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PROTEIN SYNTHESIS BY CHLOROPLASTS ISOLATED FROM GAMETOPHYTES OF THE FERN, *TODEA BARBARA**

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Abstract—Characteristics of the amino acid incorporating activity of chloroplasts isolated from gametophytes of the fern *Todea barbara* are described. Maximum incorporation of ^{14}C-amino acid mixture into chloroplasts is obtained in an incubation medium devoid of nucleotide triphosphates or an energy-generating system. Incorporation of the isotope is Mg-dependent. *In vitro* protein synthesis by isolated chloroplasts is relatively insensitive to RNase, DNase, actinomycin D, chloramphenicol and cycloheximide. Indirect evidence indicates that chloroplasts of the fern differ from other chloroplasts in maintaining their integrity under conditions where ordinarily chloroplasts are disrupted.

INTRODUCTION

GAMETOPHYTES of polypodiaceous ferns normally develop from filamentous protonema to two- and three-dimensional prothalli only if they are exposed to blue or white light. In red light or in darkness they continue to develop as cellular filaments.[1] Induction of prothallial growth and reversion to filamentous morphology by changes in the quality of light have been correlated with characteristic changes in the size and shape of the chloroplasts.[2] Promotion of the synthesis of structural proteins of the chloroplasts has been suggested as one of the effects of blue or white light.[3] Thus, the chloroplasts of ferns are of special interest for their structural and functional features associated with the effect of light quality on the change in morphology of the gametophytes.

In view of the apparent involvement of chloroplasts in light-induced morphogenesis of the gametophytes, a detailed investigation of the requirements of isolated chloroplasts for protein synthesis has been undertaken. Chloroplasts of several higher plants contain their own protein-synthesizing machinery[4–13] but little is known about the plastid protein-synthesizing system of lower plants, except of the algae.[14,15] Results of the present study

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show that chloroplasts isolated from fern gametophytes are unusual in having no requirement for external energy sources for protein synthesis and in being relatively insensitive to inhibitors of RNA and protein synthesis.

RESULTS

Characteristics of the Amino Acid Incorporating System

Chloroplasts isolated from gametophytes of *Todea barbara* incorporated ^{14}C-amino acid mixture rapidly (about 0·1 nmole of ^{14}C-amino acid mixture into protein/mg chlorophyll/hr). The rate of incorporation was linear up to at least 1 hr and in some experiments it extended up to 2 hr. This might indicate that integrity of the outer membrane of the chloroplasts is maintained for a long time. The level of activity of the plastid preparation was somewhat lower than that observed in the plastids isolated from gametophytes of *Pteridium aquilinum* grown in red light.[16] According to Ellis,[17] bean and tobacco chloroplasts incorporated about 0·5–1·0 nmole and 0·05–0·1 nmole, respectively, of ^{14}C-leucine/mg chlorophyll/hr; a somewhat higher rate of incorporation was reported for plastids isolated from *Phaseolus vulgaris* leaves.[18] However, in view of the different isotopes used, no direct comparison of the activity of fern chloroplasts with chloroplasts of angiosperms is possible.

The activity of the chloroplast preparation was also found to be linearly dependent upon the amount of chloroplast suspension added up to 30–35 μg/ml chlorophyll. The activity then decreased with higher plastid concentrations (Fig. 1).

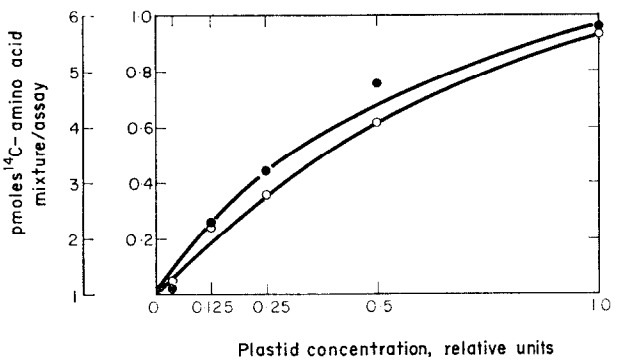

FIG. 1. INCORPORATION OF ^{14}C-AMINO ACID MIXTURE INTO CHLOROPLASTS AS A FUNCTION OF THE PLASTID CONCENTRATION.

