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ENERGY TRANSFER BETWEEN BACTERIOCHLOROPHYLL AND THE CAROTENOIDS IN BACTERIAL PHOTOSYNTHESIS

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy in the
Faculty of Science

by

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ABBREVIATIONS

The following non-standard abbreviations have been used;

A	absorbance
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
BPh	bacteriopheophytin
BSA	bovine serum albumin
Car	carotenoid
CD	circular dichroism
Chl	chlorophyll
cyt c	cytochrome c
DPIBF	1,3-diphenyl isobenzofuran
EDTA	ethylene diamine tetra acetate
kD	kilodalton
Mes	2-(-morpholino) ethane sulphonic acid
NAD	nicotinamide adenine dinucleotide
NIR	near infra red
OD	optical density
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TRIS	Tris (hydroxymethyl) aminomethane

CONTENTS

ERRATA

In the legend for Fig. 5.6 (following p.50), 'a laser intensity of 100 = 1 Joule' should read "100 = 0.4 Joule". The laser energies cited in legends for Figs. 5.2 and 5.4 should now read 0.176J.

P.28: The following sentence should be inserted at the end of the description of SDS-polyacrylaride gel electraphoresis:

'The electrophoresis was performed by passing a current of 6 milliamps through the gel for 15-18 hrs.'

SUMMARY

Carotenoids have two major functions in photosynthesis:

(1) They act as accessory light-harvesting pigments, absorbing light at wavelengths where chlorophylls do not absorb and transferring the absorbed energy to the chlorophylls.

(2) They prevent the chlorophylls from sensitizing the destructive 'photodynamic' reaction which occurs in the presence of light and oxygen.

In the photosynthetic bacteria, as in plants, the way in which carotenoids perform these functions is not well understood. In whole cells of photosynthetic bacteria the efficiency of energy transfer from carotenoids to the bacteriochlorophyll (BChl) varies from 30 - 90% depending on the species. It is not clear whether this is caused by variation in carotenoid type between species or by differences between the relative arrangement of carotenoids and BChl in pigment-protein complexes.

During this project I have developed a method for adding carotenoids to the B850 light-harvesting pigment-protein complex from the carotenoidless mutant Rps. sphaeroides, R26. This should allow investigation into the factors which affect the functions of carotenoids. Since the carotenoids bind to identical sites on the complex, a direct comparison can be made between the energy transfer and photoprotective abilities of a range of carotenoids.

During the course of this project I characterised the B850 pigment-protein complex from Rps. sphaeroides, R26. Wild-type Rps. sphaeroides contains two types of light-harvesting complex classified by the wavelengths of their near infra red (NIR) absorption spectra. These are called B800 + 850 and B875 since they show absorption bands at 800 and 850, and 875nm respectively. R26 shows only a single NIR absorption maximum at approximately 850nm (when R26 was first isolated this band absorbed at 870nm). R26 is usually thought of as containing only one light harvesting complex, B850, which is thought to be a modified B875 type. Using SDS-polyacrylamide gradient gel electrophoresis and isoelectric focussing I characterised the B850 complex. I found that the two BChls responsible for the 850nm absorption band are attached to two polypeptides, approximate molecular weight 8 and 10 kilodaltons (kD). These polypeptides appeared to be identical to the polypeptides of B800 + 850 from wild-type Rps. sphaeroides. I therefore suggest that the B850 complex of R26 is a B800 + 850 type of complex which lacks the BChl responsible for the 800nm absorption band.

On gradient gels R26 membranes showed the same three light-harvesting complex bands shown by wild-type membranes. This suggests that R26 in current laboratory use contains 2types of light-harvesting complex. I received a culture of the original strain of R26 (absorbing at 870nm). On gradient gels membranes showed only two light-harvesting polypeptides (approximately 8 and 12kD). In wild-type strains these had previously been identified as belonging to the B875 type of light-harvesting complex. I concluded that the R26 strain has gained a B800 + 850 light-harvesting complex (lacking the 800nm BChl) since it was first isolated in 1963.

I reconstituted carotenoids into the B850 complex by adding them in petroleum spirit to freeze-dried R26 chromatophores. The petroleum spirit was evaporated off, the chromatophores plus carotenoids were resuspended in buffer and treated with the detergent sodium dodecyl sulphate (SDS). The B850 complex was then isolated by hydroxylapatite column chromatography.

Two carotenoids, neurosporene and spheroidene, were reconstituted into the B850 complex in amounts approaching those which would be expected if they occurred naturally in the B850 complex. These carotenoids were bound to specific sites on the B850 pigment-protein complex in an all-trans configuration as in naturally occurring carotenoids in light-harvesting complexes.

When reconstituted, neurosporene and spheroidene performed both their light-harvesting function and photoprotective function.

Light absorbed by carotenoids sensitized BChl fluorescence showing that singlet - singlet energy transfer had occurred from the carotenoids to the BChl. The efficiency of this energy transfer was 60 - 70% for both carotenoids. These efficiencies were determined after normalization of the fluorescence excitation spectra of B850 complexes to their absorption spectra.

Laser flash photolysis was used to investigate triplet - triplet energy transfer from BChl to the carotenoids. Laser illumination (347nm) of B850 complexes without carotenoids resulted in a transient absorbance change with a decay half-time of 12.5µsec. The flash-induced difference spectrum of this absorption change allowed it to be identified as representing BChl in its triplet state. If neurosporene or spheroidene were present in the B850 complex laser flash illumination gave an absorbance change with a decay half-time of 4.3µsec. The difference spectra identified the absorbance changes as representing carotenoid triplet states. Since the laser flash excited the BChl, and carotenoids cannot be directly excited

to their triplet states, the carotenoid triplets were likely to have formed by triplet - triplet energy transfer from BChl to the carotenoids.

The quantum yields of triplet formation (ϕ_T) were calculated for BChl, neurosporene, and spheroidene in the B850 complex. For all three molecules the ϕ_T was found to range between 0.02 and 0.12 (ie. 2 - 12% efficient). There seemed to be little difference between the ranges; this suggested that ϕ_T for the carotenoids was similar to ϕ_T for bchl. It had been previously suggested that if carotenoid triplets were formed by quenching BChl triplets, then the ϕ_T for carotenoids would be determined by ϕ_TBChl, and would be almost identical to ϕ_TBChl. The ϕ_Ts obtained therefore provided more evidence to show that the carotenoid triplet states were formed by triplet - triplet energy transfer from BChl, ie. the carotenoids quenched triplet BChl.

The quenching of triplet BChl by carotenoids is thought to be the main method by which carotenoids prevent the photodestruction of the photosynthetic bacteria in the presence of light and oxygen. Triplet BChl can sensitize the formation of damaging singlet oxygen or superoxide from O_2 in its normal triplet state.

I investigated the photodestruction of the B850 BChl in the presence of bright light and found that this destruction was a photodynamic process ie. required the combined presence of light and oxygen. When neurosporene and spheroidene were present in the B850 complex this photodestruction was greatly reduced, probably because the carotenoids quenched BChl triplets before they could sensitize formation of damaging species of O_2.

CHAPTER ONE

INTRODUCTION

1.1 The Photosynthetic Bacteria : A General Introduction

There are two major groups of photosynthetic prokaryotes; the Cyanobacteria (or blue-green algae) and the anaerobic photosynthetic bacteria, the Rhodospirillales. The pattern of photosynthesis in the cyanobacteria is the same as that found in plants - the major chlorophyllous pigment is chlorophyll a, they oxidise water to molecular oxygen and they have two photosystems (Avron 1967, Stanier 1974).

In contrast the Rhodospirillales (which I shall now refer to as the photosynthetic bacteria) contain bacteriochlorophyll a (BChl), do not evolve oxygen and have only one type of reaction centre. Photosynthetic bacteria use a wide range of substrates as electron donors eg. reduced sulphur compounds, molecular hydrogen and simple organic compounds. The corresponding oxidised products are sulphate, protons, organic compounds and carbon dioxide. The physiology and ecology of the photosynthetic bacteria have been widely described eg. Van Niel (1963), Kondrat'eva (1965), Pfennig (1967, 1975, 1977, 1978). From his studies of the pattern of photosynthesis in a variety of photosynthetic bacteria, Van Niel (1931, 1941) formulated his general equation of photosynthesis which showed the essential unity of photosynthesis in plants and bacteria ie.

$$CO_2 + 2H_2A \xrightarrow[\text{chlorophyll}]{\text{light}} (CH_2O) + H_2O + 2A$$

where H_2A represents a general oxidised substrate, (CH_2O) represents stored organic matter. In plants H_2A is H_2O and 2A is O_2, while in the sulphur photosynthetic bacteria, for example, H_2A is H_2S and 2A is S_2.

The taxonomy of the Rhodospirillales is shown in Fig 1.1, the group has been classified into suborders and families by their pigment compositions and metabolic differences (eg. Trüper and Pfennig 1978).

All of the species mentioned in this thesis, except for Chromatium vinosum, are members of the Rhodospirillaceae, the purple and brown non-sulphur bacteria.

Figure 1.1 Taxonomy of the Photosynthetic Bacteria (see Trüper and Pfennig, 1978)

ORDER	RHODOSPIRILLALES bacteria containing BChl, performing anoxygenic photosynthesis			
SUBORDER	RHODOSPIRILLINEAE BChl*a* or *b* in intracytoplasmic membranes		CHLOROBIINEAE BChl*a* only in reaction centres, BChl*c*,*d*,*e* in vesicles under cytoplasmic membranes	
FAMILY	CHROMATIACEAE 'purple sulphur bacteria'	RHODOSPIRILLACEAE 'purple and brown non sulphur'	CHLOROBIACEAE 'green sulphur'	CHLOROFLEXACEAE 'filamentous, gliding, green sulphur'

1.2 The Light Capturing Apparatus of Photosynthetic Bacteria

The photosynthetic apparatus of members of the Rhodospirillales is localized on intracytoplasmic membranes (Cohen Bazire 1963, Cohen Bazire and Sistrom 1966, Pfennig 1967, Lascelles 1968, Oelze and Drews 1972) except in the case of the Chlorobiaceae or Green Sulphur Bacteria (Cohen Bazire et al 1964, Pfennig 1967).

The part of the apparatus involved in light capture consists of two types of pigment-protein complexes - the light-harvesting complexes and reaction centres. The light-harvesting complexes (also known as antenna complexes) absorb light and pass excitation energy to the reaction centres, where the electron transfer processes begin (Duysens 1952, see Zankel 1978 for a review). Electron transport is initiated by the light-induced oxidation of the reaction centre BChl. The electrons ejected from the reaction centre Bchl are fed into a cyclic electron transport system. Free energy released by cyclic electron transfer is used to drive coupled synthesis of ATP. Cyclic electron transfer also provides reducing power (as NADH). NAD is reduced to NADH by reverse electron flow from an external donor eg. succinate (see Dutton and Prince 1978). In this way the light reactions of photosynthesis generate the assimilatory power (NADH and ATP) which is required to power the reactions of carbon fixation.

Both types of pigment-protein complex have been isolated from a range of species. So far the reaction centres have been more extensively investigated. In most of the species studied the reaction centre consists of 3 hydrophobic polypeptides with apparent molecular weights of approximately 21, 24 and 28 Kilodaltons (KD) (Feher et al 1971, Okamura et al 1974; see Feher and Okamura 1978 for a review). The pigments - 4 BChl, 2 bacteriopheophytins (BPh) and one carotenoid - are attached to the two lighter polypeptides (Clayton and Clayton 1972, Okamura et al 1974, Cogdell et al 1976). Two of the BChls interact to give an absorption band at 870nm. This dimer is called P870 and is the primary electron donor (Norris et al 1971, 1973). The carotenoid is bound in a cis-configuration (Boucher et al 1977, Lutz et al 1976), possibly di-cis (Agalidis et al 1980, Lutz et al 1978). The reaction centre also contains a quinone, usually ubiquinone (Feher et al 1972, Okamura et al 1975), and an atom of non-haem iron (Feher 1971).

In contrast, less is known about the structure of the light-harvesting complexes, mainly because less research has been carried out on them. Generally, however, there seem to be two basic antenna types classified by, and usually named after, the approximate wavelength of their near infra red (NIR) absorption bands (Sistrom 1964, Aagaard and Sistrom 1972, Feick and

Drews 1978, Thornber et al 1978, Cogdell and Thornber 1979, see Fig 1.2).

Complexes in the first class have two major NIR absorption bands at 800 and 850nm. They are therefore usually called B800+850 complexes. The B denotes 'Bulk' the term used to describe light-harvesting BChl. This can also exist as B800+820 in Chromatium vinosum. The B800+850 complex is thought to exist as aggregates of a 'minimal unit' (Cogdell and Crofts 1978, Sauer and Austin 1978). In Rhodopseudomonas (Rps.) sphaeroides and Rps. palustris this unit consists of two polypeptides, each with an apparent molecular weight of 9 - 10 KD. Three BChls and one carotenoid are attached to these polypeptides (Firsow and Drews 1977, Cogdell and Crofts 1978, Sauer and Austin 1978, Broglie et al 1980). The carotenoid is bound in an all-trans form (Lutz et al 1976a). A similar unit, with the addition of a 14KD polypeptide, exists in Rps. capsulata, but the pigments are bound only to the two lighter polypeptides (Feick and Drews 1978). Two of the BChls in these complexes are exciton coupled to give the 850nm absorption band, while the third is responsible for the 800nm band (Cogdell and Crofts 1978, Sauer and Austin 1978).

The second class of light harvesting complex has a single major NIR absorption band at 890nm or between 860 and 890nm in some species eg. Rps. sphaeroides. This type of complex has yet to be fully characterised but Broglie et al (1980) describe the minimal unit from Rps. sphaeroides as two polypeptides of 8 and 12 KD plus 2BChls and 2 carotenoid molecules. Some species (eg. Rhodospirillum (Rhs.) rubrum, Rps. capsulata strain A1a$^+$) contain only the B890 type of light-harvesting complex. Rps. sphaeroides strain R26 is also commonly described as having only the B890 type complex.

In most species the B890 complex is associated, and synthesised with the reaction centre in a ratio of approximately 30:1 (Aagaard and Sistrom 1972, Schumacher and Drews 1978). The proportion of B800+850 to reaction centre varies inversely with the light intensity at which the cells are grown (Aagaard and Sistrom 1972) and also changes with the state of membrane development in cells (Niederman et al 1976). An exception to this general rule is seen in Rhs. tenue, where the B800+850 complex does not vary with light intensity (Wakim et al 1979).

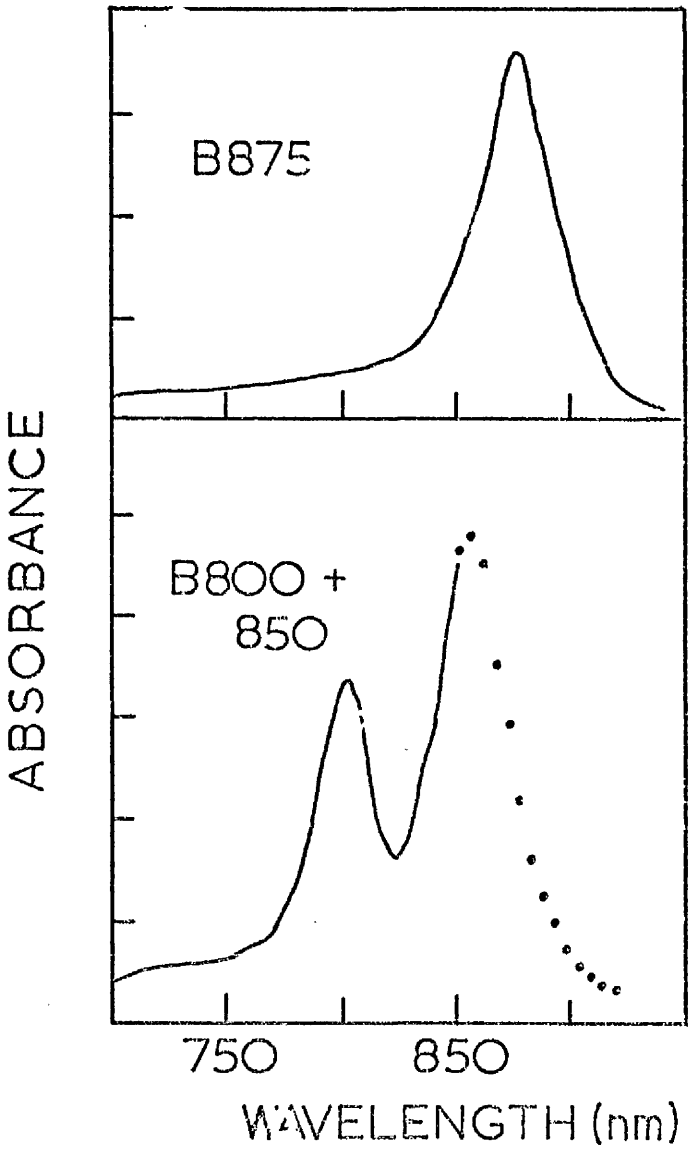
Figure 1.3 shows a possible arrangement for these pigment-protein complexes in the photosynthetic membrane (after Cogdell and Thornber 1979).

Figure 1.2

Near infra red absorption spectra of the two basic types of light-harvesting complex thought to exist in photosynthetic bacteria

(a) B800 + 850 from Rps. sphaeroides

(b) B875 from Rps. sphaeroides


B875
B800 +
850
ABSORBANCE
750
850
WAVELENGTH (nm)

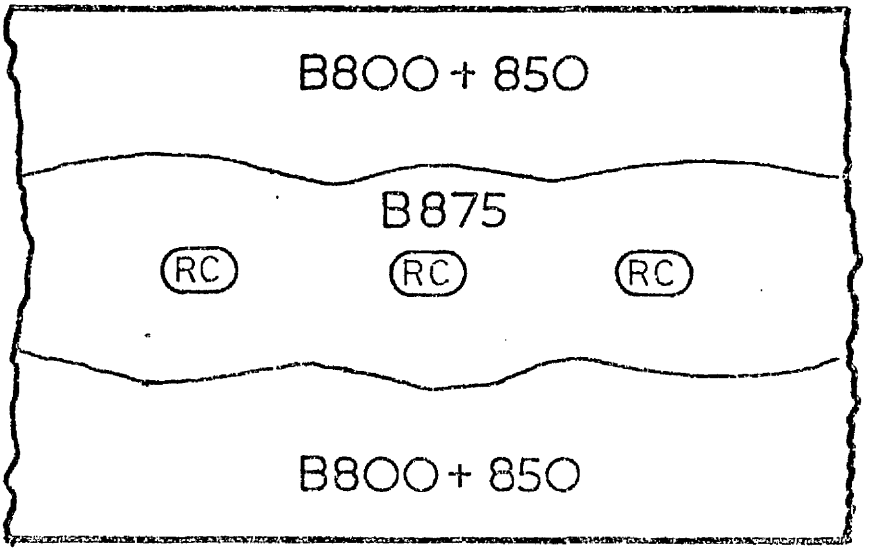


Figure 1.3

A model for a possible arrangement of the pigment-protein complexes in photosynthetic membranes (after Cogdell and Thornber 1979)

RC = reaction centre

1.3 The Function of Carotenoids

It is clear from the previous section that, except for some mutant strains, carotenoids are found in all of the pigment-protein complexes involved in the light-harvesting reactions of photosynthesis. As constituents of these pigment-protein complexes the carotenoids are thought to perform two important functions:-

1. They act as accessory light-harvesting pigments by absorbing light at wavelengths where BChl does not absorb and then passing the energy of the absorbed light to BChl. Carotenoids can therefore extend the range of light wavelengths which drive photosynthesis.
2. The more important function of the carotenoids is to protect the bacteria from the photodestructive reaction, or photodynamic effect, which occurs in the presence of oxygen and light (Griffiths et al 1955, Sistrom et al 1956, Cohen-Bazire and Stanier (1958).

In this project both of these functions were investigated using carotenoids added back to a light-harvesting complex from the carotenoidless mutant of Rps. sphaeroides, R26. Reconstitution of carotenoids into the light-harvesting complex should allow the examination of the energy transfer and quenching properties of a range of carotenoids in identical environments. I have tried to discover which factors govern the efficiency of energy transfer between the carotenoid and the BChl. Can the variations in efficiency observed among different species be due to the difference in carotenoid types in the complexes or does the efficiency depend on the orientation of a carotenoid relative to the BChl and the distance from it as determined by its binding site on the complex?

I have also tried to characterise the photodynamic reaction ie. to discover whether oxygen in its singlet state, or in its ionic form as superoxide, is responsible for the photodynamic reaction observed in carotenoidless strains of the photosynthetic bacteria.

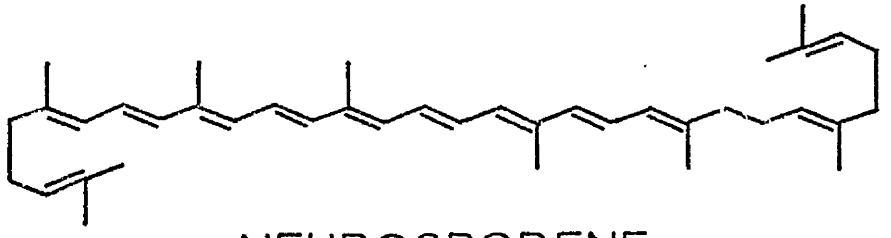
1.4 The Carotenoids

Carotenoids are long chain unsaturated hydrocarbons belonging to the terpenoids, a class of compounds which have the C_6 compound mevalonic acid as a precursor (eg. see Goodwin 1979). Their characteristic absorption spectra typically between 400 and 560nm in organic solvents (see Fig 1.4) enable them to act as accessory light-harvesting pigments in photosynthetic organisms. In the photosynthetic bacteria the carotenoids are usually C_{40} molecules, ie. tetraterpenoids, consisting of eight isoprene units. A typical carotenoid structure is shown in Fig 1.4. In all species examined so

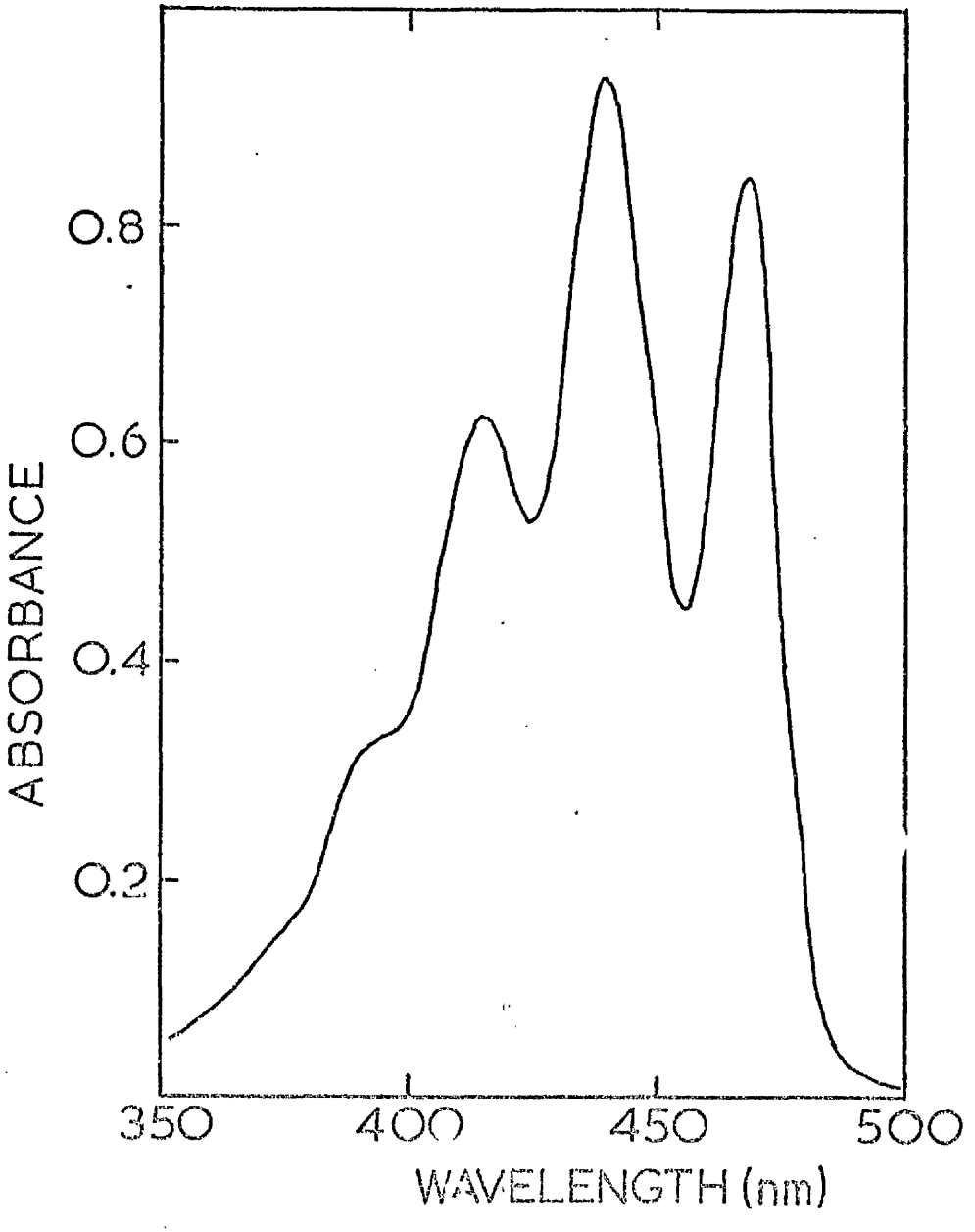
Figure 1.4

Structure and absorption spectrum of neurosporene

The absorption spectrum represents neurosporene in petroleum spirit.



NEUROSPORENE



far the first C_{40} molecule in the biosynthetic pathway is phytoene (Goodwin 1971, Schmidt 1978). Schmidt, in the most recent review of carotenoid biosynthesis in the photosynthetic bacteria, suggests that there are four main pathways of carotenoid biosynthesis (see Fig 1.5). All four pathways have the following initial steps in common:

phytoene ——→ phytofluene ——→ neurosporene

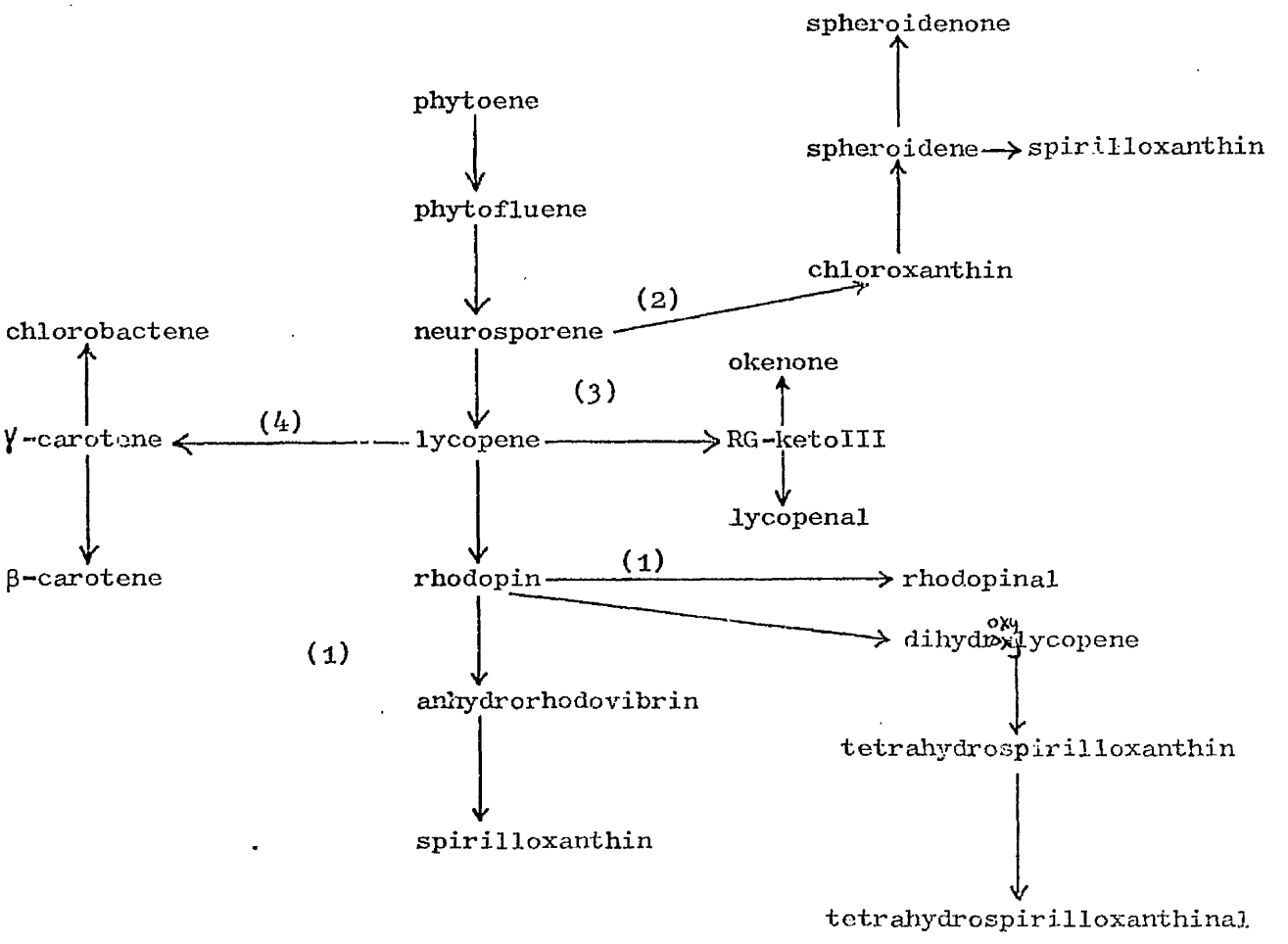
As Fig 1.5 shows, carotenoids of the rhodopin spirilloxanthin series are widespread, being found in a number of the species in the Rhodospirillaceae and Chromatiaceae. Other carotenoids are found only in Rps. sphaeroides, Rps. gelatinosa and Rps. capsulata.

1.5 Energy Transfer from Carotenoids to Bacteriochlorophyll

Duysens (1952, cited Goedheer 1959) recorded the action spectra for BChl fluorescence using whole cells from Rhs. rubrum, Rhs. molischianum and Chromatium vinosum. From these spectra he calculated the efficiency of energy transfer from the carotenoids to BChl to be 35-40% for each species. Goedheer (1959), using the method of Duysens, measured the efficiency of energy transfer in Rhs. rubrum and Rps. sphaeroides using both whole cells and chromatophores. The efficiency of carotenoid to BChl energy transfer was calculated from the ratio of a carotenoid peak height in the fluorescence action spectrum to the corresponding peak in the absorption spectrum after the two spectra were normalised at the 590nm BChl absorption peak.

The efficiency of energy transfer was 35% in Rhs. rubrum and 90% for Rps. sphaeroides. The efficiencies were the same in whole cells and chromatophores. The low efficiency of energy transfer in Rhs. rubrum agreed with previous efficiencies estimated from action spectra of photosynthetic carbon dioxide fixation and phototaxis (Thomas 1950, Manten 1948, Thomas and Goedheer 1953). Goedheer therefore concluded that the efficiency of the use of light absorbed by carotenoids could be found by measuring the fluorescence intensity as a function of the wavelength of incident light.

Since the carotenoids have been shown to be integral components of the pigment-protein complexes in photosynthetic bacteria there are two possible explanations for the difference in efficiency of carotenoid to BChl energy transfer between Rps. sphaeroides and Rhs. rubrum. The efficiency may be determined by the carotenoid present in a pigment-protein complex since different species contain different carotenoids. Variations in carotenoid type could cause changes in the overlap integral of the carotenoid and the BChl or in the lifetime of the excited singlet state of the carotenoid; both



Pathway (1) - many species of Rhodospirillaceae and Chromatiaceae

" (2) - Rps. sphaeroides, Rps. gelatinosa, Rps. capsulata

" (3) - 5 species of Chromatiaceae, 1 of Rhodospirillaceae

" (4) - Chlorobiaceae and Chloroflexaceae

Figure 1.5

Postulated pathways of carotenoid biosynthesis in the photosynthetic bacteria (after Schmidt 1978)

factors would affect the efficiency of energy transfer (Knox 1977). Secondly, the efficiency may depend on the distance and the relative orientation between the carotenoid and the BChl as determined by the carotenoid binding site on the pigment-protein complex (Knox 1977). This is probably an oversimplification since the efficiency of energy transfer will undoubtedly not depend entirely on one of these two factors. However, in the two published studies involving the effect of different carotenoids in energy transfer there is evidence that, in each case, one of these two factors is predominant (Boucher et al 1977, Cogdell et al 1981). Boucher and his colleagues reconstituted carotenoids into reaction centres isolated from the carotenoidless mutant of Rhs. rubrum, G9. The reaction centres, in detergent, were incubated with purified carotenoids deposited on the walls of test tubes. The carotenoids were bound in a cis conformation in a ratio of one per reaction centre as in wild type reaction centres (Clayton and Clayton 1972, Cogdell et al 1976). Spirilloxanthin, the major carotenoid of wild type Rhs. rubrum (Jensen et al 1958) and the carotenoid bound to wild-type reaction centres (van der Rest and Gingras 1974), transferred energy to the BChl with only 20% efficiency. Spheroidene, the major carotenoid of Rps. sphaeroides (Goodwin et al 1955), transferred energy to the BChl with an efficiency of 90%. Since it is assumed that the carotenoids were bound to the same site on the reaction centre it seems that, in this instance, the efficiency of carotenoid→BChl energy transfer depends on the carotenoid present and is therefore not a property of its binding site.

However, investigations into energy transfer in the B800+850 light-harvesting complex by Cogdell et al (1981) support the alternative possibility for determining the efficiency of energy transfer. They isolated B800+850 complexes from several strains of Rps. sphaeroides - 2.4.1 wild type, Ga, G1C, and 2.4.1 bubbled with air for 24 hours before harvesting. Strains Ga and G1C differ from Rps. sphaeroides wild type only in their carotenoid composition. Aeration of the wild type strain results in spheroidenone replacing spheroidene as the major carotenoid. In this way a number of complexes were obtained which varied only in their carotenoid content. High values (75-100%) were obtained for the efficiency of carotenoid ⟶ BChl energy transfer irrespective of carotenoid type. These values suggest that, for this pigment-protein complex the efficiency of energy transfer is determined by the binding site of the carotenoid. not by the structure of the carotenoid.

The transfer of energy from carotenoid to BChl (and from BChl to BChl) occurs as singlet-singlet transfer (Vredenberg and Duysens 1963, Borisov and Godik 1973, Knox 1977). This can be represented by the following scheme.

$$\text{Car} + \text{BChl} \xrightarrow{\text{light}} \text{Car}^* + \text{BChl} \longrightarrow \text{Car} + \text{BChl}^*$$

Car* and BChl* represent the excited singlet states of a carotenoid molecule and a BChl molecule respectively. The wavelength of the absorbed light is such that it could be absorbed only by the carotenoid. A brief description of the singlet and triplet states of molecules is given in Appendix 1.

The mechanism by which carotenoids transfer energy to the BChls is not completely understood (Razi-Naqvi 1980). Razi-Naqvi suggests that Dexter's 'electron transfer' mechanism (Dexter 1953, Forster 1959) is more suitable than the Förster dipole-dipole mechanism (Lamola 1969) proposed by Thrash et al (1979). The electron transfer mechanism requires an even closer arrangement between the two molecules involved than the 5-10nm distance over which the Förster mechanism operates (Dexter 1953, Förster 1959). The two mechanisms postulated are outlined in Appendix 2.

Recent research suggests that carotenoids and BChls may indeed be very close to each other. Dallinger et al (1981), using resonance-enhanced Raman spectroscopy, concluded that the lifetime of the excited singlet state of β-carotene was not greater than 1 picosecond (psec). Assuming that the lifetime of excited singlet state of all carotenoids is of this magnitude then BChls would require to be very close to the carotenoid for energy transfer to complete successfully with the rapid internal decay of the carotenoid excited state. The necessity of a very close interaction between the carotenoid and BChl was further demonstrated by Bensasson et al (1981) using two synthetic carotenoid porphyrins (Dirks et al 1980, Moore et al 1980). In one the carotenoid part of the molecule projected away from the porphyrin resulting in negligible energy transfer from the carotenoid to the porphyrin. In the other molecule the carotenoid conjugated double bond system lay directly over the porphyrin ring at a distance of approximately 4Å. In this molecule carotenoid to porphyrin energy transfer occurred with 25% efficiency. This suggests that carotenoids and BChl must be only a few angstroms apart for efficient energy transfer to occur.

1.6 The Photodynamic Effect

The term 'photodynamic effect' refers to the damaging action on organisms which occurs in the presence of light, oxygen and photosensitizing molecules. The presence of these three factors can result in the photosensitized oxidation of many cell constituents (Spikes and Straight 1967, Krinsky 1978, 1979). This oxidation is thought to be caused mainly by singlet oxygen ($^1O_2, \Delta^1$ g state) formed from its normal ground triplet state by energy transfer from a triplet sensitizer (Foote 1968, Gollnick 1968). Photochemical reactions are initiated by light energy absorbed by a sensitizer molecule which then forms the first electronically excited state of the molecule - the excited singlet state. The energy of this short lived state (generally around 10^{-11} sec) can be dissipated in a number of ways - radiationless internal conversion to heat, re-emission as light (fluorescence), or intersystem crossing to the triplet state (see Fig 1.6). If this intersystem crossing occurs the molecule can become a triplet sensitizer. The lifetimes of triplet states (usually 10^{-7} - 10^{-6} sec) are long enough for them to collide with other molecules and initiate photochemical reactions. BChl has the opportunity to become a triplet sensitizer when the light intensity for photosynthesis is saturating. Under such conditions the excited singlet states of BChl in the light harvesting complexes are not immediately 'trapped' by reaction centres and are therefore available for intersystem crossing to the triplet state.

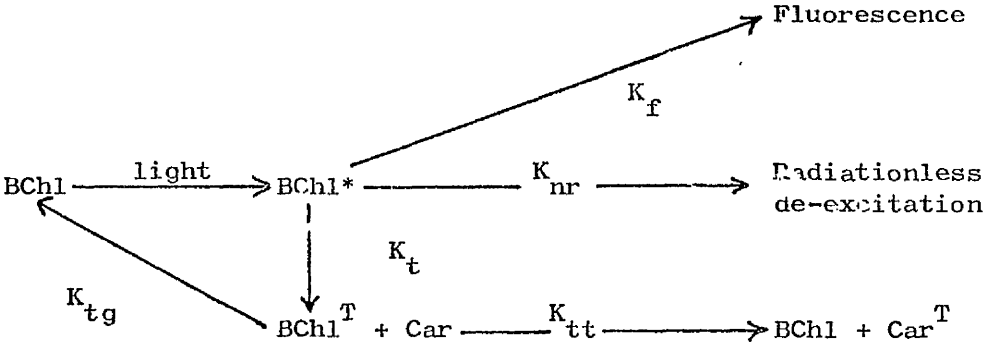
There are two general types of reaction available to the triplet sensitizer, classifiable as Types I and II (Gollnick and Schenck 1967, Foote 1968, 1976).

Type I - the sensitizer triplet reacts directly with another molecule and the resulting radicals can react with other molecules to cause biological damage.

Type II - usually the triplet sensitizer reacts with ground state (triplet) oxygen ($^3O_2$) to produce the excited singlet state ($^1O_2, \Delta^1$ g) which can react with other molecules (Corey and Taylor 1964, Foote and Wexler 1964, Foote 1968, Wayne 1969, Kearns 1971).

Singlet oxygen combines rapidly with dienes eg. in fatty acids, amino acids, purines, and, most relevant to this study, chlorophylls. $^1O_2$ can therefore inflict considerable damage, and possibly death, on living organisms. It is also possible for an electron to be transferred from the sensitizer to the oxygen, causing formation of a superoxide ion ($O_2^{\cdot -}$), leaving the sensitizer as an oxidised form (Kasche and Lindqvist 1965, Kepka and Grossweiner 1972, Balny and Douzou 1974). This reaction has been

Figure 1.6 Possible Fates of BChl in the Excited Singlet State (BChl*)



K_f = rate constant for decay as fluorescence
K_{nr} = rate constant for non-radiative decay
K_t = rate constant for intersystem crossover to triplet state
K_{tt} = rate constant for triplet-triplet transfer to carotenoid
K_{tg} = rate constant for decay from triplet to ground singlet state

Yield of $BChl^T$, $\phi_{BChl^T} = \frac{K_t}{K_f+K_{nr}+K_t}$

$$\phi_{Car^T} = \frac{K_{tt}}{K_{tg}+K_{tt}} \cdot \phi_{BChl^T}$$

described as occurring on less than 1% of the reactions between O_2 and most sensitizer triplets (Kasche and Lindqvist 1965). However, there is some evidence for greater involvement in the photoinduced bleaching of reaction centre BChl carried out by Boucher et al (1977).

1.7 Evidence for BChl as a Sensitizer of the Photodynamic Reaction in the Photosynthetic Bacteria

Griffiths et al (1955) showed that a 'blue-green' strain of Rps. sphaeroides, lacking coloured carotenoids, was killed in the combined presence of light and air. Under anaerobic conditions in the light or aerobic conditions in darkness the mutant grew normally, indicating that the destructive reaction was photodynamic. The photodestruction occurred even when only NIR light (wavelengths 800nm) was used in the presence of air. Since only BChl can absorb beyond 800nm, Griffiths et al concluded that the BChl in the cells sensitized the destructive reaction.

Sistrom et al (1956) grew the 'blue-green' mutant of Rps. sphaeroides for many generations in air and total darkness, conditions which inhibit BChl synthesis. The bleached cells of this mutant were then exposed to high light intensities in air without a change in the growth rate. This suggested that the BChl was necessary for the destruction of the cells.

Monger et al (1976) detected the triplet state of BChl ($BChl^T$) in chromatophores of the carotenoidless mutants Rps. sphaeroides, R26 and Rhs. rubrum, G9. They observed that, in the presence of O_2, the decay of the BChl triplet state was accelerated by about an order of magnitude in both species.

There is still relatively little known about the photodynamic reaction in the photosynthetic bacteria: does it proceed mainly by $^1O_2$ or also via the involvement of $O_2^{\cdot -}$?

For sensitized formation of $^1O_2$ to be possible, the triplet sensitizer (ie. BChl) must have a triplet energy level (E_T) greater than the energy difference (E_T) between ground state (triplet) O_2 and the excited $^1O_2$. It has been known for some time that O_2 quenches the triplet states of chlorophylls in organic solution (Livingston et al 1954). However, as Connolly et al (1973) pointed out, the quenching of a triplet state by O_2 does not in itself prove that energy transfer to the O_2 has occurred. Paramagnetic gases such as O_2 can quench triplet states by an enhanced intersystem crossing process in which the O_2 remains in its ground triplet state (Porter and Wright 1959, Mathis 1969, 1970).

The E_T for O_2 is approximately 94 kJ $mole^{-1}$ or 1eV (Foote and Denny 1968, Kearns 1971) and the E_Ts for Chla and Chlb are approximately 130 and

135 kJ mole^{-1} respectively (Becker and Kasha 1955, Parker and Joyce 1967, Seely 1966). The triplet states of Chl*a* and Chl*b* can therefore sensitize the formation of $^1O_2$ from its normal triplet state.

Until recently (Boucher et al 1977) it had not been shown that BChl^T could sensitize the formation of $^1O_2$. As described above, the observed quenching of BChl^T by O_2 (Monger et al 1976) does not necessarily imply that energy transfer has occurred between the two. Unfortunately there are many conflicting values for the E_T of BChl. Theoretical values of 66 and 85 kJ mole^{-1} have been predicted (Weiss 1972, Song cited Weiss 1972). Seely (1966), using phosphorescence studies on Chl*a* and Chl*b* (Becker and Singh 1960) estimated the E_T for BChl to be 108 kJ mol^{-1}. Connolly et al (1973) derived values for the E_T of BChl experimentally by comparing the rates of quenching of BChl*a*^{T} and Chl*a*^{T} by β-carotene. Their value of 65 kJ mole^{-1} is only half that for Chl*a* and is 30 kJ mole^{-1} lower than the E_T for O_2, implying that BChl^T could not sensitize the formation of $^1O_2$. Connolly et al therefore suggested that, in the photosynthetic bacteria, the photodynamic reaction does not proceed via $^1O_2$ but by electron transfer from the BChl to the O_2 forming the $O_2^{\cdot -}$ ion. The experiments of Boucher et al (1977) showed that $O_2^{\cdot -}$ was indeed involved in the reaction but also demonstrated the presence of $^1O_2$. Using 1,3-diphenylisobenzofuran as a chemical reporter for $^1O_2$, sodium azide as quencher of $^1O_2$, and the enzyme superoxide dismutase, Boucher et al showed that both $^1O_2$ and $O_2^{\cdot -}$ were responsible for the photodynamic bleaching of BChl. The amount of $O_2^{\cdot -}$ produced was roughly constant above a low light intensity of 2 x 10^6 ergs cm^{-2} sec^{-1}. $^1O_2$ was generated mostly at higher light intensities (8 x 10^6 ergs cm^{-2} sec^{-1}). It was concluded that the $^1O_2$ was generated by triplet-triplet energy transfer from the BChl^T to $^3O_2$. Recently obtained values for the E_T of BChl are high enough to permit such energy transfer. Krasnovsky (1979) placed the E_T of BChl slightly above the E_T for $^1O_2$ after studies on the photoluminescence of $^1O_2$. Lebedev and Krasnovsky (1978) calculated the E_T to lie between 83 and 95 kJ mole^{-1}. However the E_T for BChl^T is still a contentious issue.

1.8 Photoprotection by Carotenoids

The possibility that carotenoids protected cells from chlorophyll-sensitized photodestruction was first suggested by Griffiths et al (1955) after they had shown that a carotenoidless mutant of Rps. sphaeroides was killed in the presence of light and air.

Further evidence was provided by Cohen-Bazire and Stanier (1958) who grew cells of Rhs. rubrum in the dark, in the presence of diphenylamine. This chemical had previously been shown to inhibit the synthesis of coloured carotenoids in this species (Goodwin and Osman 1953) causing the accummulation of colourless carotenoids, mainly phytoene. Cells which had grown without forming carotenoids showed photosensitivity when exposed to light and air. If coloured carotenoids were restored, by removal of diphenylamine from the medium, the photosensitivity was greatly reduced. The correlation of increasing photosensitivity with a decrease in coloured carotenoid content provided strong evidence for the photoprotective function of the carotenoids in the photosynthetic bacteria.

As I mentioned in a previous section, Boucher et al (1977) bleached reaction centres isolated from the carotenoidless mutant of Rhs. rubrum, G9 and also reconstituted carotenoids into reaction centres. After reconstitution the carotenoids gave a degree of protection against this irreversible light-induced BChl bleaching. In some cases this protection was the same as that observed in reaction centres isolated from the wild type Rhs. rubrum.

Cogdell et al (1976) had suggested that, in reaction centres, the photoprotective role of carotenoids was likely to be more important than their accessory light harvesting role. They showed that a specific carotenoid is bound to reaction centres in Rps. sphaeroides strains 2.4.1 and Ga, in Ga that this carotenoid differs from the predominant carotenoid in chromatophores from these strains. They calculated that reaction centre carotenoids accounted for only 1-2% of the total carotenoids present in chromatophores and would therefore play only a very small part in transferring energy to the reaction centre BChl. It has been previously observed (Cogdell et al 1975) that reaction centre carotenoids quenched the triplet states of reaction centre BChl, Cogdell et al (1976) proposed that the main function of carotenoids in reaction centres is to prevent formation of damaging singlet oxygen by quenching its sensitizer, BChl in its triplet state.

