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THE ROLE OF THE INTRA-CYTOPLASMIC MEMBRANE SYSTEM IN
PHOTOSYNTHESIS AND DIFFERENTIATION IN
Rhodospirillum rubrum

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B.Sc. (Hons.) BATH

Thesis submitted for the degree of Doctor of Philosophy,
University of Warwick, Department of Biological Sciences.

NOVEMBER 1985

For my mother, father and nan.

"I don't like work - no man does - but I like what is in the work - the chance to find yourself. Your own reality - for yourself, not for others - what no other man can ever know. They can only see the mere show and can never tell what it really means".

Joseph Conrad, from
"A Flat Man" by Ivor Cutler
(Trigram, 1979)

CONTENTS

	Page
SUMMARY	i
LIST OF TABLES	ii
LIST OF FIGURES	iii
ACKNOWLEDGEMENTS	viii
DECLARATION	ix
ABBREVIATIONS	x
1. INTRODUCTION	1
Aims of the Project	2
1.1 An overview of the photosynthetic bacteria	3
1.1.1 The legacy of van Niel	3
1.1.2 Microbial photosynthesis	4
1.1.3 Taxonomy and phylogeny of the anoxygenic phototrophic bacteria	6
1.2 The Rhodospirillaceae	11
1.2.1 Metabolic flexibility and versatility	11
1.2.2 Physiological ecology	15
1.2.3 The structure of the cell envelope and membrane systems in the Rhodospirillaceae	18
1.2.3.1 Surface arrays and the outer membrane	18
1.2.3.2 The cell wall	21
1.2.3.3 Cytoplasmic and intra-cytoplasmic membrane systems	23
1.3 Mechanisms of energy capture and transduction	30
1.3.1 Organization, structure and composition of the photosynthetic apparatus	30
1.3.1.1 Light-harvesting	30
1.3.1.2 The photochemical reaction centre	36
1.3.1.3 Interactions between light-harvesting and reaction centre complexes	39
1.3.2 Photosynthetic and respiratory electron transport	42

	Page
1.3.3 Vectorial proton translocation and the synthesis of ATP	47
1.4 Development of the photosynthetic apparatus	51
1.4.1 The regulation of photopigment synthesis	51
1.4.2 Regulation of the assembly of the pigment-protein complexes	54
1.4.3 The development of the ICM system in batch cultures	59
1.4.4 Development of the ICM system in synchronized cultures.	62
1.5 Cellular differentiation and the role of the cell envelope	67
1.5.1 Concepts of morphogenesis and differentiation	67
1.5.2 The prosthecate and budding bacteria	70
1.5.2.1 Polar growth and cellular asymmetry	71
1.5.2.2 Intra-cytoplasmic membrane systems in polarly growing bacteria	72
1.5.2.3 The photosynthetic prosthecate and budding bacteria	74
1.5.2.4 Cell-type expression in <u>Rm. vanniellii</u>	77
1.5.3 The swarmer cell concept	81
1.6 Conclusions from the literature	85
2. MATERIALS AND METHODS	86
2.1 Organisms and media	87
2.2 Maintenance of cultures	89
2.3 Photoheterotrophic growth conditions	89
2.4 Chemoheterotrophic growth conditions	90
2.5 Chemoheterotrophic to photoheterotrophic growth shifts	91
2.6 Anaerobic dark growth of <u>Rm. vanniellii</u>	91
2.7 Culture harvesting	91

	Page
2.8 Preparation of synchronized swarmer cell populations	92
2.9 Preparation of multicellular arrays from batch cultures.	93
2.10 Cell-volume distribution analysis	94
2.11 Radioisotopic labelling of cells	94
2.12 Measurement of radioisotope incorporation into whole cells and subcelluar fractions	95
2.13 Preparation of cell-free extracts	95
2.14 Isolation of intra-cytoplasmic membranes	96
2.15 Enzyme assays	97
2.16 Measurement of oxygen uptake	100
2.17 Polyacrylamide gel electrophoresis of proteins	101
2.17.1 One dimensional completely denaturing 10-30% (w/v) polyacrylamide gradient gels	101
2.17.2 One dimensional completely denaturing uniform concentration gels	106
2.17.3 One dimensional incompletely denaturing uniform concentration gels	107
2.17.4 Two dimensional semi-denaturing gels	108
2.17.5 Two dimensional O'Farrell gels	109
2.18 Staining of polyacrylamide gels	113
2.18.1 Coomassie blue staining	113
2.18.2 Silver-staining	113
2.18.3 Staining gels for cytochromes	115
2.19 Photography of polyacrylamide gels	116
2.20 Autoradiography	116
2.21 Fluorography	116
2.22 Production of antisera	118
2.23 Immunoelectrophoresis	119
2.24 Protein immunoblotting	120
2.24.1 Preparation of iodinated protein A	120
2.24.2 Antigen transfer to nitrocellulose sheets	121
2.25 Isolation of flagella	122

	Page
2.26 Routine spectrophotometry	122
2.27 Isolation of photosynthetic pigment-protein complexes	123
2.27.1 Ion-exchange chromatography	123
2.27.2 Triton-SDS gel electrophoresis	123
2.27.3 Sucrose gradient centrifugation	124
2.28 Reversible chemical cross-linking	125
2.29 Penicillin binding protein assay	127
2.30 Preparation of proteoliposomes	128
2.31 Measurement of the proton-motive force by the redistribution of perm@ont ions	129
2.31.1 Construction of ion-selective electrodes for membrane potential and pH gradient detection	129
2.31.2 Calibration and use of the electrodes	132
2.32 Measurements of pH changes in cell suspensions	135
2.33 Measurement of membrane potential using the carotenoid bandshift	135
2.33.1 Slow time-scale measurements	135
2.33.2 Rapid electrochromic responses on a fast time-scale	136
2.34 Bacteriochlorophyll and carotenoid determination	137
2.35 Protein determination	137
2.36 Electron microscopy	138
2.37 Chemicals	138
3. RESULTS AND DISCUSSION	140
3.1 Isolation and characterization of the intracytoplasmic membranes of <u>Rm. yannielii</u>	141
3.1.1 Isolation on sucrose gradients	141
3.1.2 Polypeptide composition	145
3.1.3 Enzyme profile	153
3.1.4 Absorption spectra	155
3.1.5 Conclusions	157
3.1.6 Discussion	158

	Page
3.2 The photosynthetic pigment-protein complexes of <u>Rm. vannielii</u>	162
3.2.1 Identification of pigment-protein complexes	162
3.2.2 Absorption spectra and redox properties	169
3.2.3 Polypeptide composition of the pigment-protein complexes	175
3.2.4 Isolation of pigment-protein complexes by sucrose-gradient centrifugation and ion-exchange chromatography	181
3.2.5 Association of cytochrome with the B885-RC complex and ICM	187
3.2.6 Organization and topographical relationships of the components of the pigment-protein complexes	195
3.2.7 Reconstitution of a functional reaction centre complex from <u>Rm. vannielii</u>	202
3.2.8 Conclusions	208
3.2.9 Discussion	209
3.3 Regulation of synthesis of the photosynthetic apparatus in batch cultures	220
3.3.1 Phototrophic growth at different light intensities	220
3.3.2 Chemoheterotrophic growth under aerobic-dark conditions	227
3.3.3 Chemoheterotrophic to photoheterotrophic growth shift	237
3.3.4 Chemoheterotrophic growth under anaerobic conditions in the dark	240
3.3.5 Conclusions	241
3.3.6 Discussion	241
3.4 The role of the intra-cytoplasmic membrane system and cell envelope in the differentiation of <u>Rhodospirillum rubrum</u> swarmer cells	247
3.4.1 Cell-type and cell-cycle specific synthesis of intra-cytoplasmic membrane components	247
3.4.1.1 Results	247
3.4.1.2 Conclusions	272
3.4.1.2 Discussion	272
3.4.2 Bioenergetics of the cell-cycle	280
3.4.2.1 Results	280
3.4.2.2 Conclusions	294
3.4.2.3 Discussion	295

	Page
3.4.3 The involvement of the cell wall in swarmer cell differentiation - the detection of penicillin binding proteins in <u><i>Em. vanniellii</i></u>	300
3.4.3.1 Introduction	300
3.4.3.2 Results	300
3.4.3.3 Discussion	302
4. OVERALL CONCLUSIONS AND OUTLOOK	304
5. REFERENCES	309
6. LIST OF PUBLICATIONS	345

i

SUMMARY

Intra-cytoplasmic membranes were isolated from Rhodomicrobium vanniellii, a Bchl_a containing member of the Rhodospirillaceae which exhibits extensive cellular differentiation, in order to probe the composition and organization of the photosynthetic apparatus. Detergent solubilization of ICM followed by gel electrophoresis or sucrose gradient centrifugation led to the isolation of two native pigmented complexes, the major one (B800-865) identifiable with the "accessory" light-harvesting complex II found in other members of the Rhodospirillaceae. The minor complex (B885-RC) contained both reaction centre and light-harvesting Bchl and was unusual in containing a c-type cytochrome (c-553) bound to the 31,000 M_r subunit of the reaction centre. Bioenergetic studies of the B885-RC complex reconstituted into liposomes showed the c-553 to be an electron donor to the RC in vitro. These properties are somewhat similar to those of the Bchl_b containing Rp. viridis but unlike many Bchl_a containing Rhodospirillaceae.

The synthesis of the photosynthetic pigment protein complexes in vivo was found to be regulated by oxygen and light but the composition of the photosynthetic apparatus was the same in both swarmer cells and multicellular arrays.

However, ICM prepared from pulse-labelled swarmer cells undergoing selection synchronized growth and differentiation showed distinct changes in the pattern of proteins synthesised at different stages during the cell-cycle. During differentiation, the B885-RC pigment-protein complex was only synthesised during daughter cell formation when de novo production of new ICM is necessary. These observations were correlated with the obligate polar growth pattern of this microbe. The production of a 34,000 M_r protein identified as flagellin was also modulated during the cell-cycle, with synthesis being restricted to the swarmer cell. Surprisingly, however, few changes in penicillin-binding proteins (PBPs) in cell-free extracts could be detected during the cell-cycle but the pattern of PBPs in Rm. vanniellii was characterised by high M_r species unlike those of Rb. sphaeroides, the only other photosynthetic bacterium examined in this respect to date.

Bioenergetic studies concerning the cell-cycle were hampered by remarkable permeability properties that did not allow the use of probe ions for $\Delta\Psi$ and Δ pH. However, using the electrochromic response of the endogenous carotenoids an overall decrease in $\Delta\Psi$ during differentiation was observed but the reasons for this were unclear.

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Rhodomicrobium vannielii was considered to represent one extreme of a spectrum of behaviour in the Rhodospirillaceae, with regard to morphological and some physiological properties.

LIST OF TABLES

	Page
INTRODUCTION	
Table 1.1	5
Table 1.2	7
MATERIALS AND METHODS	
Table 2.1	88
RESULTS AND DISCUSSION	
Table 3.1	154
Table 3.2	156
Table 3.3	221
Table 3.4	228
Table 3.5	234
Table 3.6	238
Table 3.7	253
Table 3.8	269

LIST OF FIGURES

	Page
INTRODUCTION	
Figure 1.1	Intra-cytoplasmic membrane arrangements in the Rhodospirillales 24
Figure 1.2	Interactions in photosynthetic energy flow 31
Figure 1.3	The mechanism of charge separation and electron flow within the <u>Rb. sphaeroides</u> reaction centre 43
Figure 1.4	Redox potential diagram of respiratory and photosynthetic electron flow in <u>Rhodobacter</u> 45
Figure 1.5	Generalized cell-cycle in prosthecate and budding bacteria 73
Figure 1.6	A morphogenetic gradient of the cell-cycles of several prosthecate and budding bacteria 76
Figure 1.7	Cellular expression in <u>Rm. vanniellii</u> 78
MATERIALS AND METHODS	
Figure 2.1	Design of the electrodes used for measuring membrane potential and pH gradients 131
Figure 2.2	The electrode set-up for the measurement of proton-motive force parameters 133
Figure 2.3	Close-up of the electrode vessel 134
RESULTS AND DISCUSSION	
Figure 3.1	Isolation of intra-cytoplasmic membranes from phototrophically grown <u>Rm. vanniellii</u> 142
Figure 3.2	Isolation of intra-cytoplasmic membranes from chemoheterotrophically grown <u>Rm. vanniellii</u> 142
Figure 3.3	SDS-PAGE of sucrose gradient fractions 146
Figure 3.4	Effect of solubilization time and temperature on the intra-cytoplasmic membrane protein profile 148
Figure 3.5	Effect of reductant on the intra-cytoplasmic membrane protein profile at two different temperatures 149

	Page	
Figure 3.6	A comparison of the intra-cytoplasmic membrane protein profiles of some members of the Rhodospirillaceae	151
Figure 3.7	The effect of solubilization time and temperature on the polypeptide profile of the cell wall/outer membrane fraction	152
Figure 3.8	Solubilization of intra-cytoplasmic membranes using lithium dodecyl sulphate	163
Figure 3.9	Treatment of <u>Rm. vannielii</u> ICM with different detergents	167
Figure 3.10	Room temperature absorption spectra of pigment-protein complexes	170
Figure 3.11	Chemical oxidation of pigment-protein complexes	172
Figure 3.12	Detection of photosynthetic pigment-protein complexes in various species by SDS-Triton gel electrophoresis	174
Figure 3.13	Polypeptide composition of pigment-protein complexes	176
Figure 3.14	Two-dimensional gel electrophoresis of the B885-RC complex	178
Figure 3.15	Two-dimensional gel electrophoresis of the B800-865 complex	178
Figure 3.16	Two-dimensional gel electrophoresis of <u>Rm. vannielii</u> ICM	180
Figure 3.17	Isolation of pigment-protein complexes on sucrose gradients	182
Figure 3.18	Absorption spectrum of the yellow pigmented fraction from sucrose gradients used to isolate pigment-protein complexes	184
Figure 3.19	SDS-PAGE of samples from sucrose gradients used to isolate pigment-protein complexes	185
Figure 3.20	SDS-PAGE of samples from ion-exchange chromatography of pigment-protein complexes	186
Figure 3.21	Detection of cytochrome by haem-associated peroxidase activity staining	188
Figure 3.22	Dithionite reduced minus air oxidized difference spectrum of the B885-RC complex	190

	Page	
Figure 3.23	Dithionite reduced minus air oxidized difference spectra of <u>Rm. vannieli</u> subcellular fractions	191
Figure 3.24	Difference spectra of <u>Rm. vannieli</u> photoheterotrophic cell-free extracts using different reductants	192
Figure 3.25	Cytochrome composition of the ICM from selected members of the Rhodospirillaceae	194
Figure 3.26	Two-dimensional gel electrophoresis of <u>Rm. vannieli</u> pigment-protein complexes	196
Figure 3.27	Two-dimensional gel analysis of reversible chemical cross-linking patterns of <u>Rm. vannieli</u> ICM and the B885-RC complex	198
Figure 3.28	Detection of cytochrome in the cross-linked B885-RC complex	201
Figure 3.29	Reactions involved in the generation of a proton-motive force from a reconstituted reaction centre - proteoliposome	203
Figure 3.30	Generation of a membrane potential by liposomes containing the B885-RC complex	204
Figure 3.31	Generation of a membrane potential by liposomes reconstituted with ICM fragments or by ICM alone	207
Figure 3.32	Proposed organization of the components of the photosynthetic apparatus in <u>Rm. vannieli</u>	217
Figure 3.33	Absorption spectra of cell-free extracts of <u>Rm. vannieli</u> grown at different light intensities	222
Figure 3.34	Absorption spectra of cell-free extracts of <u>Rd. palustris</u> grown at different light intensities	223
Figure 3.35	Separation of native pigment-protein complexes from membranes of <u>Rm. vannieli</u> grown at different light intensities	225
Figure 3.36	ICM polypeptide profiles from <u>Rm. vannieli</u> grown at different light intensities	226
Figure 3.37	Morphology of <u>Rm. vannieli</u> grown under aerobic-dark conditions	229
Figure 3.38	Absorption spectra of ICM from <u>Rm. vannieli</u> grown under aerobic-dark conditions	230

	Page	
Figure 3.39	SDS-PAGE of soluble and ICM proteins from photoheterotrophically and chemoheterotrophically grown <u>Rm. vanniellii</u>	232
Figure 3.40	Oxygen uptake by whole cells of photoheterotrophically grown <u>Rm. vanniellii</u>	235
Figure 3.41	Changes in ICM polypeptide profiles during a chemoheterotrophic to photoheterotrophic growth shift	239
Figure 3.42	Incorporation of ³⁵ S-methionine into cell protein of <u>Rm. vanniellii</u>	248
Figure 3.43	Electron micrographs of <u>Rm. vanniellii</u> swarmer cells, stalked cells and multicellular arrays	250
Figure 3.44	Cell volume distribution analysis of <u>Rm. vanniellii</u> cell-types	252
Figure 3.45	Cell-type specific intra-cytoplasmic membrane protein synthesis in <u>Rm. vanniellii</u>	255
Figure 3.46	Detection of flagellin in ICM by immunoblotting	257
Figure 3.47	Changes in cell volume, optical density and cellular pigment and protein content in differentiating swarmer cells	261
Figure 3.48	Changes associated with the intra-cytoplasmic membrane system during the differentiation of <u>Rm. vanniellii</u> swarmer cells	263
Figure 3.49	SDS-PAGE of ICM from differentiating swarmer cells	265
Figure 3.50	ICM proteins synthesised by differentiating swarmer cell populations	266
Figure 3.51	Incorporation of label into the B885-RC complex during swarmer cell differentiation	267
Figure 3.52	Summary of the period of synthesis of ICM proteins during swarmer cell differentiation	271
Figure 3.53	Measurement of membrane potential in intact cells of <u>Rb. sphaeroides</u> and <u>Rm. vanniellii</u>	282
Figure 3.54	Measurement of the cellular pH gradient in <u>Rb. sphaeroides</u> and <u>Rm. vanniellii</u>	284
Figure 3.55	Light-induced proton translocation in intact cells of <u>Rm. vanniellii</u> monitored with a pH electrode	286
Figure 3.56	The effect of various energy transfer inhibitors on the development of the carotenoid bandshift	288

	Page
Figure 3.57 Slow time-scale changes in the carotenoid bandshift observed during swarmer cell differentiation	289
Figure 3.58 Rapid time-scale changes in the carotenoid bandshift observed during swarmer cell differentiation	292
Figure 3.59 Penicillin binding proteins in cell-free extracts of <u>Rm. vannielii</u>	301

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DECLARATION

I hereby declare that the work described in this thesis was conducted by myself, under the supervision of Dr. C. S. Dow, with the exception of those instances where the contribution of others has been specifically acknowledged.

None of the information contained herein has been used in any previous application for a degree.

All sources of information have been specifically acknowledged by means of reference.



David J. Kelly

ABBREVIATIONS

ICM	Intra-cytoplasmic membrane
OM	Outer membrane
LPS	Lipopolysaccharide
Bchl	Bacteriochlorophyll
LDAO	Lauryldimethylamine-N-oxide
DMSO	Dimethylsulfoxide
TMAO	Trimethylamine-N-oxide
DTSP	Dithithiobis (succinimidyl propionate)
TMBZ	3,3',5,5'-tetramethylbenzidine
LH	Light-harvesting
RC	Reaction centre
M_r	Molecular ratio
IR	Infra-red
pI	Isoelectric point
Em	Midpoint potential
$\Delta\psi$	Membrane potential
ΔpH	pH gradient
Δp	Proton-motive force
TPP ⁺	The tetraphenylphosphonium cation
TPB ⁻	The tetraphenylborate anion
UQ ₀	2,3-dimethoxy-5-methyl-1,4-benzoquinone
CD	Circular dichroism
NMR	Nuclear magnetic resonance
EPR	Electron paramagnetic resonance
CCCP	Carbonylcyanide-m-chlorophenylhydrazone
FCCP	Carbonylcyanide-p-trifluoromethoxyphenylhydrazone

NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
PBP	Penicillin binding protein
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
LDS	Lithium dodecyl sulphate
EDTA	Ethylenediamine tetra-acetic acid
PBS	Phosphate buffered saline
PPO	2,5-diphenyloxazole
Tris	tris(hydroxymethyl) amino methane
ATP	Adenosine 5' triphosphate
BSA	Bovine serum albumin
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
TCA cycle	Tricarboxylic acid cycle
TEMED	N,N,N',N'-tetramethylethylenediamine
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume
A	absorbance
IEF	iso-electric focussing
R	The gas constant
T	Absolute temperature
F	The Faraday constant

1. INTRODUCTION

The Aims of the Project

The major aims of the present study were threefold. (i) To characterize the photosynthetic apparatus of Rm. vannieli as an example of a member of the Rhodospirillaceae with a lamellate ICM system. (ii) To investigate relationships between the synthesis of membrane components and the events of swarmer cell differentiation. (iii) To study aspects of the growth and physiology of this microbe in relationship to photosynthesis and respiration. These aims were undertaken bearing in mind the paucity of information currently available concerning the physiology of polarly growing members of the Rhodospirillaceae, as reviewed below.

1.1 An Overview of the Photosynthetic Bacteria

1.1.1 The legacy of van Niel

"It must, however, be recognized that in nature the conditions are seldom simple. Hence we must learn to study more carefully the effects of complicating circumstances ... This will require much imaginative work and the correlation of many kinds of observations".

C. B. van Niel (1955)

Towards the end of the nineteenth century a technique was discovered which had a profound influence on the development of microbiology; that of the elective enrichment culture. Pioneered by Beijerinck in Delft and Winogradsky in Paris, this method allowed the isolation in pure culture of a wide range of bacteria which were able to utilize light as an energy source for growth. Nevertheless, the importance of the photosynthetic bacteria was generally under-estimated until well into the 1920s. This was at least partly due to the "awkward" observation that these microbes did not produce molecular oxygen during photosynthesis as green plants do - a property which was not in harmony with the idea of the "unity of biochemical processes" prevalent at this time (Gest, 1982). It was van Niel's pioneering studies on the physiology of the purple sulphur bacteria during the 1930s which provided an explanation for this apparent anomaly. He suggested that reductants other than water were used by these microbes in order to assimilate CO_2 into cell material, particularly H_2S , H_2 and simple organic compounds. Later the theory was modified to include a light-driven dehydrogenation of water but in a special way that did not

4

involve oxygen evolution. Although the details are now known to be untenable, this theory provided a conceptual framework for experiments for many years (Gest, 1982).

The strains of photosynthetic bacteria obtained in pure culture by enrichment techniques up to about 1945 were largely rods and cocci - "conventional characters, deprived of all the couleur locale" - as Winogradsky put it (Pfennig, 1984). Van Niel's advocacy of the enrichment culture technique as a tool in experimental microbial ecology (van Niel, 1944, 1955) stimulated the search for more unusual forms. One such example is the subject of this thesis, Rhodomicrobium vannielii (Duchow & Douglas, 1949).

1.1.2 Microbial photosynthesis

Prokaryotes capable of satisfying all or a part of their cellular energy demands from light are the cyanobacteria, the purple and green eubacteria and the halobacteria, which are a group within the archaeobacteria (Fox et al., 1980). These groups represent the three broad patterns of microbial photosynthesis known to date (Table 1.1) based on chlorophyll, bacteriochlorophyll (Bchl) and bacteriorhodopsin, respectively. It could be argued that the halobacteria are not true phototrophs because their growth cannot occur solely at the expense of light energy, yet light stimulated increases in growth yield clearly indicate the truly photosynthetic nature of their metabolism. Interestingly, oxygen tension appears to be a controlling factor in the synthesis of bacteriorhodopsin (Oesterhelt & Krippahl, 1982) just as it is in controlling bacteriochlorophyll levels in the purple eubacteria

TABLE 1.1 PATTERNS OF MICROBIAL PHOTOSYNTHESIS

Group	Type of Photosynthesis	Pigment associated with the primary photoact	Electron donors	Products	C-Source
Eubacteria	Anoxygenic	Bacteriochlorophyll	H ₂ , H ₂ S, S, organic compounds	ATP + NAD(P)H	CO ₂ + organics
	Oxygenic	Chlorophyll	H ₂ O (H ₂ S) ¹	ATP + NAD(P)H	CO ₂ organics ³
Archaeobacteria	Halobacterial	Bacteriorhodopsin	-2	ATP	organics

1 - only certain Cyanobacteria, e.g. Oscillatoria limnetica under aerobic conditions

2 - electron donors do not participate

3 - some cyanobacteria can grow photoheterotrophically

(section 1.4.1).

The isolation of microbes such as Erythro bacter (Shiba & Simidu, 1982) in which increased oxygen levels stimulate Bchl synthesis, may, however, indicate much greater diversity in the pattern of photosynthesis and its control than is suggested in table 1.1. Nevertheless, in each type of photosynthetic process, light energy is primarily converted to a proton-motive force which is usable in ATP synthesis. With the exception of the archaebacteria, reducing power to drive the synthesis of cell material from CO₂ (a widely used carbon source) can also be produced by photosynthetic electron flow.

In the following sections, I deal with the microbes and processes associated with one type of anoxygenic photosynthesis. A broader account of microbial photosynthesis in toto can be found in a recent review (Kelly & Dow, 1985a).

1.1.3 Taxonomy and phylogeny of the anoxygenic phototrophic bacteria

As originally defined (Gibbons & Murray, 1978) the anoxyphotobacteria comprised two orders; the Rhodospirillales containing the families Rhodospirillaceae (the purple non-sulphur bacteria) and Chromatiaceae (the purple sulphur bacteria) and the Chlorobiales which contained the Chlorobiaceae (green sulphur bacteria) and Chloroflexaceae (green gliding bacteria). These two orders are well defined with respect to fine structure and photosynthetic pigments (Table 1.2). In the Rhodospirillales, the major photosynthetic pigments are Bchl_a or _b which are contained in the cytoplasmic membrane or intra-cytoplasmic membranes

TABLE 1.2 CHARACTERISTICS OF THE ANOXYPHOTOBACTERIA

Groups	DNA G + C moles %	Motility	Membrane Glycolipids	Photosynthetic Pigments	Arrangement of Photosynthetic Membranes
ANOXYPHOTOBACTERIA					
RHODOSPIRILLALES					
Rhodospirillaceae	61-70	+	None	Bchl <u>a</u> or <u>b</u>	Vesicular, lamellate, stacks
Chromatiaceae	45-70	+	None	Bchl <u>a</u> or <u>b</u>	Usually vesicular
CHLOROBIALES					
Chlorobiaceae	48-58	-	Monogalactosyl-	Bchl <u>a+c, d, e</u>	Chlorosomes
Chloroflexaceae	53-55	Gliding	Monogalactosyl-	Bchl <u>a+c, d</u>	Chlorosomes

The light harvesting Bchl c, d, and e of the Chlorobiales is located in non-unit membrane bound chlorosomes which may consist of a galactolipid monolayer.

(ICM) derived from it. In the Chlorobiales, the light harvesting Bchl c d or e is contained within specialized structures (chlorosomes) attached to, but not continuous with, the cytoplasmic membrane.

With the advent of modern molecular sequencing methods (Fox et al., 1980), it has become clear that this taxonomic arrangement, based primarily on morphological properties, does not accurately reflect phylogenetic relationships amongst the phototrophic bacteria.

Oligonucleotide sequencing of 16S rRNA from over 400 bacteria has now been performed (Stackebrandt & Woese, 1984) and this shows that the bacteria fall into two basic groups - the archaeobacteria and eubacteria. Within the eubacteria about 10 major subgroups can be distinguished which represent separate lines of descent. The striking finding with regard to the photosynthetic prokaryotes is that the green bacteria Chlorobium and Chloroflexus are representatives of two separate subgroups and that the "purple photosynthetic bacteria" form a major phylogenetic unit, encompassing many non-photosynthetic Gram negative species (Gibson et al., 1979).

Relationships amongst the purple photosynthetic bacteria have been examined in some detail (Gibson et al., 1979; Fowler et al., 1984). The species studied thus far fall into three major phylogenetic groups, two of which contain members of the purple non-sulphur bacteria and one which contains the purple sulphur bacteria. Members of the Chromatiaceae (Chromatium, Amoebobacter, Lamprocystis, Thiocapsa, Thiocystis, Thiodictyon and Thiospirillum) form a phylogenetically coherent grouping although the species investigated do not in each case cluster according to their present classification (Fowler et al., 1984). The genus Ectothiorhodospira which is distinct from the Chromatiaceae in its external deposition of sulphur during sulphide oxidation has been

accommodated in a new family, the Ectothiorhodospiraceae (Imhoff, 1984).

The two groups of the purple non-sulphur bacteria each contain three sub-groups. In each case certain genera of non-photosynthetic bacteria show specific relationships to some of the photosynthetic species. Some examples are Rhodopseudomonas capsulata and Paracoccus denitrificans in group Ia, Rhodopseudomonas palustris and Nitrobacter winogradskyi in group Ib and Rhodopseudomonas gelatinosa and Sphaerotilus natans in group IIa (Gibson et al., 1979; Seewaldt et al., 1982). This suggests that a number of non-photosynthetic Gram negative bacteria have arisen from a photosynthetic ancestry (Stackebrandt & Woese, 1984) and that the family Rhodospirillaceae as presently defined (Pfennig & Truper, 1974) is heterogeneous.

In order to take account of this new evidence, Imhoff et al., (1984) have proposed a rearrangement of the species and genera of the purple non-sulphur bacteria. In their proposal, all species with vesicular intracytoplasmic membranes are removed from the genus Rhodopseudomonas and placed into two new genera; Rhodobacter, containing Rhodobacter capsulatus, Rb. sphaeroides, Rb. sulfidophilus (Hansen & Veldkamp, 1973), Rb. adriaticus (Neutzling et al., 1984) and Rb. veldkampii (Hansen & Imhoff, 1985); and Rhodopila, for Rhodopseudomonas globiformis. In addition, Rhodospirillum tenue, Rhodopseudomonas gelatinosa and Rhodocyclus purpureus are all united in the genus Rhodocyclus. The genus name Rhodopseudomonas is retained for those species, like the type species Rp. palustris, which have a lamellate intracytoplasmic membrane system adjacent and parallel to the cytoplasmic membrane and which exhibit polar growth. The genus Rhodomicrobium, which appears most closely related to Rp. viridis (Gibson

i

SUMMARY

Intra-cytoplasmic membranes were isolated from Rhodospirillum vannielii, a Bchl_a containing member of the Rhodospirillaceae which exhibits extensive cellular differentiation, in order to probe the composition and organization of the photosynthetic apparatus. Detergent solubilization of ICM followed by gel electrophoresis or sucrose gradient centrifugation led to the isolation of two native pigmented complexes, the major one (B800-865) identifiable with the "accessory" light-harvesting complex II found in other members of the Rhodospirillaceae. The minor complex (B885-RC) contained both reaction centre and light-harvesting Bchl and was unusual in containing a c-type cytochrome (c-553) bound to the 31,000 M_r subunit of the reaction centre. Bioenergetic studies of the B885-RC complex reconstituted into liposomes showed the c-553 to be an electron donor to the RC *in vitro*. These properties are somewhat similar to those of the Bchl_b containing Rd. viridis but unlike many Bchl_a containing Rhodospirillaceae.

The synthesis of the photosynthetic pigment protein complexes *in vivo* was found to be regulated by oxygen and light but the composition of the photosynthetic apparatus was the same in both swarmer cells and multicellular arrays.

However, ICM prepared from pulse-labelled swarmer cells undergoing selection synchronized growth and differentiation showed distinct changes in the pattern of proteins synthesised at different stages during the cell-cycle. During differentiation, the B885-RC pigment-protein complex was only synthesised during daughter cell formation when *de novo* production of new ICM is necessary. These observations were correlated with the obligate polar growth pattern of this microbe. The production of a 34,000 M_r protein identified as flagellin was also modulated during the cell-cycle, with synthesis being restricted to the swarmer cell. Surprisingly, however, few changes in penicillin-binding proteins (PBPs) in cell-free extracts could be detected during the cell-cycle but the pattern of PBPs in Rm. vannielii was characterised by high M_r species unlike those of Rb. sphaeroides, the only other photosynthetic bacterium examined in this respect to date.

Bioenergetic studies concerning the cell-cycle were hampered by remarkable permeability properties that did not allow the use of probe ions for $\Delta\psi$ and ΔpH . However, using the electrochromic response of the endogenous carotenoids an overall decrease in $\Delta\psi$ during differentiation was observed but the reasons for this were unclear.

In terms of its overt phenotypic properties, Rm. vanniellii appears to represent an extreme case; it expresses the most complex and diverse cell-cycles and cell-types but nevertheless exhibits some degree of "metabolic inflexibility". Moreover, there appears to be a general correlation between increasing morphological complexity and decreasing metabolic flexibility through the sequence: Rb. capsulatus/Rb. sphaeroides - Rp. blastica - Rp. acidophila - Rp. palustris - Rp. viridis - Rm. vanniellii. A specific example of this concerns the sensitivity to oxygen and the capacity for aerobic growth in the dark. Rhodopseudomonas viridis shows many of the same features as Rm. vanniellii in this respect (Pfennig, 1978) but contrasts with Rb. capsulatus and Rp. blastica, the latter being able to grow faster under aerobic-dark conditions (Eckersely & Dow, 1980) in addition to being remarkably insensitive to the potentially lethal effects of light and O_2 .

As far as it is possible to make conclusions, two other properties of particular concern in this study - the structure of the photosynthetic apparatus and whole cell permeability also change according to a spectrum of behaviour in the Rhodospirillaceae. The change from vesicular to lamellate ICM arrangements has been well documented previously and correlates with the apparent change in growth mode in the "budding" species. The concomitant appearance of the use of membrane bound c -type cytochromes, in particular in Rp. viridis and Rm. vanniellii, may be related to this and might merit further investigation

et al., 1979) is also retained.

Although this system is more in line with the rRNA and cytochrome c (Ambler et al., 1979a,b) sequence data, the genus Rhodospirillum remains heterogeneous and it is clear that more data is needed before a final classification that reflects the true phylogeny can be reached. Nevertheless, henceforth in this thesis, the scheme of Imhoff et al. (1984) will be adhered to.

1.2. The Rhodospirillaceae

1.2.1 Metabolic flexibility and versatility

The Rhodospirillaceae is the most physiologically diverse and best studied group of the anoxyphotobacteria. Many of its members can grow under a wide variety of growth conditions, demonstrating a high degree of metabolic flexibility (Fuller, 1978; Madigan & Gest, 1979). The predominant growth mode is that of photoheterotrophy where organic compounds are assimilated into cell carbon using light as the energy source. Under these conditions, and in most cases, the organic compound also acts as an electron donor to the cyclic photophosphorylation system, and/or to provide electrons for pyridine nucleotide reduction (Gest, 1982). The range of carbon sources usable for such purposes is large; fatty acids, organic acids, amino acids, alcohols, sugars, aromatic and C₁ compounds have been reported to support the growth of various species in the group (van Niel, 1944; Leadbetter & Hawk, 1965; Quayle & Pfennig, 1975; Sojka, 1978; Dutton & Evans, 1978). This versatility is consistent with the presence of an active tricarboxylic acid cycle, combined with a set of inducible catabolic pathways (Sojka, 1978) and this has been confirmed in several species (Ormerod & Gest, 1962; Fuller, 1978). The role of the TCA cycle under photoheterotrophic conditions is primarily to supply biosynthetic intermediates - for example amino acid precursors, and to generate the succinyl-Co-A needed for the synthesis of bacteriochlorophyll and cytochromes (Beatty & Gest, 1981a). In Rhodospirillum rubrum (Anderson & Fuller, 1967), Rhodopseudomonas palustris (Chernyad'ev et al., 1969) and Rhodobacter capsulatus (Beatty & Gest, 1981b) a complete TCA cycle is present under

anaerobic conditions but with a generally low specific activity of 2-oxoglutarate dehydrogenase (Cox et al., 1982). This activity increases dramatically under aerobic conditions in the dark, for example, where the TCA cycle assumes importance as the major route of substrate oxidation for respiratory electron transport to O₂ (Cox et al., 1983).

This type of chemoheterotrophic metabolism is a typical feature of many species of the Rhodospirillaceae, although some, for example the "brown" species of Rhodospirillum (Pfennig, 1978), show a sensitivity to oxygen that only allows microaerophilic growth. It contrasts with the Chromatiaceae where a much narrower carbon source utilization spectrum coupled with the presence of an incomplete TCA cycle has been observed (Fuller, 1978).

Another type of chemoheterotrophy can occur in certain species under anaerobic conditions in the dark with a "fermentable" carbon source. Uffen & Wolfe (1970) first observed anaerobic dark growth of purple non sulphur bacteria (Rs. rubrum, Rd. viridis, Rd. palustris, Rb. sphaeroides) using a modification of the Hungate technique to maintain strict anaerobiosis. Yen & Marrs (1977) and Madigan & Gest (1978) found that growth yields of Rb. capsulatus were markedly increased if electron acceptors such as dimethylsulfoxide (DMSO) or trimethylamine-N-oxide (TMAO) were included in media with fermentable sugars as carbon sources. Initial studies (Madigan et al., 1980; Cox et al., 1980) suggested fermentation as the sole mechanism for ATP production with the electron acceptors required as a "sink" for the management of redox balance. More recent evidence strongly suggests that electron-flow to DMSO or TMAO can generate a membrane potential (McEwan et al., 1983) which is

uncoupler and rotenone sensitive, indicating proton translocation which in principle could be coupled to ATP synthesis by electron transport phosphorylation.

The photoautotrophic growth of members of the Rhodospirillaceae, using CO_2 as sole carbon source and H_2 as the electron donor was first observed by Gaffron (1935). Under these conditions CO_2 is fixed by the enzyme ribulose biphosphate carboxylase-oxygenase (RubisCO) as the first step in the reductive pentose phosphate, or Calvin Cycle. This series of reactions results in the production of a C-3 compound from CO_2 according to the overall stoichiometry:



and is dependent upon a continued supply of reducing power and ATP from photosynthetic electron flow. The C-3 compound can "feed in" to the TCA cycle to produce biosynthetic intermediates. The properties and regulation of Calvin cycle enzymes, particularly RubisCO and phosphoribulokinase have been the subject of an increasing number of studies in recent years (Dijkhuizen & Harder, 1984). The highest specific activities of these enzymes are present under conditions where CO_2 is the sole carbon source (Slater & Morris, 1973); under photoheterotrophic conditions with malate as carbon source, for example, the specific activity is decreased (Tabita, 1981) but with butyrate - a reduced substrate where CO_2 is necessary for its assimilation - intermediate activities of RubisCO are found. This type of control has been observed in *Rb. sphaeroides*, *Rb. capsulatus* (Tabita *et al.*, 1983) and *Rp. blastica* (Sani *et al.*, 1983; A. Sani, personal communication). In these bacteria two molecular forms of RubisCO are present (M_r approx. 550,000 and 300,000) the specific activities of which are independently controlled. This enzyme duality may represent another level of

regulation of CO₂ fixation (Dijkhuizen & Harder, 1984). The reactions of the Calvin cycle are similar in photosynthetic bacteria, green plants and cyanobacteria but in certain species of the latter group (Pelroy & Bassham, 1972) some of the enzymatic machinery of the cycle is also used for the dissimilation of organic compounds in the dark, using an oxidative pathway in which NADP specific dehydrogenases generate reducing power for respiratory electron transport (Stanier & Cohen-Bazire, 1977). This makes an interesting contrast to the Rhodospirillaceae and may be due to the lack of a complete TCA cycle in the cyanobacteria. The green sulphur bacteria appear to use a reductive carboxylic acid cycle for CO₂ fixation and not the Calvin cycle (Evans *et al.*, 1966).

Remarkably, at least one species of the Rhodospirillaceae, Rb. capsulatus, has been shown to grow chemoautotrophically under aerobic conditions in darkness, with CO₂ as the carbon source and H₂ as electron donor (Madigan & Gest, 1979). Unusually high contents of Bchl and intracytoplasmic membranes were observed in such cells - components normally subject to repression by oxygen under chemoheterotrophic conditions (see section 1.4.1). Madigan & Gest (1979) drew attention to the extremely flexible metabolism of Rb. capsulatus, which allows it to assimilate carbon under all of the conditions of light and oxygen discussed above.

Two other examples of metabolic flexibility within the Rhodospirillaceae are to be found in the processes associated with nitrogen and sulphur metabolism. Nitrogen fixation is a widespread and general property of all the species within the family (Madigan & Gest, 1982). All species can also use NH₄⁺ and the enzymology associated with its assimilation

has been studied (Brown & Herbert, 1977). In addition, recent evidence suggests assimilatory nitrate reduction to be a widespread property in the group (Klemme, 1979; Jackson *et al.*, 1981). The use of reduced sulphur compounds as electron donors by the "purple non-sulphur bacteria" (an obvious misnomer) was highlighted by Hansen & van Gemerden (1972) and several species are now known which are actually dependent on such compounds (Neutzling *et al.*, 1985).

The flexibility and versatility of the Rhodospirillaceae as a group is without parallel amongst phototrophic prokaryotes. Part of the reason for this may be their complex electron transport chains, allowing the use of a plethora of donor and acceptor molecules. In addition, a large number of metabolic "sliproads" leading to the major "highway" of metabolism, the TCA cycle, appears to be a widespread feature.

1.2.2 Physiological ecology

Although the metabolic patterns exhibited by the anoxyphotobacteria to a large extent determine their natural distribution and activities, factors such as the availability of a sufficiently low redox potential to allow photosynthesis, light quality and quantity and the presence of suitable electron donors and carbon sources will all influence the types of photosynthetic bacteria able to exploit particular ecosystems. In addition, features such as the microbes "competitiveness" and its ability to survive periods of nutrient starvation, for example, may be the deciding factors which determine either multiplication or death in a particular environment (Pfennig, 1984).

The "classical" environs of the anoxygenic phototrophic bacteria are the

anaerobic muds of shallow ponds and the chemocline in stratified lakes where anaerobic conditions, established as a result of the activities of heterotrophic bacteria, allow sufficient accumulation of organic compounds to act as electron donors and/or carbon sources. Indeed, members of the Rhodospirillaceae in particular may be dependent on these activities, since with the exception of Rc. gelatinosus, they characteristically lack the ability to degrade organic polymers such as starch, cellulose or protein (Pfennig & Truper, 1974). Pfennig (1978) commented upon the association of non-photosynthetic chemoheterotrophs with members of the Rhodospirillaceae, and this may be a reflection of such dependence. Nevertheless, although the Rhodospirillaceae rarely reach the high cell densities characteristic of the "blooms" of the purple and green sulphur bacteria (Pfennig, 1977, 1978) their opportunistic use of a range of carbon sources and ability to grow under a variety of conditions might explain their rather ubiquitous distribution in habitats as diverse as antarctic sediments (Herbert, 1976) and salt-rich ponds (Drews, 1981). In contrast, the purple sulphur groups (Chromatiaceae and Ectothiorhodospiraceae) show evidence of a natural distribution that is related to the presence of reduced sulphur compounds, particularly sulphide (Truper, 1978). Many species also lack an assimilatory sulphur metabolism and so sulphide must act as both electron donor and sulphur source (Pfennig, 1978).

The light climate is clearly an important environmental factor and photosynthetic bacteria have evolved a number of mechanisms to maximize the amount of light absorbed from a given environmental light flux density.

One strategy is the absorption of wavelengths that are not used by other

phototrophs. Another mechanism is to alter the composition of the light-harvesting machinery to compensate for variation in light quality. This is a well known phenomenon in some cyanobacteria (Cohen-Bazire & Bryant, 1982) where chromatic adaptation allows modification of the phycoerythrin/phyocyanin composition of the phycobilisomes according to the incident light wavelengths. Recently an analagous process, though much less pronounced, was reported in Rb. sphaeroides (Hellingwerf *et al.*, 1982). Continuous cultures at different wavelengths of light were used to determine an action spectrum of specific growth rate *vs.* wavelength. Maxima were observed at 480, 600 and 800 + 870 nm, which correlated with peaks in the cellular absorption spectrum. However, at comparable growth rates at different wavelengths, the relative peak heights in the absorption spectrum varied, indicating a change in the photopigment content and composition. This study also demonstrated the utilization of light wavelengths above 800 nm, which was thought to be ecologically significant, because light of >600 nm penetrates well in shallow waters (Pfennig, 1967), the natural habitat of Rb. sphaeroides.

An important and neglected aspect of the ecology of the Rhodospirillaceae is their capacity for variation in cell-type expression which is related to survival and dispersal in aquatic environments (Dow & Whittenbury, 1980; Dow *et al.*, 1983). This is discussed in detail in section 1.5 but as a comparison it is interesting to note that conceptually similar cells are also formed in some species of the Chromatiaceae. Under conditions of high light and high sulphide concentrations, species such as Chromatium, Thiospirillum and Lamprocyctis form blooms consisting of large colonies of cells attached by slime. Upon a decrease in sulphide concentration and light intensity the aggregates disperse forming motile cells which show "swarming

behaviour" and diurnal movements in response to local changes in sulphide concentration (Pfennig, 1978).

1.2.3 The structure of the cell envelope and membrane systems in the Rhodospirillaceae

1.2.3.1 Surface arrays and the outer membrane. Ultrastructurally, all photosynthetic eubacteria are Gram negative (Pfennig & Truper, 1974). They have an electron dense cell-wall (3-8 nm) together with an outer membrane (7-8 nm) that appears triple-layered in electron micrographs (Weckesser *et al.*, 1972; Drews *et al.*, 1978). Outside the outer-membrane of some species there lies a regularly arranged layer of particles composed of protein (Glauert & Thornley, 1969) and this has recently been isolated from *Rs. salexigens* (Evers *et al.*, 1984). In this species, a single protein of M_r 68,000 was identified as the sole component of the surface array by both cell surface iodination and sucrose wash treatment, followed by gel electrophoresis. Certain species such as *Rb. capsulatus* (Weaver *et al.*, 1975) also form polysaccharide capsule and slime layers, external to the outer membrane, which can be visualized electron microscopically using specific antisera (Omar *et al.*, 1983). Relatively little is known about the composition and function of the various outer layers, however.

In recent years, more information has become available on the outer membrane in photosynthetic bacteria. Early studies involving separation of the inner and outer membrane fractions of *Rs. rubrum* (Oelze *et al.*, 1975; Collins & Niederman, 1976) and *Rb. sphaeroides* (Guillotín & Reiss-Husson, 1975; Ding & Kaplan, 1976) were performed with

chemoheterotrophically grown cells. These investigations employed either French press prepared cell free extracts or mechanically disrupted sphaeroplasts prepared by lysosyme-EDTA treatment, followed by sucrose density gradient centrifugation. Fractions were obtained which had a density of $1.20-1.25 \text{ g cm}^{-3}$ and which were enriched in carbohydrate (Collins & Niederman, 1976) and the lipopolysaccharide component 2-keto-3-deoxyoctonate (KDO; Ding & Kaplan, 1976). Cytochromes and succinate dehydrogenase activity were absent from such fractions.

The protein profile of the outer membrane fraction of Rs. rubrum showed relatively few, but highly abundant species upon denaturing gel electrophoresis in the presence of SDS (Collins & Niederman, 1976). This contrasted with that of the cytoplasmic membrane which was much more diverse with respect to protein composition. The protein composition of the Rb. sphaeroides outer membrane was characterised in detail by Baumgardner et al. (1980). They found a major protein of M_r 47,000 which formed three hetero-oligomers of M_r 68,000, 72,000 and 75,000 with polypeptides of M_r 21,500, 26,500 and 27,000 respectively, when membranes were not heated prior to SDS-gel electrophoresis. Upon heating to 75°C or above, the aggregates dissociated to give the 47,000 M_r common "subunit" and the lower M_r polypeptides. Supportive immunochemical evidence of the role of the M_r 47,000 polypeptide was provided by Deal and Kaplan (1983a) and it was characterized as a proteolipid containing at least 1 mole fatty acid per mole of protein in an amide linkage to the N-terminal L-alanine of the protein (Deal & Kaplan, 1983b). This protein was also isolated by Weckesser et al. (1984) from saline extracts of whole cells and it exhibited strong porin activity when reconstituted into proteoliposomes. From the swelling

rates of the liposomes with various sugars it was concluded that the pores approximated to hollow cylinders of 0.62 nm radius. In the hands of Weckesser *et al.* (1984) the porin existed as a homo-oligomer of 68,000 M_r which dissociated upon EDTA or heat treatment to the M_r 47,000 form only, with no evidence of lower M_r polypeptides associated with it. This type of behaviour is a well known feature of the porins of other Gram negative bacteria (Lugtenberg & van Alphen, 1983; Nikaido & Vaara, 1985) and despite the uncertainties regarding its oligomeric relationships, the evidence would suggest the M_r 47,000 protein to be the major porin of Rb. sphaeroides.

Of the other outer membrane proteins studied in Rb. sphaeroides, the 21,500 M_r species was thought to be associated with peptidoglycan, as evidenced by alteration of electrophoretic mobility after lysosyme treatment and an 8-10,000 M_r lipoprotein which may be equivalent to the Braun lipoprotein of Escherichia coli was also observed (Baumgardner *et al.*, 1980).

Similar studies of Rb. capsulatus (Flammann & Weckesser, 1984a) indicated that the outer membrane protein profile was dominated by a single heat modifiable protein of M_r 69,000 which, when solubilized above 70°C, altered mobility to 33,000 M_r . Again, saline extracts of cells were enriched in this protein, which showed porin activity in vitro with an estimated pore size of 0.8 nm (Flammann & Weckesser, 1984b).

The only other photosynthetic bacterium in which cell surface or outer membrane proteins have been studied is Chromatium vinosum (Lane & Hurlbert, 1980). Here a 42,000 M_r protein dominates the gel profile,

but it is not known if this exhibits porin-like activity.

The other, major, component of bacterial outer membranes is lipopolysaccharide (LPS) consisting of the endotoxic lipid A and the O-antigen side chains of sugar residues. The function of LPS is probably to endow the cell surface with hydrophilicity, while at the same time maintaining an effective permeability barrier (Nikaido & Vaara, 1985). The chemical composition of LPS from photosynthetic bacteria - especially the Rhodospirillaceae - has been very extensively investigated and was reviewed by Weckesser *et al.* (1979). The lipid A moieties of the different species exhibit different toxicities (Galanos *et al.*, 1977) and species-specific composition. This, combined with studies of the sugar residue composition of the o-antigenic side chains, has been useful in taxonomic studies of the Rhodospirillaceae (Drews *et al.*, 1978; Weckesser *et al.*, 1979). Such studies have indicated close relationships between *Rp. palustris*, *Rp. viridis* and *Rp. sulfoviridis* (Ahamed *et al.*, 1982) and between *Rc. gelatinosus* and *Rc. tenuis* (Weckesser *et al.*, 1977) which, to some extent, agree with conclusions reached from phylogenetic studies (see section 1.1.3). *Rhodomicrobium yannielli* was found to contain a lipophilic LPS of one type (Holst *et al.*, 1981) with a unique lipid A fatty acid spectrum. Interestingly, an ornithine containing lipid was co-extractable with the LPS from *Rm. yannielli* (Holst *et al.*, 1983) which was phosphate free and "of an unusual structure", when compared with those known in some other members of the Rhodospirillaceae. The role or location of this lipid in the cell envelope is unknown.

1.2.3.2 The cell wall. The cell walls of photosynthetic bacteria have received relatively little attention. In contrast to the plethora of

peptidoglycan types found in Gram positive bacteria, it is generally assumed that Gram negative cell walls contain only one major type of peptidoglycan with the "backbone" of N-acetylmuramic acid and N-acetylglucosamine and side-chains of L-alanine, D-glutamate, Meso-diaminopimelic acid and D-alanine in a molar ratio of 1:1:1:1:1 (Schleifer & Kandler, 1972). Indeed such a structure was found in Rs. rubrum by Newton (1968). However, Schmelzer et al. (1982) reported that in the peptidoglycan of Rp. viridis, at least 70% of the glucosamine residues in the glycan chains lacked N-acetyl substituents. Evidence was also provided of a similar structure in Rp. sulfoviridis and Rp. palustris but not in Rc. gelatinosus or Rc. tenuis. This represents a major modification to the accepted A1-type of Gram negative peptidoglycan and may imply a correlation between an obligate polar growth mechanism - as found in the budding and prosthecate members of the Rhodospirillaceae (see section 1.5.2.3) and peptidoglycan structure.

During the last decade there has been increasing interest in the enzymes involved in peptidoglycan synthesis and modification after the development by Spratt & Pardee (1975) of the radioactive penicillin-binding assay for such proteins. Penicillin-binding proteins have been detected in E. coli (Spratt & Pardee, 1975; Spratt, 1977), Pseudomonas (Noguchi et al., 1979), Clostridium (Murphy et al., 1981), Streptococcus (Coyette et al., 1980), Bacillus (Todd et al., 1983) and Caulobacter (Koyasu et al., 1980), amongst many others. Recently, Shepherd et al. (1981) studied the composition of the PBPs from the photosynthetic bacterium Rb. sphaeroides. Three major and several minor PBPs were detected in purified preparations of cytoplasmic membrane from chemoheterotrophically grown cells. The size distribution (M_r 35,000 - 90,000) was comparable to that of other Gram negative bacteria. The

principle PBP had a molecular weight of 42,000, equivalent to the PBP-5 of E. coli which has been identified as a D-alanine carboxypeptidase. The PBPs were localized exclusively in the cytoplasmic membrane of both phototrophically and chemoheterotrophically grown cells.

There is an obvious gap in our knowledge of the composition and function of penicillin binding proteins in photosynthetic bacteria and how this relates to peptidoglycan structure. The study of these two areas would appear to be especially important within the Rhodospirillaceae where there is evidence of major peptidoglycan modification of a type not seen in other Gram negative bacteria (Schmelzer et al., 1981).

1.2.3.3 Cytoplasmic and intra-cytoplasmic membrane systems. The early studies of Pardee et al. (1952) clearly established that the photosynthetic pigments of purple non-sulphur bacteria were associated with a discrete "particulate" fraction within the cell. These particles were named "chromatophores" and they participated in photochemical reactions in vitro (Frenkel, 1954). Morphological and fine structure analysis of Rs. rubrum revealed vesicular, chromatophore like membrane invaginations that were absent from aerobic, chemoheterotrophically grown cells and these were proposed as the location of the photosynthetic apparatus (Vatter & Wolfe, 1958; Cohen-Bazire & Kunisawa, 1963).

Today, the term chromatophore is usually reserved for vesicular membrane structures which carry out photophosphorylation and/or electron transport activities and which are oriented inside-out with respect to the cytoplasmic membrane. The general term intra-cytoplasmic membranes (ICM) will be used where these criteria are either not met or have not

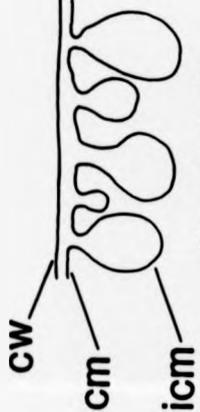
VESICULAR	OCCASIONAL INVAGINATIONS	TUBULAR	STACKS	LAMELLATE
 <p>CW cm icm</p>				
<u>Rb. sphaeroides</u> <u>Rb. capsulatus</u> <u>Rh. globiformis</u> <u>Rs. rubrum</u> <u>Chromatium</u>	<u>Rhodocyclus</u>	<u>Rs. fulvum</u> <u>Rs. molischianum</u> <u>Rs. photometricum</u>	<u>Rp. palustris</u> <u>Rp. viridis</u> <u>Rp. sulfoviridis</u> <u>Rp. acidophila</u> <u>Rhodomicrobium</u>	
		<u>Thiocapsa</u> <u>pfennigii</u>	<u>Ectothiorhodospira</u>	24

FIGURE 1.1. Intracytoplasmic membrane arrangements in the Rhodospirillales CW; cell wall.
 CW; cytoplasmic membrane. ICM; intra-cytoplasmic membrane (from Kelly & Dow, 1985a).

been investigated.

Within the Rhodospirillaceae, a variety of types of arrangements of ICM are known (Fig. 1.1). Rhodospirillum rubrum, the species of Rhodobacter and Rhodopila globiformis have a vesicular pattern while the other largest group - Rp. blastica, Rp. acidophila, Rp. palustris, Rp. viridis, Rp. sulfoviridis, Rp. rutila and Rm. vanniellii have a lamellate arrangement with the membranes lying underneath and parallel to the cytoplasmic membrane. A stacked arrangement is characteristic of the "brown" species of Rhodospirillum (Rs. fulvum, Rs. molischianum and Rs. photometricum). A small group of the Rhodospirillaceae, encompassing the genus Rhodocyclus, have what is considered to be a rather "primitive" ICM pattern which is characterized only by occasional finger-like intrusions of the cytoplasmic membrane into the cell (Imhoff et al., 1984).

From studies of thin sections, it was originally assumed that the peripheral, cytoplasmic membrane was continuous with the intracytoplasmic membrane system in the case of both vesicular (Cohen-Bazire & Kunisawa, 1963) and lamellate arrangements (Tauschel & Drews, 1967). This assumption has been largely borne out by a variety of independent experimental approaches including electron microscopy (Lampe et al., 1972; Peters & Cellarius, 1972; Golecki & Oelze, 1975), the isolation of BChl *a* depleted areas of the membrane system in phototrophically grown cells (Parks & Niederman, 1978), the identification of putative "invagination sites" of the cytoplasmic membrane on sucrose gradients (Inamine et al., 1984) and treatment of whole cells with pyridoxal 5'-phosphate - a non permeant reagent which was found to react with amino groups on the periplasmic aspect of both the CM and ICM (Francis &

Richards, 1980; Inamine *et al.*, 1984). Prince *et al.* (1975) also found cytochrome C_2 (located on the periplasmic side of the membrane) to be trapped within the chromatophores as they were "pinched off" from the CM during cell disruption. These studies support the contention of a single but dynamic membrane system which is divided into discrete areas (Oelze & Drews, 1972). Holmquist (1979) on the other hand, presented evidence of a discontinuity between CM and ICM in *Rb. sphaeroides*, based on the lack of penetration of ferrous gluconate into the ICM vesicles. It is possible that only a proportion of these vesicles are continuous with the CM and although the weight of evidence is against such an interpretation, the question of the physical continuity of the various regions of the membrane system has not been entirely resolved. This is especially true in those species with lamellate ICMs where fewer investigations have been carried out.

Isolation procedures for the ICMs of the Rhodospirillaceae largely rely on differential or sucrose gradient centrifugation (reviewed by Niederman & Gibson, 1978). The behaviour of the membranes on sucrose gradients has been the subject of much study and critically depends upon the ionic strength and divalent cation composition (Gibson, 1965; Niederman, 1974). Early studies revealed two pigmented bands in sucrose gradients. These contained Bchl and carotenoid and were designated "light" and "heavy" chromatophores. High concentrations of magnesium ions were present in these gradients, however, and it was shown by Niederman & Gibson (1971) and Niederman (1974) that the "heavy" chromatophore fraction in *Rb. sphaeroides* resulted from magnesium ion-induced aggregation between "light" chromatophores and the cell envelope. In gradients made with 1-10 mM Tris buffer alone, all the photosynthetic pigments could be isolated in a single discrete band

after centrifugation to equilibrium. Similar observations were made with ICM from Rp. palustris, Rc. gelatinosus (Hansen & de Boer, 1969) and Rp. viridis (Pucheu et al., 1973).

Isolated ICM contains 50-60% (dry weight) protein and 20-30% (dry weight) lipid, along with photosynthetic pigments and some carbohydrate (Niederman & Gibson, 1978). The majority of the lipid is accounted for by phospholipid and in the case of the well studied Rb. sphaeroides, the major components were identified as phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC) (Gorchein, 1968). However, as a group, the Rhodospirillaceae have a very heterogeneous lipid composition (Kenyon, 1978; Imhoff et al., 1982). Many species contain large amounts of ornithine-containing lipids, especially Rm. vannielii (Park & Berger, 1967a, Imhoff et al., 1982) but the relationship of these to the structure and function of the ICM system is unknown. The phospholipid composition of Rb. sphaeroides ICM was very similar whether the cells were grown phototrophically, or chemotrophically under aerobic-dark conditions (Onishi & Niederman, 1982). When grown in the presence of Tris, some strains of Rb. sphaeroides and several other bacteria were shown to accumulate a novel phospholipid, N-acylphosphatidylserine (Donohue et al., 1982) and this was proposed as a means of manipulating the phospholipid head group composition in studies of membrane structure-function relationships.

Far more work has been performed on the protein components of the ICM system in the Rhodospirillaceae although the vast majority of this knowledge has been gleaned from three species (Rs. rubrum, Rb. sphaeroides and Rb. capsulatus) which all contain a vesicular ICM arrangement. Several enzyme activities have been demonstrated in

chromatophore preparations from these species. These include succinate dehydrogenase, NADH oxidase and ATPase activity (Niederman & Gibson, 1978). In *Rs. rubrum* succinate dehydrogenase is exclusively localized in the cytoplasmic membrane during aerobic, chemoheterotrophic growth and in the chromatophores during photoheterotrophic growth (Oelze et al., 1975; Collins & Niederman, 1976). This contrasts with *Rb. sphaeroides* where it is localized in both the peripheral cytoplasmic membrane and the chromatophores in phototrophically grown cells (Niederman, 1974), although the specific activity is higher in the CM. This enzyme has a pivotal role in the transfer of electrons from organic substrates to the electron transport chain and its activity is markedly increased under aerobic, respiratory conditions (Beatty & Gest, 1981b).

NADH oxidase activity has been detected in chromatophores of all the above species and is taken to represent the presence of respiratory activity in such preparations. The specific activity of this multicomponent enzyme system therefore increases when the cells are grown chemoheterotrophically under aerobic conditions for example (Thore et al., 1969). Partial reactions such as NADH-dehydrogenase activity have also been detected and this particular enzyme may also function in NAD photoreduction in phototrophically grown cells (Knaff, 1978).

The ATPase of chromatophores is loosely bound to the external (originally cytoplasmic facing) side of the membrane and is readily removed by EDTA or sonication (Reed & Raveed, 1972; Baccarini-Melandri & Melandri, 1978). It consists of a hydrophobic portion (F_0) embedded within the membrane and a hydrophilic complex (F_1) located on the cytoplasmic side, which is thought to act as the catalytic site.

These three enzyme activities are the ones most studied and often used as "markers" for the membrane during purification. However, many other enzymes appear to be specifically associated with the intra-cytoplasmic membrane system, including glutamine synthetase (Yoch *et al.*, 1983), the proton translocating pyrophosphatase (Baccarini-Melandri & Melandri, 1978) and L-lactate dehydrogenase. This is reflected to some degree by the complexity of the protein profile when crude ICM is subjected to denaturing polyacrylamide gel electrophoresis. However, of these enzymes, only the α and β subunits of the proton translocating ATPase have been definitively identified on such gels (Post & Oelze, 1980; Drews & Oelze, 1981).

In fact, the bulk of the protein content of the ICM of those members of the Rhodospirillaceae which have been studied is accounted for by the pigment-protein complexes of the photosynthetic apparatus and these are considered below.

1.3 Mechanisms of Energy Capture and Transduction

1.3.1 Organization, structure and composition of the photosynthetic apparatus

Photosynthesis is a carefully regulated redox process which depends upon a precise spatial relationship of the components of the photosynthetic apparatus. This organization can be described in terms of the interaction of four components: (i) a light-harvesting antenna system, (ii) the photochemical reaction centre, (iii) the electron transport chain and (iv) the proton-translocating ATPase. The reactions catalyzed by these components are largely membrane bound, vectorial events directed at supplying a source of both reducing power and energy. In both anoxygenic and oxygenic photosynthesis, light harvested by the antenna pigments is transferred to a few specialized sites - the reaction centres (RCs) - where charge separation produces an oxidized form of chlorophyll and an electron which is transferred to the electron transport chain (Fig. 1.2).

1.3.1.1 Light-harvesting. Light energy harvested by the antenna pigments creates mobile electronic singlet states ("excitons") which migrate with high efficiency through the aggregates of the light harvesting pigments until trapped by the reaction centre or lost as heat or fluorescence (Borisov, 1978). Although this can be described as a random-walk process, the structure of the light-harvesting system is such that there is a high probability of transfer from the carotenoids to the different spectral forms of bacteriochlorophyll in the complexes

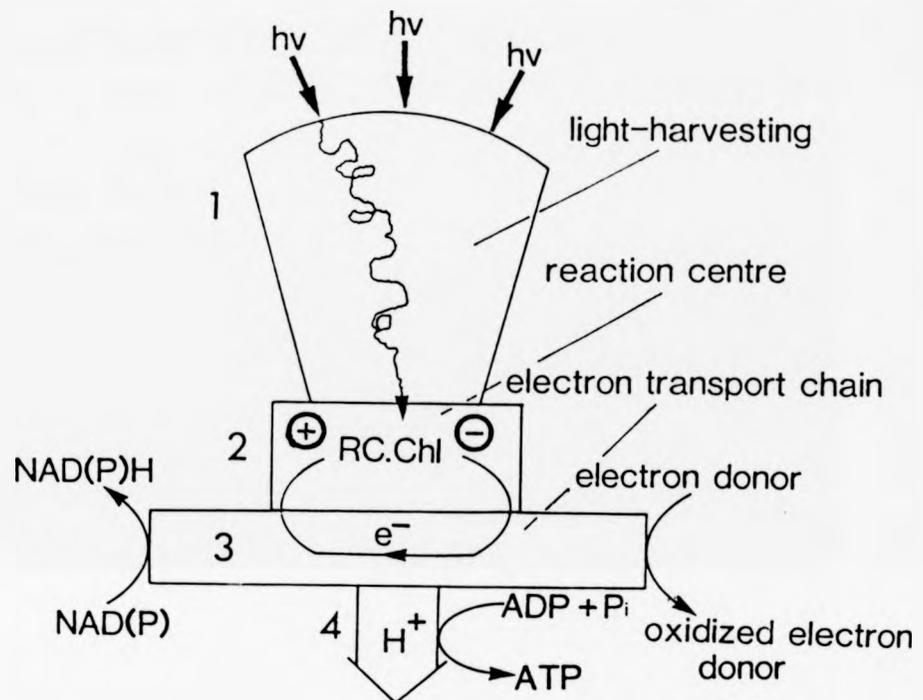


FIGURE 1.2. Interactions in photosynthetic energy flow.

Light harvested by the antenna system (1) arrives by a tortuous route to the reaction centre (2) as excitation energy. Here, charge separation and electron transport (3) are initiated and subsequently used to produce a proton-motive force. ATP synthesis then occurs via the proton-translocating ATP synthase (4). Figure form Kelly & Dow (1985a).

and then to the RC (Feick *et al.*, 1980; Drews, 1985).

Light-harvesting complexes have been studied in detail from several species of the Rhodospirillaceae (Drews & Oelze, 1981). *Rhodospirillum rubrum* produces only one type of LH complex with an absorption maximum in the infra-red at 890 nm and this dominates the *in vivo* absorption spectrum of whole cells. Most species of the genus *Rhodopseudomonas* and *Rhodobacter*, where studied, appear to contain two light-harvesting systems one with an absorption maximum at 870-890 nm, similar to that in *Rs. rubrum* ("B870" or "B890") and the other with two maxima, at 800 and 850-860 nm (B800-850). These complexes were originally designated LHI and LHII, respectively by Lien *et al.* (1973) long before they were isolated and characterized. However, it now appears that there may exist several sub-classes of both types of light-harvesting complex (Cogdell & Thornber, 1980; Thornber *et al.*, 1983). Nevertheless, all LHI type complexes appear to be closely associated with the reaction centre *in vivo* (Monger & Parson, 1977; Saeur & Austin, 1978; Broglie *et al.*, 1980) and remain in a fixed stoichiometry with it upon variation of cultural conditions (Aagaard & Sistrom, 1972). In contrast, LHII type complexes are of variable stoichiometry with the RC, and contain the bulk of the membrane bound Bchl and carotenoids (Aagaard & Sistrom, 1972; Lien *et al.*, 1973; Firsow & Drews, 1977; Broglie *et al.*, 1980).

The Bchl and carotenoid photopigments within these complexes are non-covalently bound to low-molecular weight polypeptides (Drews & Oelze, 1981; Thornber *et al.*, 1983; Drews, 1985) of M_r 6,000-12,000 as determined by SDS gel electrophoresis and 5,000-9,000 as determined by sequence analysis, in *Rb. capsulatus* (Peters & Drews, 1983a; Tadros *et al.*, 1982, 1983, 1984), *Rb. sphaeroides* (Cogdell *et al.*, 1980; Cogdell & Thornber, 1980; Bachman *et al.*, 1983), *Rp. palustris* (Firsow & Drews,

1977; Varga & Staehelin, 1985a), Rp. viridis (Jay et al., 1984; Peters et al., 1984), Rp. acidophila (Cogdell et al., 1983) and Rs. rubrum (Brunisholz et al., 1981; Picorel et al., 1983). In general, both types of light-harvesting complex contain at least two polypeptides to which the pigments are bound. Some species such as Rb. capsulatus have a third polypeptide in the B800-850 complex which does not participate in pigment-binding (Feick & Drews, 1978). Its function is unclear, as it is not present in the B800-850 complex from Rb. sphaeroides. When isolated from ICM by detergent solubilization, all LH complexes have apparent molecular weights of 80,000-200,000 in the native-pigmented state (Broglie et al., 1980; Peters & Drews, 1983a). Dissociation of this oligomeric form to yield one pair of the two different Bchl binding polypeptides in an undenatured state has not been achieved to date and this oligomerization may be necessary to stabilize the complex in the membrane (Drews, 1985).

Amino acid sequence analyses of the LH pigment-binding polypeptides indicate the presence of a hydrophobic central region of about 20 residues between the N- and C-termini which contains a conserved histidine (Brunisholz et al., 1981; Tadros et al., 1983, 1984). This is thought to be the site of ligand formation between the polypeptide chain and the Mg atom of the Bchl tetrapyrrole ring. One of the two polypeptides usually has an additional, conserved, histidine residue located at the beginning of the hydrophobic segment which in the case of the B800-850 complexes is also involved in Bchl binding (Feick & Drews, 1978; Cogdell & Thornber, 1980). The polypeptide with a single conserved histidine residue is known on the α -apo protein and is usually of a lower molecular weight than the β -apoprotein, with two conserved histidine residues (Cogdell et al., 1985). Evidence from

surface iodination (Peters & Drews, 1983b) and protease digestion studies (Oelze, 1978) indicate that the central hydrophobic region of these polypeptides spans the membrane, and it probably has an α -helical structure (Drews, 1985).

The interaction of Bchl with the light-harvesting polypeptides causes a pronounced red-shift in the near IR absorption maximum from 770 nm to 800-900 nm and is thought to result from electrostatic interactions between Bchl and charged amino-acids on the protein (Eccles & Honig, 1983). The electronic structure of these IR peaks has been extensively investigated in order to probe the organization of the pigment molecules within the complexes (Saeur & Austin, 1978; Kramer *et al.*, 1984). Thornber *et al.* (1983) concluded that two subclasses of LHI type complexes exist based on such parameters as polypeptide composition, carotenoid stoichiometry, absorption and circular dichroism (CD) spectra. These are represented by the B890 complex from *Ra. rubrum*, and the B870 complex from *Rb. sphaeroides* and *Rb. capsulatus* respectively. The B890 complex contains 2 polypeptides, 2 with Bchl, 1 mol carotenoid and exhibits an intense CD spectrum around the 890 nm IR peak. This is indicative of strong exciton coupling within a Bchl dimer (Saeur & Austin, 1978). In contrast, the B870 class contains 2 mol carotenoid per pair of polypeptides and exhibits a relatively weak CD spectrum. However, studies using resonance raman spectroscopy (Robert & Lutz, 1985) indicate that these differences are not reflected in the microenvironments of the Bchl molecules within the complexes.

The electronic structure of the pigment within the LHII type complexes also indicates at least two sub-classes to exist (Thornber *et al.*, 1983). Type I B800-850 complexes are typified by those of *Rb.*

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capsulatus, Rb. sphaeroides and possibly Rd. palustris (Feick & Drews, 1978; Thornber et al., 1983). Cogdell & Thornber (1980) discussed a model of the B800-850 complex of Rb. capsulatus in which 1 carotenoid molecule and 1 Bchl molecule responsible for the 800 nm absorption band were bound to the 8,000 M_r (α) polypeptide and two Bchl molecules responsible for the 850 nm absorption band were bound to the 10,000 M_r (β) polypeptide. The 850 nm band exhibits a strong CD spectrum indicating exciton coupling between these two Bchl molecules while the 800 nm band does not, consistent with the proposed monomeric structure (Picorel et al., 1984). The peak height of the 850 nm band is usually 1.5 times as intense as the 800 nm band in type I B800-850 complexes (Thornber et al., 1983). The type II B800-850 complex is present in some Rd. acidophila strains under certain growth conditions (Cogdell et al., 1983) and in the purple sulphur bacterium Chromatium vinosum. Here the 850 nm absorption band is of equal or lower intensity than the 800 nm band. The resonance Raman study of Robert & Lutz (1985) also revealed a difference between the B800-850 complexes of the Rhodospirillaceae and Chromatiaceae in the type of "environment" in which the Bchl is located but this included differences between Rd. acidophila and Chr. vinosum.

Clearly, the classification of the various light-harvesting complexes depends upon which parameters are used. Although the Bchl environments may be very similar in the B800-850 complexes from the Rhodospirillaceae for example, there still exists marked differences in absorption spectra, pigment stoichiometry and polypeptide composition. This is especially true for organisms such as the Bchl_h containing Rd. viridis in which a single B1020 LH complex is thought to exist (Jay et al., 1984) - how does this compare in properties with those of Bchl_g

containing LH complexes? New approaches, such as the recently reported crystallization of the B800-850 complex from Rb. capsulatus by Welte et al. (1985) should allow detailed studies by X-ray crystallography of the structure of LH complexes in general, and give new insights into how energy is transferred within them.

1.3.1.2 The photochemical reaction centre. That the photochemical reaction centre existed as a defined structure in photosynthetic bacteria was first proposed by Clayton (1963). This followed the observation that when the light-harvesting Bchl of the carotenoidless mutant R-26 of Rb. sphaeroides was destroyed chemically, light induced bleaching of a component in the absorption spectrum (at 870 nm) could still be observed. The isolation of the RC as a pigment-protein complex from Rb. sphaeroides (Reed & Clayton, 1968) precipitated intense research, both on the mechanism of primary photochemistry and subsequent electron transport (section 1.3.1.4) and on structure-function relationships within the complex.

Reaction centres from the well studied Rb. sphaeroides and Rb. capsulatus contain 3 polypeptide subunits of apparent molecular weight as determined by SDS gel electrophoresis of M_r 28,000, 24,000 and 21,000 (Feher & Okamura, 1978). They are designated H (heavy), M (medium) and L (light) respectively and occur in a 1:1:1 stoichiometry. The M and L subunits alone can carry out primary photochemical reactions and they bind 4 mol of Bchl, 2 mol of bacteriopheophytin, 1 mol of carotenoid, 2 mol of ubiquinone and 1 mol of iron (Feher & Okamura, 1978; Parson & Ke, 1982). The H subunit is not essential for primary photochemistry and its function is unclear. Recently, the application of molecular genetic techniques in Rb. capsulatus has led to the cloning and sequencing of

DNA coding for the RC subunits (Youvan *et al.*, 1984a, b; Zsebo & Hearst, 1984). The structural genes for the L and M subunits overlap by 5 nucleotides which may allow transcriptional coupling and they are located downstream from the B870 (LHI) α and β genes. This L and M gene cluster may form an operon with the production of polycistronic mRNA (Youvan *et al.*, 1984a,b; Belasco *et al.*, 1985; Zhu & Kaplan, 1985). The H subunit structural gene is located outside this cluster and has been cloned on a separate fragment. Data from DNA sequence analysis (Youvan *et al.*, 1984b) and from amino acid sequencing (Williams *et al.*, 1983, 1984) have indicated that the M subunit has an M_r of 34,400, the L of 31,319 and H of 28,534 which is considerably different to that determined on SDS gels. Hydropathy plots (Youvan *et al.*, 1984b; Drews, 1985) have shown that the M and L polypeptides each contain five stretches of hydrophobic amino acids which may span the membrane as α -helices.

The optical properties of the *Rhodobacter* reaction centre have been extensively studied (Norris & Katz, 1978). The absorption spectrum of purified RCs shows near IR peaks at 760, 800 and 870 nm. Evidence from CD and electron paramagnetic resonance spectroscopy suggests the 870 nm peak to be composed of a dimer of Bchl which is known as the "special pair" or P870 and is responsible for initiating primary photochemistry within the RC (section 1.3.1.4). The 800 nm peak is due to the remaining two "voyeur" Bchl molecules and the 760 nm peak originates from bacteriopheophytin (BPheO).

Whereas the genetic organization and optical characteristics of the *Rhodobacter* type of Bchl_a containing RC have received greatest attention, that from the Bchl_b containing species *Rp. viridis* has been

the subject of detailed and elegant structural studies. This is a direct consequence of the successful crystallization of the complex by Michel (1982). Previous biochemical studies (Pucheu *et al.*, 1976) indicated the presence of 4 polypeptides, three of which (M_r 33,000, 27,000 and 24,000) are probably equivalent to the H, M and L subunits of Bchl_a containing RCs. The remaining 38,000 M_r protein is associated with cytochrome and binds haems c-553 and c-558 (Pucheu *et al.*, 1976; Jay *et al.*, 1984). The structural studies have utilized X-ray diffraction and sequence analysis to build a complete model of the organization of the RC with regard to both chromophores and proteins (Deisenhofer *et al.*, 1985). The L and M subunits which are liganded to the special pair dimer (P960) contain α helices which span the membrane. The porphyrin systems of the special pair, the remaining Bchl molecules and the Bp_{heo} are related by a diad symmetry although electron transfer probably occurs only via one of the branches. The H subunit forms the cytoplasmic cover and as with *Rb. capsulatus*, has the shortest amino-acid chain. It has an N-terminal transmembrane helix and touches at its tip a segment of the cytochrome, which forms the periplasmic cover of the complex.

