

BIOLOGICAL ELECTRON TRANSFER MEDIATORS

IN MODEL MEMBRANE SYSTEMS

by

IAN G. LYLE

*A thesis submitted for the degree of Doctor of
Philosophy in the University of Glasgow.*

June 1978

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ACKNOWLEDGEMENTS

The work described in this thesis was carried out in the Department of Chemistry, Glasgow University. I should like to thank all those in the Department who helped and encouraged me during the course of the research.

I am particularly indebted to Dr. Russell Paterson for his friendly guidance and supervision throughout the work, and to Stephen Anderson for his pioneering studies on electron transfer across liquid membranes, and for many useful discussions. My thanks are due also to Jim Walker, to Lutfullah and to my other colleagues who helped to make my years in the Department so enjoyable.

I must express my sincere thanks to those of the technical staff who made this work possible, especially to Mr. Andy Hislop and his staff in the mechanical workshop, and to all the glassblowers. I owe a particular debt of gratitude to Mr. Fred Harvey for his skilled and painstaking work in constructing the membrane cells described in Chapter Four of this thesis. I am grateful also to Dr. A. L. Porte for his help in the recording and interpretation of EPR spectra.

Finally I should like to thank the Science Research Council for providing me with a maintenance grant.

DECLARATION

Some of the work described in Chapters Two and Three of this thesis has been published, as a short note, in the journal 'Nature'.

Dr. A. L. Porte assisted during the recording of EPR spectra (Section 2-2.7).

SUMMARY

The overall objective of this research was to create chemically well-defined liquid redox membranes, each of which would be capable of mediating a process of continuous electron transfer between an aqueous reductant on one side and an aqueous oxidant on the other. The transport of electrons between the two otherwise separate aqueous phases would be effected by membrane-bound reversible redox-active carrier molecules. It has been proposed that such molecules function in certain electron transfer processes associated with biological membranes. In particular, the isoprenoid benzoquinone coenzyme Q is thought to carry electrons (and protons) across the inner mitochondrial membrane during respiration, whilst the related naphthoquinone vitamin K acts as a carrier in photosynthetic and bacterial electron transfer. Since these two molecules were also found to possess all the attributes required of 'ideal' carriers, they were chosen for incorporation in all the redox membranes investigated during this research, in the hope that their behaviour in these simple model systems could be used to elucidate their biological function.

Preliminary investigations were carried out to find suitable aqueous reductants and reducible substrates. A wide variety of common redox reagents, and several biological molecules, were tested for their ability to reduce coenzyme Q₁₀ and vitamin K₁, or to reoxidise their quinol forms, in biphasic reactions in which the quinone/quinol in hexane solution was equilibrated anaerobically over the aqueous reagent. A number of criteria were specified defining 'ideal' reductants, substrates and carriers, and control experiments were performed to

assess the ideality of the reagents used. In general terms, coenzyme Q was found to be reduced by a wider range of reductants than vitamin K, while dihydrovitamin K was much more easily reoxidised than reduced coenzyme Q. Both quinones reacted readily at pH 7 with reduced methyl viologen and flavin mononucleotide free radicals, which behaved as ideal reductants. Electron paramagnetic resonance spectroscopy revealed the presence of semiquinone free radical species of coenzyme Q and vitamin K at the hexane/aqueous interface during these reactions.

To establish continuous membrane-mediated electron transfer processes, experiments were conducted in an H-shaped reaction vessel in which the quinone solution (in hexane) formed a bridge between the aqueous reductant and aqueous oxidant in the lower limbs. Reduced methyl viologen, in large molar excess over the quinone and substrate, was routinely used as a reductant, and the kinetics of reduction of various substrates were followed spectrophotometrically. Both coenzyme Q and vitamin K were shown to function as reversible electron carriers in these bulk membrane systems, but the rate of substrate reduction was always very much slower when coenzyme Q was used. For this reason, more detailed studies were restricted to vitamin K as electron carrier. The kinetics of reduction of methylene blue by dihydrovitamin K were first order with respect to the oxidised dye, and the measured rate constant was consistent with diffusion control on the substrate side of the interface. The reaction was inhibited to varying degrees by the addition of different amphipathic phospholipids to the membrane. With cytochrome c as substrate, the biphasic reaction with dihydrovitamin K was no longer diffusion-controlled, but was determined by mechanistic factors. The very slow, apparently zero-order reaction was greatly

stimulated by the addition of the mitochondrial phospholipid cardiolipin to the membrane, and the variation of the reaction rate with ionic strength of the aqueous phase could be explained in terms of binding between the acidic phospholipid and the basic protein. Such interactions are proposed to be of importance in the functional organisation of the mitochondrial membrane.

Having established the abilities of coenzyme Q and vitamin K to act as electron carriers across a bulk hydrocarbon phase, attempts were made to improve the biological model by reducing the thickness of the membrane to the dimensions of a lipid bilayer. The two model systems studied were planar bimolecular lipid membranes (BLM) and closed unilamellar lipid vesicles (liposomes). The stabilities and thicknesses of a large number of BLM formed from a selection of amphipathic lipids and lipid mixtures were examined, and 'recipes' were found for membrane-forming solutions which yielded stable bilayers containing either vitamin K or coenzyme Q. A membrane cell was developed, allowing electron transfer across ultrathin membranes to be followed spectrophotometrically. Unfortunately, the instability of lipid bilayers within the apparatus did not allow kinetics experiments to be performed using BLM. However, electron transfer across thicker lens membranes, mediated by vitamin K, was demonstrated.

In order to achieve redox reactions across membranes of true bilayer thickness, it was necessary to use liposomes as models. Unilamellar vesicles were prepared from mixtures of vitamin K with phospholipid, encapsulating a photosensitive reductant system which contained EDTA and flavin mononucleotide. On irradiation of the liposome suspension with blue light in the presence of a suitable external substrate such as cytochrome c, rapid reduction of the

substrate took place. Numerous control experiments were performed to ensure that the observed reaction was indeed mediated by the membrane-bound carrier. Thus the ability of vitamin K to act as an electron carrier across a bilayer membrane was verified.

SYMBOLS AND ABBREVIATIONS

A	amps
A	area (cm ²)
	constant in Debye-Hückel equation
A _λ	absorbance at wavelength λ
Å	Ångstroms
a _i	isotropic hyperfine coupling constant (gauss)
AC	alternating current
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BLM	bimolecular lipid membrane
C	capacitance (farads)
c	concentration (moles litre ⁻¹)
CCCP	carbonyl cyanide <i>m</i> -chlorophenylhydrazone
CL	cardiolipin
CoQ	coenzyme Q
CoQH ₂	reduced coenzyme Q
cyt	cytochrome
D	diffusion coefficient (cm ² s ⁻¹)
d	thickness (cm)
	atomic d-orbital
DC	direct current
DCIP	2,6-dichlorophenol indophenol
E	reduction potential (volts)
E' _o	standard reduction potential at pH 7 (volts)
E _{mN}	midpoint potential at pH N (volts)
E _o	initial voltage (volts)
E _t	voltage at time t (volts)

SYMBOLS AND ABBREVIATIONS (cont'd.)

e	electronic charge
emf	electromotive force
EPR	electron paramagnetic resonance
ESR	electron spin resonance
esu	electrostatic units
F	Faraday (9.6487×10^4 coulombs equiv ⁻¹)
f_x	activity coefficient of ion X
FAD	flavin adenine dinucleotide
ferrin	iron(III)-o-phenanthroline complex
ferroin	iron(II)-o-phenanthroline complex
Fe - S	iron - sulphur protein
FMN	flavin mononucleotide
Fp	flavoprotein
g	electronic g-factor
H	magnetic field (gauss)
h	Planck's constant (6.626×10^{-34} J s)
Hz	Hertz (cycles s ⁻¹)
I	ionic strength (moles litre ⁻¹)
I	intensity
K	equilibrium constant
k	rate constant
k_0	zero-order rate constant (mol l ⁻¹ min ⁻¹)
k_1	first-order rate constant (min ⁻¹)
l	path length (cm)
LL	lysolecithin
ln	natural logarithm (base e)

SYMBOLS AND ABBREVIATIONS (cont'd.)

<i>log</i>	logarithm (base 10)
LUV	large unilamellar vesicle
M	molar
M_I	nuclear spin quantum number
<i>m</i>	electronic mass
m_s	electronic spin quantum number
<i>M. phlei</i>	Mycobacterium phlei
MV	methyl viologen
MW	molecular weight
<i>n</i>	number of moles
	non-bonding molecular orbital
	normal
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
<i>N. crassa</i>	Neurospora crassa
nig	nigericin
NMR	nuclear magnetic resonance
<i>o</i>	ortho-
	oxidised
osM	osmolar
P_i	inorganic phosphate
PA	phosphatidic acid
PC	phosphatidyl choline
PCB ⁻	phenyldicarbundecaborane
PE	phosphatidyl ethanolamine

SYMBOLS AND ABBREVIATIONS (cont'd.)

PF	proflavine
PI	phosphatidyl inositol
pI	isoelectric pH
PMS	N-methylphenazinium methyl sulphate
ppm	parts per million
PS	phosphatidyl serine
PVC	polyvinyl chloride
Q	coenzyme Q
QH ₂	reduced coenzyme Q
R	gas constant (8.314 J K ⁻¹ mol ⁻¹)
R	resistance (ohms)
R _m	membrane resistance (ohms)
r	reduced
rpm	revolutions per minute
T	absolute temperature
t	time (minutes)
t _{1/2}	half-life (minutes)
TLC	thin layer chromatography
TLM	thin lipid membrane
TMPD	tetramethyl- <i>p</i> -phenylenediamine
TPB ⁻	tetraphenylboride
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volts
v	volume (cm ³)
val	valinomycin
Vit K	vitamin K
Vit KH ₂	dihydrovitamin K

SYMBOLS AND ABBREVIATIONS (cont'd.)

v/v	volume-to-volume ratio
z_i	valency of ion i
β	electronic Bohr magneton ($9.274 \times 10^{-24} \text{ J T}^{-1}$)
δ	diffusion layer thickness (cm)
ϵ	dielectric constant
ϵ_λ	extinction coefficient at wavelength λ ($\text{l mol}^{-1} \text{ cm}^{-1}$)
ϵ_{max}	extinction coefficient at absorption maximum ($\text{l mol}^{-1} \text{ cm}^{-1}$)
λ	wavelength (nanometers)
λ_{max}	wavelength of maximum absorption (nanometers)
μ	magnetic moment
ν	frequency (Hertz)
ν_X	stoichiometric coefficient of species X
$\xi(\sqrt{I})$	some function of \sqrt{I}
π	molecular π -bonding orbital
π^*	molecular π^* -antibonding orbital
Σ	summation sign
τ	time constant (s)

Chapter One

INTRODUCTION

The importance of membrane-associated electron transfer processes in biology has been realised for many years, but their detailed understanding has been hindered by the almost overwhelming complexity of living systems. The present research was undertaken with the intention of constructing and studying simple model systems as a means of elucidating some of these processes. The overall objective of this work was to create chemically well defined redox membranes, each of which would be capable of mediating a process of continuous electron transfer from an aqueous reductant on one side to an aqueous oxidant on the other. The transport of electrons between the two otherwise separate aqueous phases would be effected by membrane-bound reversible redox-active carrier molecules. In this context a 'membrane' may be defined simply as an intervening phase which acts as a barrier to the free diffusional exchange of molecular and ionic species between the two solutions which it separates.

1-1 EVOLUTION OF ARTIFICIAL ELECTRON TRANSFER MEMBRANES

An electron transfer membrane as defined above may be either a solid or a water-immiscible liquid. The bulk of published work to date on membrane-related phenomena has been concerned with the transport of water, ions and non-electrolytes across the membrane barrier^(1,2,3). However in recent years some synthetic redox membranes have also been investigated⁽⁴⁾. These are formed from redox polymers, which are solid, insoluble, non-porous, high molecular weight substances possessing the ability to reversibly exchange electrons with oxidising and reducing agents in the aqueous medium⁽⁵⁾. The most frequently incorporated redox active group is the quinone/quinol moiety. In the presence of, for example, an aqueous reductant, the initially reduced interfacial layer interacts with the adjacent sublayer of oxidised polymer to generate two layers of semiquinone. The surface layer is then further reduced, and reduction proceeds through the membrane, converting it to the quinol form until the electrons are passed finally to an aqueous oxidant on the opposite side. This process, which is dependent on individual redox active groups, bound covalently to the rigid polymer matrix, transferring electrons to their neighbours, is extremely slow due to the lack of mobility of the active sites, and mainly for this reason redox polymer membranes have not found widespread application. Attention was therefore turned towards liquid redox membranes, in which an electron carrier species would be free to diffuse across the membrane, thus permitting much faster mediation of the reaction. At the outset of this research, however, no such membrane, capable of sustaining a continuous electron transfer process, had been reported.

Although much less investigated than solid membranes, some work has been done on the ion exchange properties of synthetic liquid membranes, and it is useful to consider these membranes briefly here since many of their characteristics should be shared by liquid redox membranes. Sollner⁽⁶⁾ has identified two distinct types of liquid membranes: 'Nernst-Riesenfeld' or 'solvent type membranes', and 'Haber-Beutner' or 'liquid ion exchanger membranes'. Membranes of the Nernst-Riesenfeld type were the first liquid membranes studied⁽⁷⁾. These are composed of organic liquids (generally hydroxylic) in which aqueous electrolytes are 'dissolved' in a highly dissociated form, due to the fact that water itself is rather soluble in the hydrophilic solvents used. Liquid ion exchanger membranes have been developed more recently, but the original ideas came from work done by Haber⁽⁸⁾ and Beutner⁽⁹⁾ at the beginning of this century. Basically they consist of solutions, in organic solvents, (preferably of minimal water solubility) of large organic molecules which have ionogenic groups but are very sparingly soluble in aqueous electrolytes⁽⁶⁾. The ion exchange compounds normally used have strong acidic or basic groups attached to long hydrocarbon chains, and the solvents are typically benzene derivatives or higher alcohols. With an 'ideal' Haber-Beutner membrane - that is, one in which both solvent and exchanger are completely confined to the membrane phase - the only possible mechanism for transport involves association of an ion in the aqueous phase with an exchanger molecule of opposite charge at one membrane/aqueous interface, followed by diffusion of the ion-carrier complex across the membrane, and finally release of the ion at the opposite interface. This mode of action is precisely analogous to that required for an

ideal liquid redox membrane as envisaged in the present research, except that electrons and protons are carried rather than anions or metal cations.

Figure 1.1 illustrates the type of liquid redox membrane developed here. The success of such a membrane, as judged by its ability to mediate continuous electron transfer from the reductant to the oxidant without contaminating either aqueous phase, depended on the selection of a suitable solvent and carrier molecule. The main property required of the solvent, apart from an ability to solvate the carrier species, was therefore water immiscibility. In this respect, any of the solvents used in 'ideal' Haber-Beutner membranes might have been chosen. However the normal alkanes from hexane upwards were found to be generally suitable solvents: the solubility of water in these is very low, being in the range 40-150 ppm (by weight) at 25°C^(10,11). The choice of carrier molecule was somewhat restricted by the requirement for a continuous electron transfer process. Redox carriers may be grouped into two types according to whether the molecule changes or maintains its overall electrical charge during interconversions between the reduced and oxidised states. A carrier of the former type, by transporting uncompensated charges across the membrane, will generate a transmembrane electrical potential gradient: electron transfer in this case is said to be electrogenic. The membrane potential thus created is of appropriate sign to oppose further electron flow, so that a continuous electron transfer process would be impossible under these conditions. The barrier may however be removed by adding to the membrane phase a second carrier molecule which allows either the transport of cations in the same direction as the electron flow

AQUEOUS

MEMBRANE

AQUEOUS

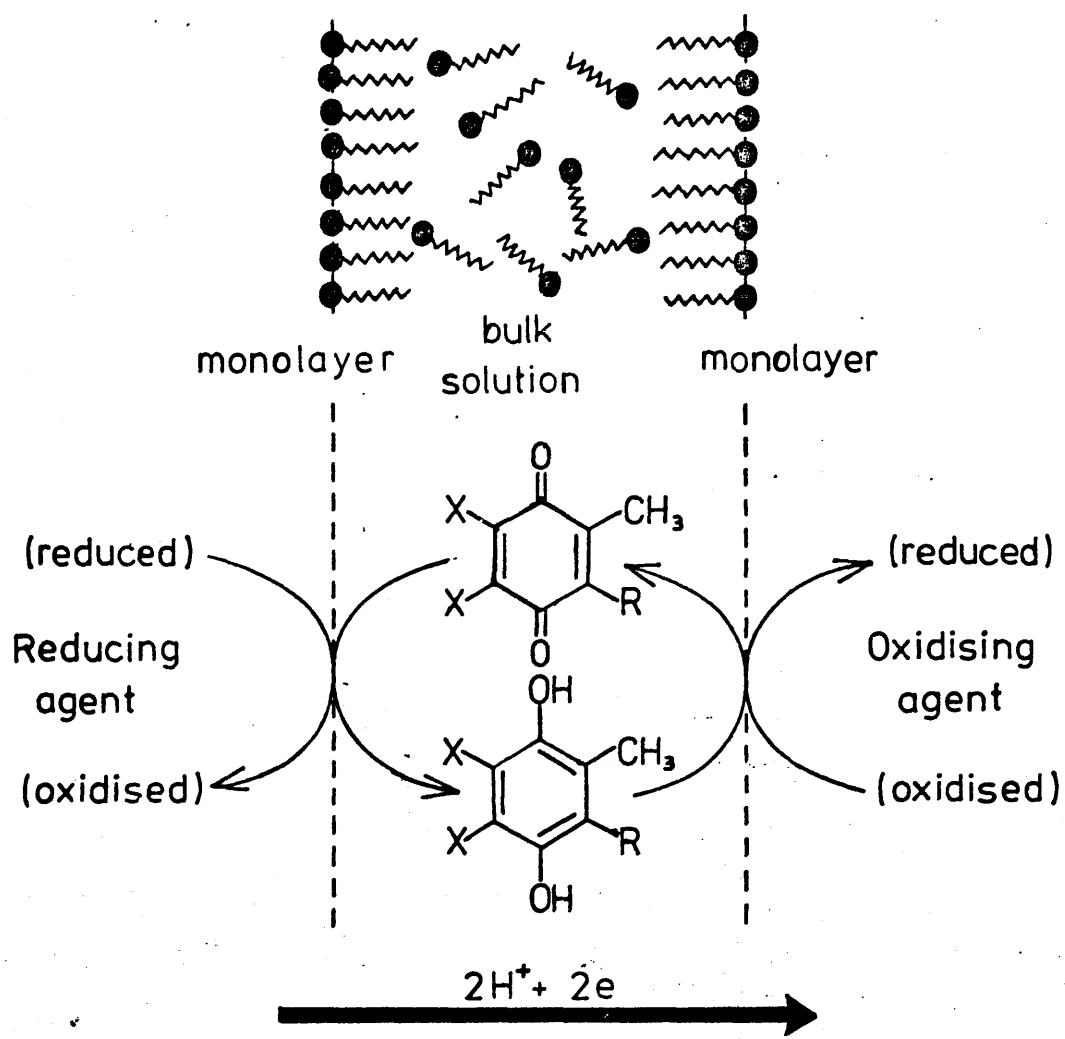


Figure 1.1 General representation of the system used to study liquid membrane mediated redox processes. The electron carrier is a membrane bound quinone. In each cycle two electrons and two protons are transported across the membrane in the direction shown.

(symport), or the transport of anions in the opposite direction (antiport). Such ionophorous compounds have been employed in various investigations on biological and model membranes: certain antibiotics, such as valinomycin, nigericin and nonactin, which are cyclic polypeptides or crown ethers, selectively induce cation permeability in bilayer lipid membranes⁽¹²⁾. For the purposes of the present work, however, an electron carrier capable of maintaining continuous (and therefore non-electrogenic) electron transfer in the absence of any additional ionophorous compound was required. It was therefore necessary that the carrier did not undergo any change in its overall charge on reduction or reoxidation, but transported an equivalent number of cations and electrons, perhaps by an ion pairing mechanism similar to that operating in the liquid ion exchanger membranes discussed above.

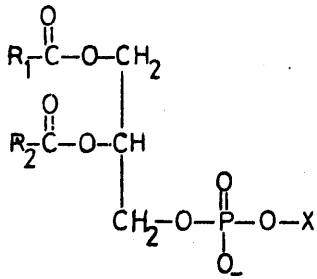
The quinone/quinol functional group, common in the redox polymers mentioned earlier, was an obvious candidate as a carrier species, since, on reduction, the quinone gains two electrons and two protons to form the corresponding dihydroquinone, or quinol. Quinone-type mediators therefore function simultaneously as carriers of both electrons and protons, with each redox cycle resulting in the translocation of two electrons and two protons across the membrane (Figure 1.1). The reversibility of reduction, which is a general property of the quinone functional group, also made this type of molecule most appropriate for incorporation into the redox membranes developed here. The other prerequisite, namely a high partition coefficient between the hydrocarbon and aqueous phases, directed attention towards quinones with long hydrophobic side-chains. Thus a natural logic led to the

conclusion that quinone-type molecules with long-chain hydrocarbon substituents would be most suitable in the role of electron carriers in this research. This conclusion was strengthened by the observation that several naturally occurring quinones (Section 1-4) possess just these required features (Figure 1.8), and it was from these that the carrier molecules were chosen.

1-2 BIOLOGICAL MEMBRANES: GENERAL STRUCTURE AND MODEL SYSTEMS

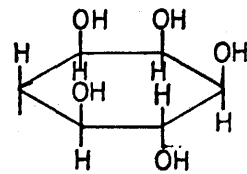
Since the ultimate aim of this research was to produce viable models for biological electron transfer membranes, it is necessary to discuss briefly the salient features of biomembrane structure and function. The first observations concerning the structure of cell membranes were made shortly before the turn of the century, when Overton⁽¹³⁾ established the lipid nature of these membranes. In 1925 Gorter and Grendel⁽¹⁴⁾ introduced the concept of the lipid bilayer, and this was later extended by Danielli and Davson⁽¹⁵⁾ who proposed that the bilayer was covered on both sides by a layer of protein. These ideas were also adopted by Robertson⁽¹⁶⁾ in his 'unit membrane' hypothesis, which was based on electron microscopic and X-ray diffraction data. However more detailed electron micrographs suggested that small globular micelles of lipid might exist in the membrane in equilibrium with the bilayer structure, and that protein too may be present in a similar form⁽¹⁷⁾.

The failure of any of these models to account adequately for all the recent experimental observations on membranes has led to their displacement over the past few years by the 'fluid mosaic' model of Singer and Nicolson⁽¹⁸⁾, which was developed mainly from thermodynamic considerations about membranes and membrane components. The principal constituents of all biological membranes are lipids and proteins. By



Phosphatidyl ethanolamine $X = \text{-CH}_2\text{-CH}_2\text{-NH}_2$

Phosphatidyl choline $X = \text{-CH}_2\text{-CH}_2\text{-N}^+(\text{CH}_3)_3$

Phosphatidyl inositol $X =$


Phosphatidyl serine $X = \text{-CH}_2\text{-CH}(\text{NH}_3^+)\text{-C(=O)O}^-$

Cardiolipin $X = \text{-CH}_2\text{-CH(OH)-CH}_2\text{-O-P-O-CH}_2\text{-CH(O-C(=O)R}_3\text{)-CH}_2\text{-C(=O)R}_4$

Lysolecithin $X = \text{-CH}_2\text{-CH(OH)-CH}_2\text{-O-P-O-CH}_2\text{-CH}_2\text{-N}^+(\text{CH}_3)_3$

Cholesterol

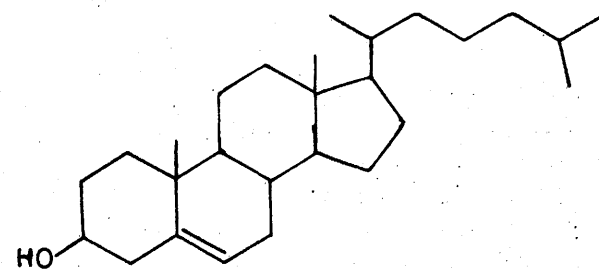


Figure 1.2 General structures of some common lipid constituents of biological membranes.

far the most important class of structural lipid is phospholipid, although in many membranes cholesterol is also present. The general structures of some of the common lipid species are shown in Figure 1.2. All of these are 'amphipathic' molecules; that is, they have both hydrophilic and hydrophobic regions. It is this property which is responsible for the bilayer configuration which they adopt in an aqueous medium⁽¹⁸⁾. In this structure the nonpolar fatty acid chains of the phospholipids are arranged together away from contact with water, thereby maximising hydrophobic interactions, whilst the ionic and zwitterionic head groups are in direct contact with the aqueous phase at the surfaces of the bilayer, thereby maximising hydrophilic interactions. Thus the lipid bilayer represents a thermodynamically stable structure, which would be substantially destabilised if the membrane surfaces were shielded from contact with water by an intervening protein layer, as proposed in the Davson-Danielli-Robertson model. Singer and Nicolson have distinguished two categories of membrane-bound proteins: 'peripheral' proteins (such as the cytochrome c of the inner mitochondrial membrane) are rather loosely attached to the membrane, mainly by electrostatic interactions, and may be removed from it free of lipid by treatment with salt solution of high ionic strength; 'integral' proteins on the other hand are strongly bound to the membrane, mainly by hydrophobic interactions, and cannot normally be removed except under rather drastic conditions, by treatment with detergents, denaturants, or organic solvents. Assuming that only integral proteins are important to the structural integrity of membranes, Singer and Nicolson proposed that these too should orientate themselves in the bilayer so as to maximise both hydrophobic and hydrophilic interactions. This results in the 'mosaic' structure

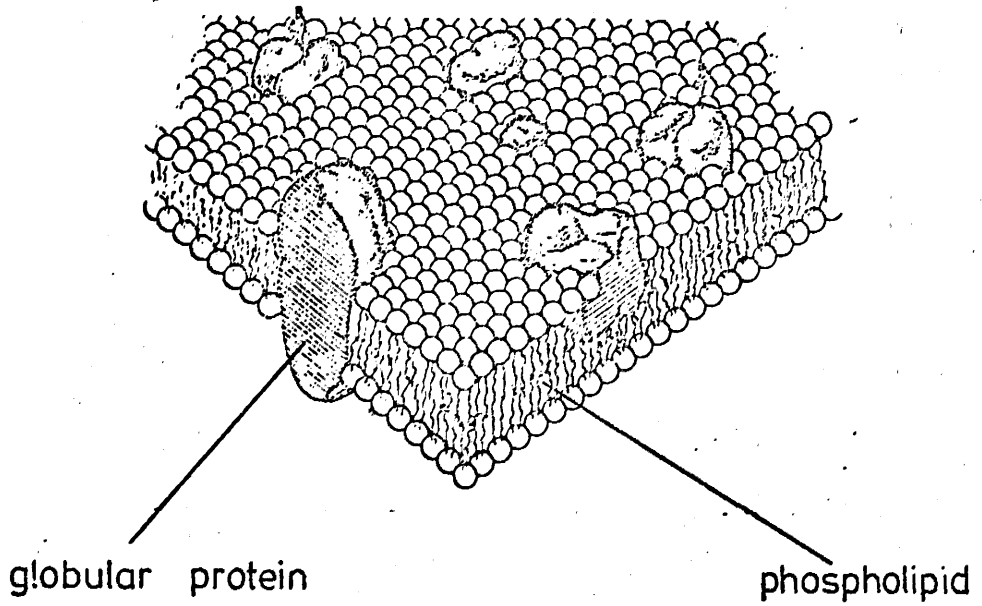
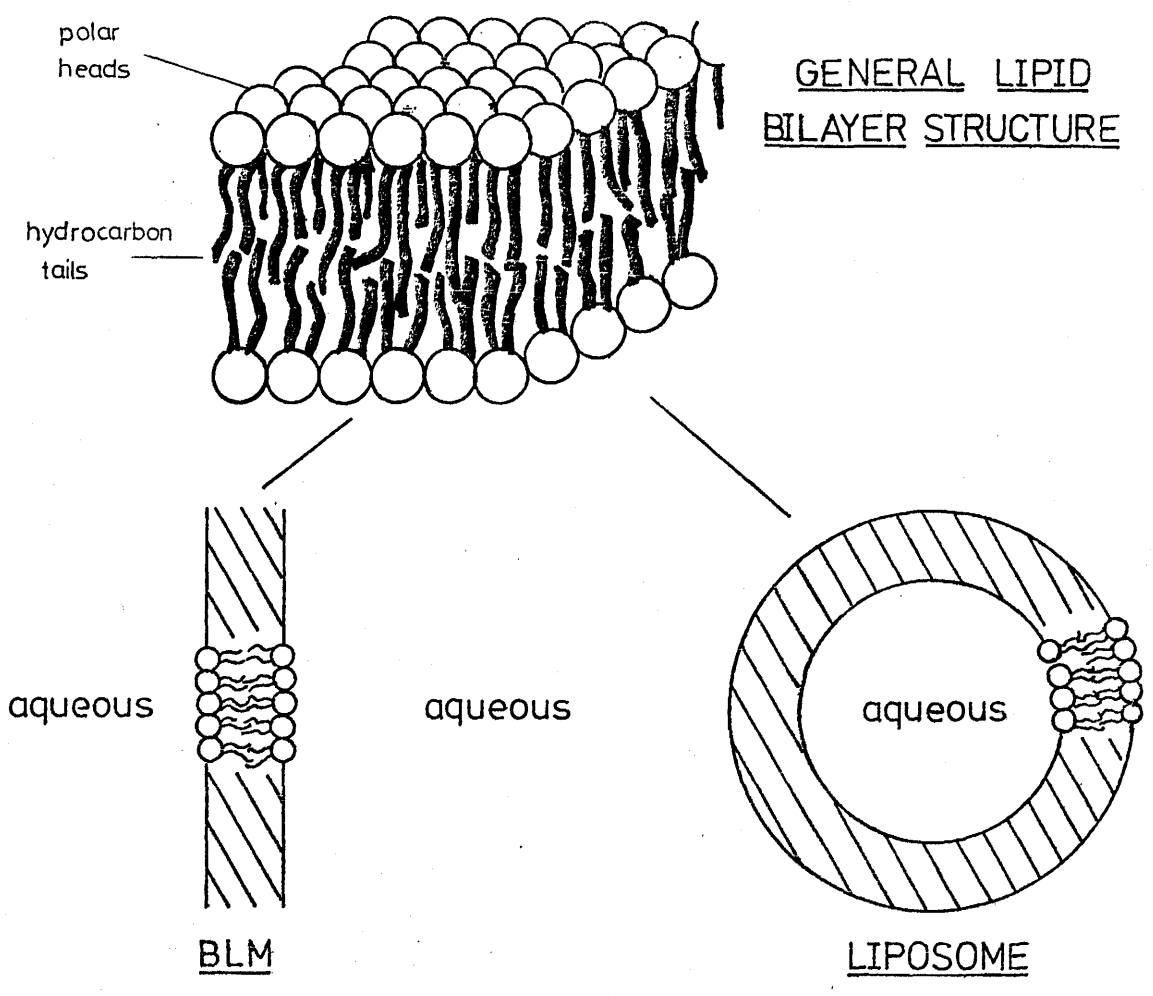


Figure 1.3 Schematic representation of the three dimensional organisation of functional membranes according to the fluid mosaic model.



denotes a lipid molecule, a typical example being :

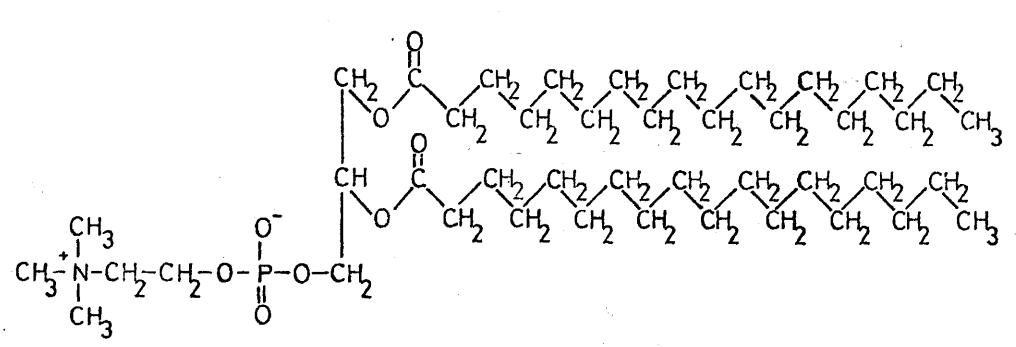


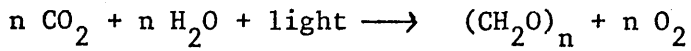
Figure 1.4 Structures of model bilayer lipid membranes. Also shown is the structure of a common phospholipid, distearoyl phosphatidyl choline.

illustrated in Figure 1.3, with some proteins penetrating the bilayer on one side only while others extend from one side of the membrane to the other. The fluidity of the structure was deduced from various pieces of evidence, such as the rate of intermixing of proteins following cell fusion⁽¹⁸⁾.

