1989

Enzymology of L-proline synthesis

Prashant John Rayapati

Iowa State University

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Enzymology of L-proline synthesis

Rayapati, Prashant John, Ph.D.
Iowa State University, 1989
Enzymology of L-proline synthesis

by

Prashant John Rayapati

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Botany
Co-majors: Botany (Physiology)
Molecular, Cellular, and Developmental Biology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Departments

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1989
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GENERAL INTRODUCTION

This dissertation comprises three parts. The literature review describes the phenomenon of proline accumulation in detail. Section I represents an article submitted to the journal Plant Physiology about the final enzyme of proline synthesis. Section II represents work that resulted from efforts to develop a cell-free system for proline synthesis from glutamic acid. Future investigations can be initiated using the information presented in Section II.
LITERATURE REVIEW

The primary fate of the imino acid L-proline is incorporation into protein. However, under a variety of stresses free proline accumulates in plants of diverse species. Proline accumulates in response to low temperature, infection, drought, salt-shock, and abscisic acid application. Because proline accumulates in a wide variety of plant species in response to these stimuli (4), proline accumulation is thought to be a general metabolic response to environmental stress in plants. The ultimate question is "Why does proline accumulate?" Is proline accumulation an adaptive mechanism or merely a symptom of water deficit? The more proximal question is "How does proline accumulate?" Progress has been made by testing hypotheses that answer this question.

Mechanisms of Proline Accumulation

Plant mechanisms

Since proline's usual fate in healthy turgid leaves is incorporation into protein, and since protein synthesis is diminished by stresses that decrease water potential (10), one might expect the decreased incorporation of proline into protein to be the primary cause of water-stress-induced proline accumulation. However, it has been demonstrated that inhibition of protein synthesis alone does not account
for the amount of free proline accumulated during stress (47). Proline released by proteolysis might also be a source of free proline, since proteolysis increases during water stress. However, its contribution of free proline directly to the accumulated proline pool is minimal (47, 98). Therefore, the source of accumulated proline should be a metabolic pool other than proline from protein. Yet, the rate of proline accumulation parallels the rate of stress induced proteolysis (25) suggesting that the two phenomena are functionally related. Carbohydrate synthesis is also impaired; thus, water stress increases the content of free sugars as well as total free amino acids (97). Studies with $^{15}$N-amino labeled amino acids suggest that the bulk of nitrogen accumulated in proline comes from leaf protein (29). Therefore, proteolysis can contribute to proline accumulation not only by releasing proline from protein, but also by providing nitrogen from deamination of other amino acids. Thus, the inhibition of carbohydrate synthesis and stimulation of proteolysis by water stress can provide precursors for proline synthesis and accumulation.

Proline can be oxidized to glutamic acid by proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase, which are located on the matrix side of the inner mitochondrial membrane (26). Protons and electrons are contributed directly to the respiratory electron transport
system. Inhibiting proline oxidation can cause proline accumulation. It has been demonstrated that proline oxidation is inhibited by water deficit in vivo and in organelle in some species (78, 88). However, the rate of proline oxidation in turgid leaves is only 10% of the rate at which proline accumulates in stressed leaves, so inhibition of proline oxidation alone does not account for its accumulation (90). When exogenous proline is applied to turgid barley leaves, the rate of proline oxidation increases (90). When proline oxidation was inhibited in turgid leaves using the proline analogue thiazolidine-4-carboxylate (T-4-C) only a slight increase in proline concentration was observed (27). The regulation of proline oxidation is unclear. Proline dehydrogenase is inhibited by chloride ions and T-4-C in vitro (27). The effect of water stress on proline transport into mitochondria is not understood. Current knowledge, however, indicates that inhibition of proline oxidation is necessary to permit proline accumulation, but such inhibition is not the primary cause of this phenomenon.

It has been well established by radioactive tracer studies that the primary cause of proline accumulation induced by drought, salt, and exogenous abscisic acid application is increased synthesis from glutamic acid (6, 8, 13, 58, 89). The time course of proline accumulation is the
same as the time course of $^{14}\text{C}$-proline synthesis from $^{14}\text{C}$-glutamic acid (88). Proline's biosynthetic pathway in plants is not certain (Appendix I). The enzymes catalyzing the first two reactions, gamma-glutamyl kinase (GK) and gamma-glutamyl phosphate reductase (GPR), have not been identified in plant extracts. Pyrroline-5-carboxylate reductase (PCR) has been purified to homogeneity from wilted barley leaves (50). Proline synthesis by a cell-free system has been reported only once (58) but has not been repeated by others (91). Activity of a proline-inhibitable glutamyl kinase from wheat has been reported, but its role in proline synthesis has not been clearly established (104). Proline inhibitability has been proposed to be the characteristic that distinguishes the enzyme gamma-glutamyl kinase (E.C. 2.7.2.11) from other glutamate-dependent kinase activities (Appendix VI, 104).

Activity of the third proline biosynthetic enzyme, pyrroline-5-carboxylate reductase (PCR), can be measured in cell-free extracts of several plant tissues (53, 57, 69, 100). The location of the three biosynthetic enzymes (GK, GPR, and PCR) appears to be in the cytoplasm and not mitochondria. Proline accumulation is greater in excised leaves transpiration loaded with sucrose than in leaves loaded with water (88). Anaerobiosis and inhibitors of glycolysis and the Krebs cycle prevent proline synthesis
from glutamate in leaves (90). These observations suggest that the oxidation of sugars supports proline synthesis. Proline accumulation is greater in excised leaves placed in light than in those placed in darkness (87), suggesting that photosynthesis supports proline synthesis. Since sugars can inhibit proline oxidation (78), they can play a significant role in the co-regulation of proline oxidation and synthesis. It has been established that the glutamate produced by proline oxidation is separated from the glutamate that gives rise to proline (13). Since P5C-dehydrogenase, which converts P5C to glutamate, is inside mitochondria, proline synthesis must occur in a compartment outside the mitochondrial matrix. Precise co-regulation of proline oxidation and synthesis determines the extent of proline accumulation and prevents futile cycling.

**Bacterial mechanisms**

The participation of the first two enzymes (GK and GPR) in plant proline biosynthesis is assumed because these enzymes have been well characterized for the same pathway in bacteria and yeast (7, 49, 99). GK and GPR may be difficult to extract because of their quaternary structure. According to a study of the *Escherichia coli* enzymes, the presence of GPR in the assay stimulates GK activity 45-fold (85). The intermediate, gamma-glutamyl phosphate, is not detectable.
The two enzymes form a complex and their genes are expressed as a single operon (24). Studies of *Pseudomonas aeruginosa* indicate that its GK and GPR form a complex but their genes are not closely linked (49). Genetic regulation of GK and GPR expression appears to be quite different in these two bacteria. In the yeast *Saccharomyces cerevisiae*, GK, GPR, and PCR may also form a complex and their genes are on different chromosomes (99). The yeast genes for GK, GPR, and PCR can functionally complement proline auxotrophs, with lesions in the corresponding genes, in *E. coli* and *Salmonella typhimurium* (99). The *E. coli* gene for GK can complement a yeast auxotroph for that enzyme (61). Thus, the GK and GPR enzyme subunits can be interchanged between a prokaryote and a eukaryote.

In certain bacteria the rate of proline biosynthesis from glutamic acid is controlled by feedback inhibition of GK by proline (85). Fifty percent inhibition is achieved by 50 mM proline with *E. coli* GK and 5 mM proline with *P. aeruginosa* GK. A similar control has been indirectly demonstrated with barley leaves. The incorporation of $^{14}$C-glutamate into proline by turgid leaves is decreased when excess exogenous proline is applied along with the tracer (12). Since this inhibition was observed in unstressed leaves but not water stressed leaves, proline accumulation is thought to be a result of increased
synthesis caused by decreased end-product inhibition. It is not known if this loss of inhibition is caused by an allosteric change in GK or the induction of GK isozymes with less sensitivity to proline.

The proline analogues L-azetidine-2-carboxylate and 3,4-dehydroproline have been used to select proline over-producing mutants in bacteria. Overproduction was caused by loss of feedback inhibition of GK, which in turn was caused by two different point mutations that effected single amino acid substitutions at residues 107 and 143 (21). Wild type E. coli does not accumulate proline in response to osmotic shock. However, E. coli, S. typhimurium and Klebsiella pneumoniae strains that were transformed with genes encoding the feedback-resistant GK gene were able to overproduce proline. These strains grew under salt and osmotic conditions that did not permit growth of the untransformed lines (52). Measures (54) has reported that gram-positive bacteria accumulate proline to three times the unstressed levels in the presence of salt, while gram-negative bacteria accumulate potassium and glutamic acid. This often-cited article implies that proline accumulation is a primitive response to osmotic stress that has been conserved by evolution among prokaryotes and eukaryotes. There is no evidence that accumulation of proline by wild-type bacteria results from increased synthesis from glutamic acid caused
by a loss of feedback inhibition by proline of gamma-glutamyl kinase.

**Proline transport mechanisms**

Regulation of proline transport may be a mechanism that effects proline accumulation in plants. The concentration of proline immediately surrounding gamma-glutamyl kinase (GK) may remain constant if the activity of a transporter that removes proline from that compartment increases during stress. An increase in the rate of proline export would increase the rate of proline synthesis without any need to alter the negative feedback regulation of GK. Plant amino acid transport systems are just beginning to be identified, and nothing is known about specific proline transport molecules in plants. In mammals, three neutral amino acid transporters have been identified: system A, system ASC and system L. System A is responsible for transporting proline across the plasmalemma (79). This is not the general amino acid transporter. System A is sodium dependent and pH sensitive.

Proline transport in *S. typhimurium* has been well defined (39). The putP gene encodes a proline permease. It is part of the proline utilization operon, which also contains putA. The putA gene encodes proline dehydrogenase and P-5-C dehydrogenase. When proline is present, putA protein associates with the membrane and donates electrons
from proline to the respiratory electron transport chain. When proline is absent, putA protein accumulates in the cytoplasm, binds the put promoter and represses transcription of the permease and the enzymes. Two other genes, proP and proU, have been implicated in proline transport, but are misleadingly named. A proline permease that is induced by amino acid limitation is encoded by proP. It also has low affinity for glycine betaine. The proU gene encodes a high affinity glycine betaine permease that is strongly induced by an increase in external osmolarity.

Patterns of Proline Accumulation

Proline metabolism in leaves

Proline can accumulate in all tissues of stressed intact plants. When excised roots and leaves are stressed, however, proline accumulates only in the leaves (82, 98). The presence of chlorophyll or photosynthesizing chloroplasts is not necessary for proline accumulation. Etiolated and albino leaves and cell suspensions can accumulate proline when supplied with sucrose and glutamate (82, 86, 87). Proline is translocated from shoots to roots (82, 101).

Proline accumulation is most commonly associated with a decrease in water potential of the accumulating tissue (83). As water potential decreases, proline accumulation tends to
increase. In comparisons of several genotypes of barley, sorghum, and rapeseed the amount of proline that accumulated varied among cultivars stressed to the same water potential (11, 71, 83). Proline accumulation is a heritable trait.

Upon rehydration, accumulated proline is readily metabolized. However, when leaves are dehydrated to an irreversible degree (firing), accumulated proline is not metabolized in the "fired" parts (101). In an experiment where intact plants were subjected to one, two, and three cycles of water deficit with rehydration between cycles, water potential dropped to -2.0 MPa in each successive deficit but proline accumulation increased with each successive cycle. More proline accumulated in plants subjected to three compared to two cycles, and more proline accumulated in plants subjected to two compared to one cycle of water deficit (83). Since free proline concentrations dropped to control levels during rehydration between deficits, the greater amount of free proline after successive deficits was not due to a residual contribution. Thus, previous exposure to water deficit causes some metabolic modification which results in greater proline accumulation during subsequent stresses.

The plant growth regulator abscisic acid (ABA) shows patterns of metabolism that seem to be associated with proline accumulation. Accumulation of abscisic acid under
water stress is a general phenomenon among plants (3, 56). The primary role of ABA during water deficit is thought to be to decrease transpiration by stimulating stomatal closure. Since this is effected by decreasing potassium-proton exchange at the plasmalemma, which causes guard cells to lose turgor, it is thought that ABA may affect other metabolic responses to water stress by regulating a potassium-proton exchange mechanism in mesophyll. For example, ABA could function in osmotic adjustment by increasing potassium transport into vacuoles.

ABA accumulates when proline accumulates under water deficiency, but this relationship is not universal (5). Endogenous ABA accumulates approximately two hours before proline accumulates in some species. ABA may function as a messenger that signals need to accumulate proline in those species. ABA can also accumulate in salt-stressed tissues that are accumulating proline, but ABA accumulation is not a prerequisite to salt-shock-induced proline accumulation (37). Exogenously applied ABA will cause proline accumulation in some species even when leaves are turgid (5, 89). Such proline accumulation is caused by a stimulation of proline synthesis from glutamate, but oxidation is not inhibited (89). Excised roots treated with ABA do not accumulate proline (3). Not all plants that accumulate proline in response to water deficit respond to exogenous
ABA (106). These observations could be artifacts if a threshold concentration for ABA induction of proline accumulation was not met (93). The wilty tomato mutant Flacca, which cannot synthesize ABA in response to water deficit, can accumulate proline in response to water deficit (92). It has been proposed that ABA accumulation is not a prerequisite to proline accumulation. However, ABA metabolism may yet play some undefined role at the subcellular level in the cascade of events leading to proline accumulation. Endogenous ABA accumulation appears to be a consequence and signal of turgor loss.

When excised barley leaves were salt-shocked with NaCl, leaf osmotic potential decreased but turgor was maintained (6, 105). Under these conditions, proline synthesis from glutamate was increased, but incorporation of proline into protein decreased 50%, and proline oxidation decreased only 60%. When leaves were removed from salt solutions, proline concentrations remained high as long as leaf ion content remained high, in contrast to rehydrated wilted leaves where proline returns to control levels. A key difference is that sodium and potassium ion levels remained high in leaves transferred from salt solutions to water. Organic solutes generally accumulate in tissues in correlation with the decrease in osmotic potential of the external medium (44). Studies of glycophytes and halophytes show that proline
accumulates in many species but its concentration does not correlate with leaf osmotic potentials in every species (109).

Gene expression patterns

Transcriptional and translational regulation have been indicated in the triggering of proline accumulation (94). The inhibitors cordycepin and cycloheximide both prevent proline accumulation, suggesting that the induction of proteins coded by nuclear genes plays a role in the perception and signalling of proline accumulation. It is not known if these events influence the feedback inhibition of gamma-glutamyl kinase or involve the induction of an isozyme that lacks feedback inhibition. Evidence for new protein synthesis of a novel pyrroline-5-carboxylate reductase in a salt-adapted cell line of *Mesembryanthemum nodiflorum* has been reported (100). Cycloheximide treatment of stressed cells prevented the stress-induced increase in PCR activity.