Such detailed information will allow the development of models for electron transfer within the RC in spatial terms in addition to providing insights into its orientation within the membrane (Miller, 1982; Deisenhofer *et al.*, 1985).

The reaction centres from other photosynthetic bacteria have received much less study, but the diversity of this type of pigment-protein complex is only just becoming apparent, as is evident from the existence of microbes such as the recently discovered *Hellobacterium chlorum* (Gest

& Favinger, 1983) which contains Bchl_a and a unique photochemical reaction centre (Fuller *et al.*, 1985). Clearly, a greater range of organisms needs to be studied to clarify this diversity, including other members of the Rhodospirillaceae where, for example, Rc. gelatinosus appears to contain an RC with only two subunits (Clayton & Clayton, 1978). Improvements in X-ray crystallography of RC complexes will also yield an increased knowledge of structure-function relationships as is currently being obtained for that of Rb. viridis (Deisenhofer *et al.*, 1985).

1.3.1.3 Interactions between light-harvesting and reaction centre complexes within the membrane. For efficient energy transfer within the ICM the proteins and bound pigments of the LH and RC complexes must be in the correct spatial orientation for maximum interaction. Kinetic studies of fluorescence emission and the migration of excited states (Monger & Parson, 1977; Feick *et al.*, 1980) indicate that B870 LHI complexes surround and interconnect the RCs within the membrane while the B800-850 LHII complexes are more peripheral, consistent with energy migration from B800→B850→B870→RC. This organization is implicit in the concept of the photosynthetic unit; a defined amount of bulk bacteriochlorophyll associated with the reaction centre.

There are at present essentially two models of the energetic organisation of these complexes; the "lake" model in which it is suggested that the reaction centres are embedded in a pool of energetically connected LH complexes (Monger & Parson, 1977) and the "puddle" model in which discrete photosynthetic units are not energetically connected to other such units. Studies with proteoliposomes, in which Rb. capsulatus chromatophore membranes fused

with liposomes were prepared (Garcia *et al.*, 1985; Takemoto *et al.*, 1985) indicate that an uncoupling of energy transfer, measured as an increase in fluorescence emission, between the B875 LH complexes and the reaction centre occurs with increasing lipid dilution of the membrane (Takemoto *et al.*, 1985). Electron transport rates also decreased under such conditions. Although not unequivocally supporting either model, such evidence does suggest an organization more consistent with the "lake" arrangement in that either physical separation of B875 complexes from the RC or dispersion of interacting photosynthetic units is possible when the membrane components are diluted with lipid.

Regardless of these uncertainties, however, it is clear that there is a high degree of order in the arrangement of such interacting complexes within the membrane. This is especially apparent in such membranes as the ICM of Rb. viridis where regular arrays of photoreceptor units are arranged as a hexagonal lattice (Stark *et al.*, 1984). Here the oligomeric B1020 LH units are organized in a ring around the reaction centres in a highly ordered fashion.

Nearest-neighbour relationships between polypeptides of the LH and RC complexes have been studied in reversible chemical cross-linking and mild detergent fractionation experiments (Peters & Drews, 1983c; Peters *et al.*, 1983, 1984). Cross-linking has been performed with a variety of different reagents (hydrophilic to hydrophobic) with rather similar results. It was found with Rb. capsulatus that the two pigment binding polypeptides of the B800-850 complex formed homo-oligomers and hetero-oligomers, while the γ polypeptide formed only homo-oligomers (Peters & Drews, 1983c). A major part of the B800-850 complex appeared to be

associated with the RC H subunit (Peters *et al.*, 1983), although they do not form complexes during detergent fractionation of membranes and are regulated separately (Drews, 1985).

Spatial relationships were also found to exist between RC subunit H and oligomers of both polypeptides of the B870 LH complex and this agreed with the association of RC and B870 complexes upon detergent fractionation (Peters *et al.*, 1983). These data provide confirmatory evidence for strong protein interactions between the RC and LHI complexes with the H subunit possibly playing a structural role. Similar studies in *Rb. viridis* (Peters *et al.*, 1984) revealed cross-linked homo-oligomers (up to the hexamer) of the B1020 LH complex and a relationship between the smaller LH polypeptide and RC subunit H. In addition, the membrane bound cytochrome $c_{553/558}$ was associated specifically with the M subunit.

A variety of evidence, then, indicates that the arrangement and molecular organization of the photosynthetic pigment protein complexes is non-random within the membrane and the rather high degree of order which appears to exist is related to the need for efficient energy transfer and trapping. The oligomeric state of the LH complexes which surround the RCs is in part a reflection of this. Moreover, other membrane bound components of the photosynthetic apparatus such as the ATPase and the cytochromes of the electron transport chain must also be accommodated in the membrane and in models of membrane structure (Oelze & Drews, 1972; Drews & Oelze, 1981; Drews, 1985). Lateral topographical relationships between such components have not been well studied as yet. However, this would appear to be important especially in view of the highly asymmetric membrane structure required for the operation of a

chemiosmotic type of energy conservation mechanism, for example, as considered in sections 1.3.2 and 1.3.3.

1.3.2 Photosynthetic and respiratory electron transport

The harvesting of light energy followed by its transfer to the reaction centre is the first step in the repertoire of events leading to the production of reducing power and ATP. In essence, the RC must act as an energy sink, containing the special pair dimer in a specialized environment within which it can convert the energy of excitation received from the LH antenna into an electron transfer event.

The operation of the Rhodobacter type of RC is illustrated in Fig. 1.3. After receiving excitation energy, the P870 Bchl enters the singlet excited state in which it is a powerful reducing agent. It rapidly transfers an electron probably via one of the 800 nm absorbing "voyeur" Bchls to a single BPheo molecule which acts as an intermediate acceptor. This initial charge separated state ($P870^+ BPheo^-$) is stabilized by electron transfer to the primary (firmly bound) quinone acceptor Qa (QI). These events all take place within the RC. Subsequent electron transfer events occur within the domain of the membrane itself and are initiated by reduction of the secondary quinone Qb (QII) which exists in the form of a "pool" of thermodynamically equivalent molecules within the membrane (Parson, 1978; Nugent, 1984). Electrons from this pool must be used to re-reduce the primary donor $P870^+$ in order to complete cyclic electron flow. This is achieved in members of the Rhodospirillaceae by transfer to a membrane-bound cytochrome b-C₁ complex followed by a "mobile" or soluble Cytochrome C₂ which acts as

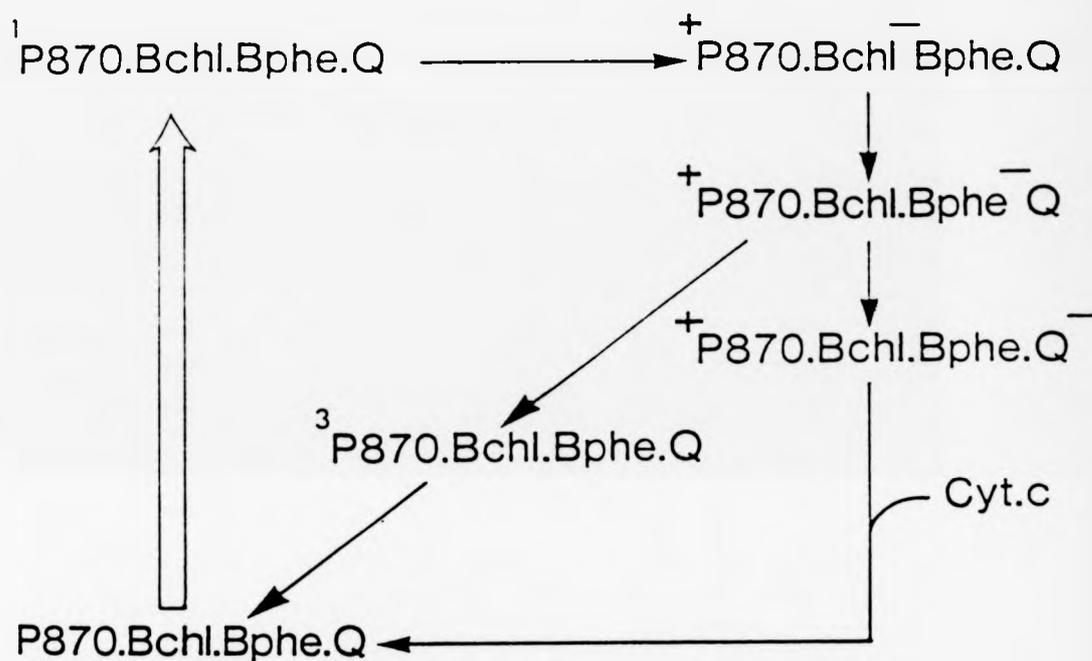


FIGURE 1.3. The mechanism of charge separation and electron flow within the *Rb. sphaeroides* reaction centre. $^1\text{P870}$ represents the singlet excited state, and $^3\text{P870}$ the triplet state, of the special pair dimer. "Bchl" represents a bacteriochlorophyll monomer absorbing at 800 nm. Bphe is bacteriopheophytin and Q, the primary quinone acceptor. (From Kelly & Dow, 1985a.)

the electron donor to P870 (Prince et al., 1975; Nugent, 1984).

The cytochrome b-C₁ complex has recently been isolated and purified from Rb. sphaeroides (Gabellini et al., 1982; Yu et al., 1983) and contains two hemes b (low and high potential) one c heme, a Rieske Fe-S centre and some bound quinone. This complex shows remarkable homology to the mitochondrial ubiquinol-cytochrome c oxidoreductase and the chloroplast cytochrome b₀-f(c) complex (Barber, 1984). It is this complex which acts as the major electrogenic proton translocating pump of the cyclic electron transport system (Bowyer & Crofts, 1981).

Cytochrome C₂ appears to be the most predominant soluble heme protein which donates electrons to P870 (Bartsch, 1978) in members of Rhodobacter and Rhodospseudomonas. Some species of the Rhodospirillaceae, e.g. Rb. viridis and also Chromatium vinosum use membrane bound cytochrome c₅₅₂₋₅₅₇ instead of, or in conjunction with, cytochrome C₂ for this purpose (Bartsch, 1978; Nugent, 1984).

The sole function of cyclic electron transport is to produce a proton gradient usable in ATP synthesis (section 1.3.3). Were it not for the fact that reducing power (NAD(P)H) is also required in order to fix CO₂ or N₂ etc. then a net input of electrons would not be needed. Unfortunately the purple photosynthetic bacteria face a potential problem in the photoproduction of NADH from certain types of exogenous electron donor which are unable to form NADH by a direct enzymic route, in that the redox potential of the primary quinone acceptor (QI/QI⁻ - - 170 mV) and that of the ubiquinone pool (approx. 0mV) is not sufficiently negative to reduce NAD directly (NAD/NADH - -340 mV). Therefore under these circumstances, a reversed electron transport

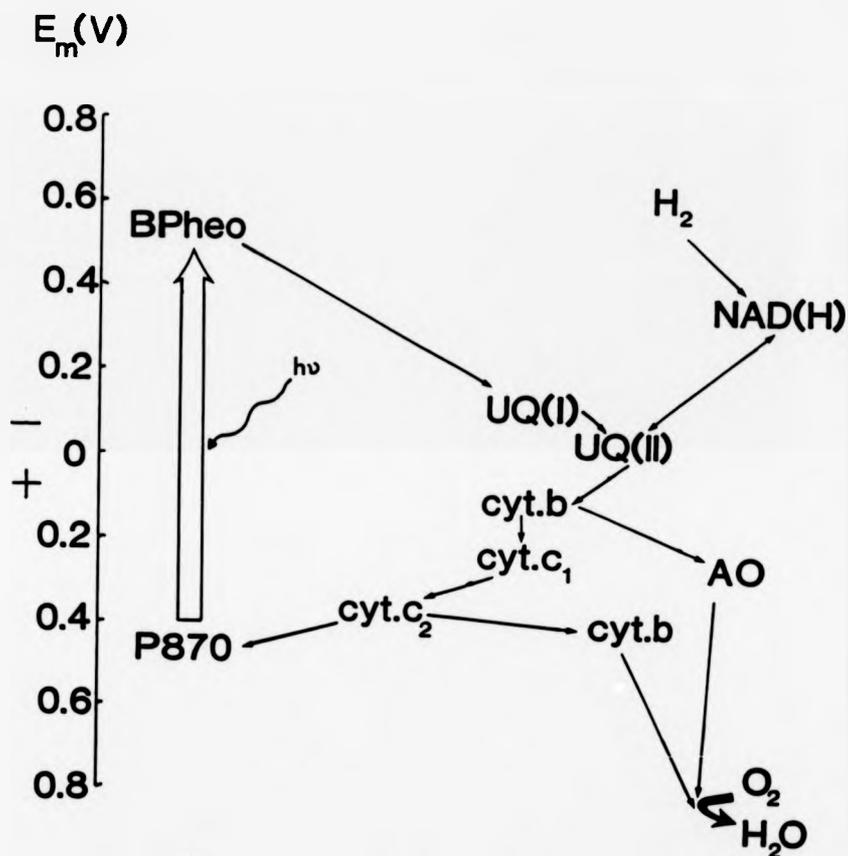


FIGURE 1.4. Redox potential diagram of respiratory and photosynthetic electron flow in Rhodobacter.

During photosynthesis, electrons produced in the reaction centre (P870-BPheo) circulate through the cytochrome b - c_1 complex via the quinone pool (UQII) and return via a periplasmic cytochrome c_2 to P870. Respiratory electron flow from NADH also occurs through the cytochrome b - c_1 region, c_2 and a terminal oxidase. However, the pathway is branched and can involve an "alternative oxidase" (AO) which bypasses the b - c_1 complex (modified from Kelly & Dow, 1985a).

pathway is used from the quinone pool to NAD via the NADH dehydrogenase (NADH-ubiquinone oxidoreductase) enzyme (Knaff, 1978). Evidence from the sensitivity of this pathway to uncouplers (Jones & Saunders, 1972) reveals that it is energy dependent.

A redox potential diagram of cyclic electron transport is shown in Fig. 1.4, where substrate derived electrons from the various dehydrogenases feed into the ubiquinone pool or the cytochrome b-C₁ complex. The central importance of the quinone pool is clearly evident, when it is considered that certain electron transport components are common to both photosynthetic and respiratory electron flow (Marrs & Gest, 1973; Keister, 1978). With O₂ as electron acceptor, the segment containing the RC is inoperative and "downhill" transport from NADH, through the NADH dehydrogenase occurs via the quinone pool, the cytochrome bC₁ complex and cytochrome C₂ to the terminal oxidase(s), of which several types are known (King & Drews, 1976; Smith & Pinder, 1978; Fenoll & Ramirez, 1984). Electrons may also leave the quinone pool along branched pathways to alternative oxidases (McEwan et al., 1983) and this emphasises the complexity of electron transport systems in at least some members of the Rhodospirillaceae.

The constitutivity of many components of respiratory electron transport under phototrophic growth conditions is illustrated by the dark oxygen uptake observable with cells grown anaerobically in the light (Keister, 1978). The fact that light inhibits such oxygen uptake is interpreted in terms of shared electron transport components (Keister, 1978) and/or to a kind of respiratory control due to an increased membrane potential in the light (Cotton et al., 1983).

1.3.3 Vectorial proton translocation and the synthesis of ATP

The chemiosmotic hypothesis (Mitchell, 1961) supposes that certain components in the chain of redox carriers within the cytoplasmic (or intra-cytoplasmic) membrane can act as proton pumps to produce a transmembrane proton gradient composed of both an electrical potential difference - the membrane potential ($\Delta\psi$) - and a chemical potential difference - the pH gradient (ΔpH). The total proton-motive force (pmf; Δp) is the algebraic sum of these parameters, thus;

$$\Delta p = \Delta\psi - Z \Delta\text{pH}$$

where $Z = 2.303RT/F$ and converts ΔpH into units of mV. This electrochemical gradient can be produced because the coupling membrane itself is generally impermeable to protons except via specific channels which allow them to "leak" back across the membrane. It is during this process that ATP can be synthesised by H^+ flux through the proton-translocating ATP synthase complex. Other proton-translocating enzyme complexes, for example the NADH-ubiquinone oxidoreductase can also utilize the proton gradient in energy requiring reactions.

Several techniques have been used to evaluate the relative contributions of $\Delta\psi$ and ΔpH to Δp in both whole cells and membrane preparations (Rottenberg, 1975, 1979; Baccarini-Melandri *et al.*, 1981) but when different methods have been employed, often conflicting values have been obtained. Such methods can be broadly divided into two groups based on the use of extrinsic or intrinsic probes. In photosynthetic bacteria, the most commonly employed extrinsic probe technique is that using the redistribution of permeant ions in response to either $\Delta\psi$ or ΔpH . Here the probe ion may be radiolabelled and its uptake by the bacteria or

membrane preparation followed using, for example, flow dialysis (Michels & Konings, 1978). The redistribution can also be followed by ion-selective electrodes with unlabelled probes (Shinbo *et al.*, 1978; Lolkema *et al.*, 1983; Keevil & Hamilton, 1984). The choice of probe depends upon the polarity of the force being measured; permeable cations, such as phosphonium ion derivatives are used in whole cells, for example, where a $\Delta\psi$, inside negative, is generated (Rottenberg, 1979; Baccarini-Melandri *et al.*, 1981) while for ΔpH determination either weak bases such as methylamine or weak acids such as benzoate or salicylate (Hellingwerf & Van Hoorn, 1985) may be used. Some potential limitations with the ion-redistribution method include (i) uncertainty about the true equilibrium distribution of the probe, (ii) permeability problems with whole cells, (iii) extensive probe binding, (iv) slow equilibration time which may not allow fast, kinetic observations. Some of these problems can be overcome with appropriate treatments (Lolkema *et al.*, 1983).

An alternative, intrinsic probe is available in photosynthetic organisms in the form of the endogenous photosynthetic pigments themselves. These can respond to the development of an electric field across the coupling membrane ($\Delta\psi$) by an alteration of their electronic structure resulting in a change in absorption properties. The most extensively studied of such changes is the light-induced red shift in the carotenoid absorption spectrum - the carotenoid bandshift (Jackson & Crofts, 1969; Wraight *et al.*, 1978a). A linear relationship exists between this electrochromic response and the value of $\Delta\psi$, the most compelling evidence in favour of which is the proportional change induced by the imposition of ionic diffusion potentials of known value (Jackson & Crofts, 1969). It also appears that it is the carotenoid associated with the LHII complex that

specifically responds to the electric field, at least in Rhodobacter.

The major advantage of the carotenoid bandshift technique is that it has a very fast response time - sufficiently rapid to enable measurements to be made of the flux of ions back across the coupling membrane after the cells are darkened (the ionic current; Jackson, 1982). It is also "non-invasive".

The chemiosmotic hypothesis clearly stipulates a relationship between the rate of ATP synthesis and the magnitude of Δp . The form of this relationship has been investigated in photosynthetic bacteria using the carotenoid bandshift method (Clark et al., 1983). It is now clear that there is a steeply non-linear dependence of the rate of ATP synthesis upon $\Delta\psi$; the rate increases dramatically over a relatively narrow range of $\Delta\psi$ values (Clark et al., 1983; Cotton & Jackson, 1984). An explanation of this relationship has come from measurements of the ionic current and its dependence on membrane potential. If whole cells or chromatophores are illuminated to "steady-state" - such that the rate of generation of $\Delta\psi$ is equal to the rate of its dissipation by ionic fluxes across the membrane - and then rapidly darkened, the decay of the carotenoid bandshift (a measure of the ionic conductance) also displays a non-linear dependence on the steady-state value of $\Delta\psi$ (Jackson, 1982; Clark et al., 1983). These results have been interpreted to mean that the membrane behaves non-ohmically (Nichols, 1974; Jackson, 1982). Such special conductance properties have implications for the regulation of ATP synthase activity as the predominant ion-flux is that of H^+ through this enzyme (Clark et al., 1983). Indeed, the dependence of the rate of ATP synthesis on $\Delta\psi$ is of the same form as that of the current/voltage curve (Clark et al., 1983). In addition, transient changes in $\Delta\psi$ upon

illumination and before a steady state is reached have been attributed to changes in the conductance properties of the membrane due to a "lag" in the development of ATP synthase activity (Cotton & Jackson, 1984). The activity of the ATP synthase may therefore be at least partly determined by the value of $\Delta\psi$.

1.4 Development of the Photosynthetic Apparatus

In growing cultures of all photosynthetic microbes, the activity and composition of the photosynthetic apparatus is carefully regulated by at least four groups of factors; light flux and quality, growth rate and nutrition, oxygen tension and temperature (reviewed by Drews & Oelze, 1981; Ohad & Drews, 1982; Oelze, 1983a). The interaction of these factors pervades every aspect of the development of the photosynthetic apparatus from the synthesis of bacteriochlorophyll and carotenoid to the formation of photochemically active membrane domains, as considered below.

1.4.1 The regulation of photopigment synthesis

The early studies of Cohen-Bazire *et al.* (1957) clearly showed that high oxygen tensions specifically repressed the formation of Bchl and carotenoids and thus prevented the formation of the photosynthetic apparatus. Transfer of cultures from dim to bright light and oxygenation of anaerobically grown cultures also inhibited Bchl formation. These effects were found to be widespread amongst the species of the purple bacteria and were initially explained by invoking the oxidation-reduction state of some carrier in the electron transport chain as a mediator in the response. From the outset it was assumed that light and oxygen acted *via* common "transmitters", a view apparently reinforced by the fact that both respiratory and photosynthetic electron flow share many components of the electron transport chain (Marrs & Gest, 1973; Jones, 1977). However, Marrs & Gest (1973) found that a

cytochrome oxidase deficient mutant of Rb. capsulatus still showed the same sensitivity of Bchl synthesis towards oxygen. In addition, although the cellular specific Bchl content is inversely related to oxygen tension in aerobic cultures and to light intensity in anaerobic cultures, low oxygen tensions alone can induce Bchl formation, while light is not necessary for its synthesis or that of any component of the photosynthetic apparatus (Aagaard & Siström, 1972). Oxygen is thus a primary and light a secondary factor in the control of Bchl synthesis. Kaplan (1978) coined the terms "governor" control and "modulator" control respectively for their effects.

Recently, new experimental approaches have been used to decide whether light and oxygen act independently or in a similar manner on Bchl synthesis. One possible cellular control site is that of the enzyme 5-aminolaevulinate (ALA) synthase which provides the Bchl precursor ALA. Oelze & Arnheim (1983) used continuous cultures to show that only oxygen and not light affected the cellular enzyme levels while both factors clearly affected Bchl formation. Further studies showed that O₂ controlled specific Bchl contents of Rs. rubrum, Rb. sphaeroides and Rb. capsulatus in a hyperbolic fashion irrespective of the presence of light but that light control itself was sigmoidal (Arnheim & Oelze, 1983a). These data indicate that light and oxygen affect Bchl synthesis by different means.

With regard to control by light, Oelze (1983b) postulated two possible sites where Bchl synthesis could be modulated in wild type cells. These were at the level of Bchl synthesis itself and, in a more general manner, by controlling cellular protein synthesis and growth rate. In the former case, there appeared to be no light-controlled step in Bchl

synthesis subsequent to ALA formation (Oelze, 1983b) which was supported by the study of Biel & Marrs (1983) who found that β -galactosidase levels in strains of *Rb. capsulatus* with *bch*-Mu d(Ap^r*lac*) fusions to the Bchl biosynthetic genes were not regulated in response to light intensity. However, as light does not affect the *in vitro* specific activity or concentration of ALA synthase, the *in vivo* control site must either be previous to this or else of a more complex nature, perhaps involving covalent modification.

The control of Bchl synthesis by oxygen regulation of ALA synthetase has yet to be investigated at the transcriptional level but presumably, since ALA is also a precursor for Fe and Co tetrapyrroles (leading to the cytochromes and corrinoids respectively), the Mg-tetrapyrrole branch, leading to Bchl, must be under strict and separate regulation (Lascelles, 1978; Hauser-Gerspach & Oelze, 1985). Indeed, Biel & Marrs (1983) using *bch-lacZ* gene fusions, showed that transcriptional regulation of the Bchl biosynthetic genes was not limited to a few key steps but that at least 5 (*bchA*, B, C, G, H) were regulated by oxygen.

Although little is known of the effects of other environmental factors on Bchl synthesis, there is some information available on the regulation of carotenoid biosynthesis in the Rhodospirillaceae. Carotenoids play two important roles in photosynthesis. They serve both to harvest light energy and to protect the cell from photo-oxidative damage (Schmidt, 1978; Cogdell *et al.*, 1981). As carotenoids are associated with proteins in the pigment-protein complexes in the same manner as Bchl it is clear that their synthesis must be regulated by either the same factors as Bchl, e.g. O₂ and light in a direct manner or by some component(s) of the photosynthetic membrane system which is itself

regulated by these factors. Biel & Marrs (1985) distinguished between these two alternatives with respect to control by oxygen by determining carotenoid contents under low and high pO_2 for strains of Rb. capsulatus in which defined lesions in one or more bch genes existed. They found that strains capable of making Bchl accumulated greater amounts of carotenoid under low pO_2 compared with high pO_2 conditions, consistent with the early observations of Cohen-Bazire et al. (1957) but that strains with a mutation in any of the bch genes did not regulate their carotenoid content in response to a change in pO_2 . This indicates that the production of Bchl or some other component of the photosynthetic system (bch mutants do not produce an ICM system; Brown et al., 1972) is necessary for the correct regulation of carotenoid levels by oxygen. This therefore represents an indirect form of control by oxygen, unlike Bchl synthesis itself (Biel & Marrs, 1983).

1.4.2 Regulation of the assembly of the pigment-protein complexes

Although an understanding of the mechanisms surrounding Bchl and carotenoid synthesis per se are important to studies of the development of photosynthetic membranes, it is clear that these photopigments must be partitioned between three (or more) types of pigment-protein complex and co-ordinated with the synthesis of their respective protein components (Takemoto & Lascelles, 1973). Early studies of the regulation of the size of the photosynthetic unit (ratio of total Bchl: RC Bchl) showed that in Rb. sphaeroides this ratio increased with increasing specific Bchl content caused by decreases in light intensity or pO_2 (Aagaard & Sistrom, 1972). Moreover, the ratio of RC Bchl: LHI Bchl remained largely constant, implicating the accessory, LHII complex

as the more "sensitive" target of regulation by O_2 and light (Aagaard & Sistrom, 1972; Lien et al., 1973). In Rs. rubrum, however, there is no accessory LH complex and the size of the photosynthetic unit remained largely constant with changes in specific Bchl content (Aagaard & Sistrom, 1972). This suggested control by environmental factors at the level of membrane synthesis and the number of photosynthetic units, rather than their composition. In the case of both Rb. sphaeroides and Rs. rubrum, the molar ratio of RC:LHI Bchl was about 1:25-35 over the range of conditions studied (Aagaard & Sistrom, 1972).

In subsequent years, essentially three types of experiment have been performed to study the temporal development of the photosynthetic apparatus. These involve transfer of cultures growing aerobically in the dark to anaerobic conditions to study the specific effect of O_2 , the transfer of high-light grown photosynthetic cultures to low-light conditions under anaerobiosis to study the specific effect of light and the transfer of cultures grown aerobically in the dark to anaerobic-light conditions - a chemoheterotrophic to photoheterotrophic growth shift - to study the interaction of both O_2 and light. The results of these investigations have been thoroughly reviewed (Oelze & Drews, 1972; Kaplan, 1978; Drews & Oelze, 1981; Kaplan, 1981; Ohad & Drews, 1982; Oelze 1983a; Kelly & Dow, 1985a).

Induction of the synthesis of the photosynthetic apparatus by a drop in pO_2 alone revealed a sequential assembly of first RC and LHI complexes and afterwards LHII complexes into the membrane of Rb. capsulatus (Nieth & Drews, 1975; Schumaker & Drews, 1978), Rb. sphaeroides (Niederman et al., 1979) and Rp. palustris (Firsow & Drews, 1977). In Rs. rubrum both RC and LHI complexes were synthesised together (Oelze & Phalke, 1976).

With growth at varying light levels, it was found that the relative amounts of LHII polypeptides were increased at low intensities while the RC and LHI polypeptides remained largely constant (Takemoto & Huang Ko, 1977), i.e. the size of the photosynthetic unit was inversely related to light intensity. This was also observed upon transfer of high-light grown cultures to low light intensities (Schumaker & Drews, 1979; Firsow & Drews, 1977) where a preferential synthesis of LHII complexes occurred, although only after a growth lag (Chory & Kaplan, 1983). In chemoheterotrophic to photoheterotrophic growth shifts (Dierstein et al., 1981; Kaufman et al., 1982; Chory et al., 1984) the sequential assembly of first RC-LHI and then LHII complexes was again found, during de novo synthesis of the photosynthetic apparatus. Although similar to the effect of O₂ alone, such abrupt changes in conditions usually resulted in a growth lag.

Explanations for these observations must account for (i) the coordinated assembly of pigment and protein components of the pigment-protein complexes, (ii) the molar excess of LHI complexes over RC complexes, (iii) the mode of regulation of LHI-RC versus LHII complexes. Only recently has it been possible to begin to answer these problems, using a combination of physiological and genetic techniques.

Using continuous cultures, Arnheim & Oelze (1983b) studied the control exerted by light and oxygen on the LHI (B875) and LHII (B800-850) complexes of Rb. sphaeroides. Steady states were established in which either the oxygen concentration or the light intensity were varied and the cellular levels of B875 and B800-850 were measured. Half-maximal inhibition of B875 and B800-850 complexes occurred at 20.4% and 0.9% O₂ saturation respectively, with hyperbolic kinetics while light controlled

the levels of both complexes in an identical fashion with sigmoidal kinetics. These patterns show light and O_2 to control the complexes via the same and different mechanisms, respectively, in a similar manner to that of Bchl itself (Oelze & Arnheim, 1983). Moreover, using a mutant in which the natural activity of ALA synthetase was replaced by exogenously supplied ALA at any given rate, Oelze (1983b) showed that the "system" assembling the B875 complexes had a much higher affinity for Bchl than that assembling the B800-850 complexes. In other words, at low rates of supply of ALA (and thus Bchl) the B875 complex would always be synthesised, whereas higher rates of supply would be needed to initiate synthesis of the accessory B800-850 complex. This also explains why LHII complex formation is more sensitive to O_2 than the LHI-RC complex, since O_2 inhibits ALA synthase (Oelze & Arnheim, 1983). The fact that light apparently does not affect this enzyme, or other enzymes of the Mg-branch of tetrapyrrole synthesis (Biel & Marrs, 1983) and that it shows the same kinetics of control of the different pigment-protein complexes, once again led Oelze (1983b) to suggest a rather indirect role, possibly related to growth rate.

In these studies, the reaction centre and LHI complexes were regulated as a unit, consistent with the observations of Aagaard & Siström (1972) and Lien *et al.* (1973) and it also correlated with their known close spatial relationship within the membrane (section 1.3.1.3). An explanation for at least some of these observations came from the cloning and sequencing of the locus designated *rxca* in *Rb. capsulatus* (Youvan *et al.*, 1984a,b). This carries the structural genes for the B870 α and β polypeptides and those for the RC L and M subunits. The observation that B870 complexes were present in 10-30 fold molar excess over the RCs in the ICM was found to be due to marked differences in the

stability of certain segments of the polycistronic rxca transcript (Belasco et al., 1985). The 3' portion was rapidly degraded to give two remnants which decayed much more slowly, both encoding only the LH polypeptides. This agreed with earlier work (Dierstein, 1984) which showed that long-lived mRNA encoded the LH polypeptides when assayed in rifampicin treated cells.

Increases - up to 40 fold - in the amounts of transcripts encoding the LHI and RC proteins were observed after a drop in pO_2 by Clark et al. (1984) and for the B800-850 LHII complex by Klug et al. (1984). It may thus be concluded that O_2 levels control both transcription of the structural genes for the protein components of the pigment-protein components and the synthesis of the pigment components themselves which, at least for Bchl, is via its rate of supply (section 1.4.1).

Taken together, the data obtained over recent years implicates O_2 levels as the primary controlling factor in the regulation of the assembly of the pigment protein complexes. This occurs at several key points on both the pigment and protein biosynthetic pathways. Control by light at the molecular level is not well understood at present. The indirect physiological role postulated by Oelze (1983b) invokes a general effect of light on growth rate and hence cell protein formation to account for differences in photopigment content and the proportions of the pigment-protein complexes. On the other hand, Zhu & Kaplan (1985) found that the cellular levels of mRNA for the LHI α and β polypeptides in Rb. capsulatus were regulated by light levels as well as oxygen partial pressure. How these environmental factors achieve their effect is not known because the true "receptors" for them have not been unequivocally identified. The results of Clark et al. (1984) strongly suggested that

a regulatory element involved in the control by oxygen of transcription of the pigment-binding proteins in Rb. capsulatus is located adjacent to the rxcA locus. Further genetic analysis of this region should prove enlightening.

1.4.3 The development of the ICM system in batch cultures

If it is assumed that the cytoplasmic membrane (CM) and the intracytoplasmic membrane do indeed form a continuous membrane system (see section 1.2.3.3) one may ask the question; how is the incorporation of ICM specific components co-ordinated with the growth of ICM during the induction of the photosynthetic apparatus? Although some of the details of the formation of photosynthetically active membranes are now known (Kaplan, 1981; Oelze, 1983a; Kaplan et al., 1983) they are largely descriptive in the sense that the underlying molecular mechanisms and changes in gene expression have not been resolved. In addition it is becoming clear that, although some general principles may apply in the processes leading to the assembly of the pigment-protein complexes (section 1.3.2), steps subsequent to this are very species-specific. Four examples will serve to illustrate this point.

Rhodocyclus tenuis is a member of the Rhodospirillaceae which does not form extensive ICM; upon transfer of aerobic cultures to anaerobiosis in the light or dark, the complexes of the photosynthetic apparatus are homogeneously incorporated into the CM. The cells increase their membrane area by a change in shape (elongation) during induction (Wakim et al., 1978; Wakim & Oelze, 1980). The changes in the near IR absorption spectra during this process were somewhat different from

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those observed in other members of the Rhodospirillaceae (Wakim & Oelze, 1980), suggesting a unique mode of regulation.

In *Rs. rubrum*, Bchl is detectable within the cytoplasmic membrane of phototrophically grown cells and during induction new photosynthetic units are incorporated first into the CM and then into the rapidly growing vesicles of the ICM. This was shown by pulse-labelling experiments which demonstrated chasing of label from CM to ICM during formation of the photosynthetic apparatus (Kaplan, 1981).

Rhodobacter sphaeroides shows yet another pattern, in which putative invagination sites of the growing ICM can be isolated as a slowly sedimenting fraction on sucrose gradients and which are enriched in RC and B870 complexes (reviewed by Kaplan, 1981). This "upper pigmented band" (Niederman et al., 1979) has a small photosynthetic unit, a low cytochrome b content and low efficiency of electron transport but a high capacity for oxidative phosphorylation (Hunter et al., 1979; Bowyer et al., 1985). Label from this band can be chased into the "mature" chromatophore band on similar gradients suggesting a precursor-product relationship (Niederman et al., 1979). As the ICM invaginations grow they become sites of assembly for the B800-850 LHII complexes (Niederman et al., 1979; Chory et al., 1984). Small "indentations" and the presence of RC subunit H in the CM were posited as playing a role in the site specific assembly of the ICM (Chory et al., 1984). Although a similar situation may exist in *Rb. capsulatus*, there is less evidence for the slowly sedimenting fraction as a topological precursor for the ICM (García & Drews, 1980; Kaplan, 1981).

A final example concerns *Rp. palustris*, which contains a lamellate ICM

arrangement. Little is known about the de novo formation of these membrane systems, although during induction caused by a drop in pO_2 , RC-LHI and then LHII complexes were found to be assembled into the ICM in sequence (Firsow & Drews, 1977). The greater complexity of this type of membrane topology is clearly evident from freeze-fracture studies in which unstacked regions of the CM and both stacked and unstacked regions of the ICM were found to contain discrete size classes of intramembrane particles corresponding to the pigment-protein complexes of the photosynthetic apparatus (Varga & Staehelin, 1983; 1985a) the diameter of which varied according to the light intensity under which the cells were grown. However, until recently, virtually nothing was known about the biogenesis of the various membrane regions, the factors controlling ICM stacking or the determinants of the sites of invagination (Kelly & Dow, 1984). Interestingly, analogies have been drawn between the phenomenon of ICM stacking in Rh. palustris and that of grana membrane adhesion in the chloroplasts of higher plants (Varga & Staehelin, 1983). Here membrane adhesion is mediated by the chlorophyll a/b binding protein of the major LH complex of photosystem II (Mullet & Arntzen, 1980) through interactions with divalent cations (Steinback et al., 1979). This complex is also reversibly phosphorylated (Bennet, 1977), the level being controlled by the redox state of the plastoquinone pool (Allen et al., 1981). When phosphorylated, excitation energy is directed more to photosystem I than photosystem II, giving this LH complex a pivotal role in the distribution of excitation energy between the photosystems (Allen et al., 1981).

As photosynthetic bacteria contain only one photosystem, it seems unlikely that the full repertoire of such events would apply. Nevertheless it has now been demonstrated (Varga & Staehelin, 1985b)

that liposomes reconstituted with pigment-protein complexes from Rb. palustris show cation induced adhesion dependent upon the presence of light-harvesting complex I (B870). Treatment of ICM with Mg^{2+} also reduced the amount of LHI extractable with detergent indicating this complex to be the main adhesion factor in vivo.

Clearly, the widely differing structural features of the different types of ICM system in the Rhodospirillaceae set particular requirements for the development of the membrane and its associated photosynthetic complexes. As yet there is no explanation, in terms of efficiency for example, for the existence of so many ICM arrangements. The complexities of membrane stacking pose particular problems for understanding heterogeneity in such membrane systems and how discrete areas form during biogenesis of these structures. Even in higher plants the cause-effect relationship between grana-stacking, phosphorylation of LH complexes and their migration in the membrane is still a matter of controversy. Some answers might ensue from studies of such phenomena in the photosynthetic prokaryotes.

1.4.4 Development of the ICM system in synchronized cultures

The way in which the development of the ICM system is co-ordinated with events during the cell-cycle cannot be studied using asynchronous batch cultures. Ideally, such investigations should be carried out by following events in individual cells through their cell-cycle. However, as this is often experimentally impractical, populations of cells synchronized with respect to the cell-cycle must be used as models for the events occurring within single cells. The problems attendant to

these methods have been reviewed by Lloyd et al. (1982) and will not be reiterated here but the chief one is that of inducing perturbations within the cultures which result in artefactual behaviour.

In recent years synchronous culture techniques have been applied to Rb. sphaeroides in an attempt to elucidate the mode of ICM synthesis during the cell-cycle (reviewed by Kaplan et al., 1983). Essentially two techniques have been used. The first employs the stationary-phase cycling method of Cutler & Evans (1966) in which cells are harvested during the stationary phase in batch cultures, re-inoculated into fresh medium and the process repeated once, or twice (Ferretti & Gray, 1968; Lueking et al., 1978). During subsequent growth, the cells were reported to show a high degree of synchrony with respect to cell-division and DNA synthesis. The second technique involves transfer of high light grown cultures to lower light intensities for several hours, and then transfer to the higher light intensity once more (Lueking et al., 1981). After the transition to low-light intensity, cell number remained constant for 2.5 h before increasing (but not doubling) while the amount of DNA remained constant throughout. Cultures transferred to high light intensity subsequent to the increase in cell number were synchronized with respect to division in that the cell number doubled over a period of about 1.4 hrs. During the period of low light intensity, Bchl continued to accumulate in the absence of large increases in cell mass giving a higher specific pigment content and an indication of adaptation to the culture light conditions (Lueking et al., 1981; Chory & Kaplan, 1983).

By following changes in the density of the ICM of cells grown and synchronized in D₂O based media, Lueking et al. (1978) concluded that

the discontinuous decrease in this parameter observed during cell-division was related to a discontinuous increase in the accumulation of phospholipid into the membrane over the same time period. However, the incorporation of protein, including components of the RC and LH complexes (Fraley *et al.*, 1978), photopigments and electron transport components (Wraight *et al.*, 1978b) into the ICM occurred continuously throughout the cell-cycle. Therefore, the ratio of protein to phospholipid within the ICM was found to undergo cyclical changes during successive cell cycles (Fraley *et al.*, 1979). Unlike other membrane components, phospholipid was not inserted into pre-existing ICM until just before cell division, when previously synthesised lipid from the CM was transferred to the ICM (Cain *et al.*, 1981). This suggested the existence of a barrier (Cain *et al.*, 1981; Kaplan *et al.*, 1983) to the equilibration of phospholipid between the CM and ICM. Chromatophores of various protein: phospholipid ratios were obtained from synchronous cultures (Kaplan *et al.*, 1983) and used to show that a linear relationship existed between this and the intramembrane particle density.

The observed uncoupling between ICM protein and phospholipid synthesis during the cell-cycle of *Rb. sphaeroides* and the movement of phospholipids between membrane systems appears to be unique in comparison to other prokaryotic systems thus far studied. In *Escherichia coli*, several studies have demonstrated continuous insertion of membrane protein through the cell-cycle (Churchward & Holland, 1976; Boyd & Holland, 1979) and it is likely that phospholipid synthesis is also continuous (Churchward & Holland, 1976) although the evidence is more equivocal (Lloyd *et al.*, 1982).

The observations reviewed above led to the formulation of a model (Kaplan et al., 1983) of how ICM biosynthesis in Rb. sphaeroides proceeded through the cell-cycle. Subsequent to cell division, the packing density of photosynthetic units within an ICM invagination was thought to be low before increasing during the current cell-cycle and reaching a maximum before cell-division. Incorporation of phospholipid at this time would lead to a decrease in packing density once more due to a dilution effect. The cell then divides and the process is repeated.

Essentially the same data with regard to the protein:phospholipid ratio, upon which the model is largely based, were obtained using both the stationary phase cycling and the light-induced method of obtaining synchronous cultures (Lueking et al., 1981). In addition, chromatophores artificially fused with liposomes in order to dilute the components of the electron transport chain showed decreases in the rate of Cyth-561 reduction and this could also be observed with chromatophores obtained post-division compared to pre-division (Snozzi & Crofts, 1984). This provided additional evidence for the basis of the observed changes in protein:phospholipid ratio.

Although the model of Kaplan et al. (1983) is consistent with the experimental data, the synchronization procedures were based on induction methods which may themselves be a source of potential artefacts. The stationary phase cycling method produced cultures with a 15-20% variation in cell-cycle time (Lueking et al., 1978) and this may be related to the physiological changes occurring during repeated transitions between exponential and stationary phase growth. The pattern of DNA synthesis was observed to be continuous by Ferretti & Gray (1968) while a discontinuous pattern was found by Lueking et al.

(1978) using the same method. In addition an accurate doubling of cell numbers was often not achieved using either this or the light-induced method and could be due to doublet or chain formation in Rb. sphaeroides under certain growth conditions (Lueking et al., 1978).

In an attempt to confirm and extend the results of Kaplan and co-workers, Knacker et al. (1985) used a "selection" method based on centrifugation, and found essentially the same pattern of discontinuous lipid synthesis during the cell-cycle. However, as in all the studies reviewed above, synchronous growth was followed only by cell count; the morphological changes accompanying progression through the cell cycle in Rb. sphaeroides have not been reported in the literature.

The importance and complexity of the interaction between the growth of the cell envelope - including internal membrane systems - and the growth pattern of the cell, as a whole is clearly evident, yet this has not been studied extensively in photosynthetic prokaryotes. This aspect is therefore next considered in a wider context.

1.5 Cellular Differentiation and the Role of the Cell Envelope

1.5.1 Concepts of morphogenesis and differentiation

The study of cell-cycles and cellular development in prokaryotic organisms is usually justified on the basis that it enables the right kind of questions to be asked about similar processes which occur in eukaryotes. In this section I briefly consider some of the terms relevant to studies of prokaryotic differentiation and then briefly discuss some of the more familiar prokaryotic "model systems", before a more detailed description of the properties of the prosthecate and budding bacteria and their utility in membrane studies.

Whittenbury & Dow (1977) defined a series of terms which are important for the description of the cell-cycles and cell interactions observable in prokaryotes. The term morphogenesis was used to describe changes in external morphology and internal cell architecture only, while differentiation described those events which lead to the production of a (functionally) distinct cell-type. Differentiation thus implies knowledge of the functional changes which occur during morphogenesis. The term development was defined as a composite process which would involve morphogenesis and differentiation under intercellular influence. Here the differentiated cells would influence the activity of the whole cellular complex. This type of interaction can be seen in filamentous cyanobacteria which form heterocysts to fix N_2 for the whole filament (Carr, 1979).

Cell-cycles of varying degrees of complexity can result from the operation of these processes. Monomorphic cell-cycles produce only one morphological type of cell under all conditions, for example E. coli (Donachie, 1979). A dimorphic cell cycle is one which produces two morphologically different cell-types at division as in Caulobacter (Shapiro, 1976) and some of the prosthecate and budding bacteria (section 1.5.2). Polymorphic vegetative cell-cycles describe those cases where alteration of the nutrient regime causes the production of two or more morphologically distinct cell-types each of which can undergo their own distinct and stable cell-cycles. Examples include the sphere-rod transition in Arthrobacter (Clark, 1979) the actinomycete Geodermatophilus (Ishiguro & Wolfe, 1970) and Rhodomicrobium vannielii (section 1.5.2.4). Spore and cyst cycles, in addition to the vegetative cell-cycles can also occur in all of these types.

The ideal prokaryote for studies of morphogenesis and differentiation could be E. coli simply because of the vast amount of biochemical and genetic information available. However, E. coli has such a simple, monomorphic cell-cycle that its use in such studies is limited. The only morphologically observable "landmark" event is that of cell-division although periodic events such as the initiation and termination of chromosome replication, nucleoid segregation and septation do obviously occur at the molecular level (Donachie, 1979). The analysis of over 750 individual proteins by 2-dimensional gel electrophoresis (Lutkenhaus et al., 1979) indicated that none were synthesised at different rates during the cell-cycle. Even in this well studied species the regulation of the events of the cell cycle have not been fully resolved.

Therefore microbes exhibiting more complex patterns of morphogenesis have been sought. The most intensively studied prokaryotic differentiation system is probably that of sporulation in Bacillus subtilis and related microbes (Chambliss, 1979; Szulmajster, 1979). The actinomycete genus Streptomyces has also been studied with respect to spore formation (Kalakoutskii & Agre, 1976) but although genetic analysis is well developed (Chater & Merrick, 1979) the inherent problems in cultivating such mycelia producing bacteria have prevented their widespread exploitation in studies of differentiation. Cellular aggregation, fruiting body formation and myxospore formation in the myxobacteria (Kaiser et al., 1979) is another prokaryotic differentiation system in which genetic and molecular biological analysis is well advanced. However it is in the Cyanobacteria that prokaryotic cellular diversity reaches its peak and the formation of specialized cell types - heterocysts, akinetes, hormogonia and tapered cells for example have all been investigated to varying degrees (see Carr & Whitton, 1982).

These few examples illustrate the essential diversity of prokaryotic differentiation. Many of the systems currently used exploit nutritional shifts to initiate the desired sequence of events. This could be problematic since changes due to this shift must be distinguished from those specific to differentiation processes per se. In addition, a majority of such systems are asynchronous and with gene expression occurring from more than one cell-type simultaneously.

The prosthecate and budding bacteria, described in detail below, present some features which circumvent such problems because their differentiation programme occurs within the cell-cycle itself.

1.5.2 The prosthecate and budding bacteria

The prosthecate bacteria are those organisms which possess integral cellular extensions of a diameter always smaller than that of the cell itself and which are bounded by the cell wall and cell membranes (Staley, 1968). They are thus distinct from microbes such as Planctomyces, Gallionella or Nevskia, which although producing cellular extensions, do not produce prosthecae (Dow & Whittenbury, 1979). All prosthecate bacteria reproduce by a polar growth mechanism, a concept expanded upon in section 1.5.2.1 and commonly called "budding" (Hirsch, 1974; Whittenbury & Dow, 1977; Dow & Whittenbury, 1979, 1980; Kelly & Dow, 1984; Schmidt & Starr, 1985). These organisms are characteristic of, but not exclusive to, oligotrophic aquatic environments (Moore, 1981; Poindexter, 1981a,b; Morgan & Dow, 1985) and they are not bound together by any criteria - taxonomic or nutritional - except that of polar growth and "prosthecateness" (Dow & Whittenbury, 1979, 1980). Reviews of the biology of the prosthecate and budding bacteria have appeared at regular intervals (Schmidt, 1971; Hirsch, 1974; Whittenbury & Dow, 1977; Moore, 1981; Poindexter, 1981a,b) and the reader is referred to these for a comprehensive treatment of the group.

Despite many years of investigations and speculation, the precise function of the prostheca as a cellular organelle is still a contentious issue. Prosthecate bacteria can be distinguished in which this structure is obligately involved in reproduction, e.g. Rm. vannielii, Hyphomicrobium and Rp. palustris, and others in which it is not, although here, the expression of the structures may nevertheless be an obligate part of the cell-cycle as in Caulobacter or non-obligate as in Ancalamicrobium (Shapiro, 1976; Whittenbury & Dow, 1977). Other roles

currently discussed include attachment to interfaces (Hirsch, 1974; Morgan & Dow, 1985), the uptake of nutrients especially from low nutrient environments (Kuznetsov *et al.*, 1979; Poindexter, 1981b; Morgan & Dow, 1985), and as a device for the promotion of colony development at interfaces in aquatic environments (Dow & Whittenbury, 1980).

It is thus clear that the prostheca functions differently in different groups of prosthecate bacteria. The wealth of morphological diversity which is a characteristic of the prosthecate and budding bacteria has opened up new vistas for the study of prokaryotic differentiation. These characteristics are now considered.

1.5.2.1 Polar growth and cellular asymmetry. Polar growth results from the insertion of new envelope material at a single active growth point such that the progeny cell produced by such a process is not equivalent to the cell from which it is formed in terms of age (Whittenbury & Dow, 1977; Kelly & Dow, 1984; Schmidt & Starr, 1985). At fast growth rates the insertion of cell envelope material in microbes such as *E. coli* occurs at multiple sites (Donachie & Begg, 1970) but at doubling times in excess of 60 minutes there is evidence from phage-attachment studies (Begg & Donachie, 1977) that cell growth is polar, although not all studies agree with this interpretation (Trueba *et al.*, 1982). As far as is known the prosthecate and budding bacteria exhibit an obligate polar growth pattern at all growth rates (Kelly & Dow, 1984) and most of them in fact exhibit rather long generation times.

From a number of investigations (Tyler & Marshall, 1967; Shapiro, 1976; Dow *et al.*, 1983) it is clear that cell-division in microbes which grow by asymmetric polar growth does not yield identical progeny, i.e.

siblings. Rather, a "mother-daughter" relationship exists (Whittenbury & Dow, 1977). This is one important consequence of the polar growth process and it results from the mother or reproductive cell being composed of structural material formed primarily in a previous cell-cycle. The daughter progeny cell is composed of new structural material formed during the current cycle (Kelly & Dow, 1984). This leads to a general property of the cell-cycles of polarly growing organisms that was initially recognized by Whittenbury & Dow (1977); that the mother cell ages beyond the division time and is therefore "mortal". Consider the generalized cell-cycle in Fig. 1.5. At division, the mother cell can immediately initiate another round of DNA replication closely coupled with polar envelope growth, to result in the production of a new progeny cell, while the daughter cell resulting from this division cannot initiate reproduction until it has passed through an obligate period of differentiation. Clearly, different mechanisms must be operating in the two progeny cells to co-ordinate DNA synthesis and continued envelope growth on the one hand and to delay DNA synthesis and initiate differentiation on the other. How the cell achieves such control is unknown, although it is known that only a limited number of reproductive cycles from any given "mature" mother cell are permitted (Whittenbury & Dow, 1977; Dow & Whittenbury, 1979, 1980; Poindexter, 1981a). The difference in the cell-cycle time between mother and daughter cell is clearly due to the time taken for the daughter cell to progress through the so-called maturation phase - the obligate differentiation events leading to the production of a reproductive unit.

1.5.2.2 Intra-cytoplasmic membrane systems in polarly growing bacteria.

An important possibility afforded to those bacteria exhibiting polar growth patterns is the formation of complex, asymmetrically arranged

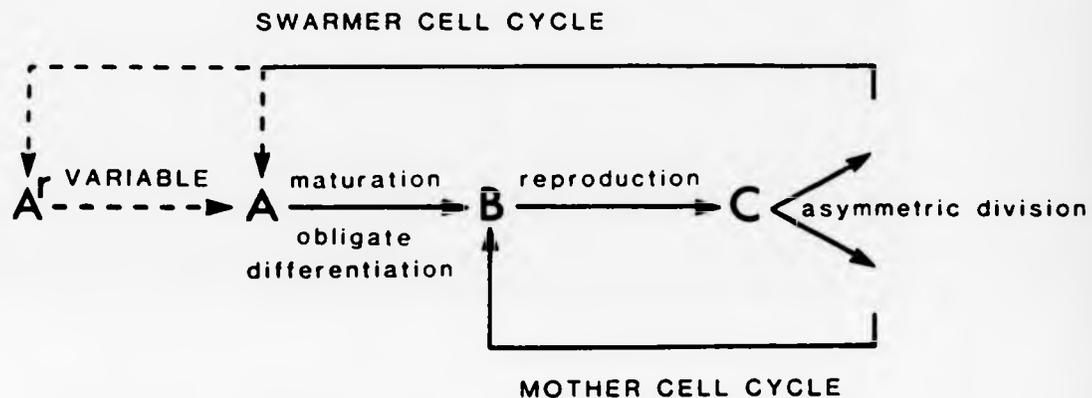


FIGURE 1.5. Generalized cell cycle in prosthecate and budding bacteria. The swarmer cell (A^r) can delay differentiation until conditions become favourable for commitment (A) to form a mature reproductive unit (B) and pre-divisional cell (C). The mother cell can immediately initiate another round of reproduction while the daughter or swarmer cell cycle is longer by a time factor equal to the maturation phase, assuming immediate commitment. (Figure from Morgan & Dow, 1985.)

intra-cytoplasmic membrane systems (Kelly & Dow, 1984). Examples of groups in which polar growth and extensive ICM development exist are the ammonia, methane and nitrite oxidizing bacteria (Dow & Whittenbury, 1980). These bacteria may have particular bioenergetic reasons for needing to increase membrane surface area with a concomitant increase in the number of membrane bound substrate oxidizing sites. However, this correlation is most clearly seen in the genus Rhodospseudomonas in which the photosynthetic prosthecate and budding bacteria all contain a lamellate ICM system, housing the photosynthetic apparatus (section 1.2.3.3). Whittenbury & McLee (1967) first noted this relationship in Rd. palustris and Rd. viridis and suggested that the polar growth process would carry the cell division point beyond the lamellate stacks in the cell body, thereby avoiding problems of dividing through them. In such species, the membrane lamellae are formed at the pole opposite to that used for reproduction and the daughter cell must therefore synthesise a new membrane system de novo. This could have a specific advantage in photosynthetic species in that it would allow the daughter cell membrane content and composition to be matched to the prevailing environmental conditions (for example oxygen concentration and light intensity) that it finds itself in at division (Kelly & Dow, 1984). This pattern of membrane development appears very different from that found in, for example, Rb. sphaeroides and related species with a vesicular ICM system which is not asymmetrically arranged. However, there are no studies in the literature which document the pattern of insertion of cell membrane components during the cell-cycle of any photosynthetic prosthecate and/or budding bacteria.

1.5.2.3 The photosynthetic prosthecate and budding bacteria. The adoption of an obligate polar growth mechanism clearly has a number of

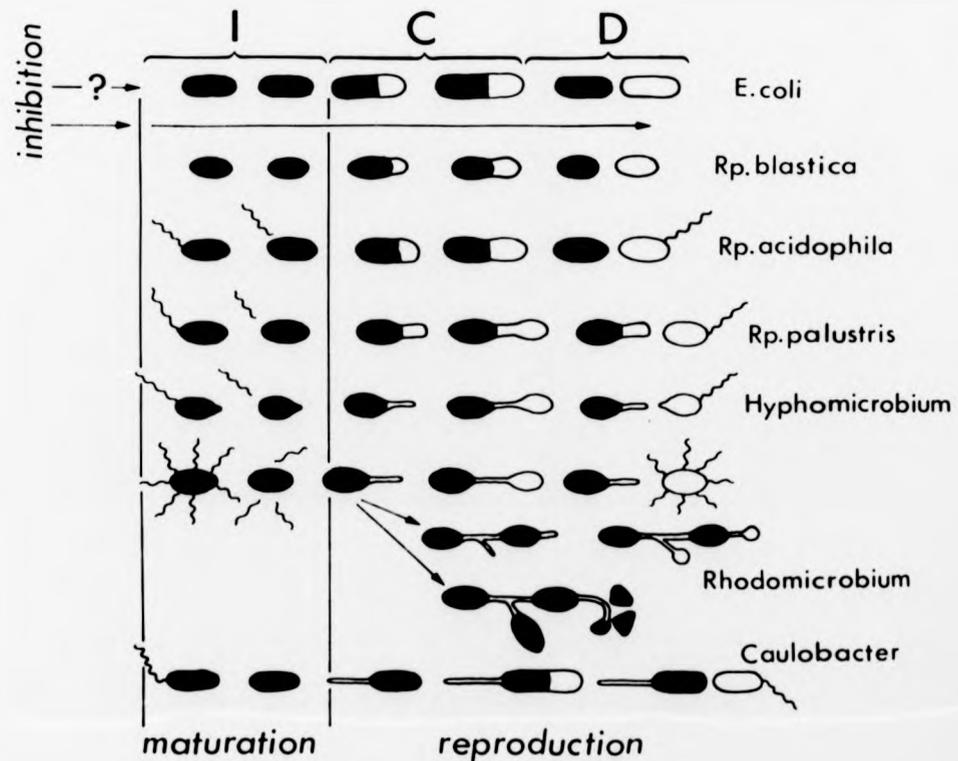
important consequences, which have led to the evolution of an asymmetric cell structure and the expression of functionally distinct cell-types (Dow et al., 1983; Kelly & Dow, 1984). Nowhere is this observed more clearly than in the photosynthetic prosthecate and budding members of the Rhodospirillaceae (Fig. 1.6). For example, in Rp. palustris cultures, there exist two major cell-types. Both show polarity in the distribution of surface structures and cellular orientation (Whittenbury & Mcleee, 1967). These cell-types are non-motile reproductive (prosthecate) cells which have the potential for attachment to surfaces via a polar holdfast and ovoid motile swarmer cells with polar flagella (Westmacott & Primrose, 1976). Reproduction occurs by extension from the polar growth point located at the distal end of the prostheca, followed by asymmetric division. Rhodopseudomonas viridis has an identical cell cycle (Fig. 1.6) but these microbes are merely points along a spectrum of morphological types within the Rhodospirillaceae.

One of the simplest cell-cycle types is exemplified by Rp. blastica (Eckersley & Dow, 1980). Here polar growth results in the production of morphologically identical progeny with division being symmetrical (Fig. 1.6). Nevertheless, the progeny exhibit different cell-cycle times with one cell - the daughter or newly produced cell - initiating envelope growth only after a "lag" period (Eckersley & Dow, 1980).

Rhodopseudomonas acidophila exhibits a more pronounced polarity in that a motile "swarmer" cell with a sub-polar tuft of flagella (Tauschel & Hoeniger, 1974) is produced at division (Fig. 1.6). These two members of the Rhodospirillaceae illustrate the point that "prosthecateness" is by no means essential to produce differential cell-type expression. It is the mode of growth that is the essential determinant. The prostheca, however may offer particular advantages in that the swarmer cell could

FIGURE 1.6. A morphogenetic gradient of the cell-cycles of several prosthecate and budding bacteria in relation to that of *E. coli*.

The cell-cycles of the former can be categorized into three characteristic stages (a) the inhibition phase, when the daughter cell is prevented from initiating differentiation as a consequence of an inadequate nutrient regime, (b) the maturation phase and (c) the reproductive phase. When a cell becomes a competent reproductive unit it undergoes repetitive rounds of replication without needing to undergo a second maturation phase. The physiological events characteristic of these phases can be compared with those of *E. coli* growing with a generation time in excess of 60 min, such that the I phase is comparable to the maturation phase, the C + D phases corresponding to reproduction (Figure from Kelly & Dow, 1984).



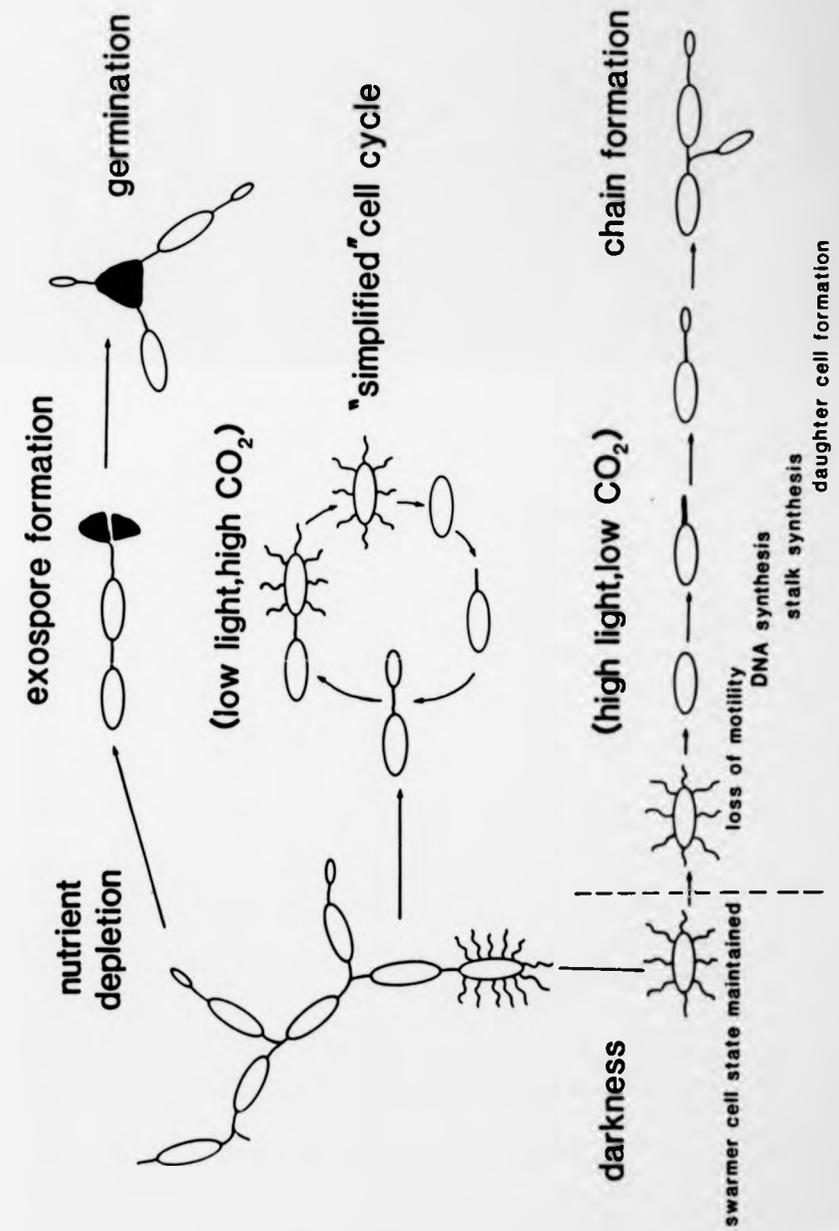
be liberated "at a distance" from the mother cell - which may be located in a potentially crowded biofilm at an interface in the natural environment. The sequence depicted in Fig. 1.6 could therefore be viewed as a kind of "morphogenetic evolution", culminating in Rm. vannieli which exhibits the most extreme degree of cell-type expression and specialization.

1.5.2.4 Cell-type expression in Rm. vannieli. Rhodomicrobium vannieli is characterized as a Bchl_a containing member of the Rhodospirillaceae which is capable of good photoheterotrophic growth on a variety of substrates but which grows less well under aerobic or microaerophilic conditions (Pfennig, 1970; France, 1978; Whittenbury & Dow, 1977). Growth factors are not required, N₂ can be actively fixed and the ribulose 1,5 bisphosphate carboxylase has been isolated and characterised (Taylor & Dow, 1980).

Rhodomicrobium vannieli was first isolated by Duchow & Douglas (1949) and was initially considered the photosynthetic counterpart of Hyphomicrobium. The reproductive mechanism as that of budding was confirmed by Murray & Douglas (1950) who also studied changes in the "nuclei" during bud formation by staining. Ultrastructural studies (Conti & Hirsch, 1965; Trentini & Starr, 1967) showed the presence of a peripheral lamellate membrane system and the presence of cross-walls or plugs within the prosthecae. These early studies were largely aimed at clarifying the taxonomic position of the organism. Later studies (Gorlenko, 1968; Dow, 1974; Gorlenko *et al.*, 1974; Whittenbury & Dow 1977) clearly indicated the variety of cellular expressions possible under defined environmental conditions, and this precipitated the use of Rm. vannieli in studies of cellular differentiation (Fig. 1.7).

FIGURE 1.7. Cellular expression in *Rm. vannielii*.

The basic cellular unit - the multicellular array - can give rise to a variety of cell types depending upon the environmental conditions. The simplified cell-cycle allows constitutive swarmer cell production in the absence of multicellular arrays, while nutrient depletion can induce the formation of angular exospores. These can germinate to form a new multicellular array (Modified from Dow *et al.*, 1983).



In batch cultures grown photoheterotrophically the characteristic cellular expression consists of multicellular arrays of cells (Fig. 1.7) linked by prosthecae from the tips of which ovoid, peritrichously flagellate swarmer cells are produced. Some strains of Rm. vannielii are characterised by a "simplified" cell-cycle (fig. 1.7) in which the constitutive production of swarmer cells occurs in the absence of multicellular arrays. This can be "induced" in "complex" cultures by prolonged sub-culturing under conditions of high CO₂ concentration and low light intensities (France, 1978; Dow & France, 1980), but once formed the condition is often stably maintained. The genetic and physiological bases for the simplified cycle are at present obscure. It is however, possible that the function of such a cell-cycle could be to ensure the dispersal of large numbers of swarmer cells upon the onset of potentially unfavourable conditions, as might occur in the centre of a dense multicellular array or microcolony, for example.

Under severe nutrient limitation, on poor carbon sources, or when strains are first isolated another cell-type can be formed; the exospore (Fig. 1.7). Exospores are angular, rather heat resistant structures produced from the prostheca tips and would appear to ensure the long-term survival of the organism. They have a complex fine structure with a membrane system but much reduced photopigment content (Whittenbury & Dow, 1977). Germination upon return of favourable conditions can occur in several ways, often sequentially from the apices by prostheca outgrowth.

Rhodospirillum rubrum is therefore unique amongst photosynthetic bacteria in producing at least three types of differentiated cell (chain cell, swarmer cell and exospore) in two vegetative cell cycles (complex

or simplified) and by two modes of reproduction (unidirectional polar growth - swarmer cell and exospore formation, or plug formation within the prosthecae - the physiological separation of cells within the arrays). The morphological differences between the multicellular arrays and the swarmer cells has been exploited to enable the production of synchronized swarmer populations by a simple filtration procedure through glass wool (Dow, 1974).

It is the swarmer cell type that has received greatest attention with regard to its production, cell-cycle and the sequence of molecular events during differentiation which lead to the production of a mature reproductive unit.

1.5.3 The swarmer cell concept

Comparative biochemical and physiological studies of the cell cycles of Caulobacter, Hyphomicrobium and Rhodomicrobium have indicated that the daughter cells produced at division are not simply "immature" versions of the mother cell with a longer cell cycle (Dow & Whittenbury, 1980). They represent a specialized cell-type - the swarmer cell - the prime function of which is dispersal. Swarmer cells are usually highly motile, show low levels of endogenous mRNA and protein synthesis (Shapiro, 1976; Potts & Dow, 1979; Dow et al., 1983; Porter, 1985) no DNA synthesis (Newton, 1972; Potts & Dow, 1979; Wali et al., 1980) and their nucleoid is in a highly condensed format with a high sedimentation value (Evinger & Agabian, 1979; Dow et al., 1985). At least in Rm. vanniellii (Dow et al., 1983, 1985) rRNA synthesis is also suppressed in the swarmer cell. These features indicate a non-growing, metabolically

quiescent state but with the facility to maintain motility and, presumably, tactic responses. From an ecological viewpoint it is the ability of the swarmer cell to remain in this condition until the environment becomes favourable for growth and reproduction - i.e. to initiate differentiation - that is its distinguishing characteristic (Dow & Whittenbury, 1980). Implicit in this view is a capacity for environmental sensing so that responses are possible over a relatively short time period. The life of a swarmer cell is thus transient and is not comparable to that of a spore, for example.

The control of swarmer cell formation and differentiation has been studied with respect to one environmental factor - light - in Rm. vannielii. During growth in batch culture, the numbers of swarmer cells increase due to the light-limitation caused by self-shading which occurs towards the late-exponential phase of growth (Dow et al., 1983; Porter, 1985). Light-limitation inhibits swarmer cell differentiation by increasing the length of the variable I phase of the cell cycle (Porter, 1985). When kept under anaerobic dark conditions, swarmer cell populations are prevented from initiating differentiation and remain motile and viable for several hours and several days respectively (Porter, 1985). However, when re-illuminated they synchronously (>90%; Whittenbury & Dow, 1977) initiate differentiation (Fig. 1.7). The major landmark events are the loss of flagella (filament and hook) and thus motility, the synthesis of the prostheca at a polar location on the cell-surface and the production of a daughter cell from the distal end of the prostheca (Fig. 1.7). Further growth results in the formation of a new multicellular array but the process beyond the two-cell stage or "pair" is asynchronous (Whittenbury & Dow, 1977; Potts & Dow, 1979).

The generality of the swarmer cell concept was discussed by Dow & Whittenbury (1980). There are a number of species which appear to have a motile cell-stage during their cell-cycles and which multiply by polar growth, for example the genus Rhodospseudomonas as presently constituted (Imhoff et al., 1984); Nitrobacter and some other ammonia or nitrite oxidising bacteria; Bdellovibrio, which produces a free-living motile cell prior to intraperiplasmic growth in other bacteria (Thomashow & Rittenburg, 1979); Sphaerotilus and Leptothrix (Dow & Whittenbury, 1980), two genera of sheathed bacteria which produce motile dispersal phases and a number of genera of the non-photosynthetic prosethcate and budding bacteria (Morgan & Dow, 1985; Schmidt & Starr, 1985). In these microbes, the swarmer cell is clearly a dispersal vehicle. However, in view of the relationship between polar growth and differential cell type expression, discussed above, one may consider a very large group of bacteria to have cell-stages which possess at least some of the distinguishing features of swarmer cells if not overt motility. The general name "shut-down" or "growth precursor" cell has been proposed (Dow et al., 1983) for such stages. An important property is the phase of the cell-cycle that these cells maintain themselves in; the I phase. In the E. coli cell cycle this has been defined as the time from cell division to the initiation of chromosome replication and is when initiation components are synthesised and complexes assembled to allow replication to proceed at all chromosome origins within the cell (Helmstetter et al., 1979). In fact E. coli itself may exhibit polar growth (Begg & Donachie, 1977) and a prolonged I phase at low dilution (growth) rates in chemostat culture (Skarstad et al., 1983). Under these conditions it has been proposed (Dow et al., 1983) that progeny cells may exhibit some of the properties of the more "classical" swarmer cells, thus indicating heterogeneity within such cultures.

Interestingly, Falkinham & Hoffman (1984) have recently drawn attention to the unique developmental characteristics of the "swarm" and "short" cells of Proteus vulgaris and P. mirabilis which differ from each other in outer membrane protein composition and enzyme complement. In their view, "swarming" in these species should be regarded as an example of differentiation.

Clearly, more evidence is needed in order to judge the validity of a generalized swarmer cell concept.

1.6 Conclusions from the Literature

From the foregoing literature survey, the following points emerge which are of relevance to the aims of the present work.

- (i) Most of the concepts of photosynthetic and respiratory energy conversion in the Rhodospirillaceae have been derived from work with two species of Rhodobacter which are assumed to be representative of the family.
- (ii) The notion that cultures of polarly growing photosynthetic bacteria may be heterogeneous with regard to cell-type has not been widely recognized.
- (iii) Far more is known about the biogenesis of vesicular ICM systems than those of a lamellate nature.
- (iv) Cell-cycle studies have only been performed with Rb. sphaeroides, largely using induction synchronization methods.

The present study was therefore aimed to redress the balance in favour of the "morphologically exciting" members of Rhodospirillaceae.

2. MATERIALS AND METHODS

2.1 Organisms and Media

Table 2.1 lists the members of the Rhodospirillaceae used in this study. Bacteria were grown routinely in pyruvate-malate mineral salts (PM) medium (Whittenbury & Dow, 1977) which contained;

	gl ⁻¹ distilled water
NH ₄ Cl	0.5
MgSO ₄ .7H ₂ O	0.4
NaCl	0.4
CaCl ₂ .2H ₂ O	0.05
sodium hydrogen malate	1.5
sodium pyruvate	1.5

For all species except Rm. vanniellii, which has no growth factor requirements, the medium was supplemented with 0.1% (w/v) yeast extract. The pH of the medium was adjusted to 6.8-6.9 with potassium hydroxide (KOH) pellets prior to autoclaving at 121°C for 15 min. After cooling, sterile phosphate buffer was added aseptically to a final concentration of 5 mM.

0.1 M phosphate buffer stock solution contained; sodium dihydrogen phosphate NaH₂PO₄.2H₂O, 7.8 gl⁻¹ and disodium hydrogen phosphate Na₂HPO₄, 7.1 g l⁻¹ pH 6.8.

For solid media, Difco Bacto-agar was added (1.5% w/v) before autoclaving and plates or agar deeps poured after cooling to 60°C.

TABLE 2.1. Sources of strains used in this study

Species	Strain number	Reference
<u>Rs. rubrum</u>	NCIB 8255	
<u>Rc. tenuis</u>	ATCC 25093	
<u>Rc. gelatinosus</u>	NCIB 8290	
<u>Rb. sphaeroides</u>	NCIB 8253	
<u>Rb. sphaeroides</u> "cordata"/81-1	ATCC 33575	Gest <u>et al.</u> (1983)
<u>Rp. acidophila</u>	ATCC 25092	
<u>Rp. palustris</u>	NCIB 8288	
<u>Rp. viridis</u>	ATCC 19567	
<u>Rm. vanniellii</u>	Rm5	Dow (1974), Whittenbury & Dow (1977)

2.2 Maintenance of Cultures

Bacteria were maintained as stab cultures in PM agar deeps contained in universals. After inoculation from exponential phase liquid cultures, they were incubated at 30°C for 3 d under an incident light intensity of $35 \mu\text{E m}^{-2} \text{s}^{-1}$ then kept at room temperature. Culture purity was checked by phase contrast microscopy and streaking to obtain isolated colonies on PM agar plates. Plates were incubated under anaerobic conditions in the light using the anaerobic bag technique described by Westmacott & Primrose (1975). Plates were placed on a tray within transparent nylon bags (Portex Ltd., Hythe, Kent) which were heat sealed and flushed with O_2 free N_2 for 15 minutes. A beaker containing saturated pyrogallol solution (15 ml) and 15% (w/v) potassium carbonate / 10% (w/v) sodium hydroxide solution (15 ml) was also included within the bag to remove traces of oxygen. Incubation was at 30°C at a light intensity of $35 \mu\text{E m}^{-2} \text{s}^{-1}$.

2.3 Photoheterotrophic Growth Conditions

Small scale cultures were grown in 100 ml PM contained in 250 ml B19 Quickfit conical flasks. After inoculation (1% v/v) the flasks were capped with rubber Suba Seals (William Freeman & Co., Barnsley, W. Yorkshire) and flushed with O_2 free N_2 for 15 min through inserted syringe needles. Flasks were incubated in a shaking water bath at 30°C under a light intensity of $25 \mu\text{E m}^{-2} \text{s}^{-1}$.

Larger scale photosynthetic cultures were grown in 5, 10 or 20 l flat-bottomed vessels (244/1350, Baird and Tatlock) with Quickfit tops which

could be sealed with Suba-Seals. After inoculation (0.2-0.4% v/v) the vessels were flushed with O₂ free N₂ for 30 min and incubated at an incident light intensity of $35 \mu E m^{-2} s^{-1}$ in a warm room at 30°C. All cultures were stirred continuously by magnetic stirrers. Growth was followed by the optical density of cultures at 540 or 650 nm in a Pye-Unicam SP500 spectrophotometer.

For growth experiments at different light intensities, PM contained in flat pyrex Roux tissue culture bottles (1 l) was inoculated (0.2% v/v) and incubated with stirring at 30°C in a warm room. Incident light intensities of 85, 35, 17 and $6.5 \mu E m^{-2} s^{-1}$ were obtained by varying the distance between the tungsten light source (100 W bulbs) and the culture vessel. At the highest light intensity, a heat-filter consisting of a water-filled Roux bottle was placed in front of the lamp. Water-filled culture vessels were initially set up to check that the temperature within them remained at 30°C.

