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OPTICAL AND PHOTOCHEMICAL PROPERTIES OF CHLOROPHYLL *a* SOLUBILIZED IN AQUEOUS SOLUTIONS OF SURFACTANTS

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SUMMARY

The phenomenon of solubilization of chlorophyll in micellar solutions of surfactants is studied by measuring the dependence of absorption, fluorescence and sensitized photoreduction of methyl orange on the surfactant concentration. Chlorophyll is solubilized well in Triton X-100 and cetyl trimethyl ammonium bromide at concentrations above the critical micelle concentration. Fluorescence and photochemical activity rise sharply in this concentration range. Absorption increases gradually with increasing surfactant concentration; it begins to increase below the critical micelle concentration and reaches a maximum above it. This phenomenon is discussed in terms of the "shadow effect" of pigment suspensions. The anionic surfactants investigated solubilize much less chlorophyll and only at high concentrations of surfactant.

The relative rates of photoreduction of 2,4-dinitrophenol and chlorophyll in solutions of different surfactants were also measured. They depend on the nature of the oxidant as well as on the surfactant.

The photoreduction of viologens in the same system is described. The reaction is reversible. The degree of reduction in the light decreases with decreasing normal potential of the viologen.

Finally, the photoreduction of $NADP^+$ is described. The reaction requires ferredoxin–$NADP^+$ reductase but is almost independent of ferredoxin and plastocyanin. The rate is much lower than with chloroplast fragments.

It is concluded that the photochemistry of solubilized chlorophyll is similar to that of chlorophyll dissolved in organic solvent and distinct from that of chloroplasts in which the structure ensures high efficiency and high selectivity for the light reactions of photosynthesis.

Abbreviations: Triton X-100, polyoxyethylene isooctylphenyl ether; CTAB, cetyl trimethyl ammonium bromide; SLS, sodium lauryl sulfate; Akypo, sodium lauryl (tri-oxapropene) oxaethane carboxylate; c.m.c., critical micelle concentration; orange OT, 1-*o*-tolyl-azo-2-naphthol; DCIP, 2,6-dichlorophenolindophenol; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; benzyl viologen, dibenzyl 4,4'-bipyridylium salt; methyl viologen, dimethyl 4,4'-bipyridylium salt; triquat, trimethylene 2,2'-bipyridylium salt.

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INTRODUCTION

Among the photochemical reactions of chlorophyll, photosensitized oxido-reductions have attracted much attention because of their possible bearing on the mechanism of photosynthesis, which can formally be described as a very complicated photoreduction of carbon dioxide by water. The chlorophylls and their derivatives sensitize many oxido-reductive reactions in organic solvents[1].

Such reactions have also been studied in an aqueous medium. Since chlorophyll is not soluble in water it was introduced into the water as a colloidal solution[2–4], absorbed on[5,6] or bound to[7] solid particles, or solubilized by addition to micellar solutions of surfactants[8–12]. In the latter case, solutions of chlorophyll are obtained which are perfectly clear and stable, have an absorption spectrum similar to solutions in organic solvents, display strong fluorescence and show strong photochemical activity.

This investigation was undertaken in order to study the phenomenon of solubilization more closely. The dependence of the absorption spectrum, the intensity of fluorescence and the rate of photochemical reduction of methyl orange on the concentration of surfactant was investigated for the following surfactants: Triton X-100, CTAB, SLS and Akypo.

In addition, the photochemical reduction of viologens and of $NADP^+$ by solubilized chlorophyll is described.

METHODS

(a) *Preparation of suspensions.* 0.25 ml of a solution of chlorophyll *a* in acetone was poured into 7.25 ml of buffer (Tris–HCl, 0.05 M, pH 7.8) containing varying amounts of surfactant, such that the final concentration of chlorophyll was 5 μM. The mixture was kept overnight in the dark at room temperature.

(b) *Absorption spectra.* The spectra of the undiluted suspensions were recorded with a recording spectrophotometer (Cary Model 14).

(c) *Fluorescence.* The emission spectra of fluorescence were recorded on the Cary equipped with attachment 1412. The built-in mercury arc, combined with a blue filter (BG 23, Schott and Gen.) was used as light source. About 0.5 % of the fluorescence of 5 μM chlorophyll in acetone was detectable.

