INVESTIGATIONS ON BACTERIOCHLOROPHYLL IN ORGANIC SOLUTIONS

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INTRODUCTION

In spite of the considerable progress which has been made in the understanding of the mechanism of photosynthesis, little is known about the way the pigments act in this process. Investigations on pigments *in vitro* in solution, pigments *in vitro* located on some structure, and pigments *in vivo*, are an important tool in the solution of this problem. Apart from their value for photosynthesis, such studies may be of interest with regard to other photobiological problems, such as photoperiodism or phototaxis.

Up to now many experiments have been done on the spectral and photochemical properties of chlorophyll *a* dissolved in organic solvents, but comparatively little information is available about these properties in bacteriochlorophyll. In the latter pigment, however, the absorption bands belonging to the different electronic transitions are more separated from each other than is the case with chlorophyll *a*, and the locations of the absorption maxima do not coincide approximately with those of related pigments (GOEDHEER[1]). Experiments on bleaching, both reversible and irreversible, also had more definite results in bacteriochlorophyll. We therefore devoted our attention to this pigment and studied some of its spectral and photochemical properties in organic solvents.

METHOD

Bacteriochlorophyll was obtained by extraction with methanol of cultures of *Rhodospirillum rubrum* strain 1. The pigment was purified by chromatography either over a sugar column, following the method described by HOLT AND JACOBS[2], or over a column of aluminum oxide. Where a "crude" extract was used in the experiments, the methanol extract was taken immediately after filtration and shaken with petroleum ether (to remove carotenoids).

In the experiments to determine the quantum yield of irreversible bleaching, the pigment was illuminated with a parallel beam of monochromatic light, obtained with an interference filter with a maximum transmission at 762 mμ. The light intensity was measured with a vacuum thermopile. The time during which the bleaching of bacteriochlorophyll proceeded to the same percentage in dependence on solvent or quinone concentration, was measured.

Fluorescence spectra were obtained with an apparatus analogous to that described by DUYSENS[3], while the effect of quinone on polarisation of fluorescence was measured with an apparatus described earlier (GOEDHEER[1]).

The temperature-dependent regeneration curves of the reversible photobleaching were registered with a Brown recorder. Light passing the above-mentioned interference filter and the cuvette containing the bacterial extract, which was placed in a thermostat, entered a CsCsO photocell. The photocell was connected with the recorder. Bleaching of the bacteriochlorophyll

References p. 490.

extract was obtained by the illumination with an Osram BL 7 flash tube of 300 W/sec. The flash time was of the order of 1 msec.

From the number of absorbed quanta (derived from measurements with the thermopile) and the number of the pigment molecules that are decomposed by the light (derived from the absorption and absolute extinction coefficients published by SMITH AND BENITEZ[4], the quantum yield of bleaching was calculated.

RESULTS

1. *Dependence on solvent and on magnesium content of the "yellow" absorption band*

The absorption band, which is due to the second electronic transition (*cf.* GOEDHEER[1]), is much more pronounced in bacteriochlorophyll than the corresponding band in chlorophyll *a*. In both pigments the maximum of this band is located at about 580 mμ in most solvents. In chlorophyll *a* this maximum has for a long time incorrectly been assumed to correspond with a vibrational level of the long-wavelength transition (main maximum at about 660 mμ). In methanol, however, this "yellow" band of bacteriochlorophyll is shifted to about 605 mμ, while in chlorophyll *a* too the band at 580 mμ is shifted towards longer wavelengths. As was indicated by measurements of fluorescence polarisation, the magnesium-free pigments bacteriopheophytin and pheophytin *a* show this band to be situated at about 535 mμ.

Nowadays the location of the maximum of this band in bacteriochlorophyll is studied as a function of the refractive index of the solvent. This was done in both polar and non-polar solvents. The results are given in Fig. 1. With the non-polar solvents the wavelength dependency is roughly as expected from Kundt's rule. This is not the case with the results in polar solvents. A purified pigment extract was used, and as far as possible the solution was kept in the dark. This was necessary because a quick bleaching reaction in light, especially in alcoholic solvents, resulted in the appearance of new bands. These bands tended to shift the apparent absorption maximum of the band studied towards longer wavelengths.

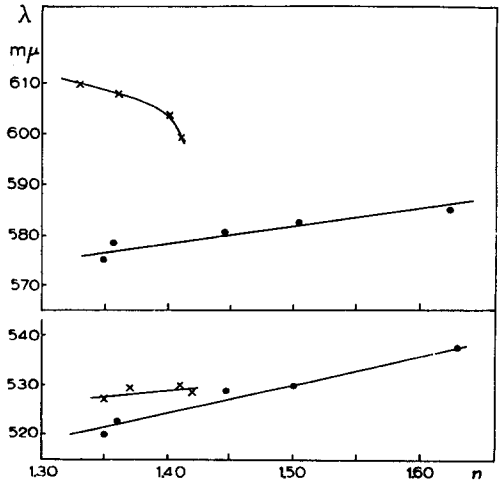

Fig. 1. Location of the maximum of the "yellow" absorption band of bacteriochlorophyll and bacteriopheophytin in solvents of different refractive index. The crosses mark the values for methanol, ethanol, amylol and hexanol, the dots the values for by ether, acetone, benzene, tetracarbonchloride and carbon disulfide, respectively.

