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Title: Development of the *alt* mutant of *Pisum sativum*.

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The *alt* (*albina-terminalis*) mutant of *Pisum sativum* L. grows normally until the fifth or sixth node. Subsequently node 6 is chlorotic, defined as a transition leaf, above which 2-3 bleached nodes are produced. Growth ceases with the appearance of these bleached nodes, and the plants eventually die. The normal nodes of the *alt* mutant have chlorophyll content, absorption spectra, and photosynthetic rates indistinguishable from the normal *Alt* plant. However, as bleaching of leaves progress, all of these parameters decline markedly.

Extracts of pea, corn and bean seeds, and of pea shoots normalize the *alt* mutant. The normalized *alt* plants have chlorophyll content, absorption spectra, and photosynthetic rates comparable to normal *Alt* plants. Extracts were partially purified using anion and cation exchange resins, and also by cellulose TLC in two solvent systems. Extracts were first applied to an anion exchange resin and then applied to a cation exchange resin which was eluted stepwise with HCl. A biologically active substance from pea seed extract, which can

normalize the alt mutant, eluted with 4 N to 7 N HCl, from corn seed extract with 4 N to 8 N HCl. This confirmed that the biologically active substance is cationic. On cellulose TLC, the biologically active substance co-chromatographs with a ninhydrin-positive zone in solvent system I, however, it does not co-chromatograph with ninhydrin-positive zone in solvent system II.

Thiamine, thiamine monophosphate and thiamine pyrophosphate normalize alt plants. Thiamine at 10^{-4} M is the optimal concentration for normalization. Thiazole and thiazole monophosphate are inactive in the bioassay. Thiamine co-chromatographs with the biologically active factor from pea seed extract using cation exchange resin and both solvent systems for cellulose TLC.

The results of these experiments demonstrate that alt causes thiamine deficiency. The normalizing factor in extracts of normal pea, corn and bean seeds, and of pea shoots appears to be thiamine.

Development of the *alt* mutant of *Pisum sativum*

by

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Typed by Wei Wen Guo

This thesis is dedicated

to my parents

with much love and appreciation

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DEVELOPMENT OF THE *alt* MUTANT OF *PISUM SATIVUM*

Literature Review

Uses of Mutants in Plant Physiology

Biochemical mutants of prokaryotic and eukaryotic microorganisms have been widely used as experimental tools in investigations of biosynthetic pathways. Because of diploidy or higher levels of ploidy in the sporophytic generation of higher plants, investigators face many difficulties in using biochemical mutants. There are detailed discussions of these problems in the reviews by Nelson and Burr (22) and Scholz and Bohme (34). However, much progress has been made by the application of newly developed screening techniques for the selection of mutants after mutagenic treatment, and also by studying existing mutants.

Using mutants as experimental tools in the analysis of photosynthetic phenomena is a well-established approach. Somerville (38) recently reviewed this area. Mutants with defects in CO_2 fixation, photorespiration, and photosynthetic electron transport have been isolated and studied. By studying these mutants, many physiological phenomena can be explained biochemically and/or molecularly. Ribulose bisphosphate carboxylase (Rubisco) is an important enzyme in photosynthetic CO_2 fixation and photorespiratory metabolism. Mutations that affect gene expression or enzymatic activity of Rubisco have been isolated. A mutant of *Arabidopsis*, *rca*, has been isolated in which Rubisco appears to be poorly activated

in vivo. This mutant could be recovered in high CO_2 levels, so the photosynthetic apparatus and the enzyme complement of the Calvin cycle are intact. However, in standard atmospheric conditions, this mutant has a very low photosynthetic rate even though it has the normal amount of Rubisco. No differences could be found in the physical or catalytic properties of Rubisco purified from the *rca* mutant compared to the wild type. It was determined, however, that stromal extracts from the wild type activated the enzyme up to five-fold, whereas stromal extract from the mutant did not have that function. The mutant lacks two moderately abundant polypeptides which faciliate activation of Rubisco. This discovery may help explain why higher CO_2 concentrations are required to activate Rubisco *in vitro* than *in vivo* and it is important to any attempts to enhance photosynthetic productivity.

By studying the waxy mutant of maize which affects starch synthesis in the endosperm (31), it was shown that ADP-glucose pyrophosphorylase is a key enzyme for nucleoside diphosphate sugars in starch synthesis. These waxy mutants lack the starch granule-bound nucleoside diphosphate sugar-starch glucosyl transferase. Thus, they cannot synthesize amylose, so soluble glucosyl transferase cannot be bound to amylose and deposited in the starch granule.

Gavazzi et al. (34) selected a mutant from the line W22 of *Zea mays*, in which the coleoptile initially develops normally, but then becomes necrotic and dies before the emergence of the first leaf. By studying the mutant in tissue culture, it was found that normal growth resumed with proline treatment. This result showed that the

mutant has a metabolic block in the proline biosynthetic pathway. Further studies (34) suggested that this block is between L-'Δ-pyrroline-5-carboxylic acid and proline.

Most investigations of the physiological roles of plant hormones have been done by external applications of hormones or synthetic growth regulators. Plant responses in such studies may not be the same as those elicited by change in endogenous levels. An alternative is to manipulate endogenous hormone levels by chemical or genetic means. There are some mutants in different species with reduced ABA levels; viviparous corn mutants, droopy potato, wilty pea and wilty tomato mutants. These mutants wilt readily compared to the wild type. The wiltiness is the result of excessive transpiration under moist soil conditions. All these mutants are reported to contain lower amounts of ABA. Regular foliar sprays of ABA restore control over transpiration and the plants grow normally. It has been suggested that the primary effect of these mutations is to lower the endogenous ABA content. Many studies have used these mutants to investigate ABA biosynthesis in plants and the primary roles of ABA as a regulator of stomatal homeostasis. Neill and Horgan (30) found that the transpiration rate of the flc tomato mutant was three times greater than that of the wild-type and stomatal resistance correspondingly lower. Stomata of both flc and wild-type respond to darkness and externally supplied ABA by closing. However, only the wild type stomata respond to water stress by closing, those of flc remained open until the leaves were severely desiccated. Thus, there is some relationship between the lack of stomatal response to water stress and

blockage of ABA biosynthesis. Bowman et al. (4) isolated a new ABA analogue from the flc. High levels of the analogue are found in the mutants compared to the normal. The analogue also accumulates in response to water stress. It was suggested that the analogue may be a metabolite of ABA. They speculated that the flc mutation results in an increase in the rate of ABA metabolism, thereby indirectly reducing endogenous ABA levels. Under water stress both ABA synthesis and metabolism are elevated, so high levels of the ABA metabolite accumulate, but much more work needs to be done to prove this.

In numerous species, there are genetic dwarf strains or cultivars: d_1 in Zea mays; dy in Oryza sativa; le in Pisum sativum. These are all single gene, simple recessive mutants. Each responds to GA_1 by growing normally. It is known that there are more than 50 gibberellins occurring naturally. By studying these dwarf mutants, it was shown that the metabolism of GA_{20} to GA_1 is blocked. The GA_1 content in these mutants is also much lower than in the normal. This leads to the conclusion that GA_1 is the main endogenous gibberellin that is active per se in the control of shoot elongation in higher plants (29).

Chloronerva, is a spontaneous, recessive, monogenic mutant selected from tomatoes. Its growth is severely retarded. The leaves are abnormal and exhibit a pale yellowish interveinal chlorosis. Only a few flower buds develop and the buds do not unfold, but simply die. This abnormal growth and development is completely normalized by grafts, whether the mutant is used as scion or as rootstock. Even compatible grafts with other species normalize the mutant. Spraying

extracts from wild-type tomato and other species also can normalize the mutants. The extracts contain a "normalizing factor" which is deficient in the mutant. This "normalizing factor" has been identified as nicotianamine (34). It is presumed, but unproven, that nicotianamine functions in iron transport across cell membranes. This is the first case in the biochemical genetics of higher plants that has led to the discovery of a previously unknown substance having an important physiological function.

Thiamine-deficient mutants in different species have been developed, and also have been studied extensively.

Arabidopsis thaliana

Langridge (21) investigated plant biosynthesis by mutational blocks. He used X-rays to induce mutation in plants, then he tested the plants for their nutritional requirements under aseptic conditions. Using this method, he was able to obtain 27 thiamine-deficient mutants from 110 X_2 families (second generation of X-ray treated seeds) (34), all of which were lethal, except one inherited as a recessive mutant. The homozygous recessives could not synthesize thiamine. The cotyledons are mottled, the leaves are white with chlorophyll at their tips. By applying 1 ug thiamine per plant to the growth medium, the growth can be normalized. But the block in thiamine synthesis is not absolute, since some plants become green again and resume growth even without thiamine treatment.

With X-rays, Redei was also able to induce two other thiamine-deficient mutants, one blocked in 2,5-dimethyl-4-aminopyrimidine synthesis, the other blocked in 4-methyl-5-(ß-hydroxyethyl)-thiazole

synthesis (34).

By treating seeds with ethylmethanesulphonate, Feenstra (34) recovered six thiamine-less mutants. The mutants have different requirements for restoring growth, however, implying that the mutations occur in different genes. Complementation experiments showed that all mutants blocked in pyrimidine synthesis belong to one locus or to two closely linked loci, similarly the thiazole-less mutants segregate together. The mutants requiring complete thiamine are distributed among at least four complementation groups.

Lycopersicon esculentum

Langridge and Brock (20) reported a spontaneous, recessive single gene mutant (py) in L. esculentum. The homozygous mutants are chlorotic, and grow abnormally. Thiamine treatment restored normal growth. Further studies showed that the genetic block is in the formation of the pyrimidine moiety, perhaps in the replacement of a hydroxyl at position 2 by a methyl position.

Boynton (5) used the x-ray-induced mutants (ten and spa) and also the spontaneous mutant tl to study the localization of their respective genetic blocks in the biosynthetic pathway of thiamine and also the manifestation of the thiamine deficiency in abnormalities of chloroplast ultrastructure (details will be discussed later). By feeding the mutants with the thiamine precursor 2-methyl-6-aminoethyl pyrimidine and 4-methyl-5-β-hydroxyethyl thiazole, the location of the blocks in thiamine biosynthesis in these mutants were found. In spa and tl the block is in the biosynthetic pathway of the pyrimidine moiety of the thiamine molecule. In ten the block is in conversion of

thiamine to TPP.

Plantago insularis

Murr and Spurr (34) reported that an albino mutant in P. insularis is partially blocked in the conversion of thiamine to thiamine pyrophosphate. They also studied the ultrastructure of the chloroplasts (28).

These examples show that using biochemical mutants of higher plants to solve problems in the field of genetic regulation of biochemical reactions and plant morphogenesis is entirely practicable. We can induce biochemical mutants, grow the plants on artificial media for selection of mutants, or we can use the substantial reservoir of existing mutants in higher plants.

Thiamine

Thiamine, also known as vitamin B_1, contains a pyrimidine moiety (2-methyl-4-amino-5-hydroxymethylpyrimidine) and a thiazole moiety (4-methyl-5-ß-hydroxyethylthiazole). These are connected by a methylene group. The chemical structure of thiamine is shown in Fig. 1. Thiamine pyrophosphate is the coenzyme form of thiamine. It is a very important coenzyme in carbohydrate and amino acid metabolism including pyruvate dehydrogenase, oxoglutarate dehydrogenase, transketolase, branched-chain α-keto acid dehydrogenase, pyruvate decarboxylase, and carboxylate carboligase (17). Breslow (2) used thiazole analogs to study the mechanism of thiamine action. He found that TPP functions as a carrier of "active aldehyde" to form the intermediates hydroxyethyl-TPP, and α-hydroxy-ß-carboxypropyl-TPP.

Since thiamine is involved in so many enzyme activities, it plays a very important role in the plant life cycle. The properties of thiamine, the biosynthetic pathway and its physiological functions have been intensively studied, primarily in animals and microorganism. Here some discussion about thiamine which relates to plants will be presented.

