ADAPTATION OF THE [redacted] THYLAKOID MEMBRANE TO LOW GROWTH TEMPERATURES

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ABSTRACT

Adaptation of photosynthesis to low temperature was studied using material taken from pea plants (**Pisum sativum** L. var. Feltham first) grown under two regimes with average growth temperatures of 7°C and 17°C which were termed "cold-grown" and "warm-grown" respectively. The fluidity of thylakoid membranes isolated from these plants was estimated using the fluorescent probe diphenylhexatriene. It was found that thylakoid membranes from the cold-grown plants were more fluid than those from warm-grown ones so that the membranes exhibited approximately equal viscosities when measured at the growth temperature of the plant from which they were isolated, consistent with the operation of a homeostatic mechanism.

Thylakoids isolated from cold-grown plants exhibited greater rates of photosynthetic full-chain electron transport under saturating conditions than those from warm-grown plants at all temperatures between 3 and 19°C. The same trend was found in assays of leaf oxygen evolution at measuring temperatures below 15°C, where faster rates were observed in cold-grown leaves than in warm-grown ones determined at saturating light and high CO_2 concentrations. At temperatures above 15°C however, the warm-grown leaves gave the faster rates.

A possible relationship between the fluidity of the thylakoid membrane and the rate of electron transport was further investigated by inducing an artificial reduction in the number of double bonds in the polar lipids of spinach thylakoids using

homogenous catalysis. It was found that the decreased level of lipid unsaturation was associated with a reduction in the rate of full-chain electron transport.

These results appear to be consistent with a positive correlation between the fluidity of the thylakoid membrane and the rate of full-chain electron transport and in particular the rate of plastoquinol oxidation. Overall, the data supports the idea of a homeoviscous mechanism operating in the thylakoid membrane of chilling-resistant plants such as pea as part of an adaptation of photosynthetic rate to low temperatures.

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CONTENTS

List of Tables

The terms "Cold-grown" and "Warm-grown" when applied to thylakoids and chloroplasts in the titles of tables and figures refer to material isolated from cold- and warm-grown pea plants.

List of Figures

Symbols and Abbreviations

ATP	adenosine triphosphate
$CF_0.CF_1$	coupling factor complex
CR	chilling resistant
CS	chilling sensitive
chl	chlorophyll
cyt	cytochrome
DBI	double bond index
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenolindophenol
DGDG	digalactosyldiacylglycerol
DPC	1,5-diphenylcarbizide
DPH	1,6-diphenyl-1,3,5-hexatriene
E_A	activation energy
EPR	electroparamagnetic resonance
Fd	ferredoxin
FeCN	potassium ferricyanide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

k	rate constant of model system reaction
k_I	light-dependent rate constant for electron transport
LHCP	light harvesting chlorophyll a/b protein
MES	2-(N-morpholino) ethane sulphonic acid
MGDG	monogalactosyldiacylglycerol
MV	methylviologen
NADP	nicotinamide adenine dinucleotide phosphate
P_{680}	reaction centre of photosystem 2
P_{700}	reaction centre of photosystem 1
PC	phosphatidylcholine
PCy	plastocyanin
PG	phosphatidylglycerol
PQ, PQH_2, $PQH^{\cdot}$	plastoquinone, plastoquinol, plastohydrosemi-quinone
PS	photosystem
Q_A	primary stable acceptor of PS2
Q_B	quinone binding polypeptide of PS2
Rubisco	ribulose bisphosphate carboxylase oxygenase
r_s	anisotropy of steady-state fluorescence of DPH
SQDG	sulphoquinovosyldiacylglycerol
TO	turnover of PQ in model system reaction
Tricine	N-tris-(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)-aminoethane
UQ,UQH_2	ubiquinone, ubiquinol
V_{max}	light-saturated rate of electron transport
Z	immediate donor to P_{680}

1.INTRODUCTION

1.1 Photosynthesis

Living organisms are almost totally dependent on photosynthesis as the process which assimilates energy into biological matter. It involves the use of light energy to drive reactions resulting in the formation of biomolecules which are far from thermodynamic equilibrium - so-called chemical energy.

In higher plants and algae photosynthesis takes place within the chloroplast organelle and is characterised by the use of chlorophyll as the primary light-harvesting pigment and the production of oxygen as a byproduct. The first three stages of the photosynthetic process can be considered to be:

1. **Energy Capture** : Light is absorbed by pigment molecules and the energy is channelled to a reaction centre where it drives a charge separation process.

2. **Electron Transport** : The strongly reducing and oxidizing species formed by the charge separation initiate a series of redox reactions which take place in a membranous structure - the thylakoid.

3. **Carbon Assimilation** : A number of aqueous enzymatic processes use the accumulated energy to fix carbon dioxide.

The work presented in this thesis deals for the most part with the electron transfer reactions of photosynthesis and the thylakoid membrane in which they occur.

1.2 Background

1.2.1 Thylakoid Membrane

Our understanding of photosynthetic electron transport has increasingly been related to the nature of its physical embodiment - the thylakoid membrane. In 1960 Hill and Bendall proposed a scheme whereby two light reactions drove reducing equivalents against the thermodynamic gradient with two cytochromes mediating the exergonic electron flow from one to the other. This model represented the process totally abstracted from structural considerations. However, the chemiosmotic hypothesis, which postulated the nature of the link between electron transport and phosphorylation (Mitchell, 1961), necessitated the vectorial arrangement of electron carriers so as to provide a transmembrane current, emphasising the importance of the topology of the chain components, the barrier properties of the membrane and the sac-like nature of the thylakoid. The elucidation of the nature of photosynthetic transport since then has taken place first by the ordering of the known components within the electron transport chain, then via the establishing of the kinetics of the main steps (reviewed in Witt, 1971). The advent of mechanical and detergent fractionation procedures (e.g. Anderson and Boardman, 1966; Sane et al, 1970) later refined by the use of phase-partition techniques (Akerlund et al, 1976) yielded evidence that the electron carriers were organised into functionally and physically separate protein complexes (Sane, 1977) which were spatially segregated into different regions of the thylakoid

(Andersson and Anderson, 1980).

The fluid mosaic model (Singer and Nicolson, 1972), which depicted biomembranes as dynamic structures in which the motion of proteins and lipids occurred continually, revolutionised the thinking about the interaction of components within the thylakoid membrane. It became possible to consider the diffusion of protein complexes and lipophilic molecules as part of the regulation of light-distribution and electron transport, particularly in the context of the spatial separation of the complexes. Since the rate-limiting step of electron transport at saturating light is the oxidation of the plastoquinol (PQH_2) molecule (Stiehl and Witt, 1969) which is generally assumed to diffuse freely within the thylakoid bilayer (Velthuys, 1982) a relationship between the fluidity of the thylakoid membrane and photosynthetic rate may exist.

1.2.2 Adaptation to Temperature

It has been proposed that when organisms experience thermal environments away from the optimum growth temperature, one of the primary sites of stress is the fluidity of the organism's membranes (Cronan, 1978). This idea is supported by the observed alterations in the constitution of biomembranes in response to changes in environmental temperature which are such so as to compensate for the effect of the change in temperature on membrane fluidity. This phenomenon, termed homeoviscous adaptation, is known to occur in prokaryotic organisms (Sinensky, 1974) including the thylakoid membranes of thermophilic cyanobacteria (Murata et al, 1979). The principle

has also been extended to higher plants, where it has been suggested that the ability of a plant to tolerate chilling temperatures is dependent on the physical nature of its membranes (Lyons et al, 1979b). There is some evidence which indicates that differences in the compositions of higher-plant non-photosynthetic membranes (Quinn and Williams, 1985) and pea thylakoid membranes (Chapman et al, 1983a) occur between plants grown at different temperatures.

The importance of processes involving diffusion in photosynthetic electron transport places emphasis on the thylakoid membrane as a fluid medium. A relationship between membrane fluidity and adaptation to temperature has been established for prokaryotes and to some extent for higher plants. It is therefore possible that the dynamic nature of the thylakoid may be modified as part of an adaptation of photosynthetic rate. This idea forms the basis for the objectives of the thesis, which are as follows :

1. To investigate the possibility of a homeoviscous adaptation of the pea thylakoid membrane to chilling growth temperature.

2. To establish whether such an adaptation, if it exists, is reflected in an enhancement of photosynthetic rate at low temperatures.

3. To try and determine whether a relationship exists between the fluidity of the thylakoid membrane and the rate of electron transport.

1.3 THYLAKOID MEMBRANE

The thylakoid membrane is a pigment-containing lipoprotein structure consisting of about 60% protein and 40% lipid by weight. The lipid molecules exist in the form of a bilayer which has the protein complexes embedded in it or associated with its surfaces, some of which contain the pigments. In addition to the polar lipids in the bilayer there is the redox-active neutral lipid platoquinone, thought to be located in the hydrophobic core (see section 1.3.1.1.2).

1.3.1 Composition and Function

1.3.1.1 Electron Transport Chain Components

Three of the membrane-spanning complexes are involved in electron flow; two of these are the sites of the light reactions, photosystem 1 (PS1) and photosystem 2 (PS2), the third being a complex of cytochromes and enzymic active sites, the cytochrome b_6/f complex (cyt b_6f). The stoichiometry of these complexes is a matter of debate; for example the PS1:PS2:cyt b_6f ratio was determined as 1:1:1:$\sim$600 chl in spinach (Whitmarsh and Ort, 1984) contrasting with a reported PS2/PS1 value of 1.5-1.8 (Melis and Anderson, 1983). The extrinsic proteins are ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase on the outside of the thylakoid and plastocyanin (PCy) on the lumenal surface of the membrane. In this section the components of the electron transport chain and the reactions they take part in are described, being dealt with in the order

that they occur in the non-cyclic chain between the oxidation of water and reduction of $NADP^+$.

1.3.1.1.1 Photosystem 2 complex

PS2 has been estimated to be a ~600kD protein (Hiller and Goodchild, 1981) containing typically about 200 chl molecules (Melis and Anderson, 1983) which effects the light-driven transfer of electrons from water to plastoquinone, a process which results in a net flow of protons from the outside to the inside of the thylakoid.

The oxidation of water occurs on the inner surface of the thylakoid membrane at a site which is part of the PS2 complex. The observation that the flash-yield of oxygen oscillated with a periodicity of four (Joliot et al, 1969) led to the S-state scheme of water oxidation (Kok et al, 1970), which is now widely accepted (Velthuys, 1980). The chemical identity of the S-states is unknown but the water-oxidase activity requires the presence of bound manganese (Velthuys, 1980, Renger and Govindjee, 1985).

There are several intermediates between the oxygen-evolving complex and the P_{680} trap (Bouges-Bocquet, 1980a; Velthuys, 1980), including the species designated "Z" which is probably a plastosemiquinol cation (O'Malley and Babcock, 1983).

The P_{680} species itself is a specialised chlorophyll a monomer or dimer (Parsons and Ke, 1982) which upon excitation donates an electron to a phaeophytin molecule (Cogdell, 1983; Parsons and Ke, 1982), which in turn is oxidised by Q_A, a semiquinone-Fe^{++} complex (Nugent et al, 1981). The electron is

then in equilibrium between Q_A and a plastoquinone molecule which is bound to the Q_B polypeptide. Following a second turnover the bound plastoquinone molecule is fully reduced and, with the uptake of two protons from the stromal phase, is released by the Q_B protein as a plastoquinol molecule into the thylakoid membrane lipid bilayer (Crofts and Wraight, 1983).

1.3.1.1.2 Plastoquinone/Plastoquinol

The thylakoid membrane contains a variety of the polyisoprenoid neutral lipid plastoquinone (PQ) species with differing chain lengths (Amesz, 1973). The most abundant of these is PQ-9 (the 9 denotes the number of isoprenoid units in the chain) and it is only this species which is thought to take part in electron transport in its free state. The terms plastoquinone and plastoquinol are used in this thesis to refer to the unbound PQ-9 and PQH_2-9 species, unless otherwise stated. The PQ molecule has many states of oxidation and protonation (see, e.g. Amesz, 1973) and is uniquely qualified for its role of proton and electron transport (Hauska and Hurt, 1982). The reduced, protonated PQH_2 molecule, formed at the Q_B protein, transfers electrons to the cytochrome b_6f complex (see below) and protons to the lumen. Both PQ and PQH_2 are highly lipophilic and are believed to move rapidly within the thylakoid bilayer, acting as a pool between the complexes (see section 1.3.3.2). There are about seven active plastoquinone molecules per P_{680} (Stiehl and Witt, 1969; Golbeck and Kok, 1979).

1.3.1.1.3 Cytochrome b_6f

This 120kD protein complex (Hurt et al, 1981) has a

plastoquinol-plastocyanin oxidoreductase activity with a mechanism very similar to the ubiquinol-cytochrome c reaction catalysed by the bc_1 complex of mitochondria and photosynthetic bacteria (Bendall, 1982; Hauska et al, 1983). It contains two cytochrome b_{563}s, a cytochrome f and a Rieske Fe_2S_2 cluster (Hurt et al, 1981; Hauska et al, 1983; Bendall, 1982). The complex may also contain a bound PQ-1 species (Hurt and Hauska, 1982).

Plastoquinol is thought to bind to a hydrophobic site close to the lumenal surface (Haehnel et al, 1983). It then transfers an electron to the Rieske Fe_2S_2 cluster and loses a proton forming a semiquinone ($PQH^{\cdot}$) (Hauska et al, 1983). The Rieske is subsequently oxidised by cytochrome f, which then passes the electron to plastocyanin (Hurt and Hauska, 1982). The route by which the second electron passes through the complex is probably dependent on the prevailing conditions (Hauska et al, 1983). In some circumstances, for every electron transferred from PQH_2 to plastocyanin more than one proton is transported across the membrane. It has been proposed that this extra stoichiometry occurs only during cyclic electron flow around the cyt b_6f and PS1 complexes (Crowther and Hind, 1980), although it has also been associated with linear electron transport under conditions of medium light intensity and pHs under 7 (Hauska et al, 1983).

At high light during non-cyclic electron flow, when the oxidoreductase activity of the b_6f complex exhibits a proton/electron ratio of 1, it has been suggested that the semiquinone is oxidised by a second rapid turnover of the Rieske

centre, both electrons passing directly to cyt f (Rathenow and Rumberg, 1980). Alternatively, pairs of semiquinones may dismutate, forming PQ and PQH_2 molecules. In either case, the b cytochromes are probably not involved under these conditions (Hauska et al, 1983).

Some or all cytochrome b_6f complexes may have associated with them a ferredoxin-$NADP^+$ reductase (Shahak et al, 1981) which is thought to catalyse the reduction of a cytochrome b_{563} by reduced ferredoxin during cyclic electron flow (Shahak et al, 1981; Clark and Hind, 1983).

1.3.1.1.4 Plastocyanin

PCy is a 10.5 kD protein containing one copper atom which transfers one electron from the cytochrome f of the cyt b_6f complex to the plastocyanin oxidase site on the PS 1 complex (Katoh, 1977). From the 3-d structure and positioning of the amino acid residues, two possible sites of electron transfer have been inferred for PCy, one hydrophobic and one negatively charged (Colman et al, 1978). It has been postulated that one of these interacts with the cyt b_6f complex binding site and the other with the site on PS1, so it is possible that it catalyses the electron transfer from cyt f to P_{700} without moving (Haehnel, 1984). Whether or not PCy acts as a pool between the cyt b_6f and PS1 complexes is discussed in section 1.3.3.2.

1.3.1.1.5 Photosystem 1

PS1 probably contains ~200 chl molecules (Melis and Anderson, 1983) and has a light-driven plastocyanin-ferredoxin oxidoreductase activity. A number of light-harvesting

chlorophyll a/b proteins (LHCP1) with a chl a/b ratio of 3.4 to 4.0 are associated with the complex (Malkin et al, 1984). The P_{700} trap is now thought to be a modified chl a monomer (Wasielewski et al, 1981), possibly a chlorinated derivative (Dornemann and Senger, 1984), which is reduced directly by plastocyanin following excitation (Haehnel et al, 1980). The electron from P_{700}^{+} is passed through a series of acceptors in the order A_0 to A_1 to A_2 to FeS centre b to FeS centre a (Malkin et al, 1984), with centre a ultimately reducing soluble Fd. A_0 and A_1 are thought to be chl a molecules (Gast et al, 1983) while A_2 may be an FeS centre (Malkin, 1982) and centres a and b are thought to be bound ferredoxins with Fe_4S_4 clusters (Cammack and Evans, 1975)

1.3.1.1.6 Ferredoxin-$NADP^+$ reductase and Ferredoxin

Fd-$NADP^+$ reductase is a 37 kD enzyme which associates with the outside of the thylakoid membrane and catalyses the reduction of $NADP^+$ or cytochrome b_{563} by Fd. This activity is switched on in the light and it has been suggested that this reaction may be a site of regulation for photosynthetic electron transport (Carrillo and Vallejos, 1983). Its substrate ferredoxin is an 11 kD soluble protein which has a one-electron carrying Fe_2S_2 cluster (Hall and Rao, 1977).

1.3.1.2 Other Integral Complexes

1.3.1.2.1 Light-harvesting chl a/b Protein 2

Unlike LHCP1, which is thought to be part of the photosystem I complex (Wollman and Bennoun, 1982), LHCP2 is probably not permanently attached to one photosystem complex and can be

considered to be a separate entity. LHCP2 has an estimated total molecular weight of ~300 kD (Mullet and Arntzen, 1980), containing about a third of the chl a and nearly all the chl b of the thylakoid. These two forms of chl exist in the complex in an almost 1:1 stoichiometry so that the chl a/b ratio can be a useful measure of the LHCP2 content of a sample (Thornber, 1975). Some LHCP2 is believed to migrate from PS 2 to PS 1 on phosphorylation (Chow et al, 1981; Kyle et al, 1983). The function of LHCP2 is to enhance the quantum efficiency of the light-reactions by increasing the light-harvesting cross-section of the reaction centres.

1.3.1.2.2 Coupling Factor

The ATP-synthetase coupling factor ($CF_0.CF_1$) is made up of a intrinsic portion and a extrinsic complex with approximate molecular weights of 100 and 400kD respectively (Nelson, 1982). Its function is to use the proton and electrical gradient generated by cyclic and non-cyclic electron flow to drive the phosphorylation of ADP.

1.3.1.3 Lipid Bilayer

1.3.1.3.1 Composition

The higher plant photosynthetic membrane has an unusual acyl-lipid composition which shows a high degree of conservation between species. There are five major polar lipid classes in the thylakoid membrane and either six or seven fatty acids depending on the species. The fatty acids are not evenly distributed between classes, some classes being much more unsaturated than others and the unusual **trans** 16:1 fatty acid occurring

exclusively in the phosphatidylglycerol class (see, e.g. Quinn and Williams, 1983). The lipid and fatty acid compositions for the thylakoid membranes of pea and spinach are shown in tables 1 and 2.

The most common lipid species within the bilayer is di-18:3 monogalactosyldiacylglycerol (MGDG), which has been extensively characterised in aqueous dispersions (Quinn and Williams, 1983) where it forms the inverted micellar Hex-II structure. Non-bilayer structures are not thought to occur in biomembranes under physiological conditions (Williams et al, 1984), but they can be induced in the thylakoid membrane by heat treatment for example with a concomitant loss of photosynthetic activity (Gounaris et al, 1983a). The predominance of this non-bilayer forming species (about 50% of the total lipid) is unusual, though it is paralleled in another bioenergetic membrane - the inner mitochondrial membrane which contains substantial quantities of the non-bilayer forming cardiolipin and phophatidylethanolamine lipids (Cullis et al, 1978).

All the other lipid classes do form bilayers in aqueous dispersions. Sulphoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) have negatively charged head groups at pH 7, while phophatidylcholine (PC) exists as a zwitterion at neutral pH.

1.3.1.3.2 Function

The thylakoid lipid matrix can be considered to have at least four roles to play in the functioning of the membrane system:

1. To provide the hydrophobic barrier necessary for the

Table 1: Thylakoid Membrane Lipid Composition

% FRACN. = % molar fraction of the total lipid extract

DBI = double bond index, which is the average number of double bonds per lipid molecule.

LIPID CLASS	SPINACH		PEA	
	% FRACN.	DBI	% FRACN.	DBI
Monogalactosyl-diacylglycerol (MGDG)	38	5.88	44	5.66
Digalactosyl-diacylglycerol (DGDG)	29	5.54	27	5.14
Sulphoquinovosyl-diacylglycerol (SQDG)	18	3.01	9	3.94
Phosphatidyl-glycerol (PG)	12	3.04	12	2.90
Phosphatidyl-choline (PC)	3	4.44	7	3.32
TOTAL	100	5.20	100	5.05

References : Pea - Chapman et al, 1983a
Spinach - Nishihara et al, 1980

Table 2: Thylakoid Membrane Fatty Acid Composition.

The first number in the fatty acid name denotes the number of carbon atoms, and the second the number of double bonds.

FATTY ACID	SPINACH	PEA
16:0	8	9.6
16:1	5	2.1
16:3	13	0
18:0	0	1.8
18:1	2	2.1
18:2	2	5.1
18:3	70	79.4

References : Pea - Chapman et al, 1983a
Spinach - Douce et al, 1973

creation of proton and other ionic gradients. The thylakoid membrane, although more permeable to ions than some membranes, represents a sufficiently great barrier to limit proton leakage to ~1% of the total flux under steady state conditions (Schonfeld and Schickler, 1984).

2. To give the thylakoid an optimal light-harvesting capability by controlling the spacing and orientation of the pigment-containing complexes.

3. To provide the structural base by packing and conferring the correct conformation on integral proteins having specific enzymic activities.

4. To act as a medium in which the lateral and rotational movements of integral proteins and the diffusion of the lipophilic proton and electron carrier PQ may take place.

The part played by individual lipid classes or species in the overall functioning of the bilayer is not known, but the work in this area has begun to indicate specific protein-lipid relationships, and separate roles for different classes have been postulated (reviewed in Siegenthaler and Rawyler, 1985; Quinn and Williams, 1985).

Lipids which adopt Hex-II structures probably do so because of their geometry, with small polar headgroups and relatively bulky acyl chains, and their role in biomembranes may be to pack proteins and stabilise highly curved regions (Israelachvili et al, 1980). It has been proposed that the highly unsaturated MGDG species functions like this in the thylakoid membrane, helping to pack the large protein complexes (Williams et al, 1984; Quinn

and Williams, 1983; Gounaris and Barber, 1983) and enabling the thylakoid to form grana by stabilising the highly curved end regions (Murphy, 1983).

The functioning of the bilayer as a medium for diffusion is discussed in section 1.3.5.

1.3.2 Structural Organisation

1.3.2.1 Stacking

The thylakoids of higher plants are characterised by regions of densely folded membranes called grana, interspersed by flat sheets of membrane, referred to as stromal lamellae. The purpose of this organisation, known as stacking, is still uncertain (Miller and Lyon, 1985).

Isolated thylakoids can be unstacked and restacked by altering the cation composition or pH of the medium (Barber and Chow, 1979; Barber 1980 a,b) and stacking has been explained in terms of the interplay between the conflicting Van der Waals attractive and coulombic repulsive forces acting on the surfaces of the membranes, the latter forces being modified by cation screening or proton neutralisation (reviewed in Barber, 1982; Barber, 1985a). It has been proposed that the structure of a stacked thylakoid is due to a heterogeneity of charge distribution on its surfaces, regions with low charge density are kept together by the electrodynamic dispersion forces (appressed membranes), while those with high charge density are separated due to electrostatic repulsion (non-appressed membranes). The appressed membranes form the basis for the

grana, pulling the thylakoids together into folds, while the non-appressed regions form the exposed regions of the grana and the stromal lamellae (see e.g., Barber, 1982). The net charge on the surface of thylakoid membranes is due principally to the acidic residues of the proteins rather than the relatively low amounts of acidic lipids (Nakatani et al, 1978). The heterogeneity of surface charge distribution therefore reflects an asymmetric lateral distribution of protein complexes.

1.3.2.2 Heterogeneity of Protein Complex Distribution

The breaking up of the thylakoid by mechanical (Sane et al, 1970) or detergent (Anderson and Boardman, 1966) treatments into fractions enriched in granal or stromal membranes indicated a difference in biochemical activities between the regions. The advent of the phase-partition technique (Akerlund et al, 1976) in which inside-out particles specifically derived from the appressed membranes are isolated, yielded convincing data which suggested that the heterogeneity of complex distribution is more extreme than previously realised. From this work it has been proposed that PS1 and PS2 are almost completely separated into the non-appressed and appressed regions respectively (Andersson and Anderson, 1980). This model was supported by electron-microscopy work which showed marked differences in particle size and density on freeze-fracture faces from stacked and unstacked membranes (reviewed in Kaplan and Arntzen, 1982).

The region into which a particular complex partitions is thought to be dependent on its surface charge (Barber, 1982); ones with low charge densities such as PS2 and

non-phosphorylated LHCP2 locating in the appressed lamellae while the highly charged PS1 and $CF_0.CF_1$ complexes reside in the non-appressed membranes.

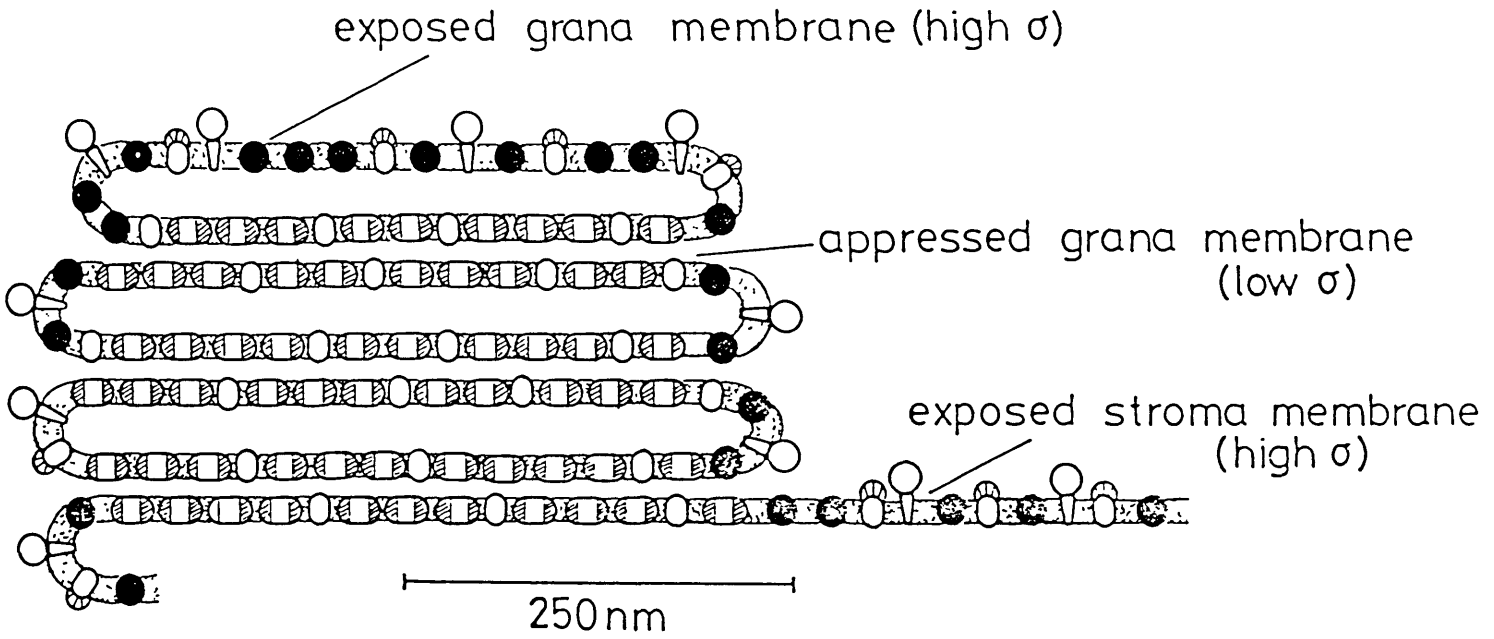
The location of the cytochrome b_6f complex is a matter of some controversy (Barber, 1985b; Whitmarsh, 1985). Some studies using inside-out particles suggest that appressed regions do contain the cyt b_6f complex (Cox and Andersson, 1981; Anderson, 1982), while another suggested they did not (Henry and Moller, 1982). It has been pointed out that phase-partition derived fractions account for only a small part of the granal material (about 15% of the total chlorophyll) so that their compostions may reflect only the centremost region of the granum (Whitmarsh, 1985). Mild detergent treatments of thylakoids yield comparatively large membrane fragments believed to be exclusively derived from the appressed areas which contain no cyt b_6f complex (Dunahay et al, 1984). The reported location of the FNR and PCy extrinsic proteins, which are believed to interact with the cyt b_6f complex in cyclic and non-cyclic electron flow, provides more evidence for a predominance of this complex in the non-appressed lamellae. Peters et al (1983) found that nearly all the PCy was associated with stromal vesicles and Fd-NADP reductase is believed to be located exclusively on the surface of the stromal lamellae (Jennings et al, 1979). On the other hand, recent evidence from an immunocytochemical study on intact chloroplasts indicated the presence of at least some cyt b_6f complex in the appressed regions (Allred and Staehelin, 1985).

In order to reconcile the data it has been proposed by Barber that the cyt b_6f complex may be located predominantly in the boundary regions between the appressed and non-appressed areas (Barber, 1983), and that only those complexes with associated FNR are in the stromal membranes (Barber, 1985b - see figure 1). These models take into account the improbability of a homogenous distribution of a complex over areas of differing surface charge (Barber, 1983). Other reviews favour a restriction of the cyt b_6f to the non-appressed membranes (Haehnel, 1984), or an even distribution between both regions (Whitmarsh, 1985), but there is no unequivocal evidence for any model.

1.3.2.3 Heterogenous Distribution of Acyl Lipids

If there are associations between certain complexes and specific lipid classes for structural or functional reasons then an asymmetry of lipid distribution laterally in parallel to that found for proteins might be expected. In fact the evidence indicates that if such a heterogeneity exists it is only partial since all lipid classes are present in both regions of the thylakoid membrane.

There has been considerable variation in the reported compositions of the lipids in phase-partition isolated inside-out particles (Gounaris et al, 1983c; Henry et al, 1983; Murphy and Woodrow, 1983) and the lipid compositions of mechanically-separated granal and stromal fractions are similar to that of the unfractionated thylakoid (Chapman et al, 1984). The only certain difference between the regions seems to be in the amount of lipid; inside-out particles and granal fractions



LHCPII-PSII complex

PSI complex

$CF_1 + CF_o$ complex

cyt b/f complex + reductase

cyt b/f complex

Figure 1 : Model of the location of the membrane-spanning complexes in the thylakoid membrane with regions of differing surface charge density (σ). Taken from Barber (1985a).

appear to have a lower lipid to protein ratio than the stromal fractions (Ford et al, 1982; Chapman et al, 1984; Murphy and Woodrow, 1983).

There have also been some conflicting reports on asymmetric distributions of lipids between the two leaflets of the thylakoid bilayer (reviewed in Siegenthaler and Rawyler, 1985; Quinn and Williams, 1985).

1.3.3 Diffusion of Thylakoid Membrane Components

1.3.3.1 Integral Protein Complexes

The lateral separation of functionally different integral complexes has important consequences for the nature of electron transport and light distribution, which must involve diffusive processes between the segregated photosystems (Anderson, 1981).

Unstacking of thylakoid membranes results in the randomisation of the complexes, so that both this and restacking by charge screening (as opposed to neutralisation) are processes which depend on the diffusion of integral proteins (Barber, 1980a,b).

Diffusion of LHCP 2 between the appressed and non-appressed regions of the thylakoid has been demonstrated as a likely mechanism behind the well-characterised State1-State2 transition during adaptation to light quality (Chow et al, 1981; Kyle et al, 1983).

1.3.3.2 Mobile Electron Carriers

The separation of the photosystems necessitates a means of communication between them in linear electron transport. The fret regions probably constitute a barrier to diffusion of

integral complexes between the stromal and granal areas (Staehelin et al, 1976). The long-range electron flux is therefore thought to be mediated by PCy and/or PQ. Since the location of the cyt b_6f complex is in doubt there is some uncertainty in ascribing roles to the two mobile carriers, but a comparison of their characteristics suggests PQ as the more probable candidate.

For PCy or PQ to act as a long-range diffusing carrier it must be able to diffuse freely between complexes and at a sufficiently fast rate to have a large range of diffusion within the observed reaction times. The Einstein random walk equation for 2 dimensions is

$$\langle r^2 \rangle = 4.D.t$$

where $\langle r \rangle$ is the mean path length, **D** is the diffusion coefficient and **t** is time. By approximating

$$\langle r^2 \rangle \sim \langle r \rangle^2$$

and using the measured times of reactions and the various estimates of lateral diffusion coefficents a range of diffusion can be calculated for a carrier.

Plastocyanin

Although there is some evidence that PCy is a mobile carrier diffusing in the aqueous phase between the cyt b_6f and PS1 complexes (reviewed in Cramer and Crofts, 1982), other data

listed below seems inconsistent with it acting as a long-range diffusing component between the segregated complexes:

1. If an electron carrier diffuses freely between complexes, the reactions that it mediates would be expected to show pool kinetics. However, the rates of steady-state electron tranport and cytochrome f oxidation are almost linearly inhibited by the inactivation of PCy (Haehnel, 1982; Haehnel, 1984).

2. The dimensions of the lumenal space suggest that the lateral motion of PCy would be too restricted to communicate between the appressed and non-appressed regions (Haehnel, 1984).

3. The lateral diffusion coefficient for PCy has been estimated from the viscosity of the lumen at $4 \times 10^{-8} cm^2 s^{-1}$ (Nesbitt and Berg, 1982) and using a fluorescently labelled PCy molecule in PC liposomes at $6 \times 10^{-8} cm^2 s^{-1}$ (Fragata et al, 1984). Using this value and the electron transfer time from cyt f to P_{700}^+ under saturating conditions of 0.3ms (Haehnel et al, 1980) gives an upper limit of ~90nm for the diffusion path length of PCy. Other estimations using kinetic measurements are 100nm (Selak and Whitmarsh, 1984) and 12nm (Takano et al, 1982). These distances seem barely adequate if PCy is shuttling between the stromal and granal membranes (radius of granum ~250nm), even ignoring the protein obstacles.

It seems that PCy plays at most a partial role in the long-distance transport of electrons from PS2 to PS1, either moving between the edge of the granum and the stromal lamellae (Whitmarsh, 1985), or being entirely restricted to the non-appressed membranes, possibly moving across the lumen

(Haehnel, 1984).

Plastoquinone

The arguments concerning plastocyanin imply that PQ and PQH_2 have to diffuse over long distances. That they act as monomeric freely diffusing carriers is supported by the following:

1. Inhibitor studies have shown that PQ and PQH_2 do act as a pool between the cyt b_6f and PS2 complexes (Siggel et al, 1972). There are probably about 6-7 PQ molecules per PS2 participating in this pool (Stiehl and Witt, 1969; Siggel et al, 1972).

2. It has been demonstrated that free PQ/PQH_2 can act as a transmembrane electron and proton carrier in a model bilayer system (Futami et al, 1979).

3. The fact that quinone analogues appear to competitively inhibit PQH_2 oxidation suggest that the PQ molecules are diffusing, unbound within the lipid bilayer of the thylakoid (Velthuys, 1982).

4. The relative amounts of PQ found in the stromal and granal regions of pea thylakoids are similar when expressed on a per lipid basis (Chapman, personal communication). This suggests that PQ is free to distribute evenly between the two regions of the thylakoid.

5. Lipid enrichment studies in the analagous mitochondrial membrane have established that ubiquinone acts as a freely diffusing, unbound mediator between protein complexes (Schneider et al, 1982). Similar studies on thylakoid membranes suggest the same is true for PQ/PQH_2 (Millner et al, 1983).

Direct measurement of quinone diffusion rates have been restricted almost entirely to ubiquinone (UQ). Using the fluorescence-recovery-after-photobleaching technique a value of 3 X $10^{-9}cm^2s^{-1}$ was determined for an UQ analogue in mitochondrial membranes (Gupte et al, 1984). On the other hand, an analysis of the fluorescence-quenching of anthrosyl-stearate (Fato et al, 1985) gave values of up to $10^{-6}cm^2s^{-1}$ for ubiquinone diffusion in phospholipid liposomes. While the presence of protein (as in biomembranes as opposed to liposomes) does generally decrease diffusion coefficients of species moving within bilayers (Vaz et al, 1984), a discrepancy of 3 orders of magnitude is very large. The work by Gupte et al (1984) suffers from the criticism that the large triplet probe moiety that was attached to the UQ molecule might hinder its movement or cause it to partition into a different region of the bilayer (which in turn affects the rate of diffusion - see below). The lateral diffusion coefficient for PQH_2 in phosholipid bilayers has very recently been provisionally determined as 3-4 X $10^{-7}cm^2s^{-1}$, using the fluorescence quenching of pyrene (M.Blackwell, personal communication).

It has been suggested that the PQH_2 diffusion rate may be closer to $10^{-6}cm^2s^{-1}$ if it is located close to the midplane of the bilayer (Millner and Barber, 1984), for which there is evidence in the case of UQ (Kingsley and Feigenson, 1981; Ulrich et al, 1985). It may be that the unsaturated thylakoid bilayer helps PQ to partition into the centre since it shows a higher degree of miscibility with unsaturated acyl chains in

galactolipid monolayers (Liljenberg et al, 1981). Faster diffusion coefficients are expected in the midplane of the bilayer since the viscosity approaches a minimum in this region as measured in both liposomes (Fato et al, 1985) and thylakoid membranes (Waggoner et al, 1985).

Although the plastoquinol-oxidation step has a total half time of about 15-20ms (Stiehl and Witt, 1969), probably only a small part of this is attributable to the diffusion step between the segregated complexes and a diffusion time of 1-2ms for PQH_2 has been inferred from the kinetics of P_{700}^+ reduction (Haehnel, 1976). Table 3 represents the distances that could be covered in 2ms or 15ms using the various values of **D** for PQ/PQH_2 discusssed above.

The average separation of the PS2 and cyt b_6f complexes has been estimated at 20nm if the appressed regions contain evenly distributed cyt b_6f (Whitmarsh, 1985), 70nm if the b_6f complex is located in the boundary regions (Millner and Barber, 1984) and > 100 nm if they are mostly restricted to the stromal membranes (Anderson, 1982). Another variable is the fact that the very large quantity of protein in the thylakoid is likely to reduce the effective lipid volume, decreasing the effective path length (Millner and Barber, 1984). The values in table 3 imply that a PQH_2 diffusion coefficient of $10^{-7}cm^2s^{-1}$ or above would probably suffice for transport between widely separated PS2 and cyt b_6f complexes even on a 2ms time scale.

In conclusion, perhaps the most plausible arrangement of the electron components is to place the average position of the

Table 3 Range of Motion of PQ/PQH_2

The ranges are calculated from the two dimensional Einstein random walk equation.

D = two dimensional diffusion coefficient.

2 ms is the time attributed to PQH_2 diffusion between PS2 and the cyt b_6f complexes (Haehnel, 1976).

15 ms is the half time for PQH_2 oxidation (Stiehl and Witt, 1969).

D/cm^2s^{-1}	2 ms	15 ms
10^{-9}	30nm	80nm
10^{-8}	90nm	240nm
10^{-7}	280nm	770nm
3×10^{-7}	490nm	1340nm
10^{-6}	890nm	2450nm

cytochrome b_6f complex between the two photosytems, but closer to PS1 than PS2. It seems likely that PQ/PQH_2 diffusion can account for most of the distance that has to be covered between the two light reactions for electron transport to occur within the observed time scale.

1.3.4 Rate of Electron Transport

Table 4 shows the half times that have been determined for the main steps in the electron transport chain by pulse spectroscopic techniques and they refer to one electron turnovers after saturating actinic flashes.

In biochemical chain processes each step exerts a degree of limitation upon the overall rate and in most cases the system is poised so that no step exerts a much greater level of limitation than all the others (Kacser and Burns, 1979). However photosynthetic electron transport at saturating light appears to be an exception in that there is a considerable bottleneck at the plastoquinol oxidation step, being more than an order of magnitude slower than the next slowest step. This is presumably because photosynthesis, unlike most biochemical pathways, is subject to an enormous variation in substrate level, i.e. light intensity, and is therefore not specifically adapted to the saturating conditions used to make the measurements in table 4.

