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PHOTOSYNTHETIC MEMBRANE MORPHOGENESIS  
IN RHODOPSEUDOMONAS SPHEROIDES

by

MICHAEL ALEC SHAW

B.Sc., London University, 1965

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY  
in the Department  
of  
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## Abstract

The pigmented membranes of photosynthetic bacteria perform functions that are analogous to those associated with the chloroplasts of eucaryotic cells. However, the composition and morphology of the bacterial organelles are by no means as complex. Thus the non-sulphur purple bacteria provide a good model system for studying the process of organelle morphogenesis. An additional advantage is that the morphogenesis can be externally controlled by varying the oxygen supply.

Rhodospseudomonas spheroides N.C.I.B. 8253 was grown at high levels of oxygen, and photosynthetic membrane synthesis was induced by reducing the oxygen supply. At various times during the adaptation, cells were harvested and disrupted. The subcellular material was fractionated by linear sucrose density gradients. During the early stages of adaptation, bacteriochlorophyll did not appear in the gradient position typical of the normal photosynthetic membrane (the chromatophore fraction), but in another fraction termed the prephore fraction. This new fraction decreased as the chromatophores developed, and thus appeared to be a chromatophore precursor. This was later supported by a ( $U-^{14}C$ ) proline labelling experiment. Electron microscopic examination, after negative staining, showed this fraction to consist of distinctive spherical structures. The prephore fraction

could be produced by various disruptive procedures and could also be separated from the other subcellular components by gel filtration.

The precursor particles were shown to dissociate into subunits. These had a simple protein composition, being composed, to a large extent, of three proteins with weights of 11, 27, and 40 + or - 2 kilodaltons. The subunits, approximately 10 nm in diameter, were shown to hydrophobically reaggregate to produce the typical prephore fraction. Parameters controlling the synthesis of the prephore material were determined, and methods of producing fractions in both the pigmented and non-pigmented state were developed.

The nature of the other fractions present in the sucrose gradient were also investigated. The protein composition of each fraction was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. In agreement with previous work, it was shown that the "heavy" (mature) chromatophores resulted from the aggregation of the "light" chromatophores; in addition, however, aggregation of a small pigmented subunit, derived from the mature chromatophore, contributed to the "heavy" chromatophore fraction.

A discrete component, termed the medium density fraction, possessed a protein composition which, if combined with the prephore subunit proteins, produce a

profile very similar to that of the mature chromatophore. This fraction appeared in electron micrographs as membranous sac-like structures. A proposal that the medium density fraction and the prephore subunits combined in vivo to form the mature chromatophores was subsequently supported by a double labelling experiment. This study also indicated that the medium density fraction was derived from the aerobic membrane, and the prephore subunits were synthesised de novo.

The site of the prephore subunit synthesis was shown to be, most probably, the periplasmic space. When chromatophore synthesis was limited, cells excreted pigment bound to lipoproteins. The protein composition of this complex was very similar to that of the prephore subunit. The process of adaptation is now believed to proceed in the following manner: a reduction in oxygen supply induces the preferential synthesis of the subunits. When pigmented, the subunits combine with a membranous derivative of the aerobic invaginations frequently seen in electron micrographs. This combination, together with additional bacteriochlorophyll synthesis, results in the formation of the bacterial photosynthetic organelles.

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List of Non-standard Abbreviations Used

Bchl	Bacteriochlorophyll a
CSU	Crude Subunit Fraction
CTAB	Cetyl Trimethylammonium Bromide
LC	Light Chromatophore Fraction
LDAO	Lauryl Dimethylamine Oxide
LH	Heavy Chromatophore Fraction
MDF	Medium Density Fraction
MSU	Mature Subunit Fraction
PHB	Poly-hydroxy Butyric Acid
PAGE	Polyacrylamide Gel Electrophoresis
S.C.F.H.	Standard Cubic Feet per Hour
SDS	Sodium Dodecyl Sulphate

## Chapter I.

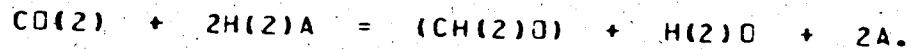
### Introduction.

#### 1. The Development of Interest in Photosynthetic Bacteria.

Pigmented bacteria appear to have been of no special interest before the studies of Engelmann in the 1880's. The pigment (the entire pigment system) had been found to be complex and was termed bacteriopurpurin by Lankester in 1873. These bacteria attained a unique status with Engelmann's finding that Bacterium photometricum was capable of photosynthesis and could utilize near infrared light. Subsequently numerous species were identified and media developed for the enrichment of particular organisms. Two main groups of pigmented bacteria emerged; the sulphur bacteria, both purple and green, capable of growing photosynthetically oxidising sulphur compounds and the non-sulphur purple bacteria which required oxidisable organic compounds. In 1907, Molisch proposed the purple bacteria be combined into the order Rhodobacteria and that it be divided into two families, Thiiorhodacea and Athiorhodacea. The green sulfur bacteria were placed in the Chlorobacteracea family. The majority of Athiorhodacea were found to be facultative phototrophs and were observed to lose their pigmentation when grown aerobically with organic substrates. A comparison of the



pigments extracted from bacteria with those from plants showed the chlorophyll and carotenoids to be similar [Schneider (1934), Fischer and Hasenkamp (1935) and van Niel and Smith (1935)]. These findings along with the extensive physiological studies made by van Niel (1941) allowed him to develop a concept which unified the process of photosynthesis in bacteria and green plants. The now classical formula for the overall process of photosynthesis was proposed:



Van Niel further believed that the photolysis of water was central to all processes of photosynthesis.

These ideas triggered a new interest in these bacteria. Although photolysis of water is not involved with bacterial photosynthesis, many features have been shown to be very similar. Van Niel's studies may have been responsible for the present widespread use of these bacteria in photosynthetic research.

## II. The Discovery of Photosynthetic Organelles in Bacteria

In 1925 Wurmser et al had showed that grinding purple bacteria released a water soluble pigmented protein. Lumbmenko (1921) had suggested that the pigments were protein bound in the cell to explain the change in

absorption maximum observed after extraction of the pigments by organic solvents. Katz and Wassink (1939) and French (1940a) also found that disruption of cells produced a soluble complex with no change in spectral properties. Still further evidence that the pigments existed in combination with protein was provided by French (1940b) in showing the pigments to be precipitated with protein by ammonium sulphate. Pardee et al (1952) appear to have made the first full investigation of material released from bacteria by grinding with alumina. These workers were surprised to find, after an ultracentrifugation study, that the pigment of Rhodospirillum rubrum (fam. Athiorhodaceae) was bound to large particles. They estimated the molecular weight to be 30 million daltons. They had expected to find a soluble low molecular weight pigment-protein complex, as initial light microscopic examination had shown the pigment not to be localized as was the case in algae. Subsequent electron microscopic examination showed, however, disk-shaped structures with an apparent diameter of 110 nm. As the sedimentation data gave a minimum value of 40 nm they assumed the disks were derived from flattened spheres of about 60 nm. They stated "Since they (the spheres) do not possess the structural complexity of typical chloroplasts, it seems most appropriate to designate them as 'chromatophores'". This work was

described in more detail in a second paper (Schachman et al, 1952).

Schachman et al (1952) had suggested that the true structure of these intracellular macromolecules could be obtained by a careful comparison of extracts prepared using different disruptive techniques. Newton and Newton (1957) found that three disruptive techniques produced the same chromatophores from Chromatium strain D (fam. Thiorhodaceae). In addition they observed that homogenization of chromatophores in media of low ionic strength released protein and pigment. They estimated the diameter of Chromatium chromatophores to be about 100 nm; however, after sonication about 25% of the pigment was contained in particles only about 40 nm in diameter. The release of chromatophores was maximum during the early period of cell rupture but the smaller particles became more abundant with increasing sonication. They suggested that the smaller particles were produced by degradation of the chromatophores. This explanation does not, however, account for the smaller '55S' component observed by Schachman et al (1952). These workers found that when R. rubrum was grown aerobically in the dark, chromatophores were not produced but smaller pigmented structures were found. The significance of these particles was not understood. The interest shown in chromatophores, following their discovery, was probably partially due to

the demonstration of their photophosphorylating ability, [Frenkel (1954) and Kamen and Vernon (1954)]; they were thus functional in vitro. Thus it was induced that the chromatophore was a membranous vesicle in vivo and was a structure characteristic of photosynthetic bacteria.

### III. Ideas as to the Structure of Photosynthetic Membrane in vivo

Advances in electron microscopic techniques allowed Vatter and Wolfe (1958) to examine sections of photosynthetic bacteria. Photosynthetically active cells of R. rubrum contained numerous membrane bound vesicles 50 to 100 nm in diameter. These were not present in aerobically grown cells. The vesicles of Rhodopseudomonas spheroides (fam. Athiorhodacea) were slightly smaller than those of R. Rubrum (40 to 80 nm in diameter). The obvious conclusion was that the structures seen in cell sections were equivalent to those isolated by Schachman et al in 1952. Karunairatnam et al (1958) found that removal of the cell wall left pigmented protoplasts. Their claim that chromatophores were bound to the cytoplasmic membrane was supported by Tuttle and Gest (1959) who found that osmotically lysed protoplasts did not release chromatophores. The question then arose as to how these studies could be reconciled with the results of Vatter and Wolfe (1958), which showed chromatophores to be dispersed

throughout the cytoplasm.

The question as to whether the photosynthetic apparatus consisted of a membranous continuum or discrete bodies was tackled by Boatman (1964). Sections of spheroplasts of R. rubrum allowed the connections between chromatophores and the cytoplasmic membrane to be visualized. Connections between chromatophores were seldom seen. Boatman suggested that if such connections existed, they were either small, or broken during sample preparation. Thus the problem was only partially resolved. Since the evidence for a continuum was based on the results of osmotic lysis of protoplasts, the possibility existed that chromatophores remained trapped inside the cytoplasmic membrane. Neither Fuller (1963) nor Gorchein (1968c) was able to exclude this possibility. Further, Gibson (1965a) showed that numerous factors would cause chromatophore aggregation. Work with R. spheroides demonstrated that the standard osmotic lysate would not produce non-aggregated chromatophores even after passage through a French pressure cell. This was also found to be the case by Gorchein (1968c). It thus threw doubt on the evidence of a continuum based on osmotic lysis techniques. There were, however, other lines of research that supported the concept of a continuous membrane system. Cohen-Bazire and Kunisawa (1963) found that brief sonication of R. rubrum yielded

inhomogenous pigmented material while a longer treatment increased the yield of typical chromatophores. They found that both the cytoplasmic membrane and the vesicles had a similar appearance in section, and further, that peripheral vesicles were continuous with the cell membrane. These workers believed that free chromatophores might be formed by breakage from the cell membrane at points of weakness. Perhaps the strongest evidence for an interconnected system was produced by Holt and Marr (1965a and b). They argued that if chromatophores were free in the cell, they should be released at a rate equal to that of cell disruption. Both sonication and ballistic disintegration studies showed this was not the case. Using stereo-electron microscopy, connections between the vesicles of R. rubrum could be seen. Tubules could be seen originating from the periphery of the cell and occasionally bulging to form spherical vesicles. The cells used for this study were, however, subjected to sonication to remove the cytoplasm so artefacts might have been produced.

There have been few ideas as to the nature of interconnections between vesicles, although the assumption has been generally made that the connecting membranes has a similar composition to the chromatophores. Gibson (1965a) found that although irreversible aggregation of R. spheroides chromatophores was produced by the standard

osmotic lysis procedures, the cells could be lysed osmotically under conditions that did not result in chromatophore aggregation. With the latter technique, however, still only about 5% of the pigment was released as chromatophores. This led Gibson to suggest (1965b, c) that chromatophores were either aggregated *in vivo* or that pigmented core particles existed in a lipid matrix. The work of Gorchein (1968c) supports the concept of some type of matrix. Electron microscopic examination of membranous fragments from lysed R. spheroides cells, after negative staining, showed discrete areas with different staining characteristics than the surrounding material. The finding that fenestrations of similar size to these areas also were present caused him to postulate that these areas were chromatophores in situ. It thus appears as if chromatophores do exist in a matrix, possibly of high lipid content. This would explain the absence of interconnections in most electron microscopy studies; the lipid being lost during the embedding procedure. The question, as to the in vivo state of the photosynthetic membranes of R. spheroides and R. rubrum, clearly is not yet fully resolved, and the possibility remains that there are significant differences between these two species.

#### IV. Terminology

Since the original term "chromatophore" was

introduced several alternatives have been suggested. Kamen (1963), finding chromatophores to be produced as discrete bodies only after vigorous cellular disruption, proposed the term "chromatophore fragment". This term has not been widely adopted, perhaps because of connotations with subchromatophore particles. In the same year Menke suggested the term "thylakoid" to describe the lamellar structure seen in some photosynthetic organisms. This term is widely used to describe in vivo pigment bearing membranes in plants. The term "pigmented membrane fragments" was proposed as an alternative to chromatophores by Stanier (1963).

I favour the terminology suggested by Peters (1970). The particulate pigmented material, isolated after disruption, is termed the chromatophore fraction. The membrane bound system of internal vesicles and invaginations of the cytoplasmic membrane, as observed in cell sections, is termed the thylakoid. I will refer to an individual pigmented vesicle in vivo as a chromatophore and employ the term thylakoid to describe the entire in vivo network.

#### V. Is the Chromatophore Pigment Content Constant?

The classic work of Cohen-Bazire et al (1957), showed the pigment content of non-sulphur purple bacteria to be related to light intensity and oxygen content of the



medium. The question then arose as to whether increased pigmentation resulted from increased thylakoid membrane, an increased pigment content of the membrane, or from both mechanisms. Cohen-Bazire and Kunisawa (1960) approached this problem by analysis of different chromatophore fractions isolated from cells of different pigment content. They found cells yielded, depending on the mode of disruption, between one third and one half of their chlorophyll as a crude chromatophore fraction. They concluded that the number of chromatophores per cell was essentially constant and that they varied in chlorophyll content. Their finding in 1963 was, however, contradictory. Examination of thin sections of R. rubrum showed an increase in chromatophores with increased chlorophyll content of the cell. This conflict between the two types of evidence was also found in R. spheroides (Bull and Lascelles 1963) and in Rhodospirillum rubrum by Gibbs et al (1965). Worden and Siström (1964), however, found that the yield of chromatophores, increased with the cellular pigment content. This was supported by Holt and Marr in 1965. These workers found that cells emptied of cytoplasm yielded membranes with a constant chlorophyll content. They concluded that cellular pigmentation in R. rubrum was regulated by changes in the quantity of membranes of a fixed composition. They attributed the high protein-to-pigment

ratio, found by previous workers with cells of low pigment content, to have been due to contamination by ribosomes. The previous paradox was investigated in R. spheroides by Gorchein (1968b). His work supported that of Holt and Marr (1965); the thylakoid system appeared to be a stoichiometric complex of proteins and pigments. Gorchein showed that, unless carefully purified, the chromatophore fraction from cells (especially those of low pigment content) were contaminated by cell wall fragments, ribosomes and cytoplasmic membrane. Ketchum and Holt (1970) found an exception to the constant ratio of pigments and proteins in a purified chromatophore fraction. Stationary phase R. rubrum cells show twice the concentration of chlorophyll normally characteristic of chromatophores from growing cells. They suggested that this was the result of preferential protein synthesis inhibition in the stationary phase. Their work implied that the concentration of pigment in the chromatophore is not regulated by stoichiometric considerations but by a close coupling of pigment and protein synthesis. Such a coupling had been demonstrated by Bull and Lascelles (1963) and more recently by Brown and Lascelles (1972).

#### VI. Heavy and Light Chromatophores

Many workers have observed that the centrifugation of disrupted cell membranes in a sucrose density gradient

results in the production of two pigmented bands. Cohen-Bazire and Kunisawa (1960) described the bands produced by material from R. rubrum as the "heavy particle fraction" and the "purified" chromatophore fraction. The heavy fraction scattered light and contained a lower specific chlorophyll content than the "purified" band. However, this fraction always contained at least half the pigment in the gradient. Treatment with pancreatic lipase followed by recentrifugation was shown to result in the formation of more "purified" (light) chromatophore fraction. This treatment caused the loss of the photophosphorylation activity, so the initial light chromatophores were considered the most highly purified functional material. Worden and Siström (1964) working with R. spheroides noted that the position of the heavy fraction in the sucrose gradient varied depending on the growth conditions they had used. Analytical ultracentrifugation showed that, unlike the light fraction, this fraction was heterogeneous. It was found to contain a large amount of RNA and higher levels of Bchl relative to the carotenoids. Interestingly, the long wavelength form of Bchl (8870) was enriched in the heavy fraction; yet conversion, by French pressure cell treatment, to the light fraction eliminated this difference. The spectral differences between the two fractions were at that time not believed to be artefacts

since they appeared to be intrinsic properties of these materials. They concluded that the photosynthetic apparatus has two distinct forms within the cell. They suggested that the heavy fraction was derived from the cell membrane as it predominated in cells of low pigment content. This rationalized the production of light chromatophores from this fraction but does not seem totally consistent with their finding that a mutant strain, (Ga) produced only small amounts of heavy fraction. Holt and Marr (1965) working with R. rubrum showed that the heavy fraction contained large membrane fragments and almost intact cell envelopes. Thus this fraction contained, rather than was derived from, cell membrane. Gibson (1965b) provided an explanation as to why the chromatophores sedimented with two apparent densities. He demonstrated that the heavy fraction obtained by previous workers was produced by chromatophore aggregation caused by divalent cations. Centrifugation using the same procedure as had been used by Worden and Sistrom, but with the omission of magnesium, produced a single (light) band. The work did not explain the different spectral properties of the two fractions but clearly demonstrated that if two forms of chromatophore existed the difference was in susceptibility to aggregation, not in buoyant density. This finding, however, did not inhibit further study of the heavy fraction. Ketchum and Holt (1968) produced a

heavy fraction using either sonication or osmotic shock treatment of light chromatophores, followed by recentrifugation on a sucrose gradient. They observed that protein was released from the light fraction material at the same rate as the heavy fraction was formed. This was interpreted as evidence for the chromatophores to be three-dimensionally complete vesicles. They did not show, however, that they had not increased the susceptibility of chromatophores to aggregate. Hansen and De Boer (1969) believed that cation concentration was not the most critical factor in heavy fraction formation. These workers believed that the pelleting of material prior to gradient centrifugation was the major cause. This was supported by the isolation by Ketchum and Holt (1970) of a heavy fraction in the absence of magnesium, using French pressure cell disrupted R. rubrum. The Bchl content of the fraction was reduced however. A similar finding was made by Niederman and Gibson (1971) with R. spheroides. In the presence of ( $10^{-2}$  M) magnesium-ion, pigment was distributed almost equally between the heavy and light fractions. In the absence of magnesium-ion, half the chromatophores were lost from the heavy fraction; further omission of Tris buffer ( $10^{-2}$  M) resulted in a total loss of chromatophores from the heavy fraction. All that remained was cell envelope material. Single chromatophore bands have also been produced by other types of gradients.

Gibson (1965a) used cesium chloride while Deize et al (1969) used Ficoll.

It appears, therefore, that there is only one type of chromatophore fraction. Much of the confusion in the literature regarding the nature of subcellular fractions stemmed from the diverse preparative techniques employed. Although correlation between results of different groups was made difficult, the problems caused the evolution of techniques producing good separation of subcellular structures. Niedermann and Gibson (1971) showed that highly purified ribosomal, chromatophore and cell envelope fractions could be isolated using a single continuous sucrose density gradient. A good separation of chromatophores from cell membrane is a prerequisite for the investigation of the relationship between these components.

## VII. Subchromatophore Particles

Many membranes have been shown to possess a subunit structure by X-ray and freeze etching studies. The existence of such subunits is obviously of great interest with regard to chromatophore morphogenesis. I shall briefly review evidence for the existence of subchromatophore particles either found after cell disruption or observed in situ. I feel that such particles are better candidates for in vivo subunits than

those produced by chaotropic agents, as the latter may not be natural associations of protein components. Comparative studies with a variety of chaotropic agents have been of great value in understanding chromatophore structure and will be discussed in the next section.

Newton and Newton (1957) proposed that the chromatophores of Chromatium consisted of identical macromolecular subunits. Sonication, grinding with glass beads or Hughes press treatment converted the 100 nm chromatophores to 40 nm particles. A five minute sonication converted about 25% of the chromatophores to this relatively large subunit. In 1959 Bergeron proposed that subunits were held together by disulphide bonding. This was supported by Newton (1960) after an immunological study of R. rubrum chromatophores. Low and Afzelius (1964) examined R. rubrum chromatophores by electron microscopy after negative staining. These chromatophores were covered with stalked particles of the same dimensions as the "elementary" particles of Green (1964). It seemed probable that the chromatophores were composed of several types of subunit.

In 1964, X-ray studies by Langridge et al indicated that R. spheroides chromatophores were hollow spheres with a mean shell radius of 29.5 nm. The data was further interpreted to suggest that the shell consisted of 5 nm subunits. Similar 5 nm particles were reported by Holt

and Marr (1965a) during studies on R. rubrum chromatophores by electron microscopy. These particles were linearly spaced at 10 nm intervals. As these workers included magnesium ions in their buffer solutions it seems possible that these particles may have been "elementary" particles. They speculated that the particles might be the loci of the photosynthetic pigments. Interest in chromatophore substructure was stimulated by the report of chloroplast subunits, the quantosomes of Park and Biggins (1964).

A pigmented particle much smaller than the chromatophore was described by Gibson (1965a). In material produced by French pressure cell disruption of anaerobic cells about 15% of the pigment sedimented with these structures. A larger percentage was noted in material prepared from cells adapting semiaerobically in the dark. Gibson suggested that this might indicate that chromatophores formed under these conditions may have been more susceptible to comminution than "anaerobic" chromatophores. It is tempting to speculate that these "semi-aerobic" subunits might be similar to the subunits described in this thesis. An interesting study of the subunits of the purple sulphur bacterium Thiocapsa floridana was made by Takacs and Holt in 1971. Freeze-fractured chromatophores showed 6 - 8 nm particles on the concave surfaces, while the convex surfaces were



relatively smooth. In an accompanying paper they describe the complete solubilization of the membrane into subunits followed by the reassociation into structures morphologically and functionally resembling the original chromatophores. The subunits were of the same size as those shown in the freeze-fracture study. Each subunit was itself composed of five spherical subunits with an average diameter of 2.0 nm. Their technique shows great potential in investigating chromatophore structure and perhaps morphogenesis.

Reed and Raveed<sup>2</sup> (1972) probably made the most detailed study of the chromatophore subunits in situ. They found that removal of the 9 nm ATPase particles had no apparent effect on the remaining subunit structure. Freeze fracturing the chromatophore membranes of R. spheroides R26 showed that a layer of 13 nm particles existed directly above a sheet of 5 nm particles. The larger subunits were of the same size as the purified reaction-center particles they had previously isolated (Reed et al 1970). The 5 nm units had been shown to contain the light-harvesting pigment and were clearly the particles described by Menke (1967) and Langridge et al (1964).

It would thus appear that there is little evidence for the existence of subunits as free entities in the cell. Chromatophore subunits, however, have been shown to

exist in situ. Their arrangement in the chromatophore membrane and functions have been now determined largely as a result of work of Reed and co-workers.

#### VIII. Further Work Towards a Model of the Chromatophore Structure

The building of a model of the chromatophore membrane requires two types of data. The composition must be known and the location of the constituents within the structure must be determined. The compositional analysis can be divided into two classes: broad quantitative studies and detailed assays for minor components and individual enzymes. This thesis is concerned primarily with the structural aspects of the chromatophore rather than its functioning. Minor components, such as ornithine lipid, will only be discussed in reference to their proposed structural or morphogenic functions.

Most workers have obtained data with different objectives in view; this makes for an untidy historical account. I propose to simply do a review by listing different types of data obtained with only one organism, R. spheroides (Table I). The use of a wide variety of organisms has diluted the data that can be used for building a model but provides a means of viewing photosynthetic bacteria with greater perspective. Another problem was pointed out by Lascelles in her review "The

Table I

Gross compositional data for the chromatophores of R. spheroides.

Component	Values	*	
Size (diameter)	40-80 nm	f	
	57 ± 3% (nm)	c	
	60 nm	g	
Wall thickness	6 nm	g	*References
	8 nm	e	
Weight (dry)	30,000 kD	c	a Bull and Iscelles (1963)
	38,000 kD	g	
Weight of protein (dry) as a % of total weight.	18,000 kD	c	b Fraker and Kaplan (1971)
	64%	b	c Gibson (1965 a and b)
	62%	d	d Gorchein (1968a)
	58%	a	e Menke and Weichan (1968)
	74% (28,000 kD)	g	f Vatter and Wolfe (1958)
Lipid Phospholipid as a % of total weight	3.6 mg/mg Bchl	c	g Worden and Sistro (1964)
	25%	b	
	20%	d	
	25%	a	
Carbohydrate as a % of total weight	1.1 mg/mg Bchl	c	
	4%	d	
	4.2%	a	
Bchl (Variable depending on light intensity)	4.6%	b	
	7.3%	d	
	4.3%	a	
	16%	g	
Carotenoids as a % of total weight	1.1%	a	
	5%	g	
Nucleic acids as a % of total weight	0.94%	a	
	0.15%	d	
	0.38%	b	
	0.005 mg/mg Bchl	c	

Bacterial Photosynthetic Apparatus" (1968); this is the lack of firmly established criteria for assessing chromatophore fraction purity. Differences in disruption techniques, harvesting procedures and buffers can profoundly alter the results, as mentioned previously. This has the advantage, however, of making agreements in data from different groups more valuable and disagreements useful in pointing to procedural artefacts.

Perhaps some of the most valuable work with regard to the structure of the chromatophore has been performed with detergents. Procedural variations are particularly pronounced, however, and have hindered the correlation of results needed for building a consistent model of the chromatophore. A model will be described based on that proposed by Sjooten (1972). It will be correlated to the subunit structure determined by Reed and Raveed (1972). For a broader picture of the properties of the bacterial chromatophore the reader is referred to reviews by Lascelles (1968) and Delze and Drews (1972).

Langridge et al (1964) attempted to unite quantitative data with the overall dimensions of the chromatophore obtained from X-ray scattering data. Little information was obtained by this approach but it was useful in that it was shown that the chlorophyll molecules could not be accommodated as a monolayer. They also observed that the number of photo-bleachable

bacteriochlorophyll molecules (P870) were constant on a chromatophore basis although the light harvesting form varied. It was proposed that chromatophores contained a fixed number of photosynthetic units with a constant reaction-center chlorophyll content. The work of Worden and Sistrom (1964) also resulted in this conclusion. Aagaard and Sistrom (1972) showed that the ratio of the total Bchl to the reaction-center Bchl varied with the specific Bchl content of the cells in R. spheroides. In R. rubrum the size of the photosynthetic unit was almost invariant. A model of the photosynthetic unit in R. spheroides must thus allow for variability in the amount of light-harvesting chlorophyll.

In 1958 Brill made a spectral study of non-ionic detergent-treated chromatophores of R. spheroides. The work indicated that pigment-protein complexes may have been liberated by this technique. Gibson (1965c) showed that treatment of R. spheroides chromatophores with bile salts reduced their sedimentation coefficient from 160 to 120 s. He calculated that this could have resulted from the removal of an outer lipid layer between 2.5 and 5.5 nm thick. The chlorophyll spectrum was almost unchanged, indicating the pigment was still attached to the protein. The work of Menke and Weichan (1968) showed that the bile salt treatment may have had a more complex effect. Their electron microscopy and X-ray studies of the chromatophore

membrane showed a 4 nm lipid layer but that it appeared to exist inside a protein shell.

In 1941 Smith used detergent to fractionate chloroplasts. Subsequently numerous studies were made with various digestive techniques resulting in the isolation of the two plant systems; these have been reviewed by Boardman (1970). These studies may have led Olson to use detergents as a means of digesting chromatophores to produce subunits. Olson isolated a pigment-protein complex from Chloropseudomonas ethylica which he showed to contain five molecules of Bchl and a protein with a molecular weight of 35 kD. Since this work a great variety of techniques have been used in the study of chromatophore structure. One of the main aims has been to produce a small, and thus analysable, unit capable of photochemical activity. By this means it is hoped that insight into the spatial relationships between the pigments, proteins and electron acceptors might be gained. Perhaps the majority of work has been performed using R. spheroides or R. rubrum (both wild type and mutant strains). The production of a model of chromatophore substructure is now possible although it is far from complete. This has been achieved by an increased understanding of the effects of different chaotropic agents.

In 1970 Loach et al developed a method in which

chromatophore lipid was almost completely displaced by Triton. The procedure employed an alkaline solution of Triton X100 and urea. The resulting subchromatophore (AUT) particles contained all of the pigments and had an apparent particle weight of  $150 \pm 50$  kD. The Bchl spectrum remained essentially unchanged and the particles possessed the ability to photooxidize P870. These workers claimed that this work was the first strong evidence for a photoreceptor subunit in vivo. The AUT particles were estimated to contain 50 Bchl molecules and were believed equivalent to the 5 - 8 nm particles seen by electron microscopic examination of chromatophores. Interestingly, removal of Triton resulted in the reaggregation of these particles to form structures of similar appearance to chromatophores. The AUT particle contained both the light harvesting and reaction-center proteins.

Various techniques have been used to digest membranes to their individual proteins. Delze et al (1969) investigated the protein composition of R. rubrum chromatophores using a phenol/formic acid mixture at low pH. After digestion the proteins were resolved by polyacrylamide gel electrophoresis. A method of calibrating this system to determine the molecular weight of the proteins was described by Biederman (1971a). In the last few years, the analysis of protein composition has been performed, almost entirely, by sodium dodecyl

sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weights of the light-harvesting and reaction center proteins, as determined by various groups, is shown (Table II).

The effects of a variety of chaotropic agents on proteins liberated and on the pigment protein interactions, was examined by Kim (1970), using C. ethylicum. A similar study was made with R. rubrum by Beiderman (1971b). These studies showed that the properties of the liberated complexes were strongly influenced by the type of digestion technique employed. This was demonstrated with a single detergent, SDS, by Feher et al (1971). At a low level (0.1%) of SDS, a reaction-center preparation was split into two components. The larger of these (37 + or - 3 kD) remained photochemically active, the other was unpigmented (28 + or - 2 kD). When 1.0% SDS was used, the larger particle was further dissociated and the photochemical activity was lost. Clayton and Haselkorn (1972) showed that the proteins of the pigmented subunit were lost if the sample was boiled with 1% SDS for longer than one minute. An interesting comparative study of chromatophore proteins from different species was also made by these workers. The reaction-center of a carotenoidless mutant of R. spheroides (R26) has been shown to contain a characteristic protein triad. The three components (19,



Table II  
Light harvesting and reaction center pigment protein complexes prepared from  
R. spheroides.

\* R. spheroides wild type. \*\* R. spheroides R-26. \*\*\* R. spheroides Y.

Workers	Method of digestion	Resolving technique	Molecular weight (kd) Reaction center	Light harvesting
* Clayton and Haselkorn (1972)	1% SDS, 100° x 1 min.	PAGE	27, 22, 19	11
** Slooten (1972)	0.3% SDS, 25° x 120 min.	Sucrose density gradient	200	9-10
" Method of Loach et al (1970)	1.5% Triton, 6M urea, pH 11	Sucrose density gradient	120	-
* Segen and Gibson (1971)	1% SDS, 100° x 10 min.	PAGE	31.5	9.9
* Fraker and Kaplan (1971)	2-chloroethanol, pH 2.2	PAGE	27	9.7
** Feher (1972)	1% LD40 then 1% SDS, 65°	PAGE	28, 23, 21	-
** Clayton, Szut and Fleming (1972)	1% Triton, 100°	Sucrose density gradient	107	-
** Reed and Raveed (1970)	1% Triton, 100°	Gel filtration	440	25
*** Jolchine and Reiss Husson (1971)	0.1% CTAB, 25° x 60 min.	Gel filtration	153	-

21 and 27 kD) were apparently present in equimolar ratios. This triad was found in R. rubrum, Rhodospseudomonas capsulata, and Rhodospseudomonas palustris but not in Rhodospseudomonas gelatinosa or in Rhodospseudomonas viridis. All of these species, however, contained a major chromatophore protein with a weight between 10 and 15 kD. This was useful as it showed that many of the organisms used in studies of morphogenesis were fairly closely related phylogenetically. Smith and Dus (1972) compared the amino acid compositions of the reaction-center protein of R. spheroides and R. rubrum and found them very similar, if not identical.

In 1972 Slooten attempted to produce a model of a chromatophore photosynthetic unit which would embrace his findings and those of other workers. The model provides a good basis for conceptualizing the chromatophore substructure. The finding of one P870 Bchl (the photo-bleachable chlorophyll molecule in the reaction-center) per 600 kD of protein defined the photosynthetic unit size in terms of protein. The assumption was made that the different digestion techniques dissociated this unit into different fragments. Reed et al (1970) had obtained subunits with a value of 440 kD per P870 while those produced by Loach et al (1970) contained between 100 and 150 kD per P870. The reaction-center particles of Slooten had a value of 200 kD per P870 and could be

reduced to 120 kD by the AUT treatment of Loach (1970). Feher et al (1971) had shown these to be convertible into two units with weights of about 20 kD. The model rationalized the otherwise confusing results obtained from these experiments. A diagram, based on the model proposed by Slooten, is shown in Figure 1. In Figure 2 the model has been rearranged to allow for the special relationships found by Reed and Raveed (1971). The ATPase particles should probably be included in Slooten's subunit, as magnesium-ion was included in the buffer used to isolate the chromatophores. Reed and Raveed estimated that the particles had a molecular weight of 300 kD and showed them to be dissociated from the chromatophore in the absence of this cation.

Slooten proposed that the reaction-center pigment-protein complex might resemble the light harvesting pigment-protein complex in its protein to pigment ratio. This would certainly be attractive from an evolutionary standpoint. Slooten found the bulk of the Bchl to be associated with protein with a weight of 150 kD. As his chromatophore preparation contained 30 molecules of light harvesting chlorophyll per P870 it seems likely that the 10 kD light-harvesting proteins are each associated with two chlorophyll molecules. Clayton and Clayton (1972) found light-harvesting protein had a chlorophyll content of 17%. This would support the figure of two chlorophylls

Figure 1

Model of photosynthetic subunit based on that  
of Slooten (1972)

Key to fig.

kD = kilo Daltons

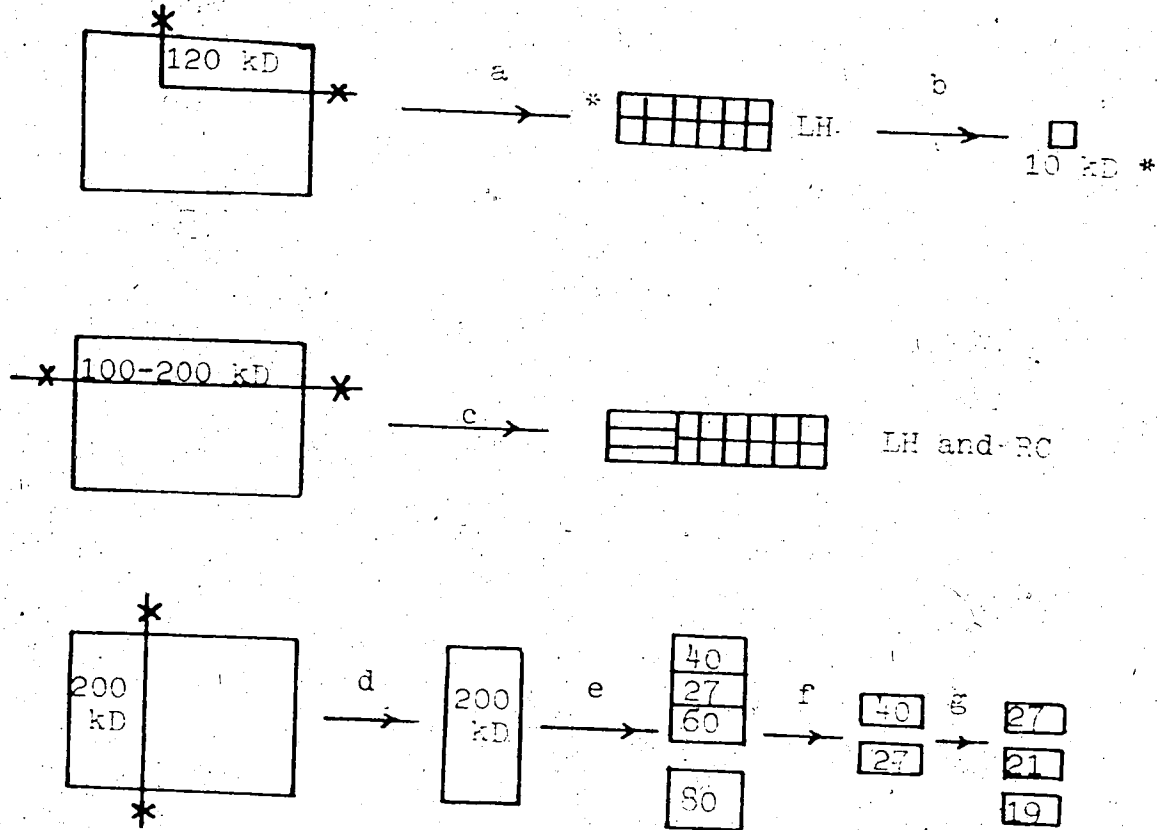
- a Digestion by 2 chloroethanol, Fraker and Kaplan (1972),  
or by Triton x 100, Reed et al (1968,1970).
- b Treatment with 1% SDS, Fraker and Kaplan (1972)
- c Digestion by urea, Triton x 100 at pH 11.0,  
Loach (1970)
- d Digestion by SDS or CTAP, Feher (1972)
- e Digestion with urea, Triton x 100 at pH 11.0, Clayton  
and Haselkorn (1972)
- f Subsequent digestion with 0.1% SDS, Feher (1972)
- g Further treatment with 1% SDS, Clayton and Haselkorn  
(1972)

LH = Light harvesting pigment proteins.

RC = Reaction center pigment proteins.

Figure 1.  
 (Molecular weights kD protein  
 per P870. Pigment \*.)

RC	LH					
21	10	10	10	10	10	10
19	10	10	10	10	10	10
27	10	10	10	10	10	10
60	280 probably ATPase.					
80						



Model showing the various ways in which digestion has been shown to disassemble the photosynthetic unit in *R. spheroides*.

per 10 kD protein. The reaction-center has been shown to contain four molecules of Bchl and two of bacteriopheophytin [ Mauzerall (1972), Reed and Peters (1972) and Straley et al (1973)]. If the value of 37 kD is used, i.e. the pigmented reaction-center protein weight of Feher et al (1971), then a similar ratio to the light harvesting protein is obtained. The idea that the reaction-center protein is a multimer of the 10 kD light harvesting protein is not supported by a comparison of the amino acid center protein (Feher 1971). The similar ratios might indicate a common evolutionary origin of these proteins.

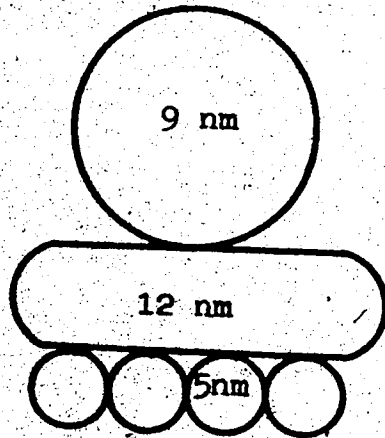
The model shown in Figure 2 is obviously far from complete: little is known as to the distribution of the lipids, the locations of the minor components, or the functions of the unpigmented proteins (other than the ATPase unit). It does, however, provide a reference point for still further analysis and is obviously useful to the understanding of chromatophore assembly.

#### IX. Ideas as to the Mechanism of Chromatophore Morphogenesis

The study of chromatophore morphogenesis in purple non-sulphur bacteria is attractive because the whole process can be manipulated by an easily controllable parameter, the oxygen supply. Cells can be grown at high

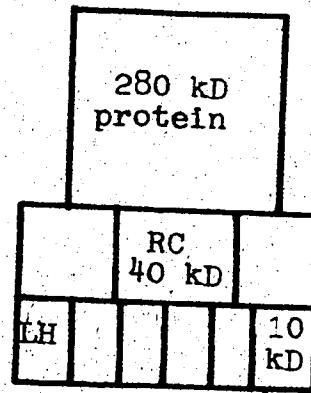
Figure 2. Models of the photosynthetic unit.

A  
Model proposed by  
Reed and Raveed (1971)

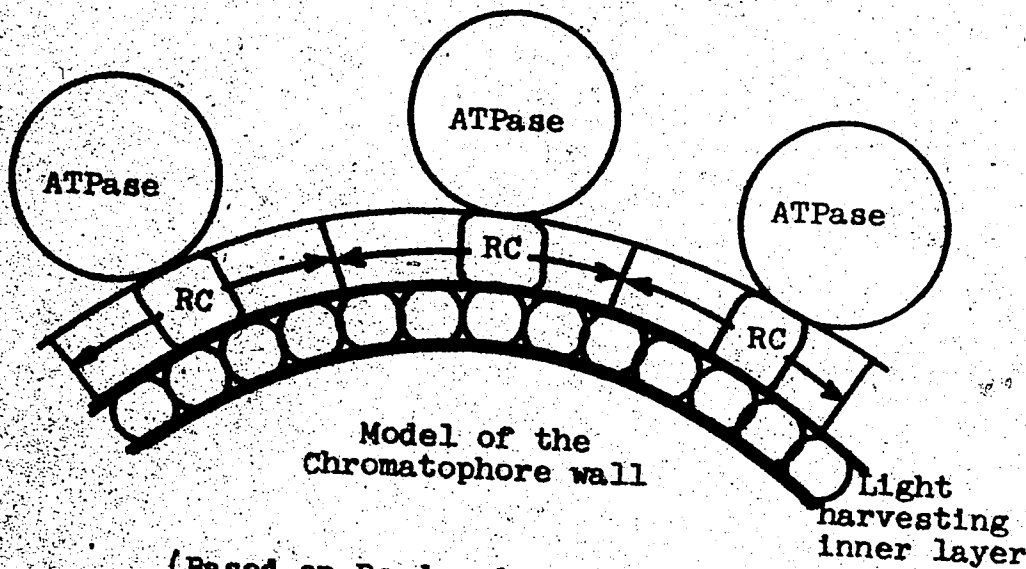


Based on electron  
microscopy

B  
Rearranged model  
of Slooten (1972)



Based on protein  
analysis after  
digestion



(Based on Reed and Raveed, 1971)

oxygen levels to produce cultures with negligible pigment content. A low level of oxygen will induce chromatophore synthesis. Cells can be studied throughout the adaptation to determine the sequence of compositional changes that result in the production of the thylakoid system. This procedure has been used by numerous workers. An alternative technique relies on chromatophore synthesis induced in photosynthetic cells by decreasing the light intensity. Most of the current ideas as to the origin and development of chromatophores have resulted from a study of adaptation by electron microscopy of the cells or by following the incorporation of a radioactive substrate. This survey will be essentially confined to work with R. spheroides and R. rubrum; species with similar chromatophores and similar pigment-bearing proteins (Clayton and Haselkorn, 1972).

The origin of the chromatophore was probably first investigated by Vatter and Wolfe in 1958. Their examination of thin sections of R. rubrum, however, provided no solution. They stated the problem that numerous subsequent workers have attempted to answer: "The mechanism of this (chromatophore) synthesis is unknown. Are chromatophores formed simultaneously throughout the cytoplasm or are they formed from one of a few sites?"

