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### Title

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July 1967

TRITIUM INCORPORATION STUDIES IN PHOTOSYNTHETIC BACTERIA<sup>†</sup>

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Although the detailed pathway of carbon, by which carbohydrates are formed from carbon dioxide during photosynthesis, has been established,<sup>1</sup> comparatively little is known about the participation and transport of hydrogen in the complex series of reactions now known to be involved in photosynthesis. The transport of hydrogen may occur concurrently with the redox reactions of photosynthetic electron transport leading to the formation of ATP, as well as in the reduction of carbon dioxide. The participation of the chlorophylls,<sup>2-5</sup> the carotenoids,<sup>6</sup> the quinones,<sup>7</sup> and other lipid components in photosynthetic hydrogen transport has been the subject of considerable speculation. In an attempt to elucidate the pathway of hydrogen transport, we are studying the tritium labeling pattern in the lipid extracts of Rhodospirillum rubrum after the bacteria have been illuminated in growth medium containing tritiated water.

In a previous communication this laboratory reported preliminary results of studies on the incorporation of tritium into bacteriochlorophyll of R. rubrum during photosynthesis.<sup>8</sup> These results suggested that the 3,4- positions of ring II in bacteriochlorophyll are specifically labeled when R. rubrum is illuminated in a tritiated medium, and that these two positions may therefore be important in photosynthetic hydrogen transfer. However, it was noted that other interpretations of the results were possible, one of these being the contamination of the bacteriochlorophyll by

a colorless, photolabeled compound which co-chromatographs with bacteriochlorophyll. In this communication we wish to report the results of further experiments which demonstrate that the bacteriochlorophyll isolated previously was indeed contaminated by at least one colorless photolabeled compound which is only one of a number of photolabeled products in the lipid extract of Rhodospirillum rubrum.

Rhodospirillum rubrum (strain S<sub>1</sub>) was grown in modified Hutner's medium in a continuous, steady-state culture<sup>††</sup> to reduce possible variations in the condition of the bacteria in different experiments. The bacteria were collected by centrifugation and resuspended in the growth medium to give an OD of ca. 30 (880 mμ). The suspension (18 ml) was placed in a flat cell with a path length of 5 mm and N<sub>2</sub> was bubbled through it. The cell was surrounded by a water jacket, and the temperature was maintained at 30°C during experiments. Tritiated water was added to give a specific activity of 10<sup>13</sup> dpm/mole, and the suspension was then illuminated from both sides with 23,000 footcandles of white light for a fixed amount of time. The bacteria were then collected by filtration and washed with several portions of water in a relatively dark environment. The wet filter mat containing the bacteria was then extracted in the dark with acetone (four 20 ml portions) until the extracts were colorless. Ether was added to the acetone extract in a separatory funnel, and the organic layer was washed with large volumes of water to remove any readily exchangeable tritium. The dark-colored ether layer was then evaporated to dryness at room temperature. The residue was redissolved in a small volume of acetone and chromatographed on powdered polyethylene thin-layer chromatogram (tlc) with an

80:20 acetone-water solvent system. The distribution of radioactivity on the tlc plate was mapped by scraping 5 mm zones from a portion of the chromatogram directly into vials containing scintillation solution.

(The radioactive material was extracted by the scintillation solvent in all samples except those containing the origin, and no variation of dpm with time was noted. Cab-O-Sil, a thixotropic gelling agent, was added to the origin samples to maintain a uniform suspension for accurate counting.) These samples were then counted and graphs of dpm vs. zone number were plotted.

Figure 1 shows the distribution of radioactivity on the polyethylene tlc from a typical 15-minute illumination experiment. At least four distinct bands of relatively high radioactivity are found.

The  $R_f$  of band A seems to be quite sensitive to the amount of material spotted on the chromatogram. (For Fig. 1 an amount of extract corresponding to 0.1 mg of bacteriochlorophyll was spotted on 17 cm of the 0.25 mm thick polyethylene tlc.) As a result, it frequently overlaps with Band B. The radioactive material can be eluted from band A with acetone. So far, attempts to identify this component have been unsuccessful. It shows no detectable absorption in the UV and visible regions of the spectrum, and co-chromatography with known materials has indicated that it is not a quinone (vitamin K, tocopherol, ubiquinone) or a phospholipid. We have been unable to pass the radioactivity through a VPC column of SE-30 at temperatures up to 280°C.

