NON-SPECIFIC INCORPORATION OF H^3-THYMIDINE INTO THE CHLOROPLASTS OF SPIROGYRA GREVILLEANA[1].

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In the last few years, DNA has been detected in the chloroplasts of a variety of species by several experimental techniques. [See reviews by Gibor and Granick (1964) and Iwamura, (1966).] One of the earliest reports of chloroplast DNA was based on autoradiographic observations of H^3-thymidine (H^3-TdR) incorporation into chloroplasts of Spirogyra sp. (Stocking and Gifford, 1959). In these experiments, however, suitable nuclease controls were not performed. We have repeated these experiments and have found that H^3-TdR is incorporated non-specifically in Spirogyra grevilleana and, therefore, is an inadequate method for demonstration of chloroplast DNA in this organism.

MATERIALS AND METHODS

Spirogyra grevilleana cultures, obtained from the culture collection of algae at Indiana University, were maintained in a soil-water medium (Starr, 1960). For light microscopy, filaments were fixed in ethanol-acetic acid (3:1) or formalin-propionic acid-ethanol for 2 hrs., embedded in paraffin and sectioned at 4μ, or squashed between slide and coverslip in 45% acetic acid followed by freezing in liquid nitrogen and removal of the coverslip.

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Some slides were stained by the Feulgen procedure while others were stained 30 min. in acridine orange (0.02 mg/ml. in 0.02 M acetate buffer at pH 4.5) and observed with standard Zeiss fluorescence equipment.

For autoradiography, filaments were incubated in 10 μC/ml. H^3-TdR (New England Nuclear Corp.) having a specific activity of 6.7 C/mM. The label was administered for 1, 6, 24, 48 or 96 hrs. at room temperature after which the cells were fixed, and embedded or squashed as described above. The slides were divided into five groups: "untreated", "DNase", "DNase buffer", "RNase" and "RNase buffer". The DNase group slides were incubated with deoxyribonuclease (Worthington, 1X crystallized, 0.3 mg/ml. in 4 x 10^{-3}M $MgSO_4$ at pH 7.0) while the DNase buffer group slides were incubated in the same solution without the enzyme. The RNase group slides were incubated with ribonuclease (Worthington, 1X crystallized, 0.3 mg/ml. in 0.1M phosphate buffer at pH 7.0) while the RNase buffer group slides were incubated in 0.1 M phosphate buffer at pH 7.0. All slides were incubated simultaneously at 38°C for 3 hrs. After washing, Kodak stripping film AR-10 was applied, and the slides were stored in black boxes at 4°C for 2 weeks and then developed in Kodak D19b.

To test the effectiveness of the deoxyribonuclease preparation, pieces of onion epidermis were fixed and treated with DNase or DNase buffer together with the corresponding slides of Spirogyra and then stained by the Feulgen procedure.

RESULTS

Examination of the autoradiographs of the squash preparations of the untreated group revealed that the cells were labelled in the cytoplasm as well as the nucleus, confirming the observations of Stocking and Gifford. The DNase-treated cells, however, showed about as much cytoplasmic label as the untreated or buffer-treated cells (Table 1). Since the possibility existed that the DNase preparation was inactive, a test was made on onion epidermis. examination of these slides showed that the enzyme did remove all of the Feulgen-positive material in the nuclei of these cells, and, consequently, it was concluded that this enzyme preparation was able to digest DNA. However,

since it was also possible that the enzyme may not have penetrated the thick cell walls of this alga, and in order to show that the label was actually

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN CELLS INCUBATED 96 HOURS IN H^3-THYMIDINE

Group	Average number of silver grains reduced in emulsion over cells*			% of total label over chloroplast
	Chloroplast	Nucleus	Non-plastid cytoplasm	
Untreated	1663	36	305	83
DNase	1510	15	320	82
DNase buffer	1526	37	354	79
RNase	768	19	207	77
RNase buffer	1608	31	287	84

*Average of three cells (squash preparations).

TABLE II

RANDOM SAMPLE GRAIN COUNTS IN SECTIONED MATERIAL

Group	Average grains per unit area*	
	24 hrs.	96 hrs.
Untreated	58	76
DNase	62	73
DNase buffer	59	73
RNase	37	55
RNase buffer	58	74

*Data obtained using an ocular containing a grid.

incorporated within the cells rather than simply adsorbed onto the surface or incorporated into surface-contaminating bacteria, sectioned material was examined. Label was found in the sections indicating that this was not a case of surface labelling. Differential grain counts on the sectioned material were not attempted, but rather, random samples of total grains were taken for each group (Table II). (Since nuclei occupy only a very small portion of the total cell volume, this analysis may be assumed to apply primarily to the cytoplasm. These data show that there was no significant difference in the total number of grains in the DNase treated samples when compared to the controls.

Examination of RNase-treated material revealed that there was 26-54% less chloroplast label than in untreated or RNase buffer-treated material (Tables I and II). A significant decrease in the number of grains was apparent over the nucleus and non-chloroplast cytoplasm as well.

Attempts to demonstrate the presence of DNA by the Feulgen reaction were unsuccessful since there was no detectable Feulgen-positive material in either the chloroplasts or the nuclei. Acridin orange fluorescence microscopy showed a definite yellow-green fluorescence in the nuclei of the cells, there was, however, no distinct fluorescence visible in the chloroplasts.

CONCLUSIONS AND DISCUSSION

The insensitivity to DNase of much of the label in our experiments, under conditions where the effectiveness of the enzyme is demonstrable, suggests that very little of the incorporated tritium is present in DNA. While the presence of nuclear DNA is confirmed by some DNase sensitive incorporation of tritium in the nuclei of Spirogyra, our inability to detect significant label reduction in the cytoplasm through DNase treatment implies the absence of detectably labelled DNA in the chloroplasts. The results of staining experiments with acridin orange are in agreement with the interpretation of the labelling data in that only nuclear DNA could be demonstrated.

Failure to demonstrate Feulgen-positive material in the nuclei of Spirogyra is difficult to explain. Perhaps the hydrolysis time in the Feulgen procedure

was not optimal for Spirogyra nuclear DNA. Similar negative results with the Feulgen reaction, however, have also been reported in Spirogyra by Stocking and Gifford (1959) and in Acetabularia by Brachet (1958a).

The grain count analyses of RNase digested cells indicate that considerable RNA labelling is present in both nuclei and chloroplasts. While the total nuclear label could be accounted for by the two nucleic acids, that in the cytoplasm cannot. The chemical form of the acid-insoluble, nuclease-resistant cytoplasmic label is not known. A reasonable explanation for RNA labelling with tritiated thymidine arises from the suggestion (Berech & van Wagtendonk, 1962; Brachet, 1958a,b), that thymidine may be converted to uridine.

The negative results on chloroplast DNA in Spirogyra reported here do not rule out the presence of DNA in these structures in this organism. They do imply, however, that if such DNA is present, tritiated thymidine labelling and Feulgen or acridin orange staining are inadequate methods for its demonstration.

These experiments further emphasize the need for proper nuclease controls to check the specificity of H^3-thymidine labelling, since H^3-TdR is not a specific label for DNA in all organisms.

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