Over the past fifteen years, further insight into the structure and function of biomembranes has been made possible by the development of two model systems: planar bimolecular lipid membranes (BLM), and liposomes. Both of these possess the basic lipid bilayer structure typical of biomembranes (Figure 1.4), but have the advantage of relatively well defined and controllable chemical compositions. The reported thicknesses of these artificial membranes, as determined by X-ray diffraction, electron microscopy, electrical and optical methods, are in the range 38-130 Å, which is in good agreement with the measured thicknesses of natural membranes^(19,20). These model membranes therefore provide simple but viable systems in which the processes occurring in biomembranes may be simulated.

1-3 ELECTRON TRANSFER IN BIOLOGICAL MEMBRANES

According to a suggestion by Szent-Gyorgyi⁽²¹⁾, the life cycle on this planet can be considered basically as a movement of electrons. The two fundamental processes of life are photosynthesis and respiration, which are carried out, respectively, in chloroplasts and mitochondria. In photosynthesis, the energy of the sun, in the form of photons, promotes an electron from the ground state of a pigment molecule (chlorophyll) to an excited state⁽²²⁾. During the transition back to the ground state, the free energy released by the electron is converted, by an enzyme system located in the thylakoid membrane of the chloroplast, into chemical bond energy. The overall photosynthetic process may be written:

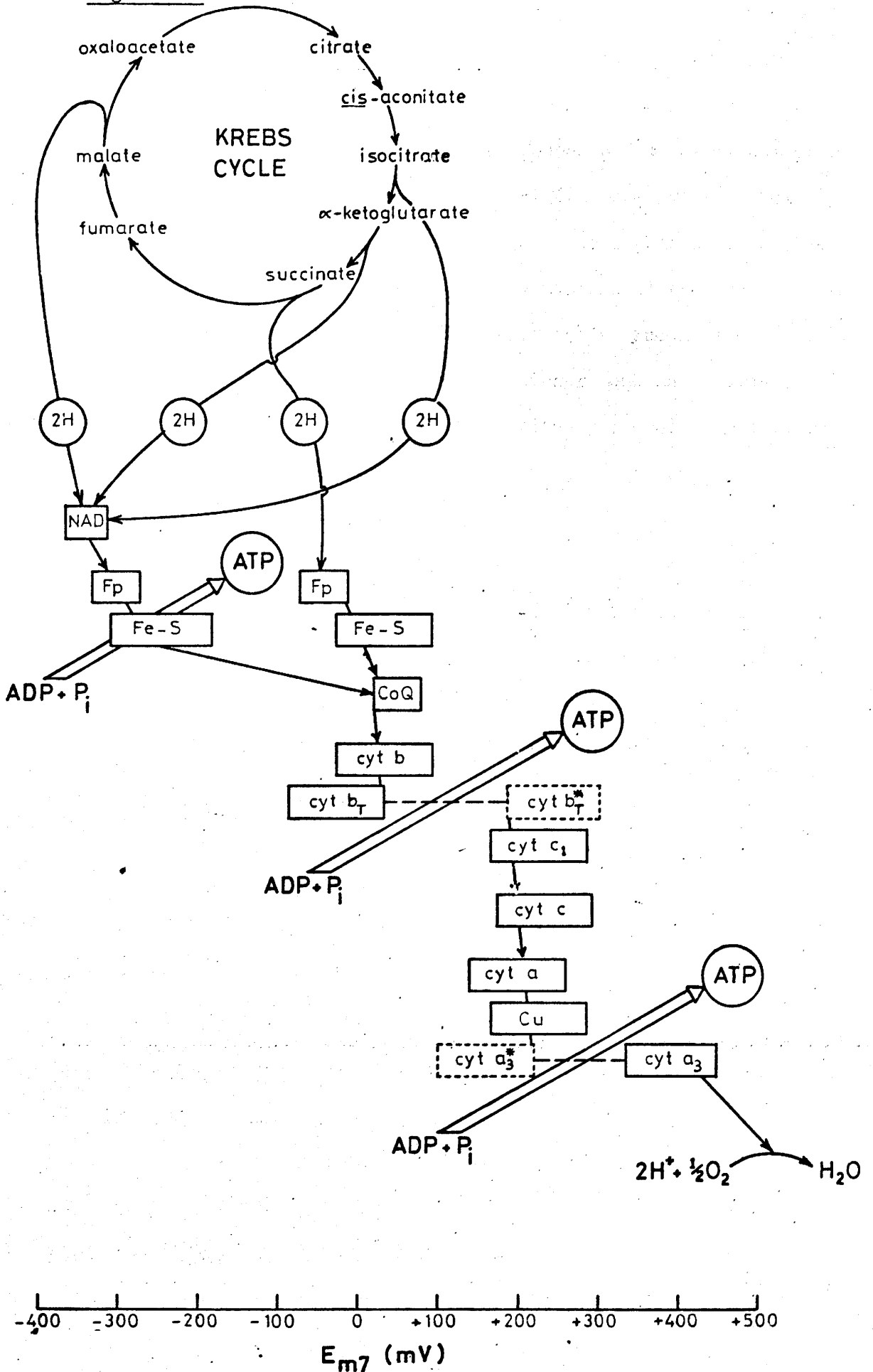


The organic compounds and oxygen produced by photosynthesis in green plants are then utilised by almost all living organisms, especially by those which are themselves incapable of making direct use of the photon energy of the sun. Respiration is the process by which all plant and animal cells derive the energy which they require for normal metabolic activity from the oxidative enzymatic breakdown of these organic molecules. During this process, the 'high-energy' compound adenosine triphosphate (ATP) is synthesised from adenosine diphosphate (ADP) and inorganic phosphate (P_i). The complex nutrients of the cell are first broken down enzymatically into three major classes of molecules - fatty acids, amino acids, and simple carbohydrates. These are then channelled into the Krebs tricarboxylic acid cycle, which is the final common pathway of oxidative catabolism for all fuel molecules in aerobic cells⁽²²⁾. In this cycle acetyl groups are dismembered to yield carbon dioxide and hydrogen atoms, or protons plus electrons. The latter are fed into the respiratory chain (or electron transport chain) which consists of a series of electron carriers of increasing redox potential (Figure 1.5). The ensuing process of electron transfer to molecular oxygen proceeds with a step-wise decline in free energy, which at several points in the chain is conserved by the coupled phosphorylation of ADP. Three molecules of ATP are synthesised for every pair of electrons passing along the respiratory chain.

In eucaryotic cells, the enzymes of the Krebs cycle, together with those associated with electron transport and oxidative phosphorylation, are located within the mitochondrion, which for this reason has been referred to as the 'powerhouse' of the cell⁽²²⁾. A

Figure 1.5 Schematic representation of the mitochondrial respiratory chain, showing the sequence and midpoint potentials of the carriers. Arrows indicate the direction of movement of electrons from the Krebs cycle intermediates along the chain to molecular oxygen. Each component in the chain is represented by a rectangle which is centred on its midpoint potential at pH 7 and extends from the potential at which the component is 9% reduced to that at which it is 91% reduced⁽²⁴⁾. Two values are shown for cytochromes b_T and a_3 , corresponding to different conformations of these proteins⁽²⁴⁾. The three sites of energy coupling to form ATP are also indicated.

Figure 1.5



typical eucaryotic cell contains several hundred mitochondria, each of which appears under the microscope as a granular organelle some $1.5\mu\text{m}$ long and $0.5\mu\text{m}$ in diameter. It is bounded by two membranes which separate the internal solution (the matrix) from the external cell cytoplasm, and which are themselves separated by a thin aqueous layer (the intermembrane space). Numerous invaginations of the inner membrane extend into the matrix (Figure 1.6). These folds, known as cristae, serve to increase the surface area of the inner membrane, which is important for the energy-transducing capacity of the mitochondrion, since it is within this membrane that the respiratory chain components are situated.

The enzymes and coenzymes of electron transfer have been the subject of numerous and detailed biochemical investigations for some considerable time, but there are still many gaps in the knowledge of protein structures and reaction mechanisms. The sequence of the various carriers has however been deduced from their thermodynamic properties, by redox titration methods using suitable mediators and specific enzyme inhibitors^(23,24), and this sequence is shown in Figure 1.5. Most of the carrier species are proteins, of which three main types are involved: namely flavoproteins, which have as their prosthetic group either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), Figure 2.15; nonhaem iron proteins, in which the redox active iron centre is linked to sulphur; and cytochromes, which contain iron-porphyrin prosthetic groups⁽²²⁾. The two other important carriers - nicotinamide adenine dinucleotide (NAD) and coenzyme Q (CoQ) - are classified as coenzymes since they are not covalently bonded to protein. The former is water-soluble and can therefore act as an electron transfer agent between the Krebs cycle intermediates contained

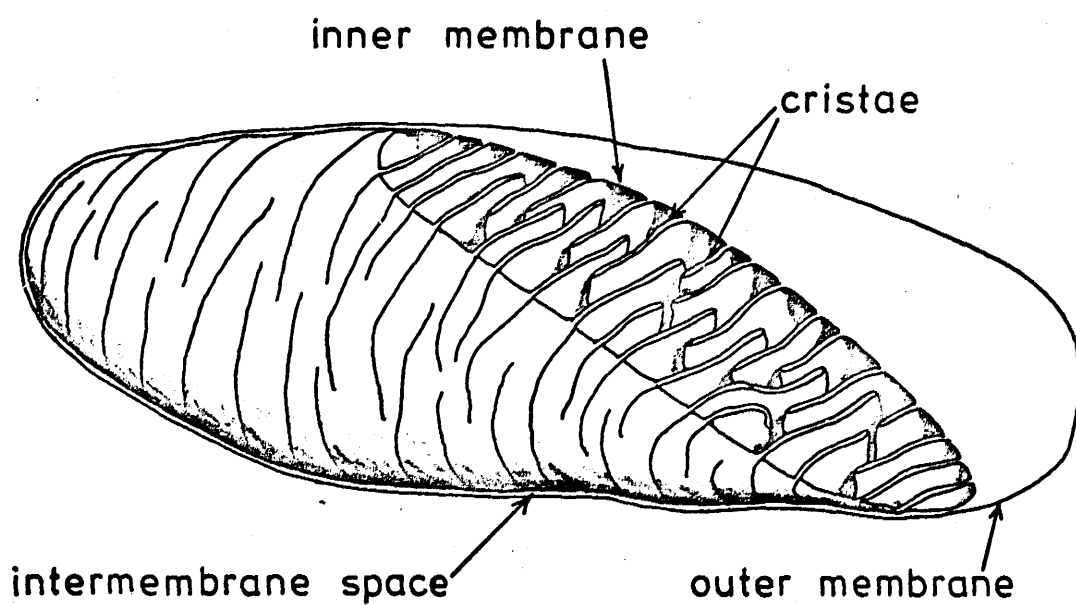


Figure 1.6 Cut-away drawing of a mitochondrion, showing the two membranes and cristae.

in the mitochondrial matrix. The latter is lipid-soluble, which allows it to carry electrons between enzymes within the membrane phase or across the membrane.

Available evidence indicates that the enzymes of the respiratory chain are arranged into supramolecular clusters, called respiratory assemblies, each of which contains a fixed number of molecules of each carrier and is by itself a complete respiratory chain⁽²²⁾. The molar ratios of the electron carriers in such complexes isolated from beef heart mitochondria are given in Table 1. Four lipoprotein aggregates have been isolated: Complex I (NADH - CoQ oxidoreductase) which contains FMN and nonhaem iron⁽²⁵⁾; Complex II (succinate - CoQ oxidoreductase) which contains FAD and nonhaem iron⁽²⁶⁾; Complex III (CoQH₂ - cytochrome c oxidoreductase) which contains cytochromes b and c₁, and nonhaem iron⁽²⁷⁾; and Complex IV (cytochrome oxidase) which contains cytochromes a and a₃, and copper⁽²⁸⁾. All of these complexes contain multiple prosthetic groups and a number of polypeptide subunits. Electron transfer between Complexes I and III, and between Complexes II and III, is effected by coenzyme Q, while that between Complexes III and IV is effected by cytochrome c. Both of these 'mobile' carriers were of particular interest in the present work and are considered in greater detail later (coenzyme Q in Section 1-4 and cytochrome c in Section 3-3.4). The sequence of the electron transfer reactions in the respiratory chain as shown in Figure 1.5 has been confirmed by *in vitro* reconstruction experiments using isolated electron carriers; thus NADH can reduce Complex I but cannot directly reduce cytochromes b, c or a-a₃; similarly, reduced Complex I cannot interact directly with cytochrome a-a₃ but requires the presence of cytochromes b and c⁽²²⁾. Furthermore the present research revealed that NADH does not directly reduce coenzyme Q, and that reduced

TABLE 1.

Molar ratios of electron carriers in beef heart mitochondria⁽²²⁾.

<u>Carrier</u>	<u>Ratio*</u>
cytochrome a + a ₃	1.0
cytochrome c + c ₁	1.5
cytochrome b	1.0
total flavins	~ 1.0
non-haem iron	6
coenzyme Q	8
NAD	12
NADP	2

* Ratios relative to cytochrome a + a₃ = 1.0

coenzyme Q is a poor reductant for cytochrome c in the absence of cytochrome b-c₁ (Sections 2-3.2 and 2-3.4).

The inner mitochondrial membrane has as its basic structure the lipid bilayer, with which the functional proteins are associated by hydrophobic and hydrophilic interactions. However, the protein content of this membrane is somewhat higher than that of most other membranes, amounting to almost 80% by weight of the total protein plus phospholipid⁽²⁹⁾. Investigation of the inner membrane by various physico-chemical techniques, most notably by electron microscopy, has revealed that the transverse distribution of proteins within the membrane is asymmetric, with a much higher particle density on the inner (matrix) than on the outer (intermembrane space) surface^(30,31,32). Moreover, the various electron transfer complexes are themselves held in specific orientations, so that some are confined to one or other of the two sides of the membrane whilst others span the entire structure (Figure 1.7). All of these proteins, with the notable exception of cytochrome c, which is readily removed from the outer surface of the membrane by washing with salt solution of high ionic strength, may be classed as 'integral' proteins since they are substantially buried in the membrane and are strongly associated with its lipid component. This asymmetric distribution of the respiratory chain components is thought to be essential for the energy coupling process (described below). The oxidation of NADH, succinate and cytochrome oxidase, together with the synthesis of ATP, all take place within the matrix of the mitochondrion⁽³²⁾.

Despite its relatively low abundance in the inner membrane, there is ample evidence, mainly from lipid depletion and reconstitution experiments, indicating the necessity of phospholipid in mitochondrial

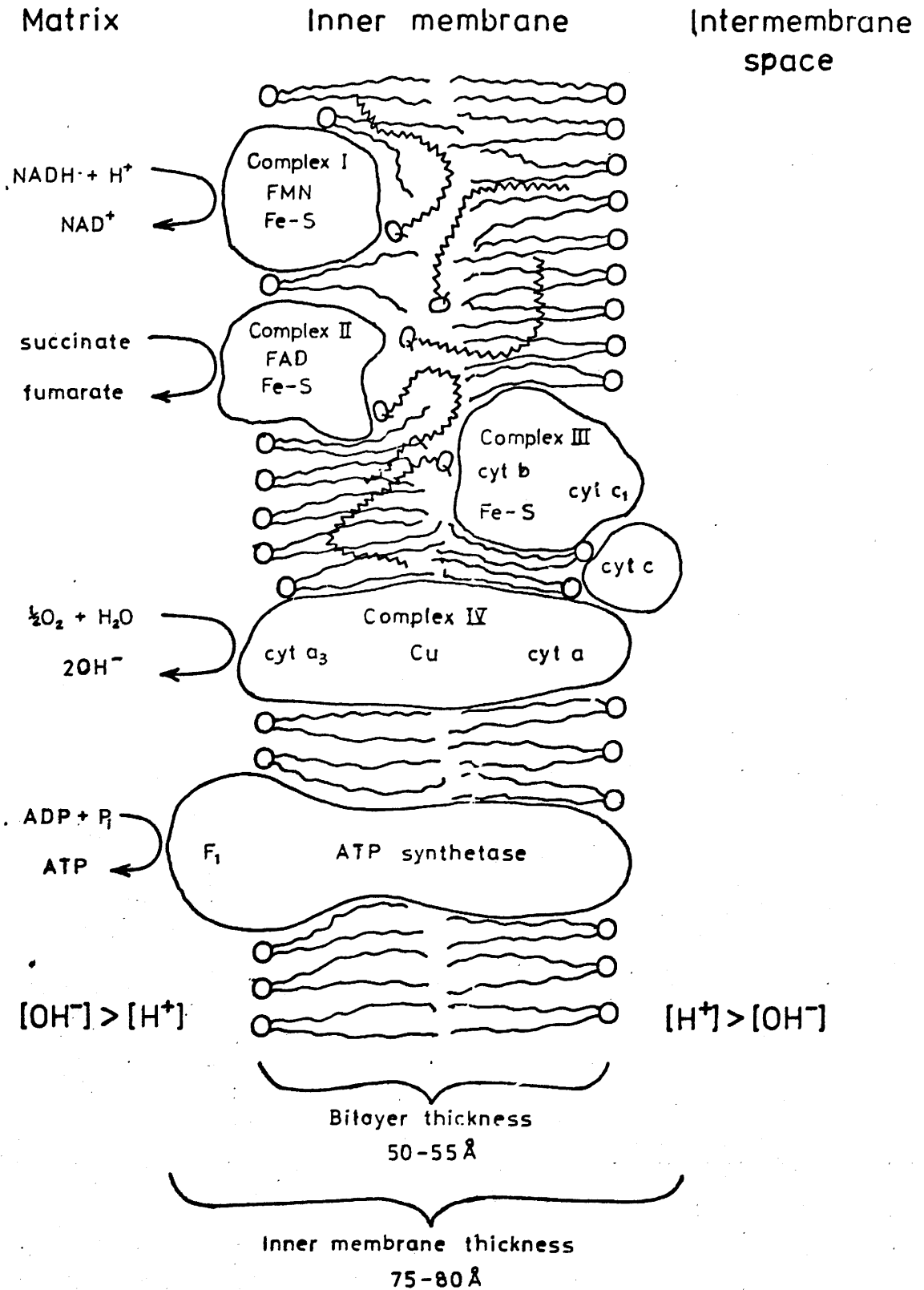


Figure 1.7 The lateral organisation of the respiratory complexes associated with the inner mitochondrial membrane. (Adapted from reference (32).)

structure and function⁽²⁹⁻³²⁾. More than 90% of the total lipid is phospholipid, the balance comprising mainly the neutral lipid coenzyme Q. Cholesterol, which is common in most biological membranes, is present in only trace amounts in the inner mitochondrial membrane. The phospholipid composition of the inner and outer membranes is compared in Table 2. Both contain large amounts of phosphatidyl choline and phosphatidyl ethanolamine, but a striking difference is apparent in their cardiolipin and phosphatidyl inositol contents, the former being concentrated in the inner membrane and the latter in the outer membrane. The special role of cardiolipin in binding with cytochrome c was studied in some detail in the model system to be described in Chapter 3. Another characteristic of mitochondrial phospholipids is the unusually high proportion of unsaturated fatty acid residues which they contain⁽²⁹⁾. It would appear that the low melting points associated with unsaturation are important for the preservation of a fluid membrane structure in which the reactions of electron transport and oxidative phosphorylation may take place^(30,31).

Many mechanisms have been proposed for the energy transduction process by which ATP synthesis is coupled to electron transfer in mitochondrial and thylakoid membranes. Three distinct types of mechanism have emerged, involving chemical^(22,23), conformational^(31,32,33) and chemiosmotic^(34,35) coupling schemes. Of these, Mitchell's chemiosmotic hypothesis has gained the most widespread acceptance^(36,37,38). According to this theory, the electron transfer molecules are arranged in loops across the membrane in such a manner that the continuous passage of electrons along the chain causes a vectorial translation of protons, so that protons are pumped out of the mitochondrion during

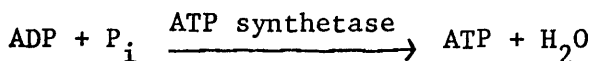
TABLE 2.

Phospholipid composition of inner and outer mitochondrial membranes⁽²⁹⁾.

<u>Phospholipid</u>	<u>Inner Membrane</u>	<u>Outer Membrane</u>
total phospholipid*	21.4%	45.1%
phosphatidyl choline	44.5%	55.2%
phosphatidyl ethanolamine	27.7%	25.3%
cardiolipin	21.5%	3.2%
phosphatidyl inositol	4.2%	13.5%
phosphatidyl serine	0.0%	0.0%
unidentified	2.2%	2.5%

* % phospholipid relative to protein + phospholipid

oxidative phosphorylation (Figure 1.7). The resulting pH gradient across the inner membrane provides the source of electrochemical free energy which drives the ATP synthetase:



In addition to its application to mitochondrial oxidative phosphorylation, the chemiosmotic hypothesis has been used to explain similar energy conservation and coupling processes occurring in bacteria and chloroplasts⁽³⁵⁻³⁸⁾. However the whole question of energy coupling in biological systems has been the subject of continuing debate over many years, and is yet to be satisfactorily resolved^(36,39,40).

1-4 QUINONES IN ELECTRON TRANSFER

Inspection of Table 1 and Figures 1.5 and 1.7 reveals an interesting point; namely the fact that coenzyme Q, the only non-protein constituent of the respiratory chain, is present in great abundance in the inner mitochondrial membrane and appears to play a central role in the electron transfer process. It will also be shown in the following chapters that this molecule possesses all the attributes required of an 'ideal' electron carrier as defined in Section 1-1. These considerations led to the adoption of coenzyme Q₁₀, and of the related naphthoquinone vitamin K₁, as electron carriers in the model membrane systems to be described in this work. It is therefore relevant at this point to outline the proposed roles of such molecules in real biological systems.

Coenzyme Q was first characterised in 1957 by two groups working independently: Morton and co-workers⁽⁴¹⁾ named the compound which they isolated from mitochondrial preparations 'ubiquinone' because they found

it to be ubiquitous in all the tissues that they examined; Crane *et al*⁽⁴²⁾ used the name 'coenzyme Q', where 'Q' stands for 'quinone'. The latter designation will be used routinely throughout this thesis. The coenzyme Q family of compounds are a series of 2,3-dimethoxy-5-methyl benzoquinones with an unsaturated isoprenoid side-chain in the 6 position consisting of from six to ten isoprenoid units (Figure 1.8 (a)). The most common type in higher animals and plants is coenzyme Q₁₀, while the shorter side-chain homologues are found in many lower organisms, as for example coenzyme Q₆ in yeast⁽²⁹⁾. Coenzyme Q is now well established as a functional member of the mitochondrial respiratory chain, fulfilling all the criteria necessary for its classification as an electron carrier⁽²⁹⁾. The most convincing experiments in this respect have involved extraction of the quinone from inner membrane preparations with concomitant loss of electron transport activity, followed by restoration of activity on reincorporation of coenzyme Q^(29,30,43,44). Studies of the steady state redox conditions of the respiratory chain components in the presence and absence of various inhibitors, which block electron transport at specific points in the chain, indicate that coenzyme Q functions as an electron carrier between the flavoprotein dehydrogenases of complexes I and II and the cytochromes b of Complex III (Figures 1.5 and 1.7)^(29,43-47). This involves movement of the reduced quinol form through the lipid membrane phase from the matrix side to the side of the intermembrane space. This apparent mobility of coenzyme Q, which is perhaps surprising considering the large size of the molecule (coenzyme Q₁₀ has a side-chain containing fifty carbon atoms), allows it to act as an inter-chain electron transfer agent, mediating between different fixed respiratory assemblies⁽⁴⁸⁾.

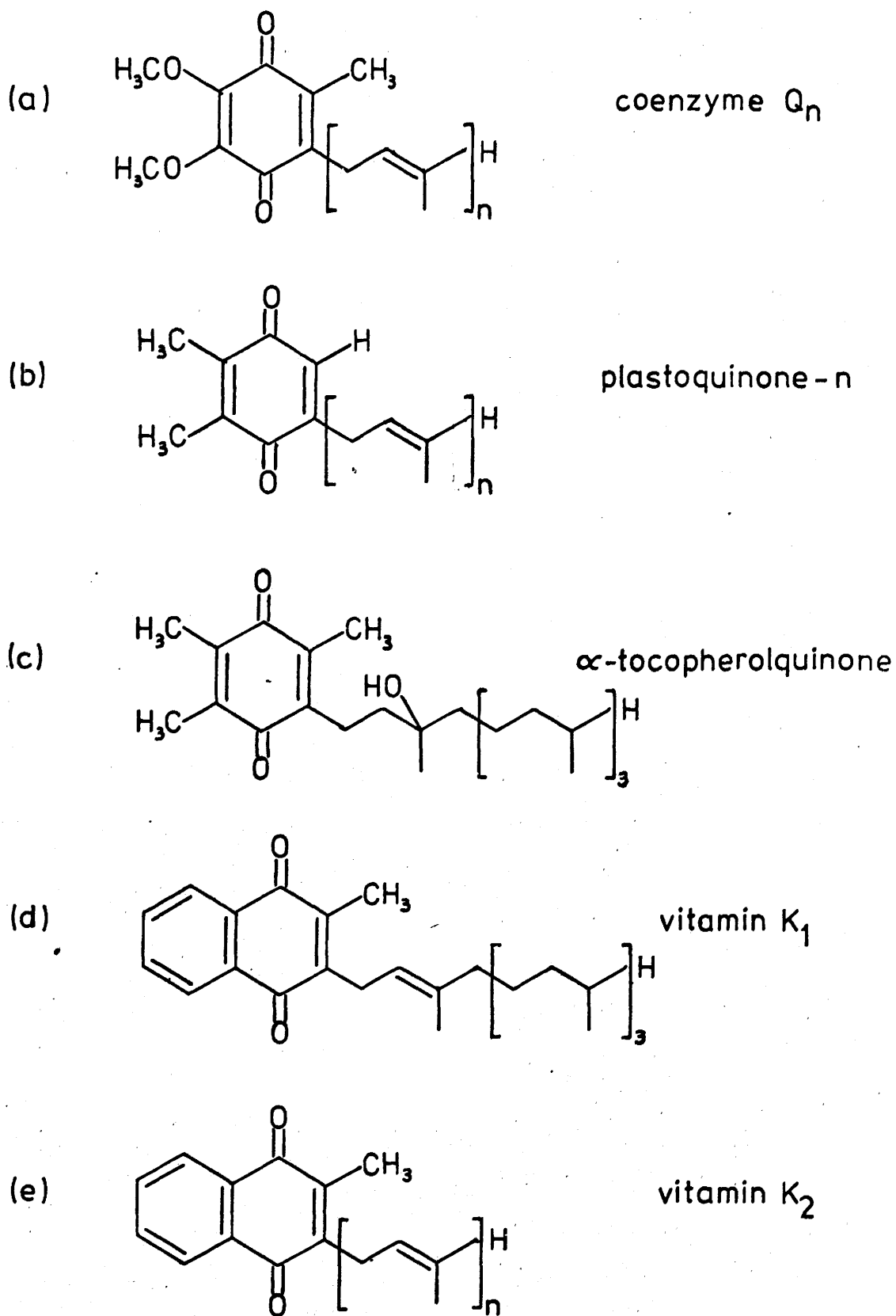
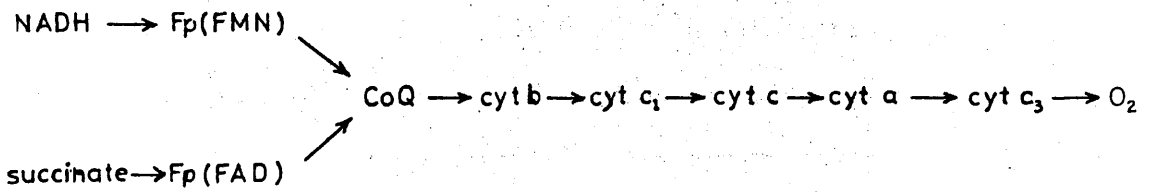


Figure 1.8 Quinones involved in biological electron transfer processes.

