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CHLOROPLAST LAMELLAR LIPOPROTEIN: <u>IN VIVO</u> AND <u>IN VITRO</u> C¹⁴ INCORPORATION

by

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A DISSERTATION

Submitted to the University of New Hampshire In Partial Fulfillment of

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ABSTRACT

CHLOROPLAST LAMELLAR LIPOPROTEIN: <u>IN VIVO</u> AND <u>IN VITRO</u> C¹⁴ INCORPORATION

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RICHARD S. BISHOP

The role of the chloroplast in the plant cell is to carry out a wide range of biosynthetic activities of which photosynthesis is but one aspect. It is thought to participate in, if not control, the maintenance, growth and replication of its own structure. Aspects of lamellar lipoprotein synthesis were investigated to further the knowledge of how the plastid and (or) the plant cell controls the processing of carbonate, acetate and leucine into this plastid structural material.

A methodology for chloroplast lipoprotein extraction and purification was established using a continuous-flow polyacrylamide gel electrophoretic cell. The C¹⁴-labeled substrate was fed <u>in vivo</u> to <u>Nicotiana tabacum</u>, L. seedlings and also to chloroplasts isolated from the same source. Patterns of substrate incorporation into the protein and lipid fractions of the lipoprotein were established from <u>in vivo</u> light and dark label incorporation measurements. These patterns were compared with similarly derived results from in <u>vitro</u> incorporations.

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The <u>in vivo</u> results gave reproducible patterns of the incorporation of all three substrates into both protein and lipid fractions under light and dark conditions. A strong light dependency was evident for carbonate and acetate incorporation but leucine incorporation was reduced by only 1/3 in the dark. An analysis of the distribution of the label after leu-C¹⁴ incorporation revealed a conversion of the leucine to other amino acids and a 20% utilization of the leucine for lipid synthesis.

The <u>in vitro</u> results established the capacity of isolated chloroplasts to incorporate all three substrates into both protein and lipid fractions. A light effect, though diminished, was observed for all three labeled materials. Analysis of the distribution of leu- C^{14} revealed only labeled leucine in the lamellar protein, but there was a 10-20% utilization of leucine for lipid synthesis.

The comparative results give further evidence of plastid-specific pathways for the synthesis of protein and lipid, in this case the lamellar lipoprotein. The substrate incorporation and conversion patterns substantiate the view that most of the necessary protein amino acids are synthesized outside the plastid. Nevertheless, the <u>in vitro</u> incorporation of carbonate and acetate into lamellar protein implies some plastid synthesis of amino acids.

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I. INTRODUCTION

Current theories propose a degree of autonomy on the part of the chloroplast and suggest that it controls the synthesis of many of its own components. Ris and Plaut (1) proposed that chloroplasts might have evolved from early symbiotic blue-green algae. Schiff and Epstein (2) have characterized the chloroplast as resembling a cell within a cell in their work with <u>Euglena</u> chloroplasts. Kirk and Tilney-Bassett (3) have surveyed the genetic and biochemical basis for the contention of plastid autonomy and concluded that there is sound cytoplasmic evidence for plastid continuity, a degree of genetic autonomy and an ability to synthesize many of their own materials.

That chloroplasts seem able to synthesize much of their own protein has been demonstrated by several successful attempts to achieve amino acid incorporation in the isolated chloroplast: among others, Goffeau and Brachet (4) with <u>Acetabularia</u> chloroplasts, Hall and Cocking (5) with tomato seedling chloroplasts, Spencer (6) with <u>Nicotiana</u> chloroplasts and Bamji and Jagendorf (7) with wheat seedling chloroplasts. These authors all reported successful isolation of intact chloroplasts which are generally agreed to be those that retain their outer 'envelope' and stroma (8).

Everson and Gibbs (9) have recently shown that both CO_2 and acetate may be taken up into protein by isolated chloroplasts. They found CO_2 to be assimilated 10 times more rapidly than acetate into their water-insoluble (lipid

and protein) fractions and asserted that this was due to the intactness of their chloroplasts.

Hall and Cocking (5) reported a significant rate of CO_2 incorporation into isolated chloroplast protein and suggested that their chloroplasts might be even more active in incorporating CO_2 into protein <u>in vitro</u> in the light than they are at incorporating amino acids.

Stumpf and James (10) used acetate- C^{14} to demonstrate that lettuce chloroplasts were capable of carrying out the synthesis of long-chain fatty acids and complex lipids. The co-factors ATP, CoA, Mg⁺², CO₂, inorganic phosphate and the presence of light were reported to be necessary for the uptake of label. The chloroplasts used in their experiments appear to have been broken or stripped of their outer envelopes (and therefore to have lost most of their stroma) which may account for the required addition of co-factors.

The incorporation of CO_2 into protein by isolated chloroplasts (9) implies that they can synthesize at least some of their own amino acids. Most investigators (11) suggest the mitochondrion as the major site of protein amino acid synthesis in the plant cell but recent work has suggested some chloroplast capacity to produce amino acids. Smith, Bassham and Kirk (12) in time-course incorporations of $C^{14}O_2$ into <u>Chlorella</u> found that alanine, aspartate, glutamate and serine seemed to exist in two or more pools; one of these was labeled rapidly and represented 20-30% of the total. It was suggested that this rapidly labeled pool might be in the chloroplast and result from chloroplast-formed amino acids.

In vitro studies have shown $C^{14}O_2$ incorporation into alanine and aspartate (13). Mudd and McManus (14), using isolated spinach chloroplasts showed radioactivity from acetate- C^{14} going into an amino acid fraction which included glutamic acid.

Glycine and serine are thought to be formed in isolated chloroplasts via a glycolic pathway (15,16). This is a pathway in which the 1- and 2-carbon atoms of pentosephosphate give rise to glycolic acid which then forms glyoxylate. Along with 3-carbon units from P-glyceric acid this pathway may account for the detected pools of glycine and serine.

Nickiporovic (17) reported that the synthesis of proteins in chloroplasts of higher plants was greatly accelerated during photosynthesis. He found that label from $C^{14}O_2$ was incorporated into protein whereas that from labeled carbohydrates was not, suggesting the utilization of intermediates of photosynthetic carbon reduction. Heber (18) working with spinach chloroplasts isolated after a period of photosynthesis with $C^{14}O_2$ reported that the incorporation of C^{14} into soluble chloroplast protein was more rapid than it was into soluble cytoplasmic protein. This also indicates a possible conversion of CO_2 to protein amino acids within the chloroplast.

A possible approach to answering the question of the chloroplast's capacity to synthesize amino acids is to analyze the plastid for appropriate enzyme content by looking for the tricarboxylic acid, glycolytic and hexose monophosphate enzymes which are associated with

amino acid synthesis. Smillie (11) using density fractionation in nonaqueous media did an extensive analysis of chloroplast enzyme content. He found no clear evidence for the existence of the above enzymes in the chloroplast.

It is clear that much more specific information is needed about chloroplast protein and lipid synthesis. A possible direction of investigation is the measurement of label incorporation into the lipoprotein of an extractable structural component of the chloroplast.

Chloroplasts are characterized by an inner structure of stacked, specialized membranes, the lamellae, which account for much of the bulk of the chloroplast. Recent success at isolating and analyzing these membranes has disclosed the presence of an homogeneous structural protein which is the largest component of these membranes (19). This protein is water-insoluble and removable from the chloroplast fraction of broken plant cells as a lipoprotein which can be made soluble in a detergent solution and then readily purified by electrophoretic techniques. The lipoprotein thereby recovered provides a chloroplast-specific protein and its associated lipids which are the end-products of structural synthesis, thus making this lipoprotein a practical subject of label incorporation investigations.

