. (1983) established that photorespiration, although present in C_4 cereals, occurs at much lower rates than in C_3 cereals. The rate of respiration in mitochondria from mesophyll cells of maize leaves that lack significant levels of GDC is not supported by Gly (Yamaya et al., 1986; Kumar and Abrol, 1989; Hänning and Heldt, 1993). From the results of Hocking and Meyers (1991), it appears that in C_4 plants, unlike C_3 plants, reductions in photorespiration that are induced by increased levels of atmospheric CO_2 (Martin et al., 1983) do not affect the nitrogen use efficiency. Thus, the C_4 metabolism appears to have a built-in control that maintains an efficient flux of NO_3^--N to protein at all times.

Table III. *Effect of CO_2 enrichment on NO_3^- accumulation and level of NR activity in wheat*

Plants were grown for 45 d with 6 mM KNO_3 in greenhouse conditions as described in Table I. C, Control; E, CO_2-enriched growing conditions. An in vivo assay was used to determine NR activity. (Adapted from Hocking and Meyer [1991] with permission.)

Days after Planting	NO_3^--N (dry wt)				NR Activity in Leaf	
	Stem base		Leaf			
	C	E	C	E	C	E
	mg g^{-1} dry wt				*μmol NO_3^- g^{-1}*	
28	7.5	4.5	4.0	1.8		
45	6.2	2.0	2.2	0.7	10.6	6.2
58	6.4	2.0	1.7	0.5		

LIMITATIONS ON NO_3^- REDUCTION

The Carbon Supply

The NH_4^+ produced by NO_3^- reduction or photorespiration is fixed primarily into the amide-N of Gln by GS and is subsequently funneled to other compounds, such as gluta-

Table II. *NO_3^- assimilation in barley and maize seedlings*

For the NR activity, NR protein, NO_3^- accumulation, and the protein nitrogen data, seedlings were grown in Kimpack (Seedburo Equipment Co., Chicago, IL) for 7 d at 28°C for a 16-h day (150 μE m^{-2} s^{-1}) and were irrigated with one-tenth Hoagland salts that contained the appropriate level of KNO_3. For the NO_3^- uptake experiments, the seedlings were planted in 0.8% agar made up in one-tenth Hoagland salts that contained no NO_3^-, and after 48 h the seedlings were transferred to a hydroponic system (one-tenth Hoagland salts with no NO_3^-) for another 7 d. At this time, the seedlings were transferred to a fresh solution that contained the appropriate level of KNO_3 in one-tenth Hoagland salts. Medium was sampled for NO_3^- periodically over the next 48 h. (Adapted from Oaks et al. [1990] with permission).

	KNO_3 (mM)					
	Barley			Maize		
	1	5	20	1	5	20
NO_3^- uptake (μmol h^{-1})	4.3	25.5	94.8	13.1	15.3	48.0
NO_3^- accumulation in shoot (μmol g^{-1} fresh wt)	29.6	102.6	169.3	26.3	46.2	76.8
NR protein (arbitrary units)[a]	15.6	100.0	248.0	95.0	100.0	110.0
NR activity (μmol $NO_2^-g^{-1}$ fresh wt h^{-1})	1.6	6.6	14.5	3.2	4.7	5.0
Protein (mg g^{-1} fresh wt)		12.2			31.5	

[a] Values for NR protein were read on a Hoeffer Scientific transmittance/reflectance scanning densitometer (model GS 300). The 100 value for barley was 2564 and for maize was 7642. The maize NR protein values have since been repeated using more highly purified antibody preparations, and essentially the same trend with increasing NO_3^- values was seen, although the NR protein levels were lower than levels of barley NR proteins (A. Oaks, unpublished results).

mate via the GOGAT reaction and Asn via Asn synthetase (Fig. 1B). The α-NH_2-N of glutamate is the major source of nitrogen for the other amino acids and, in addition, glutamate carbon is required for the synthesis of Gln, Arg, and Pro, and for Chl (see Sechley et al., 1992). Thus, when there is an active protein synthesis, there is a requirement for the net synthesis of both glutamate and Gln. Since glutamate is derived from 2-oxoglutarate, an intermediate of the TCA cycle, there is also an enhanced need for carbon derived from the TCA cycle.