Investigations into the kinetics of the redox reactions of coenzyme Q suggest that it operates according to saturation kinetics, with only a small portion of the total quinone 'pool' being involved in electron transfer at any one time^(29,43,45). It is possible that the large excess of coenzyme Q over stoichiometric requirements may serve to maintain a constant supply of the quinol in the membrane, to act as an antioxidant, preventing the peroxidation of labile haem compounds and unsaturated fatty acids⁽²⁹⁾. The location of the quinone/quinol headgroup within the membrane is also of interest. Results from a study of interactions between coenzyme Q and a fluorescent probe molecule incorporated into the membrane suggest that the headgroup is situated roughly 14 Å from the aqueous interface⁽⁴⁹⁾. However, measurement of the midpoint potential of coenzyme Q in intact mitochondrial particles (Section 2-1.1) indicates that the redox active moiety is in a more aqueous environment than this would permit, and it seems more likely that it is in fact located at the membrane surface.

In addition to coenzyme Q, which is found in the mitochondria of plant and animal cells, and in the photosynthetic electron transfer systems of certain bacteria, several other naturally occurring isoprenoid quinones are functional in biological redox processes^(29,50,51). These may be divided into three generic classes: the plastoquinones, the tocopherolquinones, and the vitamins K (Figure 1.8). The plastoquinones (Figure 1.8 (b)), which are structurally closely related to the coenzymes Q, are thought to play a very similar role to their mitochondrial counterparts, in the photosynthetic electron transfer system located in the thylakoid membrane of chloroplasts⁽⁵²⁾. The tocopherolquinones (Figure 1.8 (c)) are also restricted to photosynthetic electron transfer, but they are found in bacteria as well as in chloroplasts⁽⁵³⁾.

(a)



(b)

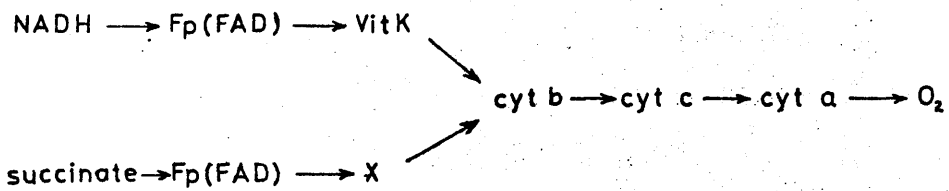


Figure 1.9 Electron transport sequences (a) in beef heart mitochondria, showing the proposed site of coenzyme Q, and (b) in *M. phlei*, showing the proposed site of vitamin K. (X is a factor destroyed by ultraviolet light.) (29,55)

The vitamins K, which are isoprenoid naphthoquinones, are involved in photosynthesis, in bacterial electron transport, and possibly in the electron transport systems of certain mitochondria⁽⁵⁴⁾. Vitamin K₁ (phylloquinone) (Figure 1.8 (d)) is common in the chloroplasts of green plants, whilst vitamin K₂ (menaquinone) (Figure 1.8 (e)) is widely distributed in microorganisms. A proposed scheme^(29,55) for electron transfer in *Mycobacterium phlei*, involving vitamin K₂, is given in Figure 1.9 (b): the electron transport system of beef heart mitochondria is also shown (Figure 1.9 (a)) to indicate the similarity between the roles of vitamin K and coenzyme Q in their respective systems. Since vitamin K₁ could act as a substitute for the endogenous vitamin in *M. phlei*⁽⁵⁶⁾, the scheme shown in Figure 1.9 (b) may be considered as applying directly to vitamin K₁.

1-5 ASSEMBLY OF MODEL REDOX MEMBRANE SYSTEMS

The principal objective of the present research was the creation of chemically well-defined liquid redox membranes across which the non-electrogenic transfer of electrons would be mediated by a membrane-bound carrier (Figure 1.1). It was also intended to design such systems so that they could be used as simple models for some of the biological membranes described above. From their structures, shown in Figure 1.8, it can be seen that the naturally occurring quinones discussed in Section 1-4 - the coenzymes Q, the plastoquinones, the tocopherolquinones and the vitamins K - possess all the attributes required of reversible electron carriers in these model membrane systems as described in Section 1-1. Of these, coenzyme Q₁₀ and vitamin K₁ were commercially available, so these two molecules were selected for use throughout this work. The 'ideality' of these carriers will be considered in greater detail in Chapter 2.

The criteria which must be met by 'ideal' carriers and solvents have been discussed (Section 1-1). It is appropriate at this point also to consider those criteria by which the 'ideality' of aqueous reducing and oxidising agents may be judged:

1. The reductant must be capable of reducing the quinone carrier cleanly to the corresponding quinol in a biphasic type of reaction, with no side reactions. Similarly the oxidant must be able to re-oxidise the quinol cleanly to the quinone.
2. Both of these reactions must proceed at measurable rates. As will be apparent later (Chapters 2 and 3) it is insufficient to consider only the relative redox potentials of the reductant, carrier and oxidant: many thermodynamically 'favourable' reactions do not proceed spontaneously due to unfavourable kinetic factors. Such reactions may however occur in the presence of a suitable enzyme or other mediator.
3. Both reductant and oxidant must be intrinsically stable in aqueous buffer at pH 7. (The physiological pH was chosen for routine experiments.)
4. Both reductant and oxidant must be water-soluble but membrane-insoluble in all their equilibrium forms (oxidised, reduced, protonated or dissociated). This will ensure that the reducing agent and substrate are kept completely separate by the impenetrable membrane barrier, so that reaction between them can only occur through the mediation of the membrane-bound carrier. It should therefore be possible to recover the reduced substrate free from contamination by the reductant at the end of the reaction.
5. In order to allow the reaction to be followed spectrophotometrically, changes in the redox states of the reactants must be accompanied by corresponding changes in their absorption spectra. A

fairly high extinction coefficient in at least one of the redox forms will also be necessary for accurate spectrophotometric determinations. The preliminary work in this research, described in Chapters 2 and 3, was concerned mainly with the search for suitable reducing and oxidising agents, their 'ideality' relative to the above criteria being tested in bulk equilibration studies.

The general form and mode of action of a bulk liquid redox membrane is indicated in Figure 1.1. The membrane may be considered as being composed of three regions: two interfacial monolayers at which the amphipathic carrier molecules are assumed to be orientated as shown, with their hydrophilic, redox-active headgroups protruding into the aqueous phases while their hydrophobic side-chains extend into the membrane interior; and an intervening bulk solution phase in which the carrier molecules are dissolved and freely mobile. Carrier molecules in the interfacial monolayers will be in a state of dynamic equilibrium with those in the bulk solution, and in thermodynamic (redox) equilibrium with the aqueous phases with which they are in contact. The overall effect is that quinone molecules on the reductant side become reduced, leave the monolayer, and diffuse through the bulk solution to the opposite interface, where the quinol gives up its two electrons and two protons to the oxidising agent. The reoxidised carrier then diffuses back to the reductant side, completing the cycle in readiness for a second journey. The electron transfer process will continue until a state of thermodynamic equilibrium among the three phases is established. By keeping the reductant in large excess, it should be possible eventually to obtain almost 100% reduction of the substrate by this method. Obviously mobility of the carrier within the membrane

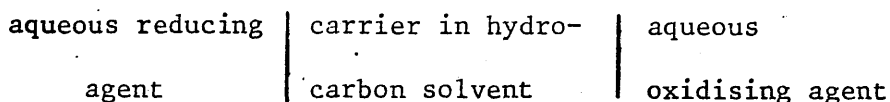
continuum is a prerequisite for this type of carrier-mediated electron transfer process. This should present no difficulties in the case of these bulk membranes, since molecules like coenzyme Q and vitamin K should be able to diffuse quite freely in a hexane solvent. If, however, the amount of bulk solution is decreased until the two interfacial monolayers are brought into contact, it is no longer certain that mobility of the carrier within such an ultrathin membrane will be retained. This is particularly doubtful when the resulting bilayer membrane is composed primarily of natural phospholipids, which have much shorter side-chains (normally sixteen to twenty carbon atoms) than coenzyme Q (with fifty carbon atoms). It might be expected that the mobility of such a large molecule would be severely restricted under these conditions. Despite this it has been proposed that coenzyme Q acts as a mobile electron carrier within the bilayer structure of the inner mitochondrial membrane (Figure 1.7). In order to test the validity of this assumption, it was necessary to turn from the bulk liquid redox membranes of Chapter 3 to develop more appropriate models for biological membranes. Both planar bilayer lipid membranes (BLM) and spherical bilayer lipid membranes (liposomes) were studied as alternative models, and these are described in detail in Chapters 4 and 5 respectively.

Chapter Two

BIPHASIC REDOX REACTIONS OF COENZYME Q₁₀ AND VITAMIN K₁

2-1 INTRODUCTION

As indicated in Chapter 1, the naturally occurring isoprenoid quinones coenzyme Q₁₀ and vitamin K₁ were immediately attractive for use as electron carriers in this research. The work to be described in the present Chapter was directed towards assessing their suitability to this role in more precise practical terms, as a first step towards generating redox membrane systems of the type



Clearly the aqueous reagents had to be carefully tailored to suit the thermodynamic and mechanistic requirements of the particular carrier incorporated in the membrane. This led to a division of the experimental effort into two distinct yet closely related halves, one concerning the redox reactions of coenzyme Q₁₀, and the other the redox reactions of vitamin K₁. The author was involved initially in investigations on the coenzyme Q₁₀ systems, while simultaneous investigations were carried out on the vitamin K₁ systems by a co-worker⁽⁵⁷⁾. For this reason, attention will be focussed on coenzyme Q when discussing experimental procedures, but results from the parallel vitamin K work will be included, both for the sake of comparison here, and as a necessary background for the work to be described in subsequent Chapters.

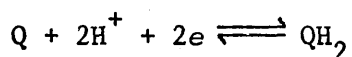
The problem of assembling functional redox membrane systems was tackled in its two constituent parts: Firstly, a search was made for aqueous reductants capable of reducing the quinone form of the carrier,

in a biphasic reaction, to the quinol. Secondly, aqueous substrates were sought which were reducible by the quinol, again in a biphasic type of reaction. It was also necessary to ensure that both of these steps were reversible, so that a continuous electron transfer process could be established across a membrane containing the carrier, and that the aqueous reagents conformed with the requirements stipulated in Section 1-5. The redox states of both carriers used were readily determined by inspection of their ultraviolet absorption spectra, which show strong peaks characteristic of their quinone and quinol chromophores. However this technique was by itself insufficient to establish the reversibility of carrier reduction since the UV spectra are relatively insensitive to changes in the isoprenoid side chain, whose double bonds, although potential reaction sites, are not in conjugation with the ring system. Proton nuclear magnetic resonance spectroscopy (NMR) was found to be a suitable technique for probing this part of the molecule for unwanted side reactions. Electron paramagnetic resonance spectroscopy (EPR) was utilised to elucidate possible mechanisms for the interfacial electron transfer between the membrane-bound carriers and certain of the aqueous reagents.

2-1.1 Midpoint potentials of coenzyme Q₁₀ and vitamin K₁

Before choosing aqueous reducing and oxidising agents for use in the coenzyme Q and vitamin K systems, it was desirable to have some estimate of the midpoint redox potentials of the two quinones, so that time would not be wasted in attempting reactions which were not thermodynamically feasible. Various values have been reported in the literature, and these, along with their sources and brief descriptions of the conditions under which they were determined, are listed in Table 3. The general reaction for reduction of a quinone Q to a

quinol QH₂ may be represented:



Applying the Nernst equation⁽⁵⁸⁾ to this half-reaction yields

$$E = E_o - \frac{RT}{2F} \ln \frac{[QH_2]}{[Q]} + \frac{RT}{F} \ln [H^+] \quad (2-1)$$

where the symbols have their usual meanings (see Abbreviations, p.15).

At the midpoint of the potentiometric titration, $[Q] = [QH_2]$, so that

$$E_m = E_o - 2.303 \frac{RT}{F} (\text{pH}) \quad (2-2)$$

Thus knowing the midpoint potential E_{mN} at pH = N, the value at any other pH can be readily calculated using equation (2-2). In this research it was desired to work in the physiological pH region, that is, in near neutral conditions, so each midpoint potential was converted to a value at pH 7: this value has been designated by E_{m7} or E_o' ⁽⁵⁸⁾.

By inspection of the literature values of E_{m7} for coenzyme Q (Table 3) it can be seen that the potentials measured for the quinone linked to the respiratory chain^(46,47) are some 30 to 60 mV lower than those obtained in free alcoholic solution^(41,58,59). It has been proposed that this difference is due to a solvent effect rather than to a specific binding of the coenzyme Q to protein in the mitochondrial preparations⁽⁴⁶⁾. The redox active benzoquinone moiety is thought to be in a more aqueous environment in the mitochondrial preparations, with the ring protruding from the membrane surface while the long isoprenoid chain remains buried in the hydrophobic interior. This explanation is supported by the observation that the redox potential of 2,6-dimethoxybenzoquinone, a simple analogue of coenzyme Q, is lowered as the amount of alcohol present is decreased (Table 3). As the present work is concerned with reactions occurring at an oil/water

Table 3 Midpoint redox potentials, adjusted to pH 7.0, of coenzyme Q₁₀, vitamin K₁, and related quinones, as reported in the literature. Temperature was 25°C unless otherwise indicated.

<u>Quinone</u>	<u>E_{m7} (volts)</u>	<u>Medium</u>	<u>Remarks*</u>	<u>Reference</u>
Coenzyme Q ₁₀	+ 0.121	ethanol:1M HCl	PT	(41)
	+ 0.112	50% n-propanol:50% 0.2M HCl, 0.2M LiCl	PT	(59)
	+ 0.066	50mM phosphate buffer	SMP	(46)
	+ 0.082	0.3M mannitol, 0.01M Tris, pH 7.2	MP	(47)
Vitamin K ₁	- 0.128	80% ethanol:20% 0.02M sodium acetate, 0.02M acetic acid	PT	(60)
	- 0.044 (20°C)	95% ethanol:5% 0.2M HCl, 0.2M LiCl	PT	(61)
	- 0.075	50% n-propanol:50% 0.2M HCl, 0.2M LiCl	PT	(62)
	- 0.078	67% propan-2-ol:33% 0.15M Tris, 1.0M NaCl, pH 7.3	PL	(63)
2,6-dimethoxy- benzoquinone	+ 0.100	0.1M HCl	PT	(64)
	+ 0.131	50% alcohol:50% 0.5M HCl	PT	(65)
1,4-naphtho- quinone	+ 0.084	67% propan-2-ol:33% 0.15M Tris, 1.0M NaCl, pH 7.3	PL	(63)
	+ 0.064	50% n-propanol:50% 0.2M HCl, 0.2M LiCl	PT	(62)
	+ 0.087	95% alcohol:5% 1.0M HCl	PT	(65)
	+ 0.056	0.1M HCl	PT	(64)

* PT denotes a potentiometric, and PL and polarographic measurement.

MP and SMP denote studies using mitochondrial and submitochondrial particles respectively.

interface, the midpoint potential values determined for respiratory chain linked coenzyme Q^(46,47) were judged to be the more appropriate: an average value of E_{m7} for coenzyme Q of about + 75 mV at 25°C was therefore adopted.

Unfortunately no comparable determination of the redox potential of vitamin K₁ at a lipid/water interface was found in the literature. All the values quoted in Table 3 were derived from measurements in aqueous alcohol solutions. Assuming the more recent results to be the most accurate, E_{m7} for free vitamin K in an alcoholic medium at 25°C would seem to be around - 75 mV. Its simple analogue, 1,4-naphthoquinone, like the benzoquinones, shows a marked variation in midpoint potential with water content of the solvent medium (Table 3), the value in pure aqueous solution⁽⁶⁴⁾ being almost 30 mV lower than that in 67% propan-2-ol⁽⁶³⁾. It therefore seems probable that the midpoint potential of vitamin K should be similarly lowered when its naphthoquinone moiety is placed in a more aqueous environment, as is the case at an oil/water interface. Although it is impossible from the rather scattered data available to assign definite values to E_{m7} for coenzyme Q and vitamin K, it can be deduced that the two values should be separated by at least 100 mV. Thus comparing the reactivities of the two carriers, in the absence of specific kinetic effects, coenzyme Q should be more easily reduced, and more difficult to reoxidise, than vitamin K. This was in agreement with the experimental findings (Sections 2-3.2 and 2-3.4).

2-1.2 Ultraviolet/visible spectrophotometry

Electromagnetic radiation impinging on matter may be transmitted, absorbed or reflected. Absorption can only occur if the energy of the incident radiation corresponds to a difference between energy levels

in the irradiated molecules. Thus the selection rule for absorption may be written

$$\Delta E = h\nu \quad (2-3)$$

where ΔE is the energy difference between the ground and excited states of the molecule, ν is the frequency of the incident radiation, and h is Planck's constant. Radiation in the ultraviolet and visible regions of the spectrum is of the energy required to effect transitions between electronic energy levels. A sample containing a chromophore will therefore absorb energy from the incident beam, the extent of absorption at any given wavelength being dependent upon the probability of the corresponding electronic transition, which in turn depends on the nature and environment of the chromophore. A graph of absorbance against wavelength therefore produces an absorption spectrum characteristic of the molecule under study. When the sample is in solution the resultant spectrum typically consists of one or more broad bands, due to unresolved vibrational and rotational fine structure underlying each electronic transition. The wavelength at which absorption is a maximum, given the symbol λ_{max} , may often be used to elucidate the molecular structure of an unknown chromophore by comparison with literature values. All chromophores in organic molecules contain unsaturation, and, in general, λ_{max} increases as the conjugation of the system increases. Highly conjugated molecules thus tend to be coloured due to strong absorption in the visible region. Normally the only electronic transitions which can be observed in organic molecules are of the $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ types. Transition metal ions and their complexes (such as the iron complexes, ferroin and cytochrome c, to be considered later) also commonly absorb in the visible region, the absorption this time being due to $d \rightarrow d$ transitions.

Ultraviolet/visible absorption spectrophotometry has proved to be a particularly useful technique in this research because it can be used quantitatively to measure solution concentrations. The absorbance, A_λ , of a solution at a given wavelength λ is commonly related to its molar concentration c by the Beer-Lambert Law:

$$A_\lambda = \log (I_0/I) = \epsilon_\lambda c l \quad (2-4)$$

where I is the intensity of the transmitted light, I_0 is the intensity of the incident beam, l is the path length through the sample in centimetres, and ϵ_λ is the molar extinction coefficient of the compound under investigation at the fixed wavelength λ ⁽⁶⁶⁾. The term $\log (I_0/I)$ is also frequently referred to as the optical density of the solution. Absorbances are normally measured at a wavelength corresponding to a peak in the spectrum, and are converted to concentrations using equation (2-4) and a known value of ϵ_{max} . Although for simple solutions at low concentrations Beer's Law usually applies, deviations from the straight line relationship between optical density and concentration are often encountered in solutions which are either more concentrated or more complex. Such deviations may be caused by a number of factors, among them formation of aggregates, complexes or polymers, or variations in pH or ionic strength. It is therefore necessary to establish the range of applicability of equation (2-4) for each new compound to be studied.

The analysis of multicomponent mixtures is more complicated since their absorption spectra may overlap, so that the measured absorbance at λ_{max} for one component is no longer directly proportional to the concentration of that component. Fortunately the only type of multicomponent system encountered here was a binary conjugate system,

in which the two species present were different redox states of a single compound. The absorbance of a mixture is simply the sum of the absorbances of its individual components, providing there is no interaction between them. Thus for a binary conjugate system, consisting of oxidised (o) and reduced (r) forms of the same compound,

$$A_{\lambda} = (\epsilon_{\lambda o} c_o + \epsilon_{\lambda r} c_r) l \quad (2-5)$$

Also, if the total concentration of the substance, c , remains constant,

$$c = c_o + c_r \quad (2-6)$$

Thus combining equations (2-5) and (2-6), and assuming that the path length $l = 1$ cm, equation (2-7) is obtained:

$$c_o = \frac{\epsilon_{\lambda r} c - A_{\lambda}}{\epsilon_{\lambda r} - \epsilon_{\lambda o}} \quad (2-7)$$

This equation will be used later in the analysis of cytochrome c solutions.

The instrumentation used in the determination of spectra and optical densities merits brief description. Ultraviolet and visible spectra of all the compounds studied here were obtained using Pye Unicam SP 800 and SP 8000 dual beam recording spectrophotometers (Figure 2.1). The former covers an absorbance range of 0 - 2.0 with an accuracy of ± 0.02 and was sufficient for routine work. The latter has 0 - 1.0 (accuracy ± 0.01) and 0 - 0.2 (accuracy ± 0.005) absorbance ranges and was used when greater sensitivity was required, as for example, when dealing with very dilute solutions. In each case wavelength readings were calibrated using holmium (200-640 nm) and didymium (570-800 nm) filters, and absorbance readings were

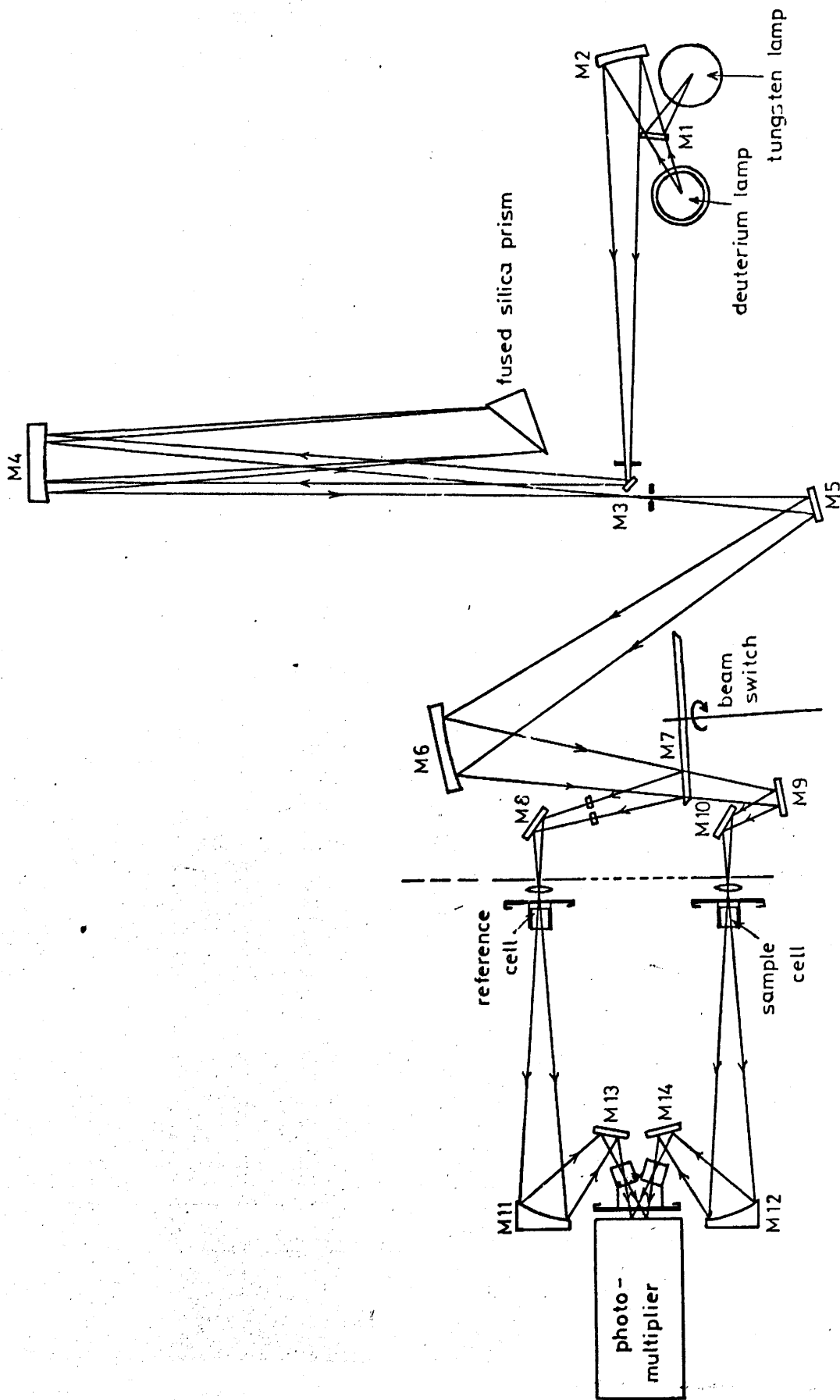


Figure 2.1 Optical layout of the Pye Unicam SP800 spectrophotometer. (The layout of the SP8000

spectrophotometer is similar.)

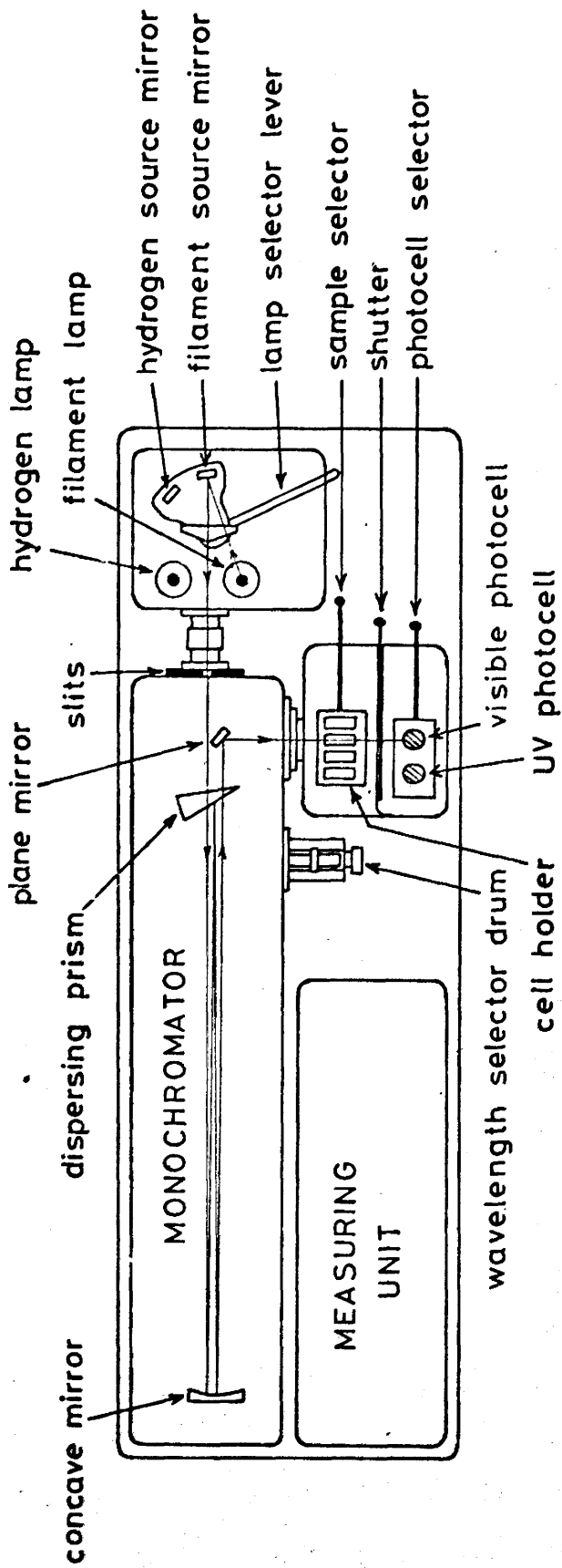


Figure 2.2 Optical layout of the Hilger-Watts H700 'Uvispek' spectrophotometer.

calibrated using a standard solution of potassium dichromate, by comparing the observed spectrum with a standard spectrum supplied by the manufacturers⁽⁶⁷⁾.

Absorbance measurements at fixed wavelength were normally determined using a Hilger-Watts H700 'Uvispek' spectrophotometer. The optical system of this single-beam instrument is illustrated in Figure 2.2. Light from one or other of the stabilised lamps (the tungsten filament lamp, for the visible region, or the hydrogen arc lamp, for the ultraviolet region) is focussed on the entrance slit to the monochromator unit. It is then dispersed by a prism into a spectrum, a selected narrow band of which is allowed to pass through the exit slit. Wavelength selection is made by rotating the prism through a small angle. The emergent beam passes through a cell containing solution and the transmitted light falls on a photocell, whose output is detected by an electronic measuring unit. The resultant electromotive force is balanced by an opposing emf from a calibrated potentiometer, the balance point being indicated by a centre zero galvanometer. Since the Hilger H700 is a single beam instrument, it is necessary to set 'zero' and 'check' controls with pure solvent in the beam immediately before each measurement on the sample solution. Using this spectrophotometer optical densities in the range 0 - 3 can be measured to an accuracy of + 0.001.

2-1.3 Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance spectroscopy, EPR (also called electron spin resonance, ESR, when applied to organic free radicals), is based on transitions between energy levels produced by the action of a magnetic field on an unpaired electron⁽⁶⁸⁾. An electron

possesses intrinsic angular momentum due to the spin and orbital closed loop type motions which it undergoes. Since it has also a negative charge, the spinning electron possesses an intrinsic magnetic moment μ which is proportional to e/m , where e is the charge on the electron and m is its mass. The magnetic moment vector will take up one of two possible orientations in the presence of an externally applied magnetic field, H_0 , corresponding to one of the allowed values of the spin quantum number m_s ($+\frac{1}{2}$ or $-\frac{1}{2}$). The applied field destroys the degeneracy of the two energy levels (Zeeman effect), and causes the magnetic moment vector μ to precess about the field vector H_0 . The frequency of precession, ν , is given by

$$\nu = g\beta H/h \quad (2-8)$$

where β is the electronic Bohr magneton, h is Planck's constant, H is the net magnetic field and g is the electronic g factor, a tensor which depends on the orbital and electronic environment of the electron, and has a value close to 2.00232 for a free electron in an organic radical. A second, small magnetic field, applied perpendicular to the main field H_0 , will effect transitions between the energy levels if it is of the correct frequency. It turns out that this correct frequency is equal to ν , the precessional frequency, and, for a typical field strength of around 3000 gauss, lies in the microwave region of the electromagnetic spectrum. The net magnetic field H is the field actually experienced by the electron, and is the vector sum of the applied field and the internal magnetic fields exerted by neighbouring spinning nuclei. In liquids this resultant field is given by

$$H = H_0 + \sum_i a_i (M_I)_i \quad (2-9)$$

where a_i is the isotropic hyperfine coupling constant for the magnetically equivalent nuclei, i , and M_I is the quantum number defining the z-axis component of their nuclear spin angular momentum. For a hydrogen nucleus, or proton, M_I can take the values $+\frac{1}{2}$ or $-\frac{1}{2}$; for nitrogen the possible values are $+1$, 0 and -1 . Thus the electronic energy levels are further split by interaction between the magnetic field of the electron and those of neighbouring nuclei, and this has the effect of producing 'hyperfine' structure in the EPR spectrum. Interaction with n equivalent nuclei of spin $\frac{1}{2}$ produces a spectrum containing $n + 1$ peaks, whose intensities follow the sequence of binomial coefficients and which are separated from each other by a distance corresponding to the coupling constant a_i . Most EPR spectrometers operate by keeping the microwave frequency ν constant and sweeping the applied magnetic field H_0 . An electromagnet with an available field range of 500-5000 gauss and with a field uniformity of $1:10^6$ is required for accurate studies. When the applied field reaches a value at which resonance occurs, the sample absorbs microwave energy, and this in turn produces a change in the microwave level at a crystal detector. Since amplifiers cannot function at high microwave frequencies, the DC signal from the crystal is normally converted to an AC one by low frequency (usually 100 kHz) modulation of the magnetic field. Providing the amplitude of this modulating field is small, the resultant spectrum shows the first derivative of the absorption spectrum.