A novel rice gene, RAB 21, may provide insight into the relationship between water stress, ABA, salt shock and proline accumulation. This ABA-responsive gene encodes a 21 kd protein which is glycine rich and accumulates in the cytosol (59). In rice, RAB 21 is induced by 10 μM ABA, 200 mM NaCl and wilting treatments. Accumulation of RAB 21 mRNA begins at 3 to 6 hours after initiation of each treatment.
Maximum accumulation of transcript and gene product occurs between 12 and 24 hours. This pattern of accumulation parallels proline accumulation, which is effected by the same treatments. The molecular genetic events that occur before proline accumulates are not well defined. RAB 21's response pattern sets a precedent for genetic response to the three principal treatments that cause proline accumulation. The RAB 21 gene product is thought to act as an inhibitor of pathogen or herbivore proteolytic enzymes, because of its sequence similarity to known protease inhibitors. The diverse responses of plants to water stress suggest that proline accumulation may be one of several strategies for responding to such environmental changes. Free accumulated proline may serve more than one function in plants.

Proline metabolism in pollen

Mature pollen and seeds are the most dehydrated living plant tissues, with water potentials of -12.5 MPa and -25 MPa (110). Although ripening seeds do not accumulate proline, maturing pollen does. Proline begins to accumulate after meiosis (15). In a survey of 200 species representing 63 families, proline comprised 1% of mature pollen dry weight for most species (41). In germinating pollen one-half of the free amino acid pool was comprised of proline (200 mM). Proline seems to be most concentrated at
the growing tip of the pollen tube. De novo synthesis of proline appears to occur during the first hour of tube growth (41). The rate of pollen tube growth was the same as the rate of proline oxidation (14). A deficiency or complete lack of proline in pollen from some male-sterile plants has been reported for the following crops: wheat, sugar beet, maize, sunflower, rice, lily, poppy, tomato, sorghum, sunflower, pea, and pepper (46). Proline in pollen may serve as a protectant during desiccation as well as a highly concentrated source of energy, carbon, and nitrogen during pollen tube growth. Proline serves these functions in yeast spores (40).

Proline metabolism of other organisms and accumulation of other compounds

A liver fluke, Fasciola hepatica, has a high proline biosynthetic capacity (65). It overproduces proline and osmotically stresses surrounding bile duct tissue causing hyperplasia. This generates a suitable habitat for its growth and reproduction. The fluke also secretes an inhibitor of proline oxidation. The fluke increases bile proline levels 1000-fold. Infusions of proline into parasite-free animals can cause the same pathophysiological changes in the bile duct. A similar proline-dependent mechanism may be used in schistosomal infections (65). Thus, proline overproduction can have osmotic effects on
living tissues. Two plant pathogens, *Agrobacterium tumefaciens* and *Meloidogyne javanica*, cause proline accumulation at infection sites (55). The function of this accumulation and the source of the proline are not known. Perhaps the plant pathogens are making use of the same mechanism as *Fasciola*.

Proline was not the only organic compound that contributes to the drop in leaf osmotic potential of wilted barley. Glycine betaine also accumulates to levels that can contribute strongly to osmotic adjustment (110). In fact, glycine betaine and potassium ions were found to be the most significantly accumulated osmotica in leaves of salt-stressed barley plants (96). Proline accumulation and turnover, however, were more rapid than that of glycine betaine (110). Organic osmotica such as proline, glycine betaine, and polyols can be synthesized to protect subcellular machinery and to assist in adjusting cytoplasmic osmotic potential. Some species accumulate neither glycine betaine nor proline, yet their leaf osmotic potentials decrease upon wilting (1, 31, 32, 96).

In the kingdom Monera, proline accumulation has not been found to occur naturally. Heterotrophic prokaryotes like *Escherichia coli* and *Salmonella typhimurium* do not accumulate proline but do accumulate glycine betaine (39). A photoautotrophic freshwater cyanobacterium, *Spirulina*
platensis, osmotically adjusts by accumulating carbohydrates such as glucosyl-glycerol and trehalose (107). Proline accumulates but does not contribute to osmotic adjustment in *Spirulina*. In several halophilic cyanobacteria, proline accumulation was not reported (68). However, *E. coli*, *S. typhimurium*, *Serratia marcescens*, and *S. platensis* will accumulate proline and become more osmotically tolerant when transformed with plasmids for proline over-production or when selected for growth in the presence of azetidine-2-carboxylic acid (A2C) (42, 70, 80). Glycine betaine accumulation, which normally functions in osmotic adjustment, has been reported for a range of heterotrophic and photoautotrophic monerans (39, 68).

In the kingdom Protista, proline has been found to accumulate in a marine chlorophyte, *Nannochloris bacillaris* (103). Selection of this organism for A2C resistance leads to enhanced proline accumulation in both the steady and stressed states, yet the enhanced accumulation did not confer increased osmotolerance. Another chlorophyte *Stichococcus bacillaris*, accumulates proline and sorbitol in response to increasing salinity. However, sorbitol accumulation accounts for most of its osmotic adjustment (17). *S. bacillaris* and *N. bacillaris* are nonmotile. The wall-less flagellated halotolerant alga *Dunaliella* osmotically adjusts by accumulating glycerol and does not
accumulate proline or glycine betaine. Glycerol, which accumulates up to 4 M in response to increasing ambient salt concentrations, is synthesized as a function of photosynthesis (30). Dunaliella parva also accumulates beta-carotene (up to 8% of dry weight) in the interthylakoid space of its chloroplasts as an osmotic stress response (9). This is a mechanism for protection against high light intensity.

In the kingdom Fungi, proline accumulation has been reported only in ascospores of the yeast Saccharomyces cerevisiae. Osmotic adjustment in vegetative cells is facilitated by sucrose and trehalose accumulation (40). In the kingdom Animalia, proline accumulation has been reported in the estuarine copepod Tigriopus californicus (66). However, the levels of accumulation are not as high as in leaves. An enzyme that catalyzes synthesis of pyrroline-5-carboxylate from glutamic acid (PC synthetase) has been reported in the intestinal mitochondria of blowfly and rat (65). These mitochondria do not contain the enzymes of proline oxidation. PCR has been found in the cytosol of erythrocytes (34).

Functions of Proline Accumulation

Proline accumulation is a well known but little understood phenomenon. It has been observed in numerous
species for 30 years, yet its function has not been elucidated.

**Drought tolerance**

An Australian research team claimed that the ability to accumulate proline should be used as a selective index for drought resistance (81). In their studies, the ability of ten varieties of barley to yield under drought conditions correlated well with their abilities to accumulate proline. When a Michigan research team performed breeding experiments with two of the same varieties used in the Australian study (81), their results revealed a negative correlation between leaf survival and proline accumulation (36). Apparently, the experimental regimes differed significantly. Perhaps a key difference is that the Australian group compared yield to proline accumulation while the Michigan group looked directly at leaf survival. A study of eight sorghum varieties showed no relationship between leaf injury caused by water deficit and the amount of accumulated proline (11). However, this study found a strong positive correlation between leaf recovery and proline content. It was concluded that the ability to recover was independent of the ability to tolerate desiccation. Since dark respiration rate and ammonia release during recovery correlated with initial proline content after stress, it was also concluded that proline is a source of respiratory energy in sorghum.
recovering from drought. A study of 14 wheat cultivars, however, found no correlation between proline accumulation ability and drought resistance or recovery ability (97). Free proline concentrations correlated only with the increase in catabolic amino acid and sugar concentrations during stress.

**Growth inhibition**

The Michigan group has asserted that proline accumulation is a symptom of stress and the selection for proline accumulation would result in selection for drought susceptibility (36). Proline accumulates in leaves when stress causes cessation of growth. If leaves are stressed severely enough, they can senesce. However, proline accumulation is not a symptom of senescence: more proline accumulated in green than in yellowing wheat leaves under water deficit (102). When barley was grown at 5°C, growth rate was slowed and proline accumulated with no signs of senescence. The same variety grown at 21°C senesced with no accumulation of proline during the same period (18).

Gibberellic acid (GA), which promotes growth, and a growth retardant, cycloceI, have no effect on proline accumulation in unstressed leaves. When barley plants were treated with these compounds and then osmotically shocked, the GA-treated plants accumulated less proline and the cycloceI-treated plants accumulated more proline than stressed plants not
treated with either compound (4). Abscisic acid (ABA) is known for its growth-arresting qualities. Abscisic acid stimulates proline accumulation. The effect of ABA on proline accumulation is antagonized by benzyladenine (BA), which is a cytokinin. In the presence of BA, ABA- and salt-shock-induced proline accumulation were inhibited (94). Cytokinins are known for their growth promoting effects. Furthermore, when the growth of cell cultures from wild salt-tolerant tomatoes (Lycopersicon peruvianum) was compared with the growth of cultures from domestic salt-susceptible tomatoes (Lycopersicon esculentum), it was found that the salt-tolerant culture accumulated less proline but grew more rapidly at the same salt concentrations that caused the domestic cultures to accumulate proline rapidly but stop growing (74). Thus, proline accumulation is associated with growth inhibition induced by water stress rather than with senescence.

**Energy storage**

It has been hypothesized that plants accumulate proline as a storage form of energy and reducing power, because its synthesis requires oxidation of two molecules of NADPH and one of ATP per proline molecule (44, 64). When proline is oxidized, this stored energy and reducing power can contribute three electron pairs to the mitochondrial electron transport system for the synthesis of ATP. The
oxidation product, glutamate, can be deaminated to alpha-ketoglutarate which can be used as a Krebs cycle intermediate. The observed low concentrations of glutamic acid, the increase in ammonium ion concentration, and the release of labeled carbon dioxide after rehydration of stressed leaves that had been given labeled proline are evidence of such events (11, 13). Proline is known to be the primary energy source in pollen cells during the rapid growth that is seen in pollen tube elongation (22). Proline is also accumulated and rapidly oxidized by the flight muscles of several insect species (4). By oxidizing proline in conjunction with a glycerol-1-phosphate shuttle, these mitochondria can generate 18 moles of ATP when oxidation is not active (66). Thus, proline may be accumulated in stressed plants as a readily accessible energy source for recovery after the stress has been terminated.

Nitrogen storage

Protein hydrolysis is not inhibited by water stress and is usually accelerated by it (4, 25, 29). Even though nitrate reductase activity is inhibited and nitrogen assimilation is decreased by water stress (84), a net accumulation of amino acids and other soluble nitrogen-containing compounds of low molecular weight occurs. Such metabolites can be toxic. Accumulated proline may, in part, be a result of some of these compounds being converted to
glutamate and then to proline. Proline accumulation can also protect enzymes and structural proteins from the inhibitory effects of toxic metabolites. Ammonia detoxification has been suggested as one of the roles of stress induced proline synthesis, since in soybean and radish ammonia levels are lowest in the leaves with the most accumulated proline (29, 64). Upon stress termination, proline may be oxidized to glutamate, which plays a central regulatory role in nitrogen metabolism. Other amino acids may be synthesized by transamination from glutamate. The newly synthesized amino acids can be used in protein and nucleotide synthesis. Proline is incorporated heavily in structural proteins in cell walls (72). Thus, proline accumulation may provide a nitrogen storage pool during water stress conditions that inhibit growth.

When leaf tissue that has accumulated proline becomes desiccated beyond recovery, proline in that tissue is unable to be remobilized and constitutes a net loss of nitrogen, carbon skeletons, and energy (35, 101). Proline began to accumulate only after its translocation had stopped in stressed, intact barley, suggesting that accumulation is the result of a transport mechanism malfunction (101). According to some calculations (101), the amount of reducing power stored in proline accumulated over three days could be generated by chloroplasts operating at ten percent of their
electron transport capacity in less than one hour. These facts support the hypothesis that proline accumulation has no particular beneficial value and is simply a symptom of stress.

**Cytoplasmic osmotic adjustment**

The concentrations to which proline accumulates under stress have been calculated to be significantly beneficial to osmotically stressed plants only if the proline is compartmentalized in the cytoplasm, which comprises 5 to 10% of mesophyll cell volume (96). Proline levels do not exceed 50 μmol/g fresh weight in leaves of stressed crop species (91). Cytoplasmic proline would function in osmotic adjustment if other metabolites were compartmentalized in the vacuole. Together, the osmotic effects of the vacuolar and cytoplasmic osmolytes would decrease cell water potential to a degree that would enable cells to retain water under hyperosmotic conditions. This hypothesis most directly applies to salt-stress-induced proline accumulation, because sodium and chloride ions are known to be compartmentalized in vacuoles (43, 95). Under other stresses that induce proline accumulation, organic acids and other metabolites would have to accumulate in the vacuole.

Most of the evidence for subcellular compartmentation is associated with salt stress. Almost all the methods of studying compartmentation are indirect. Root segments of
salt stressed and osmotically stressed corn shoots had the highest concentrations of proline in their apices with lower concentrations in segments near the leaf sheath (33). This distribution is cited as evidence for cytoplasmic compartmentation, because meristematic tissue is the least vacuolated. Apices should have the most proline, because their cytoplasm-to-total volume ratio is the greatest. However, the cytoplasm-to-total volume ratio is not necessarily the cause of higher proline concentrations in root tips. Meristematic cells may synthesize or oxidize proline at different rates from mesophyll cells.

Kinetic efflux analyses of ions from tissues of several halophytes revealed compartmentation of sodium and chloride ions in the vacuole at three times the concentration of the same ions in the cytoplasm (28). Organic osmotica also accumulated, but their cytoplasmic concentrations were not as easily determined as those of the ions. Dimethyl sulfoxide is known to increase passive diffusion across the plasmalemma but not the tonoplast. This compound was applied to pea shoots that had accumulated proline in response to exogenous abscisic acid and sodium chloride (37). When the amount of proline that leaked out of such tissue (cytoplasmic) was compared with the amount retained (vacuolar), it was concluded that most of the accumulated proline had been cytoplasmic, although some had been
transported into the vacuoles. Thus, indirect methods of measuring compartmentation indicate a primarily cytoplasmic localization of accumulated proline.

More direct methods of compartmentation analysis have also been attempted. Because of the problems of fixing tissue without changing subcellular concentrations and of low resolution, electron microscopy and microprobe analysis have not been useful in studying subcellular compartmentation of osmolytes (28). Vacuole isolation is the most direct method. When vacuoles from beet storage tissue were mechanically isolated, the cytoplasmic proline concentration was always much greater than that of the vacuole (51). Again the amount of proline in vacuoles was significant. However, this tissue had not been stressed and is not well known for its ability to accumulate proline as a stress response. Vacuoles were isolated from protoplasts of tobacco (62). When leaves were stressed with polyethylene glycol (PEG), the extravacuolar proline concentration increased 2.6-fold, while the vacuolar content increased 0.7-fold. In protoplasts from unstressed control leaves, the cytoplasmic/vacuolar proline concentration ratio was 1:1, while that in protoplasts from stressed leaves was 3:1. The results reveal significant but not exclusive cytoplasmic compartmentation of proline. Several problems exist with these compartmentation analysis methods. The most
significant one is that compartmentation of metabolites can change during protoplast and vacuole isolation. It is difficult to maintain constant stress conditions during the protoplast isolation procedure, which is itself stressful. Vacuole isolation also requires an extended period during which the contents may leak out or be diluted.

Chloroplasts comprise most of the cytoplasm's volume in mesophyll cells. Proline has been reported to accumulate in chloroplasts in two instances. In *Mesembryanthemum crystallinum*, proline accumulated in chloroplasts from salt stressed plants to 5 times the level found in chloroplasts from nonstressed plants (23). Salt-shocked spinach leaves yielded chloroplasts with fourfold higher proline levels than those from control leaves (45). When stressed tobacco leaves were fractionated, Boggess et al. (12) found no majority of cellular proline associated with chloroplasts prepared by aqueous or nonaqueous methods. Perhaps stress-induced proline synthesis occurs in chloroplasts and proline initially accumulates there before proline transport mechanisms enable it to equilibrate with the cytosol.