That PQH_2 oxidation does limit steady-state electron transport was demonstrated by Stiehl and Witt (1969). From their time resolved measurements they were able to predict the relationship between the steady-state rate and the redox state of the PQ pool as modified by different dichlorophenyl-

Table 4: Kinetics of Electron Transfer Processes

OEC = oxygen evolving complex

STEP	HALF TIME	REFERENCES
OEC → Z^+	100µs-1ms [a]	Babcock et al, 1976
Z → P_{680}^+	25-45ns 400ns [b]	Van Best and Mathis, 1978 Sonneveld et al, 1979
P_{680} → Q_A [c]	200ps	Trissl et al, 1982
Q_A^- → Q_B [d] $Q_A^-.Q_B^-$ → $Q_A.Q_BH_2$	200µs 400-800µs	Cramer and Crofts, 1982
PQH_2 → Rieske, cyt b_6	15ms	Stiehl and Witt, 1969
cyt f → PCy	40µs	Cramer and Crofts, 1982
PCy → P_{700}^+	20&200µs	Haehnel et al, 1980
P_{700} → $FeS_{A,B}$	170ns	Thurnauer et al, 1982
$FeS_{A,B}$ → FNR [e]	1µs	Bouges-Bocquet, 1980b

a Half-time depends on S state
b Steady-state conditions
c Via phaeophytin
d Electron exists in equilibrium between Q_A and Q_B
e Measured in **Chlorella** at 0°C

dimethylurea concentrations asssuming that PQH_2 oxidation was rate-limiting. This prediction was borne out by experiment.

The kinetics of the PQH_2 oxidation step could be determined by one, or a combination of, three possibilities:

1. The long-range lateral diffusion of the plastoquinol molecule.

2. The binding of the PQH_2 to the plastoquinol oxidase site on the cytochrome b_6f complex.

3. The oxidation and deprotonation steps catalysed by the Rieske FeS centre and (possibly) cytochrome b_6.

However it is generally accepted that the first possibility is not consistent with the data (Haehnel, 1984; Whitmarsh, 1985). The reasons for this conclusion are summarised below.

1. The kinetics of PQH_2 oxidation suggest that it is evenly distributed between complexes after 5 ms (Stiehl and Witt, 1969; Haehnel, 1976).

2. The rate of the oxidation is dependent on the lumenal pH (Siggel et al, 1972), which would not be expected to affect the rate of diffusion.

3. A pH-independent lag phase of 1-2ms in the step has been attributed to the diffusion of PQH_2 along and across the membrane (Haehnel, 1976).

4. Unstacking (resulting in randomisation of the complexes) has little effect on the observed half time for PQH_2 oxidation, but does reduce the lag phase (Haehnel, presentation at 3rd European Bioenergetics Conference, Hannover, 1984).

The possibility that long range diffusion of PQH_2 between the

PS2 and cyt b_6f complexes occurs relatively quickly does not necessarily mean that the oxidation rate is independent of the rate of PQH_2 diffusion. If the turnover is a function of the rate of interaction between the molecule and its binding site on the complex (i.e. a diffusion controlled reaction) then the faster the PQH_2 molecule moves within the bilayer the greater the rate of oxidation.

The Rieske-FeS centre is located within a hydrophilic part of the cyt b_6f complex, and it has been postulated that the binding site for PQH_2 may be close to the membrane-water interface (Hauska et al, 1983). If this is the case, then the binding of the molecule may be limited by the motion of the PQH_2 from the hydrophobic core to the polar headgroup region of the membrane. That plastoquinone can communicate with the aqueous surfaces of bilayers has been demonstrated by its ability to conduct electrons and protons across a membrane in a liposome (Futami et al, 1979).

If the kinetics of PQH_2 oxidation are dependent on the rate of encounter between it and its site of oxidation then the lateral and transverse fluidity of the thylakoid membrane may have a substantial influence on the rate of electron transport at saturating light. Alternatively, it has been suggested that the level of lipid order in the bilayer of biomembranes is an important factor in determining the conformation of integral protein complexes (Pottel et al, 1983). In the context of the thylakoid membrane, this could mean that the conformation, and therefore activity, of the PQH_2 oxidase site might be affected

by the physical properties of the lipid matrix.

1.3.5 Thylakoid Membrane Fluidity

The recognition of the physiological importance of biomembrane fluidity has led to the use of electroparamagnetic resonance (EPR), nuclear magnetic resonance and fluorescent probe techniques to estimate the viscosity of a wide range of membrane systems (reviewed in Stubbs and Smith, 1984; Shinitzky, 1984; Quinn and Chapman, 1980). There has been some debate about whether steady-state measurements using fluorescence probes are related more to the level of lipid order of their environment, rather than the ease of diffusion in the plane of the membrane (Van Blitterswijk et al, 1981; Pottel et al, 1983). The arguments on this point are considered in the discussion section (section 4.1.2). However, for the sake of convenience, the property of membranes which is reported by incorporated probes is termed "viscosity" and its inverse "fluidity" throughout this thesis.

Determinations of thylakoid membrane fluidity have been conducted with spin-label EPR probes (e.g. Raison, 1973; Hiller and Raison, 1980; Nolan and Smillie, 1977; Aronson et al, 1983; Waggoner et al, 1985; Strzalka and Subcyzinski, 1981; Strzalka and Machowicz, 1984; Ford et al, 1982), by measuring the polarisation of diphenylhexatriene fluorescence in steady-state (Ford and Barber, 1980; Yamamoto et al, 1981; Chapman et al, 1982; Ford et al, 1982; Ford and Barber, 1983a; Haworth 1983; Barber et al, 1984) and time-resolved measurements (Ford and Barber, 1983b) and by monitoring intra-molecular excimer

formation in the fluorescent probe diPy (Millner and Barber, 1985).

One conclusion of these studies have been that the thylakoid membrane is a relatively fluid system, of the same order of fluidity as membranes with similar protein to lipid ratios. The degree of fluidity in thylakoid membranes is drastically reduced by the presence of protein in the bilayer since liposomes made up of isolated thylakoid lipids are much less viscous than the membranes themselves (Hiller and Raison, 1980; Yamamoto et al, 1981). Model membranes composed of thylakoid lipids can be made to approach the fluidities found in the natural chloroplast membrane by reconstitution of the $CF_0.CF_1$ protein into them (Millner et al, 1984a; Millner and Barber, 1985). Work with protein-synthesis inhibitors on greening leaves showed that the lower protein to lipid ratio in the thylakoid induced by inhibitor treatment led to a more fluid membrane, despite a decrease in the level of acyl chain unsaturation (Strzalka and Subzynski, 1981; Strzalka and Machowicz, 1984). This suggests that the protein content rather than the level of fatty-acid unsaturation is the dominant factor in determining the fluidity of the thylakoid membrane. This conclusion is the same as that arrived at by other workers for biomembranes in general (Kinosita et al, 1981; Jahnig, 1981; Stubbs and Smith, 1984).

Lowering the unsaturation level of thylakoid lipid vesicles by hydrogenation does decrease their fluidity, but this trend is associated with structural changes which may not occur in the natural membrane (Gounaris et al, 1983b); nevertheless the **in**

situ hydrogenation of thylakoid lipids has been associated with a concomitant lessening of membrane fluidity (Horvath et al, 1985).

There seems to be a lateral heterogeneity of viscosity in the thylakoid membrane with granal membrane fractions being less fluid than their stromal equivalents (Ford et al, 1982; Ford and Barber, 1983b). It seems very probable that this is attributable to the higher protein to lipid ratio found in the granal lamellae (Chapman et al, 1984). The same trend was found in a lipid-depleted granal-derived PS2 fraction where a much higher membrane viscosity than the intact thylakoid was observed (Aronson et al, 1983; Waggoner et al, 1985).

As mentioned in section 1.3.3.2 the variation in membrane fluidity across the width of the bilayer may be of crucial importance in determining the rate of diffusion of PQ and PQH_2. By using EPR spin labels of different chain lengths it is possible to probe to different depths within the bilayer (Jost et al, 1971). This technique has been applied to thylakoid lipid bilayers, PS2 particles and thylakoid membranes (Hiller and Raison, 1980; Waggoner et al, 1985). In all cases the probe molecules embedded deepest in the bilayer reported the lowest levels of lipid order.

1.3.5.1 Protein Diffusion

The incorporation of cholesterol and its derivatives into lipid bilayers is a well established method of artificially reducing the fluidity of membranes. Although the thylakoid membrane has no endogenous sterol (Benson, 1964) the method has

also been applied to thylakoids (Yamamoto et al, 1981; Ford and Barber, 1983a; Haworth 1983; Briantais et al, 1984). The resulting reduction in fluidity abolishes the State 1-State 2 transition, apparently by preventing the diffusion of phosphorylated LHCP2 (Harworth, 1983). It also inhibits the Mg^{++}-induced fluorescence rise (Yamamoto et al, 1981; Briantais et al, 1984) which is due to changes in the spatial interaction between the photosystems and LHCP2 on stacking (Barber 1980a). Stacking itself does not seem to affect the average fluidity reported by probes incorporated into the thylakoid membrane (Harworth, 1983; Barber et al, 1984), despite the pronounced effect of Mg^{++} ions on the fluidity of liposomes made from total thylakoid lipid extract (Gounaris et al, 1983b).

1.3.5.2 Relationship with Electron Transport

The incorporation of cholesteryl-hemisuccinate into thylakoid membranes decreased the steady-state rate of full-chain electron transport, apparently by inhibiting a dark step (Yamamoto et al, 1981) and was shown to slow the kinetics of cytochrome f re-reduction after a saturating actinic flash (Ford and Barber, 1983a), which is consistent with a lower rate of PQH_2 oxidation due to the reduced level of fluidity. Changes in the fluidity of thylakoid membranes interpreted as indicative of phase changes have also been associated with alterations in the rates of electron transport (see section 1.4.4.2).

Recently a hydrogenation-induced increase in thylakoid membrane viscosity was correlated with a reduction in the rates of full-chain and partial electron transport reactions, the

full-chain process being the most susceptible to low degrees of hydrogenation (Horvath et al, 1985). This is in contrast to an earlier study on the effects of hydrogenation on the thylakoid membrane where fluidity was not measured and no inhibition of electron transport was found (Restall et al, 1979). The use of this technique on thylakoids will be examined in more detail in the discussion section of this thesis.

1.3.5.3 Temperature Dependence

Macroscopic viscosity is related to temperature by the empirical equation:

$$n = A.\exp(E_A/RT)$$

where n is the viscosity, E_A is the flow activation energy, R is the gas constant and T is the absolute temperature (Shinitzky and Barenholz, 1978). Phase transitions in lipid bilayers are associated with sharp changes in the value of E_A and can be detected as breaks in the lines of Arrhenius plots of (**log(n)** vs 1/T).

1.4 ADAPTATION TO TEMPERATURE

1.4.1 Adaptation

An organism which is physiologically equipped to survive under a particular set of conditions has adapted to that environment. Adaptation operates at two different levels :

Genotypic - particular species, varieties or ecotypes are genotypically adapted if they are genetically suited to a given

environment. In many cases, part of the genotypic adaptation is a capacity for phenotypic adaptation.

Phenotypic - phenotypic adaptation (or acclimation) is a response by an organism to an external parameter which results in an enhanced ability to maintain function or avoid injury under those conditions. This process may be a rapid response to a short-term fluctuation in conditions (e.g. the State 1-State 2 transition), or a change in structure and/or composition following prolonged exposure to one environment.

A study on acclimation comparing plants of the same species grown in controlled environments is probably better qualified to isolate and determine the nature of an adaptive mechanism than a study on genotypic adaptation (Graham and Patterson, 1982).

1.4.2 Adaptation to Temperature in Higher Plants

Temperature is probably the most important factor in determining the distribution of plants across the globe. The ability of plants to survive within temperature regimes is a complex subject involving every level of its organisation. In this section emphasis will be laid on the adaptation of membranes and the photosynthetic process, but this constitutes only one part of the plants overall ability to tolerate a given temperature environment.

Chilling

The term chilling is applied to the range of temperatures (below about 12°C and above 0°C) which are low enough to prevent growth or cause death in a large number of plant species (see, e.g. Graham and Patterson, 1982). The effects of chilling

stress and injury are manifested at many different levels within the plant and have been well characterised (Levitt, 1980), but it has been suggested that the primary cause of chilling damage is a change in the fluid state of the membranes within the tissue of the plant (Lyons and Raison, 1970; Lyons, 1973). This controversial view is discussed in section 1.4.4.2.1.

Plants are categorised as chilling-resistant (CR) or chilling-sensitive (CS) according to their ability to grow at chilling temperatures.

1.4.3 Adaptation of Photosynthesis to Temperature

It has been suggested that photosynthetic acclimation to sub-optimal (not necessarily chilling) temperatures takes place primarily by an increase in the total catalytic activity of the Calvin cycle enzymes (Berry and Bjorkman, 1980). However Bjorkman and Badger (1979) found that for **Nerium Oleander** plants low growth temperatures (20°C) induced a change in the photosynthetic apparatus which allowed greater light-saturated rates of carbon-fixation at that temperature, even at high CO_2 concentrations. This suggests that the maximal electron transport rate has also been adapted to the growth temperature. A comparison of the temperature dependency of isolated chloroplast electron transport and whole leaf CO_2 fixation rates has suggested that the capacities are normally similar at a given temperature, implying that both parameters are modified during adaptation of photosynthetic rate (Berry and Raison, 1982).

Comparative studies on chilling resistant and sensitive

cultivars of Alfalafa (Peoples et al, 1978) or ecotypes of Australian snow gum (Slayter and Ferrar, 1978) indicated that CR plants are capable of supporting greater photosynthetic rates at low temperatures. Ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) isolated from CR species of potato gave greater rates of CO_2 fixation at low temperatures than enzyme from CS potato plants while the reverse was true at higher temperatures (Huner et al, 1981) which is evidence of a genotypic adaptation of photosynthetic rate to temperature. It has also been suggested that CR and CS plants differ in their ability to conduct electron transport below a critical temperature (see section 1.4.4.2.1).

Some CR plants apparently acclimate their photosynthetic rate during prolonged exposure to low temperatures. The cold-hardening of winter rye induces a change in the kinetic nature of the Rubisco enzyme so that its activity is increased at low temperatures (Huner and McDowall, 1979). This acclimation also takes place at the level of photosynthetic electron transport as the rates of several reactions are increased on hardening including the full-chain light-saturated process (Huner and Hopkins, 1984). In comparing **Lolium temulentum** L. plants grown continuously at 20°C and those transferred to 5°C, Pollock et al (1983) observed higher rates of oxygen evolution from the leaves of the transferred plants under high light and CO_2 conditions at low measuring temperatures.

1.4.4 Adaptation of Membranes to Low Temperature

1.4.4.1 Bacteria

In prokaryotic organisms temperature stress is often associated with an impairment of membrane function due to a change in its dynamic state. Adaptation to temperature in these organisms occurs by alterations in the composition of the membranes which compensate for changes in temperature in order to maintain an optimal level of fluidity (reviewed in Inniss and Ingraham, 1978; Cronan, 1978; Sinensky, 1974). In bacteria this homeoviscous mechanism involves changes primarily in fatty acid composition; thus an increase in temperature evokes a lowering of the unsaturation level of the membrane acyl chains, while a decrease results in the opposite response (Cronan, 1978; Thompson, 1979).

A great deal of work has been done on the effects of a cold shock on the the membranes and photosynthetic functioning of the cyanobacterium **Anacystis nidulans** (reviews Murata et al, 1979; Quinn and Williams, 1983). The temperature dependencies of the thylakoid membrane fluidity and photosynthetic parameters of this organism vary considerably with growth temperature. Arrhenius plots of spin label motion, chl a fluorescence, rates of partial photosynthetic electron transport reactions (Murata et al, 1975) and the kinetics of the State 1-State 2 transition (Ono and Murata, 1979) all showed breaks at around 13 and 24°C in algae grown at 28 and 38°C, respectively. These breaks are believed to be associated with the start of a liquid crystalline to gel phase transition of the thylakoid membranes,

demonstrated by differential scanning calorimetry (Mannock and Williams, 1982) and freeze fracture, which shows that phase separation of the integral thylakoid proteins takes place below the critical temperature (Furtado et al, 1979; Armond and Staehelin, 1979). This data has been interpreted as demonstrating a relationship between the activation energies of photosynthetic processes and the fluid state of the membrane in which they take place (Murata et al, 1979). The lower phase transition temperature found in organisms grown at 28°C compared with those grown at 38°C is a consequence of a more fluid thylakoid membrane (Murata et al, 1975), and is thought to be an example of a homeoviscous mechanism. A similar situation at even higher growth temperatures has been reported for the obligate thermophilic cyanobacterium **Synechococcus lividus** (Fork, 1979). The adaptation in both species is believed to be brought about by the observed cold-induced increase in the fraction of unsaturated fatty acids in the membrane lipids (Murata et al, 1979; Fork, 1979). **A. nidulans** maintains its photosynthetic capacity below the temperature at which the thylakoid membrane transition starts and only suffers irreversible damage at a temperature about 10 degrees lower when the plasma membrane undergoes a phase transition resulting in the loss of the cell's K^+ ions (Ono and Murata, 1981a,b).

1.4.4.2 Higher Plants

1.4.4.2.1 Lyons and Raison Hypothesis

The postulated link between fluidity and temperature adaptation which arose from work on prokaryotes was extended by

Lyons and Raison to the chilling sensitivity of higher plants in a very specific form (Lyons and Raison, 1970; Kuamoto et al, 1971; Lyons, 1973). This theory stated that:

1. The primary event of chilling injury is a liquid crystal to gel transition in the membranes of the plant.

2. Chilling resistance in plants is characterised by membranes which do not undergo phase transitions above freezing, possibly due to a high fatty-acid unsaturation level.

The experimental basis on which the hypothesis was originally founded came from the observations that breaks apparently occurred in Arrhenius plots of succinate oxidase activity in mitochondria isolated from CS plants, but not in those isolated from CR species (Lyons and Raison, 1970; Raison et al, 1971b). These breaks were explained in terms of the conformational alterations being induced in integral enzymes by phase changes in the membrane (Kumamoto et al, 1971), and were found to be coincident with sharp changes in the motion of an incorporated spin-label (Raison et al, 1971a).

This work was later extended to the thylakoid membrane and photosynthetic electron transport, where Shneyour et al (1973) observed changes in the apparent activation energy of full chain coupled electron transport rates in chloroplasts from CS but not CR species, and Raison (1973) correlated these with breaks at the same temperatures in thylakoid membrane fluidity as reported by an ESR probe.

However recent findings seem to refute two of the basic premises; that phase transitions occur in higher plant membranes

at physiological temperatures, that breaks in Arrhenius plots are invariably associated with chilling sensitivity (see Quinn and Williams 1978,1983; Steponkus, 1982).

Phase Transitions

Since phase transitions in biomembranes generally take place over a 20-30°C range (Quinn and Williams, 1978), the idea of deriving a single transition temperature from the EPR signal of a probe incorporated into a plant membrane as originally envisaged (Lyons and Raison, 1970) seems untenable. Raison (1974) therefore proposed that the observed breaks in plots of spin label motion represented the start and end of the mixed phase.

However there is considerable doubt about whether phase transitions occur at all above freezing in the membranes of higher plants, especially in the case of the thylakoid membrane (Quinn and Williams, 1978,1983; Steponkus, 1982). A range of techniques including wide angle X-ray diffraction (McKershie and Thompson, 1978), the measurement of chl a fluorescence and other physiological parameters (Fork, 1979) have failed to detect such a transition in chloroplasts above 0°C. Moreover, there have been no reports of freeze-fracture electron microscopy detecting a phase separation of complexes in the thylakoid membranes of higher plants (Quinn and Williams, 1983). The properties of isolated thylakoid lipids indicate that the bulk of the membrane phase transition is likely to take place substantially below freezing (Quinn and Williams, 1983).

Nevertheless, almost all the data which supports the

existence of the postulated phase changes in CS higher plant membranes above zero degrees has come from work on isolated lipids. The occurrence of such phase-transitions in dispersions of lipids isolated from leaves has been correlated with the chilling sensitivity of the plants using spin-labels (Raison et al, 1979), differential scanning calorimetry (Raison and Wright, 1983) and the fluorescent probe **trans**-parinaric acid (Pike et al, 1979). Murata and Yamaya (1984), also using parinaric acid, detected gel-phase lipid above zero in dispersions of PG isolated from CS plant leaves. The PG contained a high level of saturated fatty-acid molecular species. No gel-phase was seen above 0°C in any of the leaf lipids isolated from CR plants, nor in any class except PG in those from CS plants.

Even if it is the case that the occurrence of phase transitions in lipid dispersions is dependent on the chilling sensitivity of the plant from which they are isolated, since the thermotropic phase properties of membranes are strongly influenced by their protein content (McKershie and Thompson, 1979), the inference that the solid lipid phase exists **in vivo** at physiological temperatures cannot be drawn.

It has also been argued that the interpretation of breaks in Arrhenius plots as being indicative of phase changes is not justified, whether they be plots of the motion of spin-label probes (Quinn and Williams, 1978; Cannon et al, 1975) or of physiological rate parameters (Bagnall and Wolfe, 1978; Wolfe, 1978; Patterson et al, 1979). The apparent correlation between the temperatures at which discontinuities in Arrhenius plots of

spin-label motion and physiological functions has been explained in terms of thermally-induced conformational changes in proteins (Quinn and Williams, 1983). In this view, such changes could result not only in alterations in the activation energy associated with an enzymic process, but also a modification of the apparent membrane fluidity detected by EPR probes.

Breaks in Arrhenius Plots and Chilling-Sensitivity

The correlation between the occurrence of breaks in plots of physiological activity and chilling sensitivity is not clear, particularly in chloroplasts. Although all the measurements on chloroplasts and mitochondria isolated from CS plants have shown breaks, so have some of those carried out on organelles from CR plants (Quinn and Williams, 1978,1983; Steponkus, 1982). Miller et al (1974) and McGlasson and Raison (1973) observed sharp changes in the motion of an EPR probe incorporated into mitochondria isolated from CR plants and Torres-Pereira et al (1974) and Nolan and Smillie (1976,1977) detected similar breaks in chloroplasts from CR plants (see table 5).

In table 5, work on changes in the fluidity of thylakoid membranes, as reported by EPR probes, is compared with studies on the same species' that examined the temperature dependency of full-chain photosynthetic electron transport. From this data it can be seen that :

1. The breaks in the temperature dependency of coupled photosynthetic electron transport rates and spin label motion do seem to occur at similar temperatures.

2. There is no apparent dependence of the occurrence of these

Table 5 Correlation Between Discontinuities in Arrhenius Plots of Full-Chain Photosynthetic Electron Transport and the Motion of an EPR probe Incorporated into the Thylakoid Membrane of CR and CS Plants

Adapted from Quinn and Williams, 1978,1983
E.T. = electron transport

SPECIES	CS/ CR	COUPLED / UNCOUPLED	BREAK TEMPERATURE(S) E.T.	EPR	REFER-ENCES
TOMATO	CS	COUPLED	11-12°C	10°C	1 2
PEA	CR	COUPLED UNCOUPLED	LINEAR 14,25°C	LINEAR	1 2 3
SPINACH	CR	COUPLED COUPLED COUPLED	17°C 5°C 11°C	18°C	4 5 6 7
BARLEY	CR	COUPLED COUPLED	9,20,29°C 9,20°C	10,28°C	8 8 9

References :
1 Raison et al, 1973
2 Shneyour et al, 1973
3 Nolan and Smillie, 1977
4 Torres-Pereira et al, 1974
5 Nolan, 1980
6 Cox, 1975
7 Innoue, 1978
8 Nolan and Smillie, 1976
9 Smillie et al, 1978

breaks on the chilling sensitivity of the plant used.

From these and other results it has been suggested that the occurrence or otherwise of breaks in Arrhenius plots of electron transport may simply depend on the degree to which it is limited by the rate of phosphorylation (Quinn and Williams, 1983).

Conclusion

The original Lyons and Raison theory is now regarded as simplistic by its proponents and it has been modified (Lyons et al, 1979a,b; Berry and Raison, 1982). Breaks in Arrhenius plots have been equated with minor thermotropic transitions (Raison and McMurchie, 1974), not necessarily associated with a bulk lipid phase change (Lyons et al, 1979a). Nevertheless the basic tenet that chilling temperatures induce changes in the physical nature of plant membranes which alter physiological activities remains (Lyons et al, 1979b, Berry and Raison, 1982), and has been broadly accepted by other reviewers (Levitt, 1980; Berry and Bjorkman, 1980; Steponkus, 1982; Graham and Patterson, 1982; Kuiper, 1985).

On the other hand, the occurrence of any lipid in the gel state in the vast majority of CS higher plant membranes **in vivo** at chilling temperatures is unproven. It therefore seems possible that the search for phase changes started by the Lyons and Raison hypothesis has been a distraction from the effects of the general decrease in membrane fluidity caused by chilling temperatures. Indeed, in considering the consequences of changes in membrane fluidity on protein conformation and hence on function there is no need to invoke such phase changes

(Wolfe, 1978).

1.4.4.2.2 The Thylakoid Membrane

The higher plant thylakoid membrane is probably the one for which there is the least evidence of a low temperature effect along the lines of the Lyons and Raison hypothesis. Quinn and Williams (1978,1983) have argued that the situation in higher plants is not analagous to that for the **Anacystis** thylakoid, where a homeoviscous mechanism does appear to exist.

Studies on the intact higher plant thylakoid membrane in relation to adaptation to temperature have looked for evidence of phase transitions in physiological or incorporated probe parameters. Although the breaks in plots of these parameters do not appear to correlate with chilling sensitivity (table 5), the possibility that thylakoid membranes adapted to low temperatures have a higher absolute level of fluidity than non-adapted ones has not been addressed. Unfortunately it is not possible to tell from the literature whether thylakoid membranes from CR plants are more fluid than those from CS plants due to the wide variety of probes and conditions used. However the difference in phase character observed between PG isolated from the leaves of CS and CR species by parinaric acid may reflect a difference in thylakoid membranes, since these contain nearly all the leaf PG (Murata and Yamaya, 1984). Most of the rest of the relevant evidence comes indirectly from studies on the lipid composition of leaves and thylakoids.

Composition

In 1964 Lyons et al found that polar lipids from CS plants

were less unsaturated than those from CR plants. Subsequent work has shown that although the relationship between the level of unsaturation of higher plant membrane lipids and the degree of chilling resistance is not absolute, there seems to be a degree of correlation (Harwood, 1983; Quinn and Williams, 1985).

Since the composition of leaf lipids is dominated by the thylakoid membrane, it might be expected that differences in lipids extracted from whole leaves reflected differences in the thylakoid, but this assumption is probably at least partly erroneous (see below). The data from work carried out on lipids extracted from leaves is summarised in table 6a. The only clear correlation between chilling sensitivity and the lipid composition was found by Murata (1983) in the proportion of dihexadecanoic and 1-hexadecanoic-2-(**trans**-hexadecenoic) species of phosphatidylglycerol, which ranged between 26 and 65% of the total PG in CS plants, and between 3 and 19% in the CR species. No significant difference between samples from CS and CR plant leaves were found for any other lipids (Murata, 1983; Murata et al, 1982; Murata and Yamaya, 1984; Murata and Hoshi, 1984).

Studies on phenotypic changes in the composition of leaf lipids seemed to find higher levels of fatty acid unsaturation and/or a greater proportion of phospholipids in extracts from the plants grown at low temperatures.

However the situation with lipids from thylakoid membranes is less clear, where the majority of studies have not found any evidence for differences in lipid class or fatty acid composition between plants grown under chilling and non-chilling

Table 6a Comparative Studies of Leaf Lipid Composition in Relation to Adaptation to Low Growth Temperature

(PL = phospholipid)

PLANT MATERIAL	NATURE OF STUDY (G = GENOTYPIC, P = PHENOTYPIC)	COMPOSITIONAL CHANGES/DIFFERENCES LIPID CLASS	FATTY ACID	REF.S
10 CS spp. 12 CR spp.	G : compared lipid species of leaf PG from CS and CR plants	only looked at PG	larger amount of 16:0,16:0 and 16:0, t-16:1 in CS	1
6 CS spp. 4 CR spp.	G : as above, looking at other phospholipid SQDG and galactolipid molecular species	relative amounts of classes not determined	no significant difference	2 3 4
4 Alfalfa cultivars	G : compared cultivars of differing cold sensitivity	not measured	more CR var.s were more unsaturated	5
13 var.s of Wheat	G + P : vars. of frost tolerance compared before and after hardening to sub-freezing temperatures	increase in % of PL correlated with frost hardiness of var.	no change in the composition of PL fatty acids	6
4 var.s of Citrus	G + P : comparison of hardened and unhardened plant leaves	hardened had more PC, PE, PI and PG	hardened more unsatd. : higher % of 18:2	7
7 CR spp. 11 CS spp.	G + P : compared CS and CR species and followed changes in 4 CR spp. after lowering the growth temperature to 2°C	not measured	no significant difference or change on hardening	8
Winter rye	P : compared before and after hardening at 5°C	rise in the % of PC and PE	non-specific increase in 18:3	9
Winter rye	P : followed changes after lowering the growth temp. to 8°C	increase in % of PC and classes	increase in 18:3 decrease in 18:2	10
Pea	P : compared leaves from plants grown under winter and summer conditions	no significant difference	winter-grown had more unsatd. and less satd.	11
Wheat	P : compared leaves grown at 2°C and 24°C	more PL in leaves grown at 2°C	more 18:3 in 2°C grown leaves	12
Barley	P : compared hardened (at 4°C) and unhardened leaves	no significant difference	hardened were more unsatd.	13

The references to which the numbers refer are listed opposite table 6b.

References for table 6.

1 Murata, 1983
2 Murata et al, 1982
3 Murata and Hoshi, 1984
4 Murata and Yamaya, 1984
5 Peoples et al, 1978
6 Horvath et al, 1980
7 Nordby and Yelenosky, 1982
8 Wilson and Crawford, 1974
9 Smolenska and Kuiper, 1977
10 Clarkson et al, 1980
11 Chapman et al, 1983a
12 De la Roche et al, 1972
13 Havaux et al, 1984
14 Bishop et al, 1979
15 Vigh et al, 1979
16 Vigh, 1982
17 Chapman et al, 1983c
18 Kuiper et al, 1985
19 Griffith et al, 1984
20 Chapman et al, 1983b

Table 6b Comparative Studies of Lipid and Fatty Acid Compositions of Thylakoids in Relation to Adaptation to Freezing or Chilling Temperatures

SPECIES	NATURE OF STUDY (P = PHENOTYPIC, G=GENOTYPIC)	COMPOSITIONAL DIFFERENCES/CHANGES		REF.S
		LIPID-CLASS	FATTY-ACID	
Pea (CR) Tomato (CS) Maize (CS)	G : Comparison of chloroplast lipids from CS + CR spp.	not measured	no significant difference	14
Winter Rye (CR) Wheat (4 var.s)	G + P : compared from different var.s and changes after hardening to sub-zero temp.s.	not measured	no significant difference or change [a]	15
Wheat (1 hardy, 1 non-hardy var.)	G + P : as above with hardening at 1.5°C for hardy/ non-hardy var.s	increase in DGDG, decrease in MGDG in hardy var.	no significant difference or change	16
Lolium t., Oat (CR) Cucumber, Marrow (CS)	G + P : compared thylakoid lipids from CR + CS plants + CR plants grown at 6°C and 17°C.	not measured [b]	no significant difference or effect of growth temp.	17
Wheat (1 frost-hardy, 1 sensitive variety)	G + P : followed changes in fatty acid and lipid composition during hardening at 1.5°C	hardening induced increase in % of SQDG + DGDG, + drop in % MGDG	hardening induced an increase in the unsatn. of SQDG fatty acids	18
Winter rye (CR)	P : comparison of thylakoid lipids from hardened + unhardened plants.	no significant difference	no significant difference	19
Barley (CR)	P : lipids and fatty acids from plants grown at 4 + 25°C compared.	no significant difference [b]	no significant difference	13
Pea (CR)	P : lipid + fatty acids from plants grown under chilling/ non-chilling condn.s	no significant difference [b]	no significant difference	11 20

[a] Although Vigh et al found no appreciable change in fatty-acid composition, they found that the fluidity of the isolated thylakoid polar lipids from CR varieties increased on hardening.

[b] A higher lipid to chlorophyll ratio was detected in the thylakoid membranes of the plants grown at lower temperatures.

For references see opposite page.

conditions (see table 6b). The exceptions are reports of changes induced by very low (close to freezing) temperatures on thylakoid membrane lipid composition: Vigh (1982) detected changes in thylakoid lipid class in freezing-tolerant varieties of wheat, which he used to explain an apparent increase in the fluidity of the isolated thylakoid lipids (Vigh et al, 1979), following exposure to sub-zero temperatures. Recently changes in both lipid class and fatty-acid composition were associated with the hardening of a hardy wheat variety at 1.5°C (Kuiper et al, 1985). In these particular plant species, therefore, there is evidence for a homeoviscous adaptation of the thylakoid membrane to low temperatures achieved by means of a change in lipid composition.

In other species the changes observed in leaves in response to low growth temperature far exceed those seen in thylakoids (Havaux et al, 1984; Chapman et al, 1983b). This may be partially accounted for by the fact that an unspecific increase in fatty acid desaturase activity as a response to low environmental temperature would only have a very small effect on the already highly unsaturated thylakoid membrane lipids (Harwood, 1983). The high level of phospholipids sometimes seen in cold-grown leaves may simply reflect a relative decrease in the fraction of plastid lipid (Quinn and Williams, 1985).

However, the composition of isolated thylakoid polar lipids has only a partial bearing on the physical properties of the intact membrane. The presence of neutral lipid and protein also have a pronounced effect on membrane fluidity. This relates to

the fact that the lipid to chlorophyll ratio of thylakoids isolated from CR plants was found to be higher in samples from plants grown at low temperatures in experiments on pea (Chapman et al, 1983a,b), winter oat and **Lolium temulentum** (Chapman et al, 1983c) and barley (Havaux et al, 1984). Since the protein to chlorophyll ratio is relatively independent of growth temperature, in pea at least (Chapman et al, 1983a,b), this seems to reflect an increased lipid to protein ratio in response to a chilling growth temperature. This work has led to the suggestion (Quinn and Williams, 1985) that the low level of 16:0,16:0 and 16:0,**t**-16:1 PG found by Murata (1983) in CR plant leaves may simply reflect a lower amount of protein, since these molecular species may specifically associate with LHCP2 in the thylakoid.

As lipid to protein ratio is thought to be positively correlated with the fluidity of the thylakoid membrane (see section 1.3.5), these results are consistent with the operation of a homeoviscous mechanism in response to chilling growth temperatures.

1.5 SUMMARY

The importance of the property of biomembrane fluidity to physiological processes which take place in membranous structures is well established. In prokaryotic organisms, homeostatic mechanisms have been demonstrated which are designed to maintain optimal levels of membrane fluidity as part of the adaptive response to environmental temperature. The role of the

membrane in adaptation to low temperatures in higher plants is less clear. In the case of the thylakoid membrane there is virtually no evidence for the occurrence of changes in the phase of the lipid at temperatures above freezing in either CR or CS plants. However there are apparent growth-temperature dependent differences in the compositions of thylakoid membranes isolated from CR plants which are consistent with an adaptation of membrane fluidity.

The rate of non-cyclic electron transport is limited under saturating conditions by the oxidation of PQH_2. The weight of evidence now suggests that PQH_2 transfers electrons from PS2 to the laterally separated cyt b_6f complex by diffusing within the lipid bilayer of the membrane. It is possible that the rate of PQH_2 oxidation is diffusion controlled, and hence that the rate of electron transport is dependent on the fluidity of the thylakoid membrane. An adaptation of photosynthetic rate in a higher plant to chilling growth temperature might therefore involve the operation of a homeoviscous mechanism in the thylakoid membrane. This raises several related questions concerning the physical properties of the thylakoid membrane, photosynthetic function and adaptation to temperature.

1. Do the growth-temperature dependent differences in the composition of thylakoid membranes from CR plants represent an adaptive response to maintain an optimal level of fluidity ?

2. If so, is this part of an adaptation of photosynthetic rate to growth temperature on the part of the plant ?

3. Does this reflect a general relationship between the rate

of photosynthetic electron transport and the physical nature of the thylakoid membrane ?

The work presented in this thesis set out to address these questions.

The first two questions were investigated using pea plants grown under chilling and non-chilling conditions, the thylakoid membranes of which exhibit the type of differences in composition described above. The fluidity of these thylakoid membranes were estimated over a range of temperatures. The possibility that the rate of photosynthetic electron transport was adapted to growth temperature in the pea plants was also examined using isolated thylakoids and whole leaves. The relationship between fluidity and the rate of electron transport was further investigated by experiments on the effect of **in situ** hydrogenation of the thylakoid membrane on electron transport reactions. Finally, a model system was used in an attempt to examine the interaction between the fluidity of liposome bilayers and the rate of PQ turnover in a redox reaction.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

The two plant species used in this work were pea (**Pisum sativum** L. variety Feltham first) and spinach (**Spinacea oleracea** L.). Pea plants were grown from seed at Imperial College (see below) while the spinach was purchased from a local supplier.

Growth Conditions for Pea Plants

Some experiments used material from pea seedlings grown in trays containing vermiculite in a greenhouse under supplementary lighting conditions (16 hour day). However, the majority of experiments on pea used plants that were germinated and grown under closely controlled environmental conditions. This was necessary so as to compare material from two differing thermal regimes, one of which involved chilling temperatures. Plants grown under the lower and higher temperature conditions are referred to as cold-grown and warm-grown respectively throughout this thesis.

Controlled environment growth cabinets (Fisons model 600G3/TL) were used to provide the growth conditions. A day length of 16 hours was used during which the plants were irradiated with a light intensity that varied from 210 to 290$\mu E.m^{-2}.s^{-1}$ at the level of the uppermost leaves (a distance of about 15 cm from the source). The relative humidity was maintained at 75% ± 5%. The temperature conditions were as follows :

	LIGHT	DARK
COLD-GROWN :	8°C	6°C
WARM-GROWN :	18°C	16°C

Temperatures were maintained within ± 0.75°C. Micol-dusted pea seeds were germinated in the cabinets on damp towelling, and those in which the hypocotyl had just penetrated the seed coat were selected for planting. These were then planted in trays of vermiculite, orientated with the hypocotyl facing down, at 3 cm intervals from each other and from the edge of the tray in order to standardise any local effects from neighbouring seedlings such as shading. The pea plants were watered daily and treated with "Long Ashton" nutrient solution (Hewitt, 1966) every seventh day. For chloroplast preparation, second and third leaf pairs were harvested from the seedlings at different ages, but usually at 16-19 days after germination in the warm-grown plants and at 29-37 days in the cold-grown plants, both of which correspond to the same stage of development - the full expansion of the second leaf pair. Leaves used for leaf-oxygen electrode measurements were taken from the second leaf pair from plants within the same age brackets used for thylakoid isolation.

2.2 CHLOROPLAST PREPARATION

Two types of chloroplast preparations were employed for the

work presented here. The first, used only for measurements with the fluorescent probe diphenylhexatriene and carried out on pea thylakoid membranes, used a technique to prepare thylakoids lacking the outer envelope membranes and stroma (broken chloroplasts). The majority of experiments used intact chloroplasts as their starting material, which in general retain high levels of photosynthetic activity for longer periods; these were prepared using a procedure modified from Nakatani and Barber (1977).

2.2.1 Preparation of Broken Chloroplasts

Pea leaves were harvested and immediately macerated for 10-20 seconds in a pre-cooled perspex grinding vessel containing 200ml of an ice-cold slush of grinding medium using a type PT 35 OD polytron. This medium consisted of 0.33M D-sorbitol, 0.2mM $MgCl_2$ and 20mM 2(N-morpholino) ethane sulphonic acid (MES buffer) brought to pH 6.5 with Tris (tris(hydroxymethyl)-aminoethane).