(d) *Reduction of methyl orange.* The photochemical reaction was carried out in a rectangular Thunberg cuvette with four clear silica walls. In order to free the mixture from oxygen the cuvette was evacuated 3 times to about 15 mm Hg, tapped to liberate air bubbles and filled with pure argon. The cuvette was illuminated in the sample compartment of the Cary with a beam of red light, as described earlier[11,12]. The mean light intensity in the cuvette was 30 mW/cm^2. At the concentration of chlorophyll normally used (5 μM) the absorbed flux was 60 $\mu Einstein \cdot l^{-1} \cdot sec^{-1}$. The temperature was 23° and the mixture was stirred magnetically during illumination. The course of the reaction was followed by recording the decrease of absorbance at the wavelength of maximum absorption of the dye, between 430 and 460 nm, depending on the surfactant used. The absorptivity was 20 $mM^{-1} \cdot cm^{-1}$.

(e) *Reduction of 2,4-dinitrophenol.* The reaction was carried out in the same manner as the reduction of methyl orange and followed by recording the increase of absorbance at 460 nm (ref. 12).

(f) *Reduction of chlorophyll.* The reaction was carried out in the same manner and followed by recording the bleaching at 670 nm. For the calculation, the initial absorbance of each mixture was used.

(g) *Reduction of viologens.* The viologens were reduced by the same method, but because the reduced compounds react very rapidly with oxygen a better method of deoxygenation was used. The mixture was first frozen in a special compartment of the cuvette, by immersing it in liquid nitrogen, then evacuated to about 10 μ Hg. The mixture was then thawed and stirred vigorously by means of a magnetic stirrer for 3 h. During this time argon, purified by passage through a column of hot copper wire (340°) and humidified at room temperature, was blown onto the mixture. By this method the concentration of residual oxygen could be reduced to about 0.7 μM. For benzyl and methyl viologen the course of the reaction was followed by recording the increase of absorbance at 400 nm and calculated with an absorptivity of 20 $mM^{-1} \cdot cm^{-1}$. The reduction of triquat[13] was measured at 390 nm and calculated with 16 $mM^{-1} \cdot cm^{-1}$. The molar absorptivity values were taken from KOK, RURAINSKI AND OWENS[14].

(h) *Reduction of NADP+.* This was performed by the same method as the reduction of viologens, except that the period of deaeration was reduced to 30 min because the proteins present in the reaction mixture were not completely stable during this treatment. The reaction was followed by recording the increase of absorbance at 340 nm, and the rate calculated with 6 $mM^{-1} \cdot cm^{-1}$.

The critical micelle concentration (c.m.c.) was determined by solubilization of a dye and by depression of surface tension:

(i) *C.m.c. by solubilization.* SCHOTT's[15] method was followed with minor modifications. In this method the lipid-soluble dye, orange OT, is equilibrated with aqueous solutions of the surfactant at concentrations below and above the c.m.c. In our experiments the dye was added as an acetone solution since the c.m.c. had to be determined in the medium which was used in the solubilization experiments with chlorophyll. The dye, 1 mg in 0.2 ml of acetone, was added to 5.8 ml of the surfactant, dissolved in 0.05 M Tris–HCl (pH 7.8). The mixture was shaken for at least 3 days at 25° in a dark room, then centrifuged twice for 30 min at 1300 $\times$ *g*. The absorption spectrum was recorded and the absorbance at the maximum of absorption, 490 nm, plotted against the concentration of the surfactant. The c.m.c. was calculated from the point of intersection of the regression line with the abscissa.

The molar absorptivity of solubilized orange OT was determined by equilibrating a known amount of the dye with solutions of the surfactant containing 1.5 and 2 times the amount necessary for solubilization. The results are listed in Table I (3d row).

Some of the solubilization curves are shown in Fig. 1.

(j) *C.m.c. by depression of surface tension.* The surface tension of aqueous solutions of surfactants decreases rapidly with increasing concentration until the c.m.c. is reached. Above the c.m.c. the surface tension remains constant since the micelles are not surface-active, and the concentration of free surfactant molecules remains constant. In the ideal case a plot of the surface tension against the logarithm of the concentration consists of a sloping straight line below, and a horizontal line above, the c.m.c. The point of intersection of the two lines is the c.m.c.[16]. In practice the graph often deviates from this form because of impurities and heterogeneity of

the surfactant used. The surface tension was determined by the drop weight method with a stalagmometer; since the stalagmometry of a great number of solutions is extremely tedious the measurements were made automatically by means of a gradient mixer and a fraction collector. The gradient mixer consisted of two graduated cylinders (100 ml) which were connected through a stopcock. The first cylinder contained a solution of surfactant in buffer of pH 7.8 (again with 3.3% acetone added); the second one contained buffer with acetone and was stirred efficiently by means of a spinning, twisted strip of aluminum. This device provides a constant gradient, beginning with zero concentration of surfactant and ending with the concentration present in the first cylinder. That such was the case was checked with a solution of methyl orange in the first, and buffer in the second cylinder: a plot of the absorbance at 460 nm against the volume yielded a straight line through the origin and the absorbance of the original solution at 200 ml. The mixture was delivered by a peristaltic pump to the nozzle of a stalagmometer which was mounted in the drop counter of