In 1959 Hickman and Frenkel (1959a) concluded that



the chromatophores in R. rubrum were not produced by pre-existing chromatophores. In an accompanying paper (1959b), these workers found that disruption of cells in the initial stages of adaptation did not release chromatophores but pigmented particles 2.5 nm in diameter. They postulated these structures to be the building blocks from which the chromatophores were formed. At this time, de novo synthesis was supported indirectly by the finding that pigment and protein synthesis were closely associated [Lascelles (1959) and Bull and Lascelles (1963)]. This idea was obscured by the finding of a relatively constant protein content in the chromatophore fraction of adapting R. rubrum cells [Cohen-Bazire and Kunisawa (1963)]. These workers interpreted this finding as evidence for chromatophores being produced from pre-existing membrane. Their idea was supported by their finding that cells of low pigment content contained chromatophores only around the periphery of the cell. They made the observation that vesicles appeared continuous with the cytoplasmic membrane in some sections, and proposed that a reduction in oxygen level caused a differential synthesis of special proteins. These were incorporated into the cytoplasmic membrane causing its expansion, and eventually resulted in intrusions into the cytoplasm. This idea was supported by the studies with osmotically lysed spheroplasts that were used as evidence for chromatophores being a continuous

membrane system. Gibson, as mentioned, showed that these experiments were not conclusive. Further, he was critical of the electron microscopic evidence and suggested (1965d) that the "budding" vesicles might have resulted from fusion of separate vesicles with the cytoplasmic membrane. Gibson thought that the peripheral vesicles might, alternatively, represent aerobic structures as similar invaginations could be observed in non photosynthetic bacteria. This he proposed would explain why a greater number of cytoplasmic membrane invaginations could be seen in aerobic as compared to anaerobic cell sections. The small distribution of chromatophore diameters (about 7%) and the rarity of chromatophores that were not spherical caused Gibson (1965d) to conclude that binary fission did not occur and that de novo synthesis was more probable as their mode of proliferation. This, it was suggested, could only be settled by a more dynamic approach than electron microscopy. Peters and Cellarius (1972), however, retained the idea that chromatophores were produced by the modification of the cytoplasmic membrane. They described electron microscopic observations as indicating that invagination was followed by constriction, proliferation and branching resulting in the thylakoid. They agreed with the work of Deize et al (1969) in the finding that pigment was synthesized before invaginations (presumably nonaerobic) occurred. In 1969 Drews et al had

shown that the production of Bchl did not parallel membrane morphogenesis in R. capsulata. Peters and Cellarius (1972) believed that once vesicles were being produced, the subsequent pigment incorporation was probably stoichiometric. They found, interestingly, that energy transfer and reaction-center activity occurred at very low pigment concentrations. Calculations of surface area and pigment content favoured the idea that pigment was incorporated non-uniformly into specific sites. The fact that they found the photochemical efficiency at low levels of pigmentation to be equivalent to that in fully pigmented cells also supported this idea. Gibson et al (1972) found that chromatophores, bound to the cell envelope, could be separated from chromatophores which they believed to be free in the cell. As will be further discussed, labelling studies indicated that bound chromatophores were not precursors to the free structures. The concept that chromatophores are produced by direct modification of the cytoplasmic membrane thus cannot be considered established.

The use of labelling techniques has certainly provided useful biochemical information than electron microscopic studies and has provided a picture as to the compositional modifications involved in chromatophore morphogenesis. In 1963 Bull and Lascelles showed that adaptation involved more than a reorganisation of existing

proteins. The uptake of amino acids was found to parallel pigment synthesis in adapting cells, indicating the synthesis of specific protein. Later a labelling study by Lascelles and Szilagy (1965) showed that pigmented cells contained additional phospholipid. Gorchein (1968 e, f, g,) made a thorough investigation of lipid metabolism in adapting R. spheroides. Adaptation caused an 85% increase in cellular phospholipid and a four fold increase in ornithine lipid. The incorporation studies indicated, however, that little preferential incorporation of phospholipid occurred and that the specific activity of the chromatophores was the same as that of the cells as a whole. Gorchein interpreted this to indicate that cell membrane lipid becomes incorporated into chromatophores. Drews et al (1969) used changes in light intensity to favour either chromatophore or cytoplasmic membrane synthesis. Pulse chase experiments caused them to conclude that a dynamic interaction existed between the cytoplasmic membrane and the chromatophores. Delze and Drews (1970) and Lamp et al (1972) used similar techniques and came to the same conclusion. An experiment of particular relevance to this thesis was performed by Gibson et al 1972. These workers (1972a, b) have presented evidence for a precursor-product relationship between membrane fractions of anaerobically grown R. spheroides cells. Exponentially growing cells were

sequentially pulse labelled with  $^3\text{H}$  phenylalanine or ( $^3\text{H}$ ) leucine. A membrane fraction was described, which was enriched in cytoplasmic membrane and contained chromatophore fragments. This fraction (the "small membrane" fraction) was found to be of high specific activity at the end of a "pulse" with a labelled amino acid but decreased in activity after the addition of cold "chase" amino acid. During this time the chromatophores showed a corresponding increase in activity. The proteins of the cell envelope, on the other hand, were shown not to be derived from this fraction. It was concluded that chromatophores were formed preferentially from part of the cell wall-cytoplasmic membrane complex in anaerobically growing cells. This "small membrane" fraction, rather than membrane bound chromatophore structures, was considered to be the true precursor to the mature chromatophores.

The involvement of cytoplasmic membrane proteins in chromatophore morphogenesis has been investigated by Huang (1972) and Fraker and Kaplan (1972). Huang found that cytoplasmic membrane proteins may form part of the chromatophore. The technique of Fraker (1971) was used to discriminate between aerobic membrane proteins and the chromatophore specific proteins: only the latter are soluble in 2-chloroethanol. Chromatophores, on digestion by this reagent, yielded 5% of insoluble protein (PI

fraction). This fraction was found to have an SDS-PAGE profile almost identical to that of her aerobic cytoplasmic membrane preparation. This fraction was constant after repeated purification of the chromatophores. A labelling study with adapting cells indicated almost all of the amino acids incorporated during the adaptation were found in the 2-chloroethanol-soluble (PII) fraction. The PI fraction appeared to be synthesized only during aerobic conditions. This work indicated that, although some chromatophore proteins were derived from the cytoplasmic membrane, a high percentage of chromatophore protein was synthesized de novo.

The dynamic potential of labelling experiments has thus produced valuable information as to the cellular origin of chromatophores. Numerous "static" comparisons of cytoplasmic membrane and the photosynthetic membranes have been made and are described in the reviews of Oelze and Drews (1972) and Lascelles (1968). Such comparisons have shown many similarities. Many of these may be fortuitous, however. This type of approach yields little data that is useful in determining the mechanism of morphogenesis. It appears that the cytoplasmic membrane is involved; the problem is how?

#### X. The Regulation of Chromatophore Morphogenesis

The regulation of cellular adaptation is intrinsically interesting. Ultimately a detailed picture of chromatophore assembly will require the understanding of the regulatory mechanisms involved in the coordinated synthesis of the components. At present only the broad metabolic controls are being established. This information, however, is useful as a means of translating the effects of culture conditions into biochemical terms.

The first thorough study of the factors affecting pigmentation in *Athiorhodacea* was made by Cohen-Bazire et al. (1957). These workers established that a definite relationship existed between the bacterial pigment content and the culture conditions. A number of fundamental observations were made. The degree of pigmentation was inversely related to the light intensity. Aerobically grown cells, unlike preadapted photosynthetic cells, showed a lag in the growth rate when transferred to conditions of low oxygen supply. Further, aeration of a photosynthetic culture resulted in stimulation of the growth rate. This was interpreted as being due to the effect of joint aerobic and photosynthetic metabolism. The finding that this rate enhancement was not long lasting, was believed due to the cessation of pigment synthesis and subsequent dilution by continued aerobic growth. These workers postulated that pigment synthesis was controlled by the oxidation state of a carrier in the

electron transport system. This concept was supported by many subsequent workers. In 1962 Sistrom proposed that this carrier was NAD. This was supported by the correlation between pigment synthesis and NADH-oxidase activity (Deize and Drews (1970). Recent work, however, makes it unlikely that the redox state of NAD is the key factor in the control of adaptation. Marrs and Gest (1973) studied the effect of oxygen on the electron transport components in a bacterium closely related to R. spheroides, R. capsulata. A mutant, M2, had normal cytochrome oxidase activity but lacked both NADH and succinate dehydrogenase activities. This mutant thus should be insensitive to oxygen if NADH (or NADPH) was the controlling factor. The mutant had, however, increased sensitivity to oxygen. Another mutant, M5, lacked cytochrome oxidase activity, and thus would be expected to have abnormally high NADH levels during semiaerobic growth. This strain, however, closely resembled the wild type with respect to the effect of oxygen on pigment formation. Thus NADH was unlikely to be directly involved. Marrs and Gest postulated that the oxygen effect was mediated by its inactivation of a component involved in Bchl synthesis. This factor was postulated to be redox regulated by either cytochrome c or b. Schon and Drews (1968), however, had not been able to correlate changes in the oxidation-reduction state of these



components, with the induction of Bchl synthesis, following a reduction in oxygen tension. Recently, Lien and Gest (1973) have suggested that the "energy charge" of the cell is probably controlling.

Some studies had suggested that the formation of Bchl might trigger chromatophore synthesis. Lascelles and Wertlieb (1971) described a mutant of R. spheroides (TA-R) which unlike the wild type, showed magnesium protophoryn-S-adenosylmethionine methyl transferase activity during aerobic growth. Brown et al (1972) showed that the ALA synthetase activity of this mutant was also not repressed by oxygen. These workers proposed that oxygen acted indirectly on the products of regulatory genes. They believed the effect was transmitted either via an electron transport component or by the ratio, or cellular concentration, of adenine nucleotides.

An early indication that ATP had a controlling influence on the synthesis of bacterial pigments came from the work of Gajdos and Gajdos-Torok, in 1963. Coproporphyrin excretion by R. spheroides was inhibited by adenosine nucleotides. A more widespread consideration of adenosine compounds as potential regulators probably resulted from the work of Atkinson and Walton (1968), however. They found that the ratio of the various adenosine nucleotides had a controlling influence on the activities of a large number of key enzymes that

controlled the generation or utilization of ATP in bacteria. They could empirically correlate enzyme activities to a factor they termed the "energy charge"; essentially half of the average number of anhydride bound phosphate groups per adenosine. In 1971 Zilinsky et al first showed that exogenous ATP caused a great reduction in Bchl synthesis. It was, however, found effective only in the light and appeared to have little effect on dark aerobic growth. They concluded that the ATP inhibition of pigmentation was due to an energy charge effect at the site of Bchl and membrane synthesis. This conclusion was premature as it was not based on endogenous nucleotide levels. Fanica-Gaignier et al (1971) measured the endogenous ATP level in cells of R. spheroides strain Y cultured under a variety of conditions. Cellular ATP content oscillated rapidly after either oxygenation or illumination of the culture. Bacteriochlorophyll synthesis was observed only when the endogenous ATP level fell to a low value. Exogenous ATP was effective in causing an inhibition of Bchl control by the cellular ATP concentration only, rather than by the energy charge parameter. The mechanism of the effect of exogenous ATP was uncertain, however. Gajdos et al (1968) had found cells incapable of utilizing exogenous ATP to an appreciable extent. The situation was further clarified by the work of Higuchi (1973). He found that ATP would

inhibit Bchl synthesis only to a maximum of 75% when increasing amounts were added to the cell culture. Complete inhibition, however, could be produced by exogenous adenosine. ALA synthetase activity was found to be suppressed by adenosine but not by ATP. Higuchi believed that ATP exerted its effect by binding to the outer periphery of the cytoplasmic membrane causing interference with the development of the photosynthetic membrane. It seems possible that the effects are at the metabolic level. Exogenous ATP was found to cause a marked increase in the incorporation of glycine into fatty acids (Gajdos et al 1968). Interestingly, Nultsch and Throm (1968) showed exogenous ATP to cause a steady increase in the rate of photokinesis until, after thirty hours, the rate was about a magnitude greater than the control cells. A slow stimulation of endogenous synthesis would seem more likely. This may be due to conversion of ATP to adenosine by periplasmic phosphatases. Adenosine entry into the cell would increase all of the adenosine nucleotides and thus the ATP level by the effect of mass action. The exact mechanism of ATP effect is obviously open to speculation.

The establishment of the cellular ATP level as the regulator of Bchl synthesis makes this factor a strong candidate for controlling chromatophore synthesis as a whole. Control of several synthetic activities by a

single parameter would favour a good coordination. Control is unlikely to be exerted at the assembly level or by alterations in enzyme activities, as these mechanisms would be inefficient with regard to cellular economy as all enzymes would be required to be synthesised for only periodic usage. Oxygen inhibition of ALA synthetase (Lascelles, 1960; Harriott et al, 1969) and magnesium protophorphyrin chelatase (Gorchein 1972) probably reflect a secondary fine control system. The finding that a specific ALA synthetase is induced in adapting conditions (Tuboi and Hayasaka 1972), together with the fact that many proteins are specific to chromatophores, strongly points to genetic control. I will briefly review evidence for both translational and transcriptional regulation of chromatophore morphogenesis.

Translational control has only been described in a few cases (Lavelle (1970) and Goldberg and Chargaff (1971) provide examples of this mechanism in E. coli). DeJesus and Gray (1971) found variations in four tRNA species when comparing aerobically grown with photosynthetic R. spheroides cells. Although theoretically such a variation could indicate a translational control mechanism, the finding cannot be taken as evidence for this type of regulation. Witkin and Gibson (1972a) studied the rate of unstable RNA degradation in R. spheroides with both aerobic and anaerobic cells after inhibition with

rifampin. The rate was twice as fast in anaerobic cells as it was in those aerobically grown. These workers suggested that an air-sensitive component stabilizes the ribosome-mRNA complex and could possibly facilitate the translation of selected transcriptional products. In a following paper (1972b) they reported that no quantitative or qualitative differences could be found between mRNA isolated from aerobic or anaerobic cells. They suggested that translational regulation might be an important mechanism in these bacteria. At first sight, translational control is not an attractive mechanism as a large amount of mRNA must be continually synthesized for only periodic usage. It may, however, be advantageous to photosynthetic bacteria growing in mud by combined aerobic and photosynthetic modes. This would obviously be a difficult question to answer.

There is no definite evidence for transcriptional regulation. The findings by Higuchi et al (1965) and Gr (1967), showing RNA synthesis to be necessary for the formation of the photosynthetic apparatus, does not discriminate between transcriptional and translational control. Bearden (1969) reported that her competition experiments indicated that 35 to 40% of the DNA sites expressed in light grown cells were repressed in those from oxygenated cultures. Interestingly she could find no difference between air 'grown' and light (anaerobically)

'grown' cellular DNA. This is in agreement with the findings of Witkin and Gibson (1972b). These workers used 95% air 5% carbon dioxide rather than oxygen. Bearen believed that oxygenation rather than aeration is required to fully repress the "photosynthetic" genes. This is not proof of transcriptional control as the cells are unlikely to grow in pure oxygen in their natural environment. A labelling study by Yamashita and Kamen (1968) showed that a greater percentage of uracil was incorporated into metabolically active RNA under anaerobic light conditions. It is not certain that this difference was due to the presence of photosynthetic mRNA, as it may have reflected an increase in production of ribosome precursors. As mentioned, Witkin and Gibson indicated a shorter half life of the ribosomal m-RNA complex under anerobic conditions.

Thus, at present, it is not certain as to which genetic mechanism controls chromatophore morphogenesis. It can only be stated that regulation is most likely exerted at the genetic level, directly or indirectly, by the cellular ATP-concentration.

#### XI. The Morphogenesis of Membranous Cellular Components in General

The finding that supramolecular biological structures are composed of characteristic aggregates of definable molecules, has posed two fundamental questions: what are

the relationships between the components and how are these structures assembled? The solution to these problems would provide a basis for an understanding of functioning beyond the present empirical level. The questions are somewhat interdependent and possibly the solutions will be found by studying the structures of precursors.

The cytoplasmic membrane and the eucaryotic organelles, especially the mitochondrion and chloroplast, have been the subjects of a vast number of investigations. The problems appear to be very similar to those encountered in the study of the bacterial photosynthetic apparatus. These problems result from the inherent complexity of these structures and the fact that they must be perturbed to be studied. The problems have produced numerous approaches and resulted in many models. Few generalities have emerged and certainly no universal assembly process has been found. Although the cytoplasmic membrane has been assigned many functions, initial electron microscopy studies showed it to be similar in appearance in a wide variety of cells. The early Danielli-Davson model (1935) has been both challenged and supported by many workers. Reviews of subsequent models have been made by Nystrom (1973) and Siekevitz (1972). Models of Benson (1966) and Green (1966, 1973) have been based on a lipoprotein subunit composition. This concept is not fully established, but, the perhaps questionable

evidence, from traditional electron micrographs and digestion techniques, has been supported by recent freeze-etching studies. This technique may be the most reliable to date. No prior fixation or staining processes known to produce artefacts are required. Data derived from X-ray scattering may be misleading due to the extensive dehydration required and the necessity of long exposure times to the X-ray beam (Nystrom 1972).

Mycoplasma, organisms lacking a cell wall, have been useful in examining the cytoplasmic membrane. Tourtellotte (1972) describes the membrane as containing the majority of lipid in a bilayer array around 5.0 and 8.5 nm protein subunits. The subunit proteins are believed to have external hydrophobic residues in addition to polar residues. Such proteins can associate with lipids and would be classified as intrinsic proteins by Green (1973).

The mechanism of cytoplasmic membrane formation is still largely unknown. The assembly data on the bacterial membrane has been reviewed by Fox (1972). Work with E. coli has indicated that newly synthesized lipids and proteins are preferentially associated. It appears that this organism may synthesize new membrane in a non-localized fashion (Tsukagoshi and Fox, 1971) while B. subtilis may form new membrane preferentially at the pole of the cell (Morrison and Morowitz 1970). Non-localized



membrane synthesis appears to have been more widely found, however. The biochemistry of bacterial membranes has been reviewed by Machtiger and Fox (1973).

Mitochondrial subunits appear to range in size from below 4 nm to 10 nm (Sjostrand and Barajas, 1968).

The chloroplast lamellae have been described by Branton and Park (1967) as extended bilayers containing 8.5 nm globular subunits. These subunits have since been found to be the coupling-protein complex (ATPase and carboxydismutase) which cover the outer surface of the chloroplast membranes (Arntzen et al, 1969). Freeze-fracturing techniques have shown the Photosystem I and II particles to be 11 nm and 17.5 nm respectively (Muhlethaler 1972). Detergent digestion techniques have yielded different subunits. Photosystem I appears to consist of a reaction-center subunit and an accessory complex containing light-harvesting chlorophylls and electron transport components (Vernon et al, 1971). Chlorophyll-bearing proteins of weights 100 and 35 kD have been found associated with Photosystems I and II respectively (Kung and Thornber, 1971). The asymmetrical distribution of the two photosystems as shown by the freeze-etching studies have been recently supported by conventional sectioning techniques (Nir and Pease, 1973).

With such limited information of membrane synthesis in general, it is not surprizing that little is known as

to the assembly of mitochondria and chloroplasts. These organelles are not "simple" membranes but membrane systems similar in many respects to procaryotes (Echlin, 1970; Stanier, 1970). Present work indicates that the formation of these organelles may require existing membranes to orientate the newly synthesized constituents. Mitochondria are formed from pre-existing organelles by division (Luck, 1963). The adaptation of yeast cells to aerobic conditions was shown to involve the development of a pre-existing structure, the promitochondrion (Plattner et al, 1970). Similarly, chloroplast formation involves the development of a proplastid. Another factor making the morphogenetic studies more complex is the possession by these organelles of their own genetic systems which code for some of their components. Current ideas as to assembly processes in these organelles have been reviewed by Getz (1972) and Green and Vanderkooi (1970).

## XII. Thesis Objectives

The discovery of a possible chromatophore precursor fraction presented a chance to gain insight into the question of chromatophore origin. I hoped to characterise these intermediates and follow their modification into the final photosynthetic structures. Many questions arose: how and where were these structures formed? What factors controlled their synthesis? Were these intermediates of

fixed composition or was continuous modification taking place? What was the origin of the additional components necessary for chromatophore formation? Although photosynthetic membranes are composed of many components, it was decided to study the changes in protein composition in an attempt to answer some of these questions. This approach was made since proteins are quantitatively the major component, the most important functional entities of the photosynthetic apparatus and probably the most important factors in determining the morphology of the membranes. It is obvious such an approach cannot produce a complete picture of chromatophore morphogenesis but it is hoped that the model produced will be of use to further studies in this area. Two preliminary reports of this work have already appeared (Shaw and Richards 1971, 1972).

Chapter 2

Materials and Methods

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## I MATERIALS.

### A. Organisms used

The parent strain of Rhodopseudomonas spheroides, N.C.I.B. 8253, was used for the majority of the experiments. Mutants of this organism were given as a gift by Dr. J. Lascelles. Other mutants were produced from the parent strain by ultraviolet irradiation or treatment with N-methyl-N'-nitro-N-nitrosoguanidine.

### B. Growth medium

Cells were grown using the "M-G" medium of Lascelles (1959). It was found convenient to store the medium, concentrated ten fold, in the freezer prior to use. In preparing the medium precipitation was avoided by the neutralization of the organic acids by sodium hydroxide, prior to the addition of the magnesium and calcium salts.

### C. Growth apparatus

Semiaerobic growth was obtained using Erlenmeyer flasks plugged with lint-bound cotton wool. These cultures were agitated by magnetic stirring or "wrist-action" shaking. Cultures were grown at fixed oxygen levels in 4- and 10- liter flasks. These flasks were fitted with a port for the oxygen electrode, a sampling tap, a swan-necked gas exit tube and a sintered gassing tube. The sinters were of surface areas 8 and 60 square cm for the 4 and 10 liter flasks respectively. Gas was filtered through a tube packed with cotton-wool prior to entry to the flasks. Both flasks and filters were sterilized by autoclaving (15 minutes at 10 lbs/inch<sup>2</sup>). Premixed gases were used with oxygen contents known to 0.5% in a balance of nitrogen, supplied by Liquid Carbonic Canada Ltd., Vancouver. The Beckman model 777 oxygen electrode was sterilized by brief immersion in ethanol. This procedure was found to have no effect on the

subsequent functioning of the electrode. When required, cultures were illuminated at approximately 300 foot candles, by fluorescent strip lighting.

#### D. Special reagents

Magnesium protoporphyrin was prepared using the magnesium-hexapyridine-diiodide method of Wei et al (1962).

Sucrose was purified for the gradient work by first heating a 70% (w/v) solution to above 90 degrees with 10% (w/v) 20 - 40 mesh charcoal, then filtration through Whatman No. 1 paper.

#### E. Labelled compounds

L-(U-<sup>14</sup>C) Proline (210 mCi/m mole) used in the initial labelling experiment was obtained from New England Nuclear Corp., Boston, Mas. S-Adenosyl-L-(Me<sup>14</sup>C) methionine (33 mCi/m mole) was obtained from I. C. N., Irvine, Cal. L-(4,5-<sup>3</sup>H) Isoleucine (30 Ci/m mole) and L-(U-<sup>14</sup>C) proline (285 mCi/m mole), used in the double-labelling experiment, were obtained from Amersham/Searle, Arlington Heights, Ill.

## II. Methods

### A. Isolation and culture conditions used

Uncontaminated cell cultures were prepared from "single cell" colonies using standard microbiological plating techniques. Such cultures were stored in tubes containing 5 ml slants of 1.5% agar and "M-G" medium. Slants were grown for a few days at 30 degrees prior to being stored at 4 degrees in the dark. Fresh cultures were made every few months. This method gave a greater viability than storage procedures involving freezing or freeze drying of liquid cultures. Material from the slants was grown in 250 ml. Erlenmeyer flasks and early to mid log phase cultures were then used to inoculate the large growth flasks.

Inocula with a very low pigment level were produced by oxygen gassing a culture until mid-log-phase. At this time the cells were harvested and resuspended in 50 ml aliquots of fresh "M-G" medium at a level of  $2 \times 10^{10}$  cells/ml. Stored at 4 degrees, these cultures remained viable for several months and contained less than  $10^{-8}$  g of bacteriochlorophyll (Bchl a)/ml.

Adaptation studies were performed in the large growth flasks at 30 degrees. Aerobic growth was ensured by gassing with 65% or 100% oxygen in the dark. At defined points in the growth of the organism, adaptation was induced by limiting oxygen entry to diffusion or by gassing with 4% or lower levels of oxygen. During adaptation the cultures were illuminated. The



relationship of the culture conditions used to the course of the adaptation is described in Chapter 5.

## B. Compositional analysis.

### 1. Cell number

In the initial studies a model B Coulter counter was used. It was fitted with a 30,000 nm aperture and a model M volume converter. Paper-Mulberry pollen was used to standardize the machine. Cell numbers were correlated to the turbidity of the culture at 680 nm using the Spectronic 20 colorimeter. In the last half of the work cell numbers were estimated by the turbidity at 1200 nm, using the Cary 14 spectrometer, as described by Fanica-Gaignier et al (1971). These workers state that an optical density of 1.0 corresponds to  $4 \times 10^9$  cells/ml.

### 2. Dry weight

Cells harvested by low speed centrifugation were washed twice by resuspension in distilled water. The cells were then dried to constant weight at 50 degrees. Dry weights correlated with the turbidity at 680 nm. The results agreed with the early finding of Lascelles (1956), namely, that an absorbance of 1.0 at 680 nm was equivalent to 1 mg/ml dry weight cells.

### 3. Protein

The method of Lowry et al (1951) was used. The

protein content of whole cells was determined after pretreatment with dilute sodium hydroxide, as described in the modification by Lascelles and Szilagy (1965).

#### 4. Pigment

##### (a) Bacteriochlorophyll a

The Bchl content of whole cells and membrane fractions was estimated by measurement of the extinction at 850 nm. Gorchein et al (1968) found that an absorbance of 1.0 was equivalent to 16 n moles/ml. This method was used in the majority of work with membrane fractions. In some studies the pigment was estimated after extraction with acetone-methanol (7:2), as described by Cohen-Bazire et al (1957).

##### (b) Intermediates in the bacteriochlorophyll biosynthetic pathway

These were determined from their spectrum in ether; molar extinction coefficients were obtained from Falk (1964), while that of magnesium protoporphyrin was obtained from Gibson et al (1969). Intermediates for which no extinction coefficient was available were compared by their relative peak heights at the wavelength of their maximum absorption.

##### (c) Carotenoids

The carotenoids were compared using their relative peak heights.

## 5. Nucleic acids

DNA and RNA were measured using the diphenylamine technique modification of Pederson (1969). Care was taken to completely remove sucrose from precipitated nucleic acid before the assay. This was achieved by washing the sample four times with 5% TCA. The DNA content was estimated by the absorption at 550 nm and that of RNA at 650 nm. Calf thymus DNA and yeast RNA were used as standards. An indication of the distribution of nucleic acid in sucrose gradient separations was obtained by a comparison of the absorption at 254 nm and 280 nm. These are the approximate absorption maxima of nucleic acids and proteins respectively.

## 6. Poly beta-hydroxy-butyric acid ( PHB ).

A few milligrams of cells were digested by 10 ml of alkaline hypochlorite (freshly prepared), as described by Williamson and Williamson (1958). The PHB was determined colorimetrically by the method of Slepecky and Law (1960).

## 7. Glycogen

Glycogen was extracted by a modification of the method of Seifter et al (1950). One milliliter of 60% potassium hydroxide was added to the sample and then dialysis rather than centrifugation was employed to remove low molecular weight compounds. This produced low

backgrounds in samples containing high levels of sucrose. The glycogen was assayed by the method of Ashwell (1957).

B. S-Adenosyl-methionine- magnesium-protoporphyrin-methyltransferase (E. C. 2.1.11) activity.

The activity was determined as described by Gibson et al (1963). After incubation the pigments were extracted by the method of Neuberger and Tait (1964). Samples were then counted by the liquid scintillation technique (section X this chapter).

#### 9. Divalent metal analysis

The sucrose solutions, used for the density gradient technique, were assayed with the standard EDTA titration using Eriochrome Black T as an indicator [ Lindstrom and Diehl (1960) ].

#### C. Disruption techniques

##### 1. Sonication

Cells were suspended in 50 ml of 0.05 M phosphate buffer at pH 7.0. A concentration of approximately 4 mg dry weight of cells per ml. was used. The solution in a 100 ml beaker was kept cool by crushed ice during sonication at 20 kHz using the Biosonik III at 20 watts. Two one minute periods of sonication with a one minute period between produced no appreciable heating of the sample.

## 2. French press treatment

Cells from one liter of culture were suspended in 40 ml of phosphate buffer and passed twice through a chilled French pressure cell at 20,000 psi. This method was used in the majority of experiments and is compared to the other disruptive procedures in Chapter 4.

## 3. Grinding with alumina

The method of Marmor (1961) was used. Harvested cells were ground with an equal wet weight of 500 mesh alumina in a precooled mortar. Grinding was performed by hand for 5 minutes.

## 4. Lysis by osmotic-shock

Cells were washed twice with distilled water and suspended in 0.1 mM Tris buffer at pH 8.0 to a concentration of about 0.7 mg (dry weight)/ml. To some of this suspension 7.5 mg of EDTA was added followed by 1 mg of egg white lysozyme (Repaske, 1956). The mixture was stirred magnetically at room temperature for 30 minutes. The osmotic shock procedure of Robrish and Marr (1962) was then used with the following modification: the lysozyme treated cells were brought to 3 molar in glycerol and stirred for 5 minutes. The solution was then transferred to 10 volumes of 0.05 M phosphate buffer, instead of the Tris-magnesium chloride buffer suggested by Repaske. Lysis was confirmed by examination of negatively-stained samples under the electron microscope. This technique was

found to produce less aggregation of cell membranes than an alternative technique in which protoplasts, prepared by the method of Reaveley and Rogers (1969), were osmotically lysed by the method of Tuttle and Gest (1959).

#### D. Electron microscopy techniques

##### 1. Negative staining

Negative staining was performed using 1% phosphotungstic acid neutralized with sodium hydroxide. Samples were examined on 200 mesh nickel grids coated with collodion. Contamination of material to be examined was prevented by adding one drop of 4% Iodoacetamide to a 3 ml sample. The size of the particles being examined was estimated by including latex spheres 109 (+ or -3) nm in diameter (E. F. Fullam Inc., New York) into the sample.

##### 2. Cell sections

Cells were fixed using the method of Kellenberger et al (1958) with the modification of Luft (1961). Fixed cells were embedded in Epon 812. Silver-grey sections (about 600 Angstroms) were double stained on Parlodion coated copper grids. Uranyl acetate and lead acetate were used by the method of Venable and Coggeshall (1965).

##### 3. Microscopes

The electron microscopy was performed using a Zeiss model EM9A or a Philips model 300 G. Some initial studies were made with the R.C.A. model E.M.U. 3H microscope.

## E. Preparative centrifugational procedures

### Low "g" techniques

Cells were harvested by centrifugation at 10,000 x g using the International 820 or the Sorval RC 2-B machines. After disruption, the unbroken cells and cell debris were removed by centrifugation at 12,000 x g for 15 minutes using the International 820 or 10,000 x g for 20 minutes with the ~~International 820~~.

### High "g" techniques

The supernatant from the low "g" centrifugation was carefully decanted from the pellet. To avoid contamination by debris approximately 5 ml were left over the pellet. The membrane fraction was then collected by centrifugation of the supernatant at 100,000 x g for 90 minutes using the International 860 machine. In later experiments it was found preferable to centrifuge the low "g" supernatant over 2 ml of sucrose trap (60% sucrose in 0.005 M phosphate buffer at pH 7.0). The subunit preparation was collected from the high "g" supernatant by centrifugation at 100,000 x g for 24 hours. A doubly layered trap was used (5 ml of 50% glycerol in Tris buffer (1mM) at pH 7.5 with an underlay of 3 ml of 100% glycerol). The use of traps completely eliminated aggregation of the harvested material.

#### F. Density gradient centrifugation methods

Linear sucrose density gradients were made by an I.S.C.O. model 570 gradient former. In an initial experiment methylene blue was included in the most dense sucrose solution. It was found, after plotting the optical density of the gradient fractions against the theoretical density of each fraction, that pumping speed 4 gave the most linear gradient. At this speed setting the gradient was almost perfectly linear. In most experiments a standard gradient was used between 25 and 55% sucrose (1.107 and 1.261 g/ml at 20 degrees). Other gradients were used for further purifications; all were of a total volume of 30 ml. The buffered sample was carefully added to the surface of the gradient by means of a syringe with an up-turned tip. The flow of the sample was controlled in a pipette-like manner. This procedure caused almost no disturbance of the top surface of the gradient. The high g pellet was resuspended in buffer by gentle all-glass homogenization or vortex mixing with two glass beads. The material trapped on sucrose needed no resuspension.

Following centrifugation at 23,000 RPM in the International 860, using the S8110 rotor (min.g 40,000 max.g 90,000) for 16 hours the gradient was fractionated. The I.S.C.O. model 182 fractionator was used at a pumping speed of 3 ml/min to produce twelve 3 ml fractions. Fraction peaks were established using the I.S.C.O. U-2



ultraviolet analyser at 254 nm. Fractions were subsequently analysed for protein and Bchl as described previously.

#### G. Density gradient electrophoresis technique

Several types of gradients, differing in pH, ionic strength and density, were tried as a means of purifying the protein subunit fraction. A system using 1 mM Tris buffer at pH 7.5 with a glycerol density gradient was found to give the best result. A 30 ml gradient between 40 and 70% (w/w) glycerol was used over an 80% glycerol underlay. The sample was carefully added in 5 ml of 20% glycerol/1 mM Tris. The sample and gradient were then carefully overlaid with the buffer. The preparative I.S.C.O. 630 electrophoresis system was used with the I.S.C.O. model 490 power supply. The electrode jackets were filled with 10 mM Tris buffer, pH 7.5. The run was made at about 1 mA at a constant voltage of 500 V. The sample layer, seen as a pigmented band, was allowed to migrate through the gradient, towards the anode, for 3.5 hr. The gradient was removed after the run by displacement with 90% glycerol by means of the I.S.C.O. model 182 fractionator. The fractionation was monitored using the I.S.C.O. U-2 ultraviolet analyser.

#### H. Gel filtration

A 2B Sepharose gel was used. It has an approximate protein separation range of 2 to 25 million daltons. A plexiglass 76.5 x 3.2 cm column was used with an internal capacity of 615 ml. Calibration of the column, packed with 2B gel, with 5 ml of 0.2% Blue Dextran 2000 indicated a void volume of about 200 ml. 40 ml of sample was added with a protein content of 2 mg/ml. The column was eluted with 5 mM phosphate buffer, pH 7.0, at a flow rate of 25 ml/hr. The column was run at room temperature (approximately 25 degrees) as the flow rate at 4 degrees was too slow. Fractions of 20.5 (+ or - 0.3) ml were collected.

#### I. Polyacrylamide gel electrophoresis

The protein composition of different membrane fractions was initially examined after digestion of the sample in phenol, formic acid and water (2:1:1). The method of Takayama et al (1964) was used. Membrane fractions from the parent and mutant strains were related to the findings of Biederman and Drews (1968) using this technique. Molecular weights of protein bands could not be determined accurately as mobilities were not easily related to molecular weights. For this reason the majority of the work was performed using the sodium dodecyl sulphate technique of Weber and Osborn (1969). The method of Laemmli (1970) with, basically, the

modifications of Clayton and Haselkorn (1972) was found to give the greatest resolution. The modifications of Weber and Osborn's method by Fraker and Kaplan (1971) and Segan and Gibson (1971) were also tried. The Clayton and Haselkorn-modified Laemmli technique was used in the analyses shown in Chapter 6, unless otherwise stated.

Polymerization was initiated with ammonium persulphate and N,N,N',N'-tetramethyl-1,2-diaminoethane. The levels of these reagents were adjusted to cause good polymerization in 20 min. Several factors were found to be critical in the production of gels giving good resolution; these will be described below.

#### 1. Gel formation

One of the reasons the Laemmli technique produced better resolution than the modifications of Weber and Osborn's system was due to the use of a 1 cm stacking gel (3% acrylamide) over the running gel (10% acrylamide). Degassing the monomer solutions for 15 minutes prior to the initiation of polymerization was found to increase the reproducibility of the technique. The catalyst and initiator were added to the solution which was magnetically stirred under a flow of nitrogen.

#### 2. Gel surfacing technique

The surface of the running gel was known to be critical to the resolving power of the gel. The normal procedure used by many workers was to introduce a few

drops of water onto the gel surface. This method produces a flat gel surface and reduces the entry of oxygen into the polymerizing gel. Some surface irregularities were produced using this method so another surfacing technique was developed. The method was facile and proved to be highly reproducible. A layer (2 - 5 mm) of freshly prepared carbon dioxide "snow" was added to the surface of the polymerizing solution. A few drops of distilled water were then added to the circumference of this layer by means of a long syringe needle. On addition, the water froze but melted in about two minutes to form a layer over the virtually undisturbed gel solution.

### 3. The front marker

Initially the widely used, bromphenol-blue dye was used. At the end of a run it produced a band about 5 mm in length. Hematoporphyrin was used in some runs as it was found that it caused the ion front to be visible as a sharp line in the gel. When used, front markers were prerun into the gels for 10 min. This was done to prevent the possibility of any interaction with the samples being run. Later it was found that the ion front could be observed without the use of hematoporphyrin. The gels had to be cut within a few minutes of being removed from the tubes for the front to remain visible. Bromphenol-blue was still added to allow the extent of the run to be followed easily but gels were cut at the ion

front.

#### 4. The choice of current used

The literature suggested that the current employed was critical to resolution. The resolution of gels run at various currents/tube was compared. A run at 5.0 mA/tube gave poor resolution. The best result was obtained using 1.0 mA/tube; at this current the running time was 4 hr.

#### 5. Digestion conditions used

The sample, 0.1 to 0.2 mg in protein, was digested in 1% SDS, 10% glycerol, 5% mercaptoethanol and 25 mM Tris buffer, pH 6.8. 0.2 ml of this solution was used. The sample was heated in a disposable polycarbonate tube immersed in boiling water. Various lengths of heating were compared and it was found that one minute gave the best result with regard to the completeness of digestion and the resolution of the resulting proteins.

#### 6. Staining and destaining techniques

The staining method described by Clayton and Haselkorn (1972) was used. It was necessary to make a fresh staining solution each time. Destaining was achieved by diffusion using 7% acetic acid, 5% methanol in distilled water. The gels were held in plexiglass troughs and the destaining solution changed every 10 minutes for the first hour and then at decreasing frequency for one week. A faster method of destaining was later developed: gels were held in one meter lengths of glass tubing (8.

gels/tube) and the destaining solution slowly passed through the system. The glass tubes were rocked by being attached to an I.S.C.O. model 390 dialyzer.

#### 7. Gel scanning method

The gels were scanned, after destaining, using a Joyce and Loeb Chromoscan MkII with the 595 nm filter. The optical density was traced at 1:1 and 1:3 length ratios, the latter being used for the mobility measurements.

#### 8. Protein standards

The Schwarz-Mann non-enzymatic protein standards kit was used. Routely cytochrome c, chymotrypsinogen A, bovine serum albumin and gamma-globulin were used as standards. Good mobility versus log molecular weight relationships were obtained for each run. Duplicate gels in which the standard proteins had been run always gave identical band patterns. Calculation of the protein molecular weights from the band mobilities was greatly facilitated by the use of a program for the Monroe 1670 calculator.

#### J. Radioactive counting methods

The initial L-(U-<sup>14</sup>C) proline labelling experiment used the following technique: a sample of 0.1 ml from the sucrose gradient was added to a 1.5 cm square piece of Whatman 3 mm. filter paper and allowed to dry. Counting

was performed in 10 ml of 5% P.P.O. phosphor (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) in toluene. The loss in counting efficiency, caused by different levels of sucrose was determined using uniformly L-(U- $^{14}$ C) proline as a standard. The efficiency was determined using the channels ratio method. The ratio was related to the lowering of efficiency by chemical and colour quenching for samples taken up in toluene by the use of a minimum volume of ethanol. This was achieved by means of the use of standard (U- $^{14}$ C) toluene as an internal standard.

Samples in the "methylating" enzyme assay were added in a minimum volume of ether (volumes up to 0.5 ml). These samples were counted and quenching determined as described. Aqueous samples were added to a cocktail consisting of dioxane, 0.5% P.P.O. containing 10% naphthalene; (U- $^{14}$ C) toluene was used as an internal standard. In all cases the vials were precounted before sample addition to determine the background count rate.

Samples in the double labelling experiment were carefully evaporated down to about 0.1 ml and then taken up into liquifluor cocktail (New England Nuclear Co.) by the addition of the minimum volume (about 0.5 ml) of N.S.S. tissue solubilizer (Amersham/Searle Inc.), as directed by the manufacturers. The cocktail contained 6 g. P.P.O. and 75 mg. P.O.P.O.P. per liter. Samples containing sucrose or glycerol were dialyzed prior to

counting. The  $^3\text{H}$  and  $^{14}\text{C}$  activity was measured using ( $\text{U-}^3\text{H}$ ) or ( $\text{U-}^{14}\text{C}$ ) toluene as an internal standard.

#### K. Labelling experiments.

##### 1. Single-label experiment.

Cells were grown to late log phase ( $1.35 \times 10^9$  cells/ml) in a 1 litre flask with oxygen gassing through a sinter with a surface area of  $8 \text{ cm}^2$ . The cells were harvested and resuspended in 3l. of fresh M-G medium. 4 micromoles of L-( $\text{U-}^{14}\text{C}$ ) proline (5 Ci/mole) was then added. The culture was magnetically stirred and a 500 ml sample immediately taken by displacement with nitrogen. The cells were harvested, and were estimated to have been exposed to the proline for 5 min before being pelleted. This was termed the zero hr sample. Further samples were collected by the same means at 2, 6, and 20 hr. The small membrane fraction was prepared in the normal manner and resuspended in 5ml of 0.05 M phosphate buffer (pH 7.0) and layered on top of 30 ml of a 25 to 55% sucrose density gradient. After centrifugation for 15 hr the gradients were fractionated in the normal manner.

##### 2. Double-labelling procedure

A culture of R. spheroides was doubly labelled using L-(4,5- $^3\text{H}$ )isoleucine and L-( $\text{U-}^{14}\text{C}$ )proline. An oxygen-grown innoculum was added to 5 l. of M-G medium gassed with 65% oxygen (35% nitrogen) at 2 SCFH.  $^3\text{H}$ -Isoleucine was added



at early log phase (0.169 mCi in 0.133 m moles of carrier amino acid). After a period of three hours a cold chase (22.2 m moles) of isoleucine was added and a 1 l. sample then taken as representative of aerobically-grown cells. The oxygen content was then reduced to induce adaptation. After a period of 10 min of gassing with 4% oxygen (balance nitrogen) the culture (previously grown in the dark) was illuminated at approximately 300 fc.  $^{14}\text{C}$ -Proline was then added (0.0365 mCi in 0.133 m moles of carrier amino acid). After a further 2 hr. a chase of cold proline (22.2 m moles) was added. Samples of 1 l. were taken at 2, 6 and 16 hr. of the adaptation and, in addition, 10 ml aliquots were taken every hour to monitor the uptake of radioactivity by the cells. All samples were immediately poisoned (made 0.02% in sodium azide) and stored at 4 degrees prior to further analysis. A careful record of the culture volume was kept throughout the experiment.

The assay of labelled amino acid uptake by the cells in the double-labelling experiment was performed in the following manner. Aliquots of the culture were poisoned (0.02% sodium azide) and subjected to low g centrifugation to remove the majority of cells from the medium. The supernatant was then filtered through a "220 nm" Millipore filter by means of the Swinnex filter holder/syringe system. This was to ensure the removal of any residual

cellular or subcellular material. A known volume of the filtrate was then evaporated almost to dryness and the residue suspended in a toluene cocktail using a minimum volume of N.B.S. solubilizer. The activity of each isotope was measured and the efficiency of counting determined by the use of  $^3\text{H}$ - and  $^{14}\text{C}$ -labelled toluene as an internal standard.

