BBA 46362

TEMPERATURE DEPENDENCE OF ABSORPTION AND FLUORESCENCE SPECTRA OF BACTERIOCHLOROPHYLLS *IN VIVO* AND *IN VITRO*

J. C. GOEDHEER

Biophysical Research Group, Institute of Physics, the State University, Utrecht (The Netherlands)

(Received April 27th, 1972)

SUMMARY

The "short wave" far-red absorption bands (795-825 nm) of bacteriochlorophyll in photosynthetic red bacteria are sharpened but not shifted upon cooling, the "long wave" far-red bands (840–890 nm) are sharpened less but shifted appreciably towards longer wavelengths. The fluorescence bands are shifted about as much as the corresponding "long wave" absorption bands. Warming results in changes in the opposite direction. The temperature effects are reversible.

With bacteriochlorophyll dissolved in a number of polar solvents, the temperature-induced shift of the yellow band is more pronounced than that of the far-red band; with colloidal and adsorbed bacteriochlorophyll, the 840-nm red band upon cooling shifts, by a similar amount as the 850-nm band in some, though not all, measured bacterial species, while the shift of the 780 nm and yellow band is small. The possible interference of temperature effects with the interpretation of results on absorption in terms of photochemical activity is discussed.

INTRODUCTION

The red chlorophyll absorption band in the plant is of a complex structure. Usually this structure is better resolved at the temperature of liquid nitrogen —196 °C, than at room temperature[1–4]. At first sight this appears to be caused by a sharpening of the absorption bands of the components due to cooling. But not only band sharpening, also a shift in position or a change in band shape may occur. Better spectral resolution of the various pigment "forms" *in vivo* is observed in photosynthetic bacteria than in the case of chlorophyll *a* in higher plants and algae. As a result the influence of temperature changes on the spectral properties of the bands in bacteria *in vivo* can be studied with a higher precision than in chloropyll *a*-containing organisms. Measurements of Vredenburg and Amesz[5] showed that in *Chromatium* chromaphores cooling to 77 °K had a profound effect on the far-red absorption spectrum.

For a correct interpretation of photo-induced absorption changes, knowledge of the temperature effects on shape and location of the absorption bands is required.

MATERIALS AND METHODS

Chromatophores were obtained from cultures grown in a light cabinet at about 27 °C (25 W incandescent lamps at 30 cm) and harvested 4–7 days after inoculation.

The cells were disintegrated using a French press (at 0.5 ton/cm^2). The suspension was centrifuged at low speed to remove large cell debris and whole cells. Chromatophores were precipitated by centrifugation for 30 min at 20000 $\times$ g and suspended in phosphate buffer (pH 7.3, 0.02 M).

Measurements *in vitro* were done with bacteriochlorophyll which was extracted with acetone from *Rhodospirillum rubrum*, purified by thin-layer chromatography according to the method of Hager and Meyer-Bertenrath[6], and redissolved in glass-distilled acetone or ethanol. Absorption spectra were recorded with a Cary 14R spectrophotometer provided with a Cary low-temperature attachment. Absorption difference spectra in the physiological temperature range were measured with suspensions or solutions in matched stoppered quartz cuvettes, which were kept at different temperatures. The temperature was kept constant (within 0.5 °C) by placing the cuvettes in a circulating flow thermostat. In order to ascertain that no irreversible changes had occurred, the difference spectra were also measured at equal temperatures of the cuvettes before and after the experiment.

Low temperature measurements with chromatophores were made in a mixture of 55 % gycerol and 45 % phosphate buffer, which yielded a transparant glass at —196 °C. A possible influence of glycerol on the spectra was checked for by placing concentrated samples on filter paper, which were frozen immediately with liquid nitrogen. Absorption spectra were then measured by the opal glass method. With *Chlorobium limicola* intact cells had to be used, as cell disintegration, either by French press or by grinding, results in marked absorption changes.

Fluorescence spectra were recorded with an apparatus[7] and perspex sample holder[8] described earlier. Actinic light was isolated from a high-pressure mercury lamp (HPO 125) with filters transmitting light mainly from the 366- and 437-nm Hg lines. No difference in the shape of the fluorescence spectra was observed if filters transmitting the 546-nm Hg line were used. The second derivatives of the absorption spectra were obtained with the use of a Wang 720 table computer*.