The chloroplast lamella is reported to have a gross composition of about 50% protein and 50% lipid (20). There is evidence that at least 50% of this protein is homogeneous and serves as a major lipid-binding backbone (19,20). This protein is credited with the properties of, 1) being directly associated with chloroplast pigments and lipids, 2) interacting with protein components of the oxidative process (photoxidation of water), and 3) reacting with

itself (after isolation and purification) to form a stable protein network (19).

One of the first successful separations of chloroplast lipoprotein and its purification by electrophoresis was achieved by Chiba (21). He used a detergent mixture which disrupts the noncovalent bonds credited with stabilizing the membrane units, thus allowing the solubilization through micelle formation of the membrane pigment-lipid-protein complex. The solubilized component is thought to be the globular lipoprotein membrane subunits described by Weier and Benson (22), four of which make up a quantasome (23). The molecular weight of these subunits is in dispute but when extracted with the same detergent system used in our extraction procedure, it was measured as 1.3×10^6 in ultracentrifugation sedimentation coefficient studies (21).

Chiba's success at showing the electrophoretic and purification possibilities with chloroplast lipoprotein encouraged our attempt to apply preparative acrylamide gel electrophoresis techniques to the separation of this chloroplast component after label incorporation. The works of Maizel (24) and Davis (25) detail the techniques most applicable to this investigation.

The electrophoretic techniques we employed produced a sharp band of migrating lipoprotein which could easily be detected by its green color, UV absorption at 280 µm and scintillation (after label incorporation). This band was collected and yielded quantities (1-4 mg) of electrophoretically pure lipoprotein which could be analyzed for quantitative label uptake.

The complete separation of chloroplasts from other cell components and contaminants, particularly where intact

chloroplasts are desired, is difficult if not impossible. Nuclear fragments, ribosomes, mitochondria, endoplasmic reticulum and bacteria can add protein (perhaps labeled) to the final chloroplast pellet. The electrophoretic fractionation of a chloroplast-specific lipoprotein eliminates some of the uncertainty that results from the direct analysis of the chloroplast pellet.

The broad objective of the research here reported has been to survey and compare the patterns of in vivo and in vitro incorporation of three C¹⁴-labeled substrates (C¹⁴O₂, acetate- C^{14} and leucine- C^{14}) into the lamellar lipoprotein of the chloroplasts of Nicotiana Tabacum L. seedlings. The experiments were specifically designed to determine, 1) the light-to-dark changes in incorporation levels, 2) the relative distribution of label between the protein and lipid fractions, 3) the degree of conversion of leucine to other amino acids and lipid substrate and, 4) the effect of the antibiotics chloramphenicol and cycloheximide on the in vitro incorporation of leucine into the lamellar protein. The information gained by these experiments is applied to the question of chloroplast autonomy.

 C^{14} carbonate and acetate were chosen to provide the simplest labeled substrates for the comparisons of light-to-dark (lt:dk), protein-to-lipid (P:L) and <u>in vivo-</u> to-<u>in vitro</u> incorporation capacities of chloroplasts. The choice of leucine-C¹⁴ over other labeled amino acids or a labeled homogenate was dictated by, 1) the intention of surveying, not comprehensively investigating, chloroplast lipoprotein-incorporation capacities, 2) the successful use of leucine by others (6,7), 3) the ease of detecting conversion of a single amino acid to other labeled substrates (such

as amino acids and lipid precursors) and, 4) the desire to use an amino acid for which there was no evidence of synthesis within the chloroplast.

The analysis of the extracted lipoprotein was largely limited to comparing the amounts of label incorporated into the structural protein and lipid fractions. While it is assumed that the protein is a largely homogeneous structural protein (19), the lipid fraction has been found to contain chlorophylls <u>a</u> and <u>b</u>, carotenes and xanthophylls (26) and an unanalyzed quantity of galacto-, sulfo- and phospholipids as well as sterols (20). A study of the distribution of label among these various lipids was deemed beyond the scope of this investigation.

An amino acid analysis of the hydrolysates of the lamellar protein following in vivo and in vitro incorporation of leucine- C^{14} was conducted to determine the degree to which leucine was converted to other labeled amino acids in either or both of these incorporations. The blocking effects of chloramphenicol (CAP) and cycloheximide (actidione) (ACT) on in vitro leucine incorporation were measured to determine the involvement of chloroplast-specific ribosomes in the structural protein synthesis (27).

II. MATERIALS AND METHODS

1. Preparation of plant material

The chloroplasts used in these experiments were isolated from <u>Nicotiana Tabacum</u> L. (Connecticut Broadleaf tobacco). Seedlings were grown on 1.2% agar made up with Hildebrant's media (28) plus 0.2 g $NH_4NO_3/1$. The seeds were sterilized with 20% Chlorox for one hour and transferred to the surface of the sterile agar in a sealed transfer chamber. The 9 cm by 5 cm petri dishes were covered with Parafilm (Am. Can Co., Neenah, Wisc.) and placed in the dark until germination. They were then placed on growth racks at 23°C with a light intensity of 300-400 μ w/cm² supplied by Sylvania Gro-Lux lamps and a light cycle of 16:8 hours (light:dark).

2. Chloroplast extraction

Chloroplasts were extracted by hand grinding 1-2 g of seedlings in a glass tissue homogenizer using a medium containing 0.4% methyl cellulose (MC4000), 0.3 M NaCl, 0.5% Hildebrant's solution (28), 0.44 g/1 MgCl₂, 0.31 g/1 ATP and adjusted to pH 7.4 with TRIS. The seedling homogenate was filtered through 6 layers of cheesecloth and then 2 layers of Kimwipes (Fisher Scientific) which removed much of the nuclear material and wall debris. Centrifugation at 1000 x g for 15 min at 4°C produced a pellet of chloroplasts. If not to be used for in vitro incubation, the pellet was resuspended in water (twice) and spun at 16,000 x g to remove soluble components.

For <u>in vitro</u> labeling experiments the pellet was suspended in an incubation medium which had the same composition as the above extraction medium. In 2 sets of experiments the incubation medium for the isolated chloroplasts was held constant; in the third 0.5 mM $CuSO_4$ was added to the medium. Hall and Cocking (6) suggested that $CuSO_4$ prevents ribonuclease action on isolated chloroplasts.

After incubation the chloroplasts were pelleted at 16,000 x g and washed in water twice to remove residual label and soluble components.

Other isolation procedures, extraction media and incubation formulae (8,9) were tested; the procedure and media described above, in our hands, gave a cleaner pellet and more intact chloroplasts (under light microscope examination) than the alternative methods tried. The levels of label incorporation measured were comparable to those reported by others (6,7,8). An extensive analysis of the effects of varying the incubation media was considered beyond the scope of this investigation.

3. Labeled substrate incorporation

Sodium acetate-2- C^{14} , sodium bicarbonate- C^{14} and L-leucine- C^{14} (u.1.) (New England Nuclear) were incorporated into 5-16 day old tobacco seedlings by diluting the aliquots of label with distilled water and allowing them to flow over the surface of the agar. These same labeled substrates were fed to isolated chloroplasts by adding them directly to the incubation medium.