After homogenisation the brei was filtered through 2 layers of muslin cloth, then through another eight layers, with the first two sheets being separated by a thin layer of cotton wool. The filtrate was collected on ice and then subjected to centrifugation at 2200 x g in a precooled rotor in a MSE super-minor bench centrifuge for 45 seconds. The supernatant was discarded, and the pellet resuspended in a small volume of stacking resuspension medium : 0.33M D-sorbitol, 10mM KCl, 5mM $MgCl_2$ and 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES buffer) brought to pH 7.5 with KOH. They were then

diluted into 10ml of osmotic shock medium for 30 seconds to completely lyse the envelope before the addition of 10ml double strength resuspension medium. In preparations of stacked chloroplasts the shock medium consisted of 10mM KCl, 5mM $MgCl_2$ and 40mM HEPES/KOH pH 7.5 and the double strength medium of 0.66M D-sorbitol, 10mM KCl, 5mM $MgCl_2$ and 40mM HEPES/KOH pH 7.5; in preparations of unstacked thylakoids these media were the same except for the $MgCl_2$ which was omitted. The second centrifugation was carried out at 5000 x g for 5 minutes at 4°C in an MSE chillspin bench centrifuge in order to collect a large yield of thylakoids free of envelope fragments. The resulting pellet was resuspended in a small volume of resuspension medium (as above for stacked chloroplasts, or without the $MgCl_2$ for unstacked chloroplasts) using a paint brush, trying as far as possible to tease the green material away from any starch aggregate. If there was a lot of starch, the thylakoid suspension was diluted down to about 40ml and subjected to two more spins. The first of these spins was for about 20 seconds in the MSE super minor at 500 x g, after which the supernatant was removed (the pellet being almost entirely aggregated starch) and respun in the chillspin at 5000 x g for 5 minutes at 4°C, resuspending as before.

2.2.2 Preparation of Intact Chloroplasts

The procedure up to the first resuspension was the same as for broken chloroplasts. The resuspension was carried out carefully using a paint brush into a small volume of cation-free medium : 0.33 M D-sorbitol, brought to pH 7.5 with Tris, then

diluted using the same medium to about 40ml. This was then spun in the MSE super minor bench centrifuge for 20 seconds at 2200 x g, resuspended carefully in stacking resuspension medium or, with spinach chloroplasts, in unstacking medium which was the same only lacking the $MgCl_2$.

When the intactness of the preparations were determined by measuring the electron transport capacity in shocked and unshocked chloroplasts with ferricyanide as the electron acceptor (to which the envelope is impermeable), they were in the region of 60-75%.

Intact chloroplasts were made for the quickness and convenience of the preparation and for the fact that their photosynthetic electron transport rates decline less quickly after isolation than preparations of broken chloroplasts. However all the data in this thesis was obtained with broken chloroplasts and intact preparations were ruptured with shock medium and made up with an equivalent volume of double strength assay medium before use.

Both preparations were carried out as quicky as possible with cold media, rotors and tubes. Following preparation, the chloroplasts were assayed for chlorophyll content and stored on ice in the dark until used.

2.2.3 Determination of Chlorophyll

Determinations of chlorophyll (chl) content of leaves and chloroplast suspensions was achieved by measuring the concentration of solutions of chl in 80% acetone by the method of Arnon (1949). Leaves were ground to a fine powder in liquid

nitrogen with a mortar and pestle, washed out with 80% acetone and made up to 25ml and filtered. For chloroplast preparations, 25µl of the suspension was added to 80% acetone which was then made up to 10ml and filtered. Smaller or larger volumes of chloroplast suspensions were on occasion used in order to obtain a final chl concentration of between 20 and 100µg per ml.

The solution of chlorophyll in 80% acetone had absorption readings taken at 645, 663 and 750nm, the final reading being taken to determine the scattering of the sample, and was subtracted off the other two values to correct for this. The formulae used to determine the chl a/b ratio was

$$\text{Total chl} = [8.02 \times \text{abs}(663)] + [20.2 \times \text{abs}(645)]$$

$$\text{chl a} = [12.7 \times \text{abs}(663)] - [2.69 \times \text{abs}(645)]$$

$$\text{chl b} = [22.9 \times \text{abs}(645)] - [4.68 \times \text{abs}(663)]$$

When comparative measurements were made on chl solutions from the same chloroplast suspension sample the variation in the chl concentration estimate was < 2% and in the chl a/b ratio < 5%.

2.3 LIPID PREPARATION

A variety of experiments on artificial lipid bilayers used aqueous dispersions of lipid in the form of unilamellar vesicles (liposomes). The lipids used were DGDG, total thylakoid lipid extract, soybean asolectin and pure PC. Thylakoid lipids were prepared by means of High Performance Liquid Chromatography from pea thylakoids by Dr. David Chapman and using silicic acid column and thin-layer chromatographic techniques by Dr. Kleoniki

Gounaris from Spinach chloroplasts at Imperial College. The fatty-acid composition of these lipids was determined using a gas-chromatographic technique described in section 2.8.3. In experiments using liposomes with the fluorescent probe diphenylhexatriene (see below) the lipid was further purified by thin-layer chromatography to remove neutral lipid contaminants from lipid extracts (since carotenoid interfered with the fluorescent signal of the probe). 1-3 mg of lipid in about 100µl of $CHCl_3$ were loaded onto silica gel (Kieselgel G) coated plates which were left to run in tanks containing a mixture of petroleum ether:diethyl ether:acetic acid (8:2:1, v:v:v) for 20 minutes. After drying, the plate was sprayed with 0.1 % dichlorofluorescein in MeOH and examined under ultraviolet light. The band of polar lipid, which stayed close to the origin, was scraped off and extracted with $CHCl_3$. Total thylakoid lipid extract was constructed from the purified individual thylakoid lipid classes recombined in the proportions in which they occur in the natural membrane. Other lipids were purchased from the Sigma Chemical Company. The fluidity of the liposomes made from PC was modified by decreasing the number of double bonds in the fatty acids. This was achieved by a heterogenous catalysis technique using Adams catalyst (PtO_2, bought from Johnson-Massey Chemicals Ltd.) developed by Gounaris et al (1983b). The lipid was dissolved in 10ml of N_2-saturated benzene and bubbled with N_2 for 15 minutes, then catalyst was added in a 1:1 ratio with the lipid by weight. The solution was bubbled for a further 5-10 minutes, after which the catalyst was

removed by centrifugation. The sample was then dried down, redissolved in $CHCl_3$ and analysed for fatty-acid composition in the same procedure described for the lipid extract in hydrogenation experiments (see section 2.8.3).

2.4 USE OF 1,6-DIPHENYL-1,3,5-HEXATRIENE

2.4.1 Description of the Probe Molecule

The conjugated molecule 1,6-diphenylhexatri-1,3,5-ene (DPH) has several properties which make it a highly effective fluorescent probe of the fluidity of biomembranes (Shinitzky and Barenholz, 1978):

(1) The DPH molecule has a very high fluorescent yield when it is located in a hydrophobic environment, but a much lower one when it is suspended in a polar medium such as water. This means that when the probe is added to a suspension of membranes the resulting fluorescent signal usually arises predominantly from the fluorophore incorporated into the lipid matrix.

(2) DPH has a large Stoke's shift, with excitation and emission peaks around 360 and 450nm respectively under most conditions. A consequence of this is that a fluorescent signal can usually be obtained from DPH embedded in a membrane free from other (non-probe) sources of fluorescence and light scattering by use of an emission monochromator.

(3) The simple geometry of the planar DPH molecule and its approximately parallel excitation and emission dipoles facilitate mathematical descriptions of its motion (see section 2.4.4).

(4) The rod-like nature of DPH is thought to result in it

orientating parallel to the acyl-chains in a membrane (Andrich and Vanderkooi, 1976) - see figure 2, so that only movement normal to the plane of the membrane will be reflected in the fluorescence anisotropy. Thus this parameter reports the fluidity along the plane of the bilayer which is related to the rate of lateral diffusion of components.

DPH was purchased as a pure solid from Calbiochem, and kept in a 3mM stock solution in Tetrahydrofuran at -20°C in a darkened container. Care was taken not to expose the probe to light in solution.

2.4.2 Incorporation of Probe

2.4.2.1 Liposomes

The probe was incorporated into liposomes by adding a volume of DPH stock solution in tetrahydrofuran to the lipid solution before drying down so as to give a ratio of 500:1 lipid:probe molecules. The lipid was then dried down in a chromic-acid cleaned tube at 40°C under N_2 until all the solvent was completely removed and then dispersed in a volume of basic buffer medium by vortex mixing and sonication. The sonication was carried out in a Kerry QH Ultrasonic water bath (220 W) under a stream of N_2 or Argon for a set time, or until the light-scattering (measured by the A_{550} signal) stabilised. The temperature was kept within 5° limits of a certain value (usually 20°C) by putting small quantities of ice into the bath. In the special case of the liposomes used to look at the quinone-catalysed reduction of entrapped ferricyanide this procedure was modified, as described in section 2.9.

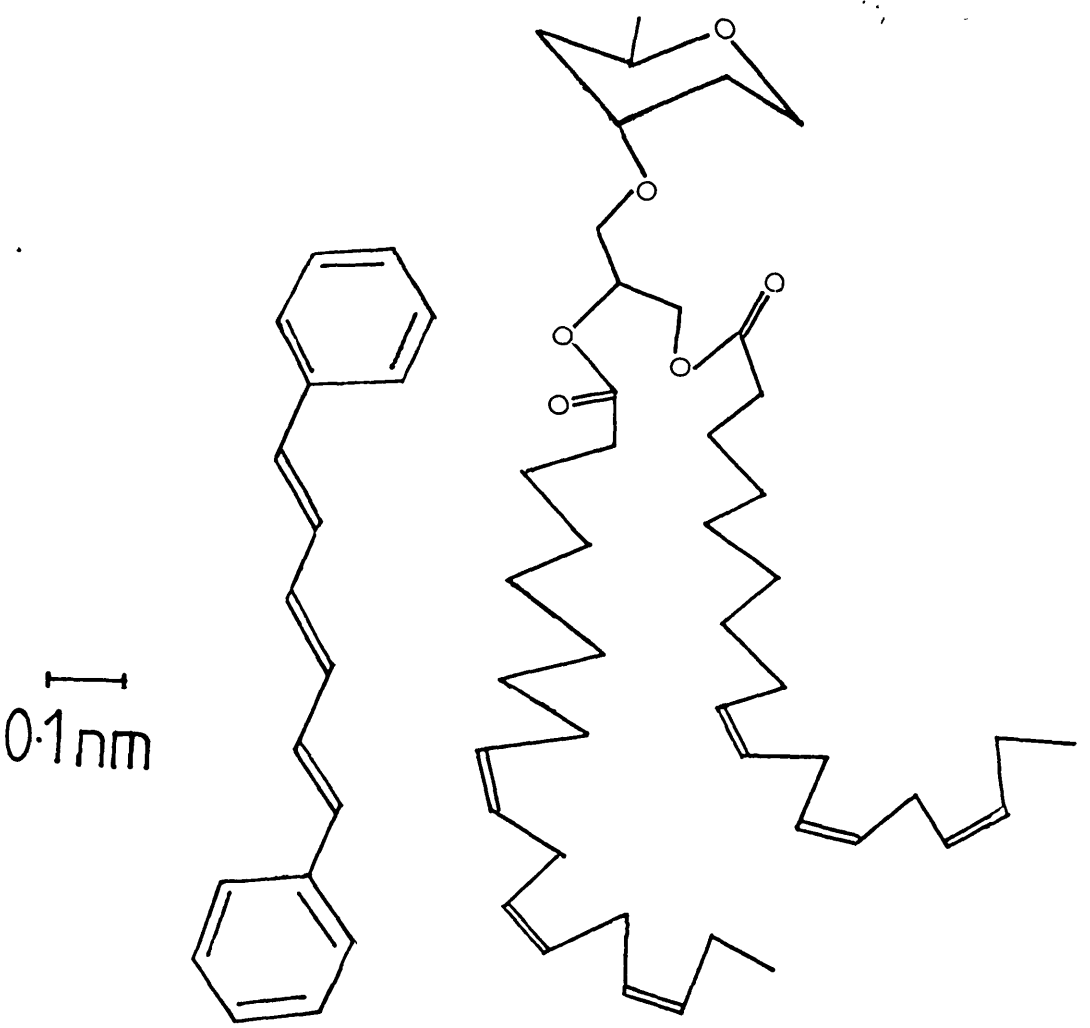


Figure 2 : Scale drawing of diphenylhexatriene and di-18:3 monogalactosyldiacylglyceride (with hydrogen atoms and hydroxyl groups omitted). The DPH molecule is believed to orientate parallel with the acyl chains when embedded in a lipid bilayer.

2.4.2.2 Thylakoid Membrane

In thylakoid membranes the original procedure (Ford, 1982) was to add DPH from the stock solution to a thylakoid suspension of 100µg chl/ml to give a concentration of 7.5µM DPH, equivalent to about 40 : 1 polar lipid to probe molecules. This solution was kept in the dark at room temperature for 30 minutes, at the end of which time the thylakoid membranes were spun down at 12,000 x g in a Beckman J-21 centrifuge for 10 minutes in order to reduce the amount of DPH still in the buffer in micellar form.

Some problems were encountered in this procedure; in particular it was found that centrifugation at this speed resulted in changes in the sample as indicated by the fact that the recombined pellet and supernatant gave a larger DPH signal than the original suspension. High speed spins were also associated with the occurrence of increasing amounts of chl and thylakoid lipid in the supernatants from successive spins, suggesting that the thylakoid was been damaged by the centrifugation process. To avoid this lower speeds were used, normally 3000 x g for 20 minutes - which was fast enough not to lose any material from the thylakoid but caused less damage to the thylakoids.

For the incorporation procedure it was important to use very clean glassware so that grease or detergent were not present in the aqueous phase, as these tended to increase the intensity of the fluorescence arising from the free DPH which had not partitioned into the membrane. Similarly, care was taken to

ensure that the suspending buffer was free of hydrophobic contaminants.

The amount of DPH in a sample was determined by subjecting it to a dual extraction with chloroform and then measuring the fluorescence in the extract and comparing with a calibration curve. Alternatively, an equivalent sample containing no DPH was used to calibrate the dependency of the fluorescent intensity on the concentration of the probe and this curve was used to estimate the amount of DPH in the sample.

Unfortunately, even though the vast majority of DPH molecules partitioned into the membrane (see section 3.1.2), the apparent fluorescent yield from the membrane embedded probe was lower than that from the proportion of DPH not associated with the thylakoid, probably because of its proximity to carotenoid pigment elements which tended to reabsorb the emitted light. When there was a high level of starch associated with the thylakoid preparation there was often a small fluorescence signal and a high background. This was partly due to a greater degree of light scattering, and possibly also because chloroplasts with a high level of starch tend to have a large amount of neutral lipid (PQ in osmiophilic globules) with which the DPH may associate and be lost during the first spin. Procedures used to minimise the starch content are described in section 2.2.1.

A number of variations on this basic protocol were tried in an attempt to determine the optimum conditions for probe incorporation using the lowest possible concentration of DPH.

The most commonly used procedure differed only in that the incubation was for 10 minutes at room temperature using 3μM DPH with 100μg chl/ml of thylakoids followed by a spin for 20 minutes at 3000 x g.

2.4.3 Temperature Control

A Grant LE8 thermostatically controlled water bath with a temperature range of -25° to 60°C circulating odourless Kerosene through the lagged cuvette holding compartment was used to control the temperature of the sample. At sub-zero temperatures the buffer used contained 33% ethane-1,2-diol (which was found to have no appreciable effect on the observed fluorescence anisotropy) and a flow of cold, dry N_2 in the sample chamber prevented condensation from forming on the cuvette surfaces. The temperature of the cuvette contents was monitored continuously with an immersed copper-constan thermocouple.

2.4.4 Measurement of DPH Fluorescence Anisotropy

The final concentration of suspension used in the cuvette was 8μg chl/ml. The cuvettes used were 4-sided quartz which were washed in chromic acid and rinsed out thoroughly with distilled water before use. An identical suspension which had been incubated with the same amount of tetrahydrofuran as the labelled sample (but without DPH) was used to ascertain the background signal due to intrinsic fluorescence and light-scattering.

Fluorescence was measured in a Perkin-Elmer MPF44A fluorescence spectrophotometer with a Hitachi polarisation accessory (polaroid type HN-P'-B). The probe was excited with

polarised light at 360nm through a Schott UG 1 cut-off filter to remove any fluorescence from the polariser and the emission was monitored at 457nm in thylakoids or 460nm in liposomes.

Fluorescence polarisation (anisotropy) measurements were routinely conducted in the energy mode, but excitation or emission spectra were carried out in the ratio mode to correct for the wavelength dependency of the Xenon lamp source intensity. The signal was detected with a Hamamatsu R777 photomultipler with a dynode voltage of between 0.5 and 0.8 kV and the gain set so that the output to the chart recorder or microcomputer analogue to digital converter was in the 10mV range. Slit widths for the excitation and emission monochromators were set at 5 and 12nm respectively for liposome measurements, and both at 20nm for thylakoids. Fluorescence intensity measurements were made with the four possible orientations of the excitation and emission polarizing filters. The signals from the background thylakoid samples were subtracted off the equivalent signals from the labelled sample of thylakoids. (This was unnecessary in liposomes where the background intensity was immeasurably small on the scale used). Usually if the background was in excess of 15% of the signal intensity from the labelled sample, the error in the derived polarisation parameter was too great for it to be of any use. The data reported here typically had to be corrected for background intensities in the region of 8%. Fluorescence polarisation (P) and anisotropy (r_s) parameters were calculated as follows:

$$P = (I_{vv} - I_{vh}.Z) / (I_{vv} + I_{vh}.Z)$$

$$r_s = (I_{vv} - I_{vh}.Z) / (I_{vv} + 2.I_{vh}.Z)$$

where I is the fluorescence intensity (corrected as necessary for background) obtained with the excitation and emission polarizers in the vertical (**v**) or horizontal (**h**) positions as designated by the first and second subscripts respectively. Z is the factor I_{hv}/I_{hh} which corrects for the incomplete depolarisation of the lamp source. For the instrument used Z had a value in the range 0.78 to 0.82. The two derived parameters are interconvertible by the formula :

$$r_s = 2.P / (3 - P)$$

Theory

When an isotropic solution of a fluorophore is irradiated with polarised light, the probability of excitation is proportional to the square of that component of the excitation dipole which lies parallel to the plane of the radiation electric vector. Since the excitation and emission dipoles of DPH lie almost parallel along the length of the molecule, the fluorescence is most intense in the same plane as the excitation light if the probe remains stationary. Therefore the degree of molecular motion within the fluorescence lifetime determines the amount of depolarisation observed in the fluorescence. Simple

geometrical integrations give theoretical limits for the ratio of the fluorescence intensities measured with the excitation and emission polarizers parallel ($I_{||}$) or perpendicular ($I_{\perp}$) for DPH in a very fluid (fluorescence completely depolarised) or frozen isotropic medium

probe immobilised : $I_{||} / I_{\perp} = 3$

very fluid medium : $I_{||} / I_{\perp} = 0.5$

which means that $-1/3 < P < 1/2$ and $-1/5 < r_s < 2/5$ for DPH immersed in an isotropic environment (Shinitzky and Barenholz, 1978).

When the probe is incorporated into a lipid matrix there is an additional factor in that the range of motion allowed to the probe is restricted by the anisotropic nature of the bilayer. This means that there is a time-independent parameter which contributes to the observed polarisation of fluorescence so that the emitted light can never be isotropic. These two components of the steady-state r_s value can be resolved by nanosecond flash techniques into the kinetics of the fluorescence anisotropy decay from the maximum of 0.4 and the minimum, time-indepedent value (Kawato et al, 1978; Kinosita et al, 1981). What these dynamic and static parameters represent in terms of the physical nature of the probe environment and their relationship with steady-state DPH fluorescence anisotropy is a matter of some controversy and is addressed in the discussion (section 4.1.2).

2.5 ELECTRON TRANSPORT ASSAYS

Measurements of the rates of electron transport reactions were mostly carried out in a Rank or Hansatech oxygen electrode with a 1ml capacity. The light source used was either a 150W tungsten-iodine projector bulb focused and heat-filtered by a flask of $CuSO_4$, or a pair of Flexilux tungsten-iodine 150W light sources with fibre optic cables. The first source provided an illumination of about 800$\mu E.m^{-2}.s^{-1}$, while the other two sources were together capable of delivering four times this intensity, though much lower levels were usually used. The unit of light intensity $\mu E.m^{-2}.s^{-1}$ used in this thesis was determined with a Li-Cor LI-185 quantum meter and refers to radiation active in photosynthesis with a wavelength between 400 and 700nm.

The concentrations of artificial donors and acceptors were chosen for the conditions so as to be saturating without exerting any significant secondary effects and are shown in table 7. Also shown is the route of electron flow that is assumed to take place in the presence of these mediators, taken partly from the accepted pathways in the literature and also deduced from the observed sensitivity to the electron transport inhibitor 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) which is believed to act on the PQH_2 oxidation step at low concentration and the PQ reduction step at high concentrations (Izawa, 1980). Hydrophobic chemicals were added in MeOH or MeOH/water and the stock concentrations were selected so that the total volume of alcohol added to the 1ml chamber did not exceed 10µl. This amount of MeOH had no effect

Table 7 Reagents Used to Mediate Electron Transport Reactions

All assays were carried out at saturating light with broken chloroplasts containing 10-20µg chl uncoupled with 1µM valinomycin and 1µM nigericin.

REACTION	CONCENTRATIONS OF REAGENTS	[DBMIB] FOR INHIBITION *	ASSUMED ROUTE OF ELECTRON FLOW
H_2O → FeCN	1mM FeCN	LOW	FULL-CHAIN
H_2O → MV	0.5mM MV 0.4mM NaN_3	LOW	FULL-CHAIN
H_2O → DCPIP	50µM DCPIP	LOW	FULL-CHAIN
DPC → DCPIP	50µM DCPIP 25µM DPC	LOW	FROM THE DONOR SIDE OF PS2 THROUGH THE REST OF THE CHAIN
H_2O → DMBQ, FeCN	1mM FeCN 0.5mM DMBQ	2 COMPONENTS ONE LOW ONE HIGH	FULL CHAIN AND FROM WATER TO THE ACCEPTOR SIDE OF PS2
DQH_2 → MV	0.5mM DQH_2 5µM DCMU 0.25mM MV 0.25mM NaN_3	2 COMPONENTS ONE LOW ONE INSENSITIVE	THROUGH THE b_6f AND DIRECTLY TO THE DONOR SIDE PS1 TO ITS ACCEPTOR SIDE
$DCPIPH_2$ → MV	0.25mM DCPIP 0.5mM Na Asc. 0.25mM MV 0.25mM NaN_3 5µM DCMU	NOT INHIBITED	FROM THE DONOR TO THE ACCEPTOR SIDE OF PS1

* Concentration of DBMIB required to substantially inhibit (>50%) the rate of the reaction. Low concentrations are below 1µM DBMIB, high concentrations are above 5µM.

on any of the measured reaction rates. All reactions were uncoupled with 1μM nigericin and 1μM valinomycin.

2.5.1 Hydrogenation Experiments

In the experiments in which spinach thylakoid preparations were hydrogenated (see section 2.8), electron transport parameters were measured at 25°C before and after the hydrogenation treatment, and in an appropriately treated control. These assays were conducted on broken chloroplasts which in some cases had been in dilute solution (20μg/ml chl) at room temperature for 5 hours in the presence of a large amount of catalyst and 5% dimethylsulphoxide. For these reasons the results were much more variable than in other experiments involving electron tranport assays.

1ml of sample was taken for each electron transport rate assay and used without dilution at 20μg/ml chl. In one hydrogenation experiment usually only one electron transport parameter was measured so that there was enough sample material for 6-7 readings. The chloroplast suspensions were completely anaerobic after treatment and the dissolved hydrogen in the hydrogenated samples tended to lower the redox poise of these suspensions to the extent that mediators such as 2,6-dichlorophenolindophenol (DCPIP) underwent a dark reduction. To avoid this situation and to introduce oxygen for oxygen uptake measurements, the treated samples (control and hydrogenated) had air bubbled through the medium using a pasteur pipette until a concentration corresponding to atmospheric O_2 was reached.

Full-chain electron transport was usually measured by oxygen uptake in the presence of the autooxidisable one electron acceptor methyl-viologen (MV), NaN_3 to inhibit catalase activity and uncoupler. Oxygen evolution with the acceptor potassium ferricyanide (FeCN) was used a few times to measure the rate of full chain electron transport in these experiments, but the results were even more variable than for MV so the latter acceptor was preferred. Measurements of the photoreduction of DCPIP were carried out on a Perkin-Elmer 557 double wavelength, dual beam spectrophotometer. The light-induced reduction was followed by monitoring the absorbance difference at 560nm - 520nm (change in extinction coefficient for DCPIP = $6.8mM^{-1}.cm^{-1}$), using an Intralux 150H light source with a Schott RG 658 cut off filter. Experiments following DCPIP photoreduction in the presence of DBMIB indicated that under most conditions the parameter represents full chain electron transport through PS1.

In some experiments 25μM 1,5-diphenylcarbazide (DPC) was used to donate electrons between the water-splitting complex and P_{680}. It was added from a freshly made 50mM stock solution in dimethylsulphoxide.

The rate of oxygen evolution was determined in the presence of the lipophilic acceptor dimethylbenzoquinone (DMBQ). Under these conditions, electron transport takes place partly from the reducing side of PS2 to DMBQ and partly through the whole chain via PS1 (Izawa, 1980). This provides a measure of PS2 activity to some extent although it is also dependent on PS1. The

presence of the catalyst in these experiments made the level of inhibition induced by DBMIB extremely variable and it was not used in these assays.

PS1 activity was determined by measuring oxygen uptake in the presence of the lipophilic electron donor duroquinol. Duroquinone in MeOH was reduced with $NaBH_4$ and the resulting alkaline solution neutralised with concentrated HCl just prior to addition. Oxygen evolution was completely inhibited with 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU).

2.5.2 Assays on Pea Material

Electron transport measurements were carried out on broken chloroplasts isolated from cold- and warm-grown pea plants as a function of temperature. Temperature control was effected by Techne C-400 circulation unit and a Tecam 1000 heat exchanger. This delivered water at a high rate through wide bore tubing to the oxygen electrode chamber which helped to minimise the heating artifact due to illumination which was < 0.3°C at the lowest measuring temperature (2.8°C). Another identical electrode in series in the water circuit with the one used for measurements was used to determine the temperature with an immersed thermometer.

The temperature was increased stepwise from around 3°C to about 19°C in 2° intervals with at least two measurements at each temperature. The electrode was recalibrated just before the assay with air-saturated water and sodium dithionite at each temperature. At the top of the range the temperature was dropped down to the starting point again in order to assess any

reduction in rate due to ageing. The chloroplasts were prepared intact and kept in the dark on ice throughout the experiment. A volume of thylakoid suspension was chosen which was on the linear part of the curve of chlorophyll concentration against electron transport rate (between 10 and 20µg/ml chl). The assay protocol was to shock chloroplasts in 0.5ml of shock medium (10mM KCl, 5mM $MgCl_2$, 2mM $MnCl_2$, 5mM HEPES/NaOH, pH 7.5) in the electrode chamber for 30 seconds, then add double strength assay medium to 1ml total volume (0.66M D-sorbitol, 10mM KCl, 5mM $MgCl_2$, 2mM $MnCl_2$, 50mM HEPES/NaOH pH 7.5), followed by the uncoupler and the reaction mediators at set intervals. The stock solutions were kept as close as possible to the assay temperature to reduce the time needed for equilibration.

Full-chain electron transport assays were conducted with MV and, more usually, with FeCN. Measurements of photosystem 2 activity were made with DMBQ and FeCN, in some cases in the presence of low concentrations (200-500nM) of DBMIB in an attempt to eliminate the component of the flux passing through PS1.

PS1 activity in pea chloroplasts was determined using $DCPIPH_2$ (kept reduced with sodium ascorbate) as a donor and monitoring the oxygen uptake due to MV reduction. The addition of 10µM DBMIB had no effect on the observed rate which implies that $DCPIPH_2$ donates directly to plastocyanin or photosystem 1.

All the concentrations of mediators used for these reactions are presented in table 7.

2.5.3 Light Dependency

Full-chain electron transport was measured as a function of light intensity in a number of experiments. The intensity of illumination was modified by the insertion of neutral density filters into the light beam. The intensity of the source was measured at the position of the leaf/thylakoid suspension and the filters were calibrated for density between 400 and 700nm on a spectrophotometer. Rates of leaf oxygen evolution or electron transport as a function of light intensity were fitted to the equation :

$$V = (V_{max}.k_I.I)/(k_I.I + V_{max})$$

using a non-linear regression routine (developed by Duggleby, 1981). V is the observed rate of full-chain electron transport, V_{max} is the rate at saturating light, I is the light intensity and k_I is a constant representing the light-dependent component of the rate of electron transport. The k_I parameter is related to the quantum efficiency of the full-chain electron transport process. The above equation is a version of that derived by Rieske et al (1959).

2.6 LEAF OXYGEN EVOLUTION MEASUREMENTS

The rate of oxygen evolution from whole leaves was determined using a Hansatech leaf disc electrode mark 2, using a version of the procedure outlined by Delieu and Walker (1981). The leaf or leaf pair was placed in the sealed chamber of the electrode over

a piece of moist capillary matting to avoid desiccation of the leaf during measurement. Another piece of matting below this was moistened with 1M $NaHCO_3$ in order to maintain a high CO_2 concentration. The electrode was recalibrated at each new temperature: the zero was determined by flushing with N_2, then the chamber was allowed to fill with air and the activity of oxygen was increased by injecting known volumes of air into the sealed chamber.

Temperature control was achieved with the same water bath and cooler as above. The actual temperature in the chamber was measured with a thermocouple and related to the set temperature on the thermostat control. The light source was either a 250W projector bulb reflected off a heat transmitting mirror or two Flexilux 150W HL sources with fibre optic light guides. A range of intensities was used up to $3000\mu E.m^{-2}.s^{-1}$. The heating artifact at the strongest illumination intensity and lowest measuring temperature (3.1°C) was < 0.8°C over the first minute and < 1.6°C in ten minutes. For many experiments low intensities ($1000\mu E.m^{-2}.s^{-1}$ - still saturating for most leaves below ~10°C) were used at low temperatures to reduce this artifact to below 0.4°C in 10 minutes.

In the majority of assays a fresh leaf was used at each temperature, and leaves were stored in the dark at -20°C after measurement until they were assayed for chl content. In the experiments on a single leaf, measurements were made at each temperature without calibrations (which were carried out at the end of the experiment at the different temperatures) so that the

effect of ageing on the rate would be minimised. This procedure seems justified since it was found that after an initial stabilisation period the sensitivity of the electrode did not change significantly with time.

2.7 LOW TEMPERATURE CHLOROPHYLL FLUORESCENCE

Broken chloroplasts from cold- and warm-grown pea plants were diluted to 2.5 and 5µg/ml chl in assay medium and transferred to glass capillary tubes in which they were then frozen in liquid nitrogen in the dark. The tubes were each placed in an optical dewar flask containing liquid nitrogen which was inserted into the cuvette holder of the MPF 44A Perkin-Elmer spectrophotometer. The 77K fluorescence from the samples was measured using excitation light at 435nm (slit width 10nm), the photomultiplier dynode voltage was set to 0.7kV and the gain adjusted to give a full scale output of 10mV to the chart recorder. Emission intensities were recorded at 660, 685, 733 and 790nm (slit width 5nm); the first and last of these values were used to calculate a baseline which was subtracted off the peak intensities measured at 685 and 733nm. The ratios of the 685nm to 733nm peaks were compared for thylakoids from cold- and warm-grown plants.

2.8 HYDROGENATION

The **in situ** hydrogenation of broken spinach chloroplasts was achieved using essentially the method developed by Chapman and Quinn (1976) and applied to thylakoid membranes by Restall et al

(1979).

2.8.1 Hydrogenation Procedure

Two sets of intact spinach chloroplasts containing 100µg chl were osmotically shocked and unstacked in 4.75mls of 5mM HEPES pH 7.5, 10mM KCl for 2 minutes and then made up to 9.5mls with double strength medium (0.66M D-sorbitol, 10mM KCl, 50mM HEPES/KOH pH 7.5). All media were bubbled for 30 minutes with nitrogen before use. The broken chloroplasts were transferred to stainless steel hydrogenation vessels which were sealed except for a small outlet and flushed with gas on ice for thirty minutes. For the control sample the gas used was N_2, and for the sample undergoing hydrogenation it was H_2. A solution of Wilkinsons catalyst (tris-(triphenylphosphine) rhodium I chloride) in dimethylsulphoxide (DMSO) (8mg/ml) was completely converted to the hydride form by bubbling with hydrogen until it acquired a clear yellow colour. At the end of the flushing period the 500µl of catalyst solution was taken directly from a vessel in which it was bubbled with hydrogen and injected into each of the reaction vessels which were then completely sealed and shaken. The vessels were transferred to a water bath at room temperature and the gas pressure was taken up to 6 atmospheres. They were then left at this pressure for a specified period (normally five hours). At the end of this period in most experiments 2ml samples were taken for fatty acid analysis, the remainder of the chloroplast suspension being used for electron tranport assays. In time-course experiments 1 or 2ml aliquots were taken at intervals from the vessels for analyses. The whole

10mls of sample were used for the fatty-acid analyses of the separated lipid classes.

2.8.2 Partitioning of the Catalyst

Seven mls of the control and hydrogenated samples were loaded onto a dicontinuous density gradient with two 2ml cushions of 60% and 30% (w/v) sucrose in buffer. These were centrifuged for one hour at 10°C, after which a green band at the interface of the two sucrose cushions and a dark pellet were obtained. Both the band and pellet were solubilised with 0.2% nitric acid and the chl content was determined. The samples were then analysed for catalyst content by monitoring the intensity of the emission band for rhodium at 369.4nm on a Perkin-Elmer Atomic Absorption/Emission spectrophotometer (model 2280). The matrix used for the standards was a sample of broken chloroplasts spun down in the same way as with the treated samples onto the interface between cushions and then treated with known amounts of the stock solution of catalyst hydride in DMSO.

2.8.3 Fatty Acid Composition Analysis

Fatty acid analysis followed the basic procedure of Williams and Merrilees (1970). The 2ml aqueous aliquots were vortex mixed with 5ml $CHCl_3$, 2.5ml MeOH to which a further 2.5ml of MeOH and 2.5ml of 0.88% KCl solutions were added. (All solvents were Analar grade purchased from BDH Chemicals Ltd.) After vortex mixing phase-separation was accelerated by slow speed centrifugation in a bench top centrifuge and the top green phase was removed by aspiration. The lower phase was then dried down

under nitrogen, 1ml of the boron-trifluoride/methanol complex methylating reagent (14% BF_3 in MeOH from BDH Ltd.) was added and the tube was sealed under N_2 and incubated in a water bath at 70°C for 25 minutes. After the tubes had cooled, a few drops of water and a hexane solution containing 0.039µmol of 15:0 fatty acid methyl ester standard were added. The samples then underwent a double extraction with a total of 3ml of hexane which was then dried down under N_2 to a volume of about 50µl. 1µl samples of this fatty-acid methyl esters solution were injected into a column of 15% Reoplex 400 on a Chromsorb 100/120 mesh in a Perkin-Elmer Sigma 2B Gas Chromatograph and the peak areas were integrated on a Perkin-Elmer 10B data handling station and corrected for relative sensitivities on a microcomputer.

2.8.4 Lipid Class Analysis

The lipids were extracted from samples of control and hydrogenated thylakoids as described above, except that the phase separation was conducted at 4°C, and the extracts were freeze-dried down for 4-5 hours in order to remove the DMSO which made resolution of the classes difficult. The classes were separated using thin-layer chromatography according to Khan and Williams (1977). Plates coated in activated NH_4Cl impreg nated silica gel G were loaded with the samples redissolved in about 100µl of $CHCl_3$. They were then allowed to run for about thirty minutes in tanks of acetone:benzene:water 91:30:8 (v/v/v), sprayed with 0.01% Rhodamine 6G dye and viewed under ultra-violet light. The bands of the different classes

were then identified, scraped off, extracted and analysed for fatty acid composition as described above. The amount of each lipid class present in a sample was calculated from the total quantity of fatty acid.

2.9 MODEL SYSTEM

These experiments employed a technique developed originally by Hinkle (1970) and extended by Hauska (1977) and Futami et al (1979). The principle is to manufacture liposomes containing FeCN in the lumen and quinone within the bilayer, add external sodium dithionite and follow the quinone-catalysed reduction of the FeCN.

2.9.1 Liposome Preparation

Lipid was dried down completely under N_2 in the presence of an amount of quinone (usually PQ) and degassed FeCN containing buffer (50mM N-Tris(hydroxymethyl)methylglycine (Tricine)/KOH pH 8.0, 0.2M $K_3Fe(CN)_6$) was added, in most experiments giving a lipid concentration of 20mg/ml. An equivalent amount of lipid without quinone was also prepared. Both suspensions were vortex mixed and sonicated until they were as completely dispersed as possible. Due to the high lipid concentrations used a powerful sonication technique was needed; samples were sonicated on ice using a "soniprobe" sonicator (Dawe Instruments Ltd.) in 30 second bursts at a 4 Amp setting until the dispersions became less turbid. The suspensions were then sonicated further in the bath sonicator under N_2 in the dark at $< 20°C$ until a sample reading of the absorbance at 550nm showed no further change. This reading was a measure of the light scattering properties of

the dispersion, which gave an indication of whether all the lipid had been dispersed into unilamellar vesicles. In some experiments the lumenal capacity of the vesicles was then increased by freezing in liquid nitrogen and thawing at room temperature.

Small gel-exclusion columns were prepared by filling disposable 5ml syringes with degassed swollen Sephadex G-50 fine packing material, equilibrating them with KCl-containing buffer (50mM Tricine/KOH pH 8.0, 0.3M KCl) and spinning them down at ~600 x g for 3 minutes. To these, 500µl of liposome suspension was added and they were again spun at ~600 x g for 3 minutes separating the liposomes from the external phase. After this treatment > 95% of the FeCN in the suspension was contained within the liposomes and the liposome concentration was virtually unchanged, as measured by the A_{550} signal. There was almost no loss of lipid on the columns as determined by gas-chromatographic fatty-acid analyses.

The average size of the vesicles could be estimated at this point by the FeCN / lipid ratio determined by the dithionite sensitive A_{420} signal. The liposomes were kept at 4°C under N_2 in the dark and used for experimentation within 24 hours of preparation.

2.9.2 Kinetic Measurements

In most cases the liposomes were diluted to 5mg lipid/ml with the degassed KCl buffer before use. The ionophore nigericin (1µM in MeOH) was added to this suspension so that the rate of the reaction was not limited by the accumulation of a pH gradient

across the bilayer (Hauska, 1977). This suspension and an anaerobic solution of (normally) 20mM dithionite in the KCl buffer were placed in the two chambers of a stopped flow apparatus connected to an Aminco DW2 dual-beam spectrophotometer. The volume of the mixing chamber allowed 8-10 measurements which were carried out at 25°C kept constant with a thermostatically controlled water bath. The change in absorbance at 420nm-550nm due to the reduction of FeCN was measured with a 5nm slit width. The kinetics of the first three seconds generally fitted a first order plot, and the rate constant of this was used to express the velocity of the reaction.

The parameters varied were the PQ content of the liposomes, the size of the vesicles, the lipid used and two factors which were thought to alter the fluidity of the bilayer; cholesterol content, and the unsaturation level of the acyl chains. Fluidity measurements were made by incorporating DPH and recording the steady-state fluorescence anisotropy as described in section 2.4.4.

3 RESULTS

3.1 USE OF DIPHENYLHEXATRIENE

1,6-diphenylhexatri-1,3,5-ene (DPH) was used in the work presented here as a fluorescent probe of lipid fluidity in liposomes and in the thylakoid membrane. The properties of the DPH molecule and the theory behind the use of its fluorescence to obtain a measure of the fluidity of the probe's immediate environment are summarised in section 2.4.

3.1.1 Fluidity of Thylakoid Lipids in Liposomes

Pure lipids isolated from pea thylakoid membranes (fatty-acid composition shown in table 8) were recombined, mixed with DPH and dispersed into the basic reaction medium to form liposomes.

Table 8 Composition of Thylakoid Lipids Used to Make Liposomes

All the numbers quoted are percentage fractions except for the DBI (double bond index) which is the average number of double bonds per lipid molecule.

	FATTY ACID						
LIPID CLASS	16:0	16:1	18:0	18:1	18:2	18:3	DBI
MGDG	0.9	-	0.3	0.2	1.9	96.8	5.89
DGDG	3.1	0.2	0.5	0.3	1.1	94.8	5.74
(SL + PG)	28.8	1.7	3.9	1.7	12.6	51.1	3.64

The purified lipid classes were used in ratios of 2:1:1 MGDG:DGDG:(SL+PG) to mimic the naturally occurring proportions

in the thylakoid membrane. The overall DBI of the recombined total lipid extract was 5.29.