2.4 Chemoheterotrophic Growth Conditions

Rhodospirillum rubrum was grown under aerobic conditions in darkness in 1 l volumes of PM contained in 2.5 l conical flasks plugged with cotton wool and covered in foil to exclude light. The flasks were inoculated (2% v/v) from late exponential phase phototrophically grown cultures, and incubated at 30°C on a gyratory shaker at 175 rpm. For growth at different O₂ tensions, the volume of PM in the flasks was varied and the inoculum size altered proportionately.

2.5 Chemoheterotrophic to Photoheterotrophic Growth Shifts

20 l PM in a foil covered aspirator (Baird & Tatlock) was inoculated with 100 ml of a phototrophically grown Rm5 culture. Air was bubbled through the medium at a flow rate of 120 ml min^{-1} via a cotton wool filter. The cultures were incubated at 30°C with magnetic stirring. At the desired time, the air-flow was stopped and changed to O_2 free N_2 for 10 min before the foil was removed, the vessel sealed, and the culture incubated at an incident light intensity of $35 \mu\text{E m}^{-2}\text{s}^{-1}$. Samples were taken through the aspirator tap at various intervals after the shift.

2.6 Anaerobic Dark Growth of Rm. vannielii

To test if Rm. vannielii could be grown under anaerobic dark conditions, the mineral salts base of PM medium was supplemented with 1.5 g l^{-1} fructose instead of the usual carbon sources and either 30 mM trimethylamine-N-oxide (TMAO) or dimethylsulfoxide (DMSO), the latter added aseptically after autoclaving (121°C , 10 min) along with the phosphate buffer. The medium was contained in foil covered bottles filled to the neck and capped with Suba-Seals. After inoculation (1% v/v) and flushing the headspace with O_2 free N_2 (15 min), the bottles were incubated at 30°C .

2.7 Culture Harvesting

Unless otherwise stated, cultures were harvested in the late-exponential phase of batch growth ($\text{OD}_{540 \text{ nm}} = 1.0\text{-}2.0$) by centrifugation at 20,000

mg for 20 min at 4°C. When necessary, cell pellets were stored at -20°C.

2.8 Preparation of Synchronized Swarmer Cell Populations

Swarmer cells of Rm. yannielii were prepared by passing a late exponential phase culture through a glass wool column using a modified version of that described by Whittenbury & Dow (1977). An 80 cm long x 7.0 cm diameter glass column was packed to a depth of 33 cm with glass wool (BDH), covered in aluminium foil and sterilized by dry heat at 160°C for 16 h. This also served to loosen impurities in the glass wool which were subsequently removed by washing the column with 2-4 litres of sterile distilled water. The column was positioned over a foil covered aspirator (20 l) in which the swarmer cells were collected. Both the aspirator and the column were continuously flushed with O₂ free N₂. The culture was poured manually into the column and the first 500 ml of eluate discarded. After the swarmer cells were collected, the aspirator was fitted with a silicone bung equipped with two metal tubes through which the headspace could be gassed with nitrogen. After 5 min the tubes were connected to two neoprene rubber gas bladders filled with nitrogen (capacity ca. 1000 ml each). This allowed successive samples to be taken from the aspirator without disturbing the gas atmosphere. The swarmer cells were allowed to remain under these anaerobic dark conditions at 30°C for 15 min subsequent to synchronization to allow equilibration of the gas atmosphere, before the foil was removed and incubation continued at an incident light intensity of 35 $\mu\text{E m}^{-2}\text{s}^{-1}$ with magnetic stirring. At the same time, an aliquot of swarmer cells was removed and maintained under dark anaerobic conditions.

This procedure was suitable for culture volumes of 5 l to 20 l. For smaller cultures (500 ml to 2 l) a smaller column was used (47 cm long, 1.7 cm dia. packed to a depth of 18 cm with glass wool).

The homogeneity of the synchronized swarmer cell population was established by phase-contrast microscopy and cell-volume distribution analysis (section 2.10). Routinely, between 5×10^7 and 2×10^8 swarmer cells ml^{-1} were obtained from the column (determined by Coulter-counting: section 2.10) representing about 10% of the initial optical density of the asynchronous culture (A540 = 0.15 - 0.25).

2.9 Preparation of Multicellular Arrays from Batch Cultures

The cells remaining on the column after synchronization could not be used as a source of multicellular arrays because of the presence of swarmer cells and the difficulties of washing the arrays free. Therefore arrays were prepared by differential centrifugation. Fifty ml culture volumes dispensed into MSE "Oakridge" polycarbonate centrifuge tubes were centrifuged at 2,000 rpm in the swing out rotor of an MSE Mistral 4L centrifuge for 3 minutes. The supernatant, containing mostly swarmer cells was removed and the loose pellet gently resuspended in 10 mM Tris-HCl buffer pH 7.4 (20 ml). The procedure was repeated three times with the final pellet resuspended in Tris buffer and stored frozen at -20°C . The homogeneity of the preparations was checked by phase-contrast and electron microscopy (section 2.9) and cell-volume distribution analysis (section 2.10).

2.10 Cell-Volume Distribution Analysis

A Coulter Counter model ZBI and Coulter Channelyzer C1000 connected to a BBC microcomputer via local interface electronics was used to determine size distributions and cell-count. Culture or cell samples (50-200 μ l) were diluted into 20 ml of balanced salt electrolyte (Isoton; Coulter Electronics Ltd.) and profiles obtained using a 30 μ m orifice probe. Profiles were stored on floppy disc and printed on a Tandy TRS-80 Plotter for comparison. The computer programme used included a calibration file which compared the cell-volumes with those of latex particle standards of known size distribution. Cell-counts were taken as the mean of five determinations, again using the 30 μ m orifice probe and an amplification setting of 0.5.

2.11 Radioisotopic Labelling of Cells

Cellular proteins in heterogeneous batch cultures or differentiating swarmer cell populations were routinely labelled using L-³⁵S-methionine of a specific activity >600 Ci mmol^{-1} . Radiolabel was diluted in distilled water and made anaerobic by passing nitrogen over the surface of a small aliquot before it was added to cultures to a final activity of 1 μ Ci ml^{-1} . Labelling was terminated by the addition of unlabelled L-methionine to a final concentration of 1 mM (Porter, 1985). Cells were then collected by centrifugation (20,000 $\times g$, 4°C, 20 min), resuspended in a small volume of 10 mM Tris-HCl buffer pH 7.4, drop frozen in liquid N₂ and stored at -20°C.

2.12 Measurement of Radioisotope Incorporation into Whole Cells and Subcellular Fractions

25 ml Aliquots of Rm. vanniellii cultures in Suba-Seal capped Quickfit flasks were labelled with $1 \mu\text{Ci ml}^{-1} \text{L-}^{35}\text{-S-methionine}$. Samples (0.5 ml) were taken at intervals and injected manually into 0.5 ml 10% (w/v) trichloroacetic acid at 4°C . After 30 min, the samples were filtered onto Whatman GF/C filter discs, washed with 5% (w/v) trichloroacetic acid, absolute ethanol and finally a mixture of equal parts of ethanol and ether. The filters were transferred to glass scintillation vials, 3.0 ml of Triton-toluene scintillation fluid added (Beckman type MP or EP) and counted in an LKB Minibeta Scintillation counter. The efficiency of counting was 90% for ^{35}S . Blanks containing unlabelled filters and scintillation fluid were also counted to determine background radioactivity.

For subcellular fractions, 2-10 μl aliquots were counted directly using 3.0 ml scintillation fluid.

2.13 Preparation of Cell-Free Extracts

Cell pellets were resuspended in ice-cold 10 mM Tris-HCl buffer pH 7.4 to a high cell density and disrupted either by two passages through a pre-cooled French Press at a pressure of 137 MPa (for volumes larger than 5 ml) or by sonication (for smaller volumes). Sonication was carried out using an MSE 12/76 MK2 sonicator equipped with a microtip probe that could be used with eppendorf tubes. Cells were cooled in a methanol/ice bath and broken by 6 separate 15 sec bursts of ultrasound

(20 KHz ; 6 μm peak to peak amplitude) with 1 min cooling periods.

Except when used in enzyme assays or for absorption spectra, 25 $\mu\text{g ml}^{-1}$ each of RNase and DNase were routinely added to cell-free extracts. Debris and unbroken cells were removed by centrifugation in an MSE HS21 centrifuge (30,000 $\times\text{g}$, 4°C, 20 min) for volumes of extract greater than 10 ml or using an Eppendorf microfuge (11,600 $\times\text{g}$, 4°C, 10 min) for smaller volumes. The supernatants were removed and, if necessary, stored at -20°C.

2.14 Isolation of Membrane Fractions

Intra-cytoplasmic membranes were prepared from photoheterotrophically and chemoheterotrophically grown cells on sucrose gradients using a modified version of the method of Garcia *et al.* (1981). Discontinuous gradients were prepared by layering 8 ml each of 50, 40, 30 and 20% (w/v) sucrose in 10 mM Tris-HCl buffer pH 7.4 on a 2 ml cushion of 60% (w/v) sucrose in Tris buffer, contained in 38 ml capacity Beckman Polyallomer centrifuge tubes. Aliquots of cell-free extracts (2.5-3.0 ml) were layered onto the gradient and centrifuged to equilibrium (100,000 $\times\text{g}$, 4°C, 16 h) in the SW28 rotor of a Beckman L8 ultracentrifuge. Gradients were fractionated from the top using a Buchler Auto-Densiflow IIc fractionator and LKB 7000 ultrorac fraction collector (1 ml fractions). Smaller gradients (1.1 ml each of 50, 40, 30, and 20% w/v sucrose in Tris buffer on a 0.4 ml cushion of 60% w/v sucrose in 5 ml Polyallomer tubes) were occasionally used for cell-free extracts of 100-500 μl . These were centrifuged to equilibrium (100,000 $\times\text{g}$, 4°C, 16 h) in the SW50.1 rotor of a Beckman L8 ultracentrifuge and

fractionated from the top by hand (200 μ l fractions) using a Gilson automatic pipette.

For the preparation of large quantities of intra-cytoplasmic membranes at high concentration from phototrophically grown cells only, 3 ml of cell-free extract was layered onto a 33 ml cushion of 25% (w/v) sucrose in 10 mM Tris-HCl pH 7.4 and centrifuged (5 h at 100,000 xg and 4°C) in the SW28 rotor of a Beckman L8 ultracentrifuge. The soluble protein remained at the top of the cushion and the ICM pellet was resuspended in 10 mM Tris buffer to a protein concentration of 15-25 $mg\ ml^{-1}$ and stored as aliquots at -20°C. A similar procedure was followed for the isolation of ICM from differentiating swarmer cells, where only small volumes of cell-free extracts were available, except that 50-500 μ l of extract was layered onto a 4.3 ml cushion of 25% (w/v) sucrose and centrifuged 2.5 h at 100,000 xg and 4°C in the SW50.1 rotor of a Beckman L8 Ultracentrifuge.

For enzyme assays soluble protein and ICM were prepared by differential centrifugation of cell-free extracts at 150,000 xg for 3 h and 4°C in the SW50.1 rotor of a Beckman L8 Ultracentrifuge. The soluble protein was removed and the ICM pellet resuspended in a small volume of cold 10 mM Tris-buffer, pH 7.4.

2.15 Enzyme Assays

All enzyme assays were performed at 25°C in 3 ml reaction volumes using 1 cm light-path quartz cuvettes in a Pye-Unicam SP8-200 double beam spectrophotometer with integral chart recorder. The NADH and NADPH

oxidase systems were assayed by measuring the decrease in absorbance at 340 nm after the addition of the appropriate sub-cellular fraction to a mixture containing (μmol in 3 ml):

Tris-HCl pH 7.4	200
NADH or NADPH	0.6

The blank, placed in the spectrophotometer reference beam, lacked NADH.

NADH dehydrogenase (NADH: acceptor oxidoreductase: EC 1.6.99.3) was assayed by monitoring the NADH dependent reduction of 2,6 dichlorophenolindophenol (DCPIP) at 600 nm as described by Oelze & Kamen (1975). The reaction mixture contained (μmol in 3 ml):

Tris-HCl pH 7.4	25
NADH	0.3
DCPIP	0.18

The blank lacked DCPIP. The reaction was started by the addition of the appropriate subcellular fraction, then NADH, and the difference between the rates obtained in the presence and absence of NADH was recorded.

Succinate dehydrogenase (succinate: acceptor oxidoreductase; EC 1.3.99.1) was assayed by the phenazine methosulphate (PMS) mediated, succinate dependent reduction of DCPIP at 600 nm in a modified version of that described by King (1963) and Flammann & Weckesser (1984a). The reaction mixture contained (μmol in 3 ml):

potassium phosphate buffer pH 7.6	150
DCPIP	0.2
PMS	1.2
sodium succinate	20.0
potassium cyanide	7.0

The blank lacked DCPIP. The reaction mixture was prepared initially without succinate, the appropriate sub-cellular fraction added and then preincubated at 25°C for 2 minutes before the succinate was added and the initial rate of absorbance decrease recorded.

Malate dehydrogenase (L-malate: NAD oxidoreductase; EC 1.1.1.37) was assayed by the oxaloacetate dependent oxidation of NADH at 340 nm (Reeves *et al.*, 1971) in a reaction mixture containing (μ mol in 3 ml):

potassium phosphate buffer pH 7.6	85.0
potassium oxaloacetate	1.3
NADH	0.4

The blank lacked NADH and the reaction started by the addition of oxaloacetate.

Isocitrate dehydrogenase (L_s -isocitrate: NAD oxidoreductase (decarboxylating); EC 1.1.1.41 and L_s -isocitrate: NADP oxidoreductase (decarboxylating); EC 1.1.1.42) activity was determined by the isocitrate dependent reduction of NAD or NADP at 340 nm (Reeves *et al.*, 1971). The reaction mixture contained (μ mol in 3 ml);

Tris-HCl buffer pH 7.4	60.0
MnCl ₂	2.0
NAD or NADP	0.5
sodium dl-isocitrate	0.5

After the addition of the appropriate sub-cellular fraction, the reaction was started by the addition of isocitrate.

Where necessary, the enzyme activities obtained were corrected for NADH oxidase activity. The following mM extinction coefficients were used; NADH, 6.22; DCPIP, 16.1 (Reeves *et al.*, 1971).

2.16 Measurement of Oxygen Uptake

Oxygen uptakes were recorded using a clear perspex Clark-type polarographic oxygen electrode (Rank Brothers, Bottisham, Cambridge) polarized at -0.6V. Reactions were performed at 30°C in 3 ml 25 mM phosphate buffer pH 7.0 which was saturated with air from an aquarium pump. A value of 225 μ M O₂ in air saturated phosphate buffer at 30°C (Chappell, 1964) was used in the calculation of results. The electrode was calibrated to zero O₂ content by the injection of 100 μ l of a saturated solution of sodium dithionite into the reaction vessel containing 3 ml phosphate buffer. Reactions were done in the dark and the vessel illuminated, where necessary, from a 60 W tungsten bulb at a distance of 5 cm.

2.17 Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

2.17.1 One-dimensional completely denaturing 10-30% (w/v) polyacrylamide gradient gels

This technique was routinely used to analyse the polypeptide composition of samples because of the excellent resolution possible across the M_r range 10,000 to 100,000 and especially the particularly good separability of low M_r polypeptides. The method was based on that described in Hames & Rickwood (1981) with several modifications. A discontinuous buffer system was employed with a stacking gel polymerized on top of the resolving gel as first described by Laemmli (1970). The gels were cast between large (20 x 25 cm) glass plates using 2 mm thick teflon spacers and run in perspex electrophoresis tanks similar to that described by Studier (1973).

The acrylamide gradient was designed to be concave (exponential) as this was found to be more reproducible than a linear gradient and gave increased resolution of low M_r polypeptides.

Two solutions, of 10% (w/v) and 30% (w/v) acrylamide were prepared from stock reagents as follows;

<u>10% (w/v) acrylamide mixture</u>	(50.0 ml)
High bisacrylamide stock	8.3 ml
distilled water	34.9 ml
resolving gel buffer	6.25 ml
10% (w/v) SDS	0.5 ml

This mixture was degassed under vacuum in a desiccator (5 min) and

immediately before pouring, 10 μ l of N, N, N', N'-tetramethylethylenediamine (TEMED) and 100 μ l of freshly prepared 10% (w/v) ammonium persulphate were added to initiate polymerization.

<u>30% (w/v) acrylamide mixture</u>	(20.0 ml)
Low bisacrylamide stock	10.0 ml
75% (v/v) glycerol	7.3 ml
resolving gel buffer	2.5 ml
10% (w/v) SDS	0.2 ml

The mixture was degassed under vacuum in a desiccator (5 min) and immediately before pouring, 4 μ l TEMED and 40 μ l of 10% (w/v) ammonium persulphate were added.

The 30% (w/v) mixture was placed in a magnetically stirred 25 ml scintillation vial, stirred slowly and a Suba-Seal with inlet and outlet tubes pressed into place so that a few ml of the acrylamide was directed down the back plate (non-bevelled) of the gel assembly. The 10% (w/v) mixture was pumped into the 30% (w/v) acrylamide via a peristaltic pump at a rate of 3.2 ml min⁻¹ with the stirrer set to maximum to ensure thorough mixing. Thus, the volume of liquid in the mixing vial remained constant, but was continuously diluted by the larger volume of the lower percentage gel mix. The gradient was poured at room temperature and until it was within 4 cm of the top of the back gel plate. This gave a gel of nominal volume 55-60 ml. To ensure an even surface after polymerization, the gel was carefully overlaid with a few ml of water-saturated butanol and allowed to set for 1-2 h. The glycerol included in the 30% (w/v) gel mix produced a density gradient which prevented convective mixing due to the heat evolved during polymerization. This

also avoided the need to include a gradient of polymerization catalyst.

After polymerization, the butanol was washed away with double-distilled water and a stacking gel polymerized on top. The stacking gel mixture (10 ml) contained;

stacking gel acrylamide stock	3.0 ml
double-distilled water	4.4 ml
stacking gel buffer	2.4 ml
10% (w/v) SDS	0.1 ml

The mixture was degassed under vacuum for 5 min and immediately before pouring, 5 μ l TEMED and 100 μ l 10% (w/v) ammonium persulphate added. Immediately after pouring into the gel assembly, a teflon gel-comb (10, 12 or 22 wells) was inserted into the stacker, avoiding air-bubbles. After the stacking gel had set (15 min) the gel-comb was removed, the wells washed with distilled water, and the gel placed in the gel tank ready for loading.

Tris-glycine running buffer was prepared from stock solutions as follows;

5X Tris-glycine reservoir buffer stock	300 ml
10% (w/v) SDS	15 ml
distilled water	1185 ml

The upper and lower reservoir tanks were filled with running buffer and air bubbles removed from beneath the gel by hypodermic syringe.

Protein samples containing known amounts of protein or radiolabel were routinely denatured in the presence of SDS and mercaptoethanol using Laemmli sample buffer (Laemmli 1970).

This was used as a 2X stock solution, containing;

stacking gel buffer	125 μ l
glycerol	100 μ l
10% (w/v) SDS	200 μ l
2-mercaptoethanol	50 μ l
0.5% (w/v) bromophenol blue	25 μ l

When added to an equal volume of protein sample this gave final concentrations of; Tris-HCl pH 6.8, 62.5 mM; SDS, 2% (w/v); 2-mercaptoethanol, 5% (v/v); glycerol, 10% (v/v) and bromophenol blue, 0.01% (w/v). Complete denaturation was effected by heating at 75°C for 2 min before application to the gel using a Hamilton glass microsyringe (100 μ l capacity).

The maximum volume loadable in each well using 10 or 12 well gel combs was about 100-125 μ l and so for some applications, 4X sample buffer was used to allow larger volumes of sample to be run.

This contained;

2X concentrated stacking gel buffer	125 μ l
glycerol	25 μ l
20% (w/v) SDS	200 μ l
2-mercaptoethanol	100 μ l
0.5% (w/v) bromophenol blue	50 μ l

10-30% gradient gels were run in a 4^oc cold room at 20 mA constant current for about 16 h. These conditions were found to give better resolution than running the gels at room temperature. In addition, the high starting current allowed fast stacking of the proteins which minimized diffusion. The exclusion limit of the high percentage end of the gel was such that electrophoresis could be continued for 2-3 h after the dye front had completely migrated off the end of the gel without protein loss. This resulted in considerable zone sharpening of low molecular weight proteins.

The stock solutions used in preparing 10-30% (w/v) concave gradient gels were as follows:

High bisacrylamide stock (acrylamide:bis = 30:0.8)

acrylamide (Eastman Kodak) 60 g

bisacrylamide (BioRad) 1.6 g

The mixture was dissolved in 25 ml double-distilled water in a hot water bath and then made up to 100 ml.

Low bisacrylamide stock (acrylamide:bis = 30:0.15)

acrylamide 60 g

bisacrylamide 0.3 g

Dissolved as above.

cast using the high bisacrylamide stock (30:0.8) solution employed for gradient gels.

<u>10% (w/v) acrylamide mixture</u>	(75.0 ml)
high bisacrylamide stock	12.5 ml
distilled water	52.3 ml
resolving gel buffer pH 8.8	9.4 ml
10% (w/v) SDS	0.75 ml

The mixture was degassed as before and polymerization initiated by the addition of 15 μ l TEMED and 150 μ l 10% (w/v) ammonium persulphate. The solution was poured manually into the same type of gel plate assembly described in section 2.17.1 to within 4 cm of the top of the back plate, overlaid with butanol and allowed to set. A 3% (w/v) acrylamide stacking gel of exactly the same composition as that used for gradient gels was then polymerized on top. Electrophoresis was carried out in a 4°C cold room either at 25 mA constant current for 6 h or at 15 mA for 16 h. The dye front was not allowed to migrate off the gel.

2.17.3 One-dimensional incompletely denaturing uniform concentration gels

For the isolation of pigment-protein complexes (section 2.27), 10% (w/v) acrylamide gels were cast with a 3% (w/v) acrylamide stacker, both gels containing SDS and the non-ionic detergent Triton X-100 (BDH) at final concentrations of 0.05% (w/v) in each case. The running buffer (section 2.17.1) also contained these detergents at the same concentration. The gels were electrophoresed at 25 mA for 6 h at 4°C. In preliminary experiments either Triton X-100, SDS or the zwitterionic detergent lauryldimethylamine-N-oxide (LDAO) alone were used, all at

concentrations of 0.1% (w/v) throughout the gel and running buffer system.

2.17.4 Two-dimensional semi-denaturing gels

This technique combined analysis of solubilized membrane protein complexes on SDS + Triton X-100 gels in the first dimension with denaturing SDS-PAGE in the second.

First dimension tube gels were cast in tubes 130 mm x 2.5 mm internal diameter, derived from glass pipettes. They were successively cleaned by soaking in chromic acid, then distilled water, then alcoholic potassium hydroxide and finally in distilled water once again before air drying. The bottom of the tubes was covered with two layers of parafilm and filled to a depth of 10 cm with a 6% (w/v) acrylamide gel mixture containing both SDS and Triton X-100;

<u>6% (w/v) acrylamide gel mixture</u>	(5.0 ml)
high bisacrylamide stock	0.5 ml
distilled water	3.82 ml
resolving gel buffer pH 8.8	0.625 ml
10% (w/v) SDS	0.025 ml
10% (w/v) Triton X-100	0.025 ml

The mixture was degassed under vacuum and polymerized with 1 μ l TEMED and 10 μ l 10% (w/v) ammonium persulphate. About 20 μ l of water saturated butanol was layered on the gel to ensure an even surface. A 3% (w/v) stacking gel containing 0.05% (w/v) each of SDS and Triton X-100 was then polymerized on top of the 6% (w/v) resolving gel, to a depth of 5 mm. The tubes were mounted in a gel tank consisting of

perspex upper and lower reservoirs, the upper reservoir adapted with bored rubber bungs to accommodate the narrow tubes. The parafilm was removed, the reservoirs filled with Tris-glycine running buffer containing 0.05% (w/v) each of SDS and Triton X-100, the samples loaded and electrophoresis carried out in a 4°C cold room for 2 h at 10 mA constant current.

The gels were removed from the tubes by water pressure from a syringe and incubated at 60°C for 5 min in Laemmli sample buffer without tracker dye (62.5 mM tris-HCl pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol). The stacking gel was removed, and the resolving gel placed on a piece of parafilm and straightened.

The second dimension was a 10-30% (w/v) polyacrylamide gradient gel cast as in section 2.17.1 but with the stacking gel polymerized to give a flat surface about 1 cm below the front gel plate level. This was achieved by the omission of the gel comb and the addition of an overlay of water-saturated butanol on the stacker. The space up to the bevel was filled with hot 1% (w/v) agarose (Sigma) dissolved in Laemmli sample buffer + bromophenol blue and the 1st dimension tube gel applied to this, avoiding trapping air bubbles. The gel was then sealed in place with more agarose solution and allowed to set. A small well was made at one end of the agarose layer to allow molecular weight standards to be run in the second dimension. Electrophoresis in the second dimension was at 14 mA constant current for 16 h at room temperature.

2.17.5 Two-dimensional O'Farrell gels

In this gel system proteins were separated by their isoelectric points in the 1st dimension and according to molecular weight in the 2nd, two

unrelated parameters that give maximal resolution of proteins in complex mixtures (O'Farrell, 1975). A stable pH gradient is formed using commercial ampholines and the proteins electrophoresed to equilibrium until their net charge is zero and migration ceases. However, the original method of O'Farrell (1975) is not suitable for the analysis of membrane proteins as no provision was made for the effects of detergent solubilization on the charge distribution of the protein. Therefore a modified version of the method of Ames and Nikaido (1976) was used. This involves membrane solubilization with SDS then 1st-dimension electrophoresis in the presence of the non-ionic detergent nonidet P-40 (NP-40) and urea to remove the SDS bound to the protein.

The isoelectric focussing gels were made in 130 mm x 2.5 mm internal diameter tubes, cleaned as described in section 2.17.4. The bottom of the tube was covered with parafilm and the gel mixture poured in using a syringe and fine tubing to give a length of 10 cm.

30% (w/v) acrylamide stock for isoelectric focussing

acrylamide (BioRad)	28.38 g
bisacrylamide (BioRad)	1.62 g
distilled water to	100 ml

Isoelectric focussing gel mixture - 10 ml

Ultrapure Urea (BioRad)	5.5 g
30% (w/v) acrylamide stock	1.33 ml
10% (w/v) NP-40	2.0 ml
distilled water	1.97 ml
Ampholines (LKB) range 5-7 pH	0.4 ml
Ampholines (LKB) range 3.5-10 pH	0.1 ml

The mixture was dissolved by warming, degassed under vacuum and 7 μ l TEMED and 10 μ l 10% (w/v) ammonium persulphate solution added before pouring.

The gel was overlaid with a solution of 8 M urea and allowed to set for 1-2 h. The overlay was removed and replaced with 20 μ l of sample dilution buffer (Ames & Nikaido, 1976) itself overlaid with water.

Sample dilution buffer - 10 ml

9.5 M Ultrapure Urea	5.5 g
1.5% (w/v) Ampholines range 5-7 pH	0.4 ml
0.4% (w/v) Ampholines range 3.5-10 pH	0.1 ml
5% (w/v) 2-mercaptoethanol	0.5 ml
8% (w/v) NP-40	0.8 ml

After a further 2 h, the buffer and water were removed, 20 μ l fresh sample dilution buffer added and the tubes filled with 0.02 M sodium hydroxide. The parafilm was removed and replaced with dialysis tubing held in place by silicone rubber 'o' rings so as not to trap any air bubbles and the tubes mounted in the electrophoresis tank (section 2.17.4). The lower chamber was filled with the anode electrolyte, 0.01 M phosphoric acid and the upper chamber filled with the cathode electrolyte, 0.02 M sodium hydroxide. The gels were pre-run at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min.

In preparing intra-cytoplasmic membrane samples for isoelectric focussing, it was sometimes found necessary to use an acetone precipitation step both to concentrate the samples and to remove lipids, which otherwise caused smearing in the second-dimension. Membrane

proteins were precipitated by the addition of 5 volumes of cold (-20°C) acetone to samples followed by microfuging (5 min). The pellets were dried by a stream of nitrogen, dissolved in Laemmli sample buffer without tracker dye and solubilized by heating at 75°C for 2 min. Other types of sample were dialyzed at 4°C for 6 h against Laemmli sample buffer before heating. Two volumes of sample dilution buffer were then added to the SDS solubilized samples.

The buffer and sodium hydroxide were removed from the pre-run isoelectric focussing gels and the upper reservoir emptied. The samples were loaded and overlaid with 10 μl of 5 M Urea/0.4% (w/v) pH 5-7 ampholines/ 0.1% pH 3.5-10 ampholines and the tubes re-filled with 0.02 M sodium hydroxide. The upper reservoir was re-filled and the proteins focussed at 400 V for 16 h and then 800 V for a further hour.

Gels were extruded by pressure from a water-filled syringe for 30 min directly into 5 ml Laemmli sample buffer. After equilibration (1 hr) the isoelectric focussing gels were loaded onto the second dimension immediately or stored frozen in sample buffer at -20°C .

The second dimension gel was a 10-30% (w/v) acrylamide gradient gel, cast as in section 2.17.1 but with the same stacking gel and agarose sealing arrangements as described in section 2.17.4. Electrophoresis in the second dimension was carried out at 15 mA for about 16 h at room temperature.

2.18 Staining of Polyacrylamide Gels

2.18.1 Coomassie blue staining

Gels were immersed in a staining solution consisting of 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) Coomassie brilliant blue R250 for 4-5 h (500 ml per gel) on a shaking platform. Destaining was effected by 3-4 successive soaks (1-2 h each) in 45% (v/v) methanol, 10% (v/v) glacial acetic acid until the background was acceptable. Further destaining was done in 20% (v/v) propan-2-ol 10% (v/v) glacial acetic acid (overnight). This method was found to be insensitive, needing > 100 µg of membrane protein to produce acceptable results, produced gels with diffuse stained bands of low contrast for photography and was time-consuming. Coomassie blue staining was therefore not used routinely. Instead, one of the several silver staining methods recently developed was adopted for routine use.

2.18.2 Silver staining

Silver staining has the considerable advantage over Coomassie blue staining of up to 100 times greater sensitivity and, because it is a positive staining method, produces gels of high contrast. In addition, the low loadings possible serve to increase the resolution of proteins. Of the plethora of methods now available (Ochs *et al.*, 1981) that of Wray *et al.* (1981) was chosen because of its simplicity and rapidity.

Gels were transferred to 50% (v/v) SLR methanol made up in double-distilled water for 8 h on a gently shaking platform. The methanol was changed 4-5 times during this period. The staining solution was prepared as follows:

Solution A; 1.6 g silver nitrate (Johnson Mathey Ltd.) dissolved in double-distilled water.

Solution B; 42 ml of 0.36% (w/v) sodium hydroxide containing 2.5 ml of "0.880" ammonia solution (BDH).

Solution A was added dropwise to Solution B with constant swirling and then made up to 200 ml with double-distilled water.

The gel was transferred to the staining solution for 15 min with agitation and then washed in two changes of double distilled water for 5 min each. The protein bands were visualized by soaking the washed gel in developer solution, prepared by the addition of 2.5 ml of 1% (w/v) citric acid and 0.4 ml of 37% (v/v) formaldehyde solution to 500 ml double-distilled water. The amount of formaldehyde was adjusted empirically so that bands appeared after 5-15 minutes of constant agitation and this was found to be a critical part of the procedure. Too much formaldehyde resulted in an overstained gel. The staining process was stopped immediately by transferring the gel directly into 45% (v/v) methanol, 10% (v/v) acetic acid, in which the gels were stored.

This procedure generally worked very well and was quite reproducible. Particular attention was given to using double-distilled water throughout and ensuring an adequate number of washes of the gel in methanol before staining. Some observations on the method are worth noting. Firstly, artefacts caused by silver staining have been found (Tasheva & Dessev, 1983) usually in the form of a doublet of lines across the gel in the 68,000 M_r region. These were also found in this study, particularly when gels had to be overstained. These bands appear

to be at least partly due to keratin contained in the dust from laboratory glassware and from human skin. Therefore all glassware was rinsed before use, gel plates cleaned with detergent followed by methanol and disposable gloves worn when preparing and handling gels. Secondly, some proteins apparently do not stain with silver, but these are easily identified as a "space" in the gel track. This was not found to be a problem in this study.

2.18.3 Staining gels for cytochromes

The method of Thomas et al. (1976) was used to identify protein bands as cytochromes by taking advantage of the ability of the haem group (which often remains attached to the apoprotein after electrophoresis even in the presence of SDS) to catalyze the peroxidation of a colourless benzidine derivative to a coloured dye. The staining solution was prepared as follows.

Solution A; 0.09 g of 3,3',5,5'-tetramethylbenzidine (TMBZ) in 60 ml of methanol (6.3 mM, freshly prepared).

Solution B; 0.25 M sodium acetate solution adjusted to pH 5.0 with glacial acetic acid.

Immediately before use, 60 ml Solution A was added to 140 ml Solution B and the gel immersed in this for 1 hour in the dark with constant agitation.

The bands were developed by the direct addition of 0.7-1.0 ml of 30% (v/v) hydrogen peroxide solution (100 vol. strength; BDH) to the staining solution. The bands became visible after 3-10 min and the gel was then transferred to 30% (v/v) propan-2-ol made up in 0.25 M sodium

acetate buffer pH 5.0 for photography.

2.19 Photography of Polyacrylamide Gels

Gels were photographed on a light box using Panatomic X (32 ASA) 35 mm monochrome film which was developed for 3 min in Kodak D19 developer and fixed in Kodafix for 5 min. A yellow filter (Pentax) was used to improve the contrast with Coomassie blue stained gels or with gels stained for haem-associated peroxidase activity.

2.20 Autoradiography

Gels were dried down onto Whatman 3 MM chromatography paper under vacuum from a filter-pump between two sheets of silicone rubber. The gel dryer was mounted on a metal plate heated to 80°C above a water bath and after 2-3 h, the dried gel was removed and placed in contact with Kodak "no-screen" X-ray film in a film cassette at 4°C. Exposure times varied with the isotope used. With ³⁵S-methionine, 100,000 cpm per track would require about 2-4 weeks exposure. Films were developed in Kodak DX-80 developer and fixed in FX-40 fixer.

2.21 Fluorography

In order to increase the sensitivity of detection of radioisotopes in gels, fluorography was undertaken in which the gel is impregnated with the scintillant PPO before drying (Bonner & Laskey, 1974). This not

only allows the detection of ^3H but also increases the sensitivity of detection of ^{35}S -methionine about 10-fold, allowing reduced loadings and/or exposure times.

Gels were fixed for 1 hour in 50% (v/v) methanol and transferred to 500 ml of DMSO for 30 min on a shaking platform in a fume cupboard. The gel was soaked for a further 30 min in fresh DMSO before being transferred to 200 ml of 22.2% (w/v) PPO dissolved in DMSO for 3 h. At the end of this period the PPO was precipitated within the gel matrix by soaking for two, 30 minute periods in distilled water followed by a short equilibration in 50% (v/v) methanol, which also reduced the size of the gel (where necessary) and removed final traces of DMSO. The gel was then dried under vacuum as in section 2.20 but at a temperature of 65°C for 3 h.

In fluorography, the X-ray film is exposed by both the β -particles of the isotope (with ^{14}C and ^{35}S) and the light generated by its interaction with the scintillant. A linear relationship does not exist between the optical density of the film image and the amount of radioactivity in a given band on a fluorograph (Bonner & Laskey, 1974; Laskey & Mills, 1975). Therefore films were pre-flashed prior to exposure (Laskey & Mills, 1975). This was done using a Vivitar 2500 flashgun mounted on a Kodak No. 2 (red) Safelight filter held in place on a retort stand. The distance from the flashgun to the film was adjusted empirically to give a background absorbance of 0.15 at 540 nm as measured against an unexposed, developed film in a Pye-Unicam SP500 spectrophotometer (Laskey & Mills, 1975). The film used was Kodak X-Omat S and after pre-flashing it was placed in contact with the dried gel in an X-ray cassette and exposed at -70°C . Films were developed in

Kodak DX-80 or LX-24 developer and fixed in FX-40 fixer.

Used PPO was recovered from solution by precipitation in water, extensive washing with water and 10% (v/v) ethanol followed by air drying at 50°C.

In most cases, the staining of gels which were to be subsequently fluorographed was avoided as silver grains cut out most of the light from the scintillant and coomassie blue severely reduces the absorbance of the fluorographic image (Harding & Scott, 1983). However, when staining was necessary, the silver staining procedure was preferred because it is possible to completely remove all the silver grains by a destaining method using Kodafix. The gels were transferred to 10% (v/v) Kodafix in 30% (v/v) methanol after staining and photography and allowed to destain for 2-3 h with 2 changes of this solution. After a 30 min wash in 50% (v/v) methanol, the gels were fluorographed as usual.

Harding & Scott (1983) also found that the absorbance of the fluorographic image for a given level of radioactivity decreased as the acrylamide concentration in the gel increased. However the relationship was hyperbolic and above 10% (w/v) acrylamide the effect was much less severe than below this value. Moreover this effect does not negate comparison of band intensity across gradient gels, where a uniform concentration of acrylamide exists.

2.22 Production of Antisera

Antibodies to Rm. vannielli intra-cytoplasmic membrane proteins were

raised in half-lop rabbits as follows. Four mg of ICM protein was solubilized in 5% (w/v) Triton X-100 + 0.8% (w/v) SDS in 50 mM Tris-HCl buffer pH 7.4 for 1 h at room temperature with gentle mixing. After microfuging (5 min) the supernatant was diluted to 1 ml with 50 mM Tris-HCl buffer pH 7.4 and emulsified with an equal volume of Freund's Complete Adjuvant (Difco Laboratories inc.). Emulsification was effected by pushing the mixture back and forth between two glass syringes connected together. After homogenization, the mixture was injected subcutaneously into the neck region.

A booster injection was given 1 month after the first but using Freund's incomplete adjuvant. Two weeks after this, rabbits were bled from a marginal ear vein. The blood was kept at room temperature for 1 h, the clot prevented from adhering to the sides of the bottle by rimming with a pasteur pipette and then incubated at 4°C overnight. The serum was pipetted from the clot and centrifuged at 1500 x g for 20 min to remove residual blood cells. Serum was stored in small aliquots at -20°C. Blood was also collected from the rabbits before administration of antigen (zero blood sera).

2.23 Immunoelectrophoresis

The presence of antibodies in sera was checked in a qualitative fashion by rocket immunoelectrophoresis. Immunoplates were prepared using 1% (w/v) agarose in barbital buffer (50 mM sodium barbital, 10 mM barbituric acid, pH 8.6) with 5 or 10% (v/v) crude antisera. Antigens were solubilized in 5% (w/v) Triton X-100 and 0.8% (w/v) SDS in 10 mM Tris buffer pH 7.4. Five -20 µl volumes were electrophoresed from 2 mm

diam wells at 8 mA constant current for 16 h at room temperature. Immunoplates were alternately washed in saline and semi-dried 3 times before being completely dried and stained with Coomassie blue.

2.24 Protein Immunoblotting

2.24.1 Preparation of iodinated protein A

Radiiodinated protein A was prepared by Miss R. Lennox of this department according to the following method.

A stock solution (1 mg ml^{-1}) of protein A from Staphylococcus aureus (Sigma) was prepared in phosphate buffered saline (PBS) which contained;

138 mM NaCl
2.8 mM KCl
7.37 mM Na_2HPO_4
1.46 mM KH_2PO_4
pH 7.4

25 μl of this solution was added to 1 mCi of $\text{Na } ^{125}\text{I}$ (Amersham International plc; specific activity $13.5 \mu\text{Ci } \mu\text{g}^{-1}$) and 10 μl of a Chloramine T solution (2 mg ml^{-1} dissolved in 25 mM potassium phosphate buffer pH 7.5). After incubation at room temperature for 2 min, 25 μl of a stock tyrosine solution (2 mg ml^{-1} in phosphate buffer), 50 μl of 10% (w/v) bovine serum albumin and 200 μl of PBS were added. The radioiodinated protein A was then separated from free iodine by gel filtration through Sephadex G50 medium grade (Pharmacia) contained in a 5 ml column of bed height 20 cm. The column was pre-washed with 10 ml

of 10% (w/v) BSA dissolved in PBS and then eluted with PBS alone. Fractions (200 μ l) were collected and aliquots counted in an LKB 1280 Ultragamma counter. Those containing radioiodinated protein A were stored at 4°C.

2.24.2 Antigen transfer to nitrocellulose filters

Protein antigens were detected on SDS gels by the "Western" immunoblotting technique of Burnette (1981). Immediately after electrophoresis, gels were transferred to the porous pad of a BioRad Transblot cell and overlaid with a nitrocellulose filter (Whatman). The gel-filter-pad assembly was then mounted in the cell which contained 3 l of transfer buffer (25 mM Tris-HCl pH 8.3 and 192 mM glycine dissolved in 20% (v/v) methanol). Proteins were transferred from the gel (cathode side) to the filter (anode side) at 50 V (ca. 250 mA) for 3.5 h at room temperature.

After transfer, the filters were soaked in 100 ml PBS containing 8% (w/v) bovine serum albumin (Sigma type V; BSA) for 1 h with gentle shaking to saturate the filter binding sites. Crude antiserum (200 μ l per filter) was added directly and incubation continued overnight. Next day the filters were washed several times with PBS and then incubated for 2 h in 100 ml PBS containing 8% (w/v) PBA, to which was added approx. 1×10^6 cpm (as determined using a hand-held counter) of ^{125}I -labelled Protein A (section 2.24.1). Protein A, a cell-wall protein from Staphylococcus aureus, binds to immunoglobulins on the filter which themselves are bound to the protein antigens of interest. The filters were next washed in PBS containing 1% (v/v) Triton X-100 with several changes, until the background radioactivity, monitored with a hand-held gamma probe, was considerably reduced. After drying with hot air, the

nitrocellulose filters were taped to cardboard, covered with clingfilm and overlaid with Kodak "no screen" X-ray film with a DuPont intensifying screen and autoradiographed at -70°C for 1-3 days. Films were developed as in section 2.20.

2.25 Isolation of Flagella

Flagella were prepared from Rm. vanniellii by Miss J. MacDonald of this department. The cells from a 5 l phototrophically grown culture of Rm. vanniellii were removed by centrifugation ($20,000 \times g$, 4°C , 30 min) and the supernatant concentrated to 50 ml using an Amicon CH4 Hollow-Fibre Concentrator (Filter type HIP-100). The concentrate was re-centrifuged ($5,000 \times g$, room temperature, 30 min), the supernatant adjusted to 35% (w/v) CsCl, 15 mM Tris-HCl pH 7.5, 2% (v/v) Triton X-100 and subjected to centrifugation ($90,000 \times g$, 4°C , 50 h) in the 8 x 25 ml titanium angle rotor of an MSE 65 ultracentrifuge. The flagella, which formed a visible turbid band at a density of 1.336 g cm^{-3} , were removed and dialyzed against 15 mM Tris-HCl pH 7.5 overnight at 4°C , and stored at -20°C .

2.26 Routine Spectrophotometry

Absorption spectra were obtained on a Pye-Unicam SP8-200 double-beam spectrophotometer at room temperature. Cytochrome difference spectra were obtained by placing the sample in both beams and recording the spectra after reducing the sample and/or oxidizing the reference as appropriate. Hydroquinone and dithionite were added directly as a few

crystals while sodium ascorbate was added from a freshly prepared solution.

2.27 Isolation of Photosynthetic Pigment-Protein Complexes

2.27.1 Ion-exchange chromatography

An attempt was made to isolate pigment-protein complexes by ion-exchange chromatography of solubilized membranes (Cogdell *et al.* 1983). A slurry of Whatman DE52 cellulose was produced in 20 mM Tris-HCl pH 7.4 and poured into a 2 ml disposable syringe (5 cm long x 1.0 cm dia.) with a layer of glass wool at the bottom, to give a bed volume of 2 ml after settling. The column was washed with 10 mM Tris-HCl pH 7.4 at a flow rate of 20 ml h⁻¹ via a peristaltic pump connected through a needle and Suba-Seal. Membranes were solubilized at 4°C with 0.8% (w/v) Triton X-100 + 0.8% (w/v) SDS (final concentrations), microfuged 5 min and the supernatant (about 1-2 µg protein) pumped onto the column. Unbound protein was eluted with 10 mM Tris-HCl pH 7.4 until the A280 nm in the eluate was negligible. Proteins were eluted with a linear gradient of KCl from 0-300 mM (10 bed volumes; 20 ml) made up in 10 mM Tris-HCl pH 7.4 containing 0.05% (v/v) Triton X-100. Fractions (1 ml) were collected every 2 min at a flow rate of 25 ml h⁻¹.

Potassium chloride concentration was measured using a conductivity meter calibrated with KCl solutions of known concentration. The absorbance at 280 nm and 870 nm was measured on individual fractions.

2.27.2 Triton-SDS gel electrophoresis

Membrane samples were solubilized at room temperature for 5 min by the

addition of an equal volume of 1.6% (w/v) SDS and 1.6% (w/v) Triton X-100 dissolved in 68 mM Tris-HCl pH 6.8 buffer. The samples were then microfuged, the supernatant adjusted to 10% (w/v) glycerol and then run on the Triton-SDS incompletely denaturing gel system described in section 2.17.3.

2.27.3 Sucrose gradient centrifugation

Larger quantities of pigment-protein complexes were prepared on sucrose gradients. Sucrose solutions (10, 20, 30 and 40% w/v) were prepared in 10 mM Tris-HCl buffer pH 7.4 containing 0.05% (w/v) each of SDS and Triton X-100, sterilized by autoclaving (121°C, 10 min) and stored at 4°C. Ten to 40% (w/v) discontinuous sucrose gradients were made in 5 ml polyallomer tubes by layering on 1.1 ml each of the above sucrose solutions. An equal volume of 68 mM Tris-HCl pH 6.8 containing 1.6% (w/v) SDS and 1.6% (w/v) Triton X-100 was added to a membrane suspension containing 15-20 mg ml⁻¹ protein. After 5 min at room temperature, the mixture was microfuged (5 min) and the supernatant (300-500 µl) layered onto the gradient.

After centrifugation to equilibrium (100,000 x g, 16 h, 4°C) in the SW50.1 rotor of a Beckman L8 Ultracentrifuge, the gradients were fractionated (150 µl aliquots) from the top. Sucrose concentrations were determined using a refractometer by reference to a calibration graph obtained using sucrose solutions of known concentration. Absorbance values were determined on an LKB Ultraspec Spectrophotometer. Haem-associated peroxidase activity in individual fractions was assayed by a modification of the method of Thomas *et al.* (1976) as described in section 2.18.3. To 0.98 ml of 1.9 mM TMBZ prepared in 30% (v/v) methanol/0.175 M sodium acetate buffer pH 5.0 was added 10 µl of

the appropriate fraction and 10 μl of 30% (v/v) H_2O_2 . The absorbance at 690 nm was measured immediately against a blank containing only TMBZ and H_2O_2 .

Fractions of interest were stored at -20°C .

2.28 Reversible Chemical Cross-Linking

Wang & Richards (1974) first introduced the technique of reversible chemical cross-linking of membrane proteins with cleavable reagents for the study of their lateral topography. Peters & Drews (1983c) and Peters *et al.* (1983, 1984) modified the original method to suit the membranes and pigment-protein complexes of photosynthetic bacteria. The method used in this study was based on these modifications.

The cross-linking reagent used was dithiobis (succinimidyl propionate) (DTSP), a hydrophobic molecule with an integral disulphide bond cleavable with reducing agents such as 2-mercaptoethanol. The functional groups at either end of the molecule can covalently bond to proteins over a distance of 1.1 nm or less. Dithiobis (succinimidyl propionate) was dissolved in DMSO (50 or 200 mM stock solution) and stored at 4°C . As the reagent reacts with Tris buffers, the triethanolamine buffer of Peters & Drews (1983c) or phosphate buffers were used throughout.

In the standard method, membranes resuspended in 50 mM triethanolamine buffer pH 8.3 (200 μg in 100 μl) were cross-linked with DTSP over the concentration range 1-10 mM for up to 1 hr at room temperature.

Pigment-protein complexes from sucrose gradients were dialyzed against 50 mM triethanolamine buffer pH 8.3 containing 0.05% (w/v) each of SDS and Triton X-100 and cross-linked in the same manner but with 1 mM DTSP and a reaction time of 10 min. In both cases the final DMSO concentration was 5% (v/v). The cross-linked products were analyzed by a modification of the two-dimensional diagonal mapping gel system of Peters & Drews (1983c) which allowed the use of 1st dimension polyacrylamide tube gels for ease of manipulation.

The first-dimension gel tubes were as described in section 2.17.4. After cleaning, the tubes were siliconized to prevent the gel from sticking to the glass when extruded. This was done by dipping them into 2% (w/v) dichlorodimethylsilane (BDH), drying with a stream of nitrogen and finally washing with distilled water. The tubes were covered with parafilm at one end and filled to a depth of 10 cm with the 10% (w/v) acrylamide gel mix described in section 2.17.2 but with reduced amounts of TEMED and ammonium persulphate. This was found necessary to avoid heat generation during polymerization which caused distortion in the gel.

<u>10% (w/v) acrylamide gel mix for cross-linking</u>	(5.0 ml)
high bisacrylamide stock	0.83 ml
distilled water	3.49 ml
resolving gel buffer pH 8.8	0.625 ml
10% (w/v) SDS	0.05 ml

After degassing:

TEMED	1 μ l
10% (w/v) ammonium persulphate	4 μ l

The gels were overlayed with butanol and set for 2 h. The butanol was removed, the surface of the gel washed and a 3% (w/v) stacking gel polymerized on top (section 2.17.1) but with 5 μ l TEMED and 70 μ l 10% (w/v) ammonium sulphate per 10 ml of stacking gel mix, again to avoid heat generation. The stacker was overlayed with butanol and allowed to set. The gel tubes were fixed into the tank (section 2.17.4), which was filled with Tris-glycine running buffer (section 2.17.1).

After the cross-linking reaction time was completed, 33 μ l of stop buffer was added to every 100 μ l of cross-linking mixture. Stop buffer contained 0.5 M Tris-HCl pH 6.8, 8% (w/v) SDS, 5% (w/v) glycerol and 0.01% (w/v) bromophenol blue but no mercaptoethanol. The mixture was heated at 75°C for 2 min and 50 μ l aliquots electrophoresed on the 1st dimension tube gels at 6 mA constant current for approx. 2 h in a 4°C cold room. When the dye front had reached the bottom, the gels were extruded by pressure from a water-filled syringe into Laemmli sample buffer in which the cross-links were cleaved in situ by 2-mercaptoethanol. After about 30 min equilibration with gentle mixing, the gels were placed on a piece of parafilm and transferred to the stacker of a 10-30% (w/v) acrylamide gradient gel sealed with hot agarose exactly as described in section 2.17.4 ready for the second dimension separation, carried out at 14 mA for 16 h at room temperature.

2.29 Penicillin Binding Protein Assay

Cell-free extracts (300 μ g protein) were incubated at 30°C for 10 min with either 0.5 μ Ci (35 μ g ml⁻¹) ¹⁴C-benzylpenicillin (55 mCi mmol⁻¹) or 10 μ Ci (22 μ g ml⁻¹) ³H-benzylpenicillin (15-30 Ci mmol⁻¹) After this

time an equal volume of 2X Laemmli sample buffer was added and the samples solubilized at 75°C for 2 min. The samples were applied to 10-30% gradient gels (section 2.17.1) which were subsequently fixed 1 hr in 50% (v/v) methanol and fluorographed as described in section 2.21 using pre-flashed film.

2.30 Preparation of Proteoliposomes

Proteoliposomes for use in bioenergetic studies were reconstituted from both intra-cytoplasmic membranes and pigment-protein complexes from Rm. vannielli. The procedure used was based upon that described by Hellingwerf et al. (1985a) as modified from Racker (1973), in which incorporation of the protein into liposome vesicles is achieved by co-sonication. Intra-cytoplasmic membranes from freshly harvested cells were prepared by differential centrifugation and used directly. However, the detergent content of the pigment-protein complexes prepared on sucrose gradients had to be reduced considerably to allow the formation of closed liposome vesicles. This was achieved by pooling the complexes from 4-5 sucrose gradients, diluting to 5 ml with ice-cold 10 mM tris-HCl buffer pH 7.4 and pelleting by centrifugation (180,000 x g, 5 h, 4°C). The pellet was gently resuspended in 250-300 µl of Tris-buffer and either used immediately, or stored at -20°C.

Liposomes were prepared from Soya Bean azolectin, a gift from Dr. K. J. Hellingwerf. The azolectin had been purified from L- α -lecithin (Sigma) by the procedure of Kagawa & Racker (1971), and stored at -20°C. For ICM-proteoliposomes, 250 µl of an ICM suspension containing about 25 mg ml⁻¹ protein was placed in a plastic centrifuge tube and 20 mg azolectin

added (80 mg ml^{-1} final concentration). The mixture was vortexed, and sonicated in an ice-bath at $6 \mu\text{m}$ peak to peak amplitude (20 KHz with 2X 3 second bursts. For reaction centre proteoliposomes, $250 \mu\text{l}$ of the largely detergent free complex was used with a final azolectin concentration of 40 mg ml^{-1} . Both preparations were stored on ice and used the same day.

2.31 Measurements of the Proton-Motive Force by the Redistribution of Permanent Ions

2.31.1 Construction of ion selective electrodes for membrane potential ($\Delta\psi$) and pH gradient (ΔpH) detection

The redistribution of permanent ions across bacterial membranes in accordance with either the electrical ($\Delta\psi$) or chemical (ΔpH) component of the proton motive force can be measured by either flow dialysis of labelled probes, or, more conveniently, by the use of electrodes which respond selectively to such probe ions. In this study, three types of electrode were constructed. These responded to membrane potential, inside negative, using tetraphenyl-phosphonium (TPP^+) as the indicator probe ion; membrane potential, inside positive, using thiocyanate (SCN^-) as the probe; and pH gradient, inside alkaline, using salicylate (sal^-) as the probe.

The electrodes were constructed essentially as described by Shinbo *et al.* (1978), using a polyvinylchloride (PVC) based membrane doped with a sensitizing agent to selectively permeabilize it. The membranes were produced from the following solutions;

TPP⁺ electrode (Shinbo *et al.*, 1978; Lolkema *et al.*, 1983; Keevil & Hamilton, 1984)

Solution A; 125 mg PVC dissolved in a mixture of 2.5 ml tetrahydrofuran and 0.3 ml dioctylphthalate

Solution B; 5 mg tetraphenylborate (TPB⁻; the sensitizing ion) dissolved in 1 ml tetrahydrofuran.

SCN⁻ and salicylate electrodes (modified from Hellingwerf & van Hoorn, 1984)

Solution A; As for TPP⁺ electrode

Solution B; 5 mg tetraoctylammoniumbromide (the sensitizing ion) dissolved in 1 ml tetrahydrofuran.

In each case, solutions A and B were mixed with gentle agitation to avoid air bubbles and poured into a glass petri-dish, 4 cm diameter, pre-washed with ethanol and tetrahydrofuran. The mixture was placed in a fume-hood at room temperature and the PVC membrane was formed by slow evaporation of the solvent (approx. 1 day).

Circular pieces of the membrane (5 mm dia.) were punched out onto greaseproof paper and glued onto one end of plastic, exchangeable electrode holders using tetrahydrofuran (Fig. 2.1). After drying at 37°C for 3-4h, or at room temperature overnight, the electrode holders were filled with the appropriate electrolyte, fitted with a platinum wire connected to a screened co-axial connector and left to equilibrate with the electrode filling solution by soaking overnight in the same solution.

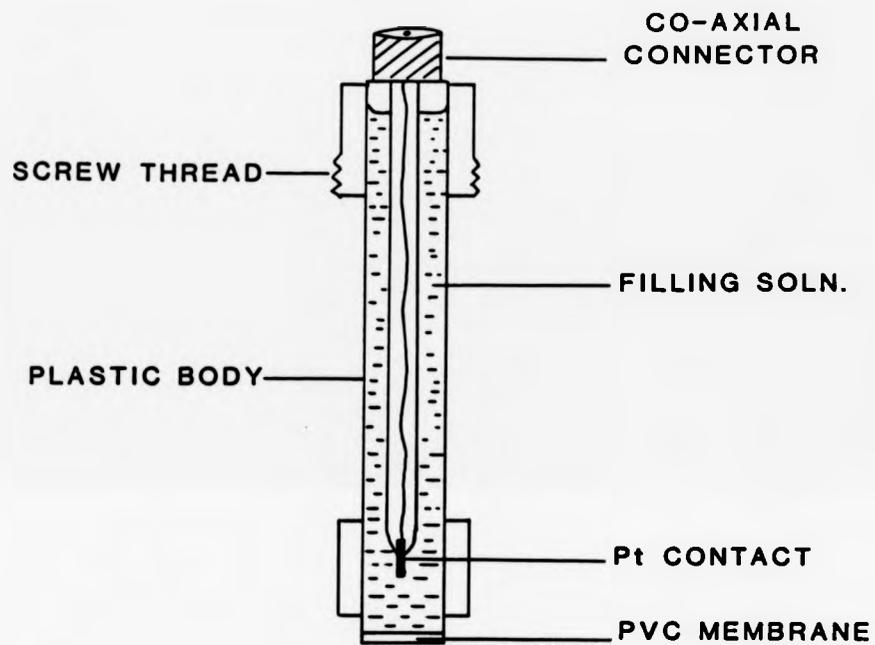


FIGURE 2.1. Design of the electrodes used for measuring membrane potential and pH gradients.

The exchangeable plastic body was equipped with a PVC membrane doped with the appropriate sensitising agent (section 2.31) and filled with electrolyte. Potentials were measured against a standard calomel electrode.

Electrolyte filling solutions

- TPP⁺ electrode: 10 mM TPP bromide dissolved in 50 mM potassium phosphate buffer pH 7.0
- SCN⁻ electrode: 10 mM KSCN dissolved in 50 mM potassium phosphate buffer pH 7.0
- Salicylate electrode: 2 mM sodium salicylate dissolved in 50 mM potassium phosphate buffer pH 7.0.

2.31.2 Calibration and use of the electrodes

The electrodes were mounted in a plastic vessel of 5 ml capacity with facilities for stirring, temperature control, energization of the sample by light, sample additions and gassing with nitrogen. A photograph of the apparatus is shown in Fig. 2.2. To improve the signal to noise ratio of the electrodes, the entire assembly was mounted on an earthed aluminium plate and the sample itself was also grounded. Potentials were measured against a standard calomel electrode (Hellingwerf & Van Hoorn 1984) filled with 3 M choline chloride in 1% (w/v) agar, also mounted in the vessel. The electrodes were provided with in-line pre-amplifiers of local design which fed to a main amplifier and chart recorder.

Prior to calibration for the first time, new electrodes were equilibrated with water or phosphate buffer for 1-2 days with repeated washing. A working volume of 2 or 3 ml was used in the sample vessel and a temperature of 30°C routinely employed, unless otherwise stated. With the chart recorder set to 50-0-50 mV, doubling microlitre additions of TPP⁺, SCN⁻ or salicylate were added, according to which electrode was being monitored, and the mV response noted. Additions were continued

FIGURE 2.2. The electrode set-up for the measurement of proton-motive force parameters.

The electrodes were inserted into a water jacketed plastic vessel with facilities for stirring, gassing with N_2 and illumination via the fibre-optic light guide. The whole system was mounted on an earthed aluminium plate upon which the in-line electrode pre-amplifiers were supported, to maximize the signal to noise ratio.



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FIGURE 2.3. Close-up of the electrode vessel.

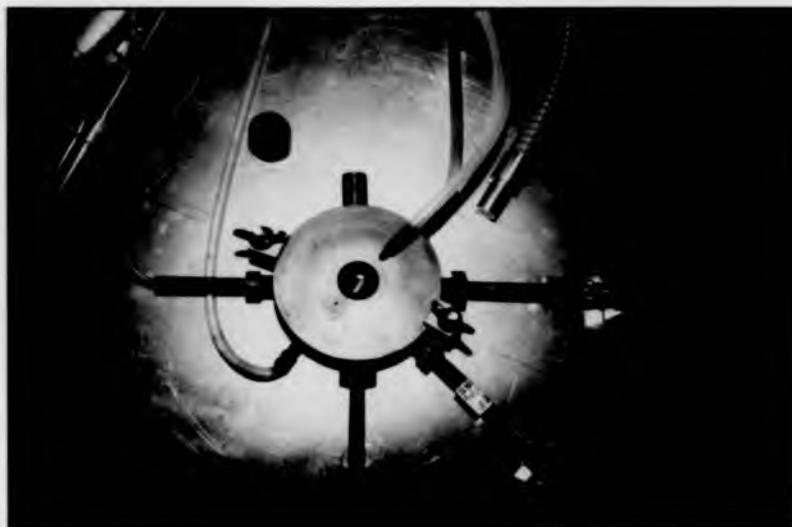


FIGURE 2.3. Close-up of the electrode vessel.

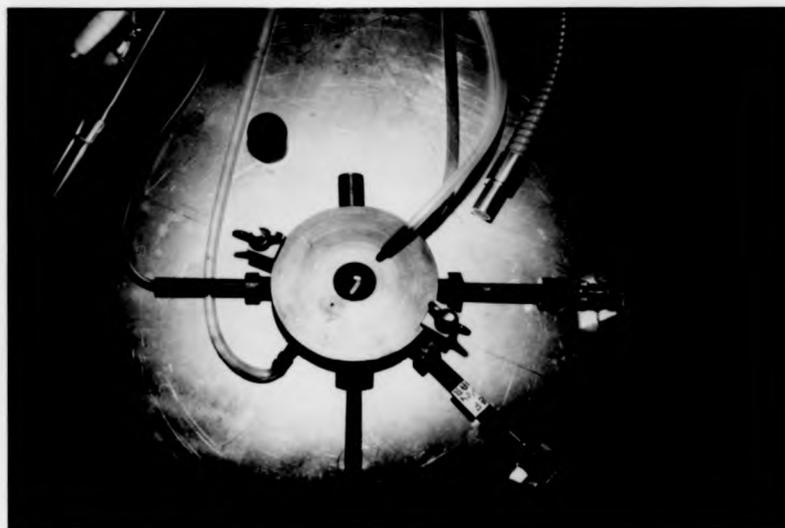
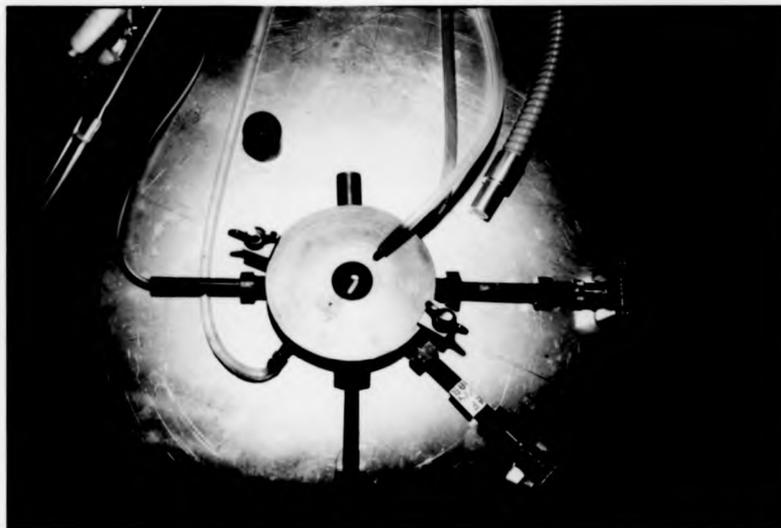


FIGURE 2.3. Close-up of the electrode vessel.



until a doubling in concentration of probe-ion produced the same change in potential difference each time. The calibration procedure was repeated each day the electrode was to be used.

For measurements on whole cells or membranes the probe ion was added first as calibrating pulses to the appropriate medium or buffer in the vessel to give a final concentration in the range of 1-5 μM (TPP^+) or 16-32 μM (SCN^- and sal^-). The samples were then added and incubated in the dark while the trace steadied. The suspension was energized via a fibre-optic light guide (Fig. 2.3) connected to a modified slide projector with a 150 W Quartz-halogen bulb of variable intensity.

2.32 Measurements of pH Changes in Cell Suspensions

An Oriel 01-2000 combination glass and reference pH electrode connected to a pre-amplifier and chart recorder were used to monitor small pH changes upon illumination of cell suspensions. The electrode was inserted into the same type of vessel described in section 2.30.2. Measurements were performed at 30°C. The electrode was calibrated with standard buffers of known pH. Experiments were routinely performed in a 2 ml reaction volume of 150 mM KCl containing 5 mM MgCl_2 .

2.33 Measurements of Membrane Potential using the Carotenoid Bandshift

2.33.1 Slow-time scale-measurements

The electrochromic response of the endogenous carotenoids of Rm. vanniellii to the development of a $\Delta\psi$ upon illumination was monitored on

a slow time scale (seconds upwards) using an Aminco DW-2A chopped dual wavelength spectrophotometer. The wavelength pair used was 522-542 nm. The sample was contained in a 1 ml quartz cuvette and energized using the Aminco side-illumination accessory equipped with a diaphragm shutter and cable release. The sample was illuminated with light of >600 nm using a Corning infra-red filter placed over the diaphragm. A blue blocking filter (cutoff approx. 600 nm) was used to protect the photomultiplier from scattered light.

For some experiments using synchronized swarmer cell cultures, performed at the University of Birmingham, a Perkin Elmer 403 chopped dual-wavelength spectrophotometer was used, with the same wavelength pair. These experiments were carried out with the assistance of Mr. Mark Taylor and Dr. J. B. Jackson.

2.33.2 Rapid electrochromic responses on a fast time scale

Rapid absorption changes were monitored on the crossed-beam spectrophotometer described by Packham & Jackson (1979). The cells were contained in a stoppered 1 x 1 cm, 4 ml cuvette and illuminated actinically from beneath. The light source was a 150 W quartz-halogen lamp which was turned on and off with a leaf-shutter with an opening and closing time of less than 6 ms. Attenuation of the light source was achieved with grey filters calibrated at 800 nm. For most experiments, the shutter was operated manually and the traces recorded on an oscilloscope. They were transferred to an Apple II microcomputer system and printed out when required. These experiments were carried out with the assistance of Mr. John Myatt and Dr. J. B. Jackson at the University of Birmingham.

2.34 Bacteriochlorophyll and Carotenoid Determination

Bacteriochlorophyll was determined in acetone:methanol (7:2 v/v) extracts using a mM extinction coefficient of 75 at 772 nm (Clayton, 1963). With whole cells of moderate to high photopigment content, it was found necessary to perform several extractions. Cells were pelleted in 1.5 ml screw-cap eppendorf tubes (Sarsdet Ltd.) and resuspended in 1 ml of acetone:methanol. The tube was vortexed for 5 s, then microfuged for 30 s and the absorbance of the supernatant at 772 nm read against an acetone:methanol blank immediately. The pellet was re-extracted with a further 1 ml of solvent and the procedure repeated until the absorbance reading was negligible. Carotenoid was determined on the same extracts, using a wavelength of 472 nm. Absorption spectra of acetone:methanol extracts showed this to be the largest peak in the carotenoid absorbance region. As no extinction coefficient is available for these carotenoids, results were expressed as the A₄₇₂ nm per unit protein.

2.35 Protein Determination

Protein was determined using the Folin-Phenol reagent (Lowry *et al.*, 1951) with crystalline bovine serum albumin as the standard.

Samples were diluted into 0.5 ml distilled water in acid-washed boiling tubes and 0.5 ml of 1 M NaOH added. The tubes were boiled for 5 min and then cooled. To 50 ml of 5% (w/v) sodium carbonate was added 0.5 ml of 4% (w/v) sodium potassium tartrate and 0.5 ml of 2% (w/v) copper (II) sulphate. After mixing, 2.5 ml of this reagent was added to the samples and incubated 10 min at room temperature. The Folin-Ciocaltau reagent

(BDH) was diluted 1:1 with distilled water and 0.5 ml added with thorough vortexing to the sample tubes. After a further 30 min at room temperature, the absorbance at 750 nm was measured against a reagent blank containing water. The protein content of the samples was determined by reference to a calibration graph of absorbance against bovine serum albumin concentration.

2.36 Electron Microscopy

Samples were examined by negative staining using phosphotungstic acid in the following manner. A drop of the suspension was placed on Formvar-coated copper grids (100 segment mesh; 3.05 mm dia.; Agar Aids) for 30 s - 1 minute before excess was removed by a strip of filter paper. The grid was allowed to dry and the sample negatively stained by placing a drop of 1% (w/v) phosphotungstic acid (pH 7.0) onto the grid and immediately removing it with a strip of filter paper. Specimens were examined using a Jeol JEM - 100S transmission electron microscope operating at an accelerating voltage of 80 kV. Photographs were taken using Kodak 4489 Estar thick base electron microscope film which was developed in Kodak D-19 developer and fixed in Kodafix fixer according to the manufacturers instructions.

2.37 Chemicals

All reagents were of "Analar" grade or the highest purity available. Unstained molecular weight protein markers for denaturing polyacrylamide gel electrophoresis were obtained from Pharmacia and prepared according

to the manufacturers instructions. The proteins were phosphorylase b (M_r 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α -lactalbumin (14,400). Pre-stained molecular weight markers were obtained from BRL and calibrated against the Pharmacia standards as described by Tsang et al. (1984). This was necessary because these proteins did not migrate according to the molecular weights given by the manufacturers. Using either set of markers, the M_r values of unknown proteins were determined on plots of $\log M_r$ against distance migrated.

Isoelectric focussing standards were obtained from Pharmacia. Acrylamide was from Kodak, bis-acrylamide, SDS, glycine and mercaptoethanol from Bio-Rad and LDAO from Fluka.

All radiochemicals were obtained from Amersham International plc.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of the Intra-cytoplasmic membranes of *Rm. vanniellii*

In this section basic procedures for the isolation of membrane fractions from *Rm. vanniellii* are described, in addition to some biochemical characterization, as a pre-requisite for work described in the later sections.

3.1.1 Isolation on sucrose gradients

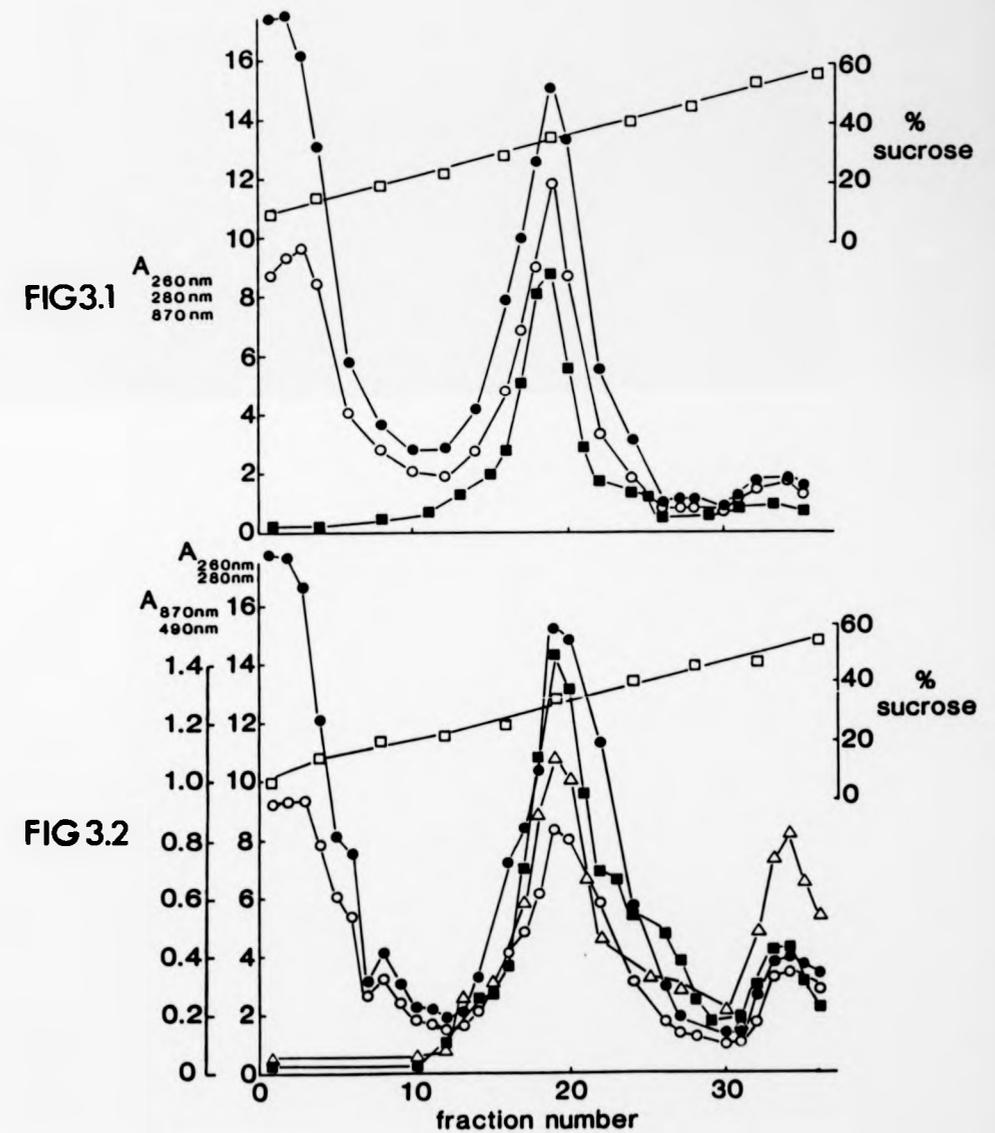
As no methods existed for the isolation of the photosynthetic membranes from *Rm. vanniellii*, the sucrose gradient technique used for *Rb. sphaeroides* (Niederman & Gibson, 1978) was initially employed. Figure 3.1 shows the absorbance profile of a 20-60% (w/v) sucrose gradient loaded with a French pressure cell prepared cell-free extract of photoheterotrophically grown *Rm. vanniellii*. Two peaks of ultraviolet absorption were observed in the gradient with A_{260:280} ratios of 1.8-2.0 and 1.2-1.3 corresponding with sucrose concentrations of 10 and 36% (w/v) respectively. The upper peak at the very top of the gradient was pale straw yellow in colour while the denser peak towards the middle of the gradient was coloured red-brown. The absorbance profile of the *in vivo* infra-red absorption maximum of Bchl in *Rm. vanniellii* at 870 nm (section 3.1.4) corresponded exactly with that of the protein absorbance of this denser, pigmented band thus identifying it as the location of the photosynthetic apparatus. No difference in the position of this intra-cytoplasmic membrane band was observed if the gradients were centrifuged for 4 h instead of 16-18 h and it corresponded to an

FIGURE 3.1. Isolation of intra-cytoplasmic membranes from photoheterotrophically grown *Rm. vannielii* by sucrose gradient centrifugation.

After loading the cell free extract (27 mg protein in 3 ml) onto a 20-60% (w/v) sucrose step gradient and centrifugation to equilibrium (100,000 x g, 16 h, 4°C), 1 ml fractions taken from the top were monitored for their absorbance at 260 nm (●—●), 280 nm (○—○) and 870 nm (■—■). Sucrose concentrations (□—□) were determined by refractometry.

FIGURE 3.2. Isolation of intra-cytoplasmic membranes from chemoheterotrophically grown *Rm. vannielii* by sucrose gradient centrifugation.

Gradients were loaded with 26 mg protein in 3 ml and run as described in the legend to Fig. 3.1. Fractions were monitored for their absorbance at 260 nm (●—●), 280 nm (○—○), 870 nm (■—■), and 490 nm (△—△). Sucrose concentrations (□—□) were determined by refractometry.



equilibrium density of 1.16 g cm^{-3} . Very similar results were obtained when cell-free extracts from either Rp. blastica or Rp. palustris - two other species with lamellate ICM systems - were subjected to sucrose gradient centrifugation. In each case a heavily pigmented band at a density of 1.16 g cm^{-3} was formed. On a few gradients with Rm. vannielli extracts, a small amount of a denser pigmented band (1.25 g cm^{-3}) could be observed but no protein was detected and it most probably represents cell envelope material (see section 3.1.2).

Intra-cytoplasmic membrane protein concentrations of $1-2 \text{ mg ml}^{-1}$ were obtained in the peak fractions from such sucrose gradients. In view of the fact that no other protein bands were observed below that of the ICM, it was possible to use a 25% (w/v) sucrose cushion for centrifugation so that the pelleted ICM could be resuspended to much higher protein concentrations, i.e. approaching 25 mg ml^{-1} .

Figure 3.2 shows a representative profile obtained when cell-free extracts of chemoheterotrophically grown Rm. vannielli were subjected to centrifugation on sucrose gradients. Three major bands formed in the gradient. The soluble protein constituted the uppermost band with an A260:280 ratio of 1.9. A peak with the same density as that of the ICM band from photoheterotrophically grown cells (1.16 g cm^{-3}) formed an orange-brown coloured band also with an A260:280 ratio of 1.8-1.9. This most probably indicates association of ribosomes with the membrane, despite RNase and DNase treatment of the extract. The A875:280 ratio was only about 0.17 in the peak membrane fractions, consistent with the low Bchl content of the cells (about $3 \text{ nmol Bchl mg cellular protein}^{-1}$). The third band (density 1.25 g cm^{-3}) appeared turbid in reflected light and contained carotenoid as evidenced by the absorbance profile at 490

nm. The A260:A280 ratio of this band was 1.15-1.20, indicating that there was no nucleic acid contamination. The turbidity probably resulted from the presence of lipopolysaccharide and the band most likely represents a photopigment depleted cell wall and outer membrane fraction (Ding & Kaplan, 1976; Niederman & Gibson, 1978).

