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HEXANE-SOLUBILISED REACTION CENTRE PROTEOLIPID COMPLEXES OF *RHODOPSEUDOMONAS SPHAEROIDES*

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Summary

Reaction centres from *Rhodopseudomonas sphaeroides*, strain R-26, have been solubilised in hexane by the use of phospholipids and cations. Two procedures have been developed: (I) a two-step technique involving the formation of detergent-free proteoliposomes from detergent solubilised reaction centres and phospholipids and mixing these with hexane in the presence of cations; (II) directly sonicating detergent-solubilised reaction centres with phospholipids before mixing with hexane and cations.

The yield of the extracted complex varied with the ratios of protein, phospholipid and cations, species of phospholipid, and sonication time. The spectral characteristics of the complex in the organic phase were similar to those of detergent-solubilised reaction centres. The stability of the reaction centres appeared to be dependent on the presence of phospholipid and water in the hexane. The usefulness of the hexane solution as a model membrane system is discussed and its possible future applications are considered.

Introduction

As more information becomes available on the electron transport components of photosynthetic organisms so it becomes increasingly important to

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Abbreviations: DAD, diaminodurene; TCBQ, tetrachlorobenzoquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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relate their structural organization to the overall function of the combined units. To this end topographical studies on the localisation and interrelation of the protein complexes in the membrane [1,2], and the reconstitution of selected components into active model membrane systems [3] are yielding information that is unavailable from the kinetic analysis of sub-cellular fractions or detergent-solubilised subfractions. During the course of these studies it has become clear that many membrane proteins are only functional when associated with specific lipids [4,5].

The isolation of proteolipids rather than detergent-solubilised purified protein complexes has taken on an important meaning since the replacement of detergent with lipids has enabled the reconstitution of complexes into chromatophores [6], liposomes [3,7—13], monolayers [14,15], bilayers [16—18], and hydrophobic solvents [16,19,20]. Hexane was proposed as an adequate model for the interior of lipid bilayers by Eisenman in 1968 [21], and many extractions of membrane proteolipids have been made using short chain aliphatic hydrocarbons as solvents [22,23]. The resultant solubilised complexes can provide interesting information on specific protein-lipid interactions necessary for their overall neutralisation in the hydrophobic phase [23]. The ability of the complexes to function in this environment may be of significance in determining the mechanisms involved. Gitler and Montal [19] described the partition of lipoprotein complexes of cytochrome *c* between aqueous and non-aqueous phases and showed that this was strongly biased toward the latter, provided that the overall charge of the complex was neutralized by cations. Here we report the extraction of reaction centres from *Rhodopseudomonas sphaeroides*, strain R26, into hexane using a procedure modified from their technique. In this paper the influence of various conditions on the extraction of the proteolipid complex into hexane, and the effect of the new environment on the spectral characteristics are described.

Materials and Methods

Buffers, inorganic and simple organic reagents were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and were of analytical or highest commercial grade. Fiske and SubbaRow reagent; 2,5,3,6-tetramethyl-*p*-phenylenediamine (diaminodurene); deoxiribonuclease I and ribonuclease IA (crude fractions) were obtained from Sigma Chemical Co. 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone); 9,10-dihydro-9-oxanthracene (anthrone); 2,3,5,6-tetrachloro-1,4-benzoquinone; *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD); 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-napthoquinone (Lapochol); 2,6-dimethylbenzoquinol, 5-hydroxy-1,6-naphthoquinol were obtained from Aldrich Chemical Co., Gillingham, Kent, U.K. Lauryldimethylamine oxide was a gift from the Onyx Corporation, Division of Millimaster, Jersey City, NJ, U.S.A. Ubiquinone-10 was a generous gift from Dr. U. Gloor and Proferssor G. Brubacher of F. Hoffmann-LaRoche and Co. Ltd., Basle, Switzerland. Xylene Brilliant Blue Cyanine G was obtained from Eastman Kodak, NJ, U.S.A. Phosphatidylcholine, purified from egg yolks and greater than 99% pure was a gift from R. Overfield (cf. Singleton et al. [24]). All other lipids were purchased from Sigma. Soybean phospholipid consisted of about

10—20% w/w phosphatidylcholine; phosphatidylethanolamine and phosphatidylserine were also present to about 10% and 5%, respectively (Sigma analysis). Partially purified soybean phospholipid was obtained by the method of Kagawa and Racker [12,25—27]. Also used were: L-α-dipalmitoylphosphatidic acid; Type V L-α-phosphatidylethanolamine; L-α-phosphatidylserine; Grade I L-α-phosphatidylglycerol; Grade I L-α-phosphatidylinositol and bovine diphosphatidylglycerol (cardiolipin).

Cell culture and reaction centre preparation

Cells of *Rps. sphaeroides*, strain R26, were grown anaerobically in the light; the growth medium was that of Cohen-Bazire et al. [28] using succinate as sole carbon source. Chromatophores were prepared by suspending harvested cells in 50 mM Tris buffer (pH 7.5), to 30% w/v and passing twice at 12 000 lb/inch^2 through a cooled French pressure cell (American Instrument Company, Silver Spring, MD, U.S.A.).

The reaction centre purification procedure was a modification of that used by Wraight [29]. The chromatophore suspension was washed by twice centrifugation and resuspension of the pellet in 10 mM Tris buffer, 100 mM NaCl (pH 7.5). This was diluted to an absorbance of 50 (at 870 nm) with salt buffer, being adjusted at the same time to a concentration of 0.5% lauryldimethylamine oxide and left stirring in the cold for 2 to 3 h. After centrifugation the supernatant contained reaction centre protein and was spectroscopically devoid of antenna chlorophyll. This was followed by a 30% saturated ammonium sulphate precipitation and the supernatant was further purified by ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals). Reaction centres were precipitated with 45% saturated ammonium sulphate and then extensively dialysed against 10 mM Tris-HCl buffer (pH 7.5) containing 0.025% lauryldimethylamine oxide. Pure reaction centres had a 280 nm to 800 nm ratio of 1.5. The reciprocal specific content (apparent molecular weight) was calculated, after protein determination, to be 71 000.

Liposomes

Lipid vesicles containing purified reaction centres (proteoliposomes) were formed by three different methods.

