FLUORESCENCE SPECTROPHOTOMETRY OF PYRIDINE NUCLEOTIDE IN PHOTOSYNTHESIZING CELLS

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INTRODUCTION

Although there is evidence that pyridine nucleotide is reduced by light during photosynthesis (*cf.*[1]). this has not yet been proved. In an attempt to detect this reduction in intact cells, we used fluorescence spectrophotometry, which has some advantages compared with the method of sensitive absorption spectrophotometry[2] formerly used.

The latter method revealed very small increases in absorption in the region around 340 mμ upon illumination of the algae *Chlorella* and *Phorphyridium cruentum*, which were tentatively interpreted as being caused by reduction of pyridine nucleotide. In this spectral region also other changes occurred; these were presumably caused by a cytochrome (and perhaps by other pigments). In purple bacteria, absorption changes in pyridine nucleotide would presumably be even more difficult to detect and to identify because of the relatively larger changes of cytochromes and other pigments. This may explain CHANCE AND SMITH'S[3] failure to obtain evidence for the occurrence of pyridine nucleotide in purple bacteria by studying absorption difference spectra.

Since only relatively few substances are fluorescent, fluorescence is a more specific property of reduced diphosphopyridine nucleotide (DPNH) than absorption. Consequently, interference by other pigments, which renders a study by means of absorption spectrophotometry difficult, is less likely in fluorescence spectrophotometry. It has been shown[4] that irradiation of suspensions of several species of unicellular organisms by the mercury line 366 mμ gives rise to a fluorescence spectrum resembling that of DPNH, and kinetic experiments with yeast strongly indicated that the fluorescence around 450 mμ is indeed mainly caused by (bound) DPNH.

Using a somewhat modified apparatus, we were able to demonstrate an increase in the fluorescence spectrum around 450 mμ upon illumination of photosynthesizing cells, which provided stronger evidence for the reduction of pyridine nucleotide than the absorption method.

METHODS

The fluorescence of a suspension of photosynthesizing cells was excited by weak radiation of 366 mμ (see Fig. 1). A wavelength band W from the fluorescence spectrum was transmitted by the monochromator and its intensity continuously recorded. The filtered light from a tungsten lamp was alternately admitted and shut of. This light,

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which was of a longer wavelength than W and did not directly cause a deflection of the recorder, brought about an increase in fluorescence; this was followed by a decrease of equal magnitude when the light was shut off. The fluorescence increase was measured for various wavelengths W, and was plotted as a function of W, after a correction had been applied for the wavelength sensitivity of the set-up. This correction (*cf.*[4]) was such as to transform the recorder deflection into fluorescence energy per wavelength interval. Since the same graphs would have been obtained by subtracting the fluorescence spectrum in darkness from that in light, the graphs may be called difference spectra (of fluorescence). The fluorescence-exciting radiation was weakened by gauzes to $2 \cdot 10^3$ ergs/(cm^2 sec), which was presumably below the intensity needed for saturation of photosynthesis under optimal nutrient conditions.

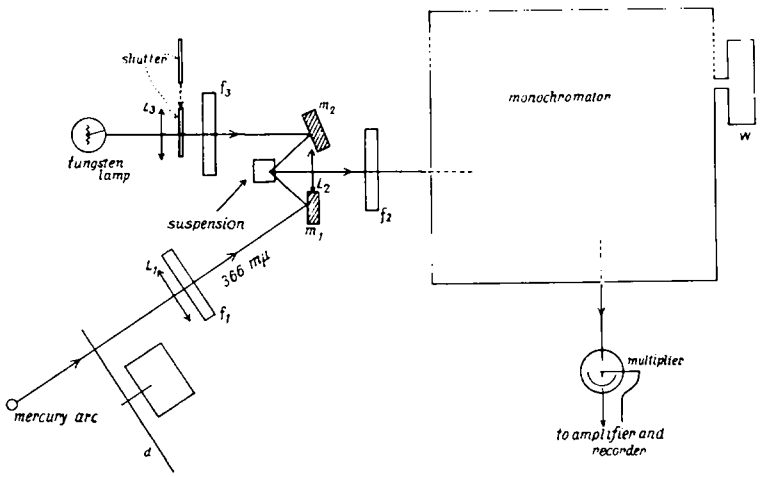

Fig. 1. Apparatus for measuring fluorescence in the blue region during photosynthesis. The filtered radiation from the tungsten lamp is used to initiate photosynthesis at a high rate. The remaining part of the apparatus is the same as used by DUYSENS AND AMESZ[4]. The L's are lenses, the f's filter sets, and the m's mirrors.

The changes in fluorescence increased with the intensity of illumination from the tungsten lamp up to a saturation value, which occurred at an intensity of the order necessary for saturation of photosynthesis. For the measurement of the difference spectra an intensity was used, which was a little below that needed for the saturation of the change in fluorescence. The changes in fluorescence in *Rhodospirillum* and *Anacystis* were completed within a few seconds, except for the decrease of *Rhodospirillum* fluorescence after darkening, which lasted ten to fifteen seconds.

The concentration of *Chlorella* and *Anacystis* was about 1 mm^3 wet cells per ml of suspension, that of *Rhodospirillum* 0.5 mm^3 per ml.

