DETECTION OF PIGMENT FORMS IN *ANACYSTIS NIDULANS* BY FLUORESCENCE REABSORPTION SPECTROSCOPY

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Received 5 April 1977

1. Introduction

Pigment forms in chloroplasts and unicellular photosynthetic organisms are not unequivocally resolved by absorption and fluorescence spectroscopy [1–7]. When obtaining true fluorescence spectra fluorescence reabsorption is a phenomenon usually corrected for (or avoided by preparing very dilute samples). In the present study, however, in contrast to common practice, the reabsorption of fluorescence was intentionally enhanced with the assumption that pigment forms involved in the reabsorption of fluorescence can be readily detected and they are the very ones the absorption of which constitute the conventional absorption spectra. The results presented show that fluorescence reabsorption spectroscopy provides hitherto unexploited advantages in discerning weakly absorbing components in complex pigment systems even if they scatter light.

2. Materials and methods

The unicellular blue-green alga *Anacystis nidulans* was cultured in Medium C of Kratz and Myers [8] in glass vessels at 38°C, illuminated by fluorescent light tubes (10 000 lux), and supplied with a mixture of air and CO_2 (95:5). Fluorescence spectra were measured at room temperature with a Perkin Elmer MPF-3 spectrofluorimeter equipped with a specially constructed integrating sphere serving as a sample chamber, to enhance both the primary excitation level and the reabsorption of fluorescence. The use of such an integrating sphere coated with magnesium oxide to facilitate diffuse scatter of light within, ensures a homogeneous radiation field in the sample, and increases the light path especially in regions of low absorption [9]. The volume of the integrating sphere was 7.8 ml and it was filled with the sample to be measured. The alga was centrifuged and resuspended to give a concentration of 10^{11} cells/ml (calculated $A = 84$). A concentration series spanning three orders of magnitude was obtained by dilution. Fluorescence of *Anacystis nidulans* was excited in the region of phycocyanin absorption at 632.8 nm, using a HeNe laser. The intensity of the exciting beam was 4.5×10^2 mW/cm^2. The fluorescence spectra were corrected for the spectral sensitivity of the apparatus. Fluorescence induction effects were avoided by illuminating the samples until stationary fluorescence was attained.

3. Results and discussion

Excitation of phycocyanin, the accessory pigment in *Anacystis*, results in phycocyanin fluorescence with a maximum between 655 nm and 660 nm and, due to effective energy transfer between phycocyanin and chlorophyll *a*, in fluorescence of chlorophyll *a* at longer wavelengths.

The set of fluorescence spectra in fig.1 can be obtained by measuring the fluorescence of a very dense sample of *Anacystis nidulans* (10^{11} cells/ml; curve No. 1) and, of samples gradually diluted to such density as to reach the well known fluorescence spectrum not distorted by reabsorption (curve No. 21). Upon further dilution spectral changes did not occur.