Chemical Properties

Thiamine is very soluble in water, less soluble in methanol and glycerol, and nearly insoluble in ethanol, ether and benzene (17). Thiamine is more stable in acidic pH than in neutral and alkaline pH. According to Mullry's report: under 130°C, the D (destruction rate) value of thiamine at pH 5 is 107.3 mins, at pH 6.5 it is only 36 mins (28).

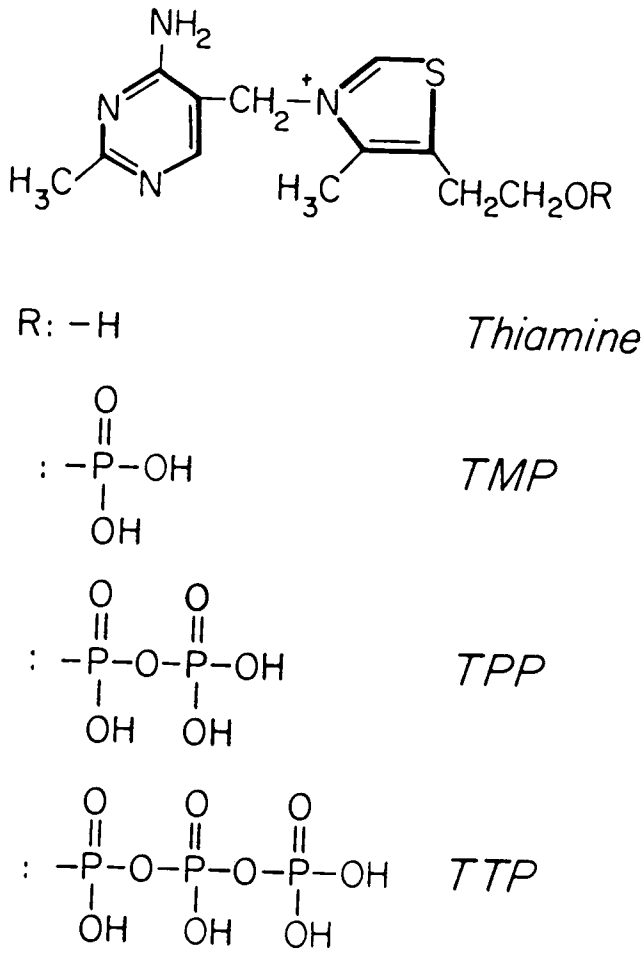

Fig. 1. Structure of thiamine and its phosphates.

Thiamine biosynthesis

Since thiamine plays an important role in cell metabolism, thiamine biosynthesis has been studied extensively. Using thiamine-deficient mutants, and tissue cultured roots, we know that thiamine biosynthesis involves the condensation of the pyrimidine and thiazole residues. From isotope studies and purified enzyme systems, investigators have elucidated the following reactions (13):

2-methyl-4-amino-5-hydroxymethyl pyrimidine + ATP=2-methyl-6-amino-5-phosphorylmethylpyrimidine (PY-P) + ADP (1)

PY-P + ATP = PY-P-P + ADP (2)

4-methyl-5(ß-hydroxyethyl)thiazole + ATP = 4-methyl-5(ß-phosphorylethyl)thiazole (Th-P) + ADP (3)

PY-P-P + Th-P = thiamine monophosphate + P-P (4)

TMP + H_2O = Thiamine + P (5)

The enzyme for reaction (4) is called TMP synthetase (thiamine phosphate phosphorylase), it has been purified 500-fold from yeast and has a pH optimum of 9.2. It is specific for the two substrates indicated above. A fungal gene, thi-10, was described which reduced the ability of one of the mutants in Neurospora crassa to condense the two residues. Spenser (38) thought reaction (3) probably was not necessary, because thiazole-P was synthesized directly.

Biosynthesis of the Pyrimidine Moiety

Although the biosynthetic pathway for the formation of pyrimidine has not yet been completely elucidated, it is known that the pyrimidine moiety of thiamine is not made by the same pathway by which

pyrimidines found in nucleic acids are made. By radioactive incorporation studies, it is found that glycine, methionine, and acetate are involved in the synthesis of the pyrimidine moiety, but different studies have different results, so the picture is not very clear yet. Brown (7) and Goodwin (14) presented a detailed discussion. It is very interesting that Newell and Tucker found a close biosynthetic relationship between purine biosynthesis and the pyrimidine of thiamine. They showed that 5-aminoimidazole ribonucleotide is a common intermediate in the biosynthesis of purine and the pyrimidine of thiamine. It is also found that yeast and bacteria use different pathways for production of pyrimidine, formate might be involved but not glycine. It was recently reported that in bacteria the purine intermediate, 5-aminoimidazole ribonucleotide whose carbon atoms are derived from glycine, formate, and ribose serves as the sole precursor of the skeleton of the pyrimidine unit of thiamine. However, in yeast the pyrimidine unit of thiamine is derived in an entirely different manner, the origin of the pyrimidine unit of thiamine is not linked to 5-aminoimidazole ribonucleotide, thus, not to purine biosynthesis. There are probably two biosynthetic pathways to the pyrimidine moiety of thiamines in yeast. In the major pathway formate is the precursor of C_4 of the pyrimidine while a hexose, metabolized to a pentose serves as the source of the remaining five carbon atoms of the pyrimidine unit. In the minor pathway, it is C_2 and not C_4 of the pyrimidine nucleus which is derived from formate, while C_4, C_5 originates from carbohydrate. The source of C_2', C_5 and C_6 of the pyrimidine unit in this minor pathway remains unknown (15,

39, 40).

Biosynthesis of the Thiazole Moiety

Similar to the pyrimidine moiety, the route to the thiazole nucleus of thiamine in bacteria appears to be somewhat different from that in yeast. In each case, the nitrogen atom and the adjacent CH carbon atom C-2 are derived as an intact unit from an amino acid. In yeast, it is glycine; in bacteria it is tyrosine. In both cases the S atom of the thiazole nucleus is derived from cysteine. In yeast, the C5 unit of the thiazole moiety originates from a pentose precursor. This pentose intermediate is generated from glucose or fructose by the nonoxidative and oxidative pentose phosphate pathways. The nonoxidative pentose phosphate pathway includes an enzymic reaction, the transketolase step which is known to require thiamine as a cofactor. Thus thiamine is required in one of the reactions of its own biosynthesis. But the oxidative pathway does not require thiamine. In bacteria, the C5 unit originates by combination of a C2 unit derived from pyruvate with a C3 unit, derived from a triose possibly via a 1-deoxy-2-pentulose (15, 39, 40).

Thiamine Biosynthesis in Microorganisms

The constitution of the medium has an important effect on the ability of bacteria to synthesize thiamine. For instance, Bacillis paralve, cultured on a medium devoid of cysteine, phenylalanine, valine and leucine, cannot synthesize thiamine, but in a reducing medium (containing thioglycocholate, $Na_2S_2O_4$ or ascorbic acid) in the absence of cysteine, phenylalanine,valine and leucine it can synthesize thiamine. Fungi are also influenced by the constituents in

the medium. Pyridoxal-phosphate inhibits the synthesis of thiamine in Neurospora. Most algae are able to synthesize their own thiamine, by the same pattern as bacteria and fungi (13).

Thiamine Biosynthesis in Higher Plants

The pathway of thiamine synthesis in plant has been studied, but not as intensively as in microorganisms. The results, however, are essentially consistent with those obtained in microorganisms. Mitsuda et al. (23, 24) obtained an enzyme preparation from acetone powders of five kinds of green leaves containing the enzyme system responsible for the synthesis of thiamine from the pyrimidine and thiazole moieties. By a bioautographic method using a thiamine-less mutant of Lactobacillus fermenti, they determined that pyrimidine is pyrophosphorylated in the presence of ATP, that thiazole is phosphorylated under the same conditions, and that these moieties are condensed to form thiamine-P with liberation of pyrophosphate. Studies of the formation of the pyrimidine and thiazole moieties have not been done in plants yet.

Physiological Properties of thiamine in Plants

There are three phosphate esters of thiamine in organisms: thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP). TMP is an intermediate in the biosynthesis of thiamine. Thiamine is pyrophosphorylated to form TPP and TTP is synthesized by phosphorylation of TPP catalyzed by thiamine diphosphate:ATP phosphoryltransferase (26). The physiological role of TTP in plants is unknown, but it has been reported that TTP might play a role in nerve conduction and transmission in animals (32). At

different developmental stages, the levels of the three forms of phosphorylated thiamine and free thiamine are different. According to Yusa (41) phosphorylated forms of thiamine are absent from mature ungerminated seeds of corn, soybean, pea and rice. It seems that phosphorylated thiamine is degraded very rapidly during seed maturation and that phosphorylated thiamine is detected only after seed germination. This indicates that an active phosphorylation takes place from free thiamine into TPP and/or TTP during seed germination. Yusa found that one third of the total thiamine in pea seed is converted into TTP in the young hypocotyl and radicle, but the function of this TTP is unknown. Molin et al. (26) reported that low levels of thiamine pyrophosphotransferase (TPTase) activity were present in preparations from unimbibed seeds. On a seedling basis, the enzyme activity increased rapidly for the first 36 h of imbibition, remained at nearly the same level from 48 h to between 96 h and 120 h, and then declined somewhat. He also found that extractable TPP content declined dramatically even though maximal TPTase activity was maintained. This might be a reflection of an increased demand and subsequent binding of TPP by apoenzymes requiring this enzyme. By using inhibitors of TPTase, they also found that TPP formation and utilization represent important facets of vigorous seedling growth, at least in soybean, and ATP and other nucleoside triphosphate concentration may represent an important factor in regulating thiamine phosphorylation in early seedling development.

Bonner (3) studied the transport of thiamine in the tomato plant in 1942. He found that the leaf is the site of thiamine synthesis in

the tomato plant. Roots require thiamine for growth, and transport of thiamine to roots occurs only in plants with intact leaves. When he girdled the plant, Bonner found that in the tops of the girdled plants, total thiamine increased, but in the roots thiamine decreased markedly by the second day. Bonner also showed that maximal synthesis and transport occur in mature leaves. Young, rapidly expanding leaves are capable of synthesis but little or no thiamine is exported at this stage. The highest concentrations of thiamine are found in the shoot apex and the youngest leaves in tomatoes, peas, cotton, maize and other plants, possibly because of translocation from the mature leaves to the apex.

Reproductive tissues of plants all contain thiamine. A large amount of thiamine is translocated to the developing seeds. Shimamoto and Nelson (35) studied the movement of [^{14}C]-compounds from maternal tissue into maize seeds grown in vitro. They found that thiamine was unique among the vitamins studied because it was concentrated in the embryo at a level that was 10 times higher than in the endosperm. They thought the absence of auxotrophic mutants requiring vitamin or cofactor supplements in higher plants may be explained by inadequate translocation of these essential metabolites into the mutant zygotes to enable their development to mature seeds.

In seeds, thiamine is bound to a protein. The thiamine-binding protein has been isolated from plants (25). During germination thiamine is phosphorylated very quickly to TPP. New thiamine synthesis also occurs in the presence of light. When Bonner (3) kept cotyledonectomized pea embryos in the dark, they did not synthesize

thiamine for at least 8 days following the onset of germination. Zinc, Cu, Mn and B stimulated thiamine synthesis.

Boynton (5) used the thiamine-deficient tomato mutants spa, tl and ten to study the ultrastructure of chloroplasts. He found that at an early stage of bleaching in leaf tissues, the chloroplasts have a normal number of grana per plastid, but a high percentage of the grana have diffuse substructure. In tl and spa, as the whitish bleached leaf tissues developed, the chloroplasts are drastically reduced in number per plastid and become abnormally large in diameter and include even higher percentages of plastids with diffuse substructure containing only primary lamellae, frequently without grana. In each instance the presence of a more normal lamellar system in the chloroplast is associated with progressively higher levels of chlorophyll. Other organelles appear to be unaffected by thiamine deficiency until a sudden and generalized necrosis of entire cells. In thiamine-treated plants, chloroplasts develop normally, the chlorophyll pigments remain light-stable and the mutant plants have a phenotype indistinguishable from the normal.