Attempts to separate putative cell wall and outer membrane fractions by recentrifugation on further sucrose gradients always resulted in the formation of a single orange and turbid band at the same density.

3.1.2 Polypeptide composition

The protein profiles characteristic of the various fractions obtained from the sucrose gradients used to prepare ICM from Rm. vannielii are shown in Fig. 3.3. As expected, the majority of the protein in the cell-free extract banded at the top of the gradient with the profile changing down the gradient to reveal an abundance of rather low M_r polypeptides in the ICM fractions.

Those fractions (30-35) corresponding to the small amount of presumptive outer membrane/cell wall material in phototrophically grown cells contained virtually no detectable protein, as judged from SDS-PAGE. Fractions taken between the soluble protein and ICM peaks in such gradients had a rather characteristic profile with significant amounts of polypeptides of M_r 53,000 and 34,000.

In Fig. 3.3. the samples were all boiled in Laemmli sample buffer before application to the gel in accordance with standard methods (Hames &

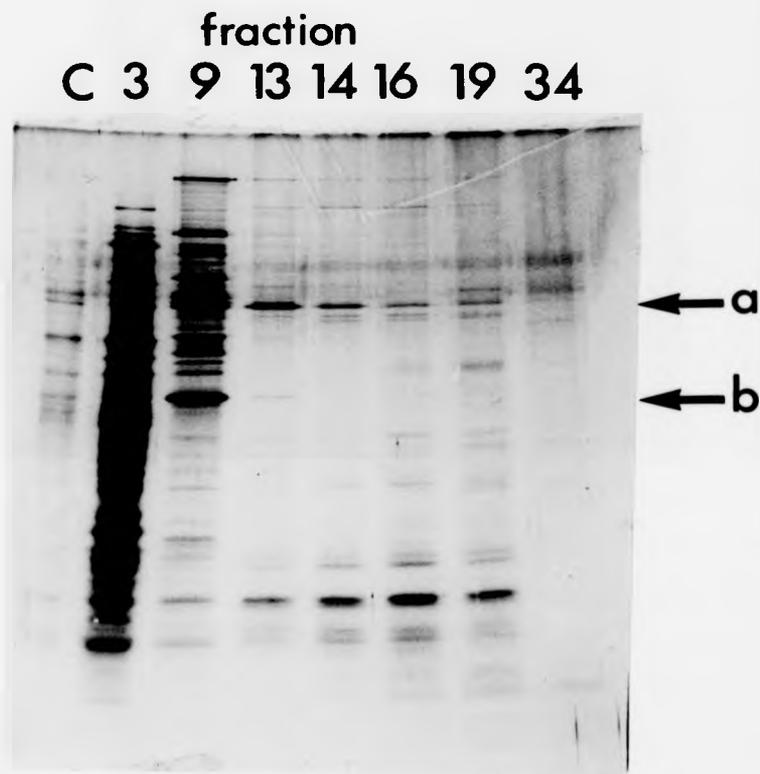


FIGURE 3.3. SDS-PAGE of fractions from the sucrose gradient shown in Fig. 3.1.

Approximately 15 μ g protein of selected fractions (3-34) or the cell-free extract before application to the gradient (C) were denatured in Laemmli sample buffer by boiling and applied to a 10-30% (w/v) gradient gel which was subsequently silver stained. The arrows indicate bands of M_r 53,000 (a) and 34,000 (b), enriched in fractions between the "soluble" and "ICM" fractions.

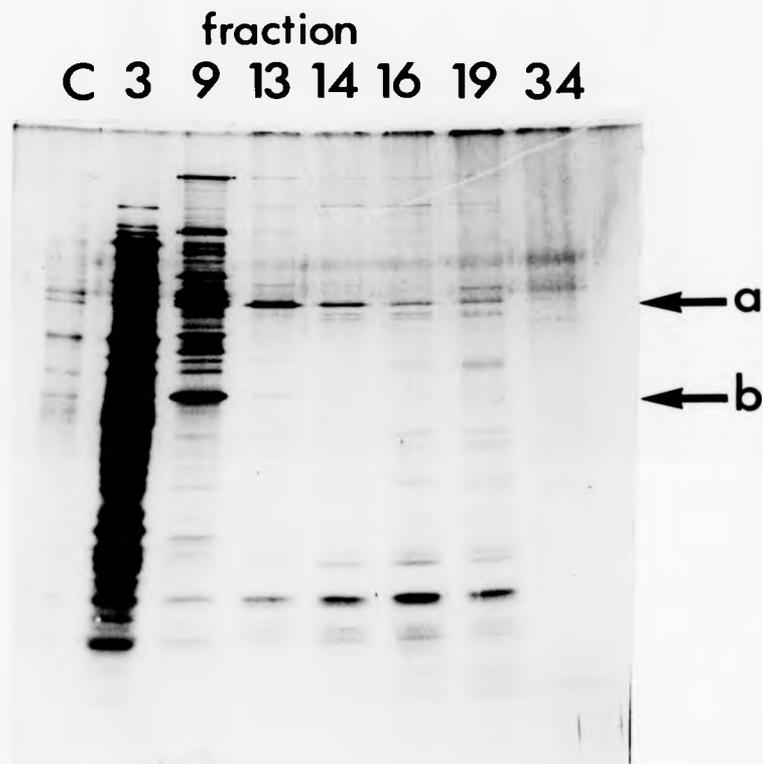


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Rickwood, 1981). However, there are several reports in the literature of ICM polypeptide profiles being affected by the temperature used for solubilization of the samples and the presence or absence of reductant (Clayton & Haselkorn, 1972; Takemoto & Lascelles, 1974; Shepherd & Kaplan, 1978). Therefore the effect of these parameters on the solubilization of ICM proteins from *Rm. vanneili* was investigated. The consequences of different solubilization times at three different temperatures were determined by incubating 50 μ g aliquots of ICM protein in Laemmli sample buffer at 25, 75 or 100°C (Fig. 3.4). Incubation of samples for up to one hour at 25°C did not result in complete solubilization of the membrane proteins. Of the four most abundant species of low M_r protein (<14,000), two (M_r 11 and 13,000) showed a gradual increase in staining intensity with prolonged incubation at 25°C or with shorter incubation times at 75°C, while those proteins of M_r 12,000 and 14,000 were satisfactorily resolved at all temperatures of solubilization. There was a correlation between the appearance of a high M_r complex at low temperatures and the absence of the M_r 11 and 13,000 polypeptides. Another protein (M_r 55,000) showed a solubilization pattern similar to that of these two proteins. One polypeptide of M_r 26,000 was particularly sensitive to heating above 25°C in sample buffer and the resolution of another (M_r 38,000) was reduced at higher temperatures. The M_r of this later species was also slightly increased at the higher temperatures. In the complete absence of mercaptoethanol during the solubilization process this was the only protein which showed band smearing and loss of resolution.

The effect of reductant on the gel profiles was studied separately (Fig. 3.5) at two different temperatures. Heating to 75°C in the absence of mercaptoethanol prevented the loss of the M_r 26,000 protein which

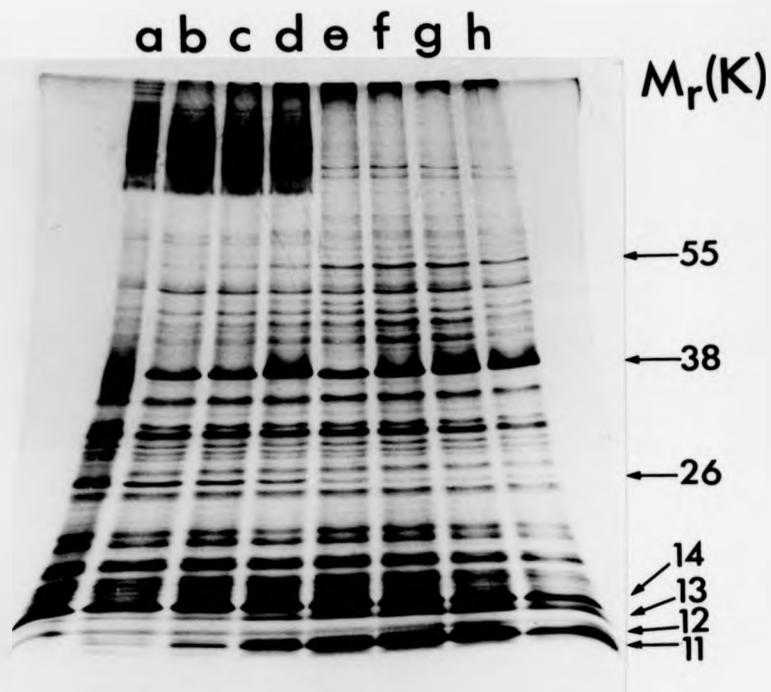


FIGURE 3.4. Effect of solubilization time and temperature on the intra-cytoplasmic membrane protein profile of *Rm. vannielii*.

Membranes (50 μ g protein) from photoheterotrophically grown cells were incubated in Laemmli sample buffer as follows:

a	5 min	} 25°C	} - mercaptoethanol	
b	5 min			
c	15 min			
d	60 min			
e	0.5 min	} 75°C		} + mercaptoethanol
f	5 min			
g	15 min			
h	5 min			

The gel (10-30% gradient) was subsequently silver-stained.

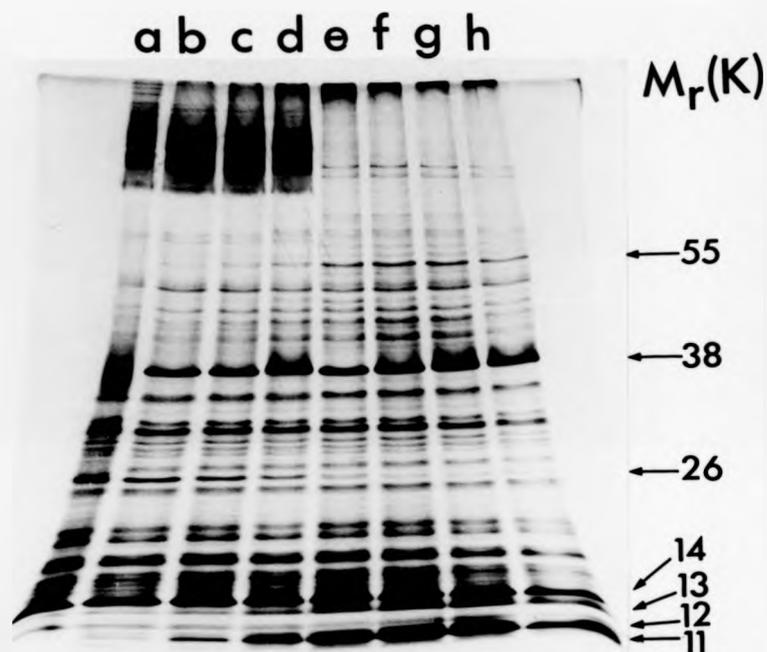


FIGURE 3.4. Effect of solubilization time and temperature on the intra-cytoplasmic membrane protein profile of *Rm. vannielii*.

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a	5 min	} 25°C	} - mercaptoethanol	
b	5 min			
c	15 min			
d	60 min			
e	0.5 min	} 75°C		} + mercaptoethanol
f	5 min			
g	15 min			
h	5 min			

The gel (10-30% gradient) was subsequently silver-stained.

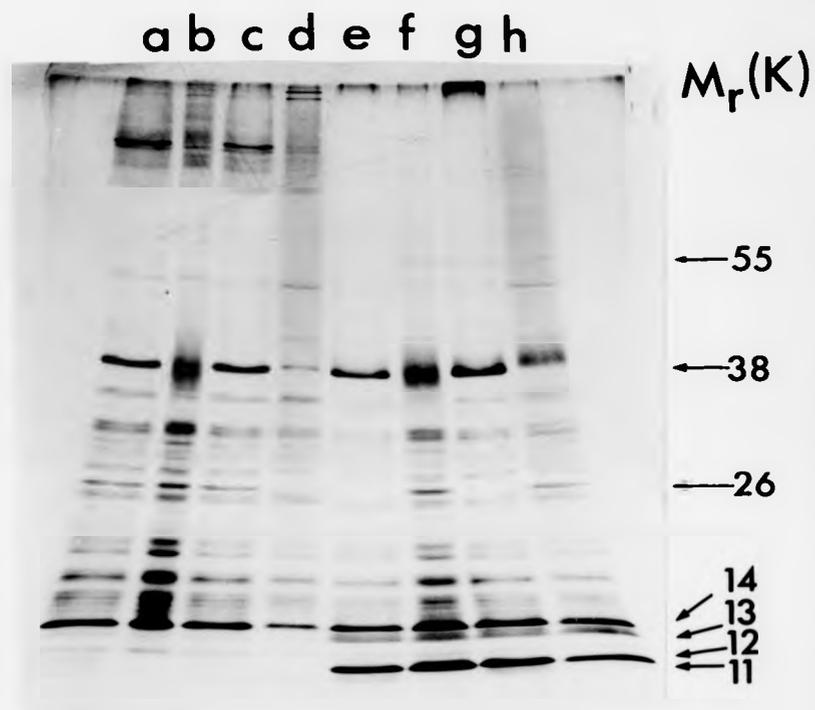


FIGURE 3.5. Effect of reductant on the intra-cytoplasmic membrane protein profile of *Rm. vannielii* at two different temperatures. Membranes (50 μ g protein) from photoheterotrophically grown cells were incubated in Laemmli-based sample buffer (modified as indicated below) at 25°C for 5 min (tracks a-d) or 75°C for 5 min (tracks e-h).

Tracks a & e + mercaptoethanol (55 v/v)
 b & f - mercaptoethanol
 c & g + mercaptoethanol & 4 M urea
 d & h + dithiothreitol (5% w/v)

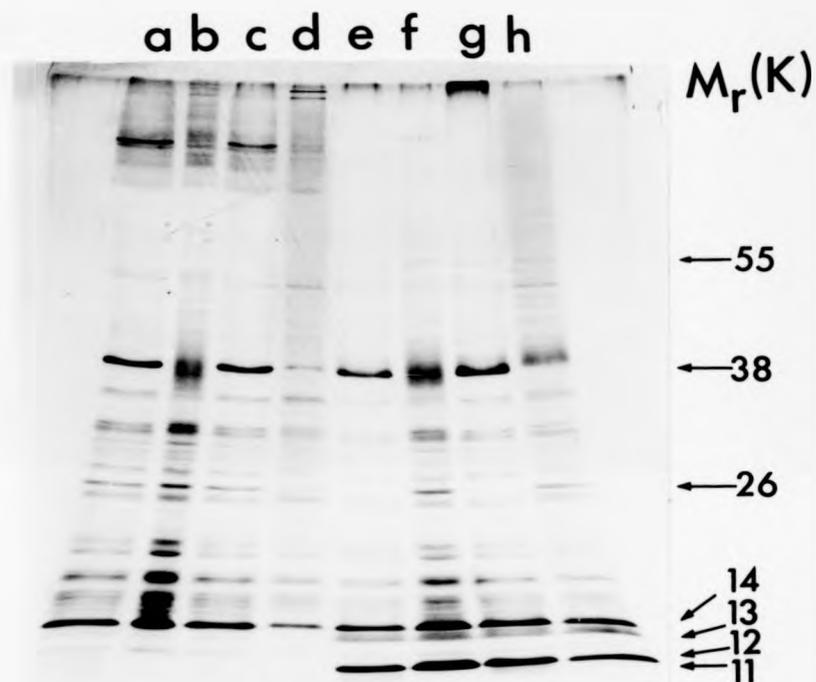


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Tracks a & e + mercaptoethanol (5% v/v)
 b & f - mercaptoethanol
 c & g + mercaptoethanol & 4 M urea
 d & h + dithiothreitol (5% w/v)

otherwise occurred in the presence of this reductant. Heating with dithiothreitol in the sample buffer in place of mercaptoethanol also prevented the loss of this polypeptide, suggesting an effect specifically due to mercaptoethanol. However, the complete absence of reductant or the presence of dithiothreitol did not provide acceptable resolution of the M_r 38,000 polypeptide at either temperature. The addition of 4M urea to the sample buffer during solubilization, which Takemoto and Lascelles (1974) found to improve resolution of certain Rb. sphaeroides ICM proteins, did not significantly alter the gel profile and did not prevent loss of the M_r 26,000 polypeptide.

From these data it is clear that the specific properties of mercaptoethanol are required for good resolution of all the ICM proteins but that heating in the presence of this reductant causes the loss of certain proteins. On the other hand, heat denaturation is essential to ensure resolution of the M_r 11 and 13,000 polypeptides. A compromise time-temperature combination of 2 min at 75°C in the presence of 5% (v/v) mercaptoethanol was therefore chosen as the standard solubilization regime.

With the establishment of conditions that resulted in acceptable resolution of the majority of the ICM polypeptides, the ICM protein profiles of several members of the Rhodospirillaceae were compared with that from Rm. yannielli (Fig. 3.6). The reaction centre polypeptide triplet of Rb. sphaeroides was readily identified (M_r values in this gel system of 24,000, 26,000 and 28,000, and similarly abundant proteins with somewhat similar apparent M_r values were observed in the ICM from Rd. blastica, Rd. palustris and Rm. yannielli. The distinctive RC composition of Rs. rubrum with subunits of M_r 24,000, 26,000 and 32,000

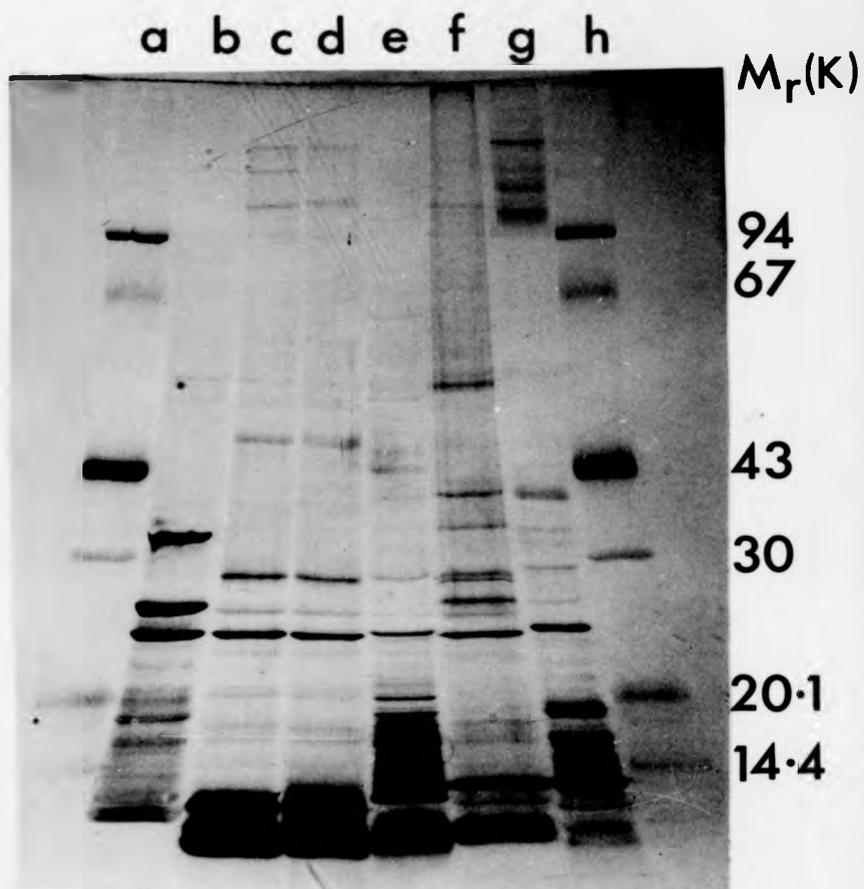


FIGURE 3.6. A comparison of the intra-cytoplasmic membrane protein profiles of some members of the Rhodospirillaceae.

All samples (30 μ g protein) were prepared by sucrose gradient centrifugation and solubilized in sample buffer for 2 min at 75°C. Tracks a & h; molecular weight markers. Track b; Rhodospirillum rubrum. Track c; Rhodobacter sphaeroides NCIB 8253. Track d; Rhodobacter sphaeroides strain 81/1 cordata. Track e; Rhodospseudomonas blastica. Track f; Rhodospseudomonas palustris. Track g; Rhodobacterium vannielii. Silver-stained 10-30% (w/v) gradient gel.

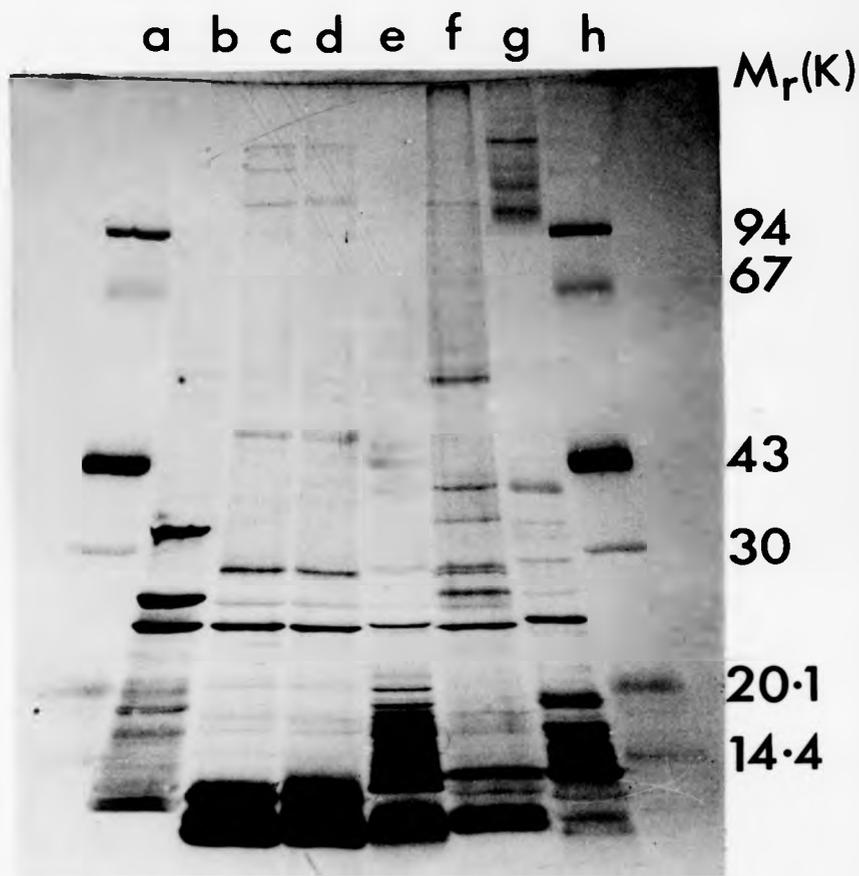


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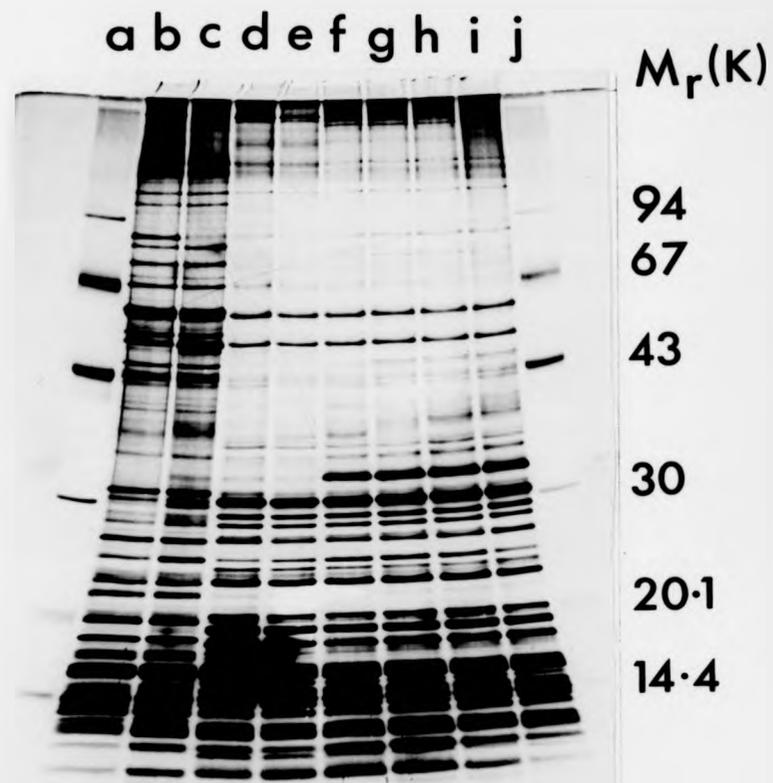


FIGURE 3.7. The effect of solubilization time and temperature on the polypeptide profile of intra-cytoplasmic membranes (tracks b & c) and a cell wall/outer membrane fraction (tracks d-i) isolated from sucrose gradients loaded with cell-free extracts from chemoheterotrophically grown *Rm. yannielii* as described in the legend to Fig. 3.2. All tracks were loaded with 50 μ g protein and solubilization was carried out using Laemmli sample buffer, except tracks a & j which contained molecular weight markers. The

time/temperature combinations were as follows: Track b	25°C,	5 min
c	75°C,	2 min
d	25°C,	5 min
e	25°C,	20 min
f	75°C,	0.5 min
g	75°C,	1 min
h	75°C,	5 min
i	100°C,	5 min

Silver-stained 10-30% (w/v) gradient gel.

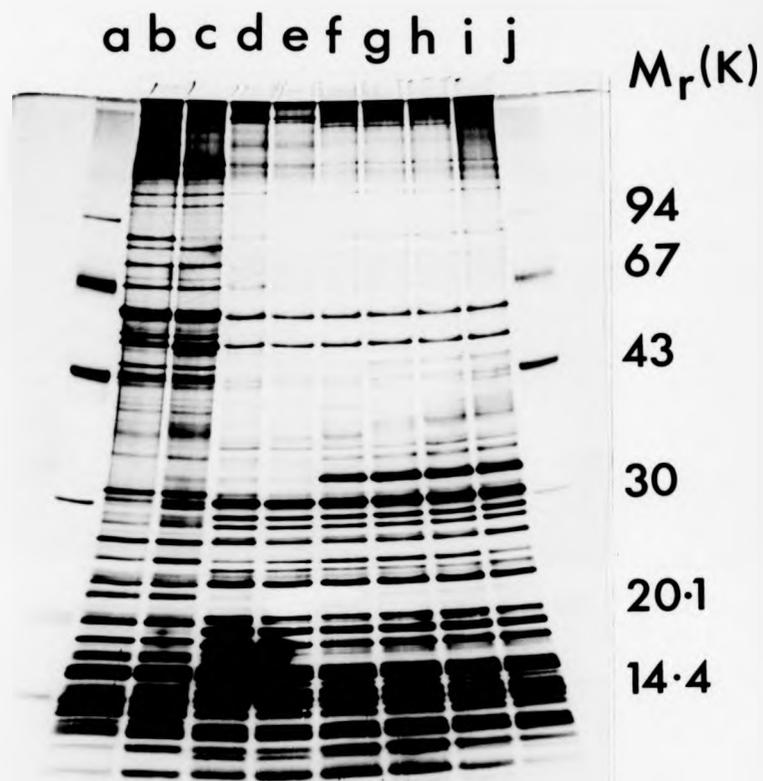


FIGURE 3.7. The effect of solubilization time and temperature on the polypeptide profile of intra-cytoplasmic membranes (tracks b & c) and a cell wall/outer membrane fraction (tracks d-i) isolated from sucrose gradients loaded with cell-free extracts from chemoheterotrophically grown *Rm. vannielii* as described in the legend to Fig. 3.2. All tracks were loaded with 50 μ g protein and solubilization was carried out using Laemmli sample buffer, except tracks a & j which contained molecular weight markers. The

time/temperature combinations were as follows:	Track b	25°C,	5 min
	c	75°C,	2 min
	d	25°C,	5 min
	e	25°C,	20 min
	f	75°C,	0.5 min
	g	75°C,	1 min
	h	75°C,	5 min
	i	100°C,	5 min

Silver-stained 10-30% (w/v) gradient gel.

was also clearly evident. All of the species also exhibited low M_r proteins which may be attributable to those of the LH complexes (Drews & Oelze, 1981).

The effect of solubilization time and temperature was also determined on the ICM and cell wall/outer membrane (OM) fractions from sucrose gradients loaded with cell free extracts from chemoheterotrophically grown cells (Fig. 3.7). The specific objective here was to identify heat-modifiable proteins which could be indicative of the presence of porins (Lugtenberg & van Alphen, 1983). The protein profiles of the ICM and OM fractions showed some similarity, as might be expected from the heterogeneity observed on the sucrose gradients, but with the OM pattern being less complex. A single major heat modifiable protein, with an M_r value of 32,000 when solubilized at high temperature, was present in the OM fraction. In the samples solubilized at 25°C, a high M_r complex of 150,000-200,000 was present which may have been the oligomeric form of the 32,000 M_r protein as there was no evidence of any other species of a higher M_r likely to fill this role.

3.1.3 Enzyme profile

The subcellular localization of some electron transport related enzymes in Rm. vannielii was determined using ICM prepared by differential centrifugation (Methods). This was done, rather than using fractions from sucrose gradients, because shorter centrifugation times could be used, thus preserving enzyme activity. Membranes could also be resuspended to high protein concentrations. As a marker the specific Bchl content, assayed in acetone:methanol extracts, was used. This

TABLE 3.1. Distribution of some dehydrogenase activities and bacteriochlorophyll in sub-cellular fractions of photoheterotrophically grown *Rm. vannielii*

	specific activity (nmol.min.mg protein ⁻¹)	
	Soluble fraction	ICM fraction
[Bacteriochlorophyll (nmol.mg protein ⁻¹)]	1.3 (5.5%)	22.0 (94.5%)
succinate dehydrogenase	1.1 (6.3%)	16.4 (93.7%)
NADH dehydrogenase	248 (56%)	193 (44%)
Malate dehydrogenase	1096 (80%)	300 (20%)
Isocitrate dehydrogenase (NADP-linked)	238 (80%)	54 (20%)
Isocitrate dehydrogenase (NAD-linked)	24 (80%)	6 (20%)

Cells were grown phototrophically at 35 $\mu\text{E m}^{-2}\text{s}^{-1}$ light intensity, harvested in late exponential phase and fractionated by differential centrifugation (section 2.14). The figures given in parentheses represent the proportion of the total activity in that fraction, as a percentage of the two fractions added together. Specific activities were measured as the mean of the determinations on the fractions from one cell-free extract.

indicated that a satisfactory fractionation had been achieved (Table 3.1).

The specific activity of succinate dehydrogenase was highest in the ICM fraction with an overall distribution which closely paralleled that of Bchl, indicating it to be a membrane bound enzyme. Dye-linked NADH dehydrogenase activity was almost equally distributed between the soluble and ICM fractions which may indicate the presence of multiple forms of this enzyme. Malate dehydrogenase, together with NADP and NAD specific isocitrate dehydrogenases appeared to be largely cytoplasmic in their subcellular localization (Table 3.1) or possibly loosely membrane bound.

3.1.4 Absorption spectra

Table 3.2 shows a comparison of the absorption maxima of ICM isolated from Rm. yanniellii and several other members of the Rhodospirillaceae grown under photoheterotrophic conditions. Peaks attributable to Bchl_a at 378, 595, 800 and 870 and those attributable to carotenoids of the normal spirilloxanthin series at 460, 490 and 525 nm were observed in Rm. yanniellii. These correspond closely to the maxima observed in whole cells (Whittenbury & Dow, 1977; France, 1978). Of the other species examined, Rp. acidophila ICM had a spectrum most similar to that of Rm. yanniellii with almost identical carotenoid maxima. That of Rp. blastica, Rp. palustris and Rc. gelatinosus were significantly different in the carotenoid region but generally similar in the near infra-red. The absorption maximum of the longest wavelength band in Rm. yanniellii at 870 nm, however, appeared red-shifted compared to these species (860-

TABLE 3.2. Absorption maxima of intra-cytoplasmic membrane preparations from some members of the Rhodospirillaceae

Species	Bacteriochlorophyll maxima (nm)	Carotenoid maxima (nm)
<u>Rs. rubrum</u>	375, 590, 800 (RC) + 875	485, 510, 550
<u>Rb. sphaeroides</u>	375, 590, 800 + 850 (875) ^a	450, 475, 510
<u>Rc. gelatinosus</u>	378, 595, 805 + 863	455, 480, 512
<u>Rc. tenuis</u>	378, 595, 798 + 865	465, 500, 530
<u>Rp. blastica</u>	376, 590, 800 + 860	450, 476, 508
<u>Rp. acidophila</u>	376, 590, 801 + 858	460, 490, 523
<u>Rp. palustris</u>	380, 595, 805 + 865	420, 450, 470
<u>Rp. viridis</u>	400, 605 - ^b - ^b	420, 450, 470
<u>Rm. vannielii</u>	378, 595, 800 + 870	460, 490, 525

^a The 875 nm LHI absorbance is visible as a "shoulder" in the spectrum.

^b The 1015-1020 nm Bchl_b IR peaks were above the range of the spectrophotometer used.

In each case, cells were grown phototrophically and ICM prepared on sucrose cushions (section 2.14).

865 nm). The near infra-red absorption maxima of all the Bchl_a containing species examined with lamellate ICM systems (Rm. vanniellii, Rp. palustris, Rp. acidophila, Rb. blastica), were significantly different to that of Rb. sphaeroides ICM in which the longest wavelength band - positioned at 850 nm - is known to be almost entirely due to the contribution of the B800-850 LHII complex (Drews & Oelze, 1981). In the organisms with a lamellate ICM system this band is broadened and red-shifted. In Rp. viridis, the spectrum is, of course, radically altered because of the presence of Bchl_b and in Rs. rubrum the near infra-red spectrum contains only contributions from the RC (760 and 800 nm) and LHI (880 nm) complexes.

3.1.5 Conclusions from membrane isolation studies

(1) *Intra-cytoplasmic membranes from Rm. vanniellii can be isolated from phototrophically or chemoheterotrophically grown cells using standard procedures developed for other members of the Rhodospirillaceae.*

(2) *Outer membrane and/or cell wall fractions were only clearly obtainable on sucrose gradients run with chemoheterotrophic cell-free extracts. Such preparations contained a 32,000 M_r heat modifiable protein and carotenoid.*

(3) *Conditions for solubilization of membrane samples for gel electrophoresis were optimized to minimize loss of RC proteins but to ensure complete denaturation of LH proteins.*

3.1.6 Discussion

The isolation of ICM from phototrophically grown Rm. vannielii cells produced very similar sucrose gradient profiles to those obtained for other photosynthetic bacteria, most notably Rb. sphaeroides (Niederman, 1974). On these gradients, the soluble protein formed a band at the top of the gradient containing a large number of polypeptides and a high A260:280 ratio, due to the presence of DNA and unfolded ribosomes. The density of the ICM band (1.16 g cm^{-3}) is within the range ($1.14 - 1.17 \text{ g cm}^{-3}$) reported for other photosynthetic bacteria when sucrose gradients have been employed (Niederman & Gibson, 1978). Shorter rate-zonal spins (section 3.1.1) of cell-free extracts did not reveal a defined "upper pigmented band" of the type described by Niederman *et al.* (1979) for Rb. sphaeroides, although a shoulder in the A870 nm profile in such gradients was sometimes observed. This may be a reflection of the lamellate, rather than vesicular ICM structure of Rm. vannielii. In addition, there was also no evidence of membrane aggregation in the gradient, which would have been indicated by the presence of multiple pigmented bands and extensive interaction of the ICM with the cell-envelope (Niederman, 1974).

The material banding in the density range expected for a cell envelope fraction ($1.20 - 1.25 \text{ g cm}^{-3}$, Niederman & Gibson, 1978) which was sometimes detected on gradients loaded with cell-free extracts from phototrophically grown cells is probably equivalent to the cell wall/outer membrane fraction which was a major component on gradients run with chemoheterotrophic-cell free extracts (section 3.1.1). The difference in the amounts of these respective fractions is likely to be a reflection of the change in the gross envelope structure under the

different growth conditions. Such differences have also been found in Rb. sphaeroides and Rs. rubrum (Ding & Kaplan, 1976; Collins & Niederman, 1976).

The enzyme activities assayed in the ICM fraction from phototrophically grown cells of Rm. vannielii represent useful markers. Membrane bound succinate and NADH dehydrogenases have been demonstrated in a number of the photosynthetic bacteria (Niederman, 1974; Parks & Niederman, 1978; Niederman & Gibson, 1978; Drews & Oelze, 1981) and are potential electron donors to the electron transport chain. Their membrane association is obviously consistent with this role but the largely soluble malate and isocitrate dehydrogenases may also be important especially considering the very high specific activities of malate dehydrogenase detected (Table 3.1) on the pyruvate-malate growth medium. This enzyme would therefore be an important source of NADH during phototrophic growth with these substrates, obviating the need for energy requiring, reversed electron transport through the NADH dehydrogenase (Knaff, 1978).

As discussed in section 3.3, it was not possible to grow Rm. vannielii under aerobic-dark chemoheterotrophic conditions, which resulted in the complete absence of Bchl from the cells. The isolation of a pigmented band on sucrose gradients loaded with extracts from aerobically grown cells which had a density (1.16 g cm^{-3}) identical to that of ICM is therefore consistent with the presence of an ICM system in such cells. Indeed, electron micrographs of thin sections of aerobically grown Rm. vannielii (France, 1978) do show evidence of a peripheral lamellate membrane system but much reduced in extent compared to cells grown phototrophically. The high A260:280 ratio and complex polypeptide

profile (section 3.1.2) of the chemoheterotrophic ICM indicates heterogeneity and the presence of carotenoid in the cell wall/OM fraction would suggest some interaction between the ICM and cell envelope. The protein profiles of these fractions were also somewhat similar (section 3.1.2). However, Jurgens & Weckesser (1985) clearly showed that some cyanobacteria contain carotenoid in their cell wall and outer membrane which may have a "structural" function. Difficulties have been encountered with other photosynthetic bacteria in obtaining pure cytoplasmic and outer membrane fractions when the cells contained Bchl (Niederman & Gibson, 1978; Shepherd *et al.*, 1981).

The search for heat-modifiable proteins in the putative outer membrane containing fractions of chemoheterotrophically grown *Rm. vannielii* revealed a likely candidate in the 32,000 M_r protein (section 3.1.2). Interestingly, a 33,000 M_r polypeptide which showed heat-modifiable characteristics was found by Flammann & Weckesser (1984a) in *Rb. capsulatus* and this showed porin-like activity when reconstituted into proteoliposomes (Flammann & Weckesser, 1984b). However, the protein existed as an oligomer of M_r 69,000 in unheated samples - considerably less than the possible oligomeric form in *Rm. vannielii* (Fig. 3.7).

In view of the apparent impermeability of *Rm. vannielii* to several metabolic inhibitors (section 3.4.2) it would be interesting to isolate the 32,000 M_r protein and assess its permeability properties *in vivo*.

Variation of the solubilization regime of ICM fractions prior to gel electrophoresis clearly showed the importance of optimizing denaturing time and temperature in order to obtain satisfactory gel profiles. Shepherd & Kaplan (1978) in a detailed study of the effect of heat and

mercaptoethanol on the ICM polypeptides of Rb. sphaeroides. found that the L and M subunits of the photochemical reaction centre (M_r 21 and 24,000) were localized in a high M_r complex ($>100,000 M_r$) which, in some cases, remained in the stacking gel when the ICM was solubilized in the presence of mercaptoethanol. This effect was apparently due to the specific properties of this reductant because dithiothreitol or glutathione did not cause the loss of the RC polypeptides from the gel profile. The M_r 26,000 polypeptide in the Rm. vannielii ICM showed similar heat and mercaptoethanol sensitivities, and, as described in section 3.2, is identifiable with one of the RC subunits in this species. The study of Shepherd & Kaplan (1978) did not identify changes in the solubilization pattern of the low M_r polypeptides ($<14,000$) which, in Rb. sphaeroides, are largely attributable to the proteins of the two light-harvesting pigment-protein complexes (Drews & Oelze, 1981). However in the paper of Shepherd & Kaplan (1978) high M_r complexes (ca. 60-100,000), similar to those observed in Rm. vannielii (Fig. 3.4), are clearly visible on some of the gels when samples were unheated prior to SDS-PAGE, which suggests incomplete solubilization of the LH complexes at low temperatures. In section 3.2.3, this is confirmed specifically for the B800-865 (LHII) complex of Rm. vannielii and thus it may be a more general phenomenon of the accessory LH complexes of the Rhodospirillaceae which clearly must be taken into account in studies utilising gel electrophoresis of membrane fractions.

3.2 The photosynthetic pigment-protein complexes of *Rm. vannielii*

In recent years, a number of methods have become available for the isolation of pigment-protein complexes from some photosynthetic bacteria and these have invariably been based on the use of detergent solubilization of ICM fractions under "mild" conditions (Cogdell & Thorner, 1980). Subsequent steps usually involve column chromatography, gel electrophoresis or sucrose gradient centrifugation (Broglie *et al.*, 1980; Hayashi *et al.*, 1982a; Cogdell *et al.*, 1983; Varga & Staehelin, 1985a). Such methods have generally been designed for specific microbes and there are few general procedures available. A number of different approaches were therefore tested using *Rm. vannielii* (Kelly & Dow, 1985b).

3.2.1 Identification of pigment-protein complexes

Delepelaire & Chua (1979) introduced a solubilization procedure using lithium dodecyl sulphate at 4°C followed by gel electrophoresis in the cold in the presence of this detergent to isolate chlorophyll *a*-protein complexes from chloroplast thylakoid membranes. The technique was adapted by Broglie *et al.* (1980) for use with the ICM of *Rb. sphaeroides*, from which a number of pigmented complexes were characterized. The use of the lithium salt was preferred because of its greater solubility at low temperature, a condition essential to prevent breakdown of the isolated complexes.

FIGURE 3.8. Solubilization of intra-cytoplasmic membranes with lithium dodecyl sulphate (LDS).

Rhodomicrobium vannielii ICM (20 μ g Bchl; tracks a-f) or *Rb. sphaeroides* ICM (20 μ g Bchl; tracks g & h) was incubated with LDS at 0-4°C for 1 min, prior to electrophoresis in the cold on a 10% (w/v) gel. Various ratios of LDS:Bchl (wt/wt) were used as follows;

Track	a	5:1
	b	10:1
	c & g	20:1
	d	30:1
	e & h	40:1
	f	50:1

giving final concentrations of 0.5% (w/v) to 5.0% (w/v) LDS. After electrophoresis, the gel was photographed unstained (a) and stained with coomassie blue (b). The M_r values given for two of the major bands are "apparent" values, calculated from the migration of initially fully denatured marker proteins in this incompletely denaturing gel system.

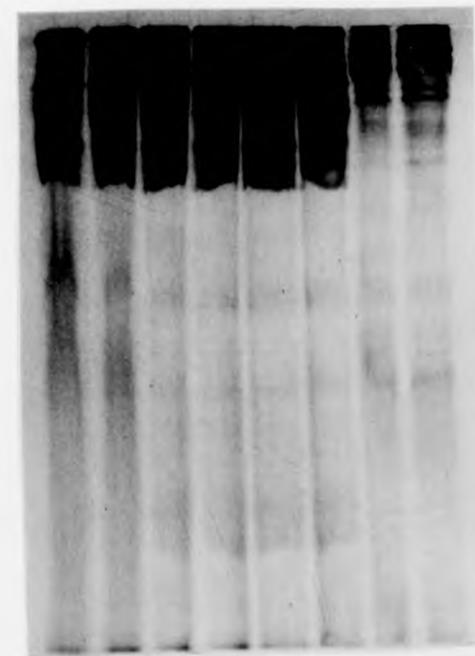
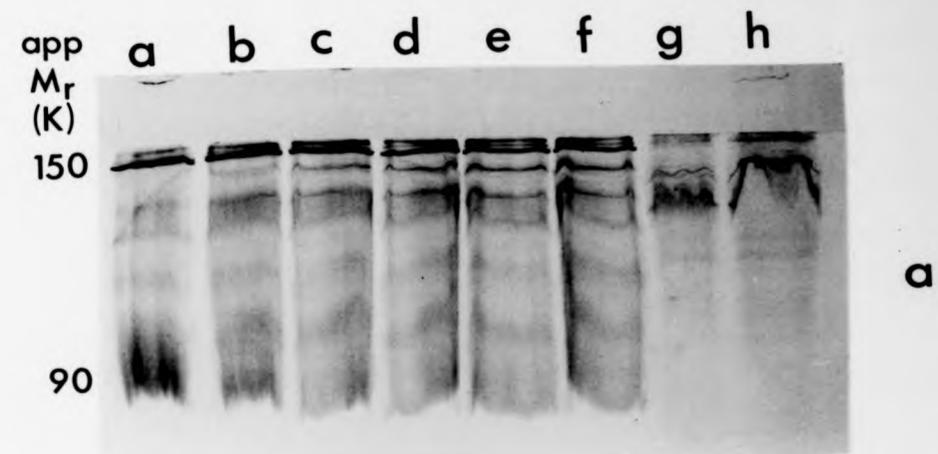
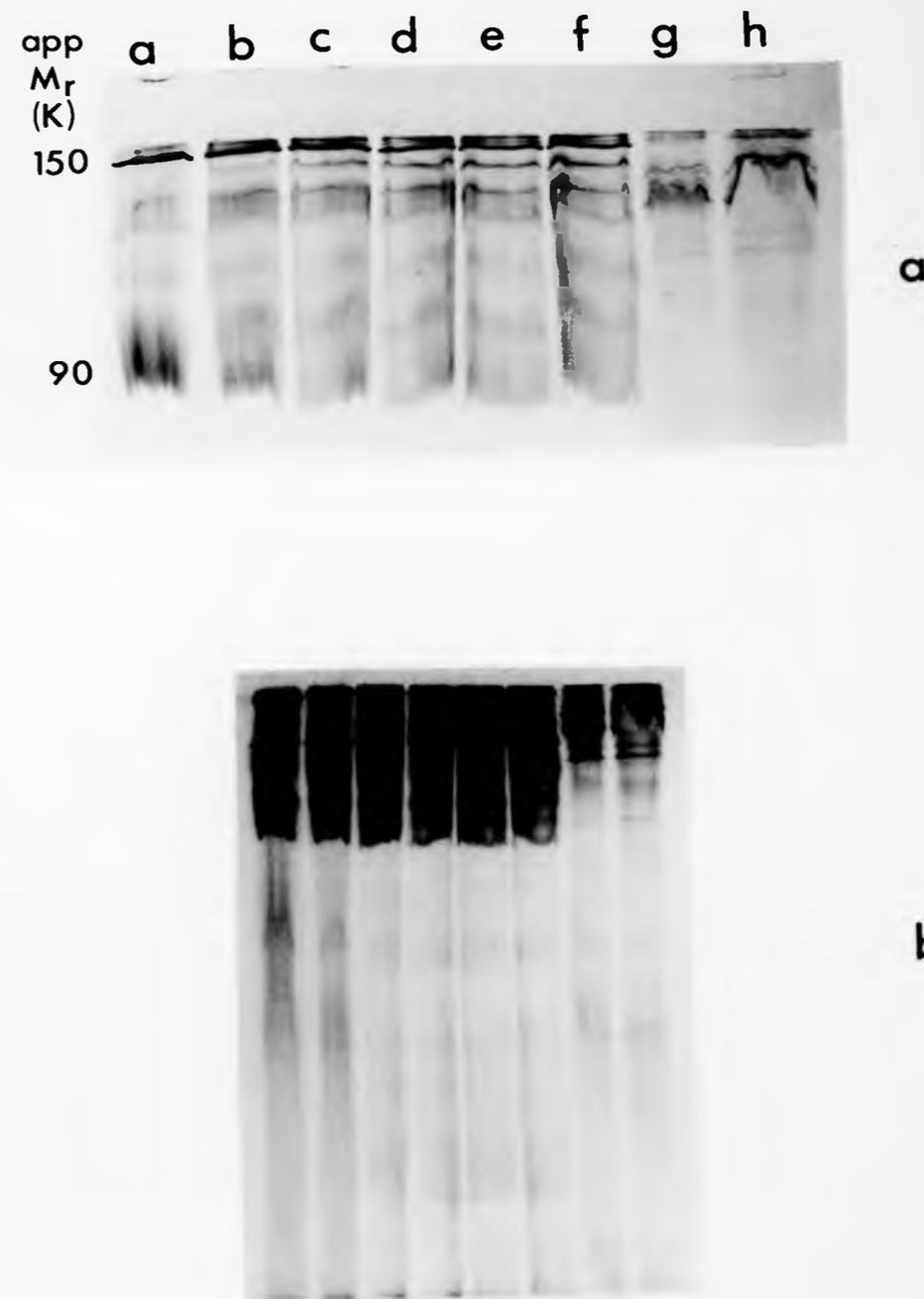


FIGURE 3.8. Solubilization of intra-cytoplasmic membranes with lithium dodecyl sulphate (LDS).

Rhodomicrobium vannielii ICM (20 μ g Bchl; tracks a-f) or *Rb. sphaeroides* ICM (20 μ g Bchl; tracks g & h) was incubated with LDS at 0-4°C for 1 min, prior to electrophoresis in the cold on a 10% (w/v) gel. Various ratios of LDS:Bchl (wt/wt) were used as follows;

Track	a	5:1
	b	10:1
	c & g	20:1
	d	30:1
	e & h	40:1
	f	50:1

giving final concentrations of 0.5% (w/v) to 5.0% (w/v) LDS. After electrophoresis, the gel was photographed unstained (a) and stained with coomassie blue (b). The M_r values given for two of the major bands are "apparent" values, calculated from the migration of initially fully denatured marker proteins in this incompletely denaturing gel system.



This technique was applied to the ICM of Rm. vanniellii (Fig. 3.8a). Membranes isolated by centrifugation on sucrose cushions were treated with various ratios of LDS:Bchl prior to electrophoresis on a 10% (w/v) gel at 4°C. Membranes from Rb. sphaeroides were treated in a similar manner with LDS:Bchl ratios of 20:1 and 40:1 as described by Broglie et al. (1980). This treatment resulted in the appearance of up to 9 red pigmented bands in Rm. vanniellii and 6-7 in Rb. sphaeroides. At an LDS:Bchl ratio of 20:1, the overall pattern of pigmented bands from Rb. sphaeroides was comparable to that observed by Broglie et al. (1980), in which the B800-850 LHII complex constitutes the major band and the other minor complexes represent mixtures of B875 and B800-850 in various preparations. At an LDS:Bchl ratio of 40:1 resolution was much poorer. A very different pattern was observed with Rm. vanniellii ICM. Variation of the LDS:Bchl ratio over a 10-fold range from 5:1 to 50:1 had little influence on the gross overall resolution but caused the disappearance of a major complex of apparent M_r 90,000 (only clearly seen at 5:1 LDS:Bchl) and the appearance of a "ladder" of other pigmented bands. A second, major complex of apparent M_r 150,000-200,000 was resolved at each treatment ratio but reduced in amount with higher detergent concentrations. Coomassie blue staining of the same gel (Fig. 3.8b) indicated the absence of most other membrane proteins from the profile, suggesting their location in high M_r complexes along with those of the photosynthetic apparatus. Indeed, most of the stained bands were also pigmented but the fact that no insoluble material was left in the stacking gel indicated complete dissolution of the membrane. However, below ratios of 5:1, LDS:Bchl the ICM was incompletely solubilized.

It is likely that the large number of pigmented bands obtained with this method represent mixtures of the various pigment-protein complexes, the

formation of which is accentuated at high detergent: Bchl ratios. At an LDS:Bchl ratio of 5:1 there was evidence of two major pigmented bands but it was not possible to extract sufficient material from the gel for spectroscopic analysis. Variation in the solubilization conditions was therefore attempted by the use of three detergents with differing properties. These were the non-ionic detergent Triton X-100, the zwitterionic detergent, lauryldimethylamine-N-oxide (LDAO) and the ionic detergent, SDS (SDS is more readily available than LDS and was found not to precipitate in the cold at the concentrations used). Gels were prepared with these detergents alone or in combination and the solubilized ICM electrophoresed in the cold.

Using a gel containing only Triton X-100 and solubilization of samples with 1% (w/v) SDS at 4°C or room temperature, 5% (w/v) Triton X-100 or a mixture of 1% (w/v) SDS and 1% (w/v) Triton X-100, resulted in poor resolution and no clear separation of pigmented bands. In a previous study, Hayashi *et al.* (1982a) reported the use of a mixture of SDS and Triton X-100 to be effective in the isolation of Bchl-protein complexes from *Rp. palustris*. When *Rm. vannielii* ICM was solubilized with final concentrations of 0.8% (w/v) each of SDS and Triton X-100 at room temperature or at 4°C and then electrophoresed in the presence of both detergents, two native pigmented bands were clearly resolved (Fig. 3.9). Essentially the same result was obtained if SDS or Triton X-100 alone were used for solubilization, provided that the gel system and running buffer contained both detergents. However treatment of ICM with SDS + Triton X-100 followed by electrophoresis on a gel containing only SDS resulted in the disappearance of the lower pigmented band (LPB) and the formation of a "ladder" of other complexes, exactly as seen with the use of LDS alone (Fig. 3.8). Under these conditions the upper pigmented

FIGURE 3.9. Treatment of *Rm. vannielii* ICM with different detergents.

Membranes (20 μ g Bchl) were incubated with 0.8% (w/v) SDS (a), 0.8% (w/v) Triton X-100 (b) or a mixture of 0.8% (w/v) SDS and 0.8% (w/v) Triton X-100 (c) for 5 min at room temperature and subsequently electrophoresed on a 10% (w/v) gel containing 0.05% (w/v) SDS and 0.05% (w/v) Triton X-100. In a separate environment, membranes (20 μ g Bchl) were incubated with 0.8% (w/v) SDS and 0.8% (w/v) Triton X-100 and the solubilized complexes subsequently electrophoresed on a 10% (w/v) gel containing only 0.1% (w/v) SDS (d). In (e), *Rm. vannielii* ICM was solubilized with 1% (w/v) LDAO and electrophoresed on a 10% (w/v) gel also containing this detergent (0.1% w/v final concentration). All gels were photographed unstained.

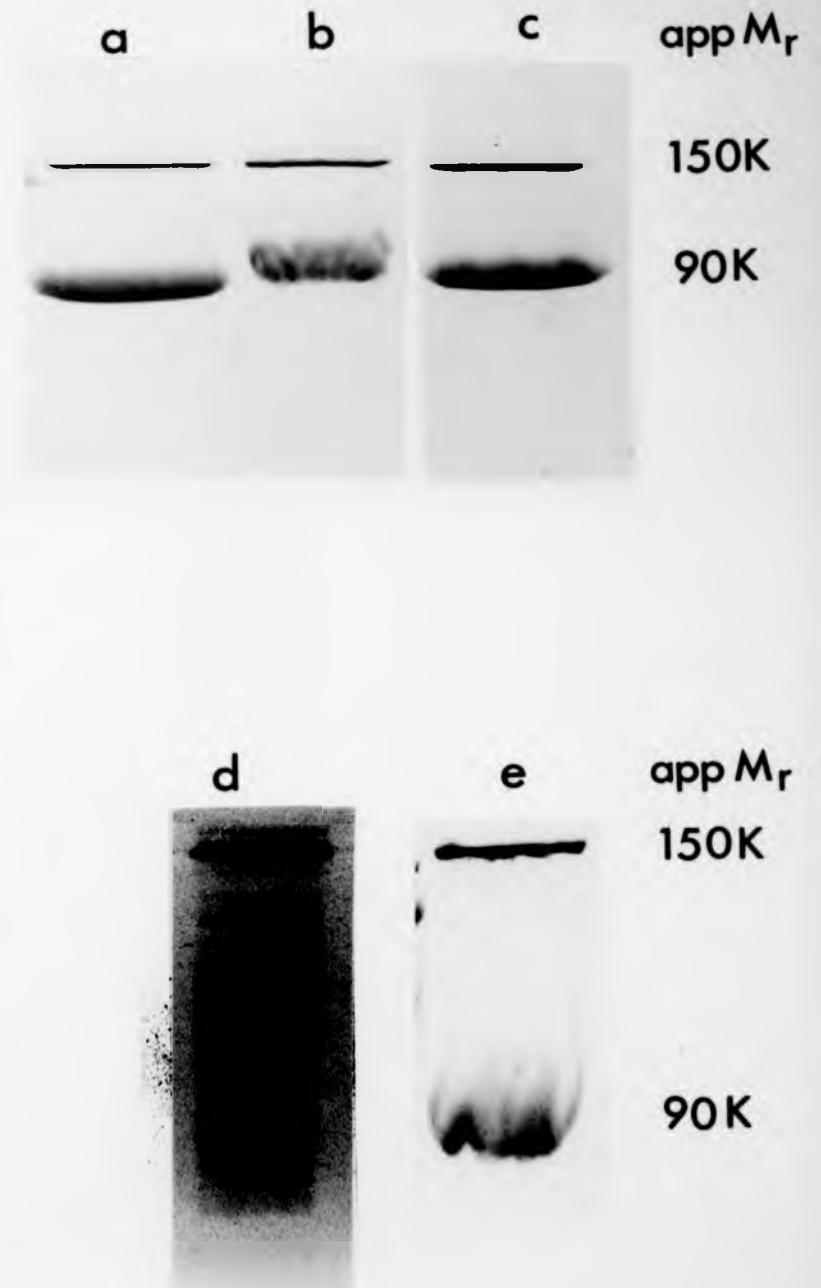
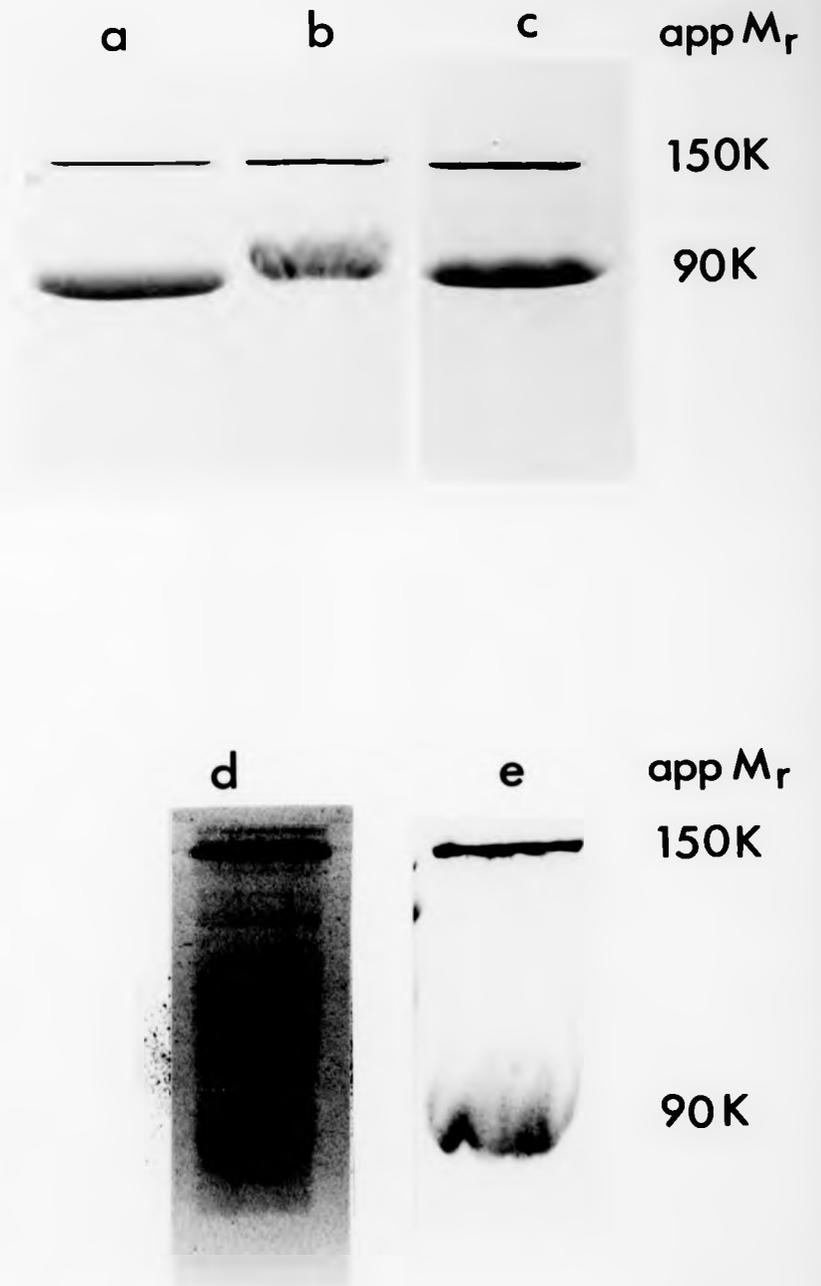


FIGURE 3.9. Treatment of *Rm. vannielii* ICM with different detergents.

Membranes (20 μ g Bchl) were incubated with 0.8% (w/v) SDS (a), 0.8% (w/v) Triton X-100 (b) or a mixture of 0.8% (w/v) SDS and 0.8% (w/v) Triton X-100 (c) for 5 min at room temperature and subsequently electrophoresed on a 10% (w/v) gel containing 0.05% (w/v) SDS and 0.05% (w/v) Triton X-100. In a separate environment, membranes (20 μ g Bchl) were incubated with 0.8% (w/v) SDS and 0.8% (w/v) Triton X-100 and the solubilized complexes subsequently electrophoresed on a 10% (w/v) gel containing only 0.1% (w/v) SDS (d). In (e), *Rm. vannielii* ICM was solubilized with 1% (w/v) LDAO and electrophoresed on a 10% (w/v) gel also containing this detergent (0.1% w/v final concentration). All gels were photographed unstained.



band (UPB) remained. The pattern obtained with the use of LDAO (Fig. 3.9) was very similar to that observed in the Triton-SDS system, with two pigmented bands being resolved.

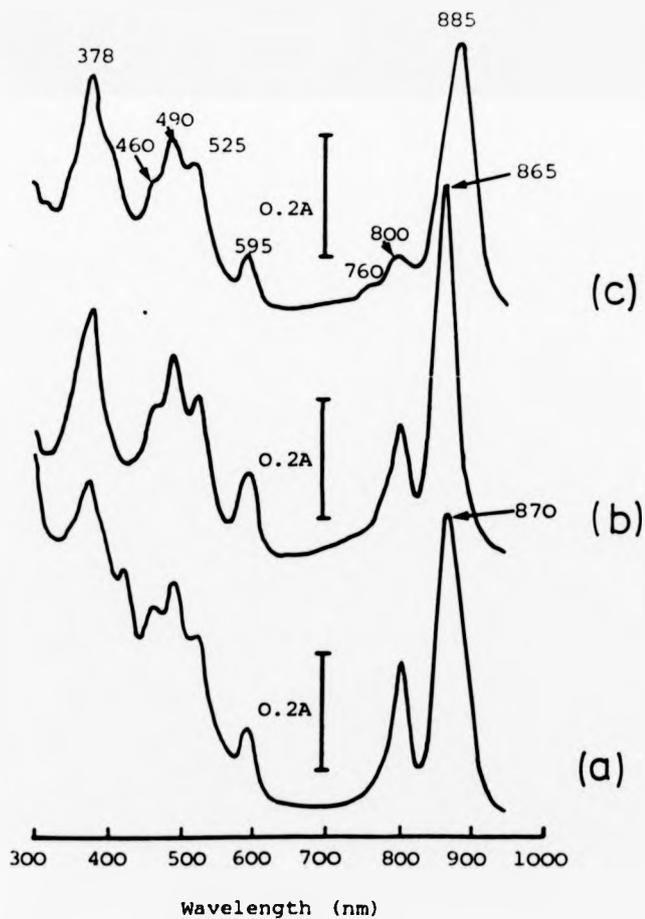
The apparent M_r values of the UPB and LPB in the Triton-SDS gel system were 150,000 and 90,000 respectively. This correlates well with the values obtained during electrophoresis with LDS (Fig. 3.8).

The fact that the same type of upper and lower pigmented bands were observed using different solubilization procedures in the Triton-SDS gel system and with the zwitterionic detergent LDAO, in addition to being present under some conditions with LDS solubilization lends credence to the view that they represent the true pigment-protein complexes of the photosynthetic apparatus. These two pigmented complexes were further characterized in order to determine their spectral identity using the Triton-SDS dual detergent method of solubilization followed by electrophoresis in the presence of both detergents. This procedure, or a sucrose gradient modification thereof (section 3.2.4) was adopted as the standard technique for their preparation.

3.2.2 Absorption spectra and redox properties

In order to identify the spectral forms of Bchl within the complexes, they were eluted from gels by homogenization in Tris buffer and room-temperature absorption spectra obtained (Fig. 3.10). The UPB, which migrated with an apparent M_r of ca. 150,000 in the Triton-SDS gel system, exhibited three peaks in the near infra-red (IR) region of the spectrum at 760, 800 and 885 nm. The ratio of the A885:800 maxima

FIGURE 3.10. Room temperature absorption spectra of (a) *Rm. vannielii* ICM, (b) the lower pigmented band (LPB) and (c) the upper pigmented band (UPB) from ICM solubilized with SDS plus Triton X-100 followed by gel electrophoresis with both detergents. Gel slices were homogenised in 10 mM Tris-HCl buffer pH 7.4 and incubated at 4°C overnight to elute the complexes. Absorption spectra were determined on the supernatants.



varied from 4 to 6 in different preparations. The LPB, which migrated with an apparent M_r of 90,000 in the Triton-SDS gel system, exhibited two infra-red peaks at 800 and 865 nm. The A865:800 ratio was between 1.5 to 3.0 and was found to be more variable from preparation to preparation than that of the UPB IR spectrum, depending upon the age of the samples and the temperature of storage.

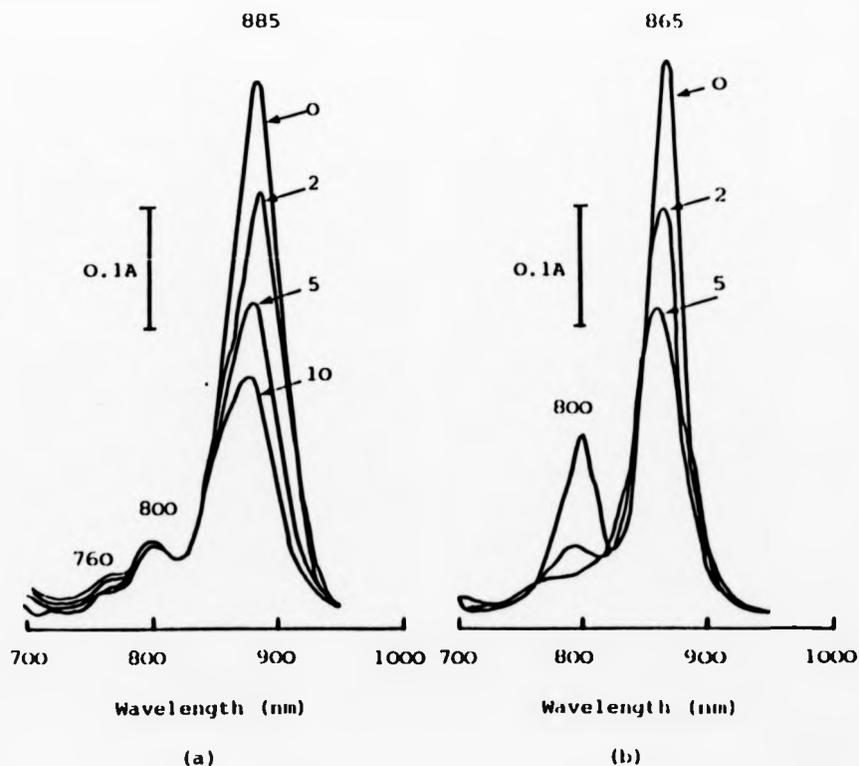
Both complexes exhibited Bchl absorption maxima in the visible region (595 nm) and ultra-violet (378 nm) which corresponded with those observed in intact ICM not subjected to detergent treatment. The carotenoid maxima of the two complexes (460, 490 and 525 nm) were also identical to those of the ICM but it was consistently observed that the 460 nm region of the UPB spectrum existed as a shoulder rather than as a discrete peak as in the LPB and ICM spectra. An additional peak in the ICM spectrum in the 415-420 nm region could not be accounted for in that of the pigment-protein complexes.

From the near IR spectrum, the UPB appeared to contain both reaction centre components - Bchl, responsible for the 800 nm peaks and bacteriopheophytin, responsible for the 760 nm absorbance - in addition to a much larger amount of light-harvesting Bchl which in this thesis has been designated B885, following the recommendations of Cogdell et al. (1985). This B885-RC complex has absorption properties similar to those described from some other members of the Rhodospirillaceae (Drews & Oelze, 1981) and in particular it resembles the spectrum of the ICM of Rs. rubrum in which only one type of light-harvesting complex is present.

The LPB contained the bulk of the membrane bound photopigments and had an IR spectrum similar to that of the "accessory" LH complexes of other

FIGURE 3.11. Chemical oxidation of (a) the B885-RC complex and (b) the B800-865 complex of *Rm. vannielii*.

Complexes in 10 mM Tris HCl buffer pH 7.4 containing 0.05% (w/v) each of SDS and Triton X-100 were adjusted to the same absorbance value of the longest wavelength infra-red peak. Potassium ferricyanide was added to a final concentration of 50 mM and spectra taken at intervals (arrows with numbers refer to the time in min. after the addition of ferricyanide).



members of the Rhodospirillaceae (Drews & Oelze, 1981). According to the near-IR absorption maxima, this complex was designated B800-865.

The redox properties of bacterial light-harvesting complexes have received increasing attention in recent years as a means of probing their electronic structure (Picorel *et al.*, 1984) in addition to confirmatory techniques such as circular dichroism spectrophotometry (Cogdell & Thornber, 1980; Thornber *et al.*, 1983). Specifically, the ease of oxidation of the IR Bchl_a peaks with ferricyanide is an indication of this structure (Picorel *et al.*, 1984). The B885-RC and B800-865 complexes from *Rm. vannielii* were oxidized with ferricyanide and the effect on the infra-red absorption spectrum noted (Fig. 3.11). Oxidation of the B885-RC complex resulted in a rapid decline of the 885 nm peak amplitude, coupled with a blue-shift of up to 10 nm, while both the 760 and 800 nm peaks remained unaltered. In contrast, the 800 nm peak of the B800-865 complex was very sensitive to oxidation and was not detectable in the spectrum a few minutes subsequent to the addition of ferricyanide but the 865 nm peak was only moderately sensitive and was blue-shifted by only 2-3 nm. This again contrasts with the longest wavelength absorption band of the B885-RC complex.

Importantly, the differences in the behaviour of the 800 nm peak of the respective complexes indicates no cross-contamination and different types of Bchl interactions.

To determine if the Triton-SDS gel system was generally applicable to the analysis of pigment-protein complexes in the Rhodospirillaceae, ICM fractions from a number of species were solubilized with the detergent mixture and electrophoresed (Fig. 3.12). The unstained gel showed clear

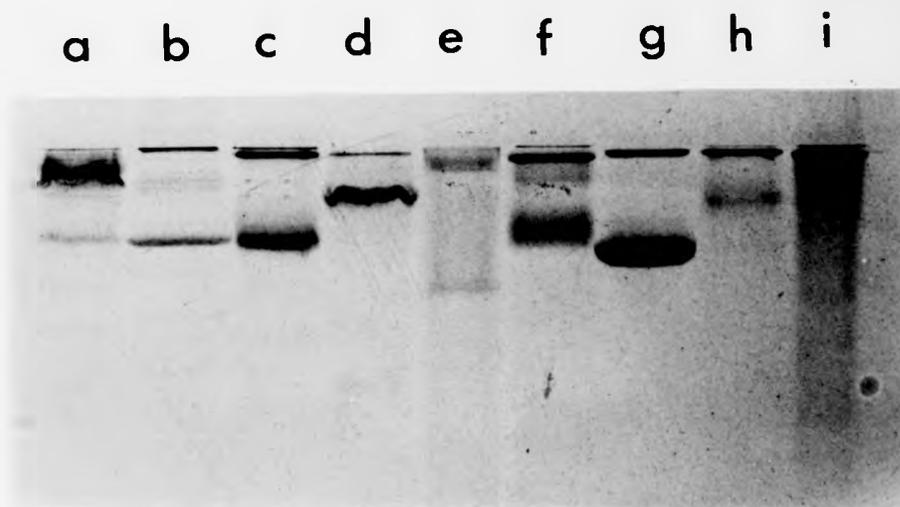


FIGURE 3.12. Detection of photosynthetic pigment protein complexes in various species of the Rhodospirillaceae by SDS-Triton gel electrophoresis.

Membranes (200-400 μ g protein) were solubilized with 0.8% (w/v) SDS and 0.8% (w/v) Triton X-100 and the complexes electrophoresed on a 10% (w/v) gel in the cold. Track (a), *Rb. sphaeroides*: (b), *Rp. blastica*: (c) *Rp. acidophila*: (d) *Rc. gelatinosus*: (e) *Rp. viridis*: (f) *Rp. palustris*: (g) *Rm. vannielii*: (h) *Rc. tenuis*. (i) *Rs. rubrum*.

The gel was photographed unstained.

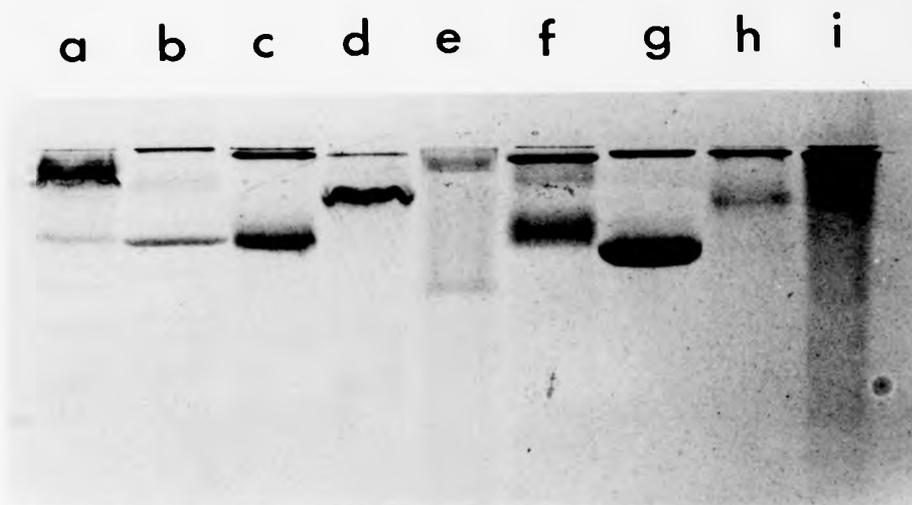


FIGURE 3.12. Detection of photosynthetic pigment protein complexes in various species of the Rhodospirillaceae by SDS-Triton gel electrophoresis.

Membranes (200-400 μ g protein) were solubilized with 0.8% (w/v) SDS and 0.8% (w/v) Triton X-100 and the complexes electrophoresed on a 10% (w/v) gel in the cold. Track (a), Rb. sphaeroides; (b), Rp. blastica; (c) Rp. acidophila; (d) Rc. gelatinosus; (e) Rp. viridis; (f) Rp. palustris; (g) Rm. vannielii; (h) Rc. tenuis, (i) Rs. rubrum.

The gel was photographed unstained.

evidence of the existence of two pigmented complexes identifiable with the UPB and LPB of Rm. vanniellii. in Rp. blastica, Rp. acidophila, Rp. viridis and Rp. palustris. The relative mobility of the LPBs was more variable than that of the UPBs in these species. Consistent with the known composition of the photosynthetic apparatus in Rs. rubrum (RC plus one LH complex) only one pigmented band was formed corresponding to the B885-RC complex (UPB) in Rm. vanniellii. The two representatives of the genus Rhodocycclus - Rc. gelatinosus and Rc. tenuis also presented two pigmented bands but with the "LPB" in each case being of a rather higher apparent M_r value than those of the genus Rhodopseudomonas. Rhodobacter sphaeroides showed clear evidence of an LPB likely to correspond to the B800-850 complex previously identified by Broglie et al. (1980) but the resolution of the putative UPB corresponding to the RC-B875 complex was compromised by the formation of other pigmented bands in the same region of the gel. For this species at least, the procedure may not be optimal and mixtures of the complexes could have been present. Nevertheless the clear-cut division of species with an accessory LH complex in addition to that associated with the RC (i.e. the majority) and those with no accessory complex (Rs. rubrum) would appear to correlate with the presence of two pigmented bands and one pigmented band respectively in the Triton-SDS gel system.

