Effect of Ultraviolet-B (280 to 320 nm) Radiation on Blue-Green Algae (Cyanobacteria), Possible Biological Indicators of Stratospheric Ozone Depletion

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The effect of ultraviolet-B (280 to 320 nm) irradiation on physiological activities of *Anabaena flos-aquae* and the water fern *Azolla caroliniana* has been studied under conditions where lethal effects of irradiation are absent. Nitrogenase activity, measured as acetylene reduction, specifically declined during irradiation with low levels of ultraviolet-B, whereas other physiological activities were unaffected. These findings indicate that measurement of acetylene reduction by cyanobacteria may serve as a specific, sensitive biochemical assay to assess environmental ultraviolet-B effects due to depletion of stratospheric ozone.

The blue-green algae (cyanobacteria) are nitrogen-fixing microorganisms found widespread in nature in soil and water and in association with a variety of plant and marine life (2). Various species can tolerate different climatic conditions and are found even in hot springs and arctic regions. These cells lack differentiated chloroplasts and contain chlorophyll in membraneous structures; consequently, they have recently been classified as blue-green bacteria, analogous to photosynthetic bacteria. However, the cyanobacteria carry out a typical plant-type photosynthesis, with water photolysis and oxygen evolution as major features. In addition to their resemblance to green plants, these ubiquitous organisms constitute a particularly useful microbial system for monitoring worldwide environmental effects as might result from enhanced solar ultraviolet (UV)-B (280 to 320 nm) irradiation due to depletion of stratospheric ozone (10). We have evaluated both *Anabaena flos-aquae* and the water fern *Azolla* as laboratory test systems for such environmental studies.

*Azolla* is an aquatic nitrogen-fixing plant that contains a symbiotic cyanobacterium, *Anabaena*, within its leaf cavity (4). This fern is also found worldwide, but it is particularly important for its use as a green manure in rice paddies in the Orient. Many species of cyanobacteria fix atmospheric nitrogen and contribute to nitrogen input into soils in a variety of ways. Both systems appear to be particularly important contributors of nitrogen to rice culture.

Our studies show that the nitrogen-fixing enzyme system in cyanobacteria is particularly sensitive to UV-B damage. Furthermore, inhibition of nitrogenase activity (measured as acetylene reduction) can take place in the absence of lethal effects or suppression of other physiological activities of the cells. These studies indicate, therefore, that measurement of acetylene reduction activity in nitrogen-fixing systems may provide a simple biochemical assay for assessing the biological effect of UV-B.

**MATERIALS AND METHODS**

*Azolla caroliniana*, a nitrogen-fixing water fern, was obtained from G. A. Peters, C. F. Kettering Foundation Laboratories, Yellow Springs, Ohio, and was grown on modified Hoagland's salts as described by Peters and Mayne (5). *Anabaena flos-Aquae* (Lyngb.) Brébisson, ATCC 22664, was grown on nitrogen-free BG-11 medium (8). Cultures of plants and cyanobacteria were grown at 25°C in light chambers under cool white fluorescent lamps at a light intensity of 10 to 20 W/m². Measurements of total light intensity were made with a Yellow Springs Instrument Co. (Yellow Springs, Ohio) model 65A radiometer equipped with a 6551 radiometer probe having a constant wavelength response from 0.28 to 2.6 μm (reduced to 65% at 0.21 μm).

UV-B irradiation of samples was obtained by using a bank of six 8-W RPR 3000A Rayonet photochemical reactor lamps (Southern New England Ultraviolet Co., Middletown, Conn.) placed above cyanobacterial and plant material at 25°C in flat dishes covered with a filter consisting of cellulose acetate film. The unfiltered RPR 3000A lamp has, in addition to UV-B, an emission in the short-wavelength region (λmax ~ 254 nm), as well as a number of minor lines in the visible region. Such lamps were used either singly or in multiples to increase irradiation. Figure 1 illustrates the emission spectrum of a single Rayonet lamp, showing the major emission in the 300-nm region, the spectrum obtained when cellulose acetate is used as a filter to
remove short-wavelength UV, and the spectrum of average noon sunlight in this region taken at Beltsville, Md. (7). It can be seen that 5-mil (ca. 127-μm) cellulose acetate effectively removes the shortwave radiation and provides a spectral quality closely approximating typical sunlight in the UV-B region. With this filtered system, visible light above 350 nm constitutes less than 21% of the total radiation emitted by the lamp.

