OXIDATION-REDUCTION POTENTIALS OF DIFFERENT CHLOROPHYLLS IN METHANOL

J. C. GOEDHEER, G. H. HORREUS DE HAAS AND P. SCHULLER

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

INTRODUCTION

One of the possible functions of chlorophyll and bacteriochlorophyll *in vivo* might be their participation in the photosynthetic process as reversible oxidation–reduction systems. It was therefore thought interesting to study the *in vitro* properties of these pigments with regard to this function.

RABINOWITCH AND WEISS[1] observed in the dark a strong decrease of the red absorption band of chlorophyll *a* in methanol after addition of ferric chloride and a regeneration of this band after the subsequent addition of ferrous salts. The equilibrium could be shifted by strong illumination of the solution. The decrease in absorption of the "red" absorption band was interpreted as indicating an oxidation of the chlorophyll molecule. The green colour returns not only on addition of ferrous salt but also when the solution is left to stand. Addition of non-reducing salts, such as sodium chloride, has the same effect. ASHKINAZI, GLIKMAN AND DAIN[2] raised objections against the interpretation of the RABINOWITCH-WEISS effect as an oxidation. They suggested that regeneration of the "red" absorption band may be due to pheophytinisation of formation of certain metal porphyrin complexes. The results of the experiments of RABINOWITCH AND WEISS were confirmed by WATSON[3]. He mentioned the complete reversibility of bleaching by $FeCl_3$ after immediate addition of Cu_2Cl_2, and attributed the slow regeneration of the red absorption band to allomerisation of chlorophyll *a*. GOEDHEER[4] observed an analogous reversible bleaching in the case of bacteriochlorophyll. With this pigment this bleaching was found to occur not only upon the addition of ferric chloride, but also upon addition of iodine or potassium permanganate. The type of solvent proved to be unimportant; with iodine a reversible bleaching was observed in methanol, acetone, and ether. No "allomerisation" seemed to occur in bacteriochlorophyll. These findings support the interpretation of the effect by RABINOWITCH AND WEISS and WATSON as a reversible oxidation.

In the present study the oxidation-reduction potentials of the systems bacteriochlorophyll–"oxy"bacteriochlorophyll, bacterioviridin–"oxy"bacterioviridin, and chlorophyll *a* and *b*–"oxy"chlorophyll *a* and *b*, were determined. The finding of analogous reversible changes in colloidal bacterial extracts, also mentioned below, may provide a link between the measured processes *in vitro* in organic solutions and processes occurring *in vivo*.

METHODS

In measuring of redox potentials in non-aqueous media several difficulties are encountered, such as weak ionisation and insufficient solubility of salts. However, if methanol containing a small

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percentage of water was used as a solvent, the potentials were sufficiently stable. The reversible bleaching proceeded normally. Theelec trode potentials wer emeasured by means of a potentiometer with an input resistance of 100 MΩ. Owing to the slight solubility of agar in methanol, an aqueous agar salt bridge of saturated KCl had to be used, in combination with the calomel and platinum electrodes. This introduced a diffusion potential at the junction salt bridge–methanol. The potential of the ferric–ferrous ion system in methanol was compared with that in water, by dissolving equal amounts of ferric chloride and ferrous sulfate (concentration in the order of 10^{-4} moles/l) in both solvents. The potentials were found to be 510 mV and 480 mV in water and methanol, respectively (versus saturated calomel electrode). According to the literature, E_0 for the ferric–ferrous system versus standard hydrogen electrode in water amounts to 760 mV, while that for saturated calomel electrode is 244 mV. This calibration shows, therefore, that the values for the potentials in methanol and water do not differ appreciably when measured in the apparatus described above.