Michael and Spurr (22) used a thiamine-deficient mutant of Plantago insularis to study the effect of TPP on the ultrastructure of the chloroplast. They found that the plastids of both mutant and normal seedlings in the dark were similar in size and lamellar content, and total nitrogen content. After 24 h of light, however, when there is a much greater difference in total nitrogen between the normal seedlings and untreated mutants, the amount of lamellar structure within the plastids is greater in the normal seedlings. So

the mutant is not handicapped in protein synthesis by TPP deficiency until after exposure to light. After such exposure the mutants were not able to synthesize as much protein as the normal seedlings. Deficiency in the ability to synthesize proteins would affect the development of plastid ultrastructure and perhaps synthesis of enzymes involved of the chlorophyll.

Cantor and Burton (10) reported altered structures in mitochondria of thiamine-deficient Polytomella agilis, a flagellate alga. In thiamine-deficient cells, they found that numerous mitochondrial profiles are evident, having a regular circular or ovoid appearance, while the inner membranes are regularly arrayed in an electron-dense matrix and generally appear elongate. But in normal cells, mitochondrial profiles are relatively few and irregular in shape, the inner membranes are randomly dispersed in a light matrix and elongated vesicular or branched in appearance. In yeast, Euglena gracilis Z. and in mammalian systems, thiamine deficiency also has a marked affect on mitochondrial structure and function.

The alt Mutant of Pisum

The alt mutant of Pisum sativum involves a recessive allele. Mutants (alt/alt) grow normally until they have 5-6 nodes, thereafter, above a rather sharply defined zone of transition, the chlorophyll degrades, a bleaching of the tissue occurs, the plant stops growing and dies later. Lamprecht (19) reported that alt is a single gene mutation, causing the chlorophyll deficiency albina-terminalis. By crossing studies he found that linkage of Alt is with the genes of chromosome III. He also found that the possible location of the Alt

gene in chromosome III is: M-Rf-Fr-Alt-St-B-Gl. By grafting experiments, Acree and Marx (1) found graft transmission of an active substance causing the formation of chlorophyll in the basal or axillary branches of the mutant. These branches ultimately produced flowers, fruits, and seeds. But when these seeds were planted, they all showed the mutant phenotype. By applying the extract of normal pea seeds to the mutant, they got the same result. These results suggested that there is a graft-transmissible substance(s) present in normal plants but deficient in the mutant. Further studies by Acree and Marx showed that the active substance in the crude exbtract is a cation and it is neither a metal ion nor a complex between a metal ion and an anion. They tested a number of naturally-occurring substances including 18 amino acids, 10 carbohydrates, 15 vitamins (including thiamine), minerals and plant hormones, but they were all inactive in bioassay.

The active substance which is deficient in the mutant plays an important role in plant growth and development, especially in regulating chloroplast development and photosynthesis. By identifying this Alt substance and using the mutant to observe its relationship to chloroplast development and photosynthesis ability, we might learn more about the regulation of chloroplast development and its relationship to photosynthesis. We can also study the biosynthetic pathway of this Alt substance, determine the biosynthetic blockage, study how the Alt gene is expressed. The specific objectives of this study were to characterize the physiology of the alt mutant and determine the identity of the normalizing compound.

Materials and Methods

Plant Material

Pea plants (Pisum sativum L.) segregating for alt were grown in a greenhouse with the natural photoperiod extended to 18 h with high pressure sodium vapor lights which provided an average photosynthetic photon flux density (PPFD) at pot level of 250 μmol $m^{-2}s^{-1}$. Seeds of different lines of pea were grown either in 6 x 250 cm plastic tubes in blocks of 20 or in 15 cm diameter plastic pots. The soil was a peat:pumice:soil mixture (2:1:1) limed to pH 6.5. All plants were fertilized twice weekly with 20-20-20 general purpose fertilizer, and were also watered according to the soil moisture.

Preparation of Extract

500 g of dry pea seeds (O.S.U S700) corn (Zea mays L. 'Jubilee') and bean (Phaseolus vulgris L. 'Oregon Trail') (each supplied by J. R. Baggett, Oregon state University) were imbibed in distilled water at room temperature for 24 h and then homogenized with a blender with 1000 ml of acetone:water (1:1). The homogenate was filtered through 4 layers of cheesecloth, then centrifuged (Beckman model J2-21) at 12000 g for 10 minutes. The supernatant was concentrated in a rotary evaporator at 45°C to 200 ml and centrifuged again at 39,000 g for 30 min. The supernatant was frozen as a crude extract for later bioassay and for purification. Normal pea shoots (200 g f.w.) were first chopped and then extracted in the same way. Methanol, acetone and water were also tested separately as extracting solvents using pea seed.

Bioassay

Mutants for bioassay of Alt plant extracts were withheld from water for 2 to 3 days (this enabled the plant to draw more of the test solution), then they were decapitated at the transition leaves, and the leaves and stipules at the two highest remaining nodes were removed. Thereafter, the stem was bent into a 2 ml vial covered with parafilm. The vial was filled with extract or other bioassay tested solutions, and refilled once or twice depending on the amount of uptake. The bioassay results were determined after two weeks by measuring the chlorophyll content of lateral branches stimulated by the decapitation. All experiments were replicated two or three times.

Chlorophyll Measurement

Chlorophyll content was measured by either of two methods. (1) The fresh leaves were weighed, then chopped and homogenized in 80% acetone:water solution with a glass homogenizer. The homogenate was filtered, and the absorbance at 663 nm and 645 nm was measured in a double beam spectrophotometer (Beckman model 34). (2) The fresh leaves were weighed and the chlorophyll extracted with N,N-dimethyl-formamide for 24 h and on a rotary shaker in the dark. The absorbance was measured at 664.5nm and 647nm with the spectrophotometer (16).

Chromatography

Crude extract (250 ml) was applied to an anion exchange column (2 x 20 cm) Dowex 1 (OH^- form), and the resin was washed with equal volumes of distilled water. The fraction was collected for bioassay, and also for further purification using a cation exchange column of Dowex 50 (H^+ form). Equal volumes of distilled water were applied and

followed successively by 250 ml each of 1 N to 12 N HCl in increments of 1 N. Each fraction was collected, evaporated, redissolved in water, adjusted to about pH 7 with 1 N NaOH, then bioassayed.

The 6 N fractions from the cation exchange column were collected, evaporated in the rotary evaporator, and redissolved in MeOH:NH_4OH (98:2) before loading onto the plates. Cellulose thin layer chromatography (TLC) plates (1000 u 20 x 20 cm, Analtech) were used. The plates were developed in solvent system I: acetonitrile:H_2O:NH_4OH (8:2:1). Then the plates were divided into five zones according to the ninhydrin positive spots and UV quenching (Fig. 2). Each region was collected, eluted with MeOH:NH_4OH (98:2), and then each fraction was evaporated and bioassayed or redissolved in MeOH:NH_4OH (98:2) and loaded onto plates. These plates were run in the second solvent system; butanol:methylethylketone (MeCOEt) :H2O:NH_4OH (5:3:1:1). The plates were again divided into 6 zones according to the pattern of UV quenching (Fig. 3). Each zone was collected and eluted by the same method, and bioassayed. The Rf of each fraction and the reaction with ninhydrin were recorded.

Measurement of Photosynthesis

The plants for photosynthetic measurement were grown in a growth chamber illuminated by fluorescent tubes supplemented with incandescent bulbs (60 W). The average photosynthetic photon flux density (PPFD) at pot level was 300 μmol m^{-2} s^{-1}. Photoperiods were 18 h with 20°C days and 15°C nights. Photosynthetic rates were determined in two ways. (1) Carbon dioxide standard curves were made

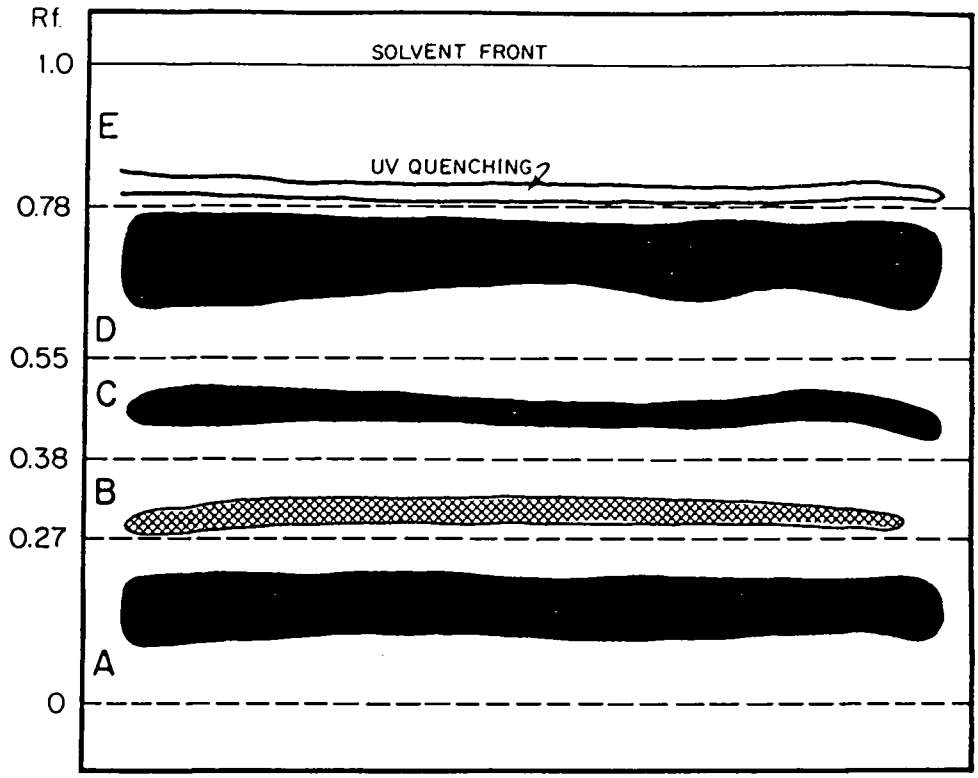

Fig. 2. TLC of 6 N fraction from cation exchange column in solvent system I. Solid black and cross-hatched bands are ninhydrin-positive.