(a) Cholate dialysis. This is essentially the procedure of Kagawa and Racker [12] with modifications by Drachev et al. [7]. Partially purified soybean lecithin was suspended in 10 mM Tris sulfate (pH 7.5) to a concentration of 50 mg/ml. Magnesium sulphate was added for reaction centre stability (5 mM). Sodium cholate was added to 5% w/v, and the suspension was then dispersed by brief bath sonication (Ultrasonic cleaner (80W) Cole Palmer, III). Purified reaction centres in 10 mM Tris-HCl (pH 8.5) were added to a final concentration of 1 mg/ml; the final ratio of lipid to protein being about 25 : 1 (w/w) with not less than 1.5% sodium cholate. The suspension was dialysed against 10 mM Tris sulphate (pH 7.5) with 25 mg/l dithiothreitol as antioxidant. The dialysis was carried out in a 2-l flask kept in the dark and at room temperature, the buffer was constantly stirred and was changed twice in 24 h. After dialysis the proteoliposomes could be collected by centrifugation at 15 000 × *g* for 2 h, or by ultrafiltration.

(b) Gel filtration. This procedure was first described by Kagawa and Racker in 1971 [12] and was modified by Brunner et al. [30]. Partially purified soybean lecithin was suspended in 100 mM NaCl, 10 mM Tris-HCl (pH 7.5) to a concentration of 25 mg/ml and the lipid dispersed by bath sonication. Purified reaction centres suspended in 10 mM Tris-HCl buffer (pH 8.5) were added to 1 mg/ml and the suspension was run down a Sephadex G-50 column (25 × 2.4 cm) at room temperature using 100 mM NaCl, 10 mM Tris-HCl (pH 7.5) as elution buffer. The cholate to lipid ratio was probably less than 0.5% in the final vesicles [30] and the ratio of lauryldimethylamine oxide molecules remaining associated with the reaction centres was 2—4 [31].

(c) Sonication. Lipid was sonicated (see Racker [10]) to clarity in 10 mM Tris-HCl buffer (pH 8.5) using an 80-W ultrasonic bath. All sonication was carried out at 45°C. Purified reaction centres were suspended in 10 mM Tris-HCl (pH 8.5) to 50 or 10 μM (0.37 or 0.74 mg/ml), lipid was added to 1 to 50 mg/ml depending on the lipid and the suspension was re-sonicated for up to 10 min.

Hexane suspensions

Hexane suspensions of reaction centres were formed by extracting proteoliposomes with hexane in the presence of cations. From 1 to 10 nmol of reaction centres were used for extraction. The suspension was diluted with a small amount of buffer (10 mM Tris-HCl, pH 8.5). Additional phospholipid was added in some cases and was either presonicated or added as a hexane solution to the hexane phase. Aqueous volumes were usually kept to between 1 and 3 ml for ease of handling. Cations were added directly to the aqueous phase, with gentle shaking to ensure complete mixing. Hexane was layered on top of the aqueous suspension and the two phases were thoroughly mixed using a vortex mixer for one or more minutes. Centrifugation (1100 × *g*) for 1 min separated the two phases.

Protein determination was by the dye binding method of Bramhall [32] and phospholipid analysis was according to Broekuyse [33].

Spectroscopy

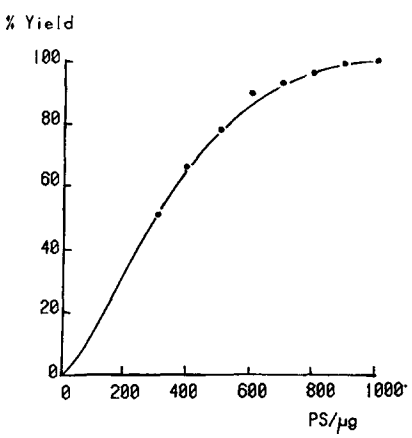
For kinetic measurements flash excitation was provided by Xe flash, 90—95% saturating, of 20 μs pulse width; absorbance changes were recorded using a rapidly responding single beam kinetic spectrophotometer [34]. Transients were averaged after being read into a PDP 11/34 minicomputer (Digital Equipment Corporation) via a Datalab DL 905 Transient Recorded (Data Labs-Mitcham, Surrey, CR4 4HR U.K.). The spectrophotometer was equipped with an automated shutter to minimise bleaching of the sample by the measuring beam between readings. The system was computer controlled through a DEC Laboratory Peripheral System 11 using an appropriate assembly language program (SPCDEC) [35]. Absorption spectroscopy was carried out using a laboratory designed single beam scanning spectrophotometer. This used a double grating monochromator (Hilger-Watts, Rank Precision Industries) with an EMI 9659B photomultiplier tube (PMT) for measurements in the far red. An orange glass filter (Corning) with cutoff below 550 nm was used to prevent second-order spectra from interferring with spectra taken above this value, and

a diffuser between the sample and PMT was routinely used to maximise light scattering [36]. The wavelength drive came from a geared-down stepping motor (Berger-Lahr) which was interfaced to a PDP 11/34 minicomputer, as was the output from the PMT, via a DEC LPS analogue to digital converter. A Fortran language program (DIFSPC) controlled the taking and storing of spectra in computer memory. Spectra subtraction, manipulation, printout, hardcopy display and signal avergaing were also governed by the same program [37].

Results

Extraction of hexane-solubilised reaction centres from detergent-free proteoliposomes

Successful extraction of hexane-solubilised reaction centres was possible using proteoliposomes formed by either cholate dialysis or gel-filtration. The cholate dialysis technique was routinely used and the vesicles formed by this method were typically 300—400 Å in diameter, single-shelled, and containing one or two reaction centres per vesicle as verified by freeze-fracture electron microscopy (Staehelin, A., personal communication). When semi-purified soybean lecithin was used, the extraction of reaction centres was dependent on the addition of small quantities of phosphatidylserine to the water or hexane phase (Fig. 1). The extraction yield is defined as the ratio of total P-800 in hexane to the initial quantity of P-800 in the aqueous phase of the extraction mixture. The substitution of pure phosphatidylcholine for partially purified soybean lecithin in the formation of the proteoliposomes abolished the extraction of reaction centres into hexane, even when 800 μg of phosphatidylserine



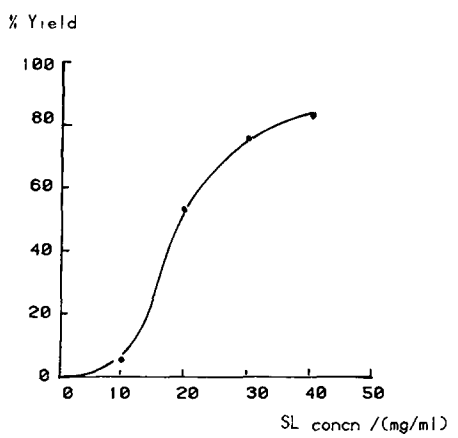


Fig. 1. Dependence of the % yield of hexane-solubilised reaction centres (HRC) from proteoliposomes on the quantity of phosphatidylserine (PS) added to the hexane phase. Proteoliposomes were 1 mg protein to 25 mg partially purified soybean lecithin. 1 nmol reaction centre liposomes were suspended in 3 ml of 10 mM Tris-HCl (pH 8.5). Hexane (3 ml) was layered onto the aqueous phase, and mixing was carried out for 2 min in the presence of 50 mM $CaCl_2$.