The quantities of label added per dish of seedlings (approximately 1 g wet weight of seedlings per dish) were:

5 μ c sodium acetate-2-C¹⁴ (2 μ c/mmole), 5 μ c sodium bicarbonate- C^{14} (5.6 μ c/mmole) and 1 μ c L-leucine- C^{14} (220 mc/mmole). For isolated chloroplast experiments the same levels of label were added to 3 ml aliquots of suspended chloroplasts: all experiments were run in trials, a single trial including all the labeled conditions to be compared (C¹⁴-carbonate, -acetate and -leucine in both light and dark incubation). All the experiments within each trial were conducted with aliquots taken from a common dense homogeneous suspension of isolated chloroplasts taken from seedlings of the same age, assuring equal numbers of chloroplasts/aliquot within that trial. Incubation periods were 8 hours light or dark for the seedlings and 30 minutes light or dark for the isolated chloroplasts.

At the end of the incubation period the seedlings were gathered by running a sterile spatula across the surface of the agar to disengage the seedlings from the agar. The seedlings were then ground and the chloroplasts isolated as described in the previous section.

4. Lipoprotein extraction and purification

The washed chloroplast pellet was placed in 1 ml of a 3:1 mixture of 1% sodium dodecyl sulfate and 1% sorbitan monooleate (20) and then gently stirred until the pellet appeared dissolved. Centrifugation at 16,000 x g left a clear green solution of solubilized lipoprotein which was decanted and stored in the dark at 10°C; the pellet, which was discarded contained small cell wall fragments, nuclear residue, bacteria (if any) and other materials not solubilized by the detergent.

The lamellar extract was further purified by preparative zone electrophoresis with polyacrylamide gel using a column and sample collecting apparatus modelled after that of Maizel (23). The total extraction procedure is outlined in Figure 1. Figure 2 diagrams the electrophoretic apparatus and monitoring equipment.

The 7.5% acrylamide gel and buffer solutions were prepared following the formulations of Davis (24). The column was loaded by mixing the lipoprotein suspension with Sephadex (G-200, Pharmacia) (2:1, Sephadex to lipoprotein) and layering it on top of the gel column to reduce the mixing of the sample with the buffer. The lipoprotein migrated through the column (electrophoretically) in a sharp band and its collection after leaving the column was monitored continuously with a UV photometer (230 mµ) and a scintillation counter (Fig. 2).

The migration period was from 2-3 hours depending upon the length of the gel column. This period provided adequate time for polypeptides, small proteins and lipoproteins, and residual label to move out of the column ahead of the lamellar lipoprotein; the small peaks (Fig. 3) resulting from these components were recorded during the first 10-60 minutes. The lamellar lipoprotein migrated as a sharp visible green band and was collected after leaving the column by taking those samples which fell within the recorded UV and scintillation peaks (Fig. 3). The lipoprotein peak displays a shoulder which indicates the existence of two components in this lamellar lipoprotein complex, a finding also reported by Chiba (20) and others (29). Fine analysis with disc electrophoresis (29) revealed two bands that migrated very close to each other: one green and one yellow-green.

Figure 1





Instruments used:

Electrophoresis cell: laboratory fabricated UV Photometer: Gilson GME Fraction collector: Gilson GME Recorders: Texas Instrument: Rectiriter llR Eric LaWhite Power supply: IKB - Type 3290 B Pump: Sigmamotor AL-4-E Preparative separations of chloroplast lipoprotein monitored by, A - UV detector, B - scintillation counter: Flow rate - 25 ml/hr. LP - lipoprotein collected for label analysis.

FIG 3



time in min

Since only one type of structural protein has been found in the complex (1,22), it is assumed that the two bands are the results of different lipid arrays bonded to the same structural protein. For the purposes of this investigation, these components are considered as a single lipoprotein complex.

The collected lipoprotein was dried at 60°C, fractionated into lipid and protein with 85% warm methanol (30 min) and centrifuged at $16,000 \times g$ for 5 min to sediment the suspended flocculent protein precipitate. The supernatant was transferred to diethyl ether; 10% NaCl and water were added until two sharp phases formed. The ether phase was washed (twice) and dried under vacuum. The lipids were resuspended in 1 ml petroleum ether and placed in 15 ml scintillation solution for counting. This total lipid fraction was quantitized as the amount of lipid which was associated with 1 mg of the protein from which it was separated. Analysis of the possible loss of phospholipid to the methanol-water fraction was made by drying and counting this fraction. A consistent loss of approximately 15% of the total lipid label was measured; the final count of the ether extract was therefore increased by this amount to compensate for this loss.

Previous chromatographic analysis (26) of the ether-soluble lipid fraction revealed all the major pigments to be still present after electrophoretic isolation; chlorophylls <u>a</u> and <u>b</u> carotene, lutein and 2-3 other xanthophylls were detected in quantity.

The protein (methanol-insoluble) fraction was washed with 80% acetone and water (twice) to remove buffer salts and detergents. The protein was dried, weighed and solubilized

(see below) for counting. A check of several of the final dried samples by washing with CHCl₃:MeOH, 2:1, and counting this wash revealed no significant residual lipid label.

5. <u>Determination of the rate of label incorp-</u> oration into the protein and lipid fractions

All samples were counted with a Packard Tri-Carb 314ES liquid scintillation counter using the solvent system: POPOP - 5g/1 and PPO - 0.5g/1 in toluene (Packard Instr. Co., Downers Grove, Ill.). Lipid samples in petroleum ether were added directly to the scintillation solvent. The final lamellar protein samples were solubilized with 1 ml of Hyamine (1M hydroxide of Hyamine: Packard).

Amino acid spots were cut out of the chromatograms (sect. II-7) and placed directly in the scintillation solvent. Blanks were prepared from the same constituents minus label for background determination of each different counting condition. Efficiency was determined with reference to quenching curves established with C^{14} quenching standards (Nuclear Chicago). In general, quenching effects were very small and could be disregarded. All results are reported as counts per min per milligram (cpm/mg) of lamellar protein, the lipid associated with this amount of protein or the amino acid recovered from this amount. The final adjusted cpm for each experiment is also the approximate disintegrations per min (dpm).

It was not possible to measure exact amounts of initial plant material due to variations in wet weight, smallness of the seedlings, their large number per gram and their attachment to the agar. Therefore, it was

difficult to maintain a constant proportion of label to plant material in the in vivo experiments. By using seedlings of the same age for each labeling trial and attempting to keep the number of seedlings per dish reasonably constant, it was possible to maintain some control over label levels. The quantity of protein recovered from each trial was used to test whether reasonably equal quantities of plant material (chloroplasts) had been subjected to equal quantities of label. Count levels were adjusted and reported as those representing the count of equal label fed to equal quantities of chloroplast protein. Only samples where the final protein was within 1 mg of the weight of the other compared were recorded; those outside this limit were discarded. Small changes in label level were found to be directly proportional to changes in uptake within the range of label levels used.

To help offset the limited control of the initial label-to-seedling levels and variations in seedlings due to minor differences in ages and batches, three separate incorporation trials (each trial a set of six experiments with the three labeled substrates, light and dark incorporation) were to run to determine each light-to-dark and protein-to-lipid ratio. The results of the three <u>in</u> <u>vivo</u> trials (a series) were totaled and lt:dk and P:L relationships expressed as the nearest small whole number ratios.

For <u>in vitro</u> experiments, equal aliquots of chloroplasts were taken from a single pooling of isolated chloroplasts extracted from seedlings of the same age. The equal aliquots were placed in equal quantities of labeled reaction media (3 ml). The six labeling experiments which

comprised each trial were thereby conducted with equal numbers of similar chloroplasts.