There has been a serious controversy concerning both the operation of the TCA cycle in the light (see Gemel and Randall, 1992) and the production of ATP and NADH by the mitochondria (Fig. 3A). Since it has been established that mitochondrial ATP is required for an effective photosynthesis (Krömer et al., 1988) and that the supply of carbon to the TCA cycle is reduced in the light, the NADH supplied by the conversion of Gly to Ser must be, as discussed by Gemel and Randall (1992), of major importance in supplying the mitochondrial ATP that is necessary for an efficient photosynthesis in C_3 plants (Fig. 3B). The photorespiratory C-N cycle, however, would not supply the carbon required for the synthesis of glutamate and Gln. In a series of papers from D. Randall's lab, of which the 1992 paper by Gemel and Randall provides an important summary, it has been established that the PDC that is localized in the mitochondria is reversibly inhibited in the light, that the degree of inhibition is related to its phosphorylation status, and that the phosphorylation is activated by monovalent cations, in particular by NH_4^+. PDC, which decarboxylates pyruvate to produce acetyl-CoA, NADH, and CO_2, is essentially irreversible. Its action is inhibited in vitro and in situ by NADH and acetyl-CoA products of its reaction as well as by a light-induced phosphorylation (Fig. 3A). The phosphorylation step with the resultant inactivation of PDC is itself enhanced by NH_4^+ and is inhibited by pyruvate. This, then, represents a very intricate regulation involving the relative concentrations of a number of metabolites.

The resultant steady-state PDC would depend on rates of supply of pyruvate and utilization of acetyl-CoA and NADH

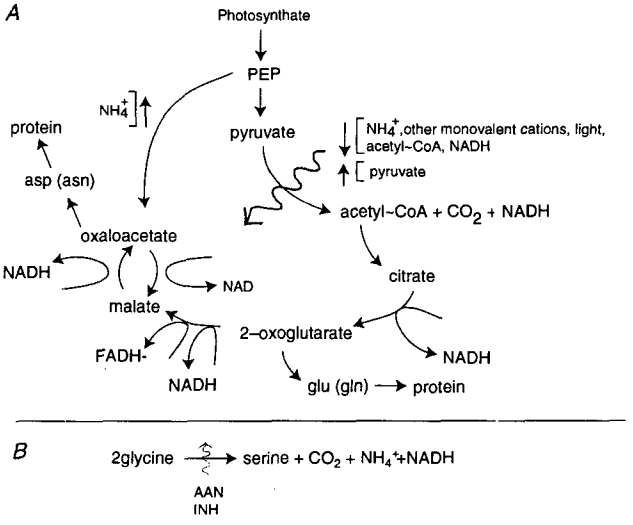

Figure 3. A, TCA cycle site of synthesis of carbon (Glu or Asp), NADH, FADH, and, by oxidation of these compounds, ATP. Regulated sites: pyruvate to acetyl-CoA + CO_2 + NADH (PDC) and PEP to oxaloacetate (PEPcase). ↓, negative effectors; ↑, positive effectors. B, Photorespiration: site of synthesis of NADH and, by its oxidation, ATP; also site of reduction (NADH) to drive the reaction oxaloacetate to malate in A.

in addition to the supply of NH_4^+ (Budde and Randall, 1988). The results in Table IV show that the mitochondrial PDC is inhibited by light, and that the inhibition is no longer apparent when DCMU, an inhibitor of the Hill reaction of photosynthesis, or when HPMS, INH, or AAN, inhibitors of photorespiration, are added to the system. Thus, the light inhibition is related to a product(s) of photosynthesis or its derivative photorespiration. Additions of Gly (Table IV) also inhibit the activity of the mitochondrial PDC, and this effect is blocked by INH or AAN, inhibitors of Gly decarboxylation (Fig. 3B). This is taken as evidence that the in situ production of NH_4^+ and NADH via photorespiration are of major im-

Figure 2. Basic steps in photorespiration. See description in text for fates of Ser, CO_2, NADH, and NH_4^+.