References p. 490.

Fig. 1 shows the wavelength dependency, in both polar and non-polar solvents, of the band due to the same electronic transition in bacteriopheophytin (and pheophytin *a*) .This graph demonstrates that there is no substantial difference between the two types of solvent in the location of the maximum of this band. The wavelength of the maximum absorption increases approximately proportionally with the refractive index of the solvent.

2. *Photo bleaching in air-saturated pigment solution*

Quantum yield of bleaching. Bacteriochlorophyll is known to be very unstable. If exposed to light, it "bleaches" much more quickly than chlorophyll *a* (VERMEULEN, WASSINK AND REMAN[5], MANTEN[6], SEYBOLD AND HIRSCH[7]). Other investigations mentioned that once it is chromatographically purified it shows rather good stability, comparable to that of chlorophyll *a* (HOLT AND JACOBS[2]). Indeed, it was found that a crude bacteriochlorophyll solution in moderately strong incandescent light could be bleached to a green pigment in a few minutes. In order to obtain a quantitative measure of the bleaching velocity the quantum yield of bleaching was determined for light absorbed in the long-wavelength absorption band (absorption maximum in methanol at about 770 mμ). These experiments were made both with "crude" and "pure" extracts.

Firstly, the effect of impurities and type of solvent was studied. Table I presents the quantum yield values in different solvents for both types of extracts. This table indicates that not the purification of the extract but the type of solvent is of primary importance in the establishment of the velocity of "bleaching". In methanol, purification decreases the quantum yield of bleaching by about 25%. It is not certain, however, that this relatively small decrease is due to an elimination of impurities. If the purified extract is stored in the dark for several days, the quantum yield of bleaching shows a further decrease. This may indicate that during purification and afterwards a change in structure of the bacteriochlorophyll molecules may occur, without an accompanying change in the absorption spectrum.

TABLE I

QUANTUM YIELD OF IRREVERSIBLE BLEACHING OF BACTERIOCHLOROPHYLL IN DIFFERENT SOLVENTS

	Crude	*Purified*
Methanol	$5.2 \cdot 10^{-3}$	$3.8 \cdot 10^{-3}$
Ethanol	$4.5 \cdot 10^{-3}$	$3.4 \cdot 10^{-3}$
Cyclohexanol	$3.1 \cdot 10^{-3}$	$3.0 \cdot 10^{-3}$
Ether	$4.0 \cdot 10^{-4}$	$4.5 \cdot 10^{-5}$
Petroleum ether	—	$8.0 \cdot 10^{-5}$
Pyridine	—	$2.4 \cdot 10^{-3}$

In ether solution the quantum yield of bleaching amounts to about 2% of the value in methanol and is of about the same order of magnitude as that of chlorophyll (a value of $\gamma = 4.5 \cdot 10^{-5}$ was measured for the latter pigment by LIVINGSTONE[8]). As "crude" bacteriochlorophyll is usually extracted from the bacteria with methanol, while chromatographically-purified bacteriochlorophyll is usually dissolved in ether, the results from Table I may explain why the results given in the literature are conflicting.

References p. 490.

The 770 mμ band was chosen since the absorption of the bleached compound is minimal in this spectral region. This property simplifies the determination of the quantum yield of bleaching. It should also be noted that with the alcoholic solvents the viscosity covers a wide range (from 0.006 poise with methanol to 0.60 poise with cyclohexanol), while the difference in quantum yield of bleaching is only relatively small.

The addition of a few percent of methanol to the ether solution results in an increase of the quantum yield values to the same order of magnitude as the ones in a pure methanol solution, although the absorption spectrum remains of the ether type.

The photo-bleaching of bacteriopheophytin proceeds much slower than that of bacteriochlorophyll. A quantum yield value of the order of $3 \cdot 10^{-5}$ was found to occur in methanol.

Secondly, the effect of temperature and pigment concentration on the bleaching velocity of bacteriochlorophyll dissolved in methanol was determined. A weak temperature coefficient was found. The bleaching proceeds slightly quicker at lower temperatures. If the bleaching velocity is set arbitrarily at 1 at 20° (corresponding to a quantum yield of bleaching of $\gamma = 3.5 \cdot 10^{-3}$), the value was found to be 1.10 at 10° and 0.90 at 37°. If the concentration was varied from $2 \cdot 10^{-6}$ moles/l to $2 \cdot 10^{-4}$ moles/l the bleaching velocity was found to increase by about 40%. This may, however be partly due to the different size of the cuvette, which caused slightly different illumination. Compared with the 100-fold increase in pigment concentration, the increase in bleaching velocity is very small.