The minimum number of unpaired electrons required in a sample before an EPR spectrum can be detected is typically of the order of 10^{12} , and for detailed analysis a free radical concentration of $10^{-6} - 10^{-5}$ M is usually required. EPR spectroscopy is therefore a

very sensitive method for the detection and, in favourable circumstances, characterisation of species containing unpaired electrons. However it is important to notice that a failure to detect an EPR spectrum for a particular sample does not necessarily mean that no such species is present: it may be due simply to the instability of the radical or to complex formation.

2-2 EXPERIMENTAL

2-2.1 Materials

Chemicals: The sources and purities of the chemicals used in this research are listed in Appendix I. In general these were used without further purification, but 2,6-dichlorophenolindophenol and methylene blue were recrystallised from water before use.

Solutions: Solutions of coenzyme Q and vitamin K were made up in spectroscopic grade hexane, and their concentrations were determined from the measured absorbances of the solutions (or accurate dilutions thereof) at their wavelengths of maximum absorption, using predetermined values of extinction coefficients and equation (2-4). Freshly distilled water was used throughout in the preparation of aqueous solutions.

Standard buffer solutions of pH 4 (phthalate), pH 7 (phosphate) and pH 9 (borate) were made up from tablets (BDH, England).

0.025M phosphate buffer pH 6.86 (25°C) was prepared from potassium dihydrogen phosphate and disodium hydrogen phosphate, following the method of Vogel⁽⁶⁹⁾.

Standard solutions of 0.1M hydrochloric acid and 0.1M sodium hydroxide were made up using 'CONVOL' CVS phials (Hopkin and Williams, England).

Routinely solutions of aqueous reducing and oxidising agents were prepared simply by dissolving the requisite weight of solid in 0.025M phosphate buffer. However this could not be done in the case of the iron (III)-*o*-phenanthroline complex (ferriin), since the ready-made complex was not available. It was therefore necessary to form the complex in solution by mixing solutions containing ferric ions and *o*-phenanthroline. Problems arose here due to the fact that Fe^{3+} is unstable to hydrolysis in solutions of pH greater than 1-2, while *o*-phenanthroline forms a very stable protonated complex at low pH, which inhibits the formation of the ferriin complex. The following procedure was found to be satisfactory: A weighed amount of ferric chloride was dissolved in 0.1M hydrochloric acid to produce a solution of known concentration. A measured aliquot of this solution was then injected by Hamilton syringe into a solution containing a slight excess of *o*-phenanthroline in 0.025M phosphate buffer (the ferriin complex contains Fe^{3+} and *o*-phenanthroline in a 1:3 stoichiometry). The resulting solution at pH 6.86 was very faintly opalescent.

Nitrogen: 'Oxygen free' nitrogen gas (British Oxygen Co. Ltd., Scotland) was further purified by passing it through a gas train. It was bubbled through a solution containing sodium anthraquinone-2-sulphonate, sodium dithionite and potassium hydroxide to remove the last traces of oxygen⁽⁷⁰⁾, and passed over blue silica gel to remove water vapour. If necessary, the gas was finally saturated with a volatile solvent: this was sometimes required to prevent loss of solvent from hexane or ethanol solutions during deoxygenation. All glass-to-glass connections were made using butyl rubber tubing (Watson and Marlow, England) since it is relatively impervious to oxygen⁽⁵⁸⁾.

When even more stringent oxygen precautions were required, the nitrogen cylinder was connected to a vacuum line. Deoxygenation was achieved by passing the gas slowly through a silica glass tube packed with copper filings — 'Cuprin' reagent (Coleman Instruments, USA) — heated to 400-450°C in an electric furnace⁽⁵⁸⁾.

2-2.2 Apparatus

Glass phials: An 18 cm length of pyrex tubing, 10 mm internal diameter, was closed at one end with a 25 mm diameter bulb, and the neck of the tube was constricted to about 3 mm internal diameter at a distance of 5 cm from the top. The tube could then be easily sealed by heating in a flame at this point and drawing out the softened glass.

Rubber septa: Due to the oxygen sensitivity of many of the reactant species studied, it was necessary always to carry out reactions under anaerobic conditions. Normally reaction vessels and solutions were flushed with oxygen-free nitrogen before generation of the oxygen-sensitive species *in situ*. This procedure necessitated a method for introducing a reactant into the vessel without simultaneously admitting air: the use of self-resealing rubber septa and hypodermic syringe needles proved to be both efficient and convenient. The septa used were turnover-type closures in red natural rubber, marketed under the trade name 'suba-seals' (W. Freeman & Co. Ltd., England) (Figure 2.4(d)) and were available in a wide range of sizes to fit most types of glass apparatus.

Needles: The stainless steel syringe needles used were of narrow bore and of various lengths, ranging from one inch to six inches. A short glass tubing adaptor, fitted with a B7 (female) Quickfit socket was cemented with 'Araldite' into the standard Luer end of each needle to

allow its connection, via a second B7 (male) fitting, to butyl tubing (and hence to the nitrogen line), or to a small gas bubbler. Ground glass joints were lubricated with 'Apiezon N' grease.

UV/visible sample cells: The cells used throughout this research were 'Spectrosil' (synthetic silica) ultraviolet/visible cells (Thermal Syndicate Ltd., England) of 1 cm path length. They were regularly cleaned, using the following procedure as recommended by the manufacturer: The cells were allowed to stand overnight in chromic acid (potassium dichromate dissolved in concentrated nitric acid) before draining and rinsing copiously, first with tap water, then with distilled water, and finally with Analar acetone. They were then oven dried at 40-50°C. During measurements, care was taken to ensure that the optical faces of the cells were kept clean and that every cell was placed in the sample holder with its engraved face towards the incident beam. Although matched cells were routinely used, in accurate work the bias between sample and reference cells was always measured with pure solvent in each at the start of an experiment.

Vacuum line: The vacuum line used in this research is illustrated in Figure 2.3. The line was provided with a gas inlet which allowed either nitrogen or hydrogen (for reconditioning the 'Cuprin' reagent after prolonged use) to be selected as required, by the use of taps T1 and T2. The gas flow rate was controlled both by these taps and by needle valves attached to the cylinder heads. 6 mm diameter copper tubing connected the gas cylinders to the selector unit, and 3.5 mm diameter tubing connected the selector outlet to the top of a silica tube, 40 cm in length and 13 mm in diameter. All metal-metal joints were welded or made using 'Swagelok' couplings and the metal-glass joint was made using a graded seal. Before connection the silica

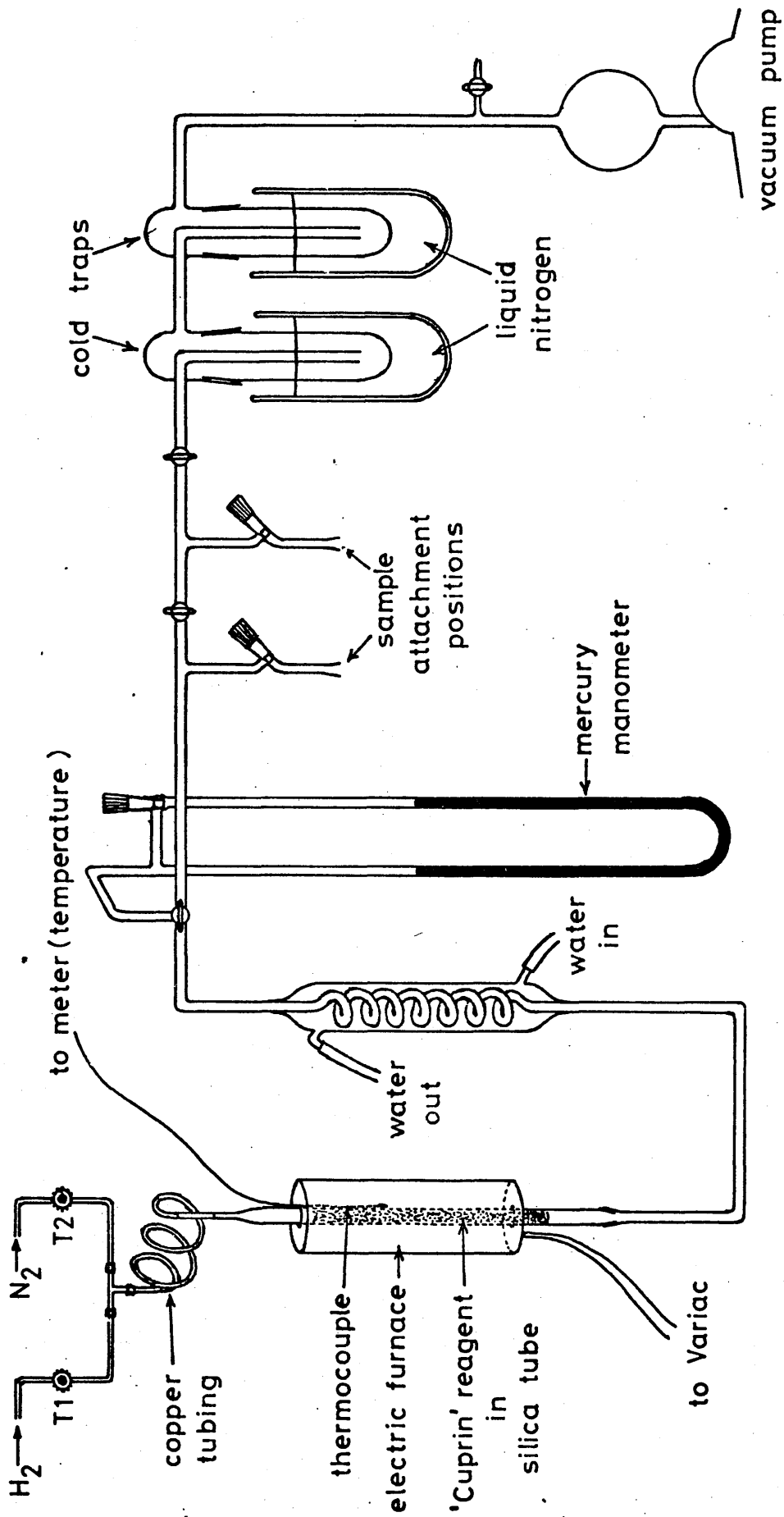


Figure 2.3 The vacuum line used in experiments where rigorous exclusion of oxygen was required.

tube was plugged with quartz glass wool at a distance of 10 cm above the Quickfit cone and was packed with a 20 cm long column of 'Cuprin' reagent. The coils in the narrow gauge copper tubing allowed the column to be manoeuvred into and out of the Quickfit socket beneath. The central portion of the coil was surrounded by an electric furnace 20 cm in length which was used to heat the 'Cuprin' reagent. Temperature control was achieved by use of a 'Variac' (Zenith Electric Co. Ltd., England) voltage regulator, the temperature inside the furnace being monitored by means of a nickel chromium/nickel aluminium thermocouple probe with an attached meter which displayed the temperature directly (Sifam Ltd., England). The gas emerging from the hot column at 400-450°C was passed through a glass cooling coil enveloped in a jacket of circulating cold water. A mercury-filled manometer indicated the gas pressure in the vacuum line, which could be reduced to less than 0.02 mm Hg by the oil diffusion pump. Two cold traps held at liquid nitrogen temperature prevented accidental contamination of the oil by volatile solvents. To avoid any chance of contamination by grease of a sample attached to the line, Teflon 'Rotoflo' taps (Quickfit and Quartz Ltd., England) were fitted to both sample side-arms: the more remote ground glass taps were lubricated with silicone high vacuum grease (Edwards, USA).

UV/visible sample cell for anaerobic spectrophotometry: To allow both qualitative and quantitative analysis of extremely oxygen-sensitive species (particularly important in the case of dihydrovitamin K), the apparatus illustrated in Figure 2.4(a) was used in conjunction with the vacuum line. The reaction vessel consisted of a 15 ml pyrex glass bulb and could be sealed by closing the Teflon 'Rotoflo' tap. The bulb was fitted with a short side-arm which allowed a solid reactant to be kept separate from the liquid in the lower section of

Figure 2.4 Apparatus used in the study of biphasic reactions under anaerobic conditions:

- (a) UV/visible sample cell for anaerobic spectrophotometry,
- (b) apparatus used in carrier reduction reversibility studies,
- (c) 'V-shaped' cell for immiscibility studies, and
- (d) a suba-seal.

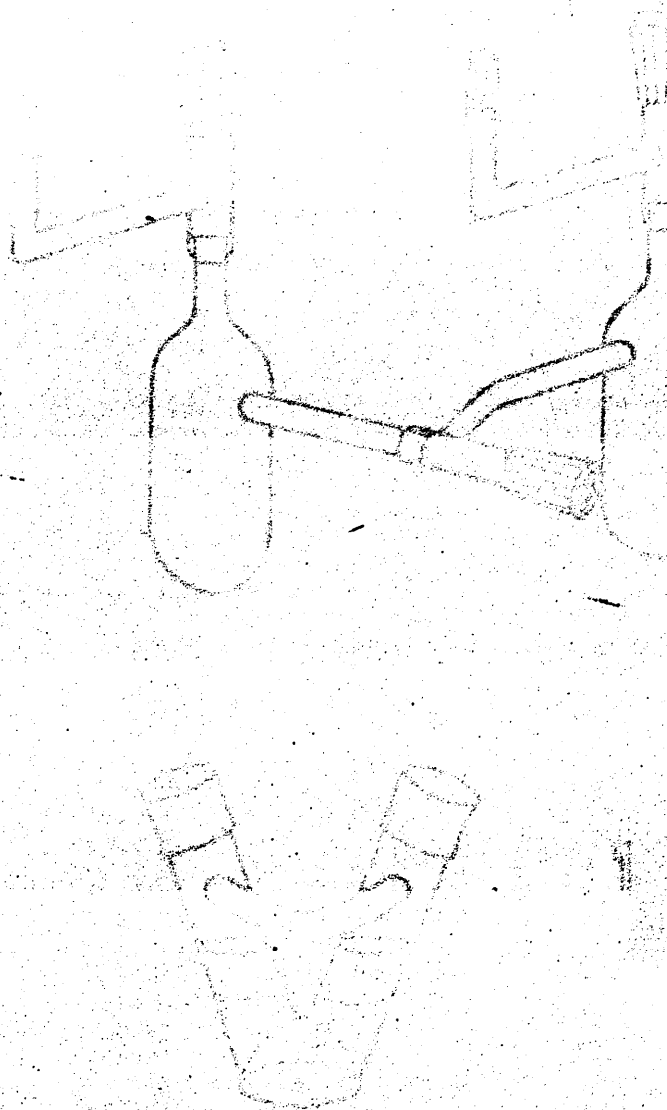
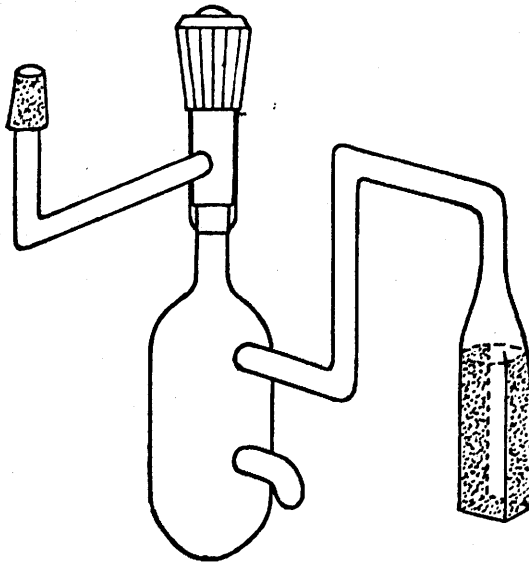
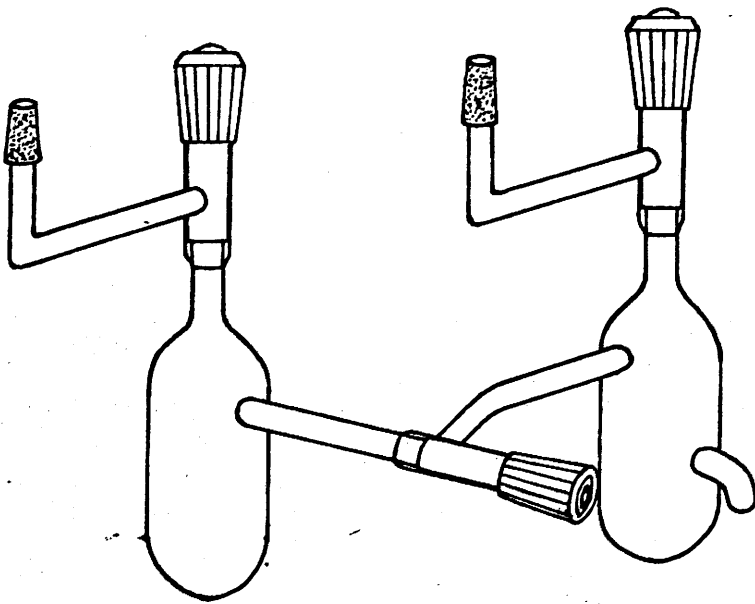


Figure 2.4

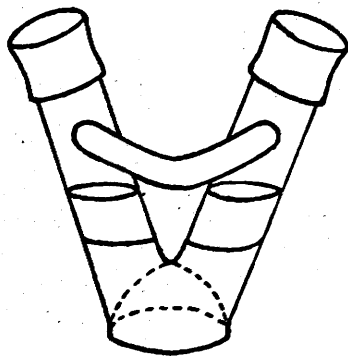
(a)



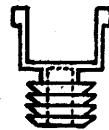
(b)



(c)



(d)



the bulb until mixing was required to initiate the reaction. A long side-arm led from the reaction vessel to an optical cell of 10 mm path length (identical to the cells described above), the link between the two being made with a graded glass joint. The remaining side-arm, fitted with a B14 (male) Quickfit cone, was used to connect the apparatus to the vacuum line.

Apparatus for use in carrier reduction reversibility studies: This apparatus is illustrated in Figure 2.4(b). Two reaction vessels, each fitted with a 'Rotoflo' tap, were interconnected by a section of glass tubing incorporating a third tap which, when open, allowed the transfer of liquid from one limb to the other, but permitted independent evacuation of the limbs when closed. One of the bulbs had a short side-arm to contain a solid reactant, and both halves of the apparatus were fitted with limbs having B14 (male) cones to allow connection to the vacuum line.

'V-shaped' cell for immiscibility studies: The apparatus devised to test the immiscibility of carrier and reductant solutions is shown in Figure 2.4(c). This consisted of two 6 cm lengths of pyrex tubing, 1 cm in diameter, joined together to form a 'V' shape, with a small flat-bottomed bulb at the base of the 'V'. The positions of the three solutions in the cell are shown in the diagram. This configuration allowed the two organic layers to be kept physically separate whilst minimising the distance for diffusion of any water-soluble species between the two oil/water interfaces. Another section, of narrower glass tubing, linked the two limbs, and allowed the suba-seals to be fitted without pushing either of the hexane solutions round the corner into the opposite limb.

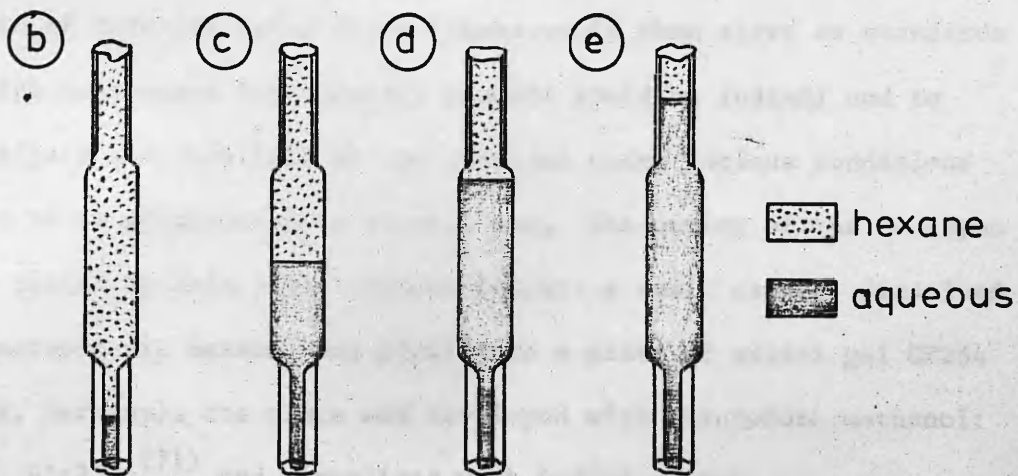
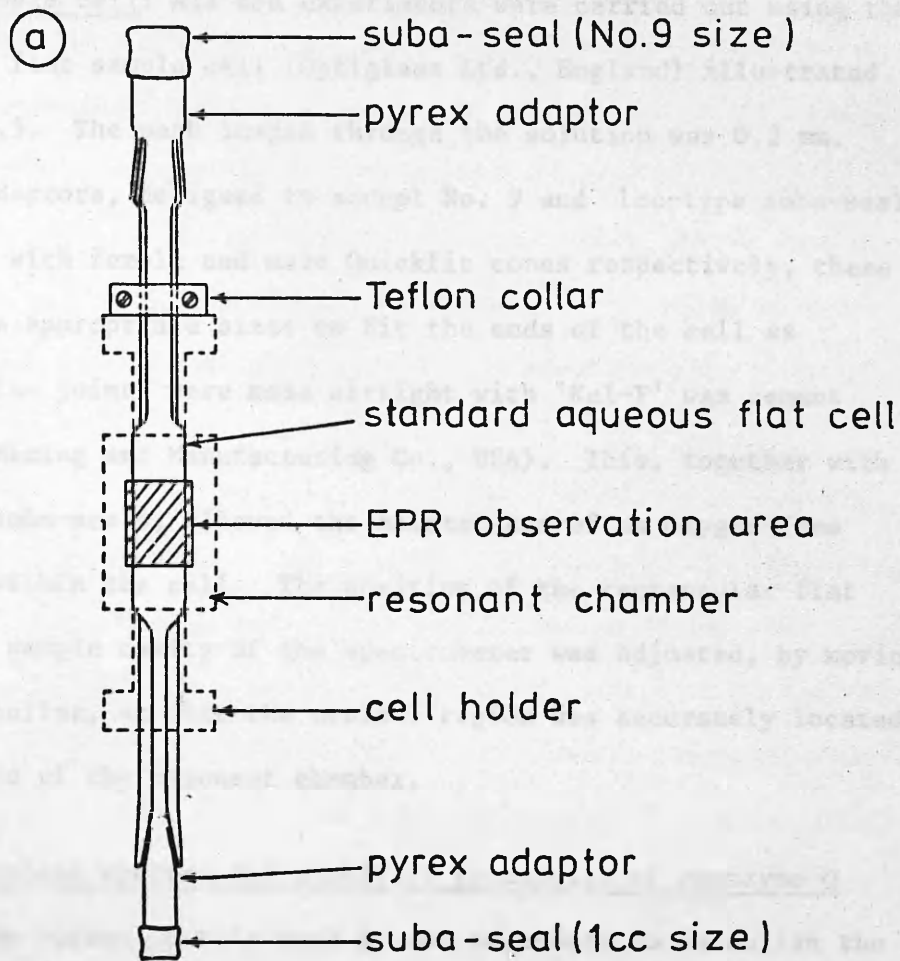


Figure 2.5 Apparatus used in EPR studies. (a) The modified aqueous flat sample cell. The positions of the hexane/water interface when (b) the bulk hexane, (c) the interface, (d) the aqueous phase just below the interface, and (e) the bulk aqueous phase, were examined, are also shown.

EPR flat sample cell: All EPR experiments were carried out using the rectangular flat sample cell (Optiglass Ltd., England) illustrated in Figure 2.5. The path length through the solution was 0.2 mm. Two glass adaptors, designed to accept No. 9 and 1cc-type suba-seals, were fitted with female and male Quickfit cones respectively, these being of the appropriate sizes to fit the ends of the cell as supplied. The joints were made airtight with 'Kel-F' wax cement (Minnesota Mining and Manufacturing Co., USA). This, together with the use of suba-seals, allowed the maintenance of an oxygen-free atmosphere within the cell. The position of the rectangular flat cell in the sample cavity of the spectrometer was adjusted, by moving the Teflon collar, so that the central region was accurately located in the centre of the resonant chamber.

2-2.3 Absorption spectra and stability properties of coenzyme Q

At the outset of this work it was necessary to establish the purity of the coenzyme Q as supplied, to record the absorption spectra of both its redox forms (these would then serve as standards by which subsequent experimental results could be judged) and to investigate the stability of the coenzyme under various conditions liable to be encountered in routine use. The purity of the coenzyme Q was tested by thin layer chromatography: a small sample, dissolved in spectroscopic hexane, was applied to a plate of silica gel GF254 (Merck, Germany); the plate was developed with chloroform:methanol:water, 65:25:4⁽⁷¹⁾ and visualised with iodine vapour.

A solution of coenzyme Q of arbitrary concentration was prepared in spectroscopic hexane, and its ultraviolet absorption spectrum was recorded on the SP800 spectrophotometer, with spectroscopic hexane as reference. This solution was then reduced by

equilibration over an aqueous solution containing a large excess of sodium borohydride⁽⁷²⁾, the reaction being carried out under nitrogen to prevent atmospheric reoxidation of the quinol. Aliquots of the hexane layer were removed at fifteen minute intervals, and their UV spectra were determined each time. When no further change in the spectrum was observed, reduction was assumed to be complete, and a final UV spectrum corresponding to fully reduced coenzyme Q was recorded.

The stability of (oxidised) coenzyme Q with respect to decomposition under conditions of prolonged exposure to the atmosphere, to daylight, to heat, and to contact with glass surfaces, was investigated. 5 ml of coenzyme Q solution in spectroscopic hexane (about 5×10^{-5} M) whose UV spectrum had previously been recorded on the SP8000 spectrophotometer, was added to each of five glass phials, labelled A-E. About 20 small glass beads were added to one of the phials (E) and all were then closed with suba-seals. Phials B, C, D and E were flushed with nitrogen (presaturated with hexane) for 45 minutes while the contents of phial A remained under air. All five phials were then cooled in liquid nitrogen and were hermetically sealed. They were then left to stand, each under its own set of conditions, as indicated in Table 4, for eight days before opening. All the phials apart from phial A, which was left beside the laboratory window, were protected from light by covering with aluminium foil. Phials A, B and C were maintained at 25°C in a laboratory whose temperature was thermostatically regulated at this value, while phials D and E were kept in a refrigerator. At the end of the eight day period the absorbance at 270 nm of each of the solutions was noted.

2-2.4 Biphasic reductions of coenzyme Q

Method A: In these experiments the glass phials described in Section 2-2.2 were used. Coenzyme Q solutions were made up in spectroscopic hexane, to a concentration of 5×10^{-5} M (determined by measurement of absorbance at 270 nm). The concentrations of the aqueous reducing agents investigated were 50-1000 times this value. All aqueous solutions were deoxygenated immediately prior to use, by degassing under vacuum at the water pump and by returning to atmospheric pressure with oxygen-free nitrogen. 3 ml of pure buffer solution, or of buffer solution containing the reducing agent, either in its oxidised form (in the case of methyl viologen, benzyl viologen, FMN and FAD) or, if stable in air, in its reduced form (NADH and NADPH) was added to the bulb of the phial using a graduated syringe with a long needle. 3 ml of the coenzyme Q solution in hexane was then similarly added and the phial closed with a suba-seal. It was then fitted with two syringe needles; a 6-inch needle attached to the nitrogen line and a 1-inch needle attached to a small gas bubbler. After flushing the phial with nitrogen for 30 minutes, 0.5 ml of a freshly prepared solution containing, in the appropriate buffer, sufficient sodium dithionite to reduce about 90% of the secondary reducing agent in the aqueous phase, was added. This step was omitted if the aqueous reductant was already in the reduced form (NADH and NADPH). The needles were then removed and the contents of the phial frozen by immersion in liquid nitrogen. This allowed the phial to be hermetically sealed without loss of solvent or possible degradation of the reactants. The phial, containing the two-phase system, was finally covered with aluminium foil to protect it from light and was

left on a vibro-mixer (Chemie-Apparatebay, Zurich) for one to five days at 25°C. In each case an identical phial was prepared, but containing pure spectroscopic hexane in place of the coenzyme Q solution. When sodium borohydride was used as reducing agent, the foregoing procedure had to be somewhat modified, since a large amount of gaseous hydrogen was evolved in the course of the reaction. In this case the phial could not be hermetically sealed, so instead it was left closed by the suba-seal and with the short needle and gas bubbler still in position.

At the end of the reaction period the phials were broken open and UV spectra of the organic layers were determined. By placing the coenzyme Q solution in the sample beam of the SP800 spectrophotometer, with the hexane from the control phial as reference, it was possible to remove any spurious absorption due to trace contaminants absorbed from the aqueous phase and hence to obtain a relatively 'clean' spectrum of the coenzyme Q. In these qualitative experiments it was unnecessary to exclude air from the optical cells used, since the reoxidation of reduced coenzyme Q by atmospheric oxygen was found to be a rather slow process. From the spectra obtained, by comparison with the spectra of pure oxidised and pure reduced coenzyme Q (Figure 2.6), the extent of reduction of the quinone was estimated, indicating the effectiveness of the aqueous reductant used.