The choice of 30 min as the incubation period for the isolated chloroplasts labeling experiments was based upon the experimental results of several investigators (5,6,7) who indicated that this period was the time limit of assimilation capacity. Our own attempts to extend the incubation period (up to 12 hours) showed no clear increase in incorporations beyond that recorded for 30 min periods.

6. Inhibition of protein synthesis

Two inhibitors of protein synthesis were chosen to give further indications of the nature of lamellar protein These experiments were limited to the measuresynthesis. ments of inhibition of leucine incorporation into isolated chloroplasts. Chloramphenicol (CAP) (Parke, Davis and Co., Detroit) and cycloheximide, also known as Actidione (ACT) (CALBIOCHEM, Los Angeles) were the inhibitors chosen. CAP is reported to sharply inhibit protein synthesis in bacteria (30) and chloroplasts (5,27). ACT is thought to inhibit protein synthesis which is controlled by cytoplasmic ribosomes (27,30). These inhibitors were fed to equal aliquots of chloroplasts isolated and incubated as described in sections II-2,3 and analyzed by the methods given in sections II-4,5. The antibiotic levels used were, CAP, 1 mg/ml, and ACT, 15 ug/ml.

7. Amino acid analysis of labeled lamellar protein

Samples of the final purified lamellar protein from in vivo and in vitro leucine- C^{14} incorporation experiments were hydrolyzed and chromatogrammed to determine the degree of leucine conversion to other amino acids. Samples of 1-2 g were hydrolyzed with 6N HCl by refluxing for 24 hours. The resulting amino acids were dried at 60° C, resuspended in distilled water, filtered and chromatogrammed on paper (Whatman No. 1: descending in one dimension for 30 hr on a 30 inch strip). The solvent system was; <u>n</u>-butanol, water and glacial acetic acid (5:4:1) (v:v:v). Leucine and phenylalanine were identified against standards and the other amino acids by Rf values (31).

III. RESULTS AND DISCUSSION

1. <u>In vivo incorporation of C¹⁴ into the lamellar</u> <u>lipoprotein of Nicotiana seedling chloroplasts</u>

The <u>in vivo</u> results are organized into 3 series of experiments established to investigate the light and dark incorporation of labeled substrates into seedling chloroplasts. Series A gives the results of sodium bicarbonate- C^{14} incorporation, series B of sodium acetate- $2-C^{14}$ incorporation and series C of L-leucine- C^{14} (u.1.) incorporation. Table 1 presents the data from experimental series A-C for protein incorporation and summarizes these data in terms of the ratio of light-to-dark (lt:dk) incorporation. Table 2 gives the parallel data and summary ratios for lipid fractions extracted from the same lipoprotein extracts as was the protein in table 1.

Table 3 displays totaled incorporations taken from Tables 1 and 2, presenting this data from the viewpoint of relative protein-to-lipid (P:L) incorporation.

Figure 4 gives a graphic summary of the 1t:dk and P:L relationships that were established by this research.

Substrate utilization

The results of tables 1 and 2 show incorporation of all three substrates into chloroplast lamellar protein and lipid. Despite the method of label feeding (the label was placed in a relatively large volume of growth media and agar and was thereby accessible to the seedling roots) and the competition of other cell components for the label, there was sufficient incorporation to give reliable

Activity of chloroplast lamellar protein: <u>in vivo</u> incorporation of sodium bicarbonate- C^{14} , sodium acetate-2- C^{14} and L-leucine- C^{14} (u.1.); 8 hours incubation in light or dark.

| series | label | trial | seedling age in days | g cpm/ prot LIGHT | mg of ein DARK | NWN* light-to- dark ratio |
|--|--|-------|---|-------------------------|----------------------|---------------------------------|
| | and the second | 1 | 5 | 8,030 | 146 | |
| А | CO ₂ | 2 | 7 | 10,800 | 332 | |
| | Z | 3 | 16 | 13,280 | 768 | |
| | | total | <u>, , , , , , , , , , , , , , , , , , , </u> | 32,110 | 1,246 | 26:1 |
| | | 1 | 5 | 12,600 | 2,140 | |
| В | acetate | 2 | 6 | 7,070 | 1,280 | |
| | | 3 | 7 | 38,100 | 4,900 | |
| | | total | | 57,770 | 8,320 | 7:1 |
| •••••••••••••••••••••••••••••••••••••• | | 1 | 5 | 8,820 | 6,930 | |
| С | leucine | 2 | 8 | 13,300 | 9,120 | |
| | | 3 | 16 | 13,900 | 10,400 | |
| | | total | <u></u> | 36,020 | 26,450 | 3:2 |

| IN | VIVO | PROTEIN | FRACTION | INCORPORATION |
|----|------|---------|----------|---------------|
| | | | | |

* NWN - Nearest Whole Number

Activity of the chloroplast lamellar ether-soluble fraction (total of chlorophylls, carotenoids and other lipids): in vivo incorporation of sodium bicarbonate- C^{14} , sodium acetate-2- C^{14} and L-leucine- C^{14} (u.1.); 8 hours incubation in light or dark.

| series | label | trial | seedlin age in days | g cpm/n prote LIGHT | ng of ein DARK | NWN light-to- dark ratio |
|----------|---------------------------------------|-------|---------------------------|---------------------------|----------------------|--------------------------------|
| <u> </u> | · · · · · · · · · · · · · · · · · · · | 1 | 5 | 9,028 | 106 | |
| А | CO ₂ | 2 | 7 | 18,700 | 469 | |
| | Z | 3 | 16 | 35,400 | 1,810 | |
| | | total | | 63,128 | 2,385 | 25:1 |
| | | 1 | 5 | 9,610 | 1,750 | |
| В | acetate | 2 | 6 | 6,870 | 845 | |
| | | 3 | 7 | 38,700 | 4,110 | |
| | | total | | 55,180 | 6,705 | 8:1 |
| | | 1 | 5 | 1,400 | 1,000 | |
| C | leucine | 2 | 8 | 3,890 | 841 | |
| | | 3 | 16 | 3,770 | 870 | |
| | | tota1 | | 9,060 | 2,711 | 3:1 |

IN VIVO LIPID FRACTION INCORPORATION

Comparison of distribution of label between protein and lipid in lipoprotein extracted from <u>in vivo</u> labeled chloroplasts: results expressed, <u>A</u> as the total count from 3 trials (tables 1 and 2), light and dark incorporation, <u>B</u> total light plus dark incorporation.

IN VIVO PROTEIN-TO-LIPID INCORPORATION

| | | | | the second s | | |
|------------------------|------------------|-----------------------------|-------------|--|------------------------|--------------|
| series and label | cpm/ PROTEIN | LIGHT mg prote: LIPID | in RATIO | DAI cpm/mg PROTEIN | RK protein LIPID | NWN RATIO |
| A-C0 ₂ | 32,110 | 63,128 | 1:2 | 1,246 | 2,385 | 1:2 |
| B-acetate | 57,770 | 55,180 | 1:1 | 8,321 | 6,705 | 1:1 |
| C-leucine | 36,020 | 9,060 | 4:1 | 26,450 | 2,710 | 10:1 |
| B-acetate C-leucine | 57,770 36,020 | 55,180 9,060 | 1:1 4:1 | 8,321 26,450 | 6,705 2,710 | 1 10 |

A

| R | |
|---|--|
| L | |
| - | |

| series | label | cpm/mg PROTEIN | protein LIPID | NWN RATIO |
|--------|-----------------|-------------------|------------------|--------------|
| A | co ₂ | 33,356 | 65,513 | 1:2 |
| В | acetate | 66,090 | 61,885 | 1:1 |
| C | leucine | 62,470 | 11,771 - | 5:1 |
| | | | | |

Figure 4

Patterns of incorporation of C¹⁴ substrate into <u>Nicotiana</u> seedling chloroplasts lamellar lipoprotein.