### Chapter 3. Results.

#### I. The Isolation and Purification of the "Small Particle" Fractions from R. spheroides.

##### A. Sucrose Density Gradient Centrifugations

##### 1. From mutant strains unable to synthesize Bchl.

A crude membrane fraction was prepared from mutants 2-73, 8-32 and 8-47 [ Lascelles (1966); Richards and Lascelles (1969) ] by removal of unbroken cells and cell debris by a low g centrifugation and harvested by centrifugation at 100,000 x g for 90 min. The pellet was resuspended and fractionated using a sucrose density gradient between 10 and 40% sucrose. A non-equilibrium fractionation of material both from mutant cells and the parent strain (100 min at 63,000 x g) showed that mutants incapable of synthesizing Bchl did not contain chromatophores. The mutants did, however, produce a pigmented fraction of greater density than the chromatophore fraction.

##### 2. From the parent strain.

A gradient between 25 and 55% sucrose was used to examine the distribution of membrane fragments from cells prepared at several times during an adaptation from aerobic to photosynthetic growth. To achieve this, cells

were grown to mid-log phase by gassing with oxygen and then induced to synthesize thylakoids by limiting their oxygen supply to that which diffused through the surface of the culture. At various times, 500 ml samples were taken and the cells harvested and stored in 0.05 M, pH 7 phosphate buffer prior to disruption by sonication. The membrane fraction was obtained by centrifugation at 100,000 x g for 90 minutes. This was termed the "small particle" fraction. This fraction was prepared from cells immediately after gassing was stopped (0 hr) and at three later times (2, 6 and 16 hrs). The material was centrifuged in linear sucrose density gradients and fractionated into 3 ml samples. The protein contents of these fractions is shown in Figure 3. At 0 hr, the protein was distributed fairly evenly throughout the gradient. The dense fraction (fractions 10-11) was prominent at 2 and 6 hrs, but was greatly reduced at 16 hrs when the chromatophore peak (fractions 6-7) contained the majority of the protein in the gradient. Bchl was found only in fractions of samples taken after six hours of incubation without oxygen gassing. At this time it was distributed almost equally between the dense fraction and the chromatophore fraction. After 16 hrs the chromatophore fraction contained almost all of the pigment.

### 3. Terminology

It appeared as if there were at least four membrane fractions that might be involved in chromatophore morphogenesis (cf. Figure 3). Although the nature of these fractions is now more fully understood it is convenient to use the names originally given to these peaks.

The peak present at 0 and 2 hr, with a density between that of 25 and 30% sucrose (fractions 4-5) was termed the "protophore" fraction. The dense fraction that appeared before the development of the chromatophores was termed the "prephore" fraction; it had a density between that of 46 and 52% sucrose (fractions 10-11). In subsequent fractionations, with less small particle fraction applied to the gradient (2 mg protein), a distinct peak at a density between 41-43% sucrose was observed. This peak was collected in fractions 8-9 and termed the "medium density" fraction, (cf. Figure 6 below). The major peak at 6 and 20 hr, with a density equivalent to about 35% sucrose (fraction 6) was termed the "light chromatophore" peak. The shoulder that appeared at the density of 39% sucrose (fraction 7) was termed the "heavy chromatophore" fraction. It was initially believed that this shoulder contained the fully mature organelles (but cf. section I.F.3. below).

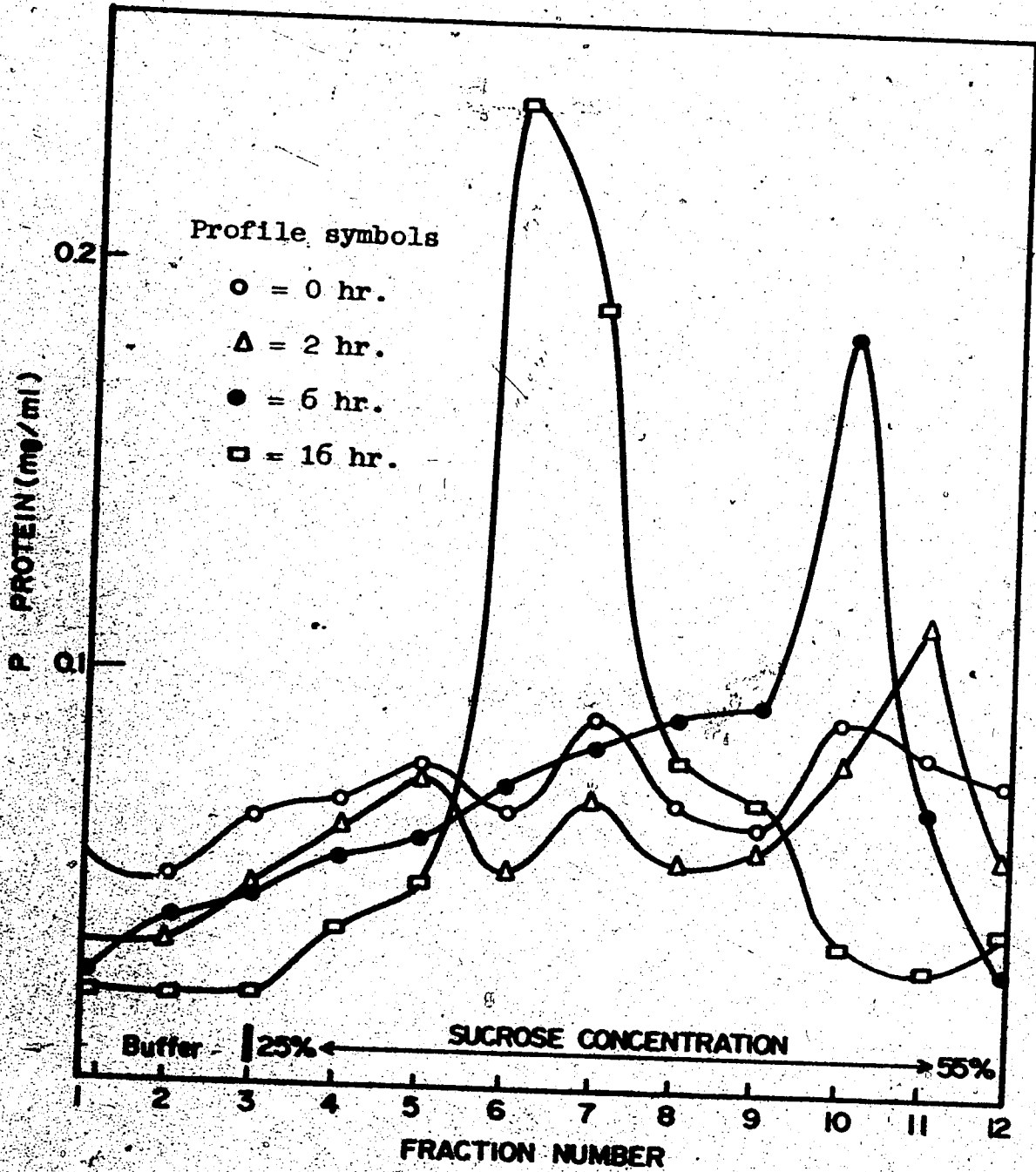


Figure 3. Protein distribution in the sucrose density gradient at various times in the initial adaptation experiment.

3. Other methods of purification.

It was thought desirable to be able to separate the fractions using a criterion other than their bouyant densities. Another stimulus was that in order to investigate the composition of each fraction, an easy large scale fractionation technique would be very useful.

1. Ammonium sulphate precipitation.

This method was not successful since an "all or nothing" precipitation of the small particle fraction occurred at low concentrations of the salt.

2. Millipore filtration.

A small particle fraction was prepared from adapting cells by French press treatment. This was then filtered through a series of millipore filters with decreasing pore sizes. Filtrates and material removed from the filters (by washing the inverted filters with buffer) was examined, after negative staining, by electron microscopy. Passage through a 450 nm pore sized filter removed the large membrane-bound storage granules (presumably PHB); however, all the pigment passed through this filter. The pigmented material was trapped on both the 300 and 220 nm pore sized filters. The first retained most of the prephore (non-collapsed) particles and aggregates of lipid (non-staining) material. The smaller filter retained all

the pigment which had passed through the the 300 nm filter; the chromatophores were split between these two filters. The 220 nm pore size filtrate contained no appreciable pigment and was composed of non-membrane-bound lipids and numerous particles between 5.0 and 20 nm in diameter. These small particles were present at most levels in the separation scheme. This technique was thus not particularly selective and it was difficult to remove material quantitatively from the filters.

### 3. Sucrose density gradient electrophoresis.

Electrophoretic mobility, I felt, might be a good basis for particle separation. Several systems were tried and, using the preparative I.S.C.O. 630 electrophoresis system, I found conditions that would separate the material into several bands: A 40 ml layer of 1 M sucrose (34.2%) was formed over the same volume of 40% sucrose with 50% sucrose being used as an underlay and chase. All solutions were made  $5 \times 10^{-3}$  M in potassium phosphate at pH 7.0. A sample (2 mg protein) of small particle fraction was added to the top of the 1 M sucrose layer and the system then run for 3 hours at 250V towards the cathode. A current of 28 mA was used. After the run, the bands (which were observed in the absorption profile at 254 nm) were collected. Bands were present at each end of both layers. After dialysis of the material, it was

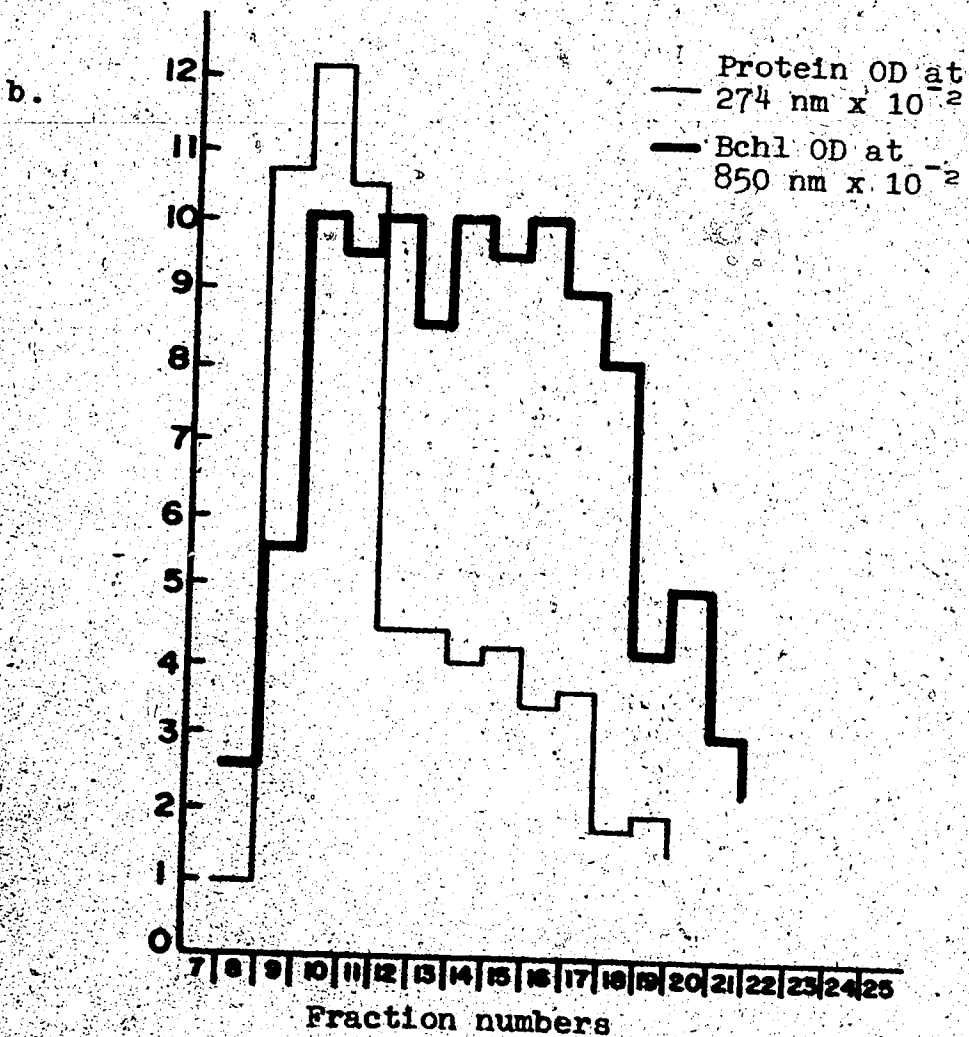
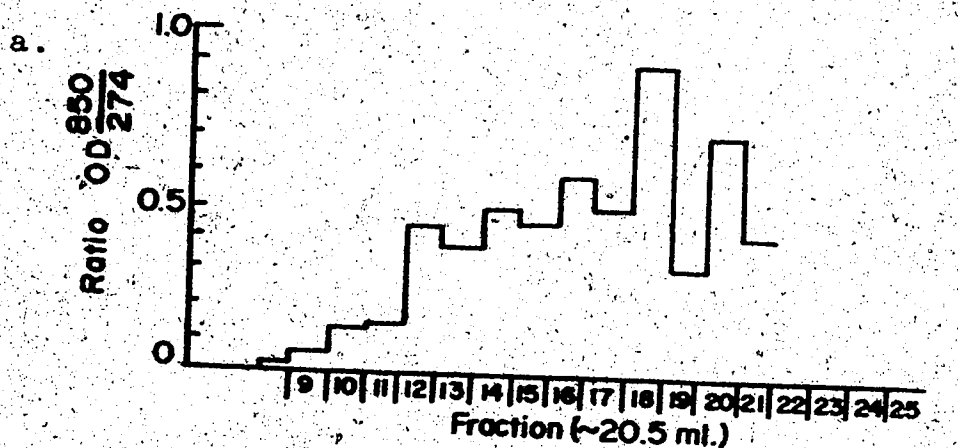


compared to the unseparated small particle fraction by the standard sucrose density gradient technique. The bands near the top of each layer contained both chromatophores and prephores. An enhancement in prephores could be seen in the upper band, but clearly, each band did not contain a unique particle type, as I had hoped.

#### 4. Sepharose 2B gel filtration.

Sepharose 2B has a separation range for proteins between 2 and 25 million daltons; in addition, the gel is somewhat hydrophobic. I hoped these properties might produce a separation of particle types. To obtain an appreciable flow rate the column had to be run at room temperature (approx. 25 degrees). Cells were adapted, after oxygen growth, by limiting oxygen supply to diffusion through the surface of the culture for 6 hr. A bulk small particle fraction was made after this time. An aliquot was fractionated using the standard sucrose density gradient technique. It contained prephore, medium density and chromatophore fractions. A sample of the small particle fraction (86 mg in protein) was run through the column. Figure 4a shows the ratio of Bchl to protein in the samples collected (absorbance ratios 850/274 nm). Figure 4b shows the absorbances of the 20.5 ml fractions. The fractions were then collected and applied to the standard linear sucrose density gradient. The profiles of

Figure 4. Adsorbance profiles of fractions from 2B Sepharose gel column.



the selected fractions are shown in Figure 5. The sharp increase in material in fraction 9 indicated that the void volume in this run was about 170 ml. This fraction consisted mainly of a prephore fraction with a small light chromatophore fraction. The prephore peak was still present in fraction 13, indicating that some of this material had been included into the gel, or had an affinity for the gel. The light chromatophore peak was present in all pigmented fractions examined but the highest concentration of these particles was found in fraction 15. Fraction 13 contained the highest level of medium density fraction. After fraction 17, heavy chromatophores were eluted. After the bulk of material was eluted (fraction 25) the column remained slightly pigmented for several days of running with buffer. This method was thus unsuccessful in completely resolving the various particle fractions but the experiment was valuable nevertheless. It demonstrated that the fractions were not artefacts of the standard sucrose gradient technique. It showed that the fractions could be separated, albeit partially, by a criterion other than their bouyant densities.

### C. Comparison of the disruptive procedures.

#### 1. Comparison of sonic and osmotic lysis.

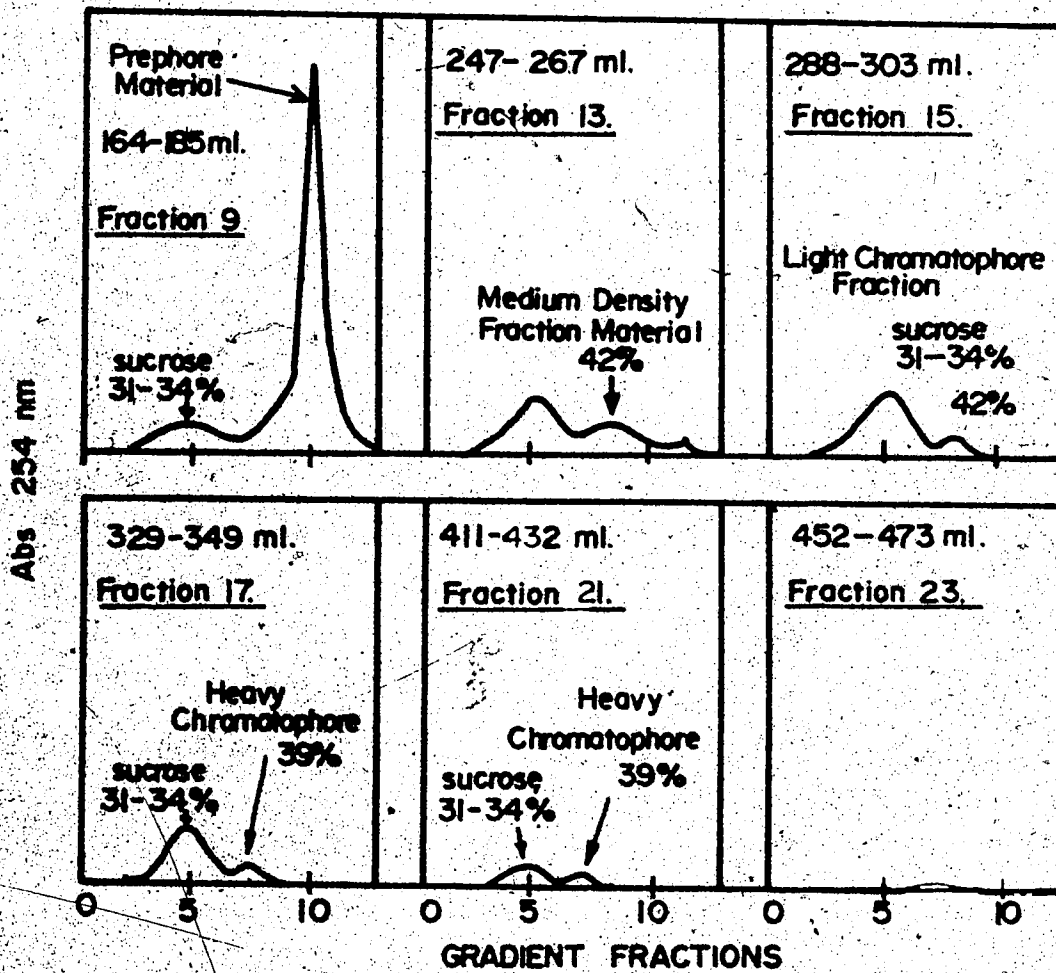


Figure 5. Sucrose density gradient resolution of small particle fraction (Culture D) material eluted from a 2B Sepharose gel column.

An oxygen grown culture was allowed to adapt with oxygen entry limited to diffusion into the medium. Aliquots were taken during adaptation and disrupted by sonication or osmotic shock (see methods, Chapter 2). After osmotic lysis the pigment could all be sedimented at  $14,500 \times g$  for 20 min. The pellet was "stringy" indicating cell lysis had occurred. This pellet was resuspended in 1M sucrose (0.05 M in pH 7.0 phosphate buffer), by vortex mixing for 10 seconds. To separate small membrane fragments from the broken cells the material was then recentrifuged at the same force for 30 min. The material in the supernatant was then collected by centrifugation for 90 min at  $100,000 \times g$ . Finally this pellet was resuspended in 0.05 M phosphate buffer and compared to the small particle fraction produced by sonic disruption by sucrose density gradient centrifugation. At the start of the adaptation, peaks were present at about 30 and 49% sucrose. The prephore fraction became pigmented after 4 hours. In both types of preparation the prephore fraction became less dense during the adaptation and attained a density equivalent to about 46% sucrose after 20 hr. The adaptation was incomplete in that a chromatophore fraction was not produced. Figure 6 shows the gradient profiles of material produced by the two techniques. The prephore and the medium density (41% sucrose) fractions from the sonicated preparation,

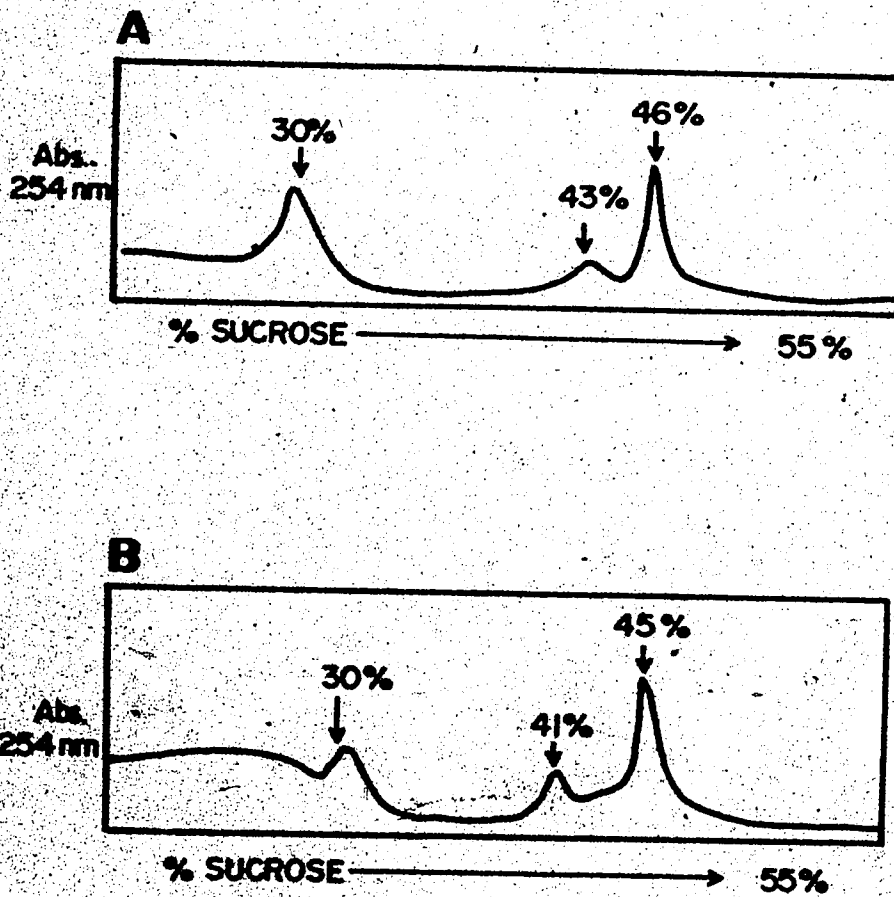


Figure 6. Gradient profiles of small particle preparations produced by A) osmotic shock and B) sonication. Partially adapted cells (+ 20 hours).

sedimented at slightly lower densities than did the equivalent fractions produced by osmotic lysis. The sonic disruption technique liberated about 40% of the pigment from the cells, while the osmotic shock procedure only yielded 20%.

## 2. Comparison of French press, sonic and alumina grinding techniques.

A culture was grown to late-log phase with oxygen gassing (2 S.C.F.H.) and then gassed with 4% oxygen (balance nitrogen) at the same flow rate. The cells were harvested after 6 hr at the low oxygen level and disrupted by the three different techniques (see methods, Chapter 2). The resulting small particle fractions (samples of 2 mg protein) were then added to the standard density gradient and fractionated. The gradient profiles are shown in Figure 7a. The majority of the pigment was found in the light chromatophore fraction. All three methods produced peaks at the heavy chromatophore and medium density fraction positions but the prephore peaks were only present in the French press and sonically disrupted material.

## 3. Peak densities of fractions resulting from French press or sonic disruption of adapting cells.

Table III shows that the five fractions described

Table III

Densities (equivalent sucrose) of fractions produced under various conditions of adaptation by density gradient centrifugation.

Disruptive technique	Protophore	Light chromatophore	Heavy chromatophore	Wad. um. density	Prephore
Sonic	26-30	34	39	-	47-50
	26-30	22-32	28-29	42	44-46
	20	-	-	43	45-49
French press	28-31	-	28-29	44	46-49
	29-30	33	29	42	47
	30	-	27-29	42	50
	30	42	28-30	42	50



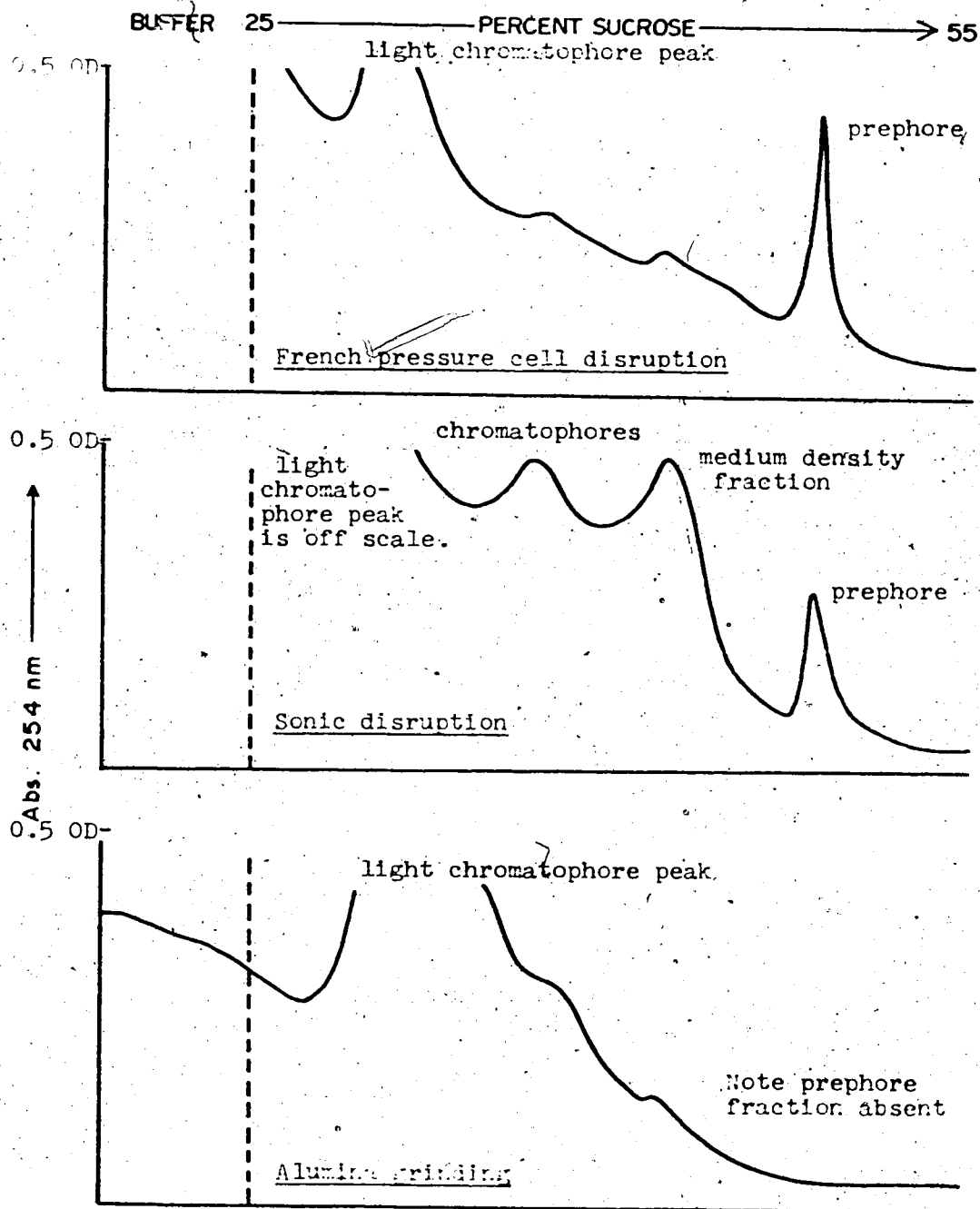


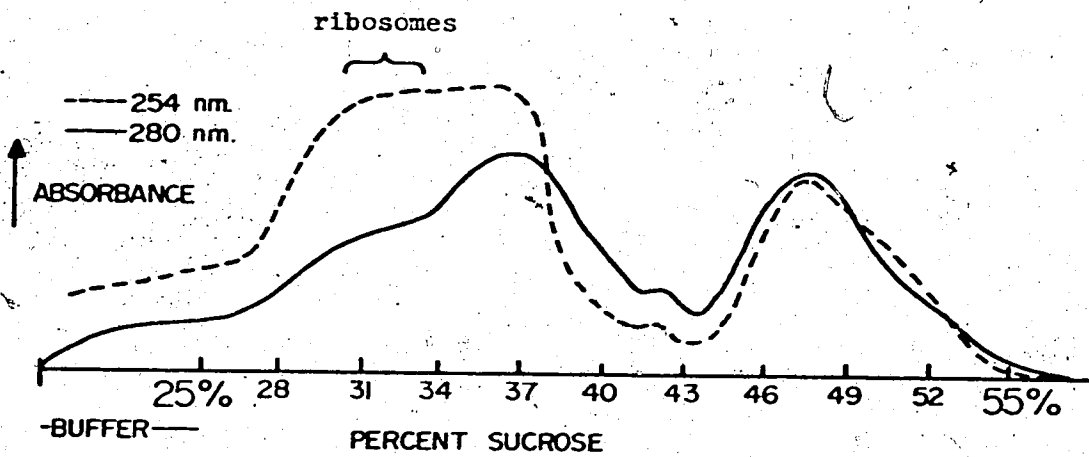
Figure 7a. Comparison of various disruptive techniques with regard to the distribution of material in the standard linear sucrose density gradient (25 - 55%).

peaked at fairly constant positions on the standard sucrose density gradient. The peaks had characteristic shapes in the 254 nm gradient profiles. The protophore peak was large and broad; the prephore peak large and narrow. The medium density fraction usually produced a small sharp peak on the profile. The ratios of the peak heights varied relative to one another during the course of adaptation, as previously described. A comparison of the 254/280 nm absorption profiles of a small particle fraction, prepared from cells adapted for 6 hr, is shown in Figure 7b. The 254/280 nm absorption ratio is highest (ca. 2) between 30 and 34% sucrose indicating the ribosomal material sedimented in this position in the gradient.

#### D. Electron microscopic examination of negatively stained small particle fractions.

Figure 8a shows the appearance of negatively stained preparations of the small particle fraction that was added to the sucrose density gradient. The sample was prepared from cells after 6 hr of adaptation. Chromatophores can be seen as characteristic ring-like structures (80 nm in diameter). The large spherical structure has the same dimension (150 to 250 nm) as PHB storage granules seen in sections of the bacterium (cf. Section III.C. below). Spherical bodies can also be seen of a size similar to the

Figure 7b.



Sucrose density gradient profiles of small particle fraction prepared from cells adapted for 6 hours.  
(Oxygen supply limited to diffusion.)

Fig. 8a

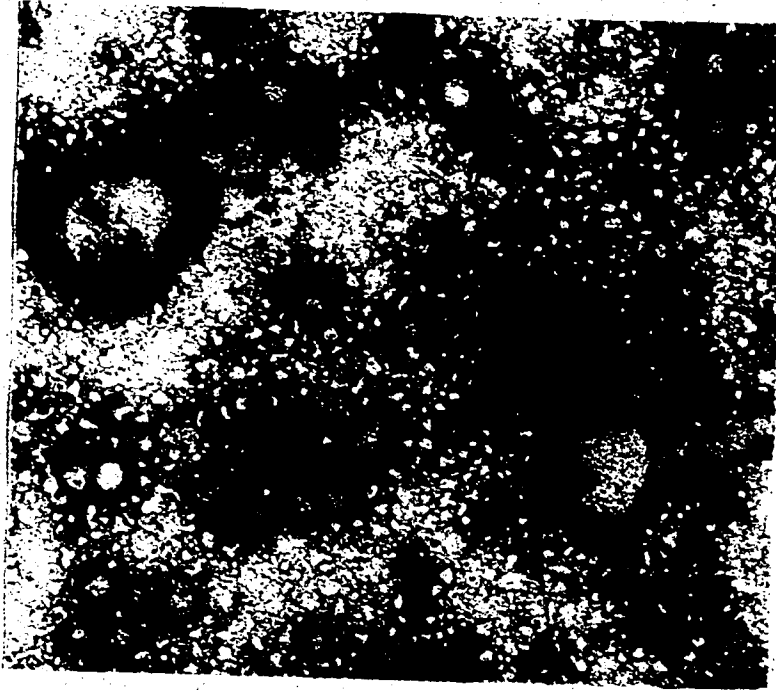
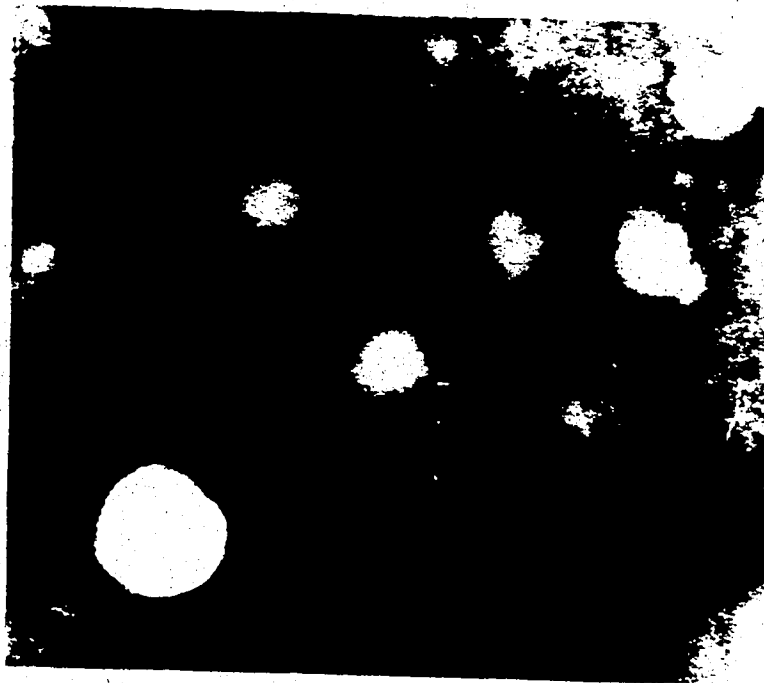


Fig. 8b



a Negatively stained small particle fraction.

b Negatively stained protophore fraction.

Scale:

1 cm = 100 nm.

chromatophore, together with smaller particles of about one-quarter the diameter.

The four major bands formed after sucrose density gradient centrifugation of this material were also examined. The sucrose was removed first by 24 hr dialysis in distilled water. The most obvious components of the protophore fraction (Figure 8b) were the large granules presumed to be PHB storage material. Less obvious irregular membrane fragments and smaller particles about 10 nm in diameter are also present. The light chromatophore fraction (Figure 9a) contained material with the characteristic chromatophore form as seen by this technique. The prephore fraction (Figure 9b) consisted of the non-collapsed particles, of a size similar to the chromatophores, and numerous small particles (approx. 10 nm dia.). The larger particles were uniform in appearance and were termed prephores. The medium density fraction had a characteristic "sac-like" appearance that was distinct both the prephores and the chromatophores (Figure 9c).

E. A study of the aggregation of the fractions.

1. Aggregation of material in the gradients by divalent cations.

In the initial sucrose density gradient separations, material in the gradient would sometimes aggregate. I

Figure 9a.

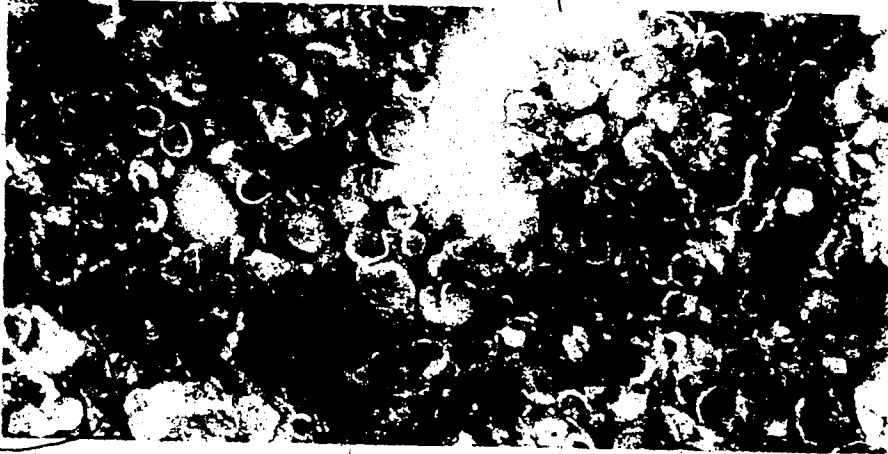
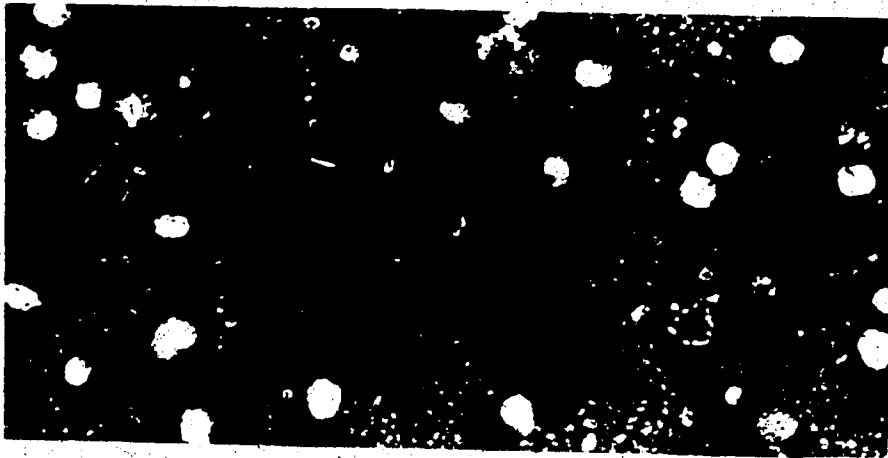


Figure 9b.



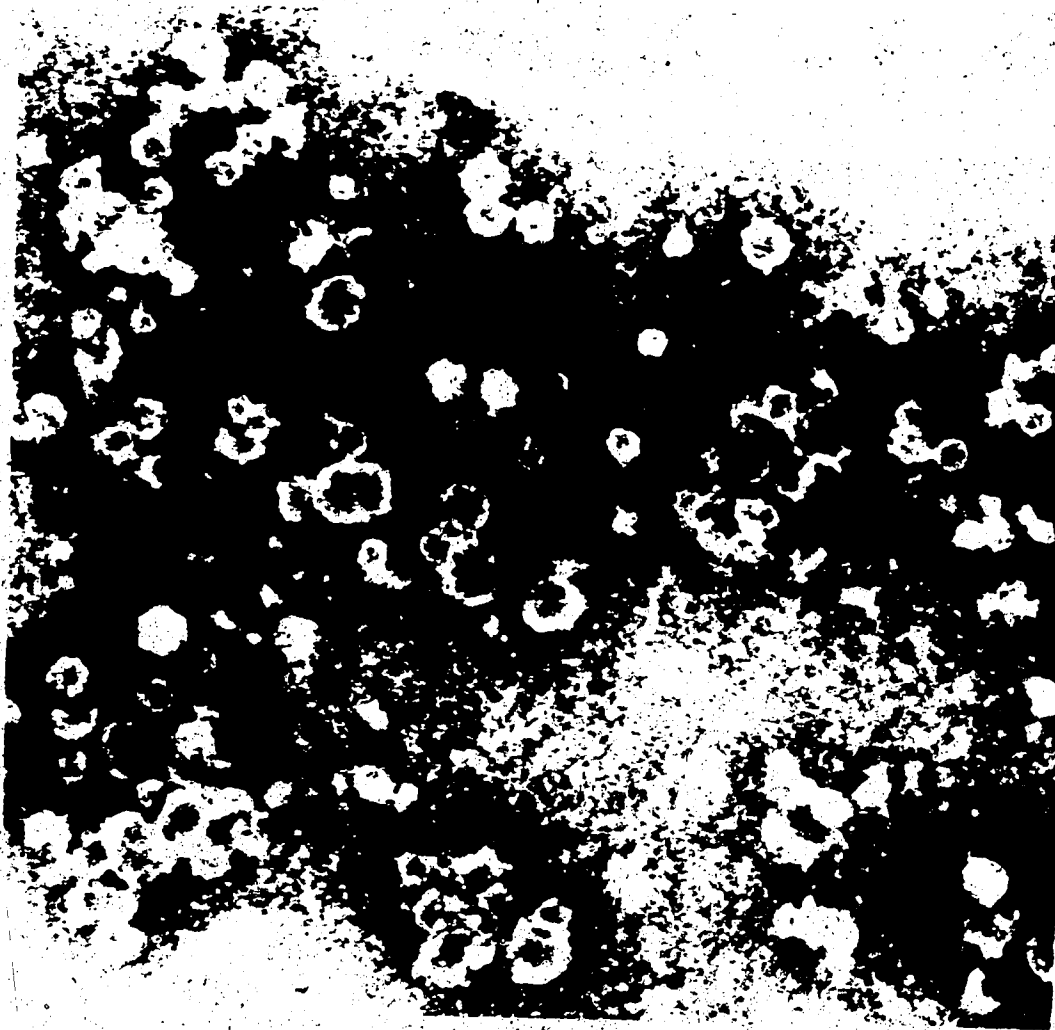
a Negatively stained chromatophore fraction.

b Negatively stained prephore fraction.

Scale:

1 cm = 100 nm.

Figure 9c.



Negatively stained medium density fraction.

scale: 1 cm = 140 nm.

thought that this might be due to the contamination of the sucrose with appreciable levels of calcium. The sucrose used for gradient formation was titrated for divalent cations by the Eriochrome Black T-EDTA method (cf. Chapter 2) The gradient contained  $4 \times 10^{-4}$  to  $1 \times 10^{-3}$  M divalent cation (25 to 55% sucrose respectively). It has been shown [Gibson (1965)] that divalent cations were a cause of chromatophore aggregation when present in concentration above  $10^{-3}$  M. EDTA was included in the phosphate buffer at a level of  $10^{-2}$  M. Subsequent runs, made with or without EDTA, showed no differences in the peak heights or densities of material in the gradient. The phosphate buffer strength was reduced; the standard buffer thus became  $0.5 \times 10^{-2}$  M in potassium phosphate, pH 7.0, and  $1 \times 10^{-2}$  M in EDTA (disodium salt). Using this modified buffer, aggregation was not a problem with samples containing 5 mg (or less) protein. The standard gradient was normally loaded with a small particle fraction containing 2 mg protein.

## 2. The prephore subunit and mature subunit fractions.

### (a) Isolation of the subunit fractions.

It seemed most likely that the prephores resulted from the characteristic aggregation of the small 10 nm particles (cf. Figure 9b) rather than the latter being a decomposition product of these structures. This



was in agreement with the finding that the prephores were only formed when the small particle fraction was pelleted, a process which could easily be imagined to favour aggregation. It was found that in the unaggregated state the so-named "prephore subunits" remained towards the top of the standard gradient during a 16 hr run. It seemed likely that the bulk of this material might not be harvested by the centrifugation at  $100,000 \times g$  for 90 min. Were unaggregated subunits, in fact, concentrated in the supernatant of the standard small particle fraction harvesting run?