3.2.3 Polypeptide composition of the pigment-protein complexes

The polypeptide composition of the isolated complexes are compared with those of the intact ICM in Fig. 3.13. When samples were unheated prior to gel electrophoresis, the native complexes migrated in a similar manner to that observed using the Triton-SDS gel system. As determined

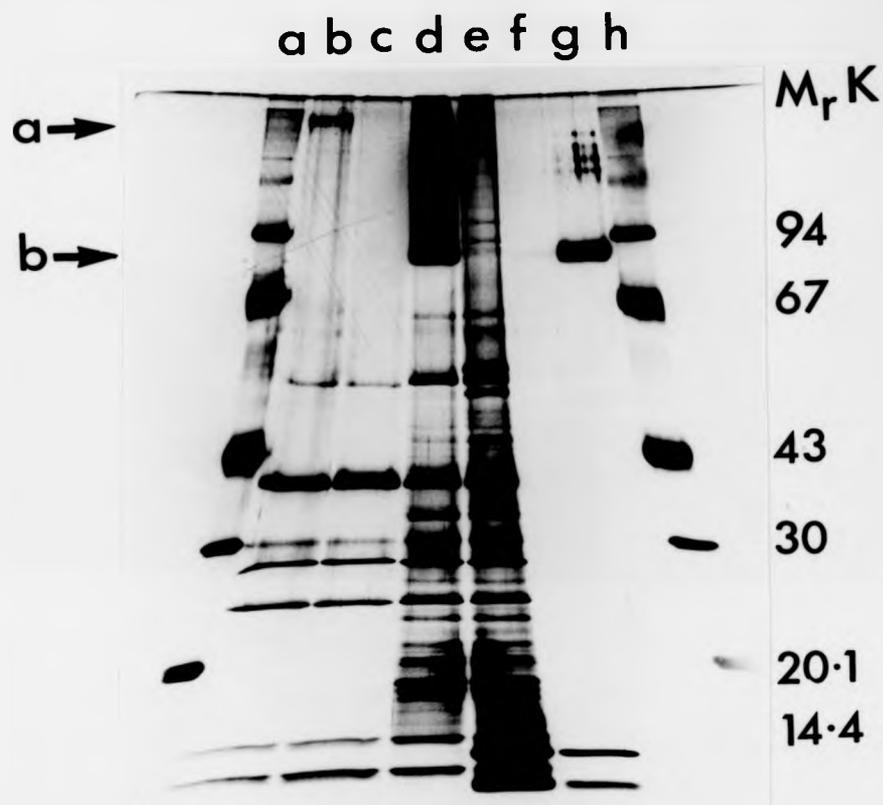


FIGURE 3.13. Polypeptide composition of the B885-RC complex (20 μ g protein; tracks b & c), ICM (50 μ g protein; tracks d & e) and the B800-865 complex (10 μ g protein; tracks f & g) from *Rm. vannieli*. In tracks b, d & g the samples were not heated in the Laemmli sample buffer before application to the gel. In tracks c, e & f the samples were heated at 75°C for 2 min to effect complete denaturation. Tracks a & h contained molecular weight markers. The arrows indicate the undenatured form of the B885-RC complex (a) and the B800-865 complex (b). Silver-stained 10-30% (w/v) polyacrylamide gel.

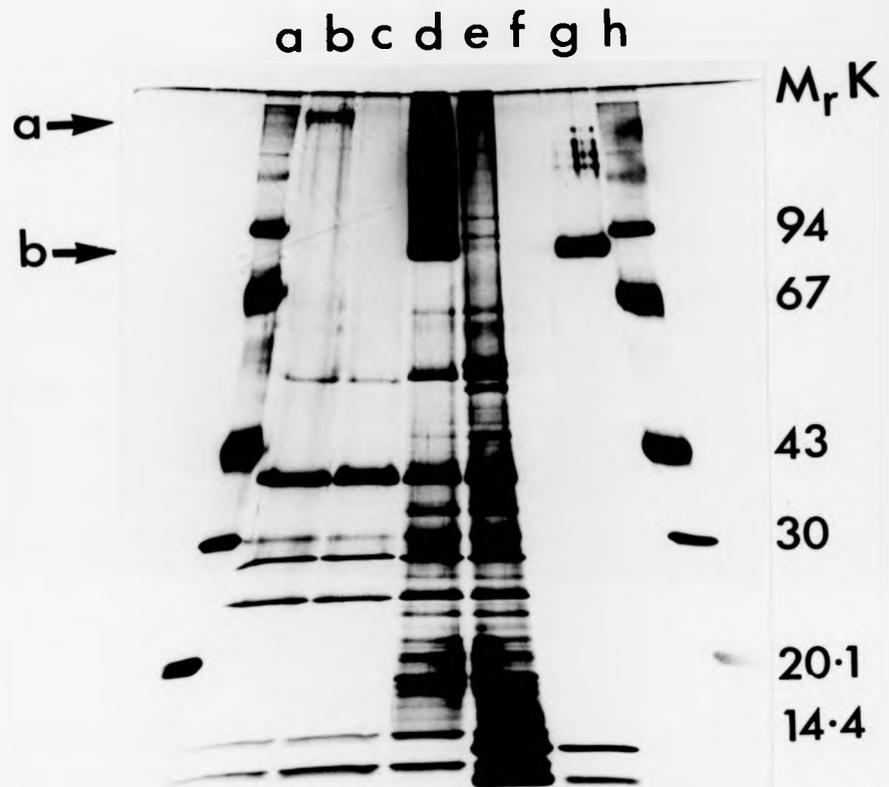


FIGURE 3.13. Polypeptide composition of the B885-RC complex (20 μ g protein; tracks b & c), ICM (50 μ g protein; tracks d & e) and the B800-865 complex (10 μ g protein; tracks f & g) from *Rm. vannieli*. In tracks b, d & g the samples were not heated in the Laemmli sample buffer before application to the gel. In tracks c, e & f the samples were heated at 75°C for 2 min to effect complete denaturation. Tracks a & h contained molecular weight markers. The arrows indicate the undenatured form of the B885-RC complex (a) and the B800-865 complex (b). Silver-stained 10-30% (w/v) polyacrylamide gel.

on 10-30% gradient gels, their molecular ratio values were 170,000 for the native B885-RC complex and 89,000 for the native B800-865 complex. These are very similar to the values obtained in section 3.2.1 and suggest that separation in the Triton-SDS gel system occurs largely according to size. Evidence for the presence of these native complexes in unheated ICM was also apparent (Fig. 3.13).

A striking difference was observed in the ease with which the pigment-protein complexes were denatured. While incubation of the B885-RC complex in Laemmli sample buffer at room temperature was sufficient to effect an almost complete breakdown of the native aggregate, the B800-865 complex required heating to 75°C or above to complete this process. Denaturation resulted in the appearance of only two polypeptides of M_r 11,000 and 13,000 in the case of the B800-865 complex and six polypeptides in the case of the B885-RC complex. Five of these proteins could be attributed to the photochemical reaction centre (M_r 26,000, 28,000 and 31,000) and the B885 complex itself (M_r 12,000 and 14,000) because their M_r values were characteristic of similar complexes found in other members of the Rhodospirillaceae (Drews & Oelze, 1981). In addition, the three RC polypeptides of *Rm. yanniellii* were clearly identifiable with the RC triplet of *Rb. sphaeroides* (Fig. 3.6) when ICM was subjected to gel electrophoresis. It should however be noted that the M_r values of the LH polypeptides had to be obtained by extrapolation off the calibration curve because the values were so low.

These results confirm those obtained with ICM solubilized under different conditions (section 3.1.2) where two polypeptides, here identified as those of the B800-865 complex, were only resolved after heating the samples above 75°C. The B885 complex polypeptides were

FIGURE 3.14. Two-dimensional gel electrophoresis of the B885-RC complex.

Forty μ g protein of the complex isolated from sucrose gradients (section 2.27.3) was subjected to isoelectric focussing (IEF) in the 1st dimension as described in section 2.17.5. The IEF tube gel was equilibrated with Laemmli sample buffer before electrophoresis in the second dimension on a 10-30% (w/v) gradient gel containing SDS. The protein spots were subsequently visualized by silver staining. Molecular weight marker proteins were also run in the second dimension.

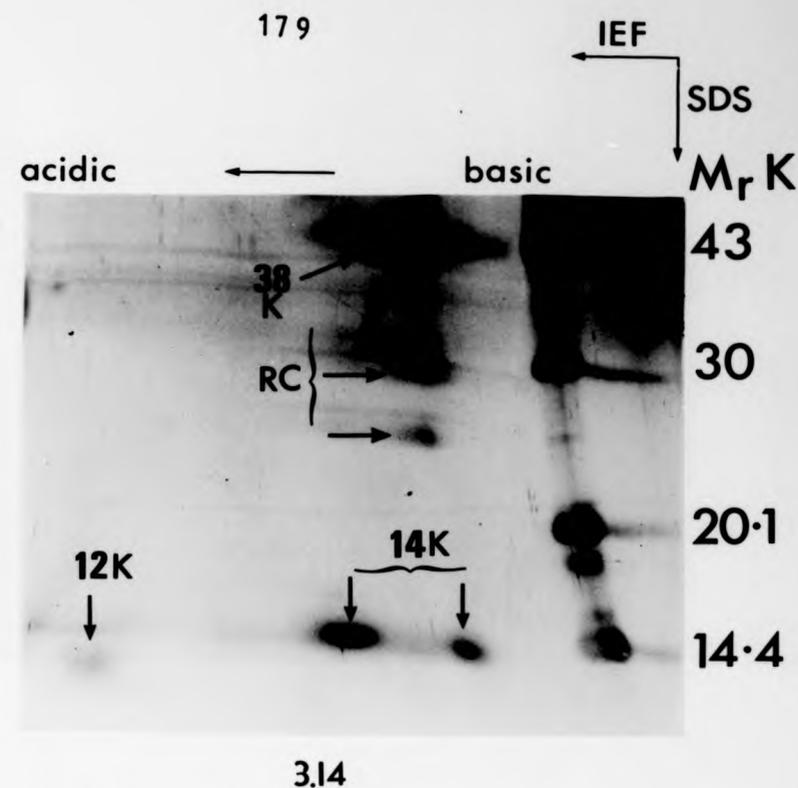


FIGURE 3.15. Two-dimensional gel electrophoresis of the B800-865 complex.

Twenty μ g protein was subjected to isoelectric focussing as described in section 2.17.5 followed by SDS-PAGE (10-30% w/v gradient gel) and silver-staining in the second dimension. Only that portion of the gel containing the proteins is shown. The purified complex (10 μ g protein) was also run in the 2nd dimension and the 11,000 and 13,000 M_r polypeptides are indicated.

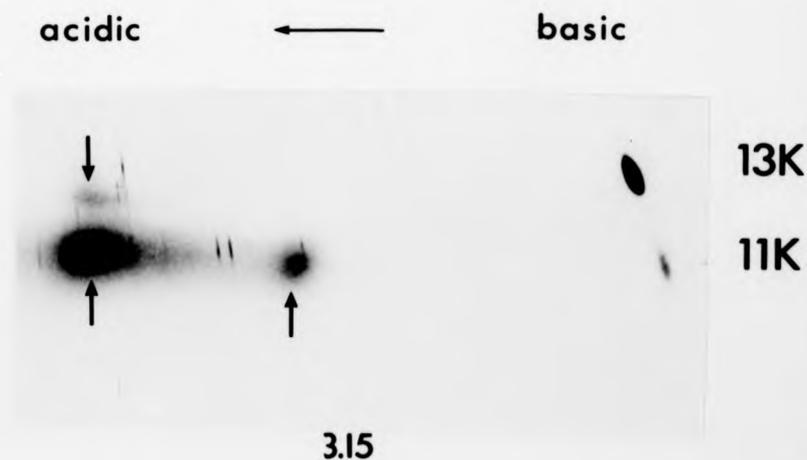


FIGURE 3.14. Two-dimensional gel electrophoresis of the B885-RC complex.

Forty μg protein of the complex isolated from sucrose gradients (section 2.27.3) was subjected to isoelectric focussing (IEF) in the 1st dimension as described in section 2.17.5. The IEF tube gel was equilibrated with Laemmli sample buffer before electrophoresis in the second dimension on a 10-30% (w/v) gradient gel containing SDS. The protein spots were subsequently visualized by silver staining. Molecular weight marker proteins were also run in the second dimension.

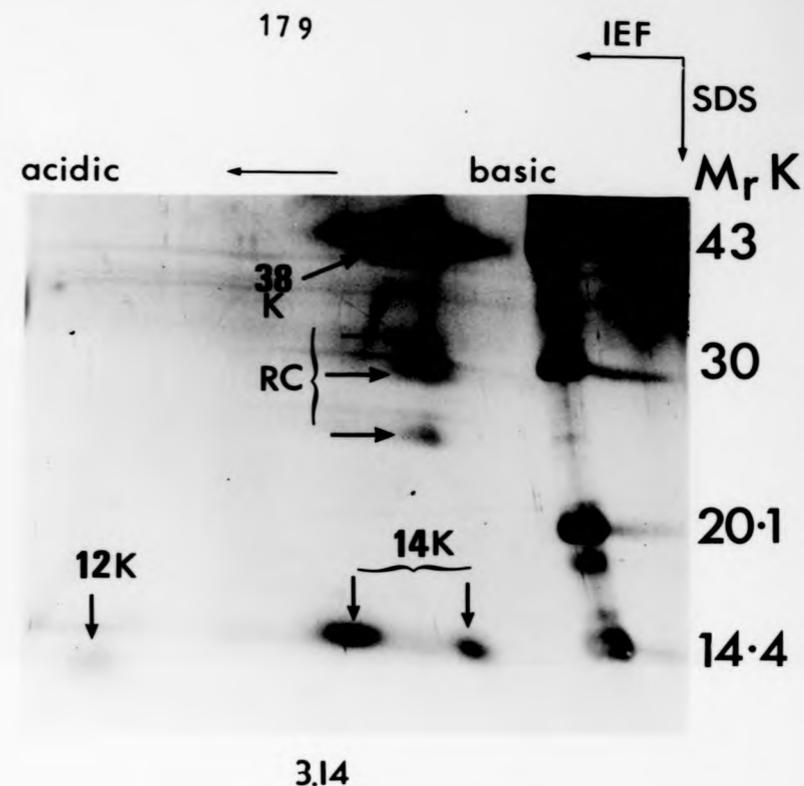
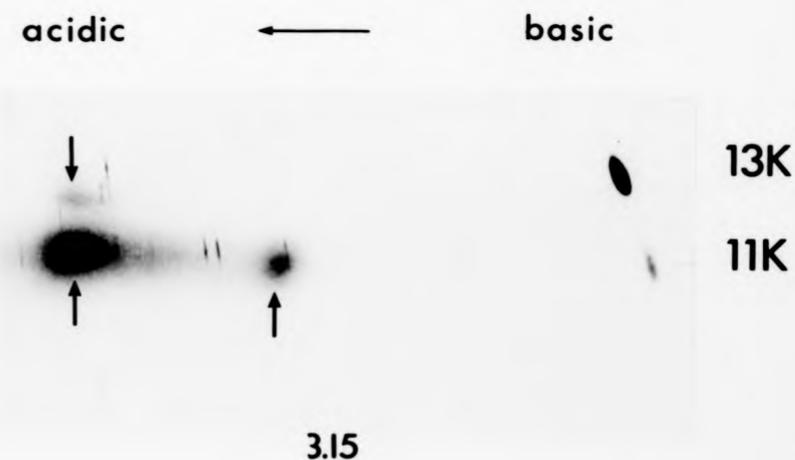


FIGURE 3.15. Two-dimensional gel electrophoresis of the B800-865 complex.

Twenty μg protein was subjected to isoelectric focussing as described in section 2.17.5 followed by SDS-PAGE (10-30% w/v gradient gel) and silver-staining in the second dimension. Only that portion of the gel containing the proteins is shown. The purified complex (10 μg protein) was also run in the 2nd dimension and the 11,000 and 13,000 M_r polypeptides are indicated.



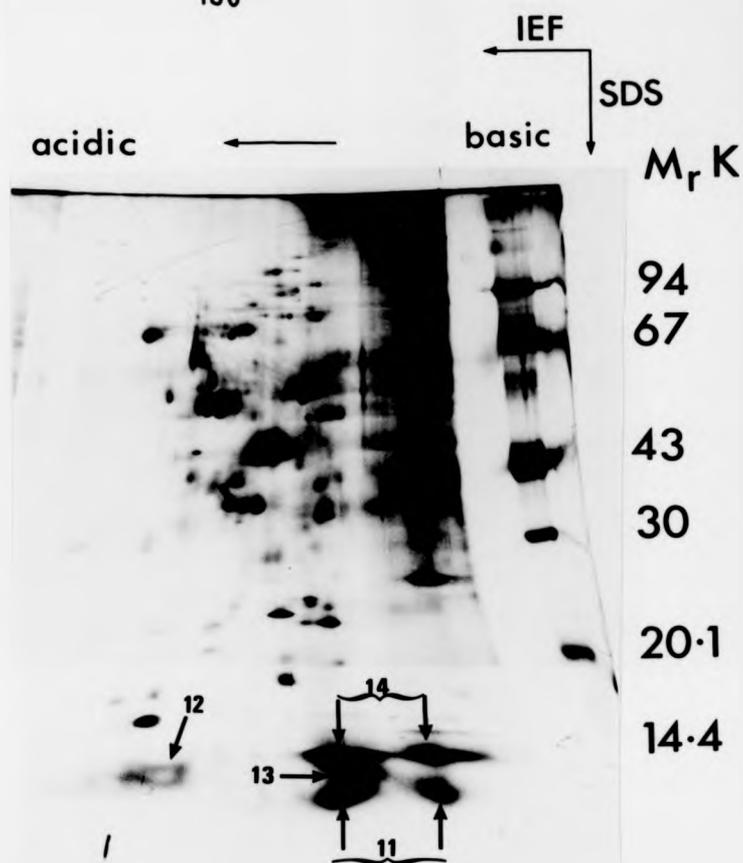


FIGURE 3.16. Two dimensional gel electrophoresis of Rm. vanniellii ICM.

About 200 μ g of ICM protein was solubilized with SDS before isoelectric focussing in the 1st dimension was performed as described in section 2.17.5. Protein spots on the 2nd dimension gel (10-30% w/v gradient) were visualized by silver staining. The arrows refer to the different isoelectric forms of the LH polypeptides.

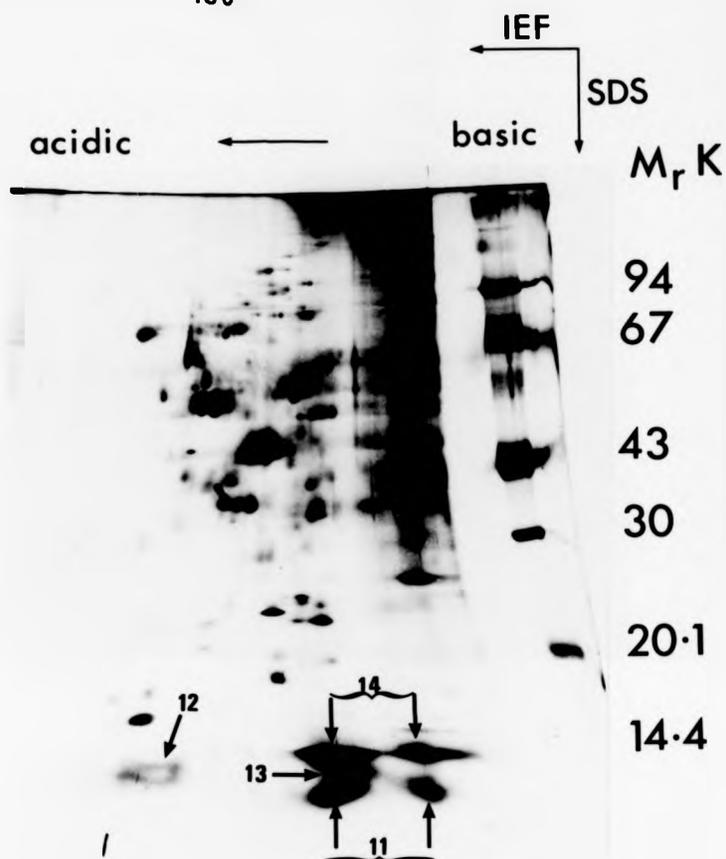


FIGURE 3.16. Two dimensional gel electrophoresis of Rm. vanniellii ICM.

About 200 μg of ICM protein was solubilized with SDS before isoelectric focussing in the 1st dimension was performed as described in section 2.17.5. Protein spots on the 2nd dimension gel (10-30% w/v gradient) were visualized by silver staining. The arrows refer to the different isoelectric forms of the LH polypeptides.

resolved at all temperatures in both ICM and the complex itself.

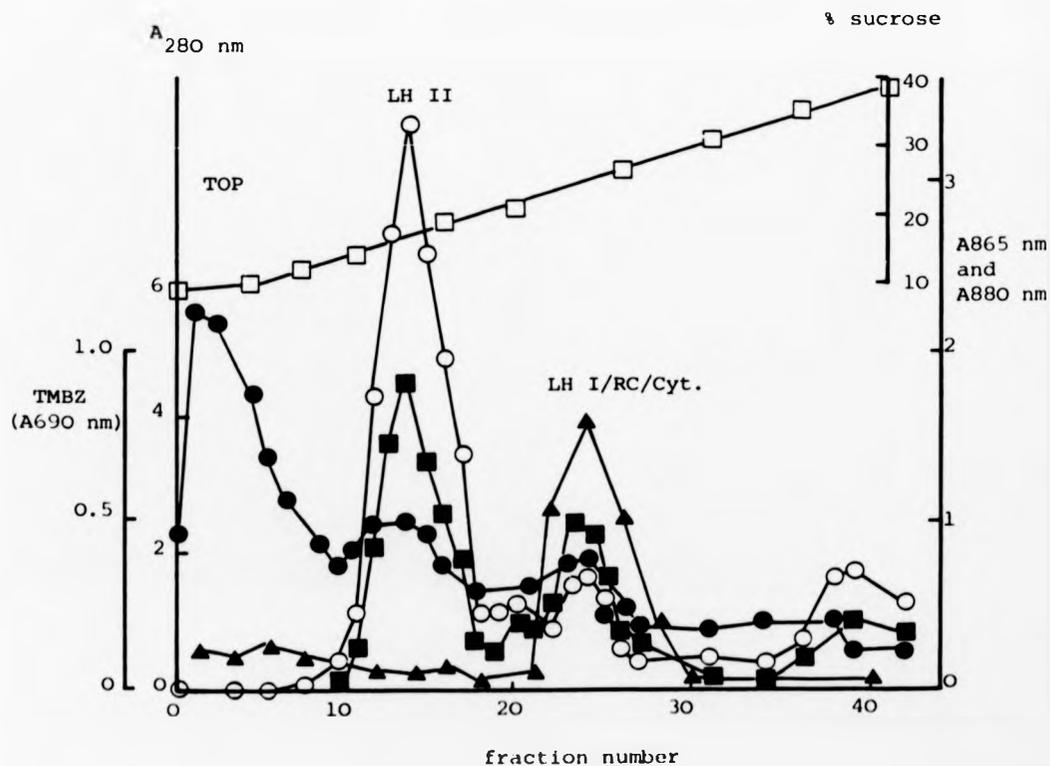
Two dimensional gel analysis with separation by charge in the 1st dimension and size in the 2nd was used to determine if the light-harvesting polypeptides existed in multiple forms (Figs. 3.14 and 3.15). Two isoelectric forms of the B885 14,000 M_r protein were detected with pI values of 6.5 and 6.8. The 12,000 M_r protein migrated as a single species in both dimensions, with an approximate pI of 5.7. The 38,000 M_r protein and the 31,000 and 26,000 M_r reaction centre polypeptides also behaved as single species with similar pI values of ca. 6.8-7.0. A similar analysis of the B800-865 complex (Fig. 3.15) revealed the presence of two isoelectric forms of the 11,000 M_r polypeptide (pI 6.5 and 6.8) and one (pI 6.5) corresponding to the 13,000 M_r species. Isoelectric focussing of SDS solubilized ICM polypeptides and subsequent size separation (Fig. 3.16) identified the spot positions of the light-harvesting and reaction centre polypeptides in relation to the other membrane proteins. Again the two isoelectric forms of the 11,000 (LHII) and 14,000 M_r (LHI) proteins were apparent, each present in approximately equal quantities.

3.2.4 Isolation of pigment-protein complexes by sucrose-gradient centrifugation and ion-exchange chromatography

Extraction of pigment-protein complexes from polyacrylamide gels was found to be inefficient for the preparation of large quantities and so an alternative procedure based on sucrose gradient centrifugation was devised. Intra-cytoplasmic membranes were solubilized with SDS and Triton as for gels but then layered onto 10-40% (w/v) sucrose step

FIGURE 3.17. Isolation of pigment-protein complexes on sucrose gradients.

Membranes solubilized with SDS plus Triton X-100 were layered (300 μ l) onto a 10-40% (w/v) sucrose step gradient, which was centrifuged to equilibrium (100,000 \times g, 4°C, 16 h) and then fractionated (150 μ l fractions) from the top. Fractions were monitored for their absorbance at 280 nm (●—●), 865 nm (○—○) and 885 nm (■—■) and assayed for haem-associated peroxidase activity (▲—▲) as described in section 2.27.3 using TMBZ. Sucrose concentrations were determined by refractometry (□—□).



gradients which contained the same detergent mixture throughout. After centrifugation, this resulted in the formation of one yellow pigmented and two red pigmented bands in the gradient (Fig. 3.17). The absorbance profile at 280 nm and the characteristic near IR absorbance maxima of the pigment-protein complexes were used to identify the fractions. The B800-865 complex banded at a sucrose concentration corresponding to a density of 1.07 g cm^{-3} and the B885-RC complex at 1.10 g cm^{-3} . Their positions were thus inverted with respect to their migration behaviour on gels.

Assay of fractions for the presence of cytochromes by their haem-associated peroxidase activity revealed a peak in the gradient corresponding to that of the B885-RC complex. The yellow pigmented fraction at the top of the gradient which corresponded with the peak protein absorbance at 280 nm did not exhibit haem-associated peroxidase activity yet its absorption spectrum (Fig. 3.18) presented a single peak at 418 nm suggestive of a cytochrome γ (soret) band. However, the addition of dithionite did not result in any change in the spectrum. This pigment most likely represents a flavoprotein and could account for the ICM 415-420 nm peak (Fig. 3.12). An additional pigmented band near the bottom of the tube was sometimes observed, which probably represented some residual unsolubilized ICM (density 1.16 g cm^{-3}).

The polypeptide profile of various fractions taken from one such sucrose gradient is shown in Fig. 3.19. This gradient was slightly overloaded, resulting in some cross-contamination of proteins. Clearly, the yellow-pigmented fraction contained the majority of the solubilized ICM proteins. Those fractions spectrally identified with the B880-865 and B885-RC complexes contained their own characteristic polypeptides

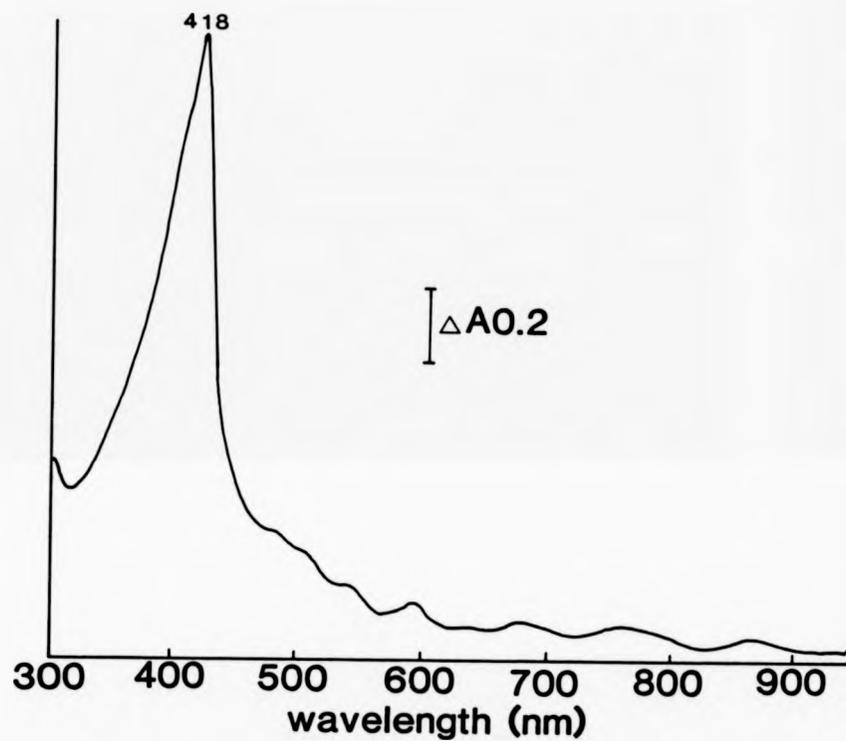


FIGURE 3.18. Absorption spectrum of the yellow pigmented fraction banding at the top of sucrose gradients for the isolation of pigment-protein complexes (Fig. 3.17).

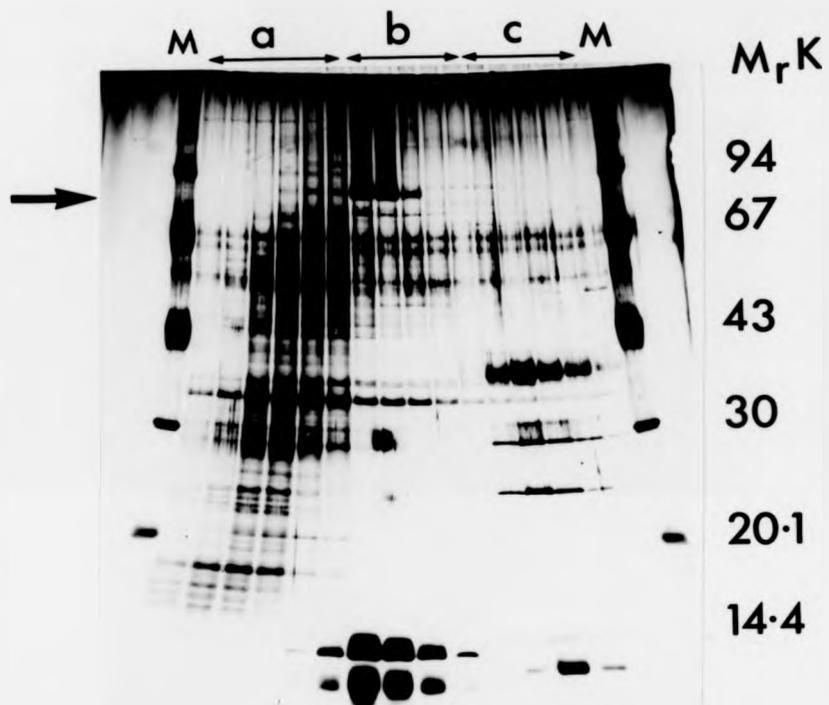


FIGURE 3.19. SDS-PAGE of samples from a sucrose gradient similar to that in Fig. 3.17.

Equal volumes ($10 \mu\text{l}$) of alternate fractions down the gradient corresponding to the yellow pigmented fraction (a), the B800-865 complex (b) and the B885-RC complex (c) were denatured in Laemmli sample buffer for 2 min at 75°C and electrophoresed on a 10-30% (w/v) gradient gel which was subsequently silver-stained. The arrow indicates some residual native B800-865 complex. The tracks labelled M contained molecular weight markers.

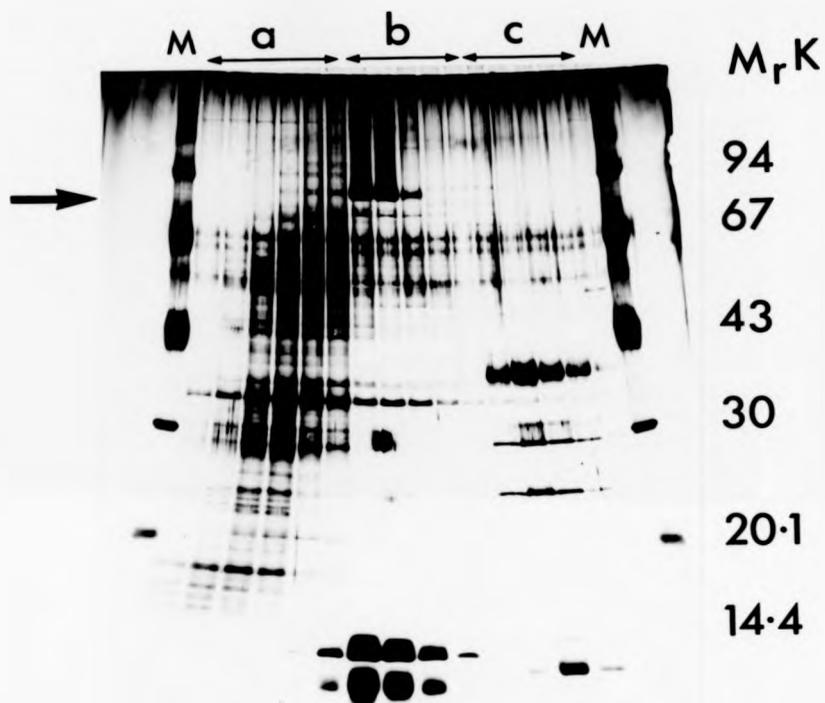


FIGURE 3.19. SDS-PAGE of samples from a sucrose gradient similar to that in Fig. 3.17.

Equal volumes ($10 \mu\text{l}$) of alternate fractions down the gradient corresponding to the yellow pigmented fraction (a), the B800-865 complex (b) and the B885-RC complex (c) were denatured in Laemmli sample buffer for 2 min at 75°C and electrophoresed on a 10-30% (w/v) gradient gel which was subsequently silver-stained. The arrow indicates some residual native B800-865 complex. The tracks labelled M contained molecular weight markers.

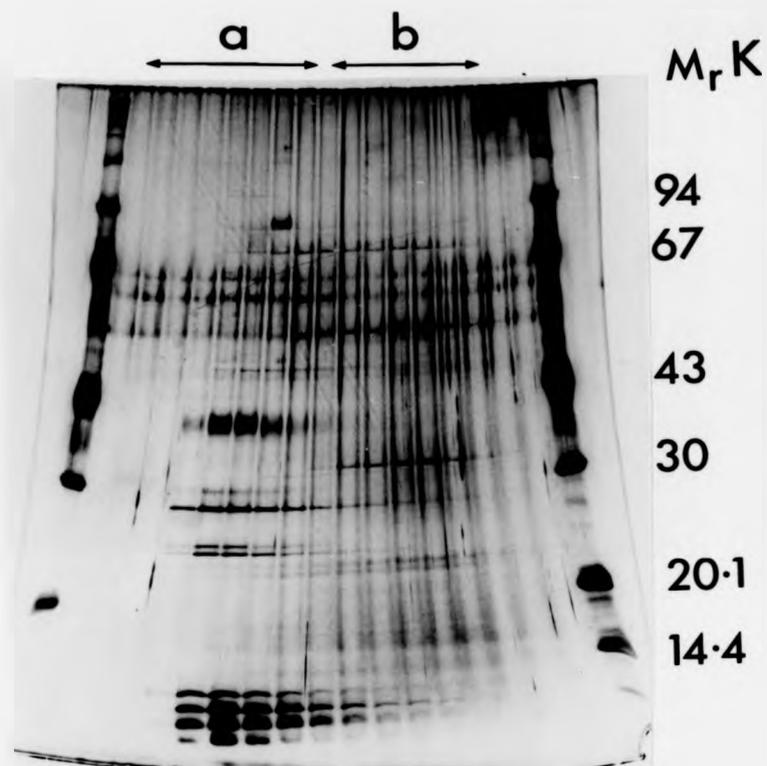


FIGURE 3.20. SDS-PAGE of samples from DE52-cellulose ion-exchange chromatography of SDS plus Triton X-100 solubilized *Rm. vanniellii* ICM.

Samples in (a) represent fractions eluting between 0.1-0.2 M KCl, and with an absorption spectrum identical to untreated ICM.

Samples in (b) represent fractions eluting between 0.2-0.3 M KCl and containing a 32,000 M_r protein. In each case equal volumes (20 μ l) were solubilized in Laemmli sample buffer (75°C/2 min) prior to electrophoresis. Tracks M contained molecular weight markers. Silver-stained 10-30% (w/v) gradient gel.

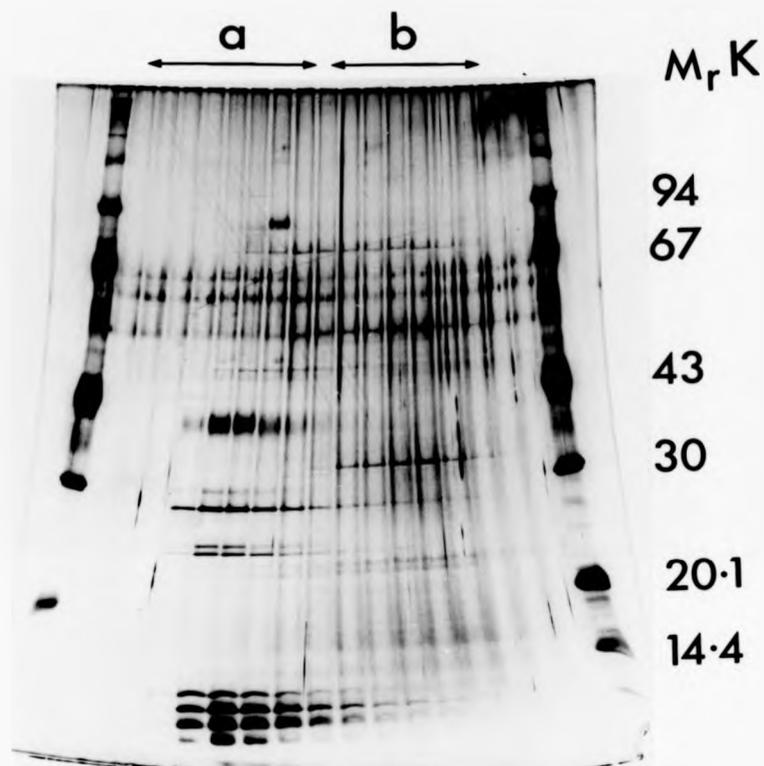


FIGURE 3.20. SDS-PAGE of samples from DE52-cellulose ion-exchange chromatography of SDS plus Triton X-100 solubilized Rm. vanniellii ICM.

Samples in (a) represent fractions eluting between 0.1-0.2 M KCl, and with an absorption spectrum identical to untreated ICM.

Samples in (b) represent fractions eluting between 0.2-0.3 M KCl and containing a 32,000 M_r protein. In each case equal volumes (20 μ l) were solubilized in Laemmli sample buffer (75°C/2 min) prior to electrophoresis. Tracks M contained molecular weight markers. Silver-stained 10-30% (w/v) gradient gel.

(section 3.2.3). As with separation of the complexes on Triton-SDS gels, the M_r 38,000 protein remained associated with the B885-RC complex.

Solubilization of ICM with SDS and Triton X-100 followed by fractionation on a DEAE-cellulose ion-exchange column resulted in a rather different polypeptide profile (Fig. 3.20). The RC, B885 and B800-850 complexes eluted as a single peak which had an absorption spectrum identical to that of intact ICM. A second peak in the A280 nm profile corresponded to fractions containing a prominent 32,000 M_r protein. Yet again, the 38,000 M_r major ICM protein was contained in the same fractions as the RC and B885 polypeptides, as observed on Triton-SDS gels (Fig. 3.13) and sucrose gradients (Fig. 3.18).

3.2.5 Association of cytochrome with the B885-RC complex and ICM

Two pieces of evidence (i) the peak of haem-associated peroxidase activity associated with the B885-RC complex on sucrose gradients and (ii) the presence of a 38,000 M_r protein in this complex, suggested that this protein was a cytochrome. This was confirmed by staining denaturing gels for haem-associated peroxidase activity (Fig. 3.21). In ICM preparations, 3 bands of M_r 38,000, 32,000 and 28,000 reacted positively while in the B885-RC complex, only a single band of M_r 38,000 was stained. Interestingly, in the absence of mercaptoethanol during the preparation and running of the samples, a decrease in sensitivity of staining was found coupled with a markedly decreased resolution of the M_r 38,000 band. This agrees with the change in resolution of this protein in the absence of reducing agents evidenced by silver staining

FIGURE 3.21. Detection of cytochrome by haem-associated peroxidase activity staining (a). Samples (200 μ g protein; ICM. 30 μ g protein; B885-RC) were solubilized in Laemmli sample buffer at 75°C for 2 min in the presence (tracks 1 and 2) or absence (tracks 3 and 4) of 5% (v/v) mercaptoethanol. Tracks 1 & 3; ICM. Tracks 2 & 4; B885-RC complex. The arrows indicate bands of M_r 38,000 (B885 complex and ICM) 32,000 and 28,000 (ICM) which stain with TMBZ. Track P contains pre-stained molecular weight markers. In (B) the same gel is shown stained with Coomassie blue.

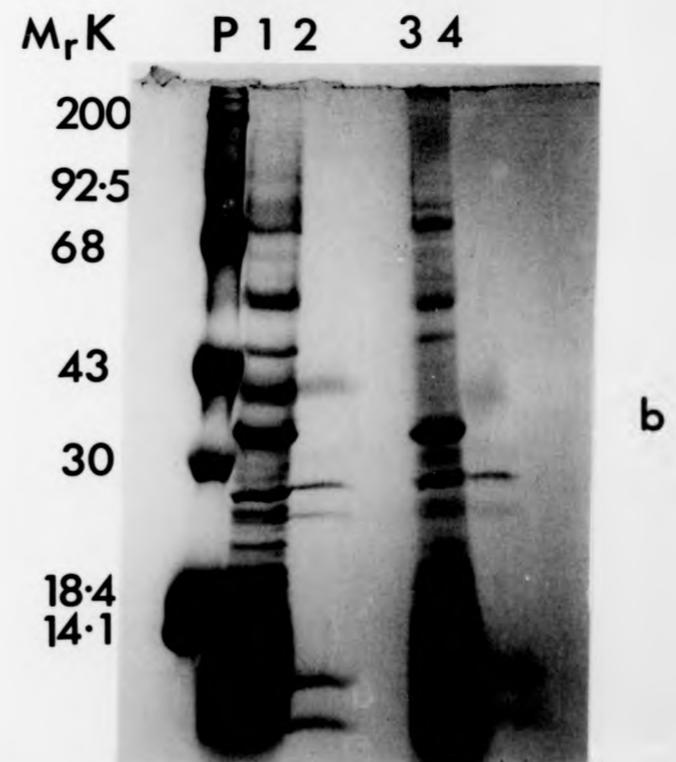
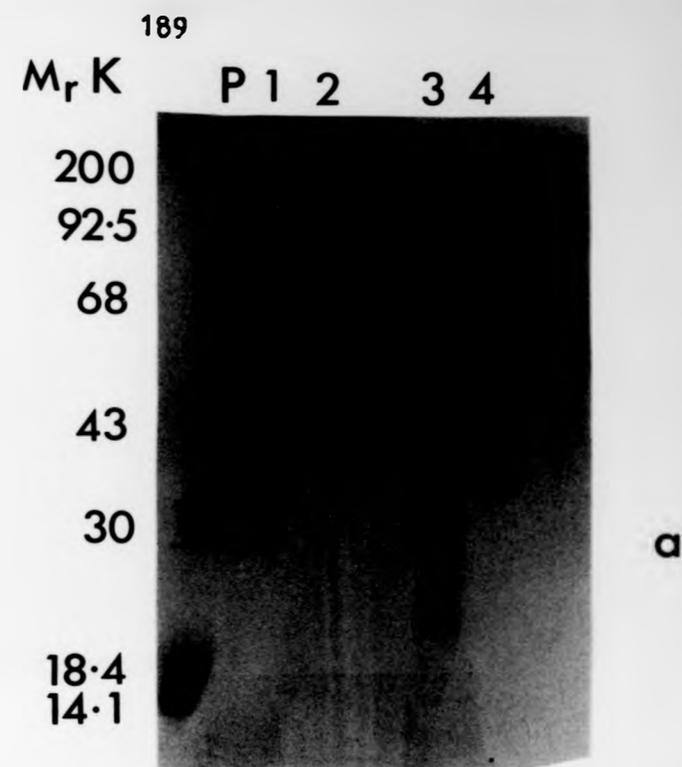
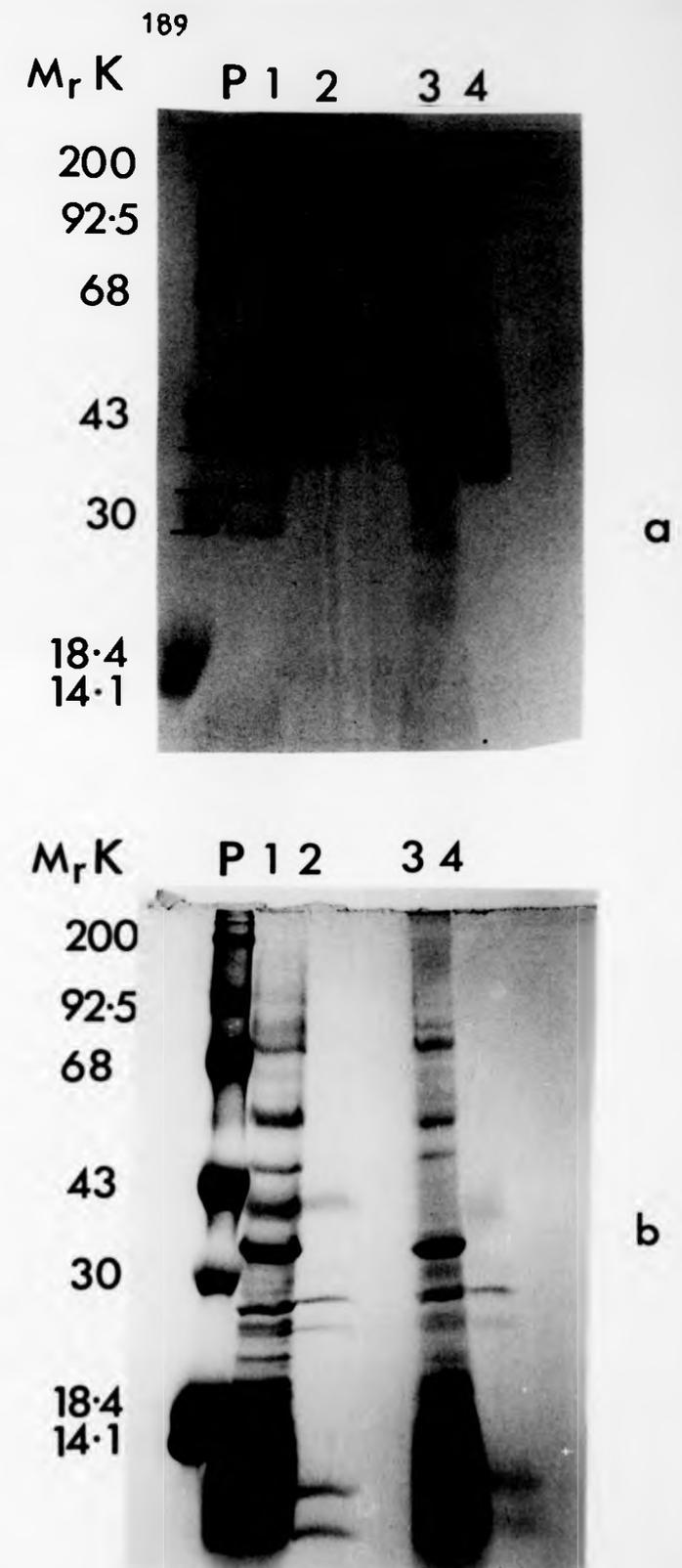


FIGURE 3.21. Detection of cytochrome by haem-associated peroxidase activity staining (a). Samples (200 μ g protein; ICM. 30 μ g protein; B885-RC) were solubilized in Laemmli sample buffer at 75°C for 2 min in the presence (tracks 1 and 2) or absence (tracks 3 and 4) of 5% (v/v) mercaptoethanol. Tracks 1 & 3; ICM. Tracks 2 & 4; B885-RC complex. The arrows indicate bands of M_r 38,000 (B885 complex and ICM) 32,000 and 28,000 (ICM) which stain with TMBZ. Track P contains pre-stained molecular weight markers. In (B) the same gel is shown stained with Coomassie blue.



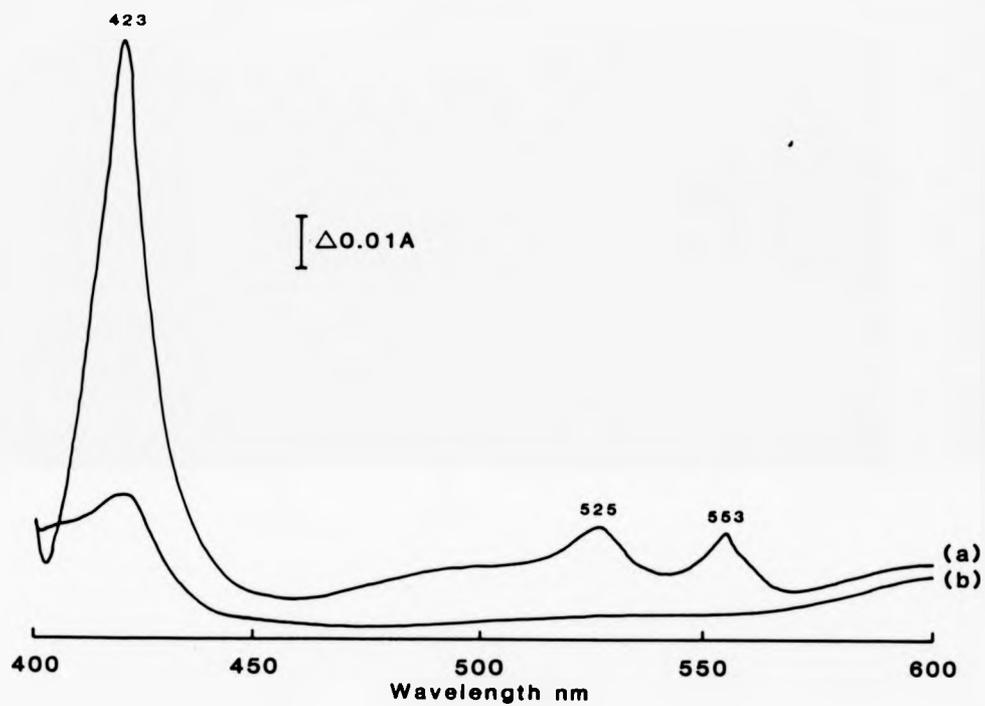


FIGURE 3.22. Dithionite reduced minus air oxidized difference spectrum of the B885-RC complex (a) and the baseline in the absence of dithionite (b). Approximately 200 μg protein in 1 ml Tris-HCl pH 7.4 buffer was used.

FIGURE 3.23. Dithionite reduced minus air oxidized difference spectra of *Rm. vannielii* subcellular fractions.

In (a) dithionite was added to a soluble protein (ICM free) fraction (15 mg ml^{-1} protein) from photoheterotrophically grown cells. Trace (b) shows the baseline in the absence of reductant. Traces (c) and (d) represent the difference spectra of chemoheterotrophic and photoheterotrophic ICM preparations respectively (3 mg ml^{-1} protein in each case).

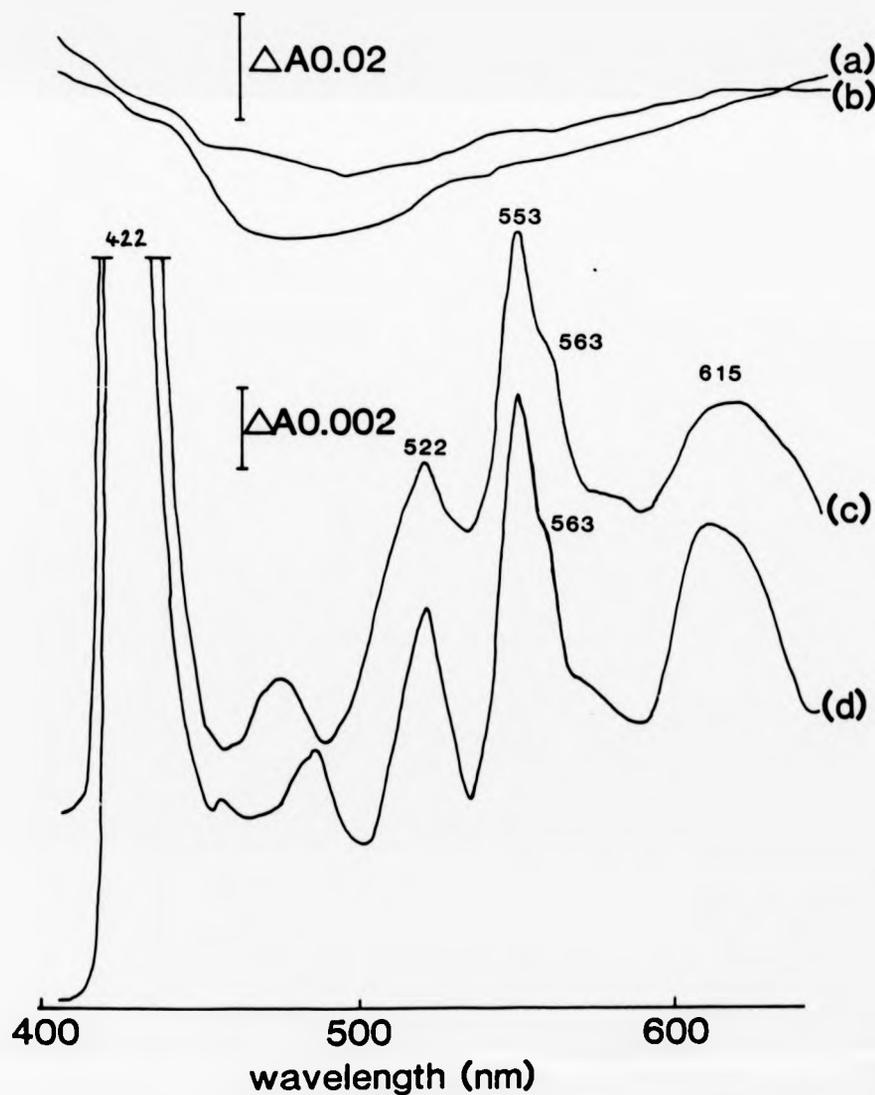
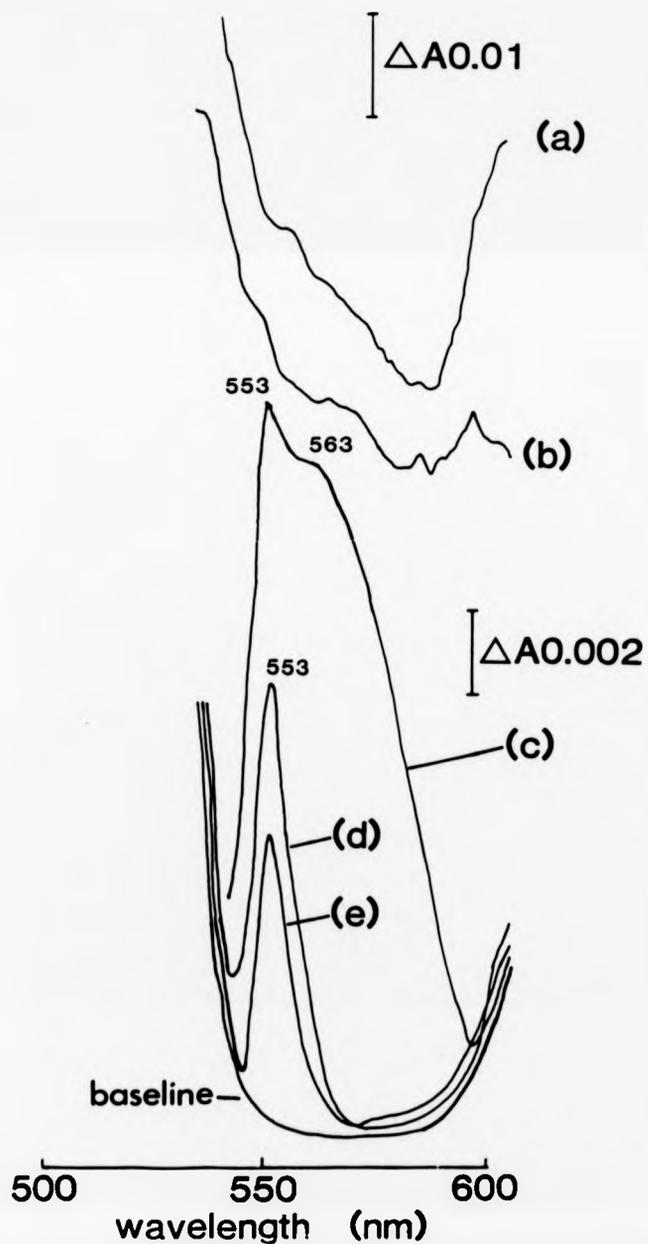


FIGURE 3.24. Difference spectra of *Rm. vannielii* photoheterotrophic cell-free extracts in the presence (a) and absence (b) of hydroquinone and dithionite reduced minus ascorbate oxidized (c), NADH reduced minus air oxidized (d) and succinate reduced minus air oxidized (e) difference spectra of similar cell free extracts. Protein concentration was 12 mg ml^{-1} in each case.



(section 3.1.2) and suggests an important role for disulphide bonds in its conformation. Dithionite reduced minus air oxidized difference spectra (Fig. 3.22) of the B885-RC complex identified the 38,000 M_r protein as a c -type cytochrome with an α -peak at 553 nm. Comparative data on the cytochrome composition of the soluble and ICM fractions of photoheterotrophically grown cells and the ICM of chemoheterotrophically grown cells (Fig. 3.23) clearly showed this to be the major cytochrome species in the cells of Rm. vannielii strain Rm5. In cell-free extracts, both NADH and succinate caused the reduction of this cytochrome c -553, indicating it to be substrate reducible (Fig. 3.24). In addition, dithionite minus ascorbate difference spectra (Fig. 3.24) which would be expected to contain contributions from any low potential ($E_m < + 50$ mV) cytochromes, showed a prominent maximum at 553 nm, indicating the c -553 species to be of the low potential type, and a shoulder at 563 nm indicative of the presence of a low potential b -type cytochrome in Rm. vannielii. Hydroquinone reduced minus air oxidized difference spectra (Fig. 3.24) would be expected to reveal any high potential cytochromes, as hydroquinone is a relatively weak reductant ($E_m + 290$ mV). However, no evidence for the presence of such cytochromes in the ICM of Rm. vannielii was obtained, although contributions from species present at low concentrations could be missed because of the masking effect of the carotenoid absorbance in the α -peak region, thus preventing the use of high protein concentrations.

As these data suggested that the major c -type cytochrome in Rm. vannielii was membrane bound and there was no spectral evidence of a large release of cytochromes into the soluble fraction during membrane preparation (Fig. 3.23) the cytochrome composition of the ICM from a number of members of the Rhodospirillaceae were compared on SDS gels stained for

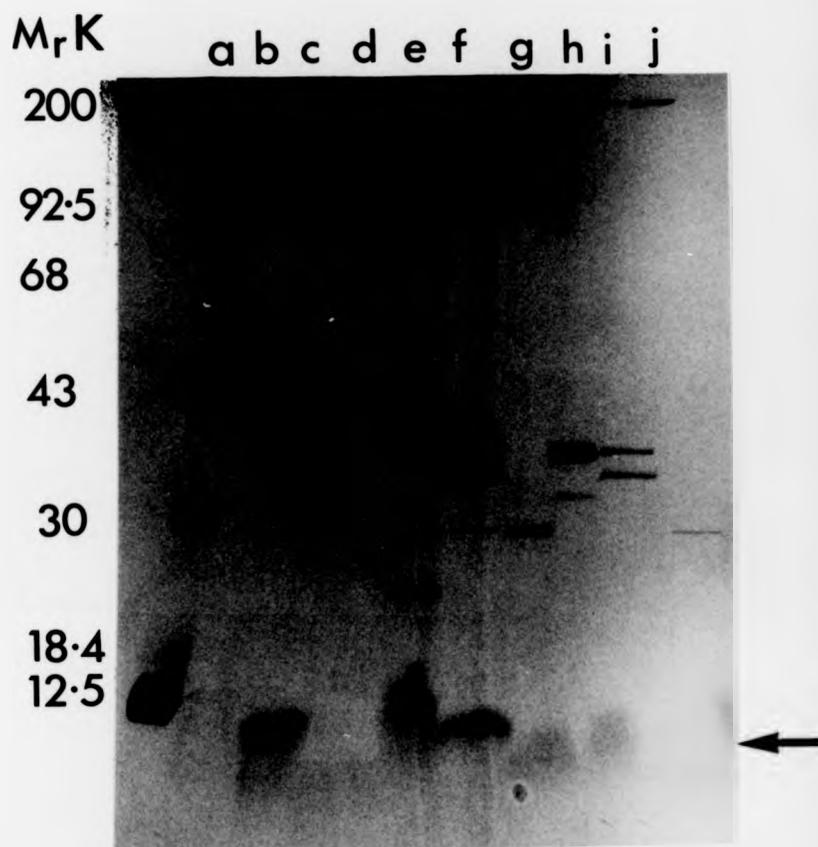


FIGURE 3.25. Cytochrome composition of the ICM from selected members of the Rhodospirillaceae.

Membranes (ca. 50 μg protein) from *Rb. sphaeroides* (b), *Rp. acidophila* (c), *Rp. blastica* (d), *Rc. gelatinosus* (e), *Rp. viridis* (f), *Rp. palustris* (g), *Rm. vannielii* (h) *Rc. tenuis* (i) and *Rs. rubrum* (j) were denatured at 75°C for 2 min in Laemmli sample buffer and electrophoresed on a 10-30% (w/v) gradient gel which was subsequently stained with TMBZ for haem associated peroxidase activity. Track (a) contains pre-stained molecular weight markers with cytochrome c_2 at M_r 12,500. The arrow indicates low M_r cytochrome c_2 type proteins to be present in most species.

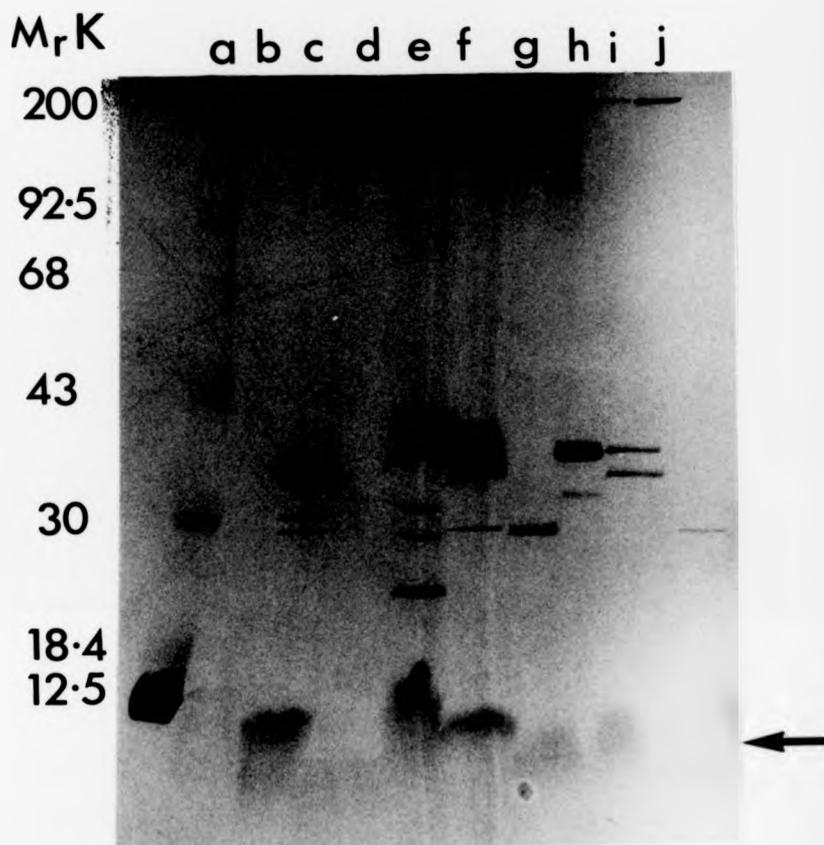


FIGURE 3.25. Cytochrome composition of the ICM from selected members of the Rhodospirillaceae.

Membranes (ca. 50 μ g protein) from *Rb. sphaeroides* (b), *Rp. acidophila* (c), *Rp. blastica* (d), *Rc. gelatinosus* (e), *Rp. viridis* (f), *Rp. palustris* (g), *Rm. vannielii* (h) *Rc. tenuis* (i) and *Rs. rubrum* (j) were denatured at 75°C for 2 min in Laemmli sample buffer and electrophoresed on a 10-30% (w/v) gradient gel which was subsequently stained with TMBZ for haem associated peroxidase activity. Track (a) contains pre-stained molecular weight markers with cytochrome c_1 at M_r 12,500. The arrow indicates low M_r cytochrome c_2 type proteins to be present in most species.

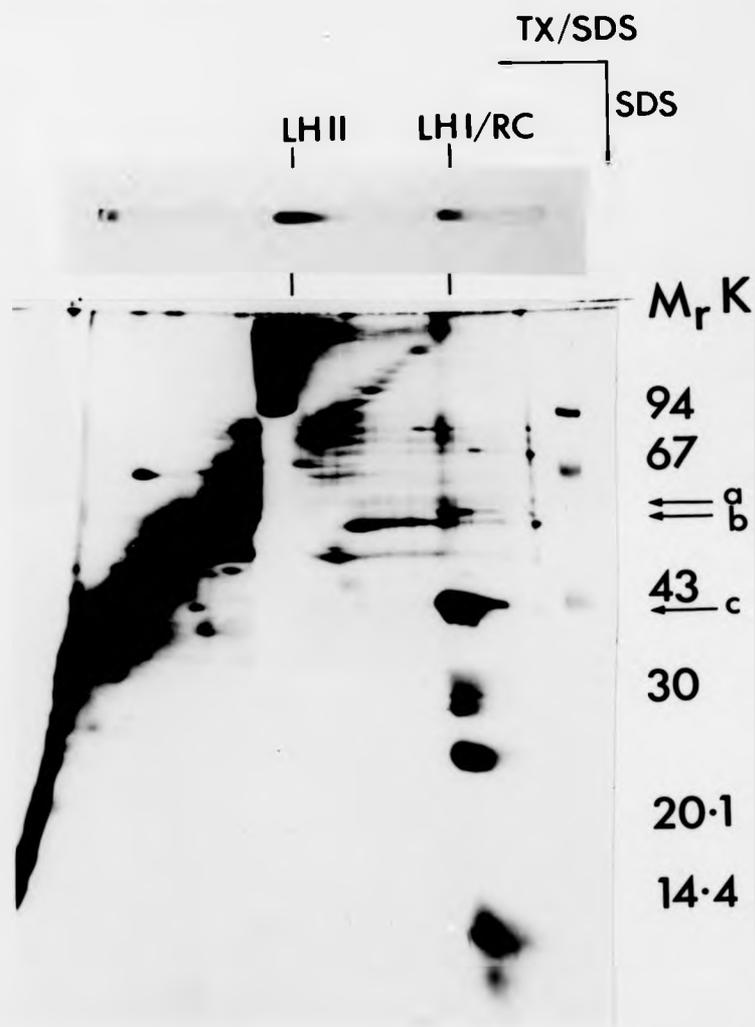
haem associated peroxidase activity (Fig. 3.25). In Rp. viridis and Rp. acidophila, a major stained band of rather similar mobility to that found in Rm. vannielli was apparent coupled with one (Rp. viridis) or two (Rp. acidophila) other cytochrome species. However, the limit of sensitivity of the stain was being approached in these cases as the 28,000 M_r cytochrome band in Rm. vannielli (Fig. 3.21) was not seen on this gel. Despite similar protein loadings, no membrane bound cytochromes stained in the tracks containing Rb. sphaeroides and Rp. blastica ICM and only one band was observed in those with Rp. palustris and Rs. rubrum ICM. Apart from stain insensitivity, this may be due to the release of loosely bound cytochromes during cell breakage (Prince *et al.*, 1975; Zannoni, 1984). On the other hand, membranes of Rc. gelatinosus displayed a minimum of 7 cytochrome species (Fig. 3.25).

The isolation of the major α -type cytochrome of Rm. vannielli along with the RC and B885 polypeptides could be merely fortuitous or it may represent a functional relationship between these components. This was tested during experiments designed to study the organization of the photosynthetic apparatus.

3.2.6 Organization and topographical relationships of the components of the pigment-protein complexes

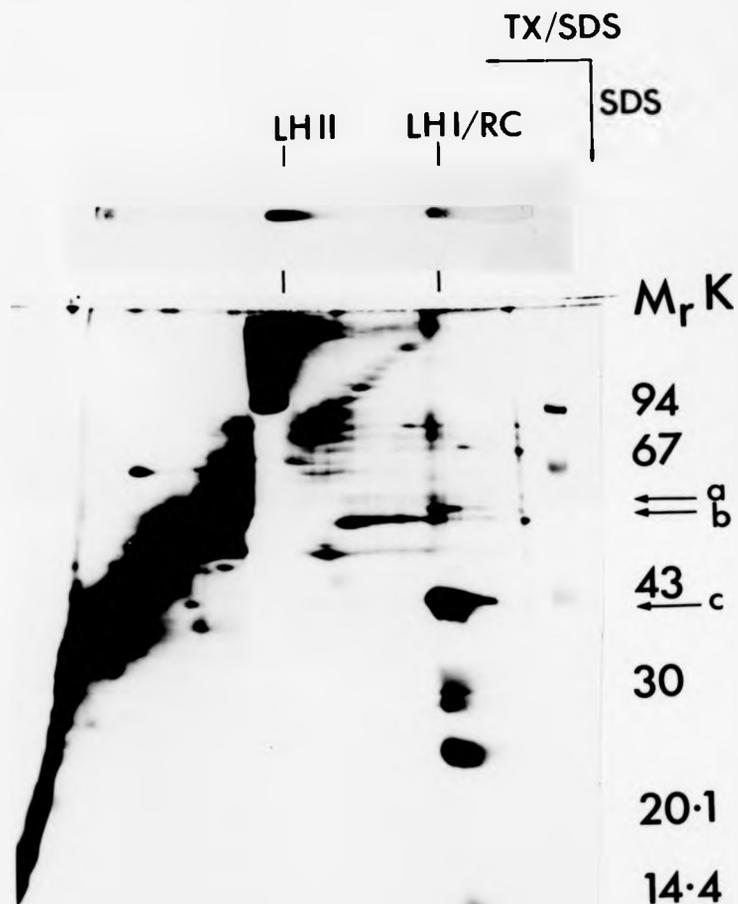
The organization of pigment-protein complexes within the membrane and the spatial relationships between their components have been probed by a variety of methods, including protease digestion studies (Oelze, 1978; Wiemken *et al.*, 1983), surface iodination (Peters & Drews, 1983b), chemical cross-linking (Peters & Drews, 1983c) and detergent

FIGURE 3.26. Two-dimensional gel analysis of *Rm. vannielii* pigment-protein complexes.



Membranes solubilized with SDS and Triton X-100 were electrophoresed (450 μ g protein) in the 1st dimension on a 6% (w/v) tube gel at 4°C. The gel was photographed unstained to reveal the position of the LHI/RC and LHII complexes and then heated in sample buffer for 5 min at 60°C before electrophoresis in the 2nd dimension (10-30% w/v gradient gel) followed by silver-staining. Proteins of M_r 61,000 (a) and 58,000 (b) which may be associated with the B885-RC complex are arrowed. The position of the M_r 38,000 cytochrome c -553 (arrow c) is also indicated.

FIGURE 3.26. Two-dimensional gel analysis of *Rm. vannielii* pigment-protein complexes.



Membranes solubilized with SDS and Triton X-100 were electrophoresed (450 μ g protein) in the 1st dimension on a 6% (w/v) tube gel at 4°C. The gel was photographed unstained to reveal the position of the LHI/RC and LHII complexes and then heated in sample buffer for 5 min at 60°C before electrophoresis in the 2nd dimension (10-30% w/v gradient gel) followed by silver-staining. Proteins of M_r 61,000 (a) and 58,000 (b) which may be associated with the B885-RC complex are arrowed. The position of the M_r 38,000 cytochrome c -553 (arrow c) is also indicated.

fractionation (Peters *et al.*, 1983). In *Rm. vannielli*, the complementary approaches of detergent fractionation, using a modification of the Triton-SDS gel system, and reversible chemical cross-linking were used.

For detergent fractionation, a 2-dimensional gel system was devised in which a first dimension separation on a Triton-SDS containing tube gel was followed by running the proteins into a second dimension denaturing 10-30% (w/v) slab gel with subsequent silver staining (Fig. 3.26). The majority of the membrane proteins formed a diagonal line across the gel indicating fractionation in the 1st dimension on the basis of size. The break point corresponding to the anodic end of the 1st dimension gel was due to proteins below an M_r of ca. 35,000 migrating at the ion-front and afterwards separating in the second dimension. Vertical alignment of spots below this diagonal would indicate polypeptides associated with each other during the Triton-SDS separation, as for example those of the B885-RC complex. Alignment of a protein spot across the gel would indicate its possible involvement in a number of complexes. The M_r 38,000 protein appeared to be specifically associated with the B885-RC pigment-protein complex as this analysis did not reveal any complex containing this species other than that also containing the RC and B885 polypeptides. A protein of M_r 58,000 did appear to be associated with other proteins in several complexes including a loose relationship to the B885-RC complex. An additional protein of M_r 61,000 was sometimes observed to be present but because such gels were deliberately overloaded with protein its quantitative significance is unclear. The difference in the susceptibility to denaturation of the two pigment-protein complexes was again illustrated by the B800-865 aggregate which migrated as a pigmented band in both dimensions. The protein "smear"

FIGURE 3.27. Two-dimensional gel analysis of reversible chemical cross-linking patterns of *Em. vannieli* ICM (a) and the B885-RC complex (b). In (a), ICM (200 μ g protein) in 50 mM triethanolamine buffer pH 8.3 was cross-linked with 5 mM dithiobis (succinimidyl propionate) for 1 h while in (b) the DTSP concentration was 1 mM with a reaction time of 10 min. After electrophoresis in the 1st dimension tube gel (10% w/v; 6 mA for 2 h at 4°C) the cross-links were cleaved *in situ* by incubation in Laemmli sample buffer containing 5% (v/v) mercaptoethanol and electrophoresis in the second dimension carried out on 10-30% (w/v) polyacrylamide gradient gels which were subsequently silver-stained. The vertical arrows refer to the most notable cross-linked polypeptides.