There are two simple mechanisms by which carotenoids could protect the photosynthetic bacteria. One is to quench triplet BChl and thus prevent the formation of $^1O_2$ or $O_2^{\cdot -}$ ie. energy transfer from $BChl^T$ to carotenoid, the other is to quench the oxygen species directly before they can cause damage in the cell.

1.9 Energy Transfer from Bacteriochlorophyll ⟶ Carotenoid

The *in vitro* quenching of triplet chlorophyll by carotenoids was first described for organic solutions by Fujimori and Livingston (1957) and subsequently by Claes and Nakayama (1959) and Claes (1960). Chessin et al (1966) showed that the quenching of triplet BChl resulted in the formation of carotenoid triplet states. This triplet-triplet energy transfer can therefore be represented as follows:

$$Chl + Car \xrightarrow{light} Chl^* + Car \longrightarrow Chl^T + Car \longrightarrow Chl + Car^T$$

The triplet-triplet energy transfer from chlorophyll to carotenoids was first shown in chloroplasts and algae by Mathis (1969, 1970) and Wolff and Witt (1969, 1971).

In the photosynthetic bacteria quenching of triplet BChl to give triplet carotenoids has been seen in chromatophores (Kung and Devault 1976, Monger et al 1976, Renger and Wolff 1977), in isolated reaction centres (Cogdell et al 1975, 1976) and light harvesting complexes (Cogdell et al 1981). Chromatophores of carotenoid containing strains of Rps. *sphaeroides* show a characteristic light-dark difference spectrum when they are excited by short, saturating light pulses (wavelength 694nm) from a laser (Kung and Devault 1976, Monger et al 1976, Renger and Wolff, 1977). The spectrum shows bleaching at three wavelengths, corresponding to the carotenoid absorption peaks, and a positive peak at 520-530nm (see Fig 1.7). This metastable state has a half-life of 5-8μsec and is identified as a carotenoid triplet state on the bleaching of carotenoid absorption bands and on the presence of a strong peak at 535nm. The presence of a major absorption band in this region of the spectrum is characteristic of the triplet states of carotenoids (Chessin et al 1966, Truscott et al 1973, Mathis and Kleo 1973). Under the same conditions chromatophores from the carotenoidless strain of Rps. *sphaeroides*, R26, exhibit a metastable state with a half-life of approximately 70μsec and giving the light-dark difference spectrum shown in Fig 1.7. The bleaching of the absorption bands below 400nm and at 590nm, with the formation of bands between 400 and 580nm are characteristic of the difference spectra of chlorophyll (in this case BChl) in its triplet state (Connolly et al 1973, Linschitz and Sarkanen, 1958).

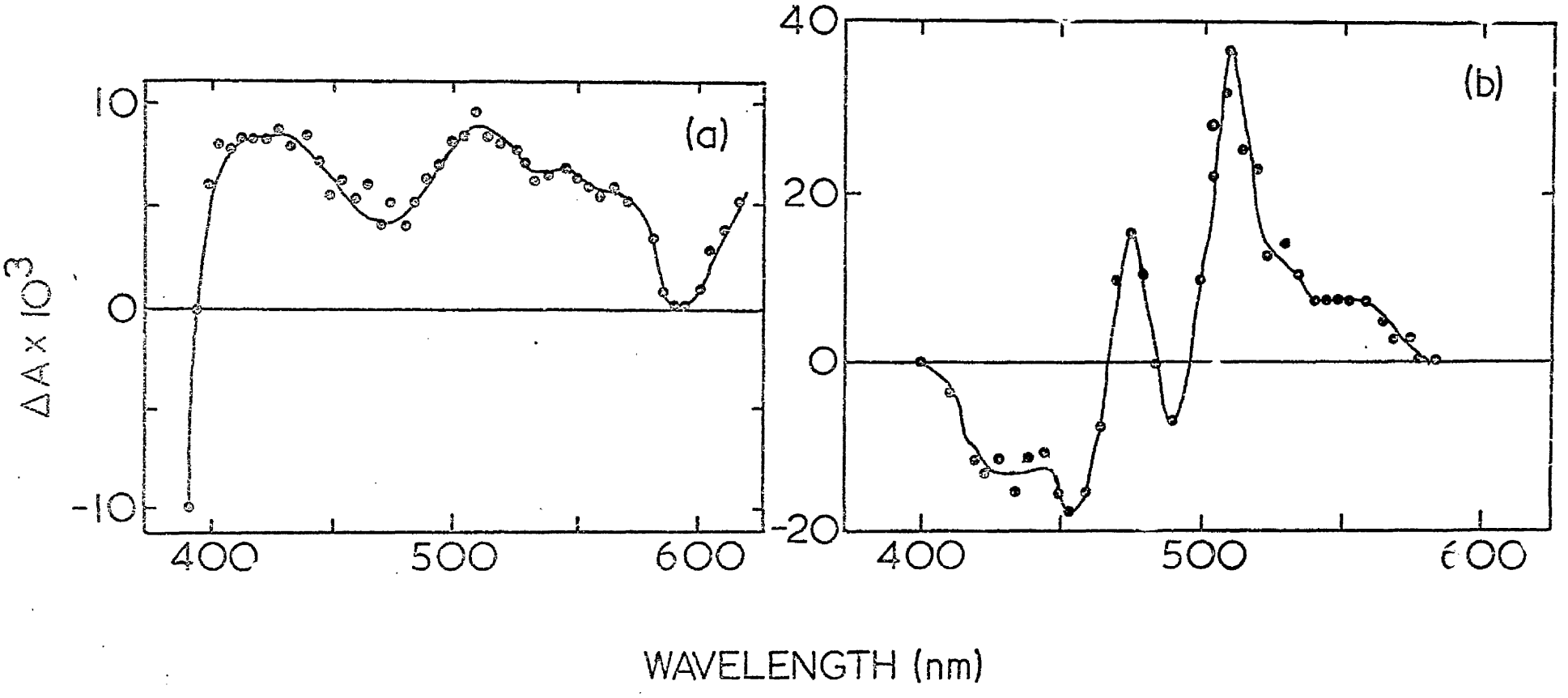
Since the actinic light pulse is absorbed only by the BChl, then the carotenoid triplet state must be formed by triplet-triplet energy transfer from the $BChl^T$. As I mentioned before, the BChl in light harvesting complexes may have the opportunity to cross to the triplet state if the

Figure 1.7

Flash-induced difference spectra for bacteriochlorophyll and carotenoids in their triplet states.

(a) Bacteriochlorophyll triplet states from chromatophores of Rps. sphaeroides, R26.

(b) Carotenoid triplet states in chromatophores of Rps. sphaeroides G.I.C.


10
0
-10
(a)
40
20
0
-20
(b)
400
500
600
ΔA x 10^3
WAVELENGTH (nm)

singlet state is not trapped by reaction centres. Renger and Wolff (1977) confirmed this when they observed an increase in the amount of carotenoid triplets formed after the 'shutting' of the reaction centres in chromatophores by the addition of the reducing agent sodium dithionite.

The half-time for the formation of the carotenoid triplet state is approximately 20 nsec (Monger et al 1976). This is 1000 times faster than the quenching of triplet BChl by O_2 (Monger et al 1976) and could explain how carotenoids prevent the photodynamic reaction - carotenoids may quench triplet chlorophylls before they can react with oxygen and sensitize the formation of the damaging species of oxygen.

This hypothesis is supported by the recent work of Beusasson et al (1981). The bleaching of diphenyl isobenzofuran was used to detect $^1O_2$ sensitized by the triplet state of the porphyrin in the carotenoporphyrins described by Dirks et al (1980) and Moore et al (1980). In the carotenoporphyrin with the carotenoid extending away from the porphyrin, the triplet state of the porphyrin was quenched very slowly. The half rise time of the carotenoid triplet was approximately 1.5μsec. Excitation of this form of the molecule resulted in extensive $^1O_2$ production. In contrast, when the carotenoid conjugated double bond system lay over the porphyrin, at a distance of 4Å, the carotenoid triplet was observed immediately after excitation of the porphyrin. No porphyrin triplet state was observed at the time resolution limit of the apparatus (30 nsec), the carotenoid quenched the triplet state very rapidly. Presumably as a result of this, no $^1O_2$ was detected when this molecule was excited.

1.10 Quenching of $^1O_2$ by Carotenoids

Quenching of $^1O_2$ by carotenoids *in vitro* was first shown by Foote and Denny (1968) for β-carotene. The sensitizer for $^1O_2$ formation was methylene blue. Further studies have shown that the ability of a carotenoid to quench $^1O_2$ seems to depend on the number of conjugated double bonds in the carotenoid.

1.11 Photoprotective Ability vs Number of Conjugated Double Bonds in a Carotenoid

There is a lot of evidence to suggest that the number of conjugated double bonds is crucial in determining the ability of a carotenoid to provide photoprotection.

Foote et al (1970) investigated the relationship between the number of conjugated double bonds in carotenoids (some of them synthetic) and their ability to quench $^1O_2$ *in vitro*. Nine or more conjugated double bonds effectively quenched $^1O_2$, seven or less conjugated double bonds quench $^1O_2$ very poorly.

The importance of nine conjugated double bonds in quenching $^1O_2$ was again demonstrated by Mathews-Roth and Krinsky (1970). They investigated the exogenously sensitized photodestruction of a non-photosynthetic bacterium, *Sarcina lutea*. The wild type strain, whose major carotenoids contain nine conjugated double bonds, was completely protected from photo-oxidation but a mutant strain, whose major carotenoids contain eight conjugated double bonds, was destroyed at the same rate as another mutant with no carotenoids. Mathews-Roth et al (1974) repeated the experiments of Foote et al (1970) using a range of naturally occurring carotenoids to quench $^1O_2$. The ability of these carotenoids to quench $^1O_2$ was compared with that of β-carotene (eleven conjugated double bonds). The major carotenoid (nine conjugated double bonds) from *S. lutea* was as effective as β-carotene, that of the mutant strain of *S. lutea* (eight conjugated double bonds) was two to three times less effective. Phytofluene (five conjugated double bonds) was 100 times less effective, and phytoene (three conjugated double bonds) 1000 times less effective.

These results agree well with those of Claes and Nakayama (1959) and Claes (1960). A range of natural carotenoids with the number of conjugated double bonds ranging from 5-11 was used to try and protect chlorophyll *a* against photodynamic bleaching *in vitro*. Carotenoids with nine or more conjugated double bonds gave relative protection of greater than 60%, those with seven or less gave under 10% protection. Again the amount of photo-protection was observed to fall sharply below nine conjugated double bonds.

Thomas et al (1981) further demonstrated that a carotenoid requires at least nine conjugated double bonds to give protection against $^1O_2$. They investigated the effects of $^1O_2$ (generated by photosensitizing dyes) on conidia from wild-type fungus *Neurospora crassa* (containing neurosporene, nine conjugated double bonds), and from two mutant strains, one containing mostly zeta-carotene with seven conjugated double bonds, the other phytoene, three conjugated double bonds. Conidia from the mutant strains were more

sensitive to photodynamic inactivation than those from the wild-type, zeta-carotene gave no more protection than phytoene.

The factor which determines the ability of a carotenoid to quench $^1O_2$ seems to be the triplet energy (E_T) of the carotenoid. This must be less than the difference in energy (also known as E_T) between the singlet and triplet states of O_2 (Bellus 1979, Krinsky 1979).

The E_T for O_2 is 94 kJ mole^{-1} (Foote and Denny 1968), the E_Ts of carotenoids vary inversely with the number of conjugated double bonds (Salem 1966, Mathis and Kleo, 1973, Bensasson et al 1976). From values calculated for the triplet energy levels of different carotenoids (Mathis and Kleo 1973, Bensasson et al 1976, see Fig 1.8), it appears that only those carotenoids with 9 or more conjugated double bonds have triplet energy levels of approximately 94 kJ mole^{-1} or less which would allow efficient energy transfer from $^1O_2$ to the carotenoid. This agrees very well with the experimental observations on carotenoid quenching of $^1O_2$.

Since most of the carotenoids in the photosynthetic bacteria have more than nine conjugated double bonds (Schmidt 1978) it is theoretically possible for protection against the photodynamic reaction to occur by quenching of $^1O_2$. However, the most likely method of protection against photodestruction would involve the quenching of $BChl^T$ before it could sensitize formation of $^1O_2$ or $O_2^{\cdot -}$; this quenching has been described in a previous section. We would therefore expect quenching of triplet Chl only if the E_T of the carotenoid (as determined by the number of conjugated double bonds) was below that of the Chl molecule.

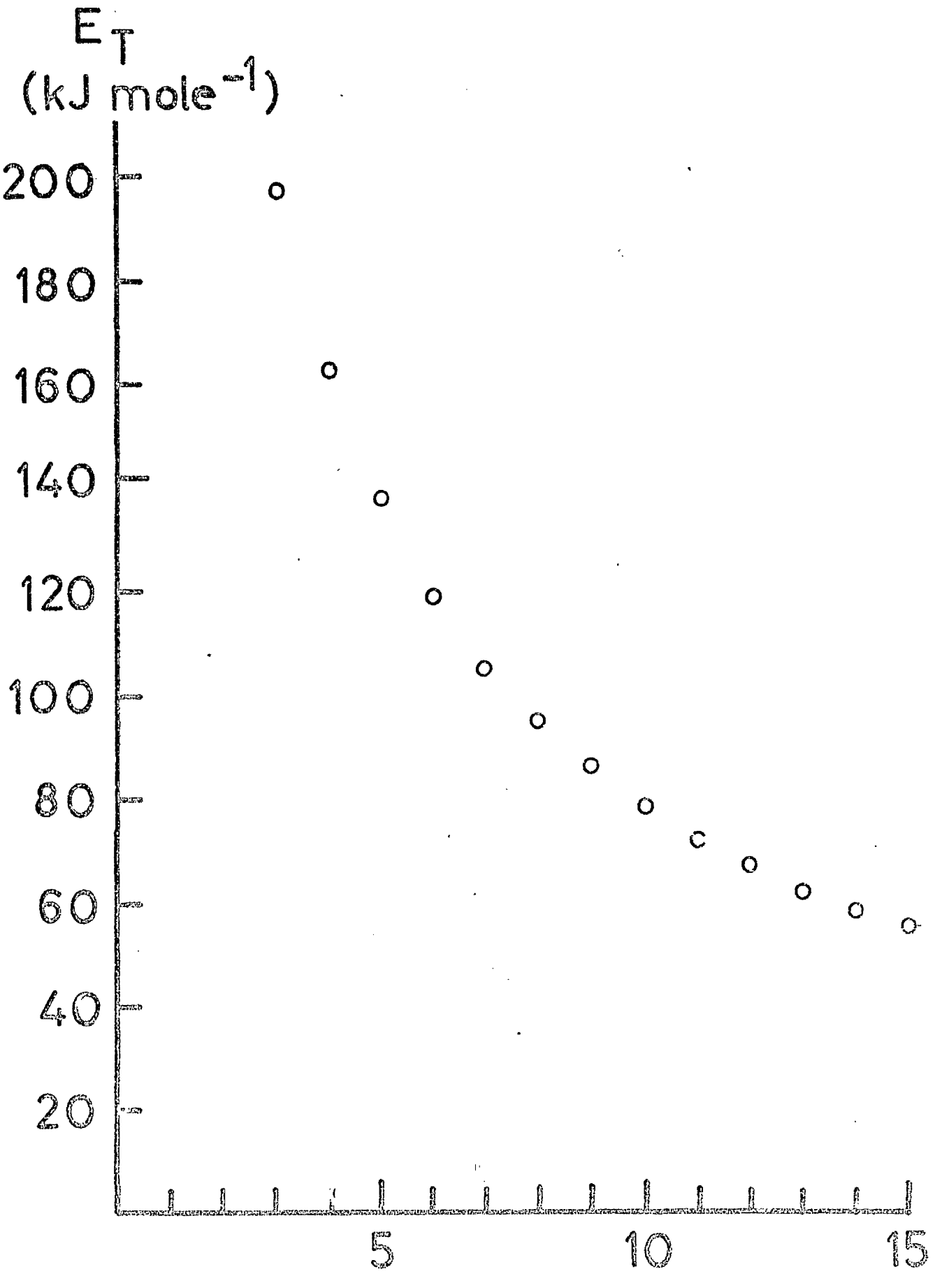
The E_T for Chl*a* is approximately 130 kJ mole^{-1} (Seely 1978). From the estimated E_Ts of carotenoids (Fig 1.8) it can be calculated that triplet Chl*a* will be quenched by (ie. will transfer energy to) carotenoids containing seven or more conjugated double bonds. This agrees with results obtained by Claes (1961) for the ability of carotenoids to protect Chl*a* (*in vitro*) from the effects of anaerobic photobleaching, a process which is assumed to occur via the Chl*a* triplet state. Carotenoids with seven or more conjugated double bonds gave good protection but carotenoids with six or less were poor protectors, presumably because their E_Ts were too high to allow triplet Chl*a* to pass energy to them efficiently.

The only *in vivo* evidence of carotenoids with few double bonds being poor photoprotectors in photosynthetic organisms comes from the work of Stanier and Cohen-Bazire (1957). Cells of *Rhs. rubrum*, grown in the dark in the presence of diphenylamine, accumulated colourless carotenoids (mainly

Figure 1.8

Estimated triplet energies for carotenoids.

These values are taken from Bensasson et al (1976) who estimated the triplet energies (E_T) by extrapolating the data for $1/E_T$ calculated by Evans (1960, 1961).


E_T
(kJ mole^{-1})
200
180
160
140
120
100
80
60
40
20
5
10
15
number of conj. double bonds

phytoene) instead of the normal coloured carotenoids. These cells showed photosensitivity in the presence of light and air, it seems likely that this was due to the inability of phytoene, E_T 197 kJ mole^{-1}, to quench $BChl^T$ (assuming an E_T of approximately 95-100 kJ mole^{-1}.)

CHAPTER TWO

MATERIALS AND METHODS

Cell Culture

The following bacteria were grown in a well defined nutrient medium using succinate as the sole carbon source (Bose 1963, see Appendix 3 for the composition of all media used).

Rhodopseudomonas sphaeroides strain ATH 2.4.1. (wild-type)

Rhodopseudomonas sphaeroides strain G1C (a green mutant)

Rhodopseudomonas sphaeroides strain GA (a green mutant)

Rhodopseudomonas sphaeroides strain R26 (a carotenoidless mutant)

Rhodopseudomonas capsulata strain Z1 (a vigorous arsenate resistant strain)

Rhodopseudomonas viridis

Rhodospirillum rubrum strain S1 (wild type)

Rhodospirillum rubrum strain G9 (a carotenoidless mutant)

Another species, Rhodopseudomonas acidophila strain 7750, was grown in a different medium (Pfennig 1969 see Appendix 3) but again using succinate as the carbon source.

Stock cultures of bacteria were kept as stabs in agar in McCartney bottles. When liquid cultures of cells were required the bottles were topped up with succinate medium and placed in the illuminated growth room. Once the cells had grown up thickly into the medium they were transferred into 300ml of medium in flat sided bottles. These were the standard bottles used to maintain liquid cultures but 10 litre bottles were used for larger amounts. To prevent contamination the tops of bottles were flamed by a bunsen before and after transfer of cells. Cultures were periodically plated out on agar and grown under nitrogen to check on their purity. Isolated single colonies of bacteria from the plates were used to make the stabs in agar. All transferring was carried out in the sterile environment of a laminar flow chamber.

The liquid cultures of bacteria were kept in a growth room at a temperature between 25°and 28°C for 2-3 days. Light was provided by banks of 3 x 100w bulbs placed 40cm from the bottles. These gave a light intensity of approximately 35Wm^{-2} at the surface of the bottles.

To prevent the photodestruction of carotenoidless strains, which occurs in the presence of light and oxygen, they were grown in the dark for 24 hours after transferring prior to exposure to the light. This allowed the cells to respire away most of the oxygen in the medium.

The cells were harvested in an MSE 6L 'Coolspin' centrifuge at 2400 x g for 1 hour 40 minutes. They were then washed by resuspending in 20mM Mes.HCl pH 6.5, 100mM KCl and spinning at 17,000 x g for 15 minutes in an MSE 18 centrifuge. The pelleted cells were resuspended in the same buffer and if not used immediately were stored at -20°C.

As I described in Chapter One, the pigment-protein complexes of photosynthetic bacteria are localized on intracytoplasmic membranes. These membranes are invaginated. Preparations of photosynthetic membranes can be obtained by breaking the cells. When this happens the invaginations form sealed, round, vesicles called chromatophores which can be isolated by differential centrifugation. Chromatophores are commonly used as raw material for the purification of pigment-protein complexes of photosynthetic bacteria.

Chromatophore Preparation (see Fig 2.1)

Harvested bacterial cells were homogenised and small amounts of DNA-ase and magnesium chloride were added. The cells were then broken by passing them through a cooled Aminco French Pressure Cell at a pressure of approximately 10 tons psi. Cells of carotenoidless strains needed to be pressed twice to ensure a good yield of chromatophores. Unbroken cells and large debris were removed, as a pellet, by centrifugation at 17,000 x g for 15 minutes (MSE18). The supernatant was spun at 100,000 x g for 1½ hours in an MSE50 centrifuge. The pellet obtained, consisting of chromatophores, was resuspended in a small volume of 50mM Tris.HCl pH8.0 and frozen if not used. Before reconstituting with carotenoids chromatophores from Rps. sphaeroides R26 and Rhs. rubrum G9 were lyophilized in an Edwards freeze drier (Model EF2) for approximately 3 days.

Extraction of Carotenoids

The required carotenoids were extracted from the particular strains of photosynthetic bacteria (see Fig 2.2) in which they are present in significant quantities (see Table 2.1). The trivial and semi-systematic names of the carotenoids are given in Table 2.2.

All stages of the carotenoid extraction were performed in dim light to minimise the isomerization and oxidation of the carotenoids (see Fig 2.2). Five volumes of acetone were added to one volume of cells in buffer in glass centrifuge tubes. The mixture was centrifuged for two minutes at 2,150 x g in an MSE super minor centrifuge. The resulting supernatant - acetone plus extracted pigments - was poured off into a separating funnel. The pelleted

Figure 2.1

Method of preparation of chromatophores and reconstitution of carotenoids

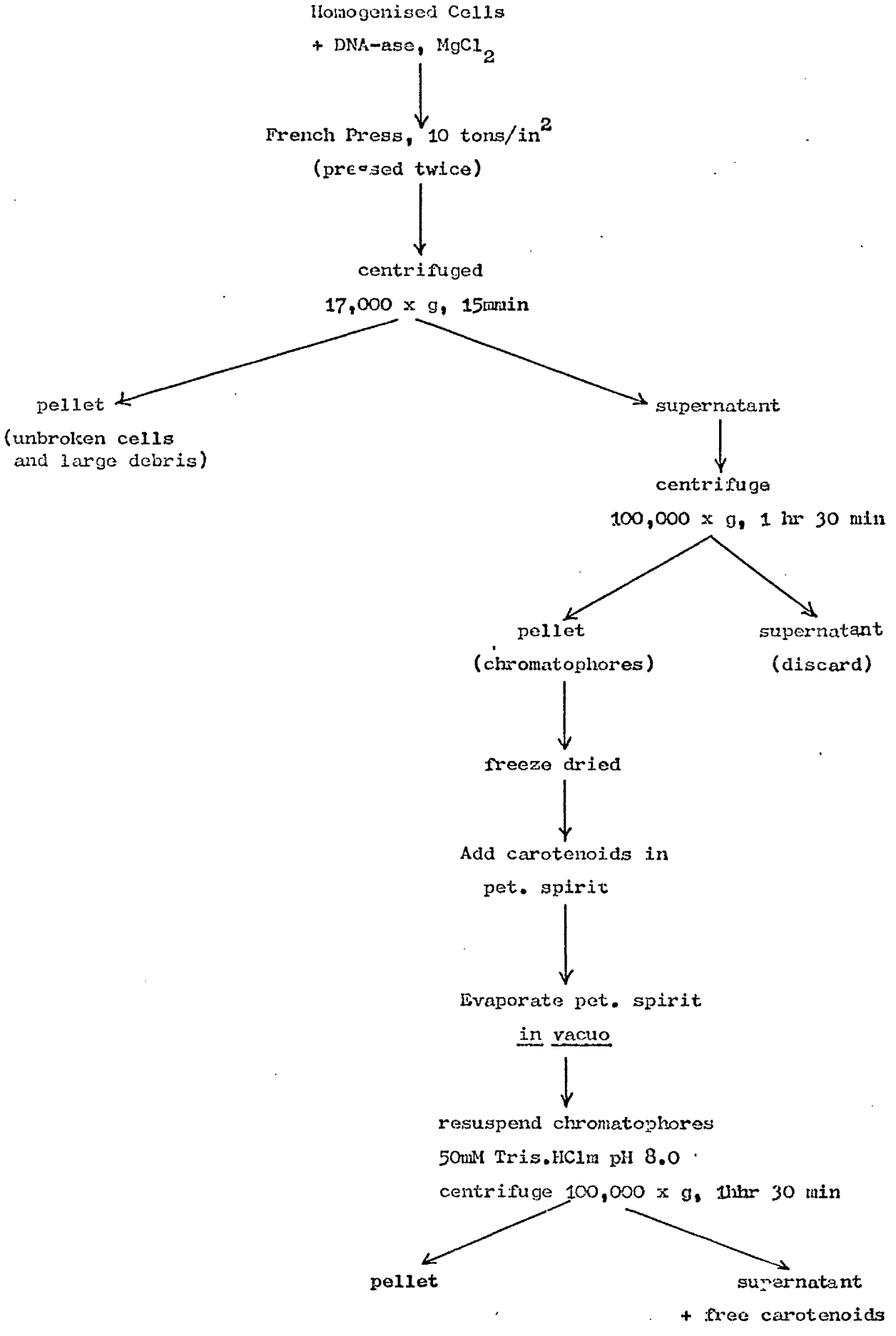

Homogenised Cells
+ DNA-ase, $MgCl_2$
French Press, 10 tons/in^2
(pressed twice)
centrifuged
17,000 x g, 15mmin
pellet
(unbroken cells
and large debris)
supernatant
centrifuge
100,000 x g, 1 hr 30 min
pellet
(chromatophores)
supernatant
(discard)
freeze dried
Add carotenoids in
pet. spirit
Evaporate pet. spirit
in vacuo
resuspend chromatophores
50mM Tris.HClm pH 8.0
centrifuge 100,000 x g, 1hhr 30 min
pellet
supernatant
+ free carotenoids

Figure 2.2

Method of carotenoid extraction from photosynthetic bacteria

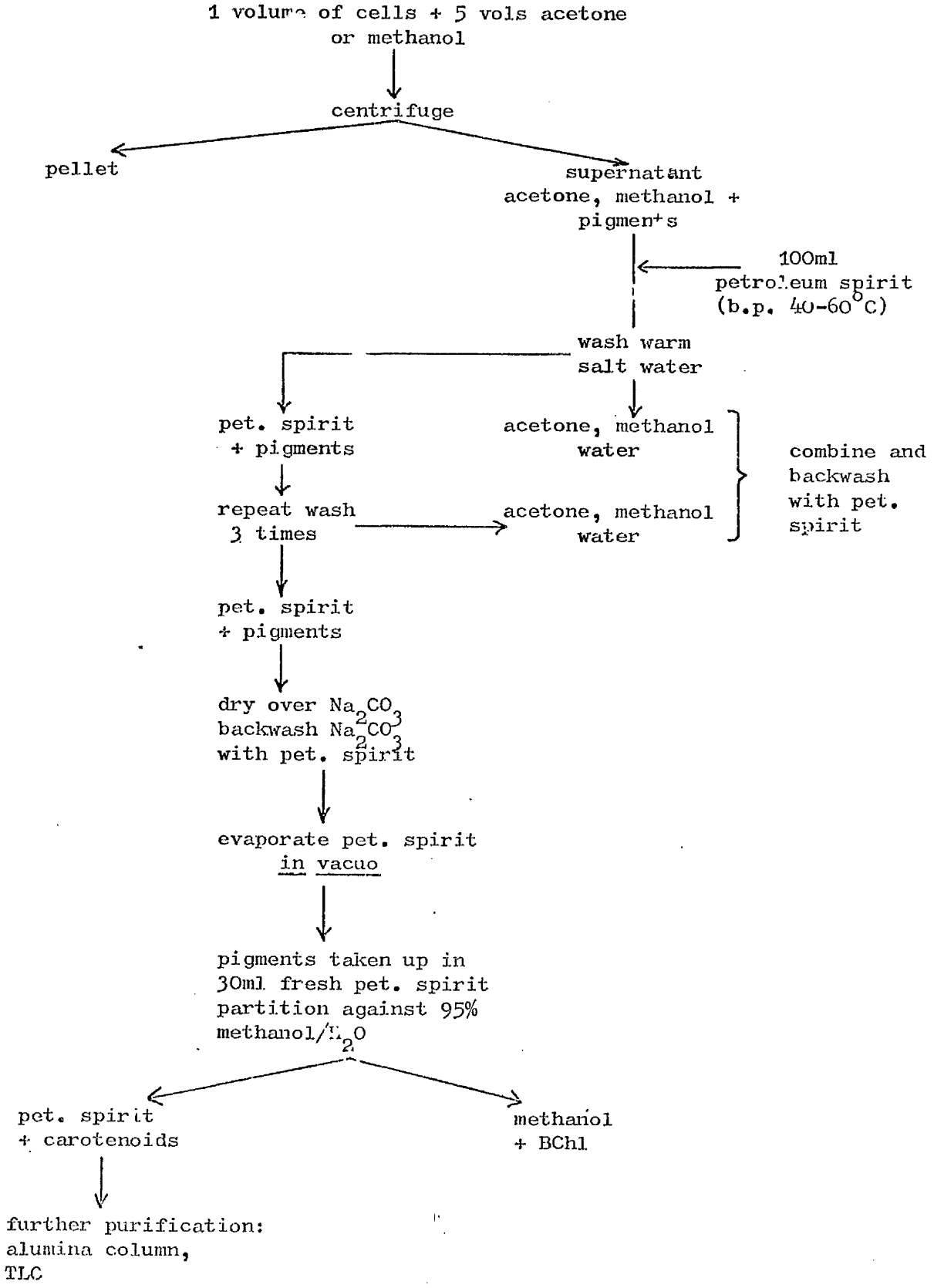

1 volume of cells + 5 vols acetone or methanol
centrifuge
pellet
supernatant acetone, methanol + pigments
100ml petroleum spirit (b.p. 40-60°C)
wash warm salt water
pet. spirit + pigments
acetone, methanol water
combine and backwash with pet. spirit
repeat wash 3 times
acetone, methanol water
pet. spirit + pigments
dry over Na_2CO_3 backwash Na_2CO_3 with pet. spirit
evaporate pet. spirit in vacuo
pigments taken up in 30ml fresh pet. spirit partition against 95% methanol/H_2O
pet. spirit + carotenoids
methanol + BChl
further purification: alumina column, TLC

Table 2.1

Sources of carotenoids used in this investigation

Species and Strain	Carotenoid	% of total	Reference
Rps. sphaeroides ATH. 2.4.1	spheroidene	90	Schneour (1962)
	spheroidenone	10	
Rps. sphaeroides ATH 2.4.1 aerated	spheroidene	70	Cogdell et al (1981)
	spheroidenone	30	
Rps. sphaeroides GIC	neurosporene	96	Holmes and Crofts (1977)
	others	4	
Rps. capsulata ZI	neurosporene	4	Webster et al (1980)
	spheroidene	79	
	OH-spheroidene		
	spheroidenone	16	
	OH-spheroidenone		
Rps. viridis	neurosporene	3	Malhotra et al (1970)
	dihydroneurosporene	72	
	lycopene	1	
	dihydrolycopene	17	
Rps. rubrum	spirilloxanthin	91	Schmidt (1978)
	rhodovibrin	6	
	anhydrorhodovibrin	2	

Table 2.2

Trivial and semi-systematic names of carotenoids (Straub 1971)

Trivial Name	Semi-systematic Name
β-carotene	β,β-carotene
zeta-carotene	7,8,7',8'-tetrahydro-ψ,ψ-carotene
lycopene	ψ,ψ-carotene
1,2-dihydrolycopene	1,2-dihydro-ψ,ψ-carotene
neurosporene	7,8-dihydro-ψ,ψ-carotene
1,2-dihydroneurosporene	1,2,7,8-tetrahydro-ψ,ψ-carotene
phytofluene	15-cis-7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene
phytoene	15-cis-7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene
spheroidene	1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro-ψ,ψ-carotene
spheroidenone	1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro-ψ,ψ-caroten-2-one
spirilloxanthin	1',1'-dimethoxy-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro-ψ,ψ-carotene

cells were then re-extracted with methanol using the same procedure. Alternate acetone and methanol extractions were continued until no colour remained in the cells. Extractions were sometimes carried out using a Soxhlet apparatus which refluxed acetone through freeze-dried cells in a cellulose extraction thimble.

Approximately 100ml of petroleum spirit (b.p. 40-60°C) was added to the pooled acetone and methanol extractions in the separating funnel. Four washes with 250ml warm salt water removed the acetone and methanol leaving the pigments in the petroleum spirit. Back washing of the salt water with petroleum spirit ensured removal of most of the pigments. After drying over anhydrous sodium carbonate the petroleum spirit was evaporated to dryness in a rotary evaporator. The pigments were taken up in 25ml of fresh petroleum spirit which was then partitioned against 95% methanol/water (v/v) in a separating funnel. Most of the bacteriochlorophyll partitioned into the methanol/water layer leaving the carotenoids in the petroleum spirit. The carotenoid fraction was then further purified or stored under nitrogen at -20°C until purification.

Other Sources of Carotenoids

The following carotenoids used in this project were not extracted from photosynthetic bacteria:

β-carotene
lycopene

Both were obtained from the Sigma Chemical Company.

These carotenoids were not further purified before being used.

Carotenoid Purification

Carotenoid purification on a large scale was achieved by using alumina column chromatography (Threlfall and Goodwin 1967). All glassware was dried thoroughly in an oven before use. Five grammes of alumina (grade 'H') in a beaker was covered with petroleum spirit and 50µl of water was added to deactivate the alumina. Any lumps in the alumina were removed by grinding with a glass rod before washing the alumina onto the column (1cm x 15cm) with petroleum spirit. The BChl-free carotenoid extract was dried down, taken up in a minimal volume of petroleum spirit and applied to the column. When

all of the carotenoid had been loaded onto the column, increasing percentages of diethyl ether in petroleum spirit were passed down the column. At least 10ml of each percentage was used. Most of the carotenoids were eluted using 1-10% diethyl ether (v/v). Fractions of the eluted carotenoid were tentatively identified by their absorption maxima in different solvents (Davies 1965

Identical fractions were pooled, dried down, taken up in petroleum spirit and stored under nitrogen in a freezer until they were used. The identities and purity of the carotenoids were confirmed by thin layer chromatography (Table 2.3) and by mass spectrometry. A typical carotenoid absorption spectrum (in this case neurosporene) is shown in Fig 1.4 and the mass spectrum of neurosporene is given in Fig 2.4. The partial mass spectra of some other carotenoids that were used are given in Appendix 5.

Dr. V.B. Math recorded the mass spectra on an AEI MS-30 mass spectrometer. The samples were inserted directly into the probe at an ionising voltage of 70eV and probe temperature of 100°C

Addition of Carotenoids to Chromatophores

The BChl content of freeze dried chromatophores of Rps. sphaeroides R26 was determined after extraction into acetone/methanol (7/2;v/v) using an extinction coefficient of 76 $mM^{-1}cm^{-1}$ at 772nm (Clayton 1963). The concentration of carotenoids were determined from their $E^{1\%}_{1cm}$ values (Davies 1965 see Table 2.3). Carotenoids, in petroleum spirit, were added to approximately 0.25g of chromatophores in ratios from 0.5 to 10:1 moles of carotenoid:mole of BChl. For purification of the reconstituted light-harvesting pigment-protein complex a ratio of 5 carotenoids:1 BChl was used.

The carotenoid-chromatophore mixture was sonicated for 30 secs. to disrupt any aggregations of chromatophores before evaporating the petroleum spirit in a rotary evaporator and resuspending the chromatophores in 20mM Tris-HCl pH 8.0. Excess carotenoid was washed free of the chromatophores by centrifugation at 100,000 x g for 1 hour 15 minutes. This left the excess carotenoid on top of the supernatant as an oily layer. Pelleted chromatophores were either washed twice more for studies on the reconstituted chromatophores, or used directly for isolation of the B850 light-harvesting pigment-protein complex.

Incorporation of Carotenoids into Liposomes

In order to observe the effect of a lipid environment on the absorption spectra of carotenoids, neurosporene and spheroidene were incorporated into lipid vesicles, or liposomes. Liposomes were made using the method of

Table 2.3

Rf values and $E^{1\%}_{1cm}$ values for carotenoids

Carotenoid	Rf[a]	Previously reported Rf[b]	$E^{1\%}_{1cm}$ value used (Davies 1965)
Neurosporene	0.96	0.94	2990 at 440nm
Spheroidene	0.57	0.6	2630 at 466nm
Spheroidenone	0.45	0.45	2065 at 499nm
Spirilloxanthin	0.42	0.39	2470 at 510nm
Dihydroneurosporene	0.95	—	Used value for neurosporene
Lycopene	0.94	—	3450 at 472.5nm
Dihydrolycopene	0.94	—	Used value for lycopene

a. The chromatography was performed on commercial thin-layer plates, with a covering of 0.5mm silica gel. The solvent used was a 50/50 mixture of chloroform and benzene (v/v).

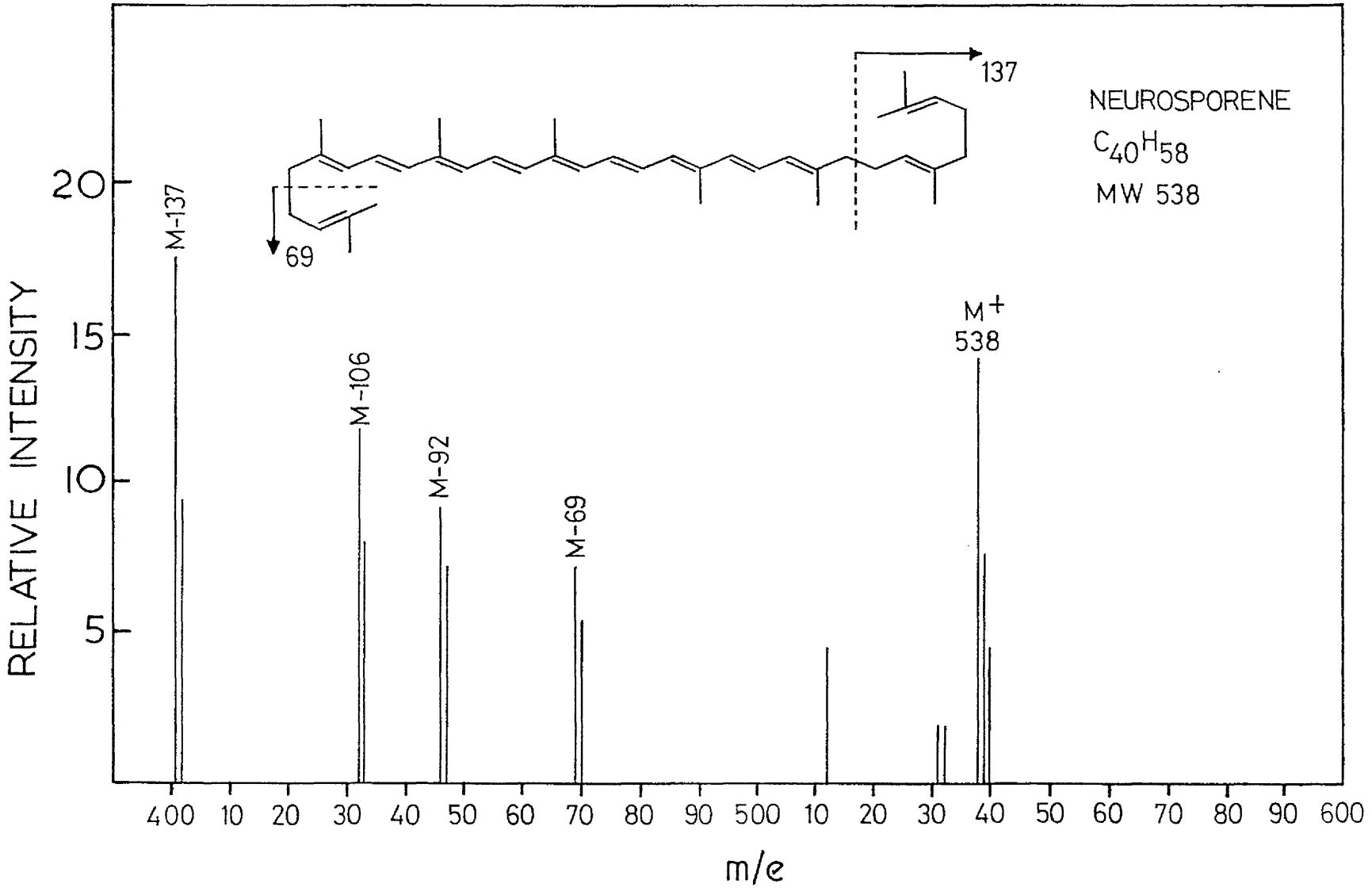
b. Rfs are those previously recorded using 50/50 chloroform and benzene. The Rfs for neurosporene, spheroidene, and spheroidenone are taken from Webster et al (1980); the Rf for spirilloxanthin is taken from the unpublished results of Karin Schmidt and Richard Cogdell.

Dihydroneurosporene and dihydrolycopene co-chromatographed with neurosporene and lycopene (from Sigma) as previously reported by Malhotra et al (1970)

Figure 2.4

Partial mass spectrum of neurosporene

The sample was inserted directly into the probe at an ionizing voltage of 70eV and probe temperature of 100°C.


NEUROSPORENE
$C_{40}H_{58}$
MW 538
137
69
M-137
M-106
M-92
M-69
M+
538
RELATIVE INTENSITY
20
15
10
5
400 10 20 30 40 50 60 70 80 90 500 10 20 30 40 50 60 70 80 90 600
m/e

Deamer et al, 1972. The carotenoids, in petroleum spirit, were added to phosphatidyl choline layered on the inside of a small flask. The petroleum spirit was evaporated off and the liposomes were then formed by adding 20mM Tris-HCl pH 8.0, 100mM NaCl and sonicating for 1 minute.

Isolation of the B850 pigment-protein complex from Rps. sphaeroides, R26

I used a method suggested by Drs. J. Bolt and K. Sauer, University of California at Berkely.

Chromatophores (OD 50cm^{-1} at 855nm, in 20mM Tris-HCl pH 8.0) were made 1 % (w/v) with respect to the detergent sodium dodecylsulphate (SDS) by addition from a 10% (w/v) solution of SDS. After stirring for 10 minutes, at room temperature in the dark, the SDS was diluted to 0.2% with 5mM sodium phosphate pH 7.0. This preparation was loaded onto a hydroxylapatite column (30ml hydroxylapatite onto a 3cm x 30cm column) which had been equilibrated with 5mM sodium phosphate pH 7.0. The column was then washed with 5mM sodium phosphate, pH 7.0, 100mM sodium chloride and 0.1% SDS. An LKB peristaltic pump provided a flow rate of 2 ml/min through the column. The phosphate concentration was increased stepwise (keeping the sodium chloride and SDS concentrations constant) from 5mM to 10mM, to 100mM, 150mM and finally to 250mM at which the B850 pigment protein complex was eluted. The complex was immediately dialysed against 10mM Tris-HCl pH 8.0 since it had been found to be unstable in high phosphate concentrations. For photodestruction and laser flash photolysis studies the complex was further dialysed against 20mM Tris-HCl, pH 8.0, 0.05% SDS. All samples therefore had the same SDS concentration, a fact which is important since the yield of triplet state formation by flash photolysis may depend on detergent concentration (Cogdell et al 1981). If the complexes were to be used within 3-4 days of isolation they were stored on ice, otherwise they were frozen.

Fluorescence Emission and Excitation Spectra

The fluorescence emission spectra of chromatophores and isolated pigment protein complexes were recorded using the home-made fluorimeter shown in Fig 2.5 (Cogdell et al 1981). Light from a 150W quartz-iodine bulb (Thorn) was passed through a Corning 4-97 and a BG 18 filter combination to give a broad band blue light source. This illuminated a 1cm quartz cuvette with four clear sides. Fluorescence was collected at right angles to the direction of excitation and was passed through a Schott RG715 cut-off filter and a monochromator (Applied Photophysics, London) before being detected by an EMI 9659 B extended S20 photomultiplier. The photomultiplier signal was

Figure 2.5

Diagrams of the fluorimeters used to record fluorescence emission and excitation spectra.

H.T. = high tension power supply

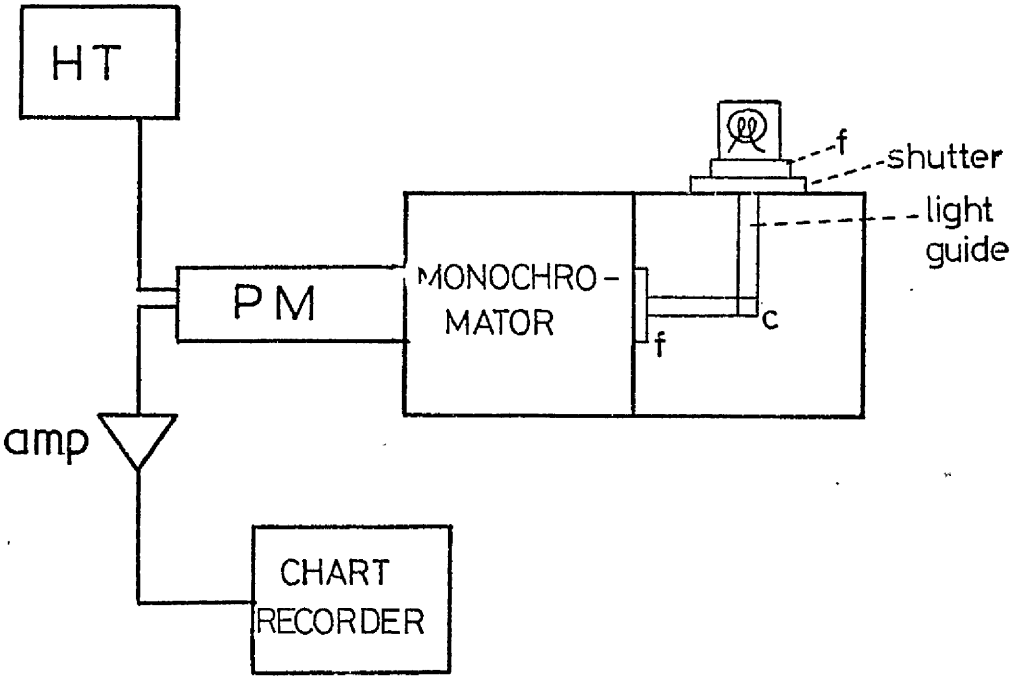
amp = amplifier

P.M = photomultiplier tube

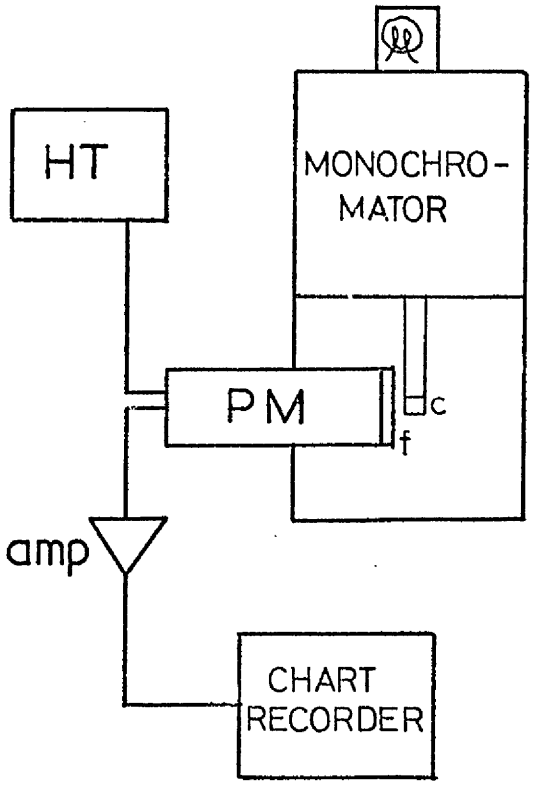
f = filter

c = cuvette

(a) FLUORIMETER FOR EMISSION SPECTRA



(b) FLUORIMETER FOR EXCITATION SPECTRA



amplified and displayed on a Linseis 038L chart recorder. The resulting emission spectra were corrected for variations in photomultiplier sensitivity at different wavelengths but not for the transmission characteristics of the monochromator.