The measurement of the potential at which bacteriochlorophyll is oxidized to a "bleached" compound proceeded as follows. In oxidized bacteriochlorophyll the long-wavelength absorption band (with a maximum at about 770 mμ in methanol) was strongly decreased, while new absorption bands arose with maxima at about 900 mμ, 525 mμ, and 420 mμ (*cf.* GOEDHEER[4]). It seemed preferable to measure the disappearance of the 770 mμ band in bacteriochlorophyll (and also the analogous bands in the other pigments) optically. A scheme of the apparatus used is given in Fig. 1. The measurements were made in light transmitted by an interference filter. After passing the cuvette with the methanolic bacteriochlorophyll solution, the light entered a photocell, which was connected with a Brown recorder. The methanolic solution was prepared by adding 35 ml methanol to 5 ml methanol (containing some water) saturated with ferrous sulfate 7 aq.; in this chlorophyll was dissolved so that the ultimate absorption amounted to 85% (corresponding to about $3 \cdot 10^{-6}$ moles/l chlorophyll). To this solution drops of a methanolic ferric chloride solution were added at a constant rate. Under vigorous stirring the potentiometer was read at constant time intervals. The potential corresponding to a 50% transition of the pigment into the oxidized state yielded reliable results, the decolorisation of ceric sulfate with ferrous sulfate was measured in an aqueous solution in a similar way. An interference filter with a maximum transmission at 437 mμ was used to obtain light absorbed by ceric sulfate. The difference absorption spectrum of the latter compound with regard to the decolorized substance had been determined beforehand. In this way a value of 1.35 V was found for the redox potential of the ceric–cerous ion system. In the literature a value of 1.40 V is mentioned. Thus the results obtained by the above method correspond reasonably well with this value. Bacteriochlorophyll and bacterioviridin were used either immediately after extraction of the bacteria with methanol or after careful chromatography of this extract on an aluminum oxide column. The spectral purity was checked by determination of the ratio of maximum and minimum absorption. In the case of bacteriochlorophyll an infrared absorption spectrum was also determined. No substantial difference from the spectrum published by JACOBS AND HOLT[5] was found. Chlorophyll *a* was obtained from a fresh methanolic *Chlorella* extract or from a solid (several years old) chromatographed product. The absorption at 665 mμ in the fresh methanolic extract due to the presence of chlorophyll *b* is about 5%. The chromatographed product was known to contain a small percentage of pheophytin. However, pheophytin does not react with ferric chloride. As the long-wavelength bands of chlorophyll *a* and pheophytin *a* nearly coincidence, the presence of the latter pigment only reduces the change in absorption.

The potential values at which the bleaching proceeded could also be qualitatively checked

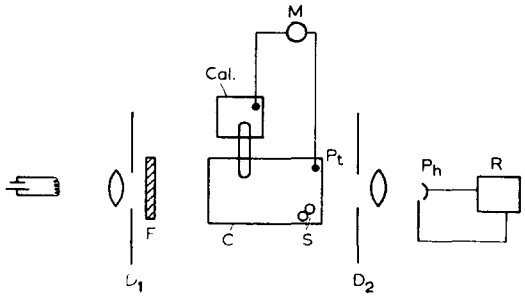

Fig. 1. Apparatus for the measurement of oxidation-reduction potentials and absorption changes. Via interference filter (F) incandescent light passes the cuvette with the alcoholic pigment solution and enters photocell (Ph). This photocell is connected with recorder (R). D_1 and D_2 represent diaphragms. An agar bridge connects cuvette C with the calomel electrode. Stirrer (S) and platinum electrode (Pt) are placed outside the light beam.

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by adding different ratios of ferric/ferrous ions to the pigment solution and observing at which ratios the bleaching of the different pigments began. Using the equation of NERNST it could be found whether these ratios give the same order of magnitude for the potential values as those found by direct potentiometry.

RESULTS

Bacteriochlorophyll

An interference filter with a maximum transmission at 762 mμ was used. Fig. 2 shows an example of the change of absorption of bacteriochlorophyll as a function of ferric chloride concentration. The potential was read at constant time-intervals. With this pigment the change in absorption proved to be most pronounced. This may be partly due to the fact that, contrary to what happens with chlorophyll *a* and *b*, no regeneration of the infrared band occurred upon standing. In these experiments 50 % oxidation corresponded to 300 ± 2 mV *versus* saturated calomel electrode. When different concentrations of bacteriochlorophyll were used, no significant change in the potential occurred. The absolute amounts of ferric and ferrous ions could also be varied without much influence on the measured potential. No difference was found to occur in the values if "crude" or chromatographically purified bacteriochlorophyll was used.

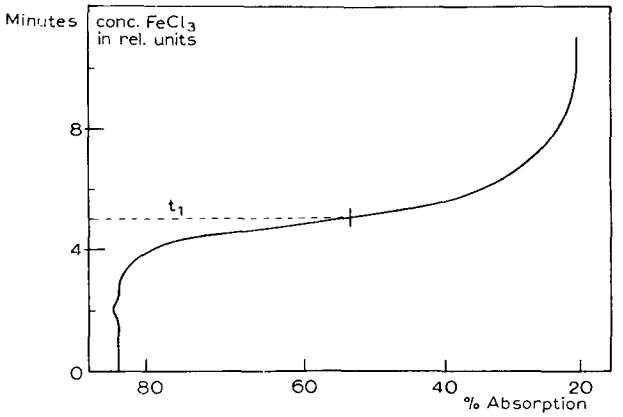

Fig. 2. Change in absorption of bacteriochlorophyll at 762 mμ as a function of time and, since this oxidant was added at a constant rate, also as a function of amount of ferric chloride. The potential at time t_1 was considered to be the redox potential of the system bacteriochlorophyll-"oxy" bacteriochlorophyll.