High density samples had fluorescence spectra

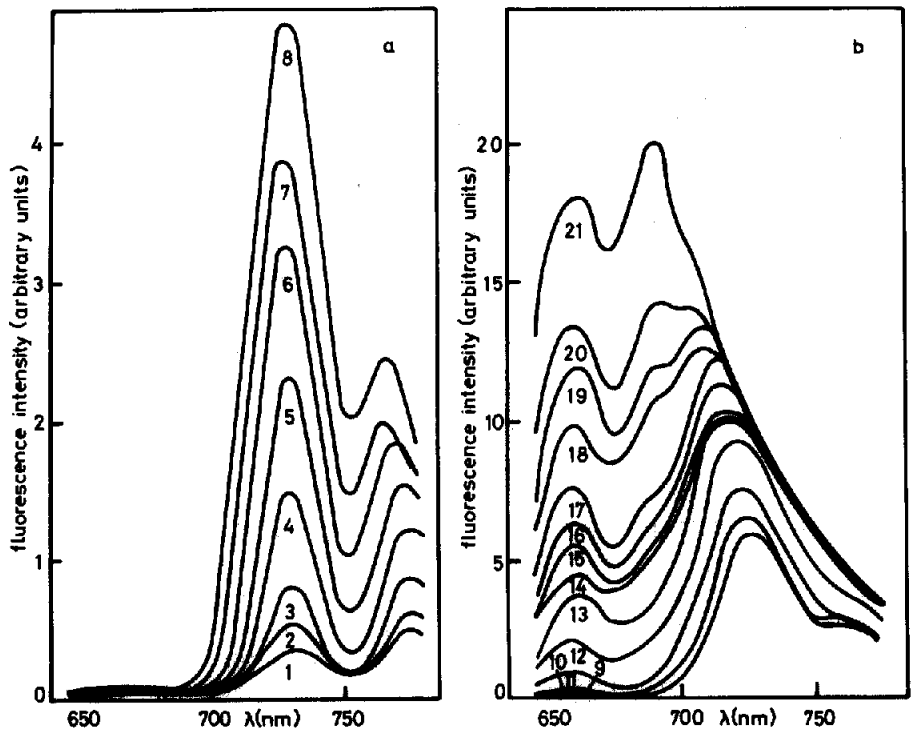

Fig.1. Fluorescence emission spectra of *Anacystis nidulans*. Curves 1–21 are members of a dilution series spanning three orders of magnitude. Curve 1 is the fluorescence spectrum of a sample of 10^{11} cells/ml having a calculated A of 84. Excitation was provided by a HeNe laser at 632.8 nm and fluorescence was measured using an integrating sphere to enhance reabsorption.

(fig.1a) affected by reabsorption in the short wavelength and the 750 nm region as well. Reabsorption at 750 nm is due to a special pigment termed P750 [10,11].

In samples of moderate density (fig.1b) the short-wavelength fluorescence was partially absorbed and the fluorescence maximum was located at wavelengths greater than 700 nm. The spectra are not unlike the low temperature spectra of *Anacystis* [6] and of photosystem fragments [12,13].

It is interesting to note that effective reabsorption of fluorescence by P750 in dense samples indicates a possible role of this pigment in better utilization of available light in photosynthetic organisms where a significant increase in optical pathlength can occur due to light scattering.

To interpret spectral changes due to reabsorption of fluorescence an analogy with measurements on a single-beam spectrophotometer offers itself. The fluorescence spectrum of a dilute sample is considered to be the spectral distribution of a reference light source I_r, whereas the fluorescence spectra of denser samples are considered to be the spectral distribution of the transmitted light I_t. Subsequently the expression $\log I_r/I_t$ as a function of wavelength, analogous to conventional absorbance spectra, may be calculated letting any one of the fluorescence spectra be I_r and any other I_t with an index t less than r.

The concentration-dependent change in the spectral distribution of fluorescence is thus attributed to absorption in the respective spectral region. Figure 2a shows a set of such fluorescence reabsorption curves when the 'transmitted' spectrum was $I_{t=1}$ and the reference spectra were I_r with $r = 3, 4, 6, 8, 9, 10, 11, 12, 13, 14$, respectively. In fig.2b the reference curve was $I_{r=16}$ and the transmitted spectra were I_t with $t = 2, 3, 5, 7, 9, 11, 14$, respectively. Here, at concentrations where reabsorption by minor compo-

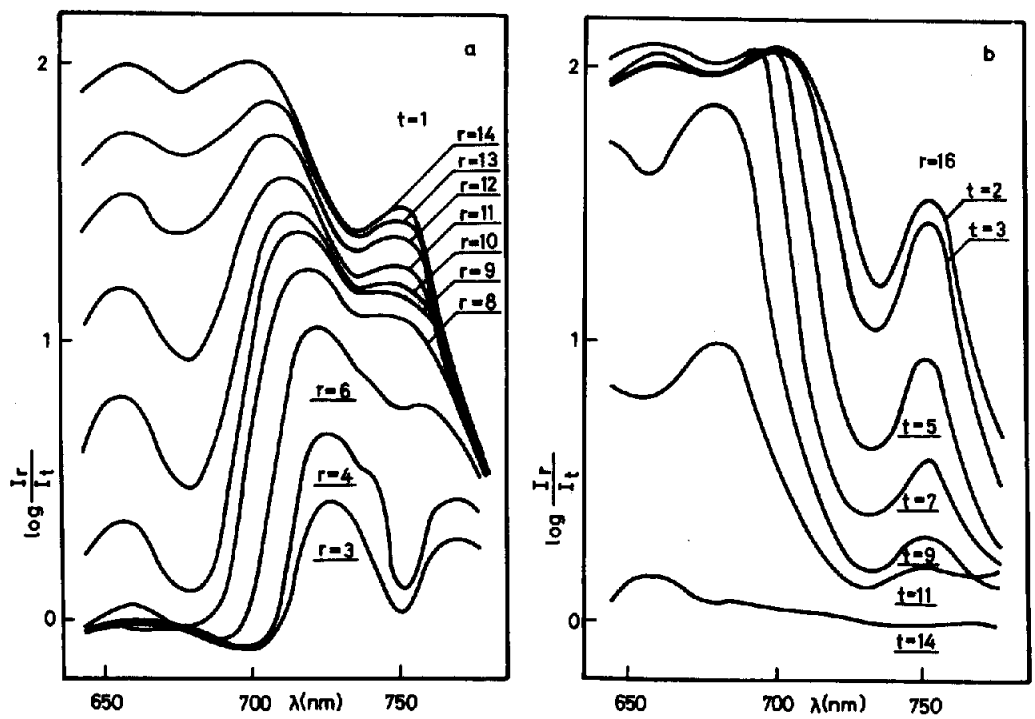

Fig.2. Fluorescence reabsorption spectra calculated from the fluorescence spectra of figure 1. Indices r and t stand for reference and transmitted intensities, respectively and refer to the curve numbers in fig.1.