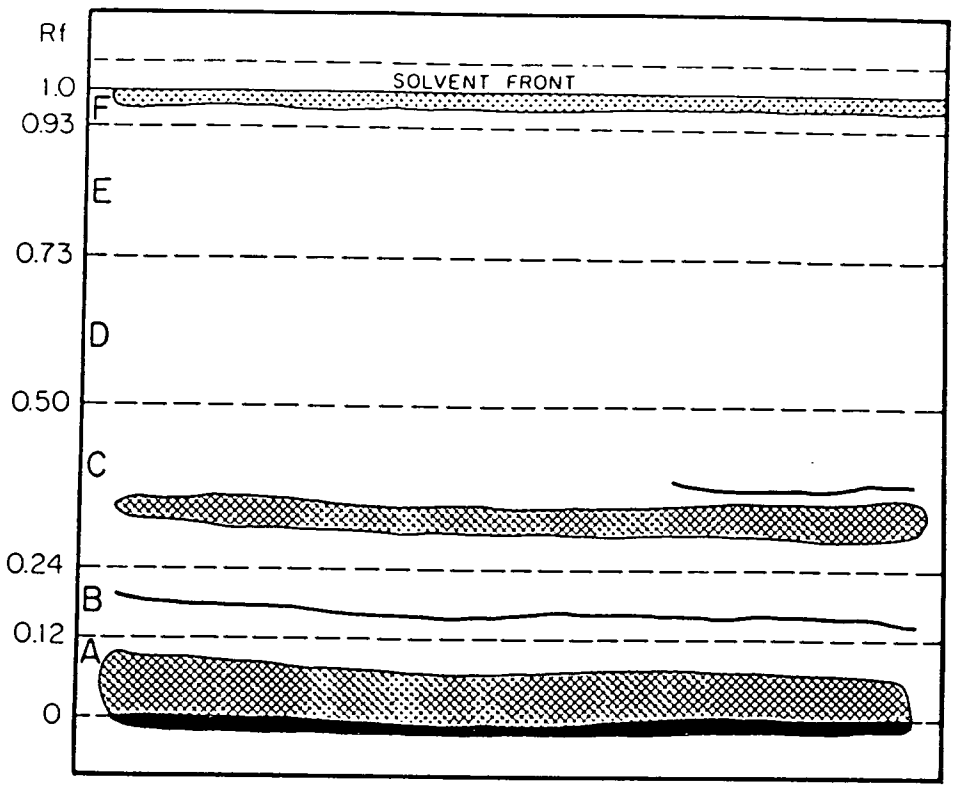

Fig. 3. TLC of zone C eluant from solvent system I in solvent system II indicating pattern of UV quenching. Zone A, B and C were also ninhydrin-positive.

with standard CO_2 (360 ppm air balance). During measurement, the environment was controlled as follows: PPFD 360 μmole $m^{-2}s^{-1}$; the intial CO_2 concentration was close to natural atmospheric CO_2 concentrations; air temperature was 20°C. The leaves whose photosynthetic rate was to be determined were put into a cuvette. The change of CO_2 concentration within one minute was measured by an infrared analyzer (Beckman model 865). The leaf area was measured by tracing the leaves on paper and cutting the paper according to the trace and measureing the area (LI-300 portable area meter, LICOR, Lincoln, NE). (2) Photosynthetic rates were determined by using a LICOR-6000 photosynthetic meter (LICOR, Lincoln, NE). For all measurements, the environment was controlled as above except that the light was composed of 90% cool white fluorescent and 10% incandescent bulbs. The leaf area was obtained by the same method.

Whole Leaf Spectroscopy

Fourth derivative spectroscopy of the whole leaf was measured in a Shimadzu 260 spectrophotometer with an integrating sphere attachment. Detached leaves from different nodes of the normal plant, alt mutant, and normalized alt were put between two glass microscope slides and clipped into the cuvette holder. Accumulated spectra equivalent to 100 uncorrected attenuation units were used for fourth derivative analysis of at least three leaves of each type. Detached leaves from a normal plant were floated on distilled water in a petri dish in the dark to induce senescence. Fourth derivative spectroscopy of these senescence leaves was measured.

Bioassay of Known Compounds

A number of growth regulators and amines and polyamines were tested for their ability to normalize alt. They were: (1) growth regulators, naphthaleneacetic acid (NAA), gibberellic acid (GA_3), zeatin, dihydrozeatin, 6-benzyladenine, absciscic acid (ABA); (2) polyamine: spermine, spermidine, putrescine, cadaverine; amines: agmatine, ∝-aminoethanol, ethylamine, iso-amylamine, DL-methionine-S-methyl sulfonium chloride. Aqueous solutions, 10^{-4}M to 10^{-6}M, of these compounds were prepared and bioassayed.

Different vitamins were also tested to see the activity to restore the alt mutant. They were: biotin, nicotine, pyridoxine, and thiamine. Different forms of thiamine were also tested: thiamine monophosphate, thiamine pyrophosphate, oxythiamine, and thiamine disulfide. Aqueous solutions from 10^{-4}M to 10^{-6}M of each compound were prepared, and bioassayed. Thiamine from 10^{-2}M to 10^{-8}M was also tested, as were the precursors of thiamine: thiazole and thiazole monophosphate. A vitamin mix was prepared according to Acree and Marx (personal communication, table 1). Different dilutions of this mix and the mix without thiamine were bioassayed.

Stability of Pea Seed Extract

Some properties of the pea seed extract eluted by 6 N HCl from the cation column were tested: (1) the fractions were heated in a boiling water bath for 10 min; (2) the fractions were autoclaved at 121°C for 15 min; (3) the fractions were ashed in an oven; and (4) the minimum amount of 6 N HCl eluant required to normalize alt mutant was tested. The eluant was dried, weighed, and redissolved in

distilled water. Before bioassay some fractions were filtered by disposable microfilters and then bioassayed, others were bioassayed directly.

Table 1. Composition of Acree and Marx's vitamin mix

Compound	g/125 g of mix
Thiamine	0.12500
Riboflavin	0.18800
Pyridoxin	0.06250
dl-Pantothenate	0.25000
Nicotinic acid	0.37500
Biotin	0.00375
Folic acid	0.02500
Menadione	0.05000
Inositol	0.93750
Vitamin B_{12}	0.50000
p-Aminobenzoic acid	0.31250
Ascorbic acid	0.02500
Vitamin A	0.25000
Vitamin D_2	0.02500
dl-tocopherol	1.50000
D-glucose	120.370

Results

The alt segregates grew normally until the 5th or 6th node. Subsequently node 6 was chlorotic, and designated the transition leaf, because above this node the leaves were bleached (Fig. 4, 5). It was observed that the chlorophyll content of nodes 3-5 of alt was normal compared to that of Alt seedling (Fig. 6). The chlorophyll content of each node was about 2.0 mg/g f.w. However, at node 6, the chlorophyll content markedly decreased. Above this node the chlorophyll was almost absent, while the chlorophyll content of the corresponding Alt leaves remained above 2.0 mg/g f.w. We were not able to distinguish Alt/alt from Alt/Alt by this chlorophyll measurement experiment.

Similar to chlorophyll content, the net photosynthetic rates of alt leaves at node 5-6 were comparable to those of Alt leaves at nodes 5-6 (Fig. 7). However, the photosynthetic rate of the transition leaves (node 7) significantly declined. At node 8 the respiration rate was higher than the photosynthetic rate. Thus, the net PS was below zero. However, in the normal plant the PS rate remained high, and slightly increased as node number increased. Because of the location and the leaf size, the leaves at node 3 were inaccessible to the cuvette used for the photosynthetic measurements. The stomatal conductance of alt leaves at each node remained constant, and did not significantly decrease as the PS rate changed, even in the bleached leaves (node 8), the stomata were still open (Fig. 8). Thus, low PS rate was not a result of stomatal closure.

When the Alt seed extract was applied to the alt mutants, within one week after treatment, the axillary buds developed as green

Fig. 4 Alt and alt seedlings, age about 30 days. Right, alt seedling with bleaching leaves on nodes 7 and 8. Left and middle, Alt seedlings having normal leaves on nodes 7 and 8.

Fig. 5. alt seedling, age about 40 days. Nodes 7 and 8 wither and die.

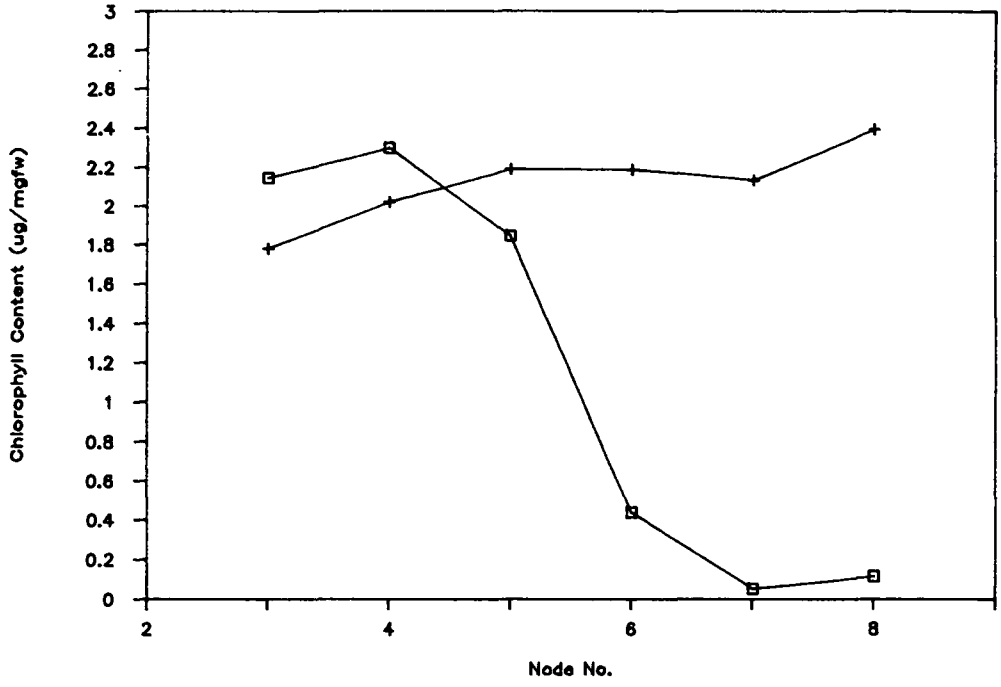

Fig. 6. Effect of homozygous alt on chlorophyll content of pea seedlings (+, Alt/-; □, alt/alt).

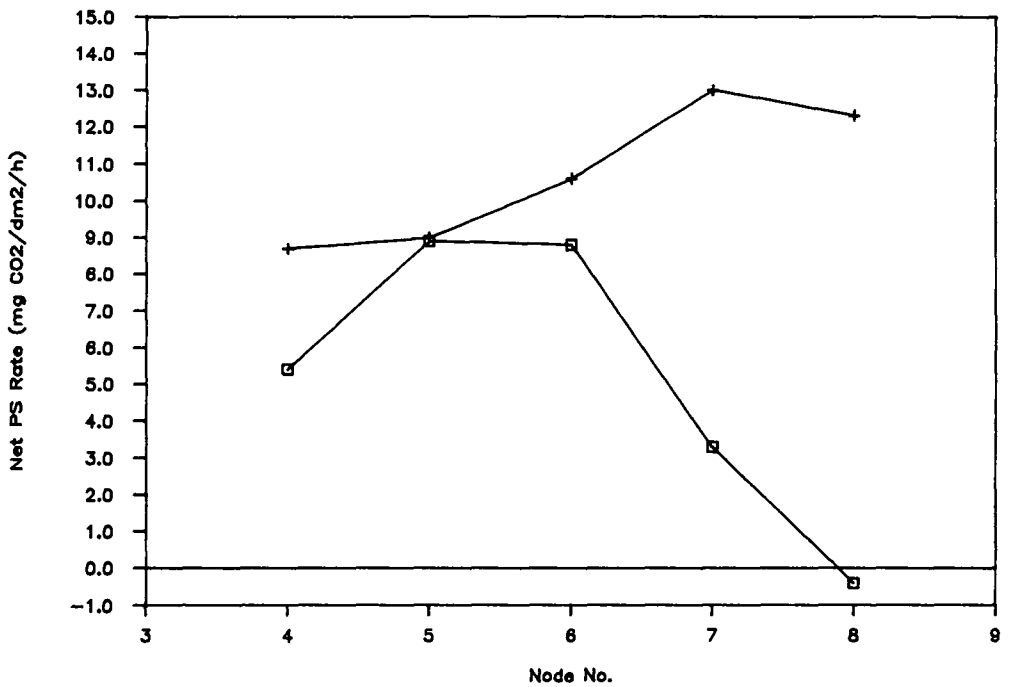

Fig. 7. Net photosynthetic rate of Alt and alt seedlings (+, Alt/-; □, alt/alt).