Fluorescence excitation spectra were recorded using the fluorimeter represented in Fig 2.5. Light from a 55W quartz-iodine bulb (Phillips) was passed through the monochromator before illuminating the sample cuvette. Fluorescence was again detected and recorded as before at right angles but in this case after passing through an RG715 filter and a Balzers 858nm interference filter. The intensity of exciting light at each wavelength was measured at the cuvette position with a light meter (United Detector Technology Model 40X) and the fluorescence was corrected for variations in intensity with wavelength. Since filters are not 100% efficient some 'leakage' of exciting light into the photomultiplier can occur. To correct for this an emission spectrum of the sample buffer alone was subtracted from that of the sample. Fluorescence excitation spectra were recorded between 400 and 630nm. The efficiency of carotenoid-BChl energy transfer was determined by normalising the fluorescence excitation spectra and the fractional absorption spectra at the 590nm BChl absorption band.

BChl→Carotenoid, Triplet-Triplet, Energy Transfer

Experiments on the triplet-triplet energy transfer using laser flash photolysis were performed with Miss Marjorie Craw and Professor George Truscott of the Chemistry Department, Paisley College of Technology. Excitation was provided by a Q-switched ruby laser (System 2000, JK Lasers, Rugby, England) giving an output of approximately 1J at 694nm, with a pulse width at half height of 20ns. Frequency doubling facilities provided a Q-switched output at 347nm, the BChl could therefore be excited at 694nm, on the edge of the 850nm absorption peak, or at 347nm in the Soret band.

The monitoring system (see Fig 2.6) consisted of a pulsed 250W xenon arc lamp (Applied Photophysics, London) with circuits modified to give an enhanced output. Flash induced absorption changes were monitored at right angles to the laser beam through a 1cm cell. The measuring beam then passed through a high radiance monochromator (Applied Photophysics) with a 2nm band pass before detection by an IP28 photomultiplier wired for fast response times and connected to an automatic back-off facility. Signals from the photomultiplier were relayed to a Tektronix 7603 oscilloscope fitted with a high sensitivity 7A13 amplifier unit and a 7B50 time base. This system allowed us to record transient absorption changes of less than 0.5% and with a

Figure 2.6

Diagram of the apparatus used in laser flash photolysis experiments

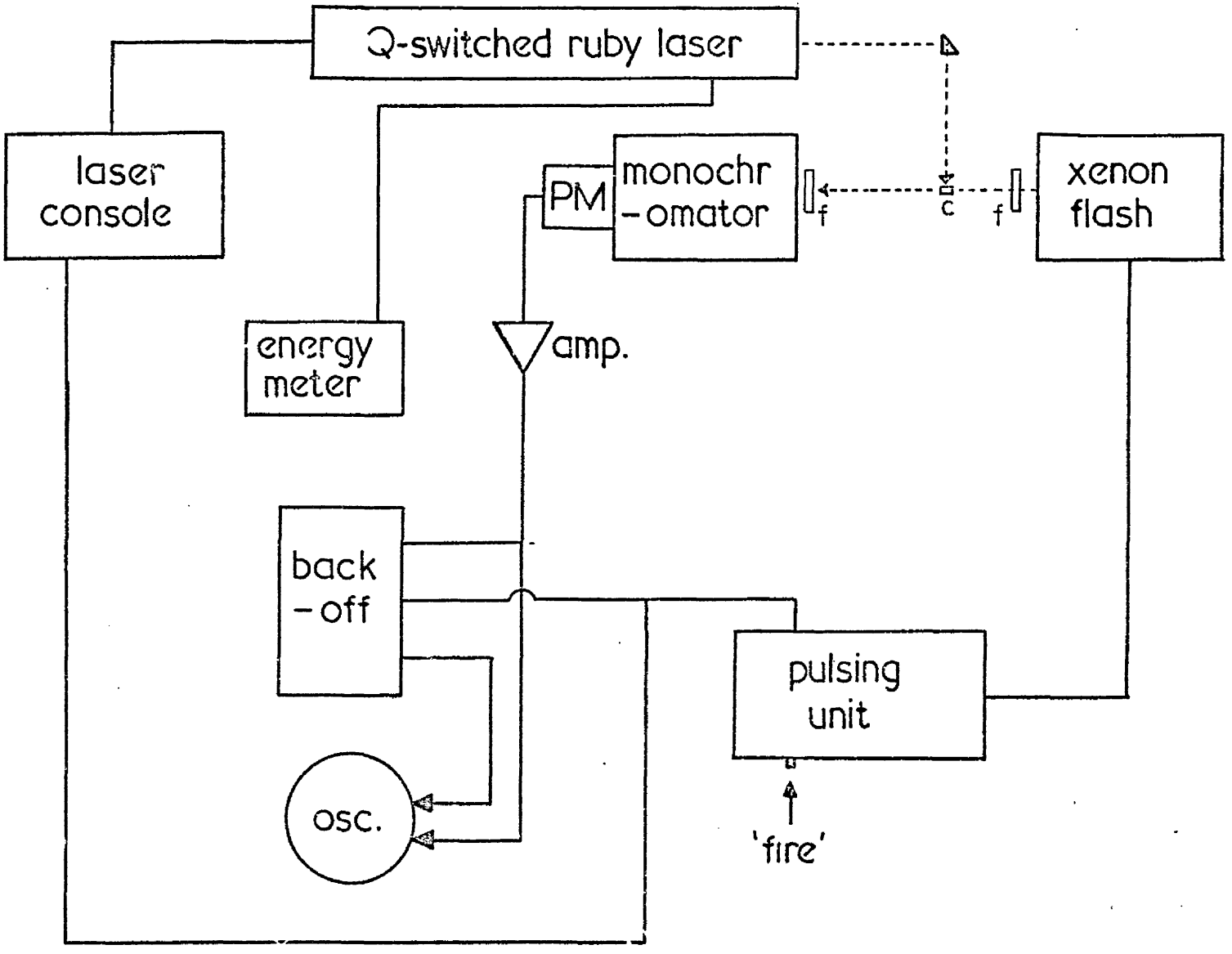
amp = amplifier

PM = photomultiplier tube

osc = oscilloscope

f = filter

c = 'flow through' sample cell


Q-switched ruby laser
laser console
PM
monochr -omator
f
c
f
xenon flash
energy meter
amp.
back -off
pulsing unit
osc.
'fire'

lifetime greater than 25ns. The intensity of each laser pulse was measured by a photodiode and displayed on an energy meter (System 2000, JK lasers). This was necessary because of the pulse to pulse variability in the energy of the laser flashes.

The laser flash intensity was reduced at 694nm by calibrated copper sulphate solutions and at 347nm by glass filters. Photodestruction of the samples due to multiple excitation was avoided by the use of a 1cm x 1mm x 1mm flow cell which allowed a fresh sample to be used for each exciting pulse. Before each experiment the sample was de-oxygenated by bubbling with nitrogen or argon for 10 minutes. The flow cell system was kept closed during the experiment, sample flow through the cell was driven by gas pressure.

The flash induced triplet state of the pigments in the pigment-protein complexes can be identified by their characteristic light minus dark difference spectra (Monger et al 1976). The spectra of complexes with and without carotenoids were recorded between 390 and 610nm, by plotting the flash induced absorbance change (ΔA) at 5nm intervals. At each wavelength ΔA following laser illumination was calculated from the following equation:

$$\Delta A = \log \frac{V_o}{V_o - x}$$

where V_o = the initial absorbance due to the xenon flash, measured (in mV) by the automatic back-off

x = extent of transient deflection, recorded on the oscilloscope after the laser pulse, normalised to a laser intensity of 1J.

The quantum yields of triplet state formation (ϕ_T) for BChl and carotenoids were obtained using a comparative technique (Armand and Bensasson 1975). The flash-induced absorbance changes, ΔA_T, due to triplet formation, were compared with those of a molecule of known quantum yield ($\phi_{STAND}{}^T$) and extinction coefficient ($\epsilon_{STAND}{}^T$). Under conditions where the sample and the standard molecule absorb the same number of photons and at low percentage conversion to the triplet state then

$$\phi_T = \phi_{STAND}{}^T \cdot \frac{\Delta A_T}{\Delta A_{STAND}{}^T} \cdot \frac{\epsilon_{STAND}{}^T}{\epsilon_T}$$

The extinction coefficients (ϵ_T) of the triplet-singlet absorptions of the BChl or carotenoids in the pigment-protein complex were estimated by the complete conversion method. The ΔA_T at a given wavelength was measured as a function of laser intensity. At high laser intensities, no change in

ΔA_T with increasing laser intensity could be taken to imply that all of the carotenoid or BChl molecules had been converted to their triplet states i.e. 'complete conversion'. The ϵ_T can therefore be obtained by dividing the maximum ΔA_T by the ground state concentration.

For laser excitation at 694nm the standard used was methylene blue in 0.03M sulphuric acid, assuming $\phi_{MB}{}^T = 0.58$ and $\epsilon_{MB}{}^T = 6000 cm^{-1}$ at 375nm (Wildes et al 1977). For 347nm excitation anthracene in cyclohexane was used taking $\phi_{An}{}^T = 0.71$ and $\epsilon_{MA}{}^T = 64,700 cm^{-1}$ at 422nm (Bensasson and Land 1971). Determination of ϕ_T for BChl involved the use of perylene in benzene as a standard, taking $\phi_{Pe}{}^T = 0.015$ and $\epsilon_{Mp}{}^T = 14,300 cm^{-1}$ at 490nm (McVie et al 1978).

Photodestruction

The photoprotection function of carotenoids was investigated by determining their ability to protect the pigment-protein complex from photodestruction. The photodestruction was measured by the irreversible bleaching of the 850nm absorption band induced by illumination with strong white light ($900 Wm^{-2}$) in the presence of oxygen. Light from a 150W quartz iodine lamp was passed through 5cm of water acting as a heat filter and was focussed onto the sample in a 1cm cuvette.

Absorption Spectra

All spectra were recorded on an SP8000 (450-850nm) or SP500 (850-950nm) spectrophotometer (Pye-Unicam).

Circular Dichroism Spectra

Circular dichroism (CD) spectra of the pigment-protein complex with and without carotenoids, and also carotenoids in various environments, were recorded at Strathclyde University on a Cary 60 spectrophotometer operated by Mr. Gordon Medlow. This facility was kindly provided by Professor G.G. Wood of the Pharmacology Department, University of Strathclyde.

Resonance Raman Spectroscopy

The resonance Raman spectra of the B850 pigment-protein complexes were recorded by Dr. Marc Lutz of the Service de Biophysique, Centre d'Etudes Nucleaires de Saclay, Gif-sur-Yvette, France. Emission lines at 363.8nm and 514.5nm of an Argon Laser (Spectra Physics) were used to selectively induce resonance Raman scattering from carotenoids. The apparatus involved has been described in Lutz (1977) and Lutz (1979).

Analysis of the Polypeptide Composition

The polypeptide composition of the purified B850 light-harvesting pigment-protein complex was determined by SDS-polyacrylamide gel electrophoresis and isoelectric focussing.

a) SDS-polyacrylamide gel electrophoresis. Electrophoresis as described by Laemmli (1970) was used to separate proteins according to their molecular weight. This method allows the molecular weight of an unknown protein to be estimated by comparing its migration on the gel system with the migration of proteins of known molecular weight.

The ingredients of all solutions used in electrophoresis are given in Appendix 4. The gels were poured as slabs (14 x 18.5cm) containing either a fixed percentage of acrylamide (usually 10%) or a gradient of acrylamide (11.5-16.5%). The acrylamide gradients were stabilized with sucrose, 5.6% sucrose with 11.5% acrylamide and 14% sucrose with 16.5% acrylamide. The gradient gels were mixed and poured on ice to slow down the polymerisation of the acrylamide and to allow a smooth gradient to be formed.

Samples, at a protein concentration of approximately 1mg/ml, were denatured by boiling in 2% β-mercaptoethanol for 1-2 minutes before being loaded onto the gel. The following proteins (from the Sigma Chemical Co. Ltd.) were used as standards.

bovine serum albumin (BSA)	mw 68KD
alcohol dehydrogenase (ADH)	mw 41KD, from yeast, E.C.1.1.1.1
myoglobin	mw 17.2KD, horse heart
cytochrome c (cyt c)	mw 12.2KD, horse heart

Gels were stained for 4-5 hours in a solution containing Kenacid blue. They were then destained to clear the gels but leave the proteins stained blue. Destained gels were photographed. Some individual gel tracks were scanned at 550nm using a Gilford 240 spectrophotometer equipped with a linear transport device.

b) Isoelectric focussing. Proteins of similar molecular weights were separated according to their charge using an isoelectric focussing method described by O'Farrell (1975) incorporating some modifications suggested by Ames and Nikaido (1976).

Electrophoresis was carried out on stick gels containing 8.5M urea, the detergent Nonidet NP-40, and ampholine buffers (LKB) which gave a pH gradient of 3.5-10.0 down the gel. Samples with protein concentrations of approximately 1mg/ml were boiled for 2 minutes in lysis buffer containing 8M urea, 2% (v/v) β-mercaptoethanol, 1% SDS (v/v). They were then loaded

on to the gels and a potential difference of 300V was put across the gels for 16 hours. During this time proteins migrated down the pH gradient until they reached positions where the pH equalled their respective isoelectric pHs. Gels were then fixed in 10% (w/v) trichloroacetic acid for 24 hours before washing in water for 3 hours to remove the ampholines. The gels were then stained, destained and photographed as for SDS-polyacrylamide gels.

Protein Assay

Protein concentrations were determined by the so-called tannin assay according to the method of Mejbaum-Katzenellenbogen and Drobryszycka (1959). The active reagent contained 10% (w/v) tannic acid, 0.33M HCl, and 2% (v/v) phenol. Bovine serum albumin was used as the protein standard.

CHAPTER 3

Characterization of the B850 light-harvesting pigment-protein complex of Rps. sphaeroides, R26

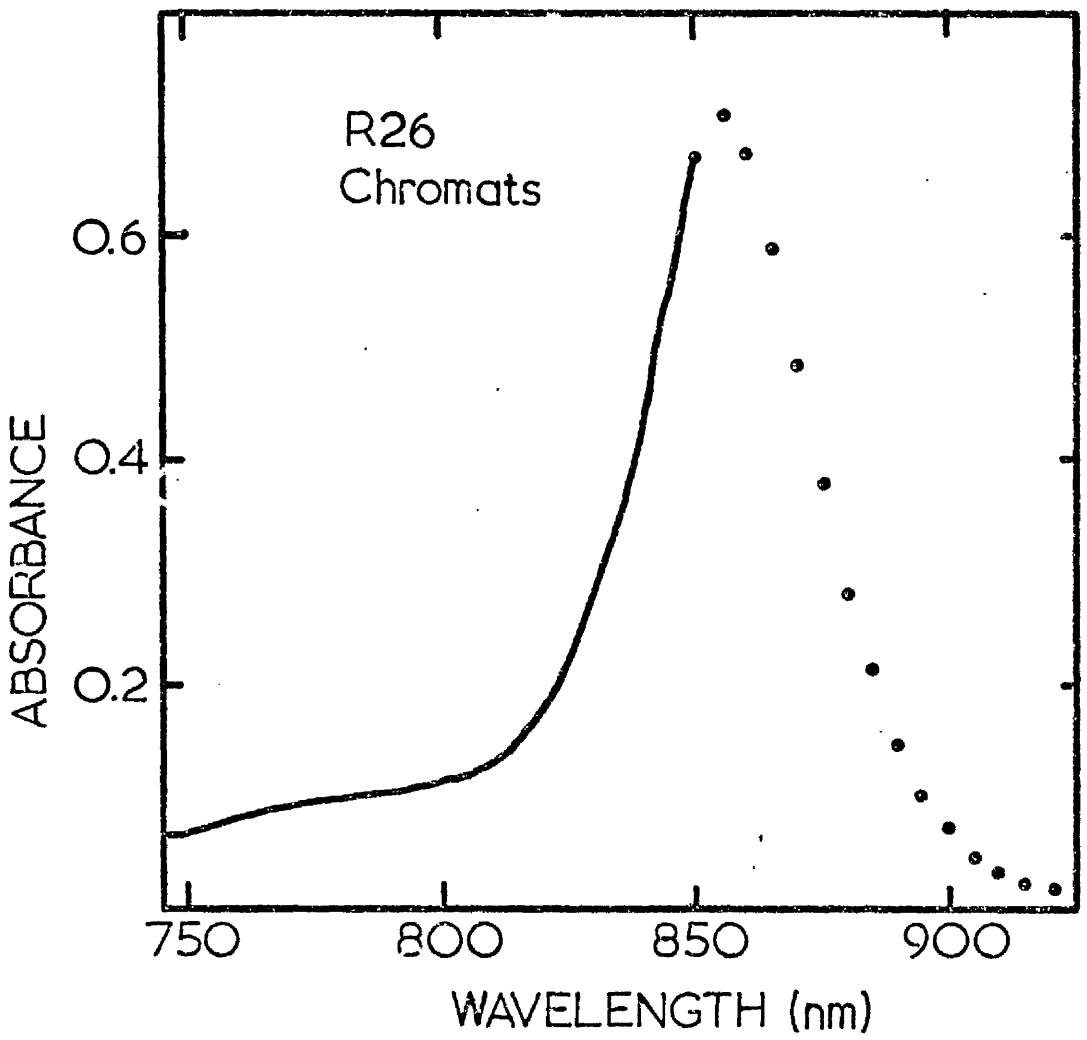
Rps. sphaeroides R26 is a blue-green, carotenoidless mutant isolated from Rps. sphaeroides 2.4.1 wild type (Clayton and Smith 1963). No polyenes are found in R26 since there is a block in the carotenoid biosynthetic pathway before phytoene, the carotenoid precursor (Crounse et al 1963). R26 is usually assumed to contain only a B890 type of pigment-protein complex (see Chapter 1, section 1.2) named B850 after the wavelength (850nm) of the only major band in the NIR absorption spectrum. However, during the course of this project it has been suggested that Rps. sphaeroides R26 contains more than one type of light-harvesting complex (Rijgersberg et al 1980). The aim of this project is to investigate energy transfer between carotenoids and BChl by adding carotenoids to the light-harvesting complex from strain R26. This strain and the isolated light-harvesting complex have also been used in a number of studies recently (Sauer and Austin 1978, Bolt and Sauer 1979, Rafferty et al 1979); it is therefore important to investigate the composition of the light-harvesting apparatus and to determine which type of complex the B850 represents.

When Rps. sphaeroides R26 was first isolated, and in subsequent years, the NIR absorption spectrum of cells and photosynthetic membranes or chromatophores showed a single absorption peak at 870nm (eg see Clayton 1963, Reed 1969). Our current understanding of the light-harvesting complexes of photosynthetic bacteria (see Cogdell and Thornber 1980) would suggest that only a B890 type of complex was present (in the form of B870).

However in recent years several publications have placed the NIR absorption band of R26 at approximately 860nm (Sauer and Austin 1978, Rafferty et al 1979, Rijgersberg et al 1980, see Fig 3.1). The isolated light-harvesting complex absorbs maximally between 850 and 855nm, it is therefore called B850 (Sauer and Austin 1978, Davidson and Cogdell 1980). To my knowledge, this 10nm difference between the absorption maxima of 'old' and 'new' R26 strains has yet to be commented upon, let alone explained. Does the R26 strain described recently contain a B870 type of complex altered to B850, or has a spontaneous 'genetic drift' occurred, resulting in the addition of a spectrally altered B800 + 850 complex? If a B800 + 850 type is present (without the chlorophyll responsible for the 800nm absorption) it will exist in greater amounts than the B870 type and will probably mask the NIR

Figure 3.1

Near infra red absorption spectrum of chromatophores from *Rps. sphaeroides* R26 used for isolation of the B850 complex


R26
Chromats
0.6
0.4
0.2
ABSORBANCE
750
800
850
900
WAVELENGTH (nm)

absorption band of the B870 complex (Aagaard and Sistrom 1972). The presence of both types of light-harvesting complex (ie B800 + 850 and B870) in Rps. sphaeroides 2.4.1 and other species, is shown by NIR absorption maxima at 800 and 850nm and a shoulder at approximately 875nm (Fig 3.2). In strains of bacteria which contain only the B870 type of complex, the ratio of total BChl:reaction centre BChl is roughly constant at 30 - 40:1 (Aagaard and Sistrom 1972). If both types of complex are present then this ratio varies inversely with the light intensity at which the cells are grown. The ratio can then range between 50:1 and 300:1 (Aagaard and Sistrom 1972). The BChl: reaction centre BChl ratio in R26 was found to be approximately 30:1 (Reed 1969). This indicated that only the B870 type of complex was present. It appears to be a characteristic of carotenoidless mutant strains of bacteria that they contain only the B870 type of light-harvesting complex (eg Rps. sphaeroides, R22 (Aagaard and Sistrom 1972) and Rps. capsulata, A1a^{+} (Nieth et al 1972). It therefore seems that the original form of Rps. sphaeroides R26, absorbing at 870nm, contained only a B870 light-harvesting pigment-protein complex.

Since membranes of photosynthetic bacteria contain large amounts of 3 photosynthetic pigment-protein complexes, the constituent polypeptides of different types of complexes can be identified by means of polyacrylamide gel electrophoresis. The initial attempts at electrophoresis on chromatophores of Rps. sphaeroides, using a fixed percentage of acrylamide, were unable to resolve the several low molecular weight polypeptides (10-11KD) which we now know to be the constituents of the light-harvesting complexes. No difference could be detected between the polypeptide compositions of wild type and R26 strains (Clayton and Clayton 1972, Clayton and Haselkorn 1972).

The only published characterisation of the light-harvesting complex from Rps. sphaeroides R26 is that of Sauer and Austin (1978). The complex had an NIR absorption maximum at 853nm. Gel filtration was used to isolate the complex as an aggregate with a molecular weight of approximately 360KD. This aggregate contained 40 BChls and after it was further treated with SDS and subjected to gel electrophoresis Sauer and Austin suggested that the complex existed as a minimal unit. The proposed minimal unit consisted of a BChl dimer attached to 2 copies of a polypeptide (approximately 8.5KD). Using the same techniques the minimal unit of the B800 + 850 complex from Rps. sphaeroides wild type was found to be 3 BChls and one carotenoid attached to 2 copies of a 10KD polypeptide. One BChl is responsible for the 800nm absorption band, the other 2 BChls are coupled to give the 850nm absorption band.

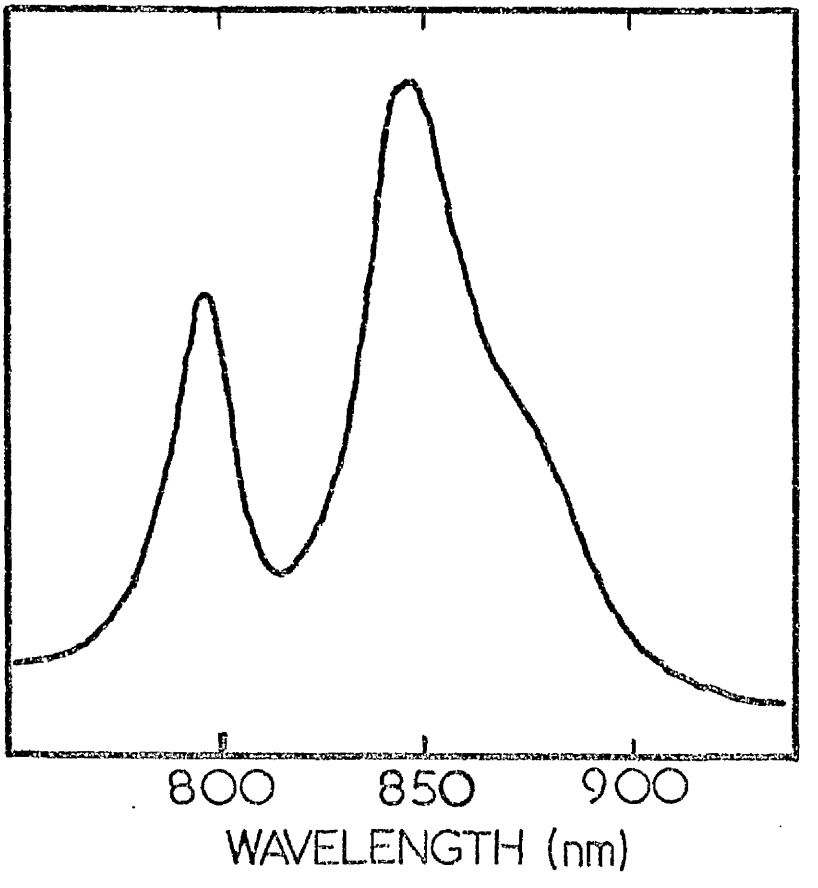


Figure 3.2

Near Infra-red absorption spectrum of chromatophores from Rps. sphaeroides 2.4.1.

The minimal unit for the B800 + 850 complex from Rps. sphaeroides has recently been completely characterised by Broglie et al (1980) using lithium dodecyl sulphate (LDS) gradient gel electrophoresis and by Cogdell et al (1980) using SDS gradient gel electrophoresis and isoelectric focussing. Both groups showed that the pigments are attached to two different polypeptides with similar molecular weights, 8-10KD. Since Sauer and Austin (1978) were unable to resolve these two B800 + 850 polypeptides they may also be incorrect in their suggestion that the B850 complex from strain R26 contains two copies of the same 8.5KD polypeptide. Broglie et al also found that the minimal unit of the B870 complex from wild type Rps. sphaeroides has 2 BChls bound to 2 polypeptides of approximate molecular weights 12 and 8KD.

It is interesting to note that the polypeptide compositions of the two light harvesting complexes in Rps. capsulata are very similar to those of Rps. sphaeroides. The only major difference is an extra polypeptide (14KD) attached to the two polypeptides bearing the B800 + 850 pigments (Feick and Drews 1978). The B870 type complex in Rps. capsulata contains 2 polypeptides weighing 12 and 8KD (Webster and Cogdell, unpublished results); these are very similar to those in the B870 of Rps. sphaeroides (Broglie et al 1980). In Rps. capsulata the 8KD polypeptide of the B870 complex seems to be easy to miss since the B870 complex has previously been described as having only a 12KD polypeptide (Feick and Drews 1978, Schumacher and Drews 1978). However both polypeptides can be seen by polyacrylamide gel electrophoresis of chromatophores from strain $A1a^+$ (Yu et al 1981).

From the characterisation of the B870 from wild type Rps. sphaeroides (Broglie et al 1980), and by analogy to Rps. capsulata, the light-harvesting complex from the R26 strain of Rps. sphaeroides would be expected to have a similar polypeptide composition to the B870 from the wild type, ie two polypeptides of approximate molecular weight 8 and 12KD.

It has recently been suggested that strain R26 contains more than one type of light-harvesting pigment-protein complex (Rijgersberg et al 1980). At a temperature of 4K the NIR absorption maximum of R26 cells shifted from its normal 858nm to 870nm. The second derivative of the absorption spectrum showed that two absorption bands, at 866 and 875nm, contributed to the broad absorption at 870nm. Since Sauer and Austin (1978) observed only one NIR absorption band at low temperatures, Rijgersberg et al suggested that strain R26 may contain a B870 complex in addition to the B850 complex. This suggestion prompted my investigation into the polypeptide composition of strain R26, but a very recent study (Bolt and Sauer, in press) has undermined the basis for Rijgersberg's idea. Bolt and Sauer found that at 90K, the NIR

absorption spectrum of the isolated B850 complex shows a peak at 850nm with a shoulder at 865nm. The presence of this shoulder means that the two bands observed in R26 chromatophores by Rijgersberg et al need not necessarily imply the presence of two types of light harvesting complex. In the light of this, the suggestion of Rijgersberg et al could probably be disregarded but, as I shall show, this suggestion was correct.

Figure 3.3 shows the polypeptide composition of the B850 complex from R26. On a gradient gel (11.5-16.5% acrylamide) the undenatured complex runs mainly as a pigmented band with an apparent molecular weight greater than 100KD (Fig 3.4). When the complex is denatured this aggregate dissociates into its constituent polypeptides. These are two low molecular weight polypeptides, both running below cyt c (12.2KD).

Polypeptides from the light harvesting complexes of strains 2.4.1 and R26 were compared on a gradient gel (Fig 3.5). Chromatophores from strain 2.4.1 (track D) showed 3 principal bands (1-3) at low molecular weights. Isolated B800+850 from 2.4.1(C) gave only bands 2 and 3. In Rps. sphaeroides bands 1-3 have previously been shown to be the polypeptides of the light-harvesting complexes (Broglie et al 1980, Cogdell et al 1980). Band 1 (12KD) and part of band 3 (8KD) are the polypeptides of B870, band 2 (10KD) and part of band 3 represent B800+850. R26 chromatophores (track B) show all three bands and must therefore contain both types of light-harvesting complex. B850 from R26 (track A) contains bands 2 and 3 and appears very similar to B800+850 (Track C) from strain 2.4.1. The B850 complex commonly isolated from R26 is therefore a B800+850 type of complex which lacks the BChl responsible for the 800nm absorption band. Scans of the low molecular weight region of the gel (Fig 3.6) show that in the isolated B800+850 type of complex, band 3 is much less intense than band 2. In chromatophores band 3 is more intense, or the two are of almost equal intensity. This confirms the observation of Broglie et al (1980) that band 3 is composed of a polypeptide from B800+850 and a polypeptide from B870.

The two polypeptides from the B800+850 complex (bands 2 and 3) are very close together on an SDS-polyacrylamide gel which separates proteins purely by differences in molecular weight. These two polypeptides can be more clearly resolved by isoelectric focussing (Cogdell et al 1980) which separates proteins according to differences in their net charges. As I have shown above, the B850 complex from R26 is an altered B800+850 type, it is possible that the amino acid content of one polypeptide has changed in such a way as to alter the binding site of the BChl responsible for the 800nm

Figure 3.3

Comparison of denatured and non-denatured samples of isolated B850 complex by gradient gel electrophoresis

11.5 - 16.5% acrylamide gradient

Track ND: 50µg Isolated, non denatured B850 complex, showing large molecular weight aggregate.

Track D: Isolated B850 complex denatured by boiling for 2min. in presence of mercaptoethanol

Track S: Standard Proteins BSA, 68kD. ADH, 41kD. myoglobin, 17.2kD. cyt. c, 12.2kD.

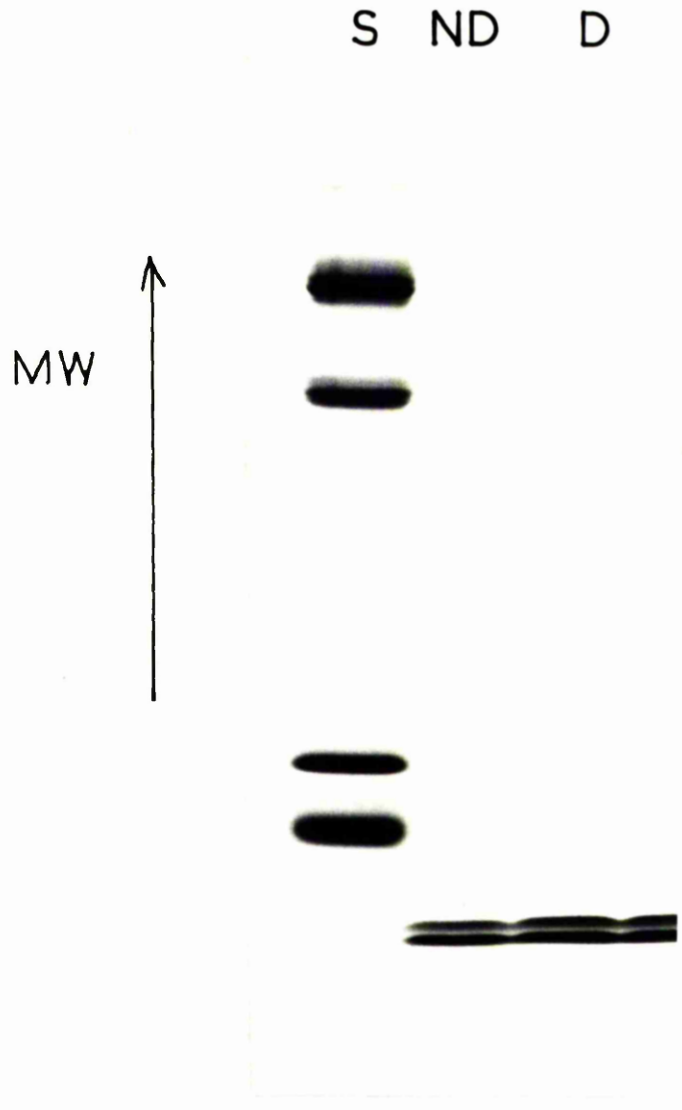

S ND D
MW

Figure 3.4

Determination of the molecular weight of the non-denatured aggregate of the B850 light-harvesting complex

The semi-logarithmic plot of molecular weight versus band Rf was determined for the following standard proteins: BSA 68kD, ADH 41kD, myoglobin 17.2kD, cyt c 12.2kD. The mobility is represented by the Rf values for protein bands on an SDS polyacrylamide gradient gel (11.5 - 16.5% acrylamide).

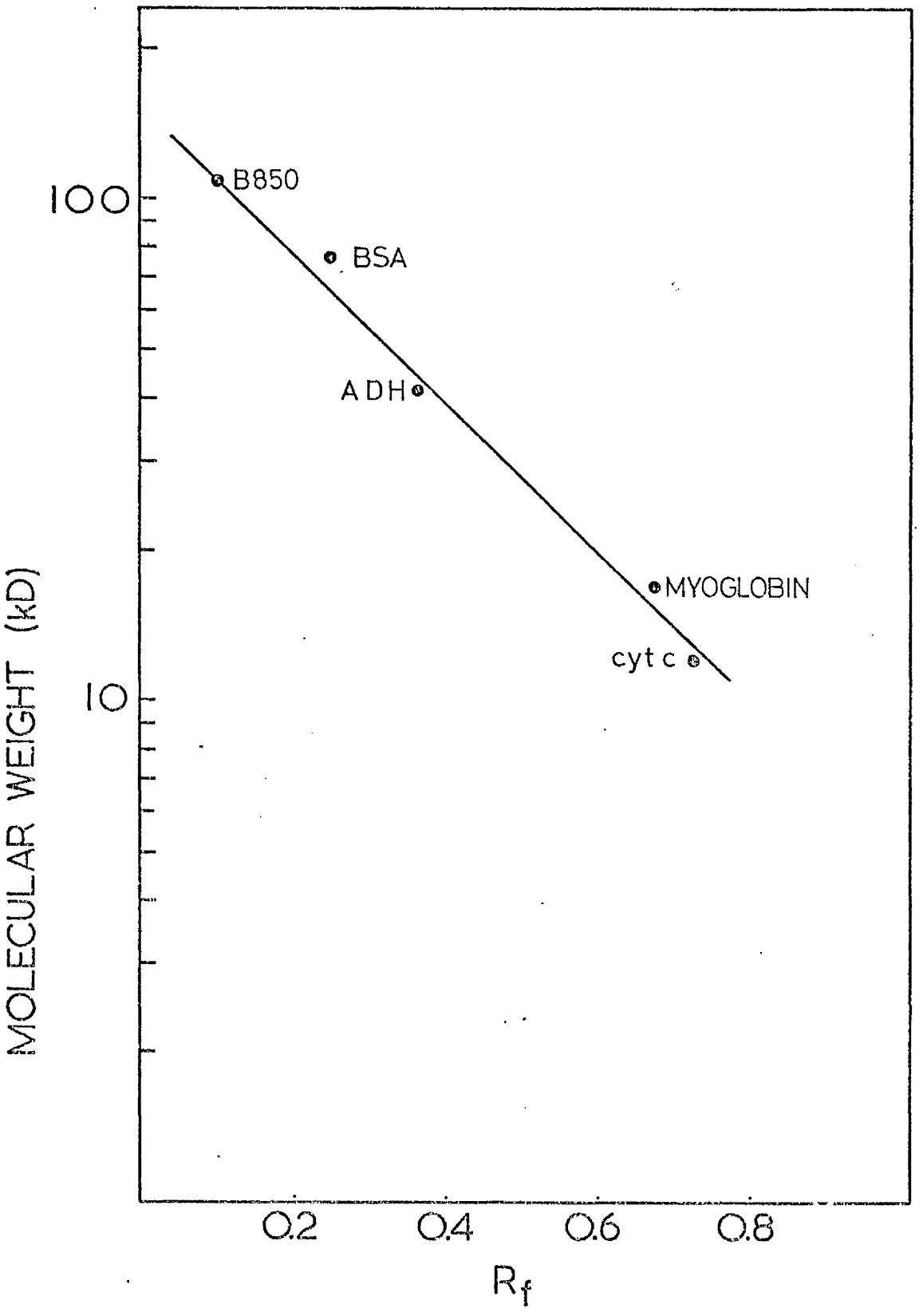

B850
BSA
ADH
MYOGLOBIN
cyt c
100
10
MOLECULAR WEIGHT (kD)
0.2
0.4
0.6
0.8
R_f

Figure 3.5

Comparison of polypeptides from Rps. sphaeroides 2.4.1. and R26 on an SDS-polyacrylamide gradient gel

11 -16.5% acrylamide. Bands 1 - 3 represent polypeptides from the light-harvesting complexes.

Track A: B850 complex from R26.

Track B: R26 chromatophores

Track C: B800 + 850 complex from 2.4.1.

Track D: 2.4.1. chromatophores.

Track S: standard proteins BSA 68kD, ADH 41kD, myoglobin 17.2kD, cyt.c 12.2kD

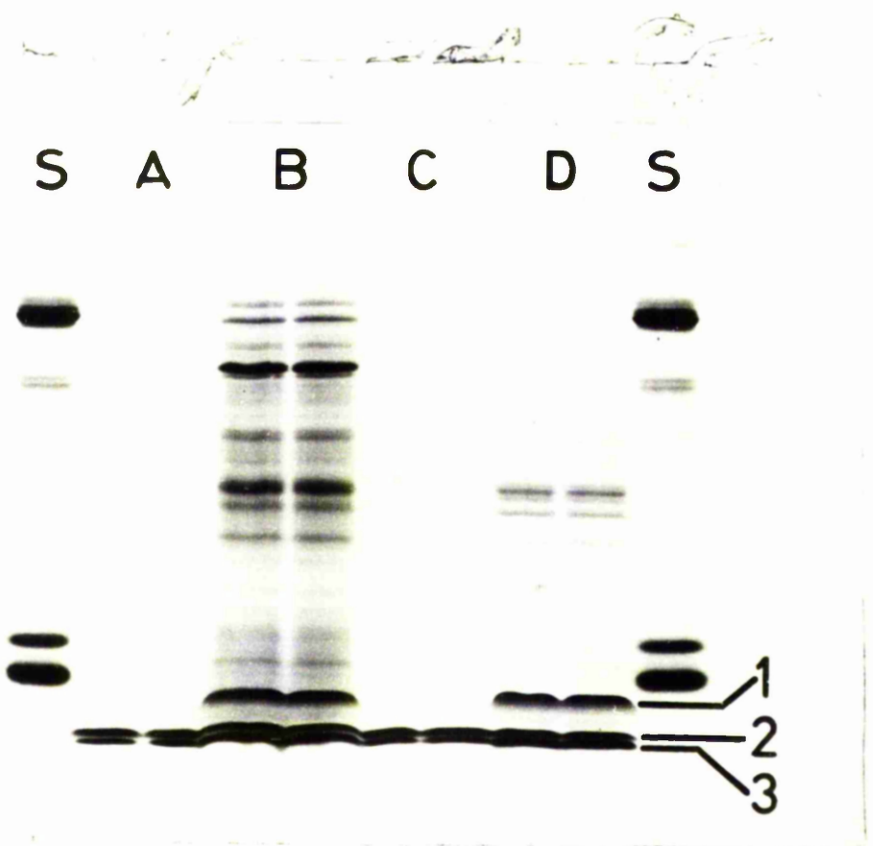

S
A
B
C
D
S
1
2
3

Figure 3.6

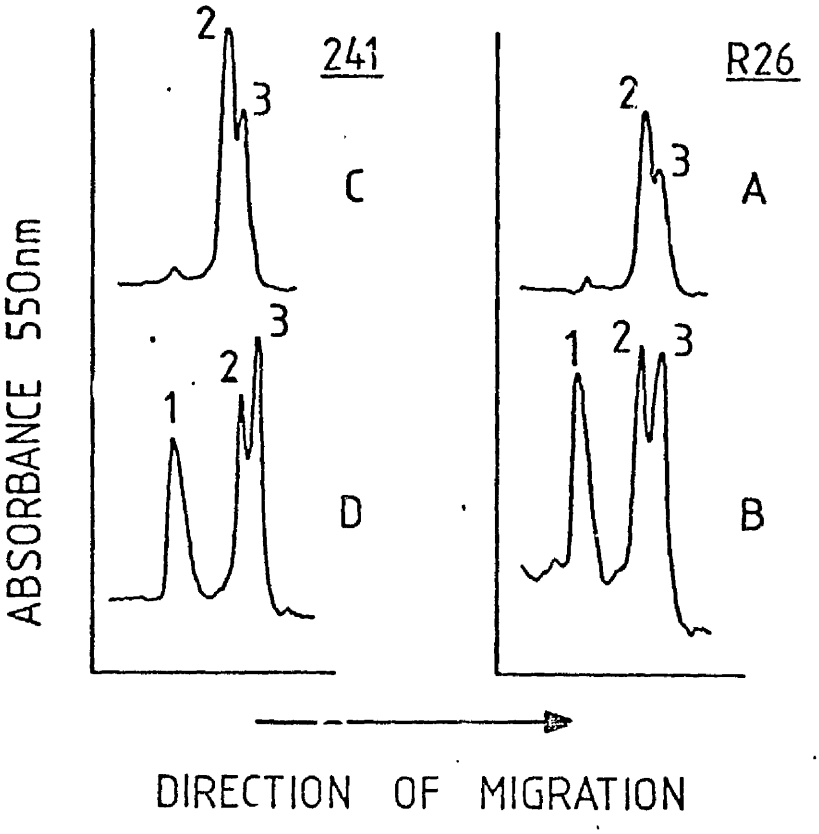
Scans of the low molecular weight region of the gel shown in Figure 2

A: B850 light-harvesting complex from R26.

B: R26 chromatophores.

C: B800 + 850 complex from 2.4.1.

D: 2.4.1. chromatophores.


241
R26
2
3
C
2
3
A
3
2
1
D
1
2
3
B
ABSORBANCE 550nm
DIRECTION OF MIGRATION

absorption band. If a change has altered the net charge of the polypeptide then the old and new forms of the polypeptide will be separated by isoelectric focussing, even although the molecular weights are almost identical. The B850 and B800+850 complexes were focussed separately and together (i.e. on the same gel) to allow a direct comparison of their polypeptides (Fig 3.7). The migrations of the two main bands appears to be the same for each complex, and only two bands are resolved on the gel containing a mixture of both complexes. This suggests that either the polypeptide compositions of both complexes are identical or that any change in the amino acid content has been conservative i.e. amino acids have been replaced by a similar type of amino acid. The net charge of the polypeptide would therefore be unchanged.

There can be no doubt that the strain of R26 in current use contains both types of pigment-protein complex, and that the B850 light-harvesting complex is an altered B800+850 type. Professor W. Sistrom (University of Oregon) kindly supplied a culture of the original strain of R26. This has a maximum NIR absorption at 870nm (Fig 3.8). As might be expected, on a gradient gel, chromatophores from the original R26 strain show only bands 1 and 3 in the low molecular weight region of the gel (Fig 3.9). Chromatophores from strain 2.4.1 and the 'new' R26 show all three bands. The original R26 therefore contains only the B870 type of light-harvesting complex.

Figure 3.7

Comparison of polypeptides from R26, B850 and 2.4.1 B800 + 850 on isoelectric focusing gels

R26 : isolated B850 from Rps. sphaeroides, R26

2.4.1 : isolated B800 + 850 from Rps. sphaeroides 2.4.1.

R26 + 2.4.1 : mixture of B850 and B800 + 850

All complexes were denatured before isoelectric focussing.

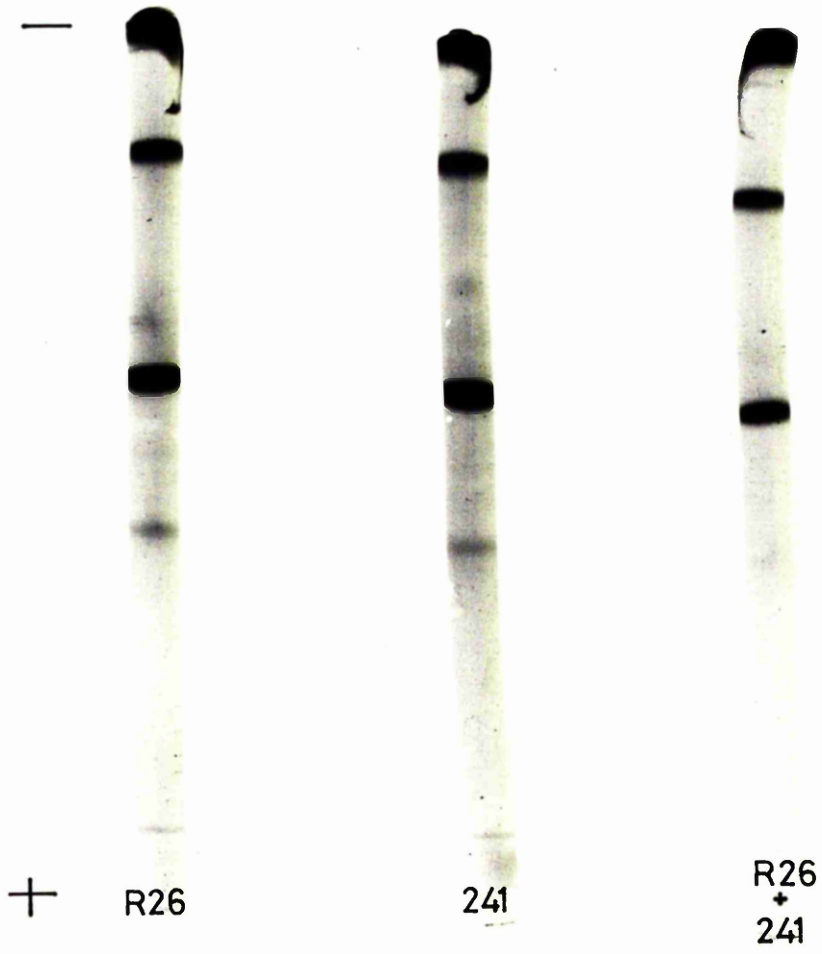

—
+
R26
241
R26
+
241

Figure 3.8

Near infra-red absorption spectrum of chromatophores from a culture of the original R26 strain of Rps. sphaeroides

This culture was supplied by Prof. W. Sistrom.