Thirdly, the effect of the addition of quinone and ferrous sulfate was considered. A marked slowing-down of the velocity of bleaching is caused by the addition of small amounts of quinone; the quantum yield of bleaching can be reduced to less than 1% of its original value. The concentration of quinone in the cuvette needed to reduce the quantum yield of bleaching in methanol to ½ of its value in the absence of quinone is 10^{-3} moles/l. The quinone effect was found to be approximately linear with the concentration in the range studied (Table II). Addition of 0.1 mole/l ferrous sulfate also resulted in a decrease of the quantum yield of bleaching to ½ of its original value.

TABLE II

RELATIVE BLEACHING VELOCITY FOR DIFFERENT AMOUNTS OF QUINONE ADDED TO A SOLUTION OF BACTERIOCHLOROPHYLL IN METHANOL

Concentration moles/l	*Relative bleaching velocity*
0	1
$1.6 \cdot 10^{-3}$	0.35
$3.3 \cdot 10^{-3}$	0.22
$5.0 \cdot 10^{-3}$	0.14
$6.5 \cdot 10^{-3}$	0.09

Absorption spectra of photo-bleached bacteriochlorophyll. In Fig. 2 the absorption spectra of bacteriochlorophyll and the bleached compound are given, using methanol as a solvent. It follows from this figure that the spectrum has changed considerably as a result of photo-bleaching. The original absorption band with a maximum at 770 mμ has nearly disappeared. At about 900 mμ absorption is increased; this suggests

References p. 490.

the occurrence of a new absorption band. Other new absorption maxima are present at about 640 mμ, 585 mμ, 545 mμ and 410–390 mμ. In the ultraviolet, the absorption spectrum also differs from that of the original bacteriochlorophyll.

The bleached compound is not stable. On prolonged illumination, and also after standing in the dark, the spectrum changes. Fig. 3 presents the difference spectrum, obtained from the absorption spectrum measured directly after bleaching and the one measured eight hours after standing in the dark. It is seen from this figure that the long-wavelength absorption band (at about 900 mμ) decreases in intensity. The

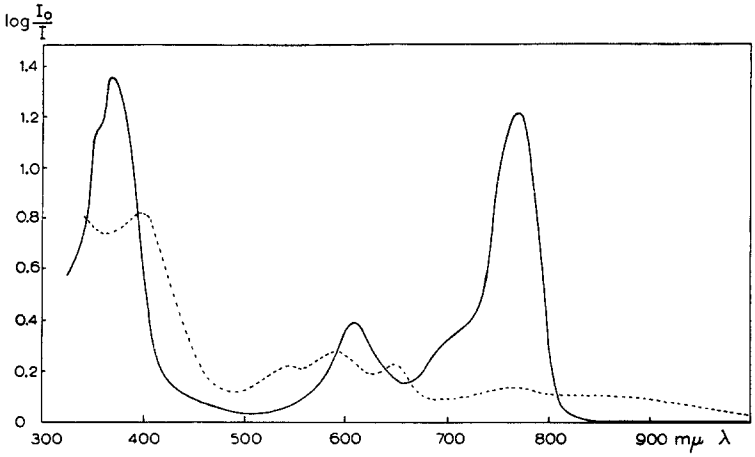

Fig. 2. Absorption spectrum of bacteriochlorophyll dissolved in methanol. The dotted line shows the spectrum after the pigment has been bleached in light mainly absorbed in the infrared absorption band.

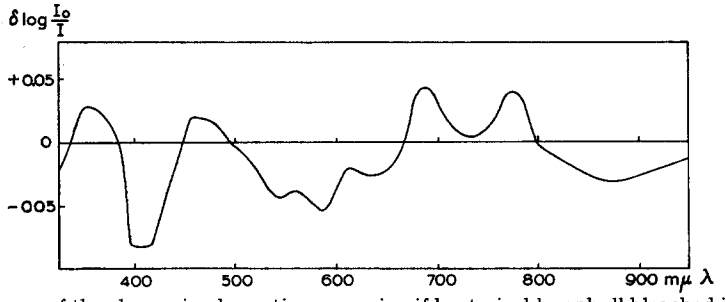

Fig. 3. Spectrum of the change in absorption occurring if bacteriochlorophyll bleached in methanol is allowed to stand for eight hours in the dark. The maximum absorption in the infrared absorption band of the pigment in the non-bleached state amounted to log $I_0/I = 1.10$.