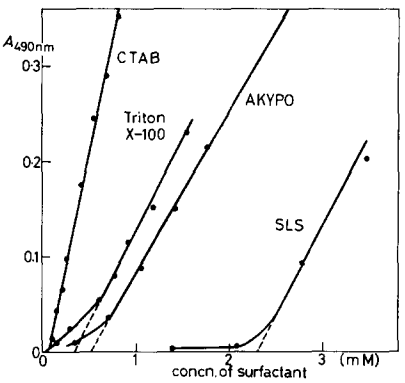

Fig. 1. Solubilization of orange OT in micellar solutions of different surfactants.

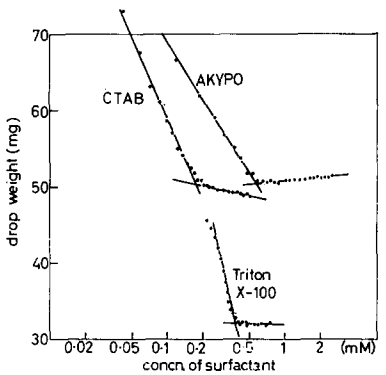

Fig. 2. Graphs of drop weight versus logarithm of surfactant concentration for the determination of the c.m.c.

a fraction collector ("UltroRac", L.K.B.). The nozzle consisted of a glass capillary, 1 and 7 mm inside and outside diameter, respectively, ground perpendicularly to the axis and polished at the lower end. It was cleaned carefully between the runs. The rate of delivery was about 0.2 ml/min, *i.e.*, 2–6 drops/min. 40–60 fractions of 80 drops each were collected in weighed tubes and these were weighed again. From the weight of the content of each tube the drop weight was calculated, and from the sum of the contents of the preceding tubes and the initial concentration of surfactant the concentration in each tube. The drop weight was then plotted against the logarithm of the concentration of the surfactant. The time needed for preparation, weighing, calculation (desk calculator) and plotting was about 4 h/run. Some of the graphs obtained in this way are shown in Fig. 2.

MATERIALS

Chlorophyll *a* was purchased from Sandoz (Basle, Switzerland) or from Fluka (Buchs, Switzerland); polyoxyethylene isooctylphenyl ether (Triton X-100) from

Rohm and Haas; cetyl trimethyl ammonium bromide (CTAB) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) from British Drug House (Laboratory grade); methyl and benzyl viologens from Mann Res. Lab.; $NADP^+$ and NADPH from Sigma Chem. Co.; clostridial ferredoxin from Worthington Biochem. Corp.; glutathione reductase from Boehringer (Mannheim); 2,6-dichlorophenolindophenol (DCIP) from Merck A.G., (Darmstadt). Trimethylene 2,2'-bipyridylium salt (triquat)[13,17] and 1-*o*-tolyl-azo-2-naphthol (orange OT)[15] were prepared in this laboratory. Sodium lauryl sulfate (SLS) was purified by recrystallization. Spinach ferredoxin, ferredoxin–$NADP^+$ reductase (EC 1.6.99.4) and plastocyanin were provided by Dr. WESSELS[18,19]. Sodium lauryl (tri-oxapropene) oxaethane carboxylate (Akypo) was provided in purified form by N.V. Chemy (Bodegraven, The Netherlands).

RESULTS AND DISCUSSION

1. *Solubilization of chlorophyll*

The results of the c.m.c. measurements are listed in Table I. The values obtained by dye solubilization and by depression of surface tension are in good agreement, with the exception of CTAB, where the solubilization method yielded a much lower value. Possibly this cationic surfactant interacts with the naphtholate ion so that the formation of micelles begins at a lower concentration. Indeed, the maximum of absorption was shifted slightly to a longer wavelength at low concentrations of CTAB.

The aggregation number n of the micelles could be estimated from the solubilization curves of orange OT (see Fig. 1). SCHOTT[15] compared the amount of solubilized dye with the micellar molecular weight calculated from light-scattering measurements, and found that in many cases the ratio of concentration of dye to micelle concentration was one. Hence, n can be calculated from the slope of the curves dA/dc (where A = absorbance and c = concentration of the surfactant) and the molar absorptivity of the dye, k, in the solution of the surfactant:

$$n = \frac{k}{dA/dc}$$

The values of k and n are also listed in Table I. The values of n found in this way do not deviate too much from values found in the literature[16], with the exception of CTAB, for which a value 4 times higher has been reported. Again, this discrepancy could be due to interaction between the dye and this surfactant. SCHOTT has not investigated cationic surfactants[15].