Fig. 2. Dependence of the % yield of hexane-solubilised reaction centres on the concentration of soybean lecithin (SL) in the aqueous phase. Reaction centres (5 nmol in 2 ml buffer) were mixed with $CaCl_2$ (75 mM).

was added to the hexane phase, or dispersed in the aqueous phase, immediately before mixing with cations.

Extraction of hexane-solubilised reaction centres by the sonication and mixing method

The majority of the work presented in this paper made use of the extraction techenique whereby reaction centres were sonicated with soybean lecithin and then mixed with hexane and cations. Not only was this method rapid, but the conditions were easily variable and the maximal yields high. The optimal component concentrations for the extraction using unpurified soybean lecithin have been determined to be as follows: 100 mg/ml of soybean lecithin, 5 nmol of reaction centres, 100 mM $MgCl_2$ or 75 mM $CaCl_2$ or 25 mM $CdCl_2$.

The effect of the alteration of the experimental conditions on the extraction yield are briefly described below.

The extraction of reaction centres into hexane was dependent on the presence of lipids. A titration curve for soybean lecithin is shown in Fig. 2. Substituting phosphatidylcholine for soybean lecithin eliminated any extraction of reaction centres into hexane, as shown with the extraction of proteoliposomes. Phosphatidylserine as sole lipid in the aqueous phase allowed the extraction of reaction centres into hexane, however 50 mg/ml were required to give a 70% yield. This is a high concentration when compared to the small proportion present in soybean lecithin additions. Also 100 mM $CdCl_2$ was necessary for the high yield. The phospholipids phosphatidylinositol, phosphatidylglycerol and cardiolipin were found to be able to substitute for phosphatidylserine but phosphatidic acid and phosphatidylethanolamine were not.

The effect of a variety of divalent cations on the extraction of hexane reaction centres was investigated and their order of effectiveness was Cd > Sr ⩾ Ba > Ca > Mg. Although the order of effectiveness did not vary under differing conditions, and the sigmoidal profile was retained, the relative yields were not

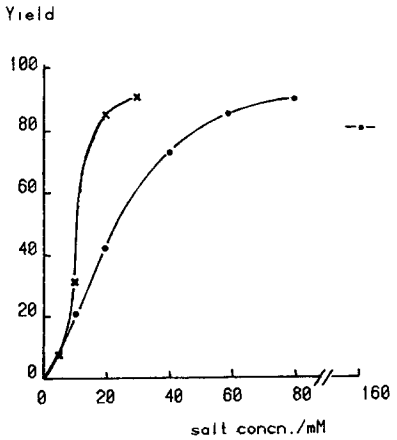


Fig. 3. Dependence of % yield of hexane-solubilised reaction centres on the cation concentration in the aqueous phase with soybean lecithin (SL) as lipid. Reaction centres (5 nmol in 2 ml buffer) were extracted with 50 mg/ml SL and $MgCl_2$ (•) or $CdCl_2$ (×).

constant. Increasing the salt concentration above that required to give maximal yields caused a decrease in the yield of P-870 (Fig. 3) and an increase in the yield of P-750. The addition of cations directly to the aqueous phase before mixing the consituents with hexane, caused precipitation of some of the lipid. There were no deleterious effects on the reaction centres and the addition at this stage was found to be more efficient than addition immediately before or during the mixing process. This was probably due to a more thorough equilibration throughout the aqueous phase before emulsifying with hexane. The yield of hexane-solubilised reaction centres was linear with respect to concentration in the aqueous phase of the extraction mixture, for the conditions used.

The effect of pH on the extraction of reaction centres in the absence of ions was investigated. The conditions were those described above, except for the lack of cations. At very low values of pH very little P-800 was extracted, but it appeared that most of the remainder had broken down to the P-750 form. Raising the pH increased the ratio of P-800 to P-750 but at pH 5 no protein was extracted at all. The effect of pH in the range of 7 to 9 on the extraction yield in the presence of cations was also examined. Although neither the % yield of hexane-solubilised reaction centres nor the ratio of P-800/P-750 changed very much over the pH range, an optimum of pH 8.5 was consistent for different extraction conditions.

Under conditions of poor interaction between lipid and protein (insufficient pre-sonication of the lipid, sonication of lipid and reaction centres, or mixing with hexane and cations) the % yield was low. This could be compensated for by the addition of detergent (lauryldimethylamine oxide). Table I shows the result of one experiment where the reaction centres were not sonicated with soybean lecithin before mixing with hexane and cations. The presence of lauryldimethylamine oxide at 0.1% concentration (w/v) was sufficient to allow over 80% of the reaction centres to be extracted on mixing. Sonication did not increase this value. The presence of excess detergent significantly reduced the yield, this was probably due to the formation of reaction centre and detergent micelles which had a higher affinity for water than hexane. Although detergent was found to give high yields, lauryldimethylamine oxide was not routinely added to the extraction mixture to minimise the detergent concentration in hexane.

TABLE I

THE EFFECT OF DETERGENT CONCENTRATION, SONICATION AND TEMPERATURE ON THE YIELD OF HEXANE-SOLUBILISED REACTION CENTRES

3 nmol of reaction centres and 100 mg of presonicated soybean lecithin in 2.5 ml of buffer were incubated for 5 min under the above conditions before mixing for 1 min with 50 mM $CaCl_2$ and 3 ml of hexane. LDAO, lauryldimethylamine oxide.

Pre-mixing conditions	% yields		Pre-mixing conditions	% yield	
No additions (<0.005% LDAO)	4°C	36	No additions (<0.005% LDAO)	25°C	57
0.05% LDAO	4°C	48	0.1% LDAO	25°C	84
0.1% LDAO	4°C	81	0.1% LDAO 5 min sonication	25°C	81
0.2% LDAO	4°C	36	5 min sonication (<0.005% LDAO)	25°C	84
0.4% LDAO	4°C	0			

The effect of temperature for less than perfect extraction conditions is shown in Table I. Raising the temperature from 4°C to 25°C increased the yield of hexane-solubilised reaction centres without affecting their stability.