To answer this question the pigmented material in the supernatants of the high g-force centrifugation was harvested into a 50% glycerol trap buffered with phosphate to pH 7.0; a prolonged centrifugation was used (24 hrs at  $100,000 \times g$ ). This technique was used to harvest material prepared from cells in various stages of adaptation, as well as cells grown photosynthetically for two days. After the glycerol was removed by dialysis, 2 mg samples were run on the standard sucrose density gradient. As can be seen from Figure 10, the gradient profiles of this harvested "supernatant fraction" from adapting cells are different from the profile produced by material from the mature photosynthetic cells. The adapting cells produced a peak with the bouyant density of that of 28% sucrose while the mature cells gave a 32% sucrose density peak. I

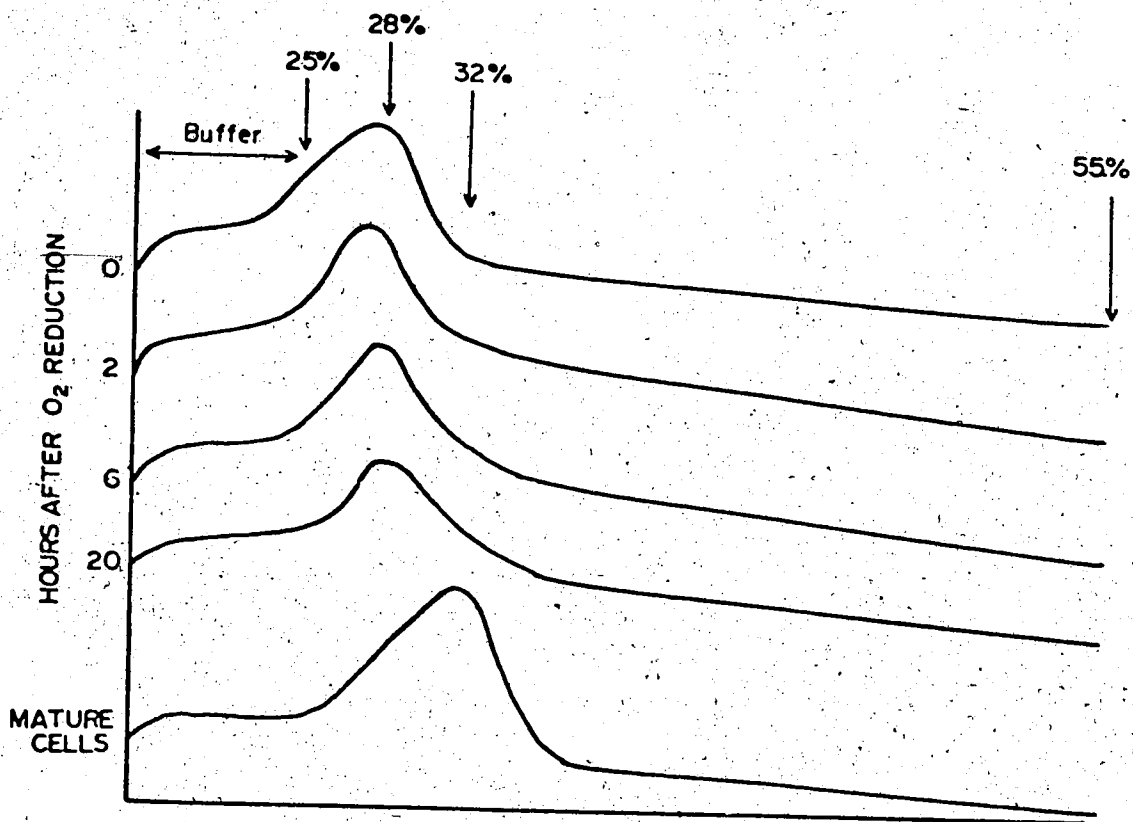


Figure 10. The 254 nm profiles of the material, harvested by centrifugation for 24 hours at 100,000 g, and run in the standard (25-55%) sucrose density gradient for 15 hours.

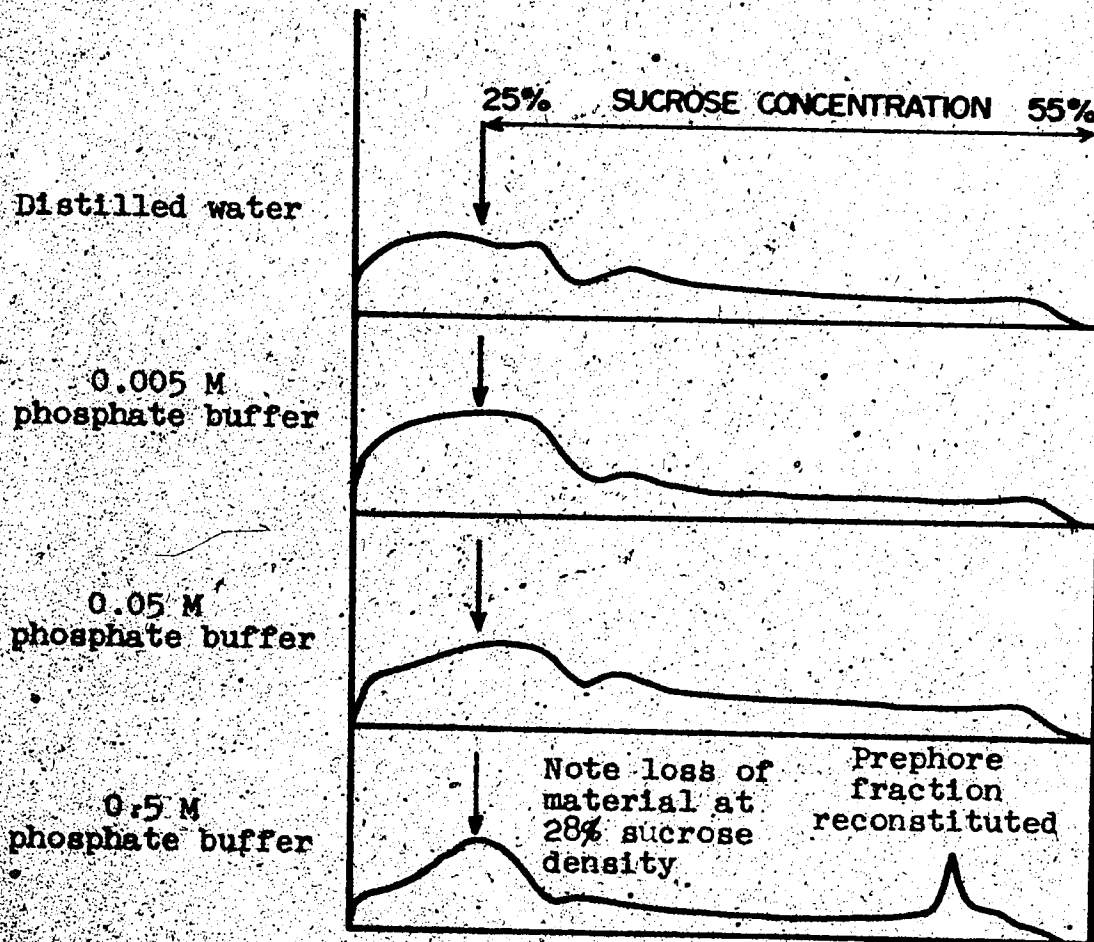
termed the material from mature cells the "mature subunit" fraction. It should be noted that the harvesting technique made it almost equivalent to the "55S" material described by Gibson (1965a)

(c) Aggregation of the subunit fractions.

Subunit aggregation might have resulted from a hydrophobic association. If this was the case then the formation of the prephore fraction should be promoted by an increase in the ionic strength of the gradients. Figure 11 shows the profiles of a standard small particle fraction when run in gradients of various ionic strengths. The material was prepared from cells after 6 hours of adaptation. It can be seen that at high ionic strength (0.5 M phosphate), a band appeared in the prephore fraction position (at 48% sucrose). The fact that the band was pigmented and formed with a concomitant loss of material at the 28% sucrose level in the gradient indicated that the material was indeed equivalent to the prephore fraction. It also showed that hydrophobic bonding was the likely cause of aggregation of the prephore subunits. Material in the 100,000 x g supernatant also produced a prephore band at 48% sucrose (not shown). This was achieved by running the sample in a gradient 0.05 M in phosphate buffer.

In a further experiment, I increased the amount of

Figure 11. Demonstration of subunit aggregation in the standard (25-55%) sucrose density gradients caused by increased ionic strength.



sample added to the gradient from 2 to 10 mg protein. This also resulted in the formation of a prephore band; however, the yield was not as great as it had been following the increase in ionic strength. The experiments showed that both the 100,000 x g supernatants and material peaking at about 28% sucrose in the standard gradient, could produce the prephore fraction.

The mature subunit fraction (the supernatant material from mature cells) also produced an aggregated band, with a density of 40% sucrose (the same position as heavy chromatophores), when centrifuged in this type of gradient. Thus both in the free and the aggregated condition the subunits from mature cells behaved differently.

(c) Glycerol density gradient electrophoresis of the subunit fractions.

I knew that the supernatant from small particle preparations isolated from adapting cells would contain two other components, namely, ribosomal proteins and varying amounts of the mature subunits. In theory, the nature of the ribosomal proteins should allow their removal from the subunits by electrophoresis. Conditions which allowed the separation of a large 254 nm peak, which was relatively low in protein content, from the prephore subunits (running as a pigmented band) are described in

Methods (Chapter 2). The unpigmented peak contained only 20 to 30% of the protein of the crude supernatant fraction. Figure 12a shows the glycerol density gradient electrophoresis profiles of material from both adapting and mature photosynthetic cells after electrophoresis. As the material from the photosynthetically mature cells had a different mobility compared to that from adapting cells, I thought the technique would also allow the separation of prephore subunits from mature subunits. The result of the two electrophoresis runs are summarized in Figure 12b.

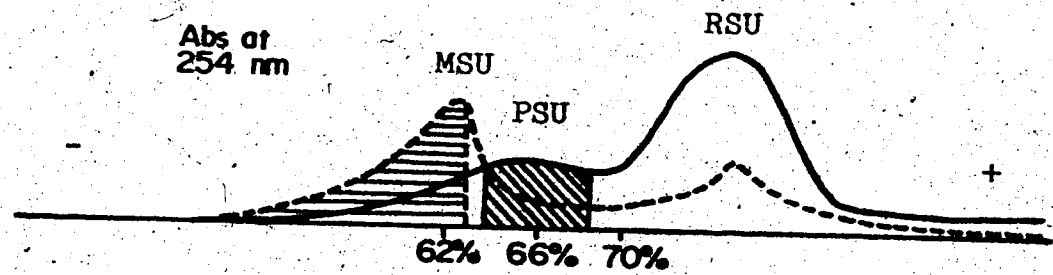
(d) Electron microscopic study of the subunit structure of the prephores.

Re-examination of electron micrographs of the negatively stained prephore fraction showed that these bodies (approx. 60 nm dia.) were regular aggregates of particles about 10 nm in diameter (Figure 13).

### 3. Light and Heavy Chromatophores.

The inclusion of  $10^{-2}$  M EDTA in the buffer and the harvesting of the small particle fraction onto a 60% w/w sucrose trap prevented the formation of the heavy chromatophore fraction. The aggregation of mature subunits may also have contributed to the heavy chromatophore fraction when the above modifications to the small particle harvesting technique were not made.

Figure 12a. Glycerol density gradient profile of purified subunits after electrophoresis.



MSU = mature subunits    PSU = prephore subunits  
RSU = ribosomal subunits

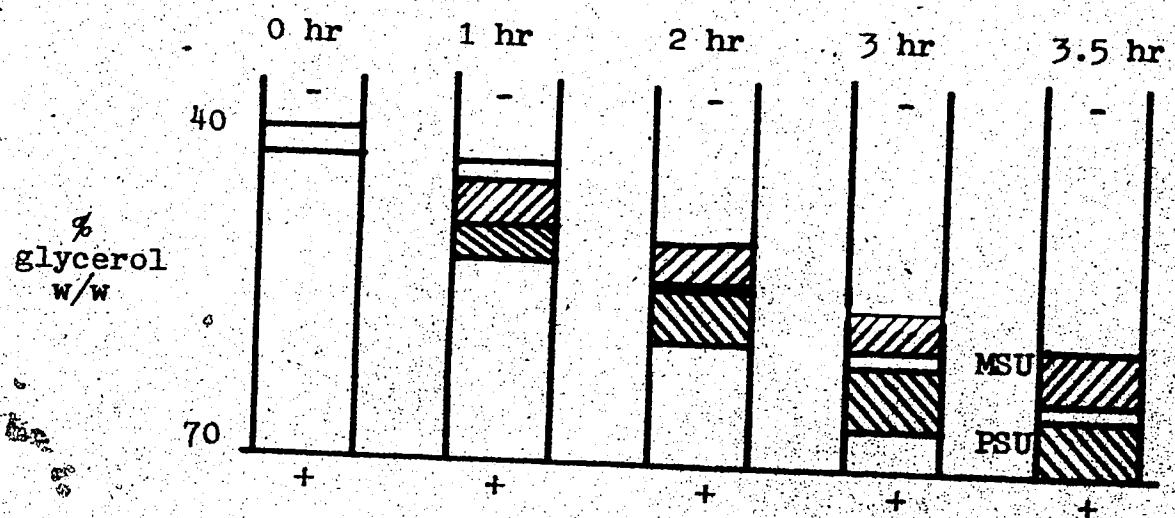
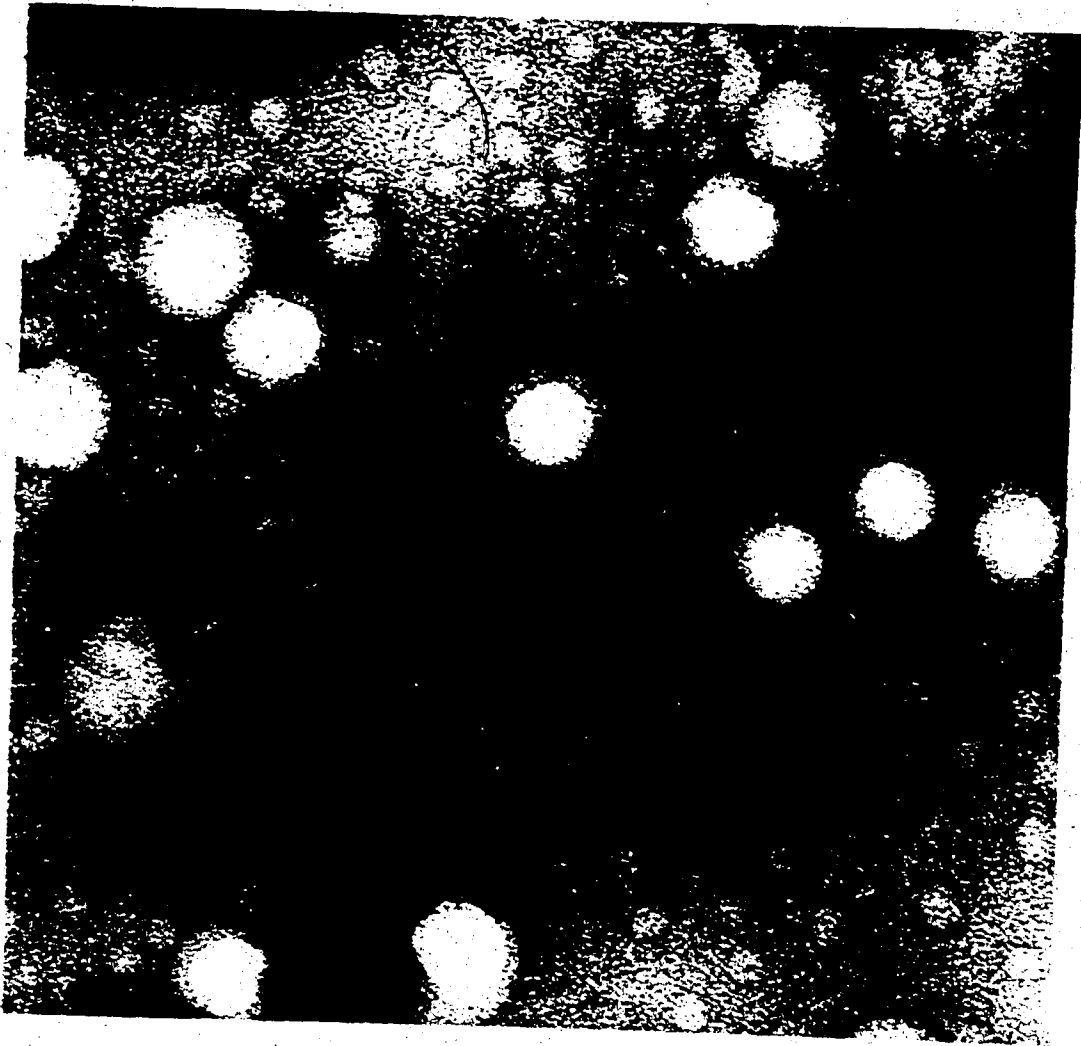


Figure 12b. Summary of the relative mobilities of the subunits from two separate (40-70%) glycerol density gradient electrophoresis runs.

Figure 13.



Negatively stained prephores and free subunits.

scale: 1 cm = 40 nm.



F. The optimal isolational and separative procedure found for the preparation of the chromatophore and prephore subunit fractions.

French press cell treatment was used in the majority of the experiments as it was found to be the most reproducible means of cellular disruption.

A buffer  $10^{-2}$  M in EDTA and  $0.5 \times 10^{-2}$  M in potassium phosphate pH 7.0 was found to prevent aggregation of material in the sucrose gradient when a 30 ml gradient was loaded with samples 5 mg or less in protein.

The initial low g force centrifugation was critical to the removal of cell envelope material from the small particle fraction. Centrifugation for 20 min at 10,000 x g allowed the small particle fraction to be decanted away from cell envelope material, which could be seen as a light-scattering layer in the 5 ml above the pelleted debris.

The use of a 2 ml 60% w/w sucrose trap in the small particle harvesting procedure eliminated the formation of the heavy chromatophore fraction in the standard sucrose density gradient. This modification reduced the prephore fraction causing a pigmented peak to form with a density of 28% sucrose.

Quantitatively, the best source of prephore subunits was the 1.5 hr, 100,000 x g supernatant derived from cells

in the first few hours of adaptation. The glycerol density gradient electrophoresis technique was used to separate the prothiore subunits from ribosomal proteins and mature subunits. The latter were predominant in cells of high chromatophore content.

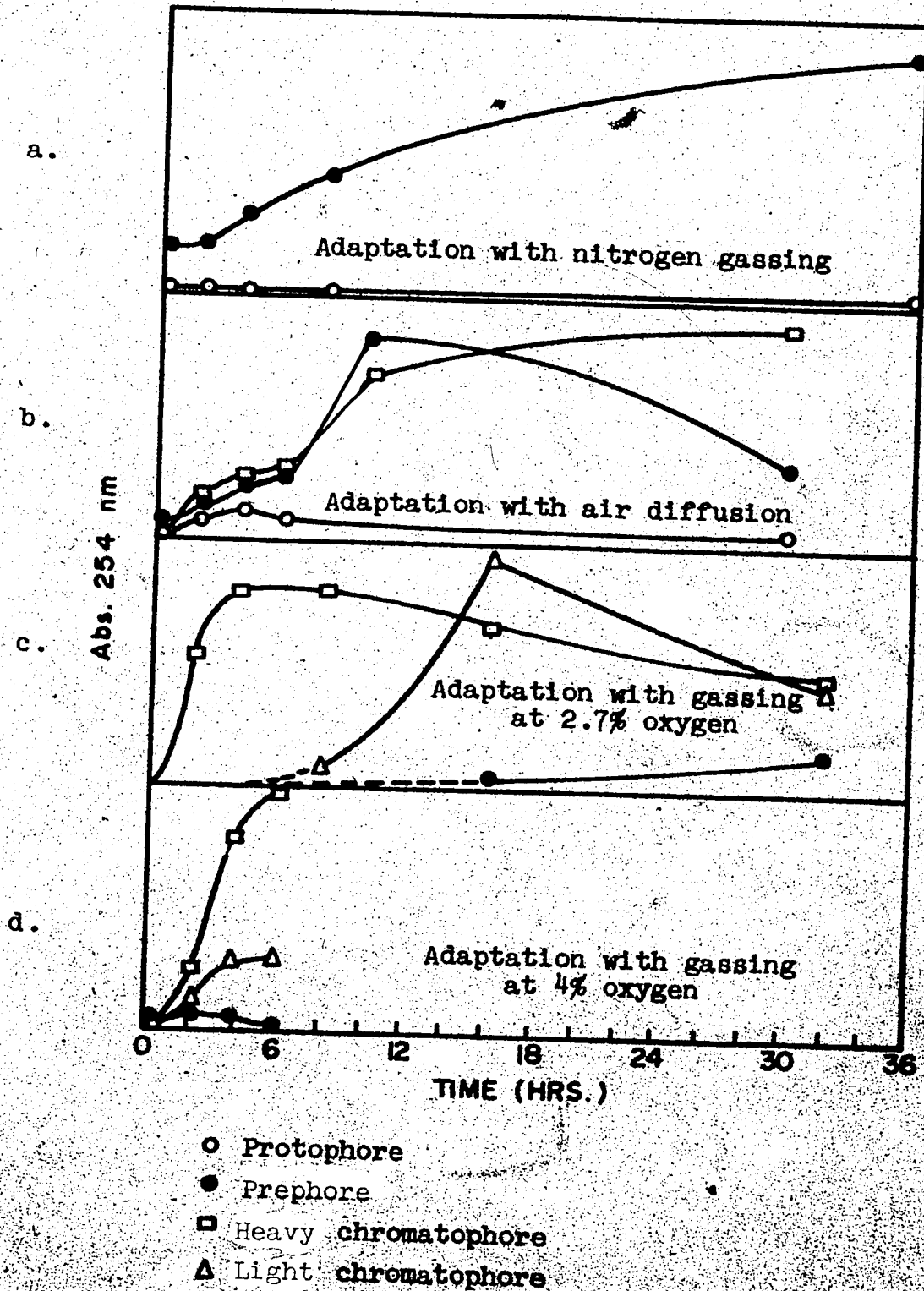
## II. A Study of R. spheroides during Adaptation from Aerobic to Phototrophic Growth Conditions.

### A. The effect of the growth phase and oxygen content on adaptation.

Most of the early work had been performed with late log phase cells. These had been diluted with fresh medium and limited in oxygen supply (diffusion through the surface of the culture) to promote thylakoid formation. As a large amount of cell lysis occurs at late log phase, I decided to work with an early log phase culture. This would show the adaptive changes in physiologically "healthy" cells.

Cells were grown to the required initial cell level aerobically with 65% oxygen at a flow rate of 4 S.C.F.H. Figure 14 shows the relative levels of the major components in the standard sucrose density gradient fractionation of material from adapting early log phase cells in the light at the oxygen levels indicated. (These experiments were performed prior to the modification of

Figure 14. Relationship of various fractions (254 nm peak heights) in the adaptation of early log phase cells under different conditions of aeration.



the buffer, described in the previous section, and thus produced a heavy chromatophore fraction). It can be seen that no adaptation occurs if unpigmented cells are gassed with nitrogen. The prephore fraction did, however, increase. Pigment was also formed, the majority being the 870 nm form of Bchl. No cell growth occurred, indicating that the prephores were not photosynthetically competent. When some oxygen was allowed into the medium through the surface of the culture, the fluctuations in the membranous fractions was similar to that described previously (Figure 3). Figure 14b shows that in such a situation, early log phase cells develop both prephore and chromatophore fractions. The prephore fraction peaked after 10 hours of oxygen limitation and the chromatophore fraction continued to increase. In the first six hours of the adaptation the cells increased one and a half times in number. Adaptation with 2.7 or 4.0% oxygen gassing (Figures 14c and d) was found to cause the rapid production of the heavy chromatophore fraction. In these conditions the cells increased three-fold in the first six hours. The prephore fraction was, however, present at much lower levels relative to the other components in the gradient.

It appeared that the ideal situation for the study of the fraction changes during adaptation to photosynthetic growth, would be one in which adaptation was neither very slow nor very rapid. The slow adaptation yields only a

small chromatophore fraction, while rapid synthesis makes it difficult to establish precursor-product relationships. To choose the optimal conditions, the amount of cell growth and pigmentation were measured under various adaptation conditions. The level of oxygen used and the growth phase of the cells at the start were varied; the results are shown in Table IV.

B. Analysis of cellular constituents during adaptation.

Figure 15 shows the concentration of PHB, glycogen, protein and  $\delta$ chl in g/cell. It appears that in the first hours of adaptation, glycogen may have been used as an energy source, presumably by a fermentative process. Later in the adaptation, the PHB level decreased.

$\delta$ chl was first detectable after two hours and increased at a nearly constant rate from 4 to 20 hr.

C. Small particle fractions produced during aerobic growth.

I thought that a method of limiting oxygen by using a slow gas flow rate might be a means of specifically producing the prephore fraction. From Figure 16 it can be seen that the oxygen level in the culture fell to a low level for about one hour towards the end of log phase.

Another experiment was performed in which samples were taken at various times during the growth of a culture

Table IV  
 Growth rate and level of Bchl synthesis of cultures adapted at different stages in log phase with different levels of oxygen (balance nitrogen) at 4 S.C.F.H.

% Oxygen in gas	Experiment	Growth phase	Cells/ml at the start $\times 10^8$	Increase in cells at 6 Hrs	Bchl formed /cell (Mx10 <sup>13</sup> ) at 6 Hrs	Bchl formed /ml (Mx10 <sup>9</sup> ) at 6 Hrs
0%	A	Early	0.46	$\times 1.0$	0.65	0.30
	B	Early	0.27	$\times 1.3$	1.80	0.64
1 ± 1/2%	C	Mid	0.82	$\times 1.0$	0.10	0.08
	D	Late	1.65	$\times 1.0$	0	0
2.7 ± 1/2%	E	Early	0.29	$\times 3.0$	1.15 <sup>a</sup>	1.00
	F	Mid	0.76	$\times 2.4$	1.48 <sub>g</sub>	2.72
4.0 ± 1/2%	G	Late	1.62	$\times 1.1$	0.09	0.16
	H	Early	0.21	$\times 3.0$	1.52	0.96
4.0 ± 1/2%	I	Mid	0.87	$\times 1.5$	0.74	0.96
	J	Late	1.73	$\times 1.0$	0	0

90b-

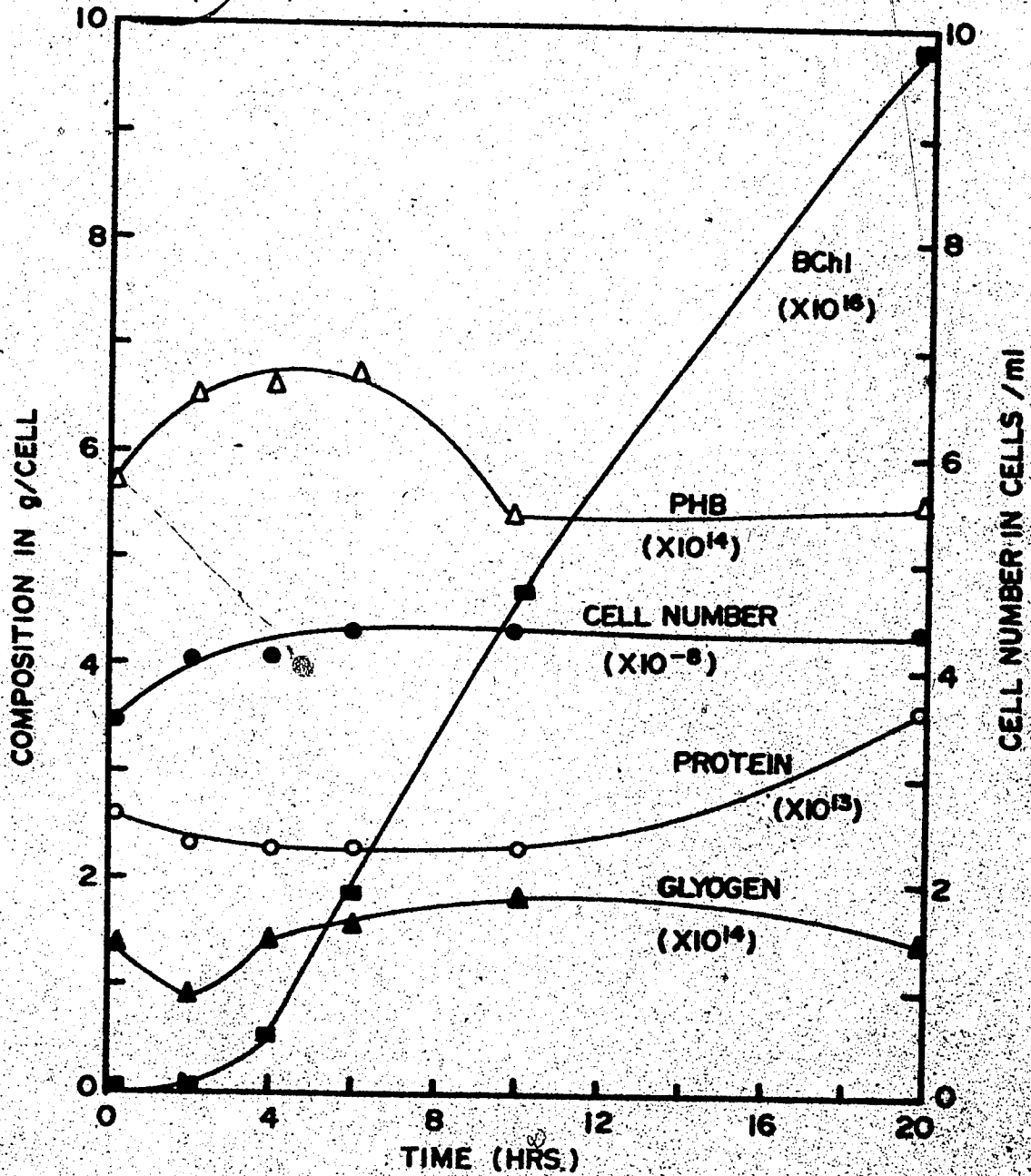


Figure 15.

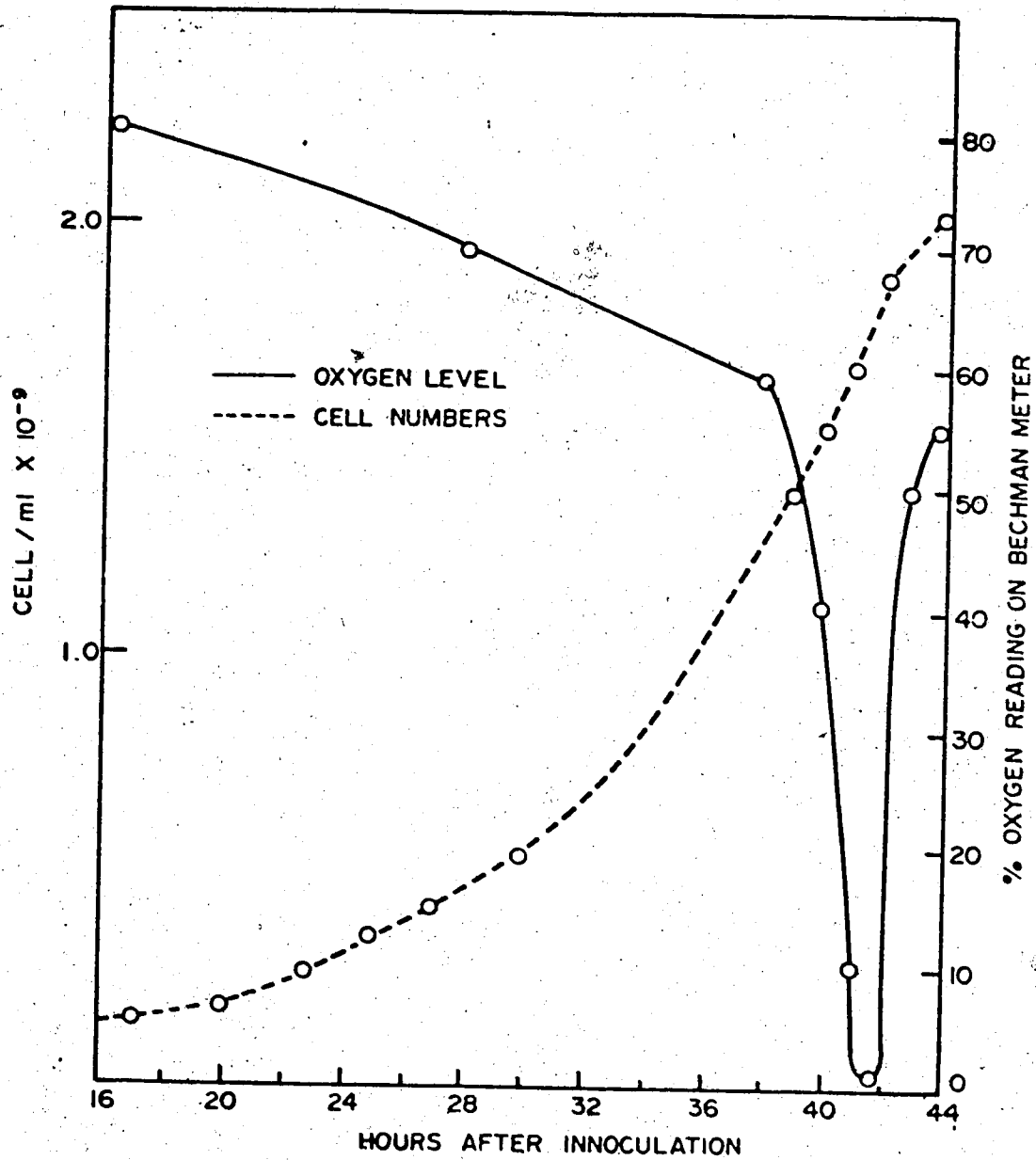
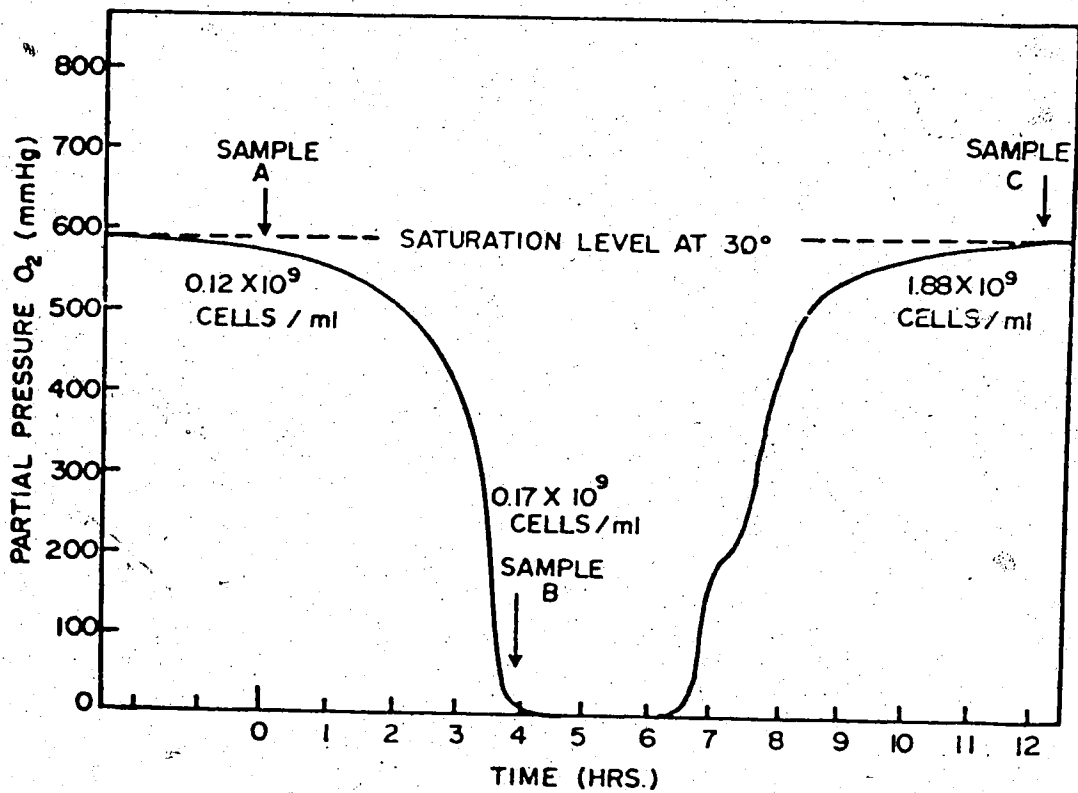


Figure 16. Relationship between cell number and oxygen partial pressure. Culture gassed at 0.5 S.C.F.H. with oxygen at 50%.



being gassed with 100% oxygen at a flow rate of 0.5 S.C.F.H. The sampling times and the oxygen level throughout the latter part of the log phase is shown in Figure 17. While the culture was at a low partial pressure, the flow rate was adjusted to 2 S.C.F.H. for a few seconds. The resulting rapid increase in the meter reading (not shown on Figure 17) showed that the dissolved oxygen level was indeed in balance with the consumption by the cells and the gas flow rate. The samples were disrupted by the French pressure cell and the small particle fraction separated by the standard sucrose density gradient technique. The gradient profiles of these samples is shown in Figure 18. Sample A produced no appreciable prephore fraction. The prephore fraction was present in sample B and had increased in sample C and become pigmented. The prephore fraction from sample C was less dense than that from sample B. Sample D (taken 48 hr after sample A but not shown on Figure 17) produced essentially the same gradient profile as sample C. The results shown (Figures 16 and 17) were obtained with the same growth apparatus and number of cells/ml ( $2 \times 10^7$ ) in the inoculum. One culture took 42 hr to reach late log phase while the other grew more rapidly requiring only 30 hr to attain the same level of cells/ml. The culture growing more slowly reduced the oxygen tension below the level of saturation only towards the end of log phase

Figure 17. Oxygen level (partial pressure mm Hg) in a 4 l. culture of *R. spheroides* gassed with 100% oxygen at 0.5 S.C.F.H. through an 8 cm<sup>2</sup> sinter.



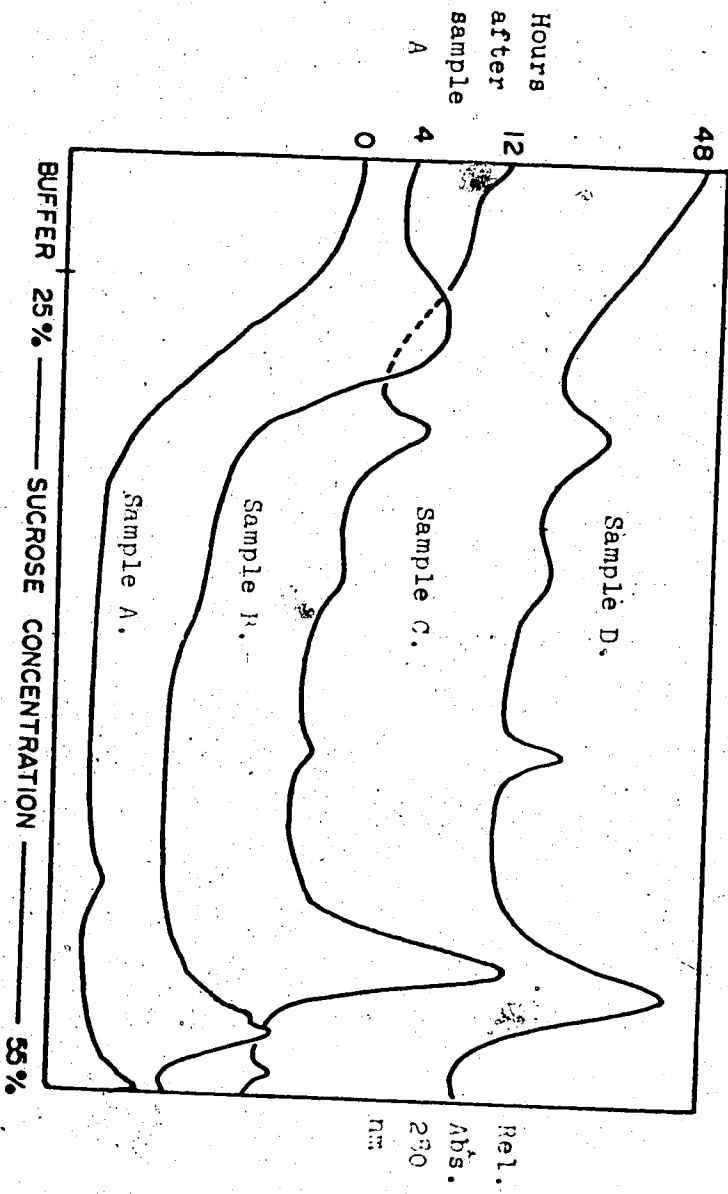


Figure 18. Sucrose density gradient profiles of the small particle fractions obtained from partially adapted cells. See figure 19 for growth curve and oxygen levels.

(Figure 16). The culture that grew most rapidly, however, reduced the oxygen level throughout the log phase (Figure 17). Although the reason for the variations in growth rates is not known the experiments show that the duration of the reduction in oxygen level in the medium is dependent on the growth rate of the culture being oxygenated.

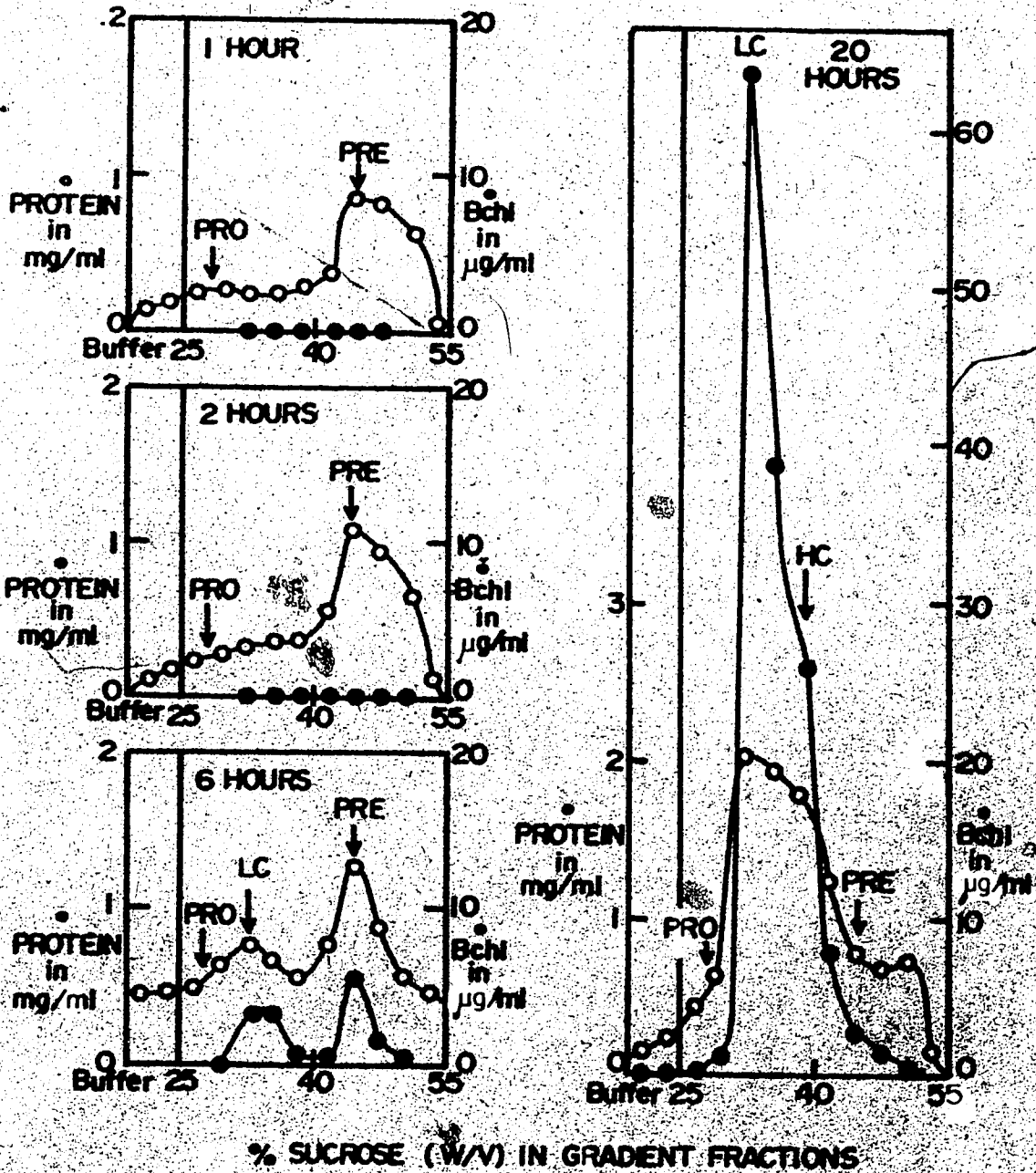
D. Bchl and protein content of small particle fractions.

