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Movement of Carbon from Vegetative Cells to Heterocysts in *Anabaena cylindrica*

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Radioactive carbon assimilated by vegetative cells of *Anabaena cylindrica* in the light passed via an intrafilamentous route into heterocysts in the dark. After several hours, label per heterocyst approximated label per vegetative cell. Much of the label entering heterocysts was not available for diffusional exchange back into vegetative cells.

Heterocysts of the filamentous blue-green alga *Anabaena cylindrica* Lemm. induce sporulation of adjacent vegetative cells (9, 10) and inhibit further heterocyst formation in their vicinity (11). As one approach to elucidation of the mechanisms underlying these developmental phenomena, the attempt has been made to determine if there are intercellular chemical interactions in this alga. Heterocysts might act (among other possibilities) by producing active substances or by serving as metabolic sinks, e.g., for photosynthate.

Baumgärtel (2) pointed out that heterocysts, being little pigmented, presumably absorb photosynthate from adjacent vegetative cells. This idea is consistent with observations of Fay and Walsby (5) that isolated heterocysts have much lower CO_2-fixing capability than vegetative cells but absorb more oxygen, per unit of nitrogen, than do vegetative cells. However, since certain reserve products appear to be consumed during heterocyst development (8), since the wall of heterocysts, reported to contain cellulose (6), might serve as respiratory substrate, and since the membranous structures occurring in heterocysts (4, 8) might permit photosynthetic assimilation of carbon and energy sufficient for maintenance of a nongrowing cell, the validity of Baumgärtel's suggestion has remained uncertain.

This paper demonstrates the movement of photosynthetically fixed carbon from vegetative cells to heterocysts. This movement is through the filaments rather than via the medium, and it does not represent merely exchange diffusion.

MATERIALS AND METHODS

A. cylindrica was grown axenically in 12-liter batches of the medium of Allen and Arnon (1) in illuminated fermentors (New Brunswick Scientific Co., New Brunswick, N. J.; *ca.* 2% CO_2 in air, 4 to 6 liters/min at 25 C), to a density of 1 to 3 mg (dry weight) per ml. Harvested algae were either concentrated [to $\leq$ 7 mg (dry weight) per ml] by centrifugation at 6,975 $\times$ *g* for 10 min or, if filaments were to be autoradiographed, were largely freed of mucilage by centrifugation at 48,200 $\times$ *g* for 20 min, followed by resuspension in fresh medium. Suspensions, dispersed into glass-stoppered Erlenmeyer flasks, stirred by magnetic stirrers, and cooled to about 18 C by flowing tap water were preilluminated for 1 to 2 hr by a fan-cooled 500-w "photo-ect" GE lamp. The subsequent program consisted of addition of $Na_2{}^{14}CO_3$ (20 mc/mmole, 100 μc/ml; New England Nuclear Corp., Boston, Mass.), or $Na_2{}^{12}CO_3$, or both, 5-min illumination with the same light source, addition of a 1,000-fold excess of unlabeled 1 M $NaHCO_3$ in H_2O, dark incubation (stirred, and cooled with flowing tap water as before), and removal of portions for processing.

To obtain autoradiographs of whole filaments, 1-ml samples from 100-ml algal suspensions were washed free of radioactivity by successive 100-ml rinses of chilled Allen and Arnon (1) medium on 0.45-μm pore size membrane filters (Millipore Corp., Bedford, Mass.); filtration was such that the algae remained bathed in medium. The final algal suspensions were streaked on microscope slides or cover glasses with glass rods, frozen on dry ice, lyophilized without thawing, and coated with Kodak NTB-3 (7) or Ilford L4 (3) nuclear emulsion.

