Cytochemical Studies on Chloroplasts I. Cytologic demonstration of nucleic acids in chloroplasts

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There is no doubt that the chloroplasts in the cytoplasm of the higher plants and green algae are the main apparatus of photosynthetic mechanism, although the cooperation of the cytoplasmic factor in this process may have recently been discussed (Frankel '41; Hill '37,'39).

The author's interest in photosynthetic mechanism suggested that cytochemical study to obtain more information on the composition of chloroplasts would lead to further progress in the elucidation of photosynthetic mechanism.

Since Brachet's ('40) and Pollister's ('47) investigations, it has generally become accepted that the elements which contain desoxyribonucleic acid (DNA) are stained green and the elements which contain ribonucleic acid (RNA) red by the differential staining of tissues with methyl green and pyronin, and that the treatment with ribonuclease will lead to the total disappearance of the basophilia due to RNA in the cytoplasm. More recent work by Kurnick ('50 a, b) has indicated that, if special handling of tissues to avoid depolymerization of nucleic acid is employed, methyl green stains selectively highly-polymerized DNA and pyronin stains preferentially both depolymerized DNA and RNA.

It seems, from these works, to be evident that the cytochemical methods with these basic dyes are available for the demonstration of the presence of nucleic acids in fixed sections of tissues. In the experiments reported here, the demonstration of depolymerized and highly-polymerized DNA and RNA in the chloroplasts of some higher plants was performed, using the staining methods with methyl green and pyronin, combined with ribonuclease digestion.

Experimental
Materials and Methods

The leaves of Selaginella Savatieri, Tradescantia fluminensis, and Rhoeo discolor were used in this work.

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The following five fixatives were tested: Cowdry's, Zenker's Helly's, Nawashin's, and Carnoy's. In the preliminary experiments with the root tips of *Allium cepa*, these fixatives seemed to cause depolymerization of DNA in the nucleus, so that it was stained red purple with methyl green and pyronin even after the ribonuclease digestion. Moreover, in all these fixative fluids except Carnoy's, the extraction of chlorophyll pigments from the leaf tissues was unsatisfactory. As a better one, 80% or 100% ethyl alcohol-glycerin (4:1) was suitable for the present purpose. It preserved nucleic acids well and showed no shrinkage of the cell.

After fixation for 48 hr at 40°-50°C, the materials were dehydrated, embedded in paraffin, and sectioned sagittally or transversally at 7-10 μ. The paraffin ribbon obtained from respective leaf tissue was cut into segments of two sections. Segments from a single leaf tissue were attached alternately to each of a pair of slides, one of which was treated with ribonuclease, trichloracetic acid, or perchloric acid etc., as will be mentioned below, while the other was given similar exposure to distilled water as a control.

Methyl green (Takeda's) was purified by successive extraction with chloroform to remove the considerable amount of crystal violet contained in the dye, and the removal of violet compounds was detected by paper partition chromatography. In staining with this dye, 0.25% solution of 0.2 M pH 4.1 acetate buffer, according to Kurnick ('50 a), was used.

Pyronin (Ishizu's) contained also a considerable amount of purple compound and a small amount of yellow substance. The purification of this dye was performed with butanol and acetone, according to the modified method of Shibatani ('49). Also in this case, the detection of these compounds was made by paper partition chromatography. In staining, 0.25% aqueous solution was used.

The method of staining used was as follows: (a) Staining with these dyes separately. (b) Double staining with methyl green and pyronin—The preparations were first stained in pyronin solution for 30 min, and after blotting with filter paper, they were then stained in methyl green solution of 0.2 M acetate buffer for the same period of time. They were blotted with filter paper, differentiated and dehydrated in tertiary butylalcohol for 2-3 hr, then clarified in xylol, and mounted.

In studying ribonuclease digestion of RNA, one of a pair of slides was placed in ribonuclease solution (0.3 mg./ml.) for 1-2 hr at

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1 The use of glycerin as fixative was suggested by Dr. O. Ichikawa, Medico-Biological Institute of Minophagen Pharmaceutical Co.

2 The sample of ribonuclease was obtained through the courtesy of Dr. O. Ichikawa.
60°C, while the other as a control was kept in distilled water for the same period of time and at the same temperature. Both slides were then rinsed in distilled water and stained with methyl green and pyronin, as described above.

Pollister and Ris ('47), Stein and Gerarde ('50) have reported that the treatment of sectioned material with 0.3 M trichloracetic acid should be successful in the extraction of nucleic acids without destroying the cellular structure. One slide was placed in 0.3 M trichloracetic acid at 90°C, washed in distilled water, and stained with methyl green and pyronin. The method of extraction of nucleic acids with perchloric acid was also employed (Erickson et al. '49). To remove RNA but not DNA, the slide material was treated with 10% perchloric acid for 20 hr at 4°C. To extract not only RNA but also DNA from the cell, 5% perchloric acid was used at an elevated temperature of 70°C. for 20 min.

Feulgen's reaction was used also to demonstrate DNA. The time of hydrolysis by 1 N HCl suitable for these materials was 15 min at 60°C.

**Results**

1. *With methyl green alone.* Chloroplasts stained blue-green and Feulgen's test was also positive. After treatment with trichloracetic acid, chloroplasts were unstained with methyl green, and Feulgen's test was negative. These facts show the presence of desoxyribonucleic acid.

2. *With pyronin alone.* Chloroplasts stained pink-red, and after treatment with ribonuclease for 2 hr at 60°C, chloroplasts still stained pink, although the color of staining became somewhat faint, showing the presence of the substance which was not digested with the enzyme and stained with pyronin, whereas chloroplasts were unstained by heating sections in trichloracetic acid solution.