RESULTS

In Figs 1a, 1b and 1c the far-red absorption of chromatophore suspensions, obtained from *Rhodopseudomonas spheroides*, *Rhodospirillum rubrum* and *Rhodospirrillum molischianum* (*Athiorhodoceae*), measured at —196 and 20 °C are given. Fig. 1d shows the spectra of *Chromatium* strain D (*Thiorhodoceae*). In general, the 800 nm absorption bands are sharpened, but little or not shifted as a result of cooling, while the bands beyond 840 nm are sharpened less but shifted markedly towards longer wavelengths. At —196 °C the absorption shoulders around 875 nm for *Rh. molischianum* and around 890 nm for *Chromatium* are seen as separate maxima around 895 and 810 nm, respectively. The 850-nm bands do not show equal long-wave shifts in all species. With *Rhodospeudomonas spheroides* this band is shifted by about 5 nm, with *Rh. molischianum* by about 12 nm.

The long-wave shift of the fluorescence band for *Rh. rubrum* is approximately equal to that of the main absorption band. This indicates that the "Stokes shift" is little altered by cooling. The fluorescence bands of the other species show a shift of a

* Thanks are due to Ir M. Sangster for providing the program and to Mr A. H. M. Haarman for performing the calculations.

magnitude similar to that of *Rh. rubrum*. In these species the position of the long-wave absorption band can be found from the second derivative spectra[9,10]. In Fig. 2 such spectra, calculated from the measured absorption spectra, are given for *Thiocystis violacea* (*Thiorhodacea*) which had absorption and fluorescence characteristics

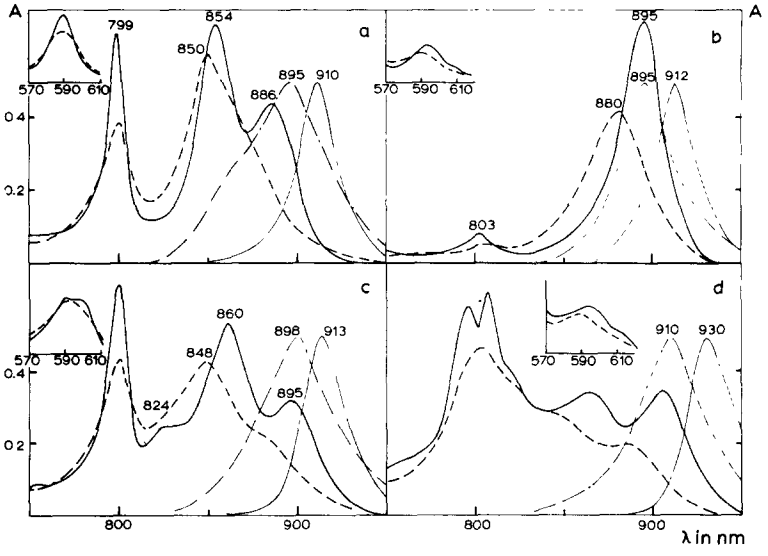

Fig. 1. Absorption and fluorescence spectra of chromatophores of the red photosynthetic bacteria, measured at 77 °K (——— and — — —), respectively and 300 °K(——— and —·—, respectively) of: (a) *Rhodopseudomonas spheroides*; (b) *Rhodospirrillum rubrum*; (c) *Rhodospirrillum molischianum*; (d) *Chromatium strain D.*

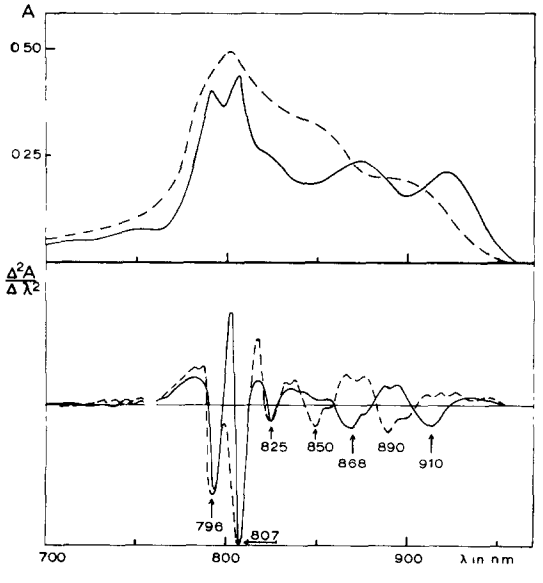

Fig. 2. Absorption spectra and second-order derivative spectra ($\Delta\lambda$ = 4 nm), measured at 77 °C (———) and 300 °K (— — —).

similar to those of *Chromatium*. The figure shows that also here the long-wave absorption maximum is shifted to the same extent as the fluorescence maximum of the various bacterial species, while no new bands are formed as a result of cooling. The maxima at 796, 808 and 824 nm are not shifted, those at 849 and 890 are shifted to 866 and 910 nm at —196 °C, respectively.