Band B contains bacteriochlorophyll and also a colorless, labeled material which has no detectable effect on the visible spectrum of bacteriochlorophyll. The position of the bacteriochlorophyll on the polyethylene tlc was determined by measuring the OD at 772 m $\mu$  (the absorption maximum for bacteriochlorophyll in the scintillation solvent) of the scintillation

solutions. The relative OD's were then plotted against zone number (dotted line in Fig. 1). It is evident from Fig. 1 that the radioactive band B does not correspond exactly with the dark blue bacteriochlorophyll band on the chromatogram.

Band C coincides with a bright orange region (possibly carotenoids) on the chromatogram, and band D corresponds to the origin of the chromatogram and contains the largest portion of the radioactivity in the lipid fraction. Preliminary results with other tlc systems indicate that band D is a mixture of several labeled components, all of which appear to be colorless. None of the components of band D have as yet been identified, because of the paucity of material, but co-chromatography experiments have eliminated ubiquinone, FMN and NADH.

The dark blue band corresponding to bacteriochlorophyll was extracted from the polyethylene and rechromatographed on mannitol tlc with iso-octane containing 3.5% methanol to further purify it. The distribution of radioactivity on the chromatogram was determined as described above and is shown in Fig. 2. The OD at 772 m $\mu$ . of each sample containing bacteriochlorophyll is also plotted in Fig. 2 (dotted line). The colorless component of band B is largely separated from the bacteriochlorophyll on the mannitol chromatogram and contains most of the radioactivity found in band B.

Any portion of band A which tails into the bacteriochlorophyll band on the original polyethylene tlc is also separated from bacteriochlorophyll on mannitol tlc. When this portion of band A is extracted from the mannitol chromatogram, mixed with unlabeled lipid extract from R. rubrum, and chromatographed on polyethylene, the radioactivity is again found slightly above, and tailing into, the bacteriochlorophyll band. When

band B is eluted from the mannitol chromatogram and rechromatographed with unlabeled lipid extract on polyethylene tlc the radioactivity is again found overlapping the bacteriochlorophyll band.

In another experiment, the radioactive lipid extract from R. rubrum was chromatographed on polyethylene tlc, the blue bacteriochlorophyll band was extracted with acetone, and its specific activity determined by measuring the radioactivity and the OD (772 m $\mu$ ) from equal aliquots of the extract. The remainder of the extract was rechromatographed on mannitol, as above, and the specific activity of the blue bacteriochlorophyll band from this chromatogram was determined. It was found that the mannitol chromatography resulted in a marked decrease in the apparent specific activity of the bacteriochlorophyll, as would be expected from the results of the zone mapping experiments. Typically, the specific activity after mannitol tlc was only 25 to 50% of the value found after the initial polyethylene tlc.

After repeated purification by these two tlc systems, the bacteriochlorophyll from the 15-minute illumination experiments showed a specific activity of 1.0 to 1.5 x 10<sup>11</sup> dpm/mole, which is only 1.0 to 1.5% of the molar specific activity of the tritiated water used in the experiments.

When a sample of the bacteriochlorophyll which had been purified by both the polyethylene and mannitol tlc systems was oxidized to the 3,4-dehydro derivative, as described previously,<sup>8</sup> there was no detectable change in the specific activity of the pigment. This indicates that there is no specific tritium labeling in the 3,4-positions of bacteriochlorophyll during illumination. The incorporation of tritium may be specific in another part of the molecule, or may be generally biosynthetic.



From the results of these experiments, it seems evident that the loss in specific activity upon oxidation reported previously<sup>8</sup> must now be interpreted as resulting from the separation on mannitol tlc of a colorless, photolabeled material that co-chromatographs with bacteriochlorophyll on powdered polyethylene tlc. The numbers (25-50%) quoted above for the decrease in the apparent specific activity of the bacteriochlorophyll from mannitol tlc are to be compared with similar numbers in the initial report<sup>8</sup> which were attributed therein entirely to oxidation at the 3,4-positions of bacteriochlorophyll.

The observation that other photolabeled radioactive components are present in the lipid extract, and apparently much more highly labeled than bacteriochlorophyll, has focused our attention on their possible role in photosynthetic hydrogen transfer. Dark control experiments show only a small amount of incorporation compared to the light experiments. This dark incorporation may be due either to the presence of a small pool of photoactivated compounds still present in the bacteria in the dark control, and necessary for hydrogen transfer, or to residue light reactions which are difficult to avoid during the manipulations of the dark experiment. Five-minute illumination experiments show essentially the same pattern of labeling as the 15-minute experiments on the polyethylene tlc of the lipid extract, except for the fact that band A appears to contain a much smaller portion of the label, and band C a significantly greater portion.

We are continuing our investigation of the tritium labeling pattern during photosynthesis, using the techniques described in this communication.