Heterocysts were separated from vegetative cells essentially by the method of Fay and Walsby (5), using a Sorvall RF-1 cell fractionator at 13,000 psi. Vessels to receive the effluent contained a volume of distilled water equal to approximately five times the volume of algal suspensions being processed. In addition, distilled water (flowing at approximately 140 ml/min) was added as diluent through the thermocouple orifice of the Ribi valve. Partially purified heterocysts were centrifuged in a solution of density of 1.4 g/ml (61.8 g of CsCl per 100 ml of water; 16,300 $\times$ *g*, 20 min). The supernatant portion was diluted to a (solution) density of 1.3 g/ml, and recentrifuged as before. The resultant pellet, containing heterocysts highly purified of contaminating wall

fragments, small particles and occasional spores ($\rho > 1.4$), and mucilaginous materials and infrequent vegetative cells ($\rho < 1.3$) found along with heterocysts before the purification steps based on density, was then washed and rinsed with distilled water (Fig. 1).

Purified heterocysts were dispersed in distilled water, and about 0.1-ml portions were counted in a Beckman CPM-100 scintillation counter, in Beckman's "Cocktail D." To determine specific radioactivity, about 0.2- to 0.3-ml portions were filtered onto tared 0.45-μm pore size membrane filters (Millipore Corp.), dried at 50 C, and weighed on a Cahn Electrobalance, model M-10. To determine counts per minute of ^{14}C fixed per milligram of alga, the algal suspension was diluted 10-fold. Quantities of 0.1 ml were then filtered, the filters were assayed for radioactivity in the scintillation counter, and quantities of 0.5 or 1.0 ml were filtered, dried, and weighed. All determinations were carried out in duplicate.

The per cent of label present in isolated heterocysts and not exchangeable by diffusion was assumed to be at least as great as the proportion of the total radioactivity that could be removed by filtration following sonic disruption. The counts per minute per milligram were determined for portions filtered from a suspension of whole heterocysts. The remainder of the suspension was cavitated with a Branson Sonifier model S-125 (setting 4 to 5) until at least 95% of the heterocysts present were disrupted, as determined with an eosinophil counter. Portions were then filtered through 0.22 μm-pore size membrane filters (Millipore Corp.), and the radioactivity on the filters was measured.

Small drops of dilute suspensions of "^{14}C," "$\frac{1}{2}$ ^{12}C + $\frac{1}{2}$ ^{14}C," etc., heterocysts were placed on marked positions on microscope slides and dried. The slides were coated with Kodak NTB-3 nuclear emulsion (7), exposed for several weeks at low temperature, and developed with Kodak Microdol X developer. Grains within one short heterocyst-diameter of heterocysts well spaced from other heterocysts were then counted.

Each of the experiments reported was performed for a minimum of three times, with concordant results.

RESULTS

Whole filaments from 100-ml algal suspensions labeled with 40 μc of $Na_2{}^{14}CO_3$, prepared so as to prevent loss of radioactivity, were autoradiographed with either a relatively thick (*ca.* 5 μm) coat of Kodak NTB-3 or a relatively thin [*ca.* 0.2 μm or less (3)] coat of Ilford L4 emulsion. By use of NTB-3 emulsion, it was observed (Fig. 2a, b, c) that 3 to 4 min after filaments were labeled, heterocysts showed very little radioactivity relative to adjacent vegetative cells. After 3 to 4 additional hr in the dark, most of the heterocysts showed approximately the same radioactivity as the vegetative cells in the same filaments. Although heterocysts infrequently still displayed less radioactivity than attached vegetative cells, many heterocysts (and these were frequently immature) had significantly more radioactivity than the vegetative cells in the same filaments (Fig. 2d, e, f). Similar results (not illustrated) were obtained with Ilford L4 emulsion.

To ascertain if the increase in heterocyst-label during dark incubation was due to assimilation from the medium, an algal suspension was "pulsed" with 40 μc (2 μmoles) of $Na_2{}^{14}CO_3$, while a second, like suspension received a corresponding amount (2 μmoles) of unlabeled sodium carbonate. After 5 min, 2 mmoles of unlabeled $NaHCO_3$ was added to each of the two suspensions. Immediately thereafter, a third suspension was constituted by mixing one-third (33.3 ml) of each of the two original suspensions. The three suspensions were then incubated for about 4 hr in the dark. Autoradiographs (NTB-3 emulsion) showed that, whereas all filaments from the ^{14}C-flask (Fig. 3a) and none of the filaments from the ^{12}C-flask were labeled, only about 50% of the filaments and their heterocysts in the third flask (Fig. 3b) were significantly labeled.