Plastids were suspended in 200 μl of the grinding medium and 1·49 nmoles of ^{14}C-amino acid mixture was added to each tube. Relative unit 1 on the abscissa is equal to either 63 μg/ml of chlorophyll (○, ordinate 0–1·0) or 298·0 μg/ml of chlorophyll (●, ordinate 1–6).

Table 1 shows characteristics of the amino acid incorporating system. In contrast to the complex requirements for protein synthesis exhibited by chloroplasts isolated from other plants, maximum incorporation of the isotope into protein is obtained in the absence of both nucleotide triphosphates and an energy-generating system. Substances such as KCl and $(NH_4)_2SO_4$ which have been reported to enhance the activity of isolated chloroplasts,[7,9,12]

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TABLE 1. EFFECT OF EXOGENOUS COMPONENTS ON ^{14}C-AMINO ACID MIXTURE INCORPORATION INTO ISOLATED CHLOROPLASTS

Additives to the incubation medium	Incorporation (pmoles ^{14}C-amino acid mixture/mg chlorophyll)
None	296·4
ATP (0·2 μmole), GTP, CTP, UTP (0·01 μmole each), PEP (1·0 μmole) and pyruvate kinase (126 mμg protein) (Complete reaction mixture)	137·5
Complete reaction mixture + $MgCl_2$ (125 μmole)	121·6
Complete reaction mixture + $(NH_4)_2SO_4$ (12·5 μmole)	140·2
Complete reaction mixture + KCl (10·0 μmole)	110·3
GTP, CTP and UTP	196·3
PEP and pyruvate kinase	246·7
ATP, PEP and pyruvate kinase	194·9

The incubation medium had 100 μl tricine buffer (pH 8·2), 250 μl chloroplast suspension containing 57·2 μg/ml chlorophyll and 2·38 nmoles of ^{14}C-amino acid mixture. When exogenous components were added, total volume of the incubation medium was kept constant by correspondingly reducing the volume of the buffer.

were inhibitory or not effective when added to the complete reaction mixture. Least inhibition was obtained when the incubation medium contained only phosphoenolpyruvate (PEP) and pyruvate kinase.

Since the compounds listed in Table 1 were originally added at a fixed concentration to the incubation medium it seemed possible that the concentrations used were inhibitory to the system. To eliminate this possibility, a range of concentrations of the nucleotide triphosphates, PEP and pyruvate kinase was added to the incubation medium and the activity of isolated chloroplasts studied (Table 2). ATP, GTP, UTP and combinations of PEP and pyruvate kinase even at the lowest concentrations tested did not promote incorporation of C^{14}-amino acids into proteins. At higher concentrations all of them were consistently inhibitory. Addition of 0·001–0·01 μmole of CTP caused a small increase in the protein synthesizing activity of the chloroplasts. It thus appears that both exogenous nucleotide triphosphates, with the possible exception of CTP, and an ATP-generating system do not participate in the synthesis of proteins in isolated chloroplasts of fern gametophytes. Similar results have been obtained with chloroplasts isolated from the gametophytes of *Pteridium aquilinum*, using ^{14}C-amino acid mixture[16] and ^{14}C-leucine[19] as precursors.

Effect of Magnesium

The addition of $MgCl_2$ to the reaction mixture inhibited amino acid incorporation into chloroplasts (Table 1). In this experiment chloroplasts were prepared in a medium containing 5 mM $MgCl_2$ and apparently an excess of $MgCl_2$ was inhibitory. The addition of exogenous $MgCl_2$ to the incubation medium of chloroplasts prepared in a medium lacking $MgCl_2$ led to a significant increase in activity. Maximum activity was obtained using 0·625–1·25 μmole $MgCl_2$/assay (Fig. 2). The extent of Mg^{2+} dependency of this system was best seen when

[19] V. RAGHAVAN, Unpublished data.