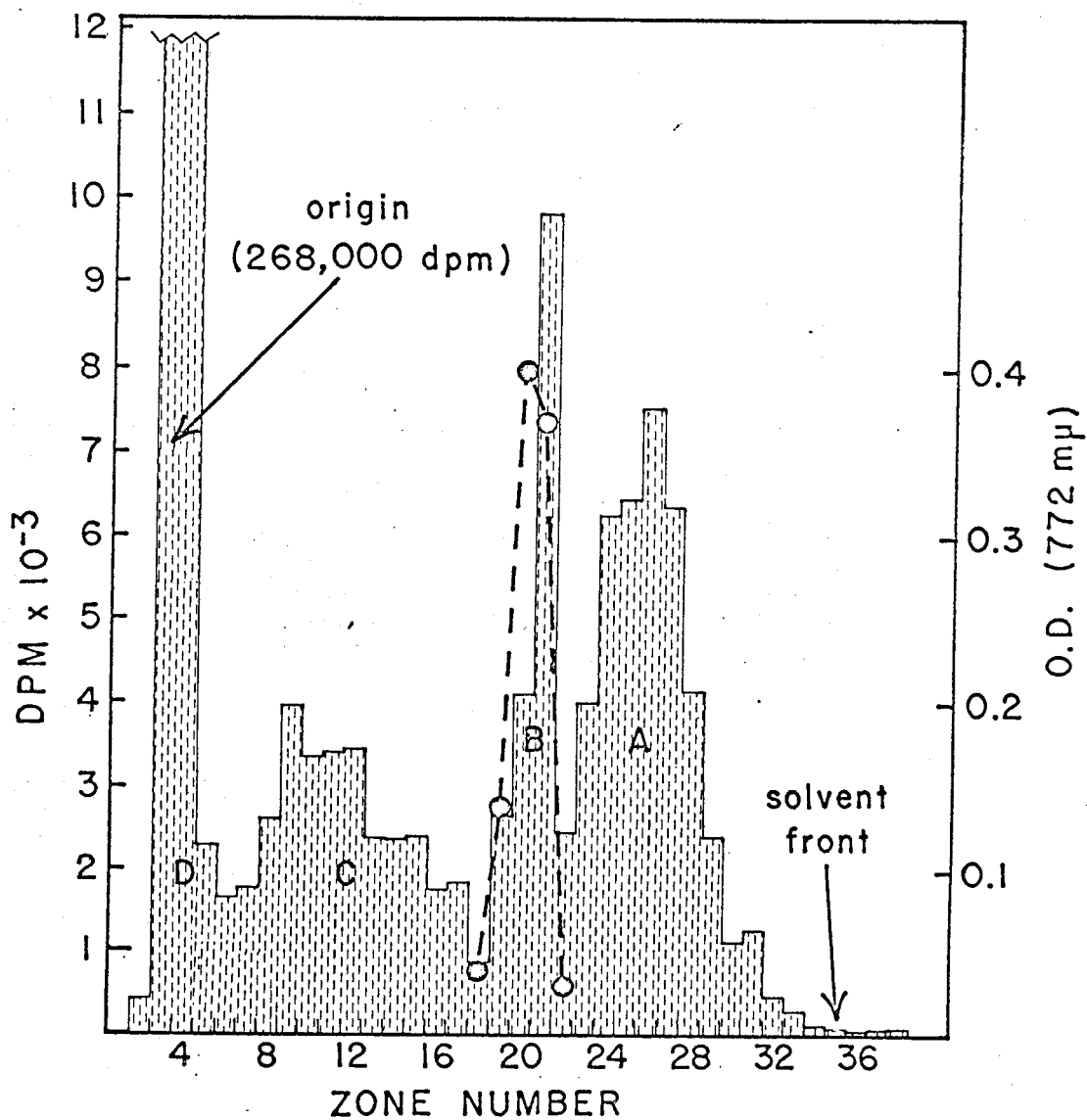
Summary

The distribution of radioactive materials in the lipid extract of Rhodospirillum rubrum photosynthesizing in a tritiated water medium has been examined by thin-layer chromatography and zone mapping techniques. The amount of tritium incorporated into bacteriochlorophyll is small compared with those of other components in the extract and is not specifically located at the 3,4-positions as previously reported. At least three other highly phototritiated materials are present which have yet to be identified.