Sonication times of up to 20 min were found to be harmless to reaction centres (using an 80-W bath sonicator) and the effect of sonication on the yield is shown in Table I. Sonication of the aqueous and hexane phases together instead of mixing, did not improve the yield, rather, the effect was to denature all the reaction centres. Sonication of the reaction centres in the aqueous phase before the addition of lipid, cations and hexane had no effect on the yield.

The mixing of the hexane and aqueous phases was carried out using a vortex mixer for 1 min. Under optimal extraction conditions about 90% of the final yield was extracted in the first 10 s of mixing. When the reaction centres were inadequately sonicated with the lipid, or when phosphatidylserine was added to the hexane phase for the extraction of cholate-dialysis proteoliposomes, longer mixing times were required to obtain good yields.

Preliminary observations on the role of the solvent in the extraction of hexane-solubilised reaction centres have indicated that solvents with dielectric constants below 2.00 can be used to extract undenatured reaction centres. The addition of 1% chloroform to a hexane solution of reaction centres caused almost instantaneous denaturation of the protein. The alkanes (pentane to decane) all extracted reaction centres with about the same degree of success.

The extraction mixture volumes were not found to be critical. Doubling the concentration of the reactants or dilution by two in the aqueous phase was found to have an insignificant effect on the extraction yield. Similarly, doubling or halving the hexane volume did not radically affect the yields obtained.

Characterisation of hexane solutions of reaction centres.

The presence of lipid. Lipid was extracted into hexane almost porportionally with reaction centres. The ratio of phospholipid to hexane-solubilised reaction centres ranged from 20 000 to 90 000 mol/mol for extractions with unpurified soybean lecithin. Large lipid micelles appear to exist in such preparations, some of which can be removed by freezing (0°C) the hexane, or brief sonication, which precipitates lipid and reduces light scattering. These methods did not remove any observable quantities of reaction centres from hexane solution. Further depletion of lipid from the preparation by methods detailed below, resulted in the gradual aggregation of reaction centres into large complexes, giving a turbid appearance to the solution; brief sonication could temporarily clear this. Dilution of a freeze-thawed hexane preparation made using soybean lecithin, with an equal quantity of hexane, and centrifugation at 150 000 × *g* for 1 h lowered the lipid to protein ratio from about 10 000 : 1 (mol/mol) to about 2000. Resuspension of the pellet in hexane (40 ml) and recentrifugation brought the ratio down to about 150 : 1 in the pellet. Even though hexane is highly hydrophobic, water saturates it to approx. 1 mM [38]. The large micelle population of freshly made hexane preparations trapped a great deal more than this. The water released by micelle disruption was usually several μl for every ml of frozen or sonicated hexane extract. Dehydration of the hexane preparations was possible with the drying agents: sodium metal, aluminium oxide,

lithium aluminium hydride, and 4A molecular sieves. When using these dessicants with hexane, the solutions were usually left at 4°C for at least one day following the removal of 'bulk' water by freezing, and centrifugation. Periodically the solutions would be put under vacuum. In the cases of sodium metal and lithium aluminium hydride this removed hydrogen gas from the solution and metal's surface, thereby increasing the rate of dessication. During the drying period sonication was found to give instantaneous increases in hydrogen production; this effect ceased after about 4 days of dehydration. Lipid accumulated at the bottom of the hexane solution during dehydration. When molecular sieves were used they were changed every 24 h for four days. Aluminium oxide was not used in large quantities, or changed, because of its affinity for reaction centres. The apparent molecular weight of the extracted reaction centres was similar to that of the purified detergent-solubilised reaction centre complexes, being about 73 000. The apparent pH of the hexane preparation as measured by a pH electrode, was the same as that of the aqueous buffer used in the extraction.

Spectroscopy of hexane-solubilised reaction centres. The absorption spectrum of freshly prepared reaction centres in hexane is very similar to that of detergent-solubilised reaction centres [2], an increase in the height of the band at 750 nm is the most prominent difference. This band was found to vary tremendously in relation to P-800 but was minimal when the extraction yield was maximal. The ratio of P-750 to P-800 for a 90% extraction yield was about equal to that for pure detergent-solubilised reaction centres. Centrifugation of the hexane solution at 150 000 $\times g$ for 30 min at 25°C sedimented the reaction centres with a great deal of lipid. The contaminating portion of P-750 remained in the supernatant with dissolved lipids and small micelles. The pellet was a rich blue and could be resuspended in hexane by gently homogenising with a hand-held teflon homogeniser. An alternative procedure, both simpler and more rapid, involved shaking about 100 mg of a aluminium oxide with 10 ml of hexane preparation, the powder was sedimented by spinning at 1000 $\times g$ for

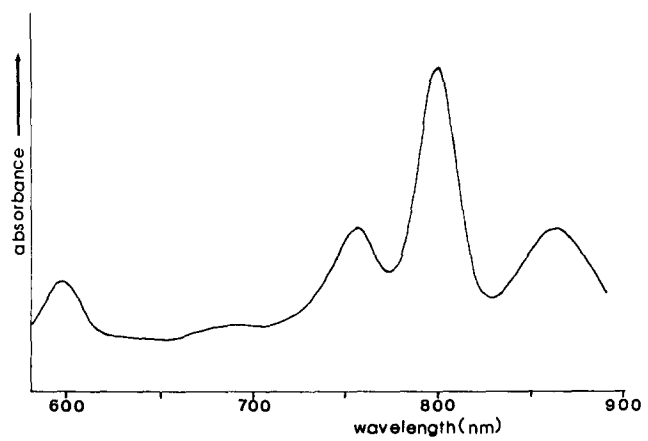


Fig. 4. Hexane-solubilised centres (HRC) after treatment with aluminium oxide. A few milligrams of aluminium oxide powder were vortex-mixed for 1 min with 5 ml of HRC (1.4 μM).

5 min; a 'clean' hexane preparation was found to remain in solution (Fig. 4). The optimal stoichiometries of aluminium oxide to hexane varied with the purity and concentration of the preparation. The aluminium oxide bound a proportion of the lipid present and when the former was in excess was also found to remove reaction centres from solution.