TABLE 2. EFFECT OF A RANGE OF CONCENTRATIONS OF NUCLEOTIDE TRIPHOSPHATES AND AN ENERGY-GENERATING SYSTEM ON THE INCORPORATION OF ^{14}C-AMINO ACID MIXTURE INTO ISOLATED CHLOROPLASTS

Additives to the incubation medium	Concentration (μmoles)	Incorporation (pmoles ^{14}C-amino acid mixture/ mg chlorophyll)
ATP	0	258·3
	0·02	225·0
	0·10	175·9
	0·20	124·2
	0·50	58·2
	1·0	64·0
	1·0	12·7
GTP	0	250·7
	0·001	238·5
	0·01	170·2
	0·05	180·4
	0·10	141·5
CTP	0	211·3
	0·001	219·1
	0·005	244·9
	0·01	220·0
	0·05	136·2
	0·10	100·7
UTP	0	212·8
	0·001	185·2
	0·005	167·3
	0·01	182·8
	0·05	178·6
	0·10	115·8
PEP	0	
Pyruvate kinase*	0	193·4
PEP	0·1	
Pyruvate kinase	12·6	170·2
PEP	1·0	
Pyruvate kinase	126	165·3
PEP	10·0	
Pyruvate kinase	1260	89·5

* The concentration of pyruvate kinase is expressed as mμg protein/assay.

The incubation medium contained 100 μl chloroplast suspension and 1·49 nmoles ^{14}C-amino acid mixture and either 100 μl tricine buffer (pH 8·2) (for ATP, GTP, CTP and UTP) or 200 μl tricine buffer (for PEP-pyruvate kinase). Chloroplast extract was added at chlorophyll concentrations of 85·3 μg/ml (for ATP and GTP) and 116 μg/ml (for CTP, UTP and PEP-pyruvate kinase).

5 mM $MgCl_2$ was added to the grinding medium. This led to a more than doubling of amino acid incorporation activity of isolated chloroplasts, compared to plastids prepared in a medium lacking Mg^{2+}.

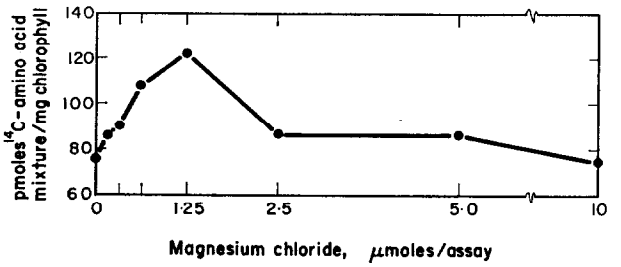

FIG. 2. EFFECT OF ADDITION OF DIFFERENT CONCENTRATIONS OF $MgCl_2$ TO THE INCUBATION MEDIUM ON THE INCORPORATION OF ^{14}C-AMINO ACID MIXTURE INTO CHLOROPLASTS.
Incubation mixture contained 250 μl of chloroplast suspension (37·2 μg/ml chlorophyll), 100 μl tricine buffer (pH 8·2) and 2·38 nmoles of ^{14}C-amino acid mixture.

Effect of Nucleases and Inhibitors

The effect of a range of concentrations of nucleases and inhibitors of RNA and protein synthesis on the activity of isolated chloroplasts is shown in Table 3. The system was virtually insensitive to DNAse, RNAse, chloramphenicol and cycloheximide (maximum inhibition 20 per cent of the control), whereas high concentrations of actinomycin D

TABLE 3. EFFECT OF INHIBITORS OF PROTEIN AND RNA SYNTHESIS ON THE INCORPORATION OF ^{14}C-AMINO ACID MIXTURE INTO ISOLATED CHLOROPLASTS

Concentration (mg/l.)	Incorporation (pmoles ^{14}C-amino acid mixture/mg chlorophyll)				
	Actinomycin D	Chloramphenicol	Cycloheximide	DNAse	RNAse
0	80·7	249·7	135·7	137·1	131·9
10	73·6	—*	136·3	—*	102·9
100	71·8	235·4	104·8	123·3	112·7
200	61·5	218·0	119·1	126·5	109·0
400	54·1	199·9	110·8	121·4	106·9

The incubation medium contained 100 μl chloroplast suspension, 50 μl tricine buffer (pH 8·2) containing the inhibitors and 1·49 nmoles of ^{14}C-amino acid mixture. The concentrations of chlorophyll in the plastid extracts for these experiments were (μg/ml), 147·3 (actinomycin D), 85·3 (chloramphenicol), 136·0 (cycloheximide and DNAse) and 100·7 (RNAse).