The anisotropy of the steady-state fluorescence from the incorporated probe (r_s) is plotted as a function of measuring temperature in figure 3. The curve is fitted from a straight-line regression of the predicted near-linear relationship (Shinitzky and Bar enholz, 1978) between $\log[(r_0/r_s-1)^{-1}]$ and $1/T$, where r_0 is the anisotropy of fluorescence immediately after excitation (value taken to be 0.39 - Kinosita et al, 1981) and T is the absolute temperature. Fluorescence anisotropy, rather than the derived $\log[(r_0/r_s-1)^{-1}]$ parameter is plotted in figure 3 in order to facilitate comparison with other results which are presented in terms of r_s.

The temperature dependence found here is similar to that reported previously for liposomes made from thylakoid lipid (Gounaris et al, 1983b), but the anisotropy values are lower than those found by other workers. Ford (1982) and Gounaris et al (1983b) reported r_s values of 0.130 and 0.135 respectively for DPH-labelled total thylakoid lipid vesicles measured at 25°C, while the value for the line in figure 3 is 0.104. This discrepancy may be due to the pigment content of the lipid extract, since energy transfer readily occurs from DPH to free carotenoid as detected by a shortening of the fluorescent lifetime (Millner et al, 1984b). This means that even very low levels of pigment contamination can result in a sizeable increase in the observed anisotropy of the DPH fluorescence from

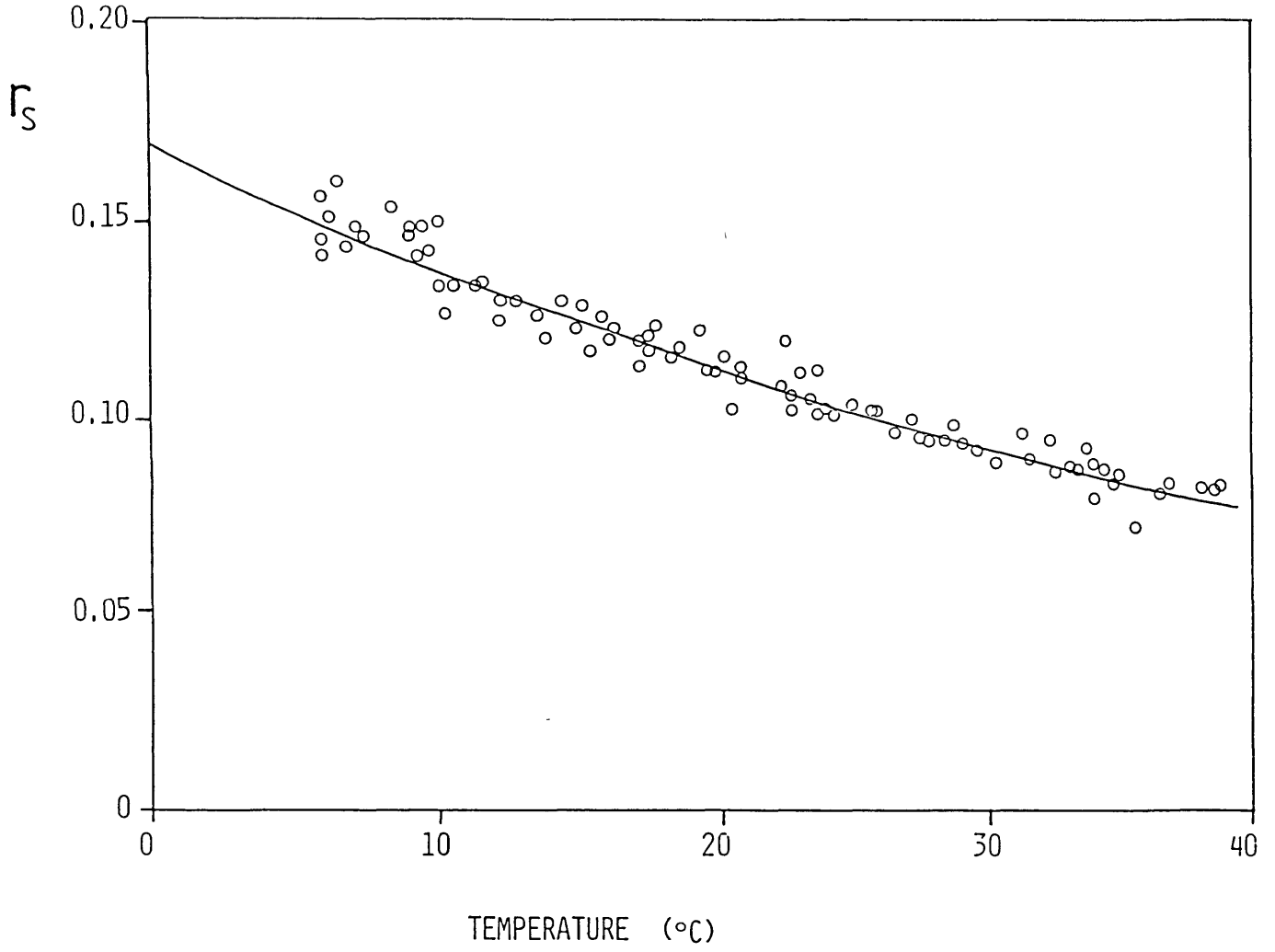


Figure 3 : Temperature dependency of anisotropy of fluorescence (r_s) from DPH incorporated into thylakoid lipid liposomes. The composition of the lipid is shown in table 8. The curve is fitted using the parameters from a linear regression of the data in the form $\log(r_o/r_s)^{-1}$ and 1/T (see text).

thylakoid lipid dispersions and particular care was taken to minimise this source of error in the preparation of the liposomes (see section 2.3). The same labelled liposome preparation for which the data is presented in figure 3 were used in experiments which carried out time-resolved measurements to determine a fluorescence lifetime of 6.1 ns for the DPH in this sample (Millner et al, 1984b). This can be compared with the value of 5.9ns determined for the same parameter in the thylakoid lipid liposomes used by Ford and Barber (1983b).

3.1.2 Fluidity of the Thylakoid Membrane

3.1.2.1 Use of DPH in Thylakoid Membranes

The use of DPH to probe the fluidity of the thylakoid membrane was pioneered by Ford and Barber (1980) and they obtained r_s values in the range 0.15 - 0.17, but a refinement of the technique in later experiments yielded values closer to 0.19 when the measurements were carried out at room temperature (e.g. Ford et al, 1982). However there are considerable problems in the use of DPH in a highly pigmented structure such as the thylakoid membrane. The fluorescence emission is attenuated to a large degree by reabsorption so that the amount of probe required to obtain a signal of sufficient magnitude for an accurate evaluation of the r_s parameter is much greater than in other membranes. The fluorescent yield of DPH embedded in thylakoid membranes is so reduced compared with that obtained with pure thylakoid lipid liposomes that it is actually lower than that for DPH suspended in the aqueous medium alone. This raises two questions about the use of DPH to measure fluidity in

the thylakoid membrane:

1 Does the probe partition into the thylakoid membrane and if so how much of it ?

2 Is the observed fluorescence derived exclusively from the probe located in the thylakoid membrane lipid bilayer ?

In order to assess the partitioning of the probe into the membrane it was necessary to separate the thylakoids from the suspending medium at the end of the incubation with DPH by centrifugation. Figure 4 shows the results from an experiment in which a thylakoid preparation was incubated with DPH in the standard procedure (100µg chl/ml envelope-free thylakoids in D-sorbitol resuspension medium for 10 minutes at room temperature) and then subjected to two centrifugation steps. The intensity of the DPH fluorescence and the derived r_s parameter were determined for the original sample and the supernatants and pellets (which were resuspended into the same volume of medium) following the spins. The amount of DPH in the supernatant was determined by an extraction procedure and the quantities of probe quoted for the resuspended pellets were calculated from these determinations assuming no loss of probe. This assumption was borne out by other experiments in which the pellet was also extracted.

The data in figure 4 shows that the vast majority (96%) of the probe is incorporated into the thylakoid by the end of the incubation period. This fraction of incorporation was independent of the probe:lipid ratio used (data not shown). The drop in the size of the fluorescent signal associated with the

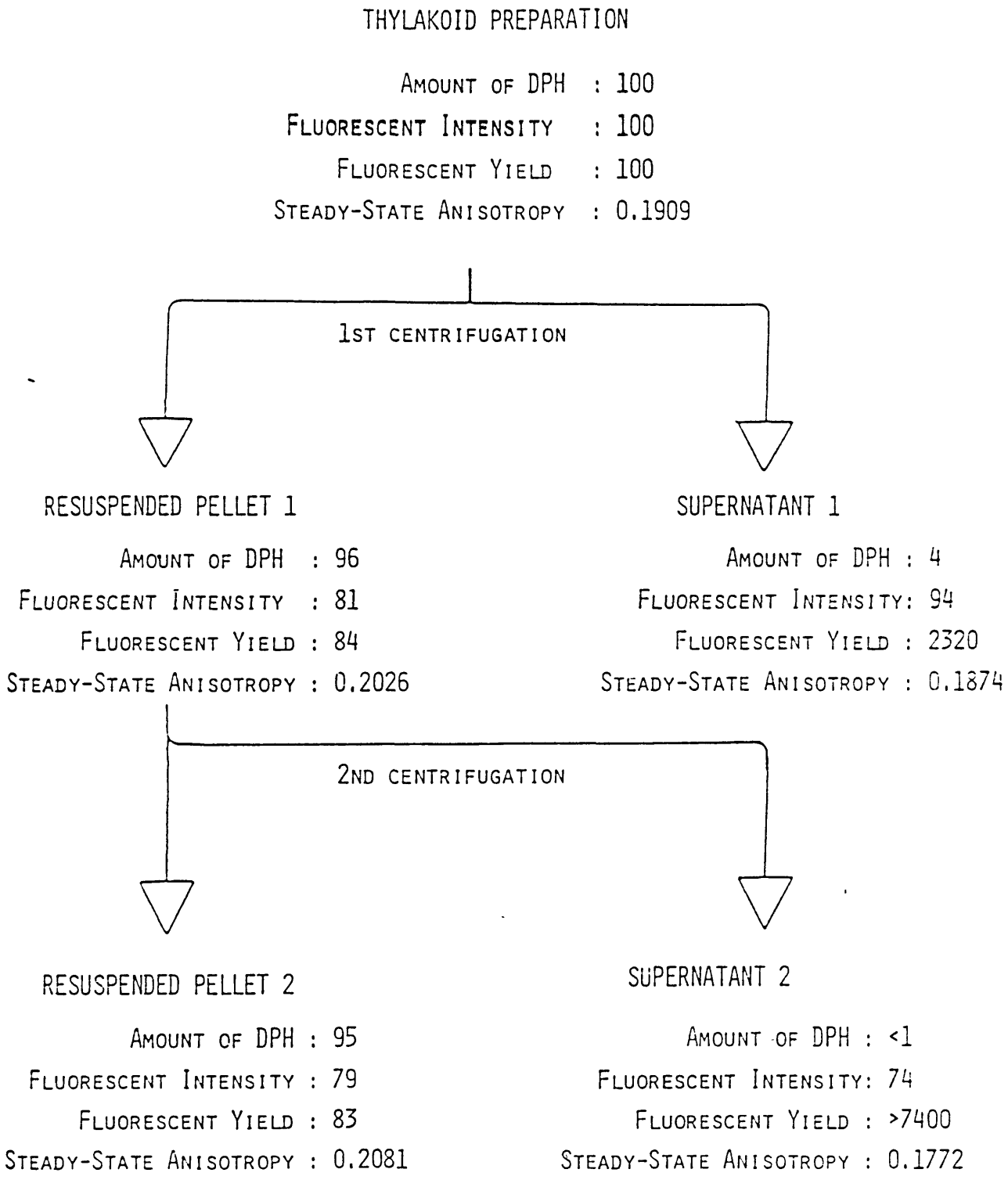


Figure 4 : Effect of centrifugation on the fluorescent signal from DPH incorporated into a suspension of thylakoids. The amount of DPH, fluorescence intensity and yield are expressed in arbitrary units. The fluorescence yield is the intensity of fluorescence per unit DPH. The fluorescence parameters are corrected for the signal obtained with a similarly treated sample with no probe.

thylakoids following the first spin indicates that the small quantity of unincorporated probe apparently accounts for 19% of the signal before centrifugation. The fact that the fluorescent signal from the supernatant is more intense than that from the resuspended pellet following the first spin is mostly attributable to the separation of this free DPH from the reabsorbing pigments. However, the fluorescent yield for DPH suspended in this supernatant is higher than that for probe in the pure resuspension medium, indicating that the presence of the thylakoids releases a hydrophobic component into the medium. Indeed, trace quantities of fatty-acids were detected in the supernatant (data not shown). The second centrifugation had much less effect than the first on the parameters measured in the thylakoids with only a slight decrease in the amount of DPH and the fluorescence intensity and an unchanged r_s value. The very high fluorescent yield of the DPH in the supernatant from the second spin is probably due to an increased concentration of hydrophobic contaminants caused by the breakup of the thylakoids during centrifugation. More spins brought about the occurrence of thylakoid lipid and chlorophyll in the supernatants.

From this data it appears that the first centrifugation step removes the unincorporated DPH, since subsequent spins do not significantly further reduce the intensity of the emission signal associated with the thylakoid. This interpretation is supported by the fact that the r_s parameter associated with the thylakoid is altered by the first centrifugation but not by the second. In addition to this it was found that if the pellet from

the first centrifugation is resuspended in media which confer widely differing fluorescent yields when DPH is freely suspended in them, the intensity of the observed signal is broadly unchanged (data not shown). In the light of these results, the fluidity of the thylakoid membrane was determined using a single spin after the addition of the DPH.

The very low yield of fluorescence from DPH incorporated into thylakoids could be due either to a high level of reabsorption of the emitted light or to direct energy transfer from the DPH to the pigment. The second of these possibilities may increase the observed fluorescence anisotropy. However, it has been claimed that energy transfer from DPH to the pigment is not thought to be significant in thylakoid membranes containing the probe since the chlorophyll excitation spectrum was not affected by the presence of large quantities of DPH (Ford, 1982). In line with this conclusion, the value of the observed r_s parameter was found to be independent of the amount of probe used in the range of lipid:probe ratios from 40 to 100 (figure 5).

3.1.2.2 The Fluidity of the Thylakoid Membranes Isolated from Warm- and Cold-Grown Pea Plants

The steady-state anisotropy parameter was determined for thylakoids isolated from cold- and warm-grown pea plants over a range of temperatures (figure 6). From this data it appears that the thylakoid membranes from cold-grown plants are more fluid than those from warm-grown plants over all temperatures between 3 and 20°C. The temperature dependencies of the r_s parameter for

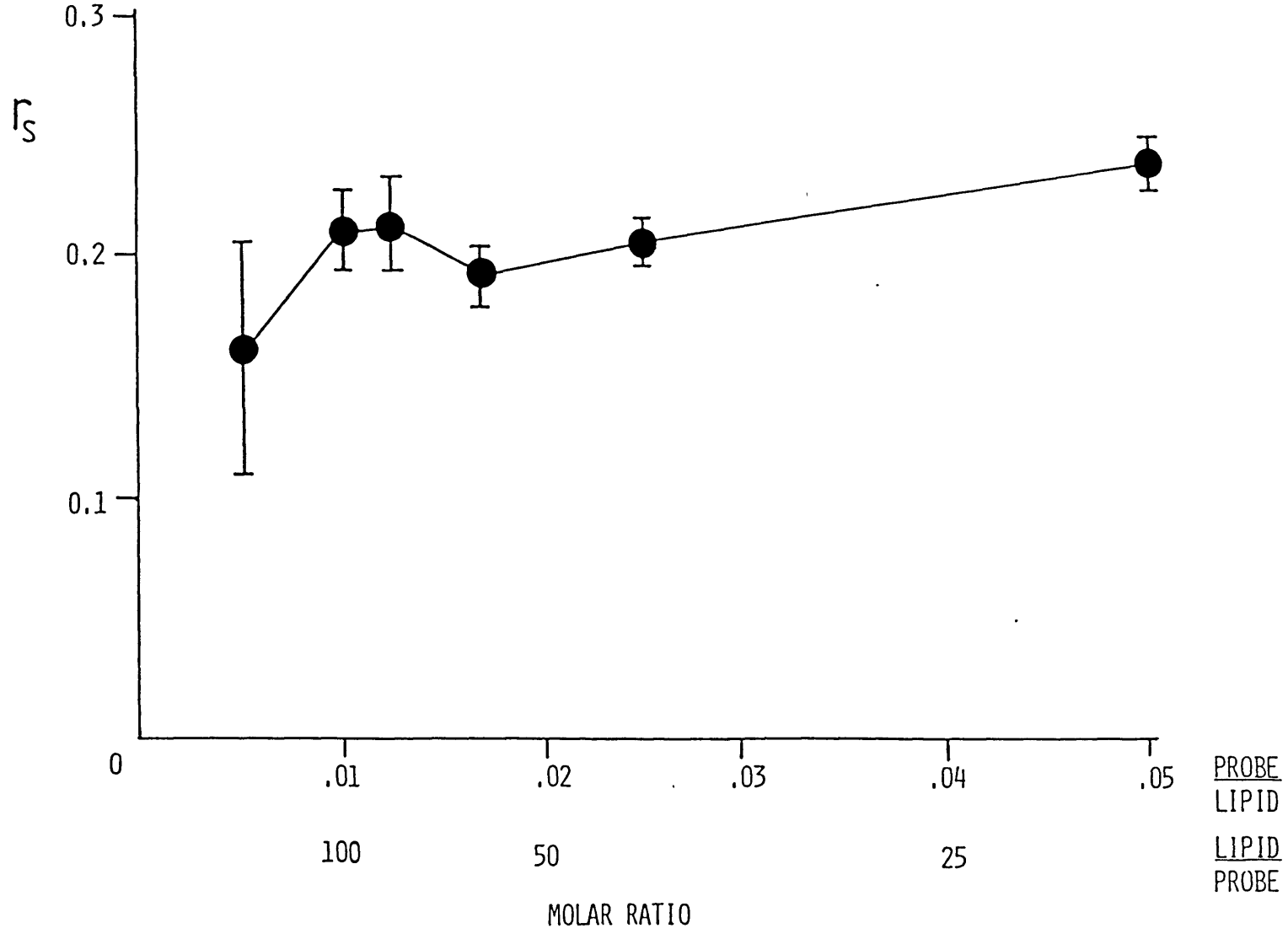


Figure 5 : The anisotropy of the DPH fluorescence observed in samples of thylakoid membranes labelled with different amounts of probe. The same thylakoid preparation was used for all the data. The error bars represent 95% confidence limits.

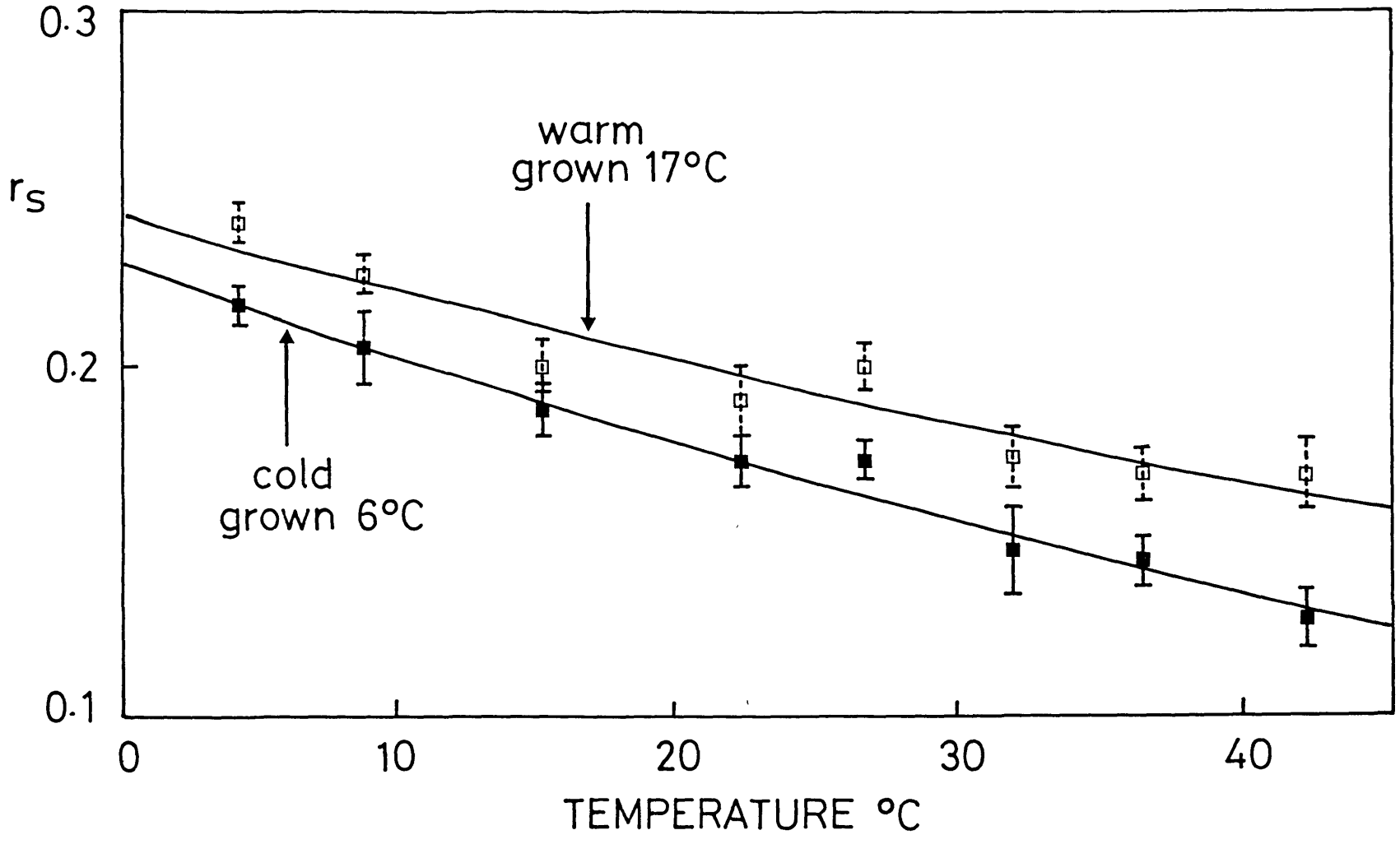


Figure 6 : Anisotropy of fluorescence from DPH incorporated into thylakoid membranes isolated from cold- and warm-grown plants as a function of measuring temperature. The error bars represent 95% confidence limits for the mean of 10 readings.

the two sets of points (as represented by the gradients of the fitted straight lines) were not significantly different within the experimental error. The points in figure 6 are drawn with a curve, although a straight line could have been used since a linear relationship fits the data just as well as the equation used to fit the results in figure 3 (see above). This is probably because the assumptions behind the non-linear relationship between r_s and temperature quoted above break down at the relatively high values of anisotropy (Shinitzky and Barenholz, 1978), like those observed in the fluorescence from DPH embedded in thylakoid membranes.

The plot shown in figure 6 is from a single experiment and reflects the trends seen in the results of a series of similar experiments where measurements of the DPH fluorescence were made only at 6 and 17°C (which correspond to the mean growth temperature for the two growth regimes). The average anisotropy values determined for cold- and warm-grown thylakoid membranes from these experiments are represented in figure 7. In the particular experiment plotted in figure 6 the r_s value associated with the preparation of cold-grown thylakoid membranes is marginally lower than that seen on average so that the difference between the two samples may be slightly exaggerated compared with the results taken as a whole.

The variation in the r_s values obtained in different experiments on thylakoids from the same thermal regime exceeded the error within an experiment, indicating that there was variation in thylakoid membrane fluidity between preparations.

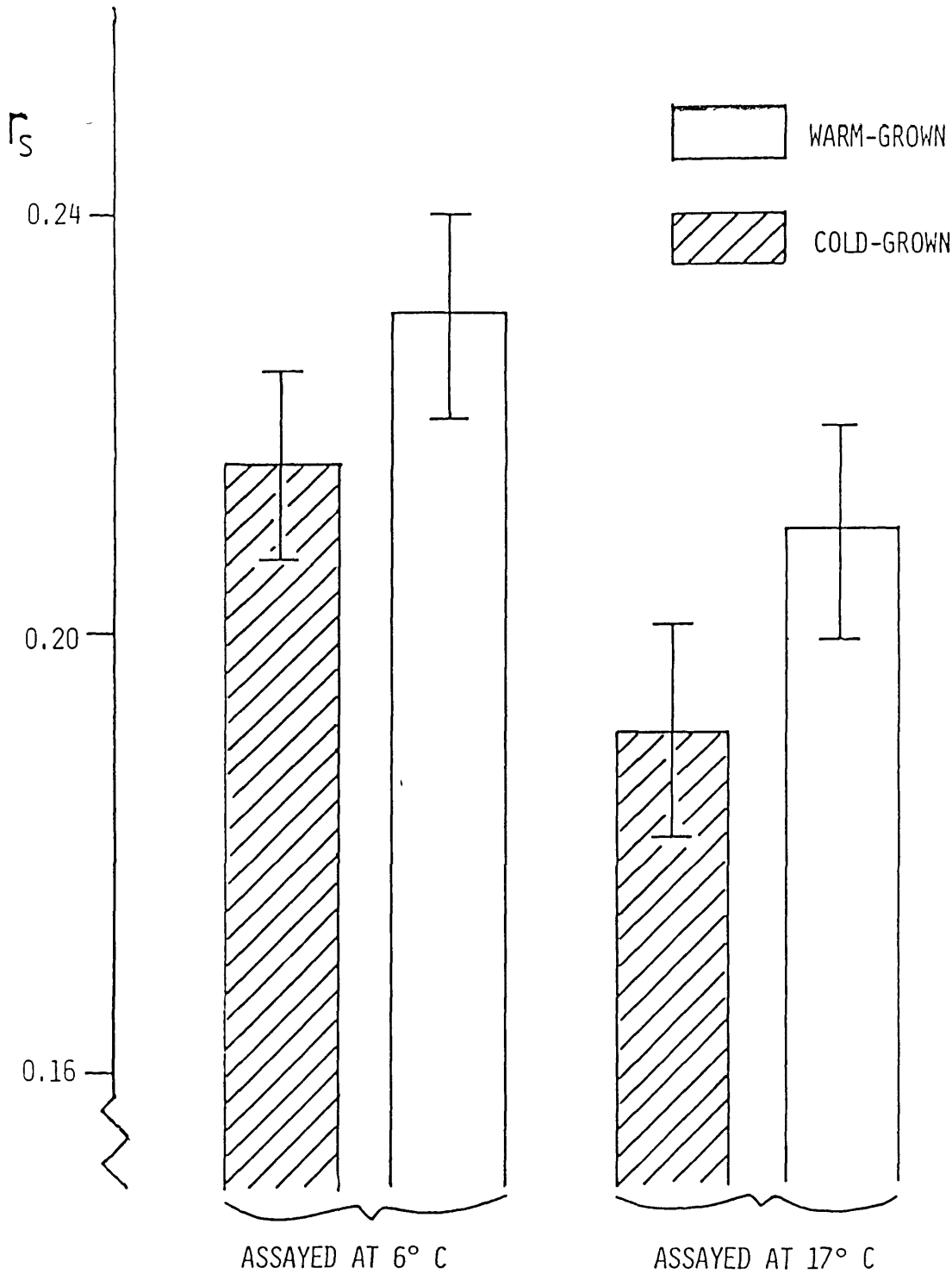


Figure 7 : Histogram of the anisotropy of DPH fluorescence observed in a number of preparations of thylakoids from cold- and warm-grown pea plants measured at 6 and 17°C. The bars depict the mean value of r_s for 11 and 8 thylakoid preparations from cold- and warm-grown plants, respectively. 95% confidence limits are shown.

This means that the confidence limits drawn in figure 7 which represent the error between different thylakoid preparations are much bigger than the limits that would be calculated taking all the r_s determinations as a whole.

The differences between the average r_s values determined for cold- and warm-grown thylakoids observed at both measuring temperatures are significant at $P < .02$. A comparison of the anisotropy values derived from measurements made on thylakoids at the average growth temperature of the plants from which they were isolated reveals a similar level of apparent thylakoid membrane fluidity. This is consistent with the existence of an adaptive mechanism which induces more fluid thylakoid membranes as a response to low growth temperatures, thus compensating to some extent for the decrease in fluidity due to the inherent temperature dependency of membrane viscosity. The results seem to indicate that the compensation is only partial since thylakoid membranes from warm-grown plants still appear to be slightly more fluid than those from cold-grown plants when measured at their respective growth temperatures (difference significant at $P < .4$).

The observed differences in the fluidity of thylakoid membranes isolated from cold- and warm-grown pea plants may be related to differences in composition (Chapman et al, 1983a), a possibility which is dealt with in the discussion section of this thesis.

3.2 ELECTRON TRANSPORT RATES IN MATERIAL FROM COLD- AND WARM-GROWN PEA PLANTS

If the apparently higher level of fluidity found in the thylakoid membranes from cold-grown plants compared with those from warm-grown plants is the result of an adaptation, it might be expected to result in a difference in the functioning of the thylakoids from the two growth regimes. This section deals with experiments which investigated this possibility by comparing photosynthetic rate parameters in material from cold- and warm-grown pea plants. The rates of a variety of uncoupled electron transport reactions in thylakoids and oxygen evolution in whole leaves were determined over a range of measuring temperatures.

3.2.1 Full-Chain Electron Transport in Isolated Thylakoids Measured at One Temperature

3.2.1.1 Relationship of Full-Chain Electron Transport Rate with Chl a/b Ratio

The original protocol for the growing of pea plants at the cold and warm growth temperatures was to plant ungerminated seeds in the vermiculite without spacing them and to harvest the seedlings when most of them had reached the stage at which the second and third leaf pairs were fully expanded. The rate of full-chain electron transport measured under uncoupling and light-saturating conditions in thylakoids isolated from plants grown in this manner varied considerably between different preparations of chloroplasts, even those isolated on the same

day from plants grown in the same growth cabinet. The spread in the observed rates tended to be paralleled by variation in the chl a/b ratio of the isolated chloroplasts with which there was a degree of positive correlation (figure 8). Since a high chl a/b ratio reflects a low level of LHCP2 per photosystem, and therefore a low amount of chl per electron transport chain, it is not surprising that it is associated with high rates of electron flow measured at saturating light expressed on a chl basis. The variation in chl a/b ratio between preparations of thylakoids might reflect differences either in the environmental light conditions or the developmental stage of the plant from which they were isolated. A number of improvements were made in the procedure for the growing of the plants (see section 2.1) in an attempt to eradicate the variation in the observed rates which masked any difference that might exist between thylakoids isolated from warm- and cold-grown seedlings. In particular, the pregermination of seeds and even density of planting led to an increase in the uniformity of the seedlings' size and developmental stage at the time of harvesting. This protocol, in combination with a closer control of the light conditions, resulted in a narrowing of the spread of chl a/b ratios.

3.2.1.2 Full-Chain Rate Against Plant Age

The cold-grown pea seedlings grew at a much slower rate than the warm-grown ones and the mature plants had smaller leaves. This made it difficult to know when to harvest the plants in order to get material at a comparable developmental stage. In order to assess this problem, the rate of uncoupled full-chain

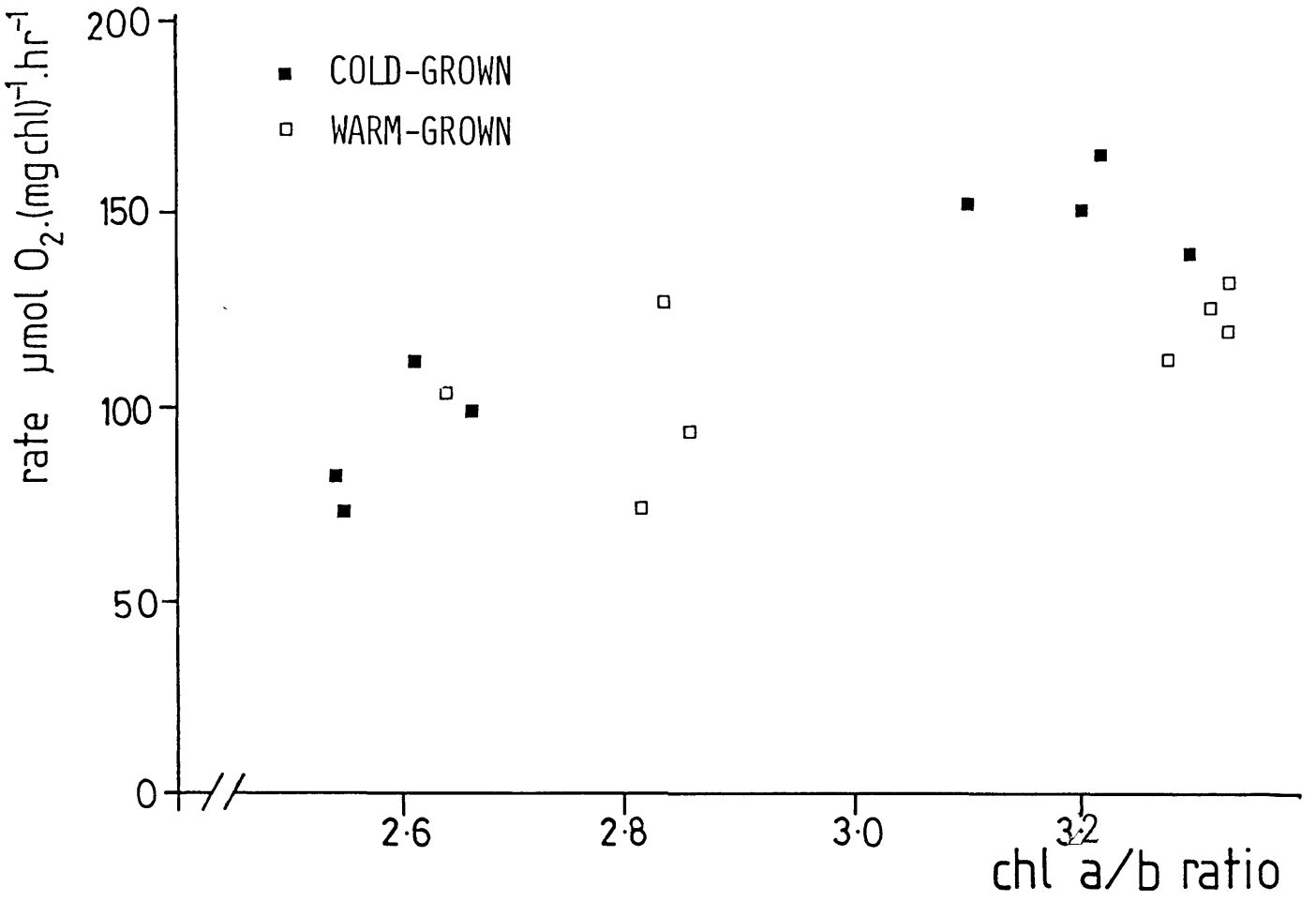


Figure 8 : Rate of full-chain electron transport in chloroplasts isolated from cold- and warm-grown plants as a function of chl a/b ratio. The rate of electron transport was measured at 17°C using FeCN as an acceptor. This data was obtained using material from plants grown before the improvement in protocol (see text).

electron transport at saturating light was determined at 17°C for thylakoids isolated from cold- and warm-grown plants of different ages (figure 9).

The shape of the curves of electron transport rate against plant age, with clear maxima at certain ages, is very similar to that found for chloroplasts from Barley plants of different ages in another study (Holloway et al, 1983). The high dependency of electron transport rate on the age of the parent plant again shows a degree of correlation with changes in the chl a/b ratios in thylakoid preparations using relatively mature leaves. In very young plants (warm-grown plants under 15 days old and cold-grown plants under 28 days old) the second and third leaf pairs were very small and the isolated thylakoids yielded relatively low rates despite their high a/b ratios. Maximal rates were observed in thylakoids isolated from 16-19 day old warm-grown plants and 29-37 day old cold-grown plants. Plants of ages within these optimal ranges (represented by brackets in figure 9) appeared to be at the same stage of development as denoted by the full expansion of the first leaf pair in both cold- and warm-grown plants. Furthermore, material harvested from cold- and warm-grown plants within these age ranges exhibited similar chl a/b ratios. From this it seemed reasonable to assume that the ages of plants which were associated with the maximal rates of electron transport were comparable for the two growth temperatures and the rest of the experiments described in this chapter were carried out using material harvested from seedlings at the appropriate times

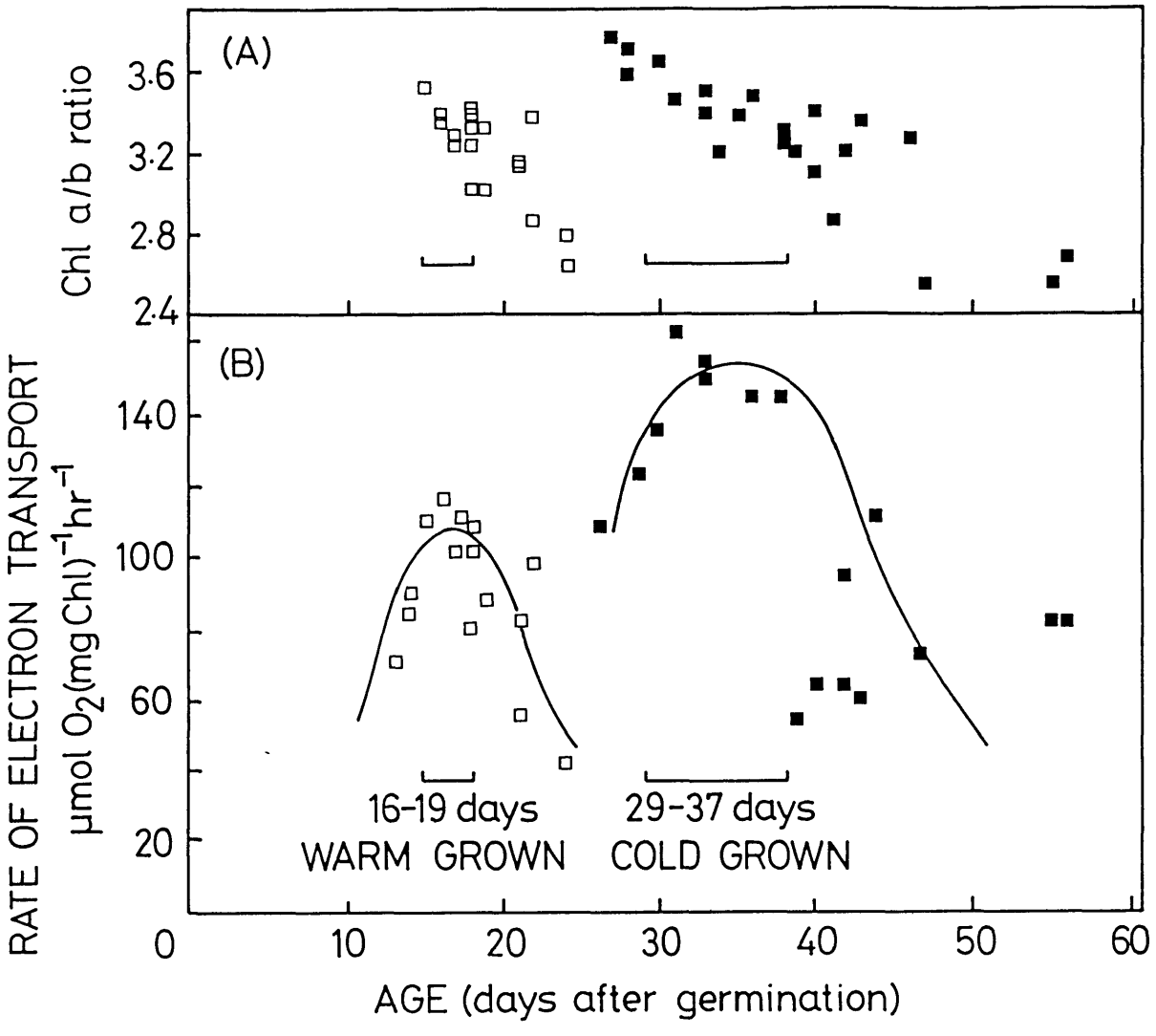


Figure 9 : Full-chain electron transport rate and chl a/b ratio in thylakoids isolated from cold- and warm-grown plants of different ages. The rate of electron transport was measured using FeCN as an acceptor at 17°C. The points shown are averages of two readings. The brackets indicate the age ranges of plants which were used in the rest of the experiments described in this section.

marked by the brackets in figure 9.