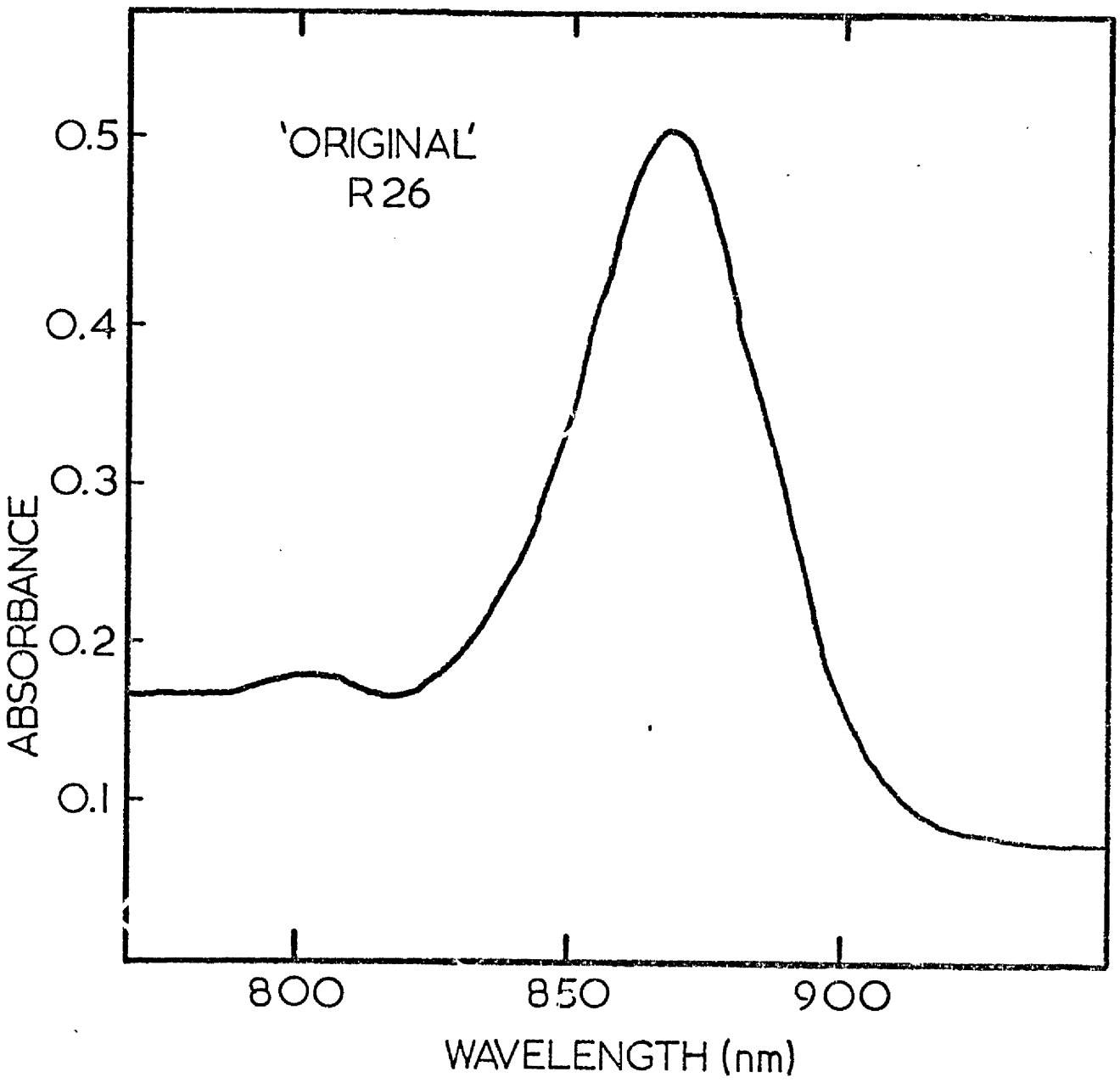

'ORIGINAL'
R 26
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
800
850
900
WAVELENGTH (nm)

Figure 3.9

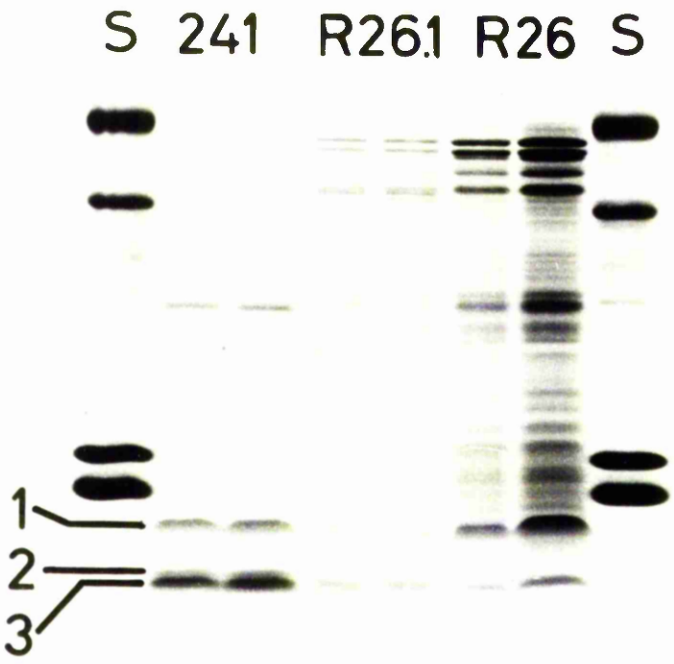
A comparison of polypeptides from chromatophores of strains of Rps. sphaeroides on an SDS-polyacrylamide gradient gel

2.4.1: Rps. sphaeroides 2.4.1

R26.1: Rps. sphaeroides, R26. This is the strain I have used during this project, NIR absorption spectrum shown in Figure 3.1.

R26: Rps. sphaeroides R26 - original R26 strain supplied by Prof. W. Sistrom, NIR absorption spectrum shown in Figure 3.8

: Standard proteins BSA 68kD, ADH 41kD, myoglobin 17.2kD, cyt.c 12.2kD.


S
241
R26.1
R26
S
1
2
3

CHAPTER FOUR

RECONSTITUTION OF CAROTENOIDS INTO THE B850 LIGHT-HARVESTING COMPLEX, SUBSEQUENT ENERGY TRANSFER FROM CAROTENOID TO BChl.

4.1 Reconstitution of carotenoids

My initial investigations into carotenoid to BChl energy transfer involved reconstitution of the carotenoids neurosporene and spheroidene into chromatophores of the carotenoidless mutant of Rps. sphaeroides, R26. I hoped that the carotenoids would be reconstituted into the pigment-protein complexes which make up the light-harvesting apparatus. By reconstituting a range of carotenoids it would be possible to investigate their respective abilities to transfer energy to the BChl of the light-harvesting complexes. The efficiency of energy transfer can be calculated after normalisation of the chromatophore absorption spectrum to the fluorescence excitation spectrum, this method was used by Goedheer (1959) to determine carotenoid to BChl transfer efficiency for Rps. sphaeroides, 2.4.1.

Light absorbed by neurosporene and spheroidene in R26 chromatophores sensitized BChl fluorescence, showing that singlet-singlet energy transfer had occurred from the carotenoids to BChl. This energy transfer was approximately 20% efficent for both carotenoids (Fig 4.1). This value is low compared to the 90% efficiency recorded for chromatophores of wild-type Rps. sphaeroides, in which the major carotenoid is spheroidene (Goedheer, 1959). Efficiencies of 75 - 100% were recorded for B800 + 850 light-harvesting complexes isolated from several strains of Rps. sphaeroides which differed only in their carotenoid composition (Cogdell et al 1981) see Chapter 1, section 1.5. I suspected that 20% was likely to be lower than the true efficiency because the turbidity of the reconstituted chromatophores made it difficult to obtain an accurate absorption spectrum for normalisation with the fluorescence excitation spectrum. I therefore decided to try to obtain a more accurate value for the efficiency of energy transfer by using B850 light-harvesting complexes isolated from chromatophores containing reconstituted carotenoids.

I obtained optically clear preparations of isolated B850 complex, Fig 4.2 shows the absorption spectrum of the complex with and without neurosporene. The presence of the reconstituted neurosporene is shown by the characteristic three carotenoid absorption bands between 400 and

Figure 4.1

Absorption and fluorescence excitation spectra from R26 chromatophores containing neurosporene

The excitation spectrum (• • •), measured at 858nm, was normalized to the absorption spectrum (——) at the 590nm BChl absorption band. For determination of the efficiency of carotenoid to BChl energy transfer the excitation spectrum was normalized to the fractional absorption spectrum. From the ratio of the carotenoid peak heights in both spectra, the efficiency was calculated to be 20%.

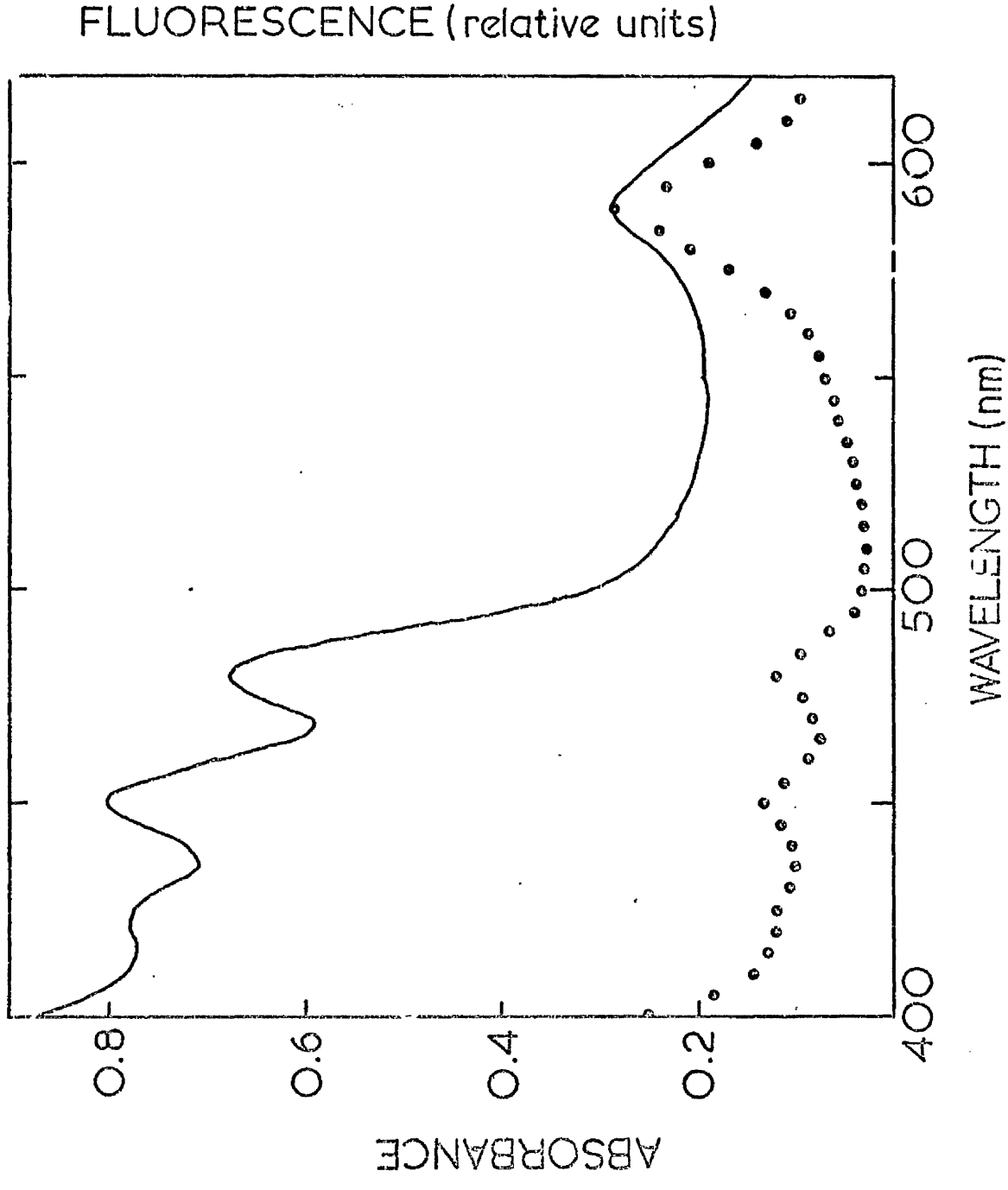
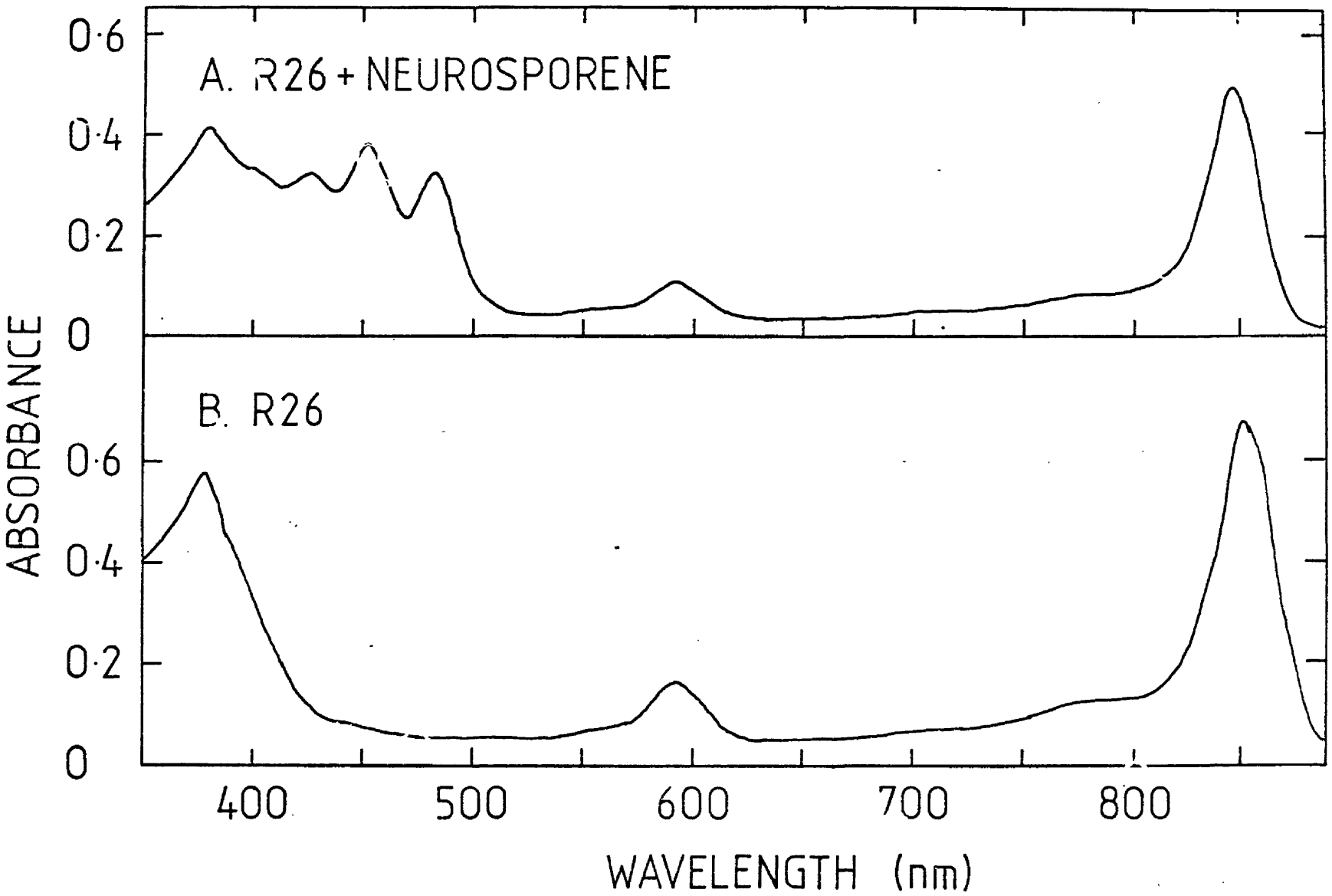

FLUORESCENCE (relative units)
ABSORBANCE
0.8
0.6
0.4
0.2
400
500
600
WAVELENGTH (nm)

Figure 4.2

Absorption spectra of B850 light-harvesting complexes with and without neurosporene

Complexes were isolated from chromatophores (B) and chromatophores containing neurosporene (A).


A. R26 + NEUROSPORENE
B. R26
ABSORBANCE
0·6
0·4
0·2
0
400
500
600
700
800
WAVELENGTH (nm)

500nm. Preparations of B850 complex without carotenoid were blue, if neurosporene was reconstituted they were green, if spheroidene they were brown-green.

If a range of carotenoids could be reconstituted into the B850 complex, the carotenoids would presumably be attached to the same binding sites. The energy transfer properties of the carotenoids could then be directly compared since they would be in identical environments. It was therefore important to show that the reconstituted neurosporene and spheroidene were bound to specific sites on the B850 pigment-protein complex and were not merely randomly attached. There were several reasons for suggesting that specific binding had occurred:

(1) In chromatophores there was a limit to the amount of carotenoid bound per BChl. The maximum carotenoid:BChl ratio was 0.4, this is within a range of values obtained for this ratio in chromatophores from carotenoid containing strains of Rps. sphaeroides.

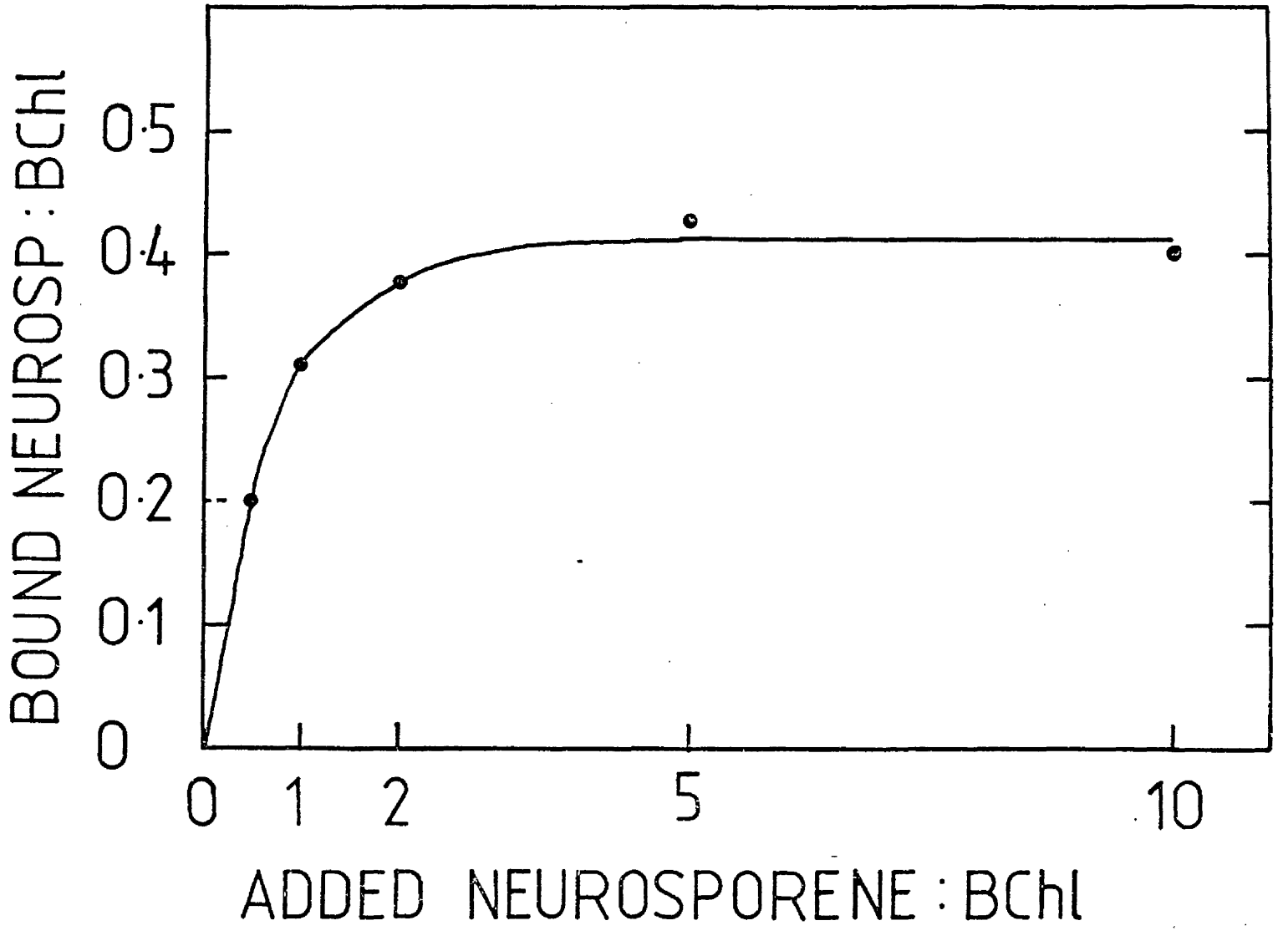
I investigated how the amount of carotenoid bound per BChl varied with the amount of carotenoid added per BChl (see Fig 4.3 for neurosporene). Carotenoids were extracted using the normal methods (see Chapter 2), the amounts present were calculated from the $E^{1\%}_{1cm}$ values. The BChl contents were determined by extraction into acetone/methanol (7/2, v/v) where the extinction coefficient is $76cm^{-1}mM^{-1}$ at 772nm (Clayton 1963). The maximum ratio of bound neurosporene:BChl was 0.4 (i.e. BChl:neurosporene ratio of 2.5:1). This value was reached when carotenoids were added at between 2 and 3 moles of neurosporene per mole of BChl. The saturation of carotenoid binding suggests that there were a finite number of sites to which the carotenoid can bind. The carotenoid:BChl ratio of 0.4 is within the range of 0.38 - 0.59 determined for carotenoid:BChl ratios in chromatophores from several carotenoid containing strains of Rps. sphaeroides (Cogdell and Crofts 1978). This suggests that most of the carotenoid in the chromatophores was bound to pigment-protein complexes.

(2) In reaction centres and light-harvesting complexes the absorption spectra of carotenoids are red-shifted compared with their absorption spectra in organic solvents. For example, in petroleum spirit (o.p. 40 - 60°C) the wavelength of neurosporene's middle absorption band is 438nm, in the B800 + 850 complex from Rps. sphaeroides G.T.C. the middle band is at 465nm (see Cogdell et al 1981). When the pigment-protein complexes are denatured the red shift is reversed as the carotenoids

Figure 4.3

Binding of neurosporene to chromatophores of Rps. sphaeroides R26 carotenoidless mutant.

Neurosporene in petroleum spirit was added to freeze dried chromatophores. After brief sonication the petroleum spirit was evaporated off under vacuum in a rotary evaporator and the chromatophores resuspended in 20mM Tris HCl pH 8.0. Excess neurosporene was washed free by centrifuging at 100,000 x g for 1h (3 times) before determination of the neurosporene: BChl ratio.


BOUND NEUROSP : BChl
0·5
0·4
0·3
0·2
0·1
0
0
1
2
5
10
ADDED NEUROSPORENE : BChl

are removed from binding sites on the protein. The absorption spectra of neurosporene and spheroidene were red shifted when they were incorporated into liposomes, liposomes containing the protein bovine serum albumin (BSA), and the B850 complex. The magnitude of this wavelength shift depended on the environment. This is shown for spheroidene in Table 4.1. When spheroidene was transferred from petroleum spirit to liposomes the spectrum was red-shifted by 11 -15nm, if the liposomes contained BSA the shift was 15 - 19nm, and in the B850 complex the shift was 19 - 22nm. In liposomes plus BSA the longest wavelength absorption band was the most intense, in the other environments the middle band was the most intense. Since spheroidene's non-specific interaction with BSA resulted in a spectral alteration and a smaller red shift than that observed for addition to B850, this suggests that binding to the B850 complex is specific.

(3) Carotenoids in reaction centres and light-harvesting complexes show strong, induced circular dichroism (Boucher et al 1977, Cogdell and Crofts 1978). Denaturation of the complexes disrupts the carotenoid-protein interaction and the carotenoid CD signal disappears. Neurosporene and spheroidene in organic solvents do not show CD but do so when bound to the B850 complex.

CD occurs due to differential absorption of left and right circularly polarised light by asymmetrical molecules (eg. Sauer 1972). Thus carotenoids which are basically symmetrical molecules, do not show CD spectra when they are in organic solvents, but exhibit strong CD spectra when they are bound to proteins. Neurosporene and spheroidene showed strong CD when they were in the B850 complex (Fig 4.4 for neurosporene). This CD was apparent only for the carotenoids in the complex, no CD was recorded for the carotenoids in petroleum spirit, detergent solution, or liposomes plus BSA (Fig 4.5). This strongly suggests that the carotenoids are bound to specific sites on the B850 complex.

(4) The best evidence for carotenoids binding to specific sites on the B850 complex is the efficient transfer of energy from carotenoids to BChl. Both neurosporene and spheroidene transfer energy to BChl with efficiencies between 60 and 70% (see below). Energy transfer between carotenoids and BChl is indicative of specific binding for several reasons. Carotenoids are thought to have excited singlet state lifetimes of less than 1psec (Dallinger et al 1981). For energy transfer to occur within 1 psec the carotenoid and BChl molecules must be held very close relative to each other and have their respective transition moments favourably aligned (Förster, 1959). In a synthetic carotenoporphyrin molecule, energy from the carotenoid part was passed to the porphyrin (equivalent to BChl) with a relatively low efficiency of 25%, but only when the carotenoid

Table 4.1

Absorption maxima of spheroidene in different environments

spheroidene environment	absorption maxima (nm)		
petroleum spirit	427	452	482
liposomes	436	464	497
liposomes + BSA	440	466	500
R26 B850	443	469	504

Figure 4.4

Circular dichroism of the B850 light-harvesting complex with and without neurosporene

(A) Complex isolated from chromatophores reconstituted with neurosporene, 6.5μM BChl. OD in carotenoid region 0.4.

(B) Complex isolated from chromatophores alone, 6.5μM BChl.

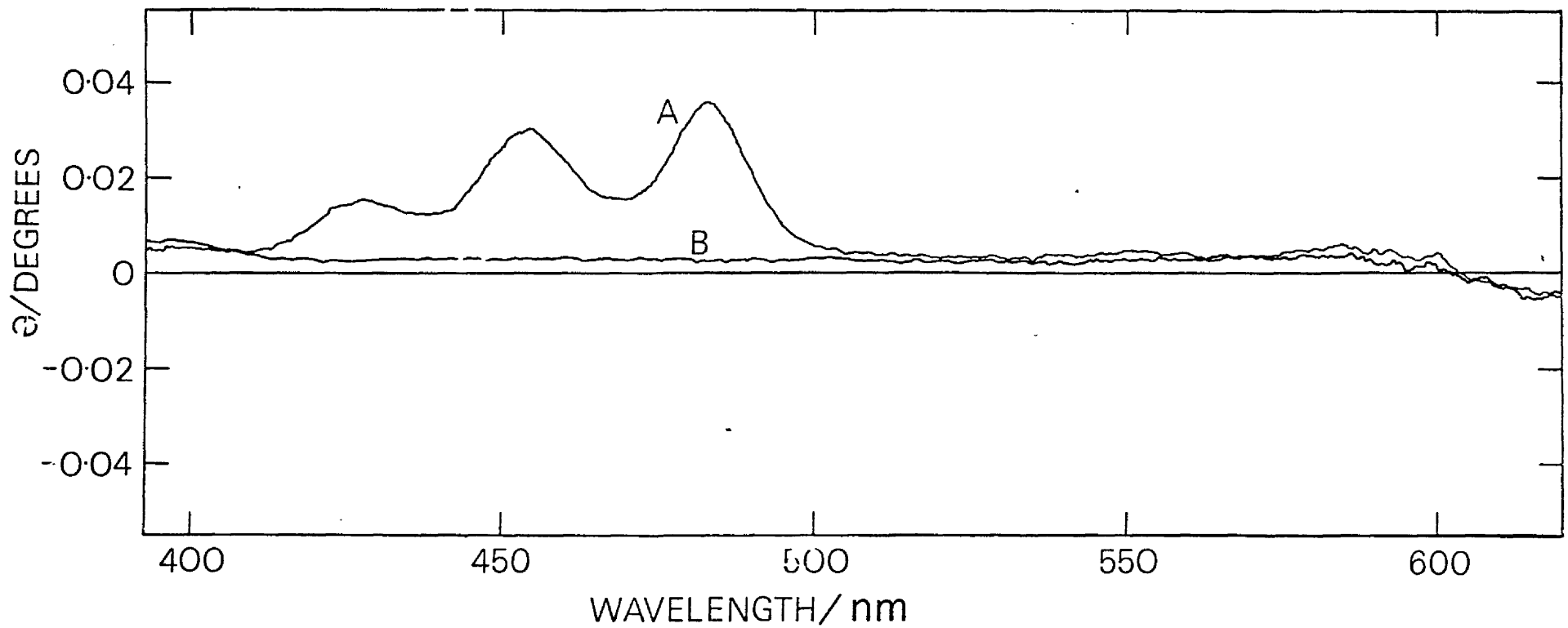

0·04
0·02
0
-0·02
-0·04
θ/DEGREES
A
B
400
450
500
550
600
WAVELENGTH/ nm

Figure 4.5

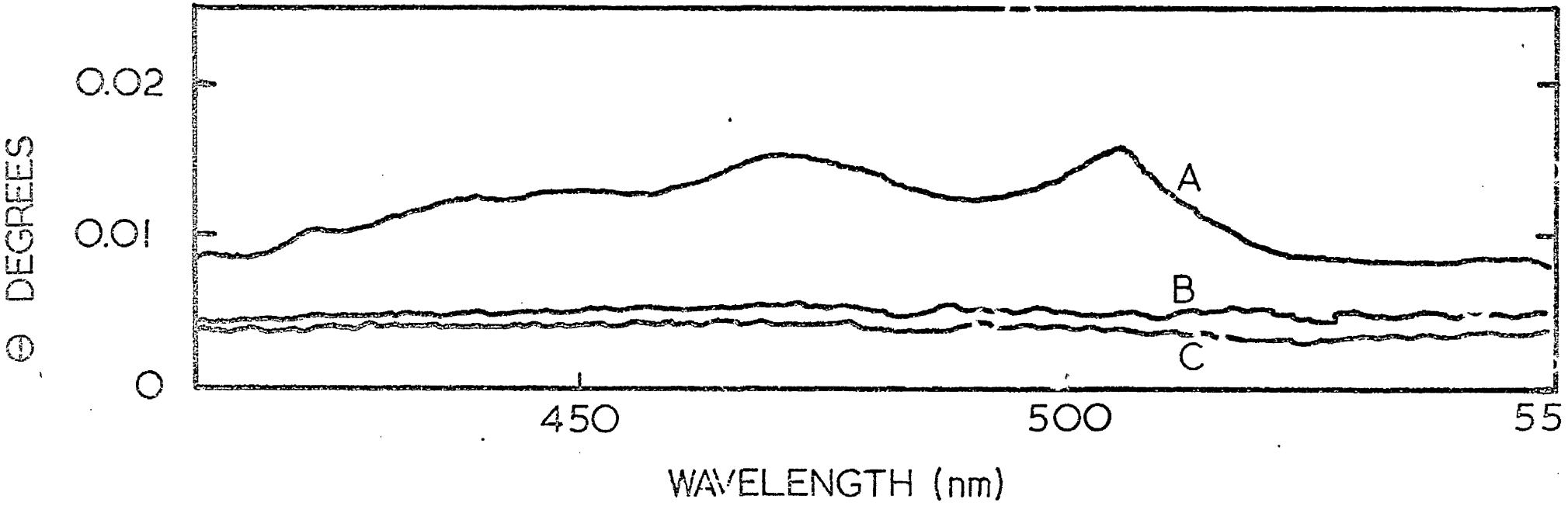
Circular dichroism spectra of spheroidene in different environments

A. R26 B850 light-harvesting complex containing spheroidene

B. liposomes containing spheroidene

C. liposomes containing bovine serum albumin and spheroidene

The optical densities of all samples were approximately 0.4 in the carotenoid region of the spectrum.


θ DEGREES
0.02
0.01
0
A
B
C
450
500
55
WAVELENGTH (nm)

conjugated double bond system lay over porphyrin ring at a distance of 4 Å (Moore et al 1980). When the carotenoid extended away from porphyrin, no energy transfer occurred. In a pigment-protein complex, a carotenoid and BChl molecule could only be held at the correct distance apart and at the correct orientation by specific binding sites.

In view of the above evidence it seems reasonable to suggest that neurosporene and spheroidene reconstituted into the B850 pigment-protein complex are bound at a specific carotenoid binding site.

Carotenoids bound to reaction centres and light-harvesting complexes assume different shapes or configurations. In reaction centres they are bound in a cis configuration ie. they are bent (Boucher et al 1977, Lutz et al 1978). In light-harvesting complexes the carotenoids are all-trans, ie. the molecules are straight (Lutz et al 1978). The configurations of these carotenoids were determined by resonance Raman spectroscopy. In order to verify that carotenoids reconstituted into the B850 complex assume the normal all-trans configuration, the resonance Raman spectra of the complexes have been recorded.

Resonance Raman spectra are recorded following laser illumination of a sample at the wavelength of one of its absorption maxima. The scattered light is collected and analysed for frequency and intensity. Some of the photons of the exciting light exchange energy with vibrational states of the sample molecules, these photons become 'Raman photons'. The difference in energy ($\Delta\nu$) between the exciting and scattered light can provide information about the vibrational energy levels of the sample molecules (see Carey 1978 for a brief introduction to resonance Raman spectroscopy).

Resonance Raman spectra of the B850 complex are shown in Fig 4.6. These spectra show that neurosporene and spheroidene are bound in an all-trans configuration to the B850 complex. The resonance Raman spectra have been compared with those of chromatophores and reaction centres, Fig 4.7, in which the carotenoids are all-trans and cis respectively.

Spectra from B850 containing spheroidene show characteristic peaks due to the presence of spheroidene:

band ν^1 = 1530cm^{-1}. This corresponds to the previously described ν^1 at 1540cm^{-1} (Lutz et al 1978). The 10 cm^{-1} difference is due to a recalibration of the recording equipment since 1978. The ν^1 band represents stretching of the C=C double bonds in the polyene chain (Rimai et al 1978).

Figure 4.6

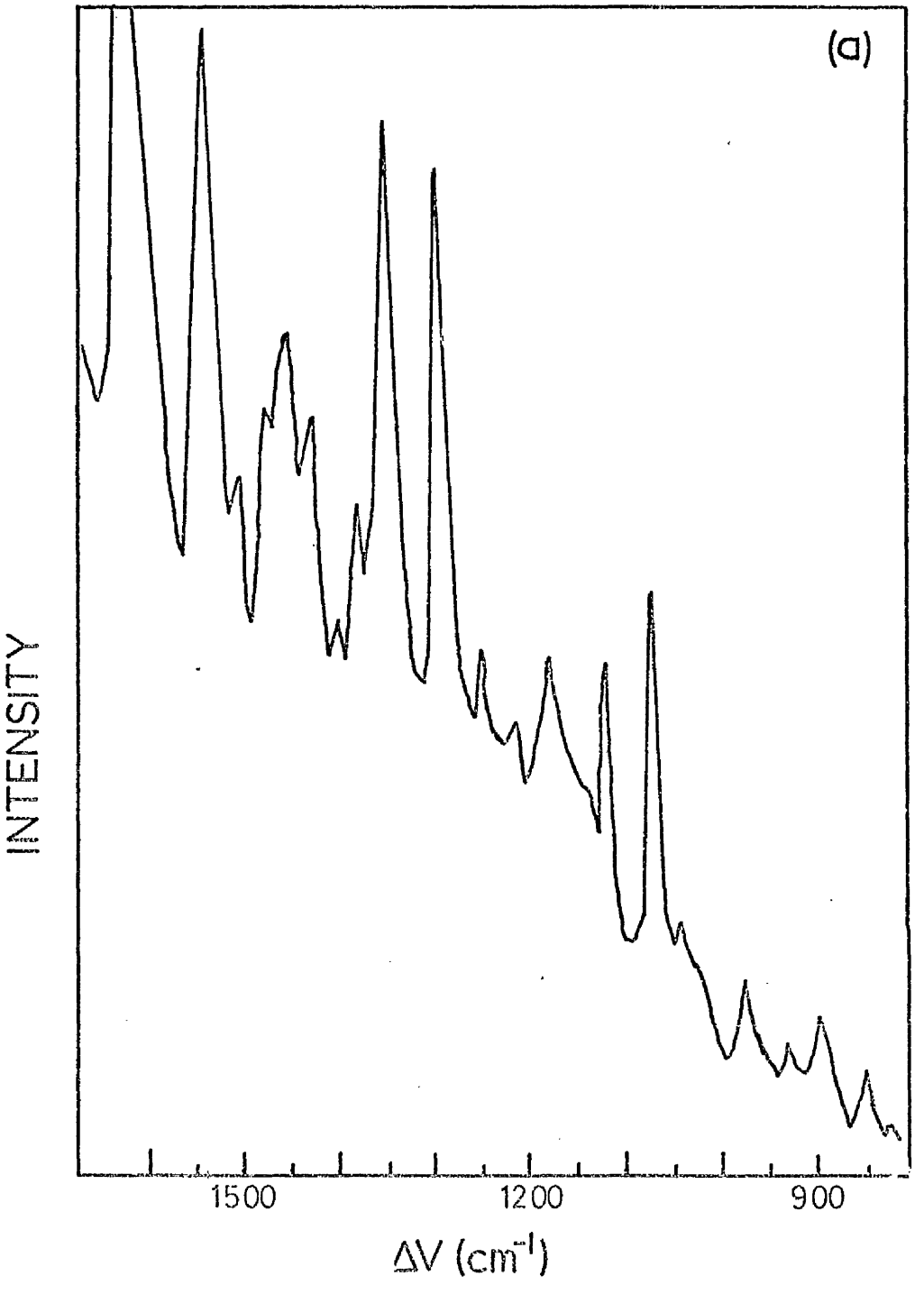
Resonance Raman spectra of B850 complexes with and without carotenoids

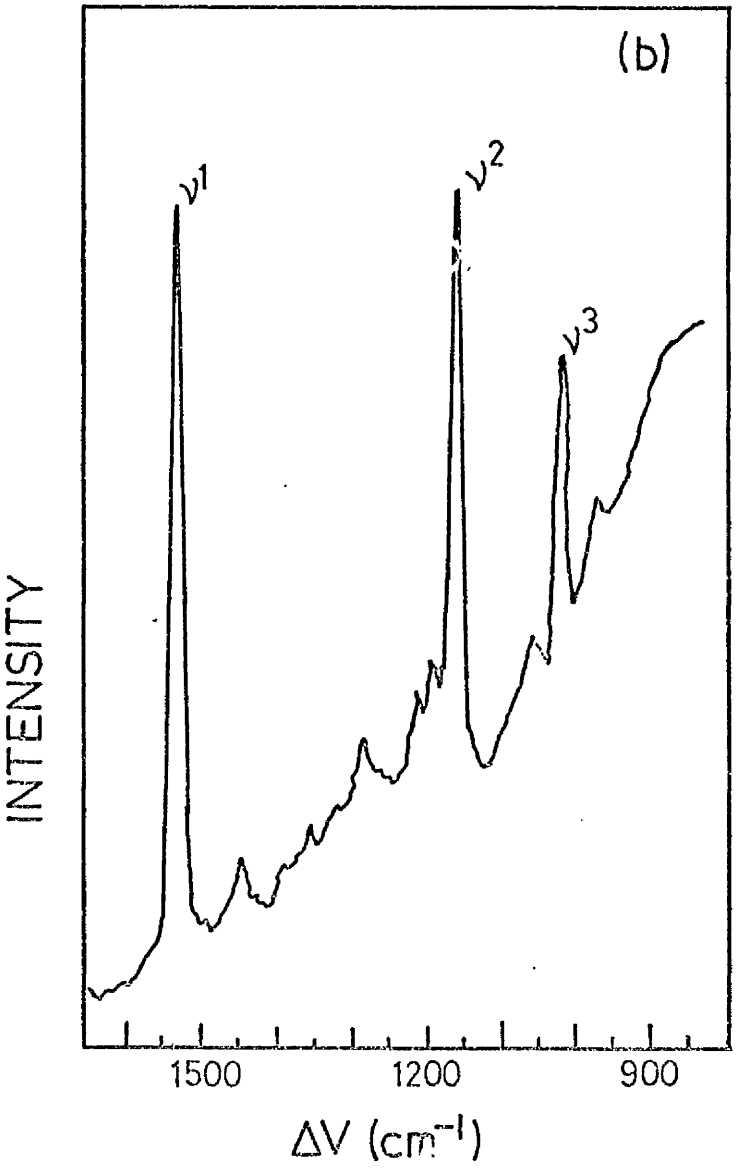
(a) B850 without carotenoids excited at 363.8nm

(b) B850 containing neurosporene, excited at 514.5nm

(c) B850 containing spheroidene, excited at 514.5nm

All spectra were recorded at a temperature of 20k


(a)
INTENSITY
1500
1200
900
ΔV (cm⁻¹)


(b)
ν1
ν2
ν3
INTENSITY
1500
1200
900
ΔV (cm⁻¹)

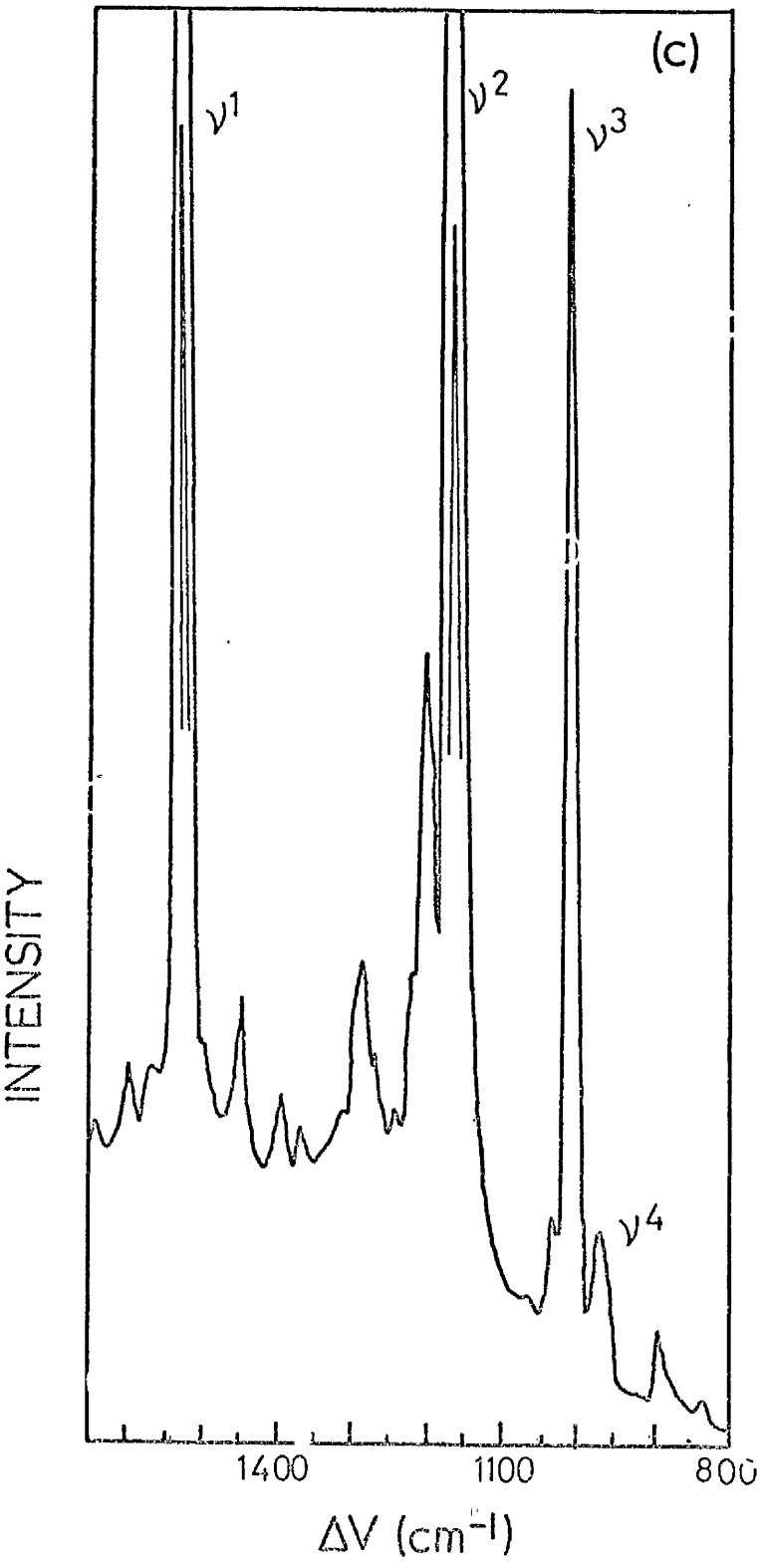

(c)
ν1
ν2
ν3
ν4
INTENSITY
1400
1100
800
ΔV (cm⁻¹)

Figure 4.7

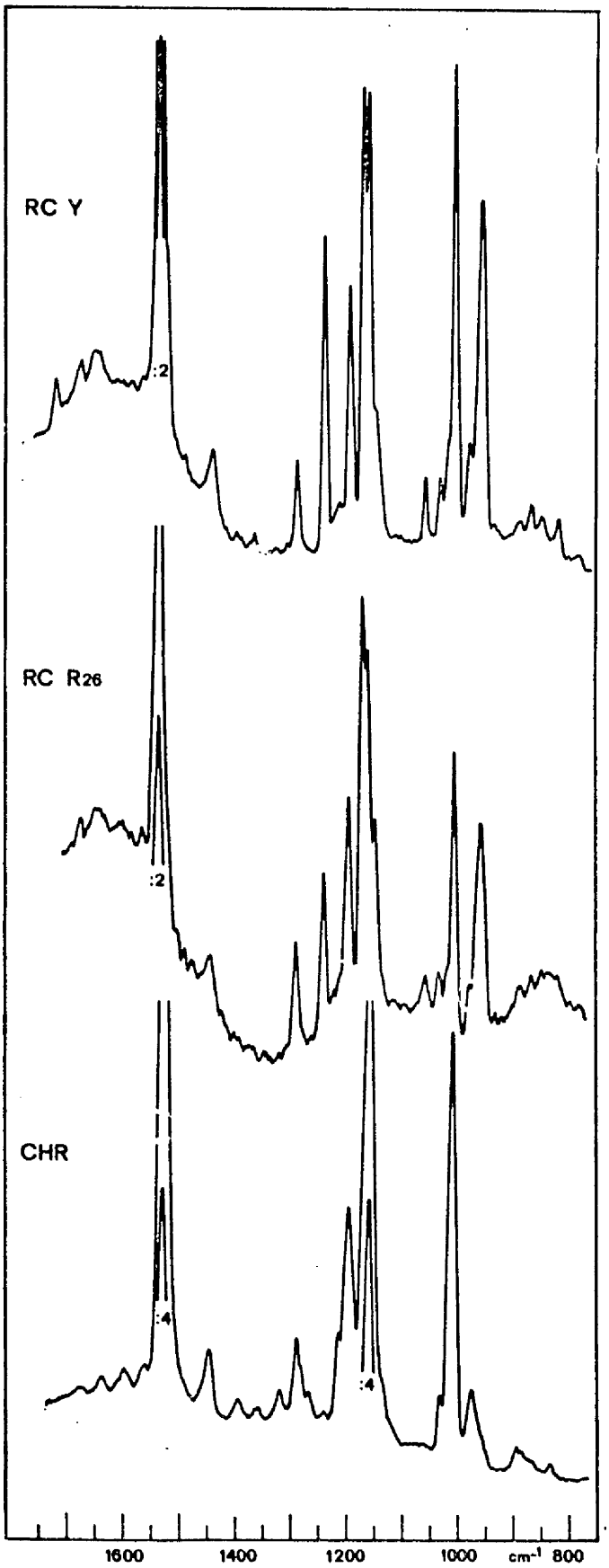
Resonance Raman spectra of spheroidene in cis and all-trans configurations in preparations from Rps. sphaeroides

RCY : wild-type reaction centres, spheroidene in cis configuration

RC R26 : spheroidene bound to R26 reaction centres, spheroidene in cis configuration

CHR : wild-type chromatophores, spheroidene in an all-trans configuration.