770 mμ band from the original bacteriochlorophyll seems to regenerate slightly. A band also occurs at 680 mμ, while the bands at 640 mμ, 585 mμ, 545 mμ and about 400 mμ disappear. The band at 680 mμ most probably belongs to the green oxidation product (with a chlorophyll *a* type of spectrum) that was described by HOLT AND JACOBS[2]. After chormatographic analysis over a sugar column this pigment (or a pigment spectroscopically identical to it) always proved to be present to some extent in bacteriochlorophyll. In a fresh "crude" extract of bacteriochlorophyll this pigment does not seem to be present either before or after bleaching. Nor do fluorescence measurements show the occurrence of such a pigment, even in small amounts.

References p. 490.

In non-alcoholic solvents, such as acetone, benzene, or ether, photobleaching results in a spectrum as presented in Fig. 4. This spectrum is similar to the one obtained from solutions in methanol after the solution has been stored in the bleached state in the dark for 24 hours. Apparently the only bands in the visible part of the spectrum are those at 680 mμ and 430 mμ, probably belonging to the above-mentioned pigment. In these solvents no change in the spectrum is observed after prolonged storage of the solution in the dark.

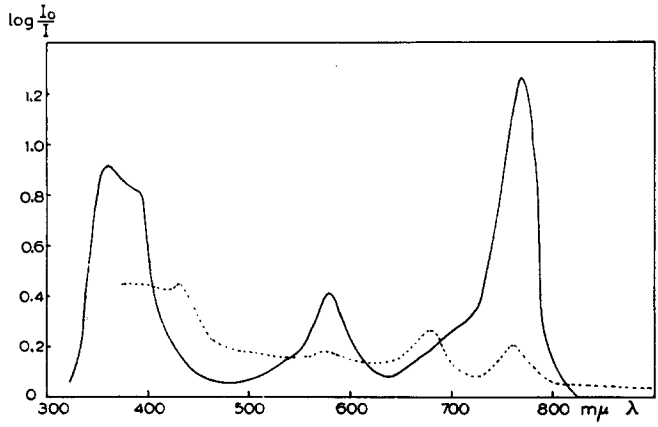

Fig. 4. Absorption spectrum of bacteriochlorophyll dissolved in acetone. The dotted line presents the spectrum after the pigment has been bleached in light mainly absorbed in the infrared absorption band.

When hydrochloric acid was added to the bleached bacteriochlorophyll in amounts that would lead to a quick pheophytinisation of the original bacteriochlorophyll, no difference in the absorption spectrum could be observed.

Reversibility of photobleaching in air-saturated pigment solutions. It was found that in methanol the bleaching of the 770 mμ band was slightly reversible. KRASNOWSKI[9] stated that after addition of ascorbic acid or hydrogen sulfide the absorption of the 770 mμ band could be regenerated to about 70% of its original intensity. This was confirmed by our measurements. Purified bacteriochlorophyll dissolved in methanol was bleached with incandescent light. Ascorbic acid was added immediately afterwards. The 770 mμ band could be regenerated to about 75% of its original intensity. When the bleached bacteriochlorophyll was allowed to stand until the absorption spectrum had changed from the type presented in Fig. 2 to that presented in Fig. 4, the percentage of the reversibility was strongly decreased. When other solvents were used, such as acetone, benzene, or ether, no reversibility of the bleaching was detected.

Moreover, experiments were made in which a solution of bacteriochlorophyll in methanol was bleached by a strong flash of light. Before the experiment an excess of ascorbic acid has been added to the solution. The regeneration of the 770 mμ band was followed by a Brown recorder. Fig. 5 shows curves obtained when the temperature of the solvent was 20° and 35°, respectively. At lower temperatures a decrease in the absorption occurs in the first seconds. Owing to the excess of ascorbic acid the curves are approximately of a shape as the curve for a monomolecular reaction. From the

References p. 490.

temperature coefficient a heat of activation of about 24,000 cal/mole can be calculated for this reaction.

Fig. 6 shows the change in absorption of the 770 mμ band after illumination with a flash of light, without addition of any ascorbic acid.

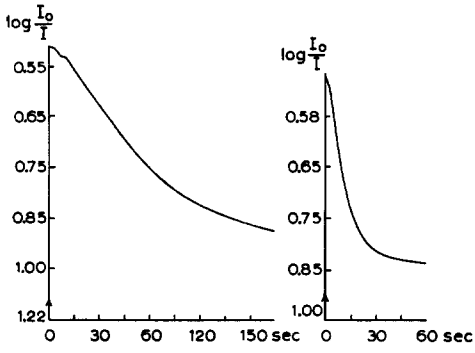

Fig. 5. Increase in absorption of the infrared absorption band of bacteriochlorophyll dissolved in methanol, owing to the addition of ascorbic acid. At time zero the solution was bleached with a strong flash of light. The temperature of the solution in the left-hand curve amounted to 20°, that in the right-hand curve amounted to 35°.