* Not determined.

inhibited chloroplast activity by about 34 per cent. Preliminary experiments indicated that preincubating the chloroplasts with actinomycin D or chloramphenicol for 2 hr before adding the isotope did not enhance the inhibitory effect of the antibiotics. Using DNAse and RNAse, maximum inhibition was obtained with low concentrations of the enzymes and higher concentrations were without further effect.

Contribution to Chloroplast Activity by Bacteria, Nuclear Material, Whole Cells and Cytoplasmic Particles

Bacterial contamination of the chloroplast preparation appeared unlikely because of the absence of noticeable bacterial colonies upon plating the reaction mixture. However, in view of the unusual properties of the amino acid incorporating system, we thought it necessary to

further investigate the possibility that contaminating bacteria, nuclear material or particles lighter than chloroplasts in some way contributed to activity in the assays.

The nonionic detergent Triton X-100 has been reported to solubilize the lamellar structure of chloroplasts leaving nuclei, bacteria and whole cells unaffected.[6,9,11] As seen in Table 4, the amount of radioactivity in 1000 *g* pellet obtained after Triton treatment due to contaminating bacteria, whole cells and nuclear material was only 2·8 per cent of the total incorporation, while radioactivity in 85,000 *g* pellet was 3·6 per cent of the total. The difference in radioactivity between the 85,000 *g* and 1000 *g* pellets is considered to be due to particles lighter than chloroplasts.

The addition of 0·1 mM KCN to the incubation medium had no inhibitory effect on amino acid incorporation (Table 4). The results indicate that this typical respiratory poison

TABLE 4. EVALUATION OF THE CONTRIBUTION OF BACTERIA, WHOLE CELLS, NUCLEAR MATERIAL AND PARTICLES LIGHTER THAN CHLOROPLASTS ON THE INCORPORATION OF ^{14}C-AMINO ACID MIXTURE INTO ISOLATED CHLOROPLASTS

Treatment	Incorporation (counts/min/mg chlorophyll)
Untreated control	29,266
1000 *g* Triton X-100 pellet	814
85,000 *g* Triton X-100 pellet	1048
Control*	17,554
Control + 0·1 mM KCN	17,202

Incubation medium contained 250 μl chloroplast suspension (49·6 μg/ml. chlorophyll), 100 μl tricine buffer (pH 8·2) and 1·19 nmoles of ^{14}C-amino acid mixture.

* Results from a different series of experiment, using chloroplast suspension containing 29·6 μg/ml chlorophyll.

did not reduce chloroplast activity and reinforce the conclusion that in our system chloroplasts are primarily responsible for the incorporation of amino acids into proteins.

DISCUSSION

Since a purified chloroplast preparation was used in this study the extent of contaminating nuclear material should have been negligible. Using Triton X-100 to solubilize the chloroplasts and including KCN in the incubation medium bacteria, whole cells, nuclei and particles lighter than chloroplasts could be eliminated as contributing to the observed incorporation.[6,9,11,12,20] There is thus convincing evidence that the amino acid incorporating activity of the fraction studied is due primarily to the chloroplasts and not to other organelles which may be present.

The characteristics of the amino acid incorporating activity of chloroplast preparation from gametophytes of *Todea barbara* contrast sharply with those reported for similar preparations from other sources with respect to cofactor requirements and sensitivity to nucleases and inhibitors of RNA and protein synthesis. A strong dependence on exogenous

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ATP, alone or together with GTP or an energy donor has been reported for plastid preparations from *Euglena gracilis*,[14,21] spinach,[7] wheat,[9,12] bean,[11,17] *Lemna*[13] and tobacco (*Nicotiana tabacum*, *N. tabacum* var. Turkish Samsun and *N. glutinosa*).[6,17] However, addition of ATP did not markedly stimulate protein synthesis in isolated chloroplasts of anucleate *Acetabularia*[15] and tobacco (*N. tabacum* var. White Barley)[22] and produced only marginal stimulation in the chloroplasts of tomato leaves.[10] Parthier and Wollgiehn,[4] Parthier[5] and Stephenson *et al.*[22] have reported incorporation of labelled precursors by isolated chloroplasts in the absence of cofactors in the incubation medium and our results are in general agreement with their findings.