The inserts in Fig. 1 show the yellow absorption bands around 590 nm. These bands are not markedly shifted as a result of cooling to —196 °C. In the case of *Rh. molischianum* a splitting of the band is observed at low temperature while with *Rh. rubrum*, *Chromatium* and *Thiocystis* a shoulder around 615 nm can be observed. With photosynthetic bacteria only a single fluorescence maximum is found. This is due to transfer of energy absorbed in the bands at shorter wavelengths to the band of longest wavelengths. With *Rhodopseudomonas spheriodes*[11] and *Chromatium*[12] the energy transfer from the 850-nm band is not 100 % efficient. A shoulder at about 872 nm can be ascribed to emission of the 859-nm bacteriochlorophyll "form". As the absorption band of this "form" in this bacterium is shifted less than the band of the long-wave "form", it should be expected that, due to this difference and band sharpening, a second band at about 878 nm should be visible at 77 °K. This is, however, not observed. Probably due to an increase in efficiency of transfer at low temperature, the shoulder becomes much lower at low temperature.

Fig. 3a shows the absorption spectrum of the bacteriochlorophyll *b*-containing organism *Rhodopseudomonas viridis*, measured at —196 and 20 °C. With this species also the far red bacteriochlorophyll *b* band at 840 nm and the weak yellow band at 600 nm are sharpened but little shifted, while the main maximum is shifted from 1010 to 1034 nm. In Fig. 3b the absorption and fluorescence spectra of intact cells of *Chlorobium limicola*, a green sulfur bacterium, measured at —196 and 20 °C are given. The main absorption band due to *Chlorobium* chlorophyll is shifted only from 740 to 744 nm, while the shoulder at 803 nm, usually ascribed to bacteriochlorophyll, is markedly increased in intensity and shows a complex structure at —196 °C (*cf.* Gulyayev and Livitin[13]). The *Chlorobium* chlorophyll fluorescence band is shifted over a similar range as the absorption maximum (from 771 to 776 nm). The fluorescence

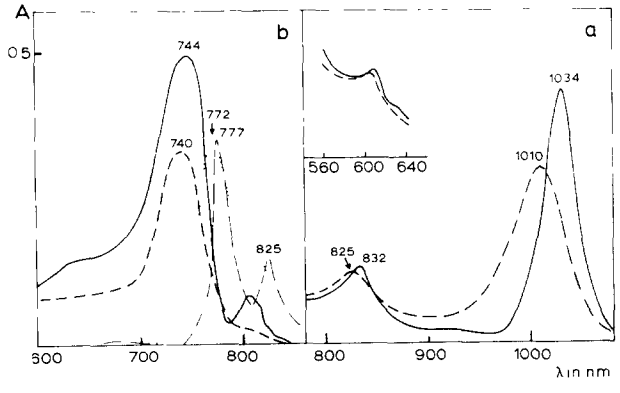

Fig. 3. a. Absorption spectrum of the bacteriochlorophyll *b*-containing organism ***Rhodopseudomonas viridis***, measured at 77 °K (———) and 300 °K (— — —). b. Absorption and fluorescence spectra of intact cells of the green photosynthetic bacterium *Chlorobium limicola*, measured at 77 °K (——— and —·—, respectively) and 300 °K (— — — and . . ., respectively).

shoulder around 810 nm at 20 °C is seen as a separate maximum at 825 nm at —196 °C, and is more likely to be emitted by the 804-nm bacteriochlorophyll "form" in this organism than by the "forms" responsible for the 813-, 825- and 833-nm absorption shoulders.

In the physiological temperature range an influence of temperature on the shape of the absorption spectrum can be measured as well. On Fig. 4 the difference spectra of a) *Rhodospirillum rubrum*, b) *Chromatium* and c) *Rhodospirillum molischianum* chromatophores kept at 37 and 17 °C are given. The largest band in the difference spectra of *Chromatium* and *Rh. molischianum* corresponds to only a shoulder in the absorption spectrum at room temperature.