From n and c.m.c. the concentration of surfactant at which the ratio of micelles to chlorophyll molecules is unity can be calculated:

$$c_{sol} = \text{c.m.c.} + n[\text{Chl}]$$

This value gives an indication of the minimum concentration of surfactant at which complete solubilization of chlorophyll can be expected (see Table I).

Fig. 3 shows the results of the experiments on solubilization of chlorophyll with Triton X-100. Fluorescence started rather sharply at about the c.m.c. and increased steeply to a maximum which was reached at about 3 times c_{sol}. Chlorophyll fluorescence shows very strong selfquenching, so that aggregated chlorophyll has little or no fluorescence (see ref. 20). The strong increase in fluorescence at the c.m.c. means

TABLE I

COLLOID-CHEMICAL PROPERTIES OF THE SURFACTANTS

For the methods of determining the c.m.c.'s see METHODS, sections (i) and (j).

Properties	*Surfactants*				
	Triton	*CTAB*	*SLS*	*Akypo*	*Akypo + 0.2 M KCl*
c.m.c. by solubilization, mM	0.32	0.064	2.3	0.53	0.19
c.m.c. from surface tension, mM	0.32	0.17	2.6	0.56	0.24
Molar absorptivity of orange OT in surfactant solution, $mM^{-1} \cdot cm^{-1}$	16	18	20	18	18
Aggregation number, n (see text)	83	41	120	105	117
c_{sol} (see text), mM	0.67	0.25	3.0	1.0	0.75

that chlorophyll is taken up into the micelles in a monomeric form, and that, at least at higher concentrations of surfactant, the molecules are spread so far apart that they do not quench each other. The peak of fluorescence was at 675 nm.

The photochemical activity paralleled the fluorescence intensity in its dependence on surfactant concentrations, except that it was still measurable in the absence of surfactant, where the rate of reduction was 16 $nM \cdot sec^{-1}$. In the presence of 0.1 mM surfactant (*i.e.*, below the threshold of fluorescence), it had increased to 60 $nM \cdot sec^{-1}$.

Such a parallelism between the rise of fluorescence and that of photochemical activity was not found by CELLARIUS AND MAUZERALL[21], who studied another form of dye dispersion. They measured absorption, fluorescence yield and photochemical activity of pheophytin *a*, adsorbed on spherical polystyrene particles. In their Fig. 4, they plotted the relative fluorescence yield and photochemical activity against surface coverage and found that the fluorescence was 50 % quenched at a coverage roughly

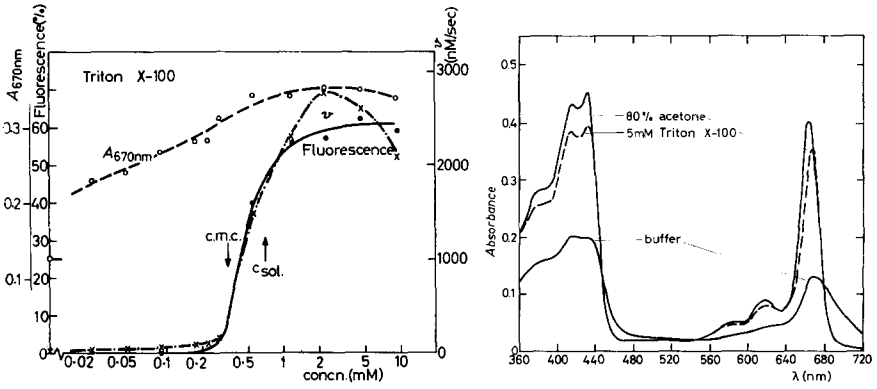

Fig. 3. Dependence of the height of the red absorption maximum ($A_{670\ nm}$) and the relative fluorescence intensity of chlorophyll, and of the rate (v) of the chlorophyll-sensitized photoreduction of methyl orange, on the concentration of Triton X-100. For conditions, see Table II.