**Membrane and protein protection**

The physiological role of proline accumulation is unclear and its value as an index of water stress tolerance is debatable. Proline's compatibility with proteins *in vitro* is indisputable. Proline has the highest solubility
among the protein amino acids. The solubility of L-proline is 1.60 g/ml. Next is L-glycine, whose solubility is 0.20 g/ml. Proline has the potential to be an osmolyte at high concentrations with few negative metabolic side effects. Proline hydrogen bonds to water molecules more strongly than water molecules hydrogen bond to each other (76). It was once thought that proline's hydrophobic regions associate with the hydrophobic regions of proteins and increase the protein's hydrophilic surface area (76). This interaction could help prevent protein denaturation. Recent experiments demonstrate that proline does not bind to protein but protects proteins through its nonosmotic interactions with water molecules (63, 111). Proline has been shown to decrease the precipitation of bovine serum albumin by ethanol and ammonium sulfate (76) and of glutamine synthetase by polyethylene glycol (63) in a concentration-dependent manner.

Reducing equivalent transfer

Evidence has been gathered for the role of proline and pyrroline-5-carboxylate (P5C) as oxidation-reduction (redox) transfer molecules between different metabolic compartments in mammalian systems (65, Appendices III and IV). The ratio of NAD⁺/NADH determines the redox state of cells and has been shown to control the rate of gluconeogenesis and urea metabolism. The NAD⁺/NADH redox state is usually oxidized.
The NADP⁺/NADPH redox state can also regulate pathways. It is usually reduced. A small change in NADP⁺/NADPH redox state can have a large effect on a redox sensitive pathway. The rate limiting step of the pentose phosphate pathway (PPP) is catalyzed by glucose-6-phosphate dehydrogenase (G6P-DH). This enzyme requires NADP⁺ and is inhibited by NADPH. It acts as a metabolic gate and has been shown to be involved in the mitotic activation of quiescent cells in culture. The PPP is tied into cell division, because a principal byproduct of this pathway is phosphoribosyl-pyrophosphate (P-ribose-PP), a rate limiting substrate of purine nucleotide metabolism (Appendix III). Phang (65) has developed a model in which P-5-C/proline interconversions modulate NADP⁺/NADPH redox states. These in turn regulate purine biosynthesis.

The regulatory effects of P5C-proline conversion on the PPP and purine nucleotide synthesis have been demonstrated with intact erythrocytes in vitro (65, Appendix III). The only P5C or proline metabolizing enzyme found in erythrocytes is P5C reductase. In normal erythrocytes, P5C caused a sevenfold increase in PPP activity, a threefold increase in P-ribose-PP synthesis, and a threefold increase in purine nucleotide synthesis. In G6P-DH deficient erythrocytes, P5C caused no change in metabolism. Thus, proline synthesis controls metabolism in erythrocytes.
The proline-P5C cycle (Appendix III) was demonstrated in a cell-free system using erythrocyte cytosol and liver mitochondria. Liver mitochondria contain high-proline dehydrogenase activity but low P5C dehydrogenase activity. In the presence of high levels of NADPH, [1-\textsuperscript{14}C]glucose, and P5C, and the absence of NADP\textsuperscript{+}, this cell-free system produced [\textsuperscript{14}C]CO\textsubscript{2}. The partial oxidation of [1-\textsuperscript{14}C]glucose, liberating [\textsuperscript{14}C]CO\textsubscript{2}, is a measure of PPP activity. When [1-\textsuperscript{3}H]glucose was substituted, [\textsuperscript{3}H]proline was found. Thus, reducing potential was transferred from glucose to proline. The same results were produced by substituting proline for P5C. The oxidation of proline in and transport of P5C out of mitochondria transferred oxidizing potential to the cytosol. The reduction of P5C back to proline produced NADP\textsuperscript{+}, which drove the oxidation of glucose by the PPP. An in vitro system containing intact erythrocytes and isolated hepatocytes yielded similar results. Thus the proline-P5C cycle can shuttle redox equivalents between cells as well as between compartments of the same cell (Appendix IV).

Kohl et al. (48) have applied the redox shuttle model to nitrogen fixing nodules. PGR activity was localized in the cytosol and not in the plastids of pea nodules. Proline oxidase activity was found primarily in bacteroids and not in the mitochondria of nodules. When the model illustrated in Appendix IV was applied to observations of nodule
preparations, Kohl et al. (48) concluded the following. Plant cytosol in nodules was the target compartment. P5C conversion to proline maintained a high NADP$^+$/NADPH ratio in the cytosol. Such a high ratio drove the PPP which in turn supported purine biosynthesis. Purine derivatives are used by several legumes as the primary transport molecule for fixed nitrogen. Proline oxidation to P5C in bacteroids provided reducing equivalents for bacteroid respiration. The P5C-proline cycle functioned to transfer reducing equivalents across membranes and to maintain a high NADP$^+$/NADPH ratio that regulated metabolism in the compartment containing PCR.

Phang's model is supported by data using cells, organelles, and extracts from mammals. This model may explain some aspects of proline accumulation in plants. Proline does not accumulate in the animal systems described by Phang unless there is a genetic deficiency in proline dehydrogenase (hyperprolinemia I). P5C concentrations are 0.4 $\mu$M and 1.2 $\mu$M for plasma and urine of normal humans. Rabbit aqueous humor (eyeball fluid) was found to have a P5C concentration of 8 $\mu$M. In the Phang model, one mole of P5C cycles with one mole of proline. Equivalent concentrations of proline would be needed for the shuttle to operate. These concentrations are 200-fold less than those found in unstressed leaves (2.2 mM) and 5500-fold less than those of
stressed leaves (up to 55 mM). There is no evidence to suggest that the activities of the pentose phosphate pathway or purine biosynthesis are increased to any great extent during proline accumulation. The movement of proline from leaves to roots does fit the model illustrated in Appendix IV. P5C should be transported to leaves from roots, if the existence of a proline-P5C cycle is to be demonstrated during stress. No reports of P5C accumulation in plant tissues are available. The identification of proline biosynthetic enzymes and their subcellular locations in plant cells is needed before the redox shuttle model can be investigated.

Summary of functions

Proline's role as an osmolyte depends upon its cytoplasmic compartmentation, which has not been thoroughly established. It has been clearly established, however, that proline accumulation is a rapid response to osmotic stress. This response is usually but not necessarily associated with turgor loss. Proline accumulation is clearly associated with a cessation of growth and is not a result of senescence. The ability to accumulate proline is not by itself a measure of water stress or salt stress tolerance. Although some studies suggest that proline accumulation is a symptom of stress, such an interpretation may be an oversimplification. The coordination of synthesis and
oxidation is not yet understood but appears to be complex. The energy inputs required for proline synthesis are significant. According to evolutionary theory, if no reproductive or metabolic advantage is conferred by an energy intensive process, natural selection should select against preserving such a phenomenon. Proline accumulates in members of most higher plant families, including the primitive gymnosperm Welwitschia mirabilis (4). If proline accumulation is not adaptive, natural selection may be in the process of selecting against this metabolism. If so, those species that do not exhibit proline accumulation (e.g., Plantago major) may represent a group of photosynthetic organisms that are better adapted to water-limiting environments.

Developing a Cell-Free Proline Biosynthetic System

The use of cell-free systems facilitates the defining the function of each component of that system. Cell-free systems have been useful for demonstrating the proline-P5C cycle in mammalian tissues. Such systems have enabled animal physiologists to develop a redox shuttle model that explains the roles played by components in vivo.

A cell-free preparation from Swiss chard leaves was reported to have synthesized proline from glutamic acid (58). The demonstration of stress-enhanced proline synthesis from glutamic acid was conducted with
radioactively labeled, intact leaves (12). Intact tissue contains several partitioned metabolic pools. The mechanism of feedback inhibition is an integral component of the current explanation of proline accumulation (12). The labeling studies with intact leaves suggest that feedback inhibition of proline synthesis is lost during water deficit. This loss of feedback inhibition causes increased proline synthesis and accumulation. For feedback inhibition to operate, the enzymes that are sensitive to proline must be in the same subcellular compartment as the pool of accumulating proline. If the enzymes are not exposed to the pool of accumulating proline, alternate hypotheses about the effects of stress on proline metabolism would need to be tested.

One primary objective of this research was to identify the three enzymes of proline synthesis from glutamic acid (Appendix I) in plant preparations so that the enzymes could be included in a cell-free system. Another primary objective of this research was to identify the subcellular location of proline biosynthetic enzymes in mesophyll tissue. Proline accumulation is a well known response to a variety of environmental stresses. The ultimate goal of this work is to develop a better understanding of plant physiological responses to environmental stress.
SECTION I. PYRROLINE-5-CARBOXYLATE REDUCTASE IS IN PEA 
(PISUM SATIVUM L.) LEAF CHLOROPLASTS
1) Supported by USDA-CRGO grant 85-CRCR-1-1671.

2) Abbreviations

MMT: MES, MOPS, Tricine
IEF: Isoelectric focusing
CAPA: 3-(cyclohexylamino) propane sulfonic acid
CHAPSO: 3-([3-cholamidopropyl]-diethylammonio)-2-hydroxy-1-propane sulfonate
GK: γ-glutamyl kinase
GPR: γ-glutamyl phosphate reductase
PCR: Δ^1-pyrroline-5-carboxylate reductase
TPDH: triosephosphate dehydrogenase
Proline accumulation is a well-known response to water deficits in leaves. The primary cause of accumulation is proline synthesis. \( \Delta^1 \)-pyrroline-5-carboxylate reductase (PGR) catalyzes the final reaction of proline synthesis. In order to determine the subcellular location of PGR, protoplasts were made from leaves of Pisum sativum L., lysed, and fractionated along a Percoll gradient. PGR activity comigrated with the activity of the chloroplast stromal marker NADPH-dependent triose phosphate dehydrogenase. We therefore conclude that PGR is located in chloroplasts. PGR activities from chloroplasts and etiolated shoots were compared. PGR activity from both extracts is stimulated at least twofold by 100 mM KCl and 10 mM MgCl\(_2\). The pH profiles of PGR activity from both extracts reveal two separate pH optima at 6.5 and 7.5. Native isoelectric focusing gels of samples from etiolated tissue reveal a single band of PGR activity with a pI of 7.8. It is known that during water deficit, the biochemical reactions that utilize NADPH for carbon fixation and nitrogen assimilation are inhibited, but NADPH generation by photosynthetic electron transport is not inhibited. Enhanced proline synthesis in chloroplasts may, therefore, function to regenerate NADP\(^+\) under these conditions.
INTRODUCTION

Induction of proline accumulation by water deficit is a well-known, but little understood phenomenon in plant stress physiology. Proline accumulation is caused primarily by increased synthesis from glutamic acid (24). Biosynthesis is postulated to be analogous to the pathway which converts glutamic acid to proline in *Escherichia coli*. The first two enzymes of this pathway have yet to be defined in plant extracts. The third enzyme, \( \Delta^1 \)-pyrroline-5-carboxylate reductase, has been measured from several plant sources (12, 13), and Krueger *et al.* (10) have purified it to apparent homogeneity from wilted barley leaves.

Interest in manipulating the proline synthesizing ability of plants by introducing genes coding for proline biosynthesis has focused the question of the subcellular location of the process. The subcellular location of proline biosynthesis has not been clearly established. The involvement of light in proline synthesis has been indicated. Noguchi *et al.* (17) have shown that inhibition of photosystem II inhibits proline synthesis in tobacco leaf disks. Rajagopal *et al.* (21) have shown that the pattern of proline accumulation in wheat parallels the pattern of diurnal change in light intensity. PGR activity has been reported in chloroplast-enriched fractions from tobacco leaves (16), but Kohl *et al.* (9) found that in soybean
nodules this enzyme is in the cytosol and not in plastids. To obtain an indication of the subcellular location of proline biosynthesis in leaves, we have investigated the subcellular location of PCR, the only proline biosynthetic enzyme for which a reliable assay is available. Our results indicate that, like many other amino acid biosynthetic enzymes, PCR is located in chloroplasts. We have also compared the properties of PCR from leaves and from etiolated shoots, and find that they are very similar.
MATERIALS AND METHODS

Plant Material

Peas (Pisum sativum L. var. Argenteum) were grown in soil flats in a growth chamber under the following conditions: 16 h light, 8 h dark, 21°C, 270 micromole s\(^{-1}\) m\(^{-2}\) combined fluorescent and incandescent light. Plants were watered with Hoagland’s solution (6) every third day and were allowed to wilt for one day each week. The wilting ensured that proline accumulation and increased proline synthesis were stimulated. The Argenteum variety was used because its leaf epidermis is easily peeled away. Protoplasts were rapidly prepared from peeled leaves. Etiolated peas (Pisum sativum L. var. Progress #9) were grown in coarse vermiculite at 30°C in darkness for 9 to 12 days and watered with deionized water. Progress #9 was used for experiments with etiolated tissue because sufficient Argenteum seed was not available.

Protoplast Preparation

Pea protoplasts were prepared from four-week-old leaves of the Argenteum variety (28). Plants were destarched by placing them in darkness for 24 hours. Adaxial epidermises were peeled and 40 leaves were floated on protoplast buffer (500 mM sorbitol, 5 mM MES-KOH, and 1 mM CaCl\(_2\), pH 6.0) containing wall digesting enzymes: 2% (w/v) Onozuka
Cellulase, 0.5% (w/v) Macerozyme, 1% (w/v) hemicellulase, and 0.2% (w/v) BSA in 8.5 cm petri dishes in darkness at 30°C. After one hour, the digestion medium was aspirated and 10 ml of protoplast buffer was added to each dish. Protoplasts were released by gentle rocking. The protoplasts were decanted into a beaker, another 10 ml of protoplast buffer was added, and the remaining protoplasts were collected. Protoplasts were centrifuged in a swinging bucket rotor at $100g_{\text{max}}$ for one minute at 4°C. The supernatant was aspirated and discarded. Protoplasts were resuspended in 5 ml of chloroplast buffer (330 mM sorbitol, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 2 mM EDTA, 0.2% (w/v) BSA, 50 mM MOPS-KOH pH 7.2).

**Protoplast Fractionation**

Protoplasts were ruptured by passing the suspension three times through 20 µm nylon mesh at the end of a syringe barrel. Microscopic examination revealed complete lysis of all protoplasts after this treatment. This crude chloroplast suspension was centrifuged in a Sorvall HB-4 swinging bucket rotor at $250g_{\text{max}}$ for 2 min. Pellets, which contained both damaged and intact chloroplasts, were resuspended in 3 ml chloroplast buffer and overlaid on a Percoll® (Pharmacia) gradient. The gradient was generated by mixing 15 ml Percoll with 15 ml 2X chloroplast buffer in 50-ml polypropylene tubes and centrifuging at $40,000g_{\text{max}}$ in
a Sorvall SS-34 fixed angle rotor for 30 min. The rotor was stopped without the brake. The overlaid gradient was centrifuged at $8,000g_{\text{max}}$ for 20 min in a Sorvall HB-4 rotor without the brake. A 12-cm long 20-gauge needle was inserted into the gradient to the bottom of the tube, and the gradient was fractionated into 1.6-ml aliquots using a peristaltic pump.

