CHLOROPHYLL AND PROTEIN INTERRELATIONSHIPS IN *ANANAS COMOSUS* (L.) MERR.1

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(WITH FOUR FIGURES)

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Studies on a quantitative separation and analysis of chloroplastic matter by different investigators have revealed that chlorophyll is held by chemical forces on proteinaceous matter. This matter, named phyllochlorin by MESTRE (17), chloroplastin by STOLL (37), photosynthin by FRENCH (8), and chloroglobin by RABINOWITCH (21), is presumably identical with the substance composing the stroma of chloroplasts.

FREY-WYSSLING (9), discussing the composition of chloroplasts, stated that, according to GUILLERMOND, MANGENOT, and PLANTEFOL (13) and SHARP (24), the structure of the chloroplasts of higher plants is homogeneous and not granular with small green particles imbedded in a colorless stroma, as postulated by MEYER (18) and SCHIMPER (22), and that the granulations are artifacts. According to HEITZ (15), the grana are not spheres but disks oriented parallel to the surface of the disk-shaped particles which alone contain the chlorophyll. NOACK (20) observed that colloidal chlorophyll does not show fluorescence except when adsorbed in monomolecular layers. Also, EULER et al. (7) have reached a similar conclusion from determinations of the quantity of chlorophyll in one single plastid.

**Methods**

The isolation of chloroplasts or of chloroplastic matter has been effected in various ways by different investigators (3, 5, 8, 11, 12, 14, 17, 19) and with somewhat similar results.

**ISOLATION OF CHLOROPLASTIC MATTER**

In former studies (25) the leaves of *A. comosus* were segregated into groups represented by old (B), mature (C), active (D), and young (E) leaves. In *A. comosus*, as in all monocotyledonous plants, the old (B) leaves occupy positions at the basal end of the stem, the young (E) at the apical end, and the mature (C) and active (D) at the medial section. Such leaves are cut for many studies into four or five cross sections in order to segregate tissues of different stages of development and physiological function. The basal sections (no. 1) of such leaves are lacking in chlorophyll, and in leaves which have not completed growth they are composed mostly of meristematic tissues. The chlorophyllous region of the blade is divided into terminal (no. 5), medial (no. 4) and basal (no. 3) sections and the neck

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or region between the non-chlorophyllous (no. 1) and chlorophyllous blade (nos. 3, 4, 5) is known as the transitional section (no. 2).

In this study the isolation of chloroplastic matter was made as follows: One hundred grams of tissues from the chlorophyllous section (no. 4) of freshly collected active (D) leaves of *A. comosus* var. smooth Cayenne (25) cut approximately 2 mm. wide were placed with 250 ml. of 0.06 N NaOH in a Waring mixer and macerated at high speed for ten minutes. The mixture was squeezed by hand pressure through 200-mesh silk cloth or Canton flannel. The residue in the cloth was wetted with 25 ml. of 0.06 N NaOH and squeezed at successive intervals until all green color was removed from the residue.

The residue was discarded and the extract placed in 100 ml. "oil" centrifuge tubes and centrifuged for the first 5 minutes at about 750 r.p.m., the next 2 minutes at about 1100 r.p.m., and the last 3 minutes at about 1500 r.p.m. The gradual increases of centrifugal speed caused starch to settle at the bottom of the container with the least admixture of chloroplastic matter, while the latter remained in the supernatant liquid.

The supernatant liquid, containing chloroplastic matter, was transferred to clean centrifuge tubes without disturbing the precipitated starch, treated with acetic acid to pH 4.0 (1 ml. or more of 6 N glacial acetic acid), agitated gently to effect mixing, and then centrifuged at different speeds as mentioned. The supernatant liquid was decanted and the precipitate examined carefully for starch granules in the bottom layer of the container. When starch was detected, the precipitate was treated with 75 ml. 0.06 N NaOH, agitated to effect uniform suspension of the precipitate, and centrifuged as described. The supernatant liquid containing chloroplastic matter was removed and the precipitated starch discarded. The chloroplastic matter was flocculated with acetic acid as before and centrifuged.

To facilitate transferring the chloroplastic matter from the tubes to volumetric flasks, 1 ml. of 0.06 N NaOH was added, the contents agitated and poured into the flask. The alkaline suspension was acidified with acetic acid to pH 4.5, the flask filled to the mark with H₂O and placed in the refrigerator. The concentration of the contaminant sodium acetate could be reduced by removal of the clear supernatant liquid at daily intervals and replacement with H₂O. The mixture was agitated before withdrawing samples for analysis.