In parallel experiments with vitamin K⁽⁵⁷⁾, spectrophotometry could not be readily used for the analysis of reaction products, due to the extreme oxygen sensitivity of the reduced form. However it was possible, using more concentrated solutions (3 mM), to judge whether or not reduction had occurred by visual inspection of the hexane layer,

since the naphthoquinone has a strong yellow colour whilst the naphthoquinol is colourless.

Method B: A second method, which allowed a more quantitative analysis of the products of these biphasic reduction reactions, and which was particularly useful when dealing with highly oxygen sensitive species, involved the use of the specially designed reaction vessel (Figure 2.4(a)) in conjunction with the vacuum line. With the 'Rotoflo' tap removed, solid sodium dithionite was manipulated into the short side-arm with the help of a piece of butyl rubber tubing attached to the end of a glass tube. A small Teflon-coated magnetic stirrer was placed in the bulb, together with 4 ml of the appropriate aqueous solution (previously deoxygenated), so that the solution level was just below the opening to the side-arm. 5 ml of coenzyme Q solution, 5×10^{-5} M, in spectroscopic hexane, was then added on top, and the 'Rotoflo' tap was fitted and the apparatus attached to the vacuum line. The contents of the bulb were frozen in liquid nitrogen before evacuating the apparatus. Once a high vacuum was achieved (below 0.1 mm Hg), the pressure was slowly returned to atmospheric by admitting a little oxygen-free nitrogen. The solutions in the bulb were then left to thaw and re-equilibrate under the new atmosphere for about fifteen minutes before repeating the cycle. After two such repetitions, with the contents of the cell finally under ultra-pure nitrogen, the 'Rotoflo' tap was tightly closed, the apparatus removed from the vacuum line, wrapped in aluminium foil, and left to return to room temperature. The dithionite was then mixed with the aqueous solution, and the biphasic system was left stirring for a period of one to five days at 25°C. At the end of the reaction period the hexane layer was examined spectrophotometrically. Careful tilting of the apparatus allowed the transfer of the hexane solution into the

attached optical cell: the separation was made easier by immersing the bulb for a few seconds in liquid nitrogen to freeze the aqueous phase.

Reversibility of carrier reduction: The apparatus described in Section 2-2.2 (Figure 2.4(b)) was used in this experiment. A large excess of silver oxide, previously washed with spectroscopic hexane to remove trace contaminants which might otherwise have complicated the interpretation of spectra, was added to bulb B along with a small Teflon-coated magnetic stirrer. Taps T2 and T3 were closed. A little solid sodium dithionite was manipulated into the short side-arm of bulb A, into which was then added 5 ml of 2.7×10^{-2} M methyl viologen in 0.025 M phosphate buffer, pH 6.86, 5 ml of 5.0×10^{-5} M coenzyme Q solution in spectroscopic hexane, and a magnetic stirrer. After freezing in liquid nitrogen, degassing at the vacuum line, and re-equilibrating with oxygen-free nitrogen as described above, bulb A was covered with aluminium foil, the dithionite mixed with the methyl viologen solution, and the biphasic system left stirring for two hours. Tap T2 was then opened to allow transfer of the reduced coenzyme Q solution from bulb A to bulb B. Taps T2 and T3 were closed to prevent hexane evaporation, and the contents of bulb B were left stirring for two hours at 25°C and protected from light. A sample of untreated coenzyme Q solution was also left under the same conditions as a control. The optical densities of the untreated and treated coenzyme Q solutions were then determined at 270 nm, using the Hilger H700 spectrophotometer.

Since UV absorption spectra tend to be rather insensitive to changes in regions of the molecule which do not affect the chromophore, the NMR technique was also used to investigate the reversibility of

coenzyme Q reduction by reduced methyl viologen. A round-bottomed glass phial containing 50 mg of sodium dithionite was closed with a suba-seal and flushed with nitrogen for about thirty minutes. 5 ml of an aqueous solution (previously deoxygenated) containing 0.1 M sodium hydroxide and 0.1 M methyl viologen was then added by syringe, followed by 5 ml of coenzyme Q solution in spectroscopic hexane, at a concentration of 5 mg ml⁻¹. The tube was then covered with aluminium foil and left on the vibro-mixer for two hours at 25°C, by which time the hexane layer was colourless. The hexane layer was then removed, washed (dilute HCl) and dried (anhydrous sodium sulphate) before mixing with a large excess of silver oxide. When reoxidation was complete (after two hours) the solution was separated off and the solvent removed by evaporation. Finally the yellow residue was dissolved in 5 ml of deuteriochloroform (C.E.A., France) and its proton NMR spectrum was recorded using a Varian T60, 60 MHz spectrometer. The spectrum of an untreated sample of the quinone was also recorded for comparison.

2-2.5 Immiscibility tests

Experiment A: 4 ml of methyl viologen solution, 2.7×10^{-2} M, in 0.025 M phosphate buffer, pH 6.86, was equilibrated with an equal volume of spectroscopic hexane under nitrogen in a hermetically sealed glass phial for a period of twelve hours at 25°C. A second phial was similarly set up, but with pure phosphate buffer replacing the methyl viologen solution. The UV spectra of the hexane layers from both phials after equilibration were recorded on the Unicam SP8000 spectrophotometer.

Experiment B: 4 ml of methyl viologen solution, 2.7×10^{-2} M, in 0.025 M phosphate buffer, was added to the bulb of the vacuum cell apparatus described in Section 2-2.2 (Figure 2.4(a)), after charging the small reservoir with excess solid sodium dithionite. 4 ml of spectroscopic hexane was then added, the liquids were frozen, and the apparatus degassed by evacuation as described above. The dithionite was finally added, and the solutions left to equilibrate under an atmosphere of pure nitrogen for about six hours. The UV spectrum of the hexane layer was recorded on the Unicam SP8000 spectrophotometer.

Experiment C: 4 ml of coenzyme Q solution, 5×10^{-4} M, in spectroscopic hexane, was added on top of an equal volume of 0.025 M phosphate buffer in a glass vial. After overnight equilibration under nitrogen, in the dark and at 25°C, the absorption spectrum of the lower aqueous layer was recorded.

Experiment D: The insolubility of coenzyme Q in an aqueous reducing layer was further checked in this experiment, involving the use of the 'V-shaped' reaction vessel described in Section 2-2.2 (Figure 2.4(c)). A small Teflon-coated magnetic stirrer was placed in the bottom of the tube, along with 6 ml of deoxygenated methyl viologen solution, 2.7×10^{-2} M, in 0.025 M phosphate buffer. This quantity was just sufficient to fill the lower part of the vessel so that the two limbs were separated by the liquid layer. 2 ml of 5×10^{-4} M coenzyme Q solution in spectroscopic hexane was then added to one limb, and an equal volume of pure hexane to the other, the two additions being carried out simultaneously and carefully, to avoid accidental transfer of solution from one limb to the other. Finally 0.5 ml of a freshly

prepared solution of sodium dithionite (sufficient to reduce about 95% of the methyl viologen) in 0.025 M phosphate buffer was added via a syringe needle through the hexane layer, and the vessel was closed with suba-seals. At no time in this experiment was a suba-seal pierced by a syringe needle or otherwise allowed to come into contact with solution, since it was found that hexane readily leached aromatic compounds out of the rubber, resulting in intense spurious absorption in the UV spectrum subsequently recorded. The vessel was then left stirring gently (to avoid emulsification of the solutions) in the dark at 25°C, for a period of 48 hours. After reoxidation of the solutions in air for five days, the UV spectrum of the 'pure' hexane solvent layer was recorded.

2-2.6 Biphasic oxidation of reduced coenzyme Q

Solutions of reduced coenzyme Q for use in these experiments were prepared in the following manner: A solution of coenzyme Q, about 5×10^{-4} M, in spectroscopic hexane, was reduced by overnight equilibration over an aqueous solution containing a large excess of sodium borohydride in pH 9 buffer. The contents of the reaction vessel were then transferred to a separating funnel, and the lower aqueous layer was removed. The hexane layer was washed thoroughly, first with 0.1 M hydrochloric acid, and finally with distilled water, until the washings were neutral to pH test paper (Johnsons Ltd., England). The reduced coenzyme Q solution was kept under an atmosphere of pure nitrogen until required.

The visible spectra of each potential aqueous substrate in both its redox forms were recorded before use, in order to check that the reduction could be followed spectrophotometrically. In every case

the oxidised form was the more stable under normal aerobic conditions in the laboratory: the reduced form was generated by addition of excess solid sodium dithionite to the substrate solution.

4 ml of the freshly deoxygenated aqueous oxidant, 10^{-5} - 10^{-4} M solution in 0.025 M phosphate buffer, pH 6.86, was added to each of two glass phials (Section 2-2.2) which were then closed with suba-seals and flushed with nitrogen for about thirty minutes. To one of the phials was added 4 ml of the reduced coenzyme Q solution, 5×10^{-5} - 5×10^{-4} M (the concentration having been accurately determined from the absorbance of the solution at 290 nm (Section 2-3.1)) and to the other was added a similar volume of spectroscopic hexane. The contents of both phials were frozen under liquid nitrogen and the phials were hermetically sealed. They were then covered with aluminium foil and left on the vibro-mixer for a period of one to eight days at 25°C, during which time the aqueous phases were regularly checked visually for any colour change indicating reaction. Finally the phials were broken open, and the UV spectrum of the coenzyme Q solution was recorded, using the similarly equilibrated pure hexane as reference. In cases where the reduced form of the aqueous substrate was relatively stable to atmospheric reoxidation, the visible spectra of the two aqueous phases after equilibration were also recorded, using 0.025 M phosphate buffer as reference.

It should be noted that the above procedure could not be followed in studies on the oxidation of dihydrovitamin K⁽⁵⁷⁾, which, due to its extreme oxygen sensitivity, had to be generated from the oxidised form *in situ*.

2-2.7 EPR studies

The two biphasic systems studied in these experiments were reduced methyl viologen/coenzyme Q and reduced FMN/coenzyme Q. In each case the concentration of the aqueous reductant, in 0.025 M phosphate buffer, pH 6.86, was 10^{-2} M, and that of the coenzyme Q, in spectroscopic hexane, was 6×10^{-3} M. The rectangular flat sample cell described in Section 2-2.2 (Figure 2.5) was used, and was flushed with nitrogen for at least fifteen minutes before filling. Small samples of the aqueous (methyl viologen or FMN) and coenzyme Q solutions were first thoroughly deoxygenated by flushing for about one hour with nitrogen, in test-tubes fitted with suba-seals. Near the end of this period, sufficient sodium dithionite, freshly prepared in 0.025 M phosphate buffer, to reduce about 80% of the methyl viologen or FMN, was added to the aqueous solution. To minimise the chances of contact with atmospheric oxygen, gas-tight Hamilton syringes (V.A. Howe, England) were used to transfer the reactant solutions from their preparation tubes into the EPR cell. A small volume of aqueous solution was first added, by way of the lower suba-seal (Figure 2.5). This was followed by addition of 0.5 ml of the coenzyme Q solution, which, by its injection just below the surface of the aqueous phase, was prevented from coming into contact with the suba-seal. Additional aqueous solution was then slowly injected until the upper hexane phase filled the flattened section of the cell and the interface was situated about 1 cm below this section. Further additions of aqueous solution allowed the interface to be raised to positions in the middle, at the top, and about 1 cm above the flattened section of the cell. In this way the bulk hexane solution, the hexane/aqueous interface, the aqueous solution just below the interface,

and the bulk aqueous solution could be observed sequentially (Figures 2.5(b), (c), (d) and (e) respectively). All spectra were recorded on a Decca X3 EPR spectrometer combined with a Newport Instruments 11-inch magnet system, using 9270 MHz radiation and a magnetic field modulation frequency of 100 kHz.

2-3 RESULTS AND DISCUSSION

2-3.1 Absorption spectra and stability properties of coenzyme Q and vitamin K

The coenzyme Q as supplied by Sigma was found to be chromatographically pure by thin layer chromatography on silica gel GF254. On development of the plate with chloroform:methanol:water, 65:25:4, and visualisation with iodine, it yielded a single spot at R_f 0.91. The vitamin K was also chromatographically pure, having an R_f value of 0.95 under the same conditions.

The ultraviolet absorption spectra of coenzyme Q and vitamin K are shown in Figures 2.6 and 2.7 respectively. Coenzyme Q in the oxidised form exhibits an absorption maximum at $\lambda_{max} = 270$ nm, with $\epsilon_{270} = 15,100$, while the reduced form has $\lambda_{max} = 291$ nm and $\epsilon_{291} = 4,470$. (These data are taken from reference (73) and refer directly to coenzyme Q solutions in light petroleum. The spectra obtained here for hexane solutions showed identical absorption maxima, so it seemed reasonable to assume that the extinction coefficients in hexane would also be similar to those measured in light petroleum.) The oxidised form of vitamin K exhibits a number of peaks, the most intense being at $\lambda_{max} = 248$ nm, with $\epsilon_{248} = 19,600$, while the reduced form shows a single peak at $\lambda_{max} = 245$ nm, with $\epsilon_{245} = 44,500$ (57).

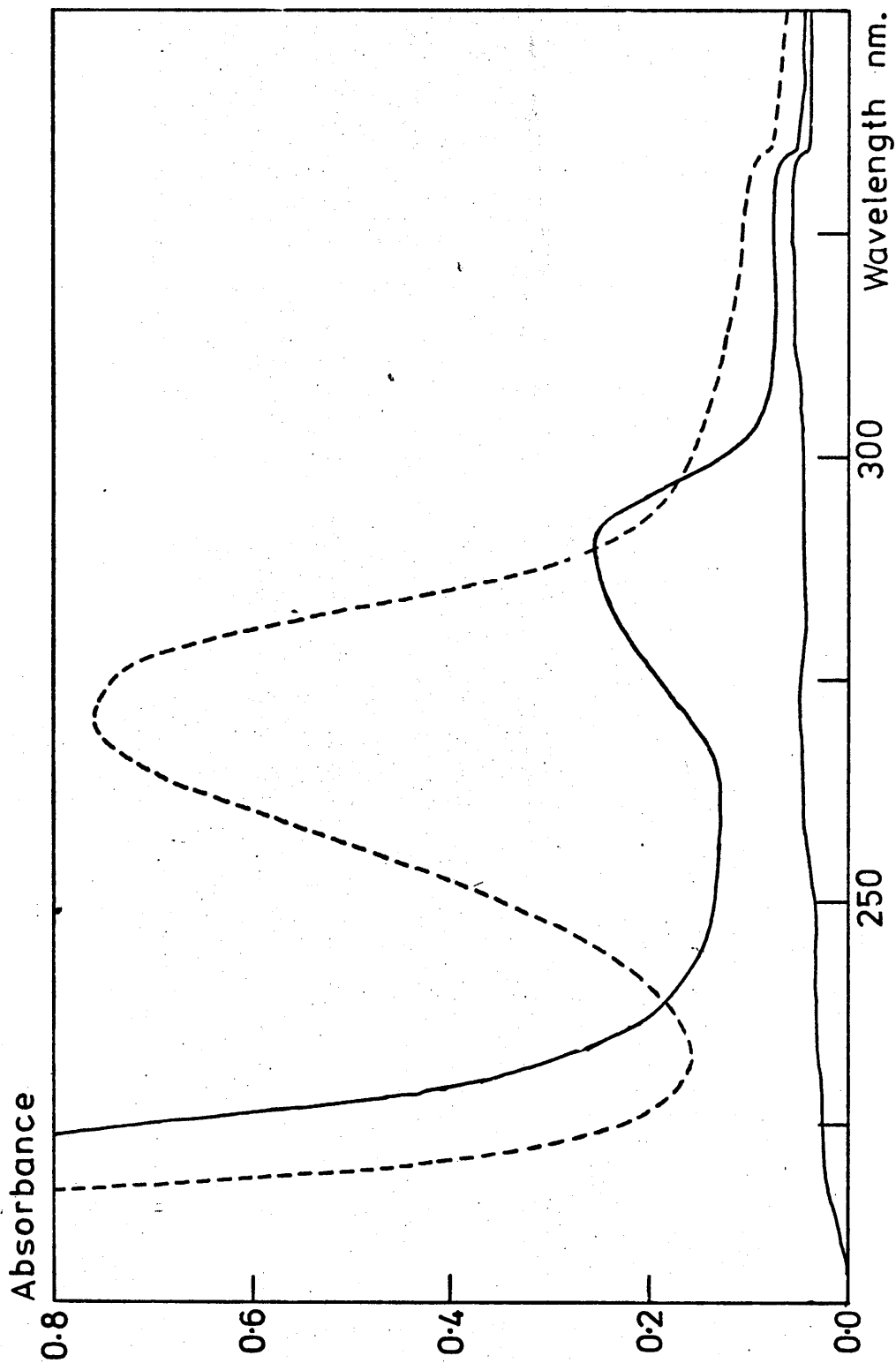


Figure 2.6 Ultraviolet absorption spectra of oxidised (-----) and reduced (——) coenzyme Q₁₀ (hexane solution, 4.7×10^{-5} M).

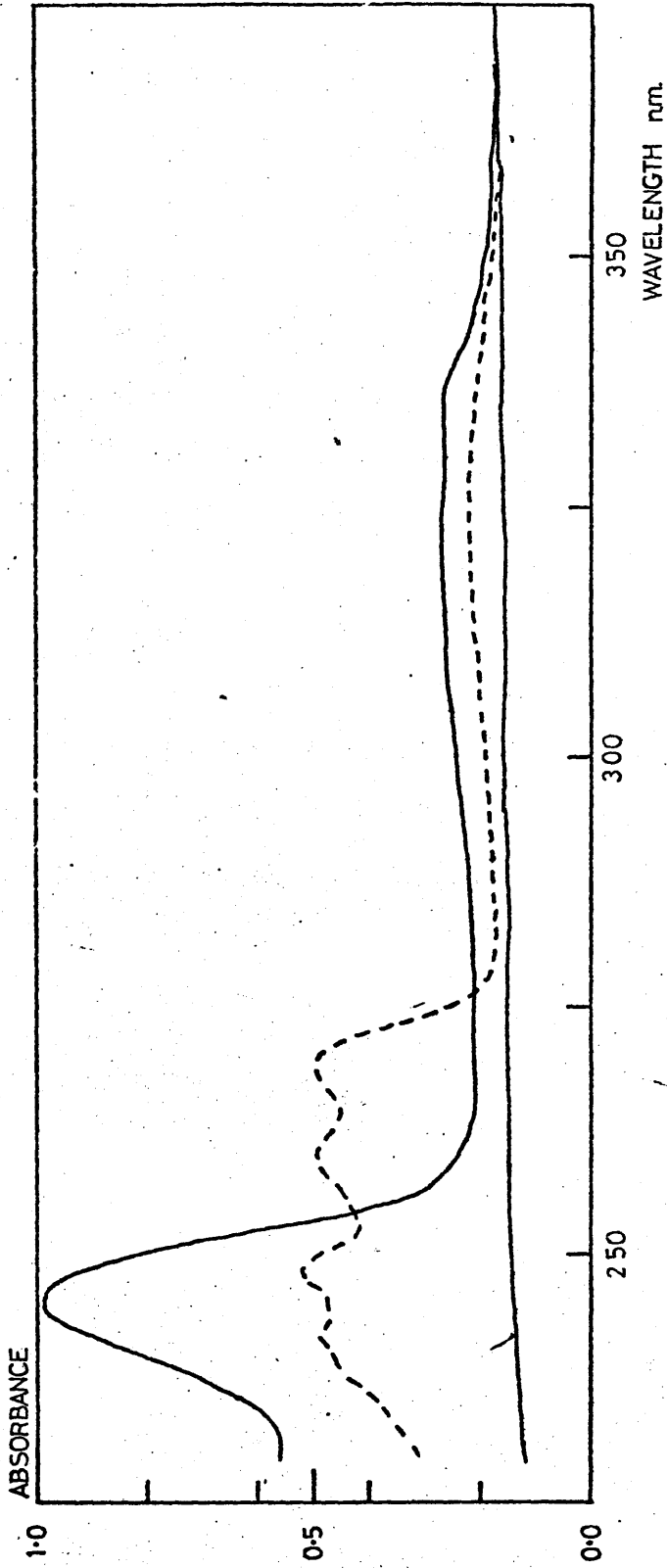


Figure 2.7 Ultraviolet absorption spectra of oxidised (-----) and reduced (—) vitamin K₁ (hexane solution, 1.9×10^{-5} M).

The results of the series of stability tests on coenzyme Q are given in Table 4. These show that, in dilute hexane solution, the quinone decomposes readily in light and slightly in heat, but can be stored without serious losses for at least eight days under nitrogen, in the dark, and in a refrigerator. The final result indicates that any absorption onto glass surfaces is minimal, and does not destabilise the molecule. Vitamin K gave similar results (57). These observations emphasise the requirement for careful protection of coenzyme Q and vitamin K solutions from light, and are in agreement with literature references which indicate the light sensitivity of these quinones (74,75).

2-3.2. Biphasic reduction of coenzyme Q and vitamin K

The results of the biphasic reductions attempted with coenzyme Q are shown in Table 5. Also included are results from the parallel work on vitamin K (57), literature values for the midpoint potentials E_m (or E^0 values) of the various aqueous reductants used, and a summary of the experimental conditions. Coenzyme Q was reduced by a wide range of aqueous reductants, including both organic and inorganic types, and both one-electron and two-electron transfer agents. Vitamin K on the other hand showed a much greater selectivity, and could not be reduced by the two-electron inorganic reductants dithionite and borohydride, although the one-electron reductant chromium (II) was effective. This difference in reactivity of the two quinones may partly reflect the difference between their redox potentials, since E_{m7} for vitamin K is at least 100 mV lower than E_{m7} for coenzyme Q, the latter being around + 75 mV (Section 2-1.1). However, all the reactions attempted were thermodynamically feasible, and it has been

Table 4. Stability of coenzyme Q solution in hexane under various conditions. In each case the solution had an initial absorbance of 0.725 at 270 nm (concentration about 4.8×10^{-5} M). Final absorbances were measured after 8 days equilibration under the stated conditions.

<u>Phial</u>	<u>Conditions</u>				<u>Final absorbance at 270 nm</u>	<u>Status of coenzyme Q</u>
	<u>atmosphere</u>	<u>illumination</u>	<u>temperature</u>	<u>surfaces</u>		
A	air	daylight	25°C	-	0.217	degraded
B	nitrogen	"	"	-	0.236	degraded
C	"	dark	"	-	0.664	slightly degraded
D	"	"	4°C	-	0.735	stable
E	"	"	"	glass beads	0.727	stable

Table 5 Tests of the reducing abilities of a number of two-electron ($n = 2$) and one- electron ($n = 1$) reductants towards coenzyme Q_{10} and vitamin K_1 in the biphasic system aqueous reductant/quinone in hexane

Reductant	n	Buffer pH or nature of aqueous solution	E_m^* , (E^0) ϕ (volts)	Results		Verification of the presence of excess reductant	Comments
				coenzyme Q	vitamin K		
sodium dithionite	2	4	(-0.08)	-	-	decolourised	unstable at pH < 7
		7		+	-	methylene blue	
		9	(-1.12)	+	slight		
sodium borohydride	2	7		+	-	decolourised	vigorous evolution of hydrogen at pH < 10
		9		+	-	methylene blue	
		0.1 M NaOH					
hydrazine	2	7	(-0.023)	-	-	generation of I_2 and N_2 on addition of IC_3^-	
		9	(-1.16)	-	-		
		0.1 M NaOH					
chromium (II)	1	4	(-0.41)	+	+	retention of blue colour	precipitate formed at pH < 7
		7		+	+		
		9	(-0.41)	+	+		
†reduced methyl viologen	1	4		-	-	retention of blue colour	precipitation at all pH's used
		7	-0.446 (30°C)	+	+		
		9	-0.446 (30°C)	+	+		
†reduced benzyl viologen	1	4		-	-	retention of violet colour	
		7	-0.359 (30°C)	+	+		
		9	-0.359 (30°C)	+	+		

Table 5 (cont'd)

Reductant	n	Buffer pH or nature of aqueous solution	E_m^* , (E ⁰) [∅] (volts)	Results		Verification of the presence of excess reductant	Comments
				coenzyme Q	vitamin K		
NADH	2	4	-0.222	-	-	ultraviolet spectroscopy	
		7	-0.311	-	-		
		9	-0.370	-	-		
NADPH	2	7	-0.313 (27°C)	-	-	UV spectroscopy	
†reduced FMN	1	4	-0.049	+	+	retention of green colour	
		7	-0.213	+	+		
		9	-0.435	+	+		
†reduced FAD	1	7	-0.213	+	+	retention of green colour	

* Reference (58); temperature 25°C unless otherwise indicated

∅ Reference (76), but values are converted to reduction potentials; temperature 25°C

† Generated by adding a slight deficiency of sodium dithionite to the oxidised form

found that both dithionite and borohydride will reduce vitamin K in homogeneous ethanolic solution^(29,77), so it seems likely that kinetic factors arising from the biphasic nature of these reactions may play a more important role here. Perhaps the extra hydrophobic aromatic ring of the naphthoquinone affects the overall polarity of the head group, changing its orientation at the interface and hence making it less accessible to the aqueous reductant. The reactivity of reduced methyl viologen, benzyl viologen, FMN and FAD may be due to the ability of these molecules to form charge-transfer complexes with the quinone (Section 2-3.5).

The fact that NADH and NADPH were ineffective in reducing either coenzyme Q or vitamin K under any of the conditions tried is also worthy of note. This at first sight contradicts the results of Ismailov *et al*⁽⁷⁸⁾ who attributed the generation of a membrane potential in their system to a reaction between NADH and coenzyme Q, but it must be remembered that the number of electrons requiring to be transferred to generate such a potential is much lower than that required to allow detection of net reaction between two bulk solutions. The failure of NADH to reduce coenzyme Q directly in the present experiments, despite the fact that both are known electron carriers in the mitochondrial respiratory chain (Figure 1.4) can be explained by the requirement for an enzyme catalyst in the reaction. In the respiratory chain, this electron transfer process is mediated by flavo-protein enzymes which have as their prosthetic groups FMN or FAD. It is interesting that the present simple system already shows this requirement for a mediator, since it was found in separate experiments that NADH reduces FMN in a homogeneous phase reaction, and that the reduced FMN can then react biphasically with coenzyme Q (Table 5).

Chromium (II), reduced methyl viologen, reduced benzyl viologen, reduced FMN and reduced FAD were all found to be effective reductants under various conditions. However, the necessity of an acidic medium for chromium (II), the unfavourable solubility characteristics of reduced benzyl viologen, and the lack of a ready source of FAD allowed only reduced methyl viologen and reduced FMN to be considered for routine use in subsequent experiments.

2-3.3 Ideality of carriers and aqueous reductants

Reversibility of carrier reduction. The reversibility of coenzyme Q reduction by reduced methyl viologen was investigated by the procedures detailed in Section 2-2.4. The ultraviolet spectrum of coenzyme Q solution after one cycle of reduction followed by reoxidation by silver oxide was compared with that of an untreated sample. The absorbances recorded at 270 nm are presented in Table 6. These results show that about 93% of the original quinone could be regenerated after two hours equilibration of the quinol with silver oxide. This represents a maximum recovery, since reoxidation was incomplete in shorter time periods, and on increasing the reaction time beyond two hours the absorbance at 270 nm decreased, with the concomitant development of an inflexion point at 252 nm in the ultraviolet spectrum.

The proton NMR spectrum of coenzyme Q after undergoing one reduction-oxidation cycle is shown in Figure 2.8(b). On comparing this with the spectrum recorded for untreated coenzyme Q, which was identical to a published spectrum⁽⁷³⁾, the former was found to contain two additional peaks at chemical shifts of 1.25 and 0.88 ppm. The integral trace indicated that only that part of the spectrum between 0.5 and 2.5 ppm was anomalous, and the chemical shifts of all the peaks other than the extra two agreed to within ± 0.02 ppm of the

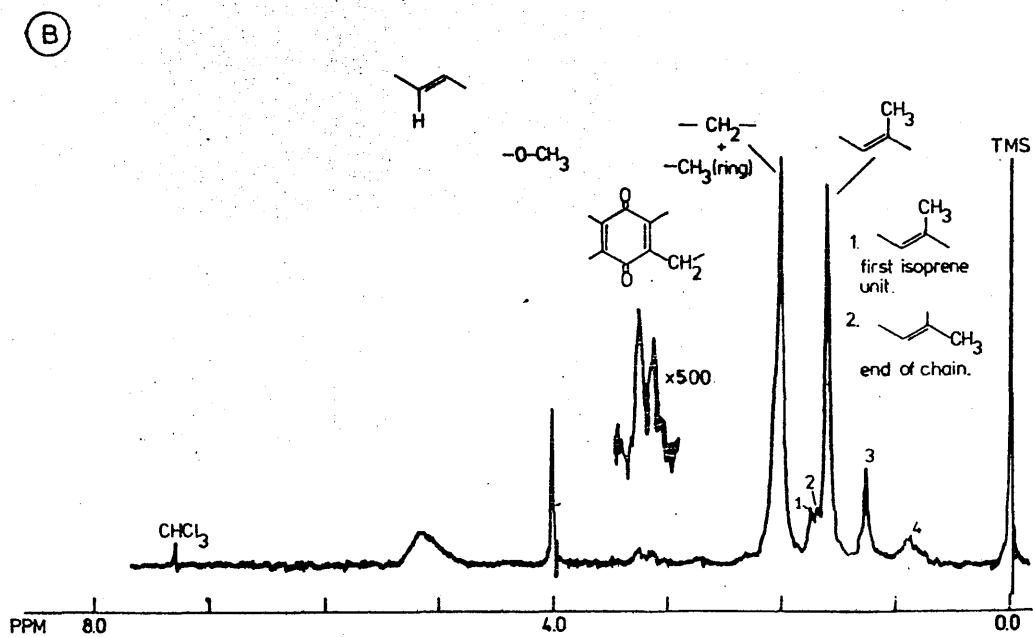
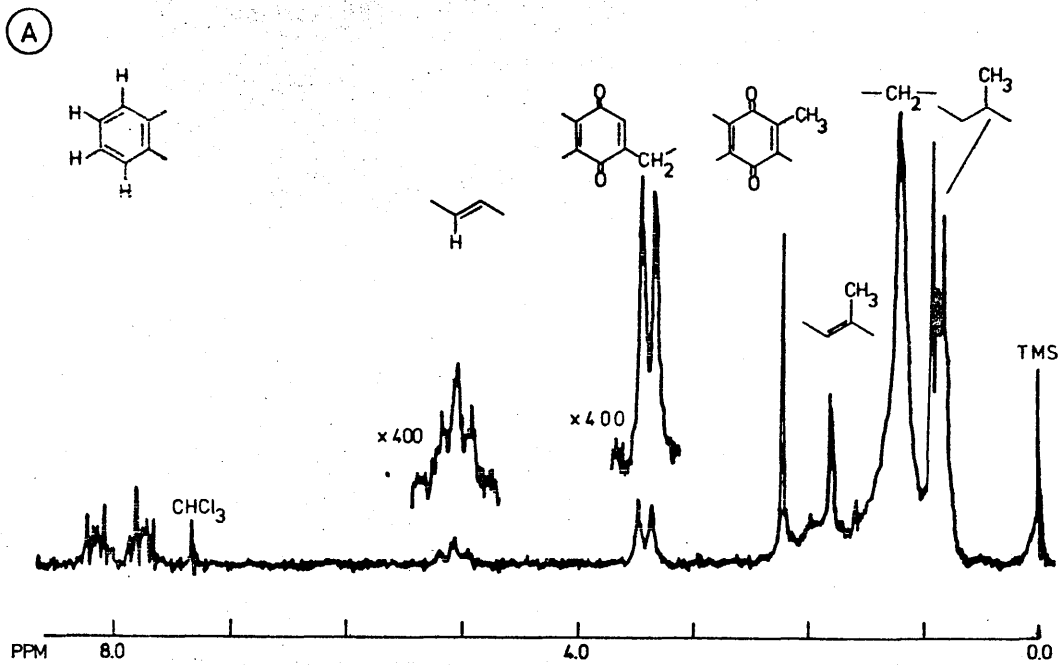


Figure 2.8 Proton NMR spectra of (a) vitamin K and (b) coenzyme Q, recorded after each quinone had undergone one reduction-oxidation cycle. For details see text (Section 2-3.3).