A slight blue shift in the spectrum was discernable when compared to that of detergent-solubilised reaction centres. The 801 nm peak (800—803 nm) was shifted by 1—2 nm to 800—802 nm and the 600 nm peak by 3 nm from 601 nm to 598 nm. The hexane solubilised reaction centres were chemically oxidisable by TCBQ (1 mM) or iodine (1 mM) [39]. Rereduction was possible by the addition of DAD (0.1 mM). Other reductants included the following: tetrachlorobenzoquinol; lapochol; anthrone; 2,6-dimethylbenzoquinol; 5-hydroxy-1,6-napthoquinol and TMPD. Flash illumination in the presence of 1 mM Q gave 100% photooxidation of P-870 when measured at 598 nm using an extinction coefficient of 26.4 $mM^{-1} \cdot cm^{-1}$ [40] and compared to the reduced absorption spectrum at 802 nm, using an extinction coefficient of 288 $mM^{-1} \cdot cm^{-1}$ [41]. The flash-induced light-minus-dark difference spectrum of the reaction centres, between 400 nm and 600 nm, was very similar to that of detergent-solubilised reaction centres. A slight blue shift was observable for the 600 nm band, the maximum absorbance being at 598 nm. The decay of the absorbance change at 598 nm after flash excitation, without added Q, was first order with a half-time of 50—60 ms. The decay of the absorbance change at

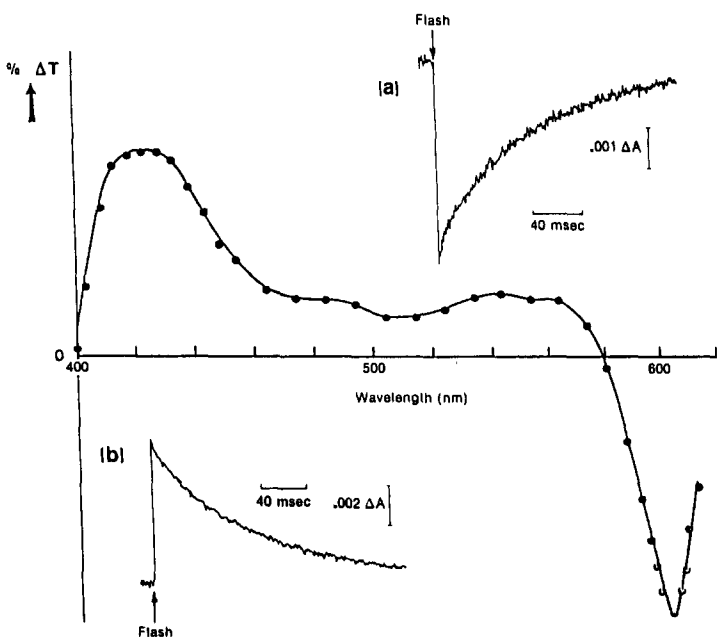


Fig. 5. Flash-induced light-minus difference spectrum of hexane-solubilised reaction centres (HRC). HRC approx. 0.1 μM. No other additions. Readings taken approx. 1 μs after the flash. Insets: Flash-induced absorbance changes at 598 nm and 445 nm for HRC. HRC 0.49 μM. No other additions. Time constant was 50 μs; average of four traces. (a) at 598 nm and (b) at 445 nm.

445 nm, due to P-870 and the primary quinone acceptor, was also first-order and had a half-time of 50—60 ms under the same experimental conditions (Fig. 5). Diaminodurene was an excellent electron donor, reducing the half-time for the rereduction of P-870 to 2—3 ms at 0.5 mM concentration. Duroquinone was an efficient substitute for Q as an acceptor, giving as much as 20% more photooxidation of P-870 as an equivalent sub-optimal concentration of Q, when added to quinone-deficient reaction centres. The $t_{1/2}$ for the back reaction in the presence of 0.1 mM duroquinone was 160 ms as opposed to 120 ms for 0.1 mM Q. Fig. 6 shows a graph of percentage photooxidation of P-870 (measured at 598 nm) for varying concentrations of quinone added to the hexane preparation.

A fresh preparation of hexane reaction centres was stable for days at room temperature and for weeks at 4°C. Although some lipid precipitated during this time, the reaction centres remained in solution. Storage at −10°C precipitated further lipid and water, but otherwise the preparation was indefinitely stable. Shaking the reaction centres with distilled water or 1 M $CaCl_2$ did not appear to affect the concentration or spectrum in any way. However, sonication for more than an instant denatured the reaction centres giving rise to a progressive change in the spectrum. After sonicating for a few seconds a spectrum was obtained in which the 800 nm and 870 nm peaks disappeared and were replaced by a peak at about 760 nm with a shoulder at 790 nm. This spectrum was very similar to that for the dimer of bacteriochlorophyll in carbon tetrachloride [42]. A peak at 590 nm was probably associated with the dimer and a peak at 525 nm seemed to be due to bacteriophaeophytin. Increased sonication changed the spectrum to that typical for the monomer with further sonication giving that for the bacteriophaeophytin [39]. The P-750 impurity seen in many fresh hexane preparations could be isolated by methods previously described, and was also identified as bacteriophaeophytin. Hexane preparations could be

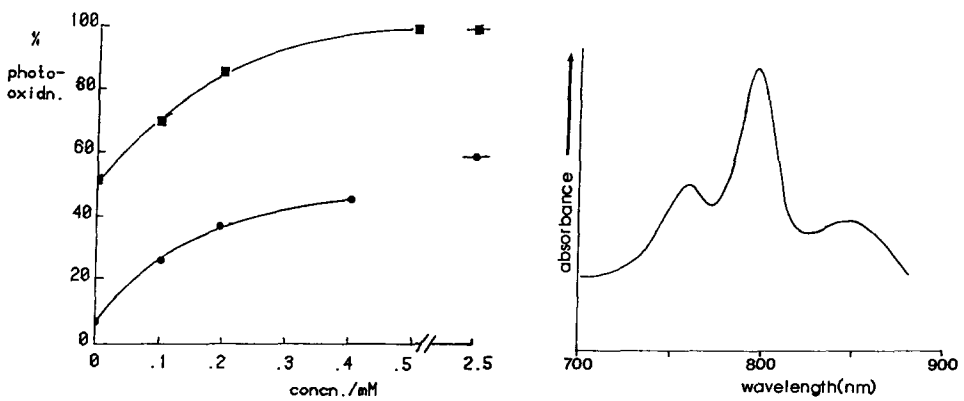


Fig. 6. Dependence of % flash-induced photooxidation of hexane-solubilised reaction centres (HRC) P-870 on the addition of Q to the hexane preparation. HRC 0.55 μM. Q was added as 100 mM solution in hexane to a fresh preparation of HRC (■) and after freezing and thawing (●).