If purple bacteria are extracted with organic solvents, only a single far-red bacteriochlorophyll band at about 770 nm is measured in organic solution. In dilute

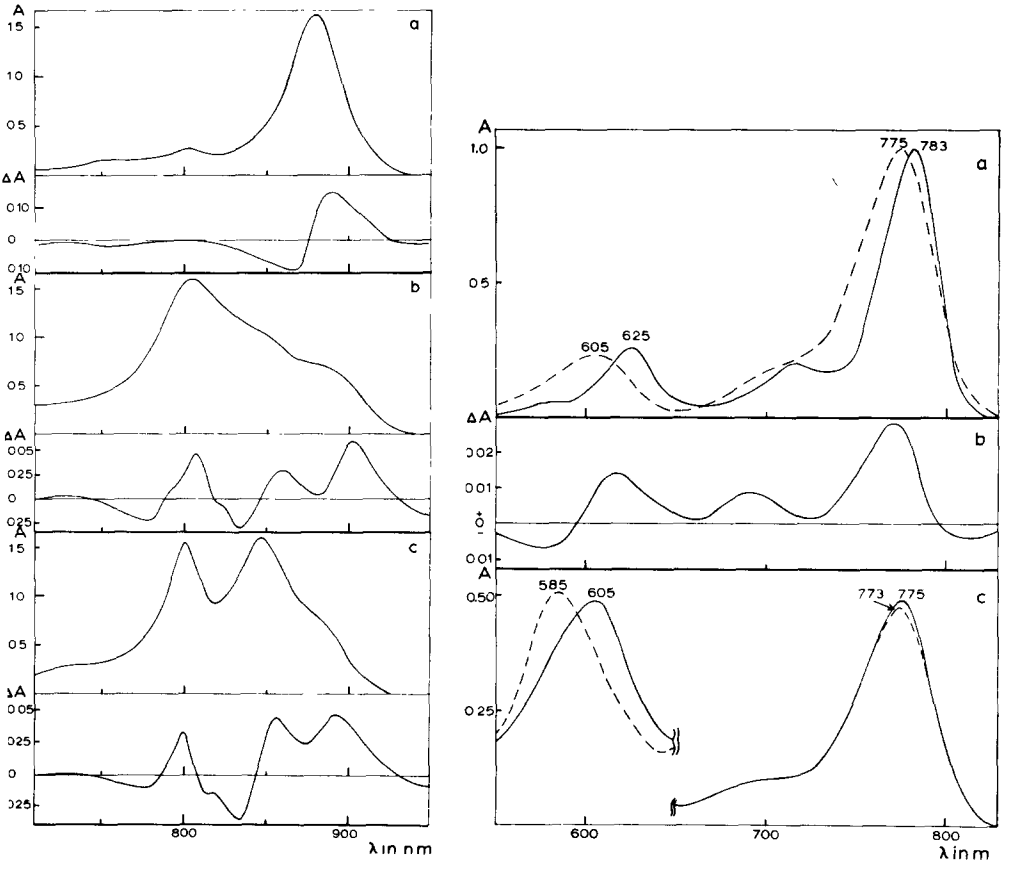

Fig. 4. Absorption spectrum at 296 °K (23 °C) and absorption difference spectrum between a cuvette kept at 310 °K (37 °C) and at 290 °K (17 °C) of chromatophores of: (a) *Rhodospirillum rubrum*; (b) *Chromatium strain D*; (c) *Rhodospirillum molischianum*.

Fig. 5. a. Absorption spectrum of bacteriochlorophyll dissolved in ethanol and measured at 77 °K (———) and 300 °K (— — —). b. Absorption difference spectrum of bacteriochlorophyll dissolved in ethanol between solutions kept at 310 and 290 °K. c. Absorption spectrum of bacteriochlorophyll dissolved in propan-1-ol (———) and propan-2-ol (— — —). The yellow band is enlarged for comparison with the far-red band.

solutions in some polar solvents, such as methanol, ethanol, propan-1-ol and pyridine, there is not much difference in position of the far-red bacteriochlorophyll band as compared with most other solvents, while the yellow band is located around 605 nm in the mentioned solvents and around 580 nm in most other solvents[14]. From fluorescence polarisation measurements it was suggested that the yellow band is due to a second electron transition, oriented perpendicularly to the one responsible for the far-red band[15,16].