**Extraction of PCR**

Protein was precipitated from each Percoll gradient fraction by addition of 2.4 ml of a 50% (w/v) polyethylene glycol (3,500 average molecular mass) solution buffered with 50 mM MOPS-KOH, pH 7.2. After vortexing and waiting 10 min, samples were centrifuged in a Sorvall SM-24 fixed angle rotor at $20,000g_{\text{max}}$ for 10 min. Supernatants were aspirated and the pellets resuspended in 0.2 ml of 20 mM Tricine-KOH, pH 8.0, 5 mM MgCl$_2$, 10 mM $\beta$-mercaptoethanol and 20% (v/v) glycerol.

Etiolated pea shoots were harvested and stored at -20°C. Five hundred grams of shoots were homogenized in 500 ml grinding buffer (100 mM MOPS-KOH, 1 mM EDTA, 20 mM MgCl$_2$, 10 mM $\beta$-mercaptoethanol, 5% (w/v) insoluble PVP) with a Polytron tissue homogenizer (Brinkmann Instruments) at 4°C for 5 min. The slurry was filtered through four layers of cheesecloth. After filtration, the extract was brought to 30% saturation with (NH$_4$)$_2$SO$_4$ at 4°C. After 15 min the
extract was centrifuged in a Sorvall GSA rotor at 25,000\textit{g}\textsubscript{max} for 10 min. The supernatants were pooled, brought to 60% saturation with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and centrifuged again. The pellets were resuspended in 5 ml 20 mM Tricine-KOH, pH 8.0, and desalted on a Sephadex G-25 column (2.5 x 28 cm). This preparation was made 30% (v/v) with glycerol and stored at -20°C.

For assays of crude chloroplast preparations, PCR was rapidly extracted from chloroplasts by the following modification of the procedure used for gradient purification of chloroplasts. The 250\textit{g}\textsubscript{max} pellet of lysed protoplast suspension was resuspended in 6 ml of chloroplast buffer and recentrifuged at 250\textit{g}\textsubscript{max} for 2 min in a Sorvall HB-4 rotor. This pellet was resuspended in 2 ml of extraction buffer (20 mM Tricine, pH 8.0, 0.1 mM PMSF, 1 mM DTT, 1% (w/v) CHAPSO). After 10 min at 4°C, the sample was centrifuged in a Sorvall SS-34 rotor at 43,500\textit{g}\textsubscript{max} for 15 min at 4°C. The supernatant, which was yellow-green, was assayed.

Assays

The following assays were performed as previously described. NADP\textsuperscript{+}-dependent triose phosphate dehydrogenase was used as a stromal marker (11), chlorophyll as a thylakoid marker (11), cytochrome \textit{c} oxidase as a mitochondrial marker (2), and catalase as a peroxisome marker (1). The general PCR assay buffer contained 50 mM
Tricine-KOH, pH 8.0, 1 mM DTT, 200 μM NADH, and 2 mM D,L-α^1-pyrroline-5-carboxylate (D,L-P5C). For the subcellular fractionation experiments (Fig. 1 and Table 1), the buffer also contained 100 mM KCl and 0.01% (w/v) Triton X-114. D,L-P5C was prepared by the method of Williams and Frank (29). P5C dependent NADH oxidation was measured at 340 nm. The extinction coefficient of NADH (6.2 mM⁻¹ cm⁻¹) was used to calculate PCR activity. Kinetic parameters were determined from Eadie-Hofstee plots.

Isoelectric Focusing

A modification of a previously described method (15) was used. Native isoelectric focusing was carried out in gels that were poured and run in a Mighty Small® electrophoresis apparatus (Hoefer). Acrylamide was 4% (w/v). Also included were 1% (v/v) NP-40, 10% (v/v) glycerol and 5% (v/v) Pharmalytes® (pH 3-10). Constant voltage was applied at 200 V for 2 h followed by 400 V for 2 h. To locate PCR, gels were rocked for 2 h in 50 ml of 50 mM CAPS-HCl, pH 9.5, 100 mM L-proline, 1 mM NAD^+, 1 mM Mg(OAc)_2, 100 mM KOAc, 300 μg/ml nitroblue tetrazolium, 20 μg/ml phenazine methosulfate. This staining system gives purple bands by the reverse reaction of PCR which is active at pH > 9. Two mm gel slices were also assayed spectrophotometrically for PCR activity in the forward reaction. Slices were incubated in assay buffer for 16 h. Assay buffer included 50 mM
Tricine-KOH, pH 8.0, 1 mM DTT, 100 mM KCl, 200 μM NADH, and 2 mM D,L-P5C. Reverse activity of the same sample was measured spectrophotometrically with a previously described method (12). In these assays forward PCR activity was fivefold greater than reverse activity.
RESULTS

Subcellular Localization of PCR Activity

Figure 1 shows that when a chloroplast enriched preparation was fractionated by isopycnic Percoll density gradient centrifugation, the maximum PCR activity coincided with the maximum NADP$^+$-TPDH activity and chlorophyll concentration. There was almost no cytochrome c oxidase activity in this region of the gradient, but some catalase activity was apparent. Table 1 shows that the recovery of catalase activity was minor relative to the recovery of stromal marker and PCR. In three of seven replicate gradients (data not shown) PCR activity was prominent in the ruptured chloroplast region (fraction #17). This distribution suggests that PCR is associated with the thylakoid membrane. Several nonionic detergents were added to the chloroplast lysis buffer to test their ability to dissociate interactions between PCR and thylakoids. PCR activity yield was increased twofold by NP-40, CHAPS, or CHAPSO (data not shown).

Whole etiolated shoots yielded 100-fold greater PCR activity than chloroplasts from green shoots. When etioplasts were purified from pea shoots on a Percoll gradient, PCR activity (42 nmol min$^{-1}$ mg protein$^{-1}$) was detected, but the yield was less than 1% of the PCR activity of whole shoot extracts. The yield of etioplasts was very
Figure 1. Distribution of pyrroline-5-carboxylate reductase (PCR) activity and subcellular markers along a Percoll gradient: PCR (○), refractive index (♦), triose phosphate dehydrogenase (△), chlorophyll (▲), cytochrome oxidase (□), and catalase (■). The overlaid samples contained 60 mg protein. Fraction 1 is the bottom of the gradient.
Table 1. Distribution of PCR and markers in subcellular fractions from pea protoplasts

<table>
<thead>
<tr>
<th></th>
<th>Total Units(^a)</th>
<th></th>
<th>Percent of Total Along Gradient(^b)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>6 ml protoplast suspension</td>
<td>6 ml chloroplast suspension</td>
<td>percoll gradient</td>
</tr>
<tr>
<td>PCR</td>
<td>1.6</td>
<td>0.4</td>
<td>0.64</td>
</tr>
<tr>
<td>TPDM</td>
<td>6.6</td>
<td>1.0</td>
<td>0.34</td>
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<td>Cytochrome C oxidase</td>
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</tr>
<tr>
<td>Catalase</td>
<td>8.4</td>
<td>1.1</td>
<td>0.09</td>
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\(^a\)One unit = 1 μmole min\(^{-1}\) for enzymes and 1 mg for chlorophyll.

\(^b\)The third column of numbers represents 100%.
low; only 1.3% of the total NADP\(^+\)-TPDH activity of etiolated shoots was present in the etioplast fraction (102 nmol min\(^{-1}\) mg protein\(^{-1}\)). Although PCR was present in etioplasts from pea shoots, our results do not provide conclusive evidence about the subcellular distribution of PCR in etiolated shoots. However, the \(V_{\text{max}}\) of PCR from both sources was greater with NADH than with NADPH (450 nmol min\(^{-1}\) mg protein\(^{-1}\) and 153 nmol min\(^{-1}\) mg protein\(^{-1}\)). PCR from etiolated shoots had apparent K\(_m\)'s for NADH and NADPH of 311 \(\mu\)M and 184 \(\mu\)M, respectively. Again the \(V_{\text{max}}\) was greater with NADH than with NADPH (2.95 \(\mu\)mol min\(^{-1}\) mg protein\(^{-1}\) and 0.83 \(\mu\)mol min mg protein\(^{-1}\)). These \(V_{\text{max}}\) values were obtained from assays using 2 mM D,L-P5C. Figure 2A shows that there are two pH optima for PCR activity in chloroplast preparations, at pH 6.5 and at pH 7.5. These optima are also present in PCR prepared from etiolated pea shoots (Fig. 2B).

Salt stress causes proline to accumulate. Light increases the Mg\(^{2+}\) concentration in the stroma in vivo. The effects of salts on PCR activity were investigated to test the hypothesis that proline biosynthesis can respond to changes in ion concentration. One hundred mM KCl (Fig. 3) or 10 mM MgCl\(_2\) (Fig. 4) caused a twofold increase in the PCR activity of both chloroplast and etiolated preparations. Sucrose and sorbitol of equal osmolalities did not cause the
Figure 2. Effect of pH on PCR activity from pea chloroplasts (A) and etiolated pea shoots (B). MMT buffer used for the pH curve contained 50 mM MES, 50 mM MOPS, and 50 mM Tricine. HCl or KOH was used to adjust pH. Except for the change in buffer, the general PCR assay conditions were used. Results are the means and standard errors of four assays.
PCR Activity
nmoles min\(^{-1}\) mg protein\(^{-1}\)

![Graph showing PCR Activity](image)
Figure 3. Effect of KCl on PCR activity from pea chloroplasts (●) and etiolated pea shoots (○). Assay was as described in Materials and Methods, but KCl was added. Results are the means and standard errors of four assays.
Figure 4. Effect of MgCl$_2$ on PCR activity from pea chloroplasts (●) and etiolated pea shoots (○). Assay was as described in Materials and Methods, but MgCl$_2$ was added. Results are the means and standard errors of four assays.
Figure 5. Monovalent cation salt, sucrose and sorbitol effects on PCR activity from etiolated pea shoots (empty bars) and pea chloroplasts (hashed bars). Assay was as described in Materials and Methods, but 100 mM salt or 200 mM sugar was added. Results are the means and standard errors of four assays.
same stimulation as KCl (Fig. 5). MnCl$_2$, CaCl$_2$, and Mg(OAc)$_2$ caused the same stimulation as MgCl$_2$ (Fig. 6). The effects of monovalent and divalent cations are not additive at high concentrations (Fig. 7).

**Isoelectric Focusing**

Separation of the pH 6.5 and pH 7.5 activities was attempted by native IEF. One zone of activity ($R_f = 0.44$, pH 7.8) contained PCR activity. Activity of the reverse PCR reaction (P5C dehydrogenase activity, pH 9.5) produced bands of purple precipitate in the same region.
Figure 6. Divalent cation salt effects on PCR activity from etiolated pea shoots (empty bars) and pea chloroplasts (hashed bars). Assay was as described in Materials and Methods, but 10 mM salts were added.
Figure 7. Effect of combinations of MgCl₂ and KCl on PCR activity from etiolated pea shoots (empty bars) and pea chloroplasts (hashed bars). To the general PCR assay (control) was added: 5 mM MgCl₂ (A), 25 mM KCl (B), 5 mM MgCl₂ + 25 mM KCl, (C), 10 mM MgCl₂ (D), 100 mM KCl (E), and 10 mM MgCl₂ + 100 mM KCl (F). Results are the means and standard errors of four assays.
Figure 8. PCR activity (○) and pH (●) in 2 mm segments of a native isoelectric focusing gel. R_f = position of proteins in segment relative to position of a pH marker, methyl red. The general PCR assay was used with the addition of 100 mM KCl. This gel was stained for proline dehydrogenase activity (pH 9.5), (A) without proline, and (B) with proline.
DISCUSSION

Noguchi et al. (16) described the localization of PCR activity in a chloroplast enriched fraction of tobacco leaves. However, Kohl et al. (9) found PCR to be localized in the cytosol and not in plastids of soybean nodules. The results of Figure 1 and Table 1 demonstrate that in green pea leaves, PCR is present in chloroplasts. PCR activity was most abundant in the same fraction in which a stromal marker, triose phosphate dehydrogenase, was most abundant. Preliminary evidence shows that PCR is present in etioplasts from etiolated pea shoots. Krueger et al. (10), Noguchi et al. (16), and Miler and Stewart (13) have shown that PCR from barley, tobacco, and soybean uses both NADH and NADPH as electron donors, but has a lower $K_m$ for NADPH. PCR's low $K_m$ for NADPH is consistent with the chloroplast localization of this enzyme. Our results demonstrate that PCR has the same subcellular localization in leaves as numerous other enzymes of amino acid biosynthesis.

The bimodal pH curve for pea PCR is similar to that reported previously for PCR from etiolated barley (*Hordeum vulgare*) and etiolated mung bean (*Vigna radiata*), both of which have optima at pH 6.4 and 8.0 (4). The pH 7 minimum is not an artifact, because the pH profile was reproducible with batches of MMT buffer prepared three different times. The bimodal curve could be due to a single enzyme or two
isoforms. If there are two forms, both are probably present in plastids. The ratio of the pH 6.5 to pH 7.5 activities in chloroplast-enriched preparations is equal to the ratio in whole etiolated shoot extracts. PCR in whole protoplast extracts from green leaves produced a broad pH curve with similar activities at pH 6.5 and 7.5 (data not shown), as in the chloroplast-enriched fraction and in etiolated shoots. There was no minimum apparent at pH 7.0, but the enzyme was difficult to assay precisely in whole protoplast extracts because of its low activity. This investigation focused on the PCR activity with the pH 7.5 optimum.

If both PCR activities are chloroplast localized, one may be more active in light, when the stromal pH approaches 8.0, and one may be more active in darkness when stromal pH is more acidic. Another possibility is that one form of PCR is in the final step of a pathway from glutamic acid to proline, while the other form is the final step of a pathway from ornithine to proline (22). Taylor and Stewart (25) used a Percoll® step gradient to separate chloroplasts and mitochondria from green pea leaves. They report that arginase and ornithine aminotransferase, which convert arginine to ornithine and ornithine to P5G, are mitochondrial. P5G produced from ornithine would have to be transported from mitochondria into chloroplasts for proline synthesis. Two P5G dehydrogenases with different pH optima
have been localized in maize mitochondria (4). One converts
P5C from ornithine to glutamic acid while the other converts
P5C from proline to glutamic acid.

The native isoelectric focusing gel revealed only one
region of PCR activity with a pI of 7.8. Thus, it appears
that if there are two isoenzymes, they have the same pI.
This activity does not represent the activity of
mitochondrial proline dehydrogenase: the mitochondrial
proline dehydrogenase is not active at pH 9.5 (4), and it
requires FAD, which was not included in these assays. No
information about the genetic differences between these two
PCR activities is established from this investigation.

The activity of PCR from both etiolated and green tissue
is stimulated twofold by 100 mM potassium chloride, ammonium
chloride, or ammonium acetate and by 10 mM magnesium,
manganese, or calcium chloride. Thus, this stimulation
appears to be a general function of ionic charge and not of
any specific ion. The stimulation is also not a function of
osmolality, because 200 mM sucrose and sorbitol had no
effect. This sensitivity of PCR activity to ion
concentrations may be involved in regulating proline
biosynthesis and accumulation in leaves, in response to both
light and stress. When chloroplasts are shifted from
darkness to light, the stromal Mg^{2+} level increases from 2
mM to 8 mM (20). Thus, the observed Mg^{2+} stimulation may
play a role in the light-stimulated increase in proline levels reported by others (5,14,17,27). Calcium, in contrast, is found at micromolar concentrations in the chloroplast stroma. Its stimulatory effect at millimolar concentrations in vitro would not be significant in vivo. Wilting, abscisic acid treatment, and salt shock can all increase ion concentrations in cells. Such increases should stimulate PCR activity. Thus, the sensitivity of PCR to changes in ion concentrations may contribute to stress-induced proline accumulation.

