This project was directed toward understanding at the physiological, biochemical and molecular levels of how photosynthetic organisms adapt to long-term nitrogen-deficiency conditions is quite incomplete even though limitation of this nutrient is the most commonly restricts plant growth and development. For our work on this problem, the unicellular green alga, *Chlamydomonas reinhardtii*, was grown in continuous cultures in which steady-state levels of nitrogen can be precisely controlled. N-limited cells exhibit the classical symptoms of deficiency of this nutrient, chlorosis and slow growth rates, and respond to nitrogen provision by rapid greening and chloroplast differentiation. We have addressed three aspects of this problem: 1) the regulation of pigment synthesis; 2) control of expression of nuclear genes encoding photosynthetic proteins; (3) changes in metabolic and electron transport pathways that enable sustained CO₂ fixation even though they cannot be readily converted into amino and nucleic acids. For the last, principle components are (a) enhanced mitochondrial respiratory activity intimately associated with photosynthates and (b) the occurrence in thylakoids of a supplemental electron transport pathway that facilitates reduction of the plastoquinone pool. Together, these distinguishing features of N-limited cells are likely to enable cell survival, especially under conditions of high irradiance stress.

**Chlorophyll Synthesis**

Severe chlorosis is the classical symptom for nitrogen deficiency but how deprivation of the nutrient attenuates pigment biosynthesis has not been clear. It might be supposed that the effect is simply due to limited amounts of δ-aminolevulinic acid as the result of deficiency of the principle substrate glutamyl tRNA. However, we found that the chloroplast-encoded transcripts for the glutamyl tRNA are unchanged under N-limitation conditions. In contrast, nuclear-encoded mRNAs for the glutamyl tRNA synthetase and WAS for several downstream enzymes in the porphyrin biosynthesis pathway are strongly down-regulated under N-deficiency conditions (Bruns and Schmidt, submitted). Thus, a primary means by which nitrogen affects chlorophyll accumulation appears to be at the early steps in the pigment biosynthesis pathway. This is not an absolute control, however, because chlorophyll synthesis still occurs to some extent and, importantly, the porphyrin synthesis pathway is also utilized for hemes necessary for respiration and other functions. Consequently, there must be more precise controls of chlorophyll synthesis, conceivably essential for prevention of the accumulation of free pigment which would sensitize deleterious photodamage events.

We discovered that more precise controls of chlorophyll accumulation and its attenuation under nitrogen-deficiency conditions is achieved at a much later step, namely at the sites of
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its major function in chloroplasts. This was accomplished through the analysis of a mutant strain of *Chlamydomonas* that is phenotypically similar to N-limited cells on account of its extreme chlorophyll deficiency and comparison of the chlorophyll synthesis capacity of this mutant with that of wild-type and another mutant with altered light-harvesting proteins Plumley and Schmidt (1995). It was found that the pigment-deficient mutant is impaired in the insertion of mature LHC proteins into thylakoids, probably because of a defect in the CPS4/ftsY pathway for stromal targeting of this class of proteins through the stroma to the photosynthetic membrane. Chlorophyll-deficiency was shown in this case to be due to the selective loss of LHCs whose absence strongly correlates with reduced rates of chlorophyll synthesis as determined by pigment pulse-chase labeling analysis. The hypothesis that LHC proteins (as might be the case to a lesser extent by chloroplast-encoded chlorophyll-binding proteins) are directly involved in chlorophyll synthesis (protochlorophyllide reduction and phytlylation) was substantiated with yet another mutant strain in which the primary lesion is in chlorophyll b synthesis, causing incomplete integration of LHC proteins into the thylakoid lipid bilayer. At elevated temperature, however, the chlorophyll b-less mutant also becomes chlorotic. Again, deficiency of LHC proteins correlates linearly with decreased rates of chlorophyll synthesis. From these studies, we attribute both the deficiency and control of chlorophyll formation in nitrogen-deficient cells as a function of the extent to which LHC proteins are produced. A striking effect of growth of *Chlamydomonas* cells in N-limited chemostat cultures is to alter the abundance of mRNAs encoding chlorophyll a/b-binding proteins such that those that are normally highly abundant are replaced by lower amounts of alternative lhc transcripts. By differential screening of genomic libraries with cDNAs created from N-limited and control cultures, we have isolated and partially characterized several members of the cab gene family, including those encoding PS I antenna proteins, that are subject to N-controlled expression. Nuclear run-on transcription analyses indicate that the genes for normally high abundance cab mRNAs are repressed under N-deficiency conditions (Bruns and Schmidt, submitted). The diminished abundance of overall lhc mRNA levels largely accounts for the depletion of antenna complexes of N-limited cells. In turn, loss of the LHCs results in loss of the primary sites of chlorophyll synthesis.