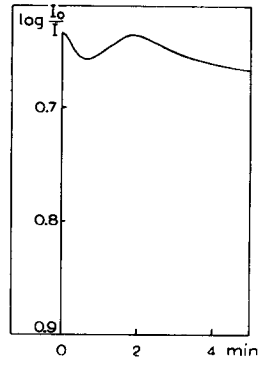

Fig. 6. Fluctuations in the absorption at 765 mμ after the bacteriochlorophyll solution has been bleached without the addition of ascorbic acid.

3. *Reversible chemical bleaching*

In accordance with the results of RABINOWITCH AND WEISS[10] on chlorophyll *a*, it was found that bacteriochlorophyll could be bleached in methanol by the addition of small amounts of ferric chloride ($5 \cdot 10^{-4}$ moles/l). If, immediately afterwards, an excess of ferrous sulfate was added, the original bacteriochlorophyll spectrum was fully restored.

The absorption spectrum of the chemically bleached bacteriochlorophyll is presented in Fig. 7. This figure shows that a new absorption band arises with a maximum at about 525 mμ, another with a maximum at about 900 mμ and a third with a maximum at about 420 mμ. In contrast to the behaviour of the "red" absorption band of chlorophyll *a* (670 mμ), the long-wavelength band of bacteriochlorophyll (770 mμ) does not show slow regeneration upon standing after bleaching with ferric chloride. The absorption spectrum of the chemically bleached bacteriochlorophyll in methanol, however, shows other changes with time. After about ten minutes the spectrum changes to one closely resembling the absorption spectrum shown in Fig. 2, produced by photo-bleaching of bacteriochlorophyll. After prolonged standing for several hours in methanol this spectrum changes into one closely resembling the curve presented in Fig. 4 in which only bands due to the green pigment with the chlorophyll *a* type of spectrum (680 mμ and 430 mμ) are seen. Actually this pigment was isolated by chromatography. In contrast to chlorophyll *a*, and also to bacterioviridin, it was not possible to bleach this green pigment at any concentration of ferric chloride.

Bacteriochlorophyll could also be reversibly bleached when dissolved in acetone. The spectrum obtained with this solvent closely resembles the one given in Fig. 7. It is, however, much more stable than the spectrum obtained with methanol as

References p. 490.

solvent. It shows no resemblance to a spectrum of the type depicted in Fig. 2, but gradually changes to a type resembling that shown in Fig. 4.

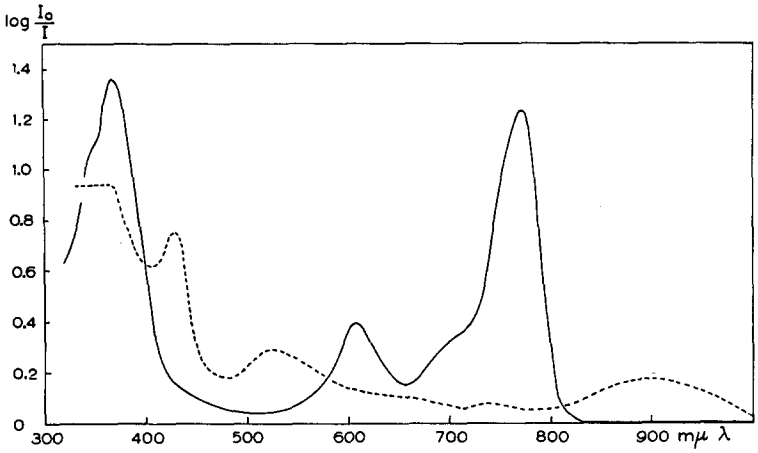

Fig. 7. Absorption spectrum of bacteriochlorophyll dissolved in methanol, before and after the addition of ferric chloride or iodine. A similar spectrum occurs if bacteriochlorophyll is dissolved in acetone.

Bleaching of bacteriochlorophyll could be produced not only by addition of ferric chloride but also by addition of iodine or potassium permanganate. Immediate reversibility could be obtained in these cases if ascorbic acid was added. This proved to be also true for iodine when ether was used as a solvent. In pure ether the reversibility was only partial, but if some water was added to increase the solubility of the ascorbic acid, total reversibility could be obtained. The absorption spectrum was found to have approximately the same shape as that obtained by bleaching with ferric chloride in acetone.

When bleaching with iodine, the 770 mμ band was found in some cases to regenerate slowly upon standing, but its maximum was found to be shifted to a shorter wavelength (750 mμ), a similar shift to that which occurs with chlorophyll *a*.

If bacteriochlorophyll in the bleached state (with ferric chloride) was transferred from methanol to ether, the absorption band at 770 mμ regenerated for about 40%, while absorption band at 680 mμ of about the same intensity also occurred, the latter probably being due to the above-mentioned green pigment.