1. During adaptation.

An oxygen-grown culture was adapted by limiting the oxygen supply to diffusion through the surface of the medium (cf. Methods, Chapter 2). Spectral analysis of the fractions showed that by 6 hr, prephore, light chromatophore and heavy chromatophore fractions contained Bchl and carotenoids (Figure 19). The Bchl/protein ratio was always low in the prephore fraction while that in the light chromatophore fraction, though similar to that in the prephore fraction at 6 hr, had increased elevenfold (to 0.028 mg/mg) at 20 hr. The Bchl/protein ratio of the heavy chromatophore fraction was only half that of the light chromatophore fraction. The protophore fraction was not pigmented at any of the times studied (Figure 19).

2. During aerobic growth.

Figure 19. Sucrose density gradient protein and Bchl profiles during adaptation.



A culture was grown by oxygen gassing at 0.5 S.C.F.H. The oxygen levels and times of sampling are shown in Figure 17. The cells harvested in samples A and B were unpigmented while those collected after the drop in oxygen level were pigmented. The experiment showed that the development of the prochlorophyll fraction into chromatophores was inhibited at the high oxygen level. Surprisingly, the synthesis of Bchl continued. The in vivo spectrum showed that the 870 nm form of Bchl predominated in sample C (Figure 20) while there was a large increase in the 850 nm form of Bchl in sample D (Figure 20). Analysis of the gradient fractions for pigment, however, failed to show the dramatic increase (Table V). This table shows that the light chromatophore fraction almost doubled its content of the 850 nm form of Bchl. This, however, represented only a small portion of the increase in the small particle fraction as a whole.

E. Analysis of individual protein components of small particle fractions during adaptation.

I. Growth conditions used prior to purification and protein analysis of the fractions.

Cultures A, B, C and D were grown from inocula of low pigment content.

Culture A.

The culture was grown at a flow rate of 2 S.C.F.H.

Figure 20. The in vivo spectra of cells grown at 0.5 S.C.F.H. oxygen gasatng.

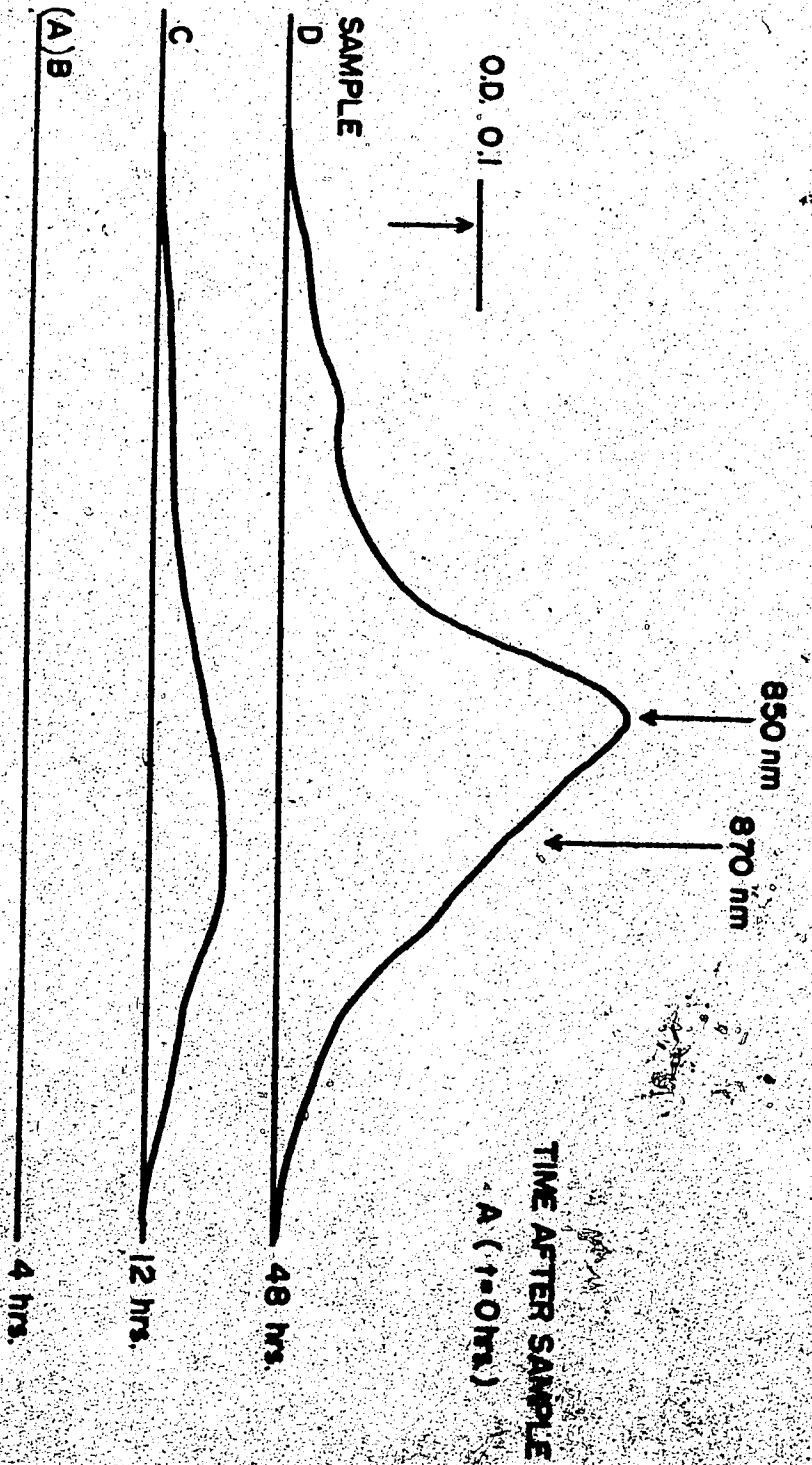


Table V

Optical density of gradient fraction at 850 and 870 nm.

(N.B. Samples A and B contained no detectable pigment.)

Fraction	Sample C		Sample D	
	O.D. 850	O.D. 870	O.D. 850	O.D. 870
Heavy chromatophore	below $5 \times 10^{-4}$	0.023	below $5 \times 10^{-4}$	0.021
Light chromatophore	below $5 \times 10^{-4}$	0.006	0.001	0.010
Prephore	0.001	0.005	0.001	0.005



using 100% oxygen. The cells were harvested while the culture was at early log phase.

Culture B.

The culture was grown at a flow rate of 0.5 S.C.F.H. using 5% oxygen. The dissolved oxygen content was monitored throughout growth by means of an oxygen electrode. Cells containing the unpigmented prephore fraction were obtained by harvesting the culture when cellular uptake had reduced the oxygen content to 0.25 m moles/liter (a meter reading of 18%).

Culture C.

In this culture 65% oxygen was used to produce a mid log phase culture. The adaptive changes were then induced by gassing for two hours with 4% oxygen at 2 S.C.F.H. Under these conditions pigmentation is rapid.

Culture D.

This culture was grown to mid log phase in the same manner as culture C and then slowly adapted by limiting the oxygen entry to diffusion through the surface of the magnetically stirred medium. The culture was harvested after 6 hours.

Culture E.

An anaerobically grown inoculum was transferred to a flask full of fresh medium and grown anaerobically for a further three days. The culture was illuminated at approximately 300 foot candles. After a slow growth the

cells were harvested at mid log phase. They were taken to represent the fully photosynthetic state.

## 2. Cell numbers and Bchl contents of the cultures.

The number of cells/ml and the degree of their pigmentation is shown in Table VI. It can be seen that cultures A and B were unpigmented, while C, D and E contained various levels of pigment. The culture induced to rapid adaptation, C, had about half the pigment/protein ratio of culture D (slowly adapted) after six hours. The photosynthetic cells (culture E) contained the most pigment. Culture C contained only the 870 nm form of Bchl, culture D contained equal amounts of the 850 and 870 nm forms, while culture E had the majority of pigment in the 850 nm form.

## 3. Separation scheme.

An early experiment had shown that material centrifuged for 24 hours in a sucrose density gradient gave an almost identical gradient profile to that obtained after the standard 16 hour run. The profile obtained after only 8 hours was different. In the standard gradient, the light chromatophore fraction reached a density equivalent to 28% sucrose (8 hr) rather than 34% (16 hr). I decided to fractionate and purify material produced from the different cultures using both

Table VI

The number of cells/ml and the degree of their pigmentation is shown for the different cultures.

Culture	A	B	C	D	E
Culture number/ml	$8.0 \times 10^7$	$9.2 \times 10^7$	$8.8 \times 10^7$	$3.4 \times 10^8$	$3.2 \times 10^8$
Pigment (Pchl)/ml of culture (g/ml*)	850 nm N.D.	N.D.	N.D.	$1.5 \times 10^{-7}$	$6.9 \times 10^{-7}$
	870 nm N.D.	N.D.	$3.0 \times 10^{-8}$	$1.5 \times 10^{-7}$	N.D.
Pchl/g whole cell	N.D.	N.D.	$3.4 \times 10^{-10}$	$8.8 \times 10^{-10}$	$21.0 \times 10^{-10}$
Pchl/protein ratio (by weight)	N.D.	N.D.	$1.3 \times 10^{-3}$	$2.2 \times 10^{-3}$	$10.8 \times 10^{-3}$

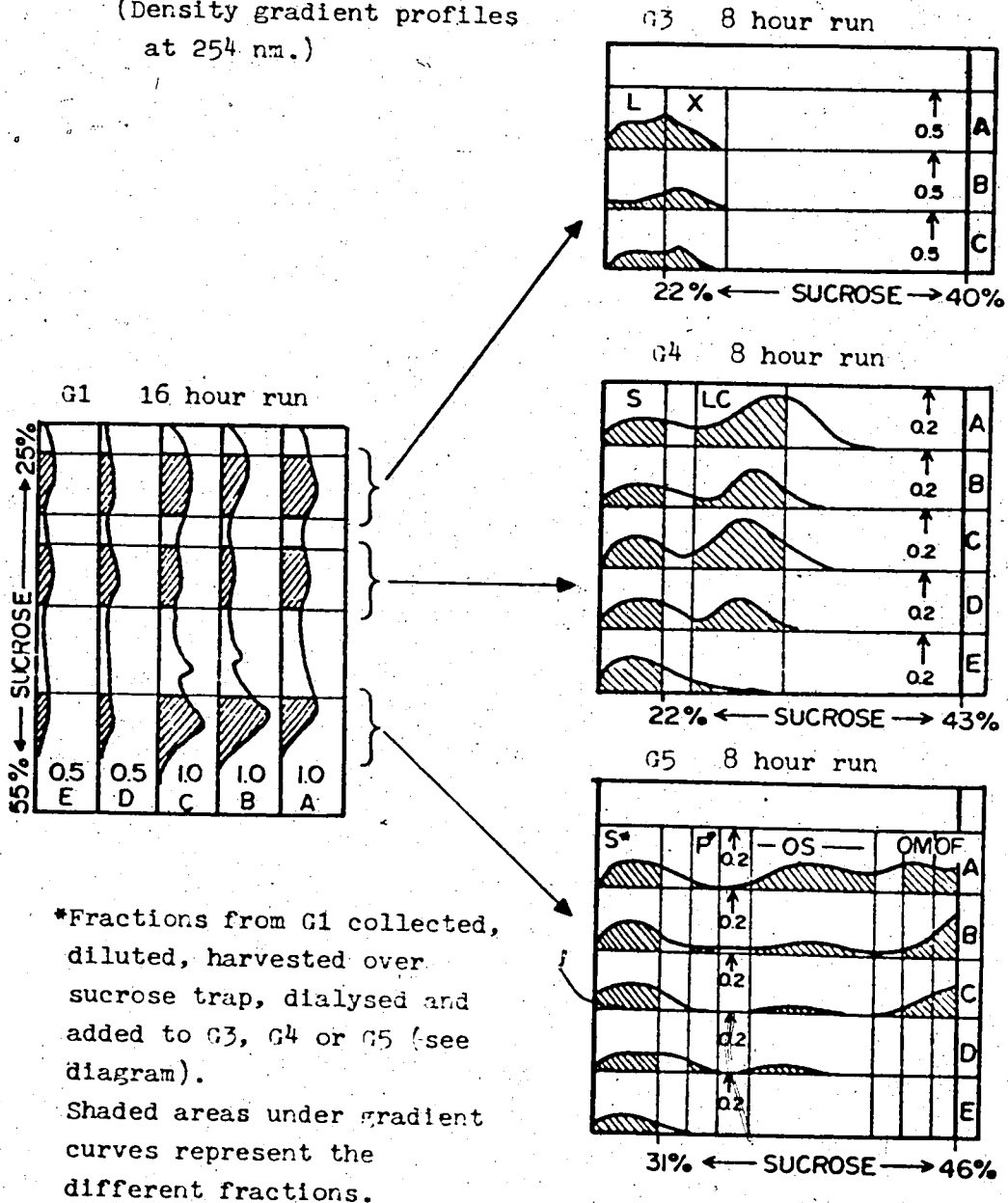
\*Assuming absorption/g of pigment at 870 is equal to that at 850 nm.

N.D. Not detectable.

equilibrium and non-equilibrium centrifugation. Fractions from the standard (16 hr) gradient were further resolved by 8 hr non-equilibrium centrifugation in less extreme gradients than the standard (25 to 55% sucrose) gradient. The separation scheme is shown diagrammatically in Figure 21. (A reverse order of the use of equilibrium and non-equilibrium centrifugations was found to give the best yield of the medium density fraction. This material was analysed along with fractions from the scheme shown in Figure 21 ).

In samples A, B and C, the prephore fraction was the largest, while samples D and E contained most material in the light chromatophore fraction (cf. run G1, Figure 21). The prephore fractions obtained from the oxygen grown cells (samples A and B), were unpigmented. The amount of material in this fraction increased from samples A to C, the latter being pigmented (in the 870 nm form of Bchl). Further purification of the prephore fractions produced an interesting result (see run G5, Figure 21). The prephore fractions of samples C, D and E in the standard equilibrium centrifugation (G1) were pigmented. After the non-equilibrium run the pigment had not sedimented but remained in the buffer layer above the gradient (S\*). The only explanation for the fact that in samples C, D and E the majority of the protein was also found in the buffer layer was that the original prephore fraction had

Figure 21. Separation scheme  
(Density gradient profiles  
at 254 nm.)



dissociated into smaller units. The new fraction has been termed the prephore subunit fraction (cf. Section I.E.2 above).

#### 4. SDS-PAGE analysis of the fractions.

Samples were analysed by the SDS-PAGE technique. Examples of the gel profiles obtained for each fraction of material from culture C are shown (Figures 22 to 26).

The pigmented material (S\*) remaining in the buffer in the gradients of run G5 (Figure 21) had a characteristically simple profile. This profile was also observed in the P\* fractions analysed from this run (Figure 29). The profiles for the S\* fraction are shown in Figures 27 and 28 for material from cells grown in all of the different culture conditions. Although material from cultures A and B in fraction S\* has a more complex profile, that from P\* from culture A showed the same typical profile as that from cultures C and D (Figure 29).

Figure 30 shows the SDS-PAGE profile of the prephore subunit material purified by glycerol density gradient electrophoresis (cf. Section I.E.2) in comparison with that of a chromatophore fraction. It can be seen that proteins B, F and H are the major proteins in the prephore subunit fraction.

Figure 31 shows that the mature subunit material purified by glycerol density gradient electrophoresis has

Figure 22.

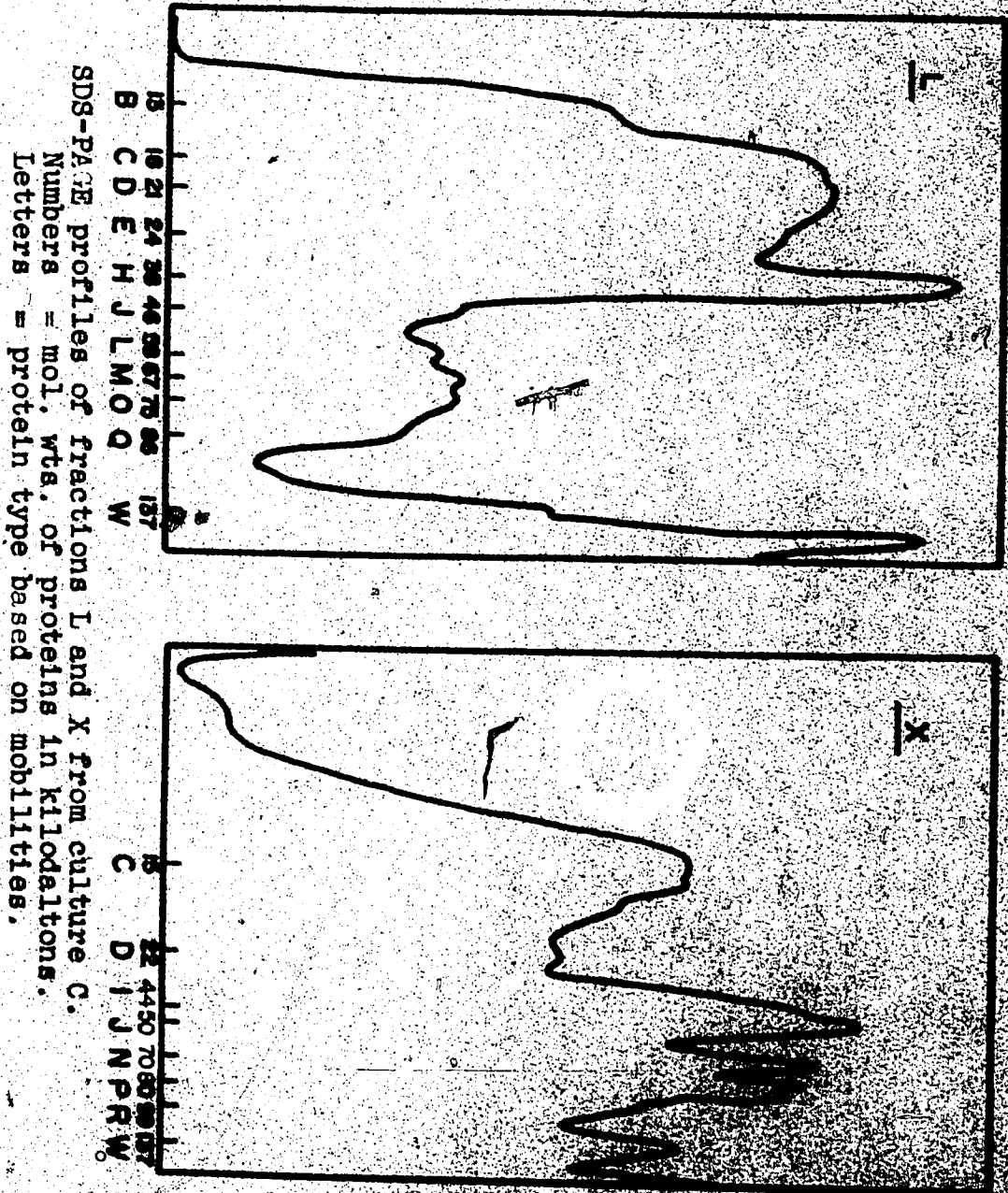
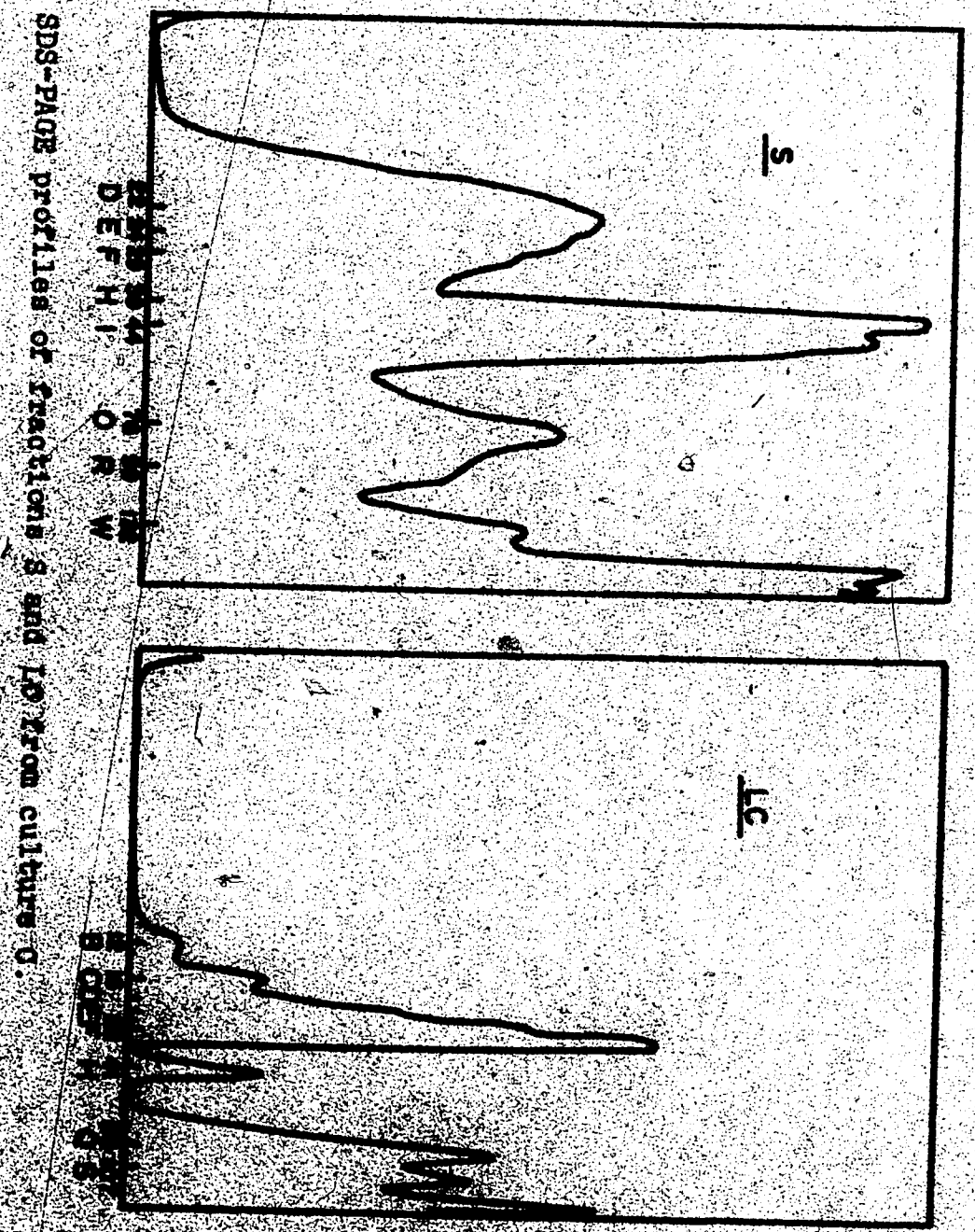


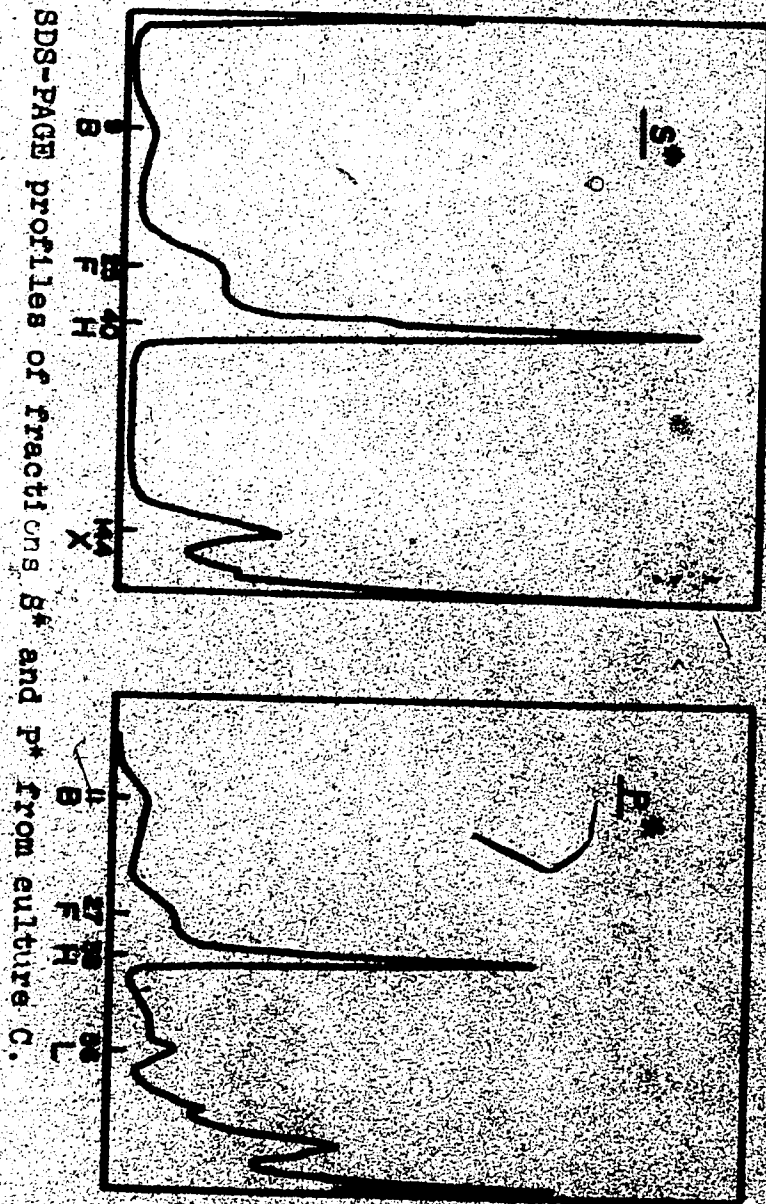
Figure 23.



SDS-PAGE profiles of fractions S and LC from cultures C.

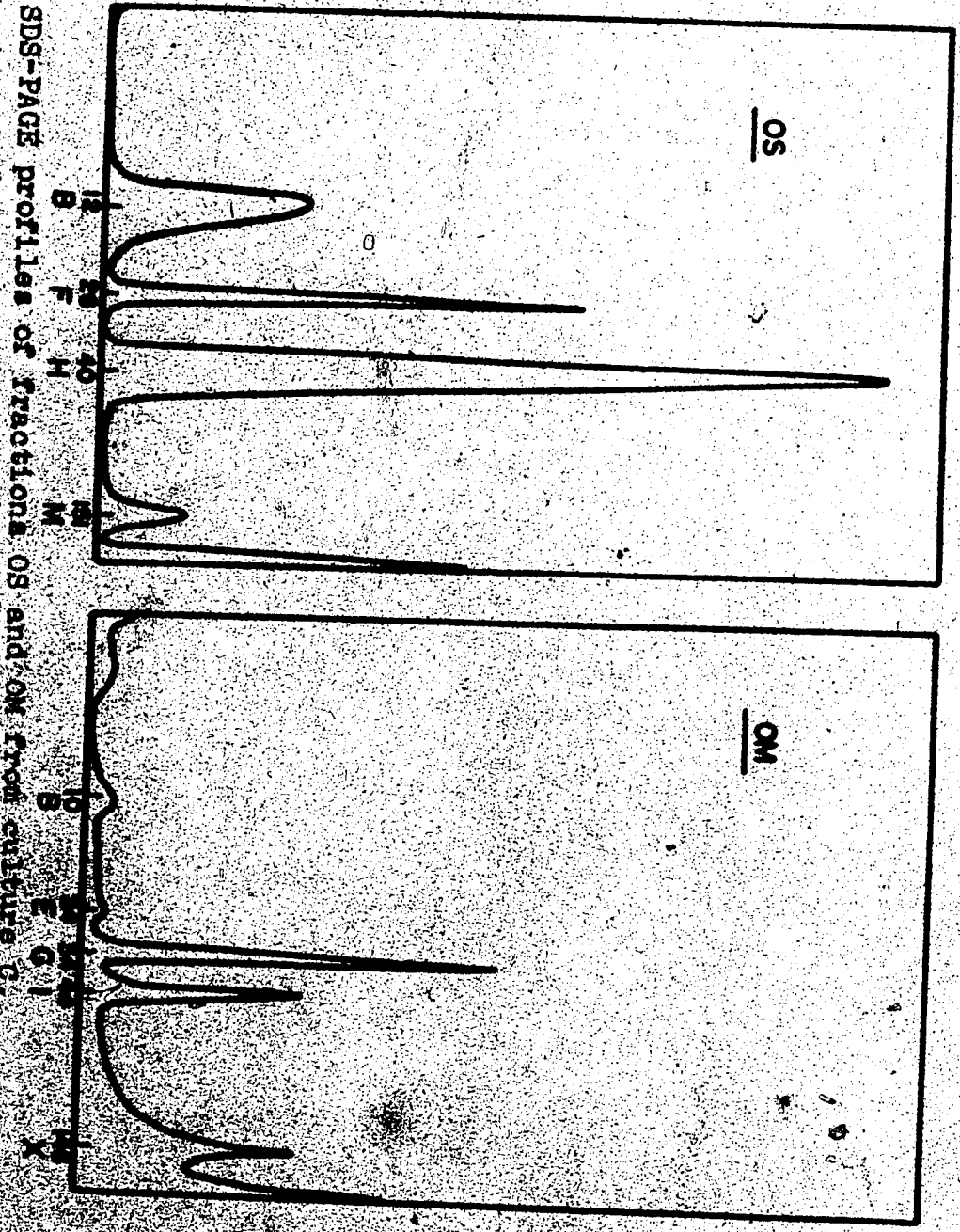


Figure 24. G



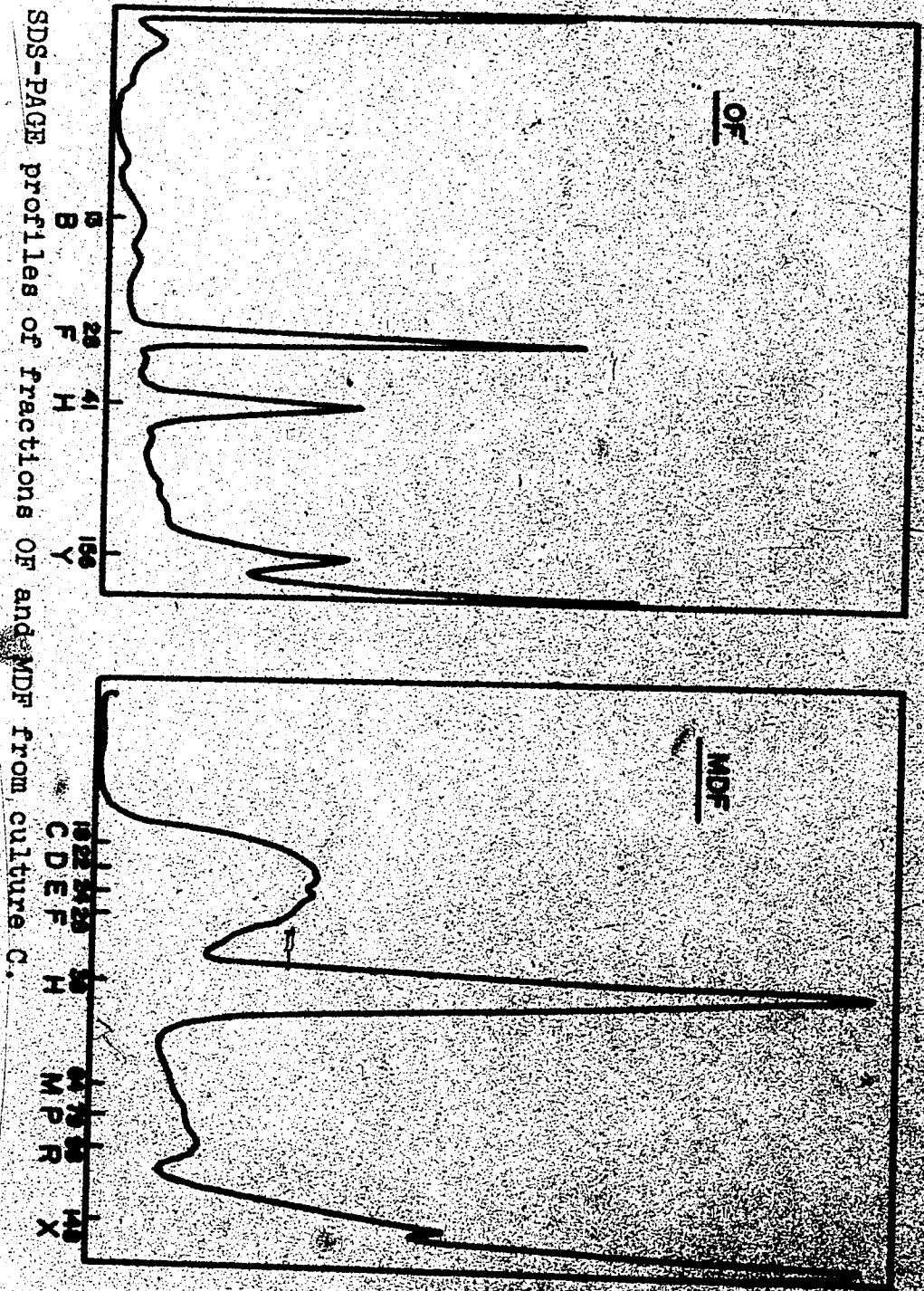
SDS-PAGE profiles of fractions  $B^*$  and  $P^*$  from culture C.

Figure 25.



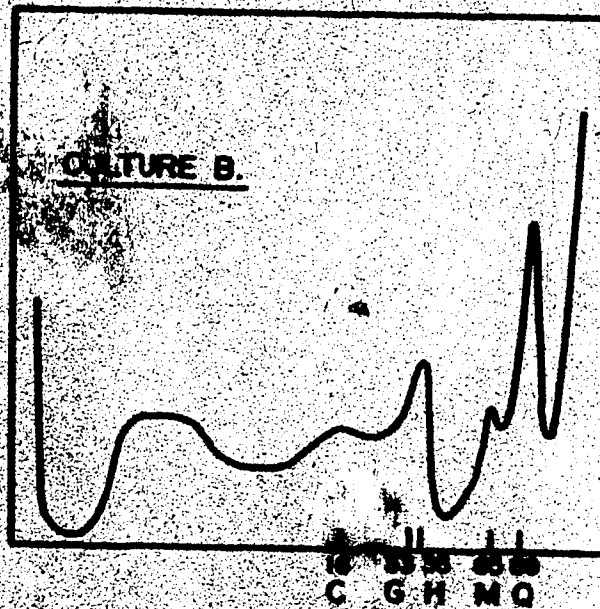
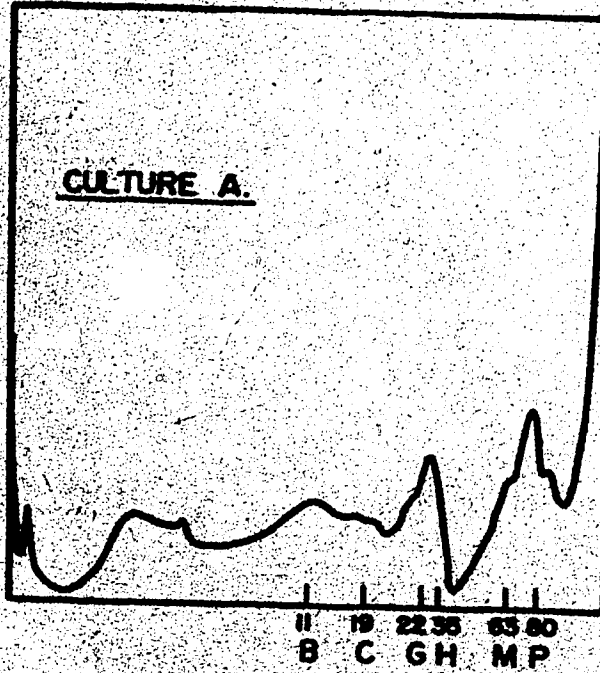
SDS-PAGE profiles of fractions OS and OM from culture 2.

Figure 26.



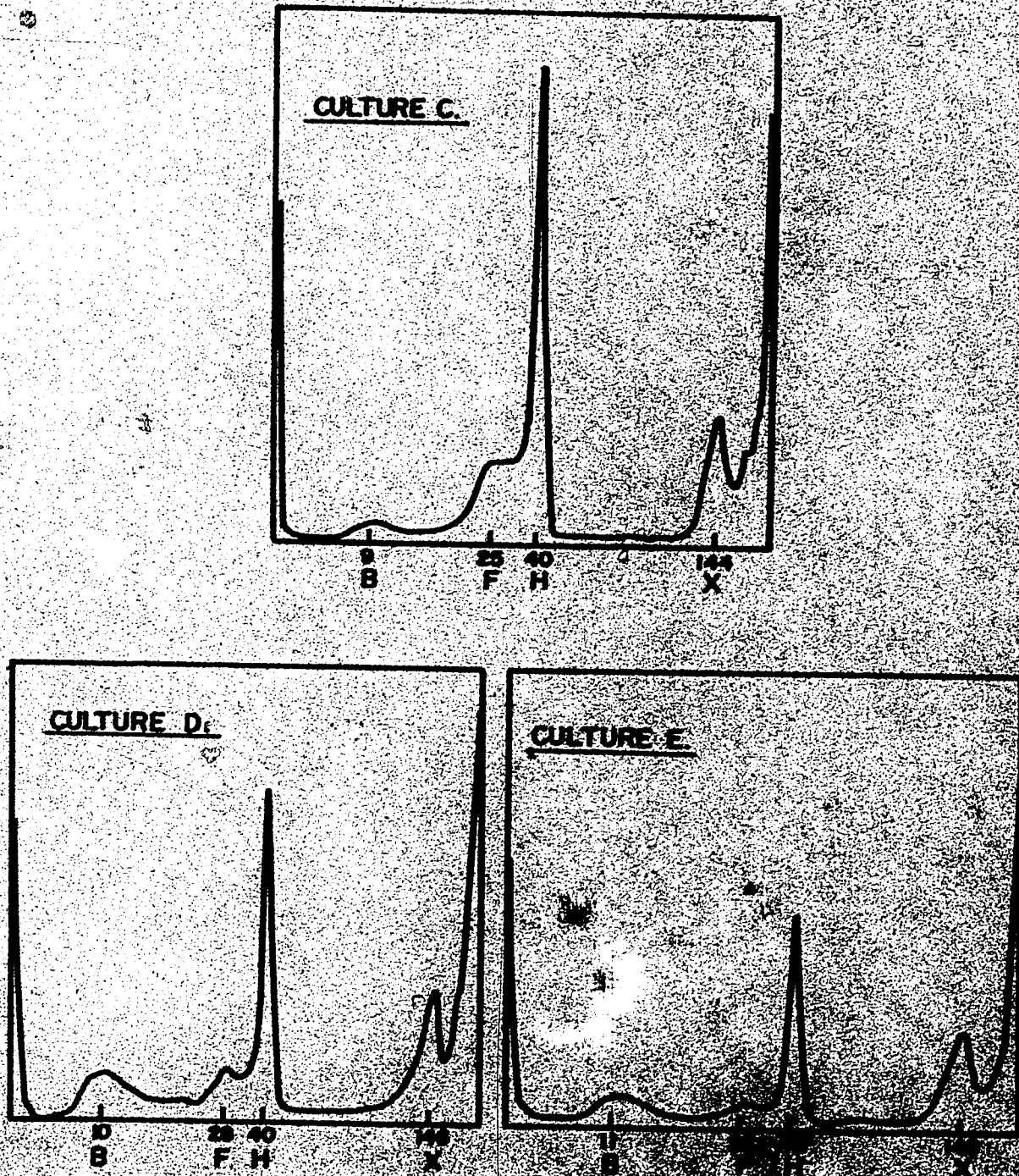
SDS-PAGE profiles of fractions OF and MDF from culture C.

Figure 27.



SDS-PAGE profiles of S<sup>2</sup> fraction from cultures A and B.

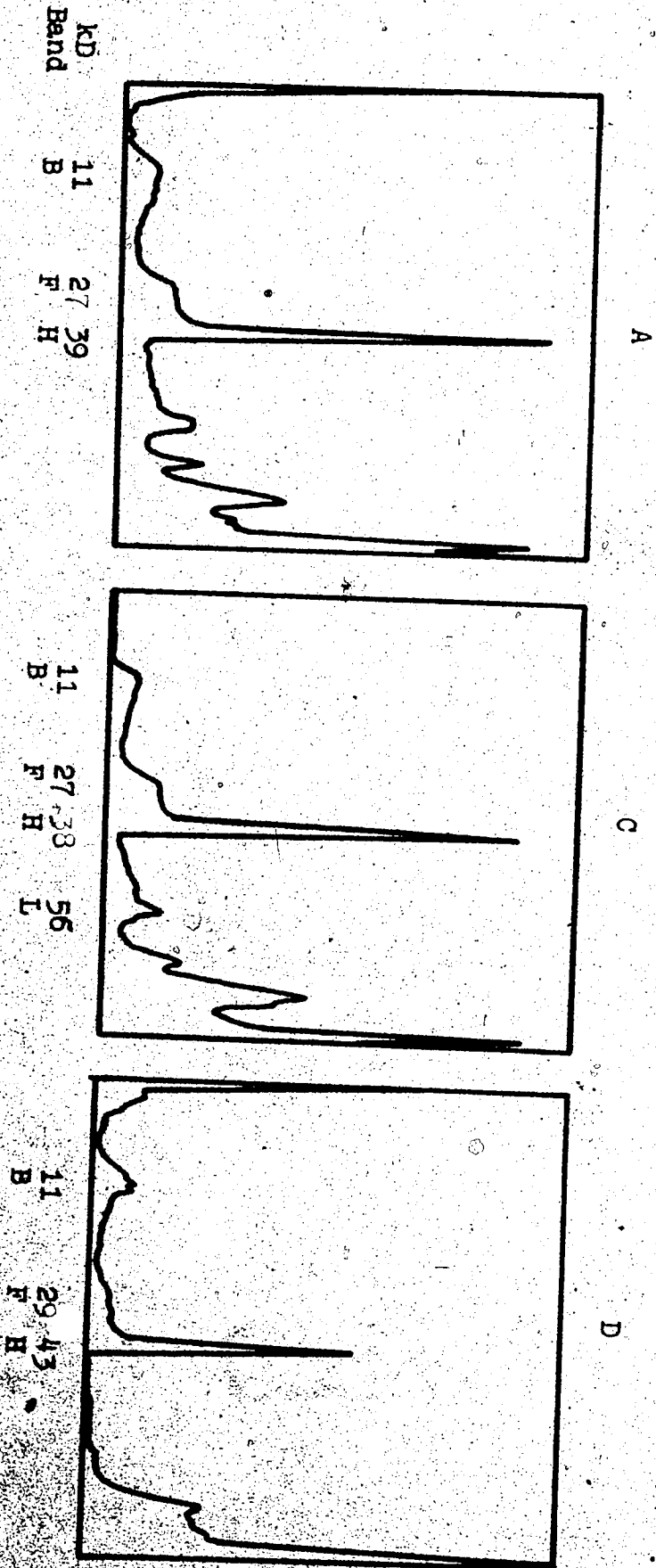
Figure 28.



SDS-PAGE profiles of S<sup>+</sup> fraction from cultures C, D and E.



Figure 29. SDS-PAGE profiles of P\* fractions.