3.2.2 Photosynthetic Rate Parameters as a Function of Temperature

This section describes experiments in which the rates of various photosynthetic reactions were determined at saturating light between about 3 and 19°C using material from both cold- and warm-grown pea plants. This type of data is usually fitted quite well assuming a simple Arrhenius relationship between rate and temperature :

$$\text{rate} = A.\exp(-E_A/R.T)$$

where A is a constant, R is the gas constant, T is the absolute temperature and E_A is the activation energy - a parameter which describes the temperature dependency of the reaction.

For the sake of simplicity and ease of comparison most of the data from these experiments are presented as plots of rate against temperature in the figures in this section. These plots are fitted with curves using the derived parameters from a single straight-line regression of an Arrhenius plot of the data. Other experiments described below indicate that the assumption of a single constant activation energy for all these reactions may not be valid, but it is used as a convenient method of obtaining an average value for the temperature dependency from the total data on a particular reaction.

3.2.2.1 Full-Chain Electron Transport Rate

It can be seen (figure 9) that there is a significant difference in the observed maximal rate of full-chain electron transport achieved by thylakoids isolated from plants grown under warm and cold growth conditions when assayed at 17°C. Thylakoids from cold-grown plants exhibited rates on average 39% higher than their warm-grown counterparts. When full-chain electron transport was measured as a function of temperature between 3 and 19°C the thylakoids from cold-grown plants exhibited greater rates over the whole range, although the percentage difference between the rates decreased at low temperatures (figure 10). This means that thylakoids from cold-grown plants exhibit greater rates of electron flow than those from warm-grown plants when assayed both at their own growth temperature (average 7°C) and at the warm growth temperature (17°C).

The calculated activation energies for all the data points taken together are 40 and 31 $kJ.mol^{-1}$ for cold-grown and warm-grown thylakoids, respectively. The variation in the rates observed in thylakoids from plants grown at the same temperature is due principally to differences between preparations, as the error within a given experiment was $< 5\%$. For this reason, a better idea of the nature of the temperature dependency of the full-chain electron transport rate can be obtained from a consideration of data from an assay on a single thylakoid preparation.

Figure 11 shows an Arrhenius plot of one set of data each

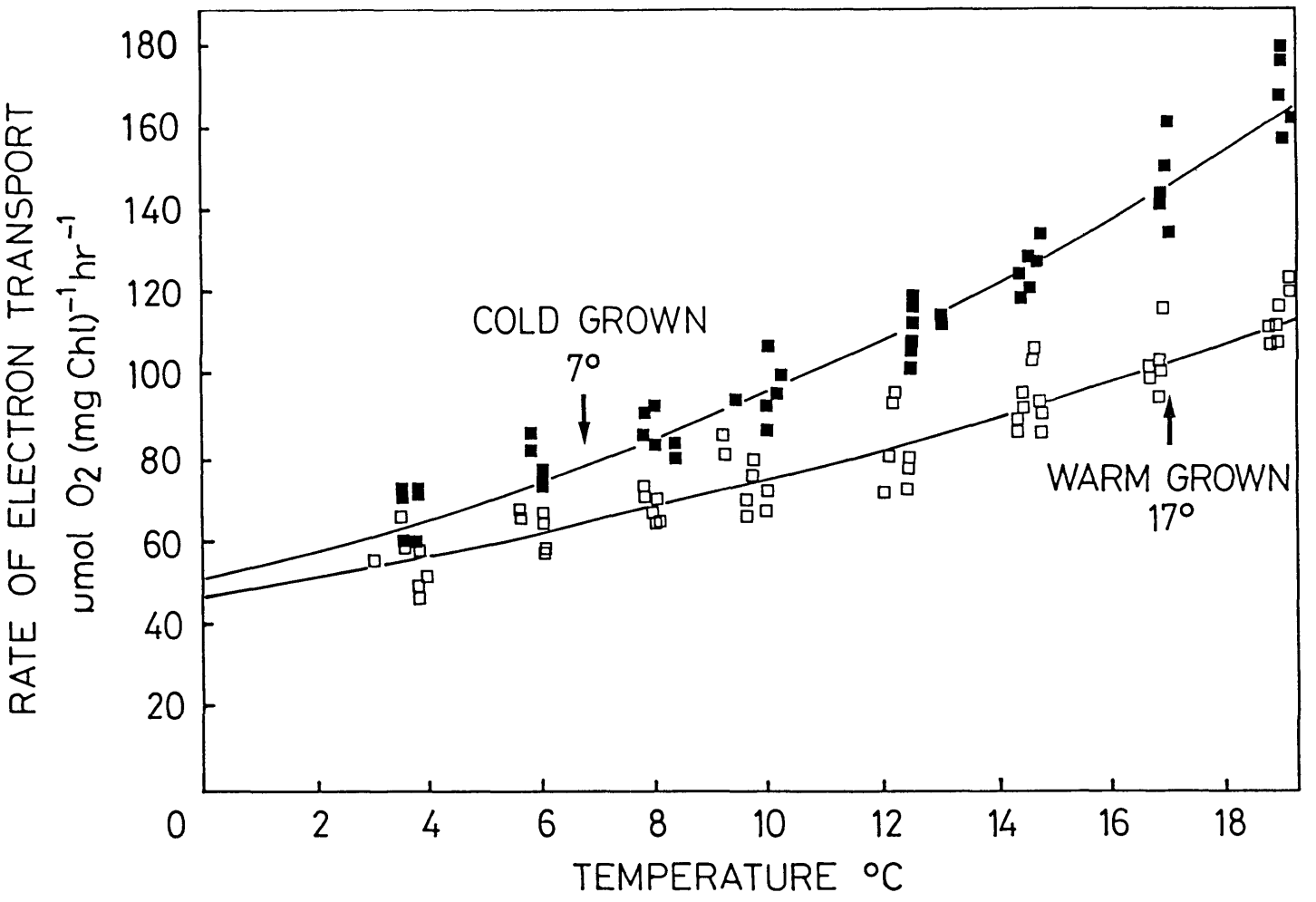


Figure 10 : The temperature dependency of full-chain electron transport rate in thylakoids isolated from cold- and warm-grown plants. The arrows indicate the mean growth temperatures for the two types of plants. Each point is an average of two readings. The curve is plotted from a single straight line regression of an Arrhenius plot of the data.

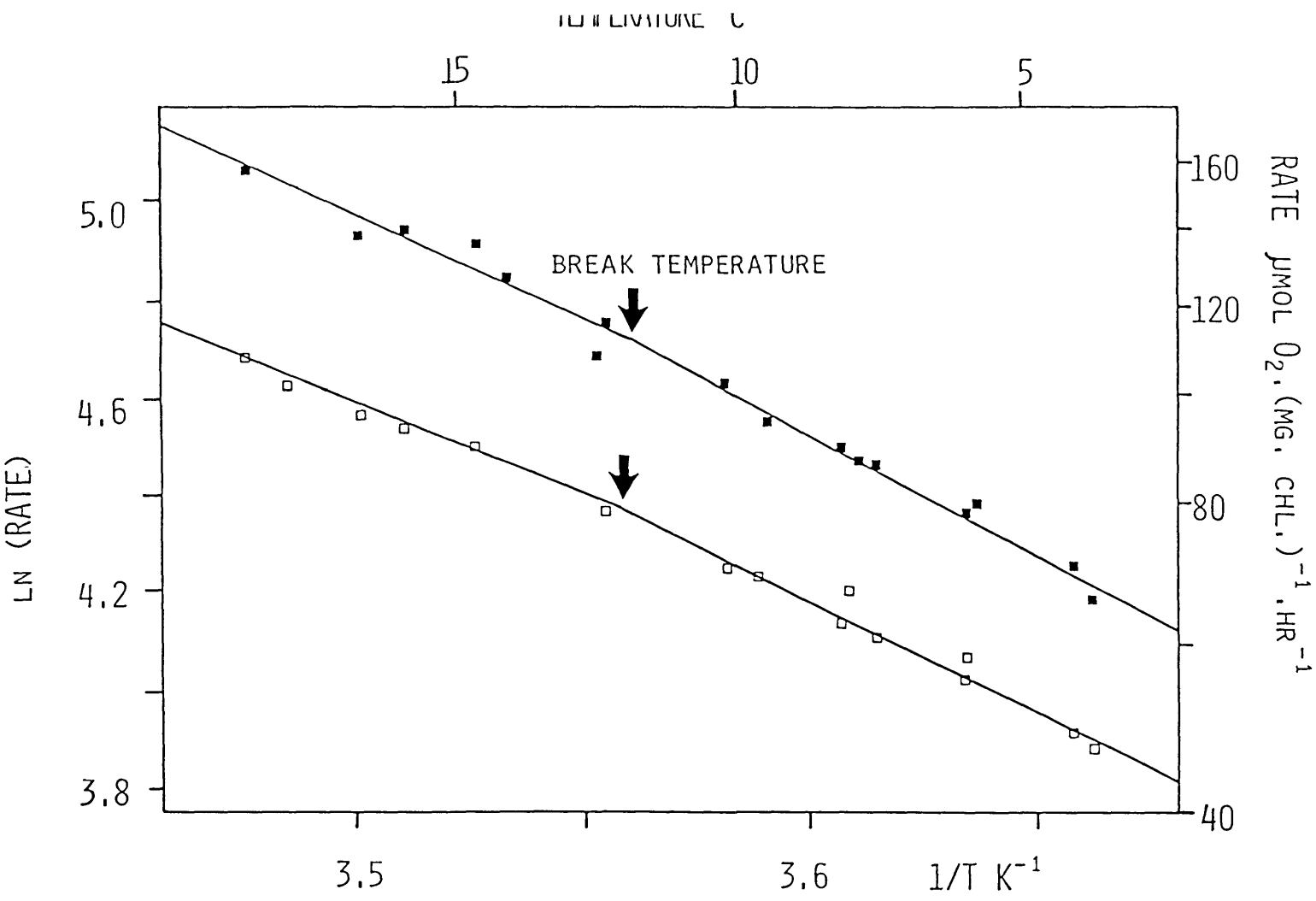


Figure 11 : Arrhenius plot of the rate of full-chain electron transport measured in individual thylakoid preparations isolated from cold- and warm-grown plants. The data is fitted to two straight lines with a break at 12°C. The break temperature was chosen so as to give the largest values of the coefficient of correlations for the two lines.

from cold-grown and warm-grown thylakoids both of which show an apparent decrease in gradient at the higher temperatures. Others have interpreted similar data for pea thylakoids (var. Massay Gem, plant growth temperature 22°C) in terms of a sharp change in activation energy (Nolan and Smillie, 1977) and the data in figure 11 has been fitted to two straight lines with a break temperature at 12°C for both types of thylakoids. However the data could have been fitted to a curve equally well (method of analysis; Wolfe and Bagnall, 1979). The apparent activation energies derived from the regressed straight lines in figure 11 are listed in table 9.

Table 9 Activation Energies of Full-Chain Electron Transport for Individual Preparations of Cold- and Warm-Grown Thylakoids Derived by Fitting the Data to Two Straight Lines

The values for the activation energies (E_A) are in units of $kJ.mol^{-1}$.

	BREAK TEMPERATURE	E_A BELOW BREAK	E_A ABOVE BREAK
COLD-GROWN	12°C	40.6	35.6
WARM-GROWN	12°C	37.6	32.4

These values can be compared with those reported by Nolan and Smillie (1977); they determined E_A values of 45 and 36 $kJ.mol^{-1}$ below and above 14°C respectively measuring uncoupled DCPIP reduction. These results are closer to those found for thylakoids from cold-grown rather than warm-grown plants, despite the much higher growth temperature used by Nolan and

Smillie.

3.2.2.2 Rate of Oxygen Evolution with Dimethylbenzoquinone

Figure 12 shows the rate of oxygen evolution from thylakoids isolated from warm- and cold-grown plants between 3 and 19°C in the presence of DMBQ and FeCN. The lipophilic acceptor DMBQ takes some of the electrons directly from PS2, thereby bypassing the rate-limiting step of linear electron flow between the photosystems and increasing the total flux (c.f. figure 10). In some experiments low levels of the inhibitor DBMIB were used in an attempt to eliminate the component of the flow which passes through the whole chain. Unfortunately the sensitivity of different chloroplast preparations to the inhibitor varied considerably and also had a large temperature dependency which made these results unreliable. However, a comparison of figures 10 and 12 shows that the difference between the rates of oxygen evolution of thylakoids from cold- and warm-grown plants is diminished by the presence of DMBQ.

3.2.2.3 Photosystem 1 Activity

Oxygen uptake was assayed in DCMU-poisoned chloroplasts with MV as the acceptor and $DCPIPH_2$ as the donor (figure 13). This parameter was completely insensitive to DBMIB which suggested that electrons passed directly from the oxidising to the reducing side of PS1. Under these conditions, the situation observed with full-chain electron transport is apparently reversed, with thylakoids isolated from warm-grown plants exhibiting significantly greater levels of PS1 activity than their cold grown counterparts over the range of measuring

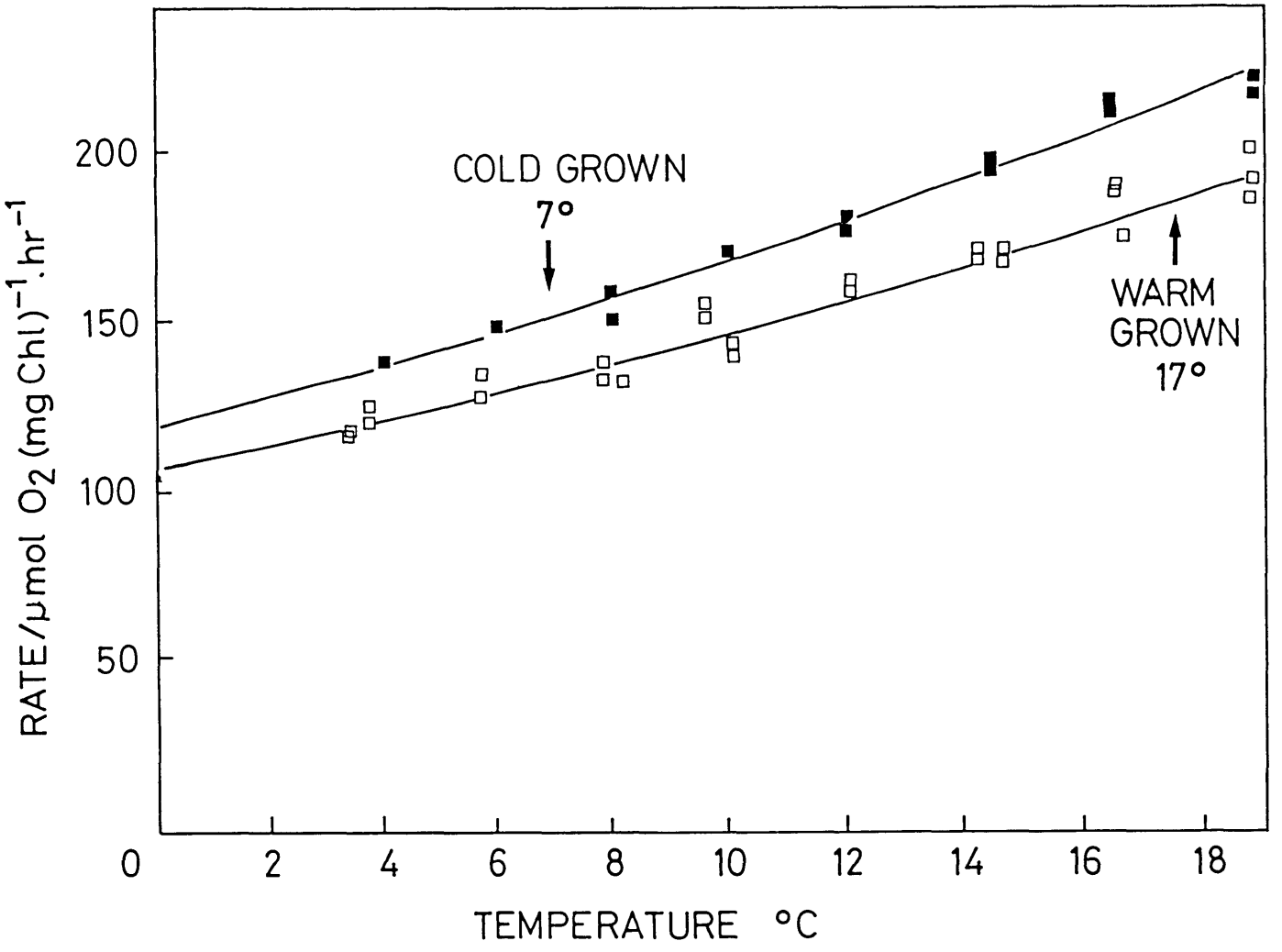


Figure 12 : The temperature dependency of the rate of oxygen evolution in the presence of FeCN and DMBQ in thylakoids isolated from cold- and warm-grown plants. The points represent averages of two readings. The curve is fitted from a single linear regression of an Arrhenius plot of the data. The arrows indicate the growth temperature of the plants from which the material was isolated.

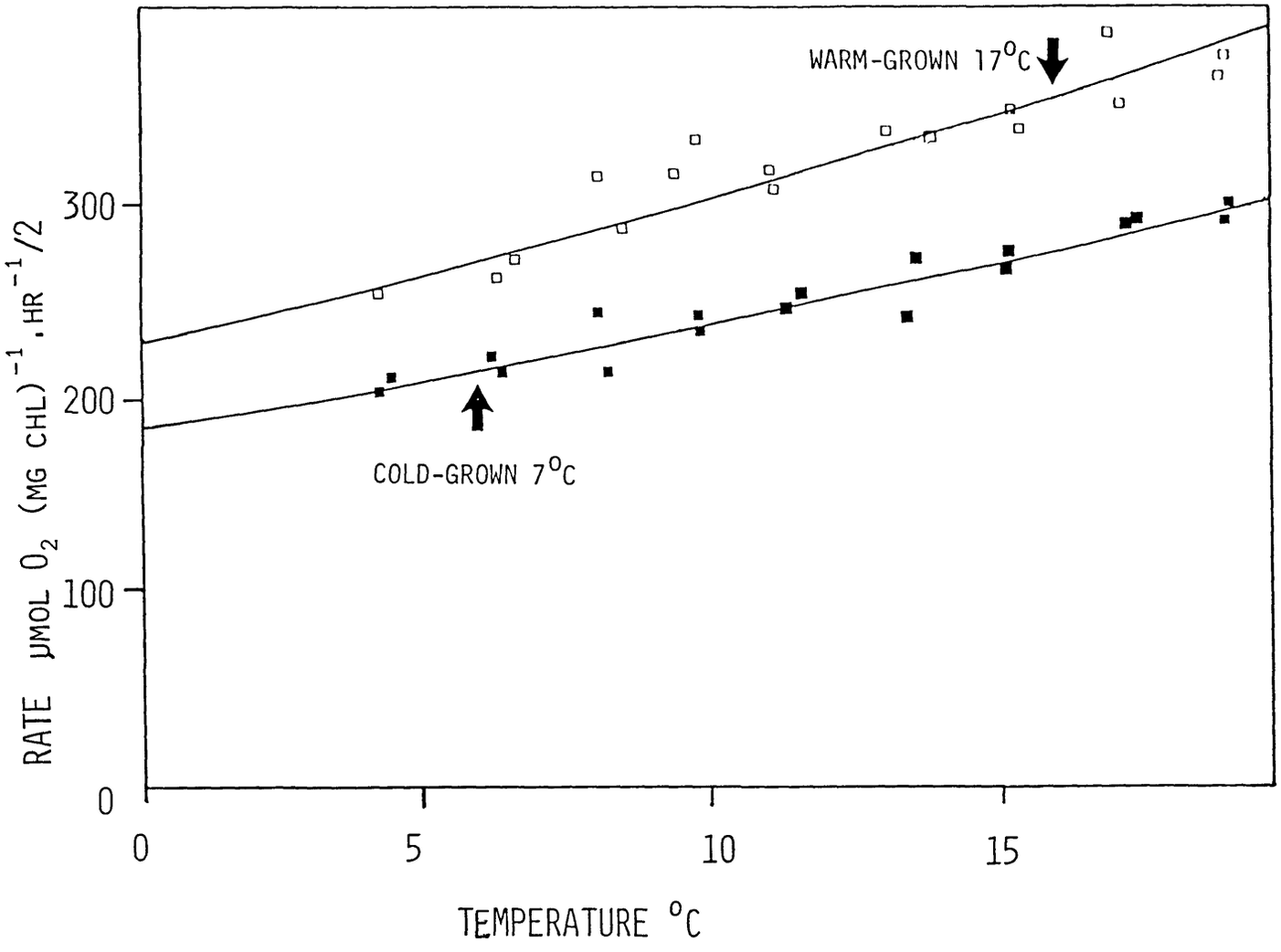


Figure 13 : The temperature dependency of PS1 activity determined in thylakoids isolated from cold- and warm-grown plants. The activity of PS1 was determined by measuring the uptake of oxygen in the presence of DCMU, MV and $DCPIPH_2$. The rate is expressed as µmol O_2. (mg chl)$^{-1}$.hr^{-1}/2 to make the values for the rates comparable to other figures.

temperatures. Both this parameter and the rate of oxygen evolution with DMBQ have much lower activation energies than the full-chain rate (see table 11 in the section below).

3.2.2.4 Rates of Oxygen Evolution from Whole Leaves

Assays of the rate of whole-leaf oxygen evolution were made using material taken from warm- and cold-grown seedlings. The chlorophyll content of each leaf was determined so that the rates could be expressed on a chl basis. In table 10 the areas, chl contents and chl a/b ratios of leaves taken from cold- and warm-grown plants are compared.

Table 10 Characteristics of Leaves from Warm- and Cold-grown Plants

The leaf area was determined for 11 cold-grown leaves and 13 warm-grown leaves. The number of leaves used to determine the chl parameters is shown as n in the table.

	AREA/mm^2	n	chl CONTENT/mg	chl a/b RATIO
COLD-GROWN	256 ± 9	71	0.128 ± .005	3.38 ± 0.02
WARM-GROWN	308 ± 11	69	0.153 ± .004	3.40 ± 0.03

This data indicates that similar amounts of chl per unit area and chl a/b ratios existed in the leaves from cold- and warm-grown plants used in these experiments. The average value of the chl a/b ratio determined in individual leaves was slightly higher than that found for preparations of thylakoids (3.29 and 3.27 for cold- and warm-grown respectively). This may have been due to the use of second and third leaf pairs for

thylakoid preparations while leaves for leaf-oxygen electrode assays were taken only from the second leaf pair.

The rates of oxygen evolution in leaves taken from cold- and warm-grown plants were measured at saturating light and high CO_2 concentrations between 3 and 19°C (figure 14). The variation in observed rate between individual leaves was greater than that found for full-chain electron transport between preparations of thylakoids, so to obtain data from the maximum sample number of leaves a new leaf was used for measurement at each new temperature. The differences between this plot and that of full chain electron transport rate in thylakoids (figure 10) are substantial. In general, with the exception of assays carried out on warm-grown leaves at low temperatures, the rates of oxygen evolution observed from leaves are greater than those from thylakoids which suggests that part of the photosynthetic electron transport capacity of the chloroplasts is lost on isolation. The most striking difference between the two plots is the difference in the temperature dependency of the observed rates (table 11); the activation energy for rates obtained with material from cold-grown plants is lower in leaves than in thylakoids, while those obtained with warm-grown material show a higher activation energy in leaves compared with thylakoids.

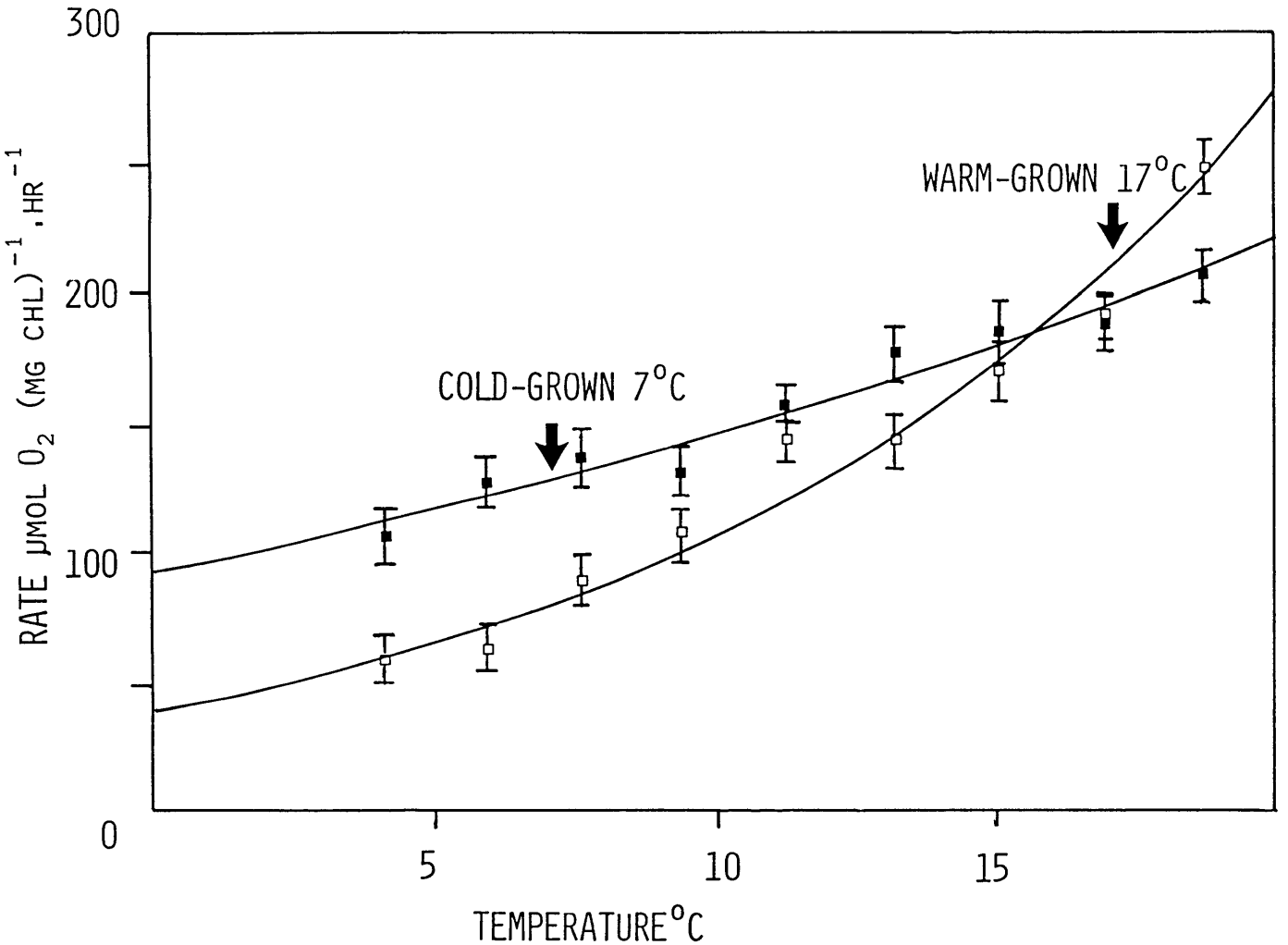


Figure 14 : The temperature dependency of the rate of oxygen evolution from the leaves of cold- and warm-grown plants. Each point represents an average of determinations made on 5-6 different leaves. A new leaf was used for every assay. The error bars represent 95% confidence limits about the mean. The curve was fitted from straight line regressions of an Arrhenius plot of the same data.

Table 11 : Activation Energies From Assays of Different Photosynthetic Rate Parameters

All activation energies are expressed in units of $kJ.mol^{-1}$, and were determined by a single linear regression of all the data for a particular reaction. Although this treatment may be simplistic (see figures 11 and 15), it does provide a means for comparing the relative temperature dependencies for the different reactions.

	COLD-GROWN	WARM-GROWN
H_2O → FeCN	40	31
H_2O → FeCN,DMBQ	22	21
$DCPIPH_2$ → MV	17	18
LEAF O_2 EVOLUTION	32	61

Despite the fairly large spread of data in the leaf oxygen evolution results, the differences in activation energy from the thylakoid results are significant at $P<.05$ and the difference between the activation energies of the data obtained with warm- and cold-grown leaves is significant at $P<.01$. The higher rates of full chain electron transport that are observed in cold-grown as compared with warm-grown material in measurements on thylakoids are paralleled in assays of leaf oxygen evolution rate at measuring temperatures below about 15°C. Since the activation energy for the rates from warm-grown leaves is greater than that for rates from cold-grown leaves (which is the reverse of the situation for full-chain electron flow in

isolated thylakoids) (table 11), the difference in rates between them apparent at low temperatures decreases with increasing temperature, and at temperatures above about 15°C warm-grown leaves appear to exhibit faster rates of oxygen evolution than cold-grown ones.

The curves fitted to the data in figure 14 are again from straight line regressions of an Arrhenius plot, but it is impossible to tell from these results whether or not this is legitimate because of the large range of rates observed in different leaves at the same temperature. In an attempt to determine the nature of the relationship between the rate of leaf oxygen evolution and temperature, individual leaves were assayed over a range of temperatures. The results from two experiments of this type are shown in figure 15, chosen because the oxygen evolution rates observed in these leaves happened to be closest to the overall average for their growth temperature. It can be seen that the data in figure 15 does not fit a single straight line so that the application of one activation energy to leaf oxygen evolution rates between 3 and 19°C is not strictly justified. The data is better fitted by a curve than by a combination of 2 or 3 straight lines. The deviation of the Arrhenius plot of leaf oxygen evolution rates from a straight line is more pronounced than it is in the equivalent plot for full-chain electron transport rates measured in thylakoids. The activation energies quoted in table 11 for the total leaf data are therefore valid only inasmuch as they represent an average for the temperature dependency of the rate over the range of

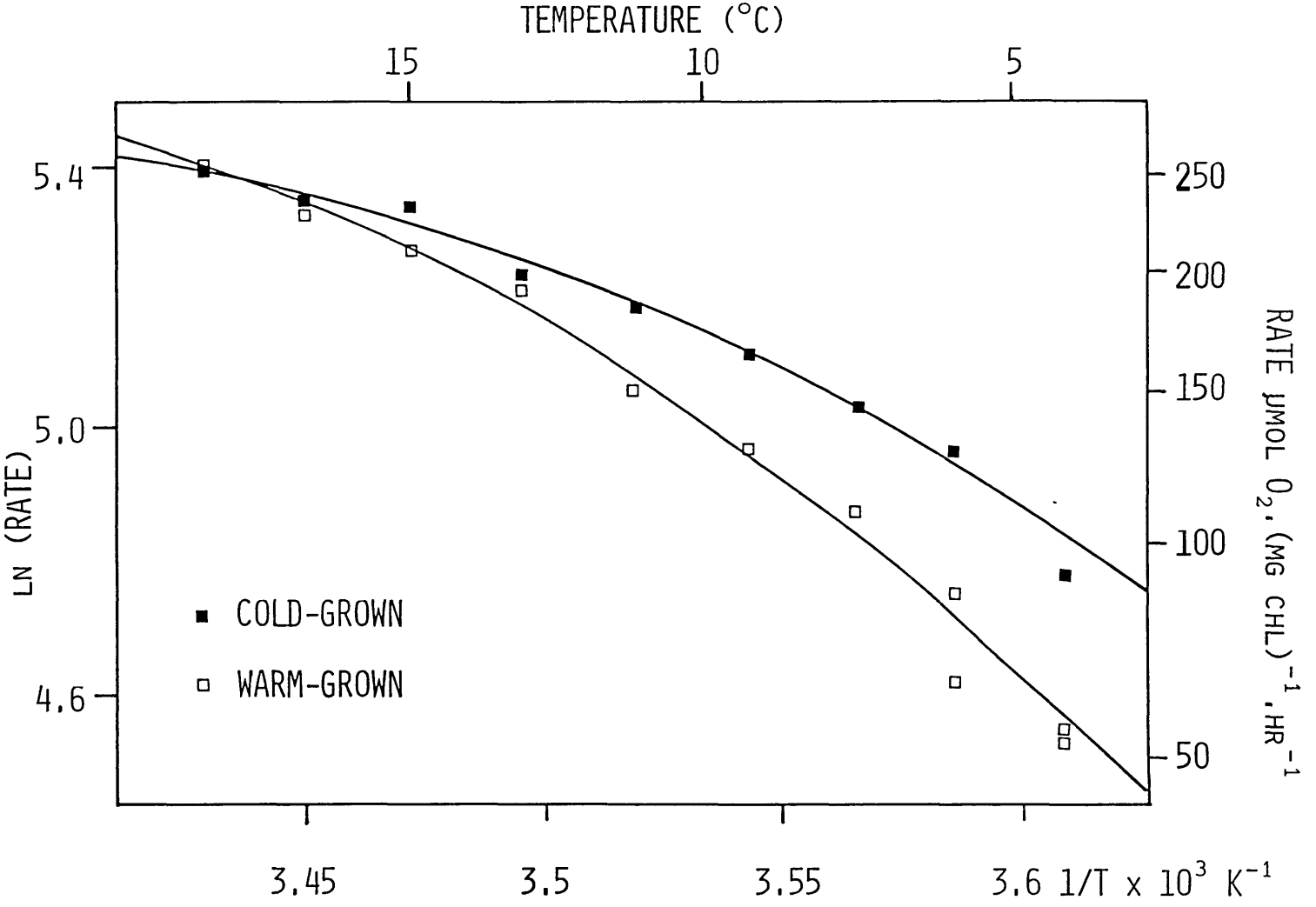


Figure 15 : Arrhenius plot of the rate of oxygen evolution determined in individual leaves from cold- and warm-grown plants. The curves are drawn by eye.

measuring temperatures.

3.2.3 Rate as a Function of Light Intensity

Assays of oxygen evolution from leaves or full-chain electron transport from thylakoids were carried out over a range of different light intensities at 7 or 17°C (chosen because they represent the average growth temperatures). The results were fitted using a non-linear regression routine (see section 2.5.3) to yield values for the light-dependent rate constant of electron transport (k_I) and the saturated rate (V_{max}).

The light-dependency of full-chain electron transport rates measured in thylakoids from cold- and warm-grown plants are presented in figures 16a and 16b which present data from experiments carried out at 7 and 17°C respectively. The equivalent data from assays of oxygen evolution carried out on individual leaves is shown in figures 17a and 17b. The curves fitted to the points plotted in figures 16 and 17 indicate that the light intensities used to generate the data presented in figures 10 and 14 above (900 and 1600$\mu E.m^{-2}.s^{-1}$ respectively) are effectively saturating at both temperatures. The derived parameters from both sets of experiments (figures 16 and 17) are tabulated together (table 12).

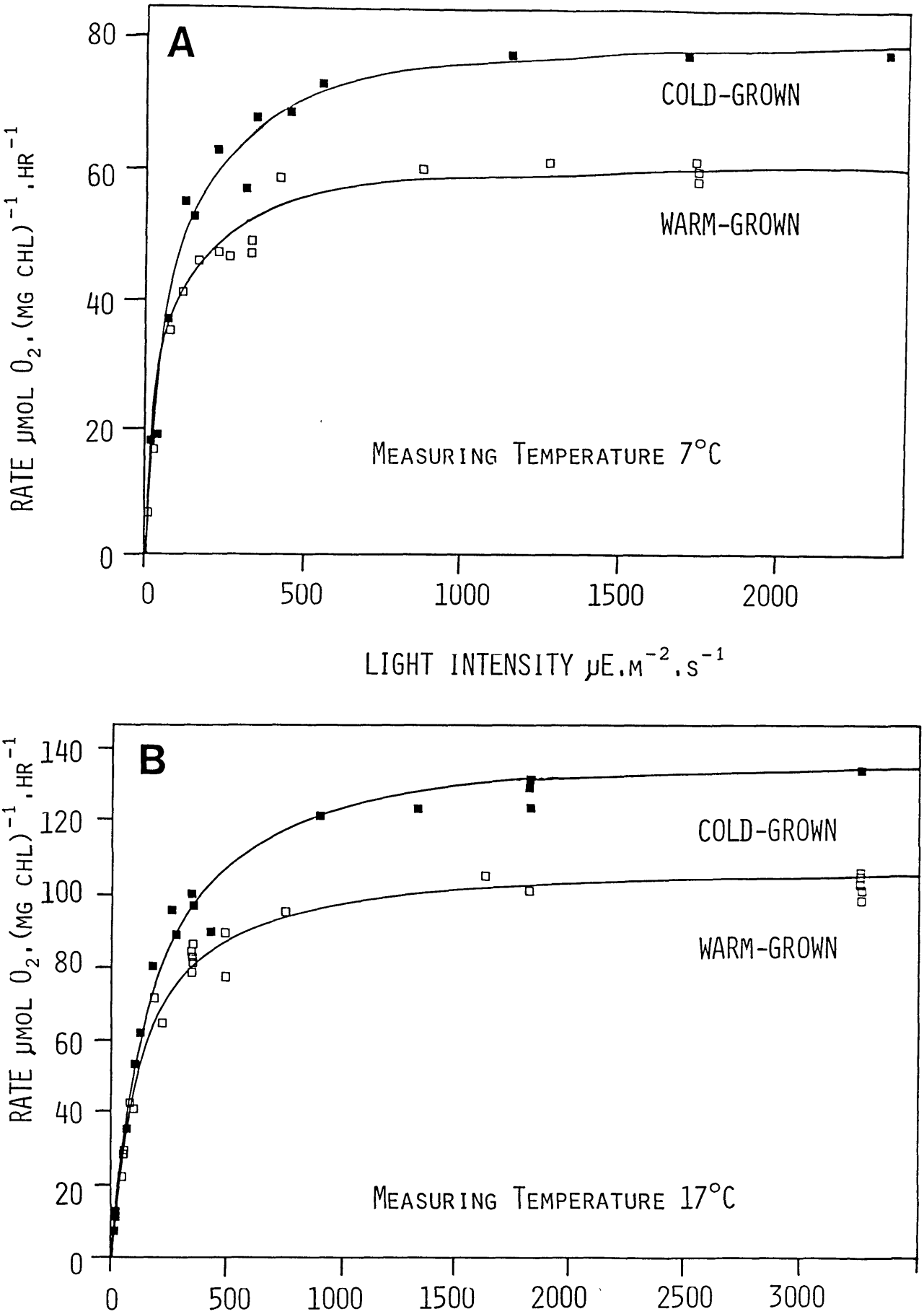


Figure 16 : Plots of the light dependency of full-chain electron transport rate measured in thylakoids isolated from cold- and warm-grown plants. The curves are fitted to the data using the equation $1/V = 1/V_{max} + 1/(k_I.I)$.
16A : measured at 7^oC. 16B : measured at 17^oC.