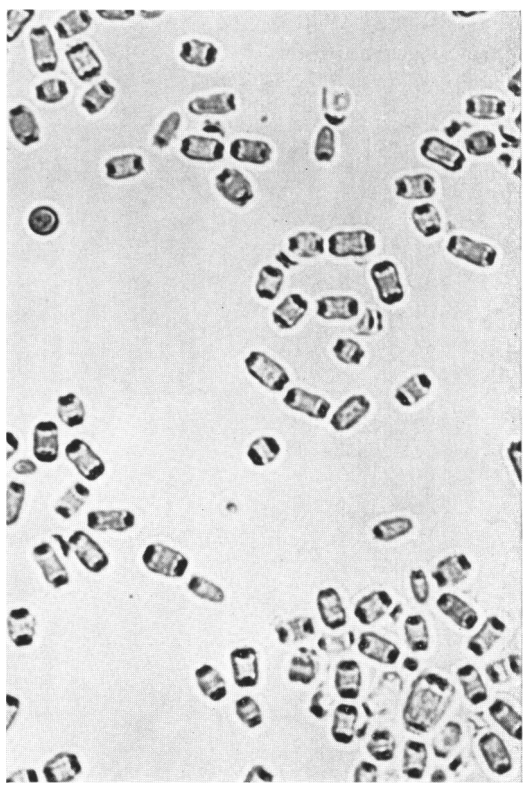

FIG. 1. *Heterocysts, isolated by a modification of the method of Fay and Walsby (5) and highly purified of contaminating materials by a procedure based principally on density differences.* × *880.*

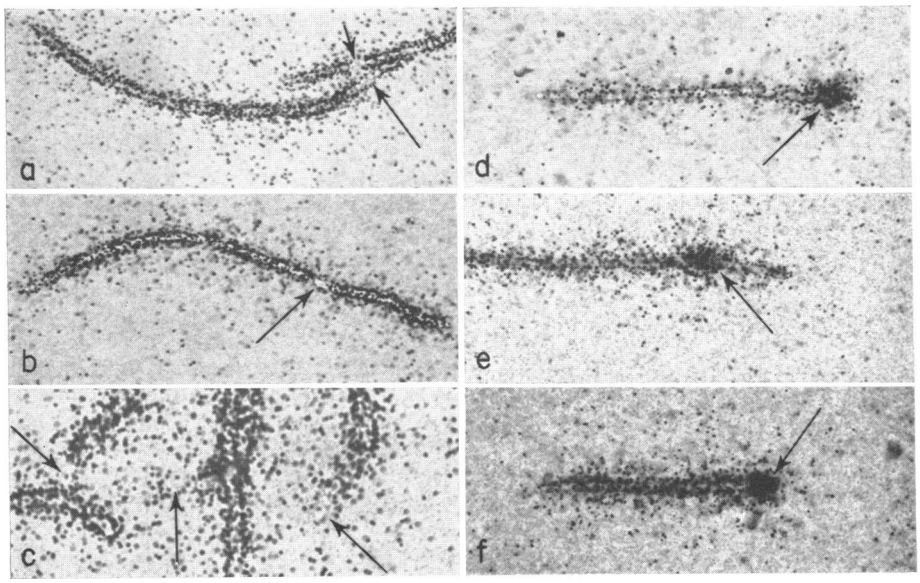

FIG. 2. *Autoradiographs (Kodak NTB-3 emulsion) of short filaments of A. cylindrica following a short exposure to* $Na_2{}^{14}CO_3$ *in the light. (a, b, c) Filaments frozen, following washing, within 3 to 4 min after photosynthetic fixation of* ^{14}C *show very little label in heterocysts (at positions marked by arrows) relative to adjacent vegetative cells. (d, e, f) After 3 to 4 additional hr in the dark, heterocysts (arrows) show radioactivity about equal to, and often (as is illustrated) significantly more than, contiguous vegetative cells. (a, b, d, e, f)* × *330. (c)* × *530.*