Failure of exogenous nucleotides and an energy-generating system to promote protein synthesis in isolated plastids might reflect the retention of high endogenous precursor and cofactor pools within the organelles; as pointed out below, this is probably due to preservation of the integrity of chloroplast membrane for long periods. Rupture of the chloroplast membrane and dilution of the endogenous energy-donors may thus account for the dependence of plastid preparations on ATP and other energy-generating systems for protein synthesis. Hall and Cocking[10] have suggested that the lack of sensitivity to ATP may be an indication of high ATPase activity, but there is no rigorous proof of this point. Dependence on the availability of ATP for effective amino acid incorporation in plastid preparations has frequently been cited as evidence against bacterial contamination.[6,9,17,20] However, nondependence on ATP for protein synthesis by isolated chloroplasts does not necessarily indicate that the preparation is contaminated with bacteria, as seen from the results of the present study using a cell-free system prepared and incubated under aseptic conditions.

Chloroplast preparations from several plants exhibit varying degrees of sensitivity to nucleases and inhibitors of RNA and protein synthesis. RNAse was extremely inhibitory to protein synthesis in isolated chloroplasts of tobacco,[6,17] spinach,[7] bean,[17,23] *Euglena*,[21] while activity of the chloroplasts of *Acetabularia* was not affected by the enzyme.[15] Chloroplasts of tobacco, spinach and *Euglena* were only slightly sensitive to DNAse and actinomycin D[6,7,21] but the latter was a potent inhibitor of protein synthesis in the chloroplasts of *Acetabularia*.[15] In testing a range of concentrations (10–400 mg/l.) of RNAse, DNAse, chloramphenicol, actinomycin D and cycloheximide we found that protein synthesis in the chloroplasts of *Todea barbara* gametophytes was not appreciably inhibited by any of these substances. The marginal sensitivity of the plastids to chloramphenicol, while puzzling, is not without precedent. Gametophytes of *Dryopteris filix-mas* exhibited normal prothallial growth in the presence of the drug, although size of the plastids was reduced due to inhibition of protein synthesis.[24] The observation that the synthetic activities of the plastids are only partially affected by chloramphenicol suggests that the drug inhibits the synthesis of only the structural proteins of the chloroplasts.

Results obtained by Sissakian *et al.*[8] and Margulies *et al.*[23] suggest that the chloroplast membrane presents a barrier to the diffusion of exogenous substrates. In seeking to explain the effect of inhibitors of RNA and protein synthesis on the activity of the chloroplasts, it is necessary to consider the extent to which integrity of the chloroplast membrane is preserved during the assay. Examination of the chloroplasts in the light microscope showed that their membranes were intact even after several hours in the incubation medium. Additional

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[23] M. M. Margulies, E. Gantt and F. Parenti, *Plant Physiol.* **43**, 495 (1968).
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observations have also suggested that membranes of fern plastids are unusually resistant to treatments which normally disrupt plastids of other plants. For example, while a single treatment with 5% Triton X-100 solubilizes chloroplasts of angiosperms[6,9,11] we have found that treatment with the detergent three times is necessary for complete disruption of *Todea* plastids. In unpublished work on the extraction of DNA from chloroplasts, DeMaggio[25] found that plastids of this species ruptured only if they were pretreated with 9M ammonium acetate and transferred to buffer, while plastids of other plants ruptured normally in buffer without any pretreatment. Finally, Gantt and Arnott[26] have demonstrated that plastids of the gametophytes of *Matteuccia struthiopteris* are unique in showing a new type of division involving invagination of the inner membrane while the outer one remains intact. These observations, although indirect in their manifestation, lead to the conclusion that we are dealing with plastids having unusual membrane properties. Further studies are currently under way to elucidate the nature of the membranes of chloroplasts of fern gametophytes.