Table 6 Absorbance of oxidised coenzyme Q solution at 270 nm before (untreated) and after (treated) one reduction-oxidation cycle

<u>Equilibration time</u> <u>with Ag₂O (hours)</u>	<u>Absorbance at 270 nm</u>	
	<u>untreated</u>	<u>treated</u>
	0.771	
2		0.715
16		0.626

literature values. From their chemical shifts the extra peaks may be tentatively assigned to $R-CH_2-R$ (1.25 ppm) and $R-CH_3$ (0.88 ppm) proton resonances. These results from UV and NMR spectroscopy suggest that a small fraction (about 7%) of the coenzyme Q is chemically altered during the reduction-oxidation cycle. This could be due to irreversible interaction with reduced methyl viologen, but this seems unlikely since the absorption spectrum of the resulting quinol showed no abnormalities when compared with the initial oxidised spectrum and with published spectra⁽⁷³⁾. It is more probable that the contaminant originated from some side reaction between the silver oxide and the quinone or quinol. These observations bring into question the claim of Lester *et al*⁽⁷²⁾ that silver oxide regenerates the oxidised form of coenzyme Q cleanly from the quinol.

Vitamin K, which was also reduced using reduced methyl viologen, but was then reoxidised in air, did not show this anomalous behaviour: the recovery after one reduction-oxidation cycle was better than 96% as judged by quantitative ultraviolet spectroscopy⁽⁵⁷⁾, and the proton NMR spectrum (shown in Figure 2.8(a), for comparison with coenzyme Q) exhibited no spurious peaks, being identical to the spectrum obtained from untreated vitamin K and to a published spectrum⁽⁷⁷⁾.

Immiscibility tests. The ultraviolet and visible spectra obtained from hexane layers after prolonged equilibration over aqueous solutions containing phosphate buffer, methyl viologen and reduced methyl viologen (Section 2-2.5, experiments A and B) showed no absorption over the range 225-700 nm, within which both redox states of methyl viologen exhibit strong maxima. Likewise the spectrum of an aqueous layer after equilibration with coenzyme Q solution showed no absorption over the entire range (Section 2-2.5, experiment C). Furthermore, the

attempted continuous extraction process using the 'V-shaped' reaction vessel (Section 2-2.5, experiment D) left the opposite hexane layer completely free of coenzyme Q, indicating its insolubility in the aqueous phase in both its redox forms. This was considered to be a particularly sensitive test, since even a slight solubility in the aqueous phase should have led to a steady buildup in concentration of coenzyme Q in the originally pure hexane layer, through a process of continuous extraction. The spectrophotometric method used should have been capable of detecting these possible contaminants (oxidised and reduced methyl viologen or coenzyme Q) at concentration levels below 4×10^{-7} M. Similar results were obtained in the case of vitamin K⁽⁵⁷⁾.

In summary, the above investigations into reversibility of carrier reduction and immiscibility of carrier and reductant solutions have indicated that vitamin K and reduced methyl viologen possess all the necessary prerequisites for use as electron carrier and aqueous reductant respectively in an 'ideal' redox membrane system (Sections 1-1 and 1-5). In addition, it seems likely that coenzyme Q and reduced methyl viologen also meet the requirements for ideality of a carrier-reductant pair. Both systems will be considered in subsequent Chapters.

2-3.4 Biphasic oxidation of reduced coenzyme Q and vitamin K

The results of the experiments carried out to find aqueous oxidising substrates for reduced coenzyme Q (Section 2-2.6) are summarised in Table 7. Results from parallel work on vitamin K⁽⁵⁷⁾ are also included for comparison, although the experimental arrangement in this case was slightly different, requiring generation of the

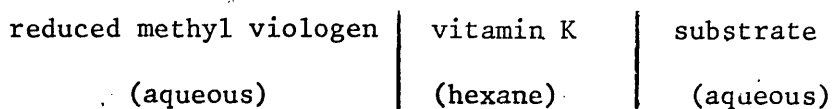
Table 7 Tests of the reducing abilities of reduced coenzyme Q₁₀ and reduced vitamin K₁ towards a variety of aqueous oxidants (substrates) in phosphate buffer, pH 6.86, at 25°C. The systems used were reduced CoQ (in hexane)/aqueous substrate, and reduced methyl viologen/Vit K (in hexane)/aqueous substrate

<u>Substrate</u>	<u>Results</u>			<u>Comments</u>
	<u>E_m[*]</u> (volts)	reduced coenzyme Q	reduced vitamin K [#]	
indophenol	0.228	+	+	considerable colour loss protonated form hexane-soluble
2,6-dichlorophenol-indophenol (DCIP)	0.217	+	+	considerable colour loss protonated form hexane-soluble
thionine	0.063	-	+	no change
methylene blue	0.011	slight	+	no change
K MnO ₄			+	slight colour loss brown precipitate formed, irreversible oxidation
K ₃ Fe(CN) ₆	0.36	+		no change oxidation partially irreversible
[Fe (o-phen) ₃] Cl ₃	1.12	+	+	slight colour gain uncomplexed o-phenanthroline hexane-soluble
ferricytochrome c (N.crassa)	0.255†	slight	+	became slightly reduced
(horse heart)	0.255 ^ø	slight	+	became slightly reduced

* Reference (58) except for: † reference (79) and ø reference (80).

Reference (57)

reduced vitamin K *in situ*:



In general it was found that reduced coenzyme Q was more difficult to reoxidise than dihydrovitamin K, the former displaying both a greater selectivity towards aqueous oxidants and, in all cases examined, a much lower reactivity towards individual common oxidants. Again this might be due in part to the difference of more than 100 mV between the redox potentials of coenzyme Q and vitamin K (Section 2-1.1). This indeed would explain the marked lack of reactivity of reduced coenzyme Q to the thiazine dyes — thionine and methylene blue — whose midpoint potentials are near that of coenzyme Q, but cannot explain the observation that methylene blue, which has the lower midpoint potential, is slightly reduced whereas thionine does not react at all. It seems likely therefore that mechanistic factors play an important part in the interfacial transfer of electrons from the quinol to the aqueous substrate. Possibly the ability of the naphthoquinol to form a more stable anion (by resonance in the conjugated ring system) increases its polarity, making it more accessible to the aqueous oxidant than the benzoquinol. It should be noticed that the order of reactivity of the two carriers towards biphasic oxidation is opposite to the order of their reactivity towards biphasic reduction, discussed in Section 2-3.2.

By inspection of the last two columns of Table 7, it can be seen that most of the substrates investigated were associated with complicating side-effects which were liable to make the accurate interpretation of results rather difficult. For example, the loss

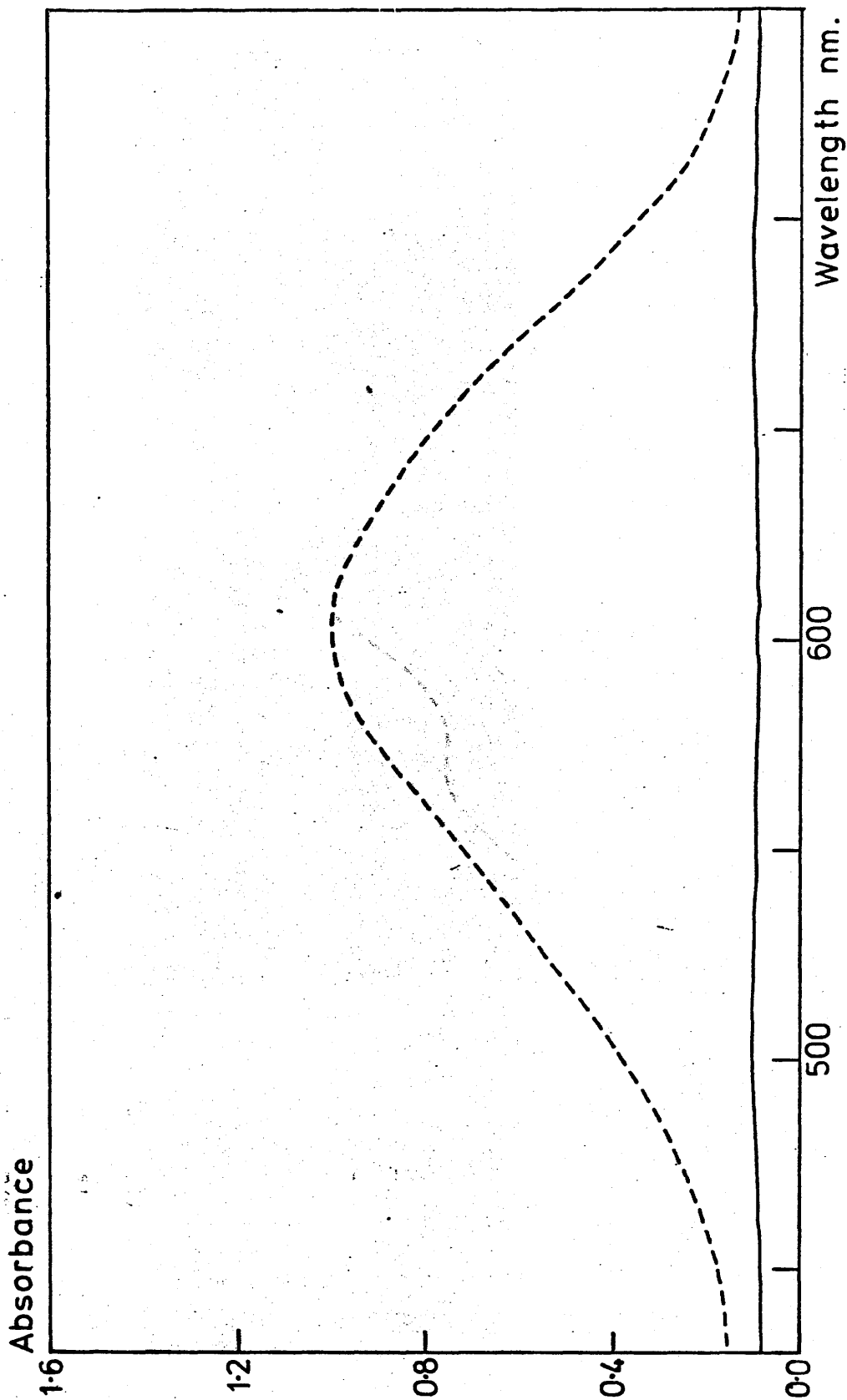


Figure 2.9 Visible absorption spectra of oxidised (-----) and reduced (—) DCIP
(in 0.025 M phosphate buffer, pH 6.86, 4.3×10^{-5} M).

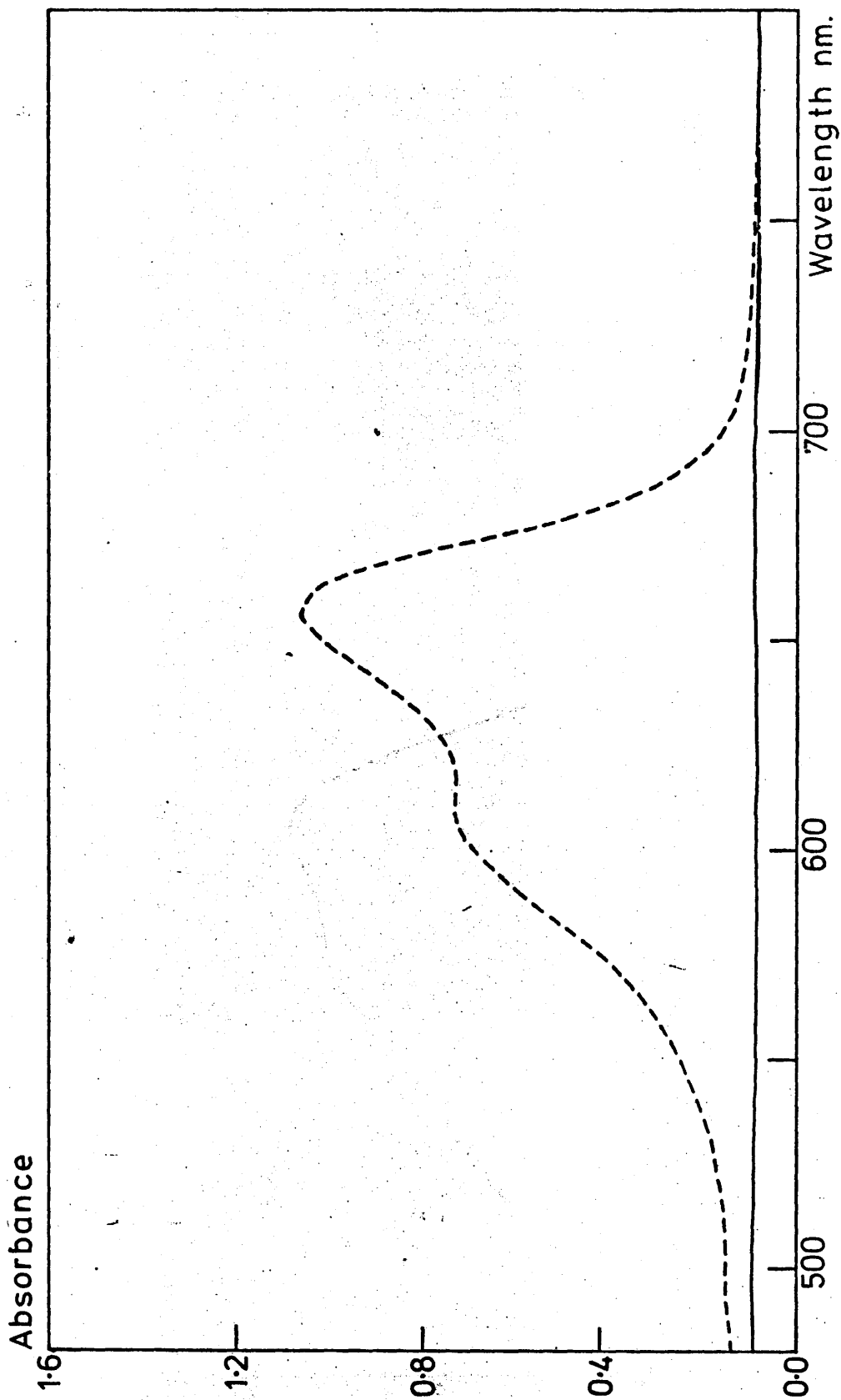


Figure 2.10 Visible absorption spectra of oxidised (-----) and reduced (————) methylene blue

(in 0.025 M phosphate buffer, pH 6.86, 1.4×10^{-5} M).

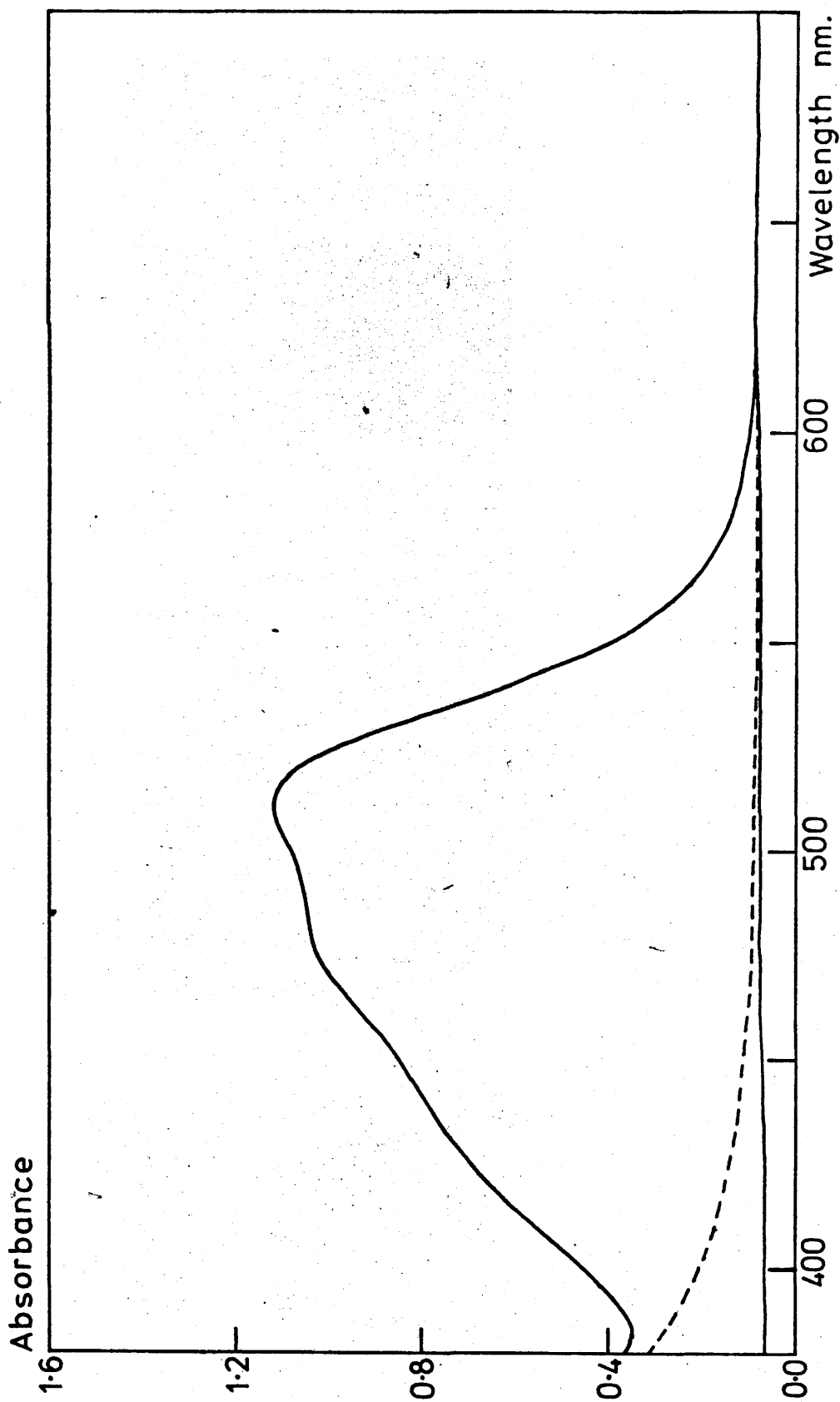


Figure 2.11 Visible absorption spectra of oxidised (---) and reduced (—) iron *o*-phenanthroline complex (in 0.025 M phosphate buffer, pH 6.86, 9.04×10^{-5} M).

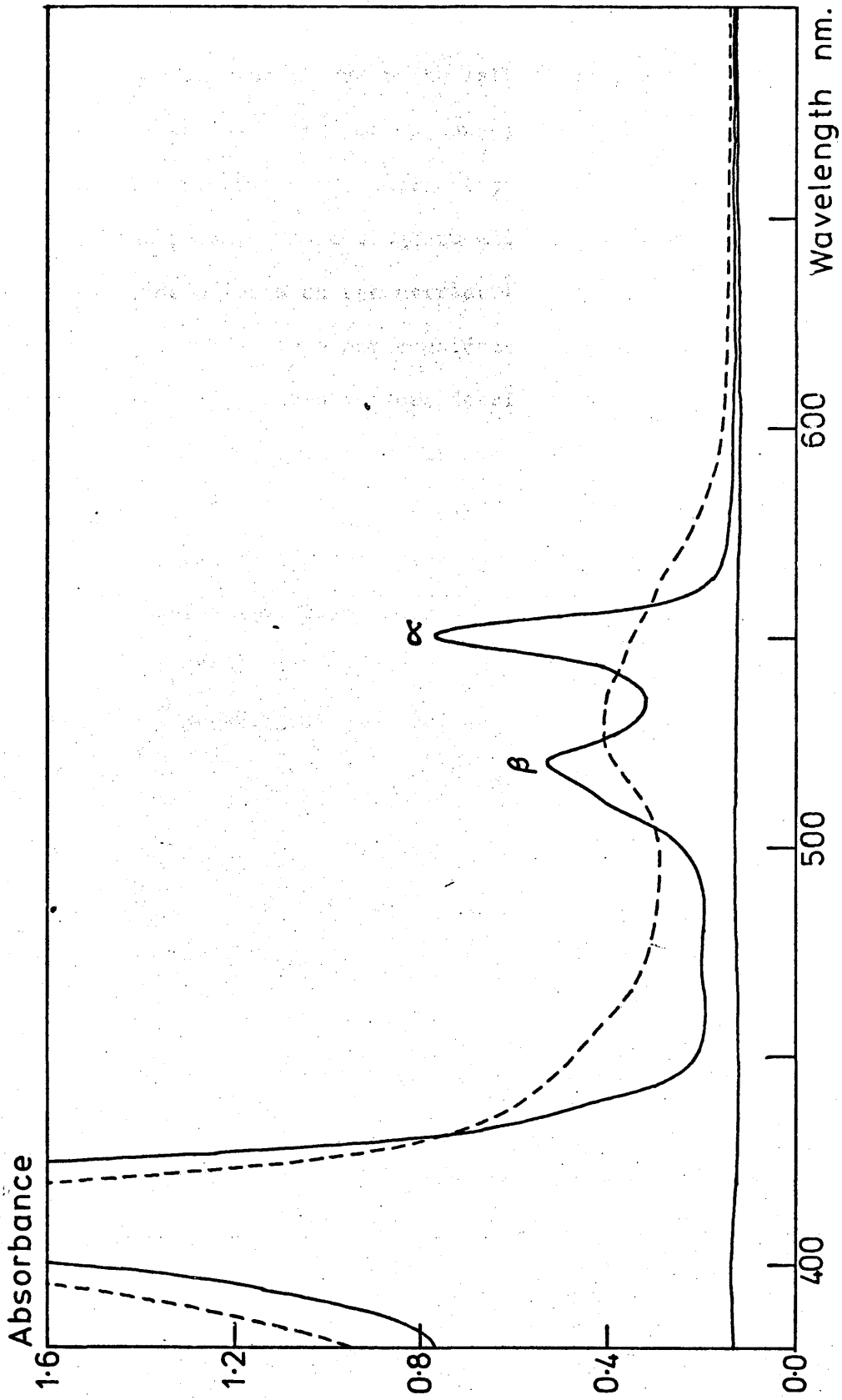


Figure 2.12 Visible absorption spectra of oxidised (-----) and reduced (——) cytochrome c (in 0.025 M phosphate buffer, pH 6.86, 2.2×10^{-5} M).

or gain of colour intensity by the control (equilibrated under pure hexane) was indicative of spontaneous reduction under anaerobic conditions (that is, due either to an intramolecular electron transfer to the active centre or to some intermolecular reaction with the solvent), or of continuous extraction of the reducing species into the hexane phase. Those reagents which were found to have irreversible side effects on the carrier (potassium permanganate and potassium ferricyanide) were not considered further. However, most of the others were subjected to more detailed investigations, using the bulk membrane systems to be described in Chapter 3. The visible spectra of the reduced and oxidised forms of these reagents — DCIP, methylene blue, iron-*o*-phenanthroline complex and cytochrome *c* — are shown in Figures 2.9, 2.10, 2.11 and 2.12 respectively: the extinction coefficients of the two redox forms of each at the monitored wavelength (corresponding to λ_{max} for one of the forms) are listed in Table 8.

2-3.5 Electron paramagnetic resonance studies

The object of these EPR experiments was to gain some insight into the mechanism of the electron transfer process occurring at the oil/water interface during reduction of coenzyme Q by reduced methylviologen and by reduced FMN. The EPR spectra obtained from the methylviologen and FMN systems are shown in Figures 2.13 and 2.14 respectively. These spectra were recorded using the methods described in Section 2-2.7, and the *g* values quoted were calculated from the spectra using equation (2-6).

By inspection of Figure 2.13 it is immediately obvious that the spectra obtained from the three distinct regions of the biphasic

Table 8 Absorption maxima and extinction coefficients for a number of aqueous substrates dissolved in 0.025 M phosphate buffer, pH 6.86. Wavelengths underlined are λ_{max} values.

<u>Reagent</u>	<u>Form</u>	<u>λ (nm)</u>	<u>ϵ</u>	<u>Reference</u>	<u>Figure</u>
2,6-dichlorophenol indophenol	oxidised	<u>605</u>	21,000	(57)	2.9
	reduced	605	0		
methylene blue	oxidised	<u>655</u>	69,800	(57)	2.10
	reduced	655	0		
iron-o-phenanthroline complex	oxidised	510	0		
	reduced	<u>510</u>	11,500	(81)	2.11
cytochrome c (horse heart)	oxidised	550	8,400	(82)	2.12
	reduced	<u>550</u>	29,500		

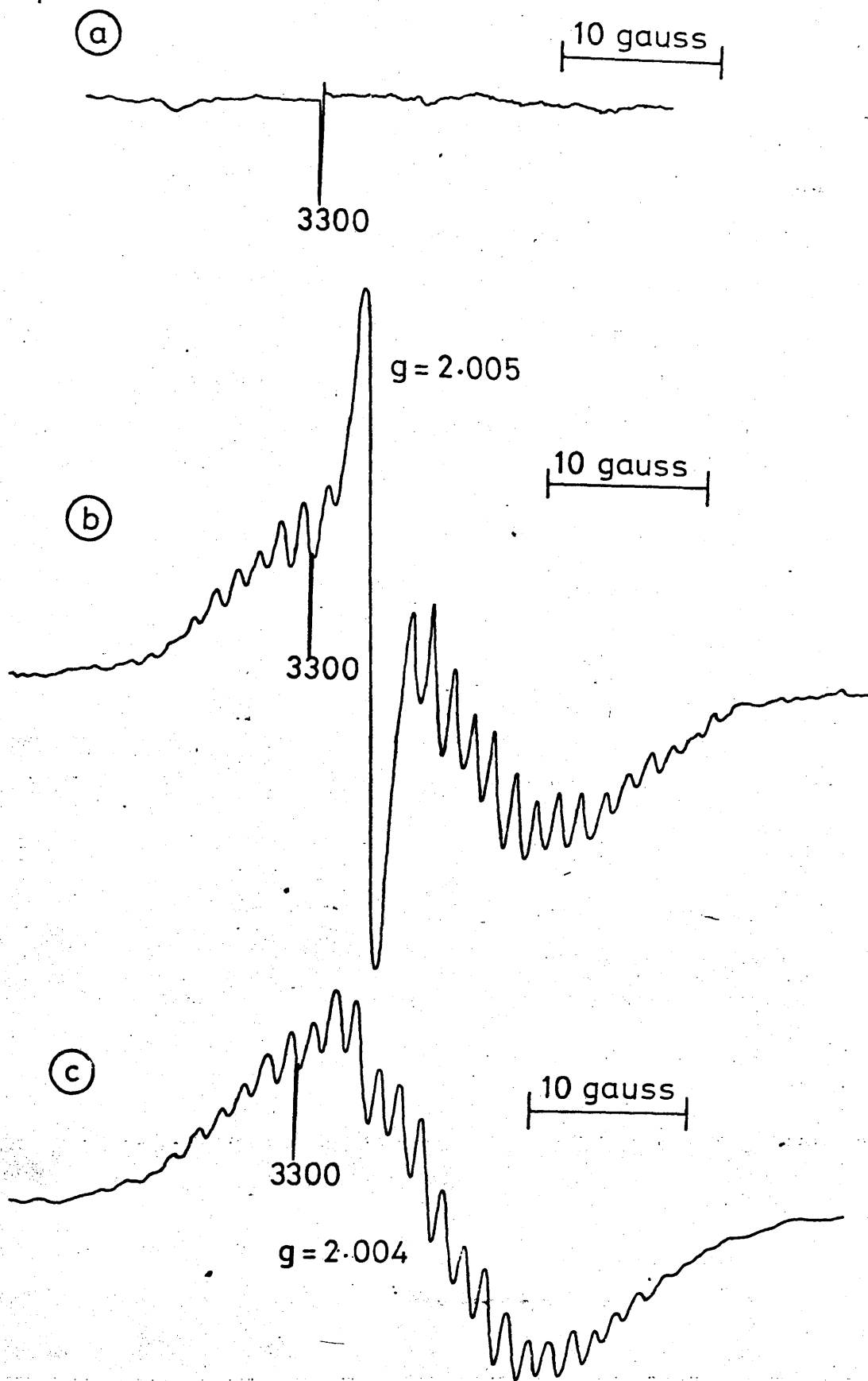


Figure 2.13 EPR spectra of the biphasic reduced methyl viologen/coenzyme Q system, obtained from (a) the bulk hexane phase, (b) the interface, and (c) the bulk aqueous phase.

system, that is, the two bulk phases and the interface, are very different from each other. This might be expected as a consequence of the proven immiscibility of the constituents of the two phases. . No EPR signal was detected from the bulk hexane phase (Figure 2.13(a)), which initially contained only oxidised coenzyme Q. In contrast, a very strong absorption centred on $g = 2.004$ was observed when the bulk aqueous phase was examined (Figure 2.13(c)). This was ascribed to the blue methyl viologen radical cation (Figure 2.15(a)) which was formed by partial reduction of the oxidised form by dithionite. (Complete reduction of methyl viologen yields a pinkish-coloured diamagnetic species.) The spectrum obtained is a broad multiplet in which about 30 peaks are resolved, and compares well with a published spectrum⁽⁸³⁾ which was determined for photochemically generated methyl viologen radicals in 95% ethanol. The strongest couplings of the unpaired electron are with the two equivalent nitrogen nuclei and with the six equivalent methyl protons, so that unpaired electron density in the radical must reside mainly on nitrogen. However, many of the weaker hyperfine interactions which were resolved in the literature spectrum were masked in the present case, probably due to a solvent effect. The EPR spectrum of the interfacial region (Figure 2.13(b)) shows a strong, sharp signal superimposed upon a fairly weak and broad multiplet. . By comparison with Figure 2.13(c) the multiplet was identified as the methyl viologen radical cation. The strong singlet, which has $g = 2.005$, was attributed to a paramagnetic species originating from coenzyme Q. The EPR spectrum of the semiquinone free radical of coenzyme Q (Figure 2.15(c)) in alkaline ethanol has been reported in the literature⁽⁸⁴⁾. In that case the signal consisted of nine evenly spaced components with a

line separation of 1.1 gauss and a g - value of 2.00467. Here, no hyperfine structure was observed, despite the fact that a splitting of 1 gauss should have been easily resolved by the spectrometer used. This implies that the unpaired electron density on the carbon atoms of the ring adjacent to the methyl and methylene groups must be very low. The most probable explanation for this is that the electron is restrained to a position near oxygen due to ion pairing or (equivalently) to formation of a charge transfer complex between CoQ^- and MV^+ or MV^{2+} .