Noguchi et al. (17) concluded that photosystem II is involved in proline synthesis from glutamic acid. Since stromal pH approaches 8 when the photosynthetic electron transport system is active, the PCR activity with the pH 7.5 optimum may be the PCR which catalyzes the final step of proline synthesis from glutamic acid. Proline synthesis from glutamic acid in water-stressed barley is doubled in light (5). If peas are similar to barley, the PCR activity with pH optimum 7.5 should be the form that participates in water-deficit-enhanced proline synthesis. The sensitivity of PCR to changes in pH may contribute to water-stress-induced proline accumulation.

The first two enzymes of proline synthesis, gamma-glutamyl kinase (GK) and gamma-glutamyl phosphate reductase (GPR), are associated as an enzyme complex in E. coli (23).
The reaction intermediate, gamma-glutamyl phosphate, is very unstable. The GK/GPR enzyme complex facilitates its conversion to P5C. A three enzyme complex has been suggested for GK, GPR, and PCR in yeast (25). P5C is unstable at physiological temperature and pH. Neither gamma-glutamyl phosphate nor P5C has been identified in plant tissues. Perhaps, a multienzyme complex catalyzes proline synthesis in plants. If this is true, then GK and GPR may also be chloroplast enzymes.

During salt-shock, proline levels increased fourfold in chloroplasts of *Spinacia oleracea* (7) and fivefold in those of *Mesembryanthemum crystallinum* (3). These proline levels were calculated by the authors to be insufficient to account for osmotic adjustment. Synthesis and accumulation of proline in chloroplasts may have direct relevance to chloroplast function. Proline has been shown to protect membranes from desiccation (22). Photophosphorylation has been shown to be very sensitive to osmotic changes *in vitro* but not *in vivo* (18). Perhaps proline accumulation in chloroplasts protects this site *in vivo*. Most amino acid synthesis occurs in chloroplasts and glutamic acid is central to amino acid metabolism. Glutamic acid has been found to comprise 33% of free amino acids in photosynthesizing chloroplasts (8). The solubility in water at 25°C of glutamic acid is 0.009 g/ml and that of proline
is 1.62 g/ml. Induction of proline accumulation by water deficit may be a mechanism of converting abundant glutamic acid into a compound which is more soluble and more compatible with subcellular metabolism at high stromal solute concentrations.

A role for proline as a redox shuttle molecule has been established for some mammalian tissues (19) and has been proposed for nitrogen fixing nodules (9). Such a shuttle may operate in leaves. According to this model proline would be synthesized in chloroplasts in order to generate NADP⁺. Proline should be transferred to another subcellular compartment or another cell to be oxidized, in effect transferring redox equivalents without the transport of NADPH across membranes. The observations that photosystem II inhibition blocks proline accumulation (17) and that proline levels are maximal during periods of maximal light intensity (21,27) can be explained by this model. NADPH is an endproduct of photosystem II driven electron transport. The rate of NADPH synthesis is dependent upon the rate of photosynthetic electron transport and the availability of NADP⁺. The activities of NADPH utilizing pathways such as carbon fixation and nitrogen and sulfur assimilation, are decreased during water deficit. Chloroplast PCR could regenerate NADP⁺ under stressful environmental conditions where light energy is available and NADP⁺ regeneration by
other pathways is inhibited. The subcellular localization of the final step of proline synthesis suggests that proline accumulation in leaves serves some metabolic function when the products of photosynthetic electron transport cannot be used by the constitutive biochemical pathways. Such a pathway could serve to prevent light damage to stressed chloroplasts by allowing photosynthetic electron transport to continue.

Acknowledgment

We are grateful to Professor G. A. Marx for supplying the Argenteum pea mutant.
LITERATURE CITED


29 Williams I, Frank L (1975) Improved chemical synthesis and enzymatic assay of Δ1-pyrroline-5-carboxlic acid. Anal Biochem 64: 85-97
SECTION II. INVESTIGATION OF RATE LIMITING ENZYMES OF PROLINE BIOSYNTHESIS
The purpose of this investigation was to identify the rate limiting enzymes of proline synthesis in plants. The glutamyl-hydroxamate assay was not specific for gamma-glutamyl kinase (GK, EC 2.7.2.11). A continuous assay which measures glutamic-acid-dependent kinase activity was developed. The continuous assay was more specific for GK than the glutamyl-hydroxamate assay, because interference by glutamine synthetase (EC 6.3.1.2) activity was minimized.

Pyrroline-5-carboxylate (P5C) synthesis was observed in extracts from etiolated mung bean (Vigna radiata L.) shoots. P5C synthesis was not inhibited by 50 mM proline or 50 mM ornithine. P5C synthesis was inhibited by 25 mM azetidine-2-carboxylate or 20 mM methionine sulfoximine. P5C synthesis was NADPH specific. Phosphate-dependent P5C dehydrogenase activity was not detected in the same preparations. Partial purification of gamma-glutamyl phosphate reductase (GPR, EC 1.2.1.41), from a proline over-producing cell line of carrot (Daucus carota L.), was unsuccessful.

P5C reductase (PCR, EC 1.5.1.2) was partially purified from chloroplasts and etiolated shoots of pea (Pisum sativum L.). PCR from both sources had apparent $K_m$'s that were smaller for NADPH than NADH. PCR from etiolated shoots was fivefold more active and much more stable than PCR activity.
from chloroplasts. Etiolated shoot PCR was inhibited by 50 mM proline, 2 mM azetidine-2-carboxylate, or 2 mM thiazolidine-4-carboxylate. Noncompetitive regulation of PCR from pea is indicated. Since three nonionic detergents caused a twofold increase in PCR yield from pea chloroplasts, membrane association of some component of proline biosynthesis is indicated.
INTRODUCTION

L-Proline is one of several metabolites that accumulate in mesophyll experiencing water deficit. The primary cause of accumulation is increased synthesis from glutamic acid (Appendix I). In vivo labeling experiments show that this biosynthetic pathway is feedback inhibited by proline in unstressed leaves but not in stressed leaves (1). In Escherichia coli the same pathway is feedback inhibited by proline. When the gene for gamma-glutamyl kinase (GK) was mutated, feedback inhibition was lost and proline was overproduced (4).

The regulation of proline accumulation in plant leaves may be explained by four different hypotheses. First, GK may be regulated in a manner that causes loss of feedback control during stress. Second, GK may retain its negative feedback characteristics. A proline transporter in a membrane surrounding the subcellular compartment in which proline is synthesized may be more active during stress, so that the pool of proline that causes feedback inhibition is separated from the pool that accumulates in leaves. Third, an increase in the amount of constitutive GK/GPR may facilitate increased proline synthesis in the presence of increased proline concentrations. Fourth, induction of GK isozymes that lack feedback inhibition may cause the change in proline biosynthetic rate. Several isozymes are known
for rate-limiting reactions of other amino acid biosynthetic pathways in plants. For example, pea has three isoenzymes for glutamine synthetase (22). Before these hypotheses can be tested, a reliable assay for the enzymes GK and GPR is required. An abundant source of activity for GK and GPR purification and characterization needs to be identified. The primary objectives of this study were to develop such an assay and identify such a source.

Treichle (24) has shown that P5C reductase activity increased in cell suspension cultures of *Mesembryanthemum nodiflorum* acclimated to 200 mM NaCl. The increase in PCR activity was accompanied by a decrease in $K_m$ (P5C) and a stimulation of proline accumulation. The increase in PCR activity that was caused by NaCl stress was prevented by the translation inhibitor cycloheximide. Treichle concluded that PCR participates in the regulation of salt-stress-induced proline synthesis. The kinetic parameters of PCR from pea were investigated in order to determine the role of PCR in regulating proline synthesis.
MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* L. var. Larker) and pea (*Pisum sativum* L. var. Progress #9 and var. Argenteum) plants were grown in soil flats in a growth chamber under the following conditions: 16 hr day length, 21°C, 270 μmol photons s\(^{-1}\) m\(^{-2}\) combined fluorescent and incandescent light. Plants were watered with Hoagland's solution every third day (7). Barley was harvested at 14 to 16 days after planting. Progress #9 peas were harvested at nine days after planting. Argenteum peas were harvested at three weeks after planting.

Etiolated shoots were grown in a lightless incubator at 30°C in coarse vermiculite beds and watered with deionized water. Mung beans (*Vigna radiata* L.) were grown for eight days. Progress #9 peas were grown for 12 days. Carrot cells were grown in a rotary shaker on Murashige and Skoog medium as previously described (27).

Enzyme Extraction from Barley Leaves and Mung Bean Shoots

One hundred grams of tissue were ground in 200 ml of grinding medium (see below) at 4°C. Solid polyvinylpyrrolidone was added during grinding (2.5 g for etiolated mung bean shoots and 5 g for barley leaves) to bind phenolic compounds. The slurry was squeezed through four layers of cheesecloth and centrifuged at 25,000g\(_{\text{max}}\) for 15 min. Solid
ammonium sulfate was dissolved in the supernatant to make it 30% saturated at 4°C. Protein was pelleted by centrifugation at 15,000 g_{max} for 5 min. This step was repeated so that protein was collected at 40%, 50%, and 65% saturation with ammonium sulfate. The pellets were resuspended in 1 ml of column buffer (see below). A 9 x 2 cm Sephadex G-25 column was used to desalt protein fractions before assaying.

Grinding medium was made up of: 100 mM MOPS-KOH, pH 7.2, 20 mM MgSO_4, 1 mM EDTA, and 10 mM beta-mercaptoethanol. Column buffer was made up of: 50 mM MOPS-KOH, pH 7.2, 20 mM MgSO_4, and 10 mM beta-mercaptoethanol.

Enzyme Extraction from Carrot Cells

Carrot (*Daucus carota* L.) cell line C63 was obtained from J. Widholm's laboratory at the University of Illinois. This cell line has been selected for resistance to hydroxyproline and accumulates proline to 30-fold higher levels than the parent cell line TC. Four hundred grams of frozen C63 cells were ground in 600 ml of buffer using a bead beater (Biospec Products) at 4°C with an ice jacket. The buffer contained 100 mM MOPS-KOH, pH 7.2, 20 mM MgCl_2, 1 mM EDTA, and 10 mM beta-mercaptoethanol. Five two-min bursts with 30-s intervals were applied. Solid polyvinylpyrrolidone was added at 1% (w/v) to bind phenolics. Glass beads were allowed to settle out and the crude preparation
was centrifuged at 21,500g_{max} for 10 min to remove debris. The supernatant was made 30% saturated with (NH₄)₂SO₄ at 4°C and centrifuged at 21,500g_{max} for 10 min again. This supernatant was made 60% saturated with (NH₄)₂SO₄ and recentrifuged at 21,500g_{max} for 10 min. This pellet was resuspended in 20 mM Tricine-KOH (pH 8) and desalted on a 40 x 2.5 cm Sephadex G-25 column. Desalted protein was loaded onto a 45 x 2.5 cm DEAE Sepharose column and eluted with a 1 L gradient of 0 to 500 mM KCl in 20 mM Tricine-KOH (pH 8). Fractions containing P5C dehydrogenase activity were pooled and concentrated in an Amicon pressure cell over a YM-30 membrane. Buffer was changed to 5 mM potassium phosphate (pH 7.5) and the solution loaded onto a 15 x 1 cm hydroxyapatite column. A 150 ml gradient of 5 to 300 mM potassium phosphate (pH 7.5) was used to elute and concentrate P5C dehydrogenase activity.

Enzyme Extraction from Pea Chloroplasts

Chloroplasts were prepared from green leaves of pea (*Pisum sativum* L. var. Progress #9) by the method of Weigle et al. (26). PCR was partially purified from chloroplasts by modifying a previously published procedure (9). The following changes were made. Tricine-KOH, 20 mM, pH 8.0 was used in place of Tris-HCl and phosphate buffers. The heat precipitation step was omitted. Polyethylene glycol (PEG, average molecular mass = 3,500) was used to precipitate
proteins in place of ammonium sulfate. PEG did not interfere with ion exchange chromatography. Thus, a desalting step was avoided. PCR activity that was partially purified from chloroplasts in this way was used to collect kinetic data.

Another chloroplast preparation method was also used. Pea chloroplasts prepared from protoplasts of *Pisum sativum* L. var. Argenteum (Section I, Materials and Methods), were used in experiments with detergents.

Enzyme Extraction from Etiolated Pea Shoots

PCR was partially purified from etiolated shoots by modifying a previously published procedure (9). The following changes were made. Tricine-KOH, 20 mM, pH 8.0 was used in place of Tris-HCl and phosphate buffers. The heat purification step was omitted. The DEAE ion exchange step was omitted. PCR activity that was partially purified from etiolated shoots in this way was used to collect kinetic data.

Glutamyl-Hydroxamate Assay for Gamma-Glutamyl Kinase

The complete glutamate hydroxamate assay included: 100 mM MOPS-KOH pH 7.2, 20 mM MgSO$_4$, 200 mM hydroxylamine-HCl, 50 mM potassium glutamate, and 20 mM Na$_2$ATP in a final volume of 500 µl. Reactions were stopped with 750 µl of iron chloride reagent, containing: 10% (w/v) FeCl$_3$·6H$_2$O,
3.3% (w/v) trichloroacetic acid, and 0.7 M HCl (16). Gamma-glutamyl-hydroxamate was detected by measuring absorbance at 540 nm after 20 min. Parallel assays were conducted in the absence of ATP or the presence of 50 mM L-proline. The difference between complete and ATP-minus samples measured total gamma-glutamyl phosphorylation. The difference between the complete and proline-plus samples measured proline-inhibitable GK activity.

Continuous Assay for Gamma-Glutamyl Kinase

A novel enzyme assay was developed for the measurement of GK. This assay is based upon an assay for aspartate kinase (15) which carries out an analogous reaction (Appendix VI). The reaction mixture contains: 150 mM MOPS-HCl, pH 7.5, 20 mM MgCl₂, 350 mM KCl, 0.5 mM semicarbazide-HCl, 200 μM NADH, 1 mM ATP, 1 mM phosphoenolpyruvate-cyclohexylammonium salt (PEP), 0.5 units lactate dehydrogenase, 0.5 units pyruvate kinase, and 5 mM glutamate-KOH in 1 ml. Kinase activity was measured by observing NADH oxidation by the coupled activities of pyruvate kinase and lactate dehydrogenase. Thus, kinase activity could be indirectly measured through the change in absorbance at 340 nm. When pyruvate kinase, lactate dehydrogenase, and enzyme extracts are desalted, stimulation of background glutamine synthetase activity by ammonium ions can be minimized.
Coupled GK-GPR Assay

The coupled GK-GPR assay measures P5C synthesis by reacting P5C with ortho-aminobenzaldehyde (oAB). The orange dihydroquinazolinium compound that is formed absorbs at 443 nm (21). The reaction mixture contained 100 mM MOPS-KOH pH 7.2, 40 mM MgSO4, 10 mM Na2ATP, 200 mM L-Na-glutamate, and 150 μM NAD(P)H in a final volume of 500 μl. Reactions were stopped with 750 μl oAB reagent and P5C was measured after waiting 1 h. The oAB reagent was comprised of 72 mg oAB, 3 ml 12 N HCl, and 21 ml 95% (v/v) ethanol.