Chlorophyll was extracted from the chloroplastic matter with acetone and determined in a Klett-Summerson photoelectric colorimeter using a light filter no. 60. The proteinaceous residue was analysed for total nitrogen.

### Results

#### Analyses of chloroplastic matter

Analyses of the chloroplastic matter from the leaves of *A. comosus* yielded 6.5 mg. of chlorophyll and 22.3 mg. of nitrogen per 20 grams of fresh tissue, the latter being equivalent to 140.0 mg. of protein (22.3 × 6.25).
Hanson (14) claimed that one tetrad of chlorophyll molecules (M.W. 900 \times 4 = 3600) is associated in the cell with one molecule of chloroplastic globulin (M.W. 68,000). Thus Hanson's estimated ratio of chlorophyll to protein is equal to 0.0530, i.e., 3600 \div 68,000, whereas that obtainable in this study was 0.0465, i.e., 6.5 \div 140.0, the latter being 14.0\% lower than the theoretical. The difference between Hanson's theoretical ratio and that obtained in our study may be due in part to incomplete extraction of chlorophyll—the extract was estimated to contain approximately 95\% of the total chlorophyll in the chloroplastic matter because the latter was faint greenish yellow after repeated extractions.

**ANALYSES OF TISSUES WITHOUT EXTRACTION OF PROTOPLASMIC MATTER**

Chlorophyll/protein ratios from other studies (26, 27) show gradients of ascending values from the basal to the terminal sections of leaves (table I). It will be noted that the ratios of chlorophyll to protein for section no. 4 of the active (D) leaves (25) were greater in the studies referred to above, where no separation of the chloroplastic matter from the tissues was effected but protein-N was estimated as the difference between total-N and soluble-N, than the value 0.0465 in the present study or the theoretical value 0.053. The results in table I indicate that the amounts of protein in relation to chlorophyll were about one-half as great as in the isolated chloroplastic matter and suggest a low rate of protein synthesis, a high rate of protein breakdown, or a low rate of chlorophyll reduction.

In sections no. 2 and no. 3, which are chronologically and physiologically less advanced than no. 4 and no. 5, the protein content of the tissues in relation to chlorophyll was less, either because of shorter periods of deposition and differences in the physiological functions of the tissues or of greater amounts of extracted protein, due to high pH values in sections no. 2 and no. 3 than in no. 4 and no. 5, which reduced the degree of association of protein with chlorophyll (23).

In general protein correlated with the chlorophyll content of tissues (figs. 1, 2, 3, and 4). The coefficient of correlation, calculated according to Snedecor (34) from \( r = \frac{sx y / \sqrt{sx^2 sy^2}} \) was statistically significant under experimental conditions which were favorable to plant growth. Chlorophyll/protein ratios in many chlorophyllous sections were in agreement with the theoretical ratio but in others, mostly partly chlorophyllous, they were either higher or lower, presumably because of the methodological shortcomings stated above.

From previous publications (27, 28), depicting relationships between chlorophyll and protein in cultures supplied with equal amounts of nitrogen as NO\(_3\) or NH\(_4\), but with different amounts of iron, correlation between chlorophyll and protein in all cultures is shown (fig. 1). The coefficient of correlation for the minus-iron cultures in the ammonium series was better than for the minus-iron cultures in the nitrate series, because the intake of iron, which occurred as impurity in the C.P. salts of the nutrient solu-
### TABLE I
Protein-N, calculated protein (protein-N x 6.25) and chlorophyll of fresh tissue; also, chlorophyll/protein ratios in different leaf sections of one-year-old *Anagro comosus*

<table>
<thead>
<tr>
<th>Leaf sections</th>
<th>Nitrate series*</th>
<th>Ammonium series†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature (C)</td>
<td>0.12</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>2.31</td>
</tr>
<tr>
<td>3</td>
<td>0.80</td>
<td>5.00</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>6.25</td>
</tr>
<tr>
<td>5</td>
<td>1.20</td>
<td>7.50</td>
</tr>
<tr>
<td>Active (D)</td>
<td>0.19</td>
<td>1.19</td>
</tr>
<tr>
<td>2</td>
<td>0.31</td>
<td>1.94</td>
</tr>
<tr>
<td>3</td>
<td>0.62</td>
<td>3.88</td>
</tr>
<tr>
<td>4</td>
<td>1.22</td>
<td>7.62</td>
</tr>
<tr>
<td>5</td>
<td>1.88</td>
<td>9.87</td>
</tr>
<tr>
<td>Young (E)</td>
<td>0.39</td>
<td>2.44</td>
</tr>
<tr>
<td>2</td>
<td>0.39</td>
<td>2.44</td>
</tr>
<tr>
<td>3</td>
<td>0.66</td>
<td>4.12</td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
<td>7.82</td>
</tr>
</tbody>
</table>

* Grown in solution cultures supplied with nitrate.
† Grown in solution cultures supplied with ammonium salts.
tions, was greater from the ammonium-N series with ultimate lower pH values than from the nitrate-N series with higher ultimate pH values than at the beginning of the absorption period.