It is interesting to note that Figure 2.13(b) bears a striking resemblance to part of the EPR spectrum obtained from the intact respiratory systems of frozen mitochondrial membranes^(85,86). A sharp, symmetric signal at $g = 2.003$ in these spectra has been attributed to the presence of small amounts of coenzyme Q semi-quinone, the absence of hyperfine structure being explained in terms of binding of the radical with protein. In this case the broader signal underlying the coenzyme Q peak was thought to be due to flavin radical species⁽⁸⁶⁾. The basic similarity between the EPR spectra obtained from intact mitochondrial membranes with that from the hexane/water interface studied here, when both are compared with the broad and highly structured signal from coenzyme Q semiquinone in a homogeneous medium⁽⁸⁴⁾, is most encouraging, for it implies that even in this very simple model system, the environment of the lipid-soluble carrier approaches that in which it finds itself in the mitochondrial membrane.

The EPR study of the reduced FMN/coenzyme Q biphasic system showed that the bulk hexane layer (Figure 2.14(a)) was again free of any paramagnetic species, whilst the bulk aqueous phase (Figure 2.14(c))

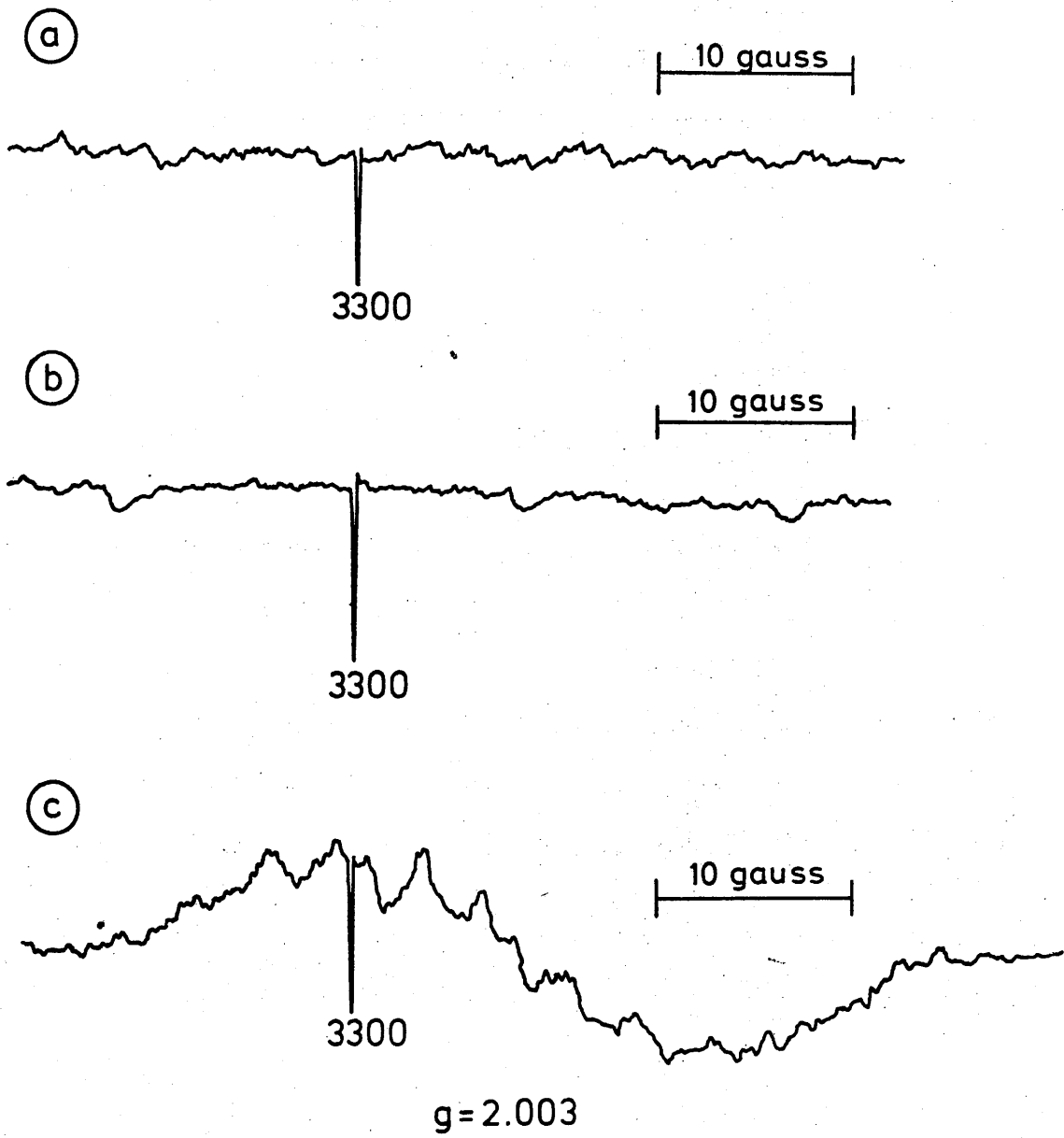


Figure 2.14 EPR spectra of the biphasic reduced FMN/coenzyme Q system, obtained from (a) the bulk hexane phase, (b) the interface, and (c) the bulk aqueous phase.

contained a free radical species which, by comparison with published spectra^(87,88), was identified as the FMN semiquinone radical (Figure 2.15(b)). The spectrum, at $g = 2.003$, was very broad and had a complex hyperfine structure which could not be clearly resolved. In contrast to the results obtained for the reduced methyl viologen/coenzyme Q system, no EPR signal could this time be detected from the interfacial region (Figure 2.14(b)). It would appear therefore that the concentration of FMN radicals immediately below the interface was much lower than that in the bulk aqueous phase, due presumably to their rapid destruction at the interface. The failure also to detect coenzyme Q radicals in this region suggests that, if indeed they were formed, their existence must have been transient. Further evidence supporting the theory that the reaction between reduced FMN and coenzyme Q can only occur via a one-electron transfer process involving radical species as intermediates comes from the observation, made during equilibration studies, that net reduction of the quinone only occurs when the green FMN free radical can be seen in the aqueous solution.

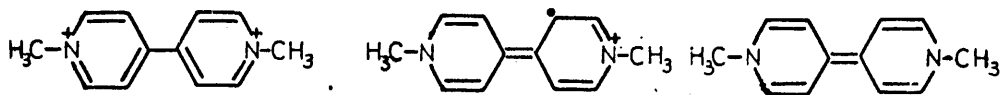
The results obtained from EPR studies of the reduced methyl viologen/vitamin K and reduced FMN/vitamin K biphasic systems⁽⁵⁷⁾ were very similar to those discussed above, except that in this case FMN free radicals were detected in the interfacial region, although still no semiquinone radicals were found. This might be accounted for by a slower rate of reaction of the FMN radicals with vitamin K than with coenzyme Q. The dihydrovitamin K/methylene blue biphasic system has also been studied⁽⁵⁷⁾, and a methylene blue radical species has been shown to exist in the interfacial region. The formation of free radical species during reduction of thionine⁽⁸⁹⁾ and indophenol⁽⁹⁰⁾

FULLY
OXIDISED

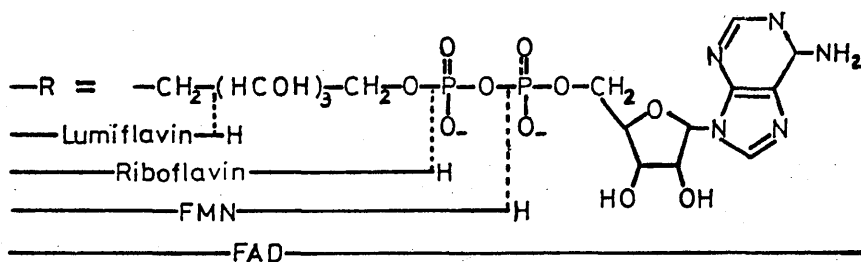
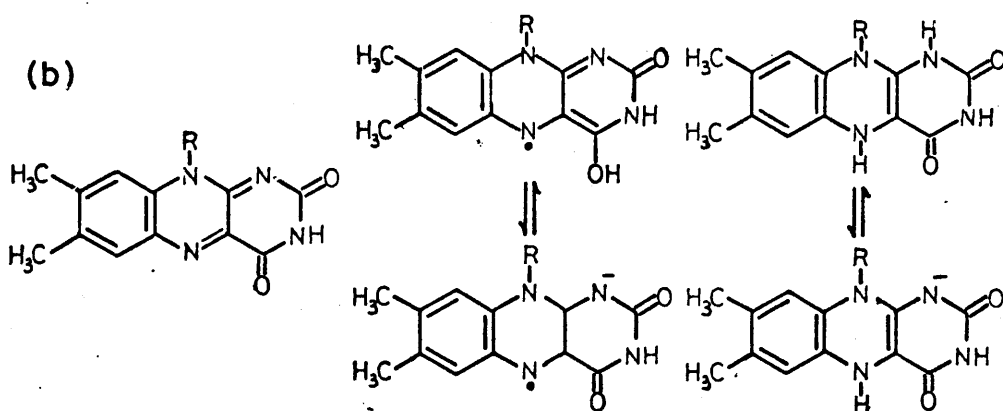
INTERMEDIATE
RADICAL

FULLY
REDUCED

(a)



(b)



(c)

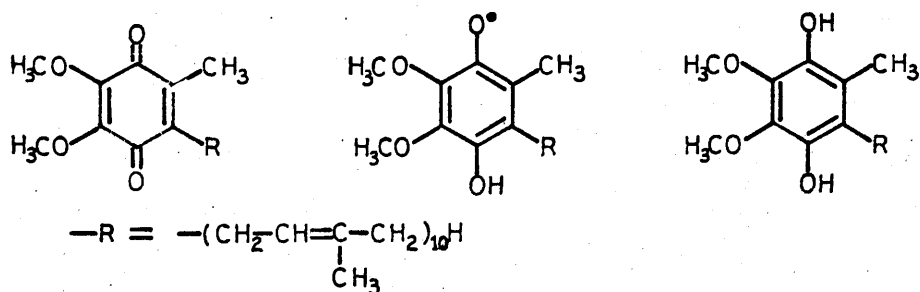


Figure 2.15 Redox states of (a) methyl viologen, (b) flavocoenzymes, and (c) coenzyme Q, showing their free radical intermediates.

also suggests that the oxidation of dihydrovitamin K by these reagents may occur in two stages involving the transfer of only one electron at each step.

In conclusion, these EPR studies have confirmed the involvement of radical intermediates and probably charge transfer complexes in a number of biphasic redox systems considered in this research. By implication it seems likely that similar one-electron transfer mechanisms will also play an important role in the remaining systems which have not as yet been subjected to EPR investigation.

Chapter Three

BULK LIQUID MEMBRANES

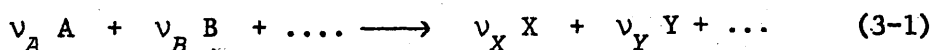
3-1 INTRODUCTION

So far attention has been focussed on the simple biphasic redox reactions of the two 'carrier' molecules under investigation — coenzyme Q and vitamin K. Aqueous reagents capable of reducing the quinones at a hexane/water interface have been found, and, in separate experiments, aqueous substrates which may be reduced by the resulting quinol forms of the carriers have been studied. It will be the first aim of the present Chapter to assemble some of these individual reactions into functional redox membrane systems, as described in Chapter 1. Although the ideality of the carriers and certain of the aqueous reductants has been demonstrated in Chapter 2, it remains to be shown that some of the aqueous substrates conform with the criteria for ideality laid down in Section 1-5. This problem will be tackled in the context of the bulk liquid membranes set up here. In addition, the factors influencing the kinetic courses of these redox reactions will be investigated, particularly with regard to the biological substrate cytochrome c, and the viability of the bulk membrane system as a model for the study of mitochondrial electron transport will be assessed.

3-1.1 Kinetics of reaction in a homogeneous phase

Since the bulk of this and the remaining Chapters of this Thesis will be concerned with the measurement and comparison of reaction rates, it is important at the outset to mention some of the general principles governing chemical kinetics, emphasising

those which are of particular interest in the present research. In a homogeneous phase, the rate at which a molecule decomposes, or a pair of molecules react, is determined principally by intrinsic properties of the molecule or the reacting pair, and, with few exceptions, cannot be predicted theoretically but must be measured experimentally. Factors which, in the absence of light, are found to influence the instantaneous rate of any reaction in solution include the reactant concentrations, the temperature, the nature of the solvent and the presence of a catalyst or inhibitor. The rate of a chemical reaction may be measured in terms of the rate of appearance of products, or, alternatively, of the rate of disappearance of reactants. Thus for a general reaction



$$\begin{aligned} \text{the Rate} &= -\frac{1}{\nu_A} \frac{d[A]}{dt} = -\frac{1}{\nu_B} \frac{d[B]}{dt} = \dots \\ &= \frac{1}{\nu_X} \frac{d[X]}{dt} = \frac{1}{\nu_Y} \frac{d[Y]}{dt} = \dots \end{aligned} \quad (3-2)$$

where $[A]$, $[B]$, ... and $[X]$, $[Y]$, ... are the molar concentrations of reactants and products, respectively. The instantaneous rate of any isothermal process in a dilute system is found to be proportional to the product of the reactant concentrations each raised to a small integral or fractional power⁽⁹¹⁾. Thus for the general reaction (3-1) a rate equation may be written:

$$\text{Rate} = k_{a+b+\dots} [A]^a [B]^b \dots \quad (3-3)$$

where a , b , ... are the orders of reaction with respect to the individual reactants A, B, ... respectively, and $a+b+\dots$ is the overall reaction order. $k_{a+b+\dots}$ is a constant of proportionality

known as the rate constant for the reaction. The rate constant tends to be very temperature dependent, and typically doubles for a 10 deg rise in temperature. In the present research, however, the reaction temperature was always kept constant at 25°C, so that the rate constants measured were all isothermal.

Equation (3-3) applies to a single step reaction: many reactions, even in a homogeneous phase, are multistep processes, in which case the overall reaction rate is proportional to the rate of the slowest step (often termed the 'rate determining step'). It should be recognised, therefore, that kinetics measurements are often of limited value in elucidating reaction mechanisms: generally the simplest mechanism which is consistent with all the available facts is accepted. Kinetic analyses of bimolecular and higher order reactions may often be simplified by keeping the concentration of one reactant constant throughout the experiment, as, for example, by using a vast molar excess of that reactant. This results in measurement of a 'pseudo' rate constant, and a reaction order which corresponds to the order with respect to the remaining reactant.

Zero-order reactions: The simplest type of kinetic behaviour is exhibited by zero-order reactions. Suppose that the concentrations of all reactants other than reactant A are held constant; then the reaction is said to be zero-order with respect to A if the rate of disappearance of A is constant:

$$- \frac{d[A]}{dt} = k_0 \quad (3-4)$$

Integration of this expression yields

$$[A]_t = [A]_0 - k_0 t \quad (3-5)$$

where $[A]_0$ is the initial concentration of A, $[A]_t$ is the

concentration of A at time t (both in mol l^{-1}), and k_0 is the zero-order rate constant ($\text{mol l}^{-1} \text{min}^{-1}$). Zero-order kinetics implies simply that the observed reaction rate is independent of the concentration of A.

First-order reactions: A first-order reaction is defined as one for which the reaction rate is directly proportional to the concentration of the reactant A, again assuming that the concentrations of other reactants are kept constant. Thus

$$- \frac{d[A]}{dt} = k_1 [A] \quad (3-6)$$

Integration of equation (3-6) gives:

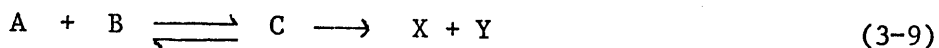
$$\log [A]_t = - \frac{k_1 t}{2.303} + \log [A]_0 \quad (3-7)$$

where k_1 is the first-order rate constant (min^{-1}). A graph of $\log [A]_t$ against time t for a first-order reaction therefore gives a straight line whose gradient is equal to $-\frac{k_1}{2.303}$. The half-life for the reaction, denoted by $t_{\frac{1}{2}}$, is defined as the time taken for the concentration of the reactant A to fall to half its initial value. Therefore, by setting $t = t_{\frac{1}{2}}$ and $[A]_t = \frac{1}{2} [A]_0$ in equation (3-7), the following expression is obtained:

$$t_{\frac{1}{2}} = \frac{0.693}{k_1} \quad (3-8)$$

The half-life of a first-order reaction is thus independent of the initial concentration of the reactant.

Ionic strength effects — the Brønsted-Bjerrum Equation: One of the factors which influences rates of reactions between charged species in solution is the ionic strength of the medium⁽⁹¹⁾. Consider a bimolecular reaction between two ionic species A and B taking place in an inert solvent. The reaction scheme may be written



where C represents an activated complex in thermodynamic equilibrium with the reactants. The reaction rate is then equal to the rate of breakdown of this complex to form the products X and Y. That is

$$\text{Rate} = k' [C] \quad (3-10)$$

Since the reactants and complex are in thermodynamic equilibrium,

$$[C] = K [A] [B] \frac{f_A f_B}{f_C} \quad (3-11)$$

where K is the equilibrium constant and the f's are activity coefficients. Substitution of equation (3-11) into equation (3-10) gives

$$\text{Rate} = k [A] [B] \quad (3-12)$$

$$\text{where } k = k' K \frac{f_A f_B}{f_C} \quad (3-13)$$

According to the Debye-Hückel limiting law

$$\ln f_i = -Az_i^2 \xi(\sqrt{I}) \quad (3-14)$$

where f_i is the activity coefficient of an ion i of valency z_i , A is a constant which depends on the solvent and on temperature, and $\xi(\sqrt{I})$ is some function of the ionic strength of the reaction medium.

Substitution of equation (3-14) into equation (3-13), with the assumption that $z_C = z_A + z_B$, yields the expression

$$\ln k = 2z_A z_B A \sqrt{I} + \ln k' K \quad (3-15)$$

Equation (3-15) is one form of the Brønsted-Bjerrum equation, describing the variation of the rate constant k with ionic strength. For the purposes of the present work it will be sufficient to note only that when the two reacting species A and B are of like charge, the reaction rate in a homogeneous solution should increase with increasing ionic strength (since screening of the ionic charges will

allow the reactants to come together more easily) whereas when A and B have opposite charges, the rate should decrease with increasing ionic strength (since screening of the charges would in that case inhibit the natural tendency of the reactants to come together).

3-1.2 Kinetics of reaction at a phase boundary — diffusion control

Thus far only homogeneous-phase reactions have been considered. However, all the redox reactions to be studied in this research took place at a liquid/liquid phase boundary. This has important consequences for the kinetic behaviour of these membrane-linked systems, since it places physical limitations on the observable reaction rates. Consider the situation where the two reactants A and B are each restricted to one of two completely immiscible liquid phases which are contained, one on top of the other, in a closed vertical tube. If the reactant B in the upper phase is maintained in large excess, so that the interfacial monolayer is always saturated with B, then the reaction with A will be dependent on at least five processes:

- (i) diffusion of A from the bulk phase to the interface
- (ii) formation of an activated complex with B
- (iii) electron transfer
- (iv) dissociation of the activated complex
- (v) diffusion of product from the interface to the bulk phase.

In practice the first of these processes is often found to be rate limiting, so that the observed reaction rate, measured as the rate of disappearance of A from the bulk solution, is equal to the rate of diffusion of A to the interface. Such a reaction is said to be 'diffusion controlled'.

The rate of fluid convection in a stirred phase decreases continuously from the bulk solution to the phase boundary, at which point, due to the action of viscous forces, it is zero. By virtue of the stirring, the concentration of solute in the bulk solution is kept uniform, but near the interface, where convection is slow, the neutralisation of concentration gradients by the stirring is much less efficient, and movement of the solute molecules must occur primarily by diffusion. Nernst⁽⁹²⁾ introduced the concept of a fictitious completely unstirred layer at a phase boundary. The transport of solute across this layer, commonly known as the 'unstirred', 'diffusion', or 'Nernst' layer, is then considered as occurring exclusively by diffusion. The thickness of the diffusion layer is very dependent upon experimental conditions, and particularly on the efficiency of stirring. At a solid/liquid interface, even under optimum stirring conditions, a diffusion layer of 20-30 microns is normally assumed to persist. In liquid/liquid systems, where the intensity of stirring may well be restricted by a need to prevent emulsification, diffusion layers are correspondingly thicker⁽⁶⁾.

If the concentration of a solute in the bulk solution is higher than its concentration at the interface, then vectorial transport of that solute across the diffusion layer towards the interface will result. The rate of diffusion is given by Fick's law:

$$-\frac{dn}{dt} = DA \frac{(c_b - c_i)}{\delta} \quad (3-16)$$

where n is the number of moles of solute transported in time t seconds, c_b and c_i are the concentrations of the solute in the bulk solution and at the interface, respectively (mol cm^{-3}), D is the

diffusion coefficient of the solute ($\text{cm}^2 \text{s}^{-1}$), A is the area of the interface (cm^2) and δ is the thickness of the unstirred layer (cm). In the reacting biphasic system considered above, where the rate of molecular interaction at the interface is much faster than the rate at which solute molecules arrive there by diffusion through the unstirred layer, the equilibrium concentration of the reactant species at the interface must be zero. Thus equation (3-16) simplifies to

$$-\frac{dn}{dt} = \frac{DA}{\delta} \cdot c_b \quad (3-17)$$

On dividing this equation by the volume V (cm^3) of the reactant solution, the rate of change of reactant concentration is obtained:

$$-\frac{dc_b}{dt} = \frac{DA}{V\delta} \cdot c_b \quad (3-18)$$

Since D , A , V and δ are normally constant throughout an experiment, it can be seen, by comparison of equation (3-18) with equation (3-6), that the observed reaction will be first-order with respect to the diffusing reactant, the rate constant k_1 being given by

$$k_1 = \frac{DA}{V\delta} \quad (3-19)$$

The parameters A , V and δ are all apparatus dependent, so the absolute rate observed is of less significance than the rate relative to those measured for other systems under comparable experimental conditions. In all the membrane systems studied in this research, the upper limit for the observable reaction rates was set by the corresponding diffusion controlled mechanism. Diffusion controlled reactions always exhibit first-order kinetic behaviour, with a rate constant which is directly proportional to the diffusion coefficient of the substrate and to the area of the interface, and inversely proportional to the volume of the substrate solution and to the thickness of the diffusion

layer. Any reaction which is not diffusion controlled must exhibit a slower rate than that predicted by diffusion control.

3-2 EXPERIMENTAL

3-2.1 Apparatus

'H-shaped' cell for generation of bulk liquid membranes: This piece of apparatus was used in initial studies to establish continuous redox processes across bulk liquid membranes. It was constructed simply by joining together two pyrex glass phials (described in Section 2-2.2) by means of a short horizontal bridge of glass tubing, 5 cm long and 1 cm in diameter, positioned about 3 cm above the bulbs.

Silica H-cell for kinetics determinations: This specially designed reaction cell (Figure 3.1) was used in all kinetics experiments involving bulk liquid redox membranes to be described here. The lower limbs of the cell consisted of 'spectrosil' optical cells of 1 cm path length and volume 4 cm^3 ; the upper limbs and cross-member were made of quartz glass tubing and were fused to the optical cells. The dimensions of the cell were chosen so as to allow it to fit into the sample holder of the Hilger H700 spectrophotometer. The aqueous solutions were held in the optical cells and the hexane 'membrane' formed the bridge between them. Stirring in the aqueous phases was effected by two specially designed magnetic stirrers (see below) turned by coupling to two external 'button' magnets, the rotational speeds of which were individually adjustable over the range 0-2,000 revolutions per minute (rpm). Turbulence and mixing in the upper hexane layer was achieved by slowly rocking a pivoted platform which held the cell and stirrer unit (Figure 3.1). A perspex wheel was attached to a low-g geared electric motor turning at 30 rpm. A second

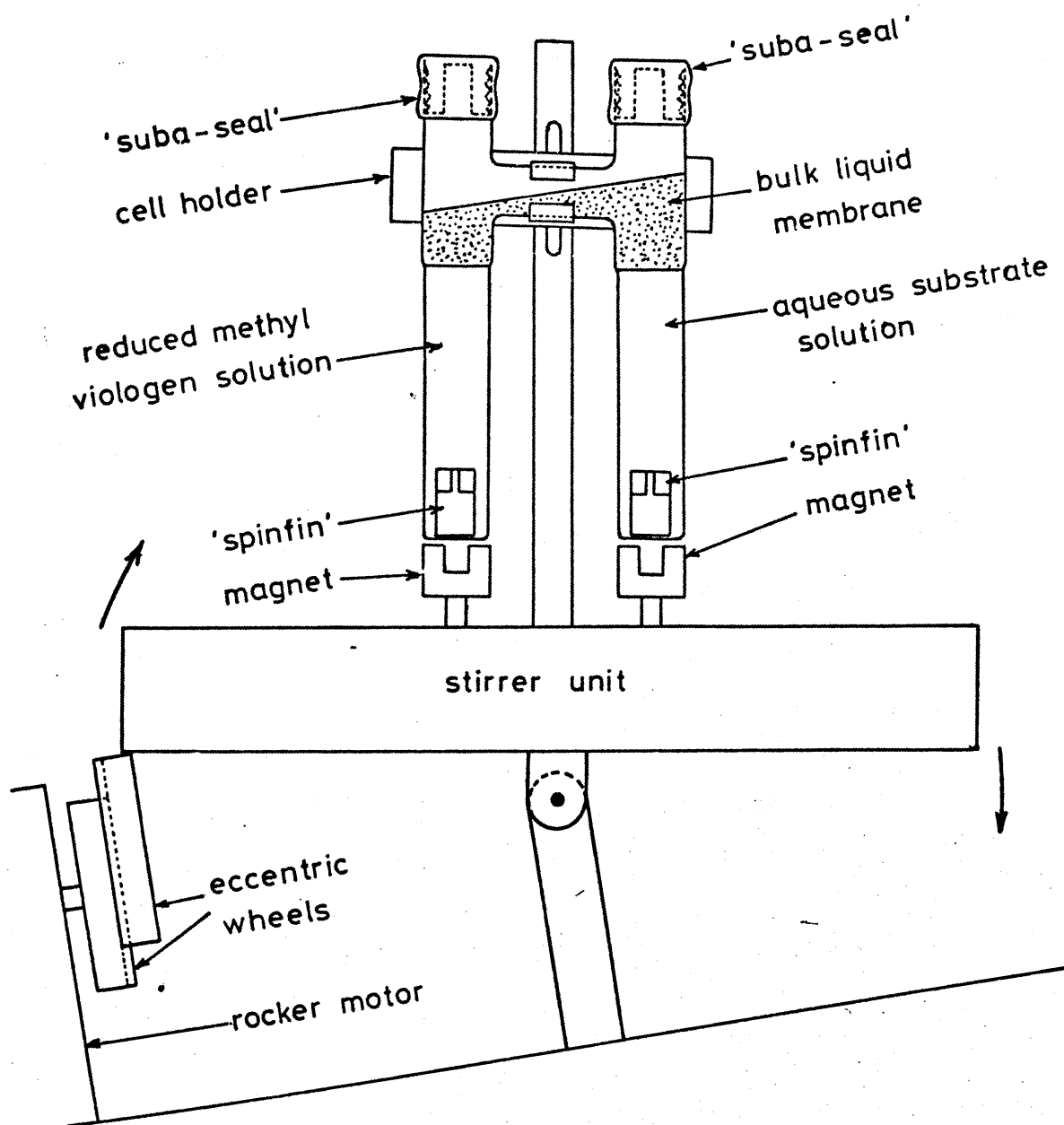


Figure 3.1 Apparatus for bulk liquid membrane kinetics experiments.
 See text for description (Section 3-2.1).

perspex wheel was positioned eccentric to the first by means of a tight tongue-in-groove type of fitting which allowed the degree of eccentricity to be varied, hence altering the displacement of the platform during rocking. The rim of the second wheel was liberally greased with 'Vaseline' to reduce friction with the perspex platform. In practice, one suitable position of the eccentric was found and this position was kept constant throughout all the experiments. The temperature of the reaction was maintained at $25.0 \pm 0.1^{\circ}\text{C}$ by enclosing the whole apparatus in an air thermostatted cabinet.

The H-cell and other reference optical cells were cleaned by the method described in Section 2-2.2 before use.