No chemical bleaching was found to occur if bacteriopheophytin was used instead of bacteriochlorophyll.

4. *Observations on bacteriochlorophyll fluorescence*

Fluorescence spectra. In order to obtain some information about the possible structure of the photo-bleached bacteriochlorophyll, fluorescence spectra were determined before and after bleaching. This was done with both non-purified and purified bacteriochlorophyll dissolved in methanol. The results are given in Fig. 8. This figure shows that, as could be expected, the main maximum of bacteriochlorophyll fluorescence is situated at about 785 mμ. Two very weak fluorescence maxima are present in the spectrum of non-purified bacteriochlorophyll, namely, one at

References p. 490.

590 mμ and one at 650 mμ. No fluorescence band at 690 mμ is seen. If illumination is with a near-ultraviolet light (366 mμ) from a mercury lamp, the colour of fluorescence is orange. In the insert the fluorescence of the wavelength region between 570 mμ and 700 mμ is drawn seven times enlarged. Bleaching results in a strong decrease of the 785 mμ band; the other two fluorescence bands remain approximately constant. It was thought at first (GOEDHEER[1]) that these two bands arose after bleaching. However, careful examination of the original spectrum showed that they were present beforehand. In the fluorescence spectrum of purified bacteriochlorophyll the bands at 590 mμ and 650 mμ are absent. Instead of these, a band at about 690 mμ is found. The latter band is probably due to the pigment with the chlorophyll *a* type of spectrum. It could not be avoided that this pigment appeared in very small amounts during purification.

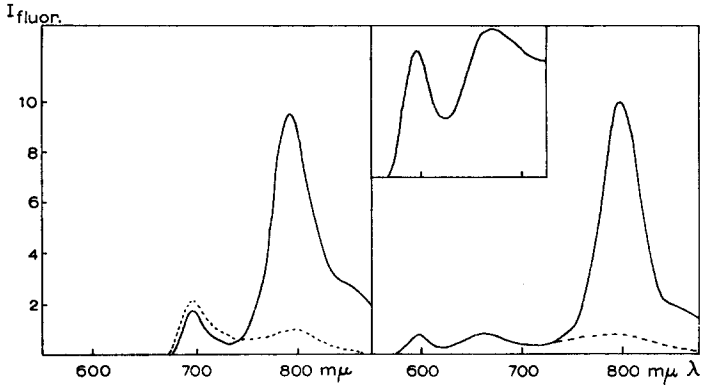

Fig. 8. Fluorescence spectrum of "pure" (left-hand graph) and "crude" (right-hand graph) bacteriochlorophyll dissolved in methanol. The solid line indicates the spectrum before bleaching, the dotted line the one after bleaching. In the insert, part of the spectrum is presented seven times enlarged.

If the "crude" extract was chromatographed over a sugar column, it was possible to isolate a small amount of a pigment with a porphyrin type of spectrum, showing small peaks at 580 mμ and 545 mμ and a Soret band at 420 mμ. This pigment may be responsible for the fluorescence band at 590 mμ.

Quenching of fluorescence. If quinone is added to a solution of bacteriochlorophyll, quenching of fluorescence occurs. In Table III the relative intensity of the fluorescence of bacteriochlorophyll, bacterioviridin and chlorophyll *a* is given for different amounts of quinone added to a methanol solution. The "50% quenching concentration" was found to be $1.8 \cdot 10^{-2}$ moles/l for bacteriochlorophyll, 10^{-2} moles/l for bacterioviridin and $0.8 \cdot 10^{-2}$ moles/l for chlorophyll *a*.

Effect of quinone on the polarisation of fluorescence. Quenching of fluorescence may be caused in two different ways: (1) part of the molecules may become non-fluorescent, while for the rest the mean lifetime of fluorescence remains unchanged (static quenching), and (2) the mean lifetime decreases for all pigment molecules (dynamic quenching).

The method of polarisation of fluorescence enables one to discriminate between these two possibilities. If quenching of fluorescence is due to the decrease in mean lifetime of all molecules, an increase in the degree of polarisation at a given viscosity

References p. 490.

TABLE III

QUENCHING OF FLUORESCENCE FOR DIFFERENT AMOUNTS OF QUINONE ADDED WITH METHANOL AS A SOLVENT

Amount of quinone added in moles/l	*Bacteriochlorophyll rel. int.*	*Bacterioviridin rel. int.*	*Chlorophyll a rel. int.*
0	1	1	1
0.0033	0.86	0.75	0.66
0.0066	0.71	0.60	0.53
0.0100	0.61	0.50	0.42
0.0528	0.22	0.17	—
0.0132	0.56	0.44	0.34
0.0246	0.40	0.28	0.20
0.0396	0.30	—	—
0.0528	0.22	0.17	—
0.0660	0.175	—	—
0.1056	0.10	0.08	—

of the solvent would result. On the other hand, the degree of polarisation may be expected to remain unaltered if fluorescence quenching is due to the non-fluorescence of part of the pigment molecules.