Fig. 7. Spectrum of hexane-solubilised reaction centres (HRC) after extensive drying with sodium metal. HRC were approximately 0.2 μM. See text for details.

TABLE II

THE EFFECT OF LIPID REMOVAL AND Q ADDITION ON THE % PHOTOACTIVITY OF HEXANE-SOLUBILISED REACTION CENTRES (HRC) AND THE HALF-TIME OF BACK-REACTION
See text for details.

Procedure	% photoactive P-870	$t_{1/2}$ back-reaction (ms)
(a) Fresh HRC (0.55 μM)	52	55
+0.5 mM Q	100	120—160
(b) 'Delipidated' HRC twice frozen, thawed and centrifuged	6—60	50
+0.4 mM Q	45—80	150
(c) 'Dehydrated' HRC 24 h with mol. sieves 4A	15	55
+0.5 mM Q	21	55
(d) Lipid depletion by centrifugation	0	0
+0.5 mM Q	0	0

evaporated down and left at room temperature for several days before resuspension into hexane without any denaturation. The dried samples could also be sonicated into 10 mM Tris-HCl buffer (pH 7.5) 0.1% lauryldimethylamine oxide to give fully photochemically active detergent-solubilised reaction centres.

Freezing and thawing increased the stability of the reaction centres to sonication, and dehydration rendered them immune to at least 30 min of sonication. The spectrum of dehydrated reaction centres became distorted (Fig. 7), but the re-addition of trace amounts of water permitted the spectrum to relax to its former state. Sonication of partially dehydrated extracts in the presence of added water, rapidly produced denaturation of the reaction centres. Vigorous dehydration with $LiAlH_2$ under vacuum produced a spectrum of monomeric bacteriochlorophyll; the addition of water did not reverse the change.

Freezing and thawing the hexane preparation also decreased the extent of flash induced photooxidation of P-870 and increased the rate of the back-reaction. Dehydration by various dessicants produced similar effects to freezing and thawing. Partial lipid depletion in the presence of water, by hexane dilution and centrifugation decreased flash-induced photooxidation to zero. The addition of quinone did not reverse this effect (Table II). The spectrum of the reaction centres was perfectly stable under the above conditions although the reaction centres were inactive.

Discussion

Sonication is a routine method for the formation of liposomes, and high extraction yields of reaction centres require good preliminary sonication of reaction centres with lipid, unless preformed proteoliposomes are used. This necessitates that the latter are an obligatory intermediate in the extraction process. A complex involvement of neutral and acidic lipids and cations is implied by both extraction techniques. Although negatively charged phospholipids were found to extract reaction centres into hexane, the concentrations of lipid

and cations were much higher than when mixed lipids were used. It is possible that residual membrane lipids, attached to the reaction centre (15 mol per mol of reaction centre) also played a role in this extraction. These results together with the observed effects of cations and pH (in the absence of cations) are consistent with previous work on solvent extractable proteolipids [18—20,23,43]. The lack of dependence of the yield of hexane solubilised reaction centers on the aqueous or hexane volumes in the extraction mixture emphasises that the proteolipid complex is formed as a result of the interaction of defined rations of the substrates, suggesting that the two solvents play only a supporting role. The situation in the hexane can be considered to be the inverse of that for lipid complexes and micelles in water (see Tanford [44]). The high concentration of lipid present in the extract is in support of this, as is the phenomenon of aggregation after extensive depletion. Thus, the hexane preparation is far from a real solution. Furthermore, pronounced scattering of X-rays and neutrons (Sadler, D., personal communication) was attributed to large lipid-protein complexes. Ultra-centrifugation studies (Creeth, J., personal communication) also indicated similar complexes. Previous work on cytochrome *c* proteolipid complexes in octane, by Shipley et al. [45] has shown that complexes occurred of particle weight greater than one million daltons, when the protein to phospholipid ratio was about 1 to 30 mol per mol. Although micelle formation in solvents is enhanced by the presence of cations (see Das and Crane [46]) it is doubtful whether the majority of reaction centres exist in hexane only because they are trapped in inverted liposomes, since methods (freezing and thawing, sonication, hexane dilution or dessicants) which destroy these structures do not precipitate any observable quantities of reaction centres from solution.

Although no measurements of water content have been made in the dehydrated systems, efficiencies for several drying agents in bezene have been reported by Burfield et al. [49]. The residual water content of benzene (saturated with 0.8 mM water), after exposure to 4A molecular sieves, sodium and lithium aluminium hydride for one day was about 0.2, 15 at 0.15 μM, respectively, and these were taken as approximations of the water content of dried hexane preparations. Assuming no tightly bound or otherwise 'unavailable' water, these figures would be overextimates since benzene saturates with water to about 20% more than hexane [38]. It is unlikely that inverted liposomes could exist in the presence of these dessicants. The spectrum of dehydrated hexane reaction centres (Fig. 7) is distored and bears a resemblance to that of the gelatin films of reaction centres of Clayton [50]. This would represent a weakening of the interaction between the 'special pair' of bacteriochlorophyll molecules. The more vigorous action of lithinium hydride could have removed sufficient water to cause irreversible dissociation of the dimer.

The dependence of flash-induced photooxidation of P-870 and half-time for the back reaction, on the concentration of quinone added to hexane preparation (Figs. 6 and 8) contributes to the evidence for Q as primary and secondary acceptor in the reaction centre complex [47,48]. These results also suggest that that the extraction procedure causes the reaction centres to lose some of their quinones, although these reassociate on evaporation of the hexane followed by solubilisation in detergent solution. Since Q is extremely soluble in hexane and lipid, it is understandable that dilution of the quinones out of the protein com-

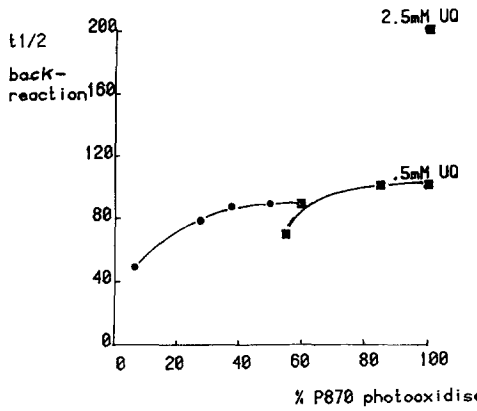


Fig. 8. Half-time for the back-reaction after flash-induced photooxidation of hexane-solubilised reaction centres P-870 against % photooxidation upon increasing the Q concentration in the hexane. Data taken from Fig. 6; Q titration for a fresh preparation of HRC (■); Q titration for HRC after freezing and thawing (●).