Solutions of bacteriochlorophyll in ethanol can be frozen to —196 °C as a clear glass. As shown in Fig. 5a, the 605-nm absorption band is shifted to 625 nm as a result of cooling, while the far-red band is shifted only from 775 to 783 nm. As can be seen in the absorption difference spectrum in Fig. 5b, warming above room temperature results in a shift of the yellow band in the opposite direction. The shift may be caused by the increase in the dielectric constant of ethanol during cooling, or decrease during warming[17], resulting in enhanced interaction primarily with the electron state responsible for the yellow band. The dielectric constant, however, is not the only property of the solvent which determines the position of the yellow band, as no simple correlation exists between band shift and the value of the dielectric constant. If, for instance, bacteriochlorophyll is dissolved in propan-1-ol, this band is located at about 603 nm, while if dissolved in propan-2-ol, which has nearly the same dielectric constant, the band occurs at 580 nm and is little or not influenced by cooling or warming. The far-red band has a similar position in both solvents (Fig. 5c). If a concentrated solution of bacteriochlorophyll in acetone or methanol is diluted 40 times with water or neutral buffer, a non- or little fluorescent colloidal bacteriochlorophyll suspension is formed with two far-red bands, one at about 780 nm and one at about 840 nm. The ratio between those bands depends to a large extent on the conditions of preparation. No fluorescence ascribed to the 840-nm bacteriochlorophyll "form" was detected, neither at 20 nor at —196 °C. Cooling a colloidal suspension to —196 °C results in a shift of the long-wave bacteriochlorophyll "form" from 840 to 858 nm, while the short wave "form" at 780–785 nm is little or not affected. The yellow band at 590 nm is shifted by only a few nanometers.

A similar situation holds if bacteriochlorophyll is adsorbed on filter paper from a concentrated acetone or methanol solution and the solvent is evaporated. The absorption spectrum is found to be similar to that of colloidal bacteriochlorophyll. Also here the 840-nm band shifts upon cooling to —196 °C, while the 785-nm band is little or not affected and no fluorescence of the 840-nm "form" could be detected at any temperature. If the sample was allowed to warm after cooling, the 840-nm band was in its pre-cooled position.

In the case of colloidal bacteriochlorophyll the ratio of intensity of the yellow band to that of the far red band depends upon the wavelength of the latter one. With some preparations the 840-nm bacteriochlorophyll "form" increased relative to the 785-nm "form" in the first 15 min after preparation, and decreased after prolonged storage (Fig 6b); the ratio of intensities of 840 nm/590 nm was found to be about 1.6 times that of 785 nm/590 nm.

The addition of Triton X-100 or a similar anionic detergent (final concentration about 1%) to the water or buffer before dilution results in the formation of a highly fluorescent bacteriochlorophyll suspension. If dilution occurs from a concentrated solution in methanol, the yellow band shifts at —196 °C from 605 to 580 nm under

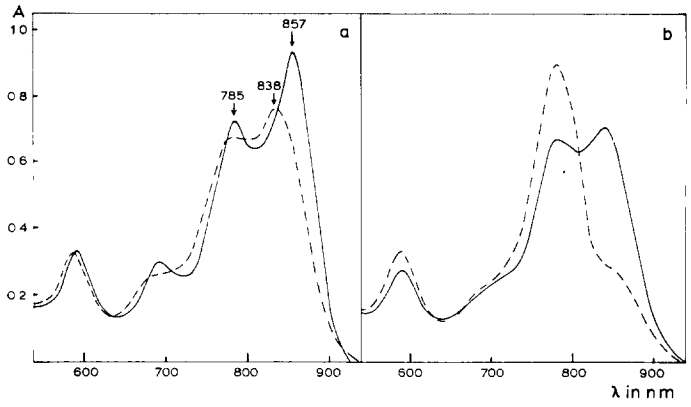

Fig. 6. a. Absorption spectrum of colloidal bacteriochlorophyll obtained by dilution of 0.4 ml of a concentrated acetone solution in 4 ml phosphate buffer (pH 7.3, 0.01 M), measured at 77 °K (———) and 300 °K (— — —). b. Absorption spectrum of colloidal bacteriochlorophyll prepared by dilution of a "crude" pigment extract, immediately after dilution (———), and after 2.5 h storage (-----) in the dark at 298 °K (25 °C).

these conditions. Cooling to —196 °C has very little effect on the position of the absorption and fluorescence bands, but the bands are markedly sharpened.

DISCUSSION

The shape of the absorption spectrum of photosynthetic bacteria and of bacteriochlorophyll *in vitro* may change markedly with variation of temperature. This effect may seriously interfere with changes due to photochemical reactions. Therefore, the temperature effect should be taken into consideration when interpreting light–dark difference spectra of pigments involved in light-induced processes.