Footnotes and References

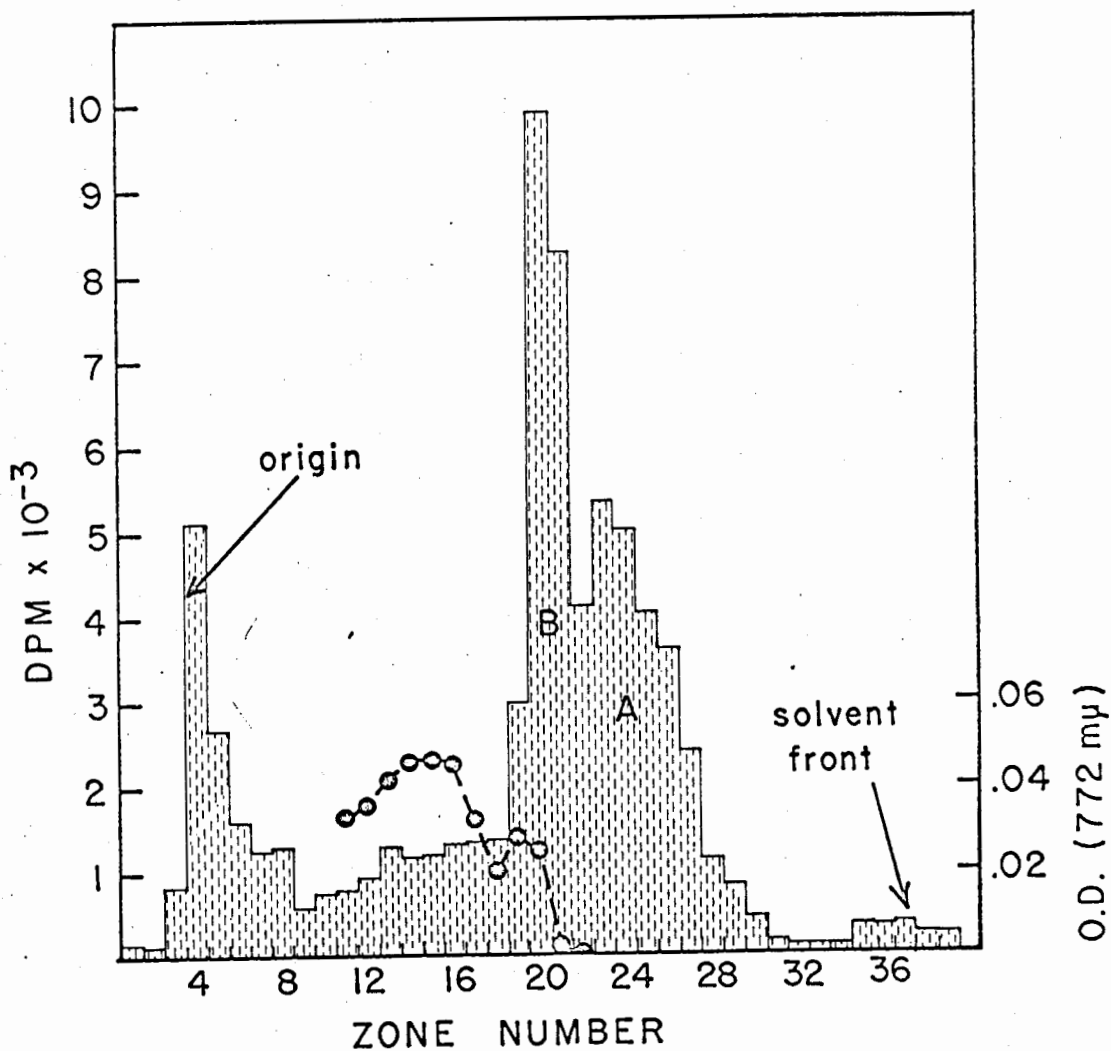
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- † The work described in this paper was sponsored, in part, by the U. S. Atomic Energy Commission.
- †† The culture was grown at 30° in modified Hutner's medium<sup>9</sup> containing 0.04 M malate. The medium supply tank was bubbled with N<sub>2</sub>, and the culture was bubbled with N<sub>2</sub> containing 5% CO<sub>2</sub> to keep the pH between 6.8 and 7.2. The glass culture tube was illuminated with six 20 W Nulite Ultralux reflector lamps to give an average intensity of 600 footcandles at the surface of the tube. The dilution system was adjusted so that the bacteria were allowed to grow to an OD of ca. 1.6 (880 mμ).
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XBL 677-4529

Figure 1. The distribution of tritium radioactivity on the polyethylene tlc of the lipid extract of R. rubrum after 15 min. Dotted line denotes the O.D. of the sample solutions measured at 772 mμ.



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Figure 2. The distribution of tritium radioactivity on a mannitol tlc after rechromatography of the bacteriochlorophyll band from the polyethylene tlc. Dotted line denoted O.D. of the sample solutions measured at 772 mμ.

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