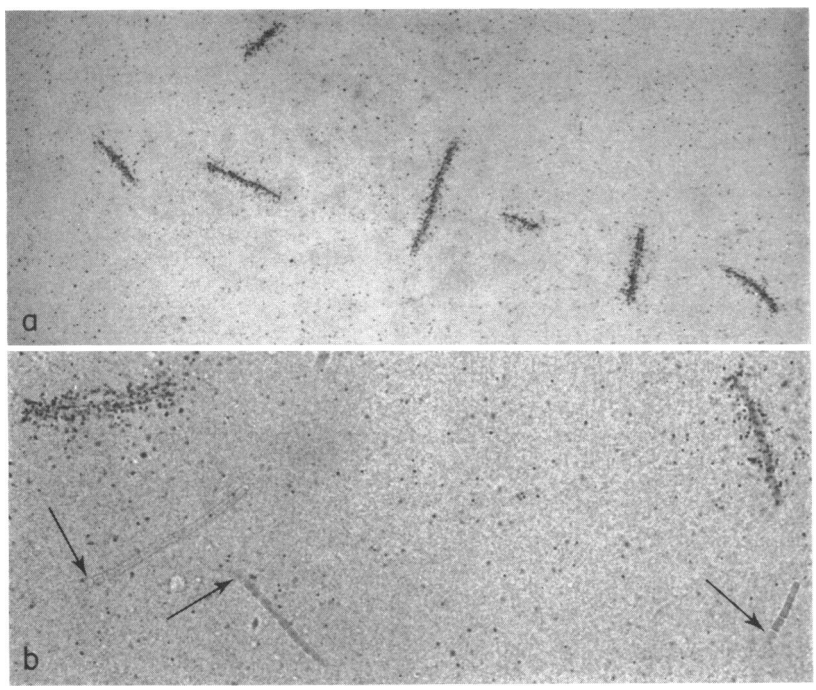

FIG. 3. *Autoradiographs (a) of filaments which have fixed* ^{14}C *and (b) of a mixture, incubated together, of such filaments with others which have fixed* ^{12}C. *There appears to be no transfer of label from radioactive to initially nonradioactive filaments; in particular, heterocysts (arrows) of the latter filaments have not become labeled. (a)* × *170. (b)* × *330.*

The above results are indicative of direct transfer into heterocysts of label fixed by vegetative cells. To determine the kinetics of transfer and the state of the label in the heterocysts, labeled heterocysts were isolated.

Following 2 hr of preillumination of 350 ml of algal suspension in each of a pair of flasks, one of the two flasks was wrapped in aluminum foil, and then 100 μc (5 μmoles) of $Na_2{}^{14}CO_3$ solution was added to each of the flasks. Following 5 min of additional illumination of the unwrapped flask and addition of 5 mmoles of $NaH^{12}CO_3$ to both flasks, the flask which had been exposed to ^{14}C in the light was wrapped in aluminum foil. At intervals thereafter, 55-ml volumes were removed from each of the flasks, and the heterocysts were isolated. The counts per minute per milligram of heterocysts were assayed and plotted as a function of the time interval between the middle of the 5-min exposure to the undiluted-^{14}C "pulse" and the midpoint of cell fractionator processing (*ca.* 2 min per 40 ml processed). The results of a typical experiment are presented in Fig. 4. They demonstrate that labeling of heterocysts is strongly stimulated by light, but that (as has already been observed) the bulk of the label appears in heterocysts in the dark following pulse-labeling of the alga in the light. Within about 20 min, the specific activity of isolated heterocysts increased to about one-half the level attained after 3 hr. During the 3.25-hr period of the dark incubation, the specific activity of algae filtered from the suspensions exposed to ^{14}C in the light and in the dark decreased from 147,300 to 92,400 counts per min per mg and from 26,600 to 17,000 counts per min per mg, respectively.