The lamps were aged 100 h and did not significantly decrease in irradiance levels during prolonged use thereafter. The cellulose acetate was preirradiated for 6 h and discarded after 30 to 40 h of use. Since we have no knowledge of the actual targets involved, other than to exclude deoxyribonucleic acid, our data are reported as total incident UV-B light over the range indicated and do not assume any biological effectiveness of a particular wavelength.

UV-B irradiance levels, in watts per square meter, were measured with an Optronics Laboratories, Inc. model 725 UV-B radiometer. We calibrated this instrument against a Rayonet lamp that had been scanned at distances of 13 and 20 cm (5-mil cellulose acetate filter) with the Beltsville Agricultural Research Center Instrumentation Laboratory spectroradiometer over the 250- to 400-nm region. Integrated watts per square meter over the range of 280 to 320 nm at these distances were taken as reference points (0.44 and 0.82 W/m², respectively) and linearly extrapolated to provide estimates of higher UV-B irradiances.

Cyanobacterial suspensions in petri dish bottoms containing 15 to 50 ml, depth 4 to 10 mm, were stirred during irradiation. Portions were removed during irradiation, rapidly agitated to separate clumped chains, and plated on BG-11 (N-free) medium, and samples were also assayed for rates of nitrogenase, fixation of 14CO2, and hydrogen evolution. The experiments reported are typical examples. At least three replicate experiments were performed for each property examined.

Acetylene reduction and hydrogen evolution were measured by gas chromatography on cyanobacterial and fern preparations incubated in light in screw-capped vials containing argon–acetylene or argon atmospheres. Samples of the gas phase were periodically withdrawn with gas-sampling syringes. The ethylene formed from acetylene was separated by gas chromatography on columns of Poropak R (9); hydrogen was measured by using a molecular sieve 5A column (1).

14CO2 fixation was measured on portions of either A. flos-aquae or fern fronds removed during irradiation and immediately exposed to NaH14CO3. After exposure, samples were collected on glass fiber papers and rinsed with 6 N HCl, and the rate of incorporation of 14C was then determined by using a liquid scintillation counter and a water-miscible scintillation fluid.

Concentrations of A. flos-aquae in irradiated suspensions were determined by measurement of densities of dilute algal suspensions and were correlated with protein content (3). With our cultures, an optical density of 1.0 at 650 nm corresponded to approximately 200 μg of algal protein (3 x 106 colony-forming units per ml). In dilute suspensions, the contribution to the absorption by algal pigments was minimal at 650 nm.

RESULTS

Cyanobacteria are known to be relatively resistant to short-wavelength UV irradiation and to possess an active photoreactivation system (11). In our early studies, we confirmed both of these effects and determined killing curves for our experimental system using an unfiltered Rayonet UV lamp (Fig. 2). Comparison of killing curves obtained by plating cell samples on plates that were immediately incubated in the light with those on plates that were allowed to incubate in the dark 24 h before illumination showed an active photoreactivation of shortwave UV killing.

It can be seen (Fig. 3), however, that when cellulose acetate is used as a filter to remove short-wavelength UV, the killing effect is virtually eliminated, even under conditions where the measured UV-B radiation intensity is increased fivefold to approximately 2.1 W/m². Note also that, although the time scale has changed from minutes to hours of irradiation, no lethal effect can be observed.

We attempted to increase the UV-B irradiation further by using a curved bank of six lamps with a reflector to impinge the light more directly on the reaction vessel. Figure 4 illustrates the results of such an experiment, in which the UV-B intensity has been approximately doubled to 5.2 W/m². These data indicate killing after prolonged high-intensity irradiation; however, a part of the population remained viable, which
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Fig. 2. Shortwave UV light killing and photoreactivation of A. flos-aquae. A single, unfiltered, Rayonet lamp was placed 15 cm from the surface of a 40-m1 stirred algal suspension containing 10^5 colony-forming units/ml which was covered with a 10-mil cellulose acetate filter to exclude short-wave-length UV. Portions of cells exposed to UV-B (2.1 W/m^2; total light, 5 W/m^2) were plated and incubated both in light and after 24-h dark preincubation to evaluate photoreactivation of UV-B damage. The control suspension was not irradiated. The curve labeled no C.A. (no cellulose acetate control) represents killing curve determined when the cellulose acetate filter was not used.