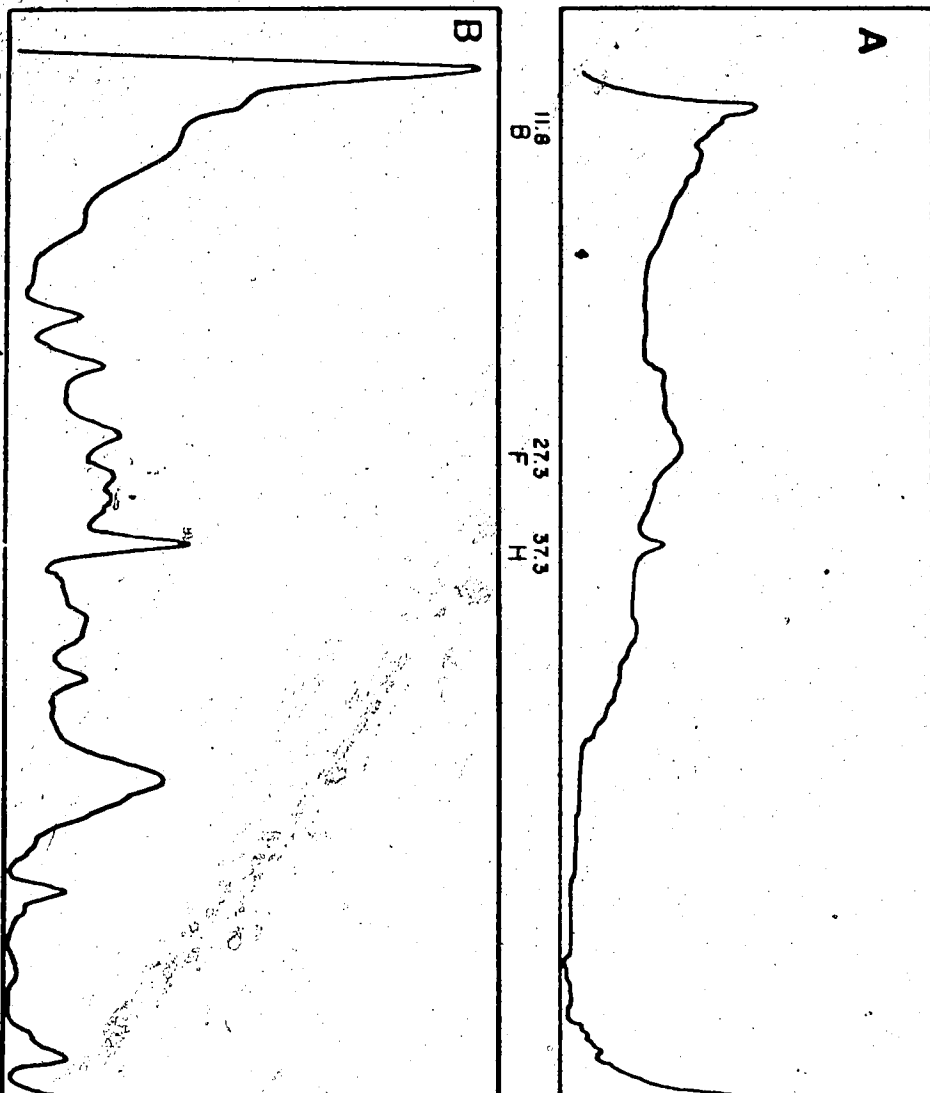


Figure 30. SDS-PAGE profiles of subunit fraction (A) and chromatophore fraction (B) prepared from cells adapted for 6 hours.

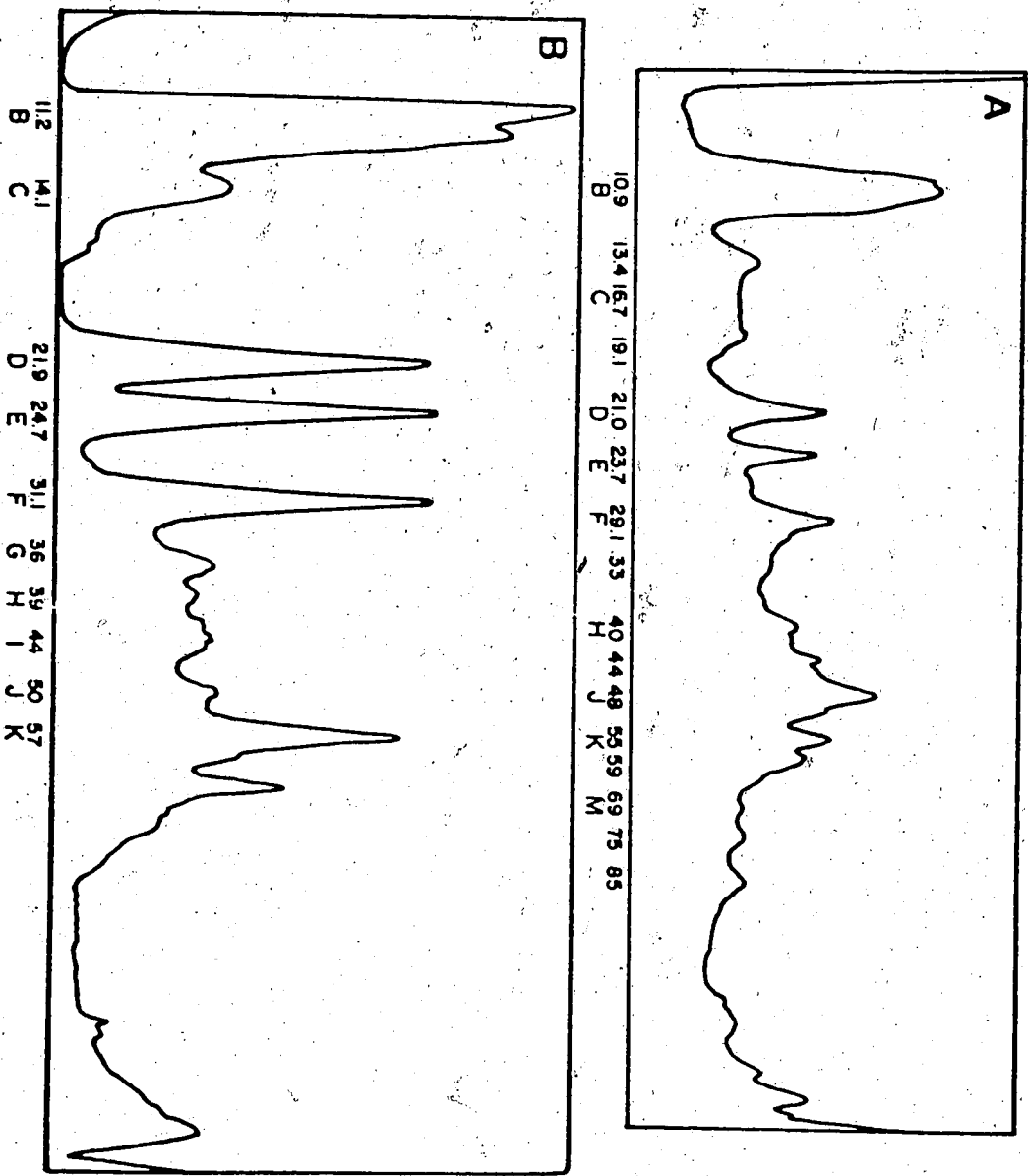


Figure 31. SDS-PAGE profiles of A. the mature subunit fraction, B. the chromatophore fraction.



a profile similar to that of the chromatophore. The typical chromatophore SDS-PAGE profile is shown in Figure 32.

SDS-PAGE of the reconstituted prephore fraction (cf. Section I.E.2) shows that proteins B, F and H are major components (Figure 33).

Figure 34 shows that a pigment-protein complex excreted into the medium by slowly adapting parent cells has many proteins in common with the reconstituted prephore material. The complex was excreted when early log phase cells were incubated for 10 hr in the dark, with oxygen entry limited by diffusion into the culture. An ether spectrum of the pigment indicated that it was magnesium protoporphyrin (or its monomethyl ester).

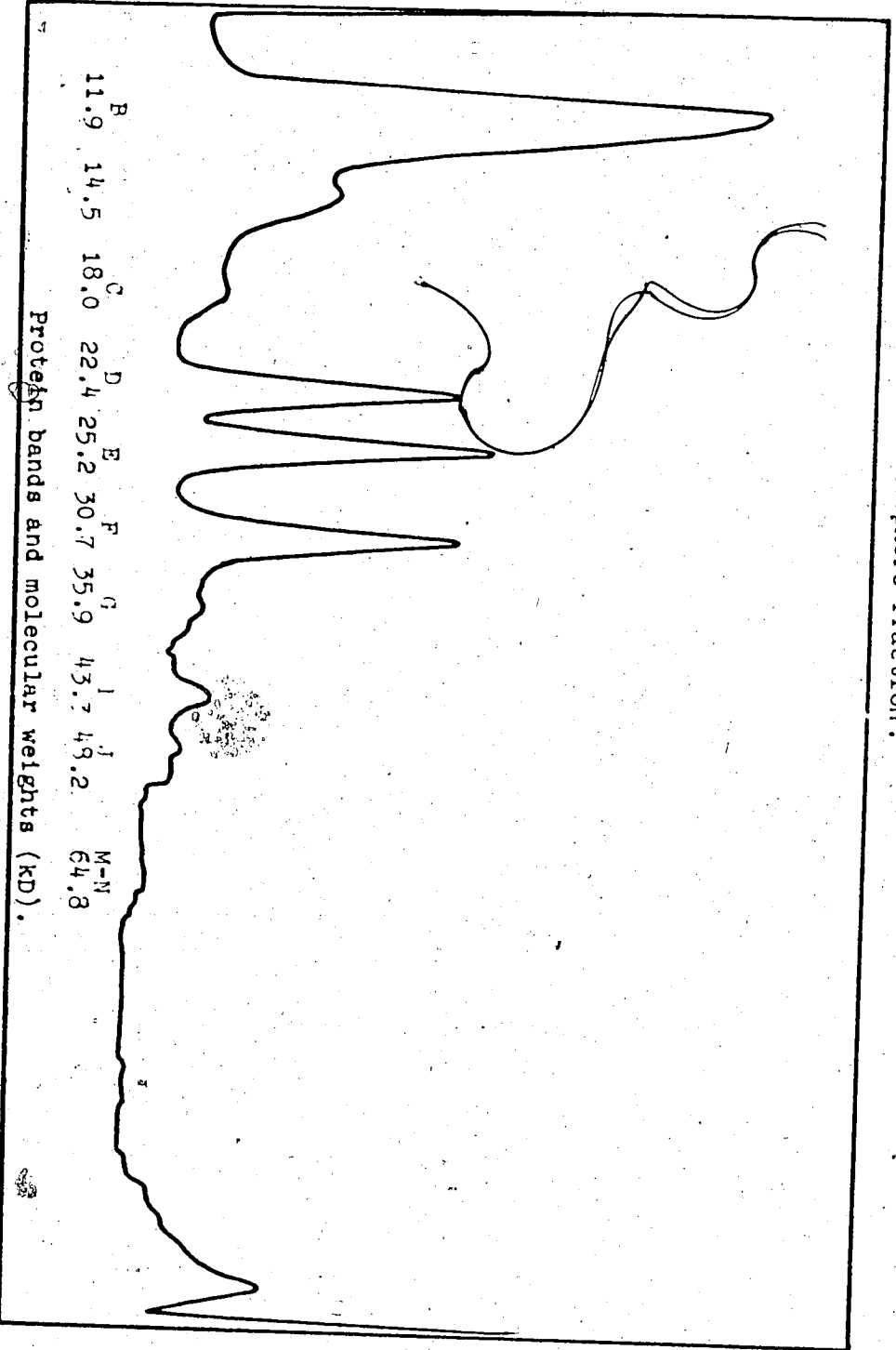
### III. Evidence for Chromatophore Fraction Precursors.

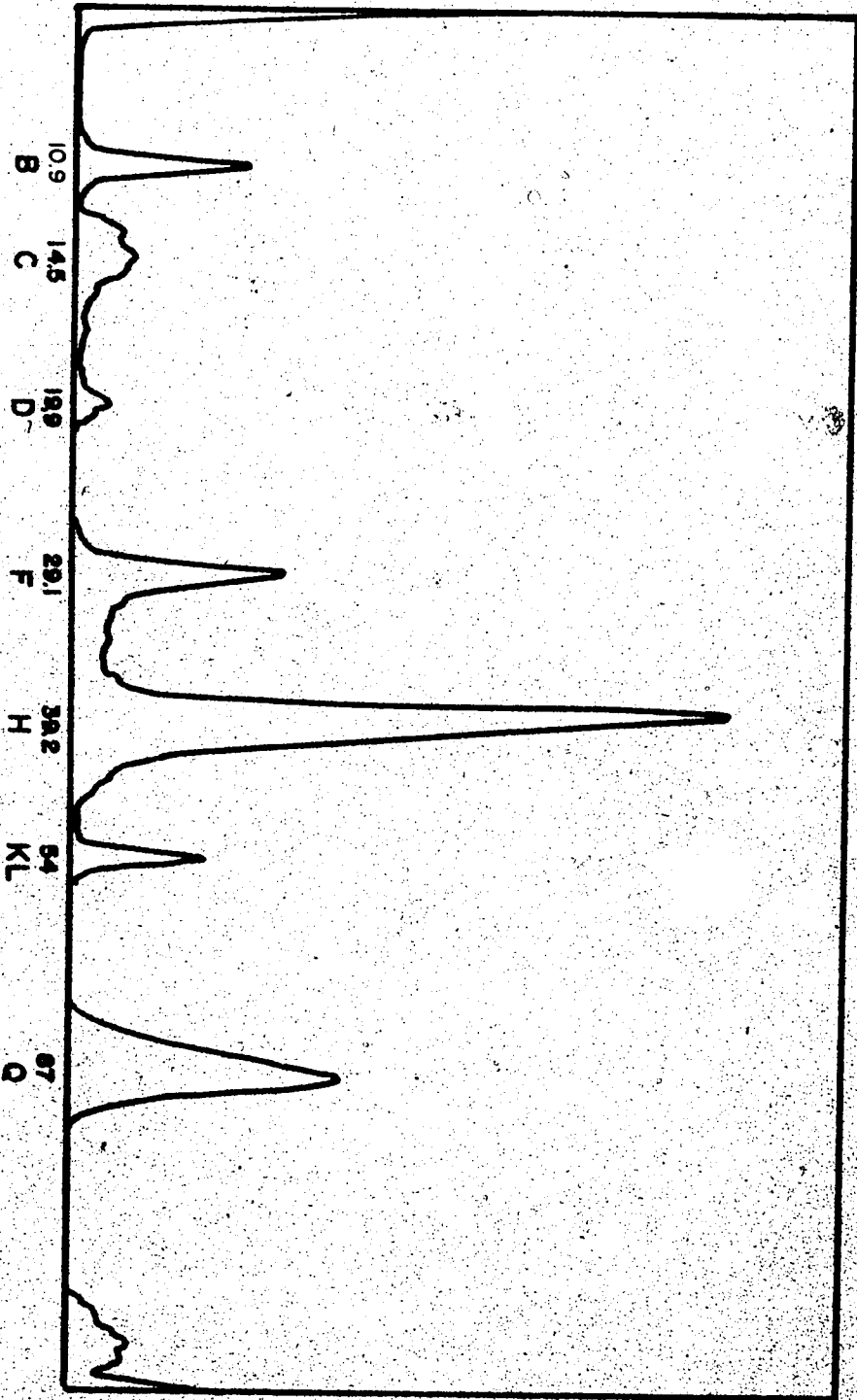
#### A. Incorporation of L-(U- $^{14}$ C) proline into the small particles during adaptation.

The adaptation experiment described in Section II.D.I. Figure 19 above was carried out in the presence of L-(U- $^{14}$ C) proline as described in the Methods section (Chapter 2, Section II.K.I). The distribution of  $^{14}$ C activity in the gradients and the specific activity of the protein in the fractions is shown in Figure 35. It can be seen that at 0, 2 and 6 hr the prephore fraction is highly labelled indicating that it contained newly synthesized

Figure 32.

Typical chromatophore fraction.

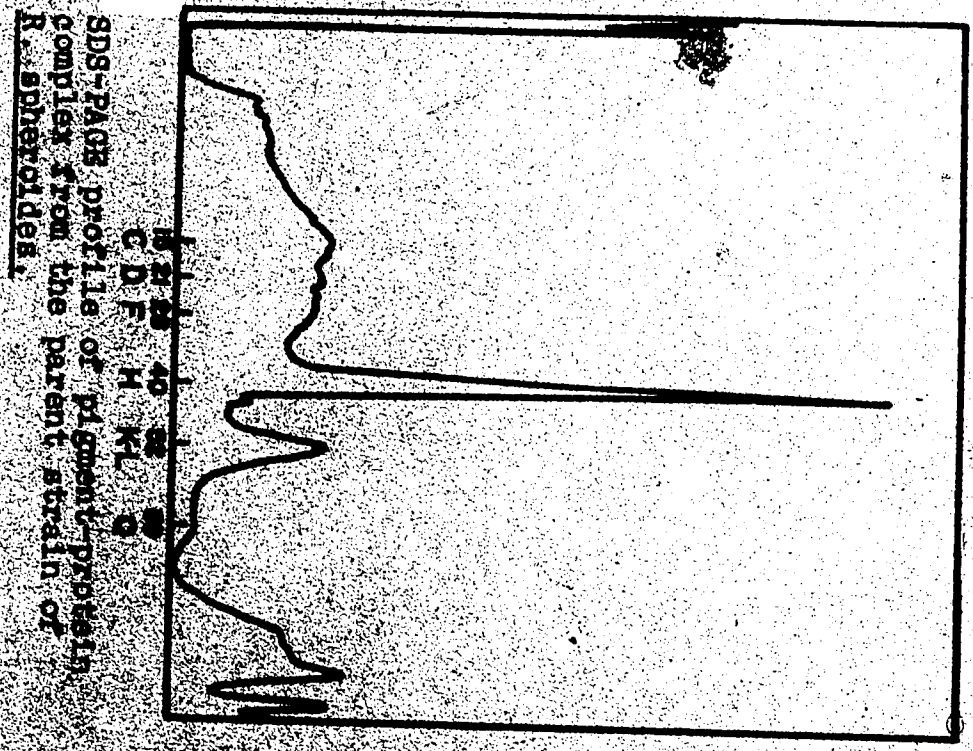




SDS-PAGE profile of reconstituted prephore fraction.

Figure 33

Figure 34.



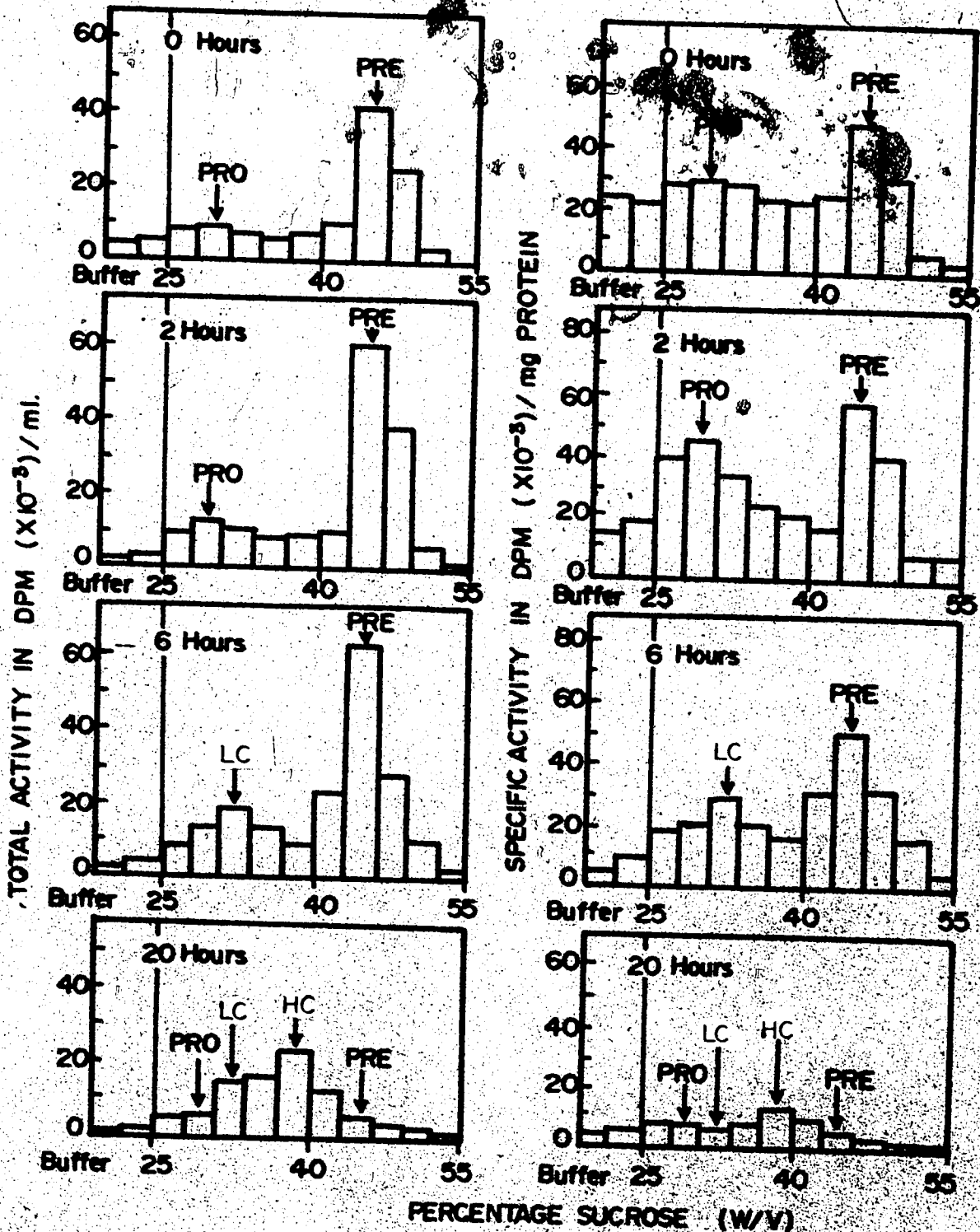


Figure 35. The total activity in dpm (x 10<sup>-3</sup>)/ml (left) and the specific activity in dpm (x 10<sup>-3</sup>)/mg protein (right) are shown for each 3 ml gradient fraction in the proline labelling experiment.

proteins. At 6 hr the majority of the activity was incorporated into the prephore fraction. The protophore fraction, at the top of the gradient, was only significantly labelled at 0 and 2 hr.

The total activity plots of Figure 35 were analysed with a Dupont Model 310 curve analyser, by assuming that the activity in all the fractions could be represented by Gaussian curves, or combinations of Gaussian curves. The area of each 3 ml segment of the curve envelope (generated by summation of all the Gaussian components) matched the activity contained in each fraction with an average deviation of + or - 3.8%. No fraction had a deviation greater than 2% of the total activity contained in the entire membrane fraction. By this method, an estimation of the total activity in each fraction was obtained. The total activity in each fraction at the four times studied is shown (Figure 36).

Activity in the soluble fraction above the gradient remained low. The activity in the prephore fraction peaked at 6 hr. The rise in activity of the prephore fraction preceded that of the light chromatophore fraction. At 20 hr, the heavy chromatophore fraction contained twice the activity of the light chromatophore fraction.

B. Double labelling study of small particle fractions during adaptation.

KEY

SOL = buffer over gradient. PRO = protophore fraction.  
BKDG = background component. PRE = prephore fraction.  
LC and HC = light and heavy chromatophore fractions.

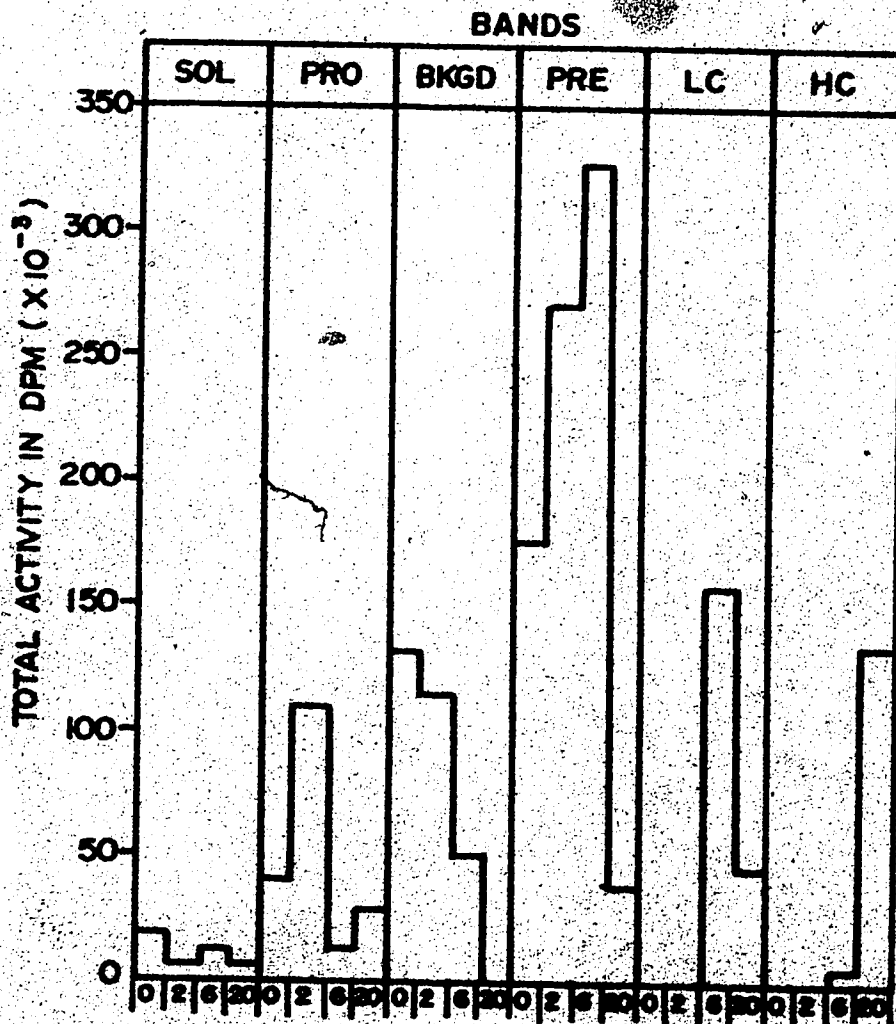


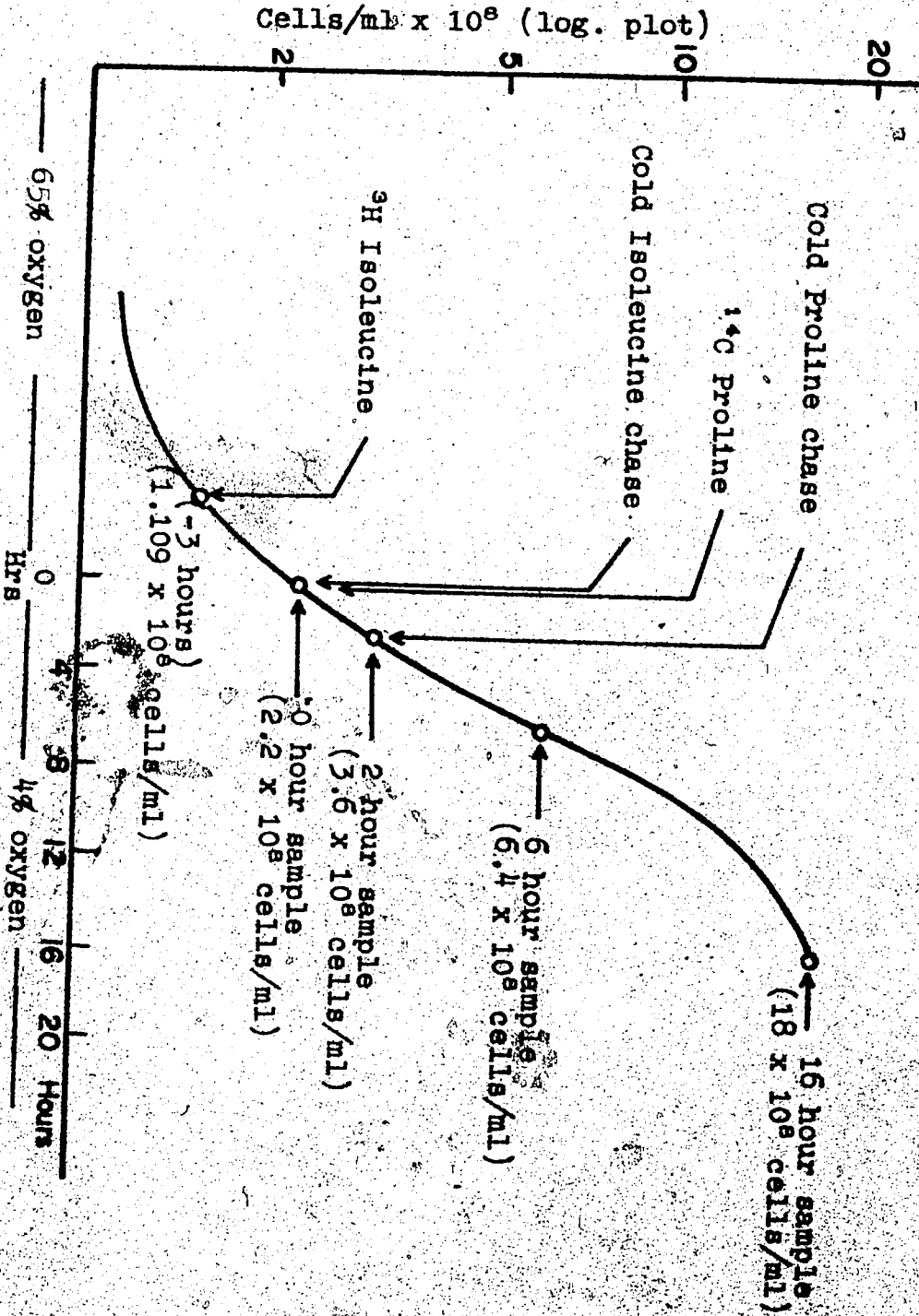
Figure 36. Distribution of  $^{14}\text{C}$  activity in the fractions at different times (hours) during adaptation in the  $^{14}\text{C}$  proline labelling experiment.

1. Growth and uptake of labelled amino acids.

A culture was grown aerobically to early log phase and incubated in the presence of  $^3\text{H}$ -isoleucine for three hours. Cold isoleucine was then added as a chase (for details see Chapter 2, Section II.K.2) and a sample taken (0 hr). Adaptation was induced by gassing with 4% oxygen (balance nitrogen), a 10 min period being allowed for equilibration of the dissolved oxygen content.  $^{14}\text{C}$ -Proline was then added and a period of 2 hr allowed prior to the addition of cold proline as a chase. The growth and uptake of the culture was monitored hourly by taking 10 ml aliquots. Samples of 1 l. were taken just before the reduction in oxygen level (0 hr) and at three subsequent times (2, 6 and 16 hr). The growth of the culture and times of additions are shown in Figure 37.

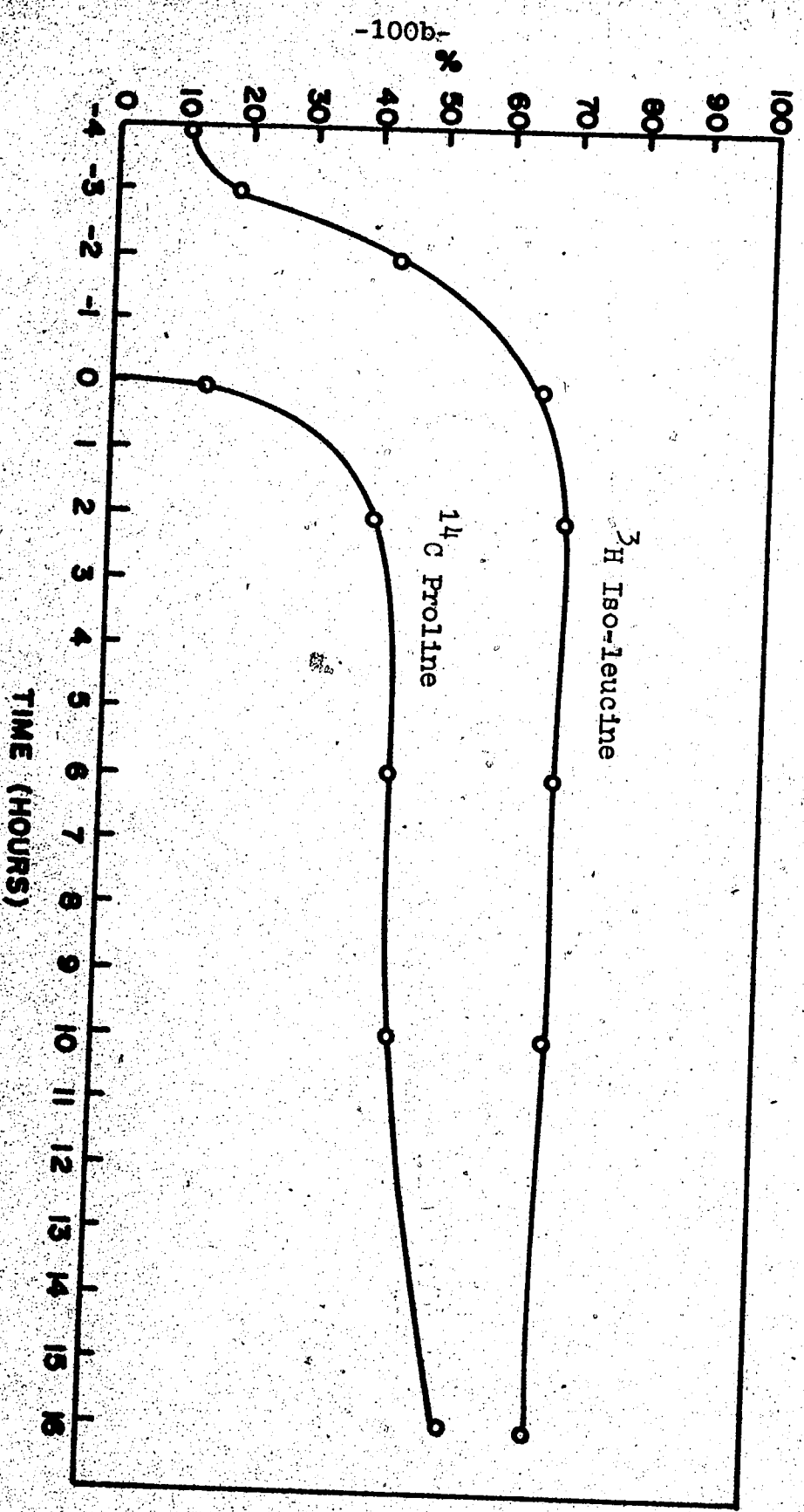
The incorporation of each label by the whole cells is shown in Figure 38 as a percentage of the amount added. It can be seen that the addition of the cold amino acids essentially prevented appreciable uptake of the labelled amino acids. The uptake of some additional  $^{14}\text{C}$ -proline that occurred between 10 and 16 hrs probably resulted from a large uptake of proline at the end of log phase when the normal substrates became limiting. The induction of adaptation at a low level of cells per ml minimized the possibility of re-utilization of "aerobic" proteins and





Growth curve in the double labelling experiment. Figure 37.

Figure 38. Uptake of labelled amino acids by cells.

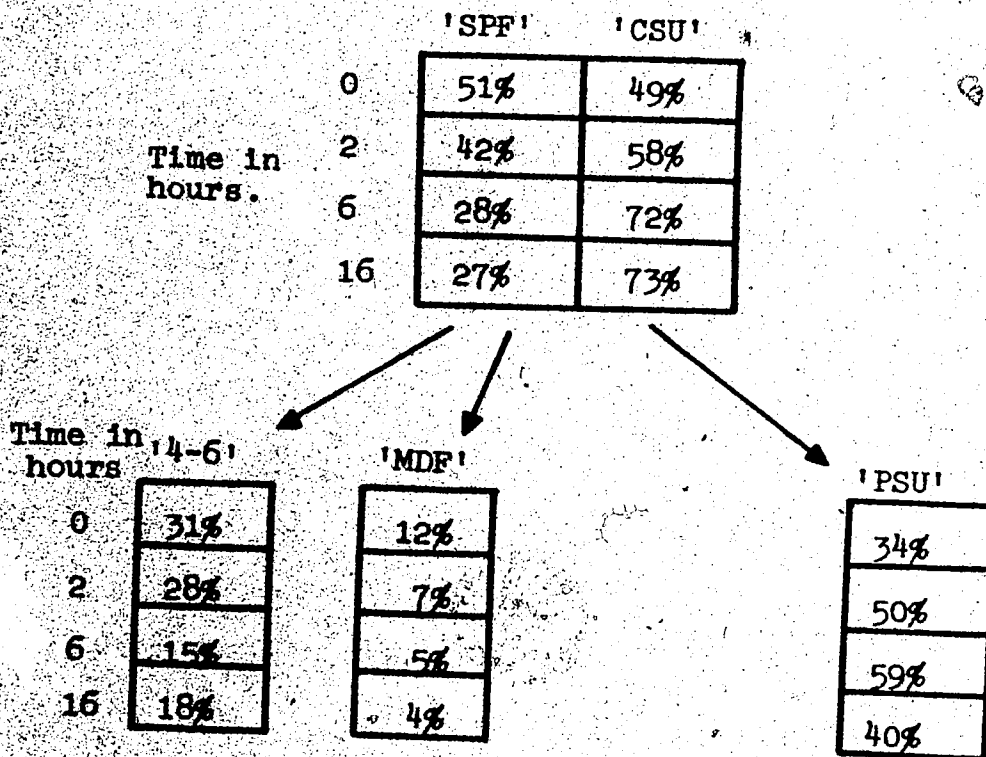


favoured the production of a large medium density fraction.

## 2. Fractionation of the cells.

After the standard preliminary centrifugations, the small particle fraction was resolved using a 25 to 55% sucrose density gradient. The subunit preparation was harvested and purified as previously described. It was analysed both before ("crude" CSU) and after glycerol density gradient electrophoresis ("purified" PSU). The purified subunit fractions and the sucrose density gradient fractions were dialysed prior to counting to remove absorbed radioactivity and glycerol or sucrose. The distribution of total protein into the different fractions is shown in Figure 39. The combination of a low initial cell level and 4% oxygen gassing allowed cells to grow aerobically at a fairly high rate (Figure 37). Almost no lag could be seen in the growth rate prior to pigment production. These conditions were used to produce a good yield of the medium density and subunit fractions. They resulted, however, in a lower level of chromatophores per cell than is obtained by adapting cells later in the log phase or with lower levels of oxygen. As a result, the aerobic membrane peak was large compared to the chromatophore peak in the sucrose gradient (29% and 34% sucrose, respectively). The aerobic membrane component

Figure 39. Distribution of sedimentable protein after low g centrifugation.



Key

- SPF = Small particle fraction
- CSU = Crude subunit fraction
- 4-6 = Fraction 4-6 material
- MDF = Medium density fraction
- PSU = Purified subunit fraction

was, however, reduced during adaptation, the chromatophore peak being dominant in the material from the 16 hr sample. The overlapping of these components prevented good resolution of the chromatophores by the standard gradient. These components, contained in fractions 4, 5 and 6 (28 to 37% sucrose) were pooled; this fraction will be referred to as the "4-6" fraction.

The culture became visibly pigmented after two hours of growth at the low oxygen level. The "4-6" fraction was highly pigmented after 6 hr indicating a chromatophore content. The 870 nm form of Bchl predominated. The absorbance of the 870 nm form of Bchl per mg protein is shown in Figure 40. A similar profile was observed from whole cell samples, but after 16 hr a peak at 850 nm was present; its absorbance was about half that of the 870 nm form. The probability that the cells were a mutant strain was low, as growth of cells from the same inoculum stock at a lower oxygen level produced the typical near-infrared absorption spectrum. The level of pigment in the crude and purified subunit fractions is also shown in Figure 40. In agreement with previous work, the subunits were relatively highly pigmented at the start of pigment synthesis but their content underwent little increase in the later stages of adaptation; at 6 and 16 hr their specific pigment content was only about 30% that of the "4-6" fraction.

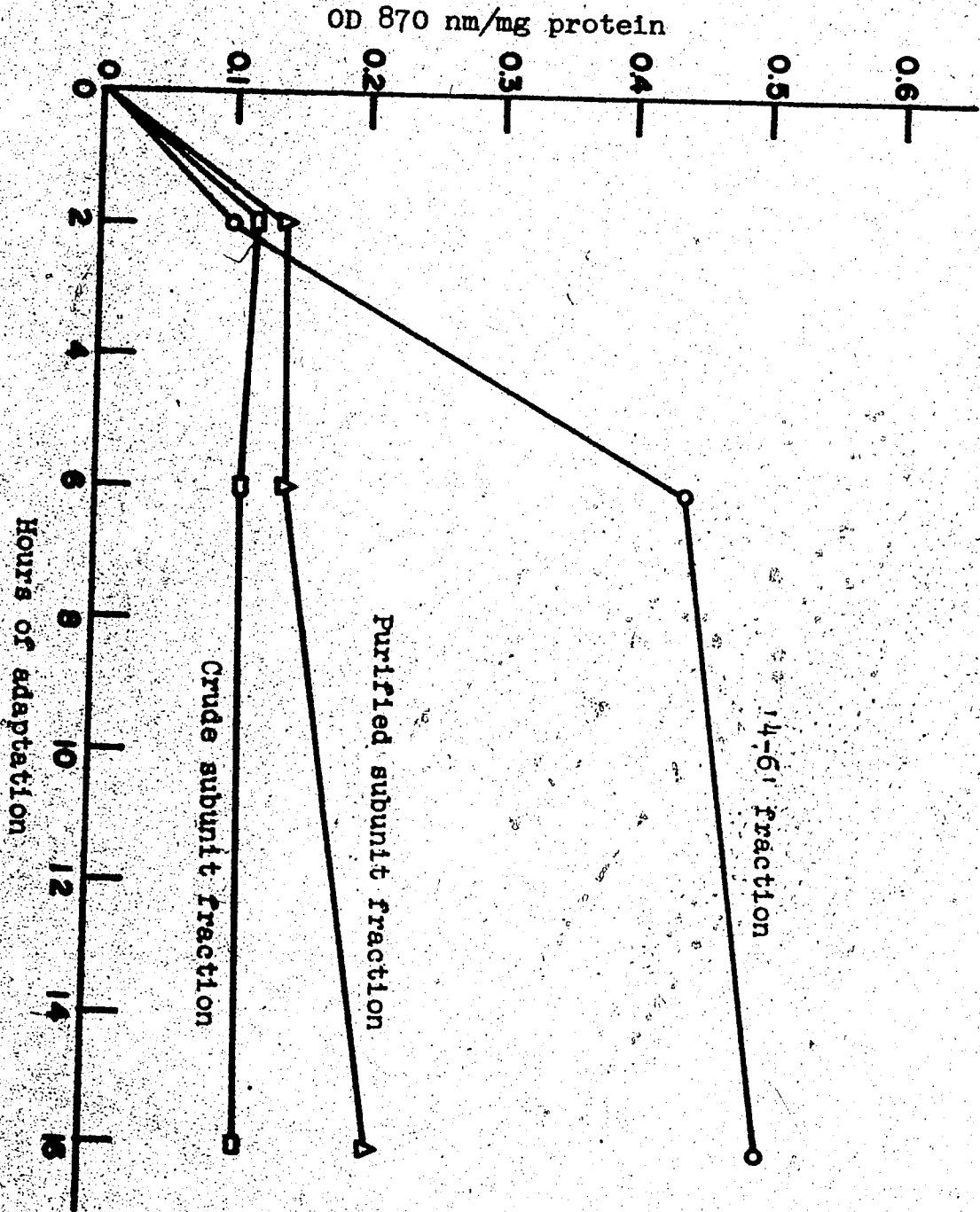


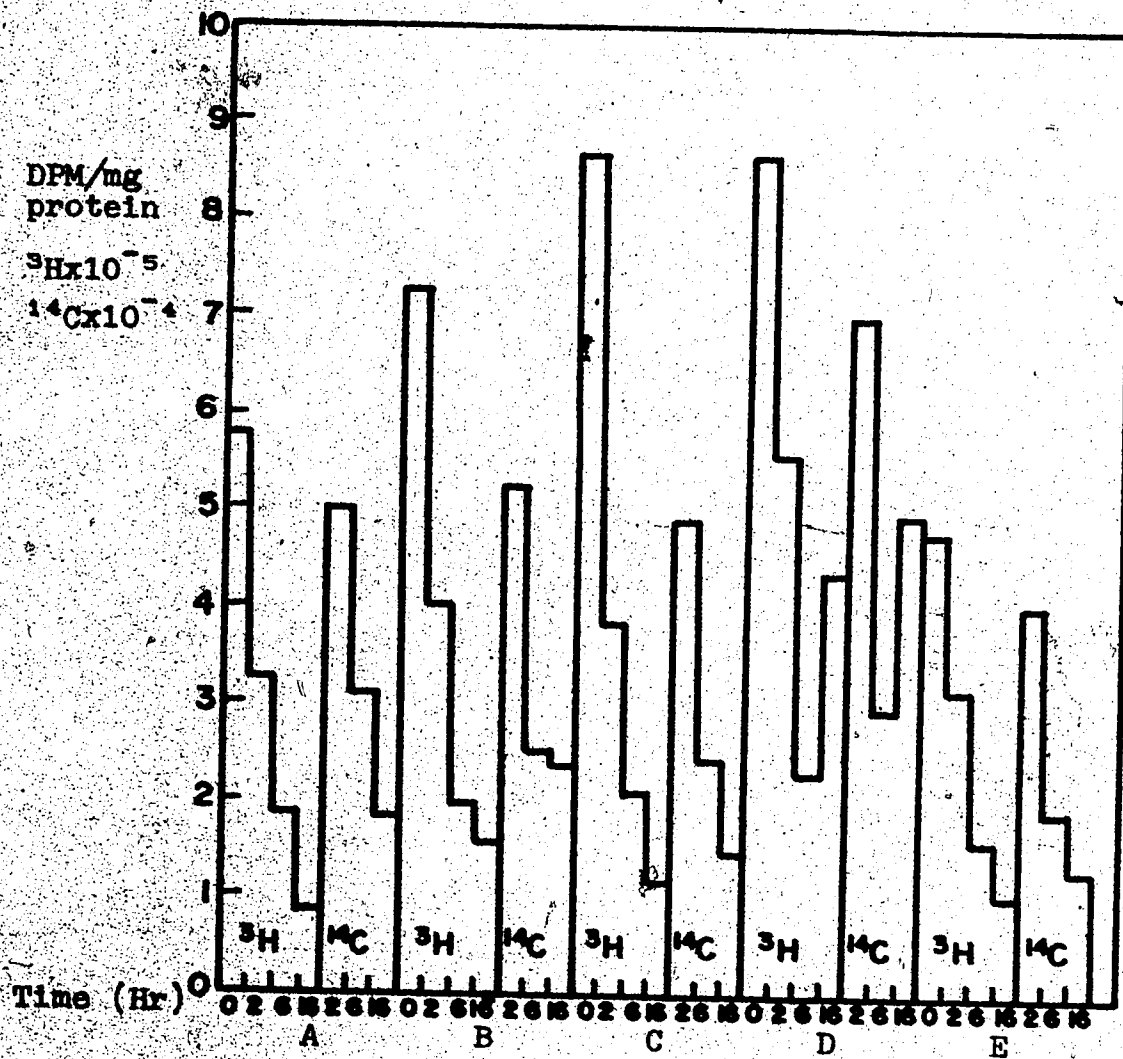
Figure 40. OD 870 nm/mg protein in the 14-61 and the subunit fractions.