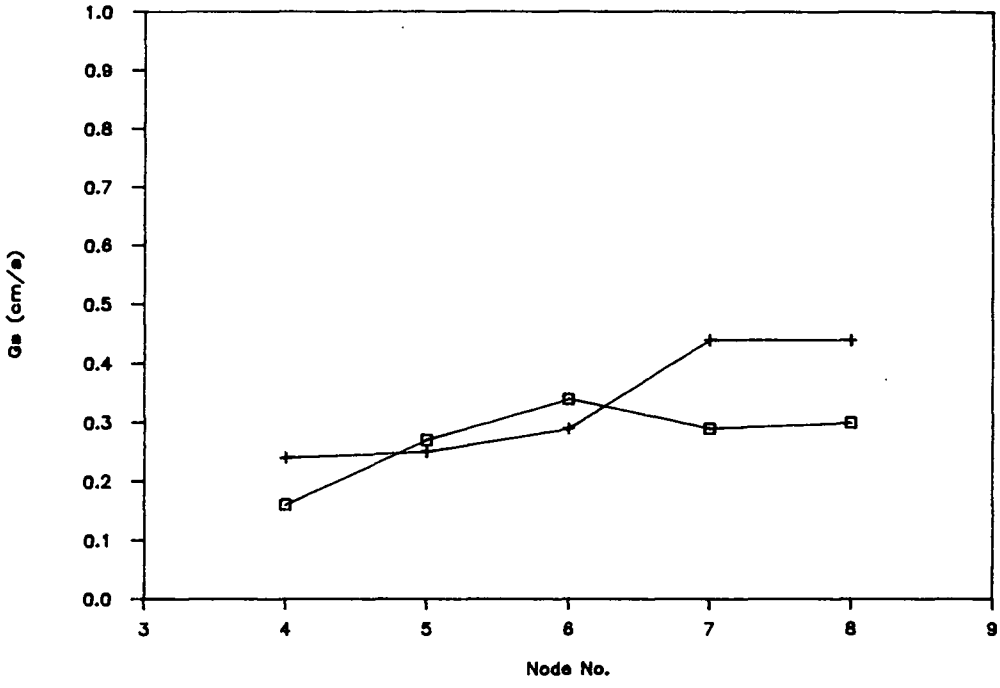

Fig. 8. Stomatal conductance of Alt and alt seedlings (+, Alt/-; □, alt/alt).

shoots for at least 2-3 nodes. We called these normalized plants (Fig 9). However, if distilled water were applied to the alt mutant, the axillary shoot emerged bleached and in a short time it senesced and died. Extracts of bean and corn seed had the same biological activity as Alt pea seed extracts, as did extracts made from Alt pea shoots. When these extracts were passed through an anion exchange column, the biological activity was not retained by the resin. However, when these fractions were passed through the cation exchange column, the biological activity was retained by the resin. When this column was washed with distilled water and 1 to 12 N HCl added stepwise, the biological activity detected by the alt bioassay was eluted from the cation column starting with 4 N HCl and spread over four fractions, 4 to 7 N (Fig. 10). The 5 N and 6 N HCl fractions contained most of the biological substance (Alt activity) recovered. With the Alt leaf extract, the same results were obtained (Fig. 11). A similar result was observed with the corn extract except that the Alt activity was spread over fractions 4 to 9 N HCl (Fig. 12). For bean crude extracts, clear results were not obtained, because the extract was very viscous. However, the crude extract of bean seeds was active, and although the biological activity was not retained by the anion resin, it was retained by the cation resin.

The normalized plants generally only grew 2-6 normal nodes then, like the alt mutant, chlorosis and bleaching occurred. These normalized plants sometimes reached the flowering stage but viable seeds were only rarely obtained. The seedlings grown from these seeds developed the alt characteristics. The green leaves

Fig. 9. Normalized plant. altseedling treated with 7 N fraction of pea seed extract from cation exchange column.

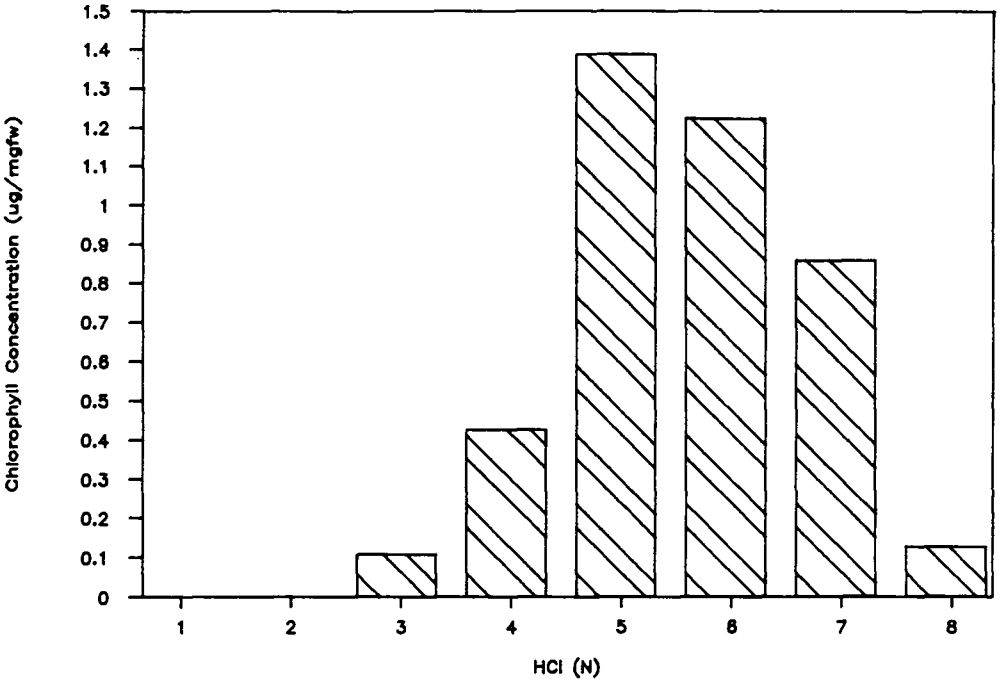

Fig. 10. Response of alt seedlings to Alt seed extracts chromatographed on cation exchange column.

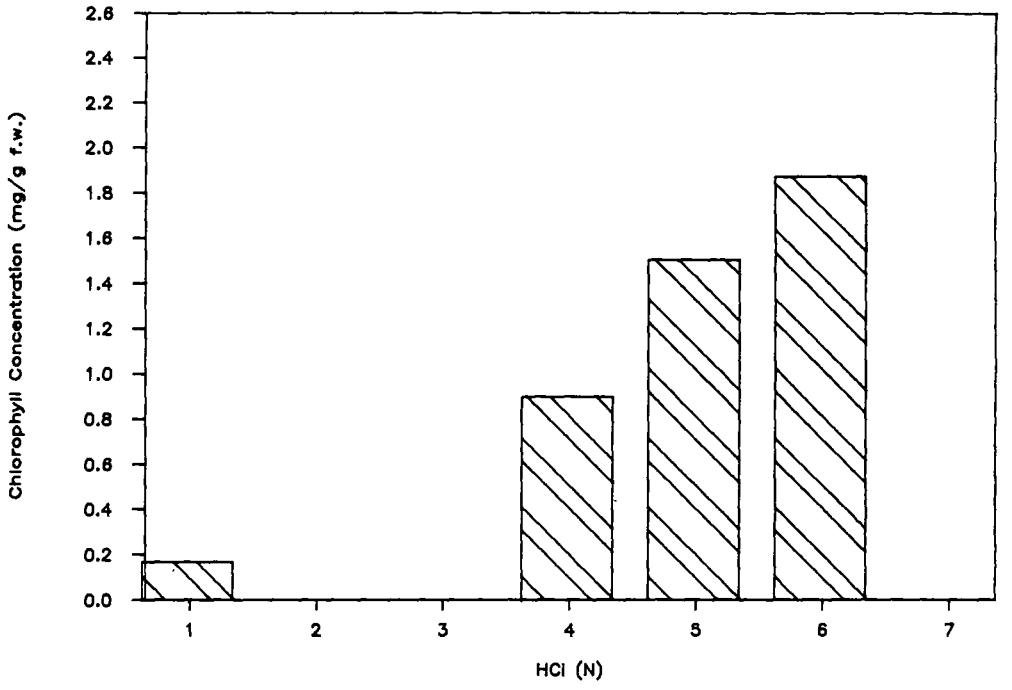

Fig. 11. Response of alt seedlings to Alt shoot extracts chromatographed on cation exchange column.

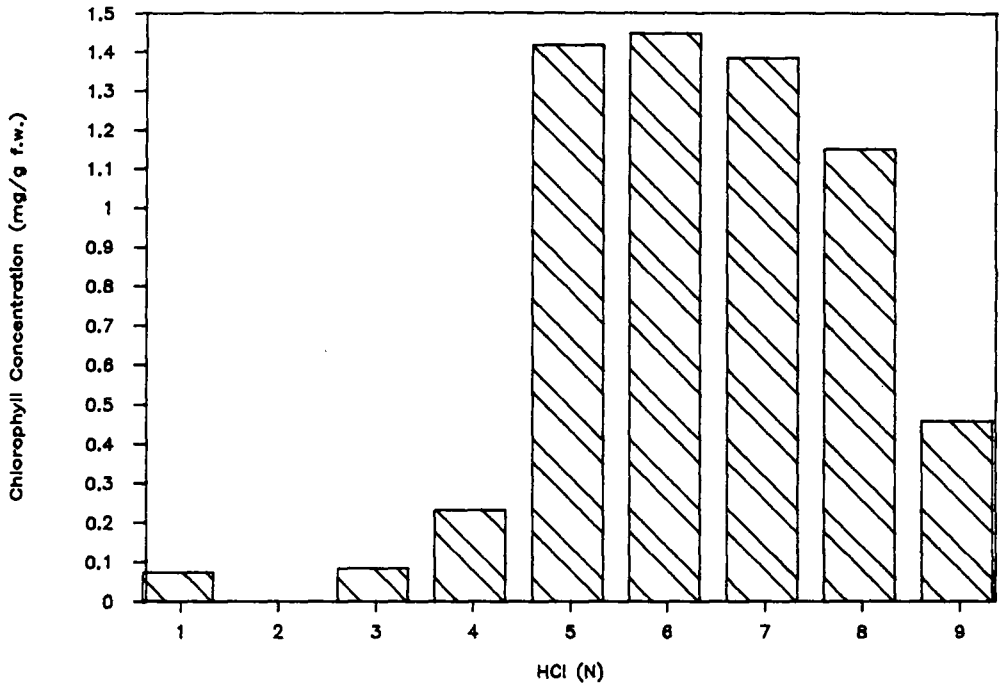

Fig. 12. Response of alt seedlings to corn seed extracts chromatographed on cation exchange column.

of the normalized plants could photosynthesize, and the photosynthetic rate was proportional to chlorophyll content above about 1 mg/g f.w. (Fig. 13). With 2-2.5 mg/g f.w., these leaves had normal photosynthetic rates compared to Alt.

The pattern of absorbance and 4th derivative of green leaves of Alt, alt, and extract-treated plants were similar (Fig. 14). However, as the leaves bleached, the absorbance of node 6 (transition leaf) and node 7 of alt declined progressively. Each component in the fourth derivative also declined. This same pattern also occurred in the normalized plants as the leaves bleached, and in the detached, senescing leaves of normal genotypes. Difference spectra of bleached mutant and green leaves of Alt revealed no difference.

The 6 N HCl fraction from the cation exchange column was loaded onto TLC plates for further purification. The bioassay results showed that the major Alt activity migrated to about Rf 0.38 to 0.55 (zone C) in the first solvent system, acetonitrile:H_2O:NH_4OH (8:2:1), some Alt activity migrated to zone D (Table 2). These zones were ninhydrin-positive (Fig. 2). When the eluant from zone C was chromatographed in the second solvent system, butanol:MeCOEt:H_2O:NH_4OH (5:3:1:1), the Alt activity migrated to Rf 0.5-0.93 (zone D and E, Table 3). There were no ninhydrin-positive compounds in these zones (Fig. 3).