**LEAVES:**

- **OLD**
- **MATURE**
- **ACTIVE**
- **YOUNG**

**NITRATE CULTURES**

**AMMONIUM CULTURES**

**PLUS IRON**

**MINUS IRON**

**CHLOROPHYLL**

**PROTEIN**

**NITROGEN**

**FIG. 1.** Correlation coefficient, $r$, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. cosmorus* grown in solution cultures supplied with or without iron and with equal amounts of nitrogen either as NO$_3^-$ or NH$_4^+$.

From previous publications (31, 32), pertaining to the effects of 140.0 vs. 2.8 mg. of nitrate or ammonium nitrogen in nutrient solutions on the chlorophyll and protein content of different leaf sections, highly significant correlation coefficient values are shown (fig. 2). In this figure as in figures 1, 3, and 4, each point represents the mean value of similar sections in the different leaf groups instead of individual sections. It will be noted that protein synthesis for the low-N cultures was greatly inhibited by nitrogen.
deficiency, which also caused a parallel reduction in the chlorophyll content of the tissues (fig. 2).

From previous publications \((28, 29)\), depicting the effects of 205 vs. 4 mg. of potassium per liter of nutrient solution on the chlorophyll and protein content of different leaf sections, it is shown that correlation coefficient values were highly significant for all cultures and may suggest that potassium played no direct role in protein or chlorophyll synthesis (fig. 3).

**Fig. 2.** Correlation coefficient, \(r\), and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages (the values are means for similar sections of leaves of different ages) of *A. comosa* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter, supplied either as \(\text{NO}_3^-\) or \(\text{NH}_4^+\).

From a previous publication \((25)\), depicting chlorophyll and protein relationships in two shoots from the same plant, of which one was exposed to seasonal light and the other was covered by a bag of black cloth for 56 days, statistically significant correlation coefficient values for the exposed
plant at $P = 0.05$ level but not at $P = 0.01$ level are indicated (fig. 4). Correlation between chlorophyll and protein was lacking for the covered plant, presumably because darkness inhibited chlorophyll formation. It may be noted, however, that some sort of relationship existed between chlorophyll and protein in the old (B) and in the mature (C) leaves which at the time of covering had approximately completed growth, but not in the active (D) and young (E) leaves which developed in darkness, lacked chlorophyll, and contained relatively little protein-N.

The findings in table II suggest that in cultures of A. comosus supplied with nitrate as the source of inorganic nitrogen, protein deposition in the
<table>
<thead>
<tr>
<th>Leaf sections</th>
<th>Exposed*</th>
<th>Covered†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prot.-N</td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>mg./gm.</td>
<td>mg./gm.</td>
</tr>
<tr>
<td>Mature (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.368</td>
<td>2.30</td>
</tr>
<tr>
<td>3</td>
<td>0.502</td>
<td>3.14</td>
</tr>
<tr>
<td>4</td>
<td>0.646</td>
<td>4.03</td>
</tr>
<tr>
<td>5</td>
<td>0.870</td>
<td>5.44</td>
</tr>
<tr>
<td>Active (D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.313</td>
<td>1.95</td>
</tr>
<tr>
<td>2</td>
<td>0.340</td>
<td>2.12</td>
</tr>
<tr>
<td>3</td>
<td>0.438</td>
<td>2.74</td>
</tr>
<tr>
<td>4</td>
<td>0.682</td>
<td>4.26</td>
</tr>
<tr>
<td>5</td>
<td>0.885</td>
<td>5.54</td>
</tr>
<tr>
<td>Young (E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.400</td>
<td>2.50</td>
</tr>
<tr>
<td>2</td>
<td>0.317</td>
<td>1.98</td>
</tr>
<tr>
<td>3</td>
<td>0.514</td>
<td>3.22</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Exposed to seasonal sunlight.
† Covered with a black cloth for 50 days.
**LEAVES:**
- OLD
- MATURE
- ACTIVE
- YOUNG

![Graph](image)

**Fig. 4.** Correlation coefficient, $r$, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. comosus* sister shoots grown on the mother plant, of which one was kept in darkness for 56 days by covering with a black cloth; and the other was exposed to seasonal light.