Fig. 4. Absorption spectra of 5 μM solutions of chlorophyll in acetone and of a suspension of chlorophyll in 5 mM Triton X-100 and in buffer alone.

one-tenth that of 50 % inhibition of photochemical activity. Their system, however, is different from ours in several respects. The diameter of their particles is about 10 times greater than that of micelles, and their rigid surface certainly interacts with the pigment molecules in a way different from that of the quasi-liquid micelles. The authors concluded from their measurements that monomers and small aggregates were present on the same particle in this region of coverage, and that the fluorescence of monomers was quenched partly by resonance transfer of the excitation energy to aggregates. In our system, at the concentration of steep rise the pigment molecules incorporated as monomers in a micelle are far enough away from the remaining non-fluorescence aggregates to exclude resonance transfer to the latter.

The height of the red absorption maximum ($A_{670\,nm}$) shows quite a different picture: in the absence of surfactant it is about 40 % of its maximum value. It increases gradually far below the c.m.c. and reaches a maximum without any break in the region of the c.m.c.

Fig. 4 shows the absorption spectra of 5 μM chlorophyll *a* in a similar solution of Triton X-100, in 80 % acetone and as a suspension in buffer (the concentration of chlorophyll has been calculated from the molar absorptivity in 80 % acetone given by VERNON[22], 82 $mM^{-1} \cdot cm^{-1}$ at 665 nm).

In the micellar solution, the red maximum was shifted to 669 nm, and both red and blue maxima were depressed by 10 % with respect to the acetone solution. In the colloidal suspension the red peak was shifted further towards the infrared and both bands were considerably flattened and broadened.

The flattening of the absorption peaks of suspensions (shadow effect) has been described by DUYSENS[23]. DUYSENS calculated the ratio of absorbance at any wavelength for a suspension and for a solution containing the same concentration of pigment, and concluded that this ratio decreases with increasing particle diameter.

The increase of $A_{670\,nm}$ in the suspension, obtained by addition of surfactant at concentrations below the c.m.c. means, therefore, that the particle size decreases. This can be explained in the following way: when a solution of chlorophyll is poured into buffer the solution becomes supersaturated. The chlorophyll molecules then aggregate into small clusters (nuclei)[24]. After this, the greater nuclei grow slowly at the expense of the smaller ones. If surfactant is present in the suspension it is absorbed on the surface of the particles and their growth is retarded or even stopped[25]. In this way the particles remain smaller and the absorbance of the suspension is increased.

To give an idea of the concentration of surfactant at which this effect may play a role, we consider a suspension of chlorophyll of 5 μM concentration in which the particles have grown to an average diameter of 100 Å. A rough calculation shows that 10 μM of surfactant is sufficient to cover all the particles with a monolayer.

Above the c.m.c. the chlorophyll molecules presumably are taken up into the micelles before the formation of nuclei starts.

In the case of chlorophyll particles the flattening is not only due to the shadow effect; inspection of Fig. 4 shows that in the suspension the loss of absorption at the peak is partly compensated for by an increase in absorption on the long wavelength side.

A similar picture is obtained with CTAB. Due to the ambiguity of c.m.c. and c_{sol}, however, the relationship between these values and the concentration of steep increase of the fluorescence and of the rate of reduction is not so clear-cut as in the case of Triton X-100. Moreover, the maximum reaction rate is much lower.

SLS gives a very different picture: although chlorophyll is solubilized to some degree, complete solubilization occurs only at a very high surfactant concentration. BAKKER[26] reported that colloidal particles of chlorophyll, obtained by mixing an acetone solution with water, are negatively charged. This charge probably repels the negatively charged molecules of SLS so that solubilization is suppressed.

Akypo, which is also anionic, gives a picture similar to that of SLS (Fig. 5), although maximum rate of reaction is much higher with Akypo. Besides its carboxyl group, the formula of Akypo resembles the one of Triton X-100. Therefore Triton X-100 and Akypo may be expected to give similar effects if the influence of the COO^--group is eliminated. This can be done by the addition of neutral electrolyte in high concentration. The "gegenions" (cations in this case) are attracted by the charged micelles and have a screening effect on the charges (see refs. 27, 28). This effect has indeed been found in micellar solutions of Akypo with added 0.2 M KCl (Fig. 6).