The spectra were recorded at 30k , excitation was at 496.5nm.


RC Y
:2
RC R26
:2
CHR
:4
:4
1600
1400
1200
1000
cm-1
800

band ν^2 = 1162cm^{-1}, associated with C-C stretching (Inagaki et al 1975).

band ν^3 = 1006cm^{-1}

band ν^4 = 960cm^{-1}, attributed to out of plane bending of C-H groups (Rimai et al 1971) and possibly also C-CH_3 stretching (Warshel and Karplus 1974).

These bands are not seen in resonance Raman spectra from B850 without carotenoids (Fig 4.6). There are several features of the bands due to carotenoids which, when compared with spectra from cis carotenoids (Fig 4.7), suggest that the carotenoid is present in an all-trans configuration:

(1) The ν^1 band (1530cm^{-1}) would be expected to have a higher frequency if the spheroidene had a cis configuration (Lutz et al 1978).

(2) The ν^2 band (1162cm^{-1}), in spectra from cis carotenoids this band shows two components.

(3) The ν^4 band (960cm^{-1}) is much less intense than the ν^3 band (1006cm^{-1}). In spectra from cis carotenoids the ν^4 band is only slightly less intense than the ν^3 band.

(4) Spectra from cis carotenoids contain bands at 1241cm^{-1} and 1058cm^{-1} these are not present in spectra from B850 containing spheroidene.

Neurosporene and spheroidene were therefore bound to specific sites and in the same conformation as carotenoids found in wild-type light-harvesting complexes. However, were they also present in the same amounts as naturally occurring carotenoids? To investigate the ratio of BChl: bound carotenoid in B850 complexes I used the extinction coefficient (ϵ) for BChl of 20mM^{-1}cm^{-1} at 590nm (Clayton 1963). I determined the extinction coefficients for neurosporene and spheroidene by extracting the carotenoids (as described in Chapter Two) from B850 preparations of known volumes and absorption spectra. Three values were obtained for each carotenoid. Neurosporene 179, 175, 166mM^{-1}cm^{-1} I used ϵ = 175mM^{-1}cm^{-1} at 453nm. Spheroidene 139, 143, 145mM^{-1}cm^{-1} I used ϵ = 140mM^{-1}cm^{-1} at 469nm. To ensure that these values were as accurate as possible I subjected a known amount of carotenoid to the extraction procedures described in Chapter Two. I found that over 96% of the carotenoid was recovered.

In Chapter Three I showed that the B850 complex is a B800 + 850 type of complex lacking the BChl responsible for the 800nm absorption band. In B800 + 850 complexes isolated from carotenoid containing strains of Rps. sphaeroides, the minimal unit of the complexes consists of three BChls and one carotenoid attached to two polypeptides ie. bchl:carotenoid

ratio is 3:1. Two of the BChls make up the 850nm absorption band, the other is responsible for the 800nm band (Cogdell and Crofts 1978, Sauer and Austin 1978). In the B850 complex, lacking the 800nm BChl the maximum BChl:carotenoid ratio would therefore be 2:1. Using the extinction coefficients described above I calculated the BChl:carotenoid ratios in B850 containing neurosporene and spheroidene, the values are shown in Table 4.2. The average ratio in B850 containing neurosporene was 2.3, when the B850 contained spheroidene the average was 2.6. This represents binding to 87% and 77% of the possible sites respectively.

I attempted to reconstitute several other carotenoids into the B850 complex - β-carotene, spheroidenone, lycopene, dihydrolycopene, dihydroneurosproene, and spirilloxanthin. However, only dihydroneurosporene bound to the B850 complex in the same quantity as neurosporene and spheroidene as is shown by the absorption spectra (Fig 4.8). The binding site therefore shows specificity. The structures of the carotenoids are given in Fig 4.9. Since all of the carotenoids had the same chain length the conformation of the chain ends and the substituents at the chain ends determined whether or not a carotenoid could bind. However it is difficult to determine the structural requirements for binding. Spirilloxanthin is similar in structure to spheroidene except that it has 13 double bonds and methoxy groups at both ends of the molecule instead of only one in spheroidene. This might suggest that carotenoids will not bind if there are substituents at both ends. However, Polgar et al (1944) found that spirilloxanthin extracted from Rhs. rubrum (where it is mostly in the all-trans form) was very unstable, most of the molecules were quickly altered to the cis form. It is therefore possible that spirilloxanthin bound poorly since it was mostly in the cis configuration. β-carotene has rings at both ends of the chain, this might again suggest that either ring structures or substituents at the end of the chain prevented carotenoids from binding. It is interesting that the addition of an oxygen atom to spheroidene - thus forming spheroidenone, greatly reduces the ability of the molecule to bind. The binding site may be highly specific. It is difficult to suggest reasons to account for lycopene and dihydrolycopene not binding to the complex since neither have ring formations or substituents which could affect binding.

4.2 Singlet - singlet energy transfer

I wanted to demonstrate that carotenoids incorporated into the B850 complex performed their light-harvesting role ie. absorbed light and transferred the absorbed energy efficiently to BChl. This was accomplished by recording the fluorescence excitation spectra. The complexes were excited at wavelengths between 390 and 620nm using the apparatus shown in Fig 2.5.

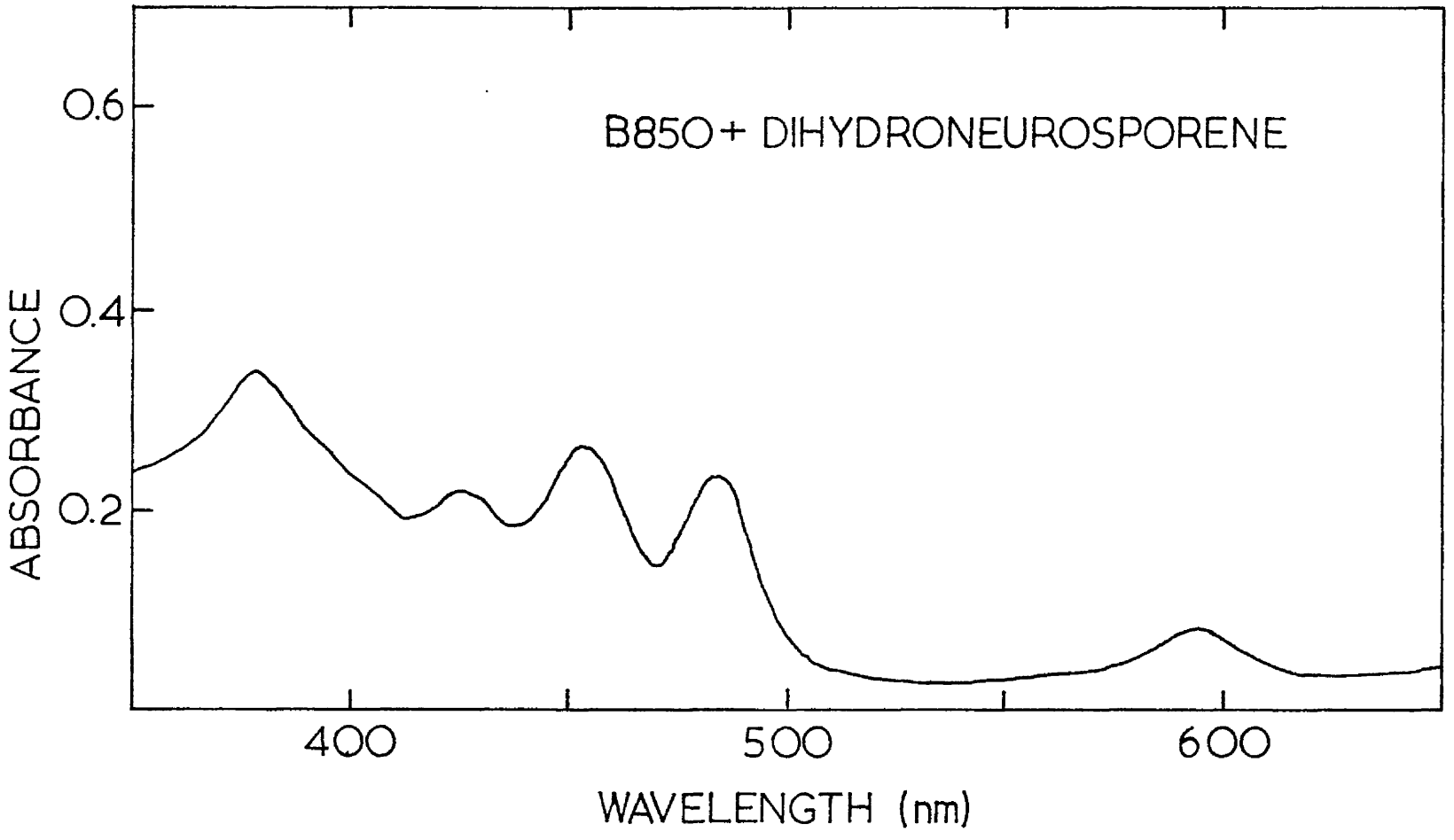
Table 4.2

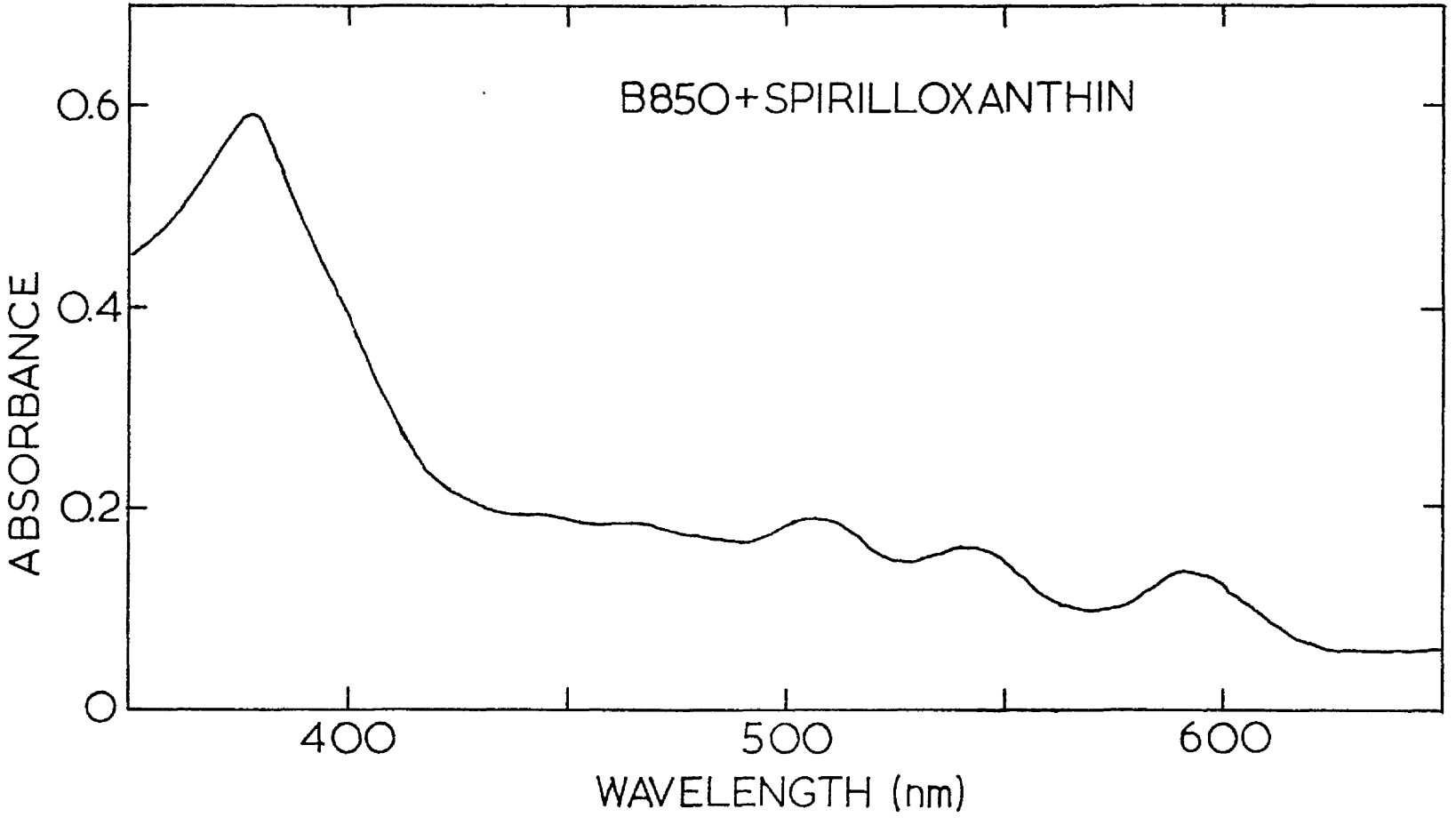
Ratio of BChl:carotenoid in B850 complexes containing neurosporene and spheroidene

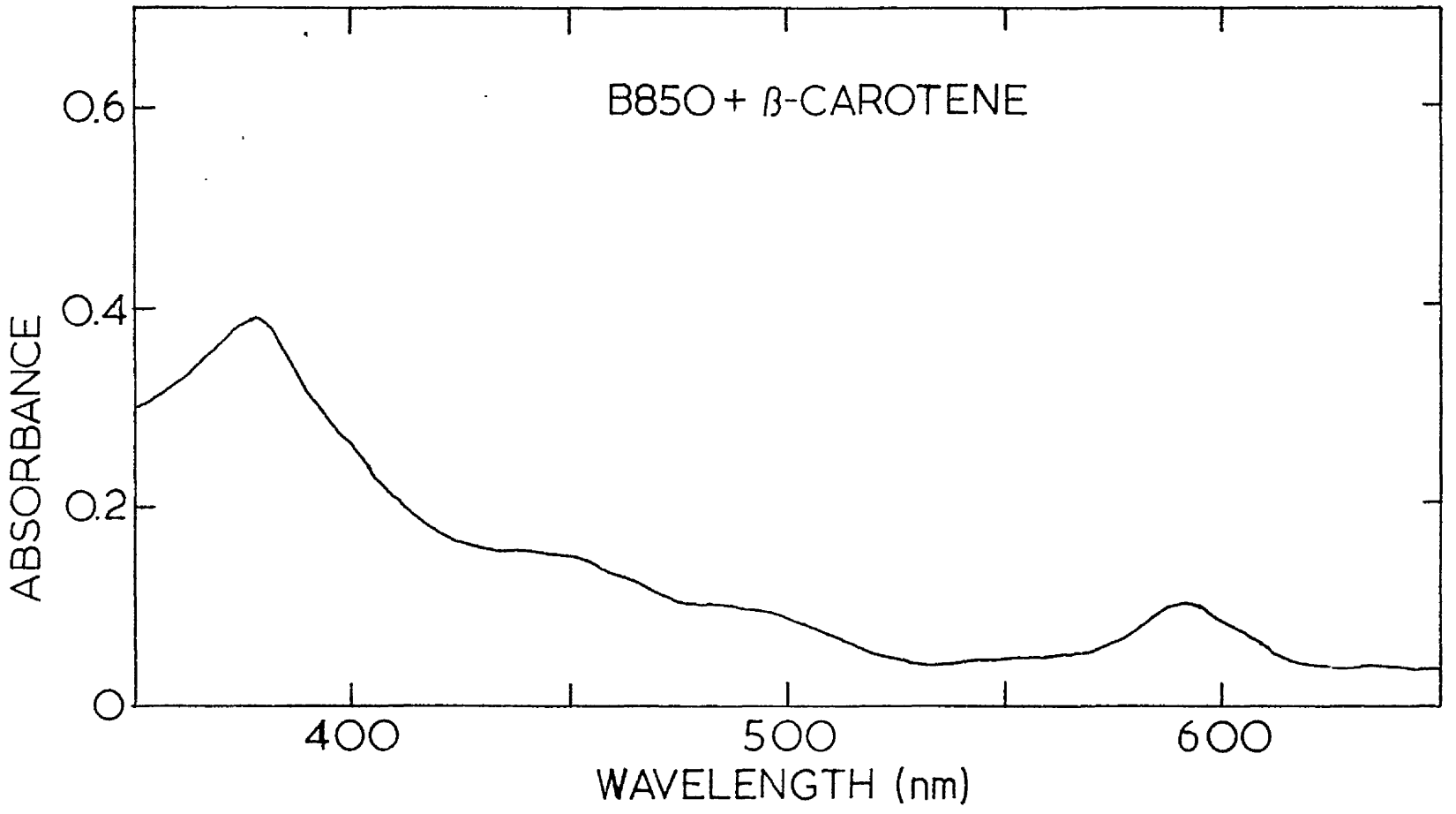
Carotenoid	BChl:carotenoid	Average BChl:carotenoid
Neurosporene	1.98 2.16 2.65 2.4 2.38	2.3
Spheroidene	2.8 2.71 2.39 2.6 2.48	2.6

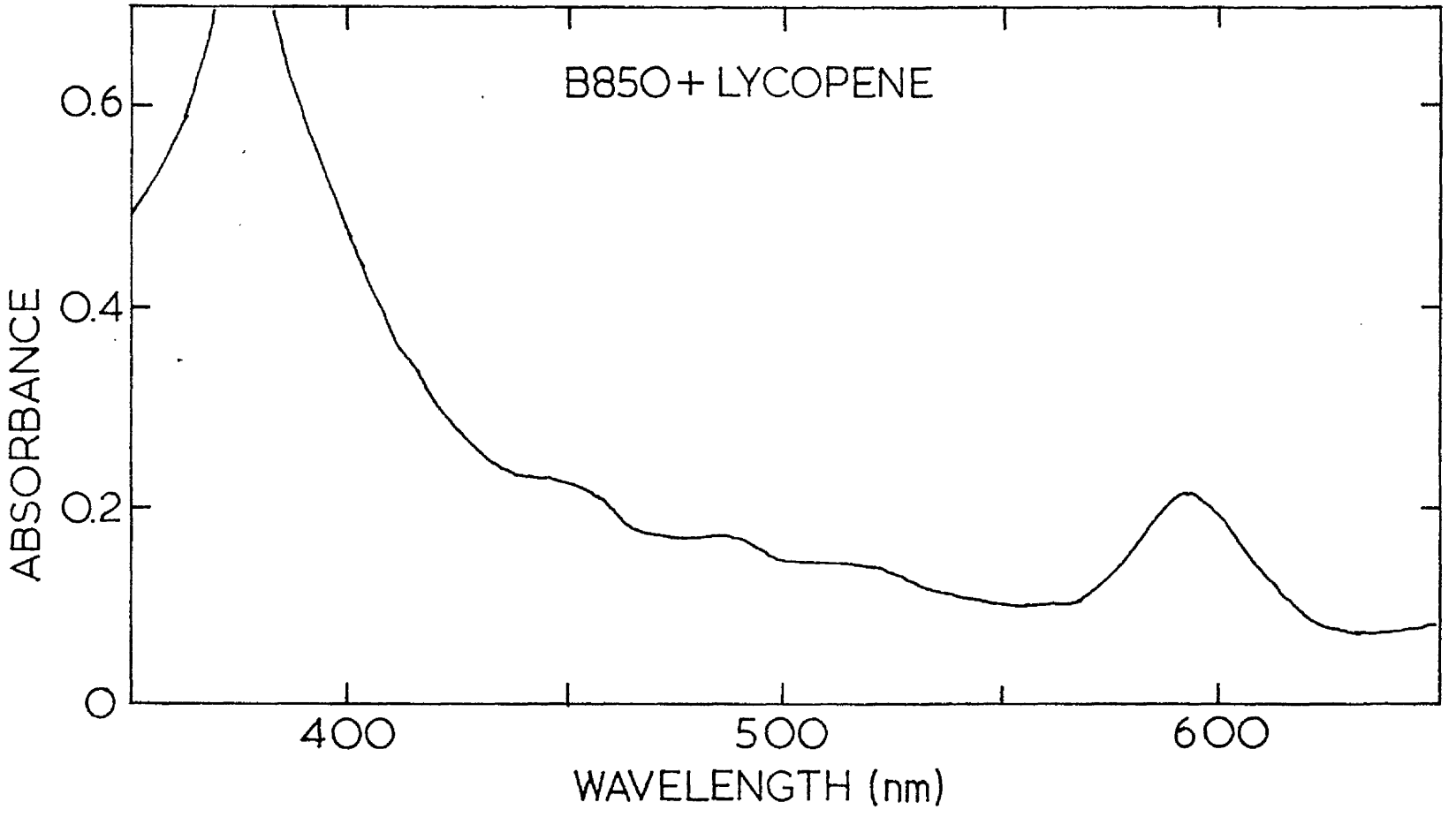
Figure 4.8

Absorption spectra of carotenoids reconstituted into the B850 pigment-protein complex from Rps. sphaeroides R26


B850+ DIHYDRONEUROSPORENE
ABSORBANCE
0.6
0.4
0.2
400
500
600
WAVELENGTH (nm)


B850+SPIRILLOXANTHIN
ABSORBANCE
0.6
0.4
0.2
0
400
500
600
WAVELENGTH (nm)


B850 + β-CAROTENE
ABSORBANCE
0.6
0.4
0.2
0
400
500
600
WAVELENGTH (nm)


B850+ LYCOPENE
ABSORBANCE
0.6
0.4
0.2
0
400
500
600
WAVELENGTH (nm)

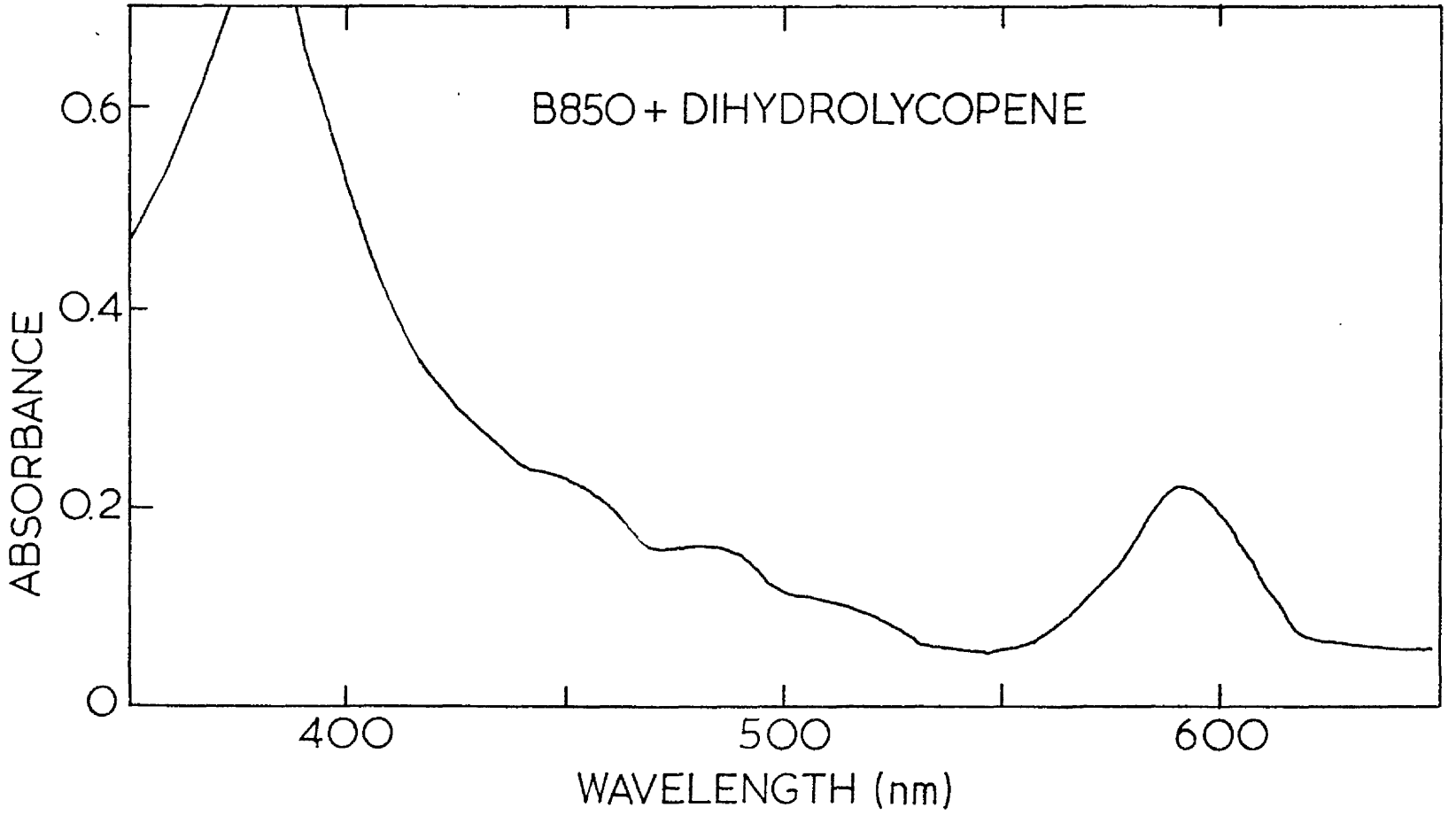
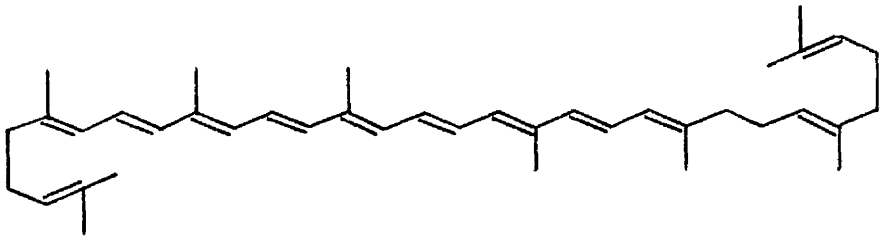

B850 + DIHYDROLYCOPENE
ABSORBANCE
0.6
0.4
0.2
0
400
500
600
WAVELENGTH (nm)

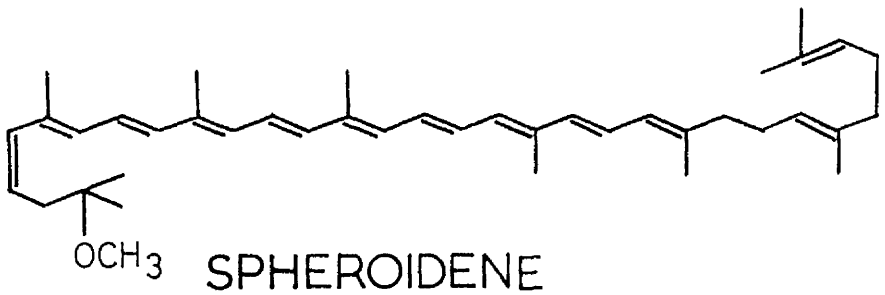
Figure 4.9

Structures of the carotenoids used in this project

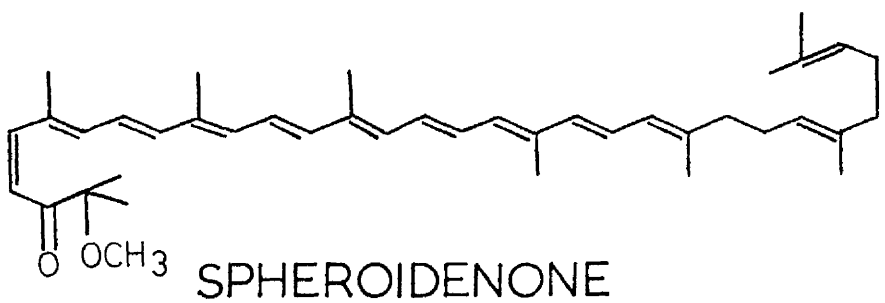
The chain-end conformations are arbitrary.



NEUROSPORENE

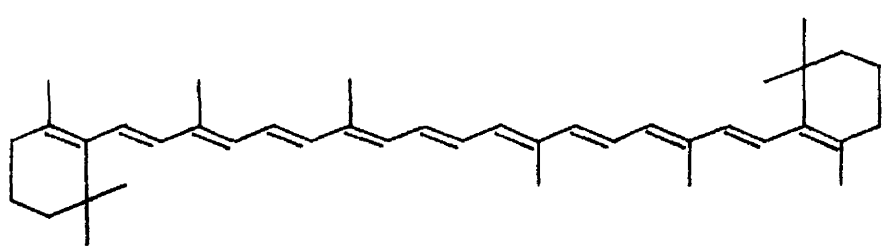


SPHEROIDENE

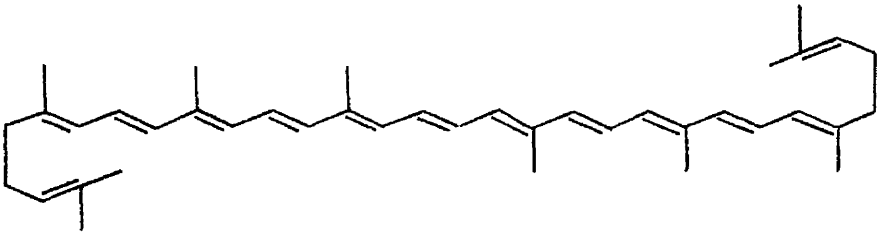


SPHEROIDENONE

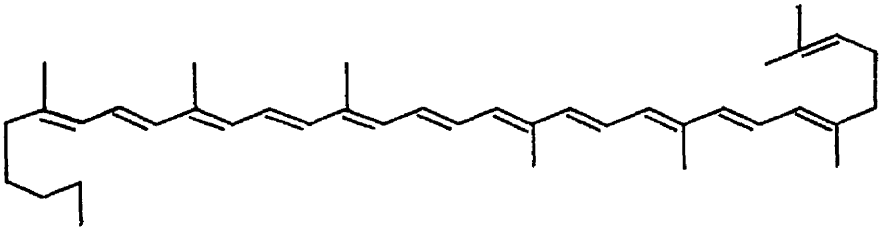
SPIRILLOXANTHIN



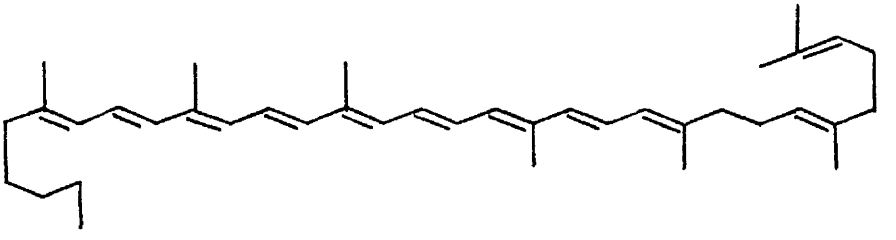
β-CAROTENE



LYCOPENE



1,2-DIHYDROLYCOPENE



1,2 DIHYDRONEUROSPORENE

The amount of fluorescence for each exciting wavelength was recorded and corrected for scattered light and variations in the intensity of exciting light at different wavelengths. The intensity of fluorescence was then plotted against the wavelength of the exciting light to give a fluorescence excitation spectrum. This showed the amount of fluorescence produced by absorption of equal quanta of light at each wavelength. Before the excitation spectrum could be recorded it was necessary to record the fluorescence emission spectrum in order to discover the wavelength at which fluorescence could be monitored for the excitation spectrum. The emission spectrum was recorded using the fluorimeter described in Fig 2.5. When a preparation of the B850 complex was excited with broad band blue light, the fluorescence emission spectra showed a single peak at 857nm (Fig 4.10); this spectrum was not corrected for the transmission characteristics of the monochromator. The emission band represents the fluorescence from the BChl dimer which absorbs at 850nm. The lack of an emission band at 780 - 790nm shows that there was very little free BChl (absorbs at 770nm) in the sample. Free BChl has a much greater fluorescence yield than BChl bound to a pigment-protein complex, so the presence of even a small amount of unbound BChl would have resulted in a large amount of fluorescence. The lack of fluorescence from free BChl is a very good indication that little degradation of the pigment-protein complexes occurred during the isolation procedure.

The fluorescence excitation spectra were recorded for fluorescence emission at 858nm, an 858nm interference filter was placed over the photo-multiplier. The fluorescence excitation spectra from B850 complexes without carotenoids shows a peak corresponding to the 590nm BChl absorption band, increasing fluorescence for excitation wavelengths below 400nm where there is absorption due to the edge of the BChl Soret Band. When the B850 complexes contained neurosporene or spheroidene the fluorescence excitation spectra contained three peaks which corresponded to the absorption bands of the carotenoids. The relative heights of the peaks in the excitation spectrum matched the relative heights of the peaks in the absorption spectrum (Fig 4.11). The presence of these 'carotenoid' peaks in the fluorescence excitation spectrum showed that light absorbed by the carotenoids sensitized BChl fluorescence. Singlet - singlet energy transfer must therefore have occurred between the carotenoids and BChl.

The efficiency of the carotenoid to BChl energy transfer was determined after the fluorescence excitation spectra had been normalised to the sample's fractional absorption spectrum at the 590nm absorption maximum. The efficiency

Figure 4.10

The fluorescence emission spectrum of the B850 light-harvesting complex from Rps. sphaeroides R26

The B850 complex was resuspended in 20mM Tris. HCl pH8.0, 0.05% SDS.

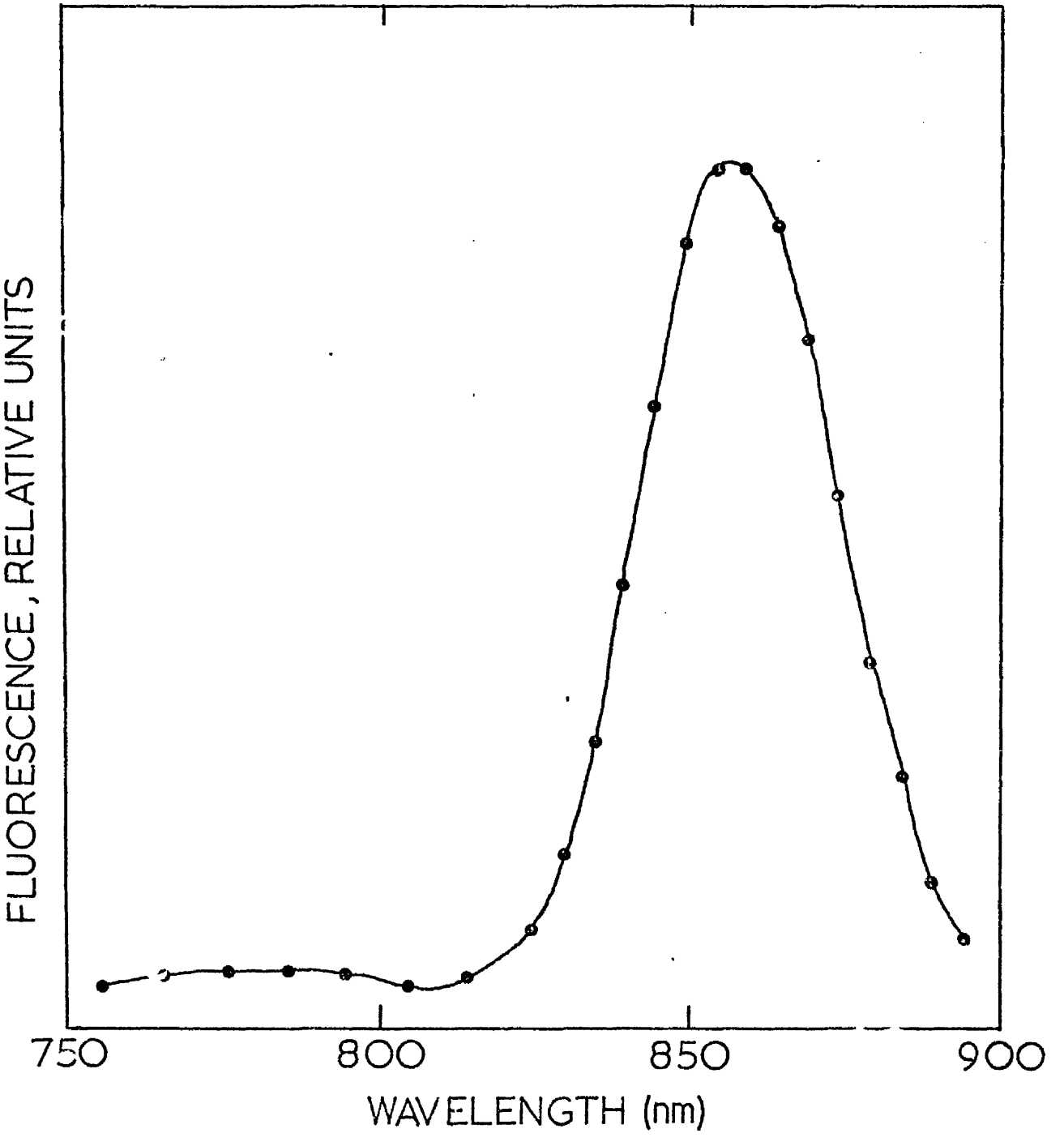

FLUORESCENCE, RELATIVE UNITS
750
800
850
900
WAVELENGTH (nm)

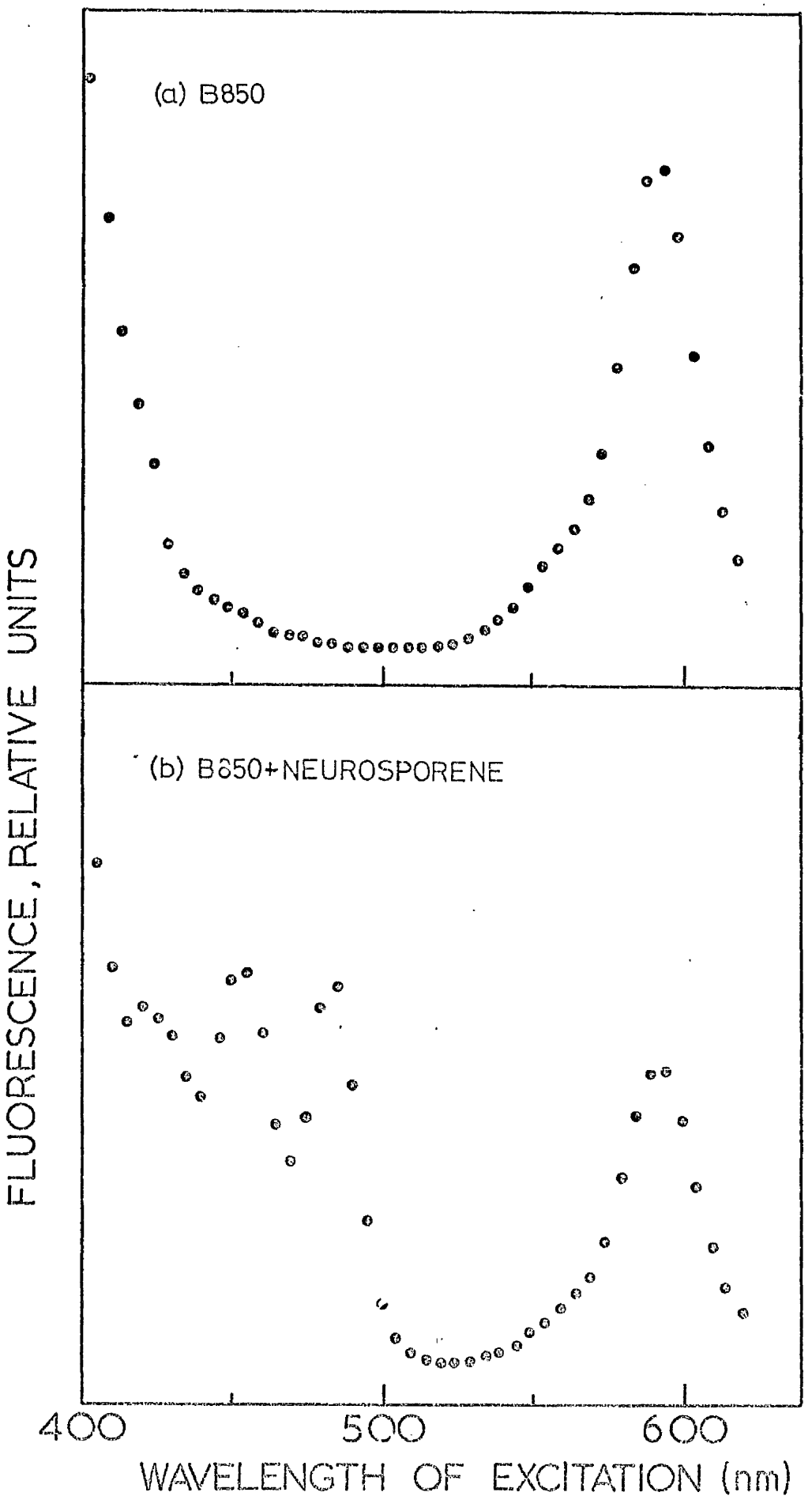
Figure 4.11

Fluorescence excitation spectra of B850 complexes

(a) B850 complexes without carotenoids

(b) B850 complexes containing spheroidene

The excitation spectra were recorded at 858nm.


(a) B850
(b) B850+NEUROSPORENE
FLUORESCENCE, RELATIVE UNITS
400
500
600
WAVELENGTH OF EXCITATION (nm)

of energy transfer was obtained by determining the ratio of the carotenoid peak height in the fluorescence excitation spectrum to the corresponding carotenoid peak height in the absorption spectrum. Figure 4.12 shows the fluorescence excitation spectrum of B850 containing spheroidene, normalised to the absorption spectrum of the complex. The ratio of carotenoid peak heights gives an efficiency of 55%. Goedheer (1959) determined efficiency of energy transfer after normalisation to the absorption spectrum. However, since the fluorescence is plotted on a linear scale and absorbance values are log values then the two cannot be directly compared.

The excitation spectrum should be normalised to the fractional absorption spectrum ie. that proportion of the exciting light which was absorbed by the sample. When this is done for the B850 complex shown in Fig 4.12 then the efficiency of energy transfer increased to 62%.

In the B850 complex both neurosporene and spheroidene transferred energy to the BChl with efficiencies between 60 and 70%. Table 4.3 shows a range of values for the efficiency from a number of preparations containing each carotenoid.

These two carotenoids showed transfer efficiencies of 95 - 100% in B800 + 850 complexes isolated from strains of Rps. sphaeroides. This difference in efficiency could be due to several factors: the B850 complexes could have been adversely affected by the reconstitution procedure - freeze-drying, addition of petroleum spirit, rehydration, isolation using detergent. However, it is likely that the absence of the 800nm BChl is the most important cause of the efficiency falling by approximately 30%. Dallinger et al (1981) showed that the lifetime of the excited singlet state of carotenoids is probably less than 1psec. Therefore if the energy of excited singlet states is not transferred to BChl within 1psec then radiationless decay to the ground state would occur. A carotenoid in its excited singlet state would probably have a greater chance of transferring energy to BChl if there were three adjacent BChl molecules (eg. B800 + 850 complex) than if there were two adjacent molecules (eg. B850).

The other carotenoids which I attempted to reconstitute into the B850 complex were generally very poor at passing energy to the BChl. Dihydroneurosporene, the only other carotenoid which bound in large amounts, had an efficiency of 60%. The others which I investigated - spirilloxanthin, β-carotene, lycopene and dihydrolycopene - gave fluorescence excitation spectra containing only very small bumps in the carotenoid region (Fig 4.13),

Figure 4.12

Absorption and fluorescence excitation spectra from B850 light-harvesting complex containing spheroidene

The excitation spectrum (o o o)$_y$ measured at 858nm, was normalized to the absorption spectrum (——) at the 590nm BChl absorption band. For determination of the efficiency of carotenoid to BChl energy transfer, the excitation spectrum was normalized to the fractional absorption spectrum of the complex.