Some properties of the Alt substance were determined. The boiled and autoclaved 6 N fraction still remained biologically active while the ashed samples were inactive (Table 4). From the response of alt to 6 N HCl eluant (fig.15), it showed that

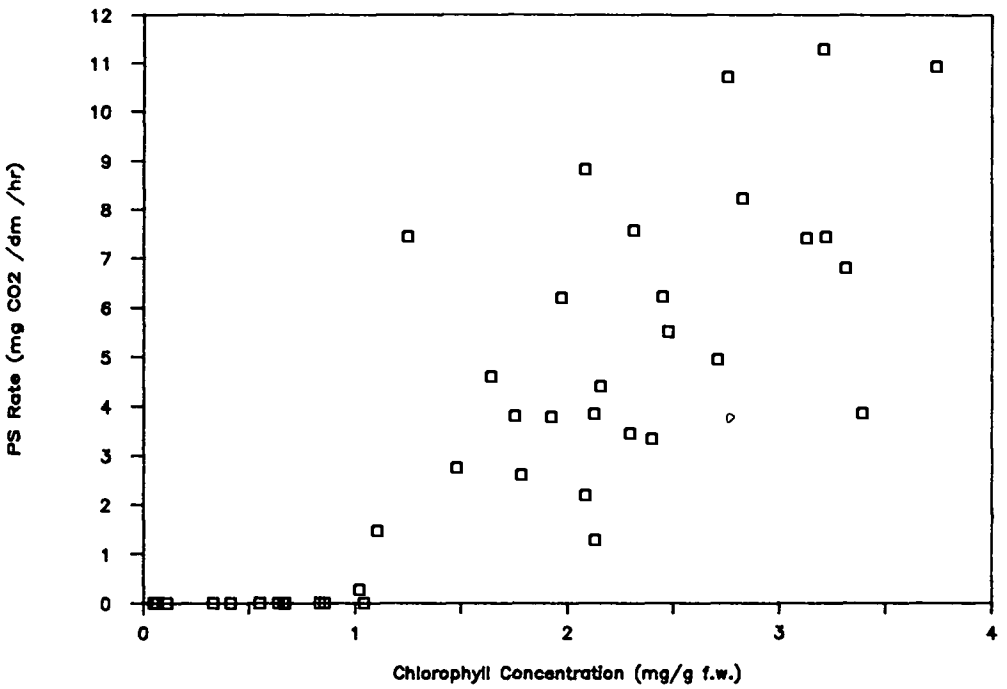

Fig. 13. Relationship of photosynthetic rate to chlorophyll content in pea seed extract-treated alt (normalized plants)

Fig. 14. Attenuance (absorbance) spectra and their fourth derivatives of normal and mutant leaves. The more abruptly changing curve, which starts at the middle of the vertical axis on the right side is the fourth derivative trace. The less abruptly changing curve, which starts at the base of the vertical axis on the right side is the attenuance trace. (a) nodes 4, 5, 6, and 7 of Alt, (b) nodes 4, 5, 6, and 7 of alt showing progressive loss of chlorophyll, (c) leaves of Alt detached and floated on distilled water in the dark for 0, 7, and 14 days to stimulate senescence, showing progressive loss of chlorophyll, (d) leaves at successive nodes of extract-treated alt showing progressive loss of chlorophyll, (e) difference spectrum of Alt and bleached alt.

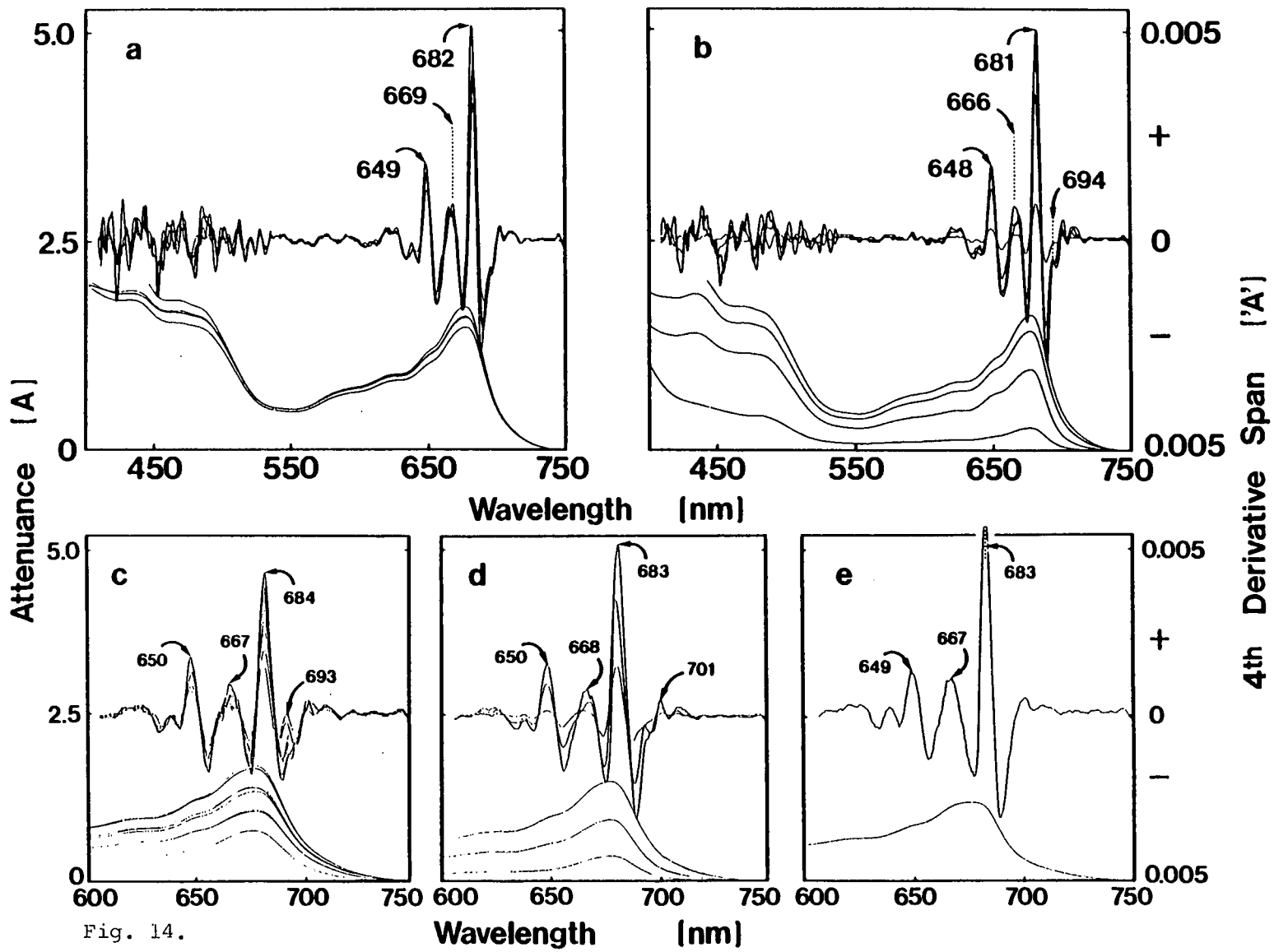
a
682
669
649
b
681
666
648
694
5.0
2.5
0
450
550
650
750
Attenuance [A]
0.005
+
0
−
0.005
4th Derivative Span ['A']
Wavelength [nm]
c
684
667
650
693
d
683
650
668
701
e
683
667
649
600
650
700
750
Wavelength [nm]

Fig. 14.

Table 2. Distribution of *Alt* activity after TLC using solvent system I

Zone	Rf	Chlorophyll Content (mg/g f.w.)
A	0.00-0.27	0.42
B	0.27-0.38	0.80
C	0.38-0.55	2.25
D	0.55-0.78	1.55
E	0.78-1.00	0.26

Table 3. Distribution of Alt activity after TLC using solvent system II

Zone	Rf	Chlorophyll Content (mg/g f.w.)
A	0.00-0.12	0.06
B	0.12-0.24	0.02
C	0.24-0.50	0.17
D	0.50-0.73	1.89
E	0.73-0.93	1.94
F	0.93-1.00	0.03

Table 4. Effect of boiling, autoclaving, and ashing on the biological activity of Alt extract

Treatment	Chlorophyll Content (mg/g f.w.)
Boiling	1.45
Autoclaving	1.40
Ashing	----

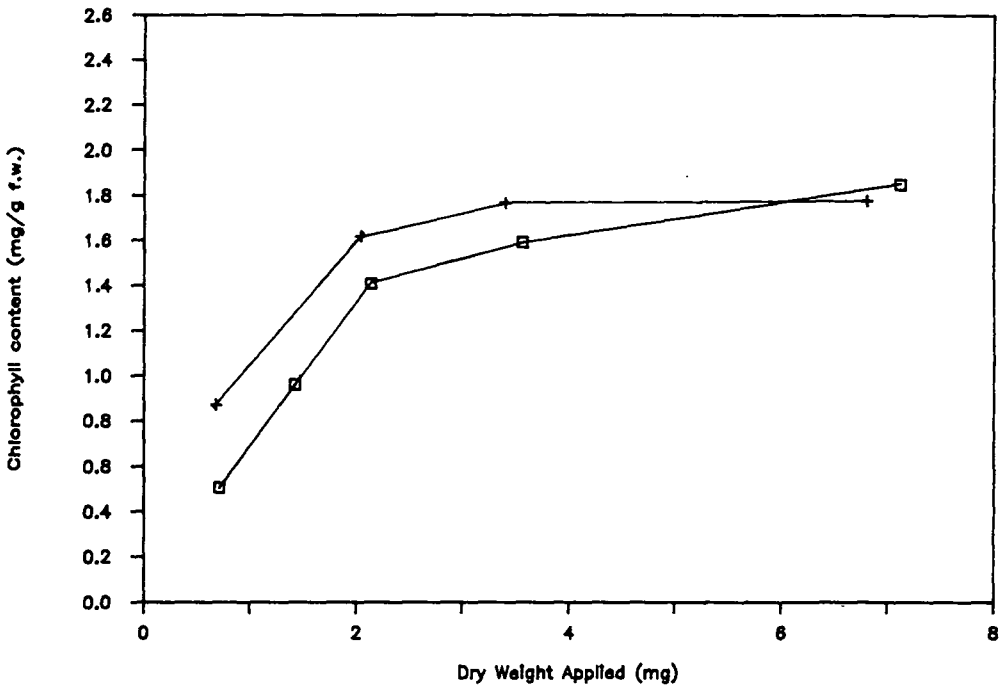

Fig. 15. Dose-response of alt seedlings to 6 N fraction (+, filtered; □, unfiltered).

approximately 2 mg of dry 6 N elutant could restore alt. This represented about 5% of the 6 N fraction. Different solvents were tested for the ability to extract the Alt activity. Since the uptake of the crude extract by the plant was not high in this experiment, results were variable. However, water was the least effective solvent to extract the Alt activity (Fig. 16).

Many polyamines, simple amines, and growth regulators were tested to determine their activity in the alt bioassay. All were inactive (Table 5). However, when different vitamins were tested, thiamine normalized the alt mutant. Different forms of thiamine: thiamine monophosphate, thiamine pyrophosphate, and thiamine disulfide, all had the same effect, except that the activity of thiamine pyrophosphate was slightly lower (table 6). However, thiazole and thiazole monophosphate, which are precursors of thiamine were not active in the bioassay. Oxythiamine, an analogue of thiamine, and other kinds of vitamins, such as biotin and pyridoxine were all inactive. In contrast to the result of Acree and Marx, Acree's vitamin mix was active in the bioassay, even diluted to 5% of full strength (Fig. 17). However, when thiamine was deleted, Acree's mix was inactive, even when diluted. The mutant reponsed to abroad range of thiamine concentration (Fig. 18). Even at 10^{-7}M thiamine was active, and 10^{-5}M and 10^{-4}M were the optimal concentrations.