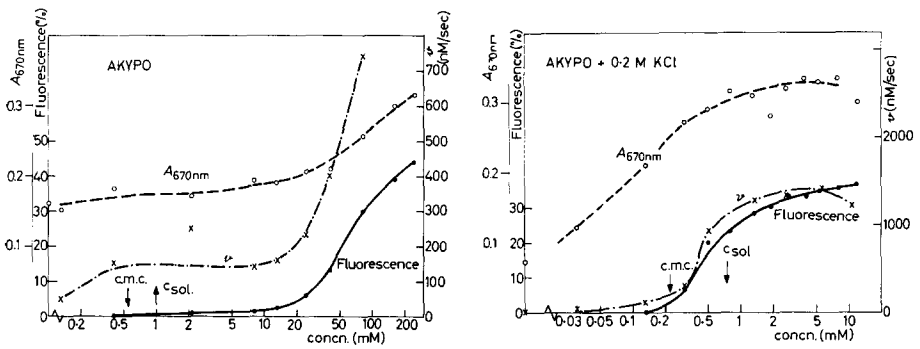

Fig. 5. As in Fig. 3, but with Akypo.

Fig. 6. As Fig. 3, but with Akypo in 0.2 M KCl.

TABLE II

THE EFFECT OF SURFACTANTS AT OPTIMUM (OR HIGHEST) CONCENTRATION ON THE PROPERTIES OF CHLOROPHYLL SUSPENSIONS

Fluorescence of chlorophyll: the emission spectrum was scanned and the peak height compared to that of an acetone solution containing the same concentration of chlorophyll. For the reduction of methyl orange the mixture contained: ascorbate, 6 mM; TMPD, 0.05 mM; methyl orange, 0.03 mM; chlorophyll, 4.5 μM; Tris–HCl buffer (pH 7.8), 50 mM; surfactant at the indicated concentration; light intensity 30 mW/cm^2. The volume was 3 ml. For procedure see METHODS, section (d). For the reduction of 2,4-dinitrophenol the mixture contained the same reagents, except that the dye was replaced by 0.03 mM 2,4-dinitrophenol and the chlorophyll concentration was 5 μM. See METHODS, section (e). For the reduction of chlorophyll the mixture contained the same reagents but omitting the oxidant. See METHODS, section (f).

	Triton	*CTAB*	*SLS*	*Akypo*	*Akypo + 0.2 M KCl*
Surfactant concentration for maximum effect, mM	2	3	>100	>200	3
λ_{max} of red band of chlorophyll, nm	669	669	671	670	670
$A_{670\,nm}$	0.35	0.30	0.28	0.31	0.32
Fluorescence of chlorophyll (% of acetone solution)	60	55	42	44	33
Rate of reduction of methyl orange, nM·sec^{-1}	3100	95	63	850	1350
Rate of reduction of 2,4-dinitrophenol, nM·sec^{-1}	125	43		120	120
Rate of reduction of chlorophyll, nM·sec^{-1}	130	1		40*	

* Another Akypo compound, containing only 2 oxyethylene groups was used in this experiment.

The curves are now similar to the curves obtained with Triton X-100 (Fig. 3), although the fluorescence intensity and the rate of reduction do not reach the same values. It is interesting to note that the effect of the salt on solubilization of chlorophyll is much stronger than on c.m.c.: the latter is decreased by a factor of 2, whereas solubilization begins at a concentration about 100 times lower.

The values of the properties of the suspensions at optimum (or maximum) surfactant concentration are compiled in Table II. For comparison, the rate of reduction of 2,4-dinitrophenol and of chlorophyll are also given in this table (last two rows). These values show that the relative rate of reduction of different dyes is influenced strongly by the surfactant: with CTAB, 2,4-dinitrophenol is reduced at an appreciable rate compared to Triton, whereas chlorophyll is reduced very slowly.

2. Reduction of viologens[29]

Viologens are quaternary, *N*-substituted bipyridyl salts which can be reduced by addition of one electron to give violet coloured products[30]. Their redox potentials are independent of pH and lie between −0.35 and −0.74 V (refs. 13, 14).

The photochemical reduction of methyl viologen by chlorophyll in organic medium has been studied by KRASNOVSKY AND BRIN[31] and by CHIBISOV AND KARYAKIN[32]. Illuminated chloroplasts are also able to photoreduce viologens[14,33,34].

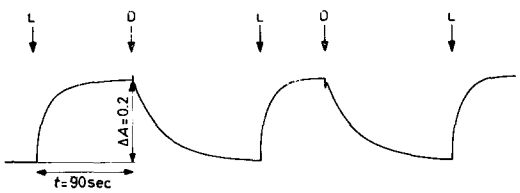

Fig. 7. Time course of the photoreduction and the dark reoxidation of methyl viologen. The reaction mixture contained: phenylhydrazine, 10 mM; methyl viologen, 0.3 mM; chlorophyll, 5 μM; Akypo, 200 mM; Tris–HCl buffer (pH 9.0), 100 mM; light intensity, 30 mW/cm^2. For procedure, see METHODS, section (g).