A - in vivo light (lt) to dark (dk) incorporation.*

B - in vivo protein (P) to lipid (L) incorporation.**



- * Ratios established from totaled protein and lipid incorporation (tables 1 and 2)
- ** Ratios established from totaled light plus dark
 incorporation (table 3-B)

measurements. Variations in levels of incorporation due to seedling age were investigated: no clear pattern of change could be detected over the range of seedling age from 5-16 days.

Light-to-dark incorporation

The light-to-dark incorporation patterns (tables 1 and 2) are clearly different for the three substrates. The expected light effect on CO_2 incorporation was clearly demonstrated, a ratio of 26:1, lt:dk, being recorded for both the protein and lipid fractions. There was some dark incorporation which may be explained by the difficulty of excluding all light during the incubation periods and transfer steps. A more careful control of dark incubation conditions would be needed to verify the possibility of some continuing low level of incorporation of CO_2 in the dark. Dark fixation of CO_2 by mechanisms such as the phosphoenol-pyruvic carboxylase reaction (32) in bacteria suggest this possibility.

Acetate incorporation was substantial both in the light and in the dark, with dark rates running at about 15% (8:1 protein and 7:1 lipid, tables 1 and 2) of light rates. The light enhancement of incorporation which gave a 7-fold greater uptake in the light than in the dark into lamellar protein suggests a possible utilization of acetate in the same pathways that utilize CO₂ in the light. The continuing dark incorporation assures that the synthesis of lamellar lipoprotein is not limited to light conditions.

The comparative light and dark incorporation rates of leucine are strikingly different from CO_2 and acetate. Leucine was incorporated into lamellar protein at dark rates which measured approximately 73% of light rates and lamellar lipid incorporation occurred at a dark rate of 33% of its light rate. The lt:dk levels of leucine incorporation into protein come close to being equal (3:2, table 1) compared with the large differentials shown with CO_2 (26:1, lt:dk) and acetate (7:1, lt:dk). The differences in the ratio of lt:dk incorporation into protein (3:2) and into lipid (3:1) are interesting when contrasted to the CO_2 and acetate incorporations where the lt:dk ratios for protein and lipid were nearly equal (table 1).

The nearly equal light and dark levels of leucine incorporation indicate that lamellar lipoprotein synthesis continues in the dark at a level comparable with its light level.

The utilization of leucine for lipid synthesis is indicative of the plant's capacity to turn over free amino acids, converting them to carbon chains which serve as lipid substrate (as well as substrate for the synthesis of other amino acids). Bassham, <u>et al</u> (33) presented evidence for the conversion of free amino acids (found in pools with high turnover rates) to protein-bound amino acids in <u>Chlorella</u> during photosynthesis. They also observed that the rate of flow of C^{14} through the free amino acid pools was much greater than the rate of movement of carbon into proteins. It was considered possible that free amino acids were being used in part for the synthesis of nonnitrogenous compounds.

The decrease in utilization of leucine as lipid substrate in the dark from 25% of the total incorporated leucine to 10% (table 1 and 2, series C) indicates a possible conversion of leucine to lipid substrate that is associated with light-activated pathways. Some substantiation of this possibility is reported in the <u>in vitro</u> results (III-2).

Protein-to-lipid label distribution

Table 3-A shows the distribution of label between protein and lipid under both light and dark incorporation conditions. Table 3-B shows the distribution in terms of total light plus dark incorporation.

CO₂ incorporation in the light is split between protein and lipid in a 1:2 ratio. The dark ratio is identical which is what would be expected if this incorporation resulted from short inadvertent exposure to light.

The similarity of the acetate incorporation ratio in both the light and dark (1:1, P:L) would seem to indicate that the utilization of acetate follows similar metabolic pathways in both the light and the dark. The acetate incorporation (1:1, P:L) when contrasted to the CO_2 incorporation (1:2, P:L) suggests that the pathways utilized for CO_2 incorporation into lamellar protein and lipid may include a more direct utilization of CO_2 for lipid substrate than for protein, since, within the 8 hr period of measurement, twice as much label appears in lipid as in protein. If the composition of the lamellar structure is 50% protein and 50% lipid (20) a more nearly one to one incorporation (as with acetate) would be expected since all carbon in the plant cell must ultimately come from CO_2 . This may suggest that CO₂ is directly utilized for lipid substrate within the chloroplast but that CO, directed toward amino acid synthesis may have to be translocated from the chloroplast as a carbohydrate and then built into amino acids elsewhere in the cell. Competition from already existing pools, 'stored' carbohydrate, and turning over amino acids may limit the amount of labeled amino acid that gets to the site of lamellar lipoprotein synthesis Parthier (34) found that $C^{14}O_{2}$ within the 8 hr period. incorporation into Nicotiana rustica leaves was more rapid into mitochondrial protein than into chloroplast protein. The specific activity of mitochondria protein continued to rise and remained higher than that of chloroplast protein for 18 hours after a 15 min period of photosynthesis in $C^{14}O_{2}$.

The distribution of label between protein and lipid following leu- C^{14} incorporation (table 3A) was 4:1 (P:L) in light and 10:1 (P:L) in the dark with an overall ratio of 5:1 (lt:dk, table 3B).

Conclusions: <u>in</u> <u>vivo</u> incorporation into lamellar lipoprotein

The plant cell seems readily able to use the three substrates fed in these experiments for the synthesis of both protein and lipid components of chloroplast lamellae. Pathways appear to exist for both the light and dark utilization of acetate and leucine in lamellar synthesis. The dark incorporation of leucine at over 65% of light uptake indicates that the synthesis of chloroplast lamellar structure may continue in the dark at rates which approach light rates. Since the full array of amino acids would be needed for lamellar synthesis it must be assumed that they are being synthesized somewhere in the cell. While the chloroplast may contribute some protein amino acids as products of its light-driven biosynthesis, its dark contribution would seem to be minimal, although some dark synthesis of amino acids cannot be ruled out.

The limited utilization of acetate for lamellar synthesis in the dark (only 12% of light utilization) may be an indication of a plastid-centered capacity for acetate utilization in lipid and protein substrate synthesis that is light dependent.

Figure 4 gives a graphic summary of the patterns of incorporation established from the data of tables 1 and 2. The comparative light effects on the utilization of these three substrates clearly show leucine incorporation as least affected by light. The protein-to-lipid distribution comparisons show the wide variance in label utilization that exists between these three substrates.

2. <u>In vitro incorporation of C¹⁴ into the lamellar</u> <u>lipoprotein of chloroplasts isolated from</u> <u>Nicotiana seedlings</u>

The <u>in vitro</u> results are organized into 3 series of experiments (tables 4-6) paralleling those of the <u>in vivo</u> incorporations. Series D are the sodium bicarbonate- C^{14} incorporations, series E the sodium acetate-2- C^{14} incorporations and series F the L-Leucine- C^{14} incorporations. Table 4 presents the data from the experimental series D-F for protein incorporations, expressed in terms of the comparative

light-to-dark label uptake. Table 5 gives data for lipid fractions extracted from the same lipoprotein fraction as the protein in table 4. Table 6 displays the same data as found in tables 4 and 5 arranged to show relative protein-to-lipid incorporation. The comparative data of each trial are expressed as the nearest whole number (NWN) ratio that can be established for each lt:dk and P:L relationship.