### 3. Distribution of radioactivity in the small particle fractions.

The specific activities of the three major purified membrane fractions at each time studied is shown in Figure 41. For comparison, two "average" values are also included: (a) the estimated average for all cellular proteins, assuming incorporation of all the amino acids taken up by the cells into proteins, and (b) the average specific activities of all the purified membrane fractions. Figure 41 shows that the specific activities of all three major fractions decreased roughly in accord with the decrease observed in the two average values. A dramatic exception, however, is the specific activity of the "4-6" fraction (both  $^3\text{H}$  and  $^{14}\text{C}$  labelled proteins) which increased at 16 hr. This was coincident with the increase in the chromatophore fraction (254 nm absorbance at 34% sucrose in the density gradient).

The changes in specific activity resulting from cellular growth can be eliminated by dividing the specific activity in the fraction by the whole cell specific activity. The resulting ratios (plotted on a log scale) for the three major fractions are shown in Figure 42. This figure shows that dilution in specific activity of the medium density fraction (between 2 and 6 hr) and the subunit fractions (between 0 and 6 hr) is indeed greater

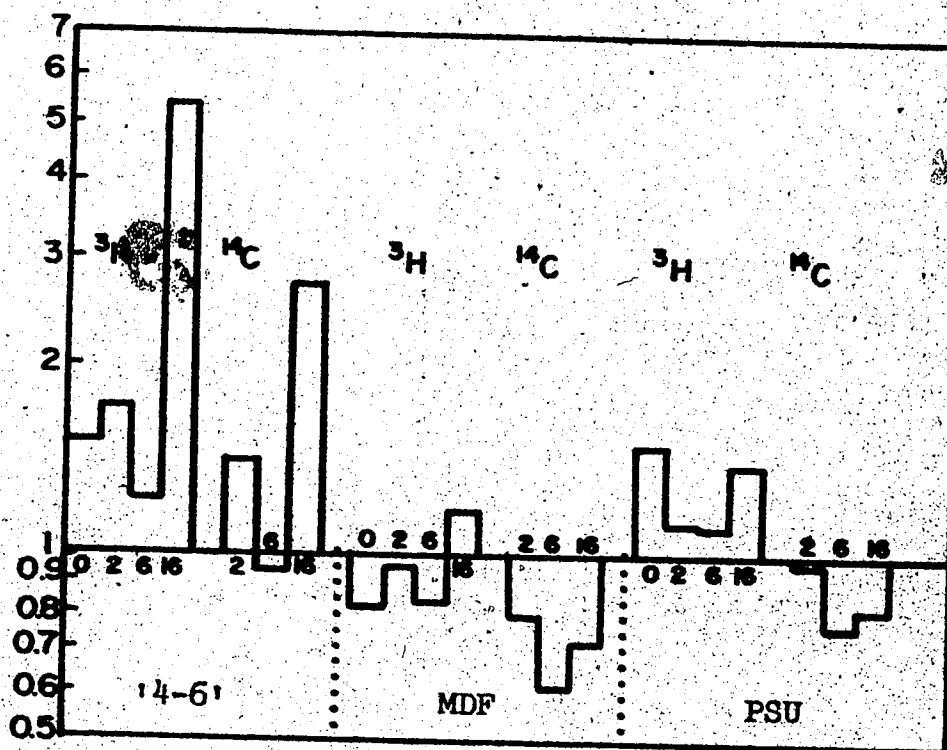
Figure 41. The specific activities of various fractions during adaptation.



- A = Whole cell average
- B = Purified membrane fraction average
- C = Purified prephore subunits
- D = The '4-6' fraction
- E = The medium density fraction.



Figure 42 . Log plot of the specific activity/fraction protein to specific activity/whole cell protein ratio.



Key

'4-61' = fraction '4-61'

MDF = medium density fraction

PSU = purified subunit fraction

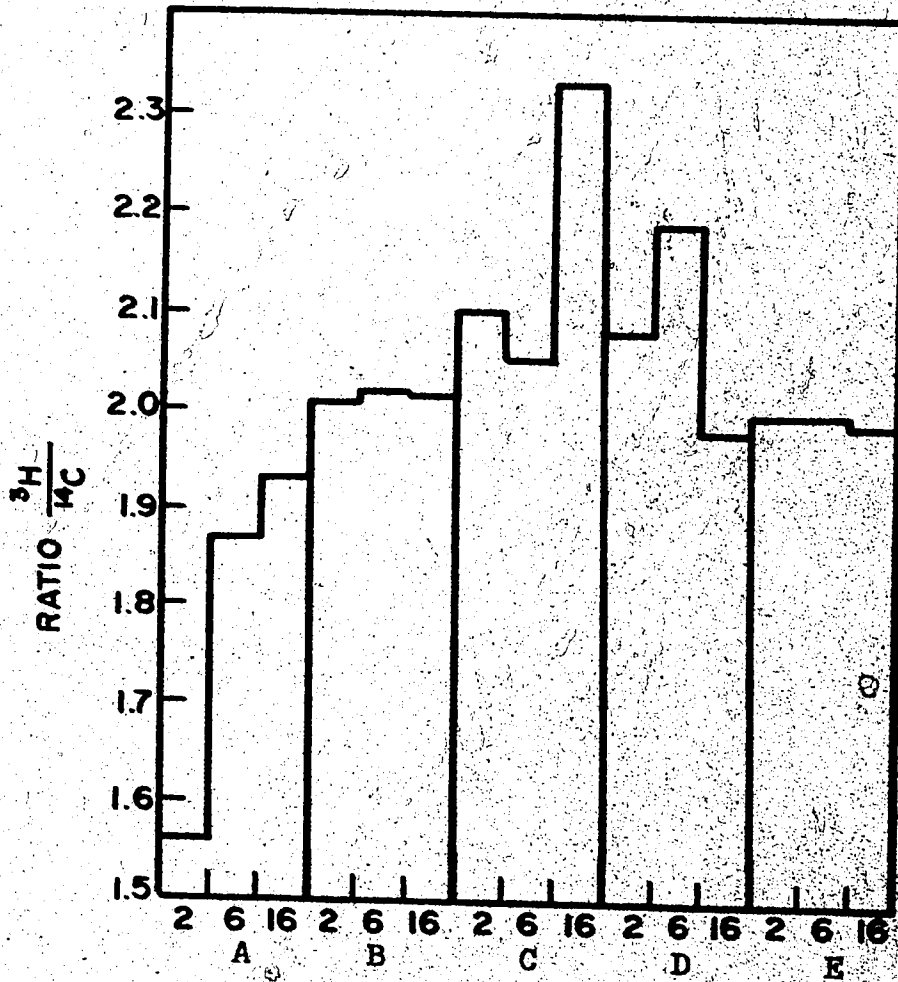
than average during adaptation.

The  $^3\text{H}/^{14}\text{C}$  ratios were also calculated. The data were corrected to reflect the ratios which would have been obtained if equal initial  $^3\text{H}$  and  $^{14}\text{C}$  activities had been added to the medium. The samples were counted to  $\pm$  or  $-$  1% and the ratios were thus determined to better than  $\pm$  or  $-$  5%. Figure 43 shows that the  $^3\text{H}/^{14}\text{C}$  ratios of the purified subunits, produced by the cell from four different stages of adaptation, were almost identical. This is in contrast to the crude subunit preparations. The variable  $^3\text{H}/^{14}\text{C}$  ratios in the crude subunits and their high specific activity was probably due to adsorbed amino acids. The similar  $^3\text{H}/^{14}\text{C}$  ratios after dialysis against 20% glycerol (1mM Tris pH 7.5) for three days shows that this fraction is probably free from dialysable contamination. The unpigmented material, separated from the subunits by the electrophoretic purification, contained less than 30% of the protein, a lower  $^3\text{H}/^{14}\text{C}$  ratio, but a similar specific activity to the subunits.

#### 4. 2-Chloroethanol digestion.

An attempt was made to separate the "aerobic" from the "photosynthetic" proteins using the 2-chloroethanol technique of Fraker (1971). The unfractionated small particle fraction was treated with 2-chloroethanol as was described in chapter 2. The material was easily

Figure 43.  $^3\text{H}/^{14}\text{C}$  ratios for the fractions.



- A = Crude subunits
- B = Purified subunits
- C = The '4-6' fraction
- D = Medium density fraction
- E = The 2-chloroethanol soluble fraction

solubilized and centrifugation (27,000 x g for 15 min) produced a white gelatinous pellet. The proteins left in solution and the pellet proteins were termed the PII and PI fractions, respectively. The soluble fraction (PII) had been found by Fraker to contain about 95% of the proteins specifically associated with the chromatophores, while the pellet material (PI) was believed to contain the majority of "aerobic" proteins. I found, however, that between 45 and 60% of the proteins were solubilized both from the "aerobic" cell small particle fraction and that from pigmented cells at 2, 6 and 16 hr. The method did not appear to be as specific as had been hoped. It was found that the  $^3\text{H}/^{14}\text{C}$  ratio obtained from the PII fraction was similar to that of the purified prephore subunits (Figure 43).

C. Electron microscopic examination of thin sections from adapting cells.

Sections of aerobically grown cells and those of cells adapted by gassing with 1% or 4% oxygen are shown in Figures 44 to 50. The staining and sectioning techniques are described in chapter 2.

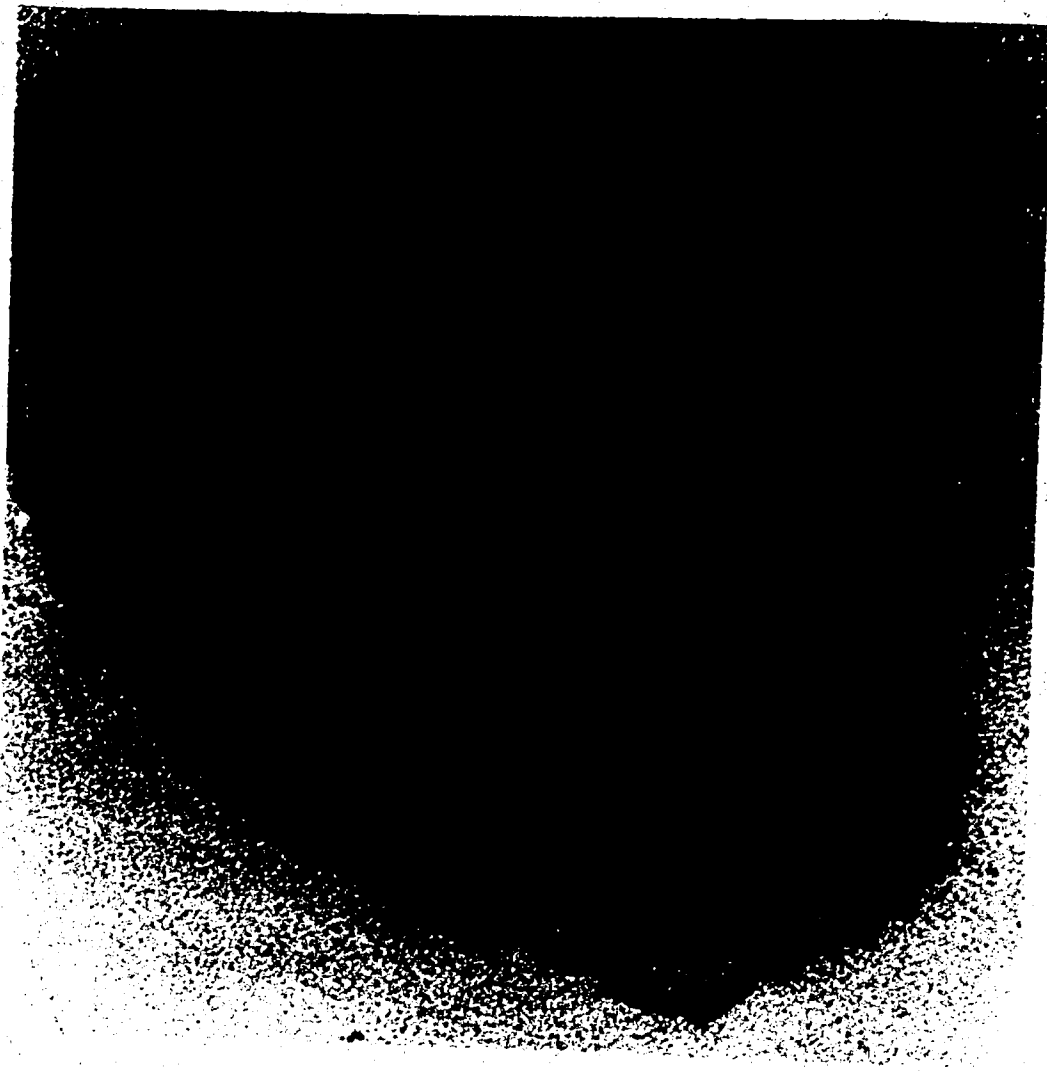
Figure 44.



Cell section of oxygen grown cells showing aerobic invaginations.

200x

Figure 45.



Section of cell with increase in periplasmic lipid.

Mid log phase aerobic cells adapted for 2 hours by gassing with 1% oxygen (balance nitrogen).

Figure 46.

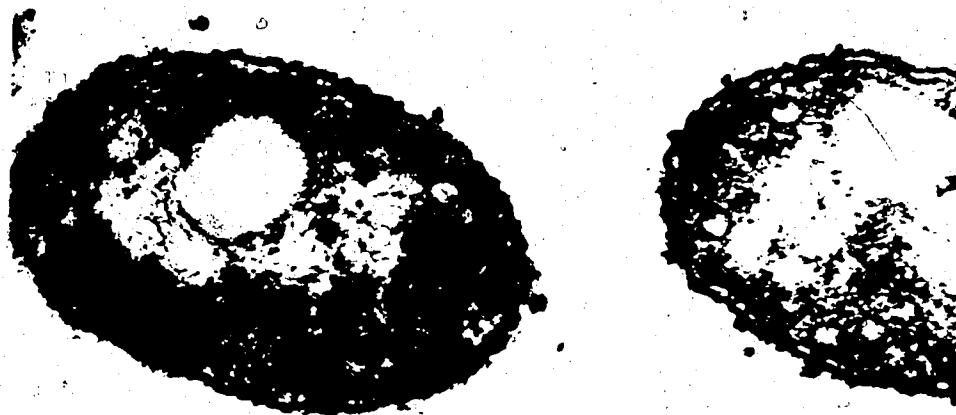


Cell section showing connection between PHP storage granule and the periplasmic space. Aerobic cells adapted at mid log phase by gassing with 1% oxygen (balance nitrogen) for 2 hours.

Figure 47.



Figure 48.



47. Cell section showing increased peripheral invaginations.  
Aerobic cells adapted from mid log phase by gassing with  
4% oxygen (balance nitrogen) for 0.5 hours.

48. Cell sections showing further increase in invaginations.  
Same culture as above after 2 hours of adaptation.



Figure 49.



Figure 50.



49. Section of cell from same culture as Figs. 47,48. Chromatophores present in the center of the cell after adaptation for 4 hours.
50. Section of cells from above culture after 8 hours showing an increase in chromatophores.

## Chapter 4. Discussion.

### I. Discussion of the Experimental Procedures.

#### A. The linear density gradient techniques.

Of all the separative techniques investigated (cf. Results, Section I.A. and B.) the linear sucrose density gradient ultracentrifugation technique was the most reproducible and gave the greatest resolution. Modifications of the harvesting technique and the gradient buffer eliminated problems of aggregation of the chromatophores with the other cellular structures. Fractions were shown to have reached their equilibrium buoyant densities after 16 hr of centrifugation, no further change being observed after 24 hr. The membrane fractions were separable by non-equilibrium centrifugation, but the resolution was not as good as was obtained by the equilibrium technique. This probably resulted from variations in the sizes of the membrane fragments being studied. The main limitation of the equilibrium technique was the small amount of material (a few mg protein) that could be added to each gradient. The method, however, provided enough material for protein analysis by SDS-PAGE and the determination of their pigment/protein ratios. The use of sucrose density gradient ultracentrifugation for the isolation of the prephore subunit and mature subunit fractions is discussed

in Section II. below. The fact that these subunits banded at the top of the gradient during the standard 16 hr centrifugation is believed due to their smaller diameters and thus their greater surface to volume ratios. The increased surface to volume ratio causes an increase in viscous drag per unit mass, thus preventing them from attaining their positions of buoyant equilibrium.

The glycerol density gradient electrophoresis technique was useful in producing a large quantity of the prephore subunit material. Separation of the prephore and mature subunits might also have been achieved by zonal centrifugation.

#### B. Electron microscopy.

This technique was useful in providing an idea as to the homogeneity of membrane fractions. The results obtained by this technique should be regarded as "indications" rather than "evidence" as the interaction of the stain and subcellular structures is well known to produce morphological artefacts. Electron microscopy does, however, provide one of the only methods of observing the macromolecular structure of cellular components.

The term, prephore subunit, was derived from studies using this technique. In addition to the large prephore structures, smaller particles about 10 nm in diameter

could also be seen in the prephore fraction. These particles appeared to form the uncollapsed prephore substructure. As the prephore fraction could be dissociated into smaller structures which, in turn, could be reaggregated to yield a prephore fraction, it seemed reasonable to suppose that the small particles were, in fact, subunits. The sac-like appearance of the medium density fraction, however, might well have been due to partial vesiculation of cytoplasmic membrane.

### C. The SDS-PAGE technique.

Various gel electrophoresis procedures were tested. The method of Laemmli (1970), modified by Clayton and Haselkorn (1972), was found the most reproducible. The current employed and the digestion conditions were optimized (Chapter 2 section II. I. 4 and 5). The chromatophore fraction produced gel profiles similar to those shown by other workers (Clayton and Haselkorn (1972); Feher (1972)). Peptides with weights less than 100 kD were used in the comparative study as many "higher-weight" bands could be reduced by a more prolonged digestion time. This suggested that they were oligomers or associated polypeptides. The SDS-PAGE technique has been found to not always abolish protein-protein interactions (Nelson 1971). Although the relationship between the logarithm of the molecular weight and the

mobility in the gel has been shown to be linear for numerous proteins [Weber and Osborn, (1969)] these were all water soluble proteins. It is possible that the actual molecular weights of membrane proteins might deviate from the apparent values calculated from their mobilities. In order to demonstrate that polypeptides with identical mobilities are the same, amino acid analysis or a double labelling experiment is required. This was not attempted with material obtained in the double labelling experiment because of insufficient material; the technique provided, however, a reproducible method of comparing the overall protein contents of the different membrane fractions. A discussion of the assignment of molecular weight values to the various fractions is given in Appendix A.

#### D. Radio-isotope labelling techniques.

##### 1. General discussion.

The use of radio-isotopes has provided a vast amount of data on the sequences of biosynthetic pathways. The technique is also a potentially powerful tool for determining precursor-product relationships among structures involved in organelle morphogenesis. The interpretation of data in such studies is hindered by the complexity of the processes involved and the occurrence of numerous events simultaneously. The demonstration that

one structure is being converted into another requires a knowledge of both the rates of breakdown and resynthesis of the structures and accurate quantitative evaluation of cellular pool sizes. During an adaptation process, metabolic state of the cell is unlikely to be constant. This may well produce a variation in the turnover rates of the intermediates. An estimation of this factor requires a knowledge of cellular pool sizes. Only with this data would it be possible to equate the quantitative changes in specific activity in all the components involved.

The study of the adaption of R. spheroides to photosynthetic growth is difficult, as some "structures" are probably part of a membranous continuum. This means that the pool size of a fraction is a function of the disruptive technique employed; pool size in this context is thus a questionable concept. Another problem is that the disruption procedure may cause the preferential liberation of one structure over another. The high level of prephore subunits liberated in the double labelling experiment may have been due to this cause. Attempts to extrapolate from the percentages of different components liberated to total pool sizes would be invalid on this account. The solution would appear to be the use of a continuous culture technique. It then might be reasonable to assume that pool sizes due to normal growth would

remain constant. A study of endogenous ATP level in the cells, if constant, would support this idea.

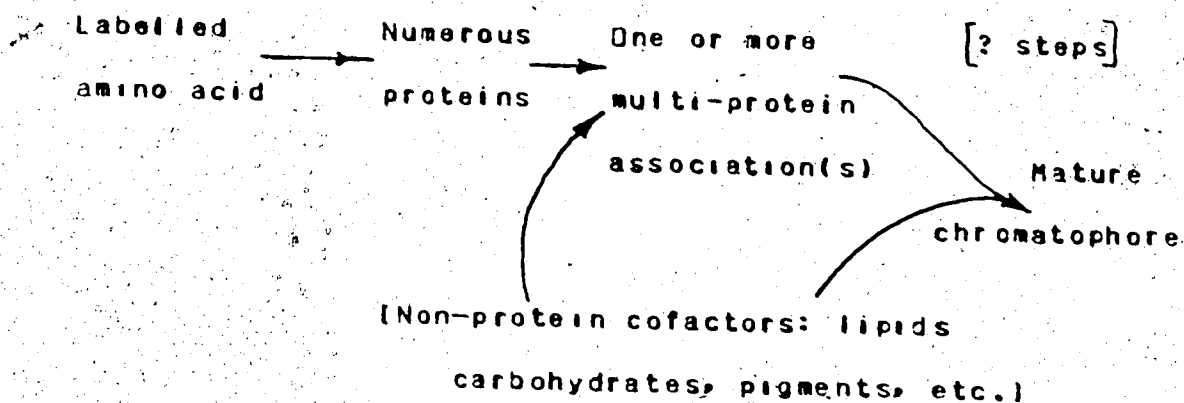
The labelling experiments described in chapter 3 were quite different with regard to the rate of cellular growth taking place during the adaptation. The first experiment, performed with a high level of cells per ml, had the advantage that the added labelled amino acids were rapidly taken up by the cells. The drastic drop in oxygen supply allowed only a limited amount of cellular growth.

In the double labelling experiment, adaptation was induced with early log phase cells to minimize the possibility of re-utilization. Cell growth was high throughout the adaptation process; however, the level of chromatophores per cell was not as high as in the first labelling experiment. Good yields of the two intermediates were obtained but, unfortunately, a clean separation of the chromatophores was not achieved.

2. The application of precursor-product relationships to the synthesis of a multi-protein-containing membrane fraction.

The application of precursor-product relationships to the synthesis of a multi-protein-containing membrane fraction is a difficult undertaking, involving either many assumptions or highly controlled experimental conditions. If degradation is negligible, the situation may be

considered as follows:



The added labelled amino acid (of known molar specific activity) would give rise to labelled proteins if the amino acid is taken into the cell, and if the protein contains that amino acid. The maximum molar specific activity of the proteins would vary from protein to protein, and would depend upon the following factors:

(a) The dilution of the labelled amino acid by the endogenous pool of the amino acid at the time of the synthesis of a detectable amount of the protein.

(b) The amount of previously existing (unlabelled) protein.

(c) The number of residues of the labelled amino acid per molecule of the protein.

The aggregation of the labelled proteins (with the possible addition of other non-protein components, eg. lipids, carbohydrates, pigments, etc.) would give rise to labelled multi-protein fractions. However, here the use of the molar specific activity would break down unless



there was a strict stoichiometric relationship between the various proteins, and their molecular weights were all known. The specific activity on a mg protein basis could be employed, however. The initial specific activity of the fraction during a pulse-chase labeling experiment would be determined by the specific activities and average amounts of each of the proteins in the fraction at the time of the addition of the chase amino acid. If either the average amounts varied with time, or if different proteins were added at different times, the specific activity of the fraction might rise or fall. If it is assumed that the fractions have a constant protein composition and the activity incorporated in the form of non-protein components is negligible, then decreases in specific activity of a fraction following the addition of the chase amino acid could be caused by several other mechanisms:

a) The expected dilution due to cellular growth, with no appreciable changes in the cellular pool sizes of the particular fractions.

b) An increase in cellular pool size of a fraction.

c) The degradation and resynthesis of a fraction from predominantly cold amino acids.

d) A turnover of proteins in a fraction by transfer to another fraction and resynthesis of and/or replacement by proteins of low specific activity.

All of these mechanisms could be superimposed, and

all would be expected to have different rates. On the other hand, increases in specific activity of a membrane fraction after the addition of the chase amino acid could only have been caused by incorporation of pre-existing highly labelled proteins into this fraction, and would indicate the involvement of other protein(s) or multi-protein-containing fraction(s).

Errors could, of course, be caused by any alteration to the proteins (such as the splitting off of labelled portions) or by the degradation of the proteins and/or labelled amino acids and the re-utilization of the labelled degradation products for the synthesis of other amino acids, lipids, or pigments and the incorporation of these into the fractions being studied.

3. The relevance of precursor-product relationships to chromatophore morphogenesis.

Three models for the utilization of one or more multi-protein-containing membrane fraction(s) for chromatophore morphogenesis will be considered:

(a) One fraction is the sole and immediate protein precursor to the chromatophore fraction.

(b) One fraction is the sole, but not the immediate, precursor to the chromatophore fraction.

(c) Two (or more) fractions are chromatophore protein precursors.

These three models would have the following implication during a time-dependent study of the changes in the specific activities (per mg protein) of the chromatophore fraction and a possible chromatophore precursor, the prephore subunit fraction:

(a) If the prephore subunits are the sole and immediate precursors to the chromatophore, the total activity in the chromatophore fraction will rise asymptotically until, at infinite time, this fraction contains all of the activity incorporated into the prephore subunits during the pulse labelling (assuming that the degradation of the fractions is negligible). If, in addition, the average amounts of the various proteins does not change during the conversion, and if all the prephore subunits were functionally and locationally equivalent such that they formed a single "compartment", then a simple relationship will exist between the specific activity of the prephore subunits and the chromatophores. The maximum specific activity of the chromatophores will be coincident with the specific activity of the prephore subunits at that time.

The actual rates of the decrease in the specific activity of the prephore subunits would depend upon the rates of all four mechanisms listed in Section I.D.2. above, whereas the rate of rise and fall of the specific activity in the chromatophore would depend upon the amount

of unlabelled chromatophores present, the rate of conversion of prephore subunits to chromatophores, and the specific activity of the prephore subunits at each time examined.

(b) If the prephore subunits were the sole, but not the immediate, protein precursor to the chromatophore fraction, that is, if the prephore subunits required a series of modifications prior to chromatophore assembly (i.e. addition of pigment and other specific non-protein components) the initially labelled prephore subunits will not be diluted in specific activity by those synthesised subsequently to the same extent as would be expected in model (a) above.

Chromatophore synthesis is kinetically a slow process and required about 6 hr under the normal adaptation conditions. Organization on a macromolecular and cellular level is probably the reason for this time requirement. The prephore fraction is thus a heterogenous collection of particles in various stages of modification. This is in agreement with the production of both pigmented and non-pigmented prephores with the same protein profiles, and also explains the decreasing density of this fraction during adaptation (from a density of 50 to 46% sucrose). The modifications would effectively introduce a series of "compartments" between the initially labelled prephore subunits and the chromatophore. As the pulse is

introduced at the time of the lowering of the oxygen tension, the system is not in a steady state and the majority of the prephore subunit and the chromatophore compartments would be empty. After the pulse labelling and the induction of adaptation, a wave of high specific activity would pass through this system of previously vacant compartments. The specific activity of the prephore fraction would decrease as an increasing number of prephore subunit compartments were filled with low activity components, following the path of the high specific activity prephore subunits.

The chromatophores, when formed, would have a greater specific activity than the average of the prephore subunit compartments at the same time. The total activity of the 'collective' prephore fraction would remain high until the formation of the chromatophores. At this time it would fall rapidly with a corresponding rapid increase in the specific activity of the chromatophore fraction. The maximum of the latter would depend upon the specific activity of the final (highly labelled) compartment at that time.

(c) If there were one (or more) fractions (in addition to the prephore subunits) which were chromatophore protein precursors, this would involve a modification of the expected results of model (b) above. In this case, the maximum of the specific activity of the chromatophores

would lie somewhere between the specific activity of the final (highly labelled) prephore subunit compartment and that of the additional precursor fraction. The actual value would depend upon the relative amounts of both fractions. In the case of more than one additional fraction, the maximum would be the weighted average of all of the specific activities of the precursors at that time. It is possible that these additional fraction(s) may have been single proteins or multi-protein-containing fractions which have not been isolated and/or analyzed and may have had specific activities much greater than that of the prephore subunit fraction.

Discussion of the Aggregation of Fractions: the prephore Subunit and Mature Subunit Fractions.

The harvesting of the small particle fraction on a sucrose trap caused a decrease in the prephore fraction with a concomitant increase in pigmented material at the top of the gradient at 28% sucrose (cf. Results, Section I.E.2.). At high ionic strength (0.5M phosphate) this new fraction (the prephore subunit fraction) aggregated to reconstitute a pigmented prephore fraction (48% sucrose). This material was shown to have a simple protein composition with proteins of the same apparent molecular weights as had the pigmented material liberated after dialysis of the prephore fraction. This method of

obtaining purified prephore subunits was limited by the low yields obtained with the modified harvesting procedure and reduced buffer strength. The material forming a pigmented band at the top of the gradient was also not a good source of purified subunits as the high 254 nm absorption to Lowry protein ratio made it likely that the fraction contained ribosomal material (see Figure 7b). It was thought likely that a large amount of unaggregated prephore material was normally lost with the 1.5 hr 100,000 x g supernatant as this was pigmented in early stages of adaptation when few chromatophores had been formed. This material was also likely to have contained ribosomal material in view of the lack of magnesium ions in the buffer. The isolation of prephore subunits from this source was quantitatively preferable to the technique of reconstituting prephore material from the material collected in the fractions at the top of the 25 to 55% sucrose gradient. An electrophoretic technique was developed which appeared to separate the subunits from the ribosomal material. Two peaks were produced in the gradient system (cf. Figures 12a and b): the peak with the greatest mobility was colourless and had a 254 nm absorption/protein ratio about four times as great as the pigmented "subunit" peak. Analysis of the material from the pigmented peak by SDS-PAGE showed that the three major subunit proteins were present (cf. Figure 30a); in

addition, however, lower levels of polypeptides were found with apparent molecular weights similar to chromatophore proteins. The latter peaks were probably caused by contamination with chromatophore fragments (mature subunits). The 1.5 hr 100,000 x g supernatant from adapting cells, when run in the 25 to 55% sucrose gradient, produced a pigmented peak centered at 28% sucrose, the same density as the peak that was produced following the modifications which lead to a reduction in the prephore fraction. This finding, together with the similar SDS-PAGE profiles of the reconstituted prephores and electrophoretically purified subunits, shows that the prephore fraction results largely from an aggregation of prephore subunits present in the supernatant of the high g-force (1.5 hr 100,000 x g) centrifugations. Aggregation presumably occurred during the pelleting of the small particle fraction, while the use of a sucrose trap resulted in material in a non-aggregated state.

The high g-force supernatant of disrupted nature photosynthetic cells, however, was different. When centrifuged in the 25 to 55% sucrose gradient it formed a peak centered at 32% sucrose. A ten-fold increase in the phosphate buffer resulted in aggregation and the production of a peak at 40% sucrose (the same as the heavy chromatophore fraction) rather than the production of a reconstituted prephore fraction. This material was termed



the mature subunit fraction; it appeared to be similar to material produced by the chromatophore fragmentation described by Gibson (1965a).

The heavy chromatophore fraction was shown to be caused either by the presence of divalent cations ( $10^{-2}M$ ), in agreement with the findings of Gibson (1965b), or by pelleting the crude membrane fraction prior to gradient separation, as was found by Hansen and deBoer (1969). It is also likely that aggregation of the mature subunits contribute to this fraction. I modified the preparative technique by harvesting the crude membrane fraction onto a sucrose trap and included EDTA in the phosphate buffer. This eliminated the formation of the heavy chromatophore fraction and drastically reduced the level of the prephore fraction with an increase in material at the top of the gradient (dissociated prephore and mature subunits).

The SDS-PAGE profile of the mature subunit fraction was similar to that of the chromatophores (cf. Figure 31). The material had a lower mobility than the prephore subunits during glycerol density gradient electrophoresis. The prephore subunit fraction, obtained from adapting cells of low pigment content, was thus distinguishable from the mature subunits by three criteria.

The contamination of the prephore subunit fraction by appreciable levels of mature subunit in the double labelling experiment was likely to have been minimal. The

cells used were of low chromatophore content as compared to cells grown at below 4.0% oxygen. The pigment level in the purified subunits was less than half that of the chromatophore-containing "4-6" fraction at both 6 and 16 hr. Contamination of the subunits with mature subunit material would have tended to obscure evidence that favoured a precursor role for the subunits.

### III. A Summary of the Criteria of Purity of the Prephore and Prephore Subunit Fractions.

The prephore and prephore subunit fractions were believed to be homogenous for the following reasons:

1) Examination of negatively stained prephore fractions by electron microscopy showed discrete structures, the majority of which had diameters between 60 and 80 nm. They had a characteristic (non-collapsed) appearance and could also be seen in the small particle fraction before sucrose density gradient centrifugation. Examination of numerous prephore fractions failed to reveal any cell wall material. Structures other than typical 'prephores' were fewer than 5%.

2) The prephore fraction appeared as a discrete band in the 25-55% sucrose density gradient with a width similar to that of the chromatophore fraction. The band was always positioned between 45 and 50% sucrose and characteristically had a concentration maximum close to

the 48% sucrose position. In addition to the electron microscopic evidence, the prephore fraction was believed not to be appreciably contaminated with cell wall material because of the position of this maximum in the sucrose density gradient. Cell wall material would have been expected to form a broad band near the bottom of the 25-55% sucrose density gradient. Such a fraction (the heterogenous cell envelope fraction) has been described by Niederman et al (1972). The peak of this band was centered at about 55% sucrose. This material was believed absent from the gradients described in this thesis because of the initial 20 minute centrifugation at 10,000 x g of the disrupted cell preparation. Niederman et al (1972) employed a 5 minute initial centrifugation. It was believed that material equivalent to the heterogenous cell envelope fraction was separated from the small particle fraction by the more prolonged centrifugation.

3) The prephore fraction was not believed to be contaminated to an appreciable extent by ribosomes as the 254/280 nm absorbance ratio of this fraction was close to unity (Figure 7b). The results shown in this figure make it probable that the ribosomes sedimented in a broad band near the top of the gradient. The profiles shown in Figure 7a were obtained before the inclusion of EDTA into the buffer as a routine procedure. The broad peaks and the presence of the heavy chromatophore fraction (at 37%

sucrose) were also caused by the addition of 16 mg of small particle fraction of the gradient rather than the 2 mg that was later adopted as a standard amount.

4) The use of a sucrose trap for harvesting the small particle fraction and a reduction in buffer concentration virtually eliminated the prephore peak and also resulted in a pigmented band with a density of about 28% sucrose (the prephore subunit fraction). Pigmented material of this density could be made to reform the prephore band at 48% sucrose by increasing the buffer concentration. It was assumed that these modifications would not have caused cell wall fragments to behave in this manner.

5) Two additional methods of purification were employed which took advantage of other physical properties of the fractions. While not used routinely, they both supported the findings of the sucrose density gradient ultracentrifugation that the prephore and prephore subunit fractions were homogeneous fractions. It was found that (a) the prephore fraction eluted with a smaller volume than did the chromatophores during Sepharose 2B gel filtration (Figure 5, Chapter 3). The majority of the prephore fraction eluted in fraction 9 and was virtually absent in fraction 13; and (b) the pigmented prephore subunits migrated as one band during glycerol gradient electrophoresis (Figure 12, Chapter 3).

#### IV. The Development of a Scheme of Chromatophore Morphogenesis.

##### A. Work which led to the discovery of the prephore fraction.

I set out hoping to demonstrate some of the enzymes of the magnesium branch of bacteriochlorophyll (Bchl) synthesis. The steps in this pathway had been postulated as a result of the identification of pigments excreted by mutant strains of R. spheroides [Richards and Lascelles (1969)]. At the time only one of the enzymes had been demonstrated: S-adenosylmethionine magnesium protoporphyrin methyl transferase, hereafter referred to as the methylating enzyme [Gibson et al (1963)]. I hoped that the addition of an intermediate produced by one mutant to a mutant blocked at an earlier or later step would result in an enzymatic conversion.

Removal of the cell walls by lysozyme caused mutant cells to lose their "bound" pigments. This indicated that pigments had accumulated in the periplasmic space. This has subsequently been supported by Peters (1970). He found that the final step in Bchl synthesis, in a green mutant of R. spheroides, was the accumulation of Bchl inside the cell wall but outside the cytoplasmic membrane. I thought that a cell membrane fraction might be prepared enriched in the magnesium-branch enzymes especially if cells actively synthesizing pigment were used. It was

anticipated that the methylating enzyme activity might provide a marker for these enzymes.

During a study of methylating enzyme activity, it was discovered that mutant strains 8-32, 8-29 and 8-47 (of *Lascelles*) did not form chromatophores (cf. Results, Section I.A.1.). This was in agreement with the finding by Deize et al (1969) that mutant strains unable to synthesise Bchl did not contain chromatophores. However, the mutants contained pigment in a fraction of greater density than the chromatophore fraction. The parent strain contained an equivalent "dense" fraction (the "prephore" fraction) but it was only slightly pigmented. I thought it possible that this fraction represented an immature form of the chromatophore fraction. If the prephore fraction discovered was a precursor to the chromatophore fraction then I thought it would most likely be prominent in preparations from cells in which chromatophore synthesis was high. Cells were then studied during the adaptation from aerobic to photosynthetic growth. The results (Figures 3 and 5, Chapter 3) indicated a reciprocal relationship between the protein content of the prephore and chromatophore fractions during adaptation and suggested a system had been found which would allow the isolation of a chromatophore precursor fraction.

B. A preliminary morphogenesis scheme based on the results of the L-(U- $^{14}$ C) proline incorporation experiment.

The measurement of specific activity in gradient fractions subsequent to the addition of L-(U- $^{14}$ C) proline to cells at the start of adaptation to photosynthetic growth gave the results shown in Figure 35. The specific activity of both the protophore and prephore fractions peaked after 2 hours while the light chromatophores were most active in the 6 hour preparation. The heavy chromatophore fraction had the highest specific activity in the 20 hour sample. The fluctuations in specific activity of the different fractions (protophore fraction excepted) were thus not parallel. This implied that the fractions were not merely different parts of a single system that was undergoing a continuous modification but rather that the fractions were separate elements involved in chromatophore morphogenesis. The assumption was also made that fractions with a characteristic position in the gradient after centrifugation had the same protein compositions at different times during the adaptation.

The variations in specific activity in the fractions could be most easily rationalized if it was assumed that the conversion of material in one fraction occurred yielding another fraction with a different density. It was assumed that the density changes resulted from changes in the protein to lipid ratios of the fractions. An

alternative explanation would have been that the proteins initially incorporated into one fraction were degraded and the labelled amino acids then preferentially incorporated into another fraction. The changes in the distribution of Bchl in the gradient during adaptation (Figure 19) favoured the first hypothesis and strengthened the postulated role of the prephore fraction as a chromatophore precursor. In addition, there was no detectable Bchl in a non-protein-bound state in the cells or the small particle fractions.

The analysis of the distribution of total activity in the fractions at different times (Figure 36) showed that the protophore fraction contained its highest activity at 2 hours, the prephore and the light chromatophore fractions at 6 hours while the heavy chromatophore fraction was most active at 20 hours. It was therefore postulated that the protophore fraction preceded the prephore fraction, which in turn was synthesized (from the specific activity data) before the light chromatophore fraction. Finally, it was postulated that the heavy chromatophore was formed from the light chromatophore fraction and represented the true "mature" chromatophore. The preliminary scheme of chromatophore morphogenesis proposed on the basis of these results is shown in Figure 51.

In terms of the models discussed previously (Section



I. D.3) the data of this experiment are not compatible with the prephore being the sole and immediate protein precursor to the light chromatophore fraction, ie model (a). The specific activity of the light chromatophores is higher than that of the prephores at the same time (20 hours). This experiment makes it likely that chromatophore morphogenesis is a multistep process and that many prephore 'compartments' are empty in aerobically grown cells. The data is insufficient, however, to allow discrimination between models (b) and (c) (Section I. D.3).