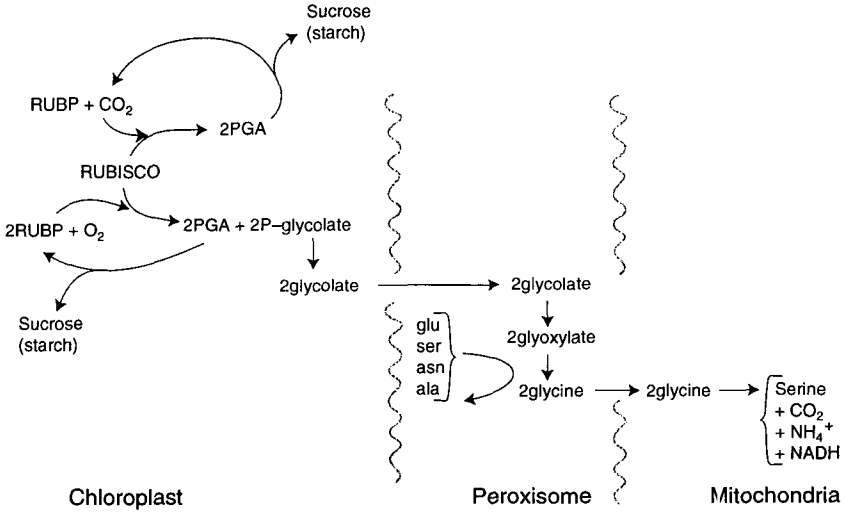

Table IV. *Effect of light and Gly on mitochondrial pyruvate dehydrogenase activity*

In Experiment A detached leaf pairs from pea seedlings were fed inhibitors for 1 h in the dark. PDC activity was determined in isolated mitochondria prepared from dark-treated tissue or from tissue that had been illuminated at 250 $\mu E\ m^{-2}\ s^{-1}$ for 1 h. In Experiment B the seedlings were kept in darkness overnight. Detached leaves were cut into 1- to 2-mm strips and incubated in the dark in the incubation solution for 1 h. PDC was then assayed using the in vivo procedure. Values were corrected for chloroplast activity (1.4 nmol $min^{-1}\ mg^{-1}$ protein). (Adapted from Gemel and Randall [1992] with permission.)

Experiment	Dark	Light	Percent Dark Control
Experiment A			
H_2O	2.4	0.6	25
+ DCMU (50 μM)	2.6	2.4	92
+ HPMS (10 mM)	2.8	2.5	89
+ INH (10 mM)	2.4	2.2	92
+ AAN (25 mM)	2.6	2.8	108
Experiment B			
H_2O	4.0		100
+ Gly (20 mM)	2.0		55
+ Gly + AAN (25 mM)	4.4		110
+ Gly + INH (10 mM)	3.8		95

portance in the regulation of mitochondrial PDC (Gemel and Randall, 1992).

In algal systems, where growth rates and the supply of nutrients can be rigorously controlled, Turpin and Weger (1990, and refs. therein) have shown that an increased demand for carbon in the synthesis of glutamate imposed by the addition of NO_3^+ or NH_4^+ is met by an equivalent increase in the steady-state conversion of pyruvate to acetyl-CoA and CO_2 and of PEP to oxaloacetate. Results with higher plant systems also indicate that light results in an enhanced conversion of PEP to oxaloacetate, and that this activation could be mediated by a phosphorylation of PEPcase (see Champigny and Foyer, 1992; Foyer et al., 1994). Superficially, the simultaneous activation of PEPcase and inhibition of PDC by light is a contradiction that should not have happened in the course of evolution. This is an oversimplification, of course, since we know that PEPcase itself is a highly regulated enzyme (Champigny and Foyer, 1992, and refs. therein), and hence that its activity, too, should be down-regulated when the activity of PDC is down-regulated. Even so, it would not have such severe implications in plants with a C_4 metabolism if NH_4^+ production via photorespiration and the PDC required to operate coordinately with PEPcase are found in different cell types.