Three separate trials were run in each series $(CO_2, acetate and leucine)$ of the <u>in vitro</u> incorporation experiments. Each trial constituted a set of 6 labeling conditions (light and dark incubation with each of the 3 labeled substrates). While measurable incorporation was achieved with all trials, the results varied sufficiently so that it was not possible to meaningfully total the three trials for the purposes of establishing representative incorporation ratios (as was possible with the <u>in vivo</u> experiments).

The risk of error in making close comparisons between trials was relatively large due to experimental variables which were difficult to control. Those recognized uncontrolled variables were, 1) the seedling age (germination times varied and new batches of seedlings were started for each series of experiments), 2) limited standardization of the numerous purification and incubation steps, and 3) variations of quantities of intact chloroplasts per ml of incubation medium (due to differences in pressure and thoroughness in the grinding, filtering and centrifugation procedures).

The choice of an incubation medium was of necessity highly arbitrary. The ideal would be to choose one exactly

Activity of chloroplast lamellar proteins: In vitro (isolated chloroplast) incorporation of sodium bicarbonate- C^{14} , sodium acetate-2- C^{14} and L-leucine- C^{14} (u.1.); 30 min incubation in light and dark; seedling age 7-8 days.

| | | ······ | | | | |
|---------|-----------------|--------|-----------------|-----------------|------------------------|-----------------------|
| series | label . | trial | cpm/mg LIGHT | protein DARK | light to dark shift | NWN ratio 1t:dk |
| | ······ | 1 | 1,170 | 792 | decrease | 3:2 |
| D | C0 ₂ | 2 | 84 | 64 | decrease | 4:3 |
| | 2 | 3* | 770 | 239 | decrease | 3:1 |
| | <u></u> | 1 | 975 | 5,920 | increase | 1:6 |
| Е | acetate | 2 | 59 | 85 | increase | 2:3 |
| | | 3* | 1,750 | 341 | decrease | 5:1 |
| | . <u>-</u> | 1 | 28,800 | 22,400 | decrease | 4:3 |
| F | leucine | 2 | 266 | 123 | decrease | 2:1 |
| | | 3* | 440 | 380 | decrease | 5:4 |

IN VITRO PROTEIN FRACTION INCORPORATION

* $CuSO_4$ added, 0.5 mM to both extraction and incubation media.

Activity of chloroplast lamellar ether-soluble fraction (total lipids: chlorophylls, carotenoids, triglycerides and sterols): <u>in vitro</u> incorporation of sodium bicarbonate- C^{14} , sodium acetate-2- C^{14} (u.1.); 30 min incubation in light and dark, seedling age 7-8 days.

IN VITRO LIPID FRACTION INCORPORATION

| series | label | trial | cpm/mg LIGHT | protein DARK | light to dark shift | NWN ratio lt:dk |
|--------|-----------------|-------|-----------------|-----------------|------------------------|-----------------------|
| | <u></u> | 1 | 687 | 625 | decrease | 1:1 |
| D | C0 ₂ | 2 | 37 | 5 | decrease | - |
| | 2 | 3* | 142 | 190 | increase | 3:4 |
| | | 1 | 606 | 2,440 | increase | 1:4 |
| Е | acetate | 2 | 21 | 31 | increase | 2:3 |
| | | 3* | 350 | 155 | decrease | 2:1 |
| | | 1 | 3,420 | 1,820 | decrease | 2:1 |
| F | leucine | 2 | 24 | 50 | increase | 1:2 |
| | | 3* | 265 | - | - | - |

* $CuSO_4$ added, 0.5 mM to both extraction and incubation media.

Comparison of the distribution of label between protein and lipid in lipoprotein extracted from <u>in vitro</u> labeled chloroplasts; data taken from tables 4 and 5.

| | ~~~~ | | | | | | | |
|------------------------|-------|-----------------------------|------------------------|--------------|-----------------------------|-----------------------|--------------|-----------------------|
| series and label | trial | LIGF cpm/mg p PROTEIN | IT protein LIPID | NWN RATIO | DARI cpm/mg g PROTEIN | K protein LIPID | NWN RATIO | OVER- ALL RATIO |
| <u></u> | 1 | 1,170 | 687 | 2:1 | 792 | 625 | 3:2 | 3:2 |
| D-CO ₂ | 2 | 84 | 37 | 2:1 | 64 | 5* | ÷ _ | - |
| 4 | 3 | 770 | 142 | 5:1 | 239 | 190 | 5:4 | 3:1 |
| | 1. | 975 | 606 | 3:2 | 5,920 | 2,440 | 5:2 | 2:1 |
| E-acetate | e 2 | 59 | 21 | 3:1 | 85 | 31 | 3:1 | 3:1 |
| | 3 | 1,750 | 350 | 5:1 | 341 | 155 | 2:1 | 4:1 |
| | 1 | 28,800 | 3,420 | 8:1 | 22,400 | 1,820 | 12:1 | 10:1 |
| F-leu | 2 | 266 | 24 | 11:1 | 123 | 50 | 2:1 | 5:1 |
| | 3 | 440 | 265 | 3:2 | 380 | <u>-**</u> | - | - |

IN VITRO PROTEIN-TO-LIPID INCORPORATION

* ratio too low to be considered significant

** sample lost

like the cytoplasmic environment from which the chloroplasts come and then to remove the components one at a time to see which are necessary to support incorporation. This was of course impossible and the choice remaining was to try a mixture of salts and identified co-factors, adjusting pH to that estimated for cytoplasm and then to try variations to establish what 'works'. An infinite number of isolation and incubation media are theoretically possible, each of which could give different incorporation results.

Tables 4 and 5 show the variations that occurred in label levels, incorporation patterns and label distribution in the three trials conducted. Despite the difficulty of achieving reproducible incorporation, the results are adequate to allow some conclusions about the metabolic capacities of isolated chloroplasts and some interesting comparisons between <u>in vitro</u> and <u>in vivo</u> results.

Substrate utilization

Tables 4 and 5 show that all three substrates are incorporated into both the protein and lipid fractions of isolated chloroplasts. The survival in these chloroplasts of pathways for the synthesis of structural lipoprotein from CO_2 , acetate and leucine seems confirmed.

Evidence for the incorporation of leucine into isolated chloroplast lamellar protein was substantial. All three trials showed incorporation (table 4) with trial 1 indicating that high levels of incorporation are possible if the right combination of conditions can be found. This level of incorporation was not again achieved in either of the other two trials reported or in any preliminary attempts

to find the 'right' conditions. Trials 2 and 3 are examples of the more typical level of measured incorporation of leucine achieved with the methods chosen for this investigation.

The incorporation of acetate into the protein fraction of the lamellae was clearly confirmed in two of the three trials (1 and 3, table 4) and occurred at a low level in trial 2. A most interesting question is what amino acids are synthesized from this labeled substrate and incorporated into the protein. Further research should confirm whether it is any of the amino acids for which there is evidence of chloroplast synthesis.