Chlorophyllous sections (nos. 3, 4, and 5) was in much greater amounts than in the basal non-chlorophyllous sections (no. 1). This may indicate that protein deposition was simultaneous with chlorophyll production, presumably because of nitrate reduction and assimilation and carbohydrate synthesis in these sections.

In variegated leaves of *Ananas bracteatus* less protein-N was present in the non-chlorophyllous than chlorophyllous areas and the ratios of chlorophyll to protein were generally lower in the former than latter areas (table III). Chlorophyll to protein ratios in the green areas of leaves no. 2 and

**TABLE III**

<table>
<thead>
<tr>
<th>Leaves</th>
<th>CHLOROPHYLL</th>
<th>PROTEIN-N</th>
<th>PROTEIN</th>
<th>CHLOR. PROT.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO.</td>
<td>COLOR</td>
<td>mg./gm.</td>
<td>mg./gm.</td>
<td>mg./gm.</td>
</tr>
<tr>
<td>1</td>
<td>White</td>
<td>0.068</td>
<td>0.28</td>
<td>1.75</td>
</tr>
<tr>
<td>1</td>
<td>Green</td>
<td>0.301</td>
<td>1.11</td>
<td>6.93</td>
</tr>
<tr>
<td>2</td>
<td>White</td>
<td>0.086</td>
<td>1.34</td>
<td>9.37</td>
</tr>
<tr>
<td>2</td>
<td>Green</td>
<td>0.729</td>
<td>2.13</td>
<td>13.30</td>
</tr>
<tr>
<td>3</td>
<td>White</td>
<td>0.083</td>
<td>0.81</td>
<td>5.06</td>
</tr>
<tr>
<td>3</td>
<td>White and green</td>
<td>0.231</td>
<td>1.07</td>
<td>6.67</td>
</tr>
<tr>
<td>3</td>
<td>Green</td>
<td>0.351</td>
<td>1.03</td>
<td>6.43</td>
</tr>
</tbody>
</table>

**CHLOROPHYLL AND PROTEIN-N OF FRESH TISSUE AND CHLOROPHYLL TO PROTEIN (PROTEIN-N x 6.25) RATIOS IN THE NON-CHLOROPHYLLOUS OR CHLOROPHYLLOUS AREAS OF VARIEGATED LEAVES OF *Ananas bracteatus* GROWN UNDER FIELD CONDITIONS**
no. 3 had attained the theoretical value of 0.055, but in leaf no. 1 the observed ratio was about 22% lower than the theoretical value.

Discussion

Stiles (36), Spoehr (35), Rabinowitch (21), Wieder (38), Burkholder (4), Gaffron (10), and Frey-Wyssling (9) have presented the various aspects of photosynthesis insofar as it concerns chlorophyll and chloroplastic proteins either as a coordinating system or as independent systems.

Sugar synthesis in green plants by photosynthetic activity is effected by reduction of carbon dioxide with evolution of oxygen. Carbon dioxide reduction takes place in other organisms lacking chlorophyll which may or may not be sensitive to light, according to Gaffron (10) and Werkman and Wood (39), suggesting that the proteinaceous matter in the cell (which may function similarly to the proteinaceous stroma in the chloroplasts), and not the chlorophyll is the locus for carbon dioxide reduction. Experimental evidence (6) on the time required for the photosynthetic mechanism corroborates the view that carbon dioxide reduction may be effected in the absence of chlorophyll proper in some other adjacent region of the system and possibly in the chloroplastic stroma. Höber (16), in an attempt to explain certain observations on studies in photosynthesis, states his point of view as follows: "The time element, possibly associated with long range forces, may be necessary to explain the observed fractional quantum efficiency for a large assembly of chlorophyll molecules in the interior of the chloroplast to absorb four light quanta simultaneously and then to transmit the electronic excitation energy from one molecule to the next by some sort of resonance effect, until it reaches a point on the surface occupied by an adsorbed CO₂ molecule."