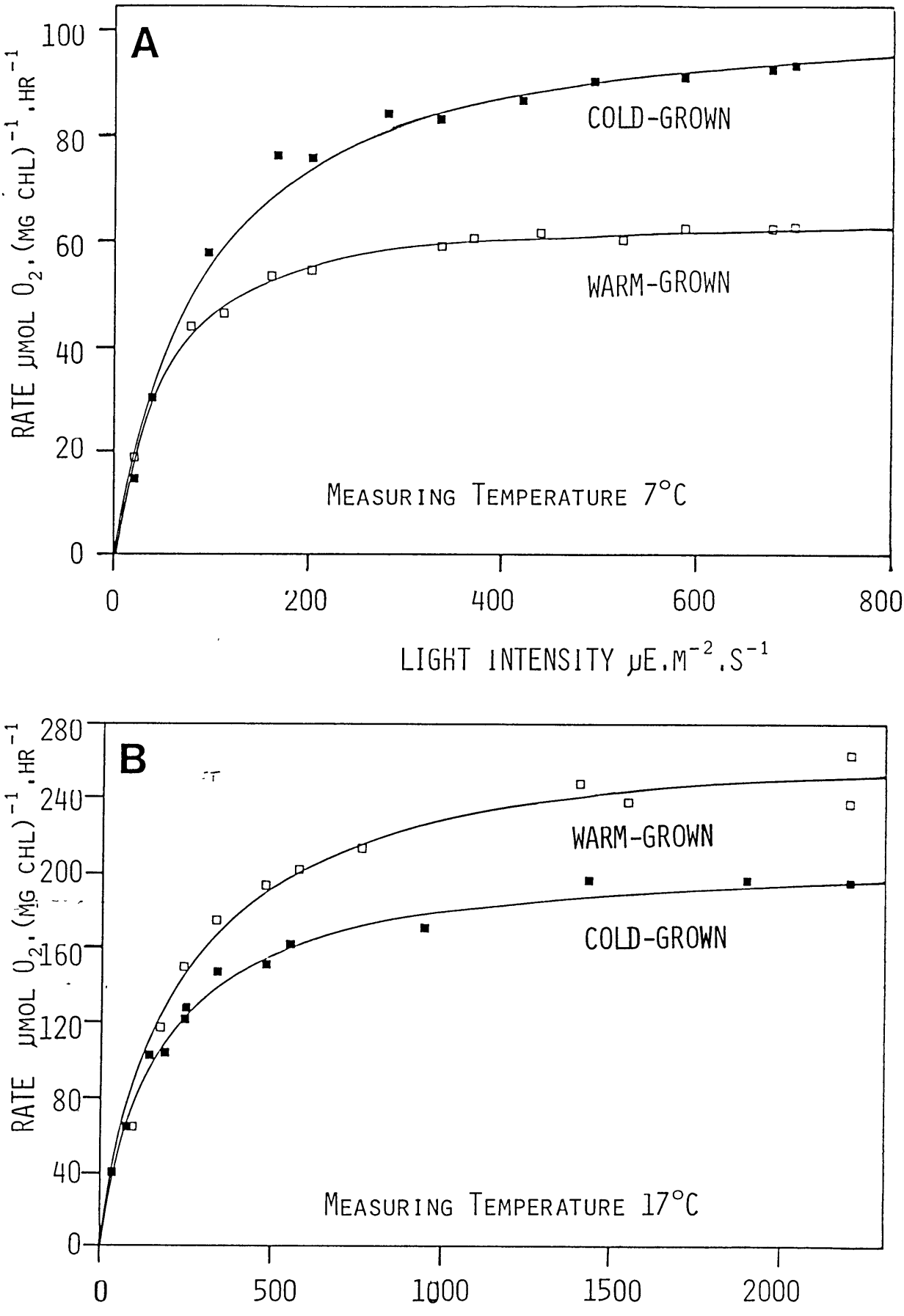


Figure 17 : Plots of the light dependency of the rate of oxygen evolution in leaves from cold- and warm-grown plants. A single leaf was used for all the measurements shown in one line. The curves were fitted in the same manner as the data in figure 16. 17A : measured at 7°C. 17B : measured at17°C

Table 12 Parameters Derived from Titrations of Leaf Oxygen Evolution and Thylakoid Full-Chain Electron Transport Rates with Light-Intensity

The V_{MAX} figures are expressed in μmole O_2.(mg chl)$^{-1}$.hr^{-1}, while the k_I values are in units of μmole O_2.(mg chl)$^{-1}$.hr^{-1}/(μE.m^{-2}.s^{-1}). Sat. light is the light intensity required to generate 90% of the V_{MAX} rate calculated using k_I and is expressed in units of μE.m^{-2}.s^{-1}.

ASSAY		LEAF		THYLAKOID	
TEMPERATURE	PARAMETER	COLD	WARM	COLD	WARM
7°C	chl a/b	3.26	3.41	3.16	3.28
	V_{MAX}	105	67	81	62
	k_I	1.24	1.52	0.98	1.02
	Sat. light	760	400	745	550
17°C	chl a/b	3.31	3.39	3.16	3.12
	V_{MAX}	208	276	140	109
	k_I	1.35	1.28	0.93	0.88
	Sat. light	1390	1940	1350	1110

The calculated V_{max} values for the thylakoid preparations used in these experiments are close to the average rates of full-chain electron transport observed in the equivalent material at the same assay temperature (figure 10). The data relating to leaves differ more from the corresponding average values for the rate of oxygen evolution (figure 14) but all except for the V_{MAX} value for the warm-grown leaves at 17°C are

within 20%. The chl a/b ratios are also similar to the averages of the values determined in thylakoid preparations and leaves (see figure 9 and table 10).

The rate constant for the light-dependent part of electron transport (k_I) is dependent on both the number of active reaction chains per chl and also the size of the light-harvesting cross-section. The values of k_I obtained here (table 12) are the same within the experimental error for measurements made using material from cold- and warm-grown at either measuring temperature in assays of both leaf oxygen evolution and thylakoid electron transport. This result is consistent with there being the same number of active electron transport chains in the material from cold- and warm-grown plants on a chl basis. The k_I values derived from the measurements carried out on leaves are higher than those made using chloroplasts, and this indicates that the higher absolute rate seen in leaves is at least in part attributable to a larger number of electron transport chains active in oxygen evolution.

3.2.4 Liquid-Nitrogen Temperature Fluorescence

The fact that the rates observed in the presence of DMBQ are greater with chloroplasts from cold-grown plants than with those from warm-grown (figure 12) while the reverse is true for the assay of PS1 activity (figure 13), suggests that there may be a difference in the stoichiometry of the photosystems between plants from the two growth temperatures. Such a difference would be expected to be reflected in the relative intensities of the peaks of fluorescence emission from chl at 77K. If frozen

chloroplasts are excited at this temperature with 435nm light, fluorescence peaks are observed at 685 and 733nm which are associated with PS2 and PS1 (possibly LHCP1), respectively (Mullet et al, 1980).

Fluorescence spectra of thylakoids isolated from cold- and warm-grown pea plants are shown in figure 18 and the ratio of intensities from the two peaks are presented in table 13.

Table 13 Ratios of Chlorophyll Fluorescence Peaks Measured at 77K in Chloroplasts Isolated from Cold- and Warm-Grown Plants

The values quoted are the ratios of the intensities of the 733nmm/685nm peaks averaged from n different thylakoid preparations.

CHL CONCENTRATION	n	COLD-GROWN	WARM-GROWN
2.5µg/ml	2	0.99	1.19
5.0µg/ml	3	1.06 ± 0.03	1.20 ± 0.01

The 733nm / 685nm ratio of peak intensities is significantly larger ($P < .05$) in chloroplasts from warm-grown plants when compared with the cold-grown equivalents. Although this is the type of result that might be expected if a larger PS1 to PS2 ratio existed in the warm-grown plants, it is not definitive evidence for a difference in stoichiometry.

In a comparison of chloroplasts isolated from unhardened and hardened Winter Rye plants Griffith et al (1984) found a drop in the 742nm / 685nm ratio of peak intensities on hardening, which is an effect of growth temperature in line with that shown

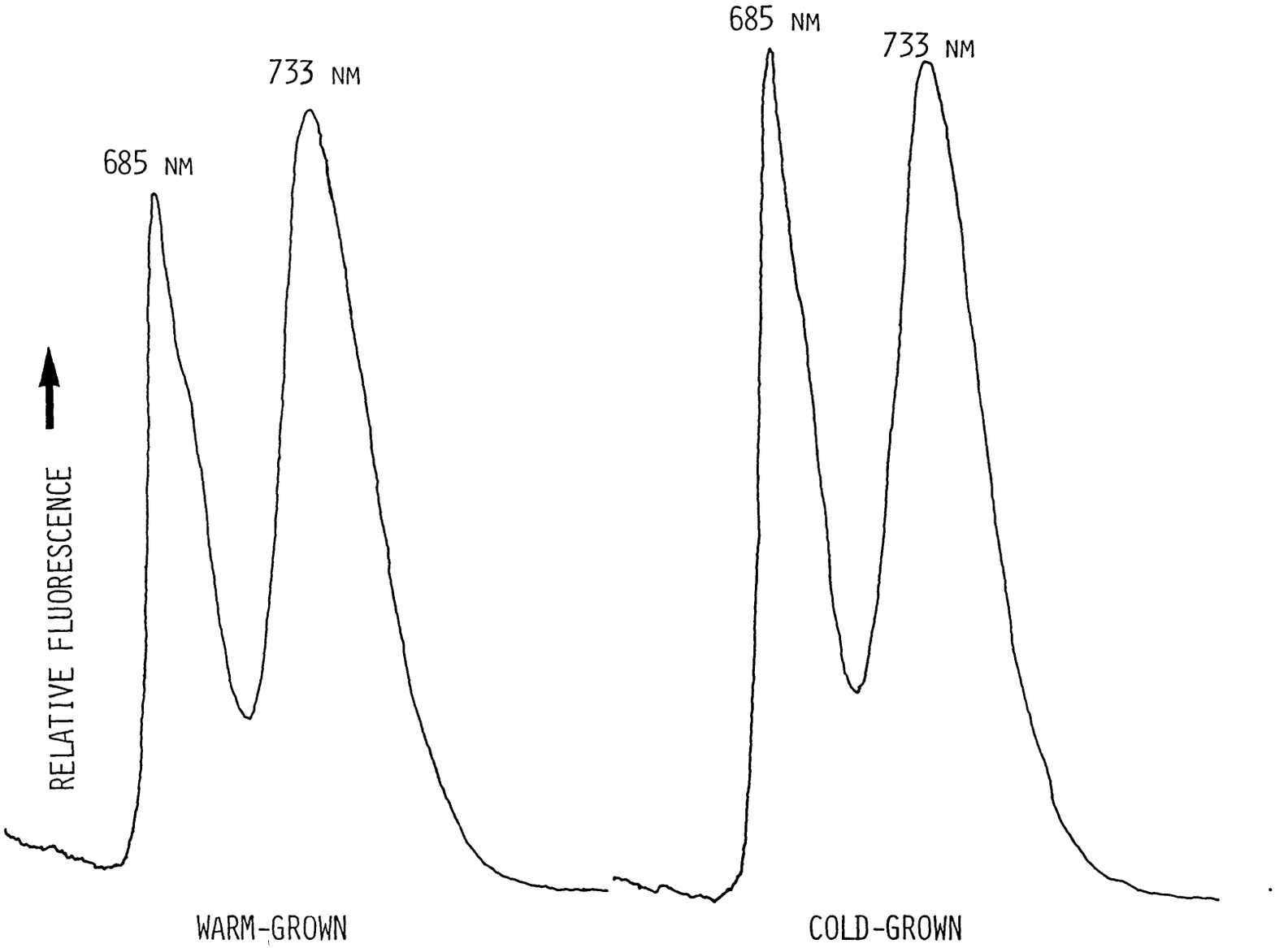


Figure 18 : Two emission specta of chl fluorescence measured at 77K. The spectra were recorded from 5µg/ml chl samples of chloroplasts from cold- and warm-grown pea plants.

in table 13 for pea. However they interpreted this as indicative of a change in the relationship of LHCP2 and PS2 rather than a modified photosystem stoichiometry.

In summary of the results presented in this section it may be said that :

1 Thylakoids isolated from cold-grown plants exhibit faster rates of full-chain electron transport than those from warm-grown plants and this situation is paralleled by the rates of oxygen evolution of whole leaves at temperatures below 15°C.

2 These differences are not observed to the same extent in the activities of the photosystems. Indeed, the activity of photosystem 1 appears to be greater in thylakoids from warm-grown plants than those from cold-grown plants.

3 The rates of full-chain electron transport in thylakoids and oxygen evolution in leaves from cold- and warm-grown plants exhibit similar dependencies on light intensity.

In the context of the results presented in section 3.1 it appears that the more fluid thylakoid membranes of chloroplasts isolated from cold-grown plants may allow greater rates of electron flow between the photosystems at saturating light when compared with the same parameter in material from warm-grown plants.

3.3 HYDROGENATION OF THYLAKOID MEMBRANES

The first half of this results section presented evidence for a more fluid thylakoid membrane and enhanced capacity for photosynthetic electron transport in material from cold-grown pea plants as compared with that from warm-grown plants. The data therefore support to some extent the hypothesis that a more fluid thylakoid membrane results in greater rates of full-chain electron transport under saturating conditions. This section describes experiments in which this possibility is further investigated.

In 1979 Restall et al reported the **in organello** hydrogenation of spinach chloroplasts using a homogenous catalysis technique. This method was adopted here to examine the effect on spinach thylakoids of artificially lowering the level of fatty-acid unsaturation in the membrane lipid bilayer. Unfortunately the amount of catalyst used in this technique caused a turbidity in the thylakoid suspension which prohibited the use of DPH to probe the effects of this treatment on the fluidity of the thylakoid membrane as was originally intended.

3.3.1 Partitioning of the Catalyst

The large quantities of catalyst needed to induce a sizeable decrease in the level of thylakoid lipid unsaturation, and the turbid appearence of the treated suspensions suggested that only some of the catalyst was associated with the membrane. This conclusion was supported by Atomic Emission analysis of the rhodium content of a thylakoid preparation which had been separated from the medium (table 14).

Table 14 Composition of Components Separated by Centrifugation of Thylakoid Membranes Treated with Catalyst

The sucrose density-gradient centrifugation (see section 2.8.2) separated the treated thylakoids into two components, a pellet and a band at the interface of two cushions. The amounts of chl and rhodium are expressed as a fraction of the total amount loaded onto the gradient and are averages of two determinations.

	CONTROL		HYDROGENATED	
	PELLET	INTERFACE	PELLET	INTERFACE
CHLOROPHYLL	3 %	86 %	5 %	88 %
RHODIUM	70 %	14 %	75 %	13 %

From the chlorophyll contents of the two components it appears that most of the thylakoid material settled at the interface between the cushions. 13-14% of the original rhodium content of the sample was accounted for in this band. The values obtained are in line with those reported by Restall et al (1979) who found 11% of the total rhodium added associated with the chloroplasts using an initial ratio of 10:1 catalyst:chl (w:w).

3.3.2 Time Course of Hydrogenation

The normal hydrogenation protocol was to expose the sample treated with catalyst to high pressure hydrogen at room temperature for 5 hours. In this experiment 1 ml samples were

extracted for fatty-acid compositional analysis at intervals within this period and the results are shown in figure 19. This plot shows a fairly linear decrease in the number of double bonds with time which is reflected in the increased proportion of saturated fatty-acids and the concomitant decrease in the fraction of the more unsaturated species. There was no measurable change in the total amount of fatty acid per chlorophyll, relative amounts of 16-C and 18-C fatty acids or the concentration of chlorophyll over the course of the treatment (data not shown).

3.3.3 Hydrogenation-Induced Changes in Fatty-Acid Composition

Figure 20 shows the average fatty-acid compositions obtained from four types of sample: untreated, treated with catalyst alone for 5 hours at room temperature, treated with catalyst and left for 5 hours under high pressure N_2 (600 kPa) and hydrogenated. (The term hydrogenated as used in the rest of this section refers to the samples which were treated under the standard conditions as described in the materials and methods section 2.8.1). The compositions of the three controls are the same within experimental error while the fatty acids in the hydrogenated sample contain on average 22% fewer double bonds. There was no difference in the amount of lipid found in any of the four sets of samples or in the relative proportions of 16-C and 18-C fatty acids.

An analysis of the relative proportions of the individual fatty acids in the hydrogenated sample as compared with the untreated material shows that all the double bonds are not

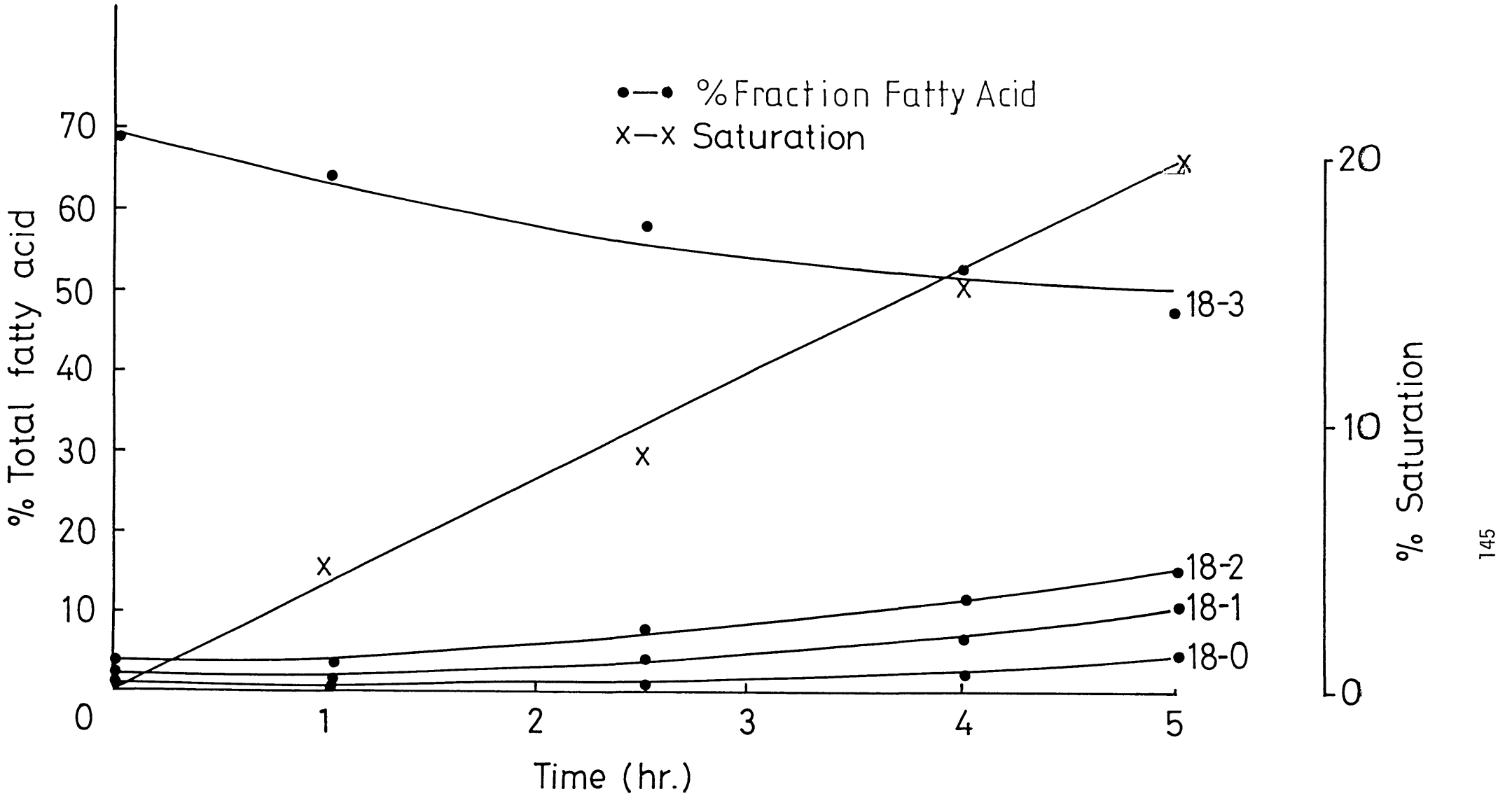
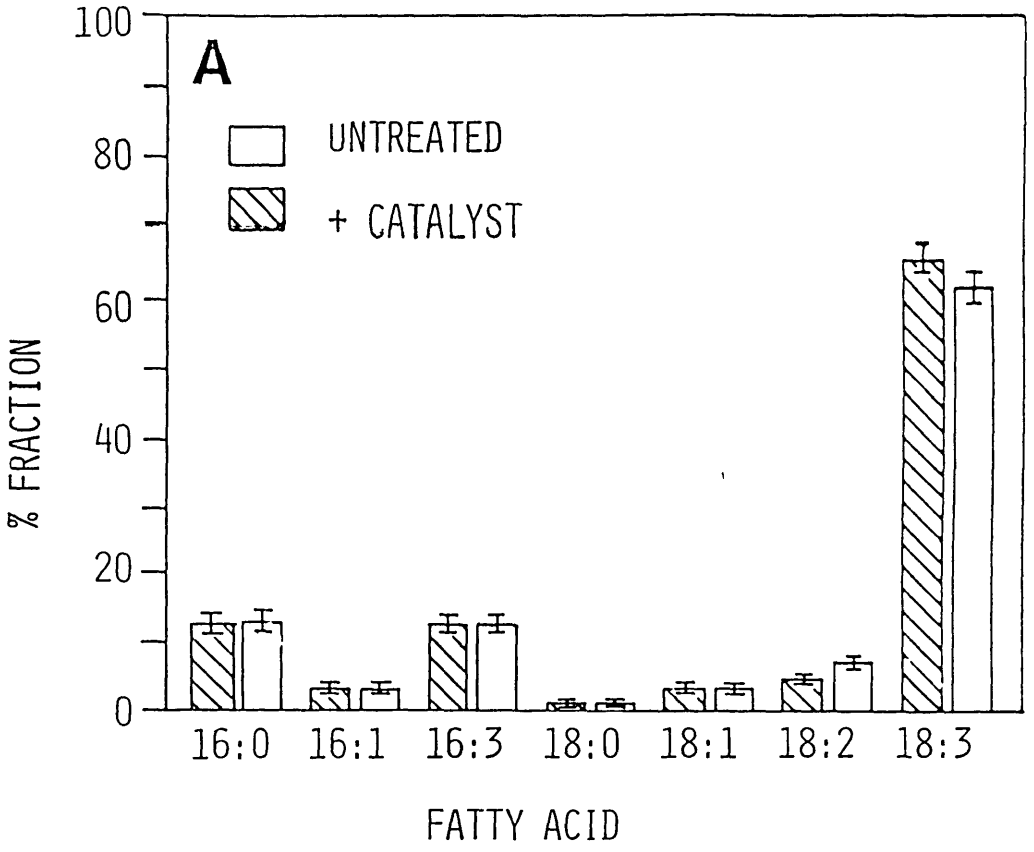


Figure 19 : Time course for the hydrogenation of thylakoid membranes. The figure shows the amount of 18-C fatty acids over the course of the five hours of the hydrogenation treatment expressed as a % of the total fatty acid. The degree of hydrogenation (saturation) is expressed as the percentage decrease in the number of double bonds.



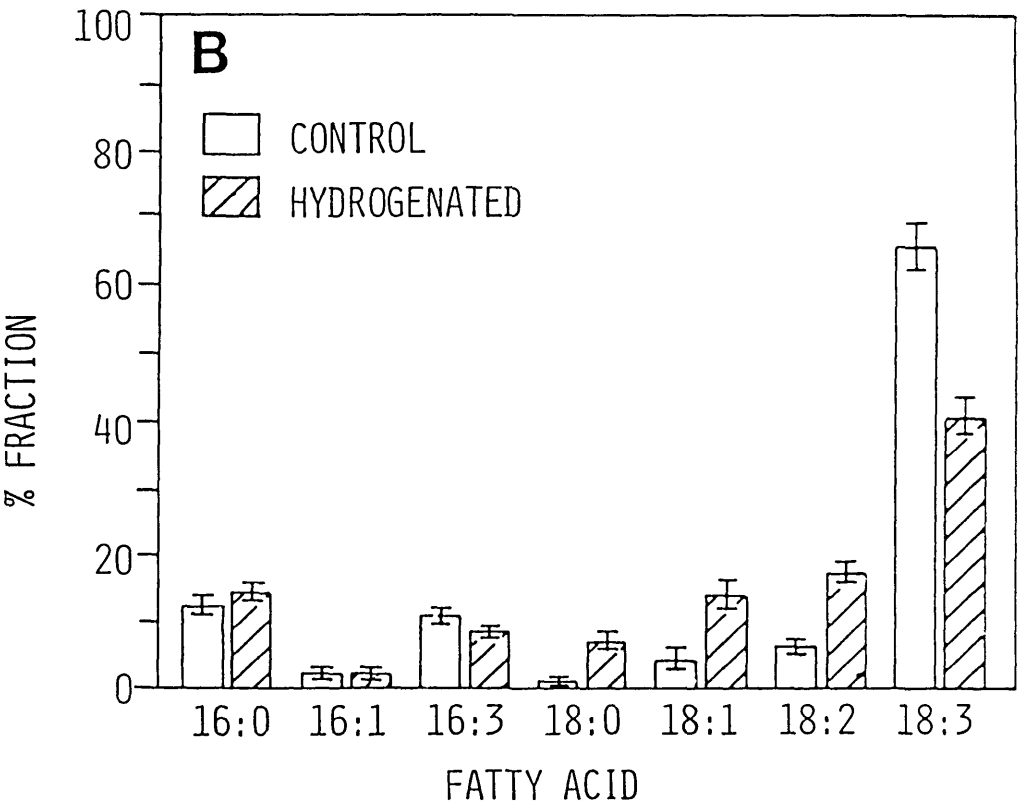


Figure 20 : The fatty acid compositions of lipid extracts from samples of thylakoids treated in different ways. The error bars represent 95% confidence limits. 20A : Comparison of total lipid extracts from an untreated sample (12 determinations) and one treated with catalyst and left at room temperature for 5 hours (14 determinations). 20B : Same as 20A with lipid samples from control (16 determinations) and hydrogenated (21 determinations) thylakoids.

equally susceptible to saturation. A comparison of the actual data with the predicted composition of a 22% hydrogenated sample assuming a first order reaction with the double bonds (table 15) shows that the fraction of 18:2 is substantially lower than predicted which suggests that the C-15 double bond on the 18:3 acyl chain may be less susceptible to hydrogenation than the others.

Table 15 Fatty-Acid Compositions of Control and Hydrogenated Thylakoids and Predicted Hydrogenated Sample Composition Assuming First Order Kinetics

All the numbers quoted are the percentage fractions of the total amount of fatty acid in the sample except for the DBI (double bond index), which is the average number of double bonds per lipid molecule.

FATTY-ACID	16:0	16:1	16:3	18:0	18:1	18:2	18:3	DBI
CONTROL	11.9	1.9	10.7	1.0	3.9	5.8	64.7	4.87
HYDROG.	14.0	2.0	7.8	6.5	13.3	16.8	39.6	3.82
PREDICTED	13.0	2.8	5.3	2.9	12.8	30.9	32.2	3.82

The hydrogenation process almost certainly results in the formation of fatty-acids which are not thought to occur naturally in the thylakoid membrane; 18:2 molecules with a double bond at the C-15 position, 18:1 species with the double bond at C-12 or C-15, the 16:2 fatty-acid and **cis**-16:1 species. From the fatty acid composition of the hydrogenated thylakoids

it seems likely that the artificially formed 18:2 and 18:1 molecules coincide with the naturally occurring species' of the same fatty-acids on the chromatograph of the methyl-esters. A peak between 16:3 and 16:1 was observed in some traces of hydrogenated samples which may have been the 16:2 fatty acid, but it was too small to significantly affect the composition and is not included in the results shown here. Since the composition of the 16:1 fatty acid was apparently unaltered by the hydrogenating conditions, it is probable that the **trans** double bond did not undergo hydrogenation.

3.3.4 Relative Sensitivity of Lipid Classes to Hydrogenation

The lipid extract from hydrogenated and control thylakoid suspensions were separated into the component lipid classes and the fatty acid composition of each was determined (figure 21). The most sensitive lipid class to the treatment was PC, probably because this class comes predominantly from the envelope contamination which may be more accesible to the catalyst. The level of hydrogenation seen is lowest in the SL class, which is also true of the hydrogenation of isolated thylakoid lipids in organic solvent using heterogenous catalysis (Gounaris et al, 1983b). The levels of hydrogenation observed in the other classes were all about 16%.

3.3.5 Effect of Hydrogenation on Electron Transport

3.3.5.1 Full-Chain Electron Transport

The effect of various stages of the control treatment on the rate of full-chain electron transport on the thylakoid samples was determined (table 16). This process was very substantially

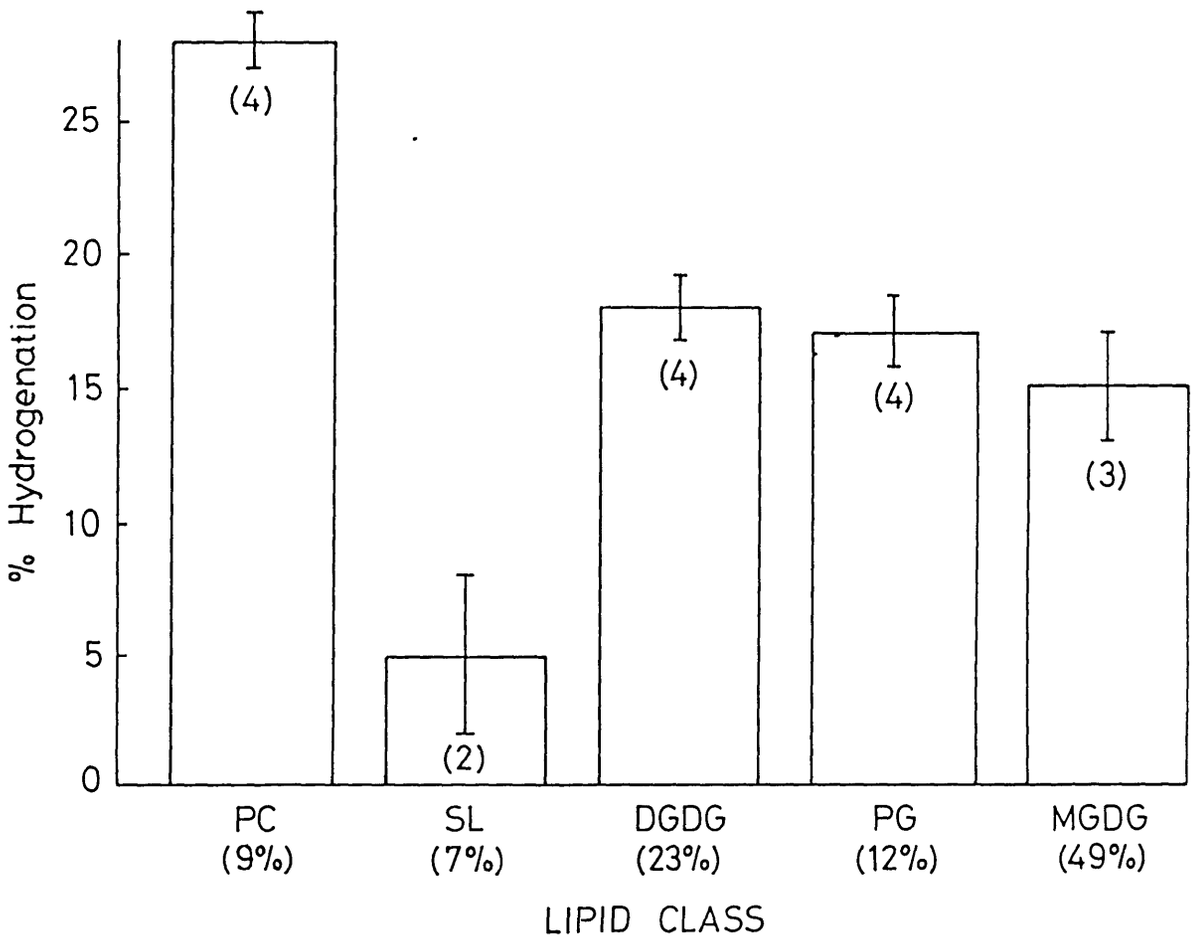


Figure 21 : The degree of hydrogenation in the indivdual lipid classes isolated from thylakoids. The hydrogenation is expressed as the percentage fewer double bonds that were observed in samples from the hydrogenated thylakoids as compared with those from the control thylakoids. The figures under the class names represent the % fraction of the total lipid that that class makes up. The figures in parentheses below the bars are the sample numbers for each lipid class. The bars depict the standard errors about the mean.

inhibited by the control treatment, particularly by the addition of the catalyst.

Table 16 Effect of Control Treatment on Full-Chain Electron Transport Rate

The rates are expressed as μmol O_2 taken up.(mg chl)$^{-1}$.hr^{-1}, measured using MV as an acceptor.

TREATMENT	n	RATE ± S.E.
Untreated	4	230 ± 6
Left at room temperature for 5 hours	6	170 ± 8
Left at room tempreature for 5 hours with catalyst	10	89 ± 12
Left at room temperature for 5 hours with catalyst under 6 atmospheres N_2	14	87 ± 11

However, hydrogenation did appear to induce a considerable degree of inhibition over and above that caused by the catalyst treatment alone (figure 22). The rates of full-chain electron transport observed for hydrogenated thylakoids were on average 36% lower than those found for the control samples (treated with catalyst under N_2 for 5 hours). Since the amount of reduction in the acyl-chain unsaturation level induced by hydrogenation was variable, the rate of electron transport was assayed in each experiment and related to the fatty acid unsaturation level. The brackets enclose the points arising from experiments in which

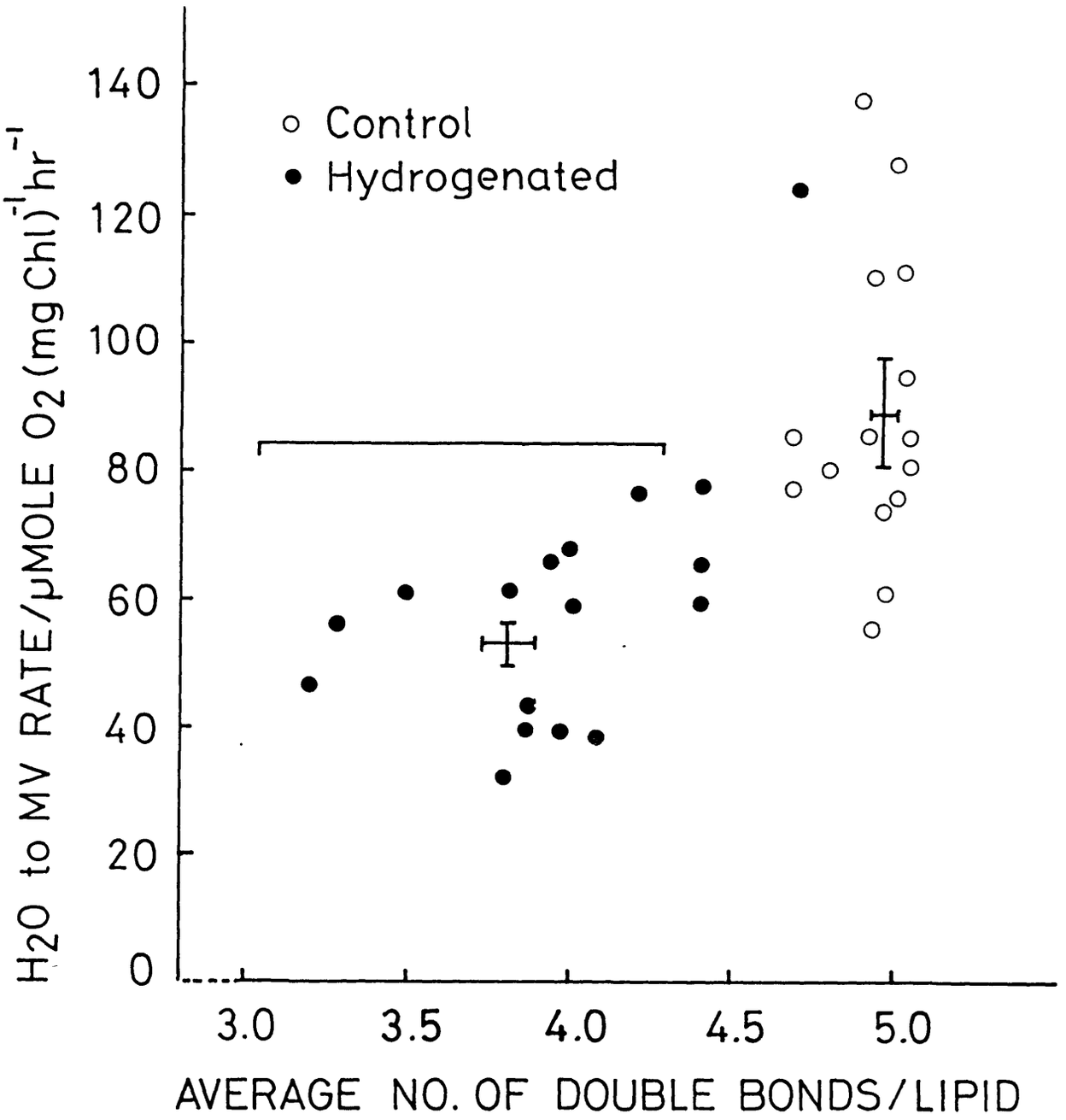


Figure 22 : The rate of full-chain electron transport as a function of the degree of unsaturation of the thylakoid membrane. The points were determined in hydrogenated and control samples of thylakoids. The bracket encloses all the points representing the samples hydrogenated under standard conditions (see section 2.4). The crosses denote the averages of the level of unsaturation and rate of electron transport for the control and standard hydrogenated samples. The size of the bars on the crosses represent the standard errors.

standard hydrogenating conditions were used. Lower levels of hydrogenation were induced with shorter periods of treatment, and in general these were associated with less inhibition of electron transport. Although there is a considerable spread of data there is a positive correlation between the degree of unsaturation of the thylakoid lipid matrix and the rate of full-chain electron transport (coefficient of correlation .61). Inhibition of full-chain electron transport associated with hydrogenation was also observed when FeCN was used as an acceptor rather than MV.

3.3.5.2 Light Dependency of Hydrogenation-Induced Inhibition

The degree of inhibition induced by hydrogenation, as represented by the percentage difference between the control and hydrogenated samples, was determined at different light intensities in a number of experiments. Since the light-dependency of the control rate of electron transport varied from experiment to experiment (probably because of variation in the turbidity of the suspension), so did the light-dependency of the calculated inhibition. To take into account the variable light response of the control rate, in figure 23 the hydrogenation-induced inhibition of electron transport is plotted against the control rate measured at that light intensity. The fact that the level of inhibition falls off at low control rates (low light intensities) implies that hydrogenation acts by inhibiting a dark step within the electron transfer chain, rather than affecting light-harvesting or eliminating a proportion of the active chains.

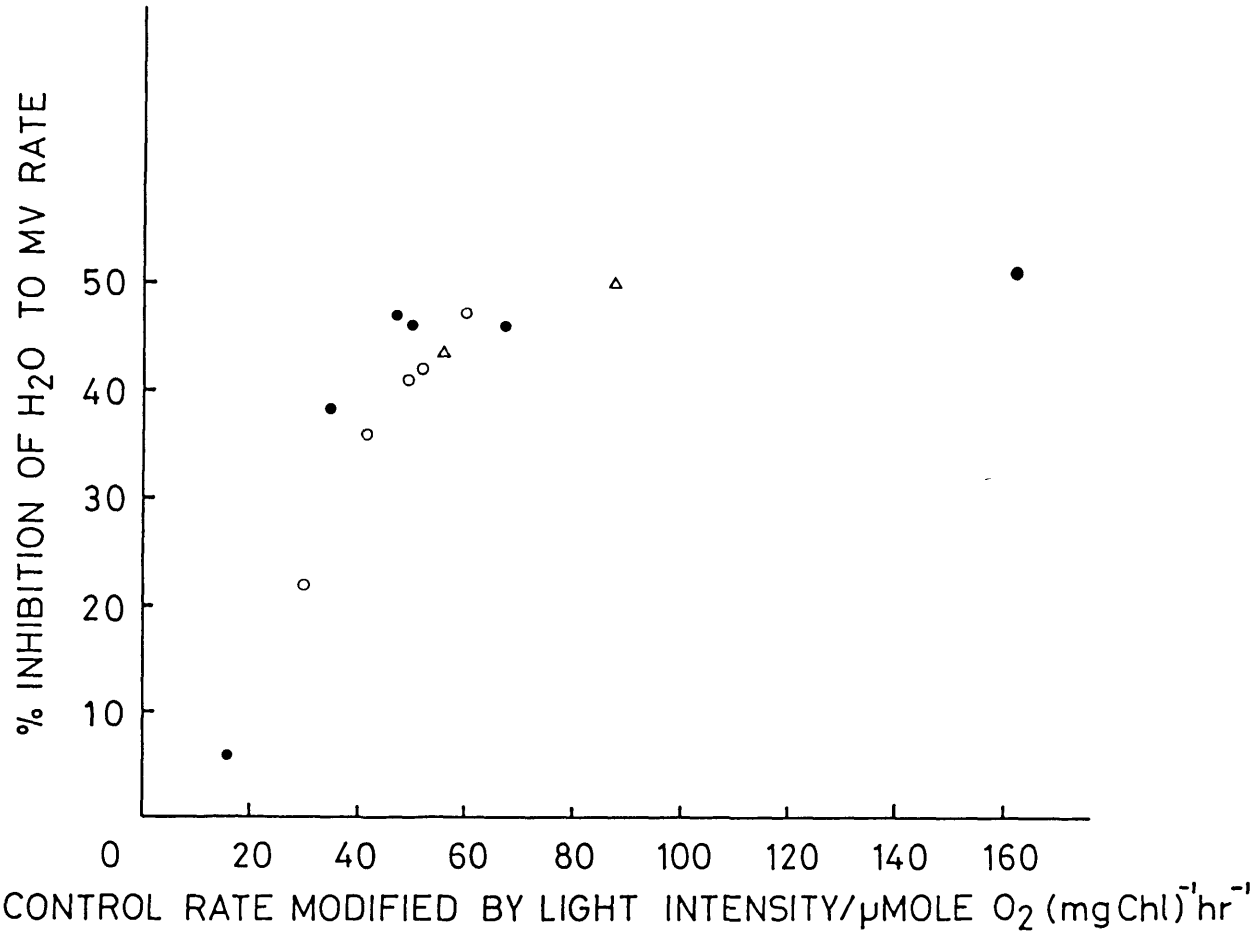


Figure 23 : The light dependency of the level of inhibition of electron transport induced by hydrogenation. The inhibition is plotted as a function of the control rate rather than the light intensity itself since the latter parameter had a variable effect on the degree of inhibition in experiments on different preparations of thylakoids. Different symbols represent data from separate thylakoid preparations.