Stirrers: In initial experiments involving use of the silica H-cell, small perspex stirrers were used to provide mixing in the aqueous limbs. Control experiments, however, demonstrated that they slowly but continuously adsorbed redox dyes onto their surfaces, which showed no tendency to become saturated at the dye concentrations used. They were therefore replaced in all subsequent kinetics experiments by Teflon 'spinfin's' (Bel-Art Products, USA) which were specially designed for use in optical cells. Dye adsorption in this case was not nearly so marked, and stopped after about thirty minutes equilibration in the dye solution, by which time only about 5-10% of the total dye present had become adsorbed. By comparison of the optical densities of the solution before and after equilibration with the stirrer (when the optical density had fallen to a stable value) the total dye uptake could be estimated. Furthermore, by assuming that a dynamic equilibrium existed between adsorbed and free dye, such that the proportional reduction in

(oxidised) dye concentration in the bulk solution during reaction was mirrored by a corresponding reduction in the amount of dye bound to the stirrer, the observed optical density readings could be corrected for dye uptake by the stirrer. However it was found⁽⁵⁷⁾ that the added correction factors made very little difference to the rate constant finally calculated, the discrepancy between corrected and uncorrected results being typically around 0.2%. Therefore, since only relative reaction rates were to be considered significant and since all the measured rates should be affected by very similar extents, it was decided that the application of stirrer uptake corrections in the present research was hardly justified.

3-2.2 Chemicals

The sources and purities of all the chemicals used in these studies are listed in Appendix I.

Aqueous solutions, unless otherwise specified, were made up in 0.025 M phosphate buffer, pH 6.86. In the case of cytochrome c, for which variations in the reaction rate due to changing ionic strength were to be studied, solutions were made up in phosphate buffer, pH \sim 6.8 or in tris-(hydroxymethyl) aminomethane — hydrochloric acid ('Tris-HCl') buffer, pH \sim 7.3, of various concentrations, and with sodium chloride added as necessary to reach the required ionic strengths. Each cytochrome c solution was prepared on the day prior to the experiment and was stored overnight at 4°C.

Spectroscopic hexane was used throughout as the solvent for coenzyme Q and vitamin K in the membrane phase.

The concentrations of coenzyme Q, vitamin K, DCIP and methylene blue solutions were determined by measurement of their

optical densities (or those of standard dilutions) at the wavelengths of maximum absorption, and by use of the extinction coefficients quoted in Section 2-3.1 and Table 8. Samples of the iron-o-phenanthroline complex and cytochrome c had first to be converted completely to their respective reduced forms by shaking with excess solid sodium dithionite before optical densities were measured.

The phospholipids used in these studies were cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and lysolecithin (LL). The purity of each of these phospholipids as supplied was first checked by thin layer chromatography on silica gel GF 254 (Merck, Germany), the plates being developed with chloroform:methanol:water, 65:25:4,⁽⁷¹⁾ and visualised with iodine vapour. In each case a single spot was obtained, although there was some evidence for 'tailing', particularly in the case of cardiolipin. Cardiolipin was supplied as a solution, 9.3 mg ml^{-1} , in ethanol, and aliquots of this solution were added to the membrane as required. Stock solutions of the other phospholipids were prepared, each about 8.3 mg ml^{-1} in 9:1 (v/v) $\text{CHCl}_3:\text{CH}_3\text{OH}$. These were stored under nitrogen in sealed ampoules at -20°C until required.

3-2.3 Generation of bulk liquid redox membranes

The 'H-shaped' cell described in Section 3-2.1 was used in these initial experiments. 4.5 ml of 2.7×10^{-2} M methyl viologen solution and 5.0 ml of the aqueous substrate solution, both made up in 0.025 M phosphate buffer, pH 6.86, and freshly deoxygenated, were introduced into opposite bulbs of the cell. The H-cell was

then closed by means of two suba-seals and was flushed with nitrogen for one hour. 0.5 ml of sodium dithionite solution, freshly prepared by dissolving 22 mg of the solid in 1 ml of deoxygenated 0.025 M phosphate buffer, was next added via a syringe needle to generate the blue radical reducing species of methyl viologen. The membrane layer was then formed by the addition of sufficient $\sim 4 \times 10^{-4}$ M coenzyme Q in hexane to almost completely fill the horizontal bridge (about 20 ml). The sealed H-cell was left on the vibro-mixer for a period of 12-48 hours at 25°C and protected from light. After the reaction period, the aqueous substrate was examined visually or (where possible) spectrophotometrically to estimate the extent of reduction. A sample of the hexane layer was also removed to determine the oxidation state of the coenzyme Q (from its UV spectrum). If at the end of the reaction the methyl viologen was colourless and the coenzyme Q substantially oxidised, it was concluded that there was an air leak in the system, the results were disregarded, and the experiment repeated.

3-2.4 Procedure for H-cell kinetics experiments

Because of the different problems which arose with each new substrate studied, it was necessary to refine experimental techniques as the work progressed. From preliminary experiments on vitamin K bulk membrane systems⁽⁵⁷⁾ it was known that, without mixing in the membrane layer (by rocking of the apparatus), long induction periods were observed preceding a reaction which was zero-order with respect to the substrate. This behaviour was indicative of diffusion control in the hexane phase and had to be avoided if meaningful results were to be obtained. Therefore in all experiments to be described here

membrane agitation was used, and the frequency and amplitude of the rocking motion were kept constant. It was also found that when the membrane-soluble carrier was initially present entirely in its oxidised form, an induction period resulted⁽⁵⁷⁾. This effect was here counteracted by initiating all kinetics runs with the carrier in the completely reduced form. Throughout these experiments, the stirring speeds in the two aqueous limbs of the H-cell were kept as nearly as possible constant, at 1650 ± 100 rpm in both oxidant and reductant solutions. Three main techniques, outlined below, were employed to fill the silica H-cell (Figure 3.1):

Method A: The aqueous methyl viologen and substrate solutions were degassed at the water pump and brought back to atmospheric pressure with oxygen-free nitrogen before use. Methyl viologen was 2.7×10^{-2} M and substrate concentrations were in the range 5×10^{-6} M - 1×10^{-4} M, all solutions being made up in 0.025 M phosphate buffer, pH 6.86.

4.0 ml aliquots of each were measured by pipette into opposite limbs of the H-cell, which already contained the stirrers, care being taken to ensure that the same stirrer was placed in a given limb throughout all the experiments. Two suba-seals were fitted to the cell, which was then flushed with nitrogen for thirty minutes before the addition of 0.5 ml of sodium dithionite solution (22 mg ml^{-1} , made by dissolving the solid in freshly degassed 0.025 M phosphate buffer) to generate the blue methyl viologen radical. 0.5 ml of coenzyme Q (or vitamin K) solution, 3.6×10^{-3} M, in spectroscopic hexane, was then added above the methyl viologen, and the cell was left stirring and rocking for about one hour, by which time the carrier was reduced to the colourless quinol form. The membrane layer was formed, and reaction started,

by addition of two 2 ml aliquots of hexane to the cell, using a Hamilton syringe. If phospholipid was to be present, then it was also added at this stage.

Method B: In initial experiments on the reduced methyl viologen/vitamin K/cytochrome c system⁽⁵⁷⁾ using method A above, it was found that the kinetics of reduction of the cytochrome were complicated by the presence of traces of oxygen in the system. It was therefore necessary, when studying reactions involving cytochrome c, to take all possible precautions to ensure that conditions within the H-cell were kept completely anaerobic. Method A was unsatisfactory since neither the hexane nor the phospholipids added were deoxygenated prior to their introduction into the cell, so some modification to the procedure was made. The limbs of the H-cell were first filled with deoxygenated methyl viologen and cytochrome c solutions, and the cell was closed with two suba-seals, secured with copper wire. Nitrogen was bubbled slowly through the cytochrome c solution for about thirty minutes before the methyl viologen was reduced by addition of 0.5 ml of sodium dithionite solution (as above). At the same time, vitamin K solution (3.6×10^{-3} M) and spectroscopic hexane were separately equilibrated over solutions of reduced methyl viologen under strictly anaerobic conditions. After about one hour, the vitamin K was fully reduced and both hexane phases were assumed to be thoroughly deoxygenated. Using gastight Hamilton syringes to transfer the solutions, 4.0 ml of deoxygenated hexane was added to the H-cell. This and subsequent additions were made just below the surface of the reduced methyl viologen solution to ensure that any traces of residual oxygen were scavenged. Any phospholipid required

was then added as 0.03 ml of solution in ethanol (for CL) or in 9:1 chloroform:methanol, and finally 0.5 ml of the reduced vitamin K solution was added to initiate the reaction. After all the required additions had been made, the puncture marks on the suba-seals were covered with 'Araldite' as a final precaution.

Method B was employed in the vast majority of the kinetics experiments described in this research when cytochrome c was used as substrate, but a disadvantage of this method eventually became apparent. The addition of phospholipid to the aqueous phase, just below the methyl viologen/hexane interface, led to the immediate formation of a layer of lipid on that interface. In order to exert any influence on the rate of cytochrome c reduction, however, some of the phospholipid had to be transferred to the opposite interface. An observation that the steady zero-order reaction rate obtained by method A after the initial long induction period⁽⁵⁷⁾ was equal to that obtained by method B in one particular experiment (involving the reduction of cytochrome c in 0.025 M phosphate buffer, by dihydrovitamin K in the presence of added cardiolipin) had been taken to indicate that transfer of phospholipid to the substrate interface was rapid in the latter case, the interface being essentially saturated with phospholipid before the first optical density reading was taken (that is, about twenty minutes after the addition). However, in view of subsequent results (discussed later) it seemed unlikely that this was the case and that there was in fact a slow buildup of phospholipid at the substrate interface. This was demonstrated in the following simple experiment, which was based on the assumption that such a buildup would cause a corresponding decrease in the interfacial tension, as indicated by the gradual flattening of the meniscus.

The H-cell was set up as for a normal kinetics run, with reduced methyl viologen in one limb and with hexane forming the membrane layer, but with phosphate buffer only in the substrate limb. The cell was carefully positioned in a firmly mounted holder in front of a vertically travelling microscope and the initial depth of the meniscus was read from the vernier scale. 0.5 ml of reduced vitamin K solution was then added to the membrane and the meniscus depth was remeasured. No change was observed even after twenty minutes on the rocker, suggesting that the naphthoquinol itself had little effect on the interfacial tension, as might have been expected from the relatively low hydrophilicity of the head-group. 0.03 ml of the stock cardiolipin solution was next added, the addition being made just below the methyl viologen/hexane interface, in accordance with method B above. The depth of the meniscus, measured immediately, showed no change. The H-cell was then placed on the stirring/rocking apparatus, operating at normal speeds, and was removed at twenty minute intervals for measurements to be made. It was found that the meniscus depth gradually decreased, but the interface only became completely flat after a period of about four hours had elapsed since the original addition of phospholipid.

Method C: In view of the above result, it was necessary to modify method B so as to speed up the formation of a stable lipid monolayer (consisting of equilibrium proportions of phospholipid and vitamin K) at the substrate interface. Direct addition of phospholipid to the substrate limb of the H-cell was ruled out because of the danger of micelle formation in the aqueous solution which was under spectrophotometric examination. Instead the phospholipid solution was injected into the methyl viologen limb, but in a position just above

the interface, which flattened immediately. On rocking the cell for a few seconds, the opposite meniscus, at the hexane/substrate interface, also flattened. This was taken to indicate rapid equilibration of the phospholipid across the membrane layer, as required. The addition of the stock phospholipid solution directly into the hexane phase necessitated previous careful deoxygenation of the solution, a function which in method B had been performed *in situ* by the methyl viologen. Deoxygenation was achieved by bubbling nitrogen very slowly through the stock phospholipid solution contained in a narrow sealed tube, the nitrogen having been pre-saturated with solvent to avoid changing the solution concentration. Apart from these alterations concerned with the pretreatment and addition of phospholipid solutions, method C was identical to method B above.

3-2.5 Generation and treatment of results

The kinetics of reduction of the aqueous substrate was followed by measurement of the changing optical density of the solution at regular time intervals throughout the reaction. All optical density measurements were made using the Hilger H700 UV/visible spectrophotometer (Section 2-1.2) at a fixed wavelength corresponding to λ_{max} for the substrate under investigation (Table 8). The cell carrier in the spectrophotometer was fitted with a small magnet which lifted the stirrer in the H-cell limb out of the light beam before measurements were made. Care was also taken to ensure that the raised stirrer did not come into contact with the hexane/water interface. Two matched optical cells, the one filled with buffer and the other with substrate solution, acted as 'blank' and 'control'

respectively in each experiment. The absorbances of the substrate solution in both the H-cell and control cell were measured at various times during the initial equilibration period: when the difference between successive readings from the substrate limb of the H-cell became insignificant (< 0.003), it was assumed that a condition of dynamic equilibrium had become established between free substrate and substrate adsorbed on the stirrer. When this condition was satisfied, reaction was initiated by completion of the membrane layer; otherwise the equilibration period was extended. In practice, when Teflon 'spinfins' were used (that is, in all but a few initial experiments), equilibrium was established within thirty minutes with every substrate studied.

After the start of the reaction, optical density readings of the substrate in the H-cell and in the control cell were taken every 20-30 minutes, depending on the reaction rate, the cell being transferred from the thermostatted cabinet through the air to and from the spectrometer for each reading. Since the time taken to make each measurement was about one minute, and the half-lives of all the reactions studied were greater than forty minutes, it was considered that this short interruption to stirring and accurate temperature regulation should not have any significant effect on the progress of the reaction. The optical density readings from the control cell served as a check on instrument reproducibility: in each experiment the control absorbances never varied by more than ± 0.005 units from the initial value.

The absorbances obtained from the H-cell substrate limb were converted to concentrations of the oxidised form of the dye by application of equations (2-4) and (2-7) as necessary. The wave-

lengths and extinction coefficients used are listed in Table 8. For each experiment the results were analysed graphically: graphs of concentration of oxidised substrate against time and $\log(\text{concentration of oxidised substrate})$ against time were drawn. From these graphs the order of reaction was deduced and the appropriate rate constant calculated, as indicated in Section 3-1.1.

3-3 RESULTS AND DISCUSSION

3-3.1 Coenzyme Q as an electron carrier

The bulk membrane systems dealt with in this Section may be represented by the general system aqueous reduced methyl viologen/CoQ in hexane/aqueous substrate. Initially the bulb-type H-cell (Section 3-2.1) was used to generate liquid redox membranes: DCIP, ferriin and cytochrome c substrates were studied by this method (Section 3-2.3). When DCIP was used as substrate, the solution, initially pale blue, was colourless after twelve hours on the vibro-mixer. This was taken to indicate that electron transfer, mediated by the membrane-bound coenzyme Q, had taken place in this system. It soon became apparent, however, that this result could also be explained in terms of back-transport of the protonated form of the dye, since, in a second experiment in which phosphate buffer alone replaced the reduced methyl viologen, a marked decrease in the colour intensity of the DCIP substrate was again observed. DCIP is a weak acid with $pK_a = 5.7^{(93)}$, so that at pH 7, about 5% is present in the protonated form. This form, which is red in colour, is very soluble in hexane and so is continuously extracted into the membrane layer. This thermodynamic equilibrium

between protonated and dissociated forms in the aqueous phase causes replacement of the red form as soon as it is extracted, and this mechanism leads to gradual loss of both forms of the dye from the substrate limb of the cell. It is evident therefore that DCIP could not be considered as an 'ideal' substrate under the criteria laid down in Section 1-5. However it was the first to be studied, and several observations of relevance to subsequent work were made on this non-ideal system.

A number of kinetics runs were also performed using DCIP as substrate. In these initial experiments perspex stirrers were employed, but these were found to adsorb large quantities of the dye. (The optical density of a 1.4×10^{-5} M DCIP solution dropped by almost half after two hours equilibration with the stirrer.) This problem was partially overcome by allowing the stirrer to equilibrate in a 1.4×10^{-5} M solution of DCIP for 24 hours prior to the run. The stirrer was washed thoroughly with distilled water immediately before use in the H-cell. Thereafter the uptake of dye was minimal, as evidenced by a fairly constant set of optical density readings obtained from the DCIP in the substrate limb. The H-cell was filled, and reaction started, according to method A above. Graphs of absorbance against time and \log (absorbance) against time were drawn in each case: the results from a typical kinetics run are shown in Figure 3.2. The reduction of DCIP by reduced coenzyme Q followed first-order kinetics, the average parameters obtained from a number of experiments being $k_1 = (3.2 \pm 0.1) \times 10^{-3} \text{ min}^{-1}$ and $t_{\frac{1}{2}} = 217 \pm 7$ minutes. These results are consistent with diffusion control on the substrate side of the interface, as will be demonstrated later with particular reference to the vitamin K/methylene blue system.

Figure 3.2 Kinetics of reduction of DCIP by reduced coenzyme Q.

Graphs of (a) absorbance at 605 nm, and (b) \log (absorbance), plotted against time for the system :

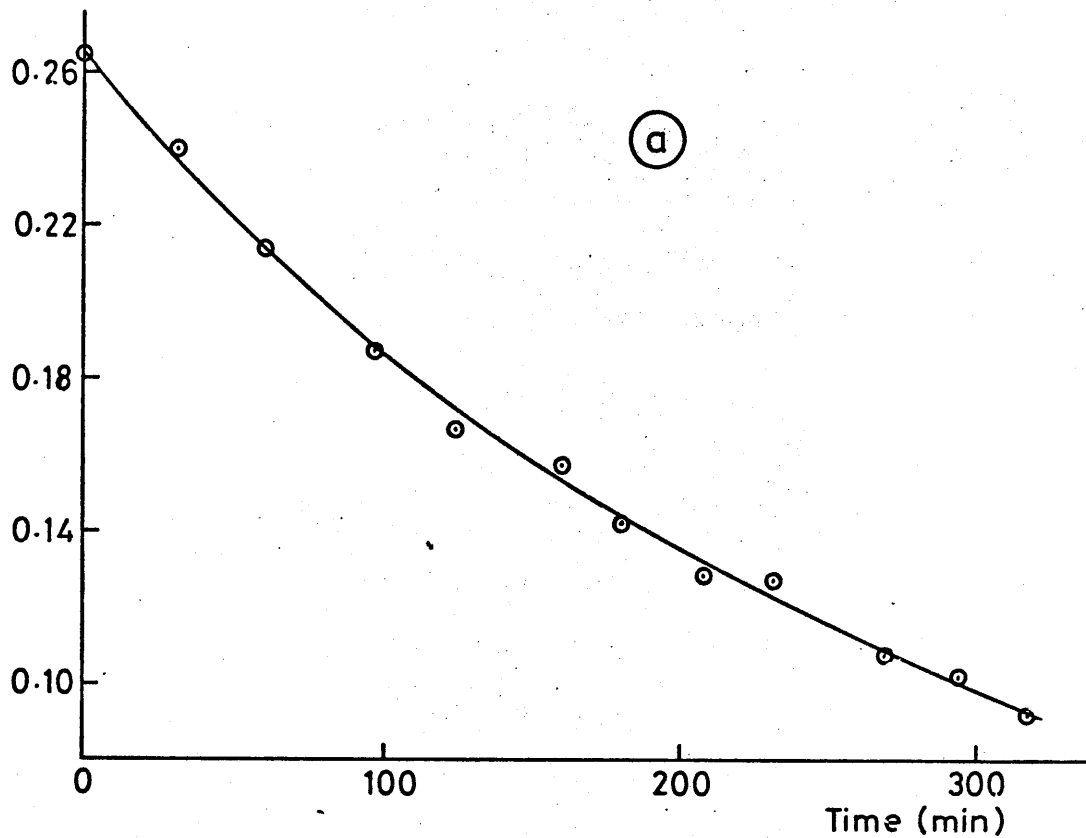
reduced methyl viologen (0.025M phosphate buffer, pH 6.86)	coenzyme Q (hexane)	DCIP (0.025M phosphate buffer, pH 6.86)
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The concentrations of the solutions were: methyl viologen — 2.7×10^{-2} M;
coenzyme Q — 4×10^{-4} M; DCIP — 1.27×10^{-5} M.

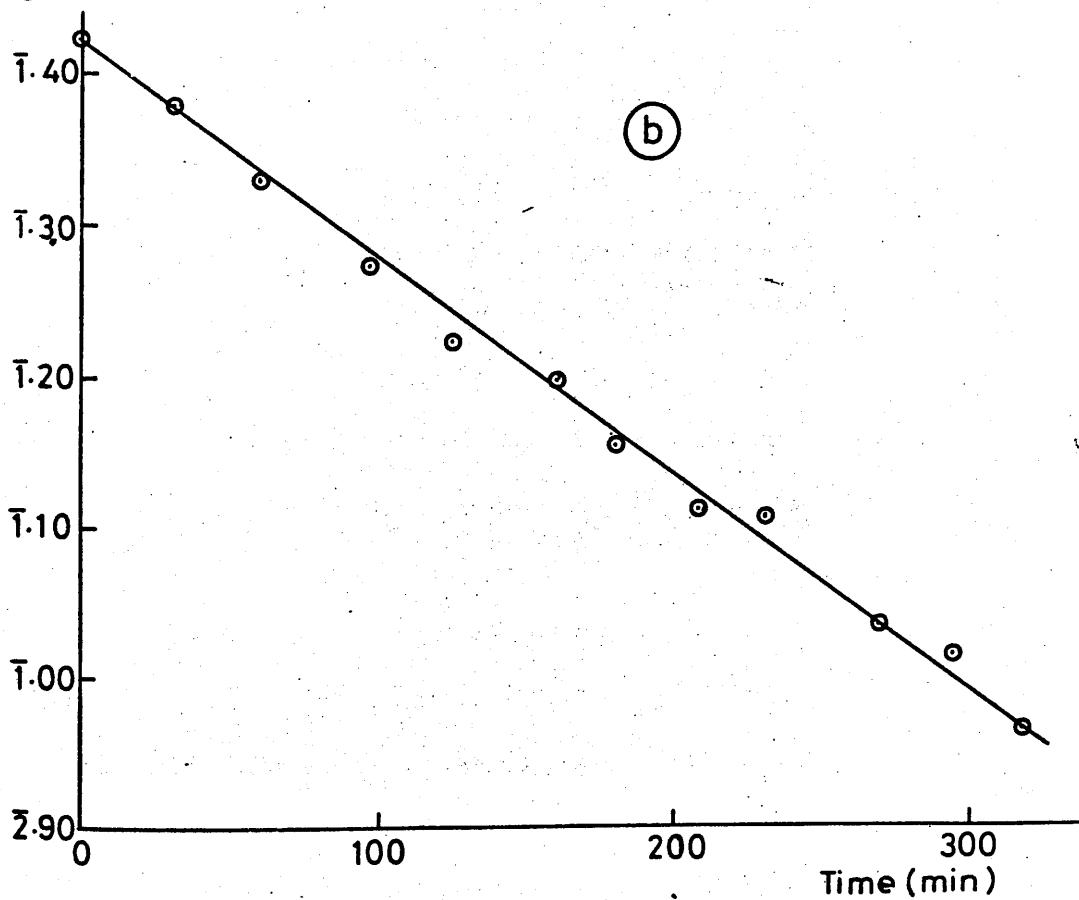
The experimental procedure followed is detailed under method A
(Section 3-2.4). Standard stirring and rocking conditions were employed.
Temperature $25.0 \pm 0.1^\circ\text{C}$.

Figure 3.2

Absorbance



log(absorbance)



In a separate experiment in which no carrier was present in the membrane layer, the rate of disappearance of DCIP from the substrate limb was found to be very much slower, with $k_1 \sim 0.7 \times 10^{-3} \text{ min}^{-1}$. This must correspond to the rate of removal of the protonated form of the dye into the bulk hexane phase. Since the overall reaction is diffusion controlled and since both forms of the dye are rapidly reduced by reduced coenzyme Q, the loss of oxidised DCIP by dissolution in the membrane should be indistinguishable from its loss by reduction at the interface. Therefore the existence of the former mechanism should not interfere with the measurement of reduction kinetics under these conditions, assuming that every DCIP molecule, whether protonated or dissociated, is reduced when it reaches the interface. In fact the measured rate constant requires to be increased by about 5% to take into account the protonated component of the equilibrium mixture which does not absorb at 605 nm (λ_{max} for the blue form). This gives a corrected rate constant $k_1 \sim 3.4 \times 10^{-3} \text{ min}^{-1}$ for the reduced coenzyme Q/DCIP system.

In a parallel series of experiments in which vitamin K replaced coenzyme Q as the electron carrier, a first-order rate constant $k_1 = 4.4 \times 10^{-3} \text{ min}^{-1}$ was obtained⁽⁵⁷⁾. Assuming in each case a diffusion controlled mechanism, the rate constants for the coenzyme Q and vitamin K systems should be identical. The observed difference was most probably due to the fact that different sets of apparatus (H-cells and stirrers) were used in the two series of experiments, since the kinetics of diffusion controlled reactions are very apparatus dependent (Section 3-1.2).

When the redox indicator methylene blue was used as substrate, the reaction with reduced coenzyme Q was extremely slow, amounting

to less than 10% in the first four hours. The slowness of the reduction must in this case be due primarily to thermodynamic factors: the midpoint potential of methylene blue at pH 7 ($E_{m7} = + 0.011$ volt, Table 7) is actually somewhat lower than that estimated for coenzyme Q under the reaction conditions ($E_{m7} = + 0.075$ volt, Section 2-1.1). The fact that reaction occurs at all may be explained in terms of a continuously shifting equilibrium between the oxidised and reduced forms of each of the participants, caused in the case of coenzyme Q by continuous reduction of the oxidised form at the opposite interface, and in the case of methylene blue by continuous extraction of the reduced reaction product into the hexane layer (see below). Despite repeating the experiment several times, it was impossible to judge from the results whether the reaction followed first- or zero-order kinetics. Certainly the results in this case were inconsistent with a diffusion controlled mechanism.

The iron (III)-*ortho*-phenanthroline complex (ferriin, $E_{m7} = + 1.12$ volts) was also investigated as a possible substrate for use in these bulk membrane systems. Initial experiments carried out in the bulb-type H-cell (Section 3-2.1) indicated that the colourless complex was partially reduced by reduced coenzyme Q to the red ferriin form on overnight equilibration. It was found that a control ferriin solution equilibrated anaerobically under pure hexane also tended to turn slightly red, but this 'spontaneous reduction', which occurred in pH 6.86 phosphate buffer in the absence of any added reductant, was much slower than that effected by the reduced carrier. Kinetic analysis of the carrier mediated reaction showed that the rate of ferriin reduction was extremely slow, only about 20% of the complex

being reduced in twenty hours under normal experimental conditions in the H-cell. Again it was impossible to tell from the results whether the reaction was first- or zero-order with respect to the oxidised substrate. A further complication became apparent when a control experiment was performed to check the immiscibility of the iron complex in the hexane layer. The H-cell was set up with ferroin solution in the 'substrate' limb, 0.025 M phosphate buffer in the 'reductant' limb, and with spectroscopic hexane forming the 'membrane' between them. The optical density of the red iron (II) complex, monitored at 510 nm, was found to decrease slowly with time, while the opposite limb, containing only phosphate buffer, showed no increase in absorbance at this wavelength. However, the ultraviolet absorption spectrum of the buffer indicated the presence of uncomplexed o-phenanthroline in the solution. Thus it would appear that, despite the high thermodynamic stability of the complex (the stability constant for ferroin is about $2 \times 10^{21} \text{ mol}^{-3} \text{ l}^3$ (94)) the ligand, which is hexane soluble, was being removed from the substrate and back-transported in its uncomplexed form to the opposite aqueous limb. Ferroin therefore could not be considered as an 'ideal' substrate as defined in Section 1-5, although it was later to prove useful in one particular experiment (described in Section 4-3.2).

The final substrate used in conjunction with coenzyme Q in these kinetics studies was cytochrome c. Horse heart cytochrome c (Sigma, Type III) was used throughout since it was the only type readily available in high purity. Method B (Section 3-2.4) was employed routinely to avoid any interference by traces of oxygen which might otherwise have been introduced into the system. Here again 'spontaneous reduction' complicated the

analysis of results. This was found to occur in 25 μM cytochrome c solution prepared in 0.025 M phosphate buffer, pH 6.86, when the solution was equilibrated in the H-cell beneath a pure hexane membrane, or when there was no membrane present and only nitrogen filled the upper sections of the H-cell. The spontaneous reduction process in each case followed zero-order kinetics, with a rate constant $k_0 = 2.7 \times 10^{-9} \text{ mole l}^{-1} \text{ min}^{-1}$. The coenzyme Q - mediated reduction process was also zero-order with respect to ferricytochrome c, and had $k_0 = 3.3 \times 10^{-9} \text{ mole l}^{-1} \text{ min}^{-1}$. The similarity of these two rates over the relatively short time period of a kinetics run cast some doubt on the ability of reduced coenzyme Q to effect reduction of ferricytochrome c. As an additional check, therefore, the 'test' (with carrier present in the membrane layer) and 'control' (with only hexane forming the membrane) systems were set up in two bulb-type H-cells which were hermetically sealed and left on the vibro-mixer for eight days before opening. At the end of this period the 'test' cytochrome c was about 25% reduced, whilst the 'control' sample was less than 10% reduced. This established the fact that reduced coenzyme Q is capable of reducing ferricytochrome c, although the reaction is extremely slow.

The autoreduction of horse-heart ferricytochrome c in aqueous solution as observed here has been noted before⁽⁹⁵⁾. Although the source of the reducing equivalents is unclear, the reduction of the haem centre is proposed to be an intramolecular process involving an electron transfer from the polypeptide chain rather than from the solvent⁽⁹⁵⁾. Autoreduction has also been observed after adsorption and elution of cytochrome c from solid particles⁽⁹⁶⁾, so it seems likely that an interfacial effect is involved. When 0.03 ml

of cardiolipin solution (Section 3-2.2) was added to the membrane containing reduced coenzyme Q, at the start of a kinetics run, the rate of reduction of ferricytochrome c was lowered, the new zero-order rate constant being 2.5×10^{-9} mole l^{-1} min $^{-1}$. This is in marked contrast to the effect of cardiolipin on the vitamin K - mediated reaction in which the rate of cytochrome reduction was substantially increased in the presence of the acidic phospholipid (Section 3-3).

It is useful at this point to summarise the results obtained from these kinetics experiments involving coenzyme Q as the electron carrier. Of the four reducible substrates investigated — DCIP, methylene blue, ferriin and ferricytochrome c — only DCIP reacts rapidly with reduced coenzyme Q, the reaction being (probably) diffusion controlled in this instance. However continuous back-transfer of the protonated form makes DCIP a non-ideal substrate. The others react only extremely slowly: in the case of methylene blue this is