EXPERIMENTAL

Isolation of chloroplasts. The detailed procedures for initiating cultures of *Todea barbara* and maintaining them *in vitro* have been described previously.[27] Mature gametophytes growing aseptically on a solidified mixture of mineral salts, trace elements and 1% sucrose[28] were used for these studies. Cultures were propagated by transferring pieces of mature gametophytes to the surface of the medium. Plants were allowed to grow for 3–4 months in a growth chamber at 25° and supplied with 12 hr daily illumination provided by fluorescent tubes and incandescent bulbs giving *ca.* 10,000 ergs/cm²/sec at the level of the cultures. Chloroplasts were isolated by grinding gametophytes with mortar and pestle in a modified Honda medium[6] consisting of 5 mM $MgCl_2$, 8 mM mercaptoethanol, 0·25 M sucrose, 2·5% ficoll, 6% dextran and 2·5 mM tricine, adjusted to pH 8·2. The homogenate was passed through 8 layers of cheesecloth and the remaining cellular material was removed by centrifugation at 770 *g* for 1 min. The supernatant cell-free extract was centrifuged at 4000 *g* for 15 min to sediment the chloroplasts. The pelleted chloroplasts were washed once with fresh grinding medium and recentrifuged at 4000 *g* for 15 min. They were then suspended in a small volume of grinding medium and layered over an equal volume of grinding medium and 0·25 M sucrose. The gradient was centrifuged at 480 *g* for 1 min to remove contaminating nuclear material and other heavy particles. The supernatant was used as the chloroplast extract. All procedures were carried out at 4°.

Assay system. Amino acid incorporation was performed by adding 0·25–0·5 μc (1·19–2·38 nmoles) of uniformly labelled reconstituted ^{14}C-amino acid mixture (specific activity 200–210 mC/mM; New England Nuclear or Searle–Amersham Corporation) to 100–250 μl of the chloroplast extract and incubating in the dark for 1 hr at 25°. When the effects of various components on the activity of the chloroplasts were studied, each substance was dissolved in 10–15 μl of tricine buffer (pH 8·2) and added to the reaction mixture. Incubation was terminated by adding 1 ml of 10% trichloroacetic acid (TCA). After standing for 15 min at 4° the TCA-insoluble fraction was sedimented at 12,000 *g* for 10 min. The pellet was suspended in 2·5 ml of 5% TCA containing 0·1% casamino acids and incubated in a water bath for 15 min at 98°. The precipitate was transferred to glass fibre filter discs (2·1 cm dia.) and washed 5 times with TCA–casamino acid mixture. Preliminary runs indicated that no further radioactivity eluted from the filters after 5 washings with TCA–casamino acid mixture. The discs were dried and placed in scintillation vials containing 5 ml of toluene-based scintillation fluid ('Liqueflour', New England Nuclear). Radioactivity was measured in a Nuclear-Chicago liquid scintillation counter. Quenching was evaluated by the channels ratio method and all counts were corrected to 70 per cent efficiency. Chlorophyll was determined spectrophotometrically on 80% acetone extracts.[29]

Assays were conducted in duplicate and controls included in each experiment had ^{14}C-amino acid mixture or chloroplast suspension added at the end of the incubation period. Results given in the text are the average of both samples. There was some variation in the activity of the chloroplasts in different experiments probably due to differences in the age of the plants used.

Triton X-100 treatment. In one experiment, chloroplasts were incubated with ^{14}C-amino acid mixture the

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usual way. At the end of the incubation period, 2 ml of 5% Triton X-100 containing 0·1% casamino acids was added and the reaction tubes placed at 4° for 20 min before centrifuging for 15 min at 1000 *g*. The supernatant was discarded and the pellet was twice treated with Triton, centrifuged and the supernatant discarded each time. The small white pellet (1000 *g* pellet) remaining was suspended in 0·5 ml of a 1 mg/ml bovine serum albumin solution. One ml of 10% TCA was added and the precipitate prepared for counting as described before. In another experiment, after Triton treatment the mixture was centrifuged in a Spinco SW 56 rotor at 85,000 *g* for three 90-min periods and the supernatant was discarded each time. The pellet (85,000 *g* pellet) was washed and prepared for counting.

Precautions against bacterial contamination. All glassware used were steam-sterilized. Reagents, including the grinding medium, were freshly prepared for each experiment and cold-sterilized through Millipore filters. All operations, except centrifugations, were done in a transfer room previously illuminated with UV sterilamps. In this way bacterial contamination was eliminated as revealed by occasional plating of the incubation medium on nutrient agar.

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