Qualitatively, it was found that with bacteriochlorophyll bleaching took place at a 1:2 ratio of ferric–ferrous ions, and not at a 1:8 ratio. Reversible bleaching of bacteriochlorophyll could be brought about not only by addition of $FeCl_3$ but also by the addition of iodine to the solution at a low concentration.

Bacterioviridin

With this pigment an interference filter with a maximum transmission at 768 mμ was used. The decrease in absorption of bacterioviridin induced by ferric chloride was somewhat smaller than that of bacteriochlorophyll. Regeneration of the red absorption band did not occur. As compared with bacteriochlorophyll, the potential values showed more fluctuation and amounted to 300 ± 10 mV for "crude" and 290 ± 10 mV

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for chromatographed bacterioviridin. With bacterioviridin also, bleaching occurred at a 1:2 ratio of ferric–ferrous ions, and not at a 1:8 ratio. This pigment could also be reversibly bleached by the addition of iodine to the solution.

Chlorophyll a

An interference filter was used with a maximum transmission at 665 mμ. In contrast to the behaviour of the red absorption band of both bacterial pigments after addition of ferric chloride, the red absorption band of chlorophyll *a* regenerated partly at, or below, room temperatures. The time in which this regeneration occurred, was of the order of some minutes. Probably owing to this effect the measured potential values showed more fluctuations upon variation of the addition rate than the values measured with the above-mentioned pigments. The potential values amounted to 395 ± 20 mV, both for a fresh *Chlorella* extract and for the chromatographed product. With chlorophyll *a* a 1:1 ratio of ferric–ferrous ions produced only a slight bleaching effect, while total bleaching occurred at a 1:2 ratio. This pigment could not be bleached by the addition of small amounts of iodine to the solution.

Chlorophyll b

The interference filter used showed a maximum transmission at 645 mμ. Only a chromatographed (several years old) preparation was available; this was slightly contaminated with pheophytin and green products. The potential values amounted to 430 ± 25 mV. Chlorophyll *b* also could not be bleached by the addition of iodine to the solution.

As was found previously (GOEDHEER[6]), reversible bleaching is independent of the type of solvent. Investigations were therefore made to discover whether such an effect could be produced in aqueous suspensions of bacterial or plant "chromophores". These suspensions were obtained by grinding bacteria or algae, or by pressing them through a needle valve at high pressure. Preliminary experiments showed that with colloidal extracts of the bacteria *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides*, and *Chromatium* strain D, a reversible bleaching could indeed be observed. The various infrared absorption bands, however, showed a different behaviour. The bleaching proceeded relatively slowly. With *Rhodospirillum rubrum*, the absorption at 880 mμ decreased by 60 % upon bleaching, while upon the addition of ascorbic acid this band could in some cases be regenerated to about 90 % of its original intensity. The spectral changes in the long-wavelength absorption band of this bacterium are presented in Fig. 3. This figure shows that a strong decrease in absorption is present at 880 mμ, while the wavelength of maximum absorption is shifted towards 872 mμ. An increase in absorption occurs around 950 mμ and also below 850 mμ. The disappearance of the weak band at about 800 mμ after addition of ascorbic acid was found to be a pH effect (*cf.* THOMAS, GOEDHEER AND KOMEN[7]).

With colloidal extracts of *Chlorella*, *Aspidistra*, and *Chlorobium limicola*, bleaching was found to proceed only at high ferric chloride concentrations (10^{-1} moles/l). After addition of ferrous salts or ascorbic acid this bleaching was found to be irreversible.

Table I gives the results obtained with the various pigments. The potential values are computed with reference to standard hydrogen electrode. Thus the measured potential values were increased by 250 mV.