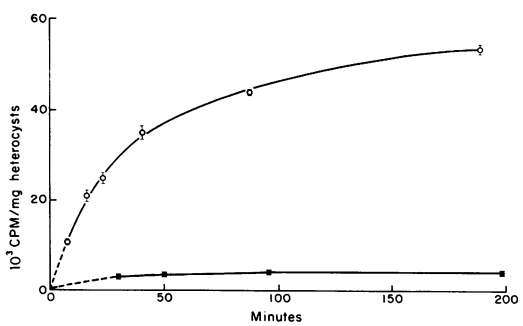

FIG. 4. *Specific radioactivity of heterocysts from algae exposed to ^{14}C in the light (○) or dark (●) following a 2-hr preillumination, as determined for heterocysts isolated at the indicated times during a subsequent dark incubation. During this period, specific activity of algae filtered from the two suspensions decreased from 147,300 to 92,400 counts per min per mg and from 26,600 to 17,000 counts per min per mg, respectively.*

In a different experiment, 144 ml of algal suspension was preilluminated 1 to 2 hr in each of three flasks. These were then supplemented with 1 ml (5 μmoles) of sodium carbonate, of which none ("^{12}C"), one-half ("½ ^{12}C + ½ ^{14}C"), or all ("^{14}C") was from a 20 mc/mmole sample of $Na_2{}^{14}CO_3$. After 5-min illumination and addition of 5 mmoles of $NaHCO_3$ in 5 ml of water to each flask, (A) a combination of 50 ml of "^{14}C" suspension plus 50 ml of "^{12}C" suspension and (B, C, D) 100 ml of each of the three original suspensions were incubated in the dark. About 3 to 3.5 hr later, heterocysts were isolated from each of the four suspensions (A, B, C, D), and their specific radioactivity was determined. In addition, the frequency distribution of grains per heterocyst was obtained from an autoradiograph of heterocysts from the several suspensions (Fig. 5).

Following sonic disruption of 95 to 97% of heterocysts labeled and isolated as above, more than one-half of the label present in the heterocysts could be recovered by an 0.22-μm pore size filter. In a typical experiment, a suspension of 2.7×10^6 heterocysts/ml, 5,860 counts per min per ml (filtered), was cavitated until only 4% of the heterocysts remained intact. Of the original radioactivity, 3,940 counts per min per ml or approximately 67%, could still be filtered from suspension.

DISCUSSION

The results of the filament-autoradiography studies show that heterocysts become labeled after contiguous vegetative cells, and that this label is not assimilated from the medium. It thus appears that label fixed by vegetative cells moves *through* filaments into heterocysts.

Results with isolated heterocysts agree with the above results as follows: (i) at early times there is little label in heterocysts, whereas after several hours, the heterocysts contain about one-half as much ^{14}C per milligram, or (in view of the greater cell mass of the thick-walled heterocysts) roughly the same amount of ^{14}C per cell, as do the vegetative cells; (ii) the label appearing in isolated heterocysts was not assimilated from the medium during the dark incubation. The latter conclusion follows from the experiment in which labeled and nonradioactive suspensions were mixed in equal proportions immediately after labeling; the mixture was incubated for several hours with stirring, and heterocysts then were isolated. Heterocysts from the mixture had, on the average, one-half as much label as heterocysts from the fully labeled culture. The frequency distribution of grains near heterocysts in an autoradiograph, and thus the distribution of radioactivity, approximated the average of the