In algal systems, mechanisms for concentrating CO_2 reduce the oxygenase function of Rubisco (Canvin, 1990) and, as a result, the photorespiratory N-cycle with its potential production of NH_4^+ is of minor importance. Thus, even if the PDC in algal cells were sensitive to NH_4^+, and this point has not yet been established, it should not be inhibited in situ, since photorespiration is not a major reaction. In the leaves of C_4 plants, photorespiration is restricted to bundle sheath cells, whereas NO_3^- assimilation is found in the mesophyll sheath cells (Edwards, 1986; Becker et al., 1993). Thus, if the PDC complex found in the mesophyll cells were inhibited by the NH_4^+ produced by photorespiration, and this point also has yet to be established, the supply of NH_4^+ in C_4 plants would not only be less than that found in C_3 leaves (Martin et al., 1983), but would also be spatially removed from the PDC complex present in the mitochondria of mesophyll cells.

The advantage of C_4 morphology and its resultant metabolism would then be 2-fold: that photorespiration is reduced relative to its contribution in C_3 photosynthesis (Ogren, 1984), and that NH_4^+ production by photorespiration and NO_3^- reduction are spatially separated in the leaves of C_4 plants. Thus, even if the PDC complexes in the mesophyll cells were inhibited by NH_4^+, the levels of NH_4^+, or rather the potential for NH_4^+ production, would be much lower than in the mesophyll cells of C_3 plants, and the negative impact of photorespiration on NO_3^- reduction would therefore also be much less.

Reductant Supply

This aspect of metabolism is harder to grasp, but from recent results in H.W. Heldt's and C. Lillo's laboratories (Lillo, 1994), it appears that it is probably crucial in regulating the flow of NO_3^--N to protein-N. The actual separation of reductive steps in the bundle sheath and mesophyll cells of C_4 plants (Leegood, 1993; summarized in Table V) would tend (a) to reduce photorespiration in the bundle sheath cells, and (b) to enhance the potential supply of NADH derived from the malate shuttles in the mesophyll cells. The distributions of the basic reactions involved in photosynthesis, photorespiration, and NO_3^- assimilation are summarized in Table V. Worthy of note is the fact that bundle sheath chloroplasts generate ATP and PGA, but not O_2 or NADPH. The PGA made in the bundle sheath cells is exported to mesophyll cells, where it is reduced to 3-phosphoglyceraldehyde. The 3-phosphoglyceraldehyde could then be used in a shuttle to produce NADH in the cytosol of mesophyll cells or be transported back to the bundle sheath cells for processing via the Calvin cycle (Leegood, 1993).

In mesophyll cells of C_4 plants, the NADPH produced in the chloroplast can be used to reduce oxaloacetate or the bundle-sheath-derived 3-phosphoglycerate. The reduction

Table V. *Division of labor between bundle sheath and mesophyll cells*

Information adapted from data in references by Edwards (1986), Hatch (1987), Leegood (1993), and Sechley et al. (1992). ME, Malic enzyme; MSX, Met sulfoximine.

	C_3 Plants	C_4 Plants	
		Bundle sheath cells	Mesophyll cells
Light reactions	PSI and PSII activity	Relative to chloroplasts from C_3 plants, chloroplasts from bundle sheath cells are deficient in PSII activity, and in Chl *b*, are relatively high in P700, generate ATP and 3-PGA, but not O_2	As in C_3 plants
Carbon assimilation	Rubisco PEPcase (some) Ribulose-5-P-kinase Rib-5-P isomerase Fru-1,6-bisP phosphatase	Rubisco PEPcase (some) NADP-ME Export PGA Export pyruvate	PEPcase (high levels) NADP-MDH pyruvate, Pi dikinase Triose dehydrogenase Export 3-P glyceraldehyde Export malate (aspartate)
Suc synthesis	Cytoplasm	—	Cytoplasm
Starch synthesis	Chloroplast	Chloroplast	—
Nitrogen assimilation	Supply of NH_4^+ by photorespiration and NO_3^- reduction occurs in the same cells GS (2) GOGAT, GDH	Photorespiratory NH_4^+, GS (1 and 2), GOGAT Protoplasts + Gly + MSX yield NH_4^+ NAD-GDH NADP-GDH	NO_3^- reduction, GS (1 and 2), GOGAT Protoplasts + NO_2^- -- MSX yield NH_4^+ NR NiR NADP-GDH