When a solution of viologen is illuminated in the presence of a reductant and chlorophyll, the oxidant is partially reduced until a steady state is reached in which the photochemical reduction is compensated for by the back reaction. In the dark after a period of illumination, the reduced compound is oxidized to the original state. The cycle of light reduction and dark oxidation can be repeated many times.

In aqueous medium the reaction rate is highest with chlorophyll solubilized by Akypo and with phenylhydrazine as the reductant. Fig. 7 shows the course of the photoreduction of methyl viologen in this system at pH 9.

In this experiment the initial rate of reduction was 3.0 μM·sec^{-1}. In the light steady state a degree of reduction of 3.5 % was reached, and in the following dark period the initial rate of oxidation was 0.66 μM·sec^{-1}. Thus, the final degree of reduction was much lower than would be expected from the ratio of the initial rates of light and dark reaction. Also, the form of the light curve suggests that a fast initial reaction was followed by a slower one; on the other hand the dark curve had the form of a first-order decay.

Other viologens could also be photoreduced in this system. Table III shows that the degree of reduction obtained in the light steady state was lower for the low

TABLE III

PHOTOREDUCTION OF VIOLOGENS

The normal redox potential E^0, the degree of reduction in the dark and in the light and the calculated redox potentials in dark equilibrium (E_d) and in the light steady state (E_l) are given in Volts. The reaction mixture contained: phenylhydrazine, 30 mM; viologen, 0.1 mM; Akypo, 200 mM; chlorophyll, 5 μM; Tris–HCl buffer (pH 9.0), 50 mM; light intensity, 30 mW/cm^2. For the procedure see METHODS, section (g).

Viologens	*Redox potential*			*% Reduction*	
	E^0	E_d	E_l	*Dark*	*Light*
Dibenzyl 4,4′-bipyridylium salt	−0.35	−0.31	—	20	100 (approx.)
Dimethyl 4,4′-bipyridylium salt	−0.45	(−0.31)	−0.41	0.4	16
Trimethylene 2,2′-bipyridylium salt	−0.55	(−0.31)	−0.46	0.01	3

potential viologens; KOK, RURAINSKI AND OWENS[14] found the same effect in the photoreduction by chloroplasts.

Under the conditions of this experiment, benzyl viologen was already partly reduced in the dark. From the degree of dark reduction the equilibrium potential in the dark E_d could be calculated by the formula:

$$E = E^\circ + \frac{RT}{nF} \ln \frac{[ox]}{[red]}$$

using the normal potentials reported by HOMER, MEES AND TOMLINSON[13].

Presumably, the dark potential was the same for the three compounds. With the two other compounds the redox potential reached in the light steady state could be calculated from the same relationship, introducing the steady state concentrations. It follows from the table that the redox potential was shifted in the light by 0.1–0.15 V towards more reducing conditions.

3. Photoreduction of NADP$^+$

The chlorophyll-sensitized photoreduction of NADP$^+$ is of special interest since NADPH is the first stable reduction product of photosynthesis. KRASNOVSKY, BRIN AND DROZDOVA[35] demonstrated the photoreduction of NAD$^+$ by ascorbate in aqueous pyridine. VERNON, SAN PIETRO AND LIMBACH[36] showed that the water-soluble porphyrins, hematoporphyrin and chlorophyllin can sensitize the photoreduction of NADP$^+$ by ascorbate. Ferredoxin–NADP$^+$ reductase was required for the reaction. Ferredoxin, which is necessary for the photoreduction of NADP$^+$ by chloroplasts, had no influence in the system of VERNON, SAN PIETRO AND LIMBACH.

In our system, NADP$^+$ was photoreduced by ascorbate–DCIP in the presence of chlorophyll, solubilized by Triton X-100. The evidence for the reduction is based on the following observations:

(a) The difference spectrum (light *minus* dark) had only one major maximum at 340 nm. Between 320 and 380 nm it coincided with the absorption spectrum of NADPH. At longer wavelengths minor maxima and minima were present; they could be ascribed to a slight photoreduction of chlorophyll.

(b) The illuminated cuvettes showed a strong, yellow fluorescence with a peak

at 460 nm. This is characteristic of the fluorescence emission spectrum of NADPH.

(c) The absorption at 340 nm of an illuminated cuvette could be reduced to the dark value by addition of oxidized glutathion and glutathion reductase[37].