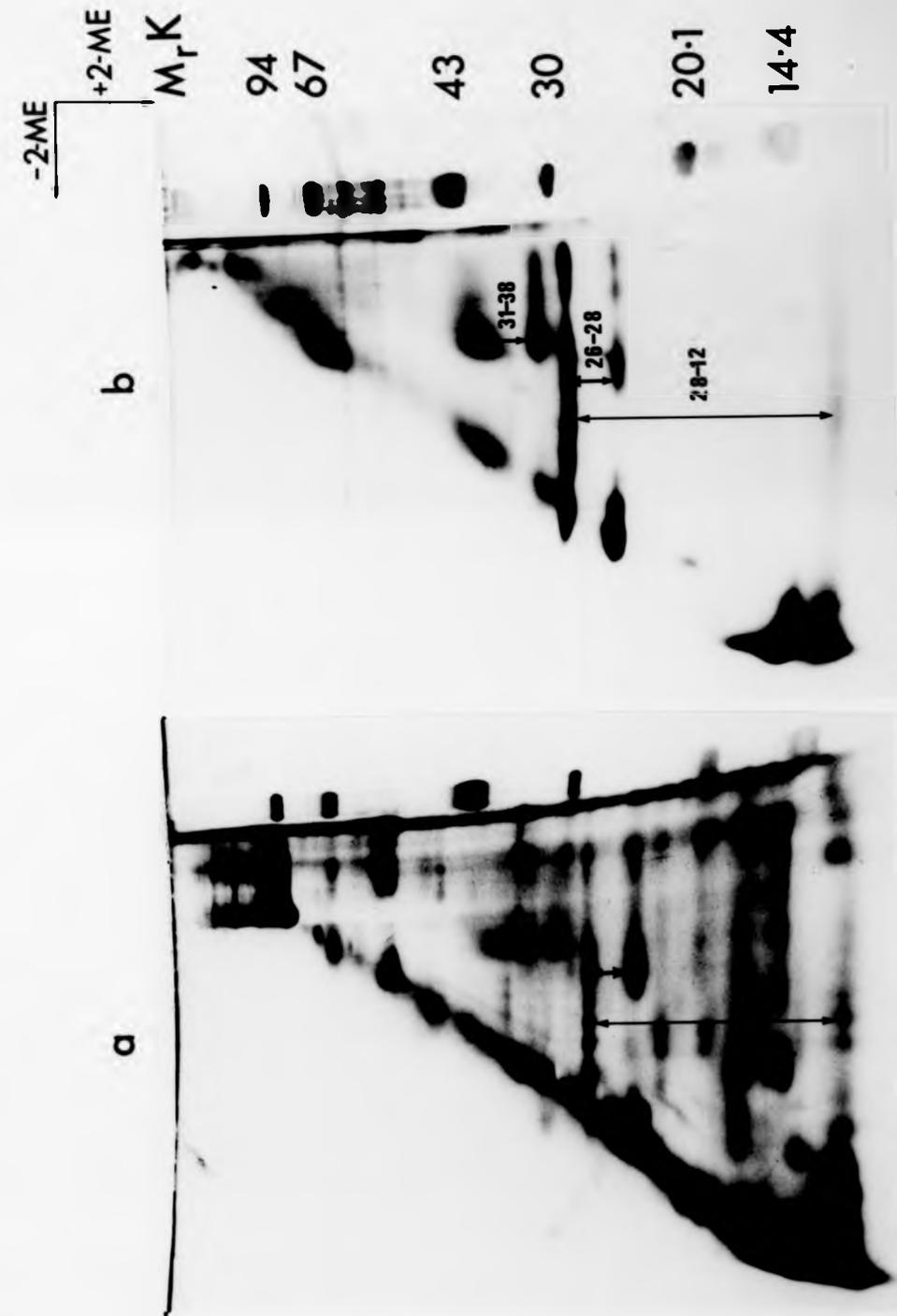
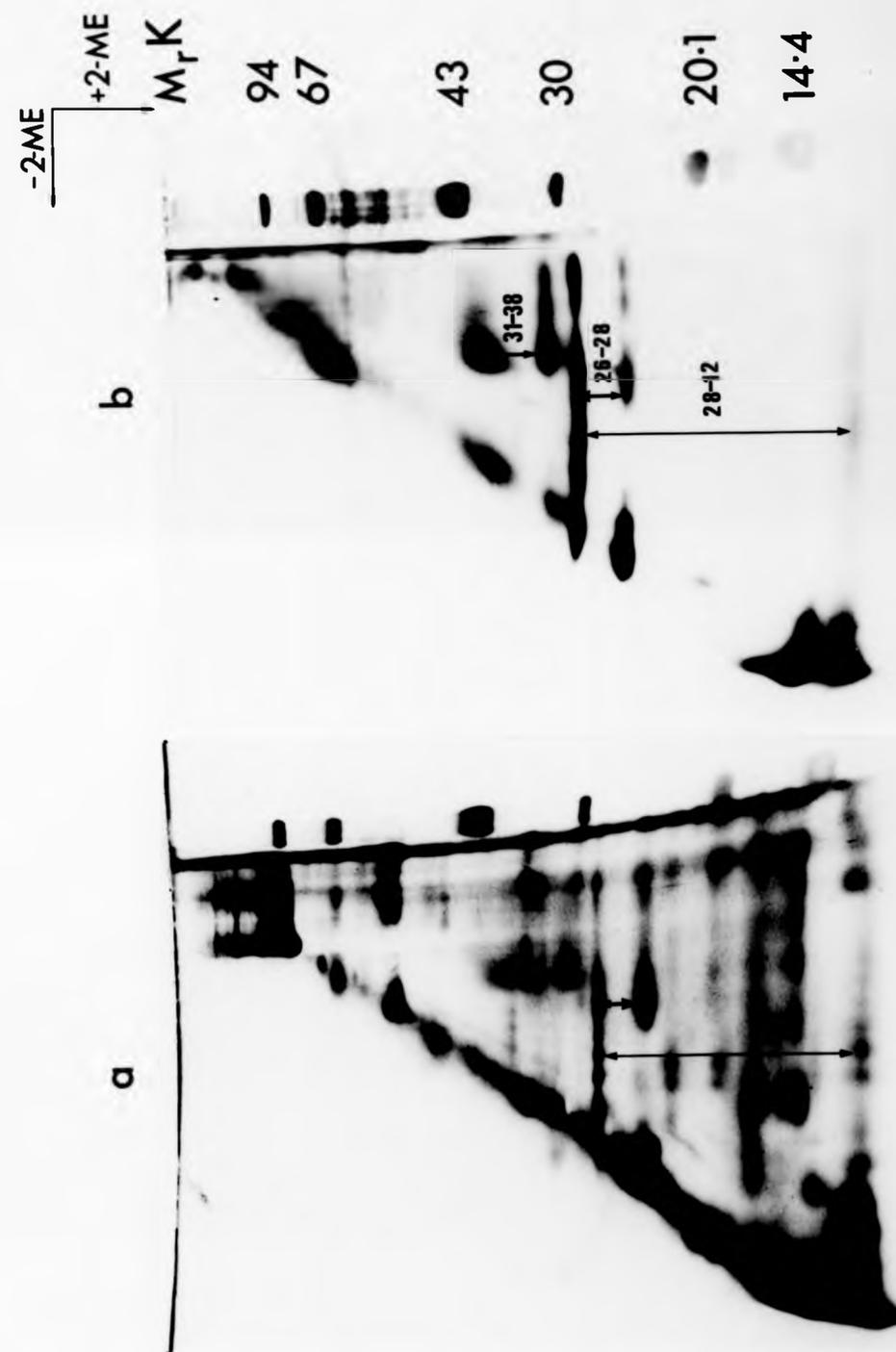


FIGURE 3.27. Two-dimensional gel analysis of reversible chemical cross-linking patterns of *Rm. vannielii* ICM (a) and the B885-RC complex (b). In (a), ICM (200 μ g protein) in 50 mM triethanolamine buffer pH 8.3 was cross-linked with 5 mM dithiobis (succinimidyl propionate) for 1 h while in (b) the DTSP concentration was 1 mM with a reaction time of 10 min. After electrophoresis in the 1st dimension tube gel (10% w/v; 6 mA for 2 h at 4°C) the cross-links were cleaved *in situ* by incubation in Laemmli sample buffer containing 5% (v/v) mercaptoethanol and electrophoresis in the second dimension carried out on 10-30% (w/v) polyacrylamide gradient gels which were subsequently silver-stained. The vertical arrows refer to the most notable cross-linked polypeptides.



above the complex in the 2nd dimension would appear to be due to aggregation induced by SDS and was also evident on one-dimensional gels of undenatured ICM (Figs. 3.4 and 3.13).

Reversible chemical cross-linking procedures are now an accepted method of probing lateral topographical relationships within photosynthetic membranes and complexes (Peters & Drews, 1983c; Peters *et al.*, 1983, 1984). The hydrophobic reagent DTSP combined with the 2-dimensional diagonal mapping technique described by Peters & Drews (1983c) was used to establish the proximity of some of the polypeptides of the pigment-protein complexes (Kelly & Dow, 1985b). In this system cross-linked polypeptides run on a 1st dimension gel were cleaved by mercaptoethanol and the products analyzed by vertical alignment of protein spots in the second dimension.

Preliminary experiments conducted at pH 7.0 produced only low yields of cross-linked ICM proteins but increasing the pH to 8.3 produced satisfactory results (Fig. 3.27). Variation of the DTSP concentration between 1 and 10 mM gave essentially the same cross-linking pattern but with increasing yields and control experiments with no cross-linker present showed no spots below the diagonal. At this pH homo-oligomers of the LH polypeptides were undetectable above the presumed dimer (Fig. 3.27b) and specific vertical relationships were observed between the smaller B885 polypeptide (M_r 12,000) and the M_r 28,000 RC subunit in both membranes (Fig. 3.27a) and the isolated B885-RC complex itself (Fig. 3.27b). A relationship between the M_r 26,000 and 28,000 RC subunit was also evident in both cases. Most interesting, however, was the degree of cross-linking observed between the 31,000 M_r RC subunit and the 38,000 M_r cytochrome c -553. This was also observed in both intact

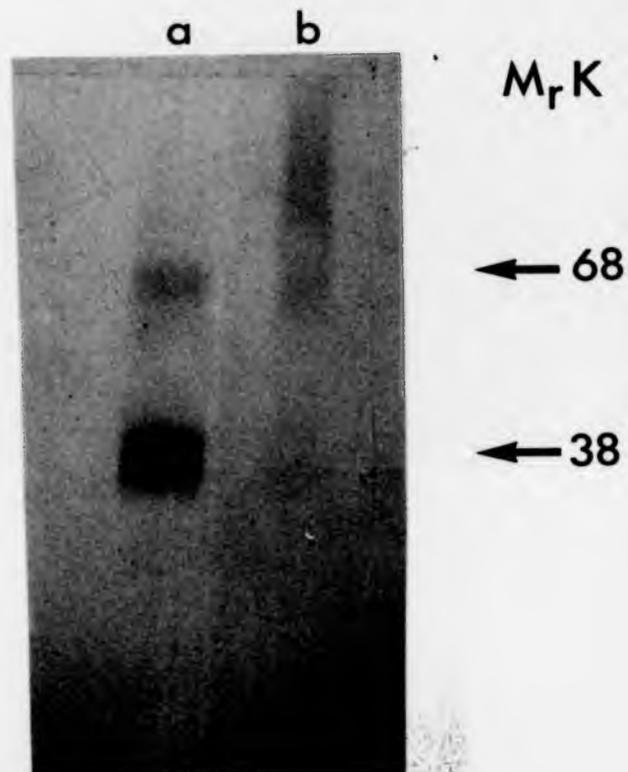


FIGURE 3.28. Detection of cytochrome by haem-associated peroxidase activity staining, after cross-linking the B885-RC complex with DTSP.

Cross-linking conditions were as described in the legend to Fig. 3.27. In (a) the cross-links were cleaved by the addition of 5% (v/v) 2-mercaptoethanol to the samples prior to electrophoresis thus giving rise to the 38,000 M_r cytochrome with some residual RC-cytochrome complex still remaining (M_r 68,000). In (b) no mercaptoethanol was present.

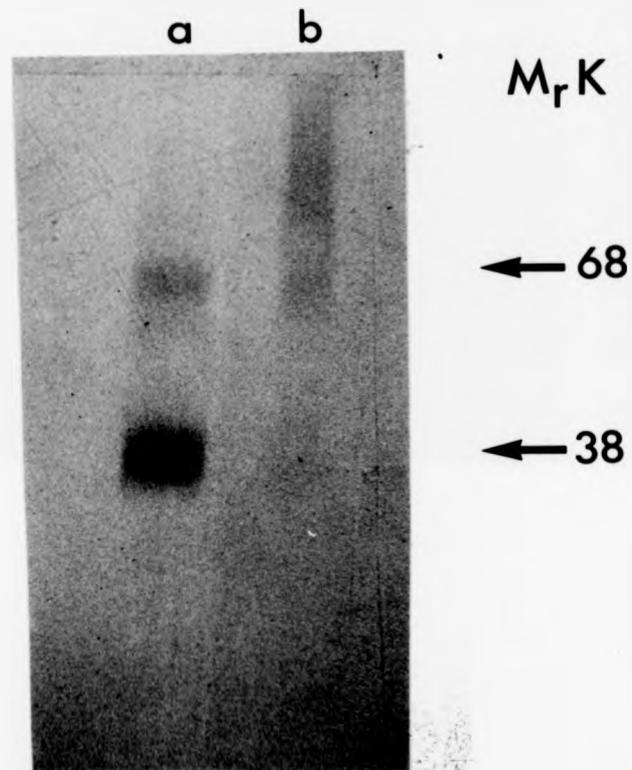


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membranes and the isolated complex. The cross-linked product had an M_r of about 68,000, from which a reaction stoichiometry of 1:1 may be deduced. The yield of this cross-linked product was quite high from the isolated complex, judging from the amount of the two polypeptides left on the diagonal and the fact that not all of it was cleaved in the 2nd dimension.

As an additional check that the 68,000 M_r cross-linked product did indeed contain the 38,000 M_r cytochrome c_{553} , the cross-linked B885-RC complex was run on a slab gel in the presence and absence of mercaptoethanol and then stained for haem associated peroxidase activity (Fig. 3.28). This showed the 68,000 M_r cross-linked product to be haem stainable under both conditions but the 38,000 M_r band was only apparent when the complex was cleaved, thus confirming the presence of cytochrome within the 68,000 M_r band.

3.2.7 Reconstitution of a functional reaction centre complex from *Rm. vannielii*

The data presented in sections 3.2.1 to 3.2.6 suggested that, as isolated, the B885-RC complex from *Rm. vannielii* contained nearly all of the components necessary for the operation of a cyclic photosynthetic electron transport system, i.e. the reaction centre pigments and proteins in addition to a bound c -type cytochrome. Incorporation of such a complex into a liposome system could be used to test if the cytochrome c_{553} acts as an electron donor to the RC by energizing the system with light and monitoring the development of a $\Delta\psi$ across the liposome membrane. The use of liposomes for such studies has been well

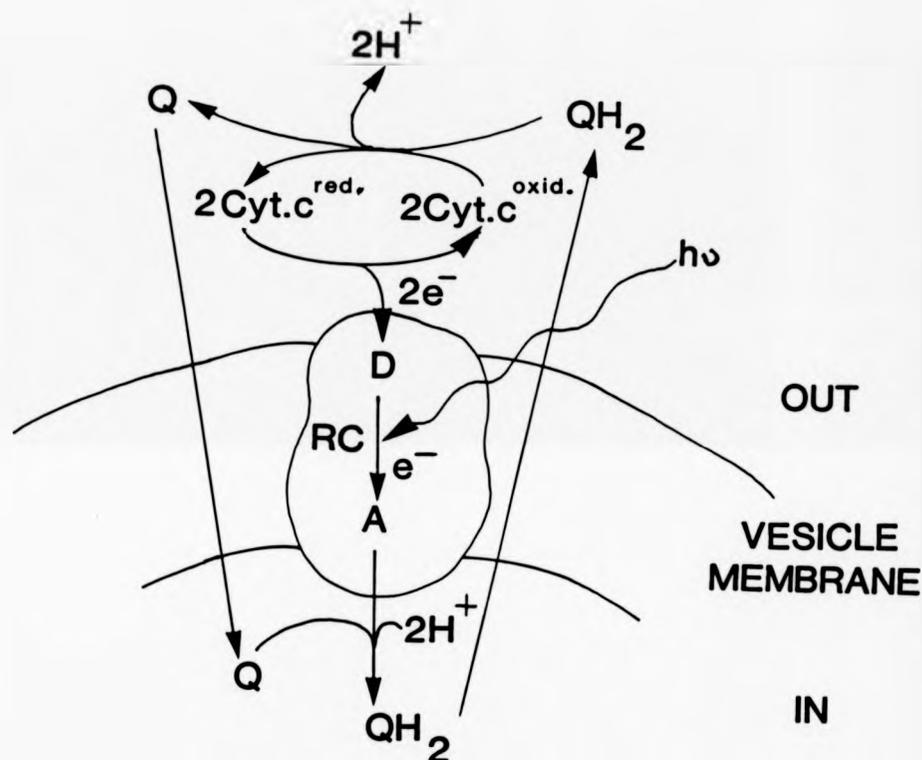
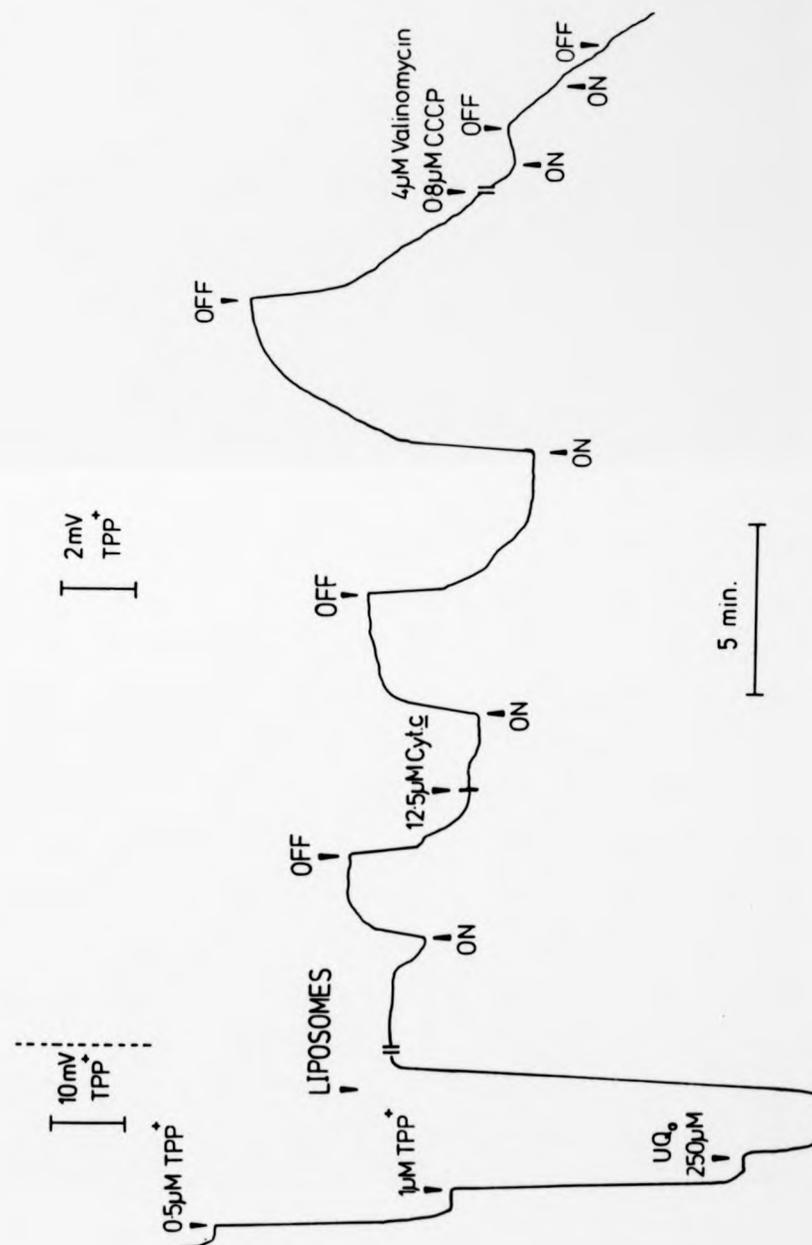


FIGURE 3.29. Reconstruction of a set of reactions which would allow the *in vitro* generation of a proton-motive force from a reaction-centre complex reconstituted into a liposome membrane. Illumination, causing electron transfer from the RC donor (D) to the acceptor (A) is followed by further transfer to a cytochrome (cyt-c) mediated by a quinone derivative (Q) which acts as the major electrogenic component of the system, binding and releasing protons as it crosses the membrane. The circuit is completed by re-reduction of the RC donor *via* the cytochrome and return of the quinone across the membrane, the net result being the generation of a proton gradient (figure modified from Hellingwerf *et al.*, 1985b).

FIGURE 3.30. Generation of a membrane potential by liposomes containing the B885-RC complex from *Rm. vannielii*.

After the addition of the quinone derivative 2,3-dimethoxy-5-methyl-1,4 benzoquinone (UQ_0) and B885-RC proteoliposomes prepared as described in section 2.30, the membrane potential generated by illumination (on/off) was monitored with the TPP^+ electrode. For the third illumination period, a light intensity twice that of the first two was used. The reactions were carried out at $30^\circ C$ in a 2 ml volume of 10 mM Tris HCl buffer pH 8.9.



documented in studies with Rb. sphaeroides RCs (Rich & Heathcote, 1983; Hellingwerf et al., 1985a,b) but with these RC-proteoliposomes, exogenous cytochrome c had to be added to allow a stable $\Delta\psi$ to be generated.

The theoretical operation of such an in vitro system is shown in Fig. 3.29. The reconstruction of a similar set of reactions was attempted with the B885-RC complex from Rm. vannielii incorporated into liposomes (Fig. 3.30). The reaction was performed at high pH to ensure that any proton translocation would be observed in the form of a membrane potential, which was monitored with the probe ion TPP^+ and the electrode set-up described in section 2.31. After the addition of calibrating pulses of TPP^+ , the quinone derivative UQ_0 was added and then the proteoliposomes. A large decrease in the free concentration of the probe ion indicated a significant binding of TPP^+ to the liposomes in the dark. This was probably due to the existence of a high surface potential, as control experiments in which liposomes containing no protein were used showed the same effect. Upon illumination of B885-RC containing proteoliposomes only, in the absence of exogenous cytochrome c, a $\Delta\psi$ was generated which could be stably and reproducibly maintained for several minutes. Addition of reduced mammalian cytochrome c did not result in a larger potential at the same light intensity. However, increasing the light intensity in either case caused the production of a larger potential. Treatment of the liposomes with an uncoupling mixture of valinomycin and CCCP resulted in the abolition of the light-induced $\Delta\psi$, thus proving the authenticity of the potentials generated.

Proteoliposomes were also prepared from intra-cytoplasmic membranes by co-sonication with azolectin and compared with the behaviour of ICM

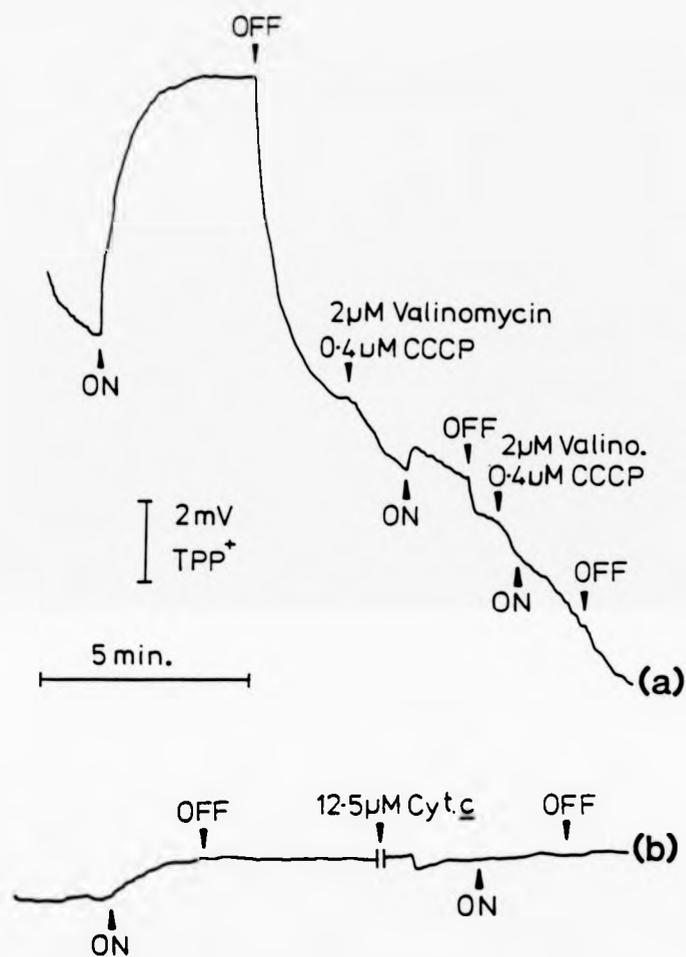


FIGURE 3.31. Development of light-induced membrane potentials, monitored with the TPP⁺ electrode, in liposomes reconstituted with ICM (section 2.30) from photoheterotrophically grown *Rm. vannielii* (a) and in ICM alone (b). After the addition of TPP⁺ and the respective preparation, illumination was provided (on/off). The reactions were carried out at 30°C in a 2 ml volume of 10 mM Tris-HCl buffer pH 8.9.

alone in the same system (Fig. 3.31). Using either the TPP⁺ or SCN⁻ electrode systems, no light-induced $\Delta\psi$ could be detected in the presence or absence of reduced cytochrome c when ICM preparations alone were assayed. However, ICM-proteoliposomes displayed a large $\Delta\psi$, inside negative, when illuminated in the absence of any exogenously added cytochrome c . The authenticity of these potentials was again demonstrated by their ionophore and uncoupler induced abolition in the light.

These data suggest that the RC bound cytochrome c -553 can act as electron donor in reconstituted systems of ICM fragments or the RC pigment-protein complex.

3.2.8 Conclusions from studies of the pigment-protein complexes

(1) Reaction centre associated (RC-B885) and reaction centre free (B800+865) pigment-protein complexes were isolated from *Rm. vannielii* using Triton X-100 and SDS as ICM solubilizing detergents.

(2) The B885-RC complex was unusual amongst those of other Bchl_a containing members of Rhodospirillaceae in being specifically associated with a 38,000 M_r c -type cytochrome. However, a similar relationship exists in the Bchl_b containing *Rp. viridis*, to which *Rm. vannielii* may be phylogenetically related.

(3) *In vitro* reconstitution of RC containing proteoliposomes resulted in the generation of a Δp upon illumination independently of exogenously added cytochrome, suggesting an *in vivo* role for the 38,000 M_r c -553 as

an electron donor to the RC.

3.2.9 Discussion

The detergent solubilization and isolation of pigment-protein complexes from photosynthetic bacteria is subject to the conflicting demands of efficient release of the complexes from the membrane combined with the necessity for the maintenance of "mild" conditions and the preservation of pigment-protein interactions. The position of the near IR absorption maxima is a sensitive indicator of the integrity of such interactions (Cogdell & Thornber, 1980). In Rm. vannielli, the complexes isolated by the Triton-SDS procedure had absorption spectra comparable to that of intact, untreated ICM. The fact that the ICM 870 nm long wavelength band was situated between the 865 and 885 nm bands of the complexes is also suggestive of the preservation of pigment-protein interactions. In addition, the carotenoid absorption maxima of both membranes and isolated complexes were identical. However, in Rb. sphaeroides, RC and B875 carotenoids are blue-shifted in their absorption maxima by up to 7-10 nm (Broglie et al., 1980) in complexes isolated by LDS solubilization. This is probably due to the association of specific carotenoids with the RC rather than evidence of loss of pigment-protein interaction.

The number of pigmented complexes observed during low-temperature PAGE of solubilized membrane clearly depends upon the conditions chosen. The greater number of such bands seen here with Rm. vannielli using LDS or SDS alone is in accord with the results of Broglie et al. (1980) who in Rb. sphaeroides, found them to consist largely of mixtures of B875 and

B800-850 complexes in different proportions. In discussing the results of their investigation, they concluded that complexes with varying proportions of the two antenna components may be derived from membrane areas in which they are intermingled. The apparent sizes in which the various complexes were isolated was thought to reflect the manner in which the photosynthetic units were expanded by the addition of an outer array of B800-850 complexes, as postulated by Monger & Parsons (1977) from energy transfer studies. Furthermore, the existence of such native Bchl- protein aggregates was taken as an indicator of the *in vivo* organization of the photosynthetic apparatus. However, these conclusions may be compromised by the formation of "micellar artefacts" - the interaction of phospholipids, proteins and/or pigments with detergent molecules in different proportions to give discrete bands. The likelihood of the formation of such mixed micelles is increased at low temperatures (Helenius & Simons, 1975) and could obscure the detection of native pigmented complexes which are true components of the photosynthetic apparatus. The differences observed here between the gel patterns of *Rm. vannielii* and *Rb. sphaeroides* ICM solubilized under essentially the same conditions (Fig. 3.8) may therefore reflect such interactions, rather than point to basic differences in the composition of the photosynthetic apparatus.

Clearly, conclusions about the *in vivo* organization of the photosynthetic apparatus based on such gel patterns must take account of possibility of micellar artefacts. Accepting this caveat, the Triton-SDS dual detergent method used here for *Rm. vannielii* suggests that the membrane bound photopigments are organized into two major complexes, one of which contains the photochemical reaction centre. One conclusion from the data presented here is that the presence of Triton X-100

(during electrophoresis or in sucrose gradients) is necessary to maintain the stability of these native pigment-protein complexes when they are solubilized with ionic detergents like SDS. By itself, however, SDS is too harsh to allow visualization of these complexes which instead may form mixed micelles with phospholipid and detergent to give a number of pigmented bands. This is supported by the observation that simply omitting Triton from the gel system (Fig. 3.9) caused the breakdown of the LPB and the appearance of other pigmented complexes, even when ICM was solubilized with SDS plus Triton. The detergent mixture is evidently essential during e.g. electrophoresis but less critical for solubilization.

Previous studies of the general structure of the photosynthetic apparatus in members of the Rhodospirillaceae (Monger & Parson, 1977; Firsow & Drews, 1977; Cogdell & Thornber, 1980; Drews & Oelze, 1981; Thornber *et al.*, 1983) have revealed the existence of both reaction centres associated (LHI) and accessory (LHII) antenna complexes with which the B885 and B800-865 components of *Rm. vannielii* may be identified. However, it is now clear that several subclasses of LHI and LHII complexes exist (Thornber *et al.*, 1983). From the near IR absorption spectrum, the *Rm. vannielii* LHI (B885) complex most closely resembles that from *Rs. rubrum*, typifying the "B890 class". The corresponding complex from *Rb. sphaeroides*, for example, typifies the "B875 class". Nevertheless, more evidence, particularly pigment composition and studies of the circular dichroism (CD) spectrum of the B885 complex (to give information on the degree of interaction of the Bchl molecules) is needed before a final assignment can be made. Indeed, in terms of the microenvironment of Bchl (Robert & Lutz, 1985) and redox properties (Picorel *et al.*, 1984) these two groups of LHI

appear similar and so they may be functionally equivalent.

The ratio of B800 to B865 in the Rm. vannielii LHII complex (1.5-3.0) indicates that it is of the "type I" group in the terminology of Thornber et al. (1983). Such complexes are characterized by the peak height of the largest wavelength form of Bchl being about 1.5-2.0 times as intense as the B800 form. In contrast, Type II B800-850 (LHII) complexes display equal intensities of the B800 and B850 forms (Thornber et al., 1983). The archaetypal type I LHII complex is that of Rb. sphaeroides or Rb. capsulatus. Most workers would probably also include that of Rp. palustris (Firsow & Drews, 1977; Varga & Staehelin, 1985a) but the B800 peak height is in fact quite variable, depending on the light intensity at which the cultures are grown (see section 3.3). As Rp. acidophila strains can contain both types of LHII complex (Cogdell et al., 1983) there would appear to be considerable heterogeneity in the accessory LH composition in members of the genus Rhodospseudomonas as presently constituted (Imhoff et al., 1984) This was also supported by a comparison of some of the species on Triton-SDS gels in this study (Fig. 3.12) where all the putative LHII complexes showed widely differing apparent M_r values.

The redox properties of the B800-865 complex from Rm. vannielii were not particularly different from those found with Rb. sphaeroides (Picorel et al., 1984), except that the 800 nm peak was highly susceptible to oxidation. In both microbes, ease of oxidation probably reflects an electronic structure consisting of a Bchl monomer and this has been confirmed in Rb. sphaeroides by CD spectroscopy (Sauer & Austin, 1978; Picorel et al., 1984). The B865 peak in Rm. vannielii is most likely dimeric but this obviously requires confirmation from CD spectra.

Interpretation of the redox behaviour of the B885-RC complex is more difficult because a rigorous comparison of CD spectra and ferricyanide induced redox changes is not available in the literature. However, the LHI Bchl IR peak in a number of other species in the Rhodospirillaceae have been found to be dimeric by CD spectroscopy (Thorner *et al.*, 1983; Picornel *et al.*, 1984).

Rhodomicrobium vannielii is generally considered physiologically similar to *Rp. palustris* from which the pigment-protein complexes of the photosynthetic apparatus have been isolated by several groups (Firsow & Drews, 1977; Hayashi *et al.*, 1982a,b; Varga & Staehelin, 1985a). Hayashi *et al.* (1982a) reported the isolation of two pigmented bands from ICM solubilized with SDS and Triton X-100. However, on gel electrophoresis in the presence of SDS alone, the corresponding LPB remained stable but the UPB disappeared from the gel profile, in contrast to the pattern obtained here with *Rm. vannielii*. The UPB and LPB of *Rp. palustris* were found to consist of RC-associated (LHI) and RC-free (LHII) Bchl. Varga & Staehelin (1985a) prepared pigment-protein complexes from *Rp. palustris* on sucrose gradients using a combination of octylglucopyranoside and SDS. They obtained RC-LHI and LHII containing fractions which had native M_r values of >95,000 and ca. 85,000 respectively. In these complexes two low M_r polypeptides (10,000 and 8,600) were attributed to LHI and three (M_r 6,500, 7,500 and 8,000) to LHII. The LHII 6,500 M_r band appeared to be a dimer. This plethora of accessory LH polypeptides was also found by Hayashi *et al.* (1982b) and if not contaminants or breakdown products, they may have a role in the assembly of the oligomeric B800-850 complex (Varga & Staehelin, 1985a). Although there are some similarities to *Rm. vannielii*, the composition and behaviour of the LHII complex in these microbes would appear to be

rather different (see also section 3.3).

Cogdell et al. (1980) and Cohen & Kaplan (1981) found the two low M_r proteins of the Rb. sphaeroides B800-850 complex to consist of one isoelectric form each, although the complex was not examined on 2-D gels and the pI values determined by the two groups were widely divergent. Indeed, no pigment-protein complexes appear to have been examined by the 2-D O'Farrell technique but the two putative isoelectric forms of the Rm. vannielii 14,000 M_r LHI and 11,000 M_r LHII proteins would appear to merit further investigation.

It is possible that a post-translational event such as phosphorylation could alone account for the charge difference. Such covalent modification of the chloroplast LHCII complex is central to the redistribution of excitation energy between the photosystems (Allen et al., 1981) and there are unpublished reports (Allen, 1983) of the phosphorylation of bacterial LH complexes. This could have implications for the regulation of energy distribution from LHII to the RC, for example.

Energy transfer efficiency is also a function of the spatial organization of the photosynthetic apparatus and information from the 1-D and 2-D detergent fractionation studies and from chemical cross-linking was used to probe this. Rhodospirillum vannielii would appear unique amongst the Bchl_a containing members of the Rhodospirillaceae so far studied in a containing an RC-bound c -type cytochrome. This was clearly indicated not only by chemical cross-linking but also on sucrose gradients, Triton-SDS gels and ion-exchange chromatography. The cytochrome composition of Rm. vannielii has been the subject of a

previous study (Morita & Conti, 1963) and a loosely bound or soluble C_2 type cytochrome (c -550) was detected in addition to a membrane bound c -553. The C_2 type cytochrome was not apparent in this study but a low M_r (ca. 12,000) stained band on some TMBZ stained gels of soluble protein could be attributable to this species, although difference spectra did not reveal its presence. The 28,000 M_r and 32,000 M_r cytochromes of the *Rm. vannielii* ICM may be components of a cytochrome p - c_1 complex (Gabellini *et al.*, 1982; Yu *et al.*, 1984).

Although the cytochrome c -553 is clearly a component of the B885-RC complex, RC or RC-LHI complexes from other Rhodospirillaceae do not appear to contain cytochromes (Feher & Okamura, 1978; Fisow & Drews, 1977; Hayashi *et al.*, 1982a,b; Cogdell *et al.*, 1983; Thornber *et al.*, 1983). Preparations from the Bchl b containing *Rp. viridis* (Pucheu *et al.*, 1976; Jay *et al.*, 1984), *Thiocapsa pfennigii* (Seftor & Thornber, 1984) and several other Bchl a containing members of the chromatiaceae (Lin & Thornber, 1975; Bartsch, 1978; Lefebvre *et al.*, 1984) do, however, contain multi-haem cytochromes, c -552-558, bound to a polypeptide of M_r 35-45,000. In *Rp. viridis* (Wynn *et al.*, 1985), the apoprotein has an M_r of 38,000 and binds a low potential c -553 and a high potential c -558 haem in a 1:1 ratio. This is remarkably similar to the *Rm. vannielii* protein except that the high potential cytochrome c could not be detected. Chemical cross-linking studies (Peters *et al.*, 1984) indicate binding of the *Rp. viridis* cytochrome c to the RC 27,000 M_r (H) subunit. In *Rm. vannielii*, cytochrome c -553 - reaction centre interactions appeared to be co-ordinated by the 31,000 M_r (M) subunit while cross-links of the 28,000 (M) protein to oligomers of the B885 12,000 M_r polypeptide were also detected. In both *Rb. capsulatus* (Peters *et al.*, 1983) and *Rp. viridis* (Peters *et al.*, 1984) RC-LHI Bchl

interactions are probably co-ordinated by the H subunit.

These comparisons suggest Rm. vannieli RC (M) and (H) subunits to have the opposite roles to those in some other photosynthetic bacteria, although the possibility of the H subunit binding electron transport chain components in Rb. sphaeroides (Feher & Okamura, 1978) and Rb. capsulatus (Peters *et al.*, 1983) has been suggested previously. Nevertheless, caution is needed because of the discrepancy between the migration behaviour of RC subunits on SDS gels and M_r values determined by sequence analysis (Williams *et al.*, 1984). The identity of "H" and "M" RC subunits in different species is therefore only valid if they migrate in a similar (anomalous) way on SDS gels.

The cross-linking data and that from detergent fractionation can be combined to form a model of the organization of the pigment-protein complexes in Rm. vannieli (Fig. 3.32). Assuming one copy each of the M_r 11,000 and 13,000 B800-865 polypeptides in a "minimal unit" of M_r 24,000, the apparent M_r value of 90,000 for the native complex on Triton-SDS gels is consistent with it being an oligomer of four copies of the minimal unit, thus containing four copies each of the two polypeptides. This complex was isolable separately from the B885-RC-cytochrome aggregate. The structure of the RC-LH-cytochrome complex is based on the equimolar cross-linking of Cyt c -553 and the 31,000 M_r RC subunit and assuming an equimolar stoichiometry (1:1:1) of the RC subunits (Feher & Okamura, 1978).

Taken together, the data suggest that the photosynthetic apparatus of Rm. vannieli has a rather unique set of properties, some of which are more akin to those of the Bchl b containing Rd. viridis than to other

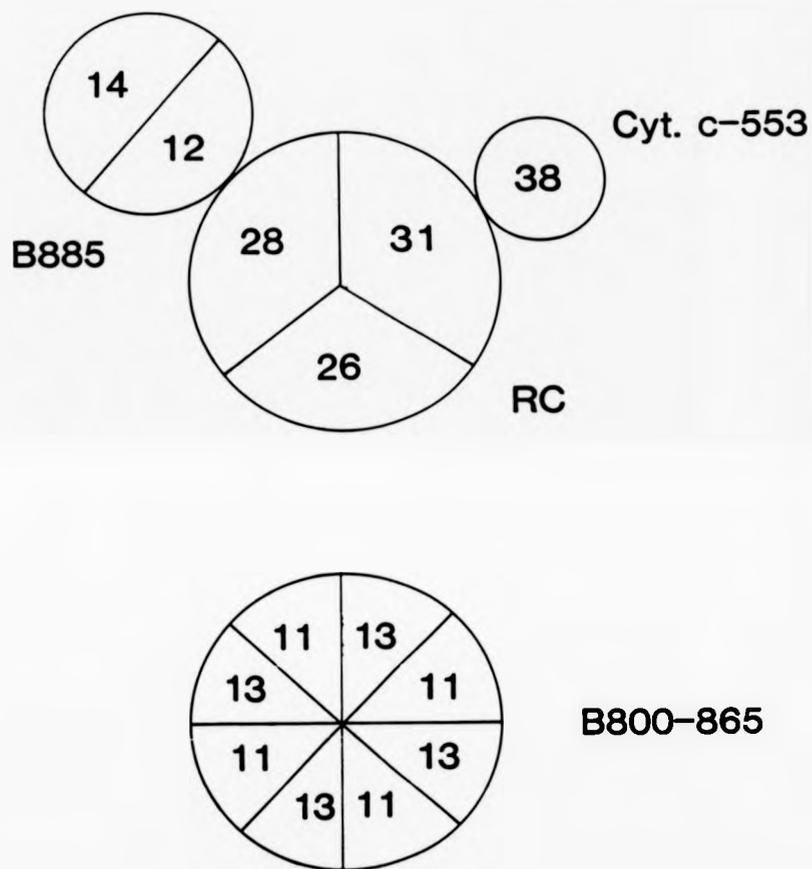


FIGURE 3.32. Proposed organization of the components of the pigment-protein complexes of the photosynthetic apparatus of *Rh. vannielii*, based on evidence from detergent fractionation and reversible chemical cross-linking. The numbers refer to the M_r values of the polypeptides.

Bchl_a containing members of the Rhodospirillaceae. This is interesting in view of the apparent close phylogenetic relationship between Rm. vanniellii and Rd. viridis revealed by 16S rRNA sequencing (Gibson et al., 1979). Nevertheless, the light-harvesting system in Rd. viridis is rather different from Rm. vanniellii, with a single RC associated B1020 antenna (Peters et al., 1984). Both microbes do, however have a lamellate ICM system which is isolable from Rd. viridis in the form of thylakoid like stacks, not obviously equivalent to the chromatophores of other species (Stark et al., 1984). In Rm. vanniellii light-induced membrane potentials were only generated when ICM preparations were incorporated into closed liposome vesicles - i.e. "artificial chromatophores" - and this could indicate a similar structure. However, this needs to be rigorously confirmed by electron microscopic analysis.

The most likely role of the RC bound α -553 in Rm. vanniellii is as the immediate electron donor to the special pair dimer during photosynthesis. This is certainly consistent with the in vitro reconstitution of light-driven $\Delta\psi$ generation in liposomes which was independent of exogenous cytochrome. The role of Cytochrome C₂ may be as an intermediate electron carrier in contrast to many other members of the Rhodospirillaceae, but as postulated for both Rd. viridis and some members of the chromatiaaceae, which have ICM bound cytochromes (Bartsch, 1978; Nugent, 1984). Reconstitution of photosynthetic electron transport from isolated reaction centres and electron transport components is an attractive way of dissecting their functional roles in vivo and has previously been achieved in Rb. sphaeroides (Rich & Heathcote, 1983; Hellingwerf et al., 1985a,b) and from an RC-LH complex in Rs. rubrum (Matsuda et al., 1984). Although the size of the light induced potentials generated in the Rm. vanniellii system were not

quantified because of the large amount of probe-binding, it is clear that the relative ease of preparation of these RC-LH-cytochrome proteoliposomes could be useful in co-reconstitution studies, where the size of the Δp can be controlled by the light intensity and its effect on other co-reconstituted complexes, such as solute transport systems, can be investigated. This has also been suggested by Driessen *et al.* (1985) who successfully demonstrated light-induced generation of a proton-motive force and calcium transport in membranes of Streptococcus cremoris fused with bacteriorhodopsin containing proteoliposomes.

3.3 Regulation of synthesis of the photosynthetic apparatus in batch cultures

The two major factors known to influence the synthesis of the components of the photosynthetic apparatus, oxygen and light, were investigated for their regulatory effects in Rm. vanniellii.

3.3.1 Photoheterotrophic growth at different light intensities

Table 3.3 shows the variation in the specific pigment content in cultures of Rm. vanniellii grown at four different light intensities. Rhodospseudomonas palustris was also included for comparison. An inverse relationship was apparent between the specific Bchl and carotenoid content and the incident light intensity in both microbes. Determinations of the specific pigment content of Rm. vanniellii swarmer cells prepared from the same batch cultures also showed this inverse relationship but the values were always slightly higher at each light intensity, compared to those of the culture as a whole. The ratio of carotenoid to Bchl did not remain constant in Rm. vanniellii cultures but underwent a slight decrease at progressively lower light intensities.

Absorption spectra of cell-free extracts prepared from such cultures are shown in Fig. 3.33. Growth at lower light intensities resulted in an increase in the size of the peaks due to carotenoid (460, 490, 525 nm) and Bchl_a (378, 595, 800 and 870 nm) and a slight decrease in the size of the 420 nm peak probably attributable to a flavoprotein (section

TABLE 3.3. Variation of pigment content and composition with growth at different light intensities in

Light intensity $\mu\text{E m}^{-2}\text{s}^{-1}$	<u>Km. vanniellii</u>				<u>Kp. palustris</u>		
	specific Bchl content $\text{nmol.mg protein}^{-1}$	Specific carotenoid content $\text{Mg/2nm mg protein}^{-1}$	Carotenoid: Bchl ratio	A870: A800 ratio	Specific Bchl content $\text{nmol.mg protein}^{-1}$	A870: A800 ratio	A870: A800 ratio
85	6.7 (8.7) ^a	0.73 (0.314)	0.108 (0.09)	1.91	8.3	2.28	
35	12.1 (17.0)	1.03 (1.40)	0.085 (0.082)	1.85	ND ^c	ND	
17	20.4 (29.0)	1.68 (2.16)	0.082 (0.074)	1.67	ND	ND	
6.5	53.0 (62.7)	3.67 (4.35)	0.069 (0.069)	1.45	42.0	1.02	

^a Figures in parentheses represent values obtained for swarmer cell populations from the same batch cultures.

^b The A870:A800 ratio (Km. vanniellii) and the A870 ($85 \mu\text{E m}^{-2}\text{s}^{-1}$) or A858 ($6.5 \mu\text{E m}^{-2}\text{s}^{-1}$): A800 ratio (Kp. palustris) were determined using cell-free extracts.

^c ND - not determined.

Cultures were grown as described in section 2.3 and harvested when the A650 nm reached 1.0.

Pigments were extracted into acetone: methanol (section 2.34).

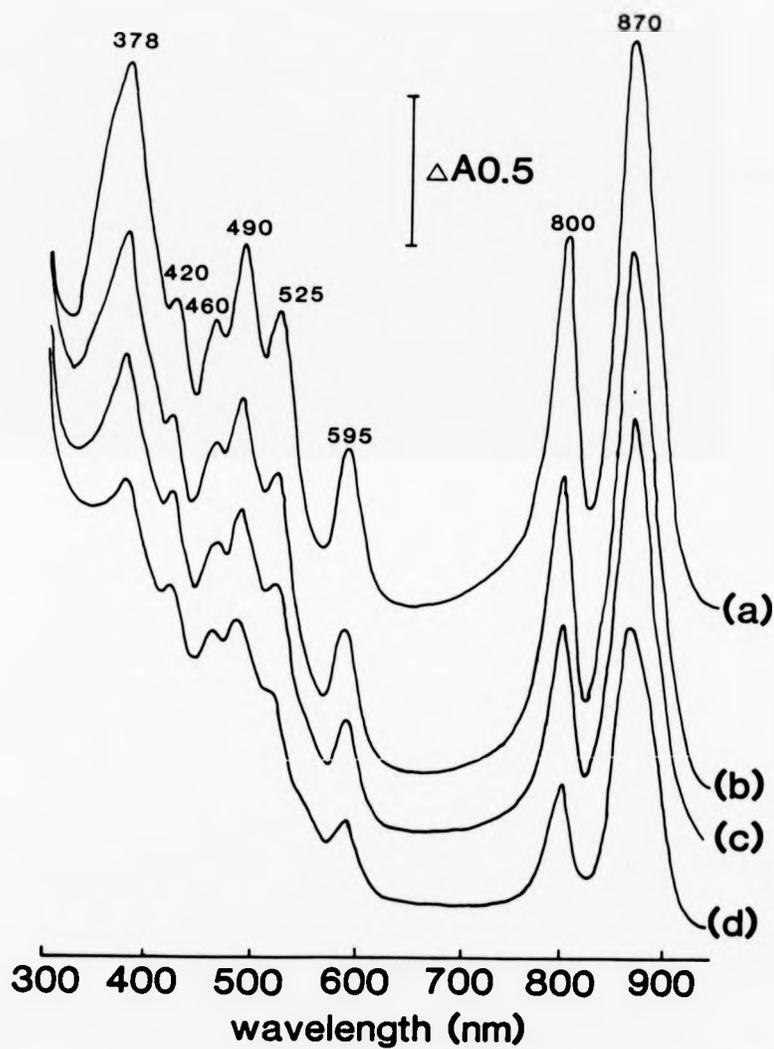


FIGURE 3.33. Room temperature absorption spectra of cell-free extracts of *Rh. vanniellii* grown at light intensities of 6.5 (a), 17 (b), 35 (c) and 85 (d) $\mu\text{E m}^{-2}\text{s}^{-1}$. Approximately equal protein concentrations were used in each case by adjusting the $A_{280\text{ nm}}$ of the extracts to the same value (ca. 2.0).

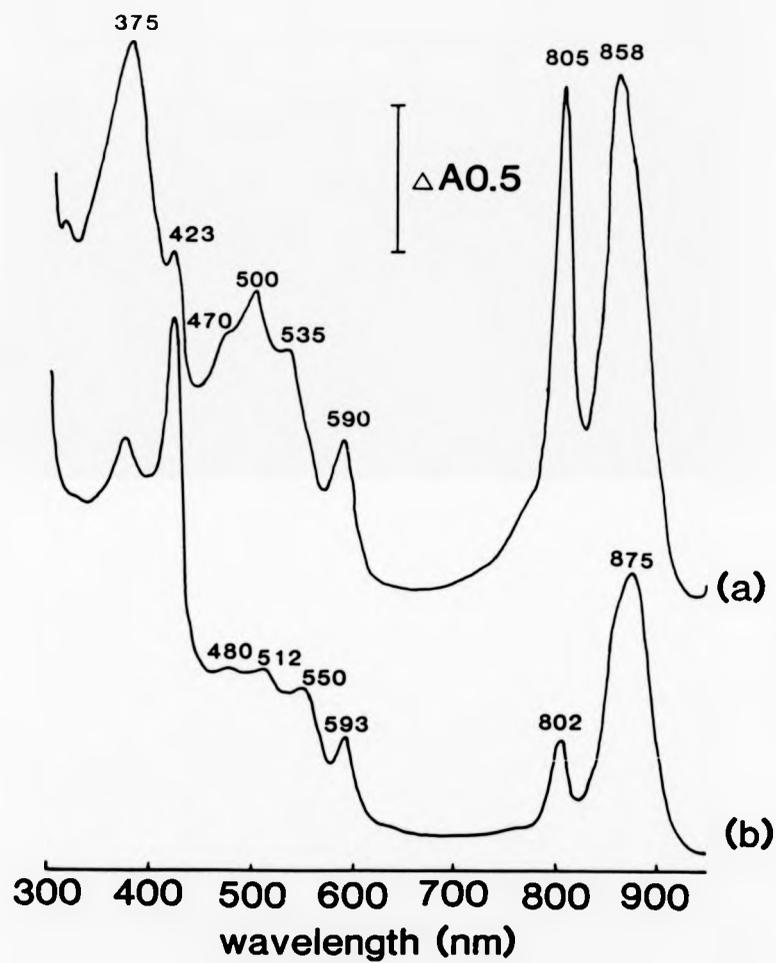


FIGURE 3.34. Room temperature absorption spectra of cell-free extracts of *Rh. palustris* grown at (a) 6.5 and (b) 85 $\mu\text{E m}^{-2} \text{s}^{-1}$. Approximately equal protein concentrations were used in both cases ($A_{280 \text{ nm}} = 2.0$).

3.2.4). A comparison with absorption spectra for Rp. palustris (Fig. 3.34) indicated a similar behaviour with regard to the carotenoid peaks and also a 423 nm band which was much more in evidence under high light intensities. However the variation in the size of the 800 nm band in Rp. palustris upon variation of light intensity was far greater than that in Rm. vannielii. The data given in Table 3.3 reveals that the B870:800 ratio varied over the range 1.9 to 1.45 in Rm. vannielii grown at these different light intensities while the corresponding ratios for Rp. palustris were 2.3 to 1.02 at the same light intensities but if anything a rather narrower spread of Bchl contents. As the 800 nm band is largely attributable to the accessory LHII complex in both microbes, the absorption spectra suggest that it is this complex which shows the greatest variation with light intensity. Also, there is a shift in the position of the longest wavelength absorption band in Rp. palustris from 875 nm at the highest light intensity used, to 858 nm at the lowest, indicating a greater contribution of the LHII complex under the latter conditions. However, in Rm. vannielii, no such wavelength shift was observed, and the peak remained at about 870 nm.

Therefore, to confirm the increase in LHII as the major cause of the increase in Bchl content with growth at lower light intensities in Rm. vannielii, ICM preparations were subjected to Triton/SDS solubilization and electrophoresis (Fig. 3.35). This clearly revealed an increase in the relative concentration of the B800-865 LHII complex under conditions of decreasing light intensity. The relative amount of the B885-RC complex remained more constant except during growth between light intensities of 17 and $6.5 \mu\text{Em}^{-2}\text{s}^{-1}$ when an increase was noticeable.

The polypeptide profiles of fully denatured ICM (Fig. 3.36) also showed

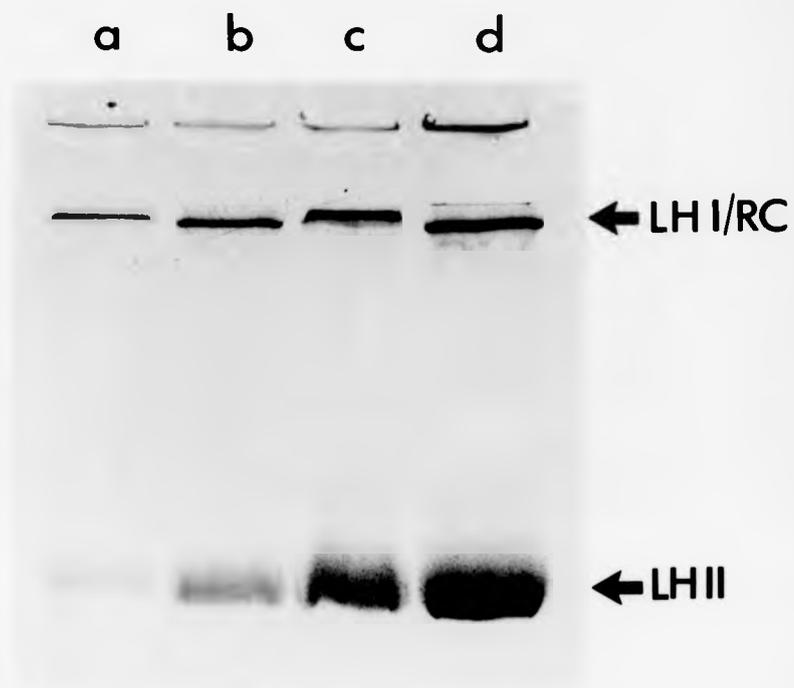


FIGURE 3.35. Separation of native pigment-protein complexes from membranes of *Rm. vanneili* cultures grown at light intensities of (a) 85, (b) 35, (c) 17 and (d) $6.5 \mu\text{E m}^{-2} \text{s}^{-1}$. Equal quantities (200 μg protein) of SDS plus Triton X-100 solubilized ICM were applied to a 10% (w/v) polyacrylamide gel containing both detergents and electrophoresed in the cold.

The gel was photographed unstained.

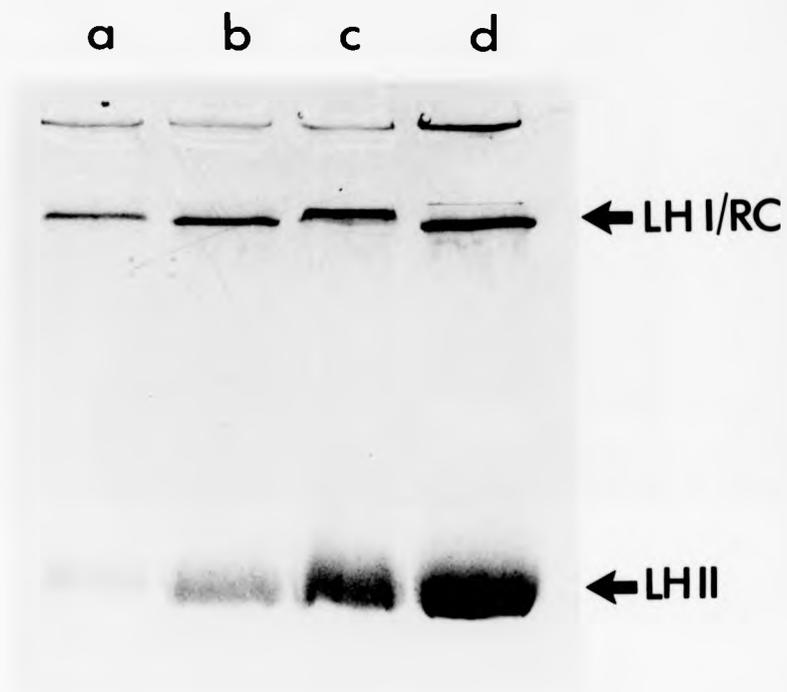


FIGURE 3.35. Separation of native pigment-protein complexes from membranes of *Rm. vannelli* cultures grown at light intensities of (a) 85, (b) 35, (c) 17 and (d) $6.5 \mu\text{E m}^{-2} \text{s}^{-1}$. Equal quantities (200 μg protein) of SDS plus Triton X-100 solubilized ICM were applied to a 10% (w/v) polyacrylamide gel containing both detergents and electrophoresed in the cold.

The gel was photographed unstained.

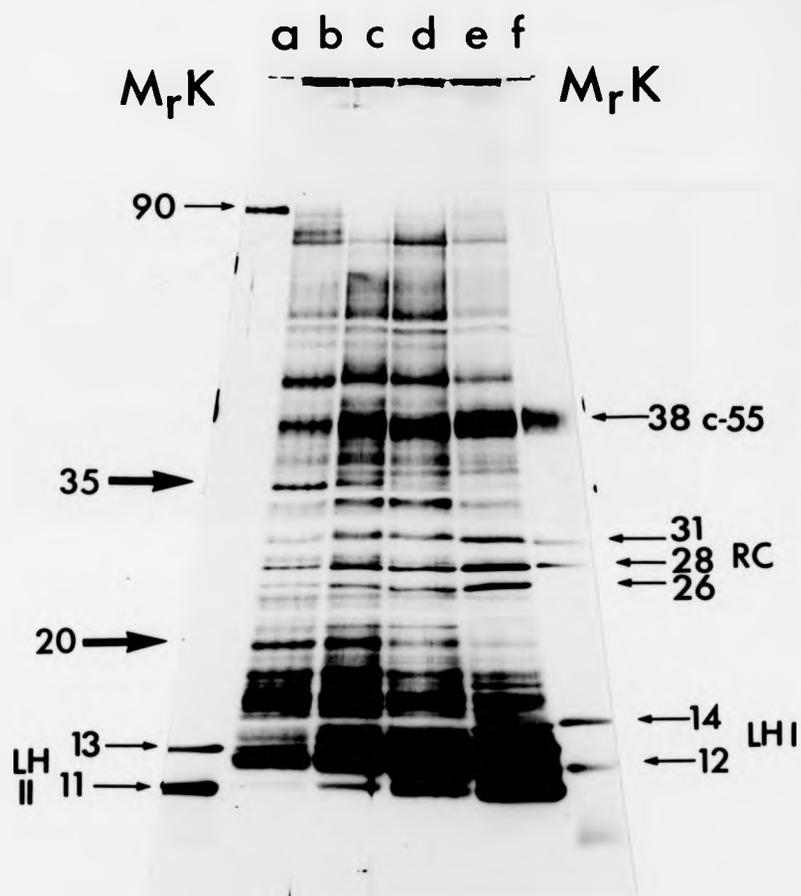


FIGURE 3.36. Intra-cytoplasmic membrane polypeptide profiles from *Rm. vannielii* cultures grown at light intensities of (b) 85, (c) 35, (d) 17 and (e) $6.5 \mu E m^{-2} s^{-1}$. In (a) the 11 K and 13 K polypeptides of the B800-865 LHII complex are indicated, with some residual undenatured complex remaining (90 K). In (f) the polypeptides of the isolated B885-RC complex are similarly indicated. In (b)-(e) the gel was loaded with $50 \mu g$ protein in each case.

10-30% (w/v) silver-stained gel.

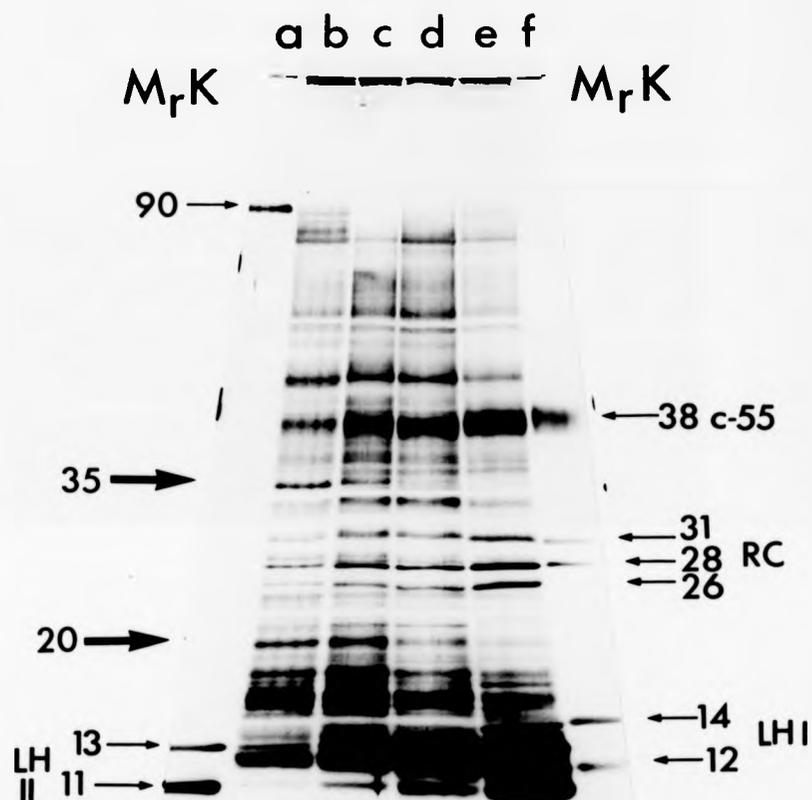


FIGURE 3.36. Intra-cytoplasmic membrane polypeptide profiles from *Rm. vannielii* cultures grown at light intensities of (b) 85, (c) 35, (d) 17 and (e) $6.5 \mu\text{E m}^{-2} \text{s}^{-1}$. In (a) the 11 K and 13 K polypeptides of the B800-865 LHII complex are indicated, with some residual undenatured complex remaining (90 K). In (f) the polypeptides of the isolated B885-RC complex are similarly indicated. In (b)-(e) the gel was loaded with $50 \mu\text{g}$ protein in each case.

10-30% (w/v) silver-stained gel.

this trend, as evidenced by an increased staining intensity of RC, B885 and cytochrome c -553 polypeptides. Two other proteins of M_r 35,000 and 20,000, however, decreased in relative abundance in the ICM from cells grown at progressively lower light intensities. The polypeptides associated with the B800-865 complex (M_r 11,000 and 13,000) clearly increased in relative abundance under these conditions of growth consistent with the behaviour of the native aggregate on Triton-SDS gels.

3.3.2 Chemoheterotrophic growth under aerobic-dark conditions

Determination of growth rates (Table 3.4) for Rm. vanniellii cultured under chemoheterotrophic conditions clearly showed the inability of this microbe to tolerate high O_2 tensions. The growth rate and final cell yield decreased as the oxygen transfer rate in batch cultures was increased in shake-flasks containing different volumes of media. The specific Bchl and carotenoid contents were markedly decreased under aerobic cultivation but the carotenoid: Bchl ratio was in fact increased under all such conditions compared to phototrophic growth (compare Table 3.3 and 3.4).

The specific pigment content varied little over the range of aeration conditions used in the shake flasks, despite a marked effect on growth, suggesting the minimum specific Bchl content obtainable with Rm. vanniellii to be in the range of 0.7-1.0 nmol (mg cell protein)⁻¹ (Table 3.4). Higher values (about 3.0-4.0 nmol Bchl (mg cell protein)⁻¹) could be obtained in batch cultures in 5 L or 20 L flasks sparged with a slow stream of air. Under both sets of conditions, an abnormal cellular

TABLE 3.4. Effect of oxygen transfer rate on growth and pigment content of *Km. vanniellii* in batch culture

Volume of aerobic dark culture (ml)	doubling time (h)	cell yield at harvest (A540 nm)	cellular Bchl content nmol.mg protein ⁻¹	cellular carotenoid content A472 nm mg protein ⁻¹	cellular carotenoid: Bchl ratio	nmol.min.mg protein ⁻¹ NADH oxidase	Isocitrate dehydrogenase
2,000	11.5	0.92	0.70	0.27	0.38	63.3	93.4 (72.8) ^c
1,000	13.0	0.63	0.81	0.31	0.38	52.1	93.8 (57.3)
500	15.5	0.51	1.0	0.33	0.33	56.8	120.6 (63.8)
250	22.0	0.24	0.9	0.31	0.34	57.4	104.3 (64.4)
125	NG ^a	0.07	ND ^b	ND	ND	ND	ND

^a NG - no growth; ^b ND - not determined; ^c values outside parentheses refer to NADP linked activity, values inside parentheses refer to NAD-linked activity.

Cells were grown under chemoheterotrophic, aerobic-dark conditions in un baffled shake flasks (section 2.4) for 4-5 days. Pigment contents were determined on whole cells by extraction into acetone:methanol. Cell-free extracts were prepared by sonication (section 2.13) and used for the enzyme-assays.



FIGURE 3.37. Morphology of Rm. vanniellii grown under aerobic dark conditions.

Cells from a 500 ml batch culture contained in a 2.5 L flask were harvested and negatively stained with phosphotungstic acid. Note the appearance of lobed and elongated cells within the multicellular arrays. Bar marker represents 1 μm .



FIGURE 3.37. Morphology of *Rm. vanniellii* grown under aerobic dark conditions.

Cells from a 500 ml batch culture contained in a 2.5 L flask were harvested and negatively stained with phosphotungstic acid. Note the appearance of lobed and elongated cells within the multicellular arrays. Bar marker represents 1 μm .

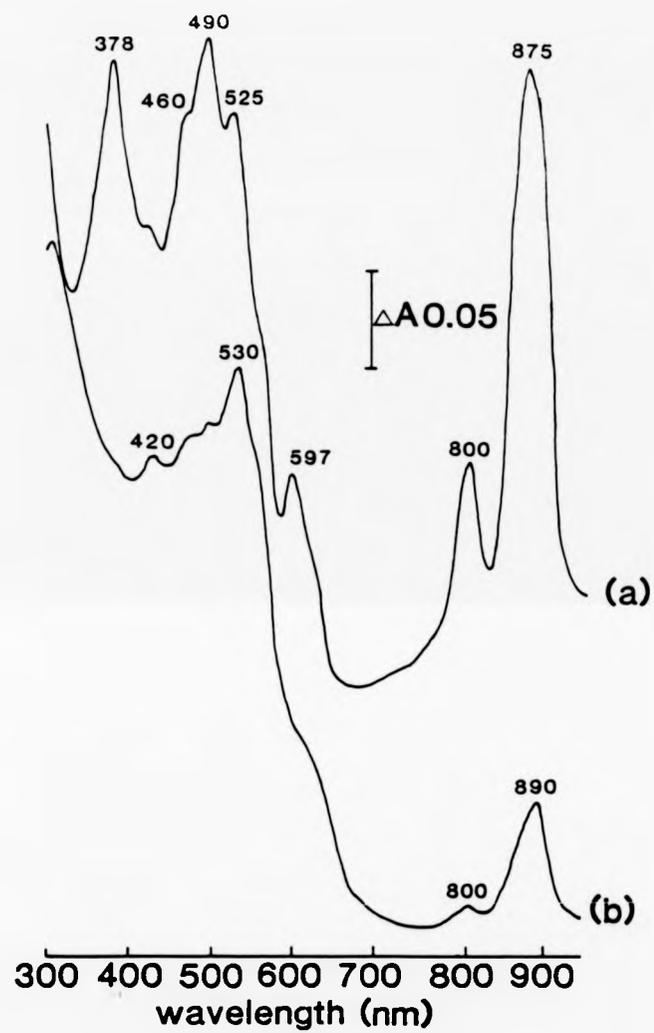


FIGURE 3.38. Room temperature absorption spectra of ICM preparations from *Rm. vannielii* grown under chemoheterotrophic conditions in the dark with cellular Bchl contents of (a) 3.0 and (b) 1.0 nmol (mg protein)⁻¹. The ICM was prepared by sucrose gradient centrifugation and the A₂₈₀ nm adjusted to ca 0.6 in each case before the spectra were recorded.

morphology was noted (Fig. 3.37) in the form of elongated and lobed cells within the multicellular arrays. Swarmer cells, however, were still observed in these cultures.

Absorption spectra of ICM preparations from batches of cells with these two ranges of specific Bchl contents are shown in Fig. 3.38. At the lowest values obtainable, the near IR Bchl_a peaks were at 805 and 890 nm, the 378 nm ultra-violet Bchl_a peak was hardly resolved and the carotenoid peaks were significantly altered in comparison to phototrophically grown cells, in that the 525 nm band was increased and the 460 and 490 nm peaks were decreased. At the higher Bchl content (3.0 nmol (mg cell protein)⁻¹) the absorption spectrum of the ICM was similar to that of phototrophically grown cells but the carotenoid peaks clearly made a much more significant contribution in the visible region. This reflects the observed increase in the carotenoid:Bchl ratio under aerobic conditions (Table 3.4). The near IR spectra suggest that the lowest Bchl contents can be accounted for by the presence of only RC and "B885" (LHI) Bchl in the membrane with no evidence of LHII. Assuming the 800 nm peak to be due only to RC Bchl, the size of the photosynthetic unit under these conditions can be estimated to be about 20 mol total Bchl per mol reaction centre, assuming an extinction coefficient of $2.8 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Straley *et al.*, 1973).

Fully denaturing polyacrylamide gel electrophoresis of both soluble and ICM fractions obtained from sucrose gradients loaded with either phototrophically grown cells or chemoheterotrophically grown cells with a Bchl content of ca. 1 nmol (mg cell protein)⁻¹ is shown in Fig. 3.39. The relative abundance of the majority of the soluble proteins remained remarkably constant under the two growth conditions with the exception

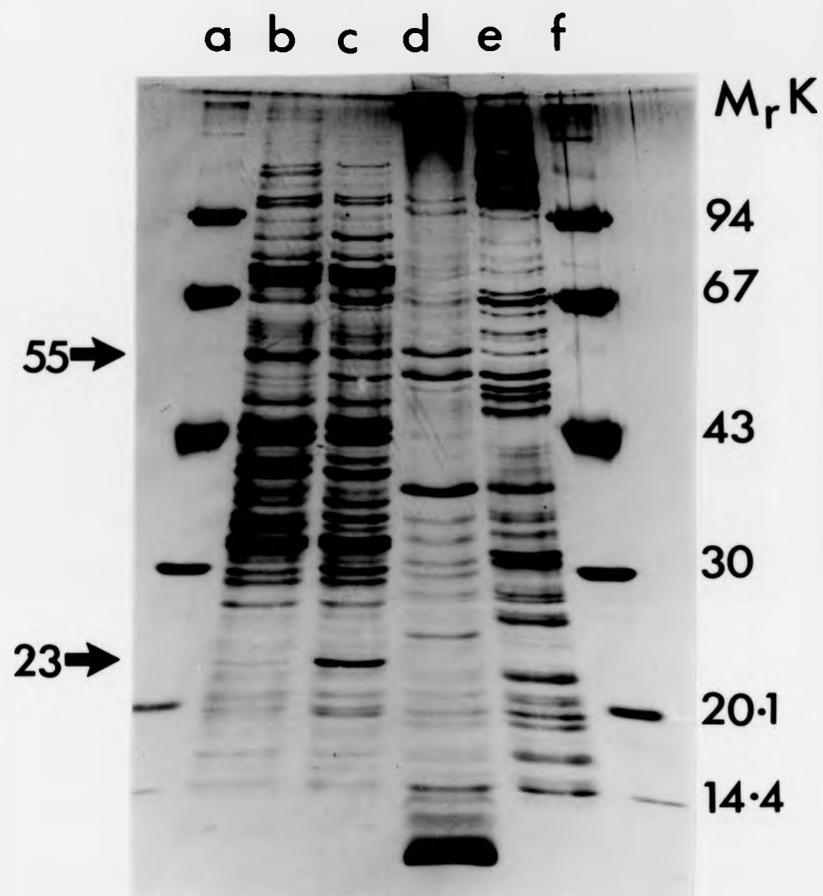


FIGURE 3.39. Polypeptide profiles of soluble (b & c) and ICM (d & e) fractions from cells grown photoheterotrophically (b & d) and chemoheterotrophically (c & e).

Fifty μg quantities of protein were loaded in each case. Arrows indicate bands of 55,000 and 23,000 M_r in tracks (b) and (c) respectively. Tracks (a) and (f) contain molecular weight markers. Silver stained 10-30% (w/v) polyacrylamide gel.

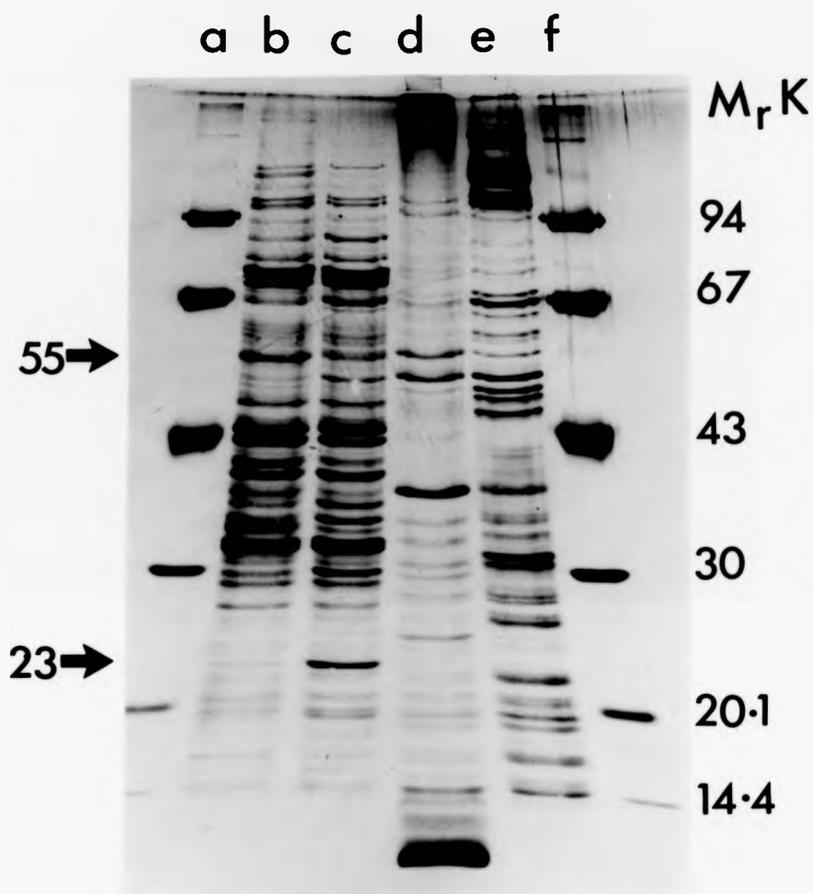


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of a 55,000 M_r species only present under phototrophic conditions and a 23,000 M_r species present under chemoheterotrophic conditions. In contrast, the majority of the ICM proteins were different. There was clearly a significant reduction in the amounts of the RC and cytochrome c -553 polypeptides and those of the light-harvesting complexes during growth under aerobic-dark conditions. Indeed, only the 14,000 M_r B885 polypeptide was detectable under such conditions and a number of new polypeptides were apparent.

A comparison of some of the enzyme activities present in cell-free extracts from chemoheterotrophically grown cells at the four different O_2 transfer rates is shown in Table 3.4. Despite the differences in growth rate of such cultures already referred to, the specific activities of NADH oxidase and both NADP and NAD specific isocitrate dehydrogenases remained remarkably constant. A more extensive comparison of such activities in phototrophically grown cells and those from cells grown in 1 L batches under aerobic conditions (Table 3.5) revealed some striking differences. Most notably, whilst NADH oxidase activity was not detectable at all in phototrophic cell-free extracts using either spectrophotometric (oxidation of NADH at 340 nm) or polarographic (NADH dependent O_2 uptake) techniques, this activity was readily apparent in those extracts from chemoheterotrophically grown cells. This was consistently observed in several separate determinations on different cell-free extracts. A fractionation study showed that about 80% of the NADH oxidase activity was located in the soluble fraction and the remainder in the membrane. The specific activities of NADH dehydrogenase, NAD-specific isocitrate dehydrogenase, malate dehydrogenase and to a lesser extent succinate dehydrogenase were all increased during aerobic-dark growth while that of NADP-specific

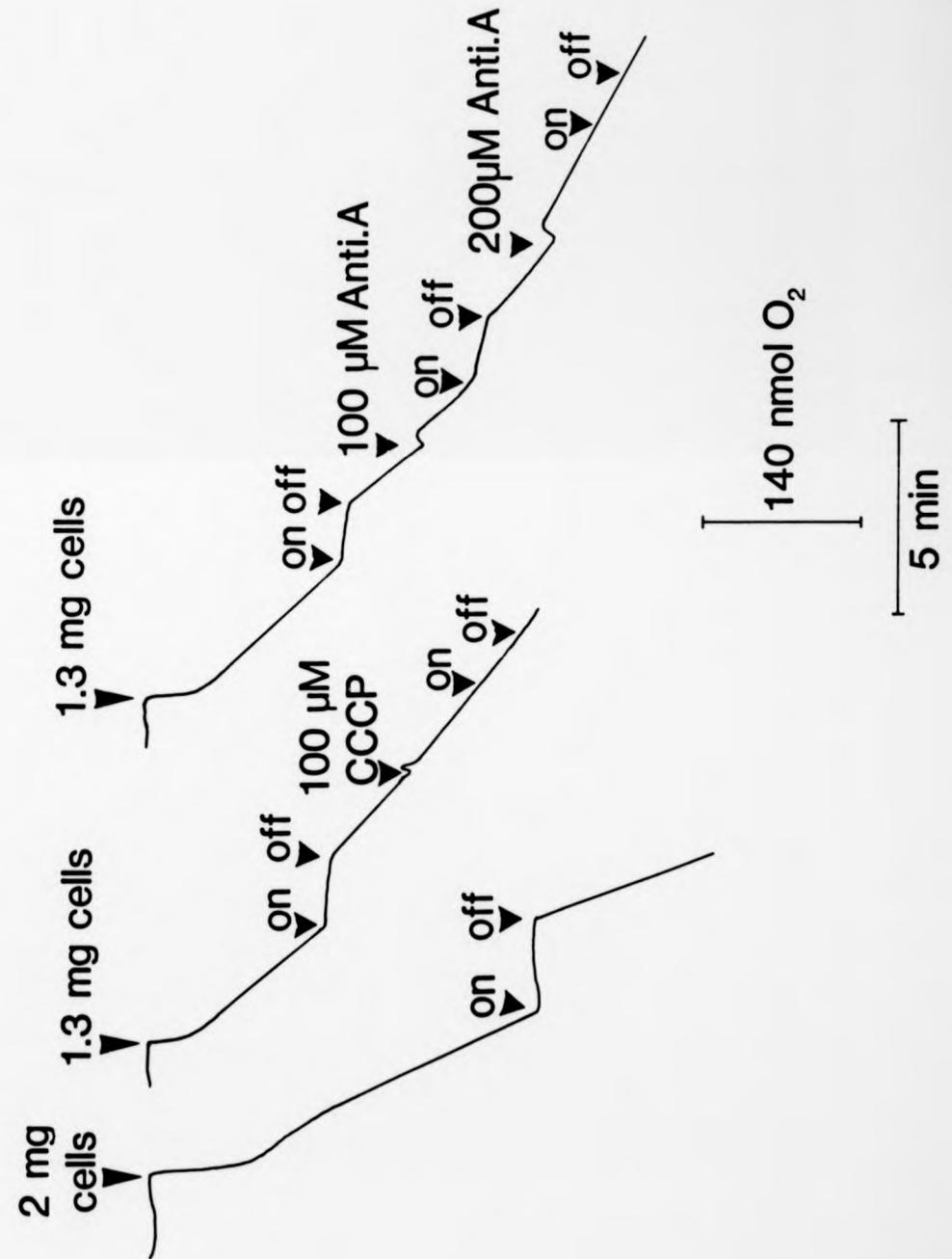
TABLE 3.5. Selected enzyme activities in cell-free extracts of
Rm. vannielii grown under different conditions

Enzyme	specific activity (nmol.min.mg protein ⁻¹)	
	photoheterotrophic	chemoheterotrophic
[Bacteriochlorophyll (nmol mg protein ⁻¹)]	8.4	2.8
NADH oxidase	0	63
NADH dehydrogenase	274	1044
Succinate dehydrogenase	5.0	10.5
Malate dehydrogenase	841	5592
Isocitrate dehydrogenase (NADP specific)	232	95
Isocitrate dehydrogenase (NAD specific)	22	73
2-oxoglutarate dehydrogenase	0	41.5

Cells were grown phototrophically at $35 \mu\text{Em}^{-2}\text{s}^{-1}$ light intensity and chemoheterotrophically in 1 L batches in 2.5 L shake flasks under aerobic-dark conditions. Cell free extracts were prepared by sonication (section 2.13). NADH oxidation was measured spectrophotometrically at 340 nm.