Phosphate-Dependent P5C Dehydrogenase Assay

This assay measures the activity of gamma-glutamyl phosphate reductase (GPR, EC 1.2.1.41) operating in reverse. It detects phosphate-dependent and P5C stimulated NAD(P)⁺ reduction at 340 nm. Reverse GPR activity requires inorganic phosphate in addition to NAD(P)⁺ and P5C to produce gamma-glutamyl phosphate. Gamma-glutamyl phosphate spontaneously changes to 2-pyrrolidone-5-carboxylic acid (Appendix I). P5C dehydrogenase (EC 1.5.1.12), in contrast, utilizes NAD(P)⁺ to convert P5C to glutamic acid in the absence of inorganic phosphate (Appendix I).

The reaction mixture contained 50 mM MOPS-KOH, pH 7.2, 150 μM NADP⁺, 2 mM D,L-P5C, and 10 mM potassium phosphate in a final volume of 1 ml. Reactions were initiated upon
addition of P5C. P5C was prepared for use as described by Treichle (24).

P5C-DH activity in gels was stained by incubating native IEF gels in P5C-DH staining mixture (50 mM MOPS-KOH, pH 7.2, 2 mM D,L-P5C, 1 mM NADP+, 300 μg/ml nitroblue tetrazolium, 20 μg/ml phenazine methosulfate). Gels were bathed in 50 ml staining mixture at 22°C for 16 h.

Other Assays

P5C reductase (EC 1.5.1.2) was assayed by measuring the rate of NAD(P)H oxidation at 340 nm. Standard assay buffer was 50 mM Tricine-KOH, pH 8.0, 200 μM NAD(P)H, and 2 mM D,L-P5C. The extinction coefficient of NADH (6.2 mM⁻¹cm⁻¹) was used to calculate P5C reductase activity.

Glutamine synthetase activity was measured by the method of Rhodes et al. (16).

Protein was quantified by the Bradford method (2).

Polyacrylamide Gel Electrophoresis

Native proteins were separated by native isoelectric focusing as previously described (Section I, Materials and Methods).

Denatured proteins were separated by discontinuous SDS-PAGE in a 12.5% (w/v) slab gel by the Laemmli procedure (10). Silver staining of proteins was performed as described by Nielson and Brown (12).
Proteins were electroeluted from gel segments with an Elutrap® concentrator (Schleicher and Schuell).
RESULTS

Gamma-Glutamyl Kinase Experiments

GK was not detected in crude extracts from any source. Using the glutamate hydroxamate assay, proline inhibitable kinase activity was detected in protein fractions concentrated by (NH₄)₂SO₄ precipitation from wilted barley leaves (Tables 1 and 2). Proline inhibition of glutamate-hydroxamate production has been proposed to distinguish GK from glutamine synthetase. GK was detected in 6 of 11 experiments with wilted barley leaves. Proline inhibited only 7% of total hydroxamate production. Background glutamate hydroxamate production can mask the presence of proline inhibitable GK activity after 10 min (Figure 1). Glutamine synthetase activity was detected in fractions in which GK was detected. Proline-inhibitable GK activity was very unstable. It was lost after storage at 4°C and -20°C in the presence and absence of 20% (v/v) glycerol. Passage of GK active fractions through DEAE-cellulose, Sephadex G-100, and Biogel A 0.5 M also caused loss of activity. GK activity was not detectable in the presence of methionine sulfoximine (MSO), which is an inhibitor of glutamine synthetase (Table 1).

A novel continuous assay was developed to measure glutamic-acid-dependent kinase activity. Activity resembling gamma-glutamyl kinase was observed in a pea
chloroplast lysate (Table 3). Azetidine-2-carboxylate (A2C) was used to determine proline sensitivity of this kinase activity, because proline has been shown to stimulate the activity of some enzymes, including glutamine synthetase (8). A2C did not inhibit glutamine synthetase activity when 20 mM A2C was added to the hydroxamate assay (data not shown). The A2C inhibition of GK activity in Table 3 represents 75% of all glutamate-dependent ADP producing activity, whereas proline inhibited only 10% of total glutamate- and ATP-dependent gamma-glutamyl hydroxamate production shown in Table 1. Furthermore, the continuous assay does not include hydroxylamine, which acts as a substrate for glutamine synthetase because it is an ammonium ion analogue. Thus, the pyruvate kinase-lactate dehydrogenase linked assay is more specific for GK than the gamma-glutamyl hydroxamate assay. This novel assay can be conducted as a continuous assay while the hydroxamate assay cannot, so that kinetic experiments are more feasible. The same chloroplast lysates that contained apparent GK activity contained PGR activity (see Section I). However, neither intact chloroplasts, nor chloroplast lysates prepared from wilted green pea leaves converted $^{14}$C-glutamic acid to $^{14}$C-proline (data not shown). Therefore, the presence of GK in pea chloroplasts is only suggested.
Figure 1. Time course of proline inhibitable gamma-glutamyl hydroxamate production by extracts from wilted barley leaves. Each point represents the mean of three replicate samples.
Table 1. The effect of methionine sulfoximine (MSO) on the detection of proline inhibitable glutamate hydroxamate synthesis in extracts from wilted barley leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>20mM MSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>201 ± 3</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>-ATP</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>+Proline (50 mM)</td>
<td>171 ± 6</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>Complete - (+Proline)</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) nmol min\(^{-1}\) mg protein\(^{-1}\).

\(^b\) Mean ± standard deviation, n = 3.
Table 2. Specific activities\(^a\) of the three proline biosynthetic enzymes in ammonium sulfate saturated fractions of extracts from green wilted barley leaves

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ammonium Sulfate Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-30%</td>
</tr>
<tr>
<td>Unstressed</td>
<td></td>
</tr>
<tr>
<td>GK</td>
<td>0</td>
</tr>
<tr>
<td>GK-GPR</td>
<td>0</td>
</tr>
<tr>
<td>PCR</td>
<td>0</td>
</tr>
<tr>
<td>Wilted</td>
<td></td>
</tr>
<tr>
<td>GK</td>
<td>0</td>
</tr>
<tr>
<td>GK-GPR</td>
<td>0</td>
</tr>
<tr>
<td>PCR</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)nmol min\(^{-1}\) mg protein\(^{-1}\). Each value is the mean of two experiments with two samples per experiment.
Table 3. Detection of gamma-glutamyl kinase activity<sup>a</sup> in a pea chloroplast lysate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete</td>
<td>9 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+A2C</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>complete-(+A2C)</td>
<td>7 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>GK was assayed with the pyruvate kinase - lactate dehydrogenase coupled assay.

<sup>b</sup>nmole min<sup>-1</sup> mg protein<sup>-1</sup>.

<sup>c</sup>Mean ± standard deviation, n = 3.
Gamma-Glutamyl Phosphate Reductase Experiments

P5C synthesizing activity was detected in the 50-65% ammonium sulfate saturated fraction of extracts from etiolated mung bean shoots (Table 4). Such activity was detectable with NADPH but not with NADH. P5C synthesizing activity was not inhibited by proline or ornithine. However, A2C and MSO inhibited P5C synthesis. The same mung bean preparation was used to assay P5C-dehydrogenase (P5C-DH) activity. Figure 2 shows that P5C-DH activity of these preparations was greater with NAD$^+$ than with NADP$^+$. Figure 2 also shows that the pH optimum for P5C dehydrogenase activity was in the alkaline range (7.5 to 8.0). P5C-DH activity from mung bean was inhibited by phosphate (data not shown).

A procedure that had been used to purify gamma-glutamyl phosphate reductase (GPR) from *E. coli* was used in an attempt to partially purify GPR from carrot cell line C63. Table 5 shows that P5C-DH activity was detectable only after an ion exchange chromatography step. Figure 3 shows that the partially purified P5C-DH activity was not dependent upon phosphate and was inhibited by phosphate. P5C-DH activity from carrot had an approximate pI of 4.5.

Pyrroline-5-Carboxylate Reductase Experiments

Pyrroline-5-carboxylate reductase (PCR) was partially purified from pea chloroplasts (Table 6). Activity in the
Figure 2. P5C dehydrogenase activity of extracts from etiolated mung bean shoots
Table 4. Effects of some metabolites and amino acid analogs on P5C synthesis in 50-65% ammonium sulfate saturated extracts from etiolated mung bean shoots as measured by the coupled GK-GPR assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity$^a$</th>
<th>Difference$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NADPH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control$^c$</td>
<td>24 ± 2$^d$</td>
<td>---</td>
</tr>
<tr>
<td>Complete</td>
<td>34 ± 2</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>+ 50 mM proline</td>
<td>39 ± 11</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>+ 25 mM azetidine-2-carboxylic acid</td>
<td>23 ± 1</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>+ 20 mM methionine sulfoximine</td>
<td>26 ± 2</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>+ 50 mM ornithine</td>
<td>35 ± 11</td>
<td>62 ± 6</td>
</tr>
<tr>
<td><strong>NADH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control$^c$</td>
<td>41 ± 6</td>
<td>---</td>
</tr>
<tr>
<td>Complete</td>
<td>50 ± 10</td>
<td>48 ± 7</td>
</tr>
</tbody>
</table>

$^a$nmol min$^{-1}$ mg protein$^{-1}$.

$^b$Treatment activity - control activity.

$^c$Control = complete reaction mix - glutamic acid.

$^d$Mean ± standard deviation, n = 3.
Table 5. Partial purification of P5C dehydrogenase activity from carrot cell line C63

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Yield</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2. 30-60% saturated &lt;sub&gt;(NH₄)₂SO₄&lt;/sub&gt;</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3. DEAE-Sepharose</td>
<td>19</td>
<td>479</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>4. Hydroxyapatite</td>
<td>70</td>
<td>281</td>
<td>59%</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>nmol min<sup>-1</sup> mg protein<sup>-1</sup>.  
<sup>b</sup>nmol min<sup>-1</sup>. 

Figure 3. Effects of phosphate on P-5-C DH activity of a partially purified preparation from carrot cell line C63. Samples had been purified to step 4 on Table 5 and were analyzed by isoelectric focusing and staining for activity. Treatments were as follows: - P5C, lanes 1 and 2; + P5C, lanes 3 and 4; - phosphate, lanes 1 and 3; + phosphate, lanes 2 and 4
Table 6. Partial purification of pyrroline-5-carboxylate reductase activity from pea chloroplast lysate

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific Activity(^a)</th>
<th>Total Activity(^b)</th>
<th>Yield %</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. chloroplast stroma</td>
<td>23</td>
<td>6256</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 10-20% (w/v) PEG(^c)</td>
<td>32</td>
<td>1705</td>
<td>27</td>
<td>1.4</td>
</tr>
<tr>
<td>3. DEAE-Sepharose</td>
<td>194</td>
<td>1040</td>
<td>17</td>
<td>8.5</td>
</tr>
<tr>
<td>4. Procion Red Affinity 241</td>
<td>241</td>
<td>365</td>
<td>6</td>
<td>11</td>
</tr>
</tbody>
</table>

\(^{a}\)nmol min\(^{-1}\) mg protein\(^{-1}\).

\(^{b}\)nmol min\(^{-1}\).

\(^{c}\)Precipitated with polyethylene glycol (average molecular mass = 3,350).
step 2 preparation was not detectable after storage in 20% (v/v) glycerol at 4°C or -20°C for 16 h. Processing from step 1 through step 4 required 48 h. PCR activity in the step 4 preparation was not detectable after storage in 20% (v/v) glycerol at -20°C for three days. Each purification step caused great losses in yield.

PCR was partially purified from etiolated shoots (Table 7). Activity in the step 2 preparation was stable after storage in 20% glycerol at -20°C for three months. Activity in the step 4 preparation was also stable after storage in 20% (v/v) glycerol at -20°C for three months. Each purification step caused great losses in yield.

Figure 4 shows the pattern of protein separation by SDS-PAGE of samples from each step of PCR purification from etiolated pea shoots. Lanes 2 through 5 of Figure 4 represent samples from steps 1-4 of Table 7. Proteins that passed through the YM-100 membrane are visible in lane 6. Lanes 7 and 8 show proteins electroeluted from segments of a native IEF gel that had been loaded with samples from step 4 of Table 7. The PCR activity bands shown in Figure 8 (Section I) were the electroeluted segments. Figure 4 shows no enhancement of a 30 kd protein during purification, and lane 8 shows no 30 kd protein at all, even though barley PCR was reported to have a monomer size of 30 kd (9). Figure 4
<table>
<thead>
<tr>
<th>Step</th>
<th>Specific Activity$^a$</th>
<th>Total Activity$^b$</th>
<th>Yield (%)</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude</td>
<td>59</td>
<td>254,400</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 30-60% (NH$_4$)$_2$SO$_4$ saturated</td>
<td>60</td>
<td>67,392</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>3. Procion Red Affinity</td>
<td>79</td>
<td>3,213</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4. Concentrated$^c$</td>
<td>162</td>
<td>2,346</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$nmol min$^{-1}$ mg protein$^{-1}$.

$^b$nmol min$^{-1}$.

$^c$Concentrated over a YM-100 membrane in an Amicon pressure cell and then precipitated by saturating to 80% (w/v) with (NH$_4$)$_2$SO$_4$. 

Figure 4. Separation of proteins from different stages of PCR purification from etiolated pea shoots, by SDS-PAGE in a 12.5% (w/v) acrylamide slab gel. Lane 1 contains molecular mass markers of 66, 45, 36, 29, 24, 20, and 14 kd. Lanes 2–8 were each loaded with 50 μg of protein. Lanes 2, 3, 4 and 5 were loaded with samples from steps 1, 2, 3 and 4 of Table 7. Lane 6 contains proteins which passed through the YM-100 membrane used in step 4 of Table 7. Lanes 7 and 8 contain proteins electroeluted from a PCR active region of a native IEF gel (Figure 8, Section I). The sample in lane 7 had been centrifuged in a Centricon-10 concentrator. The sample in lane 8 had been precipitated with 20% (w/v) trichloroacetic acid. The gel was stained initially with Coommasie blue and then four times with silver.
shows the enhancement of a 21 kd protein after the Procion red affinity step (Lanes 4 and 5). The 21 kd protein is also present in the samples that were electroeluted from the native IEF gel segments that contained PCR activity (Lane 8). Proteins that passed through a YM-100 membrane and exhibited no PCR activity (Lane 6) also include an abundance of 21 kd protein.

Table 8 compares some kinetic parameters of partially purified PCR from chloroplasts (step 4, Table 6) with partially purified PCR from etiolated pea shoots (step 4, Table 7). The apparent $K_m$ for NADH was almost twofold greater than the apparent $K_m$ for NADPH for PCR from both sources. The $V_{max}$ with NADH was at least twofold greater than with NADPH. The PCR specific activity of the partially purified preparation from etiolated shoots was approximately fivefold greater than that of the preparation from chloroplasts.

Table 9 shows the effects of proline, azetidine-2-carboxylate (A2C), and thiazolidine-4-carboxylate (T4C) on the kinetic parameters of the partially purified preparations of PCR from etiolated pea shoots. PCR activity was inhibited by 50 mM proline, 2 mM A2C, and 2 mM T4C. A2C increased the apparent $K_m$ (P5C) of PCR.