products of either reaction would then be available to generate the NADH in the cytosol, which is required for the reduction of NO_3^-. In addition, because of reduced levels of NH_4^+ in these cells, there should also be a greater abundance of NADH generated via the TCA cycle as well as more carbon for the synthesis of glutamate (Fig. 3A). Thus, whereas there is a tight competition for the NADH produced in mesophyll cells of C_3 plants, the reactions in C_4 mesophyll cells are designed for a maximum supply of NADH to the cytosol.

THE IN VIVO REGULATION OF NR ACTIVITY

There have been sporadic reports of the activation of NR in higher plant tissues over the last 20 years (see Aryan et al., 1983, for a summary of the early work; and de Cires et al., 1993, for more recent examples). Extensive work performed in B. Vennesland's laboratory and summarized in a paper by Pistorius et al. (1976) established that NR from *Chlorella* could be oxidized in vitro by ferricyanide to yield a more active enzyme and reduced by low levels of cyanide and NADH to yield an inactive NR, and that these processes were freely reversible. In Pistorius's experiments, enzyme activity was measured in the initial extracts and after activation with ferricyanide. The results showed a significant positive correlation between activation levels of NR and the growth rate of *Chlorella* (Pistorius et al., 1976).

In higher plants there are also a number of examples of activation of NR by ferricyanide and inactivation by NADH and cyanide (see de Cires et al., 1993, and refs. therein). In particular, Aryan et al. (1983) were able to demonstrate that there was an inactive NR in extracts of wheat leaves that could be activated by ferricyanide, or by light in the presence of flavin adenine dinucleotide, and that thiols were somehow implicated in the activation. The proportion of the enzyme in the inactive form was higher late in the photoperiod and in leaves exposed to salt stress. Lillo (1991) also presented good evidence for the importance of environmental factors (e.g. temperature or light) on the activation level of NR. It would be of interest to establish whether the proportion of NR in the active form is also higher in leaves of C_3 plants when photorespiration is suppressed, and whether such an effect would even be observed in the leaves of C_4 plants. The suggestion from the results of experiments performed in B. Vennesland's laboratory and those performed with higher plants is that redox potential (NADH/NAD) could be an important cue in regulating the proportion of NR found in the active form.

Recently, good evidence has emerged of a rapid and reversible light activation of NR (see Lillo, 1994, and refs. therein). For example, with higher levels of CO_2 in a nitrogen atmosphere, levels of extractable NR were also higher in the leaves of spinach and barley seedlings. Indirect evidence from

the use of inhibitors and nucleotides (ATP, AMP) suggests that this light/dark modulation of NR occurs via NR phosphorylation/dephosphorylation reactions. Results from experiments by Kaiser and co-workers, Huber and co-workers, and MacKintosh are discussed by Lillo (1994). The postulated phosphorylation was shown to be Mg^{2+} and ATP dependent (Kaiser et al., 1992) and to be mediated by two proteins (Spill and Kaiser, 1994). Earlier investigators (Hageman's, Oaks's, and Campbell's groups, for example) used buffers that contained EDTA, which removed Mg^{2+} from the extracts with the result that this very important interaction was missed. Any treatment that reduced the level of endogenous ATP in the leaf extracts (anaerobiosis, feeding Man, or 2,4-dinitrophenol), which reduced the potential for phosphorylation, resulted in a reversal of the dark inactivation of NR (Kaiser et al., 1992). A direct effect of tissue ATP levels on the phosphorylation status of NR, however, has not been established. In fact, the contrary may be true, since neither total nor cytosolic ATP fluctuates in a direction required for the activation of NR (Kaiser et al., 1992, and refs. therein). Thus, the fluctuations in NR activity are real but the actual effector molecules have not yet been clearly defined.