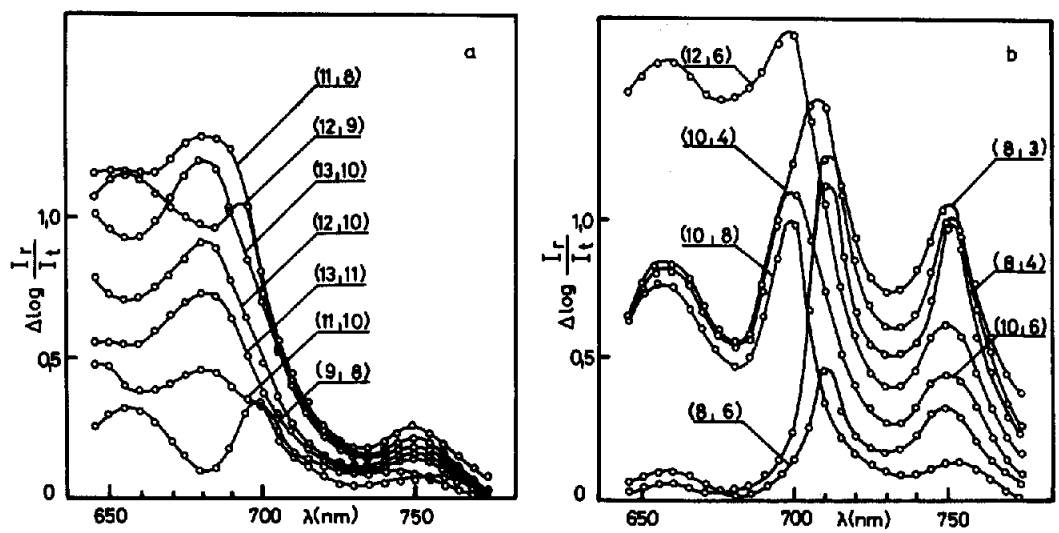

Fig.3. Difference spectra calculated from the curves of fig.2a. The numbers in brackets refer to indices r of figure 2a. and indicate which curves were used to calculate the given spectrum. The expression $\Delta\log I_r/I_t$ is equivalent to $\log I_r/I_{r'}$ in the special case above (i.e., when $t = t'$) but the former notation is preferred since it allows analysis on a broader scale.

nents is not as significant yet, the fluorescence reabsorption curves are similar to the usual *Anacystis* absorbance spectra (t = 11.9).

Figure 2a was chosen for further analysis and 'difference' spectra were constructed by subtracting one fluorescence reabsorption curve from another. A set of difference curves was obtained (fig.3). It is easy to see that the smaller the absorption, the greater increase in optical pathlength is possible and therefore due to multiple diffuse scattering within the sample chamber used, the reabsorption of fluorescence by pigment forms with small absorption can be enhanced.

A close inspection of fig.2 and fig.3 reveals absorbing chlorophyll forms at 655–660 nm, 680–685 nm, 695–698 nm, 710–712 nm, 730–735 nm and 750 nm. All of these forms with the exception of the 730–735 nm form are manifested as peaks in the fig.3, and the existence of the 730–735 nm form is strongly implicated.

Relevant data from literature are produced below. Room-temperature derivative absorption spectra of *Anacystis* [7] exhibit a peak at 680–682 nm, shoulders at 671–672 nm and 693 nm, and three pigment forms are suspected at 664 nm, 703 nm and 715 nm. Low-temperature absorption spectra [6] exhibit a peak at 679 nm, shoulders at 670 nm, 686 nm and 745–750 nm, and suggest another pigment form at 705 nm. Goedheer [4] found peaks at 673 nm and 750 nm and a shoulder at 686 nm in low-temperature absorption spectra of *Anacystis* fragments.

Comparison of the data presented here with those obtained by using standard methods indicate that the suggested method based on the reabsorption of fluorescence, in spite of its simplicity (single, monochromatic excitation providing a wide spectrum of incident light) could be a powerful tool to complement other methods of spectroscopy in resolving pigment forms under physiological circumstances.

Acknowledgements

Thanks are due to G. L. Farkas and B. Karvaly for discussions and S. Buda for technical assistance.

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