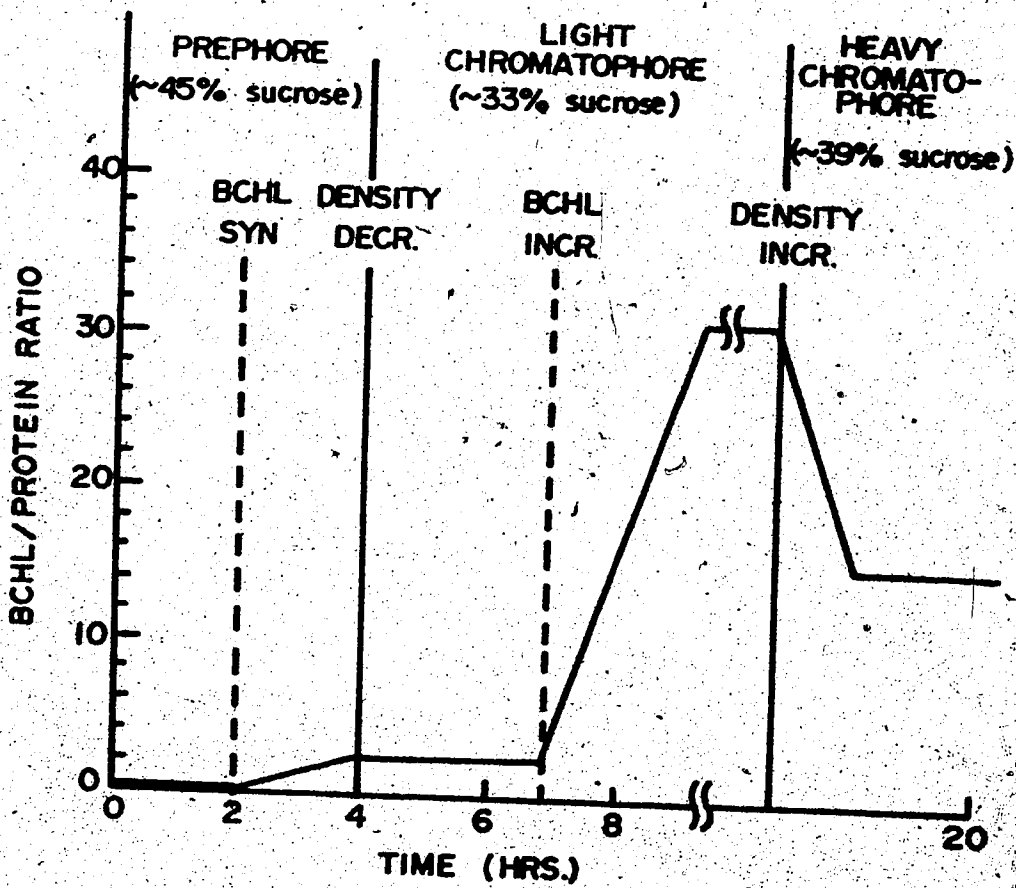
C. Implications of the discovery of the prephore subunit and mature subunit fractions.

The discovery of the prephore and mature subunit fractions had a number of implications on the preliminary morphogenesis scheme which had been proposed on the basis of results of the L-(U-<sup>14</sup>C) proline incorporation experiment (Figure 51):

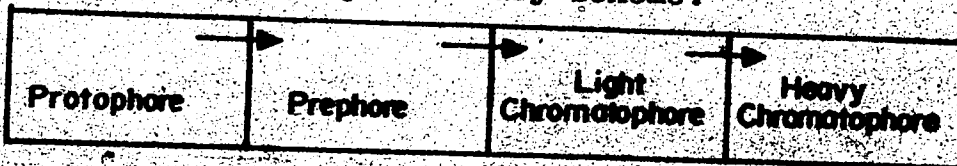
1. The prephore subunit

The finding that the prephore subunit occupied a position centered at 28% sucrose during density gradient centrifugation (the same as the protophore fraction) made it likely that a majority of the so-called protophore fraction consisted of prephore subunits. Thus the specific activity changes of the protophore fraction during the L-(U-<sup>14</sup>C) proline incorporation experiment were

Figure 51. Bchl/protein ratio in the initially proposed morphogenetic scheme.



Morphogenic relationships of the fractions in the preliminary scheme.



probably due to the presence of unpigmented pre-phore subunits in this fraction. The fact that the specific activity in this fraction was lower than that of the prephore fraction at all times studied makes it unlikely to be a distinct prephore precursor.

In addition, the reconstitution of the prephore fraction from the prephore subunits, indicated that the former fraction is an artefact of the isolation procedure resulting from the aggregation of prephore subunits. The prephore cannot thus be considered to be a discrete cellular structure. The prephore subunits, on the other hand, may exist as such in the cells, or be derived from a membranous structure by the disruption technique. The in vivo location of the prephore subunits is discussed in the next section. The prephore subunit is now believed to be the true chromatophore precursor.

## 2. The mature subunit

It was also found that the aggregated mature subunit and heavy chromatophore fractions occupied similar positions (ca. 39-40% sucrose) during density gradient centrifugation. The heavy chromatophore fraction is therefore now believed to be an artefact consisting of aggregated chromatophores and mature subunits. Its increase in specific activity from 6 to 20 hours probably is due to aggregated mature subunits produced by the breakdown of the initially labelled light chromatophores.

Possibly the 'oldest' chromatophores might be the most susceptible to aggregation and that both these mechanisms cause the increased activity in this fraction at 20 hours. Hence, the light chromatophore fraction is now believed to be the true mature chromatophore fraction.

D. Location of the prephore subunit in vivo

Peter's work (1970) indicated that the periplasmic space was the site of Bchl synthesis. As the subunit is the first structure that can be isolated specifically bound to Bchl, its most likely in vivo location would be the periplasmic space. This proposal is supported indirectly by several findings. Alumina grinding does not produce a prephore fraction unlike the more vigorous disruptive techniques. This could be the result of the subunits being trapped between the cell wall and cytoplasmic membrane. Studies of thin sections of adapting cells (see Figures 45 and 46) show that an influx of storage lipid occurs into the periplasmic space very early in the adaptation. The hydrophobic nature of the subunits could be imagined as compatible with their functioning in this location. In work with mutant strains and parent cells I have found, in agreement with other workers [Oelze and Drews (1969)] that the pigments excreted by Bchl-less mutants were protein bound. This protein, like the subunit, had a strong tendency to

aggregate. A study (M.A. Shaw, unpublished data) of the distribution of the pigment-protein complex from cells of mutant 8-47 indicated that 30% was bound by the cells and only 10% of the "soluble" complex would pass through a 100 nm millipore filter. The "soluble" complex could be harvested by centrifugation in 30% sucrose 0.005M in phosphate buffer (16 hrs at 70,000 x g). The pelleted complex could be dissociated, by stirring in 50% glycerol/50% distilled water, into a form not sedimenting at the above g force. This made it probable that hydrophobic bonding was the cause of the aggregation. SDS-PAGE analysis of the complexes produced by several mutants (Shaw, et al, 1973) and also the parent strain under Bchl-limiting conditions (Figure 34) indicated that protein H of the subunit was prominent. Unlike the subunits, the pigment-protein complex contained a large amount of a protein with a molecular weight of 15 kD (protein C). This protein and the protein with a molecular weight 40 kD daltons (protein H), were observed to carry pigment. The similar protein compositions of the pigment-protein complex from the parent strain and the reconstituted prephore fraction make it probable that these pigmented structures are related. The loss of pigment by cells to the medium after treatment with lysozyme together with the absence of the prephore fraction after disruption by alumina grinding makes the

periplasmic space the most probable in vivo location of the prephore subunits.

E. Discussion of the proteins of the prephore subunit and medium density fractions.

SDS-PAGE analysis showed the prephore fraction to have a simple protein composition in comparison with the other fractions studied. It was postulated that the pigmented prephore subunits might combine with another fraction to produce the chromatophores. The other alternatives are that the prephore subunit combines with additional proteins synthesised de novo or contained in some other fraction not isolated. The protein composition of the medium density fraction made it the most likely candidate as a fraction which combined with the prephore subunit during chromatophore morphogenesis (cf. Figure 52).

F. A discussion of the results of the double labelling experiment.

1. The incorporation of radioactivity into the fractions.

The double labelling experiment was different from the initial L-(U-<sup>14</sup>C) proline labelling experiment in that the cells were actively dividing during the adaptation. In this experiment, the fractionation technique allowed

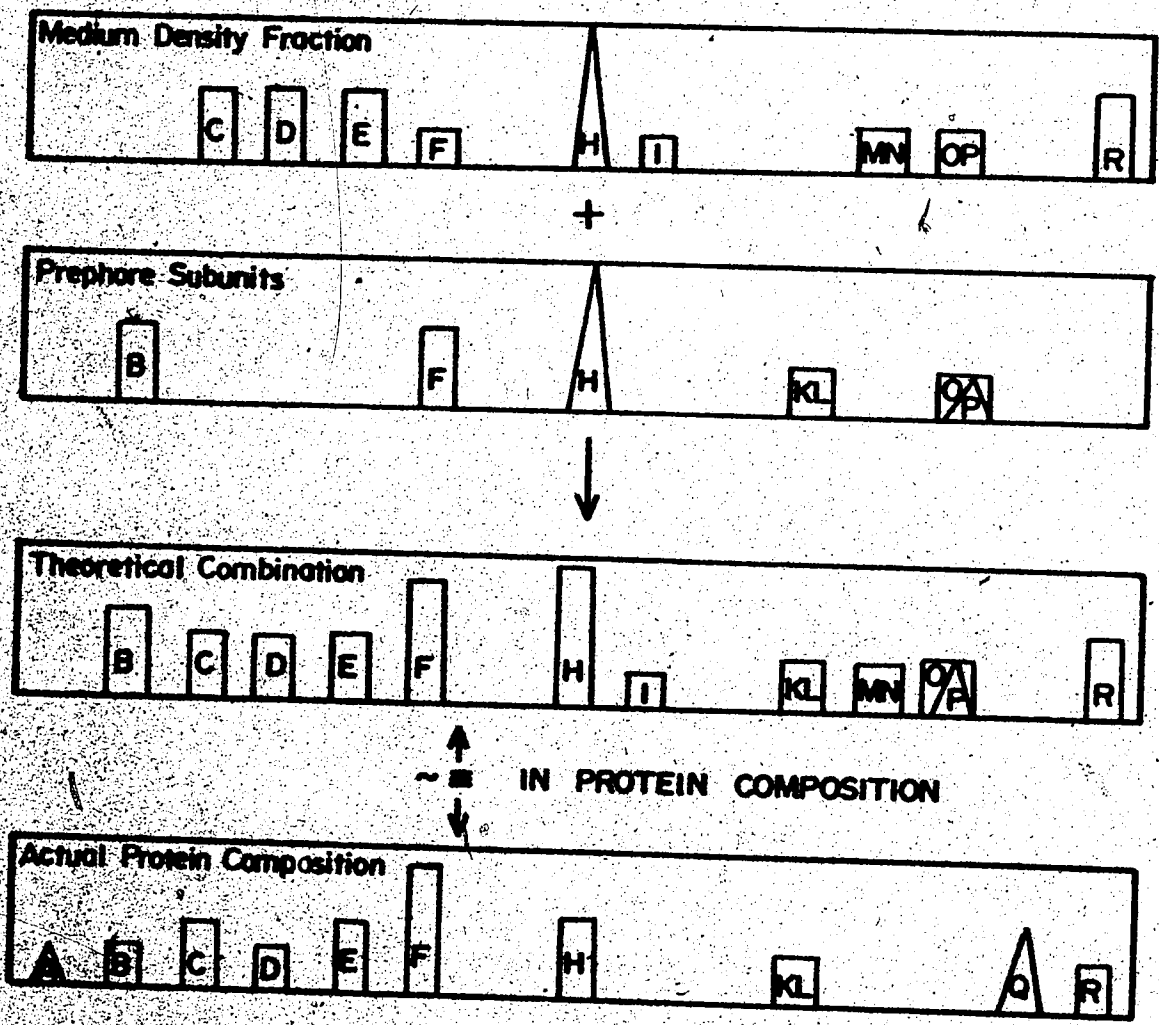


Figure 52. Theoretical combination of the medium density fraction and the S<sup>+</sup> fraction (prephore subunits) to yield a protein pattern similar to the light chromatophore pattern. Material from cells grown in condition C.

the isolation of the medium density fraction (Chapter 3, Section I. A.3). Further, the prephore subunit fraction, rather than the prephore fraction, was studied after purification by glycerol density gradient electrophoresis.

As expected, the specific activity of all but one of the fractions studied decreased steadily after the additions of the chase amino acids. The exception was the "4-6" fraction at 16 hours (see Figure 41). The increase in the specific activity of the "4-6" fraction at 16 hours shows involvement of a precursor, as it can only be accounted for by the incorporation of pre-existing proteins, formed between -3 hr and 0 hr ( $^3\text{H}$  data) and between 0 hr and 2 hr ( $^{14}\text{C}$  data). If these labelled proteins originated in the prephore subunit or medium density fractions, a change in their specific activities or pool sizes would be expected. The question arises as to whether or not the decreases in specific activity of the purified subunits and the medium density fraction (Figure 41) were the result of their utilization in chromatophore synthesis. As previously mentioned (Section IV.D.2. above), four distinct mechanisms for the dilution of specific activity are possible. Data which was corrected for dilution due to cell growth (mechanism a) was shown in the form of a log plot of the ratio of the specific activity per fraction over the specific activity per whole cell protein (Figure 42). This figure shows



that dilution in specific activity of the medium density fraction (between 2 and 6 hr) and the subunit fractions (between 0 and 6 hr) is indeed greater than average during adaptation. However, to eliminate the possibility that this increase in dilution is not due to increases in pool sizes (mechanism b) but does in fact result from utilization as a precursor (mechanism d), the absolute quantity of these fractions per cell at each time must be known. As these fractions are the product of a harsh disruptive procedure, it is not possible to evaluate the efficiency of their liberation from the cell. This could depend on their tendency to associate with other components and the vulnerability of the cells to disruption. These parameters might vary during the course of adaptation. It is also difficult to eliminate the possibility of increased degradation and resynthesis from unlabelled amino acids subsequent to the additions of the chase (mechanism c). Obviously this factor might vary with the metabolic state of the cell. If it is assumed, however, that the pool sizes of the prephore subunits and the medium density fraction are constant and further, that their degradation and resynthesis is not responsible for the increase in dilution of specific activity during adaptation (i.e. mechanisms b and c in Section IV.D.2. above are not operative), then these results are consistent with the possibility that these fractions are

being converted into another fraction (eg. the chromatophore component of the "4-6" fraction).

The maximum observed specific activity of the "4-6" fraction was at 16 hr. At this time, the specific activity of this fraction was greater than either the prephore subunit or the medium density fractions, both in the  $^3\text{H}$  and the  $^{14}\text{C}$  data. Hence, as was the case with the single  $^{14}\text{C}$ -proline labelling experiment (Section IV.8. above), the data are not consistent with either fraction being the sole and immediate precursor to the chromatophore fraction (model a of Section I.D.3. above). The protein analysis (Chapter 3, Section II.E.3.) showed that additional proteins must be added to the prephore subunits before these become chromatophores. Hence, both models a and b (Section I.D.3.) would not be favoured on this basis alone. A theoretical combination of proteins (Section IV E. above) implicated the medium density fraction as the source of these proteins. A combination of the prephore subunit and medium density fractions (together with the addition of Bchl and other non-protein components) would correspond to model c of Section I.D.3. In such a case, the maximum specific activity of the chromatophore fraction would lie somewhere between the activities of the combining fractions at the time of their formation.

The specific activity data cannot confirm the

implication of the prephore subunit or the medium density fractions as chromatophore precursors, nor can it by itself distinguish between models b and c of Section I.D.3. The maximum specific activity of the "4-6" fraction at 16 hr was less than the maximum specific activity of either the prephore subunit or the medium density fractions at 0 hr (for the  $^3\text{H}$  data). However, it was equal to that of the prephore subunit fraction and greater than that of the medium density fraction at 2 hr (for the  $^{14}\text{C}$  data). Hence, combinations of the two should yield  $^{14}\text{C}$  specific activity values for the "4-6" fraction substantially less than the maximum specific activities of the prephore subunit fraction. In order for such a combination to be in effect, one must evoke one of the following two mechanisms:

(1) A wave of high specific activity prephore subunit "compartments" formed before 2 hr (and of higher specific activity than that shown in the  $^{14}\text{C}$  data at 2 hr) may have become chromatophores between 6 and 16 hr. This mechanism is indistinguishable from model b of Section I.D.3. above.

(2) The high specific activity of the "4-6" fraction may have been due to the transfer of activity to the fraction from a source not isolated by the fractionation technique. Although such a source was not found in the small particle or the 100,000 x g supernatant fractions,

it might be strongly associated with the cell envelope material which was discarded in the preliminary centrifugations (eg. a protein or proteins of the cell membrane) and may have been very highly labelled.

As mentioned above, the specific activity data do not allow a distinction to be made between these two possibilities. Thus, all that can be stated is that the distribution of incorporated activity during adaptation is not incompatible with the roles of the prephore subunit and medium density fractions as chromatophore fraction precursors. In either case, the entirely de novo synthesis of the extra proteins from precursors of low specific activity is not likely.

## 2. The $^3\text{H}/^{14}\text{C}$ ratios of the fractions

The essentially constant  $^3\text{H}/^{14}\text{C}$  ratio of the purified prephore subunits (cf. Figure 43) has two important interpretations: 1) it confirms the previous findings that prephore material is contained in unpigmented "aerobic" cells and 2) it shows that this material is the product of de novo synthesis rather than being formed through the incorporation of pre-existing aerobic proteins formed before 2 hr and therefore containing a higher  $^3\text{H}/^{14}\text{C}$  ratio. It seems likely that the medium density fraction, on the other hand, contains a large aerobic protein component. The increase in the ratio at 6 hr and

the subsequent drop in the ratio of this fraction at 16 hr may reflect passage of aerobic protein(s) of the cell membrane (formed before 2 hr) into this fraction (by 6 hr) and from thence into the chromatophore fraction (by 16 hr). The latter possibility is supported by the increase in the  $^3\text{H}/^{14}\text{C}$  ratio of the '4-6' fraction at 16 hr.

G. The modification of the preliminary morphogenesis scheme.

The preliminary morphogenesis scheme (Section IV.B.) included four components: the protophore, prephore, light chromatophore and heavy chromatophore fractions. As mentioned in section IV.C. above, the protophore fraction contained a lower specific activity in the initial L-(U- $^{14}\text{C}$ ) proline labelling experiment than the prephore fraction. It was positioned before the prephore fraction in the preliminary scheme as its total activity peaked at 2 hours while that of the light chromatophore was highest in the fraction studied at 6 hours. The greater specific activity of the prephore fraction implied that only a component of the protophore fraction enriched in L-(U- $^{14}\text{C}$ ) proline was involved in the production of the prephore fraction.

Subsequently it was found that the prephore fraction was in fact an aggregate of smaller particles (Section II above), which in a non-aggregated state peaked at a

density of 28% sucrose in the standard gradient. This made it most likely that the activity observed in the protophore fraction in the single label experiment was due to the presence of prephore subunits at 0 and 2 hours (Section IV.C.).

The heavy chromatophore fraction was found to be produced by the small particle fraction harvesting procedure initially employed and that its formation was, in addition, promoted by the presence of divalent cations (Section I.E.2.e, Chapter 3). These findings showed that only two of the fractions in the preliminary scheme remained distinct entities: the prephore fraction and the light chromatophore fraction. Further the prephore fraction was found to be an aggregate of smaller particles, the prephore subunits.

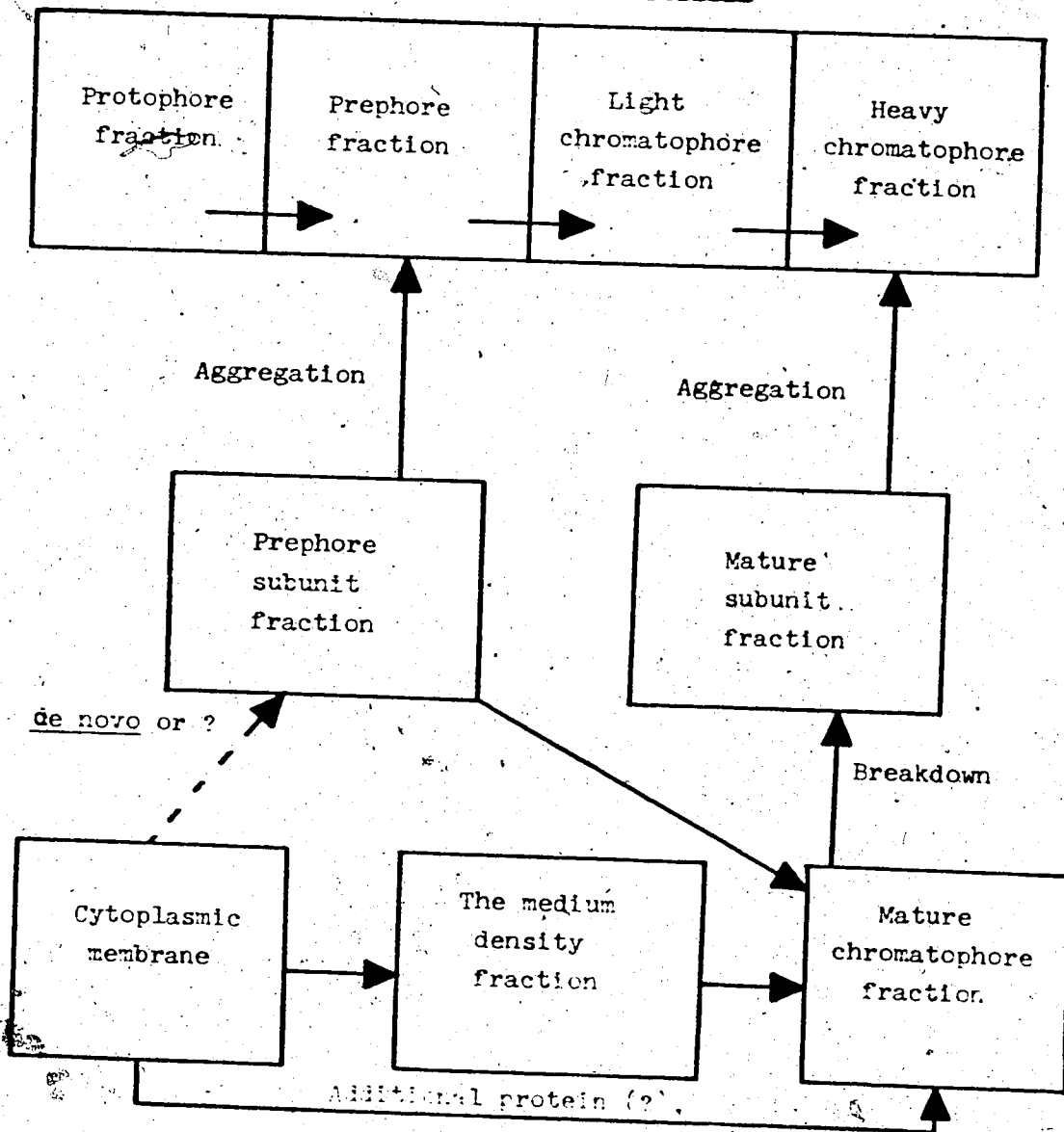
The SDS-PAGE analysis of various fractions (discussed in Section IV.E. above) showed that the prephore subunit had a simple protein composition compared to the other fractions studied. It was thought most likely that these subunits combined with some portion of the cytoplasmic membrane as many workers have shown the cytoplasmic membrane to be involved in chromatophore morphogenesis (see Section IX, Chapter I). The protein composition of the medium density fraction made it the most likely candidate to combine with the prephore subunits to form the chromatophore (see diagram in Figure 52 above). This

belief was further supported by the membranous 'sac-like' appearance of the medium density fraction as seen by electron microscopic examination of negatively stained preparations (Figure 9c). As discussed in Section IV.F.1, it is probable that the medium density fraction has an increased turnover during adaptation. However, the double label experiment could not confirm the prephore subunit and medium density fractions as the sole chromatophore precursors. It seems likely that additional proteins of this cell membrane, not isolated and analysed in this study, are also incorporated into the chromatophore fraction (Section IV.F.1.). The  $^3\text{H}/^{14}\text{C}$  ratios, however, implied that the prephore subunit fraction was formed de novo, whereas the medium density fraction may have contained a significant aerobic membrane component (Section IV.F.2.).

These factors lead to the development of the modified morphogenesis scheme shown in Figure 53; the preliminary scheme is included for comparison.

Figure 53. A comparison of the preliminary and modified schemes of morphogenesis.

The original scheme (Chapter 3)



The modified scheme.



## Chapter 5. Conclusions and Speculations.

### I The Role of the Prephore Subunits in Chromatophore Morphogenesis.

It is now possible to answer some questions with regard to the role of the prephore subunits in chromatophore morphogenesis.

#### A. How and where are the prephore subunits formed?

The constant  $^3\text{H}/^{14}\text{C}$  ratio found in the double labelling experiment showed that, unlike the other fractions, the prephore subunits were formed de novo without the incorporation of pre-existing proteins.

The question as to where the synthesis of these precursors takes place is difficult to answer. The only evidence as to their location in the cell is indirect. Peters (1970) showed that the first detectable pigment in adapting cells was probably located in the periplasmic space. The subunits could be synthesised on membrane bound ribosomes and migrate to the periplasmic space directly through the cytoplasmic membrane. Another possibility is that the migration of lipid into the periplasmic space in the early stages of adaptation is connected with the organization of these hydrophobic subunits. It could be proposed that the migration of PHB storage granule material into the periplasmic space is

triggered by their association with subunits in the cytoplasm. This might be supported by the liberation of subunit proteins from isolated PHB storage granules obtained from cells in the first stages of adaptation.

B. What factors control the synthesis and utilization of prephore subunits?

It was found that the prephore fraction can be obtained from aerobic cells, but growth of the cells at high levels of oxygen inhibited their formation. Unpigmented prephores can be produced by reducing the oxygen level for a short period of time. If low oxygen levels are maintained the prephore fraction becomes pigmented and the cells form chromatophores. An increase in the level of the prephore fraction does not however, automatically result in the production of chromatophores. When cells grown at high oxygen levels are gassed with nitrogen the prephore fraction is increased and becomes pigmented; however, chromatophores are not developed. This demonstrates that prephore utilization does not depend only on Bchl synthesis. When a culture is gassed with 4% oxygen/nitrogen the chromatophore production is rapid and a lower level of prephores is found than when cells are cultured in media gassed with less than 4% oxygen/nitrogen. It would appear that it is the utilization of prephores rather than their production which

is limited at low oxygen tensions. It would seem likely that the synthesis of components other than the prephore subunit proteins is more susceptible to metabolic inhibition under these conditions. It appears, however, that it is the synthesis of the prephore subunits which is limited at high oxygen levels (gassing the culture with greater than 65% oxygen). An appreciable unpigmented prephore component is normally present at lower oxygen tensions. Oxygen (probably via the maintenance of a high endogenous ATP level) causes the inhibition of the synthesis of some other component which is required for prephore utilization. Although this component, as mentioned, is not Bchl, it seems likely that prephore pigmentation is also required before prephores can be utilized. This assertion is based on the results of early studies with mutants unable to synthesize Bchl. In agreement with Delze et al (1969), it was found that such mutants were defective in both chromatophore proteins and Bchl-synthetic ability. I found the mutants did produce a fraction of equivalent bouyant density to the prephore fraction. In addition, spectral analysis of prephore fractions from the parent strain have not revealed precursors to Bchl. Thus it is probable that Bchl is required for insertion into the prephore subunit in addition to the synthesis of some other component(s). As discussed below, it seems likely that the medium density

fraction is one other component.

C. What is the source of the additional proteins required for the conversion of prephore subunits into chromatophores?

The analysis of the protein composition of different fractions indicated that the medium density fraction might combine with the pigment-carrying prephore subunits to form the chromatophore. This belief was supported by the "sac-like" appearance of this fraction in electron micrographs, such structures could be imagined to arise from the dislocation of invaginations. As was discussed in Section IV.F.1. above the entirely de novo synthesis of the extra proteins was shown to be unlikely. It was suggested, on the basis of the electron microscopic appearance of the fraction, that it was derived from dislocated aerobic invaginations. This remains to be demonstrated, however. Niederman and Gibson (1972) used E. coli, believed to lack aerobic invaginations, to determine whether their small particle fraction represented these structures. They found that disrupted E. coli, in fact, produced a similar fraction to the small particle fraction from R. spheroides. If E. coli cells had not produced a small particle fraction this would have indicated that the fraction in R. spheroides was produced from the invaginations. A similar approach

might provide more substantial evidence that the medium density fraction is derived from aerobic invaginations seen in electron micrographs. This would obviously be a difficult point to prove. The possibility remains that the source of the additional protein was in a cell envelope fraction which was not isolated in this study.

## II. A Morphogenic Model of Chromatophore Synthesis.

The results of the double labelling experiment are compatible with, but not absolute proof for, the role of the subunit as a chromatophore precursor. The subunit was shown not to be derived from a pre-existing membrane fraction but to have been synthesized de novo. In addition it was indicated to have had a higher-than-average metabolic turnover between 0 and 6 hr. A study of the increase in  $^3\text{H}/^{14}\text{C}$  ratio of the medium density fraction at 6 hr may indicate that, as would be expected, the medium density fraction is derived from the cytoplasmic membrane. This result, together with its turnover data, makes it likely that the medium density fraction supplies proteins derived from the aerobic cytoplasmic membrane to the chromatophore. Electron microscopic examination of this fraction after negative staining had suggested that this material may have been derived from invaginations of the cytoplasmic membrane and thus provides further support for this belief. The double

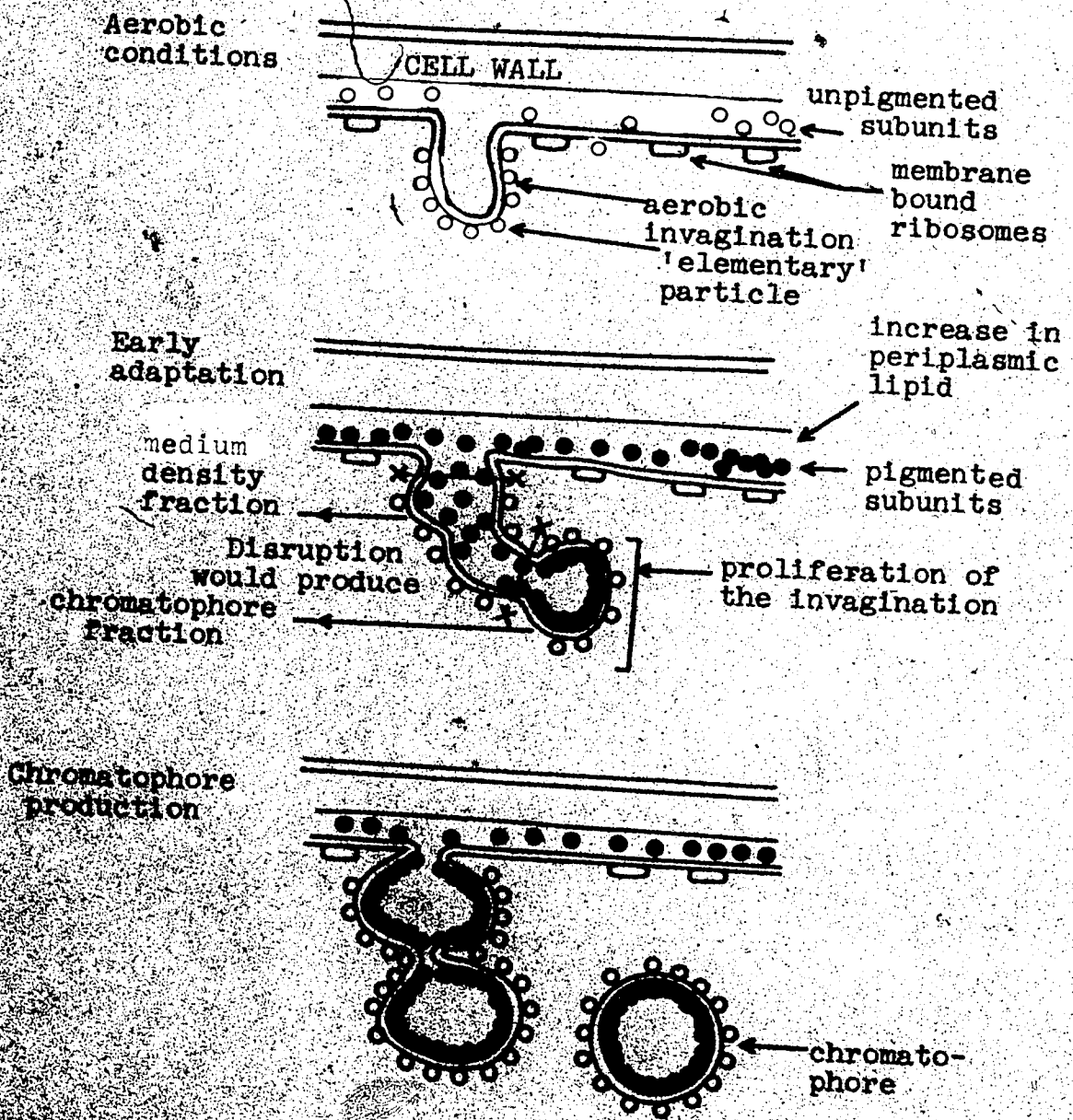
labelling experiment also showed that chromatophores are not formed entirely de novo. If this had been the case, the specific activity of the "4-6" fraction would have continually decreased with the net increase in chromatophores in the "4-6" fraction material.

The following model of chromatophore morphogenesis can now be constructed. Reduction in oxygen supply and the resulting decrease in the cellular ATP level causes the preferential synthesis of the prephore subunits and the initiation of Bchl synthesis. When the pigmented subunits reach a certain concentration in the periplasmic space they combine with the simultaneously proliferating "aerobic" invaginations. This combination and continued pigment synthesis then converts them to functional chromatophores. Figure 54 shows diagrammatically the proposed scheme of chromatophore morphogenesis.

### III. An Overview

The question as to the origin of bacterial chromatophores has been studied by numerous workers in the last twenty years and remains poorly understood. Much of this thesis has been concerned with establishing that it is possible to isolate a chromatophore precursor, which was termed the prephore subunit fraction. The effects of culture conditions and disruptive procedures on the production of this precursor fraction were described.

Figure 54. Proposed model of chromatophore morphogenesis.



Further studies provided information as to its macromolecular structure and protein composition. Some work was performed on the question of how this precursor might develop into the mature photosynthetic organelle. Problems with regard to the study of chromatophore morphogenesis, by the use of different techniques, have been discussed. Although a model of the overall process was produced, the thesis has shown that the understanding of chromatophore development is still by no means complete.

It is my hope that this thesis will provide useful groundwork for further investigations.

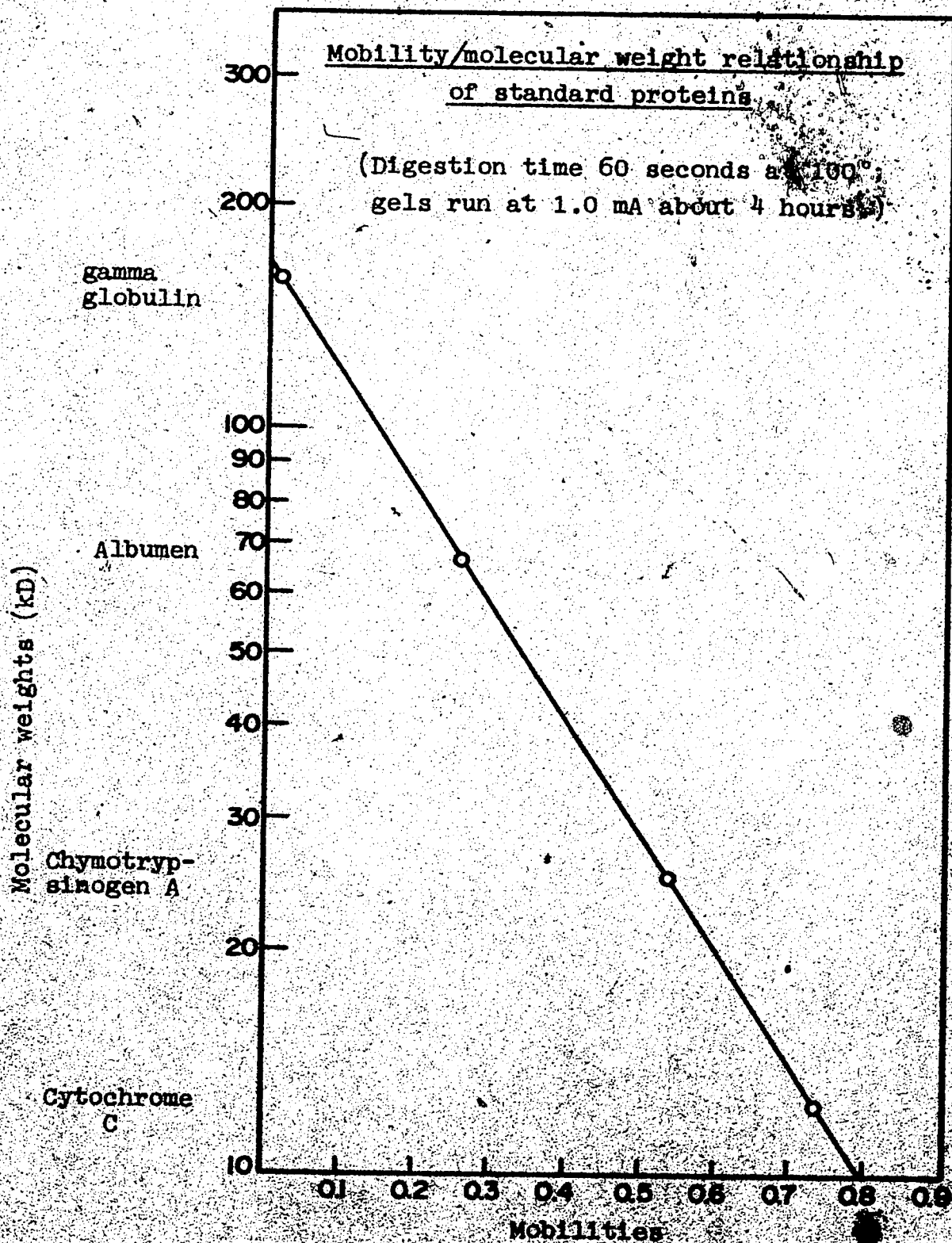


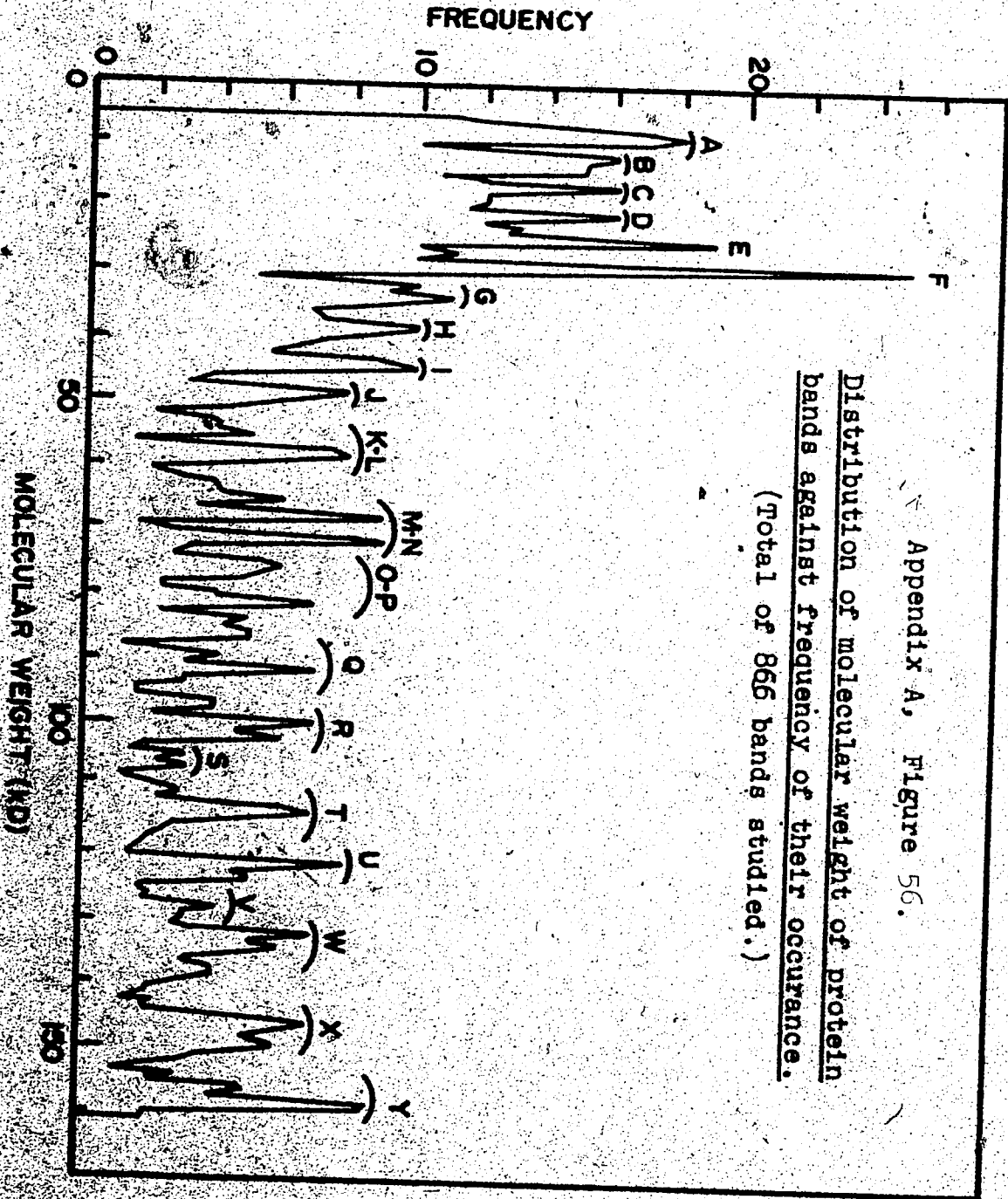
Appendix A.

The comparison of protein contents of different fractions.

To make the protein compositions of different fractions easily comparable, the stained polypeptide bands were assigned to groups depending on their apparent molecular weights. Although duplicate samples in any run gave the same gel profiles, it is probable that there were small variations between the apparent molecular weights of polypeptides in different runs. The technique is recognized to give + or - 5% variation in the apparent molecular weights. A good linear relationship was obtained with the protein standards used (Figure 55). The assignment of a polypeptide to a particular group was achieved in the following manner. The apparent molecular weights, corresponding to 866 bands obtained from the study of the protein compositions of different fractions in chapter 3, were plotted against the frequency of their occurrence (Figure 56). Distinct peaks were produced. As mentioned, two polypeptides may have the same apparent molecular weight; this technique alone is not sufficient to exclude this possibility. Classification of bands by this method does allow the protein pattern in different fractions to be compared. As can be seen from Figure 56, assignment of bands to distinct groups becomes more difficult with increasing molecular weight. Many

Appendix A, Figure 55.





Appendix A, Figure 56.  
Distribution of molecular weight of protein  
bands against frequency of their occurrence.  
(Total of 865 bands studied.)

apparently 'high molecular weight' bands may be due to the association of lower molecular weight polypeptides. For this reason, comparison of bands corresponding to polypeptide weights below 50 kD have been preferentially used; the bands corresponding to higher molecular weights were classified into groups K-L, M-N, O-P, Q and R. To compare the protein contents of different fractions, histograms were drawn, peaks being further classified on the basis of their profiles and their heights. Apparent molecular weights and band assignments were shown on the figures.

### Disolved Oxygen Content.

The Beckmann model 777 oxygen electrode was used for monitoring the dissolved oxygen concentration in growing cultures. The meter was preset to give a reading of 20% oxygen in air. The dissolved oxygen concentration for particular meter readings at 25 degrees in water were determined by Maharajh (Ph. D. Thesis, Chemistry department, Simon Fraser University, 1973). The corresponding values at 30 degrees were calculated and are shown in the following table. I would like to thank Dr. J. Walkley for his assistance in the calculation of the oxygen solubilities corresponding to the different meter readings at 30 degrees.

Oxygen concentrations corresponding to various meter readings  
of the Beckmann model 777 electrode.

Table VII

Oxygen level (%) in the saturating gas mix. (balance nitrogen).	Reading of electrode in the solution	nmoles per liter oxygen in water at 25°C	Values at 30°C
	1	15	14
4 ± ½	3	45	42
	5	75	69
	10	150	140
20 (Air)	18	290	250
	20	320	280
65 ± ½	50	800	690
100	80	1,300	1,110

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