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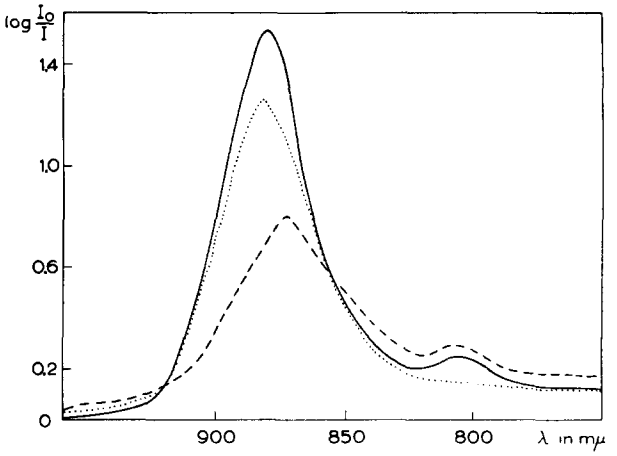

Fig. 3. Long-wavelength absorption band of bacteriochlorophyll in *Rhodospirillum rubrum*. The solid line indicates the spectrum of the colloidal extract, the dashed line the same spectrum some minutes after the addition of ferric chloride (10^{-3} moles/l), and the dotted line this spectrum after the subsequent addition of ascorbic acid.

TABLE 1

COMPARISON OF REDOX POTENTIALS AND SOME OTHER PROPERTIES FOR VARIOUS BACTERIAL AND GREEN PLANT CHLOROPHYLLS

	Redox potential mV	*Regeneration of red absorption band*	*Reversible bleaching in colloidal extract*	*Solubility in petroleum ether*
Bacteriochlorophyll	550 ± 2	—	+	—
Bacterioviridin	550 ± 10	—	—	—
Chlorophyll *a*	645 ± 20	+	—	+
Chlorophyll *b*	680 ± 25	+	—	+

DISCUSSION

The measurements demonstrate the possibility of measuring reproducible oxidation-reduction potentials for various chlorophylls in methanol. The absolute values of these potentials are not of great accuracy. The values measured in methanol were found to be about 30 mV lower than the equivalent values (equal concentrations of ferric and ferrous ions) in water. The data of Table I, however, allow an estimation of the order of magnitude for this potential.

There is a marked difference between the bacterial and green plant pigments studied. The potential difference between bacterioviridin and chlorophyll *a* is all the more striking since the absorption spectra in the visible and near-ultraviolet part of the spectrum almost coincide (*cf.* also KATZ AND WASSINK[8] and RABINOWITCH[9]). When both pigments are dissolved in methanol, the maximum of the red absorption band in bacterioviridin is located only 5 mμ further towards the red side of the spectrum than the corresponding band of chlorophyll *a*. This similarity probably indicates that the all-round conjugated ring system, which is responsible for the "electronic" absorption bands, is identical for the two pigments. The difference in behaviour after bleaching

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with ferric chloride (there is no regeneration of the red absorption band with bacterioviridin) may suggest differences in the cyclopentanone ring in bacterial and plant pigments. Also, the near-infrared spectrum arising after bleaching in methanol with ferric chloride was found to differ for bacterioviridin and chlorophyll *a*. In the former pigment a new band arose with a maximum at about 750 mμ, while the analogous maximum for chlorophyll *a* appeared at 850 mμ. Both maxima disappeared rapidly (lifetime 1.4 min at 20°). The absorption coefficient of this new maximum was about 10% of the absorption coefficient of the red maximum in the non-bleached state. Another point of difference between the bacterial and plant pigments studied is demonstrated by their solubility in petroleum ether. The solubility of both bacterial pigments in this solvent is very small, whereas the plant pigments show a reasonable solubility.

With respect to the difference found in redox potential between bacterioviridin and chlorophyll *a*, it should also be remembered that the red absorption band of bacterioviridin *in vivo* and in colloidal extracts (where it is rather unstable) is shifted 65 mμ more towards longer wavelengths than the analogous band of chlorophyll *a*. This shift corresponds to a difference of 0.150 eV on energy scale.

The possibility of obtaining analogous reversible bleaching in colloidal extracts of photosynthetic bacteria does not apply to such changes *in vivo*. The difference spectrum of this bleaching was found not to correspond with the difference spectrum of reversible bleaching upon illumination, as measured by DUYSENS[10].

SUMMARY

It was found that the reversible decolorisation of some chlorophylls upon the subsequent addition of ferric and ferrous salts, as originally measured by RABINOWITCH AND WEISS for chlorophyll *a*, proceeded at a reproducible oxidation-reduction potential.

A marked difference was found to occur between the redox potentials of the bacterial pigments and those of the green plant pigments.

Apart from these measurements in organic solvents, reversible bleaching could also be demonstrated in aqueous extracts from photosynthetic bacteria.

The possible significance of these data is discussed.

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Received October 21st, 1957