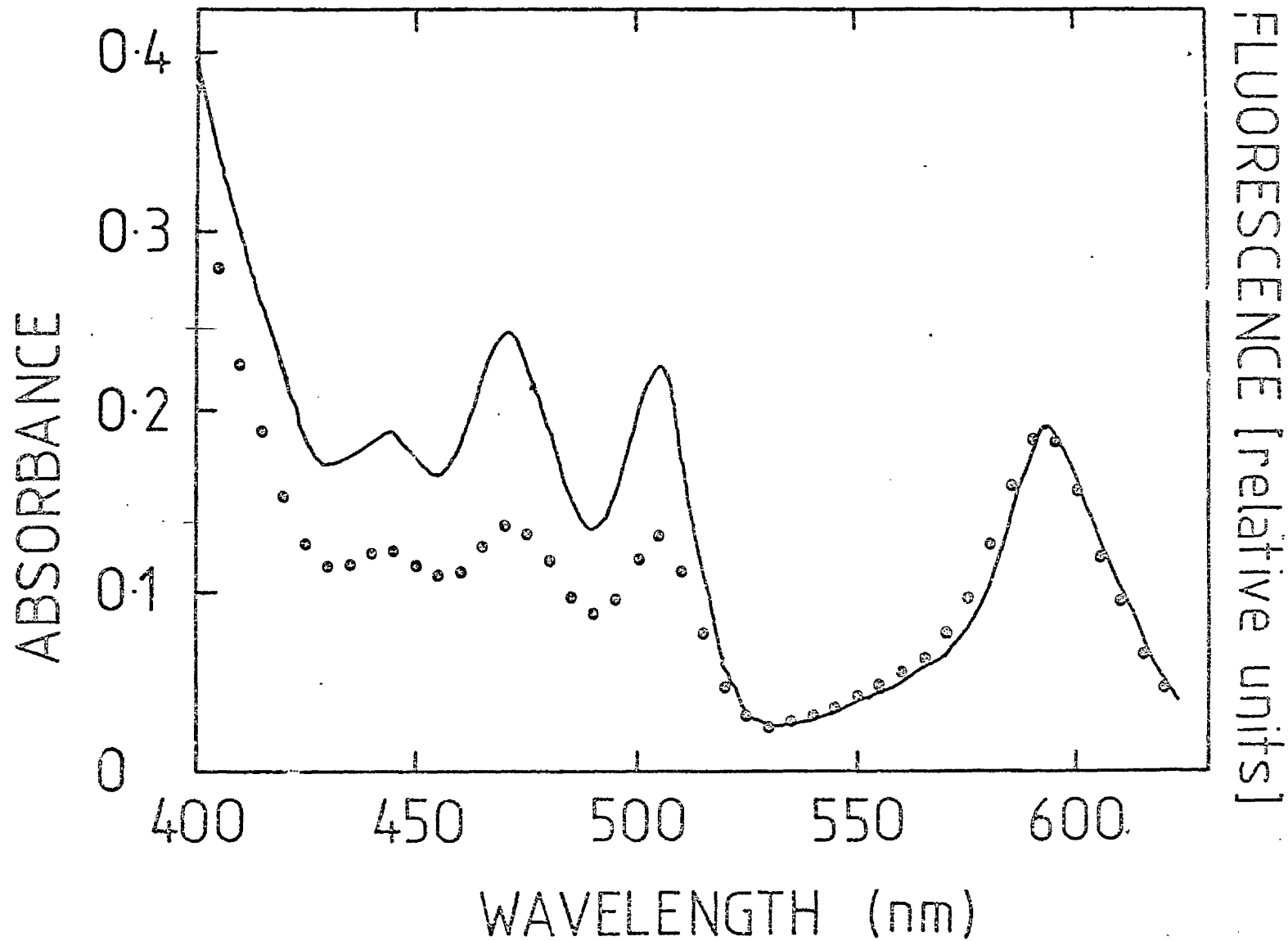

0·4
0·3
0·2
0·1
0
ABSORBANCE
400
450
500
550
600
WAVELENGTH (nm)
FLUORESCENCE [relative units]

Table 4.3

Efficiencies of carotenoid to BChl energy transfer in preparations of the B850 light-harvesting complex containing neurosporene and spheroidene

Carotenoid	% Efficiency
spheroidene	61
	62
	63
	66
	68
neurosporene	60
	62
	63
	64
	65

Figure 4.13

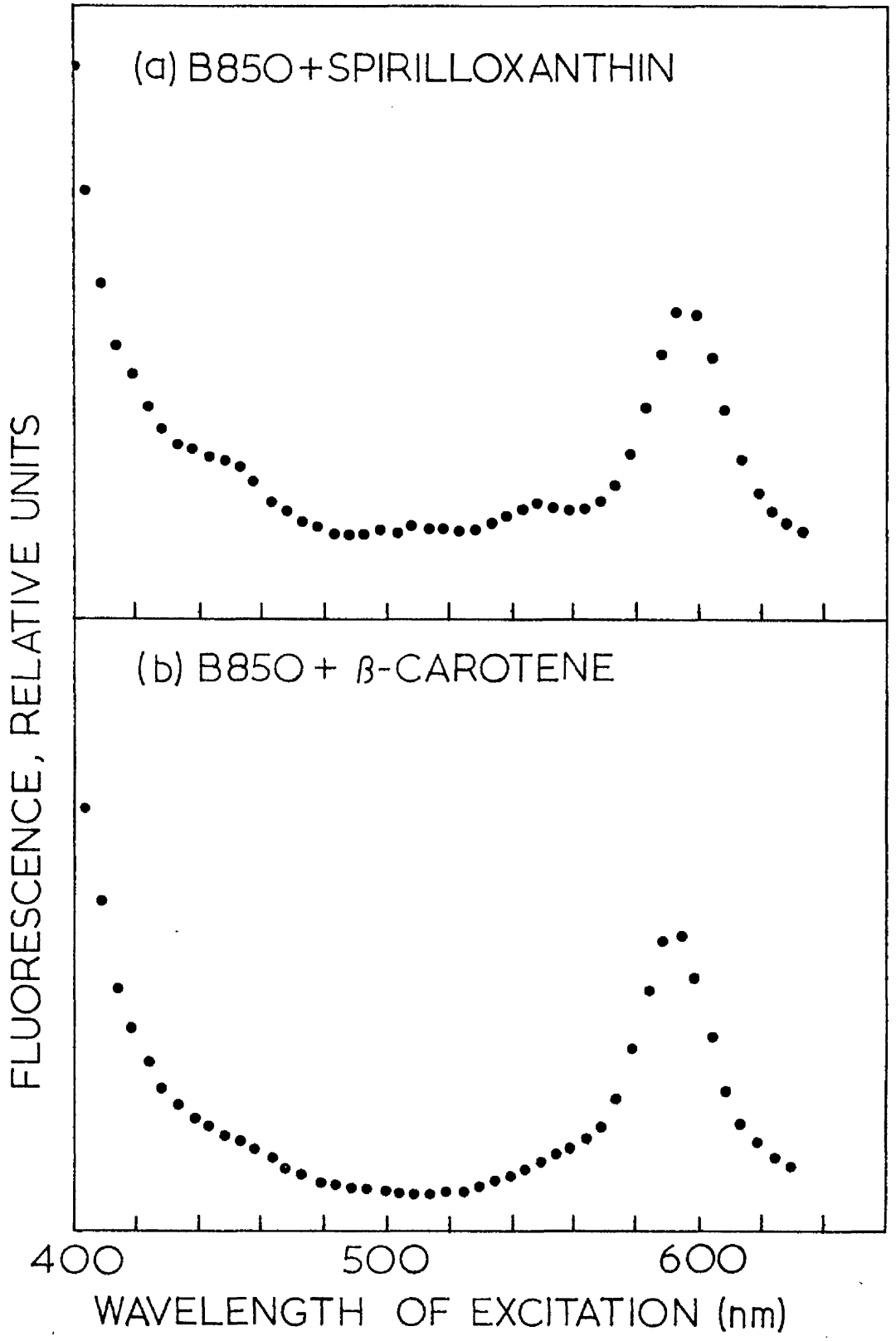
Fluorescence excitation spectra from B850 light-harvesting complexes containing carotenoids

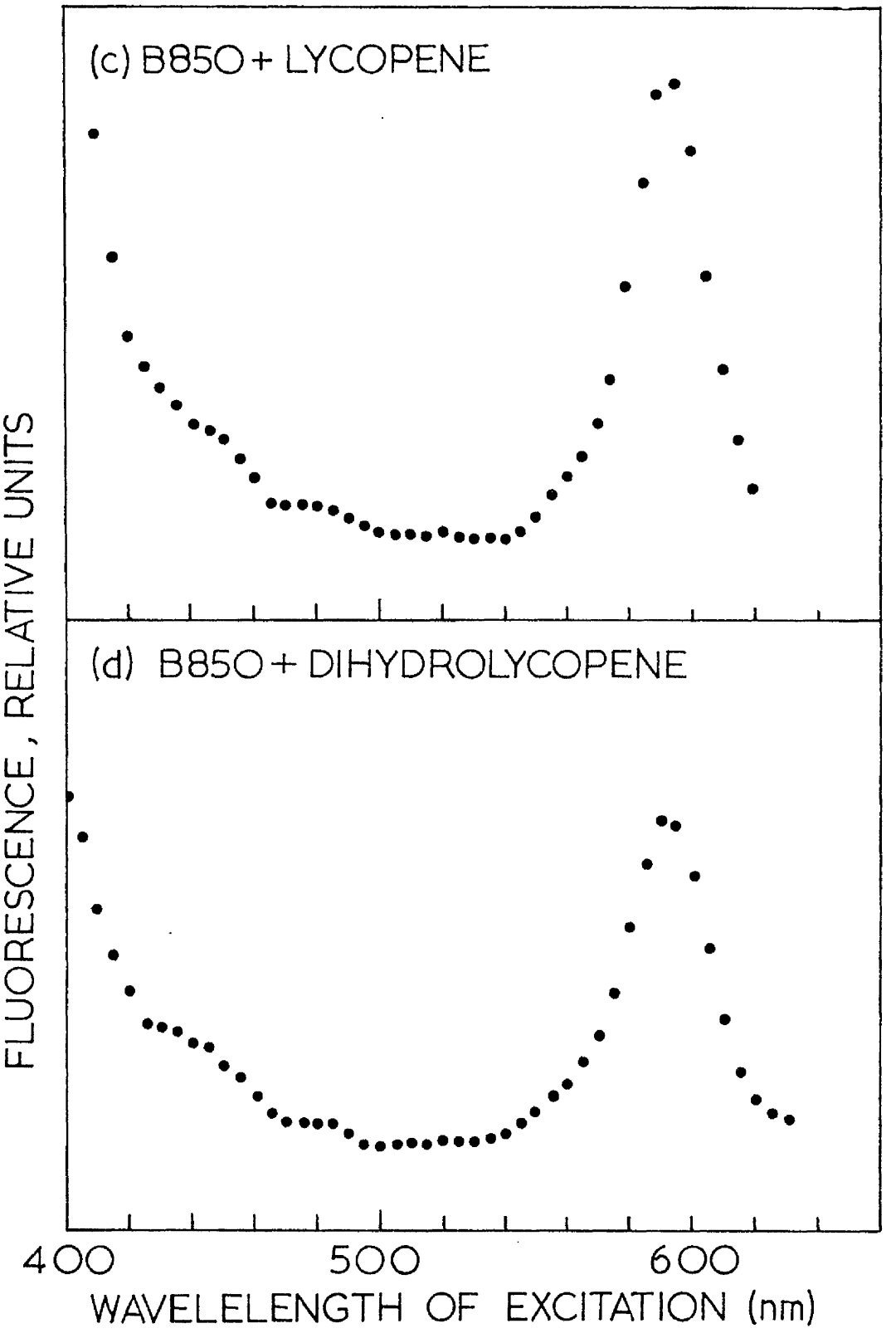
(a) B850 plus spirilloxanthin

(b) B850 plus β-carotene

(c) B850 plus lycopene

(d) B850 plus dihydrolycopene


(a) B850+SPIRILLOXANTHIN
(b) B850 + β-CAROTENE
FLUORESCENCE, RELATIVE UNITS
400
500
600
WAVELENGTH OF EXCITATION (nm)


(c) B850 + LYCOPENE
(d) B850 + DIHYDROLYCOPENE
FLUORESCENCE, RELATIVE UNITS
400
500
600
WAVELELENGTH OF EXCITATION (nm)

unlike the peaks found in the excitation spectra from neurosporene, spheroidene and dihydroneurosporene. Energy transfer from spirilloxanthin to BChl was 20 - 25% efficient, β-carotene, lycopene and dihydrolycopene were only 10 - 15% efficient.

CHAPTER FIVE

TRIPLET - TRIPLET ENERGY TRANSFER FROM BChl TO CAROTENOIDS

In Chapter Four I described the incorporation of neurosporene and spheroidene into the B850 light-harvesting complex from Rps. sphaeroides, R26. These carotenoids bound to specific sites on the pigment-protein complex and passed captured light energy to BChl by singlet - singlet transfer. This carotenoid to BChl energy transfer is recognised as one of the two main functions of carotenoids. The other main function is thought to be the quenching of triplet BChl molecules ($BChl^T$) before they can sensitize the formation of the damaging species of oxygen; $^1O_2$ and O_2^- (see Chapter 1, sections 1.6 to 1.9). Quenching of $BChl^T$ by carotenoids (ie BChl - carotenoid, triplet - triplet energy transfer) has been shown to occur in chromatophores and in isolated reaction centres and light-harvesting complexes (Monger et al 1976, Cogdell et al 1975, 1981). In order to test whether neurosporene and spheroidene were fully functional in the B850 complex I investigated their ability to quench $BChl^T$ in the complex.

Carotenoids and BChl have triplet state lifetimes of microseconds, in order to study them spectrophotometrically it is necessary to use a very brief exciting light pulse. I used a 20nsec light pulse from a Q-switched ruby laser (maximum output 1J) to excite preparations of the B850 complex. The laser could provide excitation at 347nm or 694nm, I used the 347nm output for several reasons:

(1) At 347nm excitation occurs directly into Soret absorption band of the BChl. At 694nm only the edge of the 850nm BChl band can absorb the exciting light (Fig 5.1).

(2) A sample of B850 complex excited at 694nm would have needed to be much more concentrated than another which was excited at 347nm, if both samples were to absorb equal numbers of photons. I therefore used less B850 complex by exciting at 347nm.

(3) If a preparation of B850 complex contained free BChl (absorbing maximally in the NIR at 770nm) then excitation at 694nm would excite a greater proportion of free BChl than excitation at 347nm. This would increase the chances of recording anomalous transient absorbance changes.

There was a possibility that, at 347nm, excitation could have occurred into a small carotenoid absorption band. However, since carotenoid triplet states cannot be formed by direct excitation (Truscott et al 1973) this

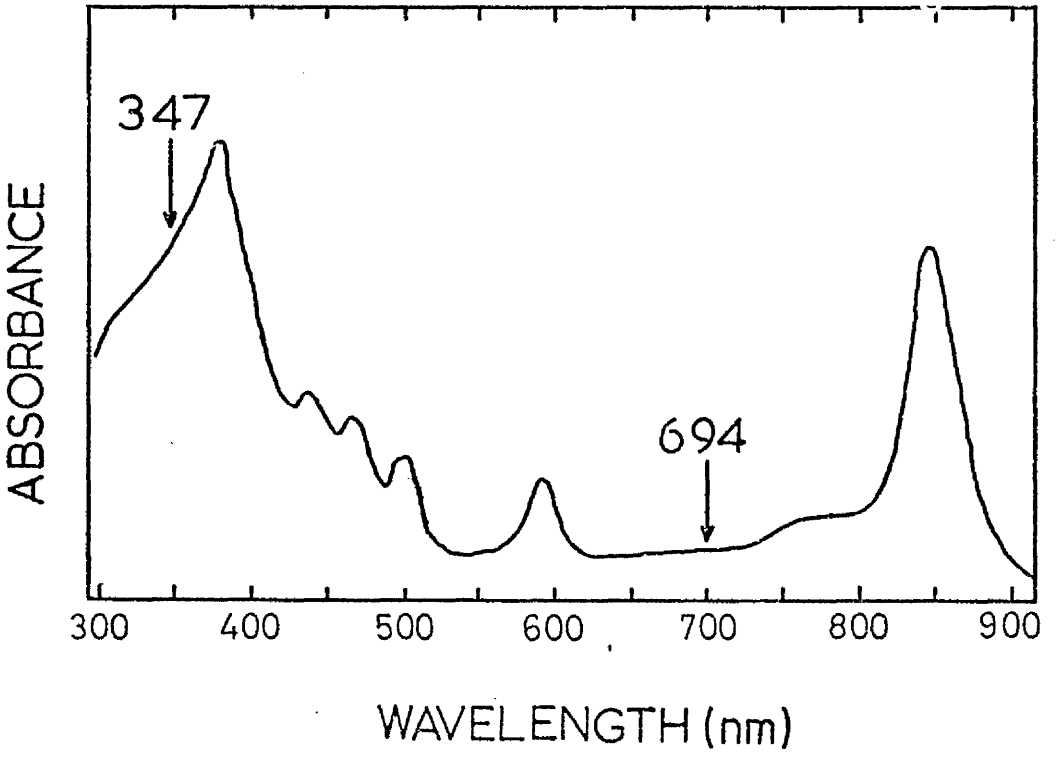


Figure 5.1

Absorption spectrum of the B850 complex showing the two possible wavelengths of laser excitation

would not affect the results which I obtained.

The laser flash photolysis apparatus is shown diagrammatically in Fig 2.6, the B850 complexes were in 10mM Tris. HCl pH 8.0, 0.05% SDS. When a deoxygenated preparation of B850 complex without carotenoids was excited by a laser pulse, a characteristic transient absorbance change (or 'transient') was recorded on the oscilloscope (Fig 5.2). This transient had a decay half-time ($t_{\frac{1}{2}}$) of 12.5μsec. Transients were recorded between 380 and 620nm to give a flash-induced difference spectrum (Fig 5.3). The absorbance changes (ΔA) were calculated as described in Chapter Two and were normalised to a laser output of 1J. The difference spectrum was very similar to previously recorded difference spectra (see Fig 1.7) which had been attributed to BChl in its triplet state (Cogdell et al 1975, Monger et al 1976). The spectrum showed a broad absorption increase from 400 to 580nm and a decrease in absorption ('depletion') between 580 and 595nm and below 395nm. The decreases are caused by bleaching of the absorption bands of ground state singlet BChl. Triplet states of BChl have been shown to have broad absorptions between 380 and 620nm (Connolly et al 1973).

The flash-induced difference spectrum therefore represents a triplet minus singlet spectrum. The previously observed transients due to BChl triplets had decay half-times between 5 and 50μsec. The flash-induced difference spectrum and the decay $t_{\frac{1}{2}}$ of the transient therefore suggested that the transient represented BChl in its triplet state. This was supported by the decrease in decay $t_{\frac{1}{2}}$ to 7μsec if air was bubbled through the solution before excitation. Triplet states are quenched by paramagnetic gases such as O_2 by the process of enhanced intersystem crossing (Porter and Wright 1959, Mathis 1969). This process does not raise the paramagnetic quencher to an excited state.

If the B850 complex contained neurosporene or spheroidene, then a different type of transient was recorded following laser excitation (Fig 5.4a).

The absorbance changes were much larger than those recorded from carotenoidless B850 preparations with the same BChl concentration, and the decay kinetics were different. The decay half-time was 4.3μsec. The flash-induced difference spectra from B850 complexes containing neurosporene or spheroidene (Fig 5.5) are similar to previously described difference spectra of carotenoid triplet states (Cogdell et al 1975, Monger et al 1976, see Fig 1.7). The difference spectra showed bleaching at

Figure 5.2

Transient absorbance change of B850 complex following laser flash excitation

Excitation at 347nm, OD_{347} of B850 complex = 0.3. ΔA recorded at 510nm, laser energy = 0.49J. The solution was deaerated by bubbling with argon.

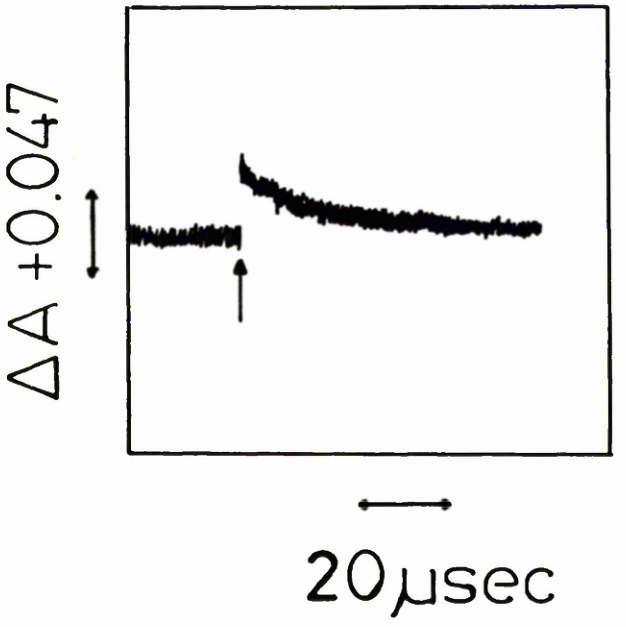

ΔA +0.047
20μsec

Figure 5.3

The flash-induced difference spectrum of the B850 light-harvesting complex from Rps. sphaeroides, R26

The B850 complex was resuspended in 20mM Tris. HCl pH8.0, 0.05% SDS, OD_{347} = 0.3. The sample was excited at 347nm, the laser intensity was 29%.

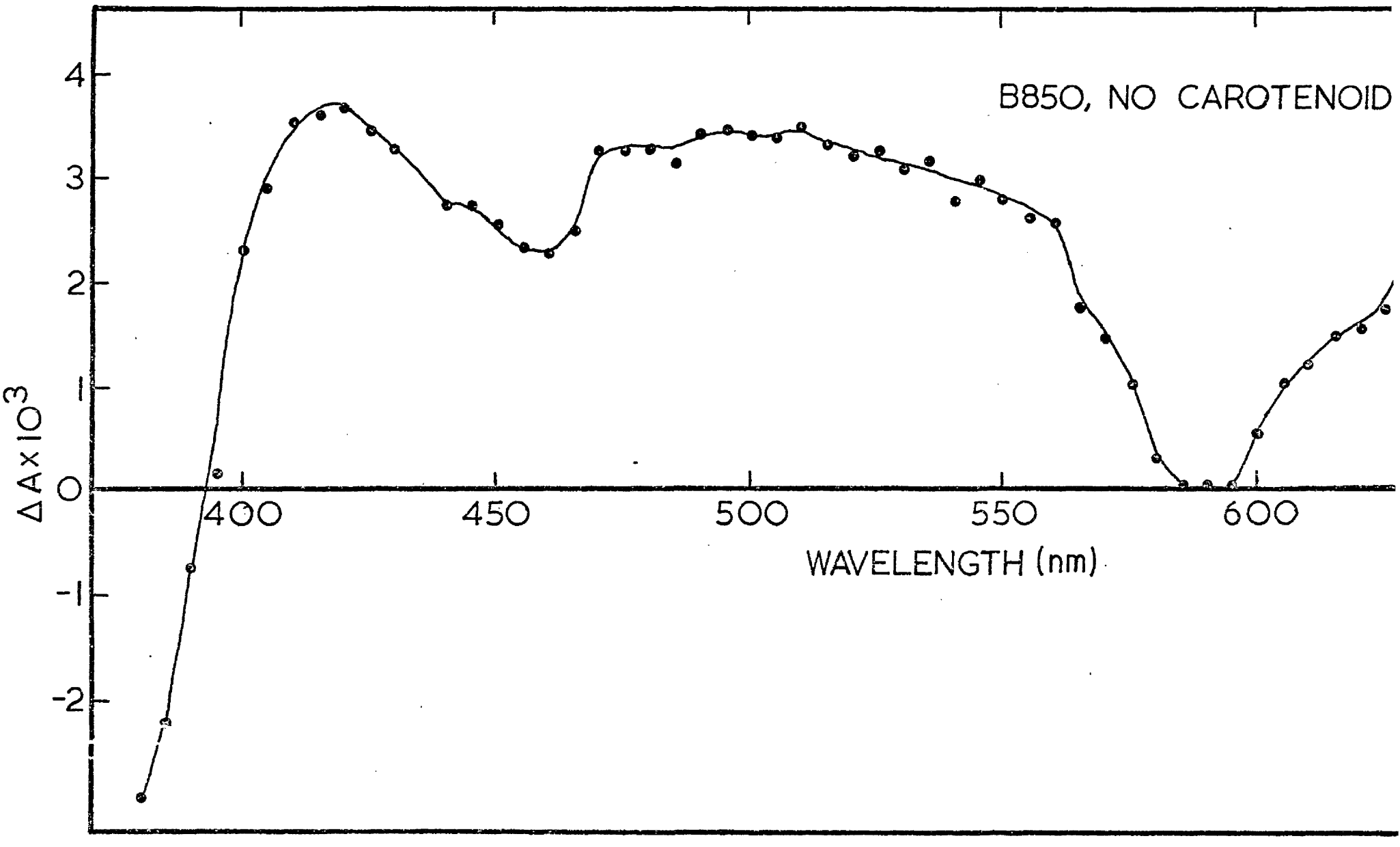

B850, NO CAROTENOID
ΔA x 10^3
4
3
2
1
0
-1
-2
400
450
500
550
600
WAVELENGTH (nm)

Figure 5.4

Transient absorbance change of B850 containing neurosporene following laser flash excitation

(a) Solution deaerated by bubbling with argon before excitation

(b) Solution aerated before excitation

Excitation at 347nm, OD_{347} of B850 complex = 0.27. ΔA recorded at 505nm, laser energy = 0.49J

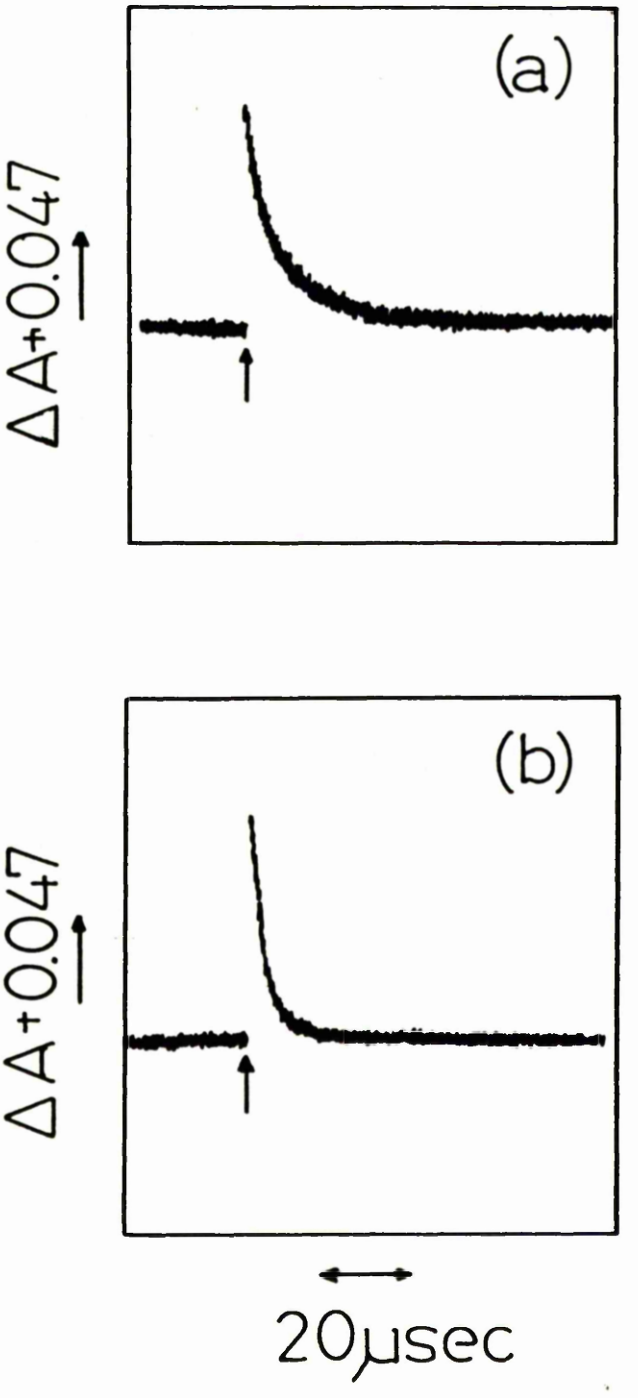
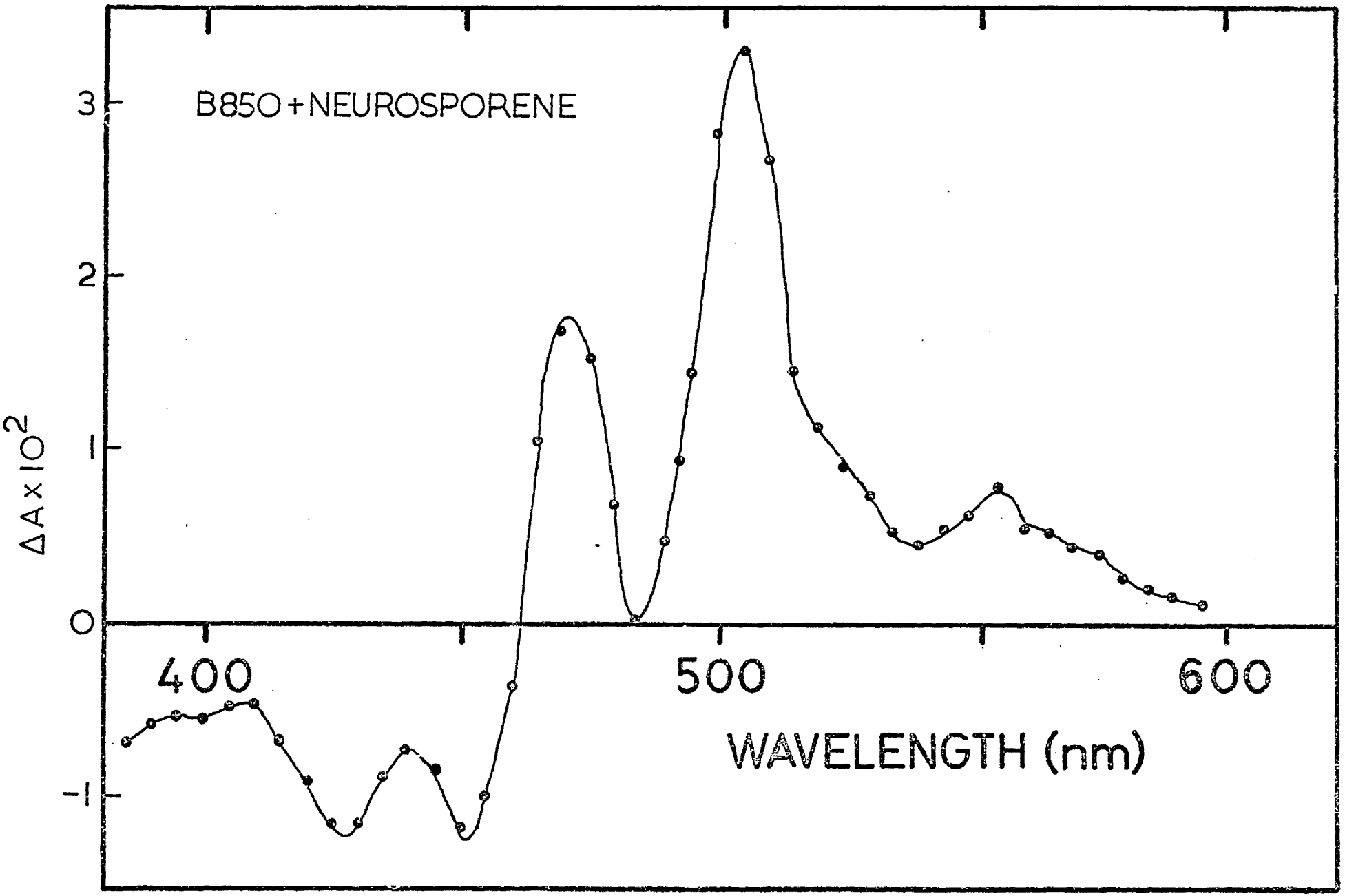
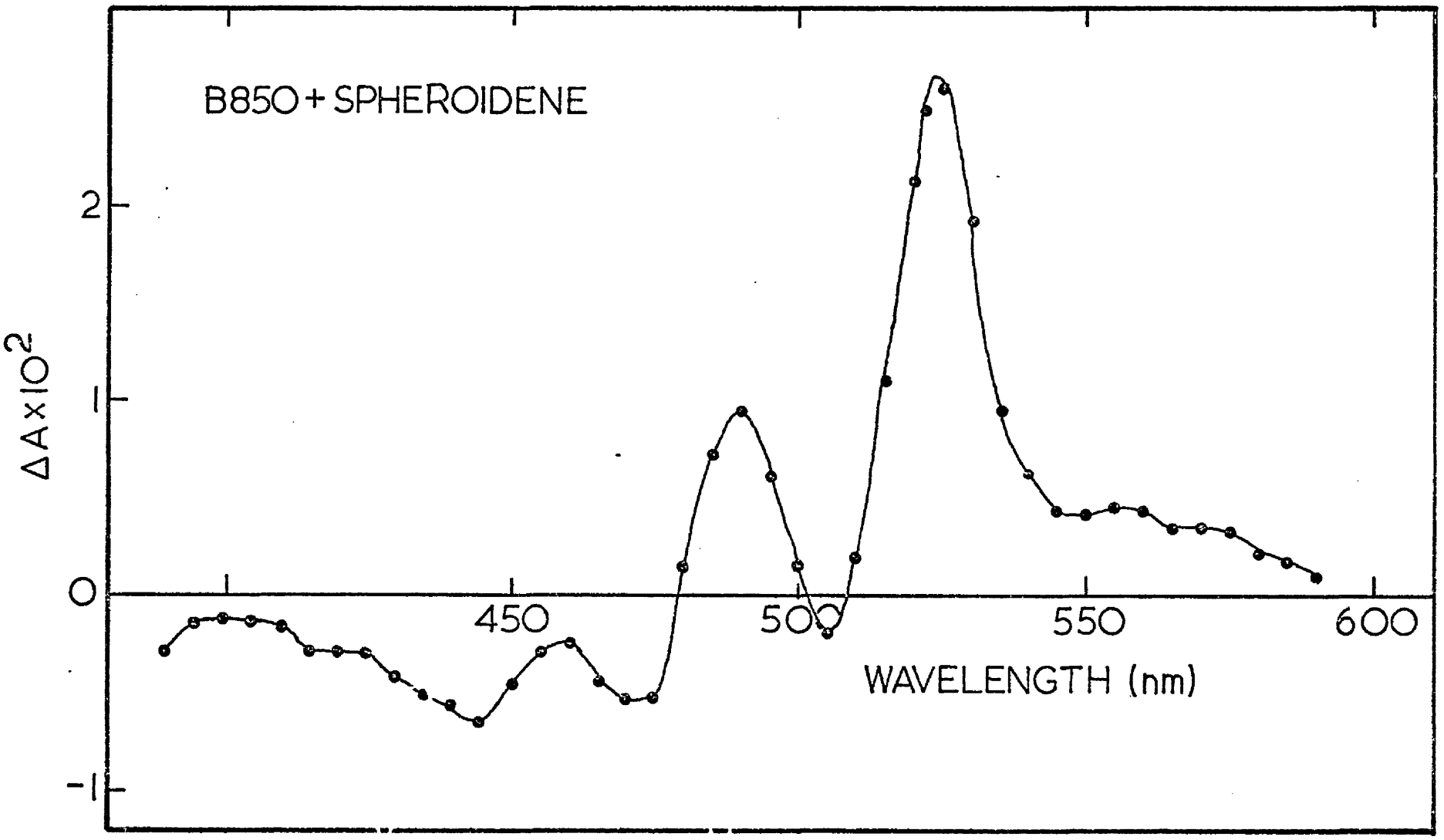

(a)
ΔA+0.047
(b)
ΔA+0.047
20μsec

Figure 5.5

The flash-induced difference spectra of B850 light-harvesting complexes containing neurosporene and spheroidene

The B850 complexes were suspended in 20mM Tris. HCl pH8.0, 0.05% SDS, OD_{347} approximately 0.3. Samples were excited at 347nm with a laser intensity of 29%.


B850+NEUROSPORENE
3
2
1
0
-1
ΔA x 10²
400
500
600
WAVELENGTH (nm)


B850 + SPHEROIDENE
ΔA x 10²
2
1
0
-1
450
500
550
600
WAVELENGTH (nm)

wavelengths corresponding to the carotenoid singlet absorption bands, and the formation of two new bands with a particularly intense band between 500 and 530nm. The absorption spectra of carotenoids in their triplet states (like the absorption spectra of singlet carotenoids) shift to longer wavelengths as the number of conjugated double bonds in the molecule increases (Truscott et al 1973, Mathis and Kleo 1973). This was also a feature of the difference spectra from the B850 complexes containing carotenoids (Fig 5.5). When neurosporene (9 conjugated double bonds) was present in the complex, the most intense absorption band absorbed maximally at 505nm. When spheroidene (10 conjugated double bonds) was present the maximum absorption was at 525nm.

When air was dissolved in the preparations of B850 containing carotenoid, the decay $t_{\frac{1}{2}}$ fell from 4.3μsec to 2μsec (Fig 5.4b). These values were similar to previously recorded decreases in carotenoid triplet decay half-times under similar conditions - 8.6μsec to 3.2μsec (Monger et al 1976) and 4.1μsec to 2μsec (Cogdell et al 1981). The larger absorbance changes in B850 complexes containing carotenoids are due to the triplet extinction coefficients of carotenoids being larger than that of BChl.

At 347nm laser excitation occurs directly into the BChl Soret absorption band; when there are no carotenoids in the B850 complex this excitation results in the formation of BChl triplet states. When neurosporene and spheroidene are present in the complex, laser excitation results in formation of triplet states of these carotenoids, the flash-induced transients, and the spectrum made up of these transients, show no evidence of $BChl^T$ on the μsec timescale (see timescale on Fig 5.4). Since carotenoids cannot be directly excited to their triplet states (Truscott et al 1973) these observations suggest that the carotenoid triplet states are formed by triplet - triplet transfer from the BChl triplet states, ie. the carotenoids quench $BChl^T$.

The flash-induced difference spectrum from B850 containing neurosporene showed no ΔA at 485nm when the ΔA's were recorded on the μsec timescale. However, on a sub - μsec timescale (20 times faster than the scale shown in Fig 5.4) a small transient was observed with a decay $t_{\frac{1}{2}}$ of approximately 500nsec. This value for the $t_{\frac{1}{2}}$ was unlikely to be accurate since it was recorded at the limits of the apparatus. It seems reasonable to suggest that this transient represented $BChl^T$ and that the rapid decay was due to quenching by neurosporene. A similar transient (deeay $t_{\frac{1}{2}}$ approximately 100nsec) has been recorded from the B800+850 complex isolated from Rps. sphaeroides strain G.1.C_9 at a wavelength where there was no absorbance

change due to carotenoid triplet formation (Cogdell et al 1981). This transient was attributed to the BChl triplet state. All of the above evidence clearly suggests that the carotenoid triplet states are formed by triplet - triplet energy transfer from BChl triplet states.

Consider the formation of neurosporene and spheroidene triplet states in the B850 complex. When these two carotenoids were present in the B850 complex, laser flash excitation caused carotenoid triplet formation, there was no evidence on the μsec timescale to suggest that BChl triplets were rapidly quenched by carotenoids, ie. there was efficient triplet - triplet energy transfer between the BChl and the carotenoids. Cogdell et al (1981) have shown that the quantum yield of carotenoid triplet formation ($\phi_{CAR}{}^T$) is determined mainly by $\phi_{BChl}{}^T$. BChl in its triplet state can either transfer energy to the carotenoid or can decay to the ground state (see Fig 1.6). These processes can be given the rate constants K_{tt} and K_{tg} respectively. The yield of carotenoid triplet formation is given by the equation:

$$\phi_{CAR}{}^T = \frac{K_{tt}}{K_{tg} + K_{tt}} . \phi_{BChl}{}^T$$

Monger et al (1976) have shown that, in the absence of carotenoids, K_{tg} was small, $10^5 sec^{-1}$ while K_{tt} (in the presence of carotenoids) was large, $0.5 - 1.0 \times 10^8 sec^{-1}$. If K_{tt} varied by as much as a factor of 100, $\phi_{CAR}{}^T$ would be essentially unchanged, and would be almost exactly the same as $\phi_{BChl}{}^T$. If the carotenoid triplet states seen in the B850 complex were formed by quenching of BChl triplets, then the $\phi_{CAR}{}^T$ and $\phi_{BChl}{}^T$ should be similar.

I compared the values of ϕ_T for BChl, neurosporene, and spheroidene. The ϕ_Ts were determined using a comparative technique (Armand and Bensasson 1975) in which the flash-induced absorbance changes due to triplet formation (ΔA_T) were compared with those of a molecule with known triplet yield (see Chapter Two). It was necessary to know the extinction coefficients for the triplet states (ϵ_T) of BChl and the carotenoids in the B850 complex. These were obtained from the 'complete conversion' method: the ΔA_T at a given wavelength was measured as a function of laser intensity. At higher intensities, no increase in ΔA_T with increasing laser intensity could be taken to imply that all of the BChl or carotenoids had been converted to their triplet states. The ϵ_T at the given wavelength was then obtained by dividing the maximum ΔA_T by the ground state concentration of the relevant molecule.

Cogdell et al (1981) found that the triplet yield in B800+850 pigment-protein complexes varied with the concentration of detergent in the preparation. I tried to overcome any problem due to variation in detergent concentration between preparations by dialysing them against 20mM Tris HCl pH8.0, 0.05%SDS. Any necessary dilutions were made using this buffer solution, whenever possible they were made several days before the preparation was used. This allowed the pigment-protein complexes to adjust to the detergent.

Fig 5.6 shows the complete conversion curves for B850 complexes with and without carotenoids. The ΔA_Ts were recorded at the wavelengths of absorption maxima in their respective flash-induced difference spectra, the laser intensity was reduced by calibrated glass slides.

All three complete conversion curves show the same features. At laser intensities up to 10% (0.1J) the plot of ΔA_T against laser intensity shows a steep rise. At laser intensity above 0.1J the ΔA_T levels off but still shows a slight increase with laser intensity. At higher laser intensities singlet - singlet fusion (eg. $BChl^* + BChl^* \rightarrow BChl + BChl^*$) and singlet - triplet fusion (eg. $BChl^* + BChl^T \rightarrow BChl + BChl^T$) can occur very rapidly (Monger et al 1976). This quenching means that not all of the BChl or carotenoids were converted to their triplet states. However, for the purposes of ϕ_T calculation, it was assumed that at 100% laser intensity almost all of the BChl or carotenoid molecules were in their triplet states. The ΔA_T at 100% laser intensity was therefore taken to represent complete conversion.

The extinction coefficients were calculated using the equation:

$$\epsilon_T \text{mM}^{-1}\text{cm}^{-1} = \frac{\Delta A_T}{\text{(BChl) or (car)}} \times \frac{1}{\text{fraction of 1cm cell illuminated by laser}}$$

The concentrations of BChl or carotenoids were calculated from the millimolar extinction coefficients given in Chapter Four. Two values for the ϕ_T of each species were calculated from the complete conversion data of two different samples. An average value was then used for ϕ_T calculation.

BChl The flash-induced ΔA_T was recorded at 510nm.

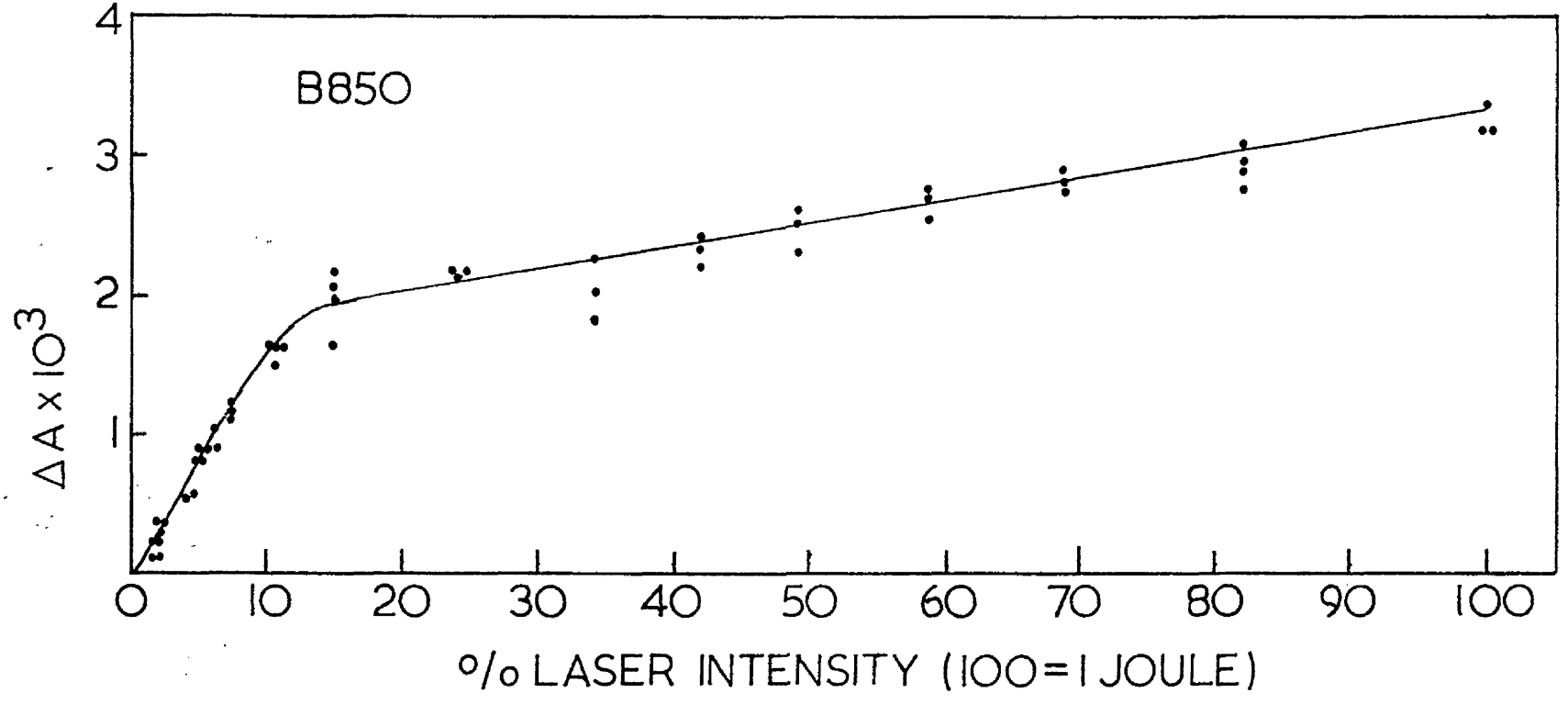
(1) $$\epsilon_{BChl}{}^T = \frac{3 \times 10^{-3}}{2.5 \times 10^{-3}} \cdot \frac{1}{0.765}$$

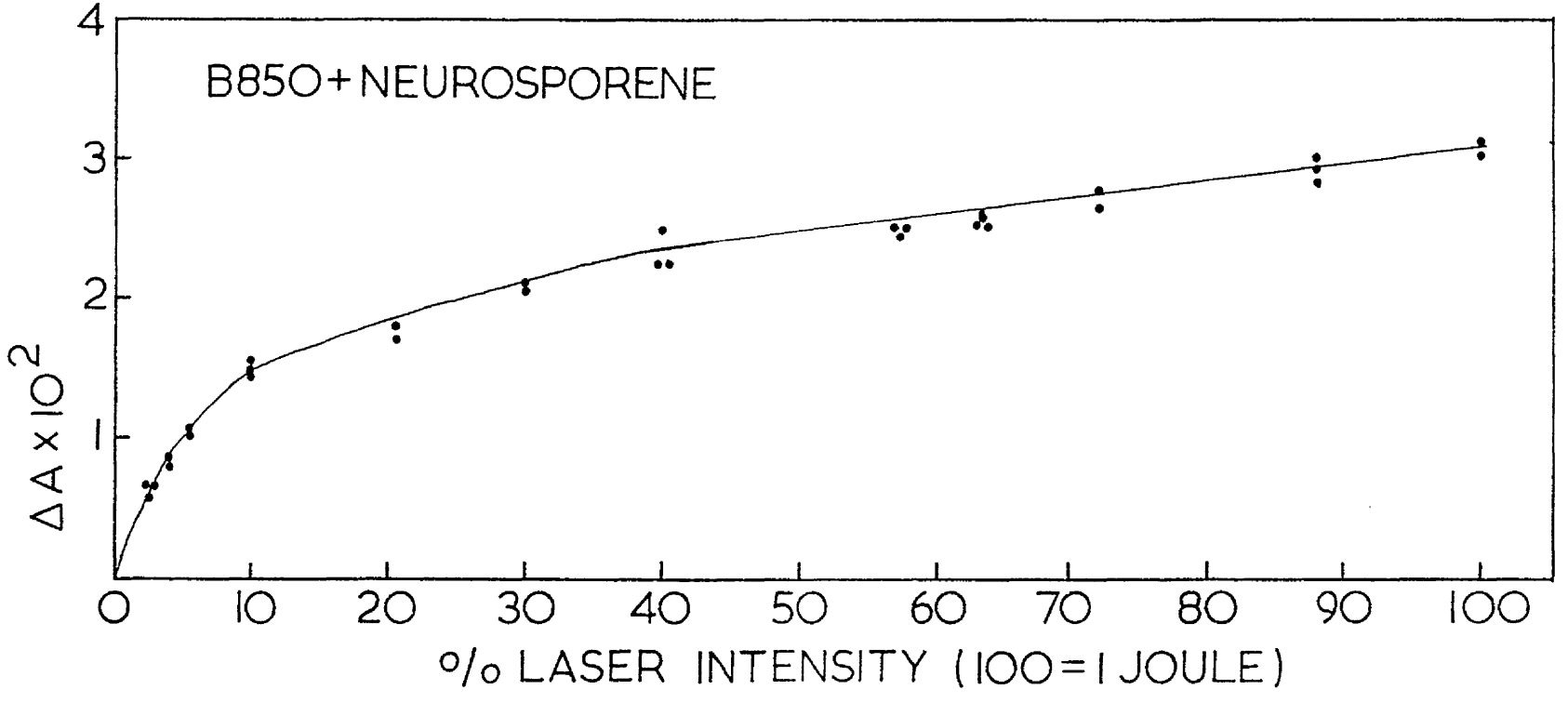
$$= \underline{1.3 \text{mM}^{-1}\text{cm}^{-1}}$$

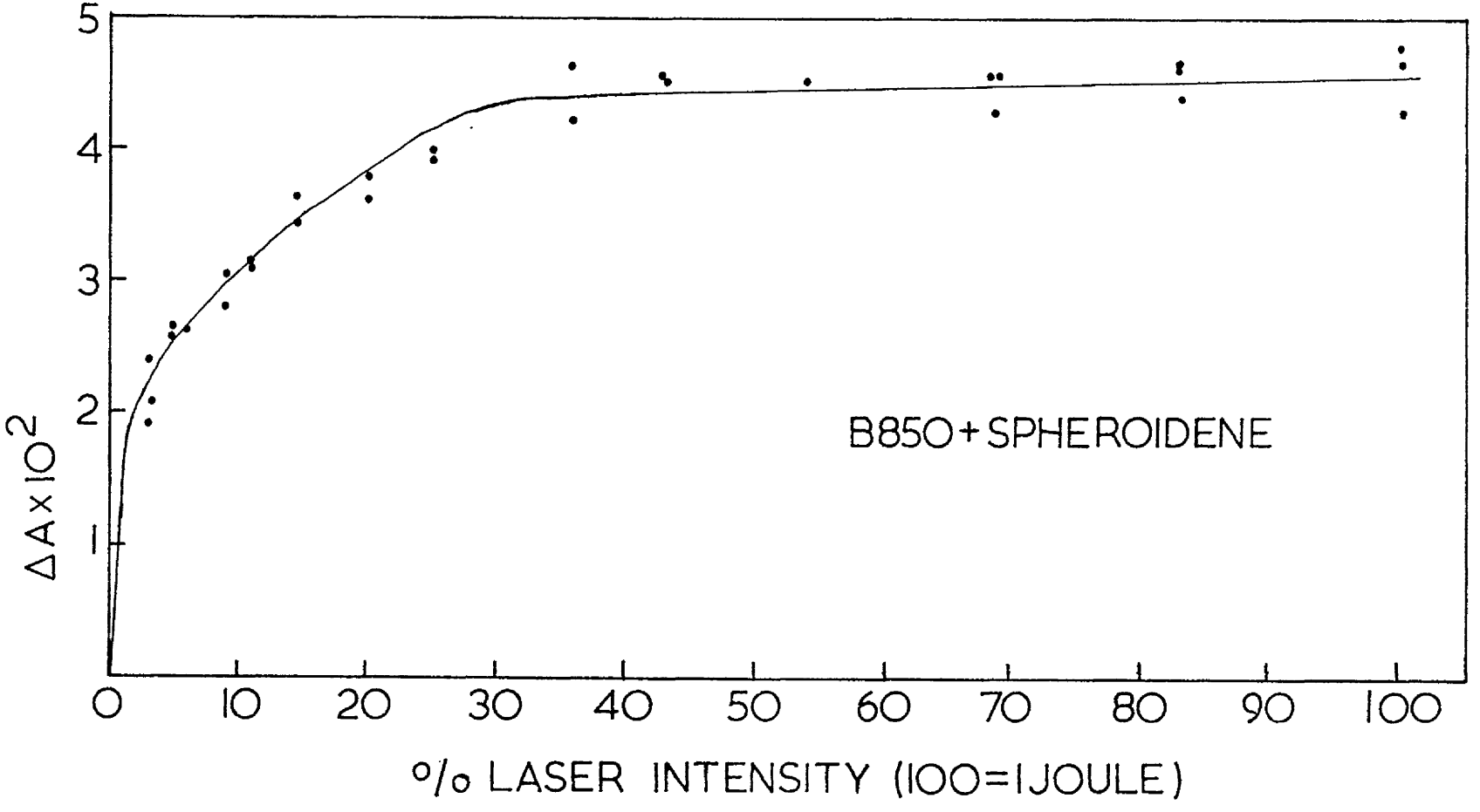
Figure 5.6

Variation in the size of triplet states with laser energy

All samples had an absorbance at 347nm of approximately 0.3. The laser intensity was varied with a series of calibrated glass slides, a laser intensity of 100 = 1 Joule.