When thiamine was applied to the anion exchange column, the biological activity was not retained on the column. However, when it was applied to the cation column, the biological activity was removed. When 1-12 N HCL was used stepwise to develop the column, the

biological activity began to elute with 4 N HCL. Similar to the crude extract, the major thiamine activity was in the 5-7 N HCL fractions (Fig. 19). Thiamine and the biological activity from the 6N fraction were compared on TLC using the solvent system: acetonitrile:H_2O:NH_4OH (8:2:1), and butanol:MeCOEt:H_2O:NH_4OH (5:3:1:1) were compared. The Rfs were the same in both systems. Neither thiamine nor the Alt activity react with ninhydrin.

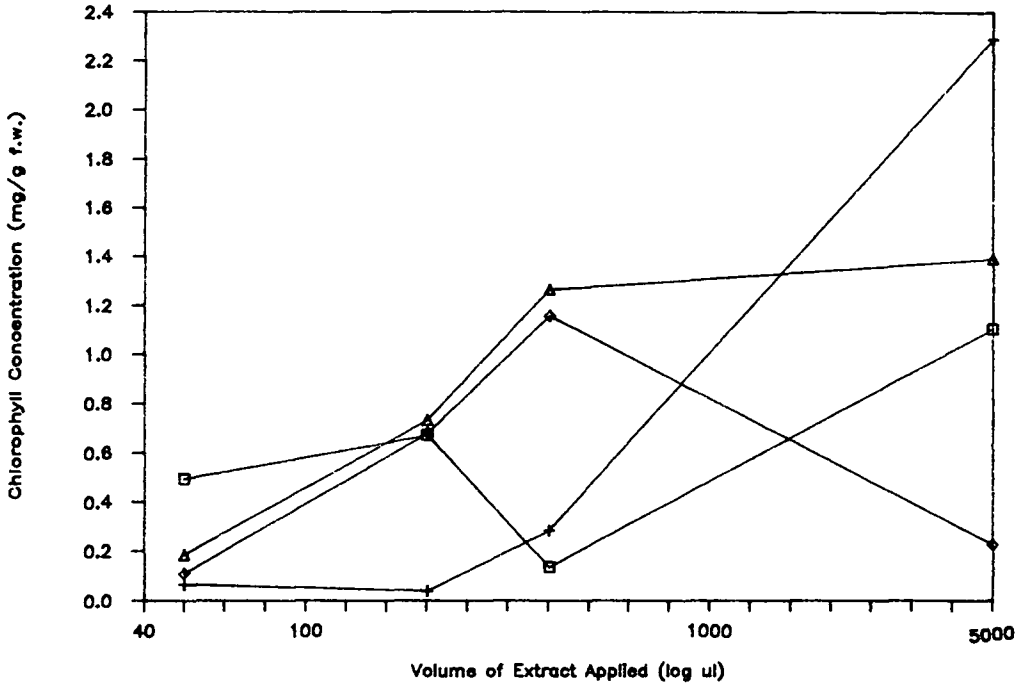

Fig. 16. Effect of various solvents on extraction Alt biological activity (+, acetone water; △, acetone; ◇, methanol; □, water).

Table 5. Cations and plant growth regulators tested on alt

Compound	Concentration		
	10^{-4}M	10^{-5}M	10^{-6}M
Agmatine	---	---	---
Ethylamine	---	---	---
∝-aminoethanol	---	---	---
Isoamylamine	---	---	---
1,6-diaminohexane	---	---	---
Cadaverine	---	---	---
Putrescine	---	---	---
Spermidine	---	---	---
Spermine	---	---	---
DL-methionine-S-methyl sulfonic chloride	---	---	---
∝-Naphthaleneacetic acid	---	---	---
Gibberellic acid	---	---	---
6-Benzyladenine	---	---	---
Zeatin	---	---	---
Dihydrozeatin	---	---	---

Table 6. Responses of *alt* to several vitamins

Coumpound	Concentration		
	10^{-4}M	10^{-5}M	10^{-6}M
	chlorophyll content (mg/g f.w.)		
Thiamine	1.95	1.95	1.87
Thiamine monophosphate	2.05	2.29	1.99
Thiamine pyrophosphate	1.18	1.64	1.62
Thiamine disulfide	2.43	2.17	1.29
Oxythiamine	----	----	----
Thiazole	----	----	----
Thiazole phosphate	----	----	----
Biotin	----	----	----
pyridoxin	----	----	----
Nicotinic acid	----	----	----

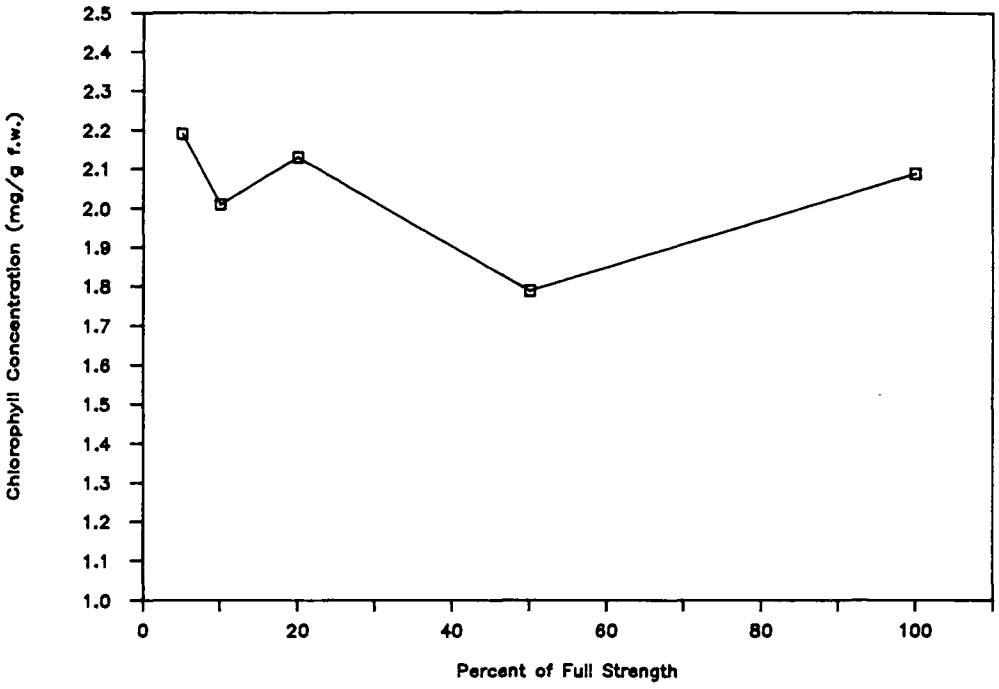

Fig. 17. Effect of Acree's vitamin mix on normalized alt.

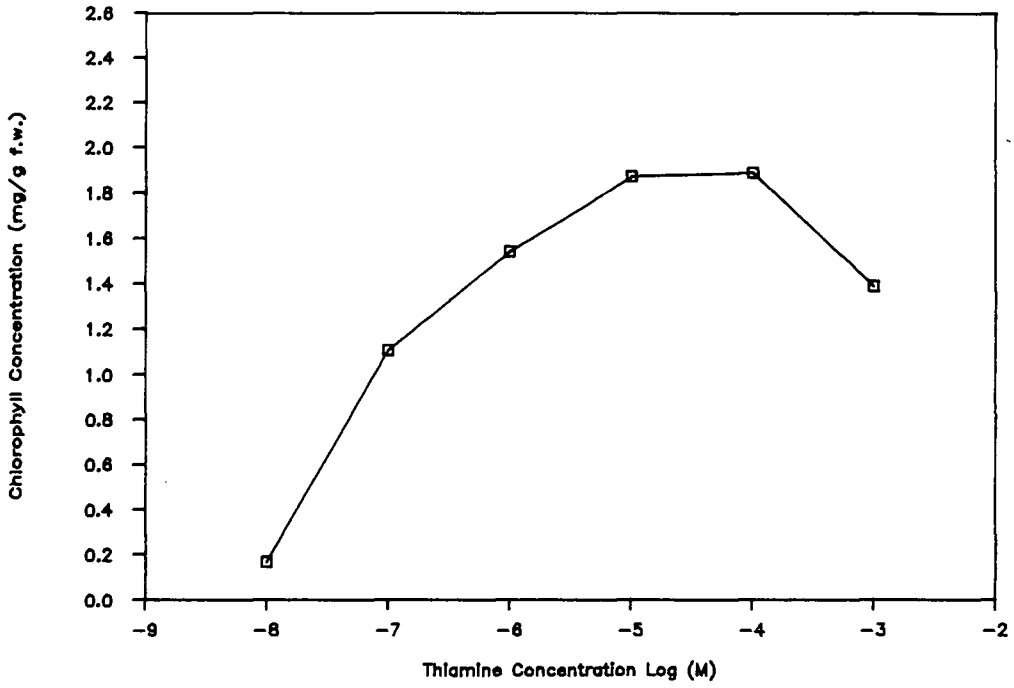

Fig. 18. Dose-response of alt to thiamine.

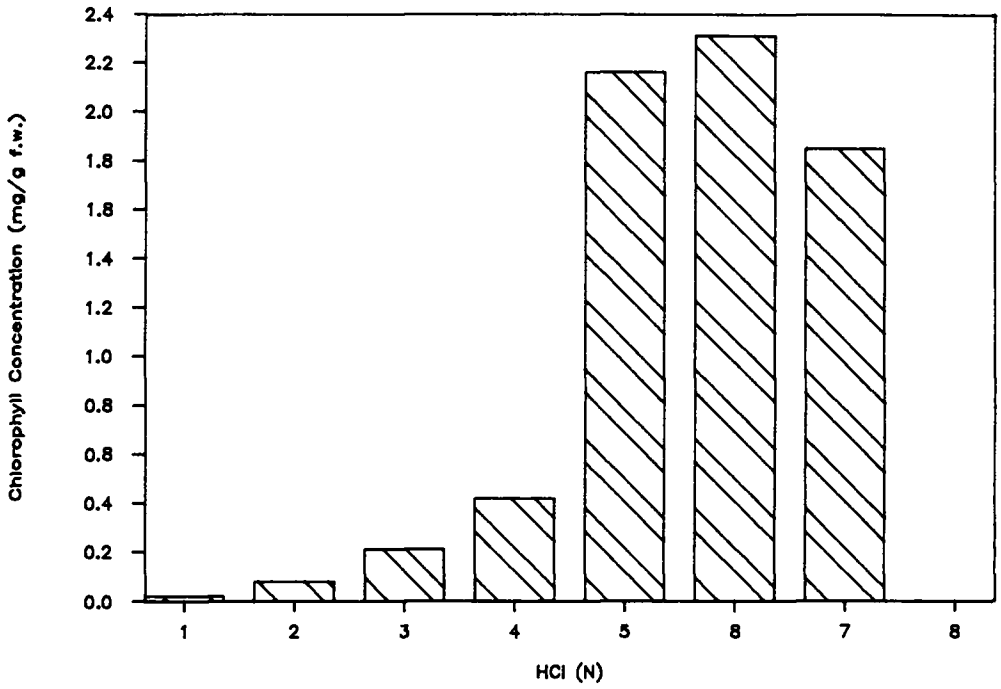

Fig. 19. Distribution of thiamine in fractions from cation exchange column.

Discussion

The alt mutant grows normally until about node 5-6. This indicates that normal chlorophyll content, chloroplast structure and photosynthetic rate are present in alt, and in fact, all these parameters are indistinguishable from the Alt plants. However, above node 6, chlorosis begins and the leaves bleach progressively. As bleaching occurs, all these parameters, chlorophyll content, chlorophyll-protein and photosynthetic rate decline to zero. This pattern of development is consistent with depletion of a maternally-derived factor in the seed.