The addition of CuSO_4 in trial 3 caused no clear shift in the overall incorporation pattern, but two minor variations involving acetate may have significance; 1) Acetate incorporation into both protein and lipid was higher than CO_2 and leucine incorporation (trial 3 compared with trials 1 and 2), and 2) there was a reversal in lt:dk acetate incorporation ratios with trial 3 (with CuSO_4) showing a decrease in the dark and with trial 1 and 2 (without CuSO_4) showing an increase in dark incorporation. Further research should establish if CuSO_4 consistently shows this effect on incorporation. The general improvement of protein incorporation in trial 3 (found for all three labeled substrates) over that in trial 2 may be an indication of CuSO_4 supression of ribonuclease activity as suggested by Hall and Cocking (5).

The utilization of labeled acetate for lipid substrate was clearly verified in two of the trials (series E, trial 1 and 3, table 5) and occurred at a low level in trial 2.

 ${\rm CO}_2$ incorporation into lamellar protein occurred at levels roughly comparable with acetate incorporation with one exception, the much higher level recorded for dark acetate incorporation in trial 1 (table 4). This may constitute evidence of similar pathways of incorporation, for whatever amino acids are formed, into the lamellar protein. Investigation of the hydrolysates of ${\rm C}^{14}{\rm O}_2$ and acetate- ${\rm C}^{14}$ labeled lamellar protein from isolated chloroplasts should indicate what amino acids become labeled. This would give substance to speculation about chloroplast capacities to synthesize protein amino acids.

Light-to-dark incorporation

There is a lt:dk shift in substrate incorporation into protein demonstrated in all three series of experiments with isolated chloroplasts (tables 4 and 5). Leucine showed dark decreases of incorporation of 25%, 50% and 20% while CO_2 showed decreases of 33%, 25% and 66%. The lipid incorporation patterns for these two substrate were inconsistent showing both increases and decreases in lt:dk comparison.

The three trials of acetate incorporation into both protein and lipid gave evidence of both increases and decreases in lt:dk incorporation comparisons, sharp evidence of the difficulties of getting reproducible results with isolated systems. Further trials are necessary to confirm a systematic lt:dk effect with acetate.

Protein-to-lipid label distribution

The patterns of distribution of label between protein and lipid for isolated chloroplast incorporations show some consistency. Leucine incorporation was predominately into protein in two of the three trials, with ratios ranging up to 11:1 (trial 2, table 6). Both the acetate and CO_2 trials also showed more label into protein than into lipid. P:L ratios were roughly comparable in both the light and the dark.

Conclusions: <u>in vitro</u> incorporation into lamellar lipoprotein

The incorporation of the substrates CO₂, acetate and leucine into both the protein and lipid fractions of the lamellar lipoprotein in isolated chloroplasts seems confirmed by these experiments. The presence, in the isolated chloroplasts, of chloroplast-specific ribosomes and the necessary enzymes to assemble the lipoprotein is implied by the successful incorporation. While the implication is that it must be intact chloroplasts which retain the necessary components to facilitate this synthesis, these experiments do not rule out the possibility that broken chloroplast systems retain these capacities or that there may be intact or fragmented mitochondrial systems present which could aid in this synthesis. The presence of cytoplasmic ribosomes is also possible if they were attached to cell fragments within the range of size and density that would separate out with the chloroplasts. However, the achievement of measurable and reproducible light enhanced CO, incorporation gave assurance that a chloroplast system

was operating. There is no direct evidence that either the acetate or leucine incorporation was assisted by extrachloroplast factors which occur as contaminants in the extracted pellet.

3. <u>Inhibition of in vitro leucine-C¹⁴ incorporation</u> with chloramphenicol and cycloheximide

The inhibition of leucine incorporation into protein by chloramphenicol (CAP) was decisive with as high as 70% inhibition measured in both trials (table 7). Chloroplast ribosomes are thought to bind CAP to a much greater degree than do cytoplasmic ribosomes (27). The evidence for the existence of chloroplast messenger RNA, ribosomes, template RNA, and polyribosomes as well as corresponding protein synthesis and CAP inhibition has been reviewed in detail by Kirk and Tilney-Bassett (3).

The inhibition achieved with CAP (table 7) would seem to assure that a chloroplast RNA system is operating in the protein incorporation experiments here reported (table 4). These inhibition results can be compared with a 70% inhibition measured by Spencer and Wildman (35) in their blocking of protein synthesis in isolated tobacco chloroplast using CAP (22 μ g/ml).

The 40-60% inhibition of leucine incorporation by cycloheximide (ACT) (table 7) is consistent with the kinds of blocking effects Smillie <u>et al</u>. (27) have recently reported for 3 photosynthetic electron transport enzymes which are thought to be lamellae-bound. They found that sharp inhibition of the production of these enzymes occurred with both CAP and ACT in <u>Euglena</u> chloroplasts. In contrast, they found that only CAP inhibited the formation of two

The influence of inhibitors on the incorporation of leucine- C^{14} into chloroplasts isolated from <u>Nicotiana</u> seedlings: seedling age 5 days, light incubation for 30 min at 23°C.

| \underline{IN} | <u>VITRO</u> | INHIBITION | OF | PROTEIN | SYNTHESIS | |
|------------------|--------------|------------|----|---------|-----------|--|
| | | | | | | |

| <u></u> | inhibitor | cpm/mg protein and per 30 min of incubation | % of inhibition |
|---------|-----------------------------|--|--------------------|
| TRIAL 1 | | · · · · · | |
| | none | 266 | 0% |
| | Chloramphenicol (1mg/m1) | 65 | 76% |
| | Cycloheximide (15 µg/ml) | 102 | 62% |
| TRIAL 2 | | | |
| | none | 420 | 0% |
| | Chloramphenicol (1mg/ml) | 101 | 75% |
| | Cycloheximide (15 µg/ml) | 247 | 41% |

Calvin cycle enzymes and Fraction I protein (the largest soluble protein component of chloroplasts). Smillie, <u>et al</u>. propose that the inhibition action of ACT suggests the involvement of cytoplasmic ribosomes in some stage of the synthesis.

One of the plausible theories of the genetic control of plastids holds that the structural genes are located in the plastid and the regulatory genes are in the nucleus (36). If this theory holds for the control of the synthesis of lamellar lipoprotein it explains how CAP could inhibit the structural assembly of lamellar protein by binding chloroplast ribosomes. The inhibitory effect of ACT might suggest an extrachloroplast-produced enzyme which controls some stage of this synthesis, although as previously explained, the possibility exists that there were some cytoplasmic ribosomes in the labeled chloroplast suspension prepared for these experiments.

Margulies (37) reporting on the effects of CAP on plastid development on <u>Phaseolus Vulgaris</u> leaves found a decreased formation of lamellae and an increased formation of vesicular structures. He also found that CAP increased the formation of a protein fraction which was soluble after osmotic shock. This work would appear to implicate CAP at the level of final assembly of protein subunits into lamellar structure, not at the level of polypeptide synthesis from amino acids.

4. The conversion of leucine to other labeled protein amino acids in in vivo and in vitro incorporations

Table 8 (parts I and II) show the results of the hydrolysis and chromatography of purified lamellar protein





after <u>in vivo</u> leucine-C¹⁴ incorporation. The label is distributed among most if not all of the amino acids indicating a rather complete conversion of leucine to some simpler substrate (acetate?) which is then utilized in the synthesis of the necessary amino acids for chloroplast lamellar synthesis. While this conversion was checked for only light incorporation, the similar levels of light and dark incorporation (table 1) make it almost certain that this conversion must also be a dark phenomenon. Since there seems little evidence that chloroplasts can produce most of the necessary amino acids for protein synthesis (there is some evidence for glycine, serine, alanine, aspartic and glutamic production) it must be assumed that this conversion is achieved outside of the chloroplast.