B850
4
3
2
1
ΔA x 10^3
0
10
20
30
40
50
60
70
80
90
100
% LASER INTENSITY (100=1 JOULE)


B850+NEUROSPORENE
ΔA x 10^2
4
3
2
1
0
10
20
30
40
50
60
70
80
90
100
% LASER INTENSITY (100=1 JOULE)


5
4
3
2
1
ΔA x 10^2
B850+SPHEROIDENE
0
10
20
30
40
50
60
70
80
90
100
% LASER INTENSITY (100=1JOULE)

$$(2)\ \epsilon_{BChl}{}^{T} = \frac{4.4 \times 10^{-3}}{4.7 \times 10^{-3}} \cdot \frac{1}{0.71}$$

$$= \underline{1.32 mM^{-1} cm^{-1}}$$

For determination of ϕ_T I used $\epsilon_{BChl}{}^{T} = 1.3 mM^{-1} cm^{-1}$ at 510nm.

Neurosporene Flash-induced ΔA_T recorded at 505nm.

$$(1)\ \epsilon_{Neur}{}^{T} = \frac{3.5 \times 10^{-2}}{1.7 \times 10^{-3}} \cdot \frac{1}{0.75}$$

$$= \underline{27.4 mM^{-1} cm^{-1}}$$

$$(2)\ \epsilon_{Neur}{}^{T} = \frac{3.1 \times 10^{-2}}{1.77 \times 10^{-3}} \cdot \frac{1}{0.71}$$

$$= \underline{24.7 mM^{-1} cm^{-1}}$$

For determination of $\phi_{Neur}{}^{T}$ I used $\epsilon_{Neur}{}^{T} = 26 mM^{-1} cm^{-1}$.

Spheroidene Flash-induced ΔA_T recorded at 525nm.

$$(1)\ \epsilon_{Spher}{}^{T} = \frac{3.25 \times 10^{-2}}{1.4 \times 10^{-3}} \cdot \frac{1}{0.765}$$

$$= \underline{30.3 mM^{-1} cm^{-1}}$$

$$(2)\ \epsilon_{Spher}{}^{T} = \frac{4.5 \times 10^{-3}}{2.26 \times 10^{-4}} \cdot \frac{1}{0.75}$$

$$= \underline{26.5 mM^{-1} cm^{-1}}$$

For determination of $\phi_{Spher}{}^{T}$ I used $\epsilon_{Spher}{}^{T} = 28 mM^{-1} cm^{-1}$

These values are of the same order as previously calculated ϵ_Ts for BChl, neurosporene and spheroidene, but are all higher eg. $\epsilon_{BChl}{}^{T} = 1.1 mM^{-1} cm^{-1}$ at 510nm in R26 reaction centres (Parson et al 1975).
$\epsilon_{Neur}{}^{T} = 17.9 mM^{-1} cm^{-1}$ at 510nm in B800 + 850 from Rps. spheroides, G.1.C. (Cogdell et al 1981).
$\epsilon_{Spher}{}^{T} = 9.5 mM^{-1} cm^{-1}$ at 535nm in B800 + 850 from Rps. spheroides, 2.4.1 (Cogdell et al 1981).

In contrast to Cogdell et al (1981), I found that the $\epsilon_{Spher}{}^{T}$ was slightly larger than $\epsilon_{Neur}{}^{T}$, although the extinction coefficient for the ground singlet states of neurosporene in the complex was greater than that of spheroidene (Chapter Four). However, the $\epsilon_{Spher}{}^{T}$ of $28mM^{-1}cm^{-1}$ is close to the reported value ($29mM^{-1}cm^{-1}$) of $\epsilon_{Spher}{}^{T}$ in cyclohexane (Bensasson et al 1976).

The extinction coefficients were used to determine the ϕ_T for BChl, neurosporene, and spheroidene. When the sample and a standard molecule absorbed the same number of photons, at a low percentage conversion to the triplet state, then ϕ_T can be calculated from the following equation (Amand and Bensasson 1975):

$$\phi_T = \phi_{STAND}{}^{T} \cdot \frac{\Delta A_T}{\Delta A_{STAND}{}^{T}} \cdot \frac{\epsilon_{STAND}{}^{T}}{\epsilon_T}$$

For estimation of $\phi_{BChl}{}^{T}$ the standard was perylene in benzene, using $\phi_T = 0.015$ and $\epsilon_{mM}{}^{T} = 14.3cm^{-1}$ at 490nm (M^{c}Vie et al 1979). For estimation of $\phi_{Neur}{}^{T}$ and $\phi_{Spher}{}^{T}$ the standard was anthracene in cyclohexane, taking $\phi_T = 0.71$ and $\epsilon_{mM}{}^{T} = 64.7$ at 422nm (Bensasson and Land 1971).

The experimental data and the values of ϕ_T calculated from this data are shown in Tables 5.1 - 5.3. For BChl, neurosporene and spheroidene a range of values was obtained for ϕ_T, $\phi_{BChl}{}^{T}$ was 0.039 and 0.075, $\phi_{Neur}{}^{T}$ varied between 0.02 and 0.08, $\phi_{Spher}{}^{T}$ varied between 0.015 and 0.12. These values fall into the range of 0.02 - 0.15 previously calculated for $\phi_{car}{}^{T}$ in B800 + 850 complexes from strains of Rps. sphaeroides (Cogdell et al 1981). This variation depended on the amount of detergent in the sample, it is likely that small differences in the SDS concentration of my samples were responsible for variations in the triplet yields. The ϕ_Ts for BChl, neurosporene and spheroidene all show this range of values, but none of the ranges is sufficiently different from the others to suggest that the ϕ_T for that molecule is slightly more or less than for the others. It therefore seems that the ϕ_T for BChl and the ϕ_T for the carotenoids were essentially the same. This would be expected if triplet → triplet energy transfer had occurred from BChl to carotenoids since $\phi_{car}{}^{T}$ should be determined by $\phi_{BChl}{}^{T}$ (Cogdell et al 1981). This is further evidence for the quenching of BChl^{T} by neurosporene and spheroidene in the B850 complex ie. energy transfer from BChl to the carotenoids.

An interesting consequence of the quenching of BChl^{T} by carotenoids is that it enables a lower limit to be set on the triplet energy level

Table 5.1

Data used in calculation of $\phi_{BChl}{}^{T}$

Standard	Laser Intensity	OD_{347} Standard	ΔA_T Standard	OD_{347} Complex	ΔA_T Complex	$\phi_{BChl}{}^{T}$
Perylene	24%	0.169	4.14×10^{-3}	0.345	2.63×10^{-3}	0.039
Perylene	2.9%	0.189	2.35×10^{-3}	0.21	1.21×10^{-3}	0.075

Table 5.2

Data used in calculation of $\phi_{Neur}{}^{T}$

Standard	Laser Intensity	OD_{347} Standard	ΔA_T Standard	OD_{347} Complex	ΔA_T Complex	$\phi_{Neur}{}^{T}$
Anthracene	3.1%	0.0504	5.46×10^{-2}	0.0409	2.17×10^{-3}	0.082
Anthracene	2.9%	0.054	2.51×10^{-2}	0.051	6.97×10^{-4}	0.05
Anthracene	5%	0.054	4×10^{-2}	0.051	1.66×10^{-2}	0.075
Perylene	24%	0.17	4×10^{-3}	0.169	8.6×10^{-3}	0.02

Table 5.1

Data used in calculation of $\phi_{BChl}{}^{T}$

Standard	Laser Intensity	OD_{347} Standard	ΔA_T Standard	OD_{347} Complex	ΔA_T Complex	$\phi_{BChl}{}^{T}$
Perylene	24%	0.169	4.14×10^{-3}	0.345	2.63×10^{-3}	0.039
Perylene	2.9%	0.189	2.35×10^{-3}	0.21	1.21×10^{-3}	0.075

Table 5.2

Data used in calculation of $\phi_{Neur}{}^{T}$

Standard	Laser Intensity	OD_{347} Standard	ΔA_T Standard	OD_{347} Complex	ΔA_T Complex	$\phi_{Neur}{}^{T}$
Anthracene	3.1%	0.0504	5.46×10^{-2}	0.0409	2.17×10^{-3}	0.082
Anthracene	2.9%	0.054	2.51×10^{-2}	0.051	6.97×10^{-4}	0.05
Anthracene	5%	0.054	4×10^{-2}	0.051	1.66×10^{-2}	0.075
Perylene	24%	0.17	4×10^{-3}	0.169	8.6×10^{-3}	0.02

Table 5.3

Data used in calculation of ϕ_{spher}^{T}

Standard	Laser Intensity	OD_{347} Standard	ΔA_T Standard	OD_{347} Complex	ΔA_T Complex	ϕ_{spher}^{T}
Anthracene	2.9%	0.057	1.45×10^{-2}	0.068	1.005×10^{-3}	0.096
Anthracene	2.9%	0.054	3.9×10^{-2}	0.049	1.44×10^{-3}	0.067
Anthracene	2.9%	0.057	1.46×10^{-2}	0.076	1.51×10^{-3}	0.127
Perylene	49%	0.169	4×10^{-3}	0.155	6.8×10^{-3}	0.014

(E_T) of BChl. Connolly et al (1973) derived a value of 65kJ $mole^{-1}$ for the E_T of BChl and observed that, since this was nearly 30kJ $mole^{-1}$ lower than the E_T of O_2, the $BChl^T$ could not pass energy to O_2 and sensitize the formation of O_2. They suggested that in photosynthetic bacteria, the destructive photodynamic reaction proceeded via the superoxide ion (O_2^-), created by transfer of an electron from BChl to O_2, rather than via $^1O_2$. Recent estimates of the E_T for BChl have been higher, approximately 95kJ $mole^{-1}$ (Krasnovsky 1979), 83 - 95kJ $mole^{-1}$ (Lebedev and Krasnovsky (1978), and greater than 100kJ $mole^{-1}$ (Shuvalov and Parsons 1981).

In the B850 complex, neurosporene quenches $BChl^T$. This demonstrates triplet - triplet energy transfer from the BChl to neurosporene, the E_T of BChl must be above the E_T of neurosporene for this to occur.

The E_Ts of carotenoids vary inversely with the number of conjugated double bonds (Mathis and Kleo 1973, Ensasson et al 1976), from the estimates of E_Ts neurosporene, which contains 9 conjugated double bonds, would have an E_T of approximately 86kJ $mole^{-1}$. The E_T of BChl in the B850 complex must therefore be greater than 86kJ $mole^{-1}$.

CHAPTER SIX

PHOTOPROTECTION BY CAROTENOIDS

There are thought to be two possible methods by which carotenoids can protect photosynthetic bacteria from the photodynamic reaction (see Chapter 1 section 1.8):

(1) The carotenoids quench $^1O_2$ or O_2^- which are responsible for photodestruction or

(2) Carotenoids quench the $BChl^T$ before it can sensitize the formation of $^1O_2$ or O_2^-.

In Chapter 5 I showed that neurosporene and spheroidene reconstituted into the B850 complex effectively quenched the $BChl^T$. I then tried to discover whether the presence of these carotenoids in the B850 complex also gave protection against photodestruction. However, firstly it was important to determine whether the photodestruction of the B850 complex was a photodynamic effect ie. required the presence of light and oxygen (see Chapter 1 section 1.6).

A preparation of B850 complex (without carotenoids) in 10mM Tris. HCl pH 8.0, 0.05%SDS was illuminated by strong white light ($900Wm^{-2}$). The actinic light was passed through a heat filter (5cm of water) before it illuminated the sample. The photodestruction of the B850 complex was measured by recording the irreversible bleaching of the 850nm absorption band. Photodestruction was plotted as OD_{850}/initial OD_{850} against time (Fig 6.1). Photodestruction started only when the complex was illuminated and it stopped when the illumination stopped. Light was therefore necessary for photodestruction. Figure 6.1 also shows that very little photodestruction occurred when the preparation was continuously bubbled with N_2 to remove dissolved O_2. This showed that the presence of O_2 was required for photodestruction. This bleaching of the BChl is therefore a true photodynamic reaction since it requires the presence of both light and oxygen.

During the course of the photodestruction, the wavelength of the 850nm absorption band shifted progressively to shorter wavelengths (Fig 6.2). Rafferty et al (1979) have shown that this shift was due to bleaching of one of the two BChl molecules which contribute to this absorption band, ie. the BChl goes from dimer to monomer.

When neurosporene or spheroidene were present in the B850 complex the destruction of the 850nm absorption band was greatly decreased (Fig

Figure 6.1

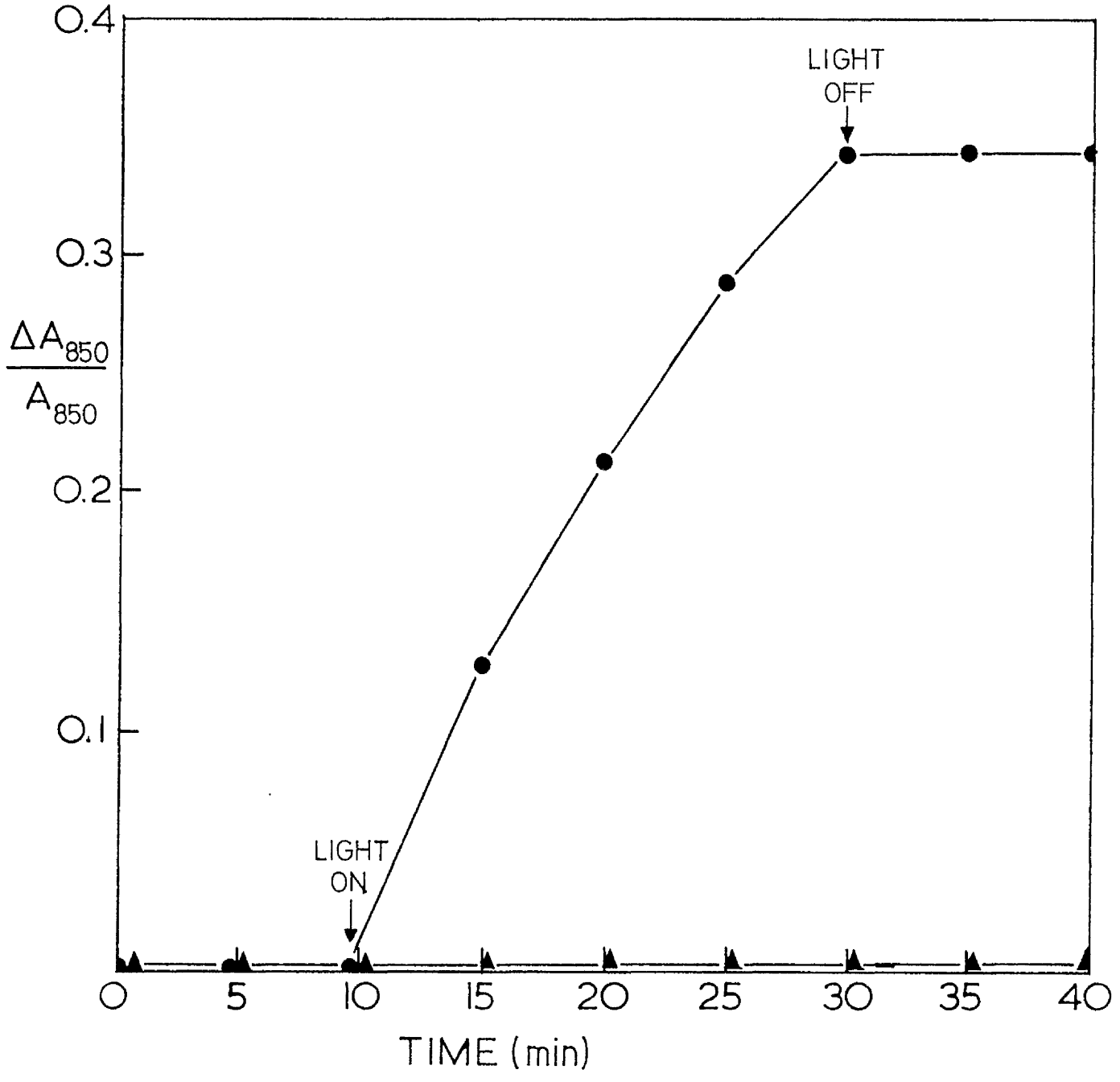
Photodestruction of the B850 light-harvesting complex

Photodestruction was measured by the irreversible bleaching of the 850nm absorption band. The complexes (in 10mM Tris. HCl pH8.0, 0.05% SDS) were illuminated in a cuvette by strong white light (900 Wm^{-2}) previously passed through 5cm of water acting as a heat filter.

●—● complex bubbled with air before and during illumination.

▲—▲ complex bubbled with N_2 before and during illumination.

In each case the BChl concentration was 6μM.


0.4
0.3
0.2
0.1
$\frac{\Delta A_{850}}{A_{850}}$
LIGHT OFF
LIGHT ON
0
5
10
15
20
25
30
35
40
TIME (min)

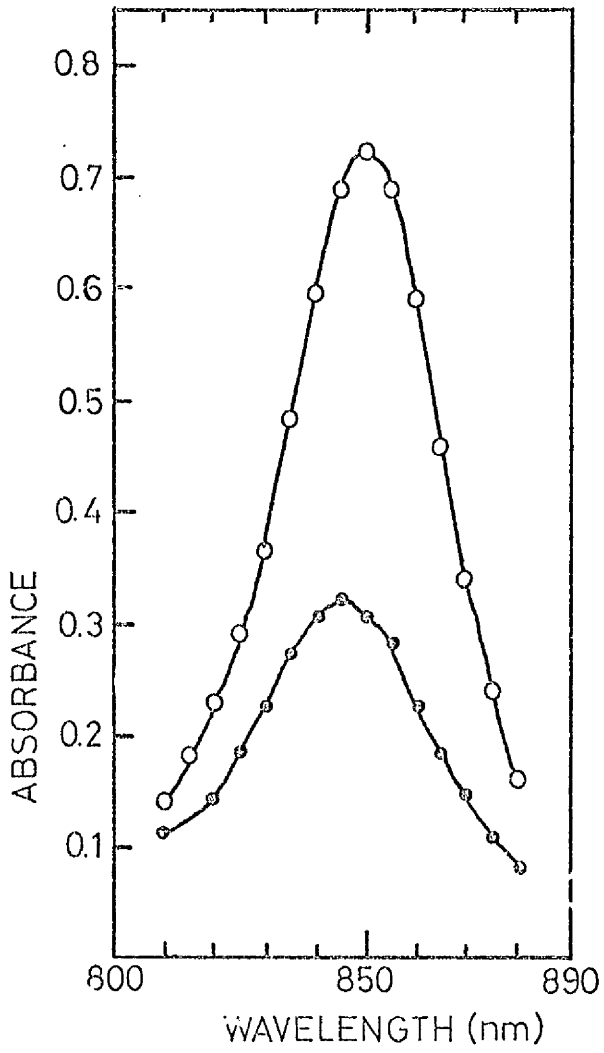


Figure 6.2

The effect of illumination on the NIR absorption spectrum of the B850 complex

o——o absorption spectrum before illumination, maximum at 850nm.

o——o absorption spectrum after 25 min. illumination ($900 Wm^{-2}$), maximum at 845nm.

6.3 for B850 containing neurosporene). These carotenoids presumably gave protection by quenching the BChl triplet states (as shown in Chapter 5) before they could sensitize the formation of $^1O_2$ or O_2^-. Monger et al (1976) found that quenching of $BChl^T$ by carotenoids was approximately 10^3 times more rapid than the reaction of the $BChl^T$ with O_2.

After showing that the presence of neurosporene and spheroidene in the B850 complex gave protection against the photodynamic reaction, I tried to characterise the photodynamic reaction more fully. I had already shown that light and oxygen were necessary, but was the damaging species $^1O_2$, O_2^- or a combination of the two? Boucher et al (1977) obained evidence which suggested that both $^1O_2$ and O_2^- were involved in the photodynamic reaction. They observed the oxidation of adrenaline to adrenochrome during the illumination of reaction centres isolated from Rhs. rubrum. These reaction centres contained the carotenoid spirilloxanthin. At low light intensities, below 8×10^6 ergs cm^{-2} sec^{-1} ($8000 Wm^{-2}$) the oxidation of adrenaline proceeded mainly via O_2^-. The oxidation was inhibited by superoxide dismutase, but not by sodium azide which quenches $^1O_2$ (Hasty et al 1972). At light intensities greater than 8×10^6 ergs cm^{-2} sec^{-1} (up to 2×10^7 ergs cm^{-2} sec^{-1}, $2 \times 10^4 Wm^{-2}$) $^1O_2$ was involved. The amount of adrenochrome formed by the action of $^1O_2$ increased with light intensity, while the amount formed due to O_2^- stayed roughly the same. The presence of both sodium azide and superoxide dismutase completely inhibited adrenaline oxidation. Boucher et al also showed the involvement of $^1O_2$ by the oxidation of 1,3-diphenylisobenzofuran (DPIBF), an indication of the presence of $^1O_2$ (Matheson and Lee 1970). When reaction centres from the carotenoidless strain of Rhs. rubrum, G9, were illuminated (beyond 690nm, 1.8×10^7 ergs cm^{-2} sec^{-1}) in the presence of DPIBF, the DPIBF was bleached. This reaction could be prevented by sodium azide ($^1O_2$ quencher) or by the presence of the carotenoid spirilloxanthin bound to the reaction centre.

I was unable to detect any decrease in photodestruction of the 850nm BChl in the presence of sodium azide or superoxide dismutase.

The effect of sodium azide on photodestruction is shown in Fig 6.4. The concentration of BChl was 6μM in both cases, for the concentrations of azide used this gave azide:BChl ratios of approximately 10,000:1 and 2,500:1. The azide:BChl ratio in the experiments of Boucher et al was 2,500:1. Figure 6.4 does not show quenching of photodestruction by sodium azide; on the contrary, photodestruction seems to increase with azide concentration. This trend was observed on several occasions.

Figure 6.3

Photodestruction of the B850 pigment-protein complex with and without neurosporene

The irreversible bleaching of the 850nm absorption band in complexes with (o—o) and without (▲—▲) neurosporene. Experimental conditions as described for Fig 6.1, air bubbled through the sample. BChl concentration was 6 μM in both cases.

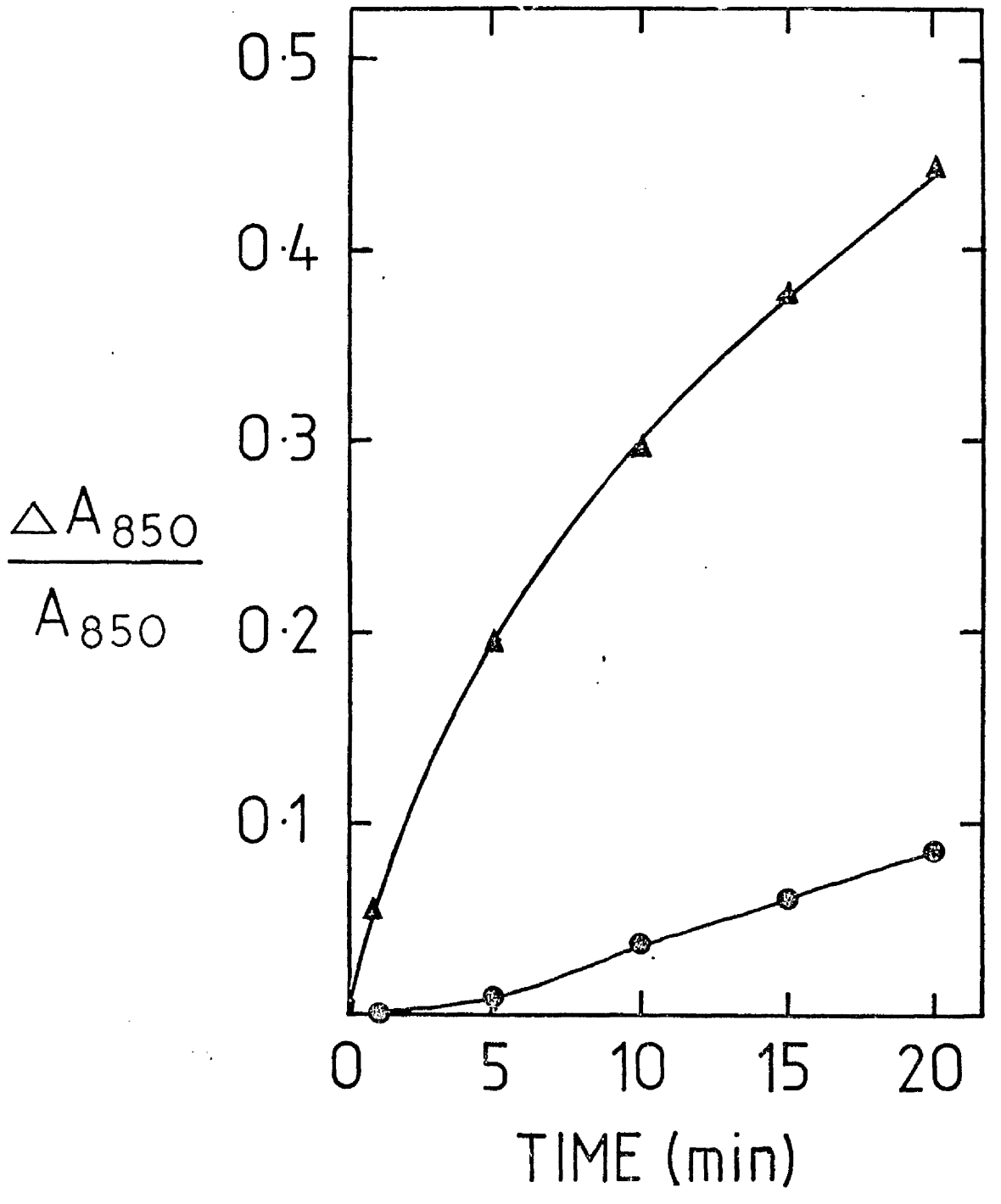

0·5
0·4
0·3
0·2
0·1
$\frac{\triangle A_{850}}{A_{850}}$
0
5
10
15
20
TIME (min)

Figure 6.4

The effect of sodium azide on photodestruction of the B850 light-harvesting complex

Experimental conditions as described for Fig 6.1, BChl concentration was 6 μM in each case.

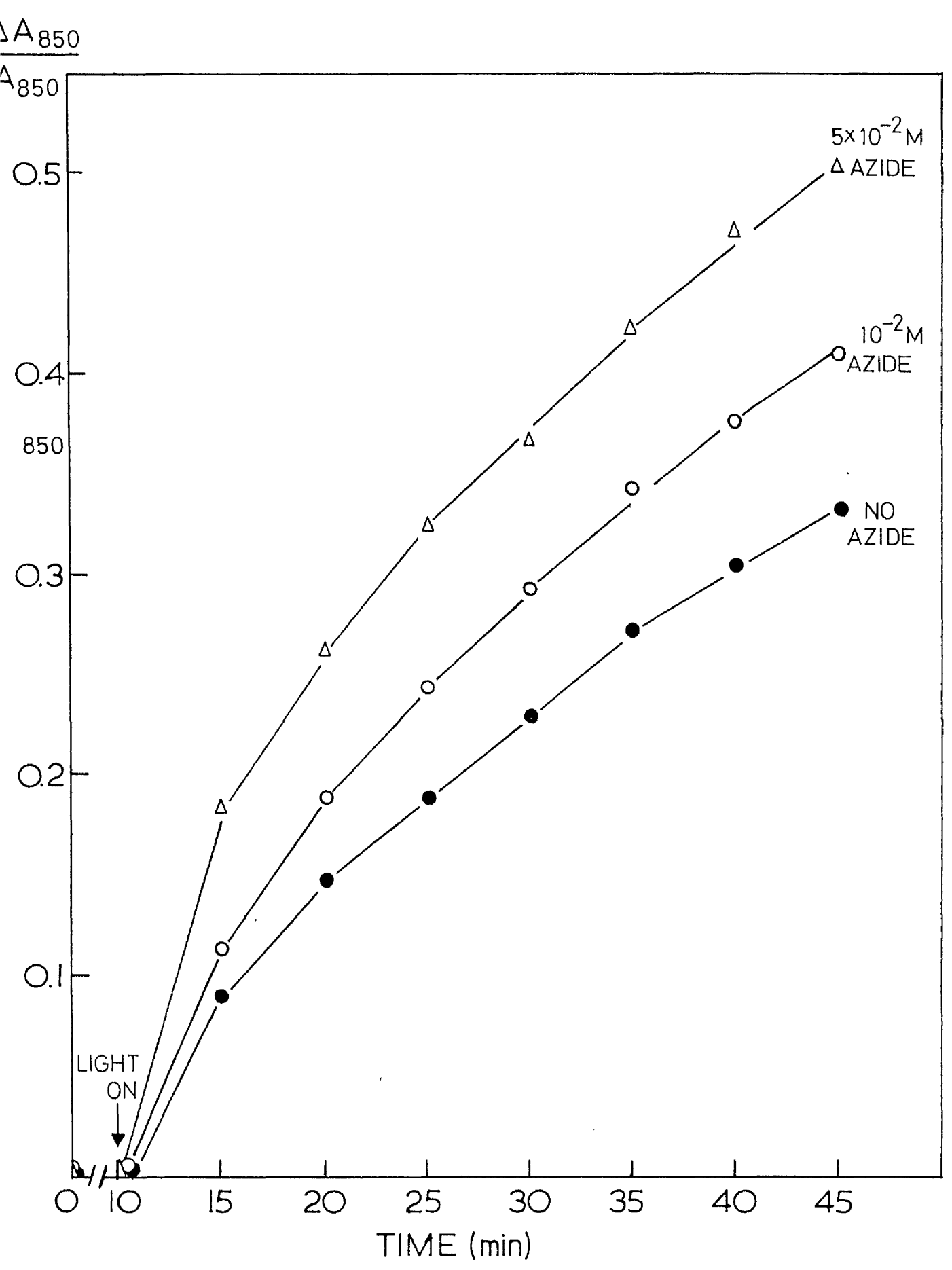

ΔA850
A850
0.5
0.4
850
0.3
0.2
0.1
5x10-2M
Δ AZIDE
10-2M
AZIDE
NO
AZIDE
LIGHT
ON
0
10
15
20
25
30
35
40
45
TIME (min)

Figure 6.5 shows the effect of the enzyme superoxide dismutase on photodestruction of the B850 complex. Superoxide dismutase was added to the B850 complexes to give 400 units superoxide dismutase (0.11g) per nmole of BChl (Boucher et al used 500units/nmole BChl). The presence of the enzyme had no effect on the photodestruction of the complex.

I also used DPBIF in an attempt to discover whether or not $^1O_2$ was involved in the photodestruction of the 850nm BChl absorption band. DPBIF showed a broad absorption band in the same region of the spectrum as the carotenoids, the oxidation of the DPBIF was measured by recording the bleaching of this band. Unfortunately the DPBIF was very labile and was bleached when the complex was not illuminated, thus DPBIF provided no evidence for or against the involvement of $^1O_2$ in the photodestruction of the BChl.

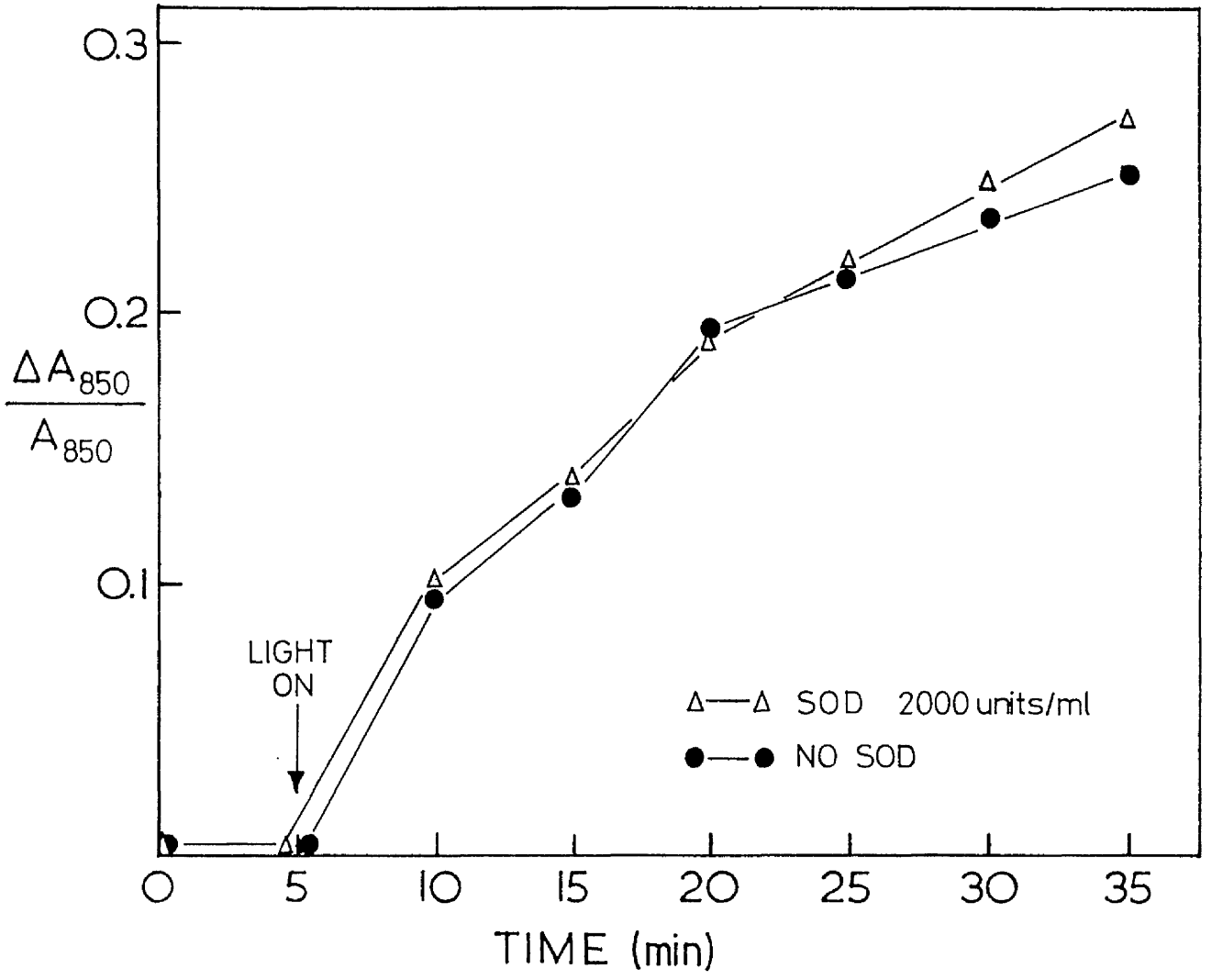
The inability of sodium azide or superoxide dismutase to decrease the photodestruction of the B850 complex does not necessarily imply that $^1O_2$ and $O_2^{\cdot-}$ were not involved. These species would be formed by sensitization from triplet BChl and would probably react rapidly with the BChl which sensitized their formation. This could occur too rapidly for quenchers to inhibit the destruction of BChl and at sites on the pigment-protein complex which were inaccessible to the quenching agents. This applies particularly to the quenching of superoxide by the enzyme superoxide dismutase.

I have shown that illumination of B850 light-harvesting complexes without carotenoids results in the BChl being progressively destroyed. This destruction is a photodynamic reaction, it requires the combined presence of light and oxygen. When neurosporene and spheroidene are present in the B850 complex the amount of photodestruction is greatly decreased. This protection can be attributed to the quenching of BChl triplets before they can sensitize the formation of the damaging $^1O_2$ or $O_2^{\cdot-}$ species.

Figure 6.5

The effect of superoxide dismutase on photodestruction of the B850 light-harvesting complex

Experimental conditions as described for Fig. 6.1. BChl concentration was 6μM in both cases.


0.3
0.2
0.1
$\frac{\Delta A_{850}}{A_{850}}$
LIGHT ON
Δ—Δ SOD 2000 units/ml
●—● NO SOD
0
5
10
15
20
25
30
35
TIME (min)

CHAPTER SEVEN

CONCLUSIONS

In this project I have investigated the energy transfer between carotenoids and BChl after reconstitution of carotenoids into the B850 light-harvesting pigment-protein complex from the carotenoidless mutant of Rps. sphaeroides, R26.

Carotenoids have two main functions in the photosynthetic bacteria:

(1) They act as accessory light-harvesting pigments, capturing light energy and passing it to BChl (singlet - singlet transfer).

(2) They give protection against the photodestruction which can occur in the presence of light and air. This photodestruction is thought to be due to either $^1O_2$ or O_2^- which is formed by sensitization from triplet BChl. The most likely way in which carotenoids protect against photodestruction is by quenching triplet states of BChl before they can react with oxygen. This quenching involves triplet - triplet energy transfer from BChl to carotenoids.

By adding carotenoids to the B850 complex I hoped to be able to investigate the efficiencies of several carotenoids in performing these two functions. Direct comparisons could be made since the carotenoids would be bound on identical sites, it should be possible to discover whether the efficiency of energy transfer is determined by the binding site or by the structure of the carotenoid.

In the course of the project I characterised the B850 light-harvesting complex from Rps. sphaeroides, R26. Chromatophores from this strain showed only a single NIR absorption maximum, at 850nm. This suggested that the R26 strain contains only one type of light-harvesting complex, a B890 type, modified to absorb at 850nm. However when the R26 was first isolated (1963) the NIR absorption maximum was at 870nm. What had caused this shift in the wavelength of the NIR absorption band? There have been recent suggestions that R26 might contain both types of light-harvesting complex (ie. B890 and B800 + 850) as in wild strains of Rps. sphaeroides, and that B850 complex was not a B890 type of complex.

Using SDS-polyacrylamide gradient gel electrophoresis I found that R26 chromatophores contained the polypeptides characteristic of both B890 and B800 + 850 light-harvesting complexes. The isolated B850 complex was found to contain the same proteins as a B800 + 850 complex isolated from wild-type Rps. sphaeroides - two low molecular weight polypeptides, approximately 10 and 8kD. These were poorly separated on gradient gels which separate according to molecular weight, however they were clearly resolved by isoelectric focussing which separates by differences in the

net charges of proteins. The B850 and B800 + 850 complexes were clearly resolved into two bands, the bands from the different complexes were co-focussed. This suggested that the amino-acid compositions of the polypeptides were identical in both complexes, or that any changes had been conservative ie. amino-acids replaced by a similar type which did not affect the net charge. I was therefore unable to determine whether alteration of the amino-acid content of the B850 polypeptides had changed the binding site of the 800nm BChl. It was clear that the B850 complex is an altered B800 + 850 type, and that the R26 strain which I used therefore contained both types of light-harvesting complex. I was fortunate to receive a culture of the original strain of R26 (from Prof. William Sistrom) which absorbed maximally at 870nm. Gradient gel electrophoresis showed that this strain contained only the light-harvesting polypeptides (12 and 8kD) associated with the B870 type of complex. Dr. Richard Cogdell and I have suggested that the 'new strain' of R26, containing the B870 and altered B800 + 850 (B850), should be designated Rps. sphaeroides R26.1. This will distinguish it from the original R26 strain containing only the B870 type of light-harvesting complex (Davidson and Cogdell 1981).

I incorporated carotenoids into the B850 complex by adding them in petroleum spirit to freeze-dried chromatophores. The petroleum spirit was evaporated, the chromatophores were resuspended in buffer and treated with the detergent SDS before the B850 complex was isolated by hydroxyapatite column chromatography.

Two carotenoids, neurosporene and spheroidene, were reconstituted into the B850 complex in amounts approaching the concentration which would be expected if they were natural constituents of the complex. Since B850 is a B800 + 850 type of complex which lacks the BChl molecule responsible for the 800nm absorption band we would expect the maximum BChl:carotenoid ratio to be 2:1. When neurosporene was incorporated into the complex the average BChl:neurosporene ratio was 2.3:1, the corresponding ratio for spheroidene was 2.6:1. Neurosporene and spheroidene were bound to specific sites on the B850 complex and were attached in an all-trans configuration expected for carotenoids in light-harvesting complexes.

The B850 complexes containing neurosporene were used in all studies on singlet - singlet or triplet - triplet energy transfer, and photoprotection I attempted to reconstitute β-carotene, spirilloxanthin, lycopene, dihydroneurosporene, dihydrolycopene and spheroidenone. Dihydroneurosporene was the only one of these carotenoids which bound in the same quantities as neurosporene and spheroidene. Since all of these have the same chain lengths as neurosporene and spheroidene it seems that the conformation of the end of the chain, and the type of substituents, determines whether or not a carotenoid can bind to the B850 complex.

In the B850 complex, neurosporene and spheroidene perform the two major functions of carotenoids in the photosynthetic bacteria. They absorbed light energy and transferred it to BChl with efficiencies of 60 - 70% as determined by the fluorescence excitation spectra. These values are lower than the 90 - 100% obtained for these carotenoids in B800 + 850 complexes isolated from strains of Rps. sphaeroides (Cogdell et al 1981). However, the B850 complex is a B800 + 850 type which lacks one of the BChl molecules, this is bound to have a significant effect on the overall efficiency of carotenoid to BChl energy transfer. When the treatment undergone by the complex is considered it is probably reasonable to suggest that 60 - 70% is a high value for the efficiency of carotenoid to BChl energy transfer in the B850 complex.

Triplet - triplet energy transfer was investigated using laser flash photolysis. Laser flash illumination (347nm) of the B850 complex resulted in the formation of BChl triplet states. If neurosporene or spheroidene was present the $BChl^T$ was rapidly quenched (probably within 500nsec) and carotenoid triplets were formed. Since laser excitation occurred at wavelength of BChl absorption, and carotenoid triplets can only be formed by sensitization from another molecule in its triplet state, it is likely that the carotenoid triplets were formed by triplet - triplet transfer from $BChl^T$. This is supported by the fact that the range of values obtained for the efficiency of carotenoid triplet formation (2 - 12%) were similar to the values for the efficiency of $BChl^T$ formation (4 - 8%). I suggest that these efficiencies are probably the same, this supports the formation of carotenoid triplets by energy transfer from $BChl^T$ since Cogdell et al (1981) showed that efficiency of carotenoid triplet formation would be the same.