The fourth derivative spectra of the chlorotic and bleached leaves of Alt and alt show that not only the chlorophyll is lost, but that the major chlorophyll-protein complexes of the chloroplasts also decline. Absorption spectra of biological materials are often a complex mixture of absorption bands which are difficult to resolve into the individual components. Use of a computer to analyze spectral data by second, third and fourth derivatives can determine the peak position of the contributing absorption band. The spectral resolution is markedly enhanced by higher derivatives (9). Spectral analyses of chlorophyll-protein complexes from higher plants have been reported. In pea and wheat, the major peak of wavelengths of chlorophyll b and protein complexes are : cb640, cb649, cb660, cb670, cb675-676; for chlorophyll a protein complexes are: ca678, ca684, ca693, ca697-699, ca703-710 (9,12). In room temperature spectroscopy, cb bands 660, 670, 675-676, and ca bands 678 and 684 form two separate complex spectral envelopes. If there are changes in the proportions of any

contributing band, then these changes can be detected very readily by difference spectra as shifts in peak wavelength. In strawberry and wheat, as the leaf senesceced, the absorbance declined and a blue spectral shift from 660 to 670 nm was observed (L. Daley, personal communication). In our experiment such changes were not observed. This suggests that a structural deficiency in the chloroplasts does not account for the development for the symptoms of the alt mutant.

Acree and Marx reported that the alt mutant could be restored by grafting alt to a normal genotype or by applying seed extracts of normal pea seed (1). In this experiment, this normalizing effect has been confirmed. It is also shown that the biological activity can be extracted from normal pea shoots, corn and bean seeds as well. The chlorophyll content, photosynthetic rate, and the fourth derivative spectra of green leaves of the normalized plants are all comparable to those of Alt. Furthermore, as the leaves of these normalized plants bleach, all these parameters decline as in the untreated alt. The occurrence of bleached leaves is due to the declining response to the Alt activity. The cause of this is probably the decreased uptake of test solution after a few days, even though I made a fresh cut in the stem every day. Acree and Marx reported that the active substance is a strong cation, because the activity in the extract was removed by the cation exchange resin and eluted with 6 N, but not 1 N HCl (1). We confirmed this result and also found that Alt activity eluted over several fractions (4 to 7 N HCl). We obtained the same results with corn extract, except that the Alt activity eluted over a wider range (4 to 9 N HCl). The 5 N and 6 N HCl fractions have the highest

biological activity. When the extracts are further purified by TLC using two solvent systems, the Alt activity has an Rf range 0.38 to 0.55 in the solvent system I, and also co-chromatographs with a ninhydrin-positive band. In solvent system II, its Rf range is 0.5 to 0.93, but does not co-chromatograph with a ninhydrin-positive band.

The plant hormones are important agents in the integration of developmental activities. Auxins and cytokinins can stimulate cell division and elongation, and retard senescence (27). If a plant were defective in the biosynthesis or use of these hormones, abnormal growth would be expected. Thus, NAA, zeatin, dihydrozeatin and GA3 were applied to the alt mutant, but were inactive. It seems that alt is not defective in the biosynthesis of these hormones.

A wide variety of amines occur in higher plants. They are derived from amino acids by decarboxylation, and transamination of aldehydes. Amines are precursors for several alkaloids (37). The diamine putrescine and the polyamines spermidine and spermine have been demonstrated in many plants and probably occur universally in eukaryotes. Polyamines appear to regulate some aspects of plant growth and key biosynthetic enzymes are under tight control. The polyamines generated by the high levels of ornithine decarboxylase repress synthesis of the decaboxylase; thus it would appear that rapid turnover of the enzyme is a mechanism to ensure appropriate polyamine concentrations under conditions of cell proliferation. Since the rise in the decarboxylase and polyamine levels often precedes increases in DNA and RNA synthesis and polyamines are often found in physical association with DNA, it has been suggested that polyamines directly

control RNA and DNA synthesis (35). Thus, several kinds of amines, diamines and polyamines were tested, but were inactive in the alt mutant.

In contrast to the report of Acree and Marx (1), we found that thiamine normalizes alt. We duplicated the vitamin mix based on Acree's instructions and found activity over a 20-fold range of concentrations, the lowest containing 2 x 10^{-5}M thiamine. However, the full strength vitamin mix is slightly toxic to the plants. This might be the reason why Acree and Marx did not detect activity. The same vitamin mix minus thiamine fails to normalize alt. Thus, thiamine plays an important role in growth of the alt plant.

Thiamine pyrophosphate is the coenzyme form of thiamine. It plays an essential role in many biochemical reactions, most prominently as the coenzyme of pyruvate dehydrogenase. This is one of the key enzymes in the tricarboxylic acid cycle, which supplies most of the energy for plant growth. If the biosynthesis of thiamine is blocked, inducing thiamine deficiency, almost all cell metabolism will be disrupted, and normal growth prevented. Thiamine also plays an important role in the structure of the chloroplasts (6, 22). In thiamine-deficient mutants, the chloroplasts degenerate first, followed by loss of other organelles (6, 10, 22). Thus, thiamine-deficient mutants are chlorotic. After thiamine treatment, normal growth is restored, chloroplasts develop normally, and the mutant has a phenotype indistinguishable from the normal. The alt mutant is restored to normal growth by thiamine treatment and the alt mutant can also be normalized by the seed extracts of normal genotypes of pea,

corn and bean, and normal pea shoots. Thus, the alt mutant is deficient in a substance which is common to normal tissues. This substance may be thiamine.

There are three forms of thiamine phosphate esters in plants: TMP, TPP, and TTP. Free thiamine also occurs in the plant. Maximal thiamine synthesis and transport occur in mature leaves (3). During seed development, large amounts of thiamine are translocated to the seeds. The storage form in seed is free thiamine, the phosphorylated thiamine being degraded very rapidly during seed maturation (26). Because of a rich supply of maternal thiamine in seed, thiamine-deficient mutants have fully green cotyledons. The requirement for thiamine is not manifested in the mutant phenotype until this supply of maternal thiamine is exhausted. The mutants in Arabidopsis (34) and in tomato (3) had fully green cotyledons and a few initially green true leaves indicating that the thiamine supplied by the seed was sufficient for normal early development. However, once this supply was exhausted, the subsequent leaves showed symptoms thiamine deficiency. The alt mutant apparently grows normally for 5-6 nodes using thiamine stored in the seed. When the thiamine is depleted, metabolism is disrupted, manifested by bleaching of the leaves. The activity of extracts of normal pea, corn, bean seeds and normal pea shoots in alt appears to be due to thiamine. In aqueous solution TTP is negatively charged, TPP is electroneutral or weakly positive, TMP and free thiamine are positively charged. Thus, thiamine in these extract is a cation, so it is retained by the cation exchange resin and eluted by 4 to 7 N HCl as is the Alt activity in seed extract.

The 6 N HCl fraction of the extract and thiamine co-chromatograph in both TLC solvent systems. Thiamine is also heat stable, and does not react with ninhydrin, both properties of the Alt activity. All this strongly suggests that the normalizing substance, which can restore the alt mutant is thiamine.

Oxythiamine, an inactive analogue of thiamine is also inactive in the bioassay. However, thiamine disulfide, which is a metabolite of thiamine (14) is active in the bioassay. Thiamine disulfide is readily reduced to thiamine in the cell environment.

Thiamine biosynthesis involves the condensation of the pyrimidine and thiazole residues. Before condensation, both moieties are phosphorylated (13, 23, 24). Thus, thiazole, thiazole monophosphate, pyrimidine, pyrimidine monophosphate, and pyrimidine pyrophosphate are all precursors of thiamine. In our bioassay, both thiazole and thiazole monophosphate are inactive. This indicates that the thiazole moiety itself is not able to restore the normal biosynthesis of thiamine. The alt mutant could be defective in the synthesis of the pyrimidine moiety. Because of the difficulty of obtaining the pyrimidine moiety, we are not yet able to test its biological activity in the alt mutant. However, the pyrimidine moiety is being synthesized in our lab. Therefore, in the near future the ability of the pyrimidine moiety to restore thiamine biosynthesis in the alt mutant will be tested.

Thiamine monophosphate is an intermediate of thiamine biosynthesis, but TMP was not a substrate for purified thiamine pyrophosphotransferase (TPTase), which catalyzes the phosphorylation

of thiamine to TPP (13). Thiamine is stored in seed in the form of free thiamine, and during seed germination free thiamine is rapidly phosphorylated (26). Thus, mutants not defective in the conversion of thiamine to TPP, can use maternal thiamine for early development. In the Plantago mutant, the conversion of thiamine to TPP is blocked, so the seedlings are albino on emergence, and only respond to TPP (29). The ability of the alt mutant to utilize maternal and exogenous thiamine indicates that the conversion of thiamine to TPP is not blocked. This is also shown in the biological activity of TMP and TPP. Highly phosphorylated compounds generally are not permeable to cell membranes. When TMP and TPP are applied to alt, they may be dephosphorylated to free thiamine first. This is probably the reason that the activity of TPP in the bioassay is slightly lower that of thiamine. However, it also seems that the system for phosphorylation and dephosphorylation of thiamine is intact.

In contrast to the alt mutant, the initial manifestation of thiamine deficiency in mutants of tomato (tl, spa, ten) was in the oldest foliage leaves, and progressed orderly upward from one leaf to the next. Thus, the progressive green-to-yellow-to-white bleaching and subsequent necrosis, was first observed in the oldest, expanding true leaves of these three mutants. Although the thiamine deficiency symptoms were manifested first in the oldest leaves in all three mutants, they are phenotypically distinct individually. Mutants tl and spa, which are blocked in the biosynthesis of the pyrimidine moiety, have a mottled appearance in their oldest leaves. However, the ten mutant, which is blocked in the phosphorylation of thiamine,

has a uniform chlorotic pattern with only a slight gradient of bleaching between young and old leaves. Treating tl with thiamine in a limited area of its oldest leaves, efficiently normalizes the mutant. In alt, bleaching only occurs at and above the transition leaf. However, the leaves at nodes 3 to 6 (below the transition leaves) remain green. It seems that when the maternal thiamine is depleted, thiamine is not mobilized from the older leaves to younger expanding leaves. This is probably due to poor translocation of TPP. Also, TPP is probably not easily dephosphorylated to thiamine. More study is needed to be done to prove this. In contrast, appearance of intial thiamine-deficient symptoms in the older leaves of tomato implies that TPP is hydrolyzed to thiamine which is translocated to the younger leaves.

Since most of our experiments were performed without knowing that the Alt substance may be thiamine, we isolated and purified the Alt substance based on methods not specific for thiamine. After it was found that thiamine can restore the alt mutant, further purification is being performed using HPLC methods developed for thiamine analysis. The Alt substance isolated from normal pea seeds has the same mobility in HPLC compared to the thiamine standard using three solvent systems on two types of columns. The Alt substance is being purified and will be analyzed by mass spectroscopy. If the Alt substance is indeed thiamine, and the alt mutant is defective in thiamine biosynthesis, further work can be done to investigate the site of blockage; how this blockage occurs; and how the alt gene itself is mutated. In our experiments we found that during summer it was easier to obtain alt

segregates. Since the plants were grown year round under long days, photoperiod is not a factor. Thus, the expression of the alt gene might be influenced by the light intensity. If this is the case, how light intensity influences this gene may be a very interesting subject. It has been reported that the thiamine-deficient mutant has an abnormal chloroplast structure. However, how thiamine influences the formation of the chloroplast is not understood. Although it is assumed that the loss of the chloroplast in thiamine-deficient plants is an indirect result of altered carbohydrate and amino acid metabolism, the possibility exists that thiamine plays a direct role in chloroplast formation. It has been suggested that thiamine may be required at some stage in the formation of the chlorophyll molecule itself, although Langridge and and Brock (20) found no chlorophyll formation by treatment of thiamine deficient plants with δ-amino levulinic acid, the precursor of the pyrrole ring, or by porphobilinogen, the precursor of the porphyrin structure. Therefore, thiamine deficiency must interfere with chlorophyll synthesis at a stage later than the formation of the pyrrole rings. This mutant system represents one method of investigating these questions.

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