FIGURE 3.40. Oxygen uptake by whole cells of photoheterotrophically grown *Rm. vannielii*.

Cells were added at a protein concentration indicated by the arrows to 3 ml 25 mM phosphate buffer pH 7.0 contained in the oxygen electrode (section 2.16). The cells were initially incubated in the dark at 30°C then illuminated (on/off) by a 60 W tungsten bulb. The effect of electron transfer inhibitors was determined by direct addition of concentrated ethanolic solutions in volumes (10-20 μ l) at which no effect of ethanol alone could be detected.



isocitrate dehydrogenase was decreased. The enzyme 2-oxoglutarate dehydrogenase could only be detected in chemoheterotrophic cell-free extracts.

The observed differences in NADH oxidase activity in Rm. vannielii could in principle be explained by the absence of a respiratory electron transport system or a terminal oxidase under phototrophic growth conditions. However, this is unlikely in view of the readily detectable O₂ uptake catalyzed by dark incubated whole cells (Fig. 3.40). In addition, illumination of the cells inhibited further O₂ uptake and this inhibition was abolished by the uncoupler CCCP and the electron transport inhibitor antimycin A, indicating an interaction between the photosynthetic and respiratory electron transport chains as found with other photosynthetic bacteria (Keister, 1978).

3.3.3 Chemoheterotrophic to photoheterotrophic growth shift

As it was not possible to grow Rm. vannielii under conditions of aeration which resulted in both high cell yield and trace amounts of (or no) Bchl, the same type of growth shift to examine the de novo formation of the photosynthetic apparatus as has been done with other members of the Rhodospirillaceae could not be performed. Nevertheless, aerobic dark growth in 20 L volumes of PM under forced aeration gave cells with a specific Bchl content of 3-4 nmol (mg protein)⁻¹ and an adequate volume from which to take successive samples without altering cultural conditions. The results of an experiment involving transfer of such a culture to photoheterotrophic conditions is shown in Table 3.6.

TABLE 3.6. Effect of a chemoheterotrophic to photoheterotrophic growth shift on pigment content and composition in *Rh. vannielii*

Time (h) after shift to phototrophic conditions	Cellular Bchl content (nmol.mg ⁻¹ protein ⁻¹)	Absorbance ratios of cell-free extracts		
		800/595	870/595	870/800
-2	3.80	1.18	2.47	2.09
0	4.00	1.20	2.55	2.05
+2	7.05	1.45	2.90	2.00
+4	10.80	1.60	3.20	2.00
+6	12.60	1.68	3.32	1.98
+8	16.50	1.78	3.42	1.92
+10	18.40	1.89	3.69	1.95
+22	27.80	2.00	3.87	1.93

The shift was performed as described in section 2.5. Zero time corresponded to 96 h after inoculation. Cellular Bchl content was determined in acetone:methanol extracts and cell-free extracts were prepared by sonication.

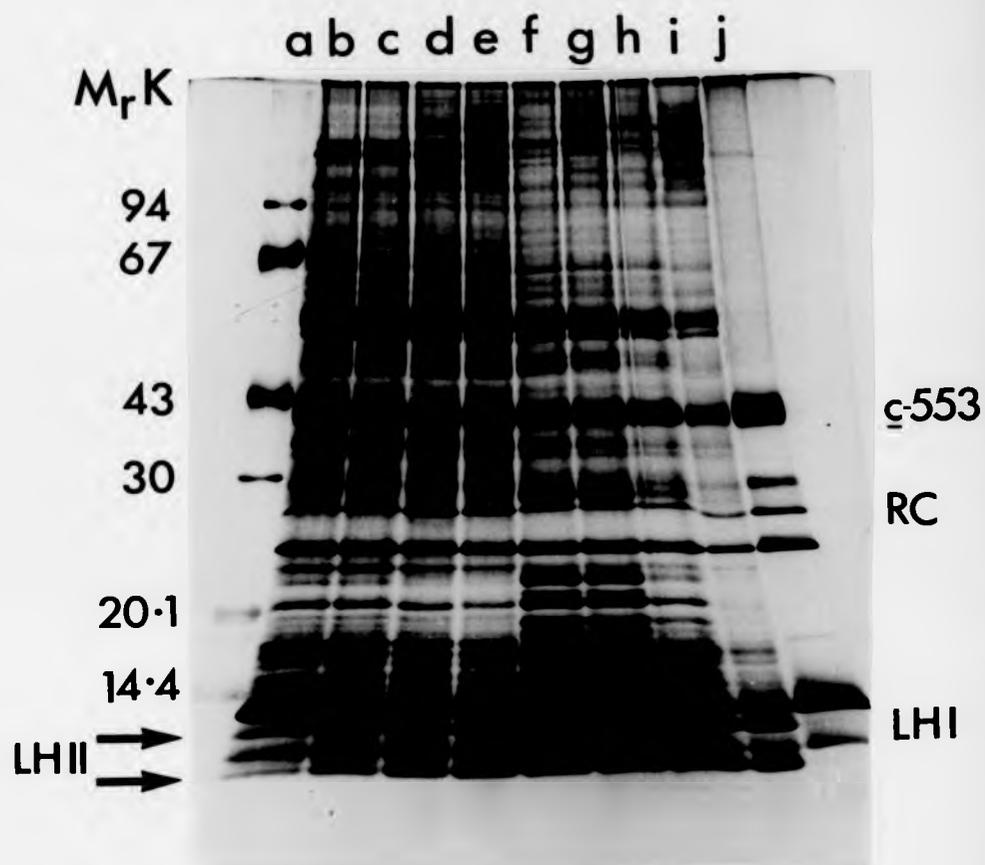


FIGURE 3.41. Intra-cytoplasmic membrane polypeptide profiles from *Rm. vanniellii* cultures undergoing a chemoheterotrophic to photoheterotrophic growth shift.

The conditions for the growth shift were as described in section 2.5. Samples (200 ml) were taken at intervals and ICM prepared on sucrose gradients (section 2.14). Fifty μg quantities of ICM protein were denatured (75°C for 2 min) in Laemmli sample buffer and electrophoresed on a 10-30% (w/v) gradient gel which was subsequently silver-stained. Track (a) contained molecular-weight markers and track (j) contained 20 μg protein of the isolated B885-RC complex to aid band identification. Samples were taken 2 h before the shift (b), at the same time as the shift (c) and at 2 h (d), 4 h (e), 6 h (f), 8 h (g), 10 h (h) and 22 h (i) subsequently.

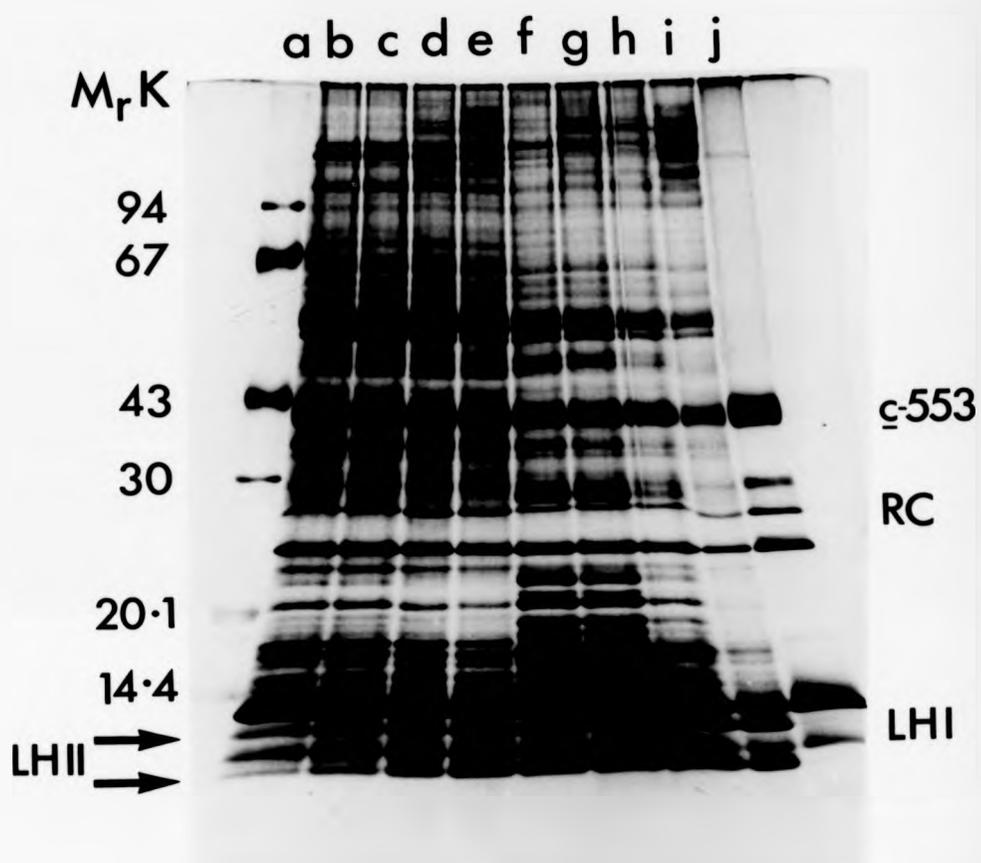


FIGURE 3.41. Intra-cytoplasmic membrane polypeptide profiles from *Rm. vannielii* cultures undergoing a chemoheterotrophic to photoheterotrophic growth shift.

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Increases in optical density and cellular protein after the shift were accompanied by an increase in Bchl accumulation but at a higher rate, so that the specific Bchl content increased from about 4 to 28 nmol (mg cellular protein)⁻¹ during the period of the experiment. Absorption spectra of cell-free extracts and measurements of absorbance ratios (Table 3.6) revealed increases in the relative amounts of B800 (largely attributable to the LHII complex) and "B870" (attributable to the LHII complex with some contribution from LHI Bchl) as a proportion of the total Bchl (measured as the 595 nm absorbance) in successive samples after the shift. However, the A870:A800 nm ratio remained largely constant. Intra-cytoplasmic membrane protein profiles on SDS-PAGE (Fig. 3.41) showed an increase in the relative abundance of at least one of the B880-865 LHII complex polypeptides (M_r 11,000) while those of the RC and B885 complexes remained more constant.

3.3.4 Chemoheterotrophic growth under anaerobic conditions in the dark

Attempts to cultivate Rm. vannielii under anaerobic dark conditions using either PM medium or PM mineral salts base supplemented with the "fermentable" carbon source fructose were unsuccessful. No growth was obtained after 3 weeks incubation in these media or in the fructose medium supplemented with either TMAO or DMSO as terminal electron acceptors. This suggests Rm. vannielii is unable to grow fermentatively or gain energy from anaerobic respiration using these substrates.

3.3.5 Conclusions from the growth studies

(1) Variation of pigment content with light intensity in Rm. vanniellii appeared largely due to changes in the relative amount of the B800-865 "accessory" LHII complex.

(2) These changes were of a different nature to those in Rp. palustris - an organism considered physiologically similar to Rm. vanniellii.

(3) Good growth under aerobic dark conditions was dependent upon a low O₂ transfer rate in batch culture. The cells remained slightly pigmented but showed an abnormal morphology. Oxygen uptake and enzyme studies indicated the presence of a respiratory system in Rm. vanniellii.

(4) NADH oxidase activity per se was unusual in being absent under phototrophic growth conditions.

(5) Anaerobic growth in the dark could not be demonstrated.

3.3.6 Discussion

The inverse relationship between the specific pigment content and incident light intensity in Rm. vanniellii agrees with data collected from a number of species of photosynthetic bacteria (Cohen-Bazire et al., 1957; Holt & Marr, 1965; Aagaard & Sistrom, 1972; Lien et al., 1973; Firsow & Drews, 1977) and with a previous study of the growth rate and ultrastructure of Rm. vanniellii as a function of light intensity by Trentini & Starr (1967). These workers also reported an inverse

variation in the extent of the lamellate ICM system with changes in light intensity, an observation subsequently confirmed by France (1978) for cells from the simplified cell-cycle. However, the absolute values of the specific pigment content determined in this study using isolated swarmer cells were always higher than those of the heterogeneous batch culture at the same light intensity. This was probably due to the fact that the prosthecae of the multicellular arrays do not contain an ICM system (Morita & Conti, 1963; Conti & Hirsch, 1965) thus giving a lower Bchl contribution on a cell protein basis. Clearly, the overall value obtained for a heterogeneous batch culture is merely an average which does not necessarily reflect the contributions of the constituent cell-types. The same could also apply to other photosynthetic prosthecae bacteria such as Rp. palustris and Rp. viridis as there is abundant electron microscopic evidence which illustrates a lack of ICM development in the prosthecae (Tauschel & Drews, 1967; Varga & Staehelin, 1983).

The variation of pigment content with light intensity in both Rm. vanniellii and Rp. palustris appeared to be largely due to changes in the relative concentration of the accessory LHII complex, as evidenced by absorption spectra, Triton-SDS and fully denaturing gel electrophoresis. This behaviour is consistent with the accepted role of the complex as the major, variable light-harvesting antenna in the Rhodospirillaceae (Lien *et al.*, 1973; Drews & Oelze, 1981). Nevertheless, whereas the B800 and B865 components of the complex were largely co-regulated in Rm. vanniellii, as evidenced by the rather narrow range of A870:800 ratios observed across the range of light intensities used, this was clearly not the case for the components of the Rp. palustris complex. The apparent independent regulation of the B800 band of the LHII complex of

Rp. palustris was also noted by Hayashi et al. (1982b) and is explicit in the several published absorption spectra of cells (or ICM therefrom) grown at different light intensities (Firsow & Drews, 1977; Hayashi et al., 1982a,b; Varga & Staehelin, 1983). This pattern is probably the result of an increase in the number of Bchl molecules specifically associated with the B800 polypeptide, thus decreasing the distance between them and resulting in stronger interactions or an increase in the B800 polypeptide-pigment concentration in toto (Hayashi et al., 1982b). The important point is that this cannot be explained by the model of the complex in Rb. sphaeroides or Rb. capsulatus (Cogdell & Thornber, 1980), where a stoichiometrically constant structure is envisaged. As a consequence, the ratio of the two IR peak heights of this type of LHII complex (Type I; Thornber et al., 1983) shows little variation as also seems to be the case in Rm. vannielli. The Rp. palustris type would therefore appear to be somewhat different to that in Rm. vannielli and Rb. sphaeroides. This is also supported by the observation of a CD signal from the 800 nm band in Rp. palustris (Hayashi et al., 1982b) indicating Bchl-Bchl interaction but no such signal from that of the Rb. sphaeroides complex (Clayton & Clayton, 1981) indicating it to be monomeric (Picorel et al., 1984) as is also likely to be the case with Rm. vannielli (section 3.2.2).

These considerations, combined with those in section 3.2, once again reinforce the view that considerable heterogeneity exists in the accessory light-harvesting complexes in the Rhodospirillaceae not only in structure and composition but also in regulatory patterns.

Variation of light intensity causes regulatory changes in the amount of the LHII complex in Rm. vannielli and not just a change in the extent of

ICM development, as concluded by France (1978). However, growth at very low light intensities was also accompanied by an increase in the amount of the B885-RC complex which contrasts with the behaviour of both Rb. sphaeroides (Aagaard & Siström, 1972; Takemoto & Huang-Kao, 1977) and Rp. palustris (Firsow & Drews, 1977; Varga & Staehelin, 1983).

Notwithstanding, in consideration of the work of Varga & Staehelin (1985b) it seems possible that this may be a consequence of the role of the B885 LHI complex in the stacking of the lamellate ICM system. Alternatively there may be a finite limit to the number of LHII complexes that can be added to the LHI-RC core in this microbe before energy transfer efficiency decreases and additional RCs are required.

When first isolated, Rm. vannieli was considered to be a strict photoanaerobe (Duchow & Douglas, 1949; Conti & Hirsch, 1965; Trentini & Starr, 1967), although Pfennig (1970) demonstrated microaerophilic growth in the dark in agar deeps. Aerobic dark growth was subsequently confirmed by Dow (1974) and France (1978). Nevertheless, the poor growth under aerobiosis and the abnormal morphology suggest a sensitivity to oxygen that is similar to that of the "brown" species of *Rhodospirillum* (Pfennig, 1978) but dissimilar to most other members of the *Rhodospirillaceae*.

In this study relatively good growth was obtained under limited aeration in liquid cultures, i.e. tantamount to microaerophilic conditions. Within such cultures, the cells were pigmented, as also found with the "brown" *Rhodospirilla* (Pfennig, 1978). The latter group of species (Rs. fulvum, Rs. molischianum and Rs. photometricum) have been reported to lack an active oxidative electron transport system and to respire only under microaerophilic conditions in the dark using a slightly modified

electron transport system (Pfennig, 1978). In contrast, Rm. vannielii cells grown under phototrophic conditions exhibited an endogenous O₂ uptake which suggested the existence of a respiratory system under photosynthetic growth conditions as is characteristic of most members of the Rhodospirillaceae. Light inhibition of respiration and its abolition by CCCP and antimycin A is also suggestive of the same kind of control mechanisms involved in the interaction between photosynthetic and respiratory electron flow as found in the majority of photosynthetic bacteria (Keister, 1978; Cotton et al., 1983). Additional evidence for an oxidative respiratory system in Rm. vannielii was obtained from the increase in respiratory dehydrogenase activities and the appearance of 2-oxoglutarate dehydrogenase activity under aerobic conditions, indicating the operation of a complete tricarboxylic acid cycle. Under photosynthetic, anaerobic conditions, however, the cycle is incomplete because of the lack of 2-oxoglutarate dehydrogenase (Morgan et al., 1985).

One unusual feature is the NADH oxidase activity, which was not detectable as such in phototrophic cell-free extracts but appeared largely in the soluble fraction under chemoheterotrophic growth conditions. The rate of oxidation of NADH is often taken as a measure of the activity of the respiratory chain and is usually measurable in phototrophically grown cells of photosynthetic bacteria because of the constitutivity of respiratory electron transport. The lack of activity in Rm. vannielii is unlikely to result from a block to NADH utilization by such an electron transport chain however, because an active NADH dehydrogenase was detectable under phototrophic conditions. The position of the block must be between this enzyme and the terminal oxidase(s) but until a more detailed analysis of the composition of the

respiratory chain is available the precise nature of this phenomenon remains obscure. It does however, suggest that some portion of the respiratory chain may be absent under phototrophic growth conditions.

The available data would suggest that the rather weak aerobic growth of Rm. vannielii is not the result of the lack of respiratory capacity as suggested by Pfennig (1978) for the brown Rhodospirilla and Rd. viridis, but rather to a more direct effect of O₂ itself on growth and/or cell-division, the latter evidenced by the consistently abnormal morphology observed in aerated cultures. As in other members of the Rhodospirillaceae, high O₂ tensions reduced the specific Bchl content of the cells with the B800-865 LHII complex being more sensitive and the RC and B885 complexes being retained in the membrane even at the highest O₂ transfer rates in batch culture. The estimate of a photosynthetic unit size of ca. 20 under such conditions is consistent with the report of Aagaard & Siström (1972) for Rb. sphaeroides and the data from a shift to photoheterotrophic conditions would indicate LHII complexes are added to this core during adaptation.

In these respects, the regulation of the composition of the photosynthetic apparatus in Rm. vannielii by O₂ and light is not atypical but compared with many species this microbe has rather limited regulatory capacities to enable good growth under a wider variety of conditions than those conducive to photosynthesis alone. This is also demonstrated by the lack of anaerobic dark growth - an alternative open to several other photosynthetic bacteria (Uffen & Wolfe, 1970).

3.4 The role of the intra-cytoplasmic membrane system and cell envelope in the differentiation of Rhodospirillum rubrum swarmer cells

The studies described thus far were performed with batch cultures in which cells at all stages of the cell-cycle were present. With the exception of the cyanobacteria, *Rm. vannielii* is unique amongst photosynthetic prokaryotes in displaying an extreme degree of cell-type specialization and it was considered important to determine the possible distinctions between the cell-types, in particular with regard to their photosynthetic apparatus and membrane physiology. This analysis was also extended to the swarmer cell-cycle itself, not least because of the possibilities afforded by the use of a selection synchronization method, but in addition because many of the overt morphogenetic events (section 1.5) must involve changes in the status of the cell-envelope. Clearly such changes could encompass not only the ICM system but also the cell wall and outer membrane/cell surface domains. Therefore some aspects of these systems and their involvement in differentiation were also investigated.

3.4.1 Cell-type and cell-cycle specific synthesis of intra-cytoplasmic membrane components

3.4.1.1 Results. The patterns of ICM proteins synthesised by the different cell-types of *Rm. vannielii* were evaluated by pulse-labelling techniques using ^{35}S -methionine. With the labelling procedure used, incorporation of the amino acid into whole cell material of a

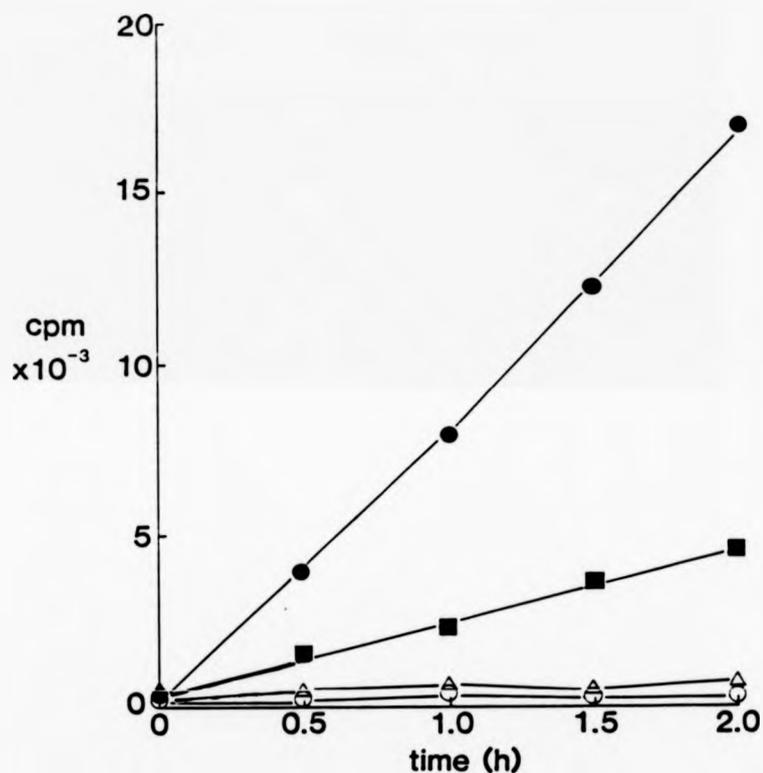


FIGURE 3.42. Incorporation of ^{35}S -methionine into cell protein of *Rh. vannielii*.

After the addition of $1 \mu\text{Ci ml}^{-1}$ ^{35}S -methionine to cells growing in PM medium (●—●), PM + 1 mM L-methionine (○—○), PM + $50 \mu\text{g ml}^{-1}$ chloramphenicol (△—△) or PM + 0.1% (w/v) yeast extract (■—■), samples (0.5 ml) were taken and added to an equal volume of 10% (w/v) trichloroacetic acid. Precipitates were collected and scintillation counted as described in section 2.12. The chloramphenicol and L-methionine only were added immediately before the isotope. Incubation was at 30°C under phototrophic conditions.

heterogeneous batch culture in PM medium remained linear well beyond the 20 min period after which labelling was normally terminated (Fig. 3.42). The addition of 1 mM unlabelled L-methionine or 50 $\mu\text{g}/\text{ml}$ chloramphenicol completely prevented incorporation. Low incorporation was also observed in PMYE grown cells. Similar results were also obtained by Porter (1985) for synchronized swarmer cells. Therefore this labelling regime was deemed satisfactory for the examination of differential protein synthesis.

Photoheterotrophic batch cultures grown to the late exponential phase were pulse-labelled under the same cultural conditions and then fractionated into swarmer cells and multicellular arrays. Electron microscopic analysis of such preparations (Fig. 3.43) revealed an adequate separation when compared to a heterogeneous batch culture and this was confirmed using cell-volume distribution analysis (Fig. 3.44). With this technique, asynchronous, heterogeneous batch cultures of Rm. yannielii displayed a peak with a modal cell volume of 0.5-0.6 μm^3 corresponding to swarmer cells and a less pronounced shoulder at 1.1-1.2 μm^3 due to cell "pairs". Further small peaks of a greater cell volume - attributable to the arrays - were also resolved at lower amplifications. The corresponding profiles for the multicellular array fraction indicated a considerable enhancement of the 1.1-1.2 μm^3 peak and the appearance of further peaks at higher cell volumes due to chains of several cells and arrays. The swarmer cell profile was characterized by a single peak with a modal cell volume of 0.5-0.6 μm^3 . Despite the indication of the presence of swarmer cells in the array fraction as judged from the coulter profile (Fig. 3.44), virtually none could be detected by phase-contrast or electron microscopy (Fig. 3.43).

FIGURE 3.43. Electron micrographs of (a) *Rm. vannielii* swarmer cells, stalked cells and multicellular arrays from a heterogeneous batch culture, (b) multicellular arrays prepared from batch cultures by differential centrifugation and (c) swarmer cells prepared by column filtration. Cells were negatively stained with 1% (w/v) phosphotungstic acid.

Bar markers represent 5 μm .

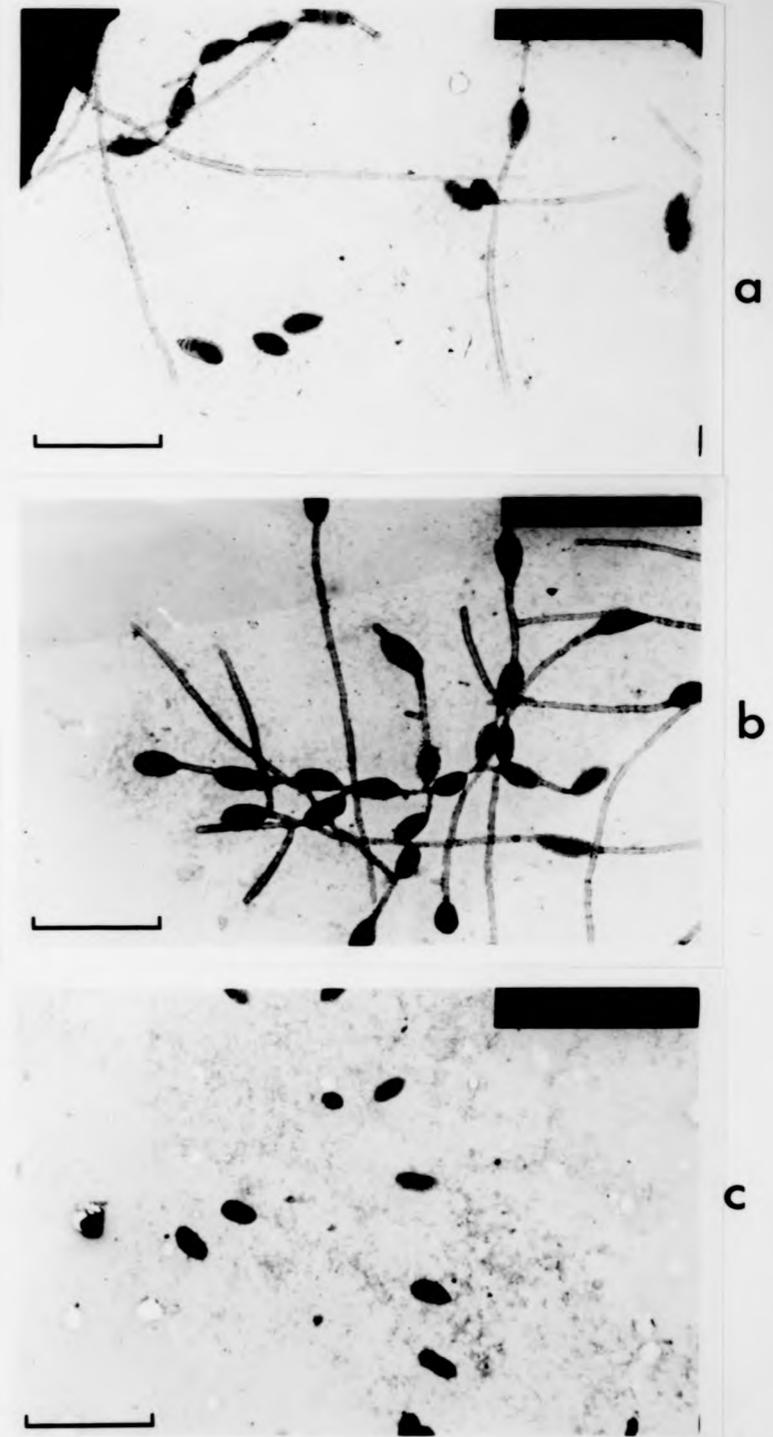


FIGURE 3.43. Electron micrographs of (a) *Rm. vannielii* swarmer cells, stalked cells and multicellular arrays from a heterogeneous batch culture, (b) multicellular arrays prepared from batch cultures by differential centrifugation and (c) swarmer cells prepared by column filtration. Cells were negatively stained with 1% (w/v) phosphotungstic acid.

Bar markers represent 5 μm .

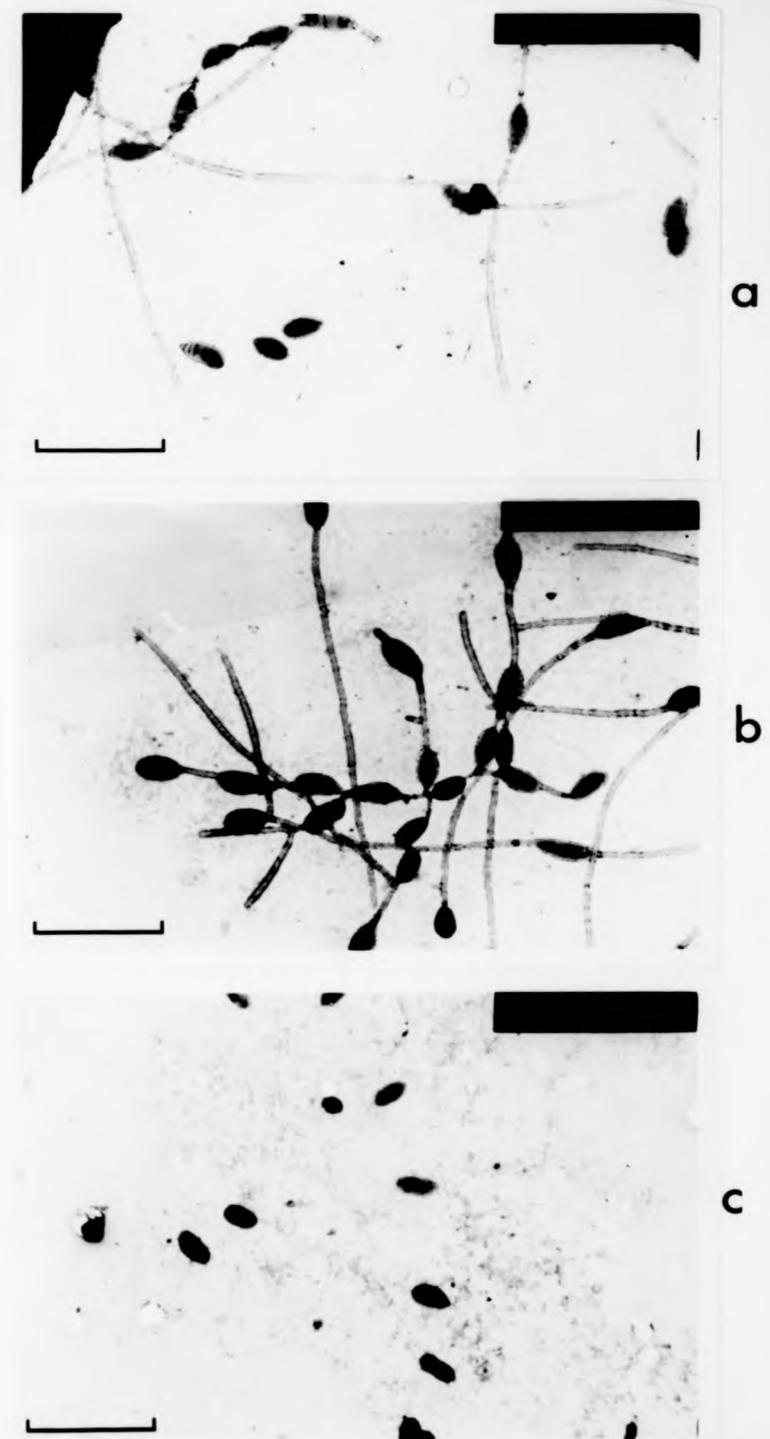


FIGURE 3.44. Cell volume distribution analysis of a *Rm. vannielii* batch culture (a & d), swarmer cells obtained by column filtration (b & e) and multicellular arrays and stalked cells obtained by differential centrifugation (c & f). In (a-c) the amplification on the ZBI coulter counter was set to 0.5, giving a scale of cell volumes from 0.3-2.3 μm^3 while in (d-f) the amplification was 1.0.

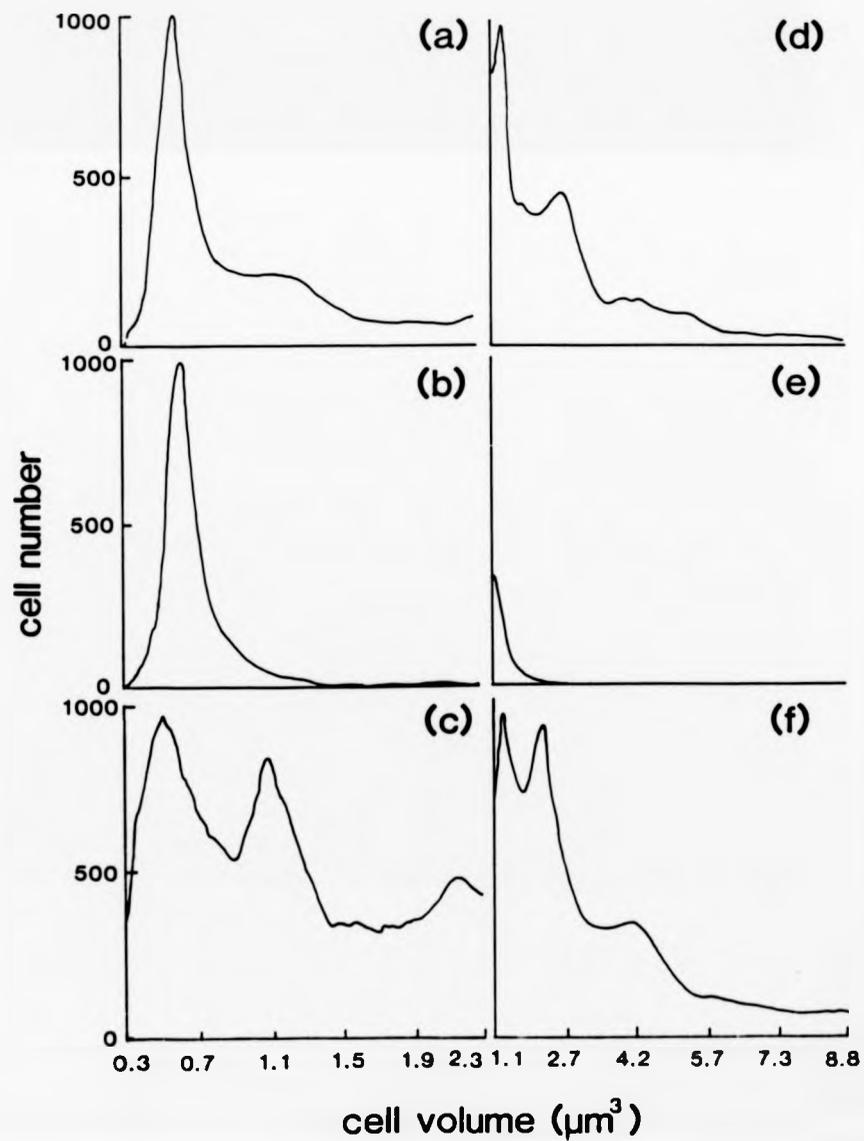


TABLE 3.7. Incorporation of ^{35}S -methionine during pulse labelling into sub-cellular fractions of *Rm. vannielii* cell-types

Cell type	dpm ^{35}S -met. incorporated per mg of;	
	soluble protein	intra-cytoplasmic membrane protein
Heterogeneous (arrays + swarmer cells)	8.7×10^5	5.4×10^5
Multicellular arrays	9.3×10^5	7.0×10^5
Swarmer cells	1.1×10^5	8.0×10^4

Cells were pulse-labelled for 20 min with $1 \mu\text{Ci ml}^{-1} \text{ } ^{35}\text{S}$ -L-methionine, labelling stopped by the addition of unlabelled methionine to 1 mM, the swarmer cells separated by column filtration and the arrays by differential centrifugation. Cell-free extracts were prepared by sonication and soluble and ICM protein separated by ultracentrifugation (10,000 x g, 4°C , 2½ h) on 25% sucrose cushions.

The distribution of ^{35}S -methionine in the soluble and ICM fractions of these pulse-labelled cell-types was not identical. On a unit protein basis, swarmer cells incorporated far less radiolabel into sub-cellular fractions than did the multicellular arrays (Table 3.7) while intermediate values for the whole batch culture - containing all cell types - were obtained.

The pattern of intra-cytoplasmic membrane proteins synthesised by these cell-types during the pulse is illustrated in Fig. 3.45(a) on a gel loaded equally with respect to both protein and radiolabel. Coomassie blue staining revealed relatively few cell-type specific proteins apart from a M_r 34,000 band which only stained prominently in the swarmer cell tracks. However, fluorography of the same gel (Fig. 3.45b) revealed qualitative differences in a number of proteins. A group of at least four polypeptides of M_r 70-85,000 and a 45,000 M_r species were specifically synthesised in the multicellular arrays while swarmer cells synthesised proteins of 68, 65, 38 and 27,000 M_r which were not detectable in the multicellular arrays. The labelling intensity of the prominent 34,000 M_r protein appeared to be much greater in the swarmer cells but it was also detectable in the other cell-types. Overall, the labelling patterns of the heterogeneous culture and arrays were very similar, reflecting the numerical dominance of the latter in batch cultures.

A comparison of the stained gel and labelling pattern clearly showed some of the "specific" proteins to be present in all cell-types but they were clearly not synthesised during the pulse - as for example the group of "array specific" proteins of M_r 70-85,000.

The identity of many of these proteins is unknown. Those of the

FIGURE 3.45. Cell-type specific intra-cytoplasmic membrane protein synthesis in *Rm. vannielii*.

A photoheterotrophically grown batch culture was pulse-labelled (section 2.11) with ^{35}S -methionine and then separated into its constituent cell-types (sections 2.8 & 2.9). Intra-cytoplasmic membranes were prepared from the whole culture, containing all cell types (tracks 2 & 5), from the multicellular array containing fraction (tracks 3 & 6) and from the swarmer cell fraction (tracks 4 & 7) and loaded equally on the gel (10-30% w/v gradient gel) with respect to protein (120 μg ; tracks 2-4) or radioactivity (60,000 cpm; tracks 5-7). Tracks 1 & 8 contain molecular weight markers. (a) shows the coomassie blue stained gel and (b) shows a fluorogram of the same gel after 2 weeks exposure.

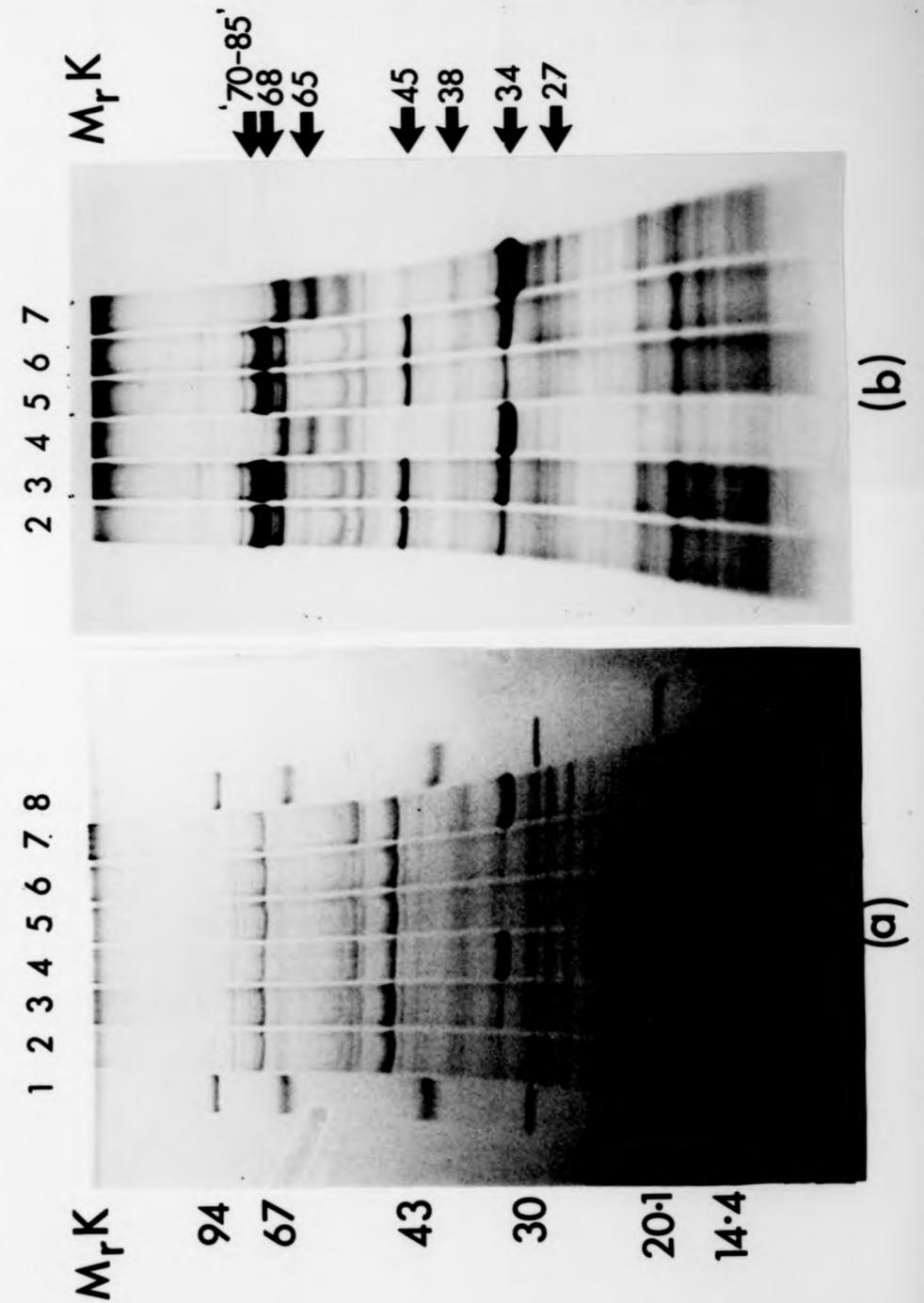


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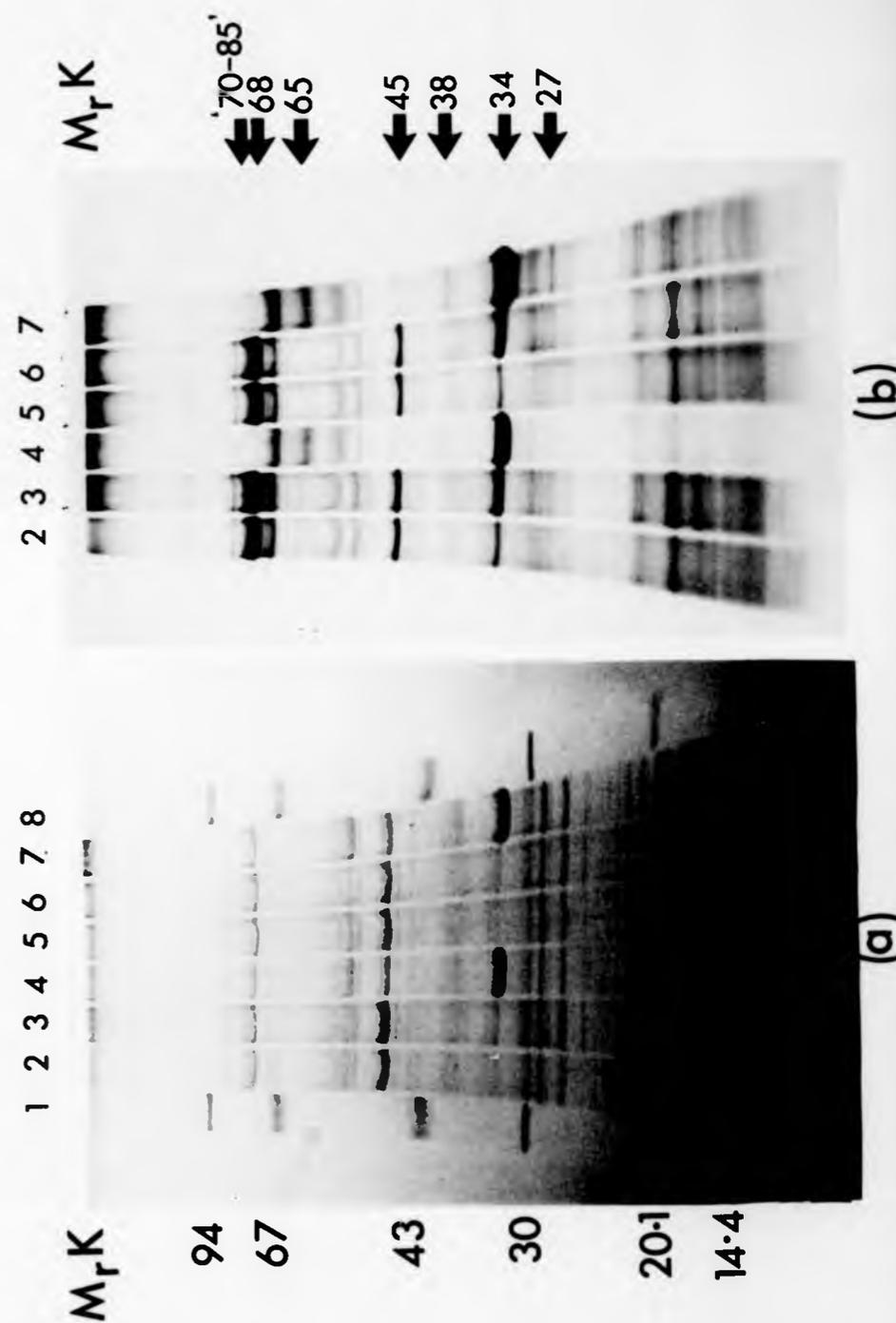


FIGURE 3.46. Detection of flagellin in the intra-cytoplasmic membranes of *Rm. vannielii* by immunoblotting.

- Track (a) purified flagella (20 μ g protein)
 Track (b) ICM from photoheterotrophically grown cells prepared on 25% (w/v) sucrose cushions (section 2.14; 50 μ g protein).
 Track (c) ICM from photoheterotrophically grown cells prepared on 20-60% (w/v) sucrose gradients (40 μ g protein).
 Track (d) ICM from chemoheterotrophically grown cells prepared on 20-60% (w/v) sucrose gradients (40 μ g protein).

The samples were denatured (75°C for 2 min in Laemmli sample buffer) and run on a 10-30% (w/v) gradient gel which was subsequently blotted onto nitrocellulose (section 2.24.2). The blot was probed with antibodies raised to detergent solubilized ICM from photoheterotrophically grown cells (section 2.22) and the antibody binding sites revealed by incubation with 125 I-labelled protein A (section 2.24.2). The figure shows the resulting autoradiograph (3-day exposure).

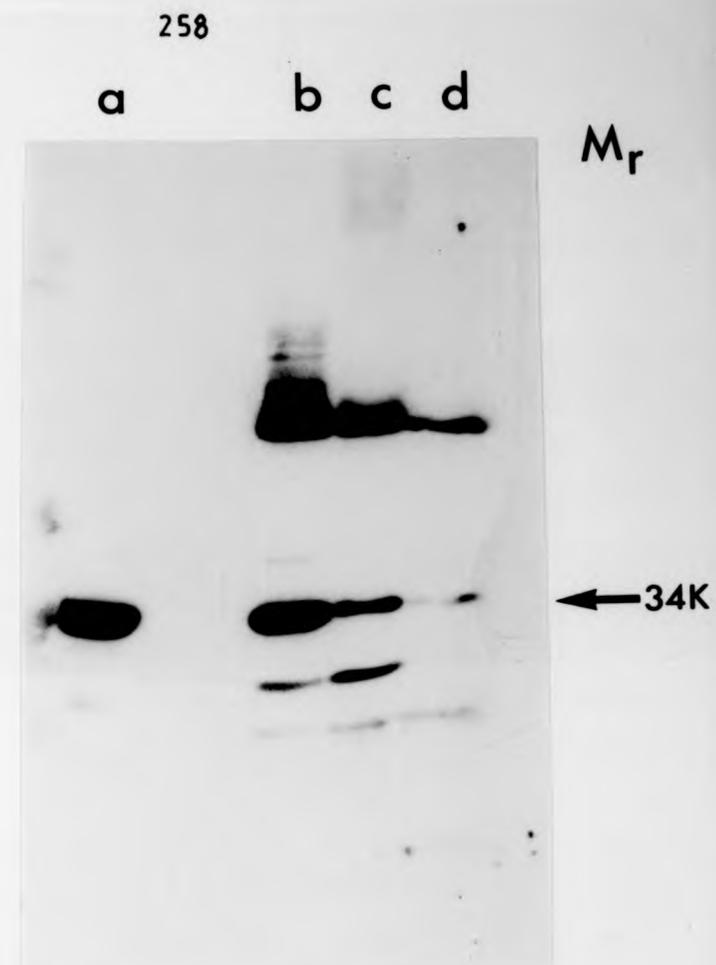
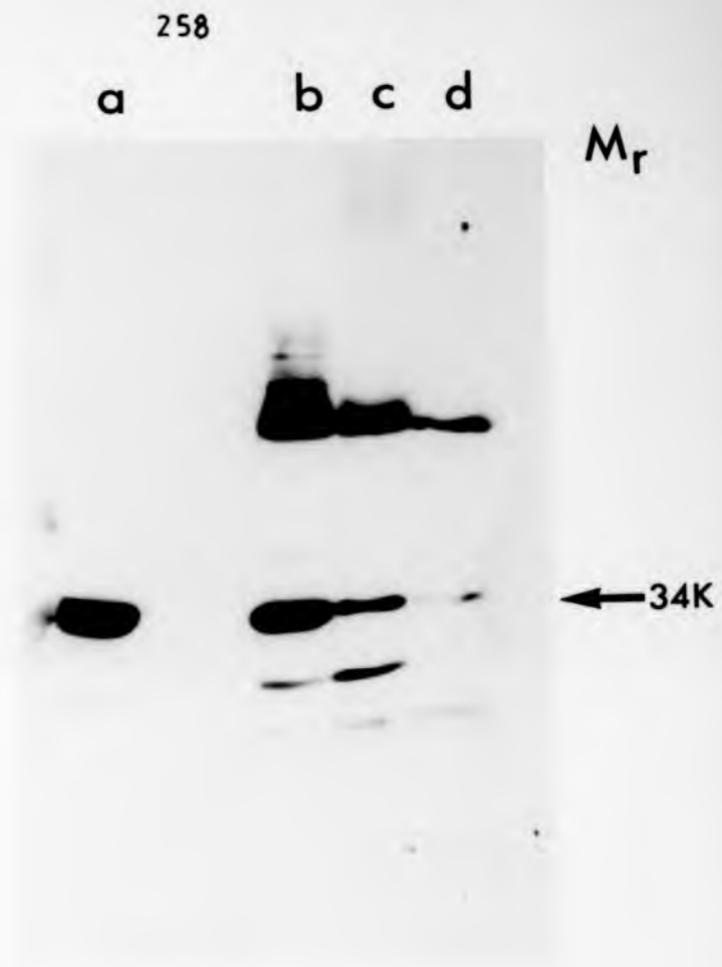


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photochemical reaction centre and light-harvesting complexes were only poorly labelled, a feature consistently noted in a number of separate experiments. There did not appear to be a significant difference in the synthesis of these proteins between cell-types in batch cultures. A possible candidate for the 34,000 M_r protein which was strongly labelled in swarmer cells could be the flagellin monomer. Purified flagella from *Rm. vannielii* show one major band of M_r 34,000 upon SDS gel electrophoresis (McDonald & Dow, unpublished) and it seemed possible that a membrane bound form of this monomer could be present. This was tested in immunoblotting experiments using antibodies raised against detergent solubilized ICM proteins (Fig. 3.46). The probe clearly recognised both the 34,000 M_r flagellin monomer and the corresponding protein in the ICM. Flagellin was present in ICM prepared by either sucrose-cushion or sucrose-gradient methods and in ICM from chemoheterotrophically grown cells. The number of other ICM proteins apparently immunogenic enough to elicit an antibody response was rather low.

In order to gain information regarding the type of membrane associated events taking place during swarmer cell differentiation, pulse-labelling was performed at intervals using homogeneous populations of synchronized swarmers under continuous illumination. An assessment of the degree of synchrony of differentiating swarmer cell populations was obtained by evaluation of the characteristic changes in the cell volume distribution profiles. Acceptable profiles were those in which the initial swarmer cell peak remained symmetrical but continuously increased in modal cell volume, particularly over the period of daughter cell formation. Profiles which became asymmetric indicated a non-differentiating sub-population, and this heterogeneity could often be observed by microscopy.

Asynchronous differentiation always occurred if illumination was insufficient (and/or if the initial swarmer cell density was too high) or if the initial gassing with O₂ free N₂ removed too much dissolved CO₂ (France, 1978; C. S. Dow, personal communication).

The changes in peak cell volume, culture turbidity, protein and pigment content are shown for a representative experiment in Fig. 3.47. Increases in optical density and whole cell protein levels occurred from time 0 without a lag and the first observable morphological landmark event was the loss of motility due to the shedding of flagella, which occupied the period from about 1-2 h after illumination. Prostheca synthesis occupied the period from 2-3.5 h and was associated with a plateau in the turbidity but not protein curve, indicating a change in the light-scattering properties of the cells. Daughter cell synthesis commenced at 3-3.5 h followed by rapid growth and enlargement, reflected in the large increase in modal cell volume from this time to the end of the experiment. No change was detected in the culture Bchl or carotenoid concentration under these conditions but the continuously increasing protein content meant that a gradual decrease in the cellular specific pigment content was observed; over a 6 h period, the specific Bchl content decreased from an initial value of 34 nmol (mg protein)⁻¹ to about 15 nmol (mg protein)⁻¹. The change in carotenoid content paralleled that of Bchl.

In Fig. 3.48, the changes associated with the ICM system are shown for a different synchronized population. However, the timing of the morphological events of the cell-cycle was quite reproducible from one experiment to another. The ICM specific pigment content showed a decline up to about 3 h after the initiation of differentiation, but

FIGURE 3.47. Changes in cell volume, optical density and cellular pigment and protein content in differentiating *Rm. vannielii* swarmer cells.

A 5 L batch culture was synchronized (section 2.8) and the swarmer cells incubated under photoheterotrophic conditions. Differentiation in 200 ml samples removed at half-hourly intervals was monitored by microscopy, cell volume distribution analysis (●—●) and optical density changes (■—■). The culture protein (○—○), bacteriochlorophyll (◆—◆) and carotenoid (◇—◇) concentrations were also determined and the specific bacteriochlorophyll (▲—▲) and carotenoid content (△—△) of the cells calculated from these.

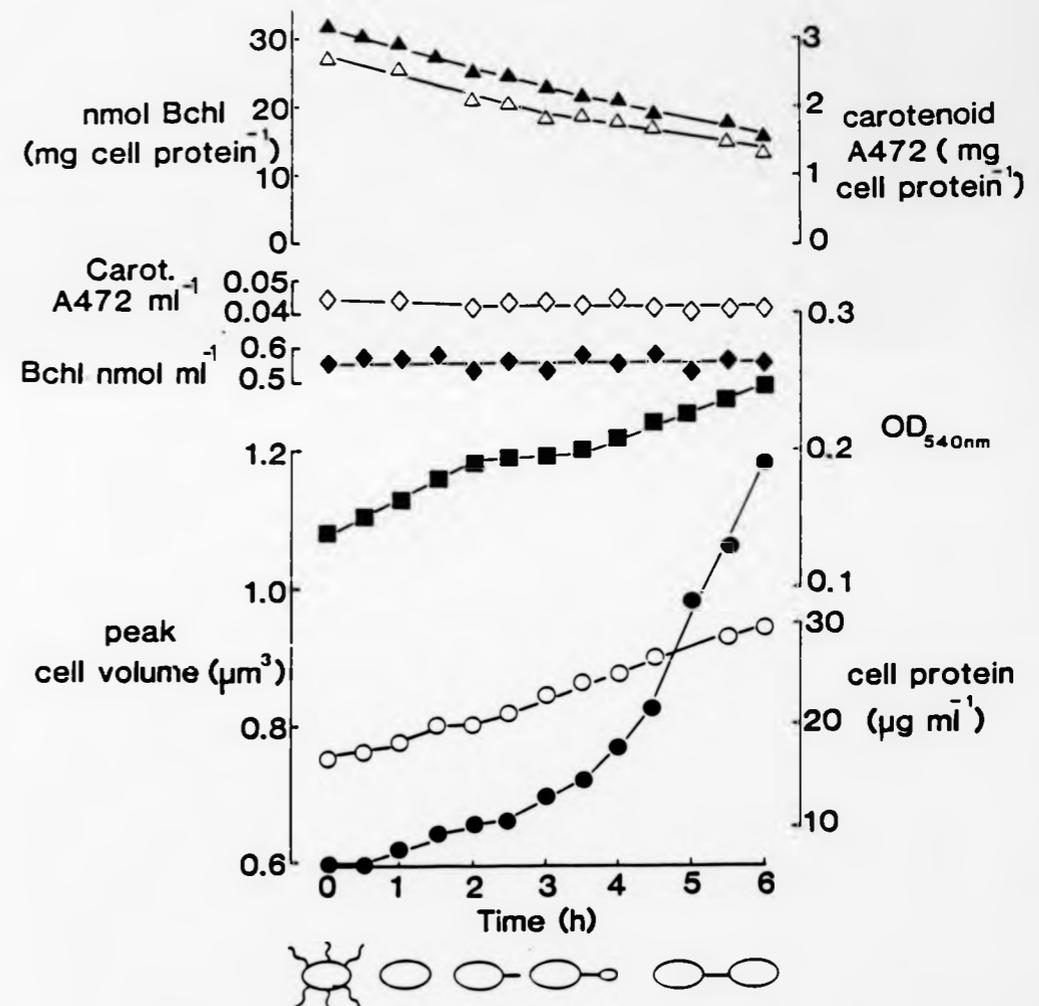
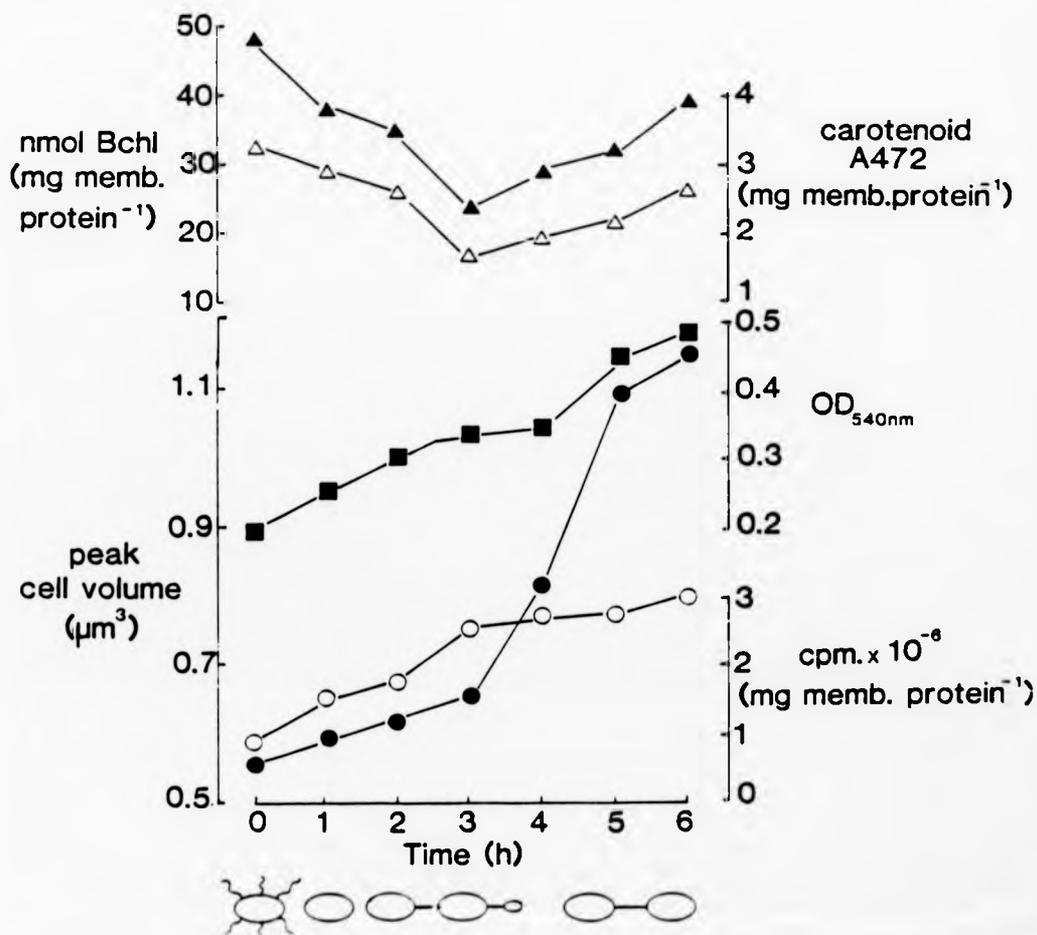


FIGURE 3.48. Changes associated with the intra-cytoplasmic membrane system during the differentiation of *Rm. vannielii* swarmer cells. Cell volume (●—●) and culture optical density (■—■) was determined as described in the legend to Fig. 3.47. At hourly intervals 100 ml samples were removed and pulse-labelled with ^{35}S -methionine (section 2.11), the ICM isolated on sucrose cushions as described in section 2.14 and the amount of radiolabel incorporated into the ICM fraction during the pulse (○—○), measured as described in section 2.12. The ICM specific bacteriochlorophyll (▲—▲) and carotenoid (△—△) content was determined by extraction into acetone:methanol (section 2.34).



thereafter an increase was observed over the period of daughter cell formation. The amount of ^{35}S -methionine incorporated per unit membrane protein was low in swarmer cells but increased in successively labelled samples during differentiation.

Swarmer cells maintained for 6 h in the dark remained motile, did not increase in biomass as evidenced by cell-volume, protein and turbidity measurements and showed little change in specific pigment content compared to time 0 cells.

The ICM proteins synthesised during pulse-labelling were analyzed on denaturing polyacrylamide gels equalized with respect to both protein and radioactivity. Those loaded with equal protein were silver stained (Fig. 3.49). The relative abundance of the majority of the 60-70 detected polypeptides showed no variation during the course of differentiation. The polypeptides of the photochemical reaction centre (M_r 26,000, 28,000 and 31,000), the cytochrome c -553 (M_r 38,000) and those of the light-harvesting complexes (11-14,000) were present in all cell-types. There was some evidence of an increase in the relative abundance of the 14,000 M_r B885 polypeptide at 6-8 h.

Analysis of silver-stained gels can give little information about changes in the period or rate of synthesis of particular protein species. Therefore fluorographs of gels loaded equally with respect to radiolabel were prepared on which the band intensity is proportional to the average rate of synthesis of the protein (Fig. 3.50). Such fluorographs revealed several ICM proteins whose rate of synthesis increased or decreased during swarmer cell differentiation. Particularly interesting was the behaviour of the 34,000 M_r protein

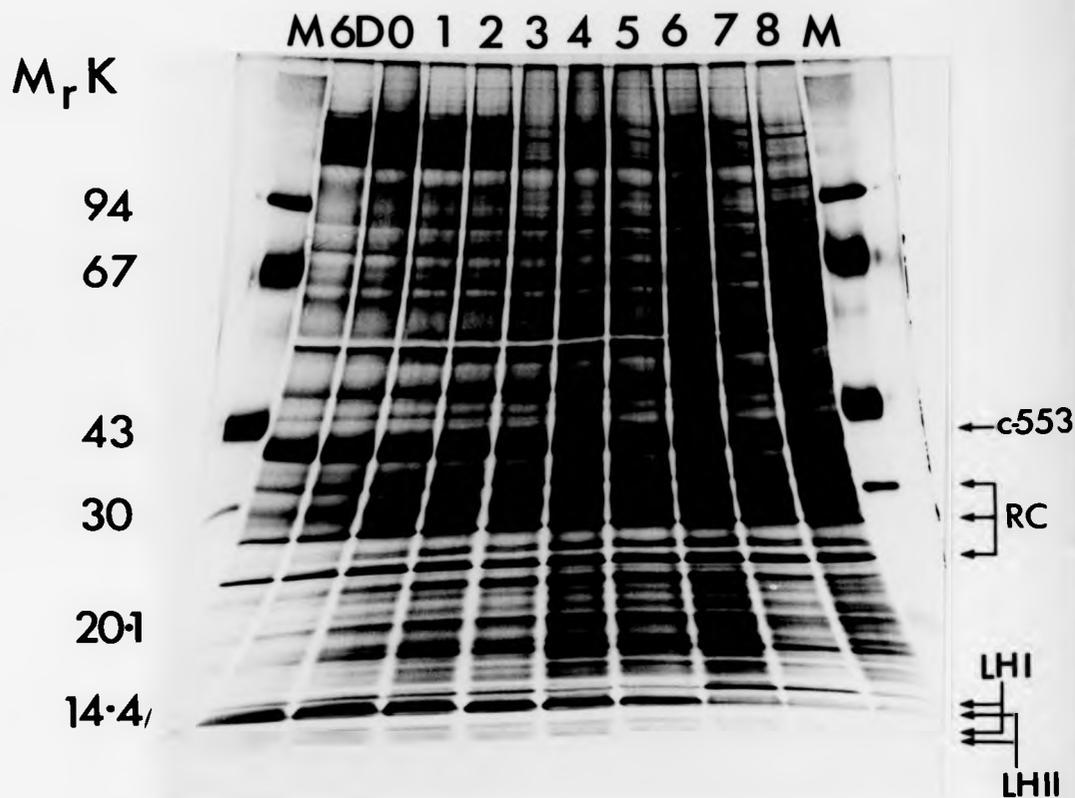


FIGURE 3.49. SDS-PAGE of ICM from differentiating swarmer cells of *Rm. vannielii*

A 5 L culture of synchronized swarmer cells was incubated under phototrophic conditions and samples taken every hour from 0-8 h. A 500 ml aliquot was also maintained under anaerobic conditions in darkness for 6 h (6D). The samples (200 ml) were used to prepare ICM on sucrose cushions (section 2.14) of which 50 μ g aliquots of protein were denatured in Laemmli sample buffer (75°C, 2 min) and applied to a 10-30% (w/v) polyacrylamide gradient gel which was subsequently silver-stained. The tracks labelled M contained molecular weight markers.

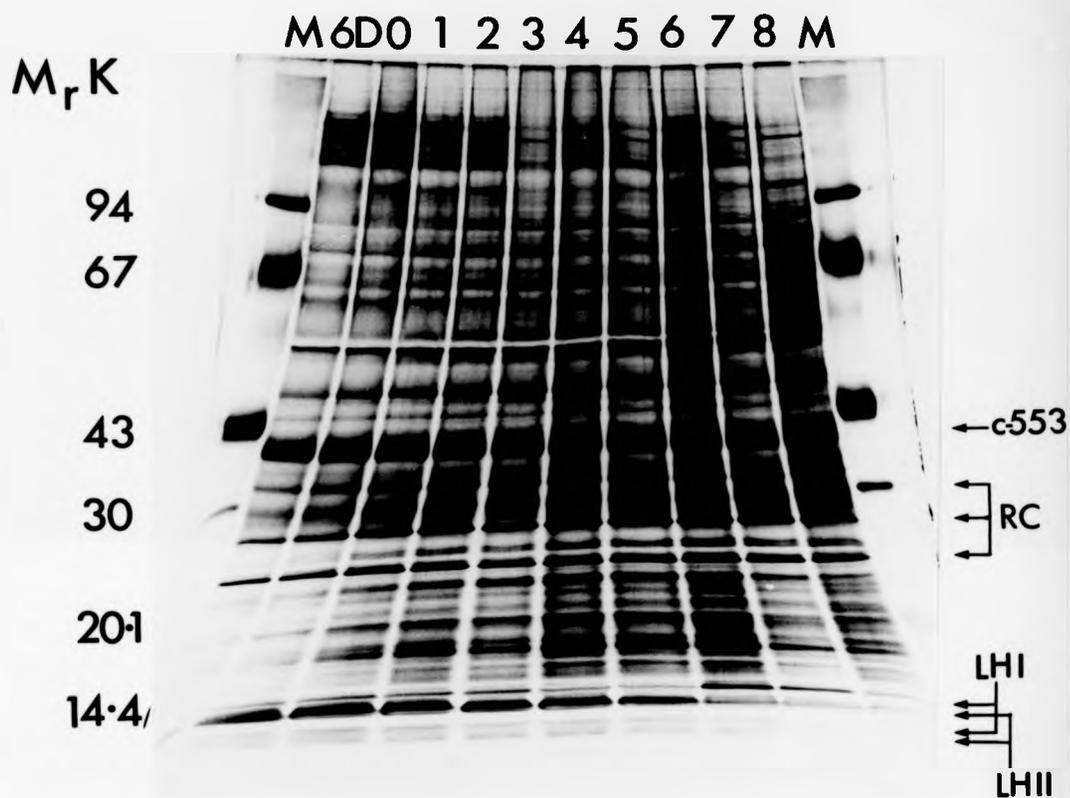


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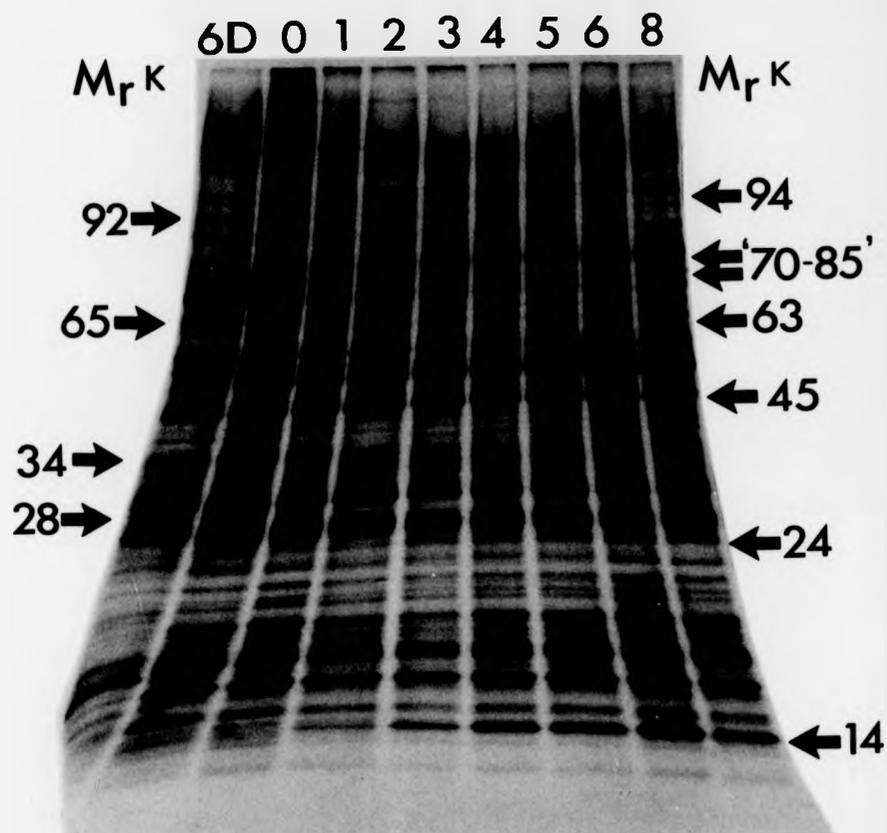


FIGURE 3.50. Intra-cytoplasmic membrane proteins synthesised by differentiating swarmer cell populations.

One-hundred ml aliquots of the same swarmer cell culture used in Fig. 3.49 were pulse-labelled at 1 h intervals for 20 min using $1\mu\text{Ci ml}^{-1}$ ^{35}S -methionine (section 2.11). Intra-cytoplasmic membranes were prepared on sucrose cushions (section 2.14) and equal quantities of radioactivity (40,000 cpm) were applied to a 10-30% (w/v) gradient gel which was subsequently subjected to fluorography (section 2.21). The figure shows the resulting fluorogram (2 weeks exposure). The designation 6D refers to cells pulse-labelled after 6 h incubation under dark anaerobic conditions.