3.3.5.3 Partial Electron Transport Reactions

The rates of a variety of partial electron transport reactions were measured in control and hydrogenated samples (table 17).

Table 17 Effect of Hydrogenation on Various Electron Transport Parameters

All the rates are expressed in terms of µmol oxygen uptake or evolution per mg chl per hour. The figure quoted for the DQH2 → MV reaction is half the observed oxygen uptake rate in order to take account of the ratio of 2 electrons transported per molecule oxygen taken up that occurs under these conditions.

REACTION	n	CONTROL	HYDROGENATED	% INHIBITION
H_2O → MV	14	87 ± 11	55 ± 5	36
H_2O → FeCN,DMBQ	11	140 ± 19	137 ± 20	∿ 0
H_2O → DCPIP	6	80 ± 5	67 ± 4	16
DPC → DCPIP	6	75 ± 5	67 ± 4	11
DQH_2 → MV	4	120 ± 20	129 ± 22	∿ 0

The variation in the rates observed with reactions using lipophilic acceptors or donors were very large, possibly because the DMSO and/or catalyst hindered the partitioning of the mediator into the membrane. For this reason the rates from the H_2O to DMBQ,FeCN and DQH_2 to MV reactions have large standard errors and there is no significant difference between values for the control and hydrogenated samples. The rates of photoreduction of DCPIP are similar to those observed for the

equivalent samples measuring oxygen uptake with MV, which suggests that the parameter is a measure of the rate of full-chain electron transport (see section 2.4). The lower level of inhibition in this reaction associated with hydrogenation compared with the $H_2O \rightarrow MV$ electron transfer rate may be due to the fact that the reduction of DCPIP was not completely light-saturated under the assay conditions. Since the rate of DCPIP photoreduction in hydrogenated thylakoids appears to be the same in the presence or absence of DPC there is no evidence for an inhibition of the donor side of PS2 by the hydrogenating treatment.

Overall, the data presented in this section suggests that the hydrogenation of the spinach thylakoid bilayer induces an inhibition of electron transport by acting on a dark step within the electron transfer process. Assuming that this hydrogenation induces an increase in membrane viscosity, these results do provide some support for a positive relationship between the fluidity of the thylakoid membrane and the rate of electron transport.

3.4 MODEL SYSTEM

The results from the experiments discussed above are consistent with the possibility that the rate of photosynthetic electron transport is dependent on the fluidity of the thylakoid membrane, as modified either by growth temperature or by the level of lipid unsaturation. Furthermore, the data from both sets of experiments suggested that full-chain electron transport was affected more than the activities of the photosystems by differences in fluidity. It is therefore possible that the rate of the PQH_2 oxidation step within the electron transfer process is influenced by the fluid state of the thylakoid membrane. The experiments described in this section represent an attempt to relate the rate of PQH_2 turnover to the fluidity of its environment.

The relationship between the rate of an electron transfer reaction catalysed by plastoquinone and the fluidity of the bilayer in which it was situated was studied using a model system developed by Hinkle (1970) and extended by Hauska (1977) and Futami et al (1979). Unilamellar vesicles containing entrapped FeCN (0.2 M) and varying quantities of PQ within the bilayer were prepared using different lipids. A suspension of these liposomes was then mixed with an anerobic solution of dithionite and the resulting reduction of the ferricyanide was followed. The kinetics of the reaction were complex, but the first three seconds were fitted quite well using an equation assuming a first order dependency of the FeCN concentration :

(1) $d[FeCN]/dt = k.[FeCN]$

The first order rate constant **k** was derived by the semi-logarithmic plot of the normalised change in absorbance at 420nm against time as shown in figure (24).

(2) $\ln(A_{420}(0)/A_{420}(t)) = k.t$

where $A_{420}(0)$ is the absorbance at 420nm (corrected for the reductant-insensitive component) at the start of the experiment and $A_{420}(t)$ is the same parameter at time **t**. Since the A_{420} signal is normalised, the fact that the constant relating it to the FeCN concentration within the liposome changes according to the lumenal volume and concentration of liposomes does not affect determination of the k value. The rate of FeCN reduction in the control liposomes containing no quinone was also plotted on a semilogarithmic plot and a pseudo first-order rate constant was obtained for the first three seconds (figure 24). This rate is presumably due to either a leakage of the dithionite into the liposomes or possibly a slow direct reduction of FeCN through the bilayer and it often did not fit first order kinetics. However the k value for the control liposomes was always less then 10% of the **k** value for the PQ-containing sample in experiments using 13nmol of PQ per mg lipid and less than 15% of the same value for liposomes containing 3nmol PQ per mg lipid. No attempt was made to correct for the rate observed in the control samples since the effect of the presence of PQ on

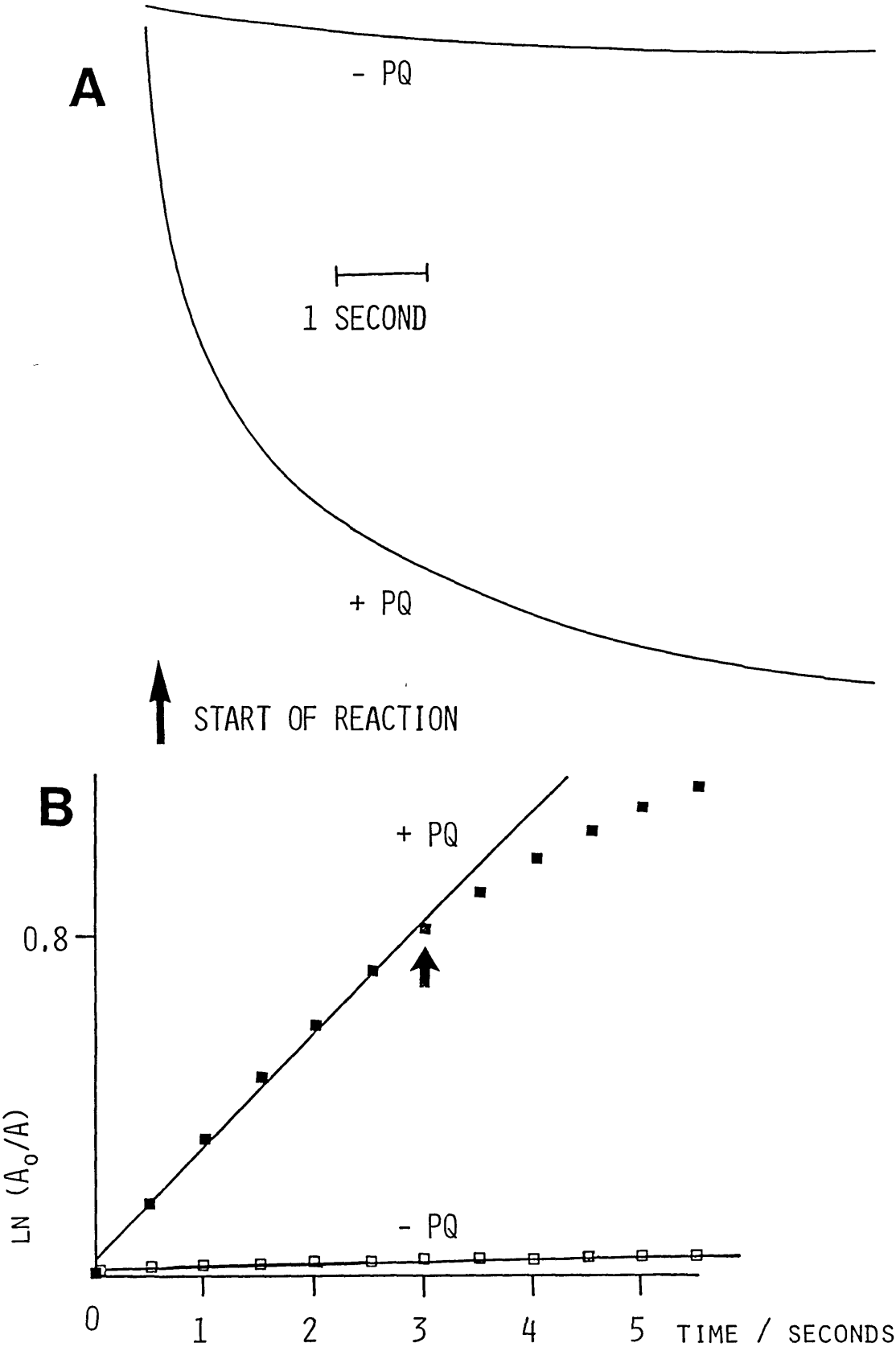


Figure 24 : Time course of A_{420} signal from liposomes containing FeCN following the addition of an external reductant. The 2 lines were obtained with liposomes containing 0 and 13nmol PQ per mg lipid. 24A : original data. 24B : the semilogaritmic plot of the digitised curves with straight lines fitted to the first three seconds to yield first order rate constants (k).

the leakage was unknown.

The aim of these experiments was to try and establish a relationship between the nature of the liposome leaflet and the catalytic efficiency of PQ in transferring electrons and protons across the bilayer, so that it was necessary to relate the observed rate of FeCN reduction to the turnover of PQ. The initial rate of the reaction was found to be approximately independent of the amount of dithionite at concentrations above about 10mM (data not shown), indicating that the turnover of the quinone was limited by the oxidation of PQH_2. If a second order reaction between PQH_2 and FeCN is assumed, and the fraction of PQ which is in the reduced state is assumed to be constant over the first three seconds then the rate can be expressed as :

(3) $\mathbf{d(FeCN)/dt = TO.(PQ)}$

where **(FeCN)** and **(PQ)** are the amounts of these reactants contained within one liposome and **TO** is the rate of turnover of PQ. Since

(4) $\mathbf{(FeCN) = [FeCN].L}$

where **L** is the average lumenal volume, **TO** can be related to the empirical parameter **k** using equations 1,3 and 4 by

(5) $\mathbf{TO = k.[FeCN].L/(PQ)}$

The ratio **L/(PQ)** is equivalent to **V/R** where **V** is the total volume enclosed within the number of liposomes made up from one mole of lipid (which can be determined experimentally by measuring the size of the reducible signal at A_{420}) and **R** is the molar ratio of PQ to lipid. Thus equation 5 becomes

(6) $TO = k.[FeCN].V/R$

Since in these experiments we are interested in the maximum rate of turnover of PQ at the start of the reaction when **[FeCN]** = 0.2M we can change equations 6 to give :

(7) $TO_{max} = 0.2 \times k.V/R$

The symbol TO is used from now on to represent this maximum turnover rate at the start of the reaction. The predicted relationship between **TO, k, V** and **R** were tested to some extent experimentally by measuring the rate of FeCN reduction in liposomes of different sizes and in liposomes with different PQ to lipid ratios. The TO parameter was then estimated in vesicles of differing lipid composition and was related to the fluidity of the vesicle bilayer, as determined by measurements of DPH fluorescence anisotropy.

3.4.1 Liposome Size

Asolectin vesicles were made with 13nmol PQ per mg lipid (which corresponds to the lipid : electron transport active PQ ratio found in thylakoids). Sometimes the liposomes were freeze

thawed which increased their size by a variable amount. When the lumenal volume per mole lipid **V** was related to the rate constant **k**, there was a distinct negative correlation (figure 25). This inverse relationship was due to the relatively constant value of **TO** with liposome size, with an average value of $2.2s^{-1}$ for asolectin liposomes with 13nmol PQ per mg lipid. A value of $2.3s^{-1}$ can be calculated for **TO** from the data reported by Futami et al (1979) for experiments using PC liposomes with the same PQ : lipid ratio.

The radius of a liposome can be estimated from the proportion of the total volume entrapped within the vesicles **V** by assuming an average surface area **A** occupied by a lipid molecule and using the equations

$$(8)\quad V = nl.(4/3).\pi.r^3$$

$$(9)\quad nl = N.A \,/\, (4.\pi.(r^2 + (r+W)^2))$$

where **nl** is the number of liposomes per mole of lipid, N is Avogadro's number, **W** is the width of the liposome bilayer and **r** is the internal radius. Equations 8 and 9 yield the polynomial

$$(10)\quad N.A.r^3/V - 6.r^2 - 6.r.W - 3.W^2 = 0$$

which was solved for r using values of $0.4nm^2$ and 4nm for **A** and **W** respectively by a Newton-Raphson iteration procedure. The average internal radius **r** was in the range 10-20nm for non

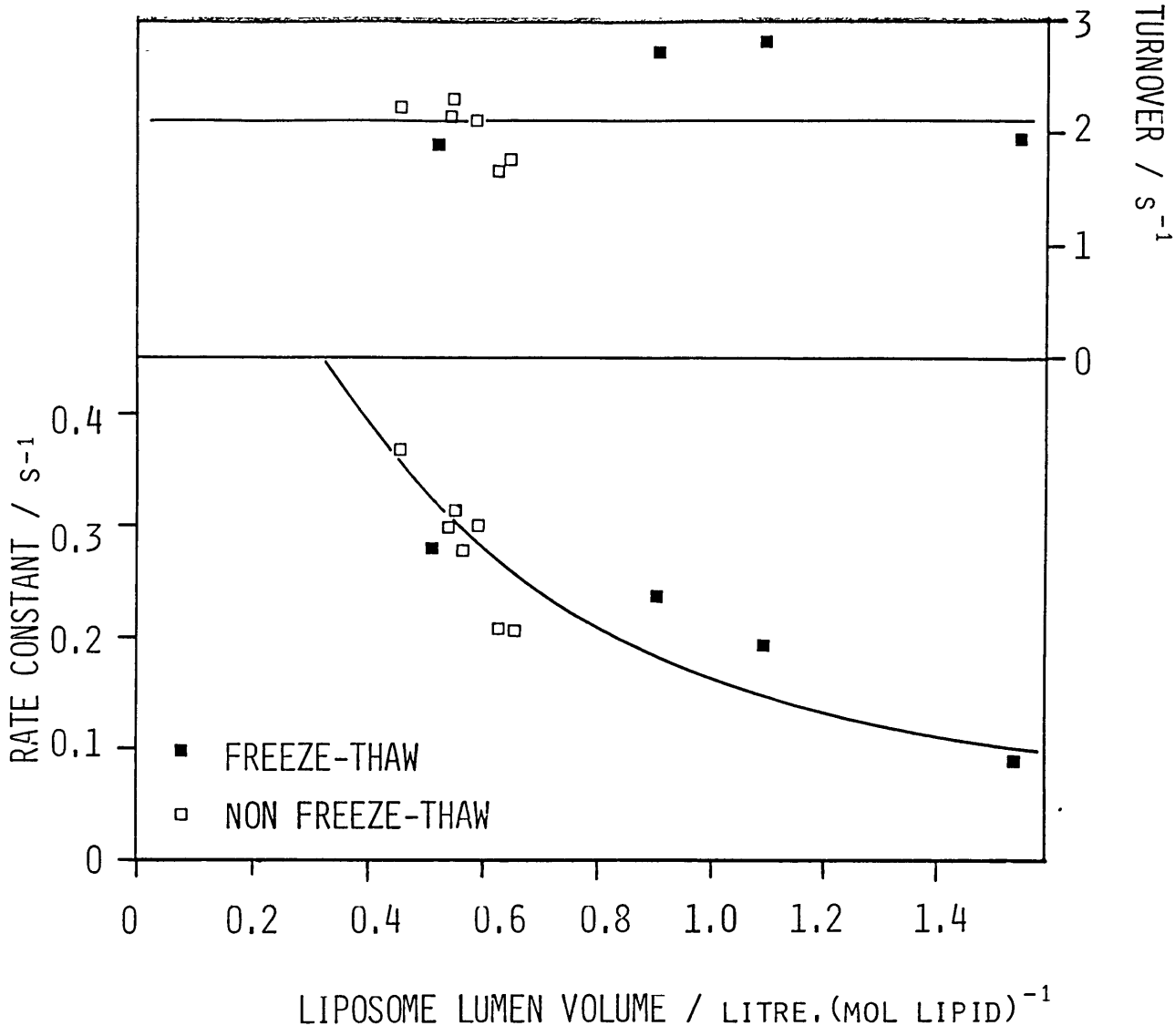


Figure 25 : The first order rate constant (k) and turnover (TO) as a function of liposome size. Some of the points were obtained using liposomes prepared with a freeze-thaw technique which tended to increase the size of the vesicle lumen. All the liposomes were made using asolectin containing 13 nmol PQ per mg lipid. The size of the liposomes is expressed as the total volume entrapped within the lumens of vesicles made from 1 mole of lipid.

freeze-thaw liposomes and 15-50nm for freeze-thaw liposomes. The size of the non-freeze thawed vesicles are very similar to those calculated by the same method for the data from Futami et al (1979) ($\mathbf{r}$ = 16nm), while the freeze-thaw treated samples are in line with those found by Pick (1981) for liposomes prepared under similar conditions ($\mathbf{r}$ = 37nm). Pick (1981) also identified the ionic strength of the medium into which the lipid is dispersed as one of the critical factors which determine the size of the liposomes. The very high ionic strength of the 0.2 M FeCN buffer means that the average size of the liposomes dispersed into it was comparatively small, and it also appeared to prevent the formation of liposomes using isolated thylakoid lipids. In the experiments reported below the freeze thaw procedure was not used in liposome preparation since this appears to result in a larger variation in the size of the vesicles.

3.4.2 Plastoquinone Content

The ratio of PQ to lipid was varied between 1 and 13nmol PQ per mg lipid in PC liposomes. The measured k parameter and derived **TO** constant are plotted as a function of PQ content in figure 26. The data presented in this figure only fits equation 6 below a PQ : lipid ratio of 4nmol per mg lipid. Above this value the turnover of PQ decreases with increasing quinone content, presumably because some step other than the PQ catalysis begins to limit the overall process. In liposomes with low concentrations of PQ the value of TO is relatively constant, remaining in the region of $3.4s^{-1}$. The experiments reported

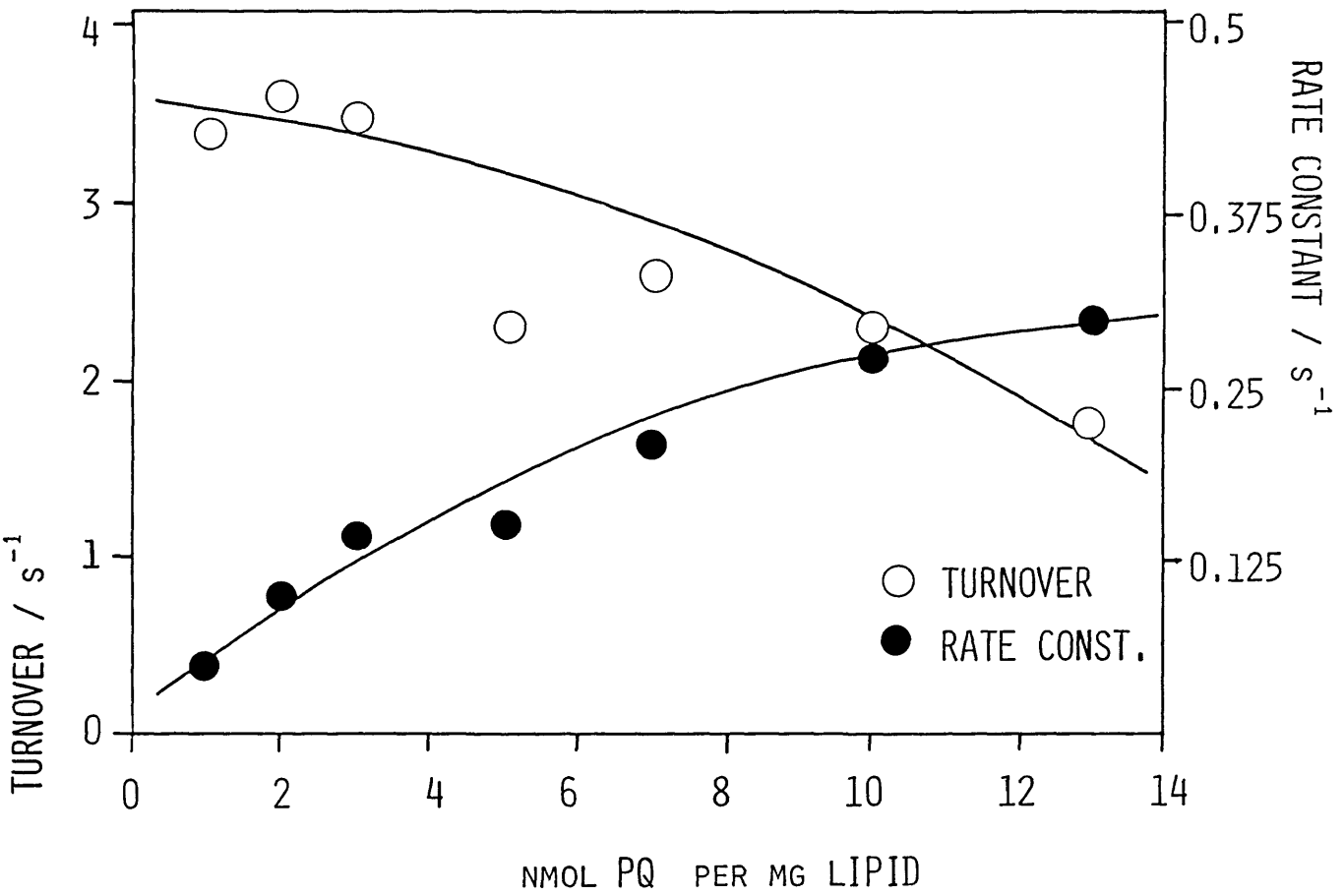


Figure 26 : The first order rate constant and turnover of PQ for entrapped FeCN reduction as a function of the plastoquinone content of the liposomes. The liposomes were prepared from asolecti n without freeze-thawing.

below were carried out using liposomes containing 3nmol PQ per mg lipid so that the rate of reaction would be limited by the turnover of PQ and would therefore reflect the efficiency of catalysis in different bilayer environments.

3.4.3 Fluidity of the Liposome Bilayer

3.4.3.1 Level of Fatty-Acid Unsaturation

Liposomes were prepared using untreated PC lipid and PC which had been hydrogenated so that the level of acyl chain unsaturation was reduced by 74%. The liposomes prepared with hydrogenated PC were considerably less fluid than vesicles made with untreated lipid as measured by DPH fluorescence anisotropy (table 18). However this lower level of fluidity did not seem to affect the rate of the reaction as measured by the value of k, which was larger in the more saturated liposomes, most probably because of the considerably smaller liposomes formed with the hydrogenated lipid (table 18). The very similar values of the TO value imply that the level of fatty acid unsaturation in the liposome bilayer has little or no effect on the rate of turnover of PQ.

Table 18 Comparison of Liposomes Prepared with Non-Hydrogenated and Hydrogenated Phosphatidylcholine

	DBI	DPH r_s	RADIUS / nm	k / s^{-1}	TO / s^{-1}
N	3.04	.096	16	.084	3.1
H	0.79	.143	11	.124	3.0

(N = non-hydrogenated, H = hydrogenated)

3.4.3.2 Cholesterol Content

Cholesterol hemisuccinate - asolectin mixtures were dispersed into liposomes with sterol : lipid (w:w) ratios between .02 and 3.0. The DPH polarisation of the fluorescence from incorporated DPH and the calculated radii are presented in figure 27. Dispersions of lipid containing cholesterol : asolectin ratios of 0.5 and above have similar r_s values and were highly light scattering (data not shown), which suggests that the liposome bilayers were saturated with cholesterol at a ratio of 0.5 and that higher ratios resulted in micelles of unincorporated cholesterol. The correlation between the DPH r_s value in asolectin liposomes and their cholesterol content is similar to that found by Yamamoto et al (1981). The presence of cholesterol appears to increase the average size of the vesicles.

The data from the experiments in which the fluidity of liposomes' bilayers were modified by hydrogenation of the lipid or incorporation of cholesterol are brought together in figure 28. This depicts the **k** and **TO** parameters calculated from the reaction kinetics plotted as a function of the DPH fluorescence anisotropy associated with a given liposome system. The decrease in the values of the kinetic parameters in liposomes containing large quantities of cholesterol may be a consequence of steric hindrance of the interaction between FeCN and PQH_2 rather than any effect due to the increased viscosity of the bilayer. In general therefore, the results summarised in figure 28 seem to indicate that the rate of turnover of PQ in this model system is independent of the degree of fluidity in the liposome leaflet.

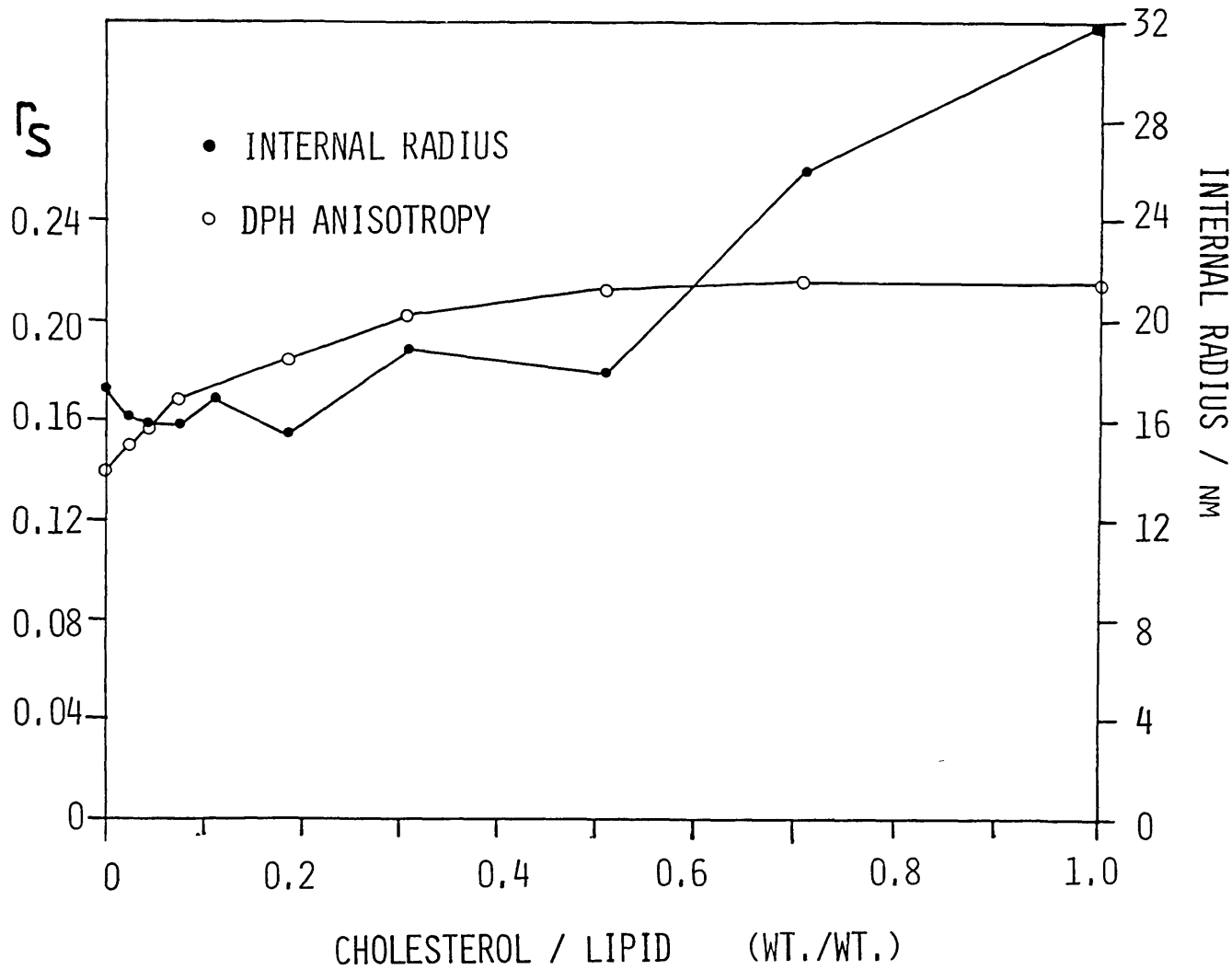


Figure 27 : The anisotropy of the fluorescence of incorporated DPH and the calculated radii of liposomes containing various quantities of cholesterol. The FeCN concentration associated with the liposomes was used to estimate their internal radius (see text).

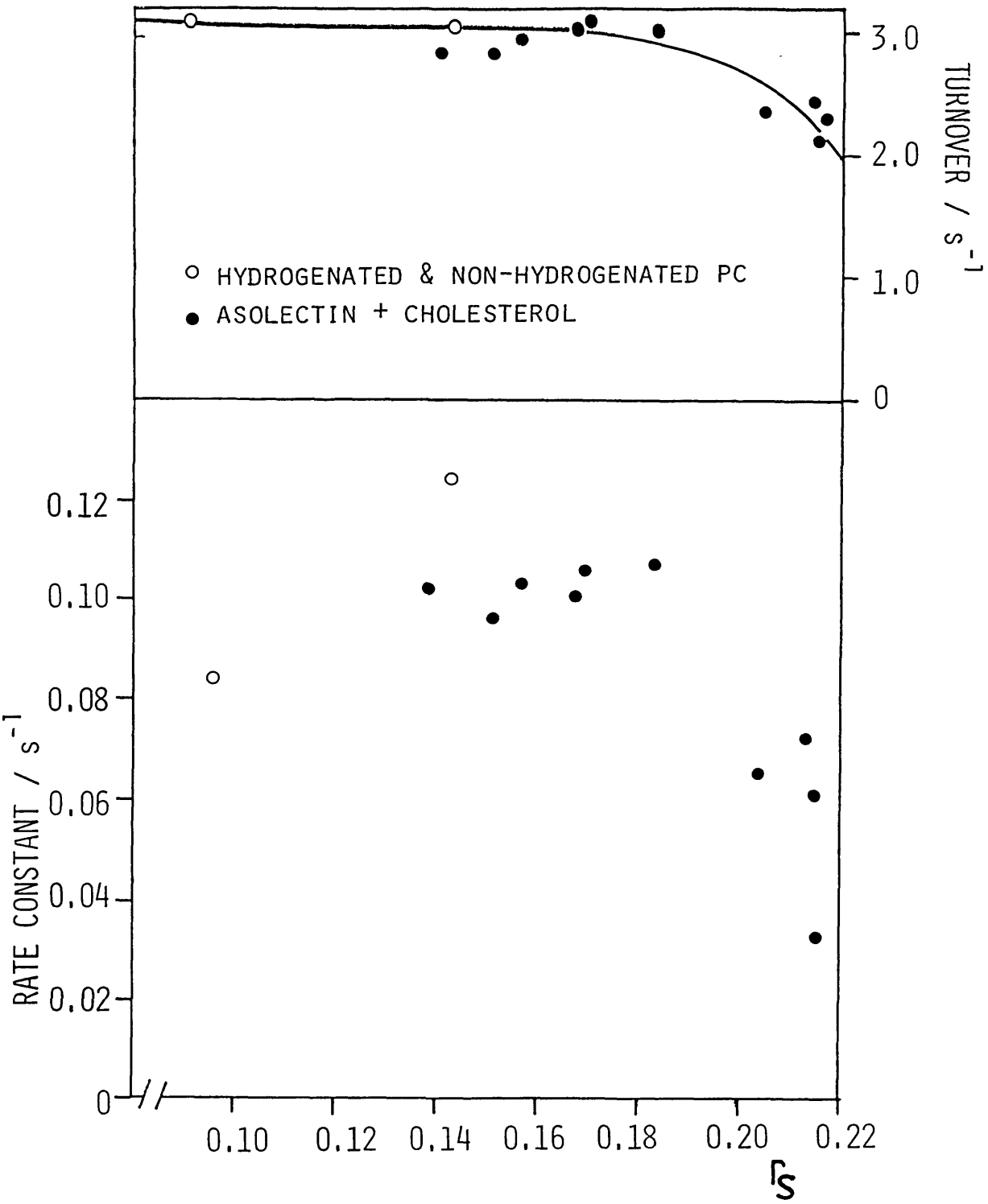


Figure 28 : The first order rate constant and turnover of PQ for entrapped FeCN reduction as a function of the fluidity of the liposome bilayer. The fluidity was modified by hydrogenation or incorporation of cholesterol and the effect was monitored by determining the r_s parameter using DPH incorporated into the liposome bilayers.

4 DISCUSSION

4.1 ANISOTROPY OF THE STEADY-STATE FLUORESCENCE FROM DPH INCORPORATED INTO THE THYLAKOID MEMBRANE

There are three questions which have to be considered before the fluorescence anisotropy data obtained with DPH-labelled thylakoids can be related to physiological function :

1 Is the signal observed with a labelled sample of thylakoid membranes from DPH embedded in the lipid matrix ?

2 What property of the membrane does the anisotropy of the steady-state fluorescence arising from the incorporated DPH report ?

3 How does this property affect physiological processes ?

These points are discussed below.

4.1.1 The Use of DPH in the Thylakoid Membrane

Other workers have provided evidence that the DPH molecule is located in the lipid bilayer of membranes (Shinitzky and Barenholz, 1978; Andrich and Vanderkooi, 1976; Stubbs and Smith, 1984). However there are particular problems with the use of the probe in the pigmented thylakoid membrane, where the signal is attenuated. The evidence that the observed signal is from DPH in the thylakoid lipid bilayer can be summarised :

1. The signal arises predominantly from the DPH associated with the thylakoid (figure 4).

2. The anisotropy of the fluorescence is affected by the incorporation of cholesterol (Haworth, 1983; Yamamoto et al, 1981; Ford and Barber, 1983b).

3. The rate at which the maximum intensity of fluorescence is reached during incubation of a sample of thylakoids with DPH is dependent on whether or not it is carried out under stacking conditions (Ford, 1982).

4. The results obtained with DPH incorporated into thylakoids correlated well with data from EPR measurements using a spin-label probe (Ford et al, 1982; Ford, 1982).

4.1.2 The Meaning of the DPH r_s Parameter

The second and third questions posed above are considered in this section. What the parameters derived from DPH fluorescence polarisation actually report about the probe's environment has been a matter of some controversy in the literature. Shinitzky and Barenholz (1978) derived a version of the Perrin relationship between the apparent microviscosity ($\underline{n}$) and the fluorescence anisotropy (r_s) in an equation which can be written :

$$(1)\quad r_0/r_s = 1 + (Cr.T.\tau)/\underline{n}$$

where **Cr** is a parameter which relates to the molecular shape of the probe and τ is the fluorescent lifetime. The **(Cr.T.τ)** factor is relatively constant for most experimental conditions so that the term $(r_0/r_s-1)^{-1}$ can be used as an approximate measure of the viscosity. The Perrin equation relates to the bulk properties of an isotropic solution so that this treatment assumes that the probe is randomly orientated within the hydrophobic core of the bilayer.

However in experiments in which the DPH fluorescence anisotropy following a nanosecond excitation pulse was resolved with respect to time (**r(t)**), it was shown that when the probe is located in a membrane there is a limiting value of **r(t)** which does not decay (Kawato et al, 1978; Kinosita et al, 1981). Thus the fluorescence anisotropy as a function of time can be expressed :

$$(2)\quad r(t) = (r_0 - r_\infty).\exp(-t/\phi) + r_\infty$$

where r_0 is the initial anisotropy (often taken to be 0.39) and ϕ is the rotational correlation time which depends on the rate of motion of the fluorophore and r_∞ is the time-independent component of the anisotropy. If the probe were to lie in an isotropic environment the value of r_∞ would be zero, so that the initial assumption of Shinitzky and Barenholz (1978) is invalidated to some extent. Kinosita et al (1981) interpreted this component as representing a restriction on the range of motion allowed to the probe which they assumed to be on average orientated parallel with the acyl chains as proposed by Andrich and Vanderkooi (1976). They were able to quantify this restriction by envisaging the DPH molecule wobbling within two cones the size of which could be determined from the r_∞ parameter. In this model the anisotropy of DPH fluorescence is composed of two completely separate parameters, one of which relates to the structural properties of the membrane (the molecular packing of the lipids and proteins), while the other

reports the kinetic properties (viscosity).

Van Blitterswijk et al (1981) examined the contribution of the two parts of fluorescence anisotropy to the observed steady-state r_s parameter using the data in the literature where both r_s and r_∞ were determined for DPH in the same lipid bilayer system. If r_s is expressed as :

$$(3)\quad r_s = r_f + r_\infty$$

then r_f represents the contribution of the steady-state parameter from the component of the anisotropy which varies with time. Van Blitterswijk et al (1981) found, by looking at the data from a wide range of reports, that at low viscosities the value of r_f exceeded that of r_∞, while in less fluid systems, including most biological membranes, the reverse was true so that the observed r_s parameter was dominated by the time-independent term. This led them to the conclusion that steady-state measurements of the fluorescence of DPH in biomembranes yielded information mostly about the degree of order of the lipid matrix.

However, Shinitzky and Yuli (1982) argued that to a first approximation the $(r_s/r_0-1)^{-1}$ parameter is a useful relative measure of viscosity at what they termed the "submacroscopic" level of resolution. In their view, this degree of resolution is of the most direct relevance to the physiological diffusive processes within the membrane. They presented evidence that shows an empirical relationship between the structural and

kinetic characteristics of bilayers, and they argue that the static and dynamic parameters reported by DPH therefore cannot be completely separated as they are in the "wobbling-cone" model of Kinosita et al (1981). However they do accept that the true viscosity will tend to deviate from that calculated from equation 1 at high values of r_s. This conclusion is supported by the data presented in section 3.1 since the fairly low r_s values obtained with DPH in liposomes (figure 3) were fitted well using equation 1 and assuming an Arrhenius relationship between viscosity and temperature while the relatively high values of r_s determined in experiments on labelled thylakoids membranes (figure 6) were not.

Pottel et al (1983) also used an empirical relationship between the kinetic and structural properties of the membrane. In their treatment the viscosity can be derived in principle from the time-independent r_∞ parameter by using the equation :

$$(4)\quad \underline{n} = V.S/(1+S)$$

where

$$(5)\quad V = Cr.T.\tau$$

and **S** is the order parameter for the lipid matrix given by (Jahnig, 1979) :

$$(6)\quad S^2 = r_\infty/r_0$$

However it turns out in practice that small errors in the determination of r_s (when the value of r_s exceeds about .08) lead to large errors in the derived value of n, while they result in only small errors in the value of S (Pottel et al, 1983). According to Pottel et al (1983), this means that in practical terms while the steady-state fluorescence anisotropy of DPH is dependent on both static and dynamic properties in very fluid systems, in biological membranes it only reports the molecular packing of the lipids. It is also argued that these static parameters depend principally on the amount of sterol and protein within a membrane, rather than the level of unsaturation (Van Blitterswijk et al, 1981; Pottel et al, 1983).

The argument as to whether the r_s parameter reflects the viscosity of the membrane or the level of order in the lipid packing has not been conclusively resolved. However both these properties may be of physiological relevance. It has been argued that the membrane viscosity will relate to the rate of diffusive processes within the bilayer (Shinitzky and Barenholz, 1978), while the static parameters may give information on the packing of, and therefore conformation of, integral proteins (Pottel et al, 1983; Kleinfeld et al, 1981).