Table 10 shows the effects of several nonionic detergents on PCR activity during chloroplast lysis. PCR
Table 8. Kinetic parameters\(^a\) of PCR from peas

<table>
<thead>
<tr>
<th>Source</th>
<th>Substrate</th>
<th>(K_m) (^b)</th>
<th>(V_{max}) (^c)</th>
<th>(R^2) (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>NADH</td>
<td>190</td>
<td>150</td>
<td>.97</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>120</td>
<td>190</td>
<td>.86</td>
</tr>
<tr>
<td>Etiolated shoots</td>
<td>NADH</td>
<td>190</td>
<td>3200</td>
<td>.88</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>430</td>
<td>834</td>
<td>.78</td>
</tr>
</tbody>
</table>

\(^a\)Parameters were calculated from Eadie-Hofstee plots.

\(^b\)Apparent \(K_m\) = \(\mu\)M.

\(^c\)nmol min\(^{-1}\) mg protein\(^{-1}\).

\(^d\)\(R^2\) = regression coefficient of plot.
Table 9. Effects of some inhibitors on kinetic parameters\textsuperscript{a} of partially purified P5C reductase from etiolated pea shoots

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Etiolated Shoots (K_m)</th>
<th>Etiolated Shoots (V_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.67</td>
<td>2005</td>
</tr>
<tr>
<td>+ 50 mM L-Proline</td>
<td>1.46</td>
<td>1000\textsuperscript{b}</td>
</tr>
<tr>
<td>+ 2 mM L-Azetidine-2 carboxylate</td>
<td>3.30\textsuperscript{c}</td>
<td>855\textsuperscript{b}</td>
</tr>
<tr>
<td>+ 2 mM L-Thiazolidine-4-carboxylate</td>
<td>1.00</td>
<td>210\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Apparent \(K_m\) = mM P5C, \(V_{max}\) = nmol min\(^{-1}\) mg protein\(^{-1}\), cofactor = NADH\(^{m}\).

\textsuperscript{b}Greater than 50% decrease below control value.

\textsuperscript{c}Greater than 50% increase above control value.
yield was doubled with the use of NP-40, CHAPS and CHAPSO. Protein extracted from chloroplasts was increased approximately sevenfold with the use of these detergents. Other detergents that did not solubilize proteins as well did not increase PCR yield.
Table 10. Detergent$^a$ effects on PCR release from chloroplasts

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Activity$^b$</th>
<th>Protein Concentration$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21 ± 0.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Triton-X114</td>
<td>27 ± 0.7</td>
<td>4.2</td>
</tr>
<tr>
<td>NP-40</td>
<td>42 ± 3.2$^d$</td>
<td>16.0</td>
</tr>
<tr>
<td>CHAPS</td>
<td>37 ± 0.5$^d$</td>
<td>20.0</td>
</tr>
<tr>
<td>CHAPSO</td>
<td>39 ± 1.1$^d$</td>
<td>19.0</td>
</tr>
<tr>
<td>BigCHAP</td>
<td>30 ± 2.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Octylglucoside</td>
<td>29 ± 1.0</td>
<td>7.7</td>
</tr>
<tr>
<td>Zwittergent</td>
<td>26 ± 1.3</td>
<td>3.4</td>
</tr>
</tbody>
</table>

$^a$0.5% (w/v) of extraction buffer.

$^b$nmol min$^{-1}$ ml$^{-1}$.

$^c$mg ml$^{-1}$.

$^d$One hundred percent increase over control yield.
DISCUSSION

Gamma-Glutamyl Kinase Experiments

The gamma-glutamyl hydroxamate assay is not specific for GK. Hydroxylamine (NH₂OH) is required to convert the gamma-glutamyl phosphate moiety to gamma-glutamyl hydroxamate (Appendix V). Any other enzymes that produce gamma-glutamyl phosphate as an intermediate will interfere with this assay (Appendix VI). Two such enzymes are glutamine synthetase (EC 6.3.1.2) and gamma-glutamyl cysteinyl synthetase (EC 6.3.2.2). Glutamine synthetase is a principal enzyme of ammonia assimilation in roots and leaves. It has cytosolic and stromal isoenzymes encoded by separate genes (22). Gamma-glutamyl cysteinyl synthetase catalyzes the first step of glutathione synthesis. Hydroxylamine is an analogue of ammonia, which can stimulate glutamine synthetase activity. In extracts from plants, glutamine synthetase activity can account for 80% of gamma-glutamyl hydroxamate synthesis (15). Clearly, a more specific assay is needed.

A more specific assay for GK was developed. This novel assay did not use hydroxylamine and did not require the detection of the unstable intermediate gamma-glutamyl phosphate. A2C was used in place of proline to distinguish GK activity from glutamine synthetase activity, because A2C interacts only with GK and proline interacts with both GK
and glutamine synthetase. The A2C inhibition of GK activity in Table 3 represents 75% of total glutamate-dependent ADP production. The proline inhibition of GK activity in Table 1 represents only 10% of total glutamyl-hydroxamate production. Thus, the pyruvate kinase-lactate dehydrogenase linked assay is more specific for activity resembling that of GK than the hydroxamate assay. Furthermore, the novel assay is continuous, while the hydroxamate assay is not. Kinetic experiments are more feasible with the novel assay. Table 3 shows GK-like activity in a pea chloroplast lysate. PCR activity was detected in the same preparation. Neither this preparation nor any other cell-free preparation from leaves synthesized $^{14}$C-proline from $^{14}$C-glutamic acid. Therefore, the A2C inhibitable kinase activity in Table 3 was not confirmed as GK activity. Gamma-glutamyl kinase has yet to be identified in plants.

**Gamma-Glutamyl Phosphate Reductase Experiments**

Bacteriologists in search of proline biosynthetic enzymes were able to identify them by generating proline auxotrophic mutants (18). These mutants, in turn, facilitated the cloning of proBA encoding GK and GPR, and proC encoding PCR. Strains with these genes cloned into expression vectors produced abundant amounts of activity from which each enzyme could be purified and characterized. GK was purified and characterized by first purifying and
characterizing GPR. Exogenous GPR became part of a standard GK assay. When the gamma-glutamyl hydroxamate assay was used, exogenous GPR enhanced the measurement of GK. Even inactive exogenous GPR enhanced GK measurement (5). When partially purified bacterial GPR was added to plant extracts, detectability of plant GK activity was not enhanced. Therefore, partial purification of plant GPR was attempted from two different plant sources that have abundant proline synthesizing activity.

P5C synthesizing activity was identified in a preparation from etiolated mung bean shoots (Table 4). The NADPH dependence of this activity is similar to the NADPH dependence of a mammalian "P5C-synthase" (13). Unlike the mammalian enzyme, the observed P5C synthesis was not inhibited by ornithine. Like the mammalian enzyme, the observed P5C synthesis was not inhibited by proline. The observation that P5C synthesizing activity was inhibited by azetidine-2-carboxylate (A2C) is consistent with previous observations of A2C inhibition of proline synthesis. A2C has been used to select proline overproducers in bacteria (3, 4), cyanobacteria (17), green algae (25), and plant cell cultures (17, 27).

Inhibition of P5C synthesis by methionine sulfoximine (MSO) is consistent with previous observations. MSO has been shown to inhibit proline accumulation in excised barley
leaves (14). MSO also inhibited GK activity measured by the hydroxamate assay (Table 1). MSO inhibited GK activity from *Pseudomonas aeruginosa* (8) but not from *Escherichia coli* (8, 18). *P. aeruginosa* GK has a \( K_i \) for proline (5 mM) which is 100-fold greater than the \( K_i \) of *E. coli* GK for proline (0.05 mM). Thus, *E. coli* GK is 100 times more sensitive to feedback inhibition. The phosphate-dependent P5C-DH assay (reverse GPR assay) did not identify GPR in mung bean preparations capable of P5C synthesis. The optimal pH range of 7.5 to 8.0 and higher activity with NAD\(^+\) than NADP\(^+\) identify the activity as belonging to the mitochondrial P5C-DH (6, 21). Phosphate-dependent P5C-DH activity could not be distinguished from P5C-DH activity. Thus, the reverse GPR assay was not useful in identifying GPR in crude preparations from plant tissue.

Partial purification of GPR from a proline overproducing carrot cell line was attempted. The same procedure that yielded GPR from *E. coli* was used. During purification of P5C-DH activity, phosphate did not appear to inhibit this activity. However, after native IEF and activity staining by the P5C-DH assay in the presence and absence of 10 mM \( \text{KH}_2\text{PO}_4 \), phosphate dependence of P5C-DH activity was not demonstrated. Figure 3 shows that phosphate inhibited P5C-DH activity. Thus, GPR was not identified in carrot cell line C63. The partially purified
enzyme is most likely the mitochondrial P5C-DH. All previous assays of the mitochondrial P5C-DH were conducted with mitochondrial preparations (6, 21). The procedure used here is a novel method of partially purifying P5C-DH from plants.

P5C Reductase Experiments

The assay for P5C reductase (PCR) was the most specific of all assays used to study proline synthesis. P5C reductase was the most reproducibly identifiable enzyme of all the proline biosynthetic enzymes studied. Thus, P5C reductase was focused upon. The localization of PCR in pea chloroplasts has been described in Section I. PCR was partially purified from peas in order to compare characteristics of pea PCR with those of previously studied PCRs. Because partially purified PCR from pea chloroplasts was very unstable and partially purified PCR from etiolated pea shoots was stable, PCR was routinely prepared from etiolated pea shoots.

Krueger et al. (9) described PCR from wilted barley leaves as a multisubunit enzyme. SDS-PAGE showed barley PCR, which had been purified to apparent homogeniety, to have a monomeric molecular mass of 30 kd. Gel filtration chromatography demonstrated that the barley PCR holoenzyme had an approximate molecular mass of 420 kd. Thus, barley PCR is composed of 12 to 16 subunits. Oligomers of 220 kd
were reported to retain activity. After six purification steps, 25% of initial activity was retained. Barley PCR activity was greater with NADPH than with NADH.

The observations of PCR purification from pea are quite different. Less than 10% of initial PCR activity was retained after only four purification steps. The lower yield suggests lower stability of pea PCR activity. SDS-PAGE separation of proteins in samples from each step of PCR purification from etiolated pea shoots (Figure 3) shows no enhancement of a 30 kd protein. Lane 8 was loaded with proteins electroeluted from a PCR active region of native IEF gels and shows no 30 kd protein.

Thus, PCR from etiolated pea shoots differs from PCR from wilted barley, because no 30 kd protein is observable. The Amicon YM-100 membrane retains molecules of molecular mass greater than 100 kd. PCR activity was detected only in the solution retained by the YM-100 membrane. Thus, PCR from etiolated pea shoots resembles PCR from wilted barley, because the native enzymes of both have a molecular mass greater than 100 kd.

Lanes 7 and 8 of Figure 4 were loaded with samples of proteins electroeluted from segments of a native IEF gel which contained PCR activity. The sample in lane 7 had been concentrated over an Amicon YM-10® membrane. The purpose was to remove ampholytes, which have an average molecular
mass of 600 daltons. The sample in lane 8 had been precipitated with 20% (w/v) trichloroacetic acid to remove ampholytes. The smear in lane 7 probably represents staining of the ampholytes. Ampholytes are known to stain like proteins.

A 21 kd protein is enhanced by step 3 (Table 7) of the purification. This protein is present in lane 8 suggesting that it may be a monomer of PCR. However, 21 kd protein is also visible in lane 6. Lane 6 was loaded with proteins that passed through the YM-100 membrane. If the 21 kd protein in lane 6 represents PCR monomers, then PCR is inactive when its oligomer size becomes less than 100 kd, because PCR activity was not detectable in the YM-100 effluent.

PCR activity from chloroplasts and etiolated shoots demonstrated lower apparent $K_m$'s for NADPH than for NADH (Table 8). This characteristic is consistent with the redox shuttle model proposed by Phang (Appendix III). The mammalian PCR's from erythrocytes and liver both have lower $K_m$'s for NADPH than for NADH. These mammalian PCR's are also feedback inhibited by proline. According to Phang's model, erythrocyte and liver PCR's drive the oxidative pentose phosphate pathway in those cells by maintaining a high NADP$^+$ concentration.
PCR activity from etiolated shoots was inhibited by proline, azetidine-2-carboxylate (A2C) and thiazolidine-4-carboxylate (T4C). The inhibition by T4C was fourfold greater than by A2C. A2C raised the apparent $K_m$ for P5C of PCR from chloroplasts and etiolated shoots. Because A2C changed the apparent $K_m$ and the $V_{max}$ of PCR activity from etiolated shoots, both competitive and noncompetitive inhibition is implied. Because proline and T4C changed only the $V_{max}$ of PCR activity from etiolated shoots, noncompetitive inhibition is implied. The observations of noncompetitive inhibition of PCR activity by proline and proline analogues in Table 9 suggest that a regulatory site distinct from the active site exists in the native PCR enzyme. If such a site exists, then proline can regulate its synthesis at the level of PCR. The inhibitory effects of proline, A2C, and T4C are consistent with Treichle's (24) conclusion that a regulatory role of PCR in proline synthesis cannot be excluded a priori. Proline levels in leaves of wilted intact plants do not usually exceed 55 mM, yet proline has a solubility capable of producing concentrations as high as 14 M. The observations in Table 9 suggest that proline accumulation may be limited by feedback inhibition of PCR by proline.

Osmotically lysed chloroplasts release most of their stromal protein. The use of detergents, which can release
active membrane proteins, increased the yield of PCR from chloroplasts (Table 10). NP-40, CHAPS, and CHAPSO increased PCR yield twofold, while protein yield was increased sevenfold. Thus, PCR may be associated with a chloroplast membrane. In three of seven Percoll® gradients, which separated ruptured chloroplasts from intact ones, PCR activity was found to migrate with the thylakoid marker (chlorophyll) as well as with the stromal marker (triose phosphate dehydrogenase) (Figure 1, Section I). Thus, PCR may be associated with the thylakoid membrane.

It has been suggested that all three enzymes of proline biosynthesis from glutamic acid may function as a multienzyme complex (23). Many multienzyme complexes are known for amino acid biosynthetic pathways in prokaryotes and eukaryotes (19). In a pathway analogous to this one, aspartate kinase and homoserine dehydrogenase are associated as a complex (Appendix VI). This would be analogous to GK and PCR being associated. Perhaps GK or GPR are thylakoid membrane proteins and PCR associates with them as an extrinsic protein. The possible hydrophobic nature of GK and GPR has not been accounted for in previous investigations of these enzymes in plants. The glutamate-dependent P5C synthesizing activity in mammals is
bound to mitochondrial membranes (13). Defining the structural relationships between enzymes and membranes is required, if the functions of such cellular components are to be understood at the physiological level.


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SUMMARY AND DISCUSSION

Increased proline synthesis from glutamic acid is a change in metabolism caused by water deficit. This investigation demonstrated the following. The enzymes that convert L-glutamic acid to L-P5C have yet to be assayed in plants. The final enzyme of the glutamate-derived proline biosynthetic pathway, pyrroline-5-carboxylate reductase (PCR), has been localized in chloroplasts of *Pisum sativum* L. This PCR activity has optima at pH 6.5 and 7.5. Its activity is affected by monovalent and divalent ion concentrations but not by osmolality. PCR activity from etiolated pea shoots has similar characteristics, but is 100-fold greater and also more stable than PCR activity from chloroplasts. The pI of the activity from etiolated shoots is 7.8. PCR from both sources has a lower $K_m$ for NADPH than NADH, but a higher $V_{max}$ with NADH than NADPH. PCR activity from etiolated pea shoots was inhibited by 50 mM proline, 2 mM azetidine-2-carboxylic acid, and 2 mM thiazolidine-4-carboxylic acid. Thus, PCR from pea tissue exhibits properties that suggest that this enzyme is sensitive to parameters that might regulate the rate of proline synthesis.