Fig. 3. Irradiation of A. flos-aquae with UV-B (280 to 320 nm) light. Six Rayonet lamps were placed 17 cm above a 40-m1 cell suspension (containing 10^4 colony-forming units/ml) which was covered with a 10-mil cellulose acetate filter to exclude short-wave-length UV. Portions of cells exposed to UV-B (2.1 W/m^2; total light, 5 W/m^2) were plated and incubated both in light and after 24-h dark preincubation to evaluate photoreactivation of UV-B damage. The control suspension was not irradiated. The curve labeled no C.A. (no cellulose acetate control) represents killing curve determined when the cellulose acetate filter was not used.

Fig. 4. High-intensity UV-B irradiation of A. flos-aquae. Six Rayonet lamps in a curved reflector were 17 cm above the stirred cell suspension (40 ml; 10^4 colony-forming units/ml) covered with a 10-mil (ca. 254-μm) cellulose acetate filter. Total light was at 12.5 W/m^2; UV-B was at 5.2 W/m^2. The control suspension was not irradiated; the curve obtained with no filter corresponds to UV killing obtained with unfiltered light.

suggests that this fraction may be resistant to high-intensity UV-B. It would be of interest to use this approach as a means of selecting strains with either enhanced resistance or sensitivity to UV-B. It should also be noted that we consistently were unable to recover all of the viable cells from control samples held for the long time periods of the experiments. Because of this, it is essential to evaluate UV-B effects by comparison with physiological activity of controls.

Two biosynthetic activities of A. flos-aquae were examined after exposure to low, nonlethal doses of UV-B (2.1 W/m^2); nitrogen fixation (measured by acetylene reduction and hydrogen evolution) and fixation of ^14CO_2. Table 1 lists the effects of total UV irradiation and UV-B on acetylene reduction by Anabaena, and indicates a decline in activity of algae irradiated with UV-B under conditions in which the number of colony-forming units remains unchanged. To assay biosynthetic activities, concentrations of suspensions of A. flos-aquae were increased tenfold. Plate counts of suspensions gave similar results over the range of 6 to 80 μg of protein per ml, i.e., no demonstrable UV-B killing during the experiment.

Data in Table 2 show that, under similar conditions of irradiation, specific effects of UV-B on CO_2 fixation were slight. From these results, it appears that the nitrogenase system is a more specific and sensitive target for UV-B damage in A. flos-aquae.

Experiments were performed to gain some insight into the nature of the nitrogenase inhibition by UV-B. Since nitrogenase is a multienzyme complex which can be assayed for in a variety of ways, we also measured the effect of UV-B on the ability of the complex to photoreduce molecular hydrogen. The effect of UV-B on nitrogenase was negligible when this assay was used (Table 3). The slow decline in hydro-
TABLE 1. Effect of UV-B on nitrogenase activity of *A. flos-aquae*

<table>
<thead>
<tr>
<th>Irradiation time (h)*</th>
<th>Acetylene reduction (nmol of C₂H₄/mg of protein per h)*</th>
<th>Control†</th>
<th>UV-B</th>
<th>UV‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1,490</td>
<td>1,490</td>
<td>945</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>—</td>
<td>—</td>
<td>370</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1,300</td>
<td>840</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1,360</td>
<td>340</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* UV-B, 2.1 W/m²; cell suspension, 40 ml; protein, 65 µg/ml.
† Portions, 5 ml each, of suspensions were incubated in light (30 W/m²) in an atmosphere of 90% argon–10% acetylene for assay.
‡ Not irradiated.
§ Rayonet lamps without cellulose acetate filter (10 W/m²) separate experiment; algal protein, 40 µg/ml.