A ROLE OF PHOTORESPIRATION IN THE REGULATION OF NR

That the in situ regulation of NR is controlled either directly or indirectly by the products of photorespiration is supported by the following evidence:

1. High levels of NO_3^- accumulation in tissues of C_3 plants (barley and wheat) relative to C_4 plants (maize and sorghum) and the reduction of tissue NO_3^- in wheat but not in maize when photorespiration is suppressed. One of three alternative processes could explain this observation: (a) the effect of CO_2 partial pressure on the rate of transpiration could result in high levels of tissue NO_3^-; (b) the enhanced synthesis of carbon intermediates when photorespiration is inhibited would lead by mass flow to more reduced nitrogen; (c) NR activity is down-regulated by the accumulation of metabolites that are derivatives of an active photorespiration. I suspect that the true cause will be the regulation of NR, but none of these possibilities has been rigorously tested.

2. The PDC complex in the mitochondria of mesophyll cells of C_3 plants is inhibited in the light and this inhibition is reduced by the addition of inhibitors of the light reaction of photosynthesis (DCMU) and of photorespiration (HPMS, AAN, or INH). In vitro (Budde and Randall, 1988) and in vivo (Gemel and Randall, 1992) evidence suggests that the potential for NH_4^+ production via photorespiration is the cause of the inhibition. My colleagues who deal directly with carbon and nitrogen assimilation or with "nitrogen use efficiency" say that the effect on the PDC complex and the resultant reduced flow of carbon into the TCA cycle is too simple an explanation for the regulation of nitrogen flow from NO_3^- to protein, and I agree. There are, for example, clear illustrations of the effect of light and NO_3^- (or NH_4^+) on other key enzymes that control carbon flow and ultimately nitrate assimilation. The activation of PEPcase and SPS (Champigny and Foyer, 1992; Foyer et al., 1994), two cytosolic enzymes, is a case in point. Each is phosphorylated in response to light and NO_3^-. PEPcase is activated and SPS is inactivated by the phosphorylation and the resultant photosynthate is directed to the TCA cycle. According to the hypothesis of Champigny and Foyer (1992), NR that can be phosphorylated (Huber et al., 1992) should be phosphorylated when PEPcase and SPS are phosphorylated to yield an active NR to supply the reduced nitrogen required for a light-enhanced synthesis of protein. But the phosphorylated NR is the inactive form. In addition, NR is phosphorylated in the dark. Does this apparent contradiction indicate unique cytosolic kinase/phosphorylase enzymes for NR? Or perhaps a regulation coupled to the regulation of mitochondrial PDC rather than the regulation of PEPcase? More work needs to be done, and perhaps with an on/off switch for photorespiration, we can sharpen the focus and actually determine which metabolites are involved in the regulation of NR activity.

3. The physiologically important regulation of NR involves the activation/inactivation of NR and not necessarily those factors that control the induction and turnover of the NR protein. The regulator cues are not necessarily NH_4^+, since NH_4^+ additions do not lead, under normal conditions, to the in vitro or in vivo regulation of NR (see refs. in Sechley et al., 1992). Rather, Gln or the Gln/glutamate ratio (Turpin and Weger, 1990, and refs. therein; Foyer et al., 1994) or the NADH/NAD ratio (Lillo, 1994), or even some other metabolite altogether, may turn out to be the true effector limiting the nitrogen flux from NO_3^- to protein. In any case, by altering levels of photorespiration, we should be able to focus on the real cues affecting the activity level of NR.

In the years 1960 to 1990, Richard Hageman's group at the University of Illinois described the importance of NR in determining both the rates of plant growth and final yields (Hageman and Lambert, 1988, and refs. therein). In light of recent observations, I think that the key to the NR effect lies neither in its in vitro nor its in vivo activity as measured experimentally, but in its true status in situ. Because of this, recent research dealing with the activation of NR is extremely important and may lead to a better understanding of the correlations or lack thereof between yield and NR activity.

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