plex might occur upon extraction [47]. This is supported by the observation that extraction of reaction centres at high cation concentrations give rise to a decreased photoactivity (not shown). Since the above extraction condition increases the micelle concentration in hexane, Q would tend to partition more into micelles and this could then be related to the effective concentration of Q in hexane reaction centres. Freezing and thawing, or the use of dessicants results in the loss of a fraction of the photoactivity (Figs. 6 and 8). More drastic removal of lipid, by hexane dilution and centrifugation, abolishes all photoactivity even in the presence of water, quinone or added phospholipid (Table II). Two effects of lipid depletion appeared superimposed on one another. Firstly, the reversible extraction of quinone from the immediate environment of the reaction centre, and secondly, the irreversible loss of photoactivity. The role of lipid is unclear, but there are three main possible interpretations; all take account of the highly hydrophobic nature of hexane. (a) The lipid may be trapping water around the hydrophilic areas of the complex, and the water is necessary for primary photochemistry. (b) The lipid may be involved in the binding of the primary quinone to the hexane reaction centre complex. (c) The lipid may be involved with the protein's conformation in hexane in a way unrelated to the previous two cases but necessary for primary photochemistry. The second case is perhaps quite likely for secondary quinone stability. The maxium rate of the back reaction ($t_{1/2}$ = 55 ms) is achieved by lipid depletion and indicates a single quinone in active reaction centres. If the irreversible lipid-depletion effect is due to a role of the lipid in trapping water around the hydrophilic areas of the reaction centre then such a micelle-like structure would be difficult to reform in hexane after disruption.

The presence of lipid (and possibly also bacteriophyaeophytin) appears to shield the complex from water, but this shielding is removed by sonication which causes rapid denaturation. The stability of the P-870 appears to be maximal under the most hydrophobic conditions, with sonication having little

or no effect after dessication of the hexane preparation. In contrast, reaction centres in aqueous solution are fairly stable to short periods of sonication. One can surmise that the binding of detergent to the bacterial reaction centres under these conditions, is sufficiently strong to prevent any direct contact between water and the more delicate hydrophobic regions of the complex. The absolute requirement for organic solvents of low dielectric constant stresses the importance of a hydrophobic environment for reaction centre stability.

Fig. 9 shows a possible model for the reaction centre proteolipid complex in hexane. The model takes into account the mainly hydrophobic nature of the protein [51], its ability to span the membrane [52] and the existence of charged groups [40]. A micellar arrangement of lipid is shown around the hydrophilic areas of the complex. Micelle disruption which prevents photoactivity would remove this lipid shield and tend to cause aggregation. A simple model of the reaction centre in a membrane is shown for comparison.

Reaction centres are intrinsic membrane proteins of a highly hydrophobic character [51]. Their solubilisation in aliphatic hydrocarbon solvents is, debateably, no more artefactual than their solubilisation in aqueous environments by the use of detergents. Moreover, the stability of hexane-solubilised proteolipid reaction centre complexes and their retention of primary photochemistry make this system an attractive one for further electron transport studies. Differences between hexane-solubilised reaction centres and detergent-solubilised reaction centres may be related to the effect of the environment on specific parts of the protein complex; and the use of hexane rather than an aqueous phase facilitates the use of hydrophobic probes. The procedures developed in this paper show how reaction centres may be solubilised in

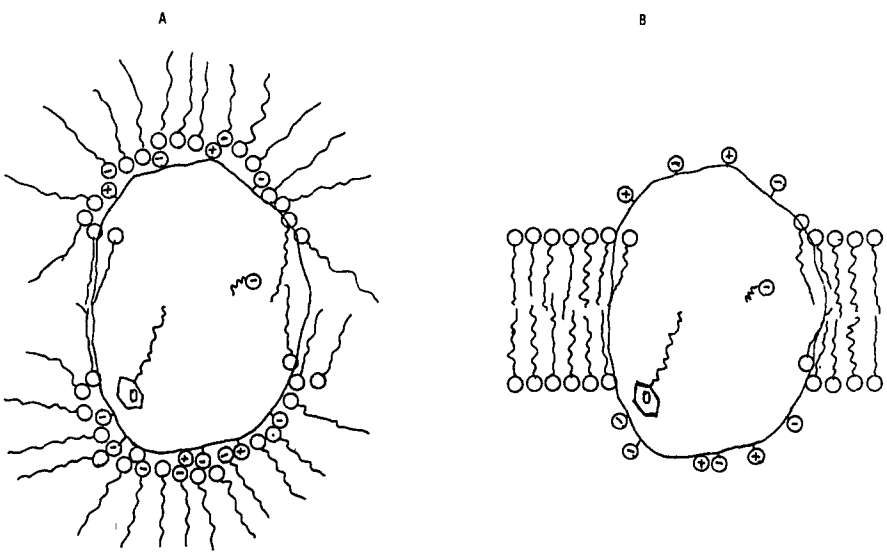


Fig. 9A. Possible model for the reaction centre proteolipid complex in hexane. Water may be trapped in micelle-like structures between lipid and reaction centre protein. See text for further details. B. Possible model for the reaction centre complex in the bacterial membrane.

aliphatic hydrocarbon solvents with high yields. Residual detergent can be removed from proteoliposomes by dialysis techniques before extraction with the solvent; thus the solvent system can be visualised as the 'negative' of the proteoliposome one. The structure of the solubilised complex is of interest in topographical studies, and the specific involvement of lipids (and possibly cations) could reveal not only direct involvement of the protein with a bilayer membrane but also the location of hydrophilic groups exposed in the natural environment, but requiring neutralisation in hexane. Detergent-free complexes in apolar solvents can be used for the construction of monolayers, which are powerful tools for the understanding of membrane phenomena [53—55]. The aposition of two monolayers over a teflon hole [56] or on a solid support [54] results in the formation of a bilayer enabling further studies not possible with proteoliposomes [3].