Leucine degradation in plants has been studied (38) and there is evidence of its reduction to acetyl CoA and acetoacetate. Mitochondrial particles prepared from germinating peanut cotyledons (39) were shown to carry on the β -oxidation of isovalerate (the suspected product of oxidative decarboxylation of leucine). There seems little doubt from the results evidenced in table 8 that leucine reduction occurs and its carbon chains are utilized to form other amino acids.

The <u>in vitro</u> results (table 8, III and IV) show striking contrast to the <u>in vivo</u> results. The isolated chloroplasts showed no capacity to convert the leucine to other labeled amino acids, and what label was incorporated was there as leucine. This would seem to confirm the assumption that the production of many of the necessary protein amino acids utilized in chloroplast lamellar lipoprotein production must come from an extrachloroplast source.

5. <u>Comparison of in vivo and in vitro results</u>: the question of chloroplast autonomy

Despite the failure to establish reproducible lightto-dark and protein-to-lipid ratios with the <u>in vitro</u> incorporations attempted in this research, there are still overall similarities of incorporation which give interesting insight into the metabolic capacities of the <u>Nicotiana</u> chloroplast.

An overall similarity of substrate utilization is evident when tables 1 and 2 are compared with tables 4 and 5. The chloroplasts both in the cell and in isolation were able to incorporate CO₂, acetate and leucine into both the lipid and protein fractions of the analyzed lamellar lipoprotein.

This similarity of substrate utilization is evidence of chloroplast autonomy in synthesizing at least some of its own structural material from simple substrate. The <u>in vitro</u> utilization of CO_2 for chloroplast protein synthesis suggests the existence of plastid-located metabolic pathways for forming at least some amino acids and processing them into protein. It also implies the direct utilization of chloroplast-formed carbon chains for lipid synthesis.

Acetate incorporation into protein by the isolated chloroplasts also (as with CO₂) implies a protein amino acid synthesizing capacity within the plastid. It also shows the utilization of 2-carbon units for both amino and lipid synthesis.

<u>In vitro</u> leucine incorporation into protein (as also does CO₂ and acetate incorporation into protein) confirms a <u>chloroplast-specific control over the synthesis of its own</u> structural protein. The conversion of leucine to lipid substrate suggests another metabolic function for chloroplasts, the reduction of amino acids to acetate or glycolate units which can be used in lamellar lipid synthesis. The comparative lt:dk results for <u>in vivo</u> and <u>in vitro</u> experiments (tables 1, 2, 4 and 5) show light effects for all trials, with the exception of trials 1 and 2 (tables 4 and 5) where an increase of acetate incorporation in the dark was measured.

The comparative P:L data for <u>in vivo</u> and <u>in vitro</u> experiments (tables 3 and 6) show widely different ratios of P:L distribution. Nevertheless, several generalizations can still be made: CO_2 <u>in vivo</u> incorporation produced a 1:2, P:L ratio (table 3B) while the <u>in vitro</u> results reversed the pattern giving 3:2 and 3:1 ratios (table 6). Acetate incorporation produced a 1:1, P:L ratio <u>in vitro</u> (table 3B) while the <u>in vitro</u> results (as with CO_2) shifted to a higher proportion of label in protein (2:1, 3:1 and 4:1, table 6). <u>In vivo</u> and <u>in vitro</u> leucine incorporations showed a more consistent pattern with an overall <u>in vivo</u> distribution of 5:1, P:L (table 3B) while <u>in vitro</u> results were 10:1 and 5:1 in the two trials where complete incorporation was recorded (table 6).

The chloramphenicol inhibition of leucine incorporation into the isolated chloroplast lamellar protein gives additional confirmation to the existence of a chloroplastspecific protein synthesizing system. The significance of the cycloheximide inhibition effect on <u>in vitro</u> protein synthesis is not clear.

The data on conversion of leucine to other protein amino acids in <u>in vivo</u> and <u>in vitro</u> labeled lamellar protein contributes to a concept of chloroplast dependence upon mitochondria and other extrachloroplast sites of synthesis to produce most of the amino acids utilized in lamellar protein synthesis.

The absence of tricarboxylic acid, glycolytic and hexose monophosphate enzymes in chloroplasts reported by Smillie, <u>et al</u>. (27), the apparent inability of isolated chloroplasts to convert leucine to other protein amino acids and the extensive dark incorporation of leucine lamellar protein (table 1) seem to substantiate extrachloroplast synthesis of many of the necessary protein amino acids for lamellar synthesis.

The limited data on incorporation of substrate into lipids does not contribute much insight into the source of lipid substrate. The extensive build-up of lipids in some chloroplasts (39) would suggest that plastids may convert photosynthetically formed carbohydrates directly to lipids. That some lipid synthesis, as well as protein synthesis, occurs in isolated chloroplasts seems adequately verified by the data measured in these investigations. That this lipid synthesis contributes to lamellar synthesis gives weight to the conclusion that chloroplasts control the synthesis of their major structural component.

SUMMARY

The <u>in vivo</u> light and dark incorporation of C^{14} -carbonate, -acetate and -leucine into the lamellar protein of <u>Nicotiana</u> seedlings established 26:1, 7:1 and 3:2 ratios, respectively of substrate uptake. The lipid fractions expressed corresponding ratios of 26:1, 8:1 and 3:1.

The relatively high levels of dark incorporation of leucine seem to confirm the continuing synthesis of lamellar structure in the dark at a level nearly that of light synthesis. The low level of dark incorporation of acetate may indicate a plastid-specific light-dependent pathway for incorporation of acetate into lamellar lipoprotein.

The <u>in vivo</u> distribution of label between protein and lipid showed ratios of incorporation of 1:2 (carbonate), 1:1 (acetate) and 5:1 (leucine) for light plus dark incorporation. The light and dark ratios for carbonate and acetate were similar but leucine showed a 4:1 (P:L) ratio in the light and a 10:1 (P:L) ratio in the dark indicating a light related conversion of leucine to lipid substrate. An analysis of the distribution of label after leu-C¹⁴ incorporation revealed a utilization of labeled carbons from leucine in the synthesis of most of the amino acids recovered by hydrolysis of the purified lamellar protein.

Similar isolated <u>Nicotiana</u> chloroplast experiments demonstrated a plastid capacity to incorporate carbonate, acetate and leucine into both the protein and lipid fractions of the analyzed lipoprotein. The ratios of incorporation

were inconsistent but the overall similarity to <u>in vivo</u> incorporations suggests that the metabolic pathways for the processing of these substrate into lipoprotein are located within the plastid.

That isolated chloroplasts utilized CO₂ and acetate for protein synthesis necessitates the conclusion that they can produce at least one protein amino acid. That plastids must depend upon extrachloroplast synthesis sites for the production and conversion of many of the amino acids needed for lamellar synthesis is suggested by 1) the failure of isolated chloroplasts to convert leu- C^{14} to other amino acids, the dark incorporation of leucine (in vivo) at rates 2) nearly as high as light rates, indicating the continuing production of all the lamellar amino acids in the dark, the lack of evidence (from other investigators) and 3)for the existence within the plastid of the enzymes necessary for the synthesis of many of the protein amino acids.

Chloramphenicol inhibition of isolated plastid protein synthesis gives additional confirmation of the existence of a chloroplast-specific RNA system which directs lamellar protein synthesis.

The data of this investigation contributes to a concept of limited plastid autonomy. Chloroplast-located metabolic pathways that can utilize CO₂, acetate and leucine for the synthesis of lamellar lipoprotein are confirmed.

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