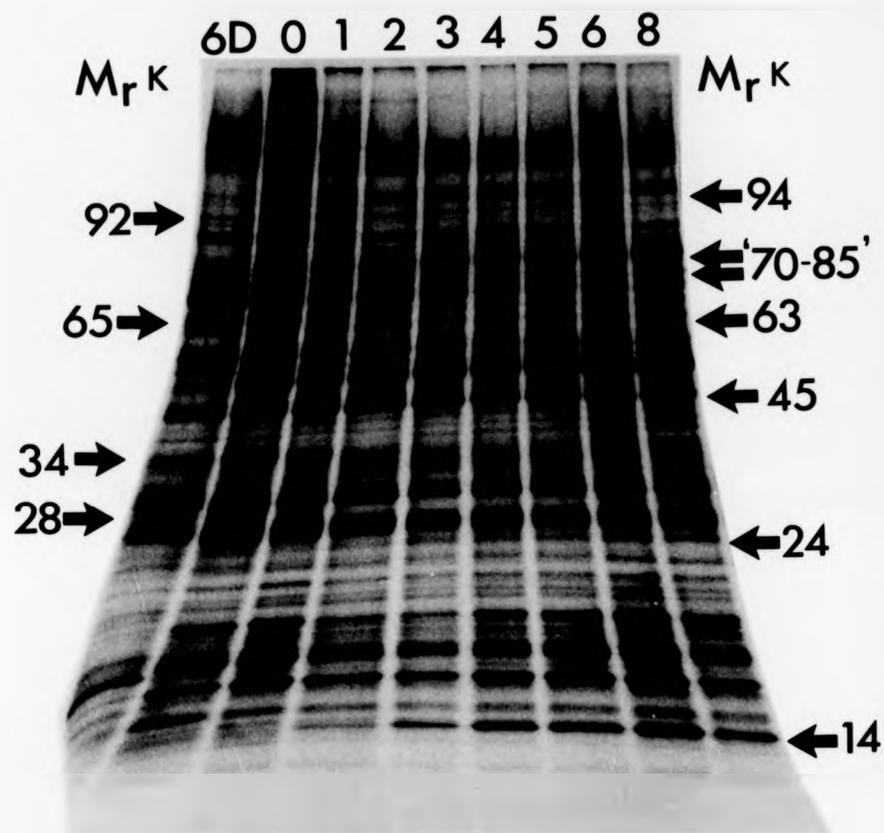
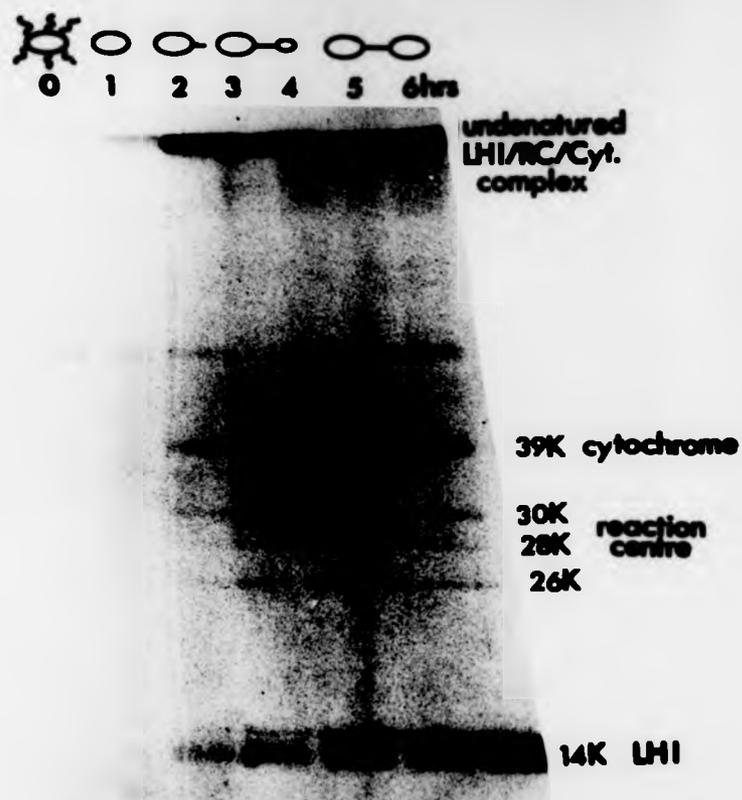


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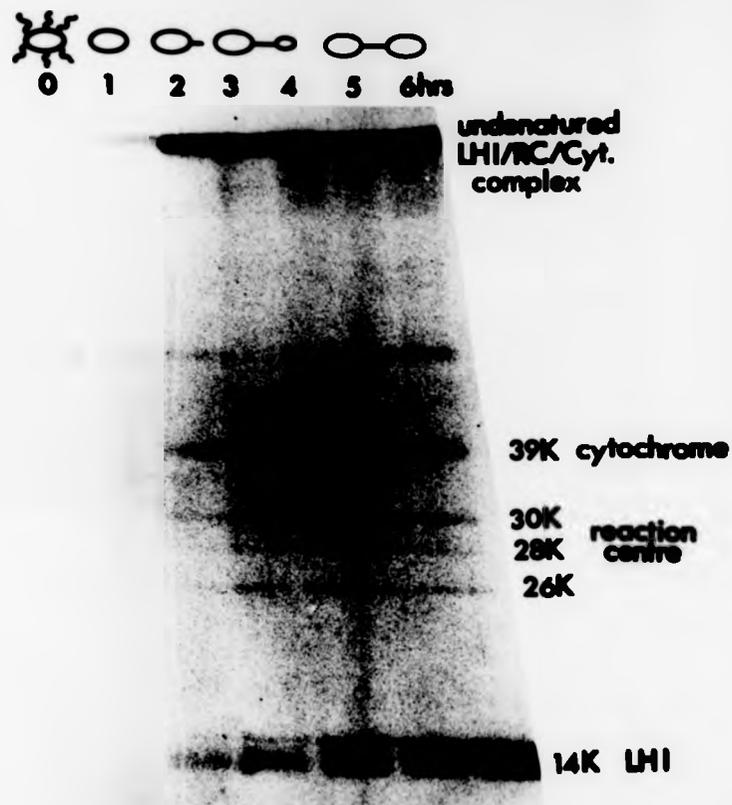
One-hundred ml aliquots of the same swarmer cell culture used in Fig. 3.49 were pulse-labelled at 1 h intervals for 20 min using 1μ Ci ml⁻¹ ³⁵S-methionine (section 2.11). Intra-cytoplasmic membranes were prepared on sucrose cushions (section 2.14) and equal quantities of radioactivity (40,000 cpm) were applied to a 10-30% (w/v) gradient gel which was subsequently subjected to fluorography (section 2.21). The figure shows the resulting fluorogram (2 weeks exposure). The designation 6D refers to cells pulse-labelled after 6 h incubation under dark anaerobic conditions.

FIGURE 3.51. Incorporation of label into the B885-RC complex during swarmer cell differentiation.



Synchronized swarmer cells were pulse-labelled as described in the legend to Figure 3.50 and intracytoplasmic membranes prepared from them (section 2.14) were solubilized with 0.8% (w/v) each of SDS and Triton x-100 in 68 mM Tris-HCl pH 6.8 (section 2.27.2). Equal quantities of radioactivity (82,000 cpm) of the solubilized membranes were then applied to a 10% (w/v) polyacrylamide gel containing both SDS and Triton X-100 and run at 4-10°C (section 2.17.3). The pigmented bands corresponding to the B885-RC complexes were cut out, incubated in Laemmli sample buffer and then re-electrophoresed on a 10-30% (w/v) gradient gel which was subsequently fluorographed. The figure shows the resulting fluorogram after 5 weeks exposure.

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identified as flagellin. This species was only synthesised in motile swarmer cells, i.e. during dark incubation or over the 0-1 h period in the light. A polypeptide of M_r 14,000 and a group of proteins of M_r 70-80,000, the latter probably corresponding to those detected in multicellular arrays (Fig. 3.45) were particularly strongly synthesised over the period of daughter cell formation and chain-cell formation (3-8 h).

The periodicity in the synthesis of the M_r 14,000 ICM polypeptide (Fig. 3.50) was considered to be most likely due to a change in the expression of the RC associated B885 LH complex I during differentiation. This was confirmed by subjecting the native B885-RC complexes isolated on Triton-SDS gels loaded with equal radioactivity of solubilized pulse-labelled membrane samples, to SDS-PAGE and fluorography (Fig. 3.51). The 14,000 M_r B885 (LHI) polypeptide showed the same pattern of synthesis as the 14,000 M_r polypeptide observed in Fig. 3.50. Under these conditions a considerable amount of the native complex was not denatured and this allowed correlation with the synthesis of the constituent polypeptides. The period of prostheca elongation and daughter cell synthesis was characterized by an increased rate of synthesis of the RC polypeptides and the 38,000 M_r cytochrome c -553 in addition to that of the 14,000 M_r B885 protein. Some label was also incorporated into a 61,000 M_r protein which appeared to be a contaminant, loosely associated with the B885-RC complex, as identified in section 3.2.6. However, this protein was synthesised throughout the cell-cycle.

Little ^{35}S -methionine was incorporated into the 12,000 M_r B885 polypeptide during differentiation as judged from Figs. 3.50 and 3.51. The same was also true of the two proteins of the B880-865 LHII complex

TABLE 3.8. Changes in pigment content and composition of whole cells of *Rm. vannielii* during swarmer cell differentiation

Time after synchronization (h)	cell stage	cellular specific Bchl Content (nmol mg ⁻¹ protein ⁻¹)	A800 mg ⁻¹ protein ⁻¹	A870 mg ⁻¹ protein ⁻¹	A870:A800 ratio
0	swarmer cells	33.0	0.149	0.315	2.11
1	loss of motility	27.0	0.110	0.255	2.31
2	non-motile	26.0	0.098	0.238	2.43
3.5	protheca synthesis + bud formation	19.1	0.074	0.177	2.39
4.5	daughter cell enlargement	19.4	0.068	0.160	2.35
5.5	chain formation	14.5	0.040	0.095	2.37

Cells were harvested at each stage and stored frozen before pigment contents were determined in acetone:methanol extracts. Absorbance ratios were performed on whole cells after correction for light-scattering, assuming a linear relationship between scattering and wavelength (Aagaard & Siistrom, 1972).

as is evident from a comparison of Figs. 3.49 and 3.50. This could be due either to a low methionine content in these particular proteins and/or to their lack of synthesis under these conditions. Conclusions about the changes in the B800-865 LHII complex during swarmer cell differentiation were therefore deduced from changes in the cellular absorption spectra.

Table 3.8 shows data obtained from an analysis of the near IR peak heights of such spectra after correction for light scattering. Although the ratio of the 870 and 800 nm peaks remained almost constant throughout the cell-cycle, the decline in whole cell Bchl_a content was accompanied by a decline in both the 870 and 800 nm absorbance, normalized on an equal cell-protein basis. The change in cellular Bchl content would therefore appear to be due largely to a decrease in the amount of the accessory LHII complex, relative to cell protein. This conclusion can be drawn because of the major contribution of this complex to the near IR spectrum, and is supported by the failure to observe any change in the 870-890 nm region correlated with the increased synthesis of the B885-RC complex indicated by pulse-labelling. Clearly, more sensitive techniques such as measuring the absorption spectra at cryogenic temperatures are needed to observe this process spectroscopically.

A summary of the changes observed in the synthesis of the various regulated ICM protein and pigment-protein complexes is shown in Fig. 3.52.

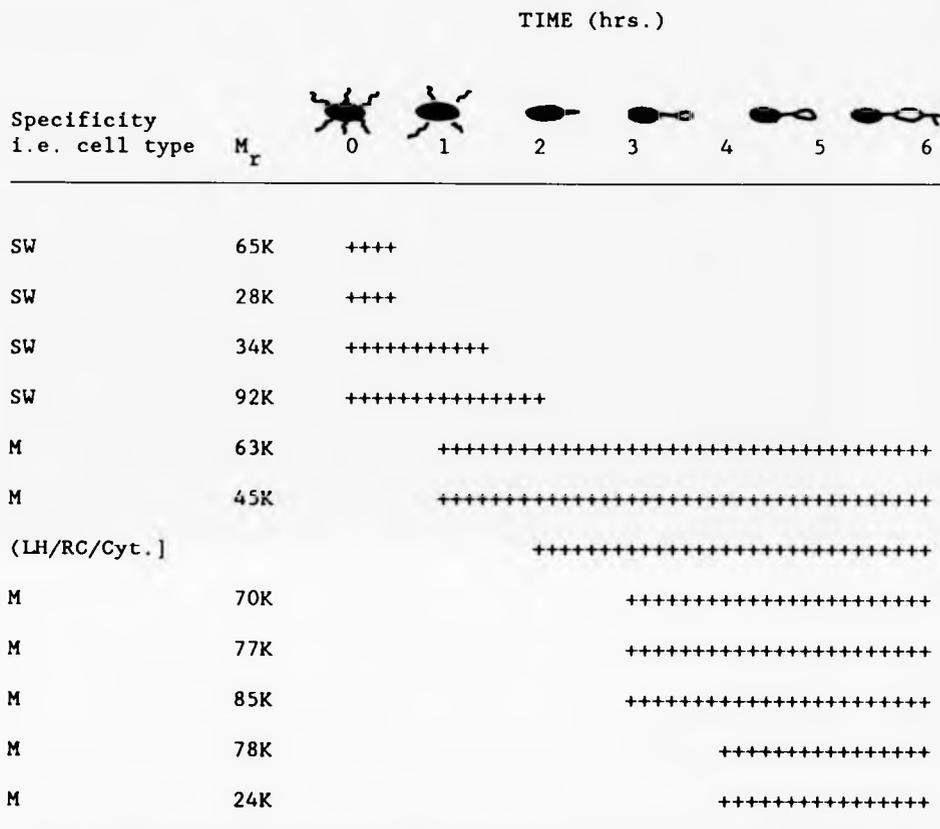


FIGURE 3.52. A summary of the period of synthesis of a number of ICM proteins during swarmer cell differentiation in *Rm. vannielii*, identified from SDS-PAGE followed by fluorography (Figs. 3.50 and 3.51). Cells were pulse-labelled for 20 min with ³⁵S-methionine and ICM prepared on sucrose gradients. "Specificity" refers to the synthesis of the protein in swarmer cells (SW) or multicellular arrays (M) as observed in batch cultures, while the period of synthesis was determined with synchronized swarmer cell populations. LH/RC/Cyt. refers to the light-harvesting-reaction centre-cytochrome complex.

3.4.1.2 Conclusions from the cell-cycle studies

(1) *Distinct patterns of ICM proteins were synthesised by swarmer cells and multicellular arrays in batch cultures and during swarmer cell differentiation.*

(2) *Flagellin was identified as a developmentally regulated polypeptide, its synthesis only occurring during the swarmer cell stage.*

(3) *Although the composition of the photosynthetic apparatus was the same in all cell-types, regulation of the synthesis of the polypeptides of the B885-RC complex was noted during the swarmer cell-cycle. This correlated with changes in photopigment content.*

3.4.1.3 Discussion. In this study a number of approaches were used to address the problem of the involvement of the ICM system in both cell-type heterogeneity in batch cultures and swarmer cell differentiation in *Rm. vannielii*. At the level of protein synthesis, the reduced rate of incorporation of ³⁵S-methionine into swarmer cell soluble and ICM protein is symptomatic of some general properties which have been noted elsewhere (Potts & Dow, 1979; Dow *et al.*, 1983, 1985; Porter, 1985); that the swarmer cell is essentially non-growing but is able to sustain protein turnover. During the cell-cycle, as differentiation ensues, the increase in biosynthetic activity - which is heralded by a change in the conformation of the nucleoid, switch on of DNA synthesis and increases in transcriptional activity (Dow *et al.*, 1983, 1985) - is also reflected in the increasing labelling of ICM proteins. However, this is not merely a quantitative distinction, as marked, qualitative differences were found to exist in the actual types of intra-cytoplasmic membrane proteins synthesised by swarmer cells and multicellular arrays in both batch culture and during differentiation. Indeed, pulse labelling through the swarmer cell-cycle clearly indicated the existence of a

temporal programme of membrane protein synthesis. A summary of the periodicity in synthesis of some of these proteins, identified from one dimensional gels (Fig. 3.52) showed that, in many cases, such proteins could be identified with those synthesised in a specific cell-type in batch culture.

Previous studies with Rm. vanniellii (France, 1978; Dow et al., 1983, 1985; Porter, 1985) and also the non-photosynthetic prosthecate bacterium Caulobacter crescentus (Cheung & Newton, 1977; Iba et al., 1978; Milhausen & Agabian, 1981) have shown variations in the rates of synthesis of a number of soluble proteins during the swarmer cell-cycle. In the study of Milhausen & Agabian (1981), 37 developmentally regulated polypeptides were identified using 2-dimensional gels, of which 7 were additionally identified as membrane proteins. Even with the use of one-dimensional gels, as here, the synthesis of a relatively large number of membrane polypeptide species appeared to be under regulation. The molecular mechanisms of regulation of the majority of these proteins are, however, unclear.

In many prokaryotic systems, gene expression is controlled at the level of transcription and in Rm. vanniellii evidence for such control has been obtained in studies using the DNA dependent RNA polymerase inhibitor rifampicin (Dow et al., 1983). Nevertheless, few changes in the subunit composition of this enzyme could be detected during the cell-cycle and similar observations have been made in Caulobacter (Bendis & Shapiro, 1973). In other differentiating bacteria, for example during sporulation in Bacillus subtilis, there are marked changes in the core associated sigma factors which change the DNA template specificity of the enzyme, thus resulting in fine transcriptional modulation (Losick &

Pero, 1981). Notwithstanding this, one possible mechanism of selective transcription is that resulting from a change in DNA structure itself, as in the relaxation in the conformation of the envelope associated nucleoid which is known to occur during the maturation phase of the Rm. vannieli cell-cycle (section 1.5).

The possibilities for post-transcriptional regulation of membrane proteins and their mRNAs are more extensive because of the additional machinery necessary to ensure the correct targeting or "spatial localization" of the gene products. The most notable expression of spatial control is the differential segregation of both particular proteins and assembled structures to only one of the progeny cells - a process in which the membrane must be involved. The observation that the functional half-life of the mRNA of several Caulobacter membrane proteins is relatively long (Agabian et al., 1979) could be due to such cell-type segregation, involving, for example, polyribosome mediated binding of mRNA to the cell membrane (Milhausen & Agabian, 1983). These possibilities may also extend to the Rm. vannieli cell-cycle where an extra level of complexity is introduced by the presence of essentially two inner membrane domains - cytoplasmic and intra-cytoplasmic. The targeting of proteins to these areas could not be distinguished in this study as there is no evidence that they are isolable separately from photosynthetic bacteria with lamellate ICM systems. However, some evidence for post-translational segregation of membrane proteins was obtained from a comparison of staining pattern vs labelling pattern for the cell-types in batch cultures (Fig. 3.45). Here, for example, the group of polypeptides of M_r 70-80,000 which were preferentially labelled in the arrays during the pulse were nevertheless detectable in the swarmer cell ICM by staining.

Once assembled into their locations, membrane protein expression can still be controlled by selective turnover and/or modulation of activity in the case of enzyme functions. Pulse-labelling procedures, as used in this study, cannot, however, distinguish between an increase in the rate of synthesis of a protein caused by true de novo synthesis and that caused by a decrease in its rate of turnover, as both possibilities could lead to increased band intensities on an autoradiograph. The contribution of specific protein turnover during swarmer cell differentiation could be investigated by pulse-chase procedures which would identify the "targets" for proteolytic action. These could be further defined using specific protease inhibitors. Indeed, radiolabelled protease inhibitors have already been used to identify changes in the pattern of serine proteases during swarmer cell differentiation in Rm. vannieli (Russell & Mann, 1984).

Of the several ICM polypeptides which were found to be regulated during the swarmer cell-cycle, flagellin was clearly identified using the Western blot technique. That the protein was present in the ICM fraction under two growth conditions and after ICM preparation on either sucrose cushions or sucrose gradients, suggests that it was not merely a contaminant. Flagellin synthesis is also developmentally regulated in Caulobacter but, in contrast to Rm. vannieli, at least two types of flagellin are produced (Lagenaur & Agabian, 1978). The hook protein and flagellin B (M_r 27,500) are synthesised together over a transient period in the pre-divisional cell, late in the cell-cycle. This is consistent with the polymerization of flagellin B subunits proximal to the hook during this period of de novo flagella assembly. Although flagellin A (M_r 25,000) is also synthesised in the pre-divisional cell, its synthesis continues in the swarmer cell until motility is lost (Shapiro

& Maizel, 1973). Flagellin A may function in flagellar elongation in the swarmer cell, as it is polymerized distal to the hook but is also likely to play a role in turnover. Remarkably, swarmer cell specific flagellin A synthesis is rifampicin insensitive (Osley *et al.*, 1977) and appears to result from continued translation of mRNA specifically segregated to this cell-type alone (Milhausen & Agabian, 1983).

In *Rm. vanniellii* the pattern of synthesis of the M_r 34,000 flagellin most closely resembled that of the *Caulobacter* flagellin A. However, it is unlikely that continued translation of pre-segregated mRNA is responsible for this pattern, as the protein could still be pulse-labelled after 6 h in dark incubated swimmers - conditions under which motility was maintained. Without invoking mRNA of extreme longevity, such an observation is consistent with *de novo* transcription. An alternative possibility is that new transcripts are specifically produced under dark or light-limited conditions in order to sustain turnover of the flagella in swarmer cells which must remain motile. However, sufficient illumination to allow differentiation halts the initiation of new transcripts and the mRNA stability then largely determines the flagellin synthetic period. These possibilities need to be examined experimentally by the determination of mRNA half-lives combined with studies of the regulation of the flagellin genes.

It seems axiomatic that the membrane bound "pool" of flagellin subunits in *Rm. vanniellii* is a precursor for the assembly of the flagellar organelle, a conclusion supported by the cell-cycle dependent correlation observed between the period of motility and the period over which ^{35}S -methionine was incorporated into the protein. Pulse-chase experiments in *Caulobacter* (Hugueneil & Newton, 1984a,b) have also

demonstrated the importance of a membrane bound pool of the monomer as an intermediate in flagellar assembly.

It remains an intriguing possibility that some of the other membrane proteins specifically synthesised in the swarmer cell may also be connected with either motility per se or a function dependent thereon, such as chemotaxis or phototaxis. Presumably, as such tactic responses cannot occur after the flagella have been shed, their molecular machinery is also under cell-cycle regulation. Evidence for this supposition has again come from Caulobacter, where the methylation of a subset of membrane proteins involved in chemotactic signal transduction is confined to those portions of the cell-cycle when the flagellum is present (Shaw et al., 1983). Unlike Caulobacter. Rm. vannieli swarmer cells normally differentiate into a "cell pair" without producing new progeny swarmer cells immediately (except in the simplified cycle) so that the patterns of regulation of both flagellar assembly and putative chemotaxis proteins are likely to be different, at least during the later stages of the cell-cycle.

In addition to the identification of flagellin as a differentially regulated polypeptide species, the identification of the components of the pigment-protein complexes of the photosynthetic apparatus also allowed a study to be made of their regulation during the cell-cycle. This was combined with an analysis of the changes in photopigment content during the same period.

One hypothesis to explain the patterns observed is based on the fact that during growth in batch cultures, swarmer cells become light-limited due to increasing self-shading and it is this which prevents them from

initiating differentiation (Dow et al., 1983). Removal of the multicellular arrays during synchronization reduces the optical density of the culture about 10-fold, thus relieving light-limitation when the swarmer cells are re-illuminated under the same conditions. The constancy of the culture pigment concentration and the consequent drop in the cellular specific photopigment content during differentiation (Fig. 3.47) can therefore be explained as a growth-dilution effect. However, although a decrease in the photopigment content of the ICM was also observed over the period of maturation, the increased synthesis of the B885-RC complex during the reproductive phase of the cell-cycle appeared to be responsible for the increase in ICM photopigment content observed over the same period. Thus, consistent with the high-light incubation conditions essential for synchronous swarmer cell differentiation, the daughter cell progeny are likely to have only limited ICM development and an excess of B885-RC complexes over B800-850 (LHII) complexes. Although conclusions about the pattern of synthesis of the LHII polypeptides (and M_r 12,000 B885 polypeptide) were precluded due to their apparent lack of incorporation of ^{35}S -methionine, the overall decrease in cellular Bchl content and the spectroscopic data suggest that this complex was not actively synthesised, again consistent with the high light availability. Low specific labelling rates of LH proteins and a lack of proportionality between methionine content and ^{35}S -methionine incorporation have also been found by Peters et al., (1983) in Rb. capsulatus.

Taken together, the pigment and protein data would seem consistent with the incorporation of new photosynthetic units into the daughter cell membrane, accompanying de novo membrane synthesis during the process of polar growth, as suggested by Whittenbury & Dow (1977) and Kelly & Dow

(1984). Because physiological but not physical separation of the two progeny cells is a feature of the normal Rm. vanniellii cell-cycle it was not possible to analyze the composition of the photosynthetic apparatus in each of these cells individually. However, this could be done using synchronized swarmer cell populations from strains exhibiting the simplified cycle were physical cell separation is the terminal event (Dow & France, 1980). This would allow an analysis to be made of the distribution of synthesised complexes, for example, between the progeny stalked and swarmer cells. Similarly, electron-microscopic data such as freeze-fracturing would also reveal differences in the architecture of the membrane system in the newly formed (daughter) cell as compared to the "old" (mother) stalked cell.

This type of synthetic pattern of the components of the pigment-protein complexes is rather different to that observed in induction synchronized cultures of Rb. sphaeroides (Fraley *et al.*, 1978; Wraight *et al.*, 1978b; Leuking *et al.*, 1981; Kaplan *et al.*, ;1983) in which a continuous insertion of such components was found. This must be at least partially due to the different cultural conditions which have to be used for Rm. vanniellii swarmer cell differentiation but must also reflect the different mode of growth of the cell envelope and thus reproduction per se. Therefore despite the light-replete conditions used throughout the cell-cycle, the requirement for the formation of a membrane in the progeny daughter cell de novo dictates the production of new photosynthetic units presumably independent of environmental conditions. Nevertheless, although de novo membrane synthesis and reproduction by polar growth are obligately coupled during the cell-cycle in Rm. vanniellii, such environmental conditions must exert controlling effects. For example, the high-light incubation conditions did not result in

synthesis of the accessory LHII complex which might be expected under light-limiting conditions. In synchronized Rb. sphaeroides cultures, this complex is, however, inserted into the membrane at a rate greater than that of the RC or B875 complexes (Knacker et al., 1985).

3.4.2 Bioenergetics of the cell-cycle

The overriding feature of the differentiation of Rm. vannielii swarmer cells is the control exerted by light. A dark to light transition sets in train a series of events which, as shown in section 3.4.1, must involve changes in gene expression. Some aspects of the bioenergetic consequences of illumination were therefore investigated.

3.4.2.1 Results. A simple experiment was designed to determine if light merely acted as a "trigger" for differentiation or if continued illumination was necessary throughout the process. Transfer of cells from any period of an on-going cell-cycle in the light to anaerobic dark conditions resulted in the immediate cessation of differentiation as evidenced by microscopy, cell volume distribution analysis and turbidity. However, if after the dark period the cells were again returned to the same illumination conditions for a further 12 h, multicellular arrays were formed in each case. These observations suggest that while illumination allows differentiation to be initiated, a continued input of light energy is necessary in order for the process to be completed. The transfer of swarmer cells to aerobic dark conditions at a variety of O₂ tensions did not result in differentiation over an 8 h period. Similarly, cells which had already initiated the cell-cycle as a consequence of illumination, did not complete the

process over a comparable time period after transfer to aerobic-dark conditions. Nevertheless, microscopic examination after 24 h under such conditions did reveal the presence of a few developing cells with daughter buds suggesting a very slow differentiation cycle. In general these observations are consistent with the notion that differentiation only occurs under the same range of rather limited growth conditions available to Rm. vannielli and is thus heavily dependent on photosynthesis as the energy source.

The most obvious candidate which could act as an intermediate between the reception of the light stimulus and events leading to an alteration in the programme of gene expression during differentiation is modulation of the proton-motive force (Δp) or some component(s) thereof. One objective was therefore to measure Δp during differentiation in Rm. vannielli. Ideally a system was required in which measurements could be made continuously without disturbing the cellular environment during synchronous growth. Methods based on permeant ion redistribution methods appeared most suitable, largely because electrodes designed in recent years would allow both continuous monitoring and the possibility of simultaneous determinations of $\Delta \psi$ and ΔpH with the appropriate probe-ion (Shinbo *et al.*, 1978; Lolkema *et al.*, 1981; Keevil & Hamilton, 1984). Minimal culture disturbance is also a feature, because such electrodes could be permanently inserted into a differentiating cell-population. The experiments described below were therefore designed to test the feasibility of this method.

The major probe-ions used were TPP^+ ($\Delta \psi$) and salicylate (ΔpH). In preliminary experiments, the presence of $1 \mu M$ TPP^+ or up to $500 \mu M$ salicylate was found to have no deleterious effect on the progress of

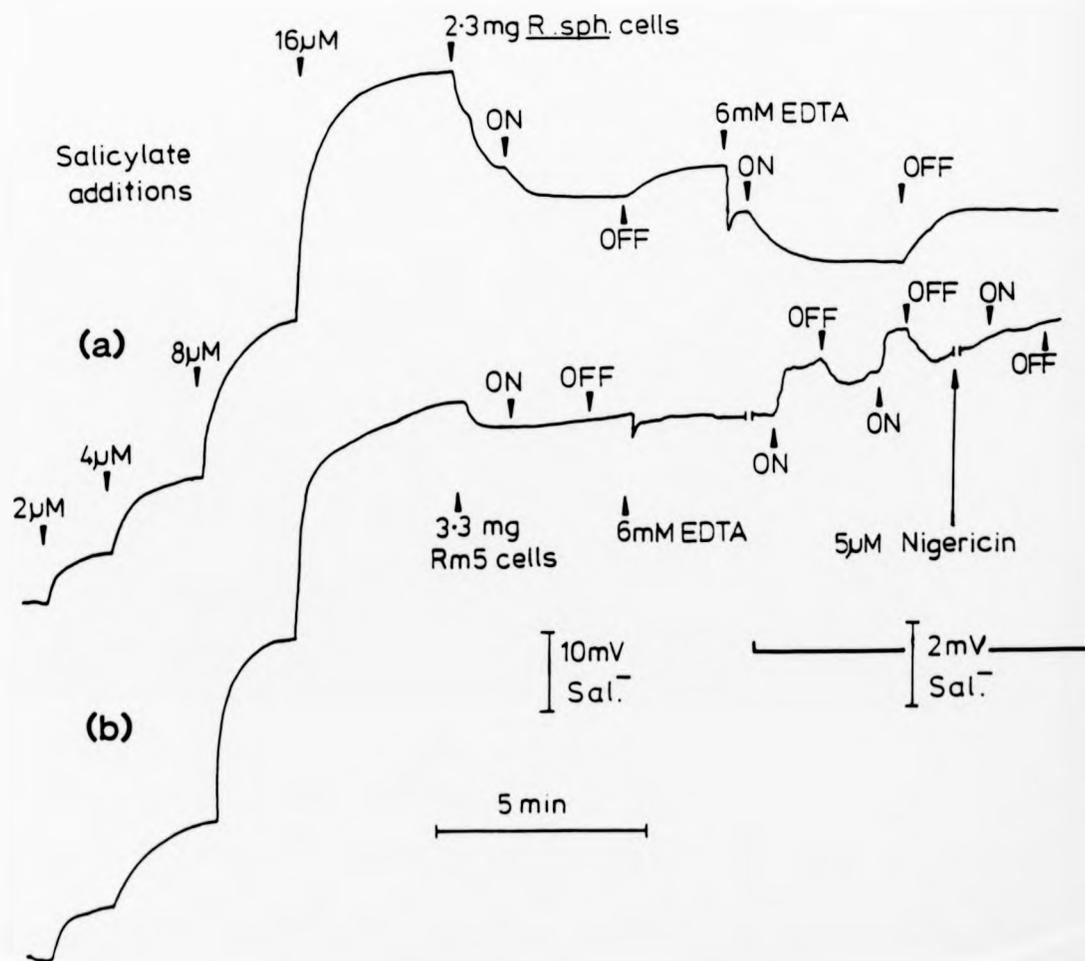
swarmer cell differentiation as assayed microscopically and by cell-volume distribution analysis.

Typical results obtained with the TPP⁺ electrode are shown in Fig. 3.53. Evidence that the electrode was responding correctly was obtained in control experiments using whole cells of *Rb. sphaeroides* which were added after the addition of calibrating pulses of TPP⁺. Binding of the probe to the cells (i.e. passive binding to cell components and uptake) was evidenced by a decrease in the free concentration of TPP⁺ as indicated by an upward deflection of the trace. Illumination of the cell suspension resulted in the generation of only a small response but this was greatly enhanced by the addition of EDTA which is known to permeabilize the outer membrane (Nikaido & Varra, 1985). Under these conditions, light induced membrane potentials were reproducibly obtained. The size of the potentials was increased when the cells were initially incubated with 20 μ M venturicidin, an inhibitor of the ATPase (Cotton *et al.*, 1981).

The results obtained with *Rm. vannielii* cells were, however, rather different (Fig. 3.53). Under identical conditions, little probe binding occurred upon addition of cells and no light-induced response could be reproducibly demonstrated in the presence or absence of EDTA, the latter at concentrations between 1-10 mM. The same result was obtained with cells from heterogeneous batch cultures, swarmer cells or swarmer cells which had been allowed to differentiate in the light for 3 h before being harvested and tested. Performing the experiments at pH 6.5, 7.5 or 8.0 or using complete PM growth medium instead of phosphate buffer with 5 mM Mg²⁺, also gave negative results. Attempts to permeabilize the cells with EDTA and Tris by pre- incubation with these reagents at

FIGURE 3.54. Measurement of the cellular pH gradient in *Rb. sphaeroides* (a) and *Rm. vannielii* (b) with the salicylate electrode system.

Cells from late exponential phase batch cultures were resuspended in 50 mM phosphate buffer pH 6.0 containing 5 mM $MgCl_2$ and kept on ice until used. Measurements were performed in the same buffer system. Calibrating pulses of salicylate were then added followed by the cells. Illumination ($300 Wm^{-2}$) was provided as indicated (on/off).



30°C for up to 1 h were also unsuccessful, as was the addition of 20 μ M venturicidin, to inhibit ATPase activity, or 10 μ M nigericin to collapse any pre-existing pH gradient, thus increasing the chance of detecting a membrane potential. The treatment of cells with the reagent that was used to selectively permeabilize the PVC membrane of the electrode-tetraphenylborate (TPB⁻) - was also tested, in the hope that this would allow TPP⁺ uptake, but although binding was apparently increased (albeit probably as a result of complexing between the TPP⁺ and TPB⁻), no light induced response was detectable, even at high (3-5 mg ml⁻¹) cell densities. These results suggest that Rm. vanniellii is not permeable to the TPP⁺ probe-ion.

Typical results obtained with the use of the salicylate electrode system are shown in Fig. 3.54. Again, Rb. sphaeroides cells were used as a positive control. The light-induced development of a pH gradient in this species was clearly observable especially under acidic conditions (pH 5.5-6.5) where Δ pH makes a greater contribution to the total Δ p (Hellingwerf & van Hoorn, 1985). The inclusion of 5 mM EDTA in the assay increased the size of the response obtained. With Rm. vanniellii, the assay pH was varied between 5.0 and 7.5 and, in the absence of EDTA, no light-induced pH gradient could be detected. At pH 5.0 or 5.5 only, with a rather high concentration of cells from a heterogeneous batch culture (Fig. 3.54) and in the presence of 6 mM EDTA, a small reversible response was observed, but in the opposite direction to that obtained with Rb. sphaeroides. This would suggest that at least some salicylate is taken up by the cells in the dark but that it is extruded upon energization by light. Some evidence for the authenticity of the potentials generated was obtained by the addition of a low concentration of nigericin, which catalyzes the electroneutral exchange of K⁺ and H⁺,

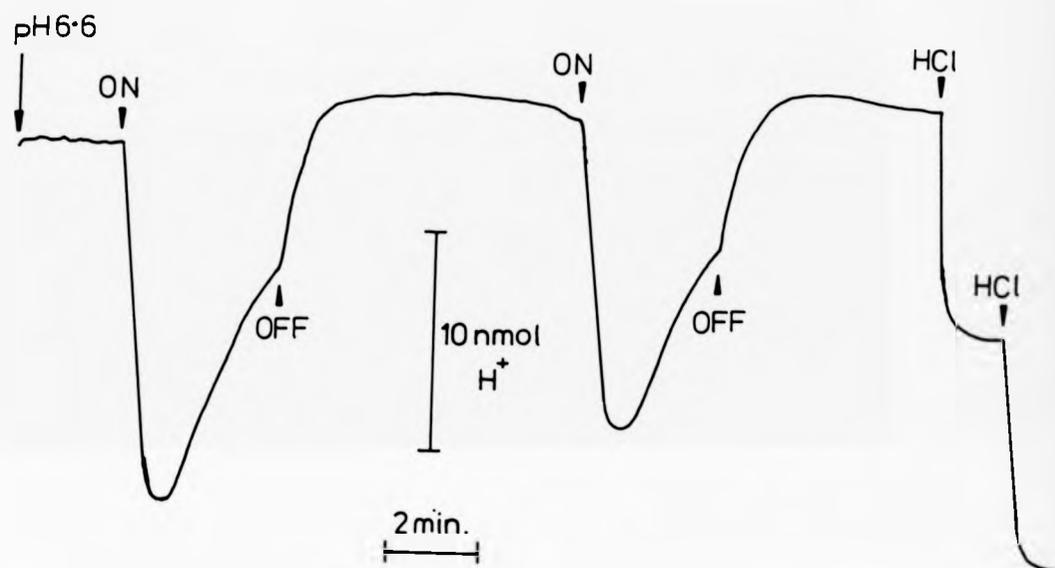


FIGURE 3.55. Light induced proton translocation catalysed by intact cells of *Rm. vannielii*. Cells from a late-exponential phase batch culture were washed once and then resuspended in 150 mM KCl containing 5 mM MgCl₂. Two ml of this salt solution was then placed in the electrode chamber (section 2.32), the cells added (about 3.0 mg protein) and the pH allowed to stabilize at about 6.6. Illumination (300 Wm^{-2}) was provided as indicated (on/off) and calibrating pulses of HCl added at the end of the experiment from a 1 mM solution.

thus affecting only ΔpH (Nicholls, 1982). In the presence of this ionophore, the light-induced effect was abolished. Although these data could point to the development of a small ΔpH of the opposite sign to that normally encountered, i.e. inside acid, it seems far more likely that the probe is behaving anomalously in Rm. vannielii, as independent measurements of the internal pH of this microbe using ^{31}P NMR (Porter & Hellingwerf, unpublished) indicate a value well above neutrality. In addition, direct measurements of light-induced proton translocation, obtained by monitoring pH changes in unbuffered cell suspensions (Fig. 3.55) clearly showed an initial, transient acidification of the external medium followed by a prolonged period of alkalization, the rate of which was accelerated when the light was turned off. This behaviour has been observed in a number of photosynthetic bacteria including the genus Rhodobacter (Cotton *et al.*, 1981).

Clearly, the use of the TPP^+ and salicylate ion-selective electrodes with whole cells of Rm. vannielii is largely negated because of the unusual behaviour of the probe ions in this microbe. As an alternative method of gaining information on the electrical component of Δp and its relationship to differentiation, the utility of the electrochromic response of the endogenous carotenoids was investigated. Using the wavelength pair 522 and 542 nm (found empirically by observing light induced changes in this region of the spectrum) an uncoupler and antimycin A sensitive shift could be obtained with whole cells resuspended in PM medium. The effect of increasing concentrations of various inhibitors on the size of the light-induced potentials is shown in Fig. 3.56. Although titratable with CCCP and antimycin A, even high concentrations did not abolish the light induced $\Delta\psi$ completely and this was much more pronounced with myxothiazole, which, like antimycin A,

FIGURE 3.56. The effect of various energy transfer inhibitors on the development of the carotenoid bandshift in intact cells of *Rm. vannielii*.

All experiments were carried out on late-exponential phase cells (1.2 ml volume) taken directly from a growing culture and placed in the cuvette of the Aminco DW2 spectrophotometer. The maximum change in the size of the carotenoid bandshift at 522-542 nm upon illumination was noted and compared with that elicited in the presence of increasing concentrations of CCCP (●—●), antimycin A (○—○), myxothiazole (◆—◆), valinomycin (◇—◇), venturicidin (▲—▲) and TPP^+ (■—■). Inhibitors were prepared as concentrated stock solutions in absolute ethanol and added in quantities (1-10 μ l) at which no ethanol effect was observed.

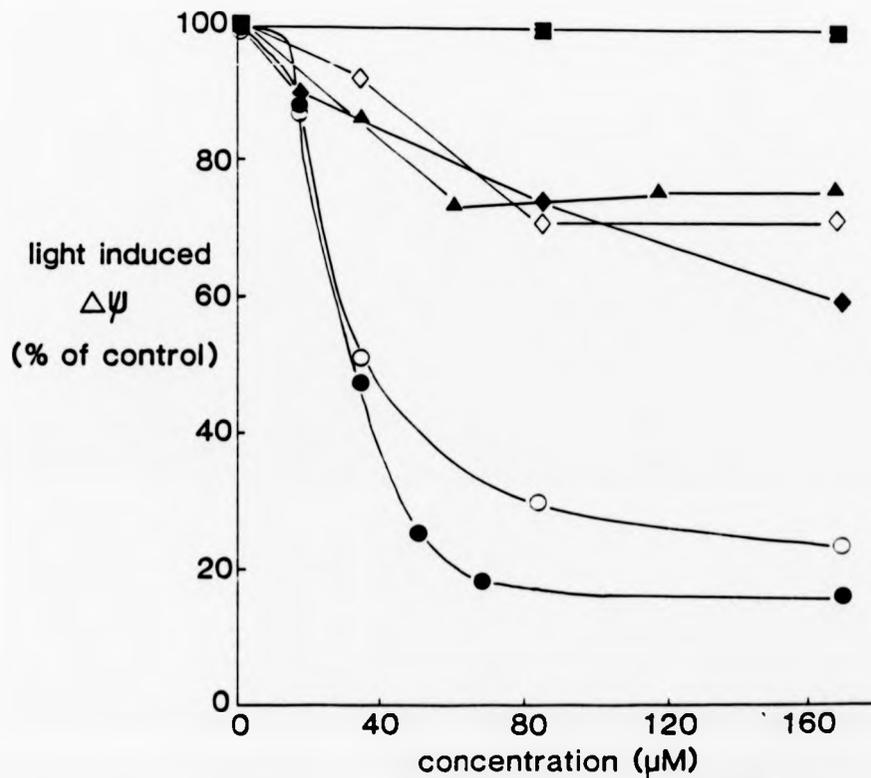
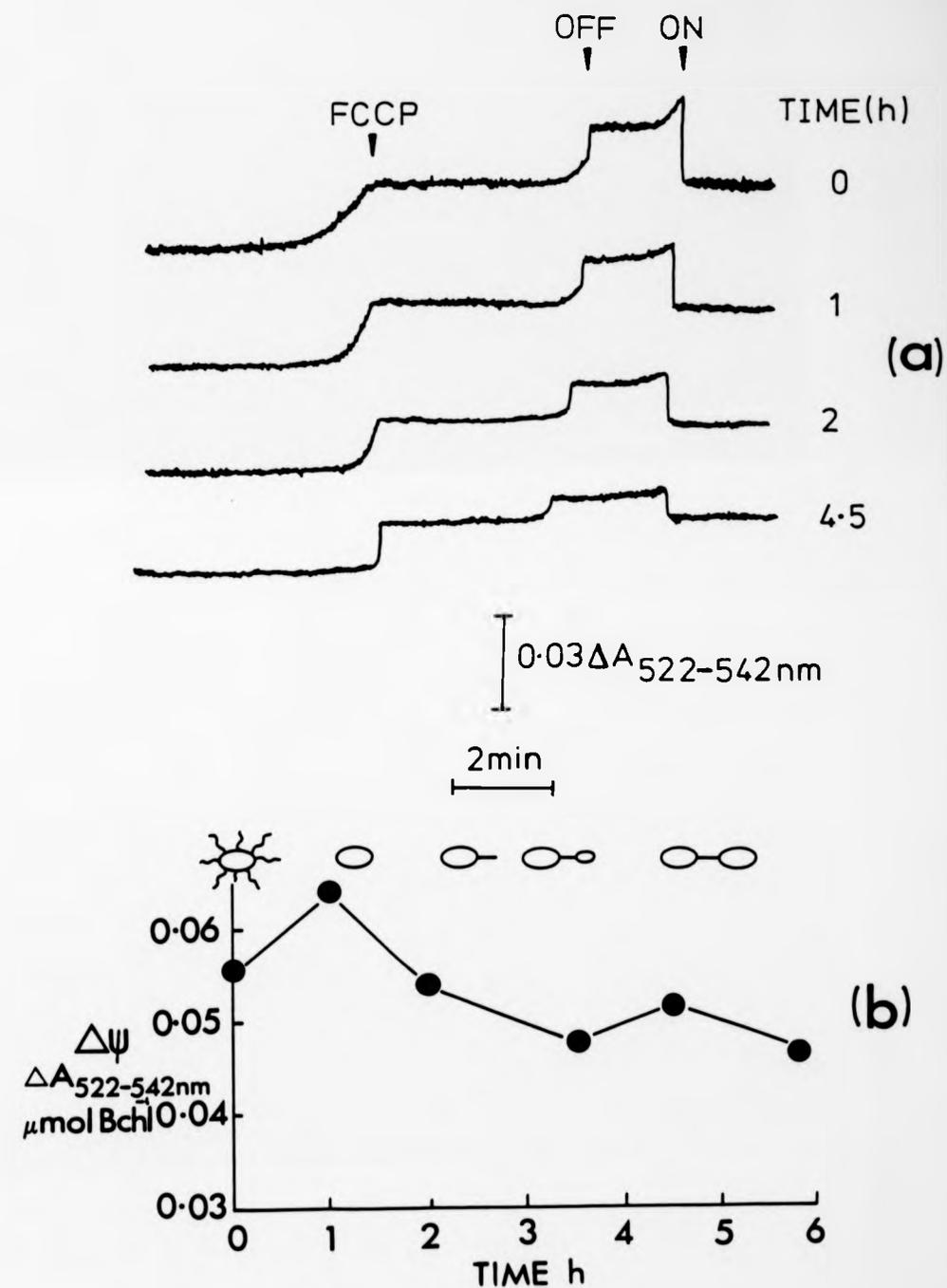


FIGURE 3.57. Changes in the carotenoid bandshift observed during swarmer cell differentiation in *Rm. vannielii* using a slow time-scale spectrophotometer.

Cells taken at intervals from a synchronously differentiating swarmer cell population were resuspended in fresh PM growth medium and stored on ice in the dark prior to the experiments. In (a) the recorder traces are shown after the addition of cells (to give only approximately equal Bchl concentrations by the same A870 nm value) and the responses observed after illumination under anaerobic conditions. The light intensity was the same in each case. The uncoupler FCCP was added to 5 μM final concentration. In (b) the size of the light-induced membrane potential after a steady state was reached is plotted after correction for the change in pigment content during the swarmer cell-cycle. For this analysis, Bchl contents were estimated accurately by extraction into acetone:methanol.

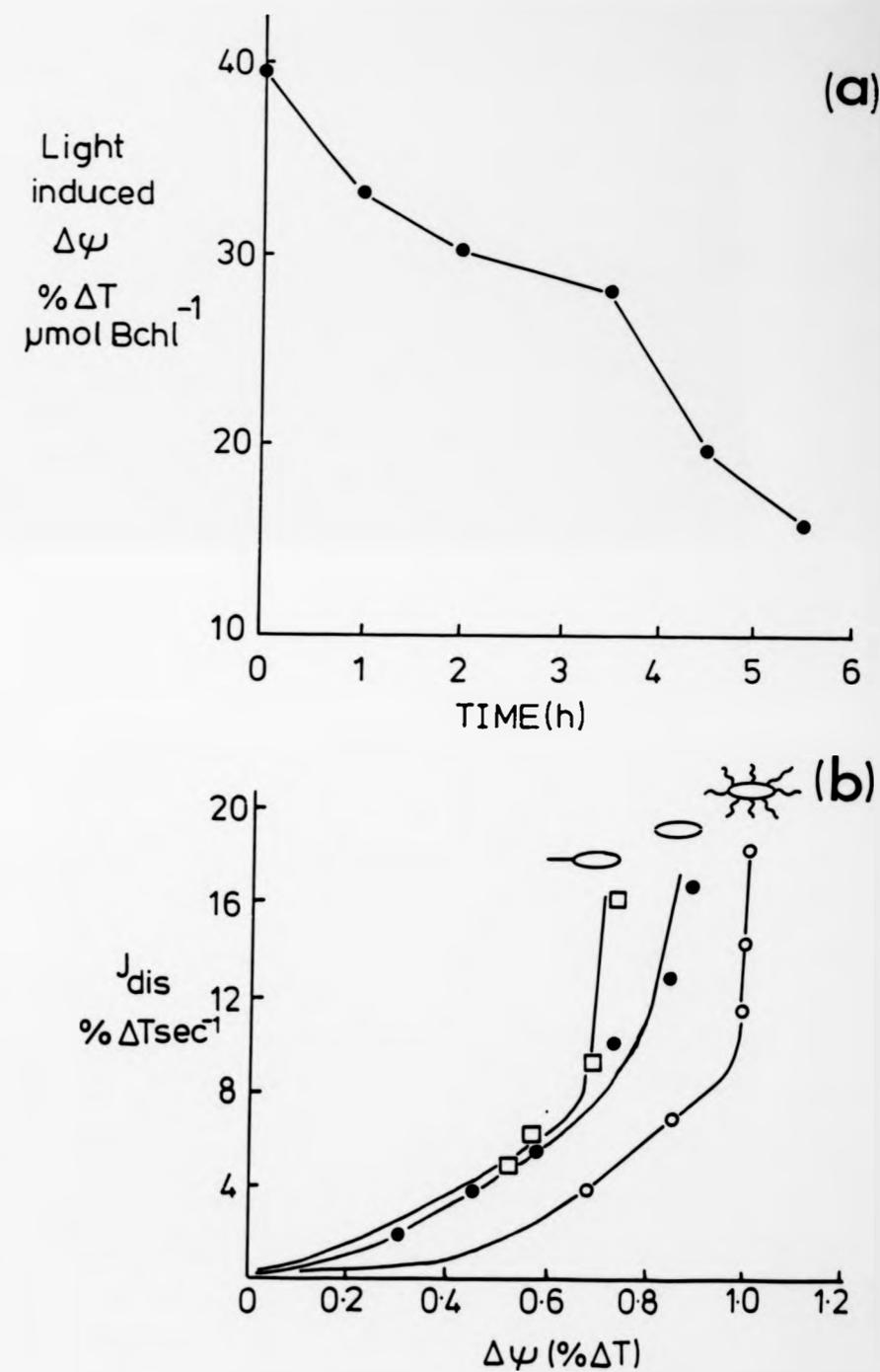


acts at the cytochrome b-c₁ region of the electron transport chain, valinomycin (an ionophore) and the ATPase inhibitor venturicidin. Addition of TPP⁺ did not affect the size of the bandshift at any concentration tested.

In assessing $\Delta\psi$ by this method with differentiating cell populations, samples had to be taken at various time periods from an illuminated culture vessel, harvested and resuspended in fresh growth medium before measurements were made under saturating light intensity and anaerobiosis in the spectrophotometer cuvette. Fig. 3.57(a) shows the characteristics of the shifts observed on a slow-time scale. In successive samples, from motile swarmer cells to the stage of daughter cell formation, a consistent decrease was observed in the size of the light-induced $\Delta\psi$ and with the initial overshoot before the establishment of a steady state becoming less apparent. Addition of the potent uncoupler FCCP led to the establishment of a lower ΔA value in each case under dark conditions suggesting the presence of a pre-existing dark membrane potential. Interestingly, the rate at which this potential was collapsed was much faster in cells from the later stages of differentiation than with swarmer cells, for example. The data in Fig. 3.57a were not corrected for the change in carotenoid content which occurred during swarmer cell differentiation (section 3.4.1). As there was no significant change in the carotenoid: Bchl ratio during the cell-cycle (section 3.4.1) the bandshifts were normalized to equal Bchl concentrations, using the appropriate extinction coefficient. After this procedure, an initial increase (up to 1 h) and then a gradual decrease in the size of the light-induced $\Delta\psi$ was observed (Fig. 3.57b) during swarmer cell differentiation.

FIGURE 3.58. Changes in the carotenoid bandshifts during swarmer cell differentiation using a rapidly responding crossed-beam spectrophotometer.

Conditions and cell concentrations were exactly the same as in Fig. 3.57. In (a) the size of the light-induced membrane potential after correction for Bchl content is shown. In (b) a current/voltage curve was constructed using data from three of the stages during differentiation (swarmer cells, non-motile cells and stalked cells). For this analysis the cells were illuminated to steady state at a range of different light intensities to give a range of values for $\Delta\psi$. At each light intensity, the initial rate of decay of the carotenoid bandshift after the cell suspension was rapidly darkened was determined as a measure of the ionic current (J_{dis}) flowing back across the membrane. The graph shows the value of J_{dis} as a function of the membrane potential reached at the different light intensities.



This pattern of decreasing $\Delta\psi$ during differentiation was also observed using a rapidly responding crossed-beam spectrophotometer (Fig. 3.58a), again, after correction for Bchl content. The initial rate of decay of the carotenoid bandshift signal upon darkening the cell suspension is known to be a measure, in other photosynthetic bacteria, of the ionic current (J_{dis}) flowing back across the membrane (Jackson, 1982). The form of the relationship between the value of $\Delta\psi$ and the ionic current in *Rm. vannielii* was investigated by progressively lowering the rate of cyclic electron transport by lowering the actinic light intensity and then plotting the steady state values of $\Delta\psi$ obtained in each case against the rate of decay of the signal after the cells were rapidly darkened (Fig. 3.58b). When this was done with populations of swarmer cells, non-motile cells or cells in the process of prostheca synthesis a similar, non-linear dependence of J_{dis} on the value of $\Delta\psi$ was obtained in each case. However the curves became progressively shifted to the left, reflecting the overall decrease in $\Delta\psi$ during differentiation.

3.4.2.2 Conclusions from the bioenergetic studies

- (1) Measurements of membrane potential using TPP^+ as probe-ion were unsuccessful in *Rm. vannielii* due to an apparent impermeability to this compound.
- (2) pH gradient measurements using salicylate as probe-ion gave some responses but these appeared anomalous when compared to the direction of proton translocation (i.e. outward, as expected) in illuminated whole cells monitored with a pH electrode.
- (3) The light-induced development of a membrane potential could be monitored using the electrochromic response of the endogenous carotenoids. This indicated a decrease in this parameter during the cell-cycle.

This pattern of decreasing $\Delta\psi$ during differentiation was also observed using a rapidly responding crossed-beam spectrophotometer (Fig. 3.58a), again, after correction for Bchl content. The initial rate of decay of the carotenoid bandshift signal upon darkening the cell suspension is known to be a measure, in other photosynthetic bacteria, of the ionic current (J_{dis}) flowing back across the membrane (Jackson, 1982). The form of the relationship between the value of $\Delta\psi$ and the ionic current in *Rm. vannielii* was investigated by progressively lowering the rate of cyclic electron transport by lowering the actinic light intensity and then plotting the steady state values of $\Delta\psi$ obtained in each case against the rate of decay of the signal after the cells were rapidly darkened (Fig. 3.58b). When this was done with populations of swarmer cells, non-motile cells or cells in the process of prostheca synthesis a similar, non-linear dependence of J_{dis} on the value of $\Delta\psi$ was obtained in each case. However the curves became progressively shifted to the left, reflecting the overall decrease in $\Delta\psi$ during differentiation.

3.4.2.2 Conclusions from the bioenergetic studies

- (1) *Measurements of membrane potential using TPP^+ as probe-ion were unsuccessful in *Rm. vannielii* due to an apparent impermeability to this compound.*
- (2) *pH gradient measurements using salicylate as probe-ion gave some responses but these appeared anomalous when compared to the direction of proton translocation (i.e. outward, as expected) in illuminated whole cells monitored with a pH electrode.*
- (3) *The light-induced development of a membrane potential could be monitored using the electrochromic response of the endogenous carotenoids. This indicated a decrease in this parameter during the cell-cycle.*

(4) *The conductance properties of the membrane did not appear to change appreciably during the cell-cycle as judged by the similarities in the current/voltage curves.*

3.4.2.3 Discussion. The close link between photosynthetic energy conversion and differentiation in *Rm. vannielii* is illustrated by the dependence of the completion of cell-cycle events upon continuous illumination. This supports the view that the differentiation of swarmer cells is an energy demanding process. In the dark the current cell-cycle appears to be aborted but can be re-initiated upon re-illumination. These observations are similar to those made in a number of other phototrophic microbes, both prokaryotic (Leuking *et al.*, 1981; Asato, 1983) and eukaryotic (Lloyd *et al.*, 1982; John, 1984). However, in *Rm. vannielii*, such a dramatic control by light is of importance to the ecological role of the swarmer cell as a dispersal agent, since some cellular activities - such as motility - must be maintained. This begs the question of the "bioenergetic state" of the swarmer cell and how this changes during differentiation. The nature of the energy source for the maintenance of dark associated events is not known, although reserve materials such as poly- β -hydroxybutyrate are known to be accumulated under phototrophic growth conditions (Trentini & Starr, 1967). These may be metabolised in the dark to produce a sufficient Δp to sustain flagellar rotation, for example, but not sufficient to initiate differentiation. Upon illumination a sufficiently high Δp would become available through cyclic electron transport to allow such a commitment.

However, measurements of the carotenoid bandshift indicated a decrease in the value of the membrane potential - which may reasonably be assumed

to be an energy related parameter - during the cell-cycle. There are several possible interpretations of these data.

One explanation is that the decreasing $\Delta\psi$ reflects an increase in the activity of various dissipative processes, particularly those energy requiring reactions such as ATP synthesis. Increased H^+ flux through the ATP synthase should be reflected in a change in the form of the relationship between the membrane ionic current and the value of $\Delta\psi$. However, little difference was apparent in this relationship at three stages of the cell-cycle except a shift of the curves to the left, reflecting the overall decrease in $\Delta\psi$. In a previous study, Porter (1985) showed that illumination of swarmer cells resulted in a transient increase in cellular ATP levels (up to 1 h post initiation of differentiation) followed by a decrease up to at least the phase of stalk formation. This observation is difficult to reconcile with the carotenoid bandshift data. Simultaneous measurements of the rate of ATP synthesis and its relationship to the current/voltage properties of differentiating swarmer cells would be desirable to resolve this point.

Another possibility is that the rate of cyclic electron transport decreases during the cell-cycle. The most obvious mechanism for this change would be an increased incorporation of lipid into the ICM, thus diluting the components of the photosynthetic apparatus and decreasing interactions between them. This effect can be reproduced *in vitro* in liposomes fused with chromatophores of *Rb. sphaeroides* (Snozzi & Crofts, 1984). Also there is evidence of a change in the protein:phospholipid ratio in the ICM of *Rb. sphaeroides* undergoing induction synchronized growth (Kaplan *et al.*, 1983), although the bioenergetic implications of this have not been determined as yet.

A further alternative is that because the "electrochromic carotenoids" appear to be those specifically associated with the accessory LHII complex, at least in Rb. capsulatus (Webster et al., 1980), the dilution in the cellular concentration of this complex during differentiation alters the way in which the pigments respond to the electronic field, bearing in mind that a decrease in $\Delta\psi$ was still apparent after the raw data were corrected for the change in pigment content during differentiation.

To distinguish between these various possibilities requires agreement between at least two independent methods of measuring components of the proton-motive force. This requirement could not be satisfied in this study because of the unexpected behaviour of the probe-ions TPP^+ and salicylate. It could be argued that such behaviour is related to the presence of a lamellate - rather than a vesicular - ICM arrangement in Rm. vanniellii which might not allow correct equilibration of the probe between the internal and external bulk phases. However this seems untenable in view of successful results obtained with Rd. blastica and Rd. palustris using both TPP^+ and salicylate (Porter & Hellingwerf; unpublished data). The most likely explanation is that related to the permeability properties of the cell envelope. Whereas EDTA treatment of Rb. sphaeroides clearly exerted a marked permeabilising effect in allowing TPP^+ uptake, for example, no such effect was obtained with Rm. vanniellii. In addition, the FCCP mediated dissipation of the "dark" $\Delta\psi$, as indicated by the carotenoid bandshift, was slow in swarmer cells but almost instantaneous in reproductive cells (Fig. 3.57) suggesting a change in permeability properties during differentiation.

In view of these observations, it is clear that a batch culture

containing all the cell-types will only give an averaged response in experiments using inhibitors to which the individual cell-type permeability varies. This may be invoked to explain the widely differing effects of the various energy transfer inhibitors on the size of the membrane potential as indicated by the whole cell carotenoid bandshift (Fig. 3.56). Thus, over a low concentration range only the sensitive cells will be affected. This may be one reason why even at very high inhibitor concentrations the light-induced bandshift could not be completely abolished due to the presence of a "resistant", i.e. impermeable, cell population. The fact that TPP⁺ had no effect at all on the $\Delta\psi$ indicated by the carotenoid bandshift suggests that all cell-types are impermeable to it, and this agrees with the results obtained from the TPP⁺ electrode studies.

These conclusions are in fact supported by a number of disparate observations described elsewhere (Whittenbury & Dow, 1977; Dow *et al.*, 1983, 1985). It has been known for some time (Whittenbury & Dow, 1977) that the sensitivity of the swarmer cell to a number of inhibitory agents is markedly increased when differentiation has proceeded to the stalk cell-stage. Defined concentrations of penicillin cause lysis of the prosthecae cell but allow growth up to this stage. Similarly, explicit changes in the sensitivity of cells to rifampicin occur through the cell-cycle and these have been attributed to modification of the DNA dependent RNA polymerase (Dow *et al.*, 1983, 1985) yet the holoenzyme isolated from various cell-types is extremely sensitive to rifampicin in all cases (Scott & Dow, unpublished).

The molecular basis for such permeability properties in Rm. vannielii is not known. The outer membrane would be the most obvious candidate,

being the main permeability barrier in Gram negative bacteria (Nikaido & Vaara, 1985). The presence of lipopolysaccharide (LPS) is known to contribute to this property. Rhodomicrobium vannielii is known to contain only one major type of LPS (Holst et al., 1981) which can be co-extracted with an unusual ornithine containing lipid component (Holst et al., 1983). This may be identical to one of the numerous triterpenoid compounds recently identified in this microbe (Howard et al., 1984; Neunlist et al., 1985). Triterpenoids of the hopane family are arousing increasing interest as structural equivalents and phylogenetic precursors of sterols in prokaryotic cells, and Rm. vannielii appears to be a good source of such compounds (Neunlist et al., 1985). Their structural characteristics suggest they could play a role as membrane reinforcers, a concept which has received some experimental support (Kannenberg et al., 1985). From the viewpoint of permeability, it is perhaps significant that hopanoid derivatives can increase molecular order in various lipid systems by strengthening interactions between lipid molecules and have been shown to decrease the permeability of glycerol through phosphatidylcholine bilayers in vitro (Kannenberg et al., 1985). As pointed out by Neunlist et al. (1985) hopanoids are amphiphilic molecules and can be present in the inner or outer membranes of Gram negative bacteria, although, as yet, their precise intracellular localization is not known in any species.

This constellation of properties could make the hopanoids attractive candidates to explain the remarkable permeability properties of Rm. vannielii but it is clear that more detailed investigations of these and the LPS and outer membrane components will be needed in order to arrive at a satisfactory conclusion.

3.4.3 The involvement of the cell wall in swarmer cell differentiation - the detection of penicillin binding proteins in *Rm. vannielii*

3.4.3.1 Introduction. The process of flagella shedding, followed by the initiation of prostheca synthesis, must result in a considerable amount of "restructuring" of the cell envelope. Preliminary studies (Dow, unpublished) demonstrated a marked uptake of the peptidoglycan constituent diaminopimelic acid to occur after the loss of motility, suggesting major changes in the structure of the cell wall. Alteration of the peptidoglycan structure might be expected a priori because of the unique shape requirements of the prostheca. One way to probe possible changes in peptidoglycan structure is to assay the enzymes involved in such cell wall modification. Many of these specifically bind penicillin, which inhibits the terminal transpeptidation reactions in Gram negative bacteria (Blumberg & Strominger, 1974). Penicillin binding proteins may be bound to the cytoplasmic membrane or they may occur in the soluble fraction of the cell. Therefore, cell-free extracts were used in this study.

3.4.3.2 Results. Preliminary experiments to assay PBPs by the incubation of ¹⁴C-benzylpenicillin with membranes or cell-free extracts of *Rm. vannielii* were largely unsuccessful because of the low specific activity of this probe. However, using ³H-benzylpenicillin combined with fluorography, the sensitivity was increased sufficiently to allow their detection. Figure 3.59 shows the pattern of PBPs present in cell-free extracts of *Rm. vannielii* prepared at various stages through the swarmer cell-cycle. Five major species were detected, all with rather high M_r values. Two of these (M_r 70,000 and 96,000) were far more

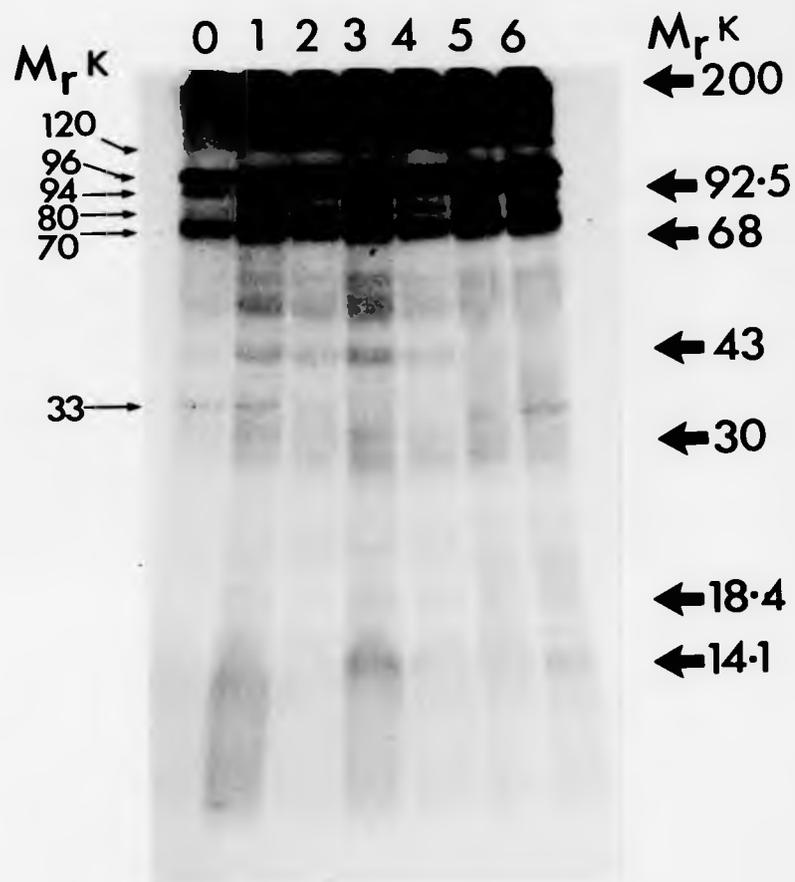


FIGURE 3.59. Penicillin-binding proteins in cell-free extracts of *Rm. vannielii*.

Samples taken at hourly intervals from a synchronously differentiating swarmer cell population (0-6 h) were used to prepare cell-free extracts (section 2.13). Penicillin binding proteins were detected in the extracts as described in section 2.29 using ^3H -benzylpenicillin and the figure shows the resulting fluorogram after 6 months exposure. Equal quantities of protein (300 μg) were loaded in each track.

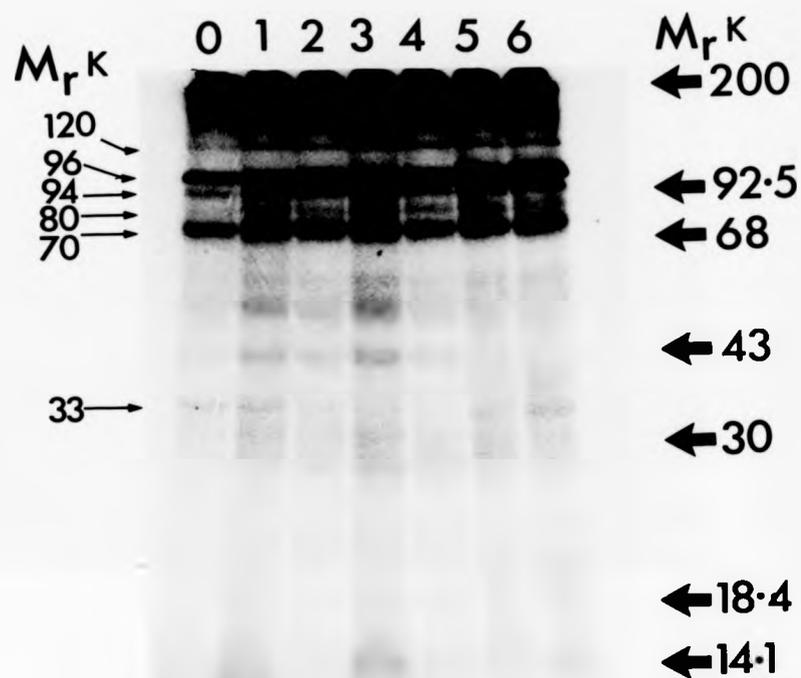


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heavily labelled than the other proteins. No changes were detectable in the types of these major PBPs during the cell-cycle as evidenced by their largely constant band intensity when equal protein loadings were applied to the gel. A number of other minor radiolabelled bands were also detected on the gel and at least one of these (M_r 33,000) appeared only to be present in swarmer cells and again at 5-6 h when some newly produced swarmer cells may have been formed from the growing multicellular arrays.

3.4.3.3 Discussion. The pattern of the penicillin binding proteins detected in Rm. vannielii was rather unusual compared to several other bacterial species. In the only other photosynthetic bacterium examined to date, Rb. sphaeroides (Shepherd et al., 1981), three major and several minor bands were detected using an ^{125}I -labelled furazlocillin probe. The major species corresponded in terms of M_r value to the PBP 1A/1B (M_r 90,000), PBP5 and PBP6 (M_r 45,000) of E. coli and, indeed, the overall pattern of those PBPs detected was remarkably similar in the two organisms. The major, low M_r PBP5 in E. coli has been identified as a D-alanine carboxypeptidase (Nishimura et al., 1980) and analogous proteins have been found in a variety of gram negative (Curtis et al., 1979) and Gram positive bacteria (Coyette et al., 1980; Murphy et al., 1981; Todd et al., 1983). In contrast, the "major" PBPs detected in Rm. vannielii were of a much higher M_r value, although a 33,000 M_r PBP may be regulated during the swarmer cell-cycle. One explanation for this unique complement of PBPs could be the obligate polar growth pattern of this microbe and comparison with some other obligately polar growing photosynthetic bacteria such as Rp. viridis or Rp. palustris would prove interesting in this respect. In the non-photosynthetic prosthecate bacterium Caulobacter crescentus the stalk peptidoglycan is richer in

glucosamine (Poindexter & Hagenzieker, 1982) and has a different PBP complement than the cell body (Koyasu *et al.*, 1983) but the stalk in this microbe is not involved in reproduction and so may not be equivalent in structural terms to that in *Rm. vannieli*. Nevertheless differential PBP localization is an attractive way of explaining how, for example, the initiation of prostheca outgrowth can occur at a defined site. Thus, although few gross changes in the pattern of PBPs could be detected during differentiation, this "topological control" exerted by the spatial localization of the enzymes could also be important in remoulding the sacculus.

Additional controls such as the possibility of modulation of enzymic activity by covalent modification or changes in the rates of synthesis of particular PBPs may also be posited. However, these are difficult to verify because of the rather low copy number of PBPs in bacterial cells (Blumberg & Strominger, 1974). Despite these considerations, marked changes in the gross pattern of PBPs have been identified during sporulation in *B. subtilis* (Todd *et al.*, 1983) and have been attributed to involvement in biosynthesis of the unique spore coat peptidoglycan. Clearly, more work is warranted on the role of PBPs in the morphogenesis of *Rm. vannieli* and related species.

4. OVERALL CONCLUSIONS AND OUTLOOK

In this thesis, I have chosen to concentrate upon essentially two aspects of the physiology of Rm. vanniellii, related to photosynthesis and differentiation. Throughout the study, attention has been drawn to the similarities or differences which exist between this microbe and other photosynthetic and/or prosthecate budding bacteria in the general area of membrane physiology. This was considered important, not merely as a means of cataloguing specific peculiarities, but to emphasise the general implications arising from cell-type heterogeneity as a consequence of polar growth and hence cellular asymmetry.

There has been a general trend in recent years of classifying members of the Rhodospirillaceae according to rather few but nevertheless "phylogenetically significant" phenotypic characteristics, in particular the arrangement of the ICM system and the mode of envelope growth. This stemmed from the opinions of Pfennig (1977) who identified three "species groups" of the genus Rhodopseudomonas (sic) in which the sphaeroides - capsulata group (vesicular ICM, non-budding) were sharply separated from the palustris - acidophila group (lamellate ICM, budding) with Rp. gelatinosa (sic) forming the third cluster, and has culminated in the proposal (Imhoff et al., 1984) for the establishment of new genera (Rhodobacter, Rhodopseudomonas and Rhodocyclus respectively) as discussed in section 1.1. Whilst this arrangement may be justified in taxonomic terms, it does tend to obscure the fact that there exists a spectrum of behaviour within this family, not only in terms of the gradual change in morphological complexity as discussed in section 1, but also in terms of physiological properties. Thus, the "morphological gradient" (Whittenbury & Dow, 1977; Kelly & Dow, 1984; section 1.5.2) can also be viewed as a "physiological gradient". How far do some of the observations made in this study support this conclusion?

In terms of its overt phenotypic properties, Rm. vanniellii appears to represent an extreme case; it expresses the most complex and diverse cell-cycles and cell-types but nevertheless exhibits some degree of "metabolic inflexibility". Moreover, there appears to be a general correlation between increasing morphological complexity and decreasing metabolic flexibility through the sequence: Rb. capsulatus/Rb. sphaeroides - Rp. blastica - Rp. acidophila - Rp. palustris - Rp. viridis - Rm. vanniellii. A specific example of this concerns the sensitivity to oxygen and the capacity for aerobic growth in the dark. Rhodopseudomonas viridis shows many of the same features as Rm. vanniellii in this respect (Pfennig, 1978) but contrasts with Rb. capsulatus and Rp. blastica, the latter being able to grow faster under aerobic-dark conditions (Eckersely & Dow, 1980) in addition to being remarkably insensitive to the potentially lethal effects of light and O_2 .

As far as it is possible to make conclusions, two other properties of particular concern in this study - the structure of the photosynthetic apparatus and whole cell permeability also change according to a spectrum of behaviour in the Rhodospirillaceae. The change from vesicular to lamellate ICM arrangements has been well documented previously and correlates with the apparent change in growth mode in the "budding" species. The concomitant appearance of the use of membrane bound c-type cytochromes, in particular in Rp. viridis and Rm. vanniellii, may be related to this and might merit further investigation

In terms of its overt phenotypic properties, Rm. vanniellii appears to represent an extreme case; it expresses the most complex and diverse cell-cycles and cell-types but nevertheless exhibits some degree of "metabolic inflexibility". It may therefore lie at the "end-point" of the spectrum observable within the Rhodospirillaceae. From a teleological viewpoint such metabolic characteristics may be the price to be paid for a variety of cellular expressions which allow dispersal and survival in environments possibly unsuited for less well endowed species. Moreover, there appears to be a general correlation between increasing morphological complexity and decreasing metabolic flexibility through the sequence: Rb. capsulatus/Rb. sphaeroides - Rp. blastica - Rp. acidophila - Rp. palustris - Rp. viridis - Rm. vanniellii. A specific example of this concerns the sensitivity to oxygen and the capacity for aerobic growth in the dark. Rhodopseudomonas viridis shows many of the same features as Rm. vanniellii in this respect (Pfennig, 1978) but contrasts with Rb. capsulatus and Rp. blastica, the latter being able to grow faster under aerobic-dark conditions (Eckersely & Dow, 1980) in addition to being remarkably insensitive to the potentially lethal effects of light and O₂.

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in Rb. palustris and Rb. blastica.

However, despite these correlations, there does exist some strong evidence that cell growth in at least some strains of Rb. sphaeroides is not by a simple diffuse, intercalary mechanism but that some form of polar growth may occur (Gest et al., 1983; Pellerin & Gest, 1983). Cells of such strains divide asymmetrically with one progeny "daughter" cell being motile by means of a sub-polar flagellum, in addition to displaying a different morphology to that of the "mother" cell which is non-motile. If this phenomenon is of general occurrence, it will have important implications because batch cultures will be heterogeneous with respect to cell-type and induction synchronization procedures - as presently used with Rb. sphaeroides - could have different effects on each cell population. It may, however be possible to devise selective synchronization methods to recover one cell-type alone. It will also prove enlightening to assess how far the properties of the motile cells of such strains of Rb. sphaeroides resemble those of "swarmer cells". This is especially important, because it would indicate the swarmer cell to be a more universal cellular expression than is presently acknowledged.

With Rm. vannieli as an example it seems clear that the morphologically different cell-types characteristic of cultures of photosynthetic prosthecate and budding bacteria are distinct with respect to several aspects of membrane physiology. Previous studies of asynchronous batch cultures of such organisms, for example Rb. palustris (Varga & Staehelin, 1983, 1985a,b) have only given averaged values for the whole population. It thus becomes important that cell-type heterogeneity be taken into account in studies on photosynthetic bacteria, especially

since the kinetics of and mechanisms for the assembly of the photosynthetic apparatus may not be the same in the different cell-types.

Similar reasoning also applies to studies in which the permeability barrier of the cell is involved, as the sensitivity of cell-type sub-populations may differ in this respect, as appears to be the case with Rm. vanniellii. Here again, this microbe appears to be at one end of the spectrum with somewhat surprising permeability properties certainly uncharacteristic of the Rhodobacter group.

In conclusion, Rm. vanniellii clearly demonstrates that despite apparent fundamental similarities in the cell-cycles and physiology of many members of the Rhodospirillaceae, individuality is expressed in the precise nature of the interactions between photosynthesis, cell growth and differentiation. In terms of studying these interactions, particularly the development of the photosynthetic apparatus in selection synchronized culture, the photosynthetic prosthecate and budding bacteria appear to possess distinct advantages and as such will assume increasing importance in the future as alternatives to Rb. sphaeroides and Rb. capsulatus, where culture heterogeneity may be problematic.

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6. LIST OF PUBLICATIONS

Some of the work and ideas in this thesis have been prepared for publication.

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