**Respiratory Control and “Chlororespiration”**

Another feature of N-limited cells is that there is a great alteration in oxygen-dependent metabolic activities as compared to cells with sufficient levels of the nutrient. We had noted that N-limitation enhances an activity noted in 1947 by Bessel Kok in which there is a non-linearity in the extent of oxygen exchange driven by the onset of photosynthetic water splitting to override respiratory oxygen consumption (Peltier and Schmidt, 1991). Initially, we attributed this to elevated amounts of thylakoid components that oxidize NAD(P)H and reduce the plastoquinone pool (discerned from the occurrence of high chlorophyll fluorescence in dark-adapted cells) followed by plastoquinol reduction by an oxidase. As light intensities are increased, photosystem I would progressively drive oxidation of the plastoquinone pool, resulting in the biphasic oxygen exchange curve. This hypothesis was put forth to be consistent with the sum of data on the phenomenon which we obtained and would account for the pervasive occurrence of genes for NAD(P)H dehydrogenase subunits in the chloroplast genomes of land plants. However, we and others have not found ndh genes in the
chloroplast genome of *Chlamydomonas* even though this organism exhibits the most pronounced activities (e.g. the Kok effect) that can be attributed to this oxidative electron transport component. Subsequently, we assessed the extent to which the *ndh* genes might have been moved in evolution from the chloroplast to the nucleus. Although no direct evidence for gene transfer in *Chlamydomonas* was found, we were able to demonstrate that organellar gene fragments are highly abundant in nuclear genomes and different modes of the gene transfer process could be deduced (Blanchard and Schmidt, 1995, 1996).

Further work that was initiated during the funding period but still is under investigation is that of a direct role of mitochondria as remarkably efficient sinks for chloroplast-generated reducing equivalents in N-deficient cells. While we are still certain that there is a chloroplast activity for reduction of the plastoquinone pool in the dark because the chlorophyll fluorescence data are irrefutable, the oxidative pathway that is subject to competition by increasing illumination is undoubtedly due to mitochondrial respiration. This was deduced from studies of *Chlamydomonas* mutants with a deletion of the mitochondrial cytochrome *b*c *cob* and cytochrome *c* oxidase *cox* genes. The mutants still exhibit mitorespiration because of the existence of an alternative, cyanide-insensitive oxidase that directly oxidizes ubiquinol with oxygen as an electron acceptor. In addition, the mutants display a greatly enhanced Kok effect when grown under nitrogen-deficiency conditions. However, unlike wild-type cells, the Kok effect is not cyanide-sensitive but it is inhibited by salicyl-hydroxamic acid (SHAM) and propyl gallate, inhibitors of the alternative oxidase. The latter inhibitors do not impair the Kok effect in N-limited wild-type cells. Therefore, "chlororespiration" in *Chlamydomonas* is definitively due to rapid shuttling of oxidizable substrates, presumably carbohydrate, from chloroplasts to mitochondria where either the conventional or alternative electron transport pathways are responsible for oxygen uptake. Remaining mysteries under investigation are a) the nature of the shuttled substrate b) the question of its synthesis and/or mitochondrial utilization under relatively low irradiance but not high irradiance hand c) the significance and biochemical basis for dark reduction of the thylakoid's plastoquinone pool in N-limited cells.

**Publications Resulting from the Grant Support:**