3. *Double staining with methyl green and pyronin.* Chloroplasts stained red-purple, and after the ribonuclease digestion, chloroplasts stained light purple. Also in this case, chloroplasts did not stain by treatment with trichloracetic acid.

Kurnick ('50a) has observed that staining with pyronin, as well as methyl green, is a function of the state of polymerization of the nucleic acid substrates and depolymerized DNA, RNA, and RNP (ribonucleoprotein) stain similarly. In experiments with the root tips of *Allium cepa*, when the same fixative as the chloroplastic materials was used and similar treatment was applied, chromatin was stained blue-green and cytoplasm was unstained in the ribonuclease digestion, and thus depolymerization of DNA was not observed. Therefore, it is unlikely that the depolymerization of DNA was caused in the leaf tissue materials during the fixation or other treatments. It appears from these facts that depolymerized DNA, as well as highly-polymerized
DNA may originally localize in the chloroplasts.

So far as our present knowledge of cytochemical analysis with these basic dyes is concerned, it seems necessary to conclude that highly-polymerized and depolymerized DNA and perhaps RNA are localized in the chloroplasts of some higher plants. Although the presence of RNA is not yet decisive from this experiment, Menke ('38, '40) has suggested, as will be discussed below, that pentose nucleic acid might localize in chloroplasts from his analytical data of chloroplastic material.

Discussion

In recent years, Caspersson ('39) and Brachet ('40) have pointed out that there exists some link between the accumulation of nucleic acids (mainly ribonucleic acid) and protein synthesis from their localization in the cell, although the actual role played by nucleic acids in protein synthesis is not yet solved. Furthermore, the transformation of ribonucleic acid into desoxyribonucleic acid has been observed by Brachet's investigation in which the comparison of ribonucleic acid in cytoplasm between dividing and non-dividing cells was made, and also, the content of both type of nucleic acid was quantitatively estimated.

On the other hand, Schmidt et al. ('48) has more recently reinvestigated the Brachet's experiments and found that DNA increased but RNA did not decrease in the developing sea urchin embryo. Villee et al. ('49) has also observed that DNA increased with time whereas RNA remained constant or increased slowly in the same material and concluded by an experiment using P32 that a precursor to DNA was contained in the acid-soluble fraction and that DNA was not transformed from RNA. Furthermore, according to Marshak's investigation ('48), there is the precursor to both DNA and RNA which has properties in common with each in isolated nuclei from the liver of rats which was given P32, and in both mitotic and non-mitotic cells, the P32 might appear first in this precursor; later, in mitotic cells the P32 is accumulated in DNA, while in non-mitotic cells it passes into RNA of the cytoplasm. From this he suggested that plasmagenes and other cytoplasmic constituents containing nucleic acid cannot be independent of nuclear activity. Although further experimental work is needed in order to test these opposite hypotheses, these works, cited above, seem to provide us with many suggestions in regard with the origin of nucleic acids localized in chloroplasts.

According to Spiegelman and Kamen ('46), flow of phosphate from the ribonucleoprotein in yeast was found only when the cells were induced to synthesize protein, but in the rapidly metabolizing cells, such transfer of phosphate was not observed. Thus, these authors
have suggested that ribonucleoprotein can act as a specific energy
donator for the particular synthetic reactions of protein and that the
cytoplasmic self-duplicating entities as well as genes, plasmagenes,
and viruses etc. are these phosphorus containing proteins which have
the ability to transform and accumulate energy leading the reaction
to protein synthesis.

From this point of view, it would appear that chloroplasts of
higher plants and green algae are self-duplicating or autocatalytic
systems enclosed in the cytoplasm of the cells, and thus it seems
reasonable to consider that chloroplasts may contain nucleic acids as
well as various enzymes.

Menke ('38, '40) extracted phosphorus- and pentose-containing pro-
tein from the lipid-free chloroplast material of Spinacea with water
which was reprecipitated with acid, and suggested that this phosphorus
was contained in nucleic acid and thus nucleoprotein localized in chloro-
plasts. Later on, he also found strong absorption of grana with slices
from Selaginella grandis and Phaseolus multiflorus by 2750 Å, and
supposed that nucleic acid may contribute to its absorption. However,
considering the fact that both types of nucleic acids show strong
characteristic absorption in the ultra-violet light only near 2600 Å,
according to Caspersson ('39), there seems to remain some doubt con-
cerning Menke's description.

In the experiment reported here, not only ribonucleic acid but also
depolymerized and highly-polymerized DNA were demonstrated by the
cytochemical method—staining with basic dyes combined with ribo-
nuclease digestion and with Feulgen's reaction. These findings may
provide us with the following possibilities about the localization and the
physiological role of nucleic acids in chloroplasts: (a) some link bet-
ween the localization of nucleic acids and protein or enzyme synthesis
in the chloroplasts as a autocatalytic system; (b) some correlation
between photosynthetic reactions and nucleic acids in chloroplasts; (c)
or some probable relation between nucleic acids and the biosynthesis
of chlorophylls. Needless to say, these hypotheses mentioned above
must be checked experimentally.

Summary

The localization of depolymerized and highly-polymerized DNA and
RNA in the chloroplasts of some higher plants was demonstrated, using
cytochemical methods with methyl green and pyronin and Feulgen's
reaction, combined with ribonuclease digestion. The methods of the
extraction of nucleic acids from the sectioned materials with trichlor-
acetic and perchloric acids were also employed.
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LITERATURE CITED