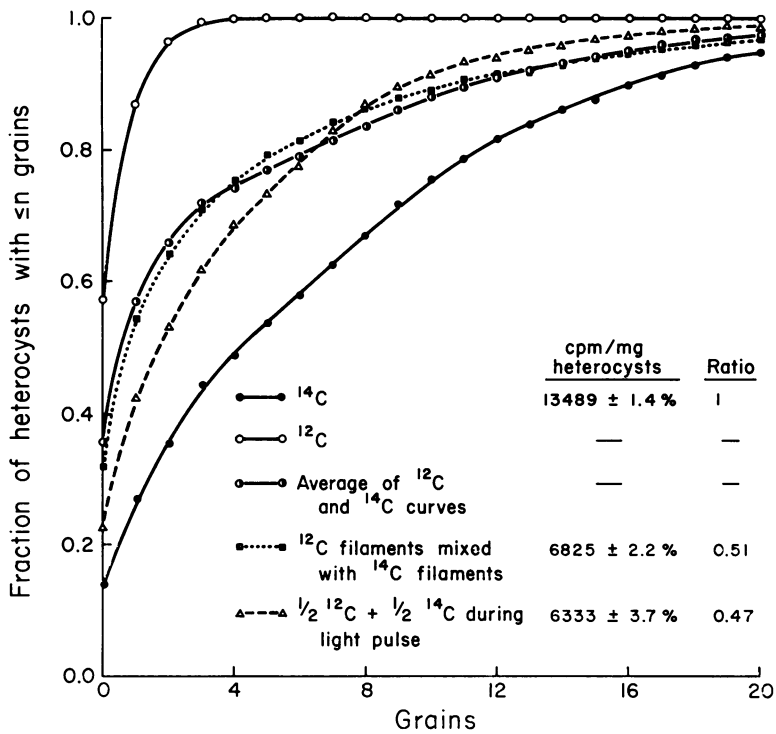

FIG. 5. *Integrated frequency distributions of silver grains per heterocyst for heterocysts isolated from suspensions exposed to $Na_2{}^{12}CO_3$ and $Na_2{}^{14}CO_3$ as indicated. Although the specific activity of heterocysts isolated from a mixture of filaments which have fixed ^{12}C and filaments which have fixed ^{14}C is approximately equal to the specific activity of heterocysts isolated from filaments which have fixed a mixture of ½ ^{12}C and ½ ^{14}C, the corresponding distribution much more closely resembles the mean of the distributions for heterocysts from ^{12}C filaments and heterocysts from ^{14}C filaments. Each curve was derived from grain counts for over 700 heterocysts.*

distributions for heterocysts from unlabeled and fully labeled cultures, and deviated much more (relative χ^2-test probability for nonintegrated distributions: 10^{-11}) from the distribution obtained for heterocysts from a culture, labeled with a mixture of ^{12}C and ^{14}C, which had approximately the same average amount of label per heterocyst. From an experiment of this type, one cannot argue that no cross-labeling occurs, but the results of repeated trials permit the assertion that any such contribution must be very limited.

It follows that (i) labeling of heterocysts is not due to a light-activated $^{14}CO_2$-fixation during the dark period: the light requirement appears rather to be for photosynthetic assimilation by vegetative cells; and (ii) the label found in isolated heterocysts is not due to substances secreted by vegetative cells and reassimilated by heterocysts, and it also does not arise from the radioactive lysate during processing.

That the labeling of isolated heterocysts is not due to any contaminating fraction from vegetative cells follows from the observed purity of the heterocyst preparations (Fig. 1), the high specific activity of the purified heterocysts relative to whole, filtered algal filaments, and the fact that in the autoradiographs of heterocyst preparations, the great majority of grain clusters was associated with recognizable heterocysts. Since less than 1% of vegetative cells would be expected to begin transforming into heterocysts during the experimental periods employed, and these cells, being still thin-walled, would have little chance of surviving the isolation procedure, at most a very small fraction of labeled, isolated heterocysts could have arisen from labeled vegetative cells.

Finally, it might be that label is merely exchanged, by diffusion, between vegetative cells and heterocysts. Instead, it seems clear that a significant part of the label moving from vegetative cells to heterocysts is bound to filterable structures.

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