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References

1 DePierre, J.W. and Ernster, L. (1977) Annu. Rev. Biochem. 46, 201—262
2 Feher, G. and Okamure, M.Y. (1978) in The Photosynthetic Bacteria (Clayton, R.K. Sistrom, W.R., eds.), pp. 349—386, Plenum Press, New York
3 Montal, M. (1976) Annu. Rev. Biophys. Bioeng. 5, 119—175
4 Coleman, R. (1973) Biochim. Biophys. Acta 300, 1—30
5 Gennis, R.B. (1977) Annu. Rev. Biophys. Bioeng. 6, 195—238
6 Hunter, C.N. and Jones, O.T.G. (1979) Biochim. Biophys. Acta 545, 325—338
7 Drachev, L.A., Jasaitas, A.A., Jaulen, A.D., Kondrashin, A.A., Liberman, E.A., Nemacok, I.B., Ostroumov, S.A., Semenov, A.Y. and Skulachev, V.P. (1974) Nature 249, 321—323
8 Crofts, A.R., Crowther, D., Celis, H., Alamanza de Celis, S. and Tierney, G. (1977) Biochem. Soc. Trans. 5, 491—495
9 Drachev, L.A., Frolov, V.N., Kaulen, A.D., Liberman, E.A., Ostroumov, S.A., Plakunova, V.G., Semenov, A.Y. and Skulachev, V.P. (1976) J. Biol. Chem. 251, 7059—7065
10 Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224—230
11 Eytan, G.E., Matheson, M.J. and Racker, E. (1975) FEBS Lett. 57, 121—125
12 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477—5487
13 Kondrashin, A.A., Mikel'saar, K., Semenova, E.G. and Skulachev, V.P. (1976) Biokhimya 40, 911—918
14 Hwang, S.-B., Korenbrot, J.I. and Stoeckenius, W. (1977) J. Memb. Biol. 36, 115—135
15 Hirsch, R.E. and Brody, S.S. (1979) Photochem. Photobiol. 29, 589—596
16 Chen, C.-H. and Berns, D.S. (1976) Photochem. Photobiol. 24, 255—260
17 Trissl, H.-W., Darszon, A. and Montal, M. (1979) Proc. Natl. Acad. Sci. U.S.A. 74, 207—210
18 Gitler, C. and Montal, M. (1972) Biochem. Biophys. Res. Commun. 47, 1486—1491
19 Gitler, C. and Montal, M. (1972) FEBS Lett. 28, 329—332
20 Darszon, A., Philipp, M., Zarco, J. and Montal, M. (1978) J. Memb. Biol. 43, 71—90
21 Eisenman, G., Ciani, S.M. and Szabo, G. (1968) Fed. Proc. 27, 1289—1304
22 Segrist, H., Nelson, S.K. and Gitler, C. (1977) Biochem. Biophys. Res. Commun. 74, 178—184
23 Hart, C.J., Leslie, R.B., Davis, M.A.F. and Lawrence, G.A. (1969) Biochim. Biophys. Acta 193, 300—318
24 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Chem. Soc. 42, 53—56
25 Darszon, A., Montal, M. and Philipp, M. (1977) FEBS Lett. 74, 135—138
26 Darszon, A., Philipp, M., Zarco, J. and Montal, M. (1978) J. Memb. Biol. 43, 71—90
27 O'Brien, F., Costa, L.F. and Otta, R.A. (1977) Biochemistry 16, 1295—1303
28 Cohen-Bazire, G., Sistrom, W.R. and Stanier, R.Y. (1957) J. Cell. Comp. Physiol. 49, 25—68

29 Wraight, C.A. (1979) Biochim. Biophys. Acta 548, 309—327
30 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322—331
31 Overfield, R.E. (1978) Ph.D. Thesis, University of Illinois
32 Bramhall, S. (1969) Anal. Biochem. 31, 146—148
33 Broekhuyse, R.M. (1978) Biochim. Biophys. Acta 152, 307—315
34 Jackson, J.B. and Crofts, A.R. (1971) Biochim. Biophys. Acta 357, 175—181
35 Crowther, D. (1977) Ph.D. Thesis, University of Bristol, U.K.
36 Shibata, K. (1959) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. 7, pp. 77—234, Interscience, New York
37 Kendall-Tobias, M.W. (1979) Ph.D. Thesis, University of Bristol, U.K.
38 Stephen, H. and Stephen, T. (1963) Solubilities of Inorganic and Organic Solvents, Vol. I, McMillan, New York
39 Goedheer, J.C. (1966) in The Chlorophylls (Vernon, L.P. and Seely, G.R., eds.), pp. 147—185, Academic Press, New York and London
40 Prince, R.C., Dogdell, R.J. and Crofts, A.R. (1974) Biochim. Biophys. Acta 347, 1—13
41 Straley, S.C., Parson, W.W., Mauzerall, D.C. and Clayton, R.K. (1973) Biochim. Biophys. Acta 305, 597—609
42 Sauer, K., Lindsay Smith, J.R. and Schultz, A.J. (1966) J. Am. Chem. Soc. 88, 2681—2688
43 Das, M.L., Haak, E.D. and Crane, F.L. (1965) Biochemistry 4, 859—865
44 Tanford, C. (1973) The Hydrophobic Effect, pp. 36—81, Wiley, New York
45 Shipley, G.G., Leslie, R.B. and Chapman, D. (1969) Biochim. Biophys. Acta 173, 1—10
46 Das, M.L. and Crane, F.L. (1964) Biochemistry 3, 696—700
47 Cogdell, R.J., Brune, D.C. and Clayton, R.K. (1974) FEBS Lett. 45, 344—347
48 Okamura, M.Y., Isaacson, R.A. and Feher, G. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3491—3495
49 Burfield, D.R., Lee, K.H. and Smithers, R.K. (1977) J. Org. Chem. 42, 3060—3064
50 Clayton, R.K. (1978) Biochim. Biophys. Acta 504, 255—264
51 Steiner, L.A., Okamura, M.Y., Lopes, A.D., Moskowitz, E. and Feher, G. (1974) Biochemistry 13, 1403—1410
52 Feher, G. and Okamura, M.Y. (1977) Brookhaven Symp. Biol. 28, 183—193
53 Phillips. M.C. (1972) Prog. Surf. Memb. Sci. 5, 139—221
54 Kuhn, H., Moebius, D. and Bucher, G. (1972) in Techniques of Chemistry (Wassenberger, A. and Rossiter, B.W., eds.), Vol. 36, pp. 577—702, Wiley, New York
55 Trissl, H. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 207—210
56 Montal, M. (1974) in Perspectives in Membrane Biology Estrada, (S. and Gitler, C., eds.), pp. 591—622, Academic Press, New York