With bacteriochlorophyll and chlorophyll *a* the degree of fluorescence polarisation was measured as a function of the amount of quinone added. The results are given in Table IV. It follows from this Table that at a quinone concentration causing a fluorescence quenching of 75% the degree of polarisation changes from 0.18 to 0.20 for bacteriochlorophyll and from 0.039 to 0.052 for chlorophyll *a*. It may be concluded from calculation (*cf.* GOEDHEER[1]) that if the fluorescence quenching is supposed to be caused by a decrease of the mean lifetime of all pigment molecules, the polarisation degree may be expected to increase from 0.18 to 0.25 for bacteriochlorophyll and from 0.039 to 0.072 for chlorophyll *a*. Hence the polarisation experiments indicated that most probably the quenching is caused mainly by the non-fluorescence of part of the molecules owing to the addition of quinone to the solution.

TABLE IV

QUINONE DEPENDENCY OF POLARISATION AND QUENCHING OF FLUORESCENCE OF BACTERIOCHLOROPHYLL AND CHLOROPHYLL IN AMYL ALCOHOL

Concn. quinone moles/l	*Fluorescence bacteriochl. p*	*Polarisation chl. a p*	*Fluorescence bacteriochl. I/I_0*	*Quenching chl. a I/I_0*
0	0.17	0.039	1	1
0.0033	—	0.039	—	0.90
0.0066	—	0.041	—	0.81
0.0132	0.17	0.041	0.78	0.69
0.0246	0.18	0.048	0.62	0.56
0.0528	0.20	0.058	0.50	0.38
0.1056	0.24	0.066	0.35	0.26

DISCUSSION

The results of the measurements about the location of the "second" absorption band (Fig. 1) suggest that the influence of the polarisability of the solvent molecules

References p. 490.

on the spectrum of bacteriochlorophyll (and probably also on that of chlorophyll *a*) depends on the presence of the central magnesium atom. *In vivo* the absorption band due to the "second" electronic transition is situated at 590 mμ. If it is taken into account that a small shift towards longer wavelengths occurs owing to the high pigment concentration, this location corresponds approximately to the one in non-polar solvents. This might be an indication that the reversible photo-bleaching, which was found to occur only in alcoholic solvents, does not occur in the bacteria.

The possibility of obtaining a reversible chemical bleaching of bacteriochlorophyll in widely different solvents suggests that the change in absorption is essentially a property of the pigment molecule itself, and not due to the interaction of the pigment with the solvent molecules. (Especially in ether, the sharp absorption bands and the low quantum yield of bleaching indicate a weak pigment-solvent interaction.) This suggestion will probably be true for chlorophyll *a* also. The bleaching of this pigment, however, does not proceed in iodine, and the weak solubility of ferric chloride in ether is too small to indicate the effect. The difference in behaviour towards bleaching suggested that if it were possible to measure an oxidation-reduction potential of the system bacteriochlorophyll-bleached bacteriochlorophyll, this value would be lower than that of chlorophyll *a*. Combined potentiometric and absorption measurements indicated that this was indeed the case (GOEDHEER, DE HAAS, SCHULLER AND WELLE[14]): it was found that reproducible values could be measured.

The absorption spectrum of bacteriochlorophyll bleached by addition of ferric chloride looks similar to that of chlorophyll *a* under the same conditions. In both pigments a new absorption band arises, with a maximum at about 525 mμ. The increase in absorption in a wavelength region beyond the infrared absorption band in bacteriochlorophyll (around 950 mμ) was also found to correspond with an increase in absorption beyond the red absorption band of chlorophyll *a* (around 740 mμ). In the region of the Soret band too, the behaviour seems analogous for both pigments. For chlorophyll *a* a spectrum of a similar shape was found to occur after: (1) photochemical bleaching in air-free pyridine to which ascorbic acid was added (EVSTIGNEEV AND GAVRILOVA[11]); (2) photo-bleaching in air-free methanol (LIVINGSTON AND RYAN[12]); (3) reversible photo-bleaching at liquid-air temperatures (LINSCHITZ AND RENNERT[13]). It seems possible that a single modification of chlorophyll *a* or bacteriochlorophyll (here called *r*-chlorophyll for simplification) accounts for the occurrence of these spectra under different conditions.

The spectrum obtained by photo-bleaching in methanol (Fig. 2) closely resembles that occurring after addition of ferric chloride and standing in air for 10 min. Thus, spectroscopically, it seems that oxidation in the dark of the *r*-bacteriochlorophyll shows the same result as photo-oxidation of bacteriochlorophyll. That the photo-bleaching in air was indeed a photo-oxidation was found by measuring oxygen uptake in light as a function of time[14].