TABLE 2. Effect of UV-B on fixation of ¹⁴CO₂ by *A. flos-aquae*

<table>
<thead>
<tr>
<th>Irradiation time (h)*</th>
<th>¹⁴CO₂ fixed (cpm/mg of protein per min in light)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>UV-B</td>
</tr>
<tr>
<td>0</td>
<td>9,300</td>
</tr>
<tr>
<td>2</td>
<td>9,800</td>
</tr>
<tr>
<td>4</td>
<td>9,200</td>
</tr>
<tr>
<td>6</td>
<td>7,800</td>
</tr>
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</table>

* Cell suspension, 37 ml; protein, 50 µg/ml; UV-B, 2.1 W/m².
† Not irradiated with UV-B.
§ Rayonet lamps without cellulose acetate filter, 10 W/m².

Discussion

From a practical standpoint, it is obvious that assessment of the environmental effects of enhanced UV-B irradiation on biological material is going to require development of simple assay procedures with wide applicability. Our studies have consistently revealed a surprising sensitivity of the nitrogenase complex to UV-B irradiation. The irradiation level of approximately 2 W/m² UV-B, which we find inhibitory to nitrogenase, is similar to that of noon sunlight in the

TABLE 3. Effect of UV-B on photoevolution of H₂ by *A. flos-aquae*

<table>
<thead>
<tr>
<th>Irradiation time (h)*</th>
<th>H₂ evolution (nmol/h per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>UV-B</td>
</tr>
<tr>
<td>0</td>
<td>460</td>
</tr>
<tr>
<td>3</td>
<td>350</td>
</tr>
<tr>
<td>6</td>
<td>265</td>
</tr>
</tbody>
</table>

* Cell suspensions (40 ml; 80 µg of protein per ml), exposed to UV-B at 2.1 W/m².
† Portions (5 ml each) of suspension incubated anaerobically (argon atmosphere); 30 W/m² white light for assay.
‡ Not irradiated with UV-B.

TABLE 4. Effect of irradiation with UV-B on ¹⁴CO₂ fixation and acetylene reduction by *Azolla*

<table>
<thead>
<tr>
<th>Irradiation time (days)*</th>
<th>¹⁴CO₂ fixed †</th>
<th>Acetylene reduced †</th>
<th>¹⁴CO₂ fixed ‡</th>
<th>Acetylene reduced ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24,000</td>
<td>450</td>
<td>20,200</td>
<td>300</td>
</tr>
<tr>
<td>2</td>
<td>17,800</td>
<td>380</td>
<td>15,200</td>
<td>100</td>
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<tr>
<td>4</td>
<td>7,200</td>
<td>320</td>
<td>5,900</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>4,350</td>
<td>350</td>
<td>4,700</td>
<td>100</td>
</tr>
</tbody>
</table>

* Visible light (10 W/m²) supplemented with UV-B (2 W/m²).
† Not irradiated.
‡ Values indicate counts per minute per gram of plants (wet weight) per minute in visible light (30 W/m²).
§ Values indicate nanomoles per gram of plants (wet weight) per hour; 90% argon–10% acetylene atmosphere; visible light was at 30 W/m².
280- to 320-nm region. This sensitivity would account for the survival value of the migration of the blue-green algae to deeper, light-limited water during daytime observed by Peterson et al. (6) in studies of algal populations in lakes.

It should be emphasized that, by performing microbiological plate counts on a population of irradiated cells, we have ruled out the possibility that the UV-B effect observed on nitrogenase is due to lethal nucleic acid damage. This finding suggests that the cellular target may be a component associated with the nitrogenase complex or its electron transport system. Further studies on the action spectrum of this effect may help to reveal the cellular component involved as UV-B receptor.

Information now available (12) on the biochemical effects of short-wavelength UV irradiation on biological material has come virtually exclusively from studies of microorganisms. It seems likely, therefore, that microorganisms may again prove to be the material of choice to study UV-B effects. Nitrogen fixation consumes a substantial fraction of the energy of a cell in which it occurs; consequently, it is possible that a minor physiological disturbance would be expressed more readily in such a system. Furthermore, this assay (acetylene reduction) is readily adaptable to use in the field and could serve as a convenient assay for a variety of environmental studies. The main drawback to this approach to assess environmental effects is that it requires the use of those limited systems that possess nitrogenase activity.

ACKNOWLEDGMENTS

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LITERATURE CITED