The location of PCR in the chloroplast has significance. According to Phang's redox shuttle model (Appendices III and IV), PCR is located in a compartment in which NADP$^+$
concentration serves a regulatory function. In mammalian fibroblasts, hepatocytes, and erythrocytes, different PCR isoenzymes have been identified and they are all cytosolic. Mammalian PCR functions to drive the pentose phosphate pathway (PPP), which is stimulated by high NADP⁺ concentrations. The PPP in turn drives ribose synthesis and nucleotide synthesis. In mammalian tissues, P5C and proline concentrations do not reach millimolar levels as they do in plants. Perhaps the NADP⁺/NADPH ratio regulates some chloroplast enzymes. The P5C-proline driven redox shuttle may down-regulate such chloroplast enzymes by maintaining a high NADP⁺/NADPH ratio in chloroplasts of stressed leaves. The shuttle may also transport reducing equivalents out of chloroplasts to the cytosol and mitochondria where energy-requiring reactions that participate in stress-induced metabolism would be active.

The presence of PCR in chloroplasts suggests another possible function. During water deficit, CO₂ assimilation and NO₃⁻ reduction are inhibited. These are the primary pathways of NADPH oxidation. Wilted leaves in the light are still capable of photon absorption and the photosynthetic electron transport system (PETS) is still active. If oxidized NADP⁺ is not available, while light absorption continues, electrons can accumulate in the PETS. If this energy is not dissipated electrons can reduce molecules that
are not PETS components. The reaction of electrons with $O_2$ is well known (2, 60, 75). Several toxic oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl ion can be formed. These can damage proteins and membranes irreversibly (75). Stress-induced proline synthesis may provide a sink for such electrons and function to prevent metabolic imbalance by regenerating NADP$^+$ under conditions when the rate of NADP$^+$ regeneration by other pathways is limited.

Two other phenomena related to water stress are associated with chloroplasts and thylakoid membranes. The first of these is abscisic acid (ABA) accumulation. ABA accumulation occurs in most plant species within two hours of wilting. ABA accumulation is caused by increased synthesis from violaxanthin (20). Violaxanthin is a carotenoid which functions as an accessory light-harvesting pigment in the thylakoid membrane. An oxygenase enzyme is involved in this ABA synthetic pathway (20). Most of the enzymes of ABA synthesis from violaxanthin are located in the chloroplast.

N,N,N-Trimethylglycine or glycine betaine is a quarternary ammonium compound that accumulates in leaves of some species in response to water deficit (16, 108). This molecule has the same solubility as proline and can contribute equally well to osmotic adjustment. Two enzymes,
choline oxygenase and betaine aldehyde dehydrogenase, synthesize trimethyl-glycine from choline in spinach and both enzymes are localized in chloroplasts (16). Betaine aldehyde dehydrogenase appears to be associated with the thylakoids (108). Ferredoxin appears to participate in trimethyl-glycine synthesis (16). Perhaps proline synthesis, trimethyl-glycine synthesis, and ABA synthesis all occur in chloroplasts. Perhaps perception of osmotic stress can also occur in chloroplasts. Perhaps these molecules serve a specific function in chloroplasts of stressed leaves. Such hypotheses ought to be tested.

Toxic oxygen derivatives are produced in unstressed leaves and chloroplasts. Mechanisms for detoxifying them exist constitutively (78). During water deficit, the activity of detoxification mechanisms should be maintained or increased, as the need for them may increase. All of the enzymes involved in detoxification are assayable. The effect of water deficit on such detoxification mechanisms should be investigated. Perhaps proline synthesis in chloroplasts participates in metabolism that prevents damage to chloroplast membranes and proteins caused by toxic oxygen species during water deficit.

The location of PGR in chloroplasts also suggests that proline must be transported across the chloroplast envelope to reach the cytosol. The presence of a chloroplast-
envelope-localized proline transporter should be investigated. It is possible that regulation of such a transporter may control proline concentrations within chloroplasts. It is possible that GK and GPR are also chloroplast enzymes, and they may be feedback inhibited by proline. An increase in proline export caused by water deficit could increase the rate of proline synthesis from glutamic acid without changing the proteins that catalyze proline synthesis. Proline oxidation is diminished during water deficit, but respiration increases. Perhaps proline transport into mitochondria controls proline oxidation. The co-regulation of proline synthesis and oxidation may occur at the level of transport across chloroplast and mitochondrial membranes.

Proline has a very high solubility in water (Table 1). It can contribute greatly to osmotic potential (-12.5 MPa) and osmotic adjustment because of its solubility and because of its biocompatible qualities. Proline protects membranes and enzymes against desiccation in vitro (76, 77). The compatible cytoplasmic osmoticum hypothesis predicts that proline should accumulate in the cytoplasmic portion of mesophyll cells. This region comprises the cytosolic, mitochondrial, and chloroplast volumes (approximately 5 to 10% of total cell volume). The vacuole comprises 90 to 95% of total cell volume. Isolation of vacuoles from mesophyll
Table 1. Qualities of some organic osmolytes

<table>
<thead>
<tr>
<th>Osmolyte</th>
<th>Formula</th>
<th>MW</th>
<th>Solubility&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MPa&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glycerol</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>92</td>
<td>∞</td>
<td>-11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-proline</td>
<td>C&lt;sub&gt;5&lt;/sub&gt;NH&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>115</td>
<td>162</td>
<td>-17.4</td>
</tr>
<tr>
<td>N,N,N-Trimethyl glycine</td>
<td>C&lt;sub&gt;5&lt;/sub&gt;NH&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>117</td>
<td>157</td>
<td>-16.5</td>
</tr>
<tr>
<td>α-D-glucose</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>180</td>
<td>82</td>
<td>-11</td>
</tr>
<tr>
<td>β-D-glucose</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>180</td>
<td>154</td>
<td>-21</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>180</td>
<td>14</td>
<td>-2</td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>180</td>
<td>237</td>
<td>-11.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;11&lt;/sub&gt;</td>
<td>342</td>
<td>204</td>
<td>-15</td>
</tr>
<tr>
<td>α,α-Trehalose</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;11&lt;/sub&gt;</td>
<td>342</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> g osmolyte per 100 ml H<sub>2</sub>O at 25°C.

<sup>b</sup> Calculated osmotic potential at maximum solubility.

<sup>c</sup> Based on 4.5 M glycerol observed in Dunaliella.
protoplasts reveals that proline accumulates primarily in the cytoplasm (62). However, the concentrations accumulated there do not contribute greatly to osmotic adjustment of the tissue. Table 1 describes some other osmolytes that have been shown to contribute significantly to osmotic adjustment in various photosynthetic organisms.

Table 2 depicts the calculated contribution of proline to leaf osmotic potentials if proline was present throughout the cell, only in the cytoplasm and only in chloroplasts. These calculations are based upon observed proline levels in excised barley leaves treated as previously described (67), and in leaves of intact plants that had been exposed to drought in the field for greater than 24 h (91). The contribution of proline to osmotic potential in any compartment is not great for leaves stressed less than 24 h. Leaf water potentials vary from -0.3 to -2.5 MPa. The cytoplasm (-0.66 MPa) and chloroplast (-1.66 MPa) calculated values for proline's contribution to osmotic potential in leaves of droughted plants fall within this range. The contribution of these proline concentrations to osmotic potential of stressed intact plants is significant. However, no investigation has shown that proline accumulation accounts for all of the osmotic adjustment observed in stressed leaves. Since proline is capable of contributing -17 MPa to osmotic potential in vitro (Table
Table 2. The calculated contribution of proline to osmotic potential in excised barley leaves

<table>
<thead>
<tr>
<th>% of mesophyll vol</th>
<th>Proline Content</th>
<th>Mesophyll Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umole Proline</td>
<td>Cell</td>
</tr>
<tr>
<td></td>
<td>g fresh weight</td>
<td></td>
</tr>
<tr>
<td>Wilted b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mM</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>MPa</td>
<td>-0.01</td>
<td>-0.05</td>
</tr>
<tr>
<td>Salt-shocked c</td>
<td>12</td>
<td>65</td>
</tr>
<tr>
<td>mM</td>
<td>13</td>
<td>65</td>
</tr>
<tr>
<td>MPa</td>
<td>-0.03</td>
<td>-0.16</td>
</tr>
<tr>
<td>Wilted d</td>
<td>50</td>
<td>275</td>
</tr>
<tr>
<td>mM</td>
<td>55</td>
<td>275</td>
</tr>
<tr>
<td>MPa</td>
<td>-0.13</td>
<td>-0.66</td>
</tr>
</tbody>
</table>

\(^a\) In excised second barley leaf of 14 day old seedlings transpiration loaded with 50 mM sucrose and 1 mM glutamic acid.

\(^b\) Wilted to 75% original fresh weight and held for 8 hrs.

\(^c\) Shocked with 205 mM KCl (-1.0 MPa) for 24 hrs.

\(^d\) Wilted in the field for 24 h or longer (91).
1), and the maximum calculated contribution was $-1.7 \text{ MPa in vivo}$ (Table 2), proline does not reach the concentrations at which it can make its maximum contribution to osmotic adjustment.

These calculations suggest that proline accumulation can serve a function unrelated to its osmotic properties. Perhaps, proline's biocompatible characteristics protect cellular machinery at the observed concentrations. Alternatively, proline accumulation could be a result of an uncoupled redox shuttle mechanism. If such a redox shuttle operates constitutively, and water deficit causes it to become uncoupled by decreasing proline oxidation without decreasing proline synthesis, then proline accumulation may be a symptom of metabolic dysfunction. Because proline has biocompatible characteristics and proline accumulation is not toxic, metabolism may be temporarily shunted in this direction until homeostasis is regained. Natural selection may have allowed such a malfunctioning mechanism to be conserved in the plant kingdom, because the side effects contributed positively to growth and reproduction.
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ACKNOWLEDGMENTS

He who cares not for fruit of the action,
He is a yogi a true sanyasin.

The Baghavad Gita

This dissertation is dedicated to
Sangeetha Ann Rayapati

Classrooms and labs and loud boiling testubes,
sing to the Lord a new song.
Knowledge and truth and loud sounding wisdom;
I too will praise him with a new song.
APPENDIX I

PATHWAYS OF PROLINE SYNTHESIS AND OXIDATION
A. Pathway of Proline Synthesis

\[
\begin{align*}
\text{L-Glu} & \xrightarrow{\text{ATP, ADP, Mg}^{2+}} \gamma\text{-Glu-P} \\
\end{align*}
\]

GK \quad \gamma\text{-Glutamyl Kinase} \quad \text{EC 2.7.2.11}
GPR \quad \gamma\text{-Glutamyl-Phosphate Reductase} \quad \text{EC 1.2.1.41}
PCR \quad \Delta^1\text{-Pyrroline-5-carboxylate Reductase} \quad \text{EC 1.5.1.2}

B. Pathway of Proline Oxidation

\[
\begin{align*}
\text{O}_2 & \xrightarrow{\text{RETS}} \text{NAD}^+ & \text{NADH} \\
\text{L-Pro} & \xrightarrow{2e^-} \text{Pro DH} \quad \text{L-P5C} \\
\end{align*}
\]

Pro DH \quad \text{Proline Dehydrogenase} \quad \text{EC 1.4.3.}
P5C DH \quad \Delta^1\text{-Pyrroline-5-Carboxylate Dehydrogenase} \quad \text{EC 1.5.11.12}
RETS \quad \text{Respiratory Electron Transport System}
APPENDIX IIA

ALTERNATE ROUTE OF PROLINE SYNTHESIS
APPENDIX IIB

IUPAC NAMES OF ENZYMES IN APPENDIX IIA
1. ATP: L-glutamate-5-phosphate transferase  
   E.C. 2.7.2.11
2. L-glutamyl-5-phosphate: NAD(P)+ oxidoreductase  
   E.C. 1.2.1.41
3. Spontaneous cyclization
4. L-Proline: NAD(P)⁺ 5-oxidoreductase  
   E.C. 1.5.1.2
5. L-Proline: O₂ oxidoreductase  
   E.C. 1.4.3.
6. L-Pyrroline-5-carboxylate: NAD(P)⁺ oxidoreductase  
   E.C. 1.5.1.12
7. Acetyl-CoA: L-glutamate N-acetyl transferase  
   E.C. 2.3.1.1
8. ATP: N-acetyl-L-glutamate-5-phospho transferase  
   E.C. 2.7.2.8
9. N-acetyl-L-glutamate-5-semialdehyde: NADP⁺ oxidoreductase  
   E.C. 1.2.1.38
    E.C. 2.6.1.11
11. N²-acetyl-L-ornithine: L-glutamate N-acetyl transferase  
    E.C. 2.3.1.35
12. L-ornithine: 2-oxoglutaric acid 5-amino transferase  
    E.C. 2.6.1.13
13. L-Ornithine: 2-oxoglutarate 2-amino transferase  
    E.C. 2.6.1.
14. Spontaneous cyclization
15. L-Proline: NAD(P)⁺ 2-oxidoreductase  
    E.C. 1.5.1.1
APPENDIX III

THE PROLINE-PS REDOX SHUTTLE
D-glucose-6-P → D-glucono-δ-lactone-6-P

ProDH

NADP⁺ → NADPH

PCR

NADP⁺ → NADPH

Lactonase

H₂O → 6-phosphogluconic acid

6-P-GDH

CO₂

D-ribose-5-P

NUCLEOTIDES

Purines
Pyrimidines
APPENDIX IV

REDOX EXCHANGE BETWEEN COMPARTMENTS
If A = Plant, then B = leaf, and C = roots
A = mesophyll cell, then B = chloroplast, and C = mitochondria
or B = cytosol, and C = mitochondria
APPENDIX V

STRUCTURES OF RELEVANT MOLECULES
APPENDIX VI
PATHWAYS WITH SIMILAR REACTIONS
Glutamine Synthetase EC 6.3.1.2

\[
\text{Glu} \xrightarrow{\text{Mg}^{2+}} \gamma\text{-Glu}-\text{PO}_4 \xrightarrow{\text{NH}_4^+} \text{Gln} + \text{PO}_4^{-3}
\]

Gamma-Glutamyl Cysteinyl Synthetase EC 6.3.2.2

\[
\text{Glu} \xrightarrow{\text{Mg}^{2+}} \gamma\text{-Glu}-\text{PO}_4 \xrightarrow{\text{Cys}} \gamma\text{-Glu-Cys} + \text{PO}_4^{-3}
\]

Aspartate Derived Amino Acid Biosynthesis

\[
\text{Asp} \xrightarrow{\text{AK}} \beta\text{-Asp}-\text{PO}_4 \xrightarrow{\text{ASDH}, \text{HDH}} \text{Homoserine} \xrightarrow{\text{Met}} \text{Cys} \xrightarrow{\text{Thr}} \text{Lys}
\]

AK = Aspartate Kinase EC 2.7.2.4
ASDH = Aspartyl Semialdehyde Dehydrogenase EC 1.2.1.11
HDH = Homoserine Dehydrogenase EC 1.1.1.3