It was formerly thought possible that the photo-bleached product of bacteriochlorophyll might consist of several components[1]. Some of these components might be fluorescing and have a porphyrin type of absorption spectrum. The measurements of the fluorescence spectra, however, do not support such a hypothesis. The bleached product does not show any fluorescence immediately after bleaching in the spectral region from 550 mμ to 950 mμ. On the other hand, photo-bleaching was always found to be not completely reversible even if short light flashes were used, and the ultra-

References p. 490.

violet part of the spectrum suggests that part of the bacteriochlorophyll bleached in methanol may directly show the spectrum (without the 680 mμ and 430 mμ fraction) of bacteriochlorophyll bleached in other solvents. Inhibition of photo-bleaching by excluding oxygen could only be obtained by bubbling purified nitrogen through the solution for 1 ½ hours.

The weak temperature coefficient, as was measured for photo-bleaching of bacteriochlorophyll, was also found to be reported for the irreversible bleaching of chlorophyll *a* (*cf.* RABINOWITCH[15]).

Contrary to chlorophyll *a*, bacteriochlorophyll does not show signs of allomerisation in methanol. After several days of storage at 35° in the dark the spectrum remains unchanged. In ether, however, spectral shifts resembling those due to allomerisation may be obtained. If bacteriochlorophyll is bleached by light or, slowly, by ferric chloride or iodine, in some cases an absorption shift may be obtained for the long wavelength band from 760 mμ to 750 mμ. No reversibility of this effect could be perceived.

Whether the effect of quinone on bleaching is correlated with the fluorescence bleaching is not clear. The "50% quenching concentration" is about 20-fold higher than the concentration needed to double the time of bleaching. The "50% quenching" concentration" was found to be twice as small for chlorophyll *a* than it was for bacteriochlorophyll, while the lifetime of fluorescence of the former pigment exceeds that of the latter by a factor of two (GOEDHEER[1]). It was also found that the "50% quenching concentration" in more viscous solvents was higher than that in less viscous solvents (*cf.* Tables III and IV). Both facts can be explained by assuming that if an excited chlorophyll molecule meets a quinone molecule, the excitation energy is dissipated (dynamic quenching, *cf.* FÖRSTER[16]). The measurements of polarisation, however, are contradictory to this. They indicate more a quenching of the static type.

SUMMARY

Various spectral and photochemical properties of bacteriochlorophyll were studied. It was found that the spectral shift of the second absorption band of this pigment in polar solvents is nearly absent in the magnesium-free pigment.

The quantum yield of irreversible bleaching was determined with respect to the type of solvent and the purity of the pigment. A difference of a factor of 100 was found to occur between the values in methanol and those in ether. The quantum-yield values were found to be slightly higher at lower temperatures. Addition of quinone resulted in a strong decrease of the quantum yield of bleaching.

The absorption spectrum was determined for photo-bleached bacteriochlorophyll in different solvents. Furthermore, the effect of reversibility of photo-bleaching was studied as a function of temperature.

A reversible "chemical" bleaching was found to occur after addition of ferric salts, iodine, or potassium permanganate. The absorption spectrum was found to be analogous to that of chlorophyll *a* under similar conditions. This reversible "bleaching" proved to occur not only in methanol, but also in acetone or ether.

Fluorescence spectra were determined before and after bleaching of "pure" and "crude" bacteriochlorophyll solutions. The quenching of fluorescence after addition of quinone and the effect of this addition on the degree of polarisation for bacteriochlorophyll and chlorophyll *a* was also investigated.

References p. 490.

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SIALIDO-LACTOSE OF COW COLOSTRUM

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Sialic acid[1], also known as acetyl-neuraminic acid[2] or gynaminic acid[3], was first isolated from bovine submaxillary mucoid, and subsequently shown to be widely distributed in mammalian tissues[4]. Mammary gland extracts and colostrum also contain a substance similar to or identical with sialic acid[5,6,7]. A crystalline compound obtained by KUHN from cow colostrum seemed to differ in some respects from these, and was therefore named lactaminic acid. This laboratory in attempts to elucidate the mechanism of the receptor destroying enzyme[8], which catalyzes the hydrolysis of sialic acid in certain mucoids, explored the possibility of using cow colostrum as a source for less complex substrates than those encountered in submaxillary gland and urine. Upon finding dialyzable sialic acid in cow colostrum, an investigation of its chemistry was undertaken. Preliminary results of this study have been reported[9].

METHODS

Sialic acid was assayed by the Direct Ehrlich's and Bial's Orcinol tests as described by WERNER AND ODIN[10]. Its acid-labile COOH group was determined manometrically by TRACEY's method for glucuronic acid[11]. *Lactose* was analyzed by the anthrone method[12]. *Reducing values* were

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References p. 494.