TABLE IV

PHOTOREDUCTION OF $NADP^+$: REQUIREMENT, STIMULATION AND INHIBITION

The standard system contained: ascorbate, 3 mM; DCIP, 0.05 mM; $NADP^+$, 0.5 mM; ferredoxin–$NADP^+$ reductase, diaphorase activity, 400 units/l (refs. 18, 19), Triton X-100, 5 mM; chlorophyll *a*, 5 μM; Tris–HCl buffer (pH 7.5), 50 mM; light intensity, 30 mW/cm^2. For procedure see METHODS, section (h).

Addition or omission	*Reduction rate* ($nM \cdot sec^{-1}$)
Standard system	92
— reductase	0
— ascorbate	0
— DCIP	14
— $NADP^+$	0
— Triton	0
+ TMPD instead of DCIP	0
+ benzyl viologen, 1 μM	0
+ aerobic	0
+ plastocyanin, 20 mg/l	78
+ ferredoxin (spinach), 33 mg/l	87
+ plastocyanin and ferredoxin	76
98 units/l reductase	39
+ ferredoxin (clostr.), 33 mg/l	67
33 units/l reductase	14
+ ferredoxin (clostr.), 33 mg/l	39

The experiments on requirement, stimulation and inhibition of reaction are summarized in Table IV. DCIP was required for maximum rate and could not be replaced by TMPD. This is a characteristic difference from the system of VERNON, SAN PIETRO AND LIMBACH[36] in which both DCIP and TMPD inhibited the reaction. The enzyme ferredoxin–$NADP^+$ reductase was required, and ferredoxin had no influence with optimum enzyme concentration, in accordance with the finding of VERNON, SAN PIETRO AND LIMBACH. However, a certain stimulation by ferredoxin could be found with suboptimum amounts of the reductase. This enzyme is not specific with respect to the first substrate, and apparently it can accept electrons from both ferredoxin and chlorophyll in this system. Plastocyanin, which is required with chloroplast fragments[19,38,40] had almost no effect in our system. Benzyl viologen inhibited the reaction at a very low concentration, presumably by creating a blackflow for the electrons.

Fig. 8 shows the result of a comparison of the reduction rates obtained with solubilized chlorophyll or with chloroplast fragments, with varying light intensity. At low intensities the rate was at least 70 times higher with chloroplast fragments. However, with fragments the reaction became light saturated at about 10 mW/cm^2, whereas with chlorophyll the rate increased linearly up to the highest intensity measured. Yet, at this intensity the fragments still supported a rate 14 times higher than chlorophyll *a* solubilized by Triton X-100.

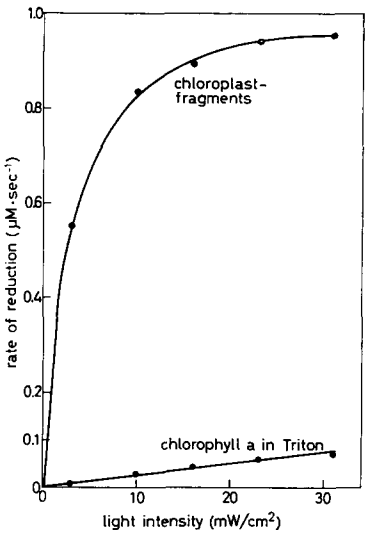

Fig. 8. Dependence of the rate of photoreduction of $NADP^+$ by chloroplast fragments and by solubilized chlorophyll on the light intensity. The fragments were prepared by treatment of spinach chloroplasts with 1.3% digitonin[39]. The supernatant of centrifugation at 80000 × *g* was used. The reaction mixture contained: ascorbate, 3 mM; DCIP, 0.033 mM; $NADP^+$, 1.3 mM; NH_4Cl, 2 mM; plastocyanin, 20 mg/l; spinach ferredoxin, 30 mg/l; ferredoxin–$NADP^+$ reductase, diaphorase activity, 33 units/l; Tris–HCl buffer (pH 7.0), 50 mM; fragments containing 5 μM chlorophyll. With solubilized chlorophyll the mixture contained: ascorbate, 3 mM; DCIP, 0.05 mM; $NADP^+$, 0.5 mM; ferredoxin–$NADP^+$ reductase, diaphorase activity, 400 units/l; Triton X-100, 5 mM; Tris–HCl buffer (pH 7.5), 50 mM; chlorophyll, 5 μM.

Conclusion

It can be concluded from these experiments that the photochemistry of solubilized chlorophyll is similar to that of chlorophyll dissolved in organic solvents and that of water-soluble derivatives of chlorophyll, but it is very distinct from the photochemistry of chloroplasts or their fragments in which the molecular surrounding of the pigment seems to provide high specificity as well as high efficiency of the essential light reactions occurring during photosynthesis.

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