The presence of neurosporene and spheroidene in the B850 complex also gave protection against photodynamic destruction. When the B850 complex without carotenoids was illuminated by strong white light the 850nm BChl absorption band was irreversibly bleached and the wavelength of the absorption maximum decreased up to 5nm reflecting the BChl changing from a dimer to a monomer. This destruction of BChl required the combined presence of light and oxygen and was therefore a photodynamic reaction. When neurosporene or spheroidene were present in the complexes the amount of photodestruction was greatly decreased. Since these carotenoids had been shown to quench BChl triplets this was the most probable method of photoprotection. Monger et al previously reported that the quenching of

$BChl^T$ by carotenoids was 10^3 times faster than the reaction of $BChl^T$ with oxygen.

I attempted to identify the species of oxygen which was responsible for photodestruction. B850 without carotenoid was illuminated in the presence of sodium azide and superoxide dismutase which are known to quench $^1O_2$ and O_2^- respectively. No decrease in BChl destruction was observed, in fact at higher concentrations sodium azide promoted the destruction of BChl. It is likely that, after formation of the damaging species of oxygen, the destructive reaction occurred almost immediately between the oxygen and the BChl molecule which sensitized its formation. This reaction might also have occurred at sites on the pigment-protein complex which were inaccessible to azide, and in particular to the enzyme superoxide dismutase.

To sum up, I have shown that neurosporene and spheroidene can be reconstituted into specific sites on the B850 light-harvesting pigment-protein complex. They were bound in amounts close to those which would be expected if they occurred naturally in the B850 complex. Neurosporene and spheroidene performed both of the major functions of carotenoids in photosynthetic organisms; they acted as accessory light-harvesting pigments and protected the complex against the photodynamic reaction, probably by quenching BChl triplets before they could sensitize the formation of damaging species of O_2.

The reconstitution of carotenoids into the B850 complex should provide a useful experimental technique with which to investigate the function of carotenoids in photosynthetic bacteria. Several areas of the research covered by this thesis would benefit from further studies.

(a) The accessory light-harvesting function of the carotenoids. It should be possible to extend the range of carotenoids which can be incorporated into the B850 complex. A comparison of the efficiencies of carotenoid to BChl energy transfer will then help to identify the factors which govern this efficiency. Does the structure of carotenoids determine the efficiency, as suggested by Cogdell et al (1981) for B800 + 850 complexes from Rps. sphaeroides, or is the binding site of the carotenoid the controlling factor as suggested by Boucher et al (1977), for reaction centres of Rhs. rubrum.

(b) BChl to carotenoid, triplet - triplet energy transfer. As for carotenoid to BChl energy transfer a range of carotenoids should be investigated. Does the ability of a carotenoid to quench triplet BChl depend entirely upon the number of conjugated double bonds in the carotenoid?

The triplet - triplet transfer to carotenoids may also help to determine the energy level (E_T) of triplet BChl. If $BChl^T$ is to sensitize the formation of singlet oxygen from oxygen in its normal triplet state (E_T = 94kJ $mole^{-1}$), it should have an E_T greater than 94kJ $mole^{-1}$. Connolly et al (1973) estimated the E_T of BChl to be approximately 65kJ $mole^{-1}$. Since this value would not allow $BChl^T$ to sensitize formation of singlet oxygen, Connolly et al suggested that the photodestruction of carotenoidless photosynthetic bacteria in presence of light and O_2 must proceed by O_2^- rather than $^1O_2$. Although recent estimates for the E_T of BChl *in vitro* have ranged between 94 and 108kJ $mole^{-1}$ (Krasnovsky 1979, Shuvalov and Parson 1981), a definitive value has yet to be obtained. Quenching of $BChl^T$ by carotenoids will not give an accurate value but should provide good reliable values for a range of values in which the E_T lies. Neurosporene (9 conjugated double bonds, E_T approximately 86kJ $mole^{-1}$) in the B850 complex quenched $BChl^T$, the E_T of BChl must therefore be above 86kJ $mole^{-1}$. Zeta-carotene (7 conjugated double bonds) has an E_T of approximately 103kJ $mole^{-1}$, see Fig 1.8. If this carotenoid bound to the B850 complex and quenched $BChl^T$ then a lower limit of 103kJ $mole^{-1}$ could be given to the E_T of BChl. If zeta-carotene did not quench $BChl^T$ then the E_T for BChl will be between 86 and 103kJ $mole^{-1}$.

(c) Photoprotection and photodynamic reaction. There is greater scope for further experimentation into these areas since relatively little is known about the photodynamic reaction. For example, it is not known whether the destructive species is $^1O_2$, O_2^-, or a combination of the two. Boucher et al (1977) suggested that both $^1O_2$ and O_2^- were involved. Reaction centres from Rhs. rubrum were illuminated in the presence of adrenaline, the oxidation of adrenaline was recorded in the presence and absence of quenchers of $^1O_2$ and O_2^-. At low light intensities (less than 8 x 10^6 ergs $cm^{-2}sec^{-1}$) O_2^- was the damaging species, at higher light intensities $^1O_2$ also had effect. Above 8 x 10^6 ergs $cm^{-2}sec^{-1}$ the activity of $^1O_2$ increased with light intensity while the activity of O_2^- stayed roughly constant. I suggest, however that these results give little information of the photodynamic reaction against which the carotenoids protect the photosynthetic bacteria. These experiments were performed at the non-physiological, high pH of 10. At this pH the relative activities of $^1O_2$ and O_2^- could be different from their activities at the normal physiological pH range of 6 - 8. The reaction centres used in the experiments contained carotenoids, the $^1O_2$ and O_2^- were therefore not necessarily produced in the same proportions as they would be if the carotenoids were absent. Although the results showed that $^1O_2$ or O_2^- could be generated in the presence of carotenoids, the light intensities used were much greater than cells would be exposed to normally.

It may be possible to demonstrate sensitization of $^1O_2$ formation from $BChl^T$ by laser illumination of the B850 complex without carotenoids and monitoring emitted light from $^1O_2$ at 1268nm(Khan and Kasha 1979). This technique has previously been used to show sensitization of $^1O_2$ formation by $BChl^T$ in organic solution.

If $^1O_2$ can be formed by illumination of the B850 without carotenoids, repetition using B850 containing carotenoids should allow characterisation of the photoprotective method ie. quenching of $BChl^T$ or quenching of $^1O_2$. If the main method involves quenching of $BChl^T$, little $^1O_2$ emission would be expected.

APPENDIX 1

The Electronic States of Molecules (see Nobel 1974, Clayton 1980).

The light absorbing characteristics of a molecule, and the fate of the energy from absorbed light, depend on the arrangement of the electrons in the absorbing molecule. The quantum state of an electron in a molecule can be specified by a series of terms which describe its energy and the angular momentum, or "spin", associated with its orbital motion.

The magnitude of the spins of all electrons is the same, the mathematical treatment of quantum mechanics assigns a value of $\frac{1}{2}$ to the spin of an electron. Spin however, is a vector, and has two possible values depending on the spin being aligned either parallel or antiparallel to a local magnetic field. In a molecule such fields are provided by the relative motion between the positively charged nuclei and the mobile, negatively charged electrons. The spin of an electron is designated $+\frac{1}{2}$ when it is parallel to a local magnetic field, $-\frac{1}{2}$ when it is antiparallel.

The Pauli Exclusion Principle specifies that two electrons cannot be in the same quantum state. Two electrons in the same orbital therefore have the same set of quantum numbers except that their spins are in opposite directions. In a simple treatment a molecule can exist in one of three types of electronic state as determined by the net spin of the molecule. This net spin is given by the sum of the spins of all of the electrons in the molecule (see Fig A.1).

1. Net Spin of the molecule = $\frac{1}{2}$; ie. an unpaired electron (eg. an organic radical) which can interact with a magnetic field in two ways; ie. molecule is doublet. When the molecule is placed in a magnetic field this doublet state is split into two substates, one parallel, the other antiparallel to the magnetic field.

2. Net spin = 0; the electron spins are antiparallel as with most organic molecules in the ground state, a magnetic field has no effect on the electronic states. The molecule is said to be in a SINGLET state.

3. Net spin = 1 eg. in an excited molecule with two parallel electron spins. In a magnetic field the electronic state is split into three substates, parallel, antiparallel and perpendicular to the magnetic field, respectively. The molecule is in a TRIPLET state.

The two most important electronic states in biological molecules are the singlet and triplet states.

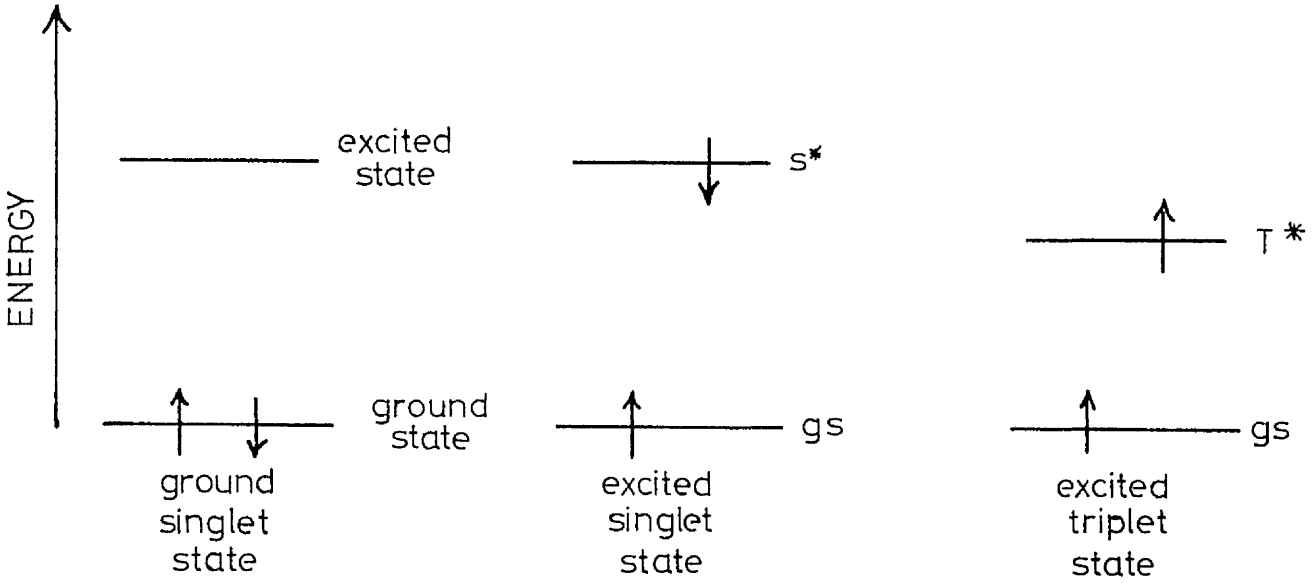


Figure A.1

Electronic States of Molecules

The diagram can be used to refer to a pair of electrons in an orbital. The arrows represent electrons, the direction of the arrow indicates a spin of + or $-\frac{1}{2}$.

When a molecule (eg. PChl) in its ground singlet state absorbs light an electron is promoted to an excited singlet state ie. the spin of the excited electron remains antiparallel to that of its partner in the ground state. A triplet state is unlikely to be formed directly from a ground singlet state since the spin of the promoted electron must be reversed. This requires a magnetic interaction with the surroundings. A triplet state is more likely to be formed from an excited singlet state where electron distribution is more widespread than in the ground state. This increases the interaction with neighbouring magnetic fields and decreases the magnetic coupling by which a pair of electrons tend to keep their spins antiparallel.

The lifetime of a triplet state (eg. several μsec for β-carotene Mathis 1970) is much greater than that of the corresponding singlet state (1psec for β-carotene, Dallinger et al 1981). This is because the decay of a triplet state to a ground singlet state involves the relatively unlikely or forbidden reversal of spin orientation. A molecule in its triplet state therefore has a long time in which to react with other molecules (see Chapter 1, section 1.6). A molecule in its triplet state is also more likely to exchange electrons with other molecules because the electron distribution is more widespread than in the singlet state.

The absorption spectra obtained by conventional spectrophotometers reflect the transition of molecules from the ground to the excited singlet state. This state can also be recognised by the light emitted (fluorescence) when an electron decays from the excited singlet state to the ground singlet state. The wavelength of light emitted as fluorescence is slightly longer than that of the absorbed light which raised the electron to the excited state. This shift occurs because absorbed light can promote an electron to one of a number of vibrational sublevels of the excited state, but decay to the ground state always takes place from the lowest vibrational sublevel. The energy of the fluorescence is therefore always slightly lower than that of the absorbed light and, since wavelength is inversely proportional to energy, the wavelength of the fluorescence is longer than that of the absorbed light.

Molecules in their triplet states can be identified by their triplet-singlet absorption spectra obtained by laser flash photolysis. A short, intense light pulse is used to promote molecules to triplet states via the excited singlet states. Carotenoids, because of the very rapid radiationless decay of the excited singlet state (1psec, Dallinger et al 1981), have very low efficiencies for singlet triplet crossover. Spectra of triplet

states obtained by laser flash photolysis show characteristic bleaching at wavelengths corresponding to ground state (ie. ground state to excited singlet state) absorption bands and formation of new absorption bands representing the triplet states of molecules.

Triplet states can also be identified by the light emitted (phosphorescence) when they decay to the ground (singlet) state. Phosphorescence can be distinguished from fluorescence since it occurs a relatively long time (10^{-3}-10^{0}) sec after excitation and has a longer wavelength than fluorescence. The energy level of a triplet state is lower than that of the corresponding singlet state, the wavelength maximum of phosphorescence will therefore be longer than that of fluorescence.

Triplet states of BChl and carotenoids have been detected by electron paramagnetic resonance or EPR (Frank et al 1980). When a triplet state is placed in a magnetic field the triplet state is split into three substates. These substates can be observed if the molecule is raised from the lower to the higher substates by supplying quanta of suitable energy. The energy difference between the states depends on the strength of the applied magnetic field. In EPR spectroscopy a fixed frequency of microwave radiation is supplied to the molecule while the strength of the magnetic field is varied. It is possible to identify the energy differences between the magnetic substates by observing the values for the magnetic field at which strong absorption of quanta occur.

APPENDIX 2

The Mechanism of Singlet-Singlet Energy Transfer From Carotenoids to Chlorophyll

Carotenoids have long been recognised as accessory light-harvesting pigments in photosynthesis, but the way in which they transfer captured light energy to chlorophyll molecules is not well understood.

Such energy transfer is usually assumed (e.g. Thrash et al 1979) to occur by a 'resonance' transfer process proposed by Förster (e.g. Förster 1959, 1960, 1965). This is thought to be the mechanism responsible for chlorophyll to chlorophyll energy transfer in photosynthetic membranes. A chlorophyll molecule absorbs light (or receives energy from an accessory pigment) which causes the molecule to be promoted to its first excited singlet state. If this energy is not transferred to another molecule the excited state decays radiatively or non-radiatively to the ground state after approximately 10^{-9} sec. If the difference in energy between the excited singlet state and the ground singlet state corresponds to the energy difference of a possible absorption transition in an adjacent chlorophyll molecule, then there is an opportunity for energy transfer from one chlorophyll to the other. If there is sufficient 'interaction' between the two molecules, the return of the donor molecule to the ground state is coupled to a transition to an excited state in the acceptor molecule. There is usually enough coincidence between the donor and acceptor transitions if the absorption spectrum of the acceptor overlaps with the fluorescence spectrum of the donor. Since energy transfer requires interaction between the two molecules, the transfer can only take place over limited distances. Strong interaction will occur if the corresponding transitions in both molecules are allowed for electric dipole radiation, this enables the transitions to be coupled to each other. If the 'interaction energy' is of a dipole-dipole nature, then its strength varies inversely with the third power of intermolecular distance (R). The probability of energy transfer is proportional to the square of the interaction energy and is therefore inversely proportional to the sixth power of the intermolecular distance (i.e. probability varies with R^{-6}) Förster (1959).

The requirements for efficient energy transfer by a Förster mechanism are therefore an overlap between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, correct intermolecular orientation, and a small distance between the molecules.

Förster (1960) recognised three types of resonance transfer on the basis of three possible strengths of interaction between molecules. Interaction

could be strong, medium or weak, depending on the intermolecular distance. These interactions were later redefined as strong, weak and very weak (Förster 1965). For the reasons outlined above, transfer between chlorophylls would be expected to vary with R^{-6} if the molecules interact very weakly. However, if energy transfer occurs between strongly interacting molecules, then the efficiency of energy transfer will vary with R^{-3} (Knox 1977). Is the in vivo Chl-Chl distance consistent with a Förster mechanism for energy transfer?

Beddard and Porter (1976) calculated that chlorophyll molecules must be at least 10Å apart to avoid concentration quenching of excited singlet states. The intermolecular distance (i.e. Chl-Chl) in plant membranes has been estimated to be 11-17Å (Thomas et al 1956, Bay and Pearlstein 1963, Colbow 1973) although these values are based on unreliable microscope observations, estimates of photosynthetic unit size and chlorophyll content of membranes. An average distance of 15Å between chlorophylls in photosynthetic membranes would imply that energy transfer by Förster resonance would take place according to those parameters which define a very weak interaction between the molecules (Forster 1965).

The most accurate value for the intermolecular distance has been obtained for BChla molecules in a BChl-protein complex from the green photosynthetic bacterium Prosthecochloris aestuarii (Matthews et al 1979). X-ray crystallography was used to determine the three-dimensional structure of this water soluble complex. The basic unit of the complex is a protein which contains 7 BChl molecules. Each BChl is at least 12Å from the nearest BChl on the same protein, however the closest distance between BChl molecules in adjacent complexes is 24Å. It may therefore be possible for two types of energy transfer to occur; one between BChls on the same complex and another between less strongly interacting BChls on different complexes. In this way the efficiency of energy transfer might vary with both R^{-3} and R^{-6} since energy could be passed between chlorophylls on the same or separate complexes. There has been much theoretical discussion on the nature of energy transfer however, experimental investigations into the mechanism of in vivo Chl-Chl energy transfer are still at an early stage. The most valuable approach seems to be the study of fluorescence decay kinetics in algae or chloroplast preparations after picosecond laser pulse excitation.

Harris et al (1976) found that, in the green algae Chlorella pyrenoidosa, the fluorescence decay curve appeared to be non-exponential. The decay could be described by an expression which included a $t^{\frac{1}{2}}$ term (t = time after excitation) as would be expected from a random diffusion of excitations

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The most accurate value for the intermolecular distance has been obtained for BChl*a* molecules in a BChl-protein complex from the green photosynthetic bacterium Prosthecochloris aestuarii (Matthews et al 1979). X-ray crystallography was used to determine the three-dimensional structure of this water soluble complex. The basic unit of the complex is a protein which contains 7 BChl molecules. Each BChl is at least 12Å from the nearest BChl on the same protein, however the closest distance between BChl molecules in adjacent complexes is 24Å. It may therefore be possible for two types of energy transfer to occur; one between BChls on the same complex and another between less strongly interacting BChls on different complexes. In this way the efficiency of energy transfer might vary with both R^{-3} and R^{-6} since energy could be passed between chlorophylls on the same or separate complexes. There has been much theoretical discussion on the nature of energy transfer however, experimental investigations into the mechanism of in vivo Chl-Chl energy transfer are still at an early stage. The most valuable approach seems to be the study of fluorescence decay kinetics in algae or chloroplast preparations after picosecond laser pulse excitation.

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(Chl-Chl) via Förster energy transfer. In further experiments using Chl. pyrenoidosa and enriched Photosystem I and II particles from spinach chloroplasts, it appeared that most fluorescence decays were non-exponential and could be described by a $t^{\frac{1}{2}}$ expression (Porter et al 1977, Searle et al 1977). These results supported the proposal that Chl-Chl energy transfer occurred by a Förster mechanism. However, in similar experiments using improved equipment and more accurate fluorescence recording techniques, it was found that fluorescence decays could be described equally as well by a double exponential expression as by a $t^{\frac{1}{2}}$ expression (Beddard et al 1979, Searle et al 1979). This need not imply that energy transfer does not occur by Förster's mechanism as it is difficult to interpret the fluorescence decay data in terms of energy transfer mechanisms (see Breton and Geacintov 1980). As was suggested above, there may be two different types of energy transfer i.e. between chlorophylls on the same pigment-protein complex and between chlorophylls on separate complexes. There is also some doubt as to whether or not a $t^{\frac{1}{2}}$ expression for fluorescence decay signifies transfer by a Forster mechanism. The $t^{\frac{1}{2}}$ dependence is obtained for excitation diffusion in three dimensions, but Breton and Geacintov (1980) suggest that two dimensional excitation diffusion may be more likely in photosynthetic membranes. This would result in a $t^{\frac{1}{3}}$ expression for fluorescence decay.

Although currently available experimental data give conflicting views on the nature of energy transfer, it should be possible for advances in laser spectroscopic techniques (e.g. subpicosecond pulse times) to help relate theoretical treatments to the in vivo situation.

Energy transfer from carotenoids to chlorophylls may proceed by a different mechanism to chlorophyll-chlorophyll transfer. Since β-carotene is the most common carotenoid in plants, the transfer of energy from β-carotene to chlorophyll a is usually taken as a typical example of carotenoid to chlorophyll transfer.

Thrash et al (1977) showed that the lowest excited singlet state in β-carotene is not the strongly allowed ^1B_u state (which is 'responsible' for the orange colour of β-carotene), but a dipole forbidden ^1A state which is approximately 3450cm^{-1} lower in energy. Similar 'low-lying' states are found in other polyene molecules, it is therefore likely that all carotenoids possess such states (Thrash et al 1979). The dipole-allowed ^1B_u state ranges in energy from 24000cm^{-1} down to about 20,700cm^{-1} (Jurkowitz et al 1959). Since the fluorescence emission of large molecules extends to approximately

3000cm^{-1} to the red of the singlet absorption band, energy transfer from the ^1B_u state might occur down to 17,500cm^{-1}. This would provide a very poor overlap with the red absorption band of Chl*a* (Fig A.2). Also, since β-carotene is non-fluorescent (Tric and Lejeune 1970), radiationless decay from the excited singlet state results in the population of the lowest excited singlet state (Kasha 1950), in this case the ^1A_g state. The ^1A_g state represents a range in energies from 20,000cm^{-1} down to about 17,200cm^{-1} (Thrash et al 1977) and should therefore be able to transfer energy down to 14,200cm^{-1}. Since this gives a good overlap with red absorption band of Chl*a* (Fig A.2), efficient energy transfer to Chl*a* should take place from the ^1A_g state of β-carotene. Thrash et al (1979) concluded that the ^1A_g state was important for energy transfer and that, although it is a dipole forbidden state, the Forster transfer efficiency is not diminished.

On the other hand, Razi-Naqvi (1980) argues that although the ^1A_g state provides overlap with the absorption band of Chl*a*, the Forster mechanism is unlikely to account for carotenoid to chlorophyll energy transfer. He considers energy transfer between a donor-acceptor pair (D-A) of separation R. The rate constant (k_{ET}) for energy transfer by a dipole-dipole mechanism is given by the following equation (Förster 1959):

$$k_{ET} = AK^2 \Omega k_D^o R^{-6} \qquad (1)$$

A is a constant, K depends on the mutual orientation of D and A. k_D^o is the rate constant for radiative decay (a measure of the dipole strength of the donor transition), this is related to the emission quantum yield (ϕ_D) and the overall decay rate constant (k_D) for D by the equation

$$k_D^o = \phi_D k_D \qquad (2)$$

In equation (1) the spectral overlap is represented by Ω where

$$\Omega = \int F_D(\nu) \epsilon_A(\nu)\, \nu^{-4}\, d\nu \qquad (3)$$

F_D is the fluorescence spectrum of D normalized to unity on a wave number (ν) scale, ϵ_a is the molar decadic extinction coefficient of A.
If K is substituted for AK^2R^{-6} in equation (1), then the efficiency (η) of energy transfer is given by the following (Razi-Naqvi 1980):

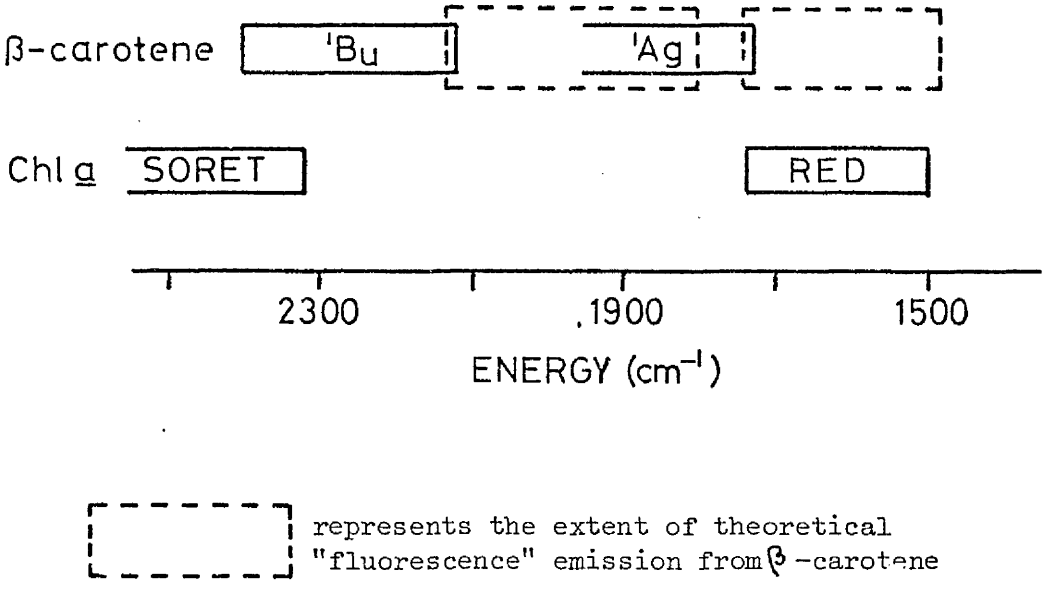


Figure A.2

Energies corresponding to Chl a absorption bands and β-carotene singlet states.

$$\eta = \frac{k_{ET}}{k_{ET}+k_D} = \left(1+\frac{1}{K\phi_D\Omega}\right)^{-1} \qquad (4)$$

This suggests that ϕ_D is as important as Ω in determining the efficiency of energy transfer.

Razi-Naqvi had argued previously that a resonance transfer mechanism would not apply to energy transfer between molecules which are very close together or 'contiguous' (Razi-Naqvi and Steel 1970). For Förster transfer over a distance of for example 50Å, the dipole-dipole term predominates in a series of terms describing molecular interaction. This relatively large distance means that smaller multipole terms and 'electron exchange' effects can be conveniently ignored. However, if a molecule passes energy to "a contiguous acceptor", then the proximity of the molecules requires that multipole terms in the interaction must be considered, along with electron exchange effects. Razi-Naqvi(1980), with no evidence to support him, assumes that carotenoids and chlorophylls are much closer together than the relatively large distances (5-10nm) over which he states Förster's dipole-dipole resonance mechanism operates. This, in conjunction with the fact that carotenoids are non-fluorescent (Tric and Lejeune 1970), leads him to propose that energy transfer occurs by an 'electron exchange' process described by Dexter (1953).

There are several sources of indirect evidence to suggest that carotenoids and chlorophylls are closer to each other than the chlorophyll-chlorophyll intermolecular distance:

(1) Triplet-triplet energy transfer from Chl*a* to β-carotene has been shown to occur *in vivo* (Mathis 1969). Triplet-triplet transfer can only take place by an electron exchange mechanism. Dexter (1953) implies that, for a crystalline system, the efficiency of energy transfer by an electron-exchange mechanism will be high for transfer between adjacent molecules on a crystal lattice, but very low between molecules separated by twice this distance. Since this could mean the difference between 2Å and 4Å, the electron exchange mechanism will probably require a smaller intermolecular distance than the typical 15Å between chlorophylls.

(2) The lifetime of the excited singlet state of β-carotene is likely to be less than 1psec (Dallinger et al 1981). For transfer to occur on this time scale, molecules of β-carotene and Chl*a* must surely be closer than the distance between chlorophylls. The lifetime of Chl*a* in its excited singlet state is 10^3-10^4 times greater than that of β-carotene and yet Chl*a* molecules are only approximately 15Å apart.

(3) Carotenoid to chlorophyll energy transfer (25% efficient) and chlorophyll to carotenoid triplet-triplet transfer have been shown to occur in a synthetic carotenoporphyrin molecule (Dirks et al 1980, Moore et al 1980). In the molecule the conjugated double bond system of the carotenoid lay over the porphyrin ring at a distance of approximately 4Å.

In photosynthetic pigment-protein complexes carotenoids and chlorophylls are therefore likely to be only a few angstroms apart, a distance small enough to favour involvement of the electron exchange theory to account for energy transfer between the two pigments.

The electron exchange theory accounted originally for triplet-triplet energy transfer in which the two molecules involved exchanged electron spins (Dexter 1953). Apart from requiring the molecules involved to be contiguous, it also meets Razi-Naqvi's requirement of the transfer efficiency being determined by spectral overlap. According to Dexter (1953) the rate constant for energy transfer by electron exchange can be given by:

$$k_{ET} = (2\pi/h)U^2 \, F_D(\nu) \, {}_A(\nu)\mathcal{E}d\nu$$

$\mathcal{E}_A$ is the spectral distribution of the acceptor, normalized to unity. U depends on R but is independent of the dipole strengths of transitions in D and A.

APPENDIX 3

Growth Media

1. C-succinate medium

20ml conc. base (see below)

20ml KH_2PO_4 + K_2HPO_4 (1/1, molar solutions)

5ml ammonium sulphate (10% w/v)

10ml potassium succinate (see below)

1ml growth factors (see below)

1g casamino acids

Add one litre of water, pour into bottles. Autoclave at 15lb/in^2 for 15 minutes.

Conc. Base

10g nitroacetic acid, pH to 6.8 with 5N KOH

14.45g magnesium sulphate

3.4g calcium chloride.$2H_2O$

9.25mg ammonium molybdate.$4H_2O$

99mg ferrous sulphate.$7H_2O$ — Add slowly, check pH

50mg nicotinic acid

25mg aneurine HCl

0.5mg biotin

50ml Metos 44 (see below)

Make up to 1 litre with distilled water

Potassium succinate

To solid succinic acid (for 1M) add KOH until pH 6.8, then add water.

Growth Factors

2mg biotin

50mg $NaHCO_3$ — Dissolve in 100ml water

100mg nicotinic acid

50mg aneurine HCL — Add to solution above and boil to dissolve

100mg 4-amino benzoic acid

C-succinate medium (continued)

Metos 44

0.5g EDTA

2.19g zinc sulphate.$7H_2O$

1.0g ferrous sulphate.$7H_2O$

308mg manganous sulphate.$4H_2O$

78.4mg copper sulphate.$5H_2O$

49.6mg cobalt nitrate.$6H_2O$

35.4mg disodium tetraborate.$10H_2O$

Add 100ml distilled water and two drops conc. sulphuric acid

APPENDIX 4

(a) Stock solutions used in SDS-polyacrylamide gel electrophoresis

Stacking Gel Buffer	0.5M Tris.HCl pH 6.8, 0.4% SDS (w/v)
Running Gel Buffer	1.5M Tris.HCl pH 8.8, 0.4% SDS (w/v)
Acrylamide	30% acrylamide (w/v), 0.8% methylene-bis-acrylamide
Electrolyte	25mM Tris 0.192M glycine 0.1% SDS
Boiling Solution	50mM Tris.HCl pH 8.0 2% SDS 10% glycerol (v/v) 2% β-mercaptoethanol (v/v) 0.1% bromophenol blue (w/v)
Staining Solution	25% isopropanol (v/v) 10% acetic acid (v/v) 0.04% kenacid blue (w/v) (Coomassie brilliant blue)
Destaining Solution	10% methanol (v/v) 10% acetic acid (v/v)

(b) Composition of SDS-polyacrylamide gels

	Stacking gel	Running gel, % acrylamide			
		10%	11.5%	15%	16.5%
Stacking Gel Buffer	.5.0ml	-	-	-	-
Running Gel Buffer	-	5.0ml	5.0ml	5.0ml	5.0ml
30% acrylamide	3.0ml	6.6ml	7.5ml	10.0ml	11.0ml
H_2O	12.0ml	-	-	-	-
sucrose*	-	8.4ml	7.5ml	5.0ml	4.0ml
10% ammonium persulphate (v/v)	60µl	100µl	100µl	100µl	100µl
TEMED	20µl	12.5µl	12.5µl	12.5µl	12.5µl

* For gradient gels the higher % acrylamide was supplemented by 70% sucrose (w/v), the low % acrylamide by 15% sucrose. For gels containing a fixed % acrylamide the sucrose was replaced by water.

(c) Stock solutions used in Isoelectric focussing

Acrylamide	30% acrylamide, 1.6% methylene bis-acrylamide
Overlay buffer	4M urea 20µl ampholytes, pH 3.5-10 for 10ml 80µl ampholytes, pH 4-6
Nonidet NP-40	10% (w/v)

Stock solutions used in Isoelectric focussing continued.....

Lysis buffer	9.5M urea 2% Nonidet NP-40 400µl ampholytes, pH 4-6 100µl ampholytes, pH 3.5-10 5% β-mercaptoethanol (v/v) 1% SDS (w/v)	for 10ml
Anode solution	0.02M NaOH, freshly degassed	
Cathode solution	0.01M H_3PO_4	

(d) Composition of isoelectric gels

9.8g urea (8.5M)	
2.68ml of 30% acrylamide	degassed before adding
4ml Nonidet NP-40	20µl riboflavin
3.96ml H_2O	14µl TEMED
320µl ampholytes, pH 4-6	
80µl ampholytes, pH 3.5-10	(riboflavin 15mg/ml)

APPENDIX 5

The partial mass spectra of carotenoids (see Vetter et al 1971)

The partial mass spectra of neurosporene, spheroidene and spirilloxanthin are shown in Fig. A.3. Carotenoid samples were inserted directly intothe probe at an ionising voltage of 70eV and a probe temperature of 100°C.

All three spectra show the molecular ion (M^+) peaks at m/e values corresponding to the appropriate molecular weights, and all three show peaks at M-92 and M-106. These peaks are highly characteristic of the carotenoids and are due to fragmentation of the polyene chain. M-92 and M-106 represent the loss of toluene and xylene respectively.

Spheroidene and spirilloxanthin give peaks at M-32 and M-73 caused by fragmentation of the chain ends which contain methoxy groups (see Fig. A.3). Spirilloxanthin has methoxy groups at both ends of the chain. Spheroidene has a methoxy group at one end only, and its spectrum shows a peak at M-137 corresponding to fragmentation of the other chain end.

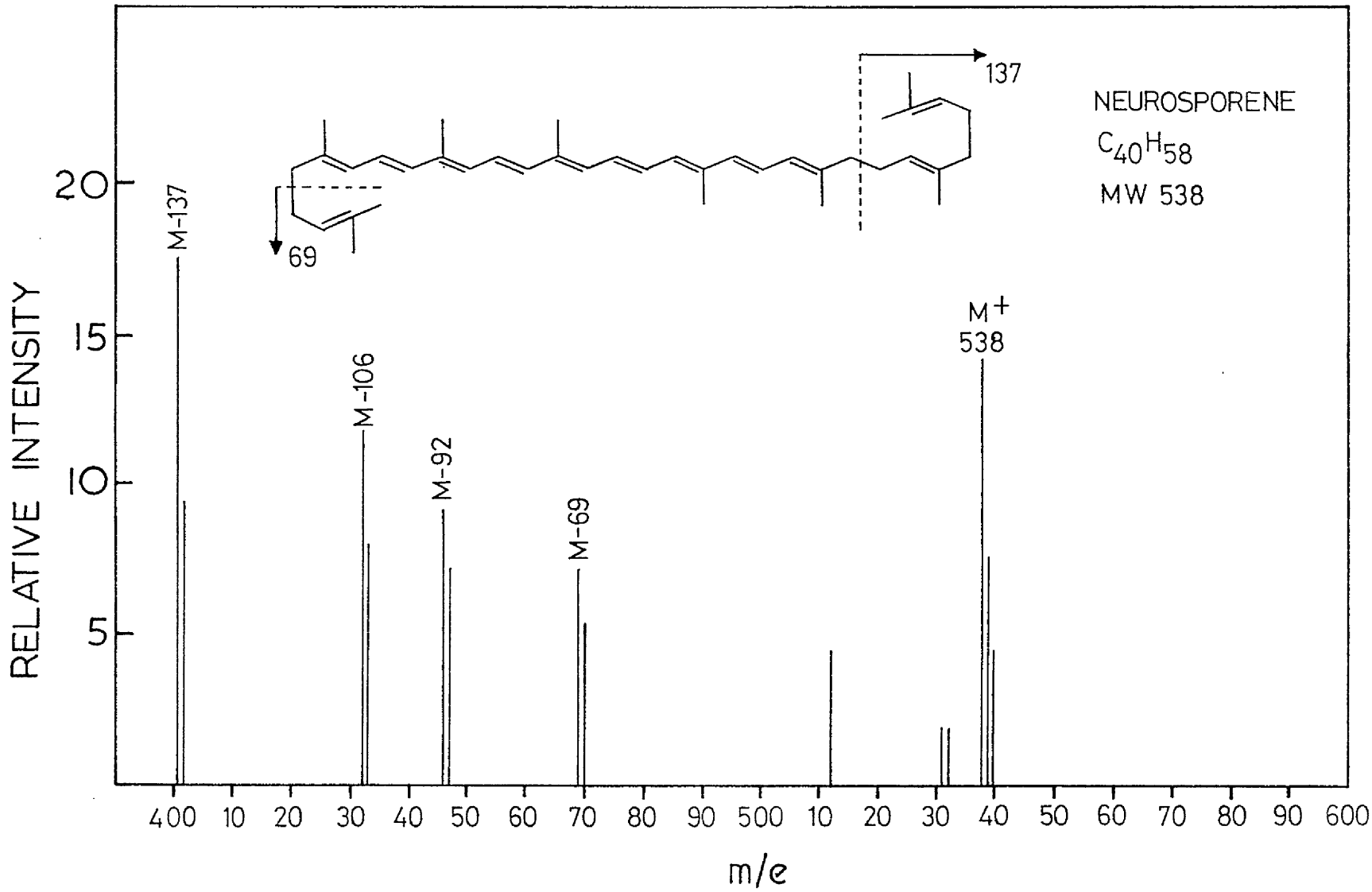
Neurosporene, with no methoxy groups, gave peaks at M-69 and M-137 due to fragmentation of the chain ends.

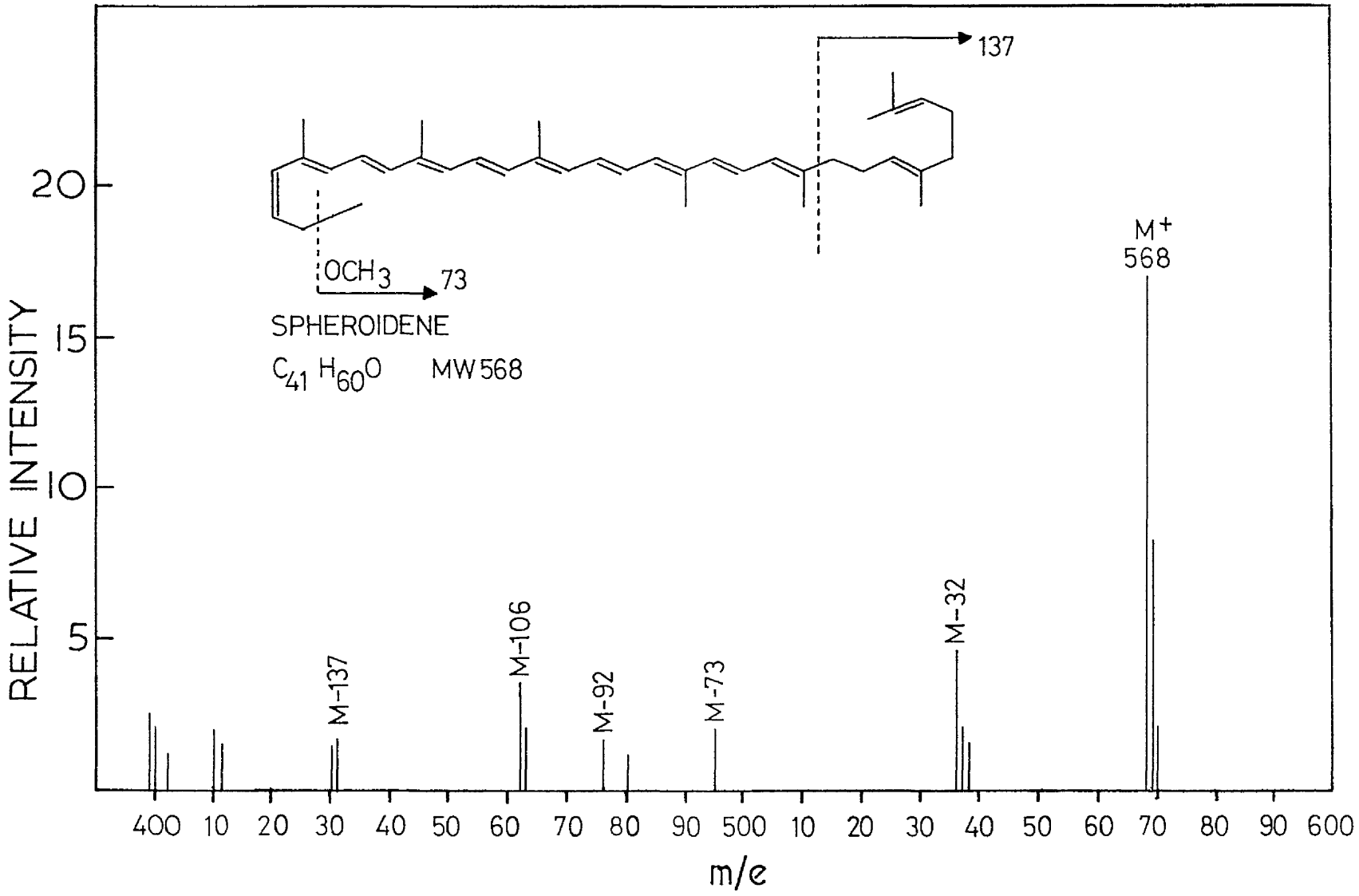
These mass spectra confirmed the identities of carotenoids which I had tentatively identified as neurosporene, spheroidene and spirilloxanthin.

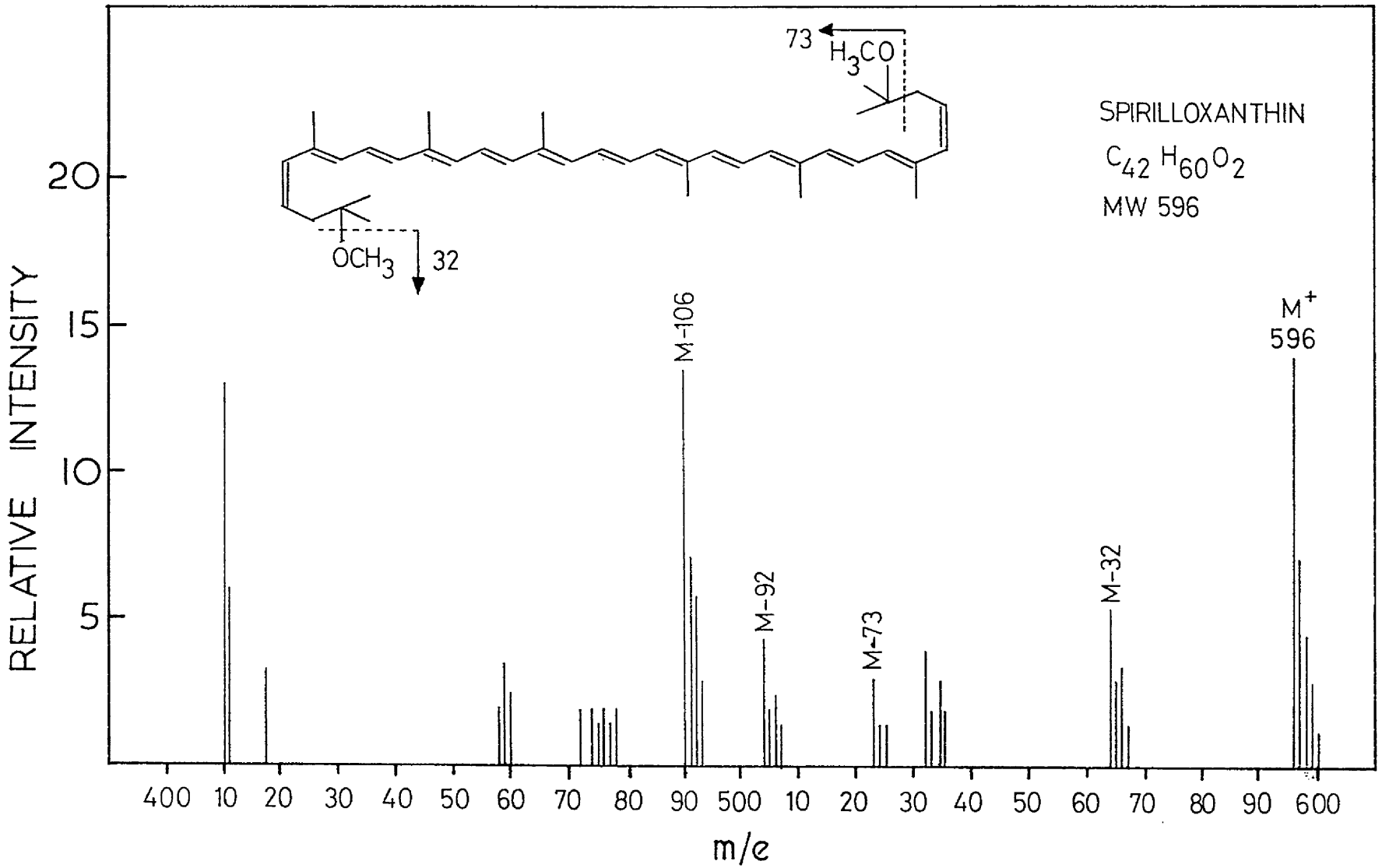
Figure A.3

The partial mass spectra of neurosporene, spheroidene, and spirilloxanthin

The spectra were recorded at an ionising voltage of 70eV and a probe temperature of 100°C.


NEUROSPORENE
$C_{40}H_{58}$
MW 538
137
69
M-137
M-106
M-92
M-69
M+
538
RELATIVE INTENSITY
20
15
10
5
400 10 20 30 40 50 60 70 80 90 500 10 20 30 40 50 60 70 80 90 600
m/e


137
OCH3
73
SPHEROIDENE
C41 H60O MW 568
M+
568
M-32
M-73
M-92
M-106
M-137
RELATIVE INTENSITY
20
15
10
5
400 10 20 30 40 50 60 70 80 90 500 10 20 30 40 50 60 70 80 90 600
m/e


SPIRILLOXANTHIN
$C_{42}H_{60}O_2$
MW 596
73
H_3CO
OCH_3
32
M-106
M-92
M-73
M-32
M^+
596
RELATIVE INTENSITY
20
15
10
5
400 10 20 30 40 50 60 70 80 90 500 10 20 30 40 50 60 70 80 90 600
m/e

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