The data on protein-N and chlorophyll content in various leaf sections of A. comosus, in previous publications (27, 28, 29, 30, 31, 32), indicate in general gradients which increased from the basal chlorophyll-free semiseriostematic no. 1 section to the terminal more or less senescent no. 5 sections. In plants supplied with insufficient amounts of iron, nitrogen, or light the chlorophyllous sections of the leaves contained relatively equal or slightly greater amounts of protein-N than the non-chlorophyllous basal no. 1 sections. Inhibition of chlorophyll synthesis by deficiencies in iron, light, or nitrogen caused a parallel reduction of protein depositions in the chlorophyllous tissues.

In plants adequately supplied with iron, light, or nitrogen, chlorophyll to protein ratios were approaching the theoretical value, although they fluctuated considerably for different sections of the leaves. Lowest ratios and ratios nearer the theoretical value were obtained mostly in the distal more mature regions of the leaves, e.g., sections 4 and 5, but in the proximal sections 2 or 3 which are less mature, the ratios were ordinarily higher. It is probable, therefore, that protein depositions in the latter were either
limited by unknown physiological causes or that some protein might have been incorporated in the soluble organic-N fraction and the reported protein values were not sufficiently high to yield the theoretical ratio, 0.055. It was observed in subsequent studies, which will be reported elsewhere, that from 12.0% to 28.0% of the total water-extractable nitrogen is proteinaceous and may be precipitated by acetic acid, while from 1.0% to 12.0% is proteose-N which is precipitable by trichloroacetic acid. In many analyses reported above, all proteose-N was incorporated in the soluble organic-N fraction. The subtraction of the latter from total-N to yield protein-N by difference lowered the actual proteinaceous values from 2.0% to 25.0% on account of the inclusion of proteose-N in the soluble organic-N fractions. If this correction be taken into consideration, many of observed chlorophyll/protein ratios approach the theoretical value.

Studies of protein structure by Astbury (1, 2) indicate that the fibrous proteins, have as the unit of structure a polypeptide grid whose normal equilibrium configuration is not flat, but buckled, that is, the main chains are thrown into a series of intramolecular folds lying in planes transverse to the cross-linkage of the grid, and its basis of the long-range elasticity is the capacity to unfold and refold. Moreover, additional evidence pointing to a simple system of close packing of the side chains, the polars on one side of the main chain and the non-polars on the other, suggests that such close packing results in the formation of protein laminae which may appear alone or sandwiched with accessory groupings, such as lipid laminae. Schulman (23) has shown that, in lipoproteins, the pH of the solution with values above or below the isoelectric point of the protein may increase or decrease the relative degree of association or combination of lipoids with proteins.

In A. comosus the pH and titrable acidity values of the sap from different leaf sections vary considerably (26, 27, 29, 31). Gradients of titrable acidity and pH of the various leaf sections, influenced by diurnal changes and rates of respiratory activity (33), increased in the former but decreased in the latter from the basal to the terminal sections during the hours of darkness or low light, while at times of full sunlight, when photosynthetic activity was at maximum, they either disappeared or became slightly reversed. Thus the different conditions provided in the cell by the constantly changing acidity or pH may increase or decrease the degree of association between chloroplastic proteins and chlorophyll. Hence, the divergence between observed and theoretical chlorophyll/protein ratios may be explained by the effects of pH on the chlorophyll combining power of the chloroplastic proteins of different leaf sections.

Summary

There was positive correlation between chlorophyll and protein in the chlorophyllous regions of plants supplied with adequate amounts of nutrients and seasonal light.

Insufficiency of iron, light, and nitrogen decreased considerably the amounts of chlorophyll and protein in the chlorophyllous regions of leaves.
Potassium in the nutrient solutions supplied in small or high amounts had no measurable influence on the relations of chlorophyll to protein.

Ratios of chlorophyll to protein in the distal relatively mature leaf sections, where both substances reached maximal content, approached the theoretical value of 0.055 of Hanson, but in the proximal less mature sections chlorophyll/protein ratios indicated greater amounts of chlorophyll in proportion to protein.

Protein-N/soluble organic-N ratios were approximately 1:1 in the basal (no. 1) non-chlorophyllous sections and 3:1 in the chlorophyllous (no. 4) sections, indicating that protein-N rather than soluble organic-N is directly related to chlorophyll.

These findings suggest that chlorosis, excluding variegation, in plants is a state of incompatible chlorophyll and protein relations in the chloroplast due to deficiencies of substances essential for the synthesis of chlorophyll or proteins, or to conditions (high Mn) inhibitory for the synthesis of such substances or for the proper operation of the chlorophyll-protein system. This state may be restored to normalcy either by the elimination of the deficiencies or the adverse conditions.

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