As follows from Fig 4, the shift of the long-wave bands in bacteria results in a $\Delta\varepsilon/\varepsilon = 5 \cdot 10^{-4}$ per °C at the long-wave side of the absorption maximum. When studying light–dark absorption difference spectra using constant illumination, thermostating the cuvettes carefully will give sufficient guarantee against temperature increase. Only with very high illumination intensities will conversion of absorbed light into heat give a measurable absorption difference. (Absorption of $2 \cdot 10^5$ ergs/cm^2 per s corresponds with a $\Delta\varepsilon/\varepsilon$ of at most $1.5 \cdot 10^{-4}$/min, which is close to the limit of detection of most absorption difference spectrophotometers.) Also, temperature equilibrium after the end of illumination usually is a slow effect as compared to reversible photoreactions. Temperature effects at a fixed wavelength then will primarily affect the baseline of the curves.

When absorption differences obtained with flash illumination (especially with short, intense Laser flashes when all emitted light is brought into the sample) are studied, the temperature effects may seriously interfere with photochemical effects. In view of the chromatophore dimensions, the time of reaching temperature equilibrium between a chromatophore or pigmented chromatophore fraction and its near surroundings can be of a similar order of magnitude as the time in which reversible photochemical absorption changes occur. Absorption changes brought about by temperature differences in general can be due to:

1. A change in pigment concentration or change in optical path length due to expansion or contraction of solvent or cuvette. The difference spectrum has a shape identical to that of the absorption spectrum. The effect can be considered as an artifact and can be corrected for.

2. A shift in position of the absorption bands. The difference spectrum caused by this effect shows maxima at one side and minima at the other side of the absorption bands.

3. Sharpening or broadening of the bands. The difference spectra show a maximum or minimum at the top of the absorption bands and minima (or maxima) at both sides of it.

4. Chemical changes of the pigment. The difference spectra may have any form. As, however, the difference spectra disappear after the experiment, the changes, if any, should be reversible.

As follows from Figs 1 and 4, both (2) and (3) play an important role in temperature-induced absorption difference spectra *in vivo*. Although the temperature-induced shift of the far-red bands always exceeds that of the shorter wave bands in red bacteria, there is no direct correlation between band location and temperature-induced shift (840-nm band in *Rh. spheriodes* and *Rh. molischianum*). This suggests that the position of the band *in vivo* is not caused by the same kind of interaction in all bacteria. In this respect it should be remarked that the high *in vitro–in vivo* shift of *Chlorobium* chlorophyll corresponds to only a weak temperature-induced absorption shift.

REFERENCES

1 Y. F. Frei, *Biochim. Biophys. Acta*, 57 (1961) 82.
2 C. S. French, J. S. Brown, C. Prager and M Lawrence, *Carn. Inst. Wash. Yearb.*, 67 (1968) p. 536.
3 W. A. Cramer and W. L. Butler, *Biochim. Biophys. Acta*, 153 (1968) 889.
4 J. M. Briantais, *Photochem. Photobiol.*, 6 (1957) 155.
5 W. J. Vredenberg and J. Amesz, *Biochim. Biophys. Acta*, 126 (1966) 244.
6 J. C. Goedheer, *Biochim. Biophys. Acta*, 88 (1964) 304.
7 C. Bril. D J. van der Horst, S. R. Poort and J. B. Thomas, *Biochim Biophys. Acta*, 172 (1969) 345.
8 A. Hager and T. Meyer-Bertenrath, *Planta*, 69 (1966) 189
9 B. A. Gulyayev and F. F. Livitin, *Biofizika*, 15 (1970) 760.
10 W. L. Butler and D. W. Hopkins, *Photochem. Photobiol.*, 12 (1970) 439.
11 J. C. Goedheer and A. K. van der Tuin, *Biochim. Biophys Acta*, 143 (1967) 399.
12 C. Bril, *Biochim. Biophys. Acta*, 66 (1963) 50.
13 B. A. Gulyayev and F. F. Livitin, *Biofizika*, 12 (1967) 845.
14 J. C. Goedheer, *Biochim. Biophys. Acta*, 27 (1958) 490.
15 J. C. Goedheer, Doctoral Thesis, Utrecht, 1957.
16 T. G. Ebrey and R. K. Clayton, *Photochem. Photobiol.*, 10 (1969) 109.