RESULTS

The difference spectrum of the non-sulfur purple bacterium *Rhodospirillum rubrum* strain 4 was measured in 0.5% sodium chloride and 0.16% sodium butyrate, the pH being adjusted to 7.0 by adding phosphate. The bacteria were irradiated with radiation of 880 mμ, which is strongly absorbed by the photosynthetically-active bacterio-

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chlorophyll; its intensity was $1.5 \cdot 10^4$ ergs/(cm²sec). The increase in fluorescence around 450 mμ was about 20% of the total fluorescence. The difference spectrum (Fig. 2) has a maximum around 440 mμ. The change in fluorescence is much smaller if butyrate is omitted from the medium.

The difference spectrum of the sulfur purple bacterium *Chromatium* strain D, irradiated with 880 mμ, also showed a maximum between 430 and 460 mμ in a complete nutrient medium of pH 7.7. If from this medium the "hydrogen donors" or carbon dioxide were omitted, no light-induced change in fluorescence was observed; however, if before irradiation this deficient medium was flushed with a mixture of 95% hydrogen and 5% carbon dioxide, changes in fluorescence occurred.

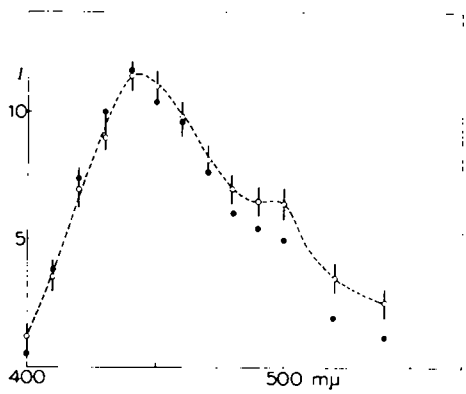

Fig. 2. Difference spectrum of fluorescence of *Rhodospirillum rubrum*. *I* is the relative energy flux per wavelength interval. The points and circles are runs on different days and with different cultures. The vertical lines indicate the possible errors for the circles. Concentration: 0.5 μl wet cells per ml medium.

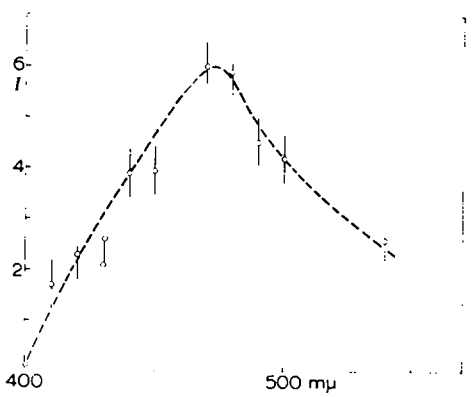

Fig. 3. Difference spectrum of fluorescence of *Anacystis nidulans*. *I* is the relative energy flux per wavelength interval. Concentration: 1 μl wet cells per ml medium.

In a few experiments with *Chlorella*, which was illuminated with radiation of 680 mμ, the change in fluorescence was too small to allow the measurement of a difference spectrum with sufficient precision.

The fluorescence of the blue alga *Anacystis nidulans*, when flushed with 5% carbon dioxide during growth, increased roughly 10% at 450 mμ upon irradiation. The wavelength of the exciting radiation was 614 mμ, its intensity $5.5 \cdot 10^4$ ergs/(cm² sec). The difference spectrum (see Fig. 3) has a maximum at 460–470 mμ. No measurable change in fluorescence was observed, when this alga was grown in an undisturbed medium exposed to air.

DISCUSSION

The facts that the changes in fluorescence are reversible, that they are influenced by the suspension medium in a similar way as photosynthesis, and approach a maximum at roughly the same intensity as photosynthesis, indicate that the changes are caused by a process related to photosynthesis.

The difference spectra resemble, but are not identical to the fluorescence spectrum

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of DPNH[4]. The cause of the relatively small deviation from the DPNH spectrum may partly be the same as in yeast and other colorless cells: namely the binding of pyridine nucleotide to cell constituents; another cause of the deviation must be selective "self-absorption" of fluorescence light, for which no correction was applied. The relative smallness of the observed changes may be caused by a simultaneously increased rate of oxidation of pyridine nucleotide in photosynthesis, or by the inactivity of part of the fluorescing material in photosynthesis.

Our observations are consistent with the hypothesis that light drives the reduction of pyridine nucleotide in photosynthesis. The fluorescence method seems at present the most suitable to check this hypothesis in experiments on intact cells, since interference by other pigments is smaller than in absorption spectrophotometry.

SUMMARY

An increase in the blue fluorescence of suspensions of purple bacteria and of a blue alga was observed upon illumination with photosynthetically active infrared or red radiation. The spectrum obtained by subtracting the fluorescence spectrum in the "dark" from that in the "light" was similar to that of reduced pyridine nucleotide. This and other evidence obtained supports the hypothesis that during both algal and bacterial photosynthesis an accelerated reduction of pyridine nucleotide occurs.

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THE ISOLATION OF PYRIDOXAL-5-PHOSPHATE FROM CRYSTALLINE MUSCLE PHOSPHORYLASE*

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Phosphate analyses of crystalline muscle phosphorylase *a* were carried out on several occasions in the last 12 years during which time the method of preparation of the enzyme has been improved. The data are summarized in Table I. When the enzyme is recrystallized from cysteine-glycerophosphate buffer, followed by exhaustive washing of the crystals with 0.03*M* KCl in the cold (prep. 1, Table I), there still adheres to the crystals an impurity which gives a pentose reaction and which

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