4.1.3 Cold- and Warm-Grown Pea Thylakoid Membranes

In light of the above, the data from experiments using thylakoids from cold- and warm-grown plants labelled with DPH presented in section 3.1.2.2 (figures 6 and 7) can be interpreted in two different ways. Thylakoid membranes from cold-grown pea plants are either more fluid than those from

warm-grown plants or they have a lower level of order. The hypothesis that the difference between the two types of thylakoid membrane represents an adaptive response to growth temperature is valid for both possibilities. In one interpretation, a compositional difference between the membranes may exist to compensate for the lower rate of diffusion of species within the bilayer of the thylakoids from cold-grown plants. The other view would be that the difference in composition is a means of ensuring that the structural characteristics of the membranes are similar in the thylakoids from cold- and warm-grown plants at their respective growth temperatures, possibly so that the bilayer confers the optimal conformation on the integral proteins. From the data in this thesis it is not possible to eliminate either of these possibilities.

Figure 29 shows the compositions of the thylakoid membranes isolated from cold- and warm-grown pea plants taken from the data of Chapman et al (1983a). It can be seen that the only significant difference between them is in the amount of lipid on a chl basis. It should be noted that the amount of PQ as a fraction of the total lipid in the thylakoid is not dependent on growth temperature, so that there is no dilution of this species in the bilayer of cold-grown thylakoids. Given the wealth of reported data which shows the profound influence of the lipid to protein ratio on the parameters reported by probes incorporated into membranes, it is perhaps not surprising that the greater quantity of lipid in the thylakoid membranes from cold-grown

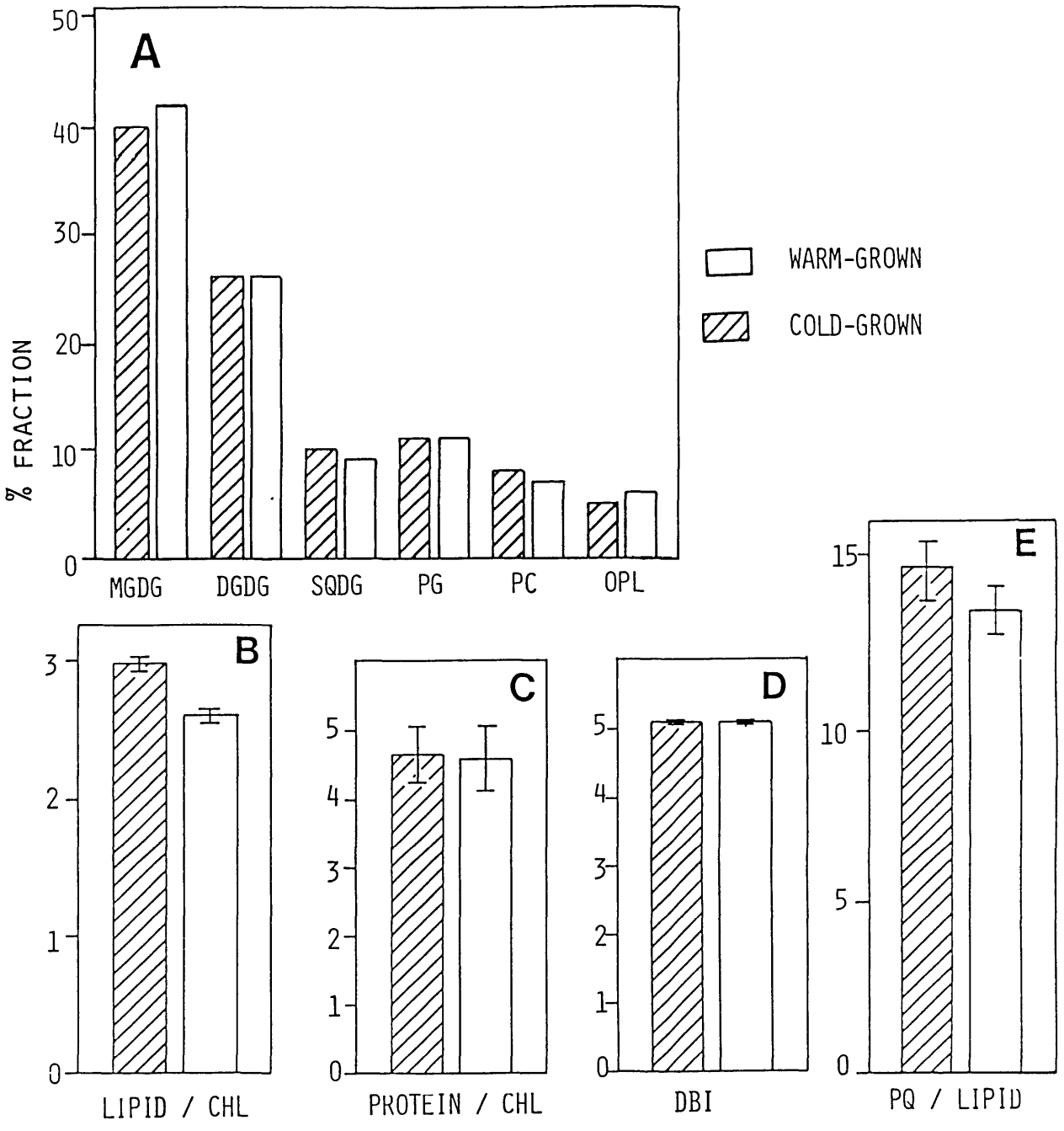


Figure 29 : The lipid class composition (A), amount of lipid (B), amount of protein (C), level of unsaturation (D) and amount of PQ (E) in lipid extracts from cold- and warm-grown pea plant thylakoids. The values given are % fraction, wt/wt ratio, wt/wt ratio, number of double bonds per lipid molecule and molar ratio/for figures A,B,C,D and E, respectively. Data from Chapman et al, 1983a.

plants apparently results in a lower value of r_s.

If the results presented in section 3.1.2 do reflect a homeoviscous adaptation of the pea thylakoid membrane, they represent the first reported example of such a mechanism in a higher plant membrane. The most comparable study in the literature is probably that of Vigh et al (1979), who found that lipids isolated from the thylakoids of freezing-tolerant cultivars of wheat were more fluid after the plants had been hardened to 5°C.

It must be said that there is no definitive evidence that the differences between cold- and warm-grown thylakoid membranes in composition and in physical properties as detected by DPH are due to an adaptive mechanism rather than merely being a direct consequence of the difference in growth temperature. However the data on the photosynthetic rates obtained with material from plants grown under the two regimes gives some support to the former interpretation.

4.2 PHOTOSYNTHETIC RATE IN MATERIAL FROM COLD- AND WARM-GROWN THYLAKOIDS

The question that was addressed in the experiments described in section 3.2 was : Is the apparent adaptation of the physical properties of the thylakoid membrane to low growth temperature reflected in an enhanced photosynthetic capacity in the cold-grown plants at their growth temperature ? The evidence from these experiments showed that an enhancement of rate did occur, both at the level of uncoupled full-chain electron transport and whole leaf oxygen evolution. This result is placed

in the context of the comparable studies in the literature below.

4.2.1 Comparison with Similar Studies

A number of studies which investigated the possibility that breaks in Arrhenius plots of photosynthetic electron transport rates are associated with the chilling sensitivity of the plant under investigation were reviewed in the introduction section of this thesis (section 1.4.4.2.1). However in general it appears that the occurrence of such breaks depends more on the coupling state of the thylakoids than the chilling-sensitivity of the species (Quinn and Williams, 1983 - see table 5). In common with the work of Nolan and Smillie (1977), it was found that an Arrhenius plot of the rate of uncoupled electron transport in thylakoids from (chilling resistant) pea plants can be fitted using two straight lines (figure 11, table 9). The apparent break temperature in the work reported here was the same for cold- and warm-grown thylakoids - 12°C. However it was not possible to discriminate statistically between the fitting of two straight lines and the fitting of curves to this data. For this reason the significance of the apparent breaks and the temperatures at which they occur is uncertain.

There have been relatively few reported studies investigating the possibility of a phenotypic adaptation of photosynthetic rate to chilling growth temperatures, such as that described in section 3.2. What reports there have been in the literature have presented a variety of findings some of which lend support to the hypothesis of an adaptation and others which appear to be at

odds with the existence of such a mechanism.

At the level of the rate of light-saturated, uncoupled electron transport, Sawada et al (1974) found that the rate of FeCN reduction was much lower in broken chloroplasts isolated from wheat plants which had been grown in field conditions (mean air temperature 5-7°C) than those from the same species but grown in a growth box (mean air temperature 20-25°C). However this study failed to take into account differences in light intensity and nutrient supply between the two growth conditions.

The experiments reported by Smillie et al (1978) are of a more comparable design to those presented here. They assayed the rate of full-chain electron transport at 24°C in thylakoids as a function of the growth temperature of the barley plants (**Hordeum vulgare** L. cv. Abyssinian) from which they were isolated. They found that this parameter increased with the growth temperature of the plants up to 27°C, which is in contrast to the results obtained with thylakoids from pea plants presented in figure 10. Huner and Hopkins (1984) compared the rates of a variety of electron transport parameters in chloroplasts isolated from hardened (grown for 7 to 9 weeks at 5°C) and unhardened Winter Rye plants (**Secale cereale** L. cv Puma). They found that the rate of full-chain electron transport assayed at 25°C was 30-50% higher in chloroplasts from hardened plants, while the PS2 activities were similar for both over a range of measuring temperatures. These results for the full-chain and PS2 reactions are in line with those found here for material from pea plants, but they also observed similar PS1 activities in the thylakoids

isolated from hardened and unhardened rye plants at 25°C, which differs from the trend seen in figure 13 where thylakoids from warm-grown pea plants exhibited greater PS1 activites than those from cold-grown plants.

Sawada and Miyachi (1974) compared the rates of CO_2 exchange measured at 25°C in leaves taken from plants grown in the field at ∿5°C with those taken from plants grown in a growth box at 25°C for a variety of species, including **Pisum sativum** L. cv. Issun. They found lower rates in the material grown at low temperatures, the values being 10% of those determined for the leaves grown at 25°C in the case of the pea plants. This study differs from the experiments on photosynthetic rate in whole leaves described here (section 3.2.2.2) in that the determinations were carried out at atmospheric CO_2 concentration and it again suffers from the lack of control of factors other than temperature between the two sets of growth conditions.

Pollock et al (1983) compared the rates of light-saturated oxygen evolution in leaves taken from **Lolium temulentum** L. plants which had been grown continuously at 20°C and those from plants which were transferred to either 5 or 2°C at 4th leaf maturity. They carried out the assays under high CO_2 concentrations at the three average growth temperatures and expressed the results on a leaf area basis. It was found that the leaves from the plants transferred to the 5°C regime exhibited greater photosynthetic rates than those from the plants grown continuously at 20°C when measured at 5° or 20°C and that the difference between them was greater at the lower

assay temperature.

These results can be compared (assuming that the chl per unit area was not affected by the growth temperature) to those found in the similar experiments on pea leaves reported here (figures 14 and 15). They show the same trends to the extent that :

(1) larger rates are observed in leaves from plants grown at the lower temperature when measurements are carried out at this temperature and

(2) this difference in rate decreases at higher assay temperatures.

The two sets of results differ in that in the experiments on pea leaves the rates of oxygen evolution determined in the material from the warm-grown plants appear to be higher than those measured with cold-grown leaves when assayed at the warm growth temperature (figure 14). However, when **L. temulentum** plants were exposed to the low temperature (5°C) for longer periods no difference in photosynthetic capacity between the transferred and non-transferred plants was observed when the measurements were carried out at 20°C (Pollock et al, 1984). The rates were not determined at any other temperature in this study.

4.2.2 The Site of the Adaptation Within the Photosynthetic Process

The results of the oxygen evolution assays carried out on leaves from cold- and warm-grown pea plants are consistent with an adaptation of photosynthetic rate to growth temperature. The data from the experiments which determined full-chain and

partial electron transport parameters in thylakoids give some indication as to the level within the complete photosynthetic process at which the differences dependent on growth temperature occur.

4.2.2.1 Comparison of Photosynthetic Rates in Leaves and Thylakoids

The differences between the rates of full-chain electron transport observed in isolated thylakoids (figure 10) and those of oxygen evolution in leaves (figure 14) imply that the degree to which the electron transport process limits the overall photosynthetic process under the conditions used to assay leaf oxygen evolution differs in the cold- and warm-grown plants and is dependent on temperature.

Since the rates of photosynthesis observed in leaves are greater than those found in thylakoids (presumably due to a reduction in activity during isolation) the absolute rates of leaf oxygen evolution and full-chain electron transport are not directly comparable. However the difference in the activation energies of the photosynthetic rates determined in the thylakoids and leaves of cold and warm-grown plants (table 11) give some information on the degree to which the overall process is limited by electron transport under saturating conditions. In going from the level of the isolated thylakoids to that of the intact leaf, there is a small decrease in the activation energies obtained with cold-grown material and an increase in those obtained with warm-grown material. This suggests that the rate of oxygen evolution in cold-grown leaves at high

temperatures and in warm-grown leaves at low temperatures is limited by some process other than electron transport. That different processes limit whole leaf oxygen evolution at different temperatures is consistent with the observation that an Arrhenius plot of this parameter does not fit a straight line regression (figure 15). The limiting process under these conditions could be the dissipation of the proton gradient across the thylakoid membrane by the ATP-synthetase, or the carbon-fixation reactions. The rates of electron transport obtained with thylakoids isolated from pea plants in their coupled (Shneyour et al, 1973) and uncoupled states (Nolan and Smillie, 1977) do not differ substantially, which suggests that the carbon-fixation process is more likely to be rate-limiting.

If cold-grown leaves are more limited by carbon-fixation than warm-grown leaves at the higher temperatures used, it explains why faster rates were observed in warm-grown leaves above 15°C (figure 14), even though cold-grown thylakoids sustain greater rates of electron flow than warm-grown ones at all temperatures between 3 and 19°C (figure 10).

There is some support for this idea from the results of a study (Kuraishi et al, 1968) where it was found that pea plants which had been hardened at 5°C had higher NADPH/NADP ratios than unhardened plants when they were illuminated at 25°C. This is the result that would be expected if photosynthesis in cold-grown (or hardened) pea plants was comparatively more rate-limited by the fixation of carbon, and less by electron transport, than it was in the warm-grown plants so that the

reduction of NADP occurred faster than the oxidation of NADPH. If this is the case for the data obtained above 15°C shown in figure 14, it means that these points reflect a greater CO_2-fixing capacity in the warm-grown plants compared with the cold-grown plants. This may be due to a higher temperature optimum for the ribulose bisphosphate carboxylase activity in the warm-grown plants. A lower temperature optimum for carbon-fixation has been observed in rubisco isolated from hardened winter rye plants when compared with enzyme from unhardened plants (Huner and MacDowall, 1979).

It should be pointed out that the results of the leaf oxygen evolution measurements described in section 3.2.2.2 do not represent a physiological situation since they were carried out at high (presumably saturating) concentrations of CO_2. This means that differences between the rates of electron transport and of oxygen evolution in leaves can be attributed only to differences in the V_{max}s, and not the k_ms, of the carboxylase activity.

The general conclusion from the discussion above is that the cold-grown pea plants are able to maintain greater rates of CO_2- and light-saturated photosynthesis at low temperatures than warm-grown ones, partly by virtue of their greater capacity for electron transport. However there is evidence that there is also an adaptation of the ribulose bisphosphate carboxylase activity to growth temperature.

4.2.2.2 Difference Between the Rates of Full-Chain Electron Transport in Cold- and Warm-grown Thylakoids

There remains the question of the nature of the difference between the thylakoids which enables those from cold-grown plants to support greater rates of full-chain electron transport than the ones from warm-grown plants at all the measuring temperatures used. The only two possibilities are that :

(1) The rate of turnover of individual electron transport components within the photosynthetic apparatus of cold-grown plants exceeds that of those in warm-grown plants.

(2) There are a larger number of active electron transport chains per chl in the cold-grown thylakoids than in the warm-grown ones.

Since the chl a/b ratios (figure 9) and the light dependency of the rates (see section 4.2.3) are practically the same for the thylakoids isolated from plants grown under the two regimes, the available evidence argues against a difference in the number of chains per chl. This therefore suggests that the flux of electrons per chain is greater in the thylakoids from cold-grown plants than in those from warm-grown plants. If this is so, it means that the turnover of the step which is usually considered to be rate-limiting to the full-chain electron transport process under light-saturating conditions - the oxidation of PQH_2 - occurs at a faster rate in the cold-grown thylakoids. The assumption that PQH_2 oxidation is rate-limiting is consistent with the greater rates of oxygen evolution observed with both cold- and warm-grown thylakoids in the presence of a PS2

acceptor (figure 12) and when determining PS1 activity (figure 13), compared with the whole-chain process (figure 10).

There is no evidence for a greater activity of either of the photosystems in the thylakoids from cold-grown plants since :

(1) the presence of a PS2 acceptor reduced the difference in the rate of electron transport between the two types of thylakoids (figure 12) and

(2) the activity of PS1 was apparently greater in the thylakoids from warm-grown plants compared with those from cold-grown plants (figure 13).

4.2.3 Light-Dependency

The parameters derived from the titrations of photosynthetic rate in leaves and thylakoids with light intensity (table 12) can be analysed to give evidence concerning :

(1) the number of PS2 complexes active in electron transport per chl,

(2) the source of the difference between rates observed in leaves and isolated thylakoids from the same growth regime when measured at the same temperature and

(3) the intensity of light at which the photosynthetic rate in material from cold- and warm-grown plants is saturated.

The information that can be obtained from the value of k_I, which is the steady-state oxygen evolution yield per unit chl at infinitely low light, is equivocal because the parameter is dependent on both the number of PS2 complexes per chl and the efficiency of the light-harvesting per PS2 (Rieske et al, 1959; Satoh et al, 1972). Since this second variable correlates with

the antenna size of PS2, it will tend to offset the effect of the first factor on the k_I parameter. However, the influence of a larger amount of active PS2 per chl will usually outweigh any effect due to a smaller average antenna size of these PS2 complexes, so that the k_I parameter shows a positive relationship with the PS2/chl ratio under most circumstances (Satoh et al, 1972). On top of this the similar chl a/b ratios which are observed in the material from cold- and warm-grown plants (figure 9, table 10) indicate that there is probably not much difference in the amount of LHCP2 per photosystem, but does not exclude the possibility of a difference in the relative amount of the photosynthetically active PS2. In light of this, the similar values of k_I calculated indicate that the active PS2 content per chl is relatively independent of growth temperature (table 12).

The fact that both the k_I and the V_{MAX} (especially at higher measuring temperatures) parameters are larger in the leaves than in the thylakoids of both cold- and warm-grown plants (table 12) suggests that the thylakoid isolation procedure results in a reduction in the number of active electron transport chains per chl. Since the reduction in the value of k_I in going from leaves to thylakoids is similar for cold- and warm-grown material, it seems that the loss of activity associated with the isolation of thylakoids is independent of the growth temperature of the plants used.

The fitted curves plotted in figures 16 and 17 were used to estimate the intensity of (nearly saturating) light required to

give a photosynthetic rate which is 90% of the calculated theoretical maximum at that temperature (table 12). Since the values of k_I are similar for a given type of assay, these intensities depend directly on the value of V_{max}; the larger the saturated rate, the greater the intensity of light needed to generate it. This trend means that oxygen evolution from cold-grown leaves is saturated at higher light intensities than that from warm-grown leaves when measured at the cold growth temperature and **vice versa.** An important point is that because of the effect of temperature, the cold-grown leaves are apparently saturated by much lower intensities at their growth temperature than the warm-grown ones are at theirs (760 compared with 1940$\mu E.m^{-2}.s^{-1}$). This means that under the growth lighting conditions (200-300$\mu E.m^{-2}.s^{-1}$) the cold-grown plants are probably much closer to light saturation than the warm-grown ones, although this does not take account of the fact that the leaf-oxygen evolution assays were carried out at high CO_2 concentrations. It is therefore possible that the cold-grown plants are adapted to effectively higher light intensity conditions than the warm-grown plants.

4.2.4 Stoichiometry

Although the similar chl a/b ratios suggest that the relative amounts of the photosystems and LHCP2 are nearly the same in cold- and warm-grown plants, there is some indication that the ratio of PS1 to PS2 may differ between plants grown under the two regimes. The evidence comes from the apparently greater activity of PS1 observed in thylakoids from warm-grown

plants (figure 13) and from the greater 733nm/685nm peak intensity ratio found in the 77K fluorescence from warm-grown chloroplasts compared with cold-grown ones (table 13). On the other hand there is little evidence for relatively less PS2 in the thylakoids from cold-grown plants as judged from the rate of electron transport in the presence of a PS2 acceptor (figure 12) or from the k_I values derived from the light-intensity curves (table 12).

There is some preliminary data which suggests that there may be a difference in the amount of cyt b_6f complex per chl in the cold- and warm-grown pea plants. The amount of cyt f and cyt b_{559} high potential (HP) was spectrophotometrically determined in thylakoids isolated from cold- and warm-grown plants (figure 30 - from Millner, personal communication). This shows that the amount of both cytochromes per chl was apparently greater in the cold-grown thylakoids than the warm-grown ones. Since the quantity of cyt b_{559} HP is purportedly stoichiometrically related to the amount of PS2 (2 b_{559} HP : 1 PS2 - Anderson, 1982), a lower level of this cytochrome in the warm-grown plants is in line with a higher PS2:PS1 ratio in the cold-grown plants. The greater quantity of cyt b_6f complex in the cold-grown plants which is implied by the cyt f assays, may be partly responsible for the faster rate of full-chain electron transport which is observed in the thylakoids from cold-grown plants. However the trends which are suggested by the results shown in figure 30 have not been demonstrated with statistical certainty because of the relatively few replicates used.

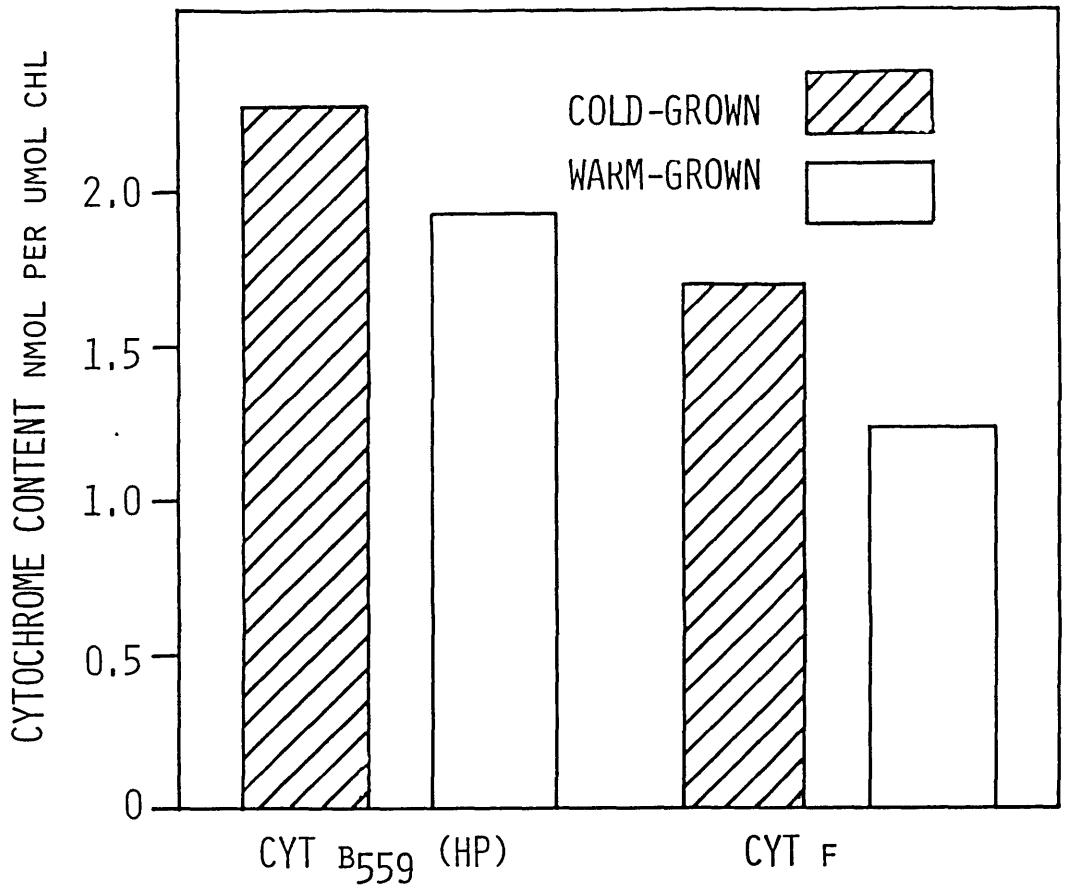


Figure 30 : High potential cytochrome b_{559} and cytochrome f content of thylakoids isolated from cold- and warm-grown pea plants. Data from Millner, personal communication.

The stoichiometric relationship of the three electron transport complexes was also found to alter in Barley plants during ontogeny (Holloway et al, 1983). In the case of pea plants, it may be that the changes in the stoichiometry occur at different rates in cold- and warm-grown plants so that the relative quantities of the three thylakoid membrane complexes differ between them at the same developmental stage.

Huner and his coworkers determined the relative amounts of a variety of thylakoid membrane components between the hardened and unhardened winter rye plants. As mentioned above, they found a greater rate of full-chain electron transport in hardened thylakoids than in unhardened ones, but similar PS1 and PS2 activities for both (Huner and Hopkins, 1984). They also found similar chl a/b ratios (Griffith et al, 1982; Huner et al, 1984), P_{700} to chl levels (Huner et al, 1984), photosynthetic unit sizes for PS2 (Huner and Hopkins, 1984) and polypeptide compositions (Griffith et al, 1982) in the two types of thylakoids. Their conclusion was that the stoichiometry of the electron transport complexes was broadly unaltered by the hardening treatment (although they did not directly determine the amount of cyt f). On the other hand, they observed the same trend of differences in the 77K fluorescence emission spectra between the chloroplasts of hardened and unhardened winter rye plants (Griffith et al, 1984) as was observed for those of cold- and warm-grown pea plants (table 13). This suggests that the results in table 13 may not reflect a difference in PS1:PS2 ratio, but rather a more subtle difference in the relationship

between the photosystems and the LHCPs. The difference in the apparent activities of PS1 shown in figure 13 may be due to a greater rate of turnover in the thylakoids from warm-grown plants rather than a greater quantity of PS1.

The evidence concerning the stoichiometry of the photosystems and cyt b_6f complexes in the chloroplasts of cold- and warm-grown plants is inconclusive. If there is a difference it seems likely that there is a greater PS2:PS1 ratio and more cyt b_6f per chl in the cold-grown plants.

4.2.4 Summary of Conclusions on the Work on Cold- and Warm-grown Pea Plants

The most plausible interpretation of the data from the assays of photosynthetic rate discussed above is that there is an adaptation of photosynthetic rate to growth temperature, and that the adaptative mechanism involves a greater rate of PQH_2 oxidation per chl in the thylakoids from cold-grown plants. However, if there is an adaptation, the data on full-chain electron transport raises the question as to why the thylakoids from warm-grown plants exhibit slower rates than the ones from cold-grown plants at the warm growth temperature. There are several factors which may partly or wholly explain this phenomenon :

(1) It might be that the warm-grown plants are so far from light saturation under the growth conditions that light harvesting, and hence the amounts of the PS2 and LHCP2

complexes, are more important in determining the rate of photosynthetic electron transport than the turnover of PQH_2. However there is little evidence for this theory in the chl a/b ratios or in the k_I parameters which are similar for cold- and warm-grown plants.

(2) The differences in the PS1 activities observed in cold and warm-grown thylakoids (figure 13) may be indicative of differing demands for ATP and NADPH on the electron transport system - so that there might be a relatively greater need for cyclic, as opposed to linear, electron flow in the warm-grown plants.

(3) It may be that at physiological concentrations of CO_2, the overall photosynthetic rate in cold-grown plants is limited by carbon fixation at the warm growth temperature. This is probably the most likely explanation of the three.

The idea of an enhanced electron transport rate as being part of an adaptation to low temperatures runs against some of conventional wisdom on the limitation of photosynthetic rate at sub-optimal temperatures. The temperature dependency of electron transport is often considered to be less than that of carbon fixation, so that low temperature stress involves a greater inhibition of the latter step than the former (Graham and Patterson, 1982; Berry and Bjorkman, 1980). In this view, adaptation of photosynthetic rate to sub-optimal temperature is a matter solely of increasing the ribulose bisphosphate carboxylase activity of the plant (Graham and Patterson, 1982). While the discussion above (section 4.2.2) led to the conclusion

that such an adaptation probably does occur in pea, the evidence is that the rate of electron transport is adapted too. Although this result is in conflict with data from some similar studies on other species (e.g. Smillie et al, 1978 on Barley), other studies have indicated that an adaptation of photosynthetic rate to sub-optimal temperatures does involve an enhanced capacity for electron transport, as well as a change in the CO_2 fixation step (Bjorkman and Bjorkman, 1979 on **Nerium oleander**; Huner and MacDowall, 1979; Huner and Hopkins, 1984 on winter rye).

This is consistent with the idea that the physical properties of the thylakoid membrane adapt to growth temperature in an attempt to maintain high levels of electron transport at low temperatures. On the other hand, the faster rate of full-chain electron transport observed in thylakoids from cold-grown plants at saturating light could be due partly or wholly to a relatively larger amount of cyt b_6f complex per chl in this material. It could be that the apparent adaptation of the fluid properties of the thylakoid membrane serves some purpose other than the optimisation of the electron transport rate. It has been found that cold-grown pea leaves undergo the state 1-state 2 transition more rapidly than warm-grown leaves at 20°C (Mitchell et al, 1984). This process, which is thought to involve the diffusion of the LHCP2 complex, may be facilitated by the more fluid thylakoid membrane of cold-grown plants.

In common with the work described here on the effect of growth temperature on pea thylakoids (figure 29), similar lipid class and fatty-acid compositions (Griffith et al, 1984) and

protein to chl ratios (Griffith et al, 1982) were found in the thylakoids from hardened and unhardened plants. Unfortunately they have not reported any data on the physical properties or the lipid to chl ratios of the winter rye thylakoids.

The increased rates of light-saturated oxygen evolution found in the leaves of **Lolium temulentum** L. following a transfer to chilling temperatures (Pollock et al, 1983) are in line with those described here for pea. The thylakoid membranes of these plants show a substantial increase in lipid to chl ratio after the transfer (Chapman et al, 1983c), which also parallels the situation observed in pea plants (Chapman et al, 1983a - figure 29).

There is then some circumstantial evidence for a relationship between the amount of lipid per chl and the rate of full-chain electron transport. Assuming that the amount of lipid correlates with the physical parameters of the thylakoid membrane of **Lolium** as it appears to for that of pea, this could be explained in terms of an optimal environment for the PQH_2 oxidase site on the cyt b_6f complex, or a faster rate of PQH_2 diffusion.

However there is no direct evidence in any of this for a correlation between the physical properties of the thylakoid membrane and the rate of PQH_2 oxidation. The experiments described in the second half of the results section (sections 3.3 and 3.4) were intended to establish whether or not such a correlation existed.

4.3 HYDROGENATION OF THE THYLAKOID MEMBRANE

The results of the application of the hydrogenation technique

described in section 3.3 show that a substantial level of hydrogenation can be consistently achieved (figure 20b) and that all lipid classes are susceptible, although some more so than others (figure 21). Assays of the effect of this treatment on the functioning of the thylakoid appeared to demonstrate a positive correlation between the level of unsaturation and the rate of full-chain electron transport (figure 22). The fact that the hydrogenation-induced inhibition of full-chain electron transport decreased at lower light intensities (figure 23) showed that the effect was not simply a consequence of eliminating a proportion of the electron transport chains. The rates of partial electron transport reactions, although subject to a large degree of variation in the presence of the catalyst, were apparently not significantly inhibited by hydrogenation (table 17). This data is therefore consistent with the hypothesis that the rate of PQH_2 oxidation is inhibited by an artificially induced decrease in the level of fatty acid unsaturation in the thylakoid membrane lipid bilayer.

However there are several obstacles to drawing conclusions about the **in vivo** situation from the results of these experiments. One difficulty is that the presence of the catalyst alone profoundly affects the functioning of the thylakoids (table 16), so that there is no way of telling whether the control samples are representitive of the physiological situation (for example whether full-chain electron transport is limited by PQH_2 oxidation at saturating light). In addition to this the presence of the catalyst prohibited the use of DPH so

that the effect of hydrogenation on the fluidity could not be determined.

There have been a number of other studies on the effects of subjecting the thylakoid membrane to hydrogenation. In the original work carried out by Restall et al (1979), they found no significant decrease in the rate of any of the electron transport reactions following a treatment which was found to induce a substantial decrease in the level of fatty acid unsaturation. However their work differed from that described here in that they used intact chloroplasts for the hydrogenation treatment. It may therefore be that the thylakoid membrane was inaccessible to the catalyst because of the envelope and that the observed hydrogenation was due mostly to the lipids from the envelope membranes and the thylakoids from the proportion of broken chloroplasts.

This technique which relied on the use of large quantities of a water insoluble catalyst added to the thylakoid sample in a vector, has been superseded to some extent by the advent of water-soluble catalysts (Palladium organometallics) which can be removed at the end of the treatment (Vigh and Joo, 1984; Vigh et al, 1985; Szalontai et al, 1985; Horvath et al, 1985). Using this technique it is possible to generate thylakoid membranes with a lower level of unsaturation which are virtually free of catalyst (Vigh and Joo, 1984). Many of the assumptions underlying the work described above have been confirmed according to reports which describe experiments using this improved method :

(1) It was found that the catalytic treatment hydrogenates the double-bonds of the polar lipid acyl chains exclusively and that the conjugated double bonds on neutral lipids such as carotenoid and PQ were not susceptible (Szalontai et al, 1985).

(2) The reduction in the level of fatty acid unsaturation induces an increase in the degree of lipid order, as shown by the use of spin-labelled fatty acids (Vigh et al, 1985; Horvath et al, 1985).

(3) That the rate of full-chain electron transport is affected more profoundly by hydrogenation than are the activities of the photosystems (Horvath et al, 1985).

It seems therefore that the hydrogenation of thylakoid membranes does provide evidence consistent with the hypothesis that an increase in the viscosity of the lipid bilayer inhibits the rate of PQH_2 oxidation. However the process of reducing the number of double bonds in the acyl chains of the thylakoid lipids alters properties of the membrane other than the overall level of fluidity. For example isolated MGDG only forms non-bilayer structures at physiological temperatures above a certain level of unsaturation (Quinn and Williams, 1983; Gounaris et al, 1983b). This shows that the number of double bonds in a lipid molecule profoundly affects its structural behaviour, so that the specific roles postulated for lipids in the interaction with proteins (reviewed in Siegenthaler and Rawyler, 1985; Quinn and Williams, 1985) might be severely affected by the hydrogenation treatment.

However the inhibition of electron transport parameters by

hydrogenation described here and reported by Horvath et al (1985) is in line with that found by adding cholesterol to the thylakoid bilayer (Yamamoto et al, 1981; Ford and Barber, 1983a). There is therefore some basis for the hypothesis that the overall level of fluidity of the thylakoid membrane affects the rate of electron transport.

4.4 MODEL SYSTEM

The experiments which employed the model system were designed to test the possibility of a relationship between the rate of a transmembrane flow of electrons and protons catalysed by PQ and the fluidity of the bilayer. The fact that this reaction fits first order kinetics with respect to the oxidant (figure 24) shows that the reaction is limited by the oxidation of PQH_2.

It was found that the reaction kinetics in liposomes made from lipid with different levels of unsaturation were not significantly altered, even though the viscosity of the liposomes' bilayers differed substantially (table 18). The incorporation of cholesterol into liposomes only inhibited the reaction at very high levels which could have reduced the accessibility of the PQH_2 to the oxidant (figure 28).

The conclusion that the rate of this reaction is probably not dependent on the fluidity of the bilayer does not imply that the same is true for the oxidation of PQH_2 in the thylakoid membrane. The reaction in the model system is not analogous to the physiological situation since :

(1) it does not depend on the lateral motion of PQ,

(2) it does not involve an interaction with the active site

of a protein and

(3) the turnover of PQ occurs at a much slower rate. (The turnover of PQ/PQH_2 in saturated full-chain photosynthetic electron transport is probably in the order of $20s^{-1}$, as opposed to the 2-3 s^{-1} observed for the reaction in the liposomes.)

However this system could provide a basis for future studies on the interaction between the rate of PQH_2 oxidation and the fluidity of the environment in which it occurs (see section 4.6).

4.5 SUMMARY

This thesis represents an attempt to link a physical property of the thylakoid membrane to the complete process of photosynthesis in plants. Although the occurrence of such a link has not been demonstrated with certainty, the results of the experiments have in general supported the theory that it exists. The conclusions of the work are summarised below :

1 There is an adaptation of photosynthetic rate to chilling growth temperature in pea plants.

2 This adaptation involves

(a) an increased capacity for full-chain electron transport in thylakoids from cold-grown plants and probably

(b) a shift in the temperature optimum of the ribulose bisphosphate carboxylase activity.

3 There is a higher level of fluidity in the thylakoid membranes of cold-grown plants compared to those of warm-grown plants, probably as a consequence of the larger amount of lipid present in these membranes.

4 This difference in fluidity may also be part of the adaptation of photosynthetic rate, representing a homeoviscous adaptation on the part of the plant. This implies a positive relationship between the fluidity of the thylakoid membrane and the rate of electron transport.

5 There is some evidence for such a relationship from the inhibition of full-chain electron transport which is induced by the **in situ** hydrogenation of the thylakoid membrane.

4.6 FURTHER WORK

The experimental work presented here has touched on several diverse areas of research into photosynthesis and it has raised more questions than it has answered. Some of these questions and the possible lines of investigation which might be applied to them are listed below.

1 Is the rate of PQH_2 oxidation in the thylakoid membrane dependent on the fluidity of the bilayer, and if it is, is it due to a faster rate of PQH_2 diffusion or a change in the conformation of the cyt b_6f complex ?

The investigation of this point could be approached by several different routes. By following the kinetics of PQH_2 oxidation following a saturating flash in the thylakoids of cold- and warm-grown pea plants, it should be possible to determine whether the observed difference in membrane fluidiy has any effect on the reaction. This technique would have to be related to the total amount of cyt b_6f in the two sets of material.

Using the improved catalyst technique developed by Vigh and Joo (1984) it may be possible to follow PQH_2 oxidation in hydrogenated thylakoids and relate this to any increase in the viscosity of the membrane.

If the model system were extended by including a reconstituted cyt b_6f complex in the liposomes, and by replacing the FeCN with PCy, this could prove an ideal way of studying the PQH_2 - plastoquinol-oxidase interaction in isolation. The composition of the bilayer in this system could be modified in

terms of lipid class, fatty acid and protein content and the effect on the fluidity could be related to the rate of PQH_2 oxidation. This system could also be used to study the second half of the question posed above. By varying the amount of PQ/PQH_2 in the bilayer it should be possible to determine whether the rate of the reaction is affected by the fluidity of the bilayer at saturating concentrations of PQH_2. If it is, it suggests that the effect is on the conformation of the protein, while if it is not affected the inference would be that the rate of PQH_2 diffusion is the critical factor.

2 Is there an adaptation of photosynthetic rate in cold- and warm-grown pea plants at atmospheric CO_2 concentrations ?

The rate of CO_2 exchange under physiological conditions can be determined in leaves and plants by a variety of techniques including infra-red gas analysis. Given the results presented here, it would be very surprising if there were not an adaptation of photosynthetic rate to growth temperature under these conditions.

3 Is there a growth-temperature dependent difference in the stoichiometry of the integral protein complexes in the thylakoid membranes of pea plants ?

There are a variety of techniques which have been used in order to determine the relative quantities of PS1 and PS2. Anderson and Melis used spectroscopic, fluorescence and steady state electron transport measurements (Melis and Anderson, 1983; McCauley et al, 1984), while others have used photochemical yields after saturating flashes (Whitmarsh and Ort, 1984). The

estimation of the amount of cyt b_6f complex is generally achieved by the spectroscopic determination of cyt f. However the application of these techniques to spinach chloroplasts has yielded some conflicting evidence on the relative amounts of the electron transport complexes. It is therefore possible that they may not be capable of resolving the postulated differences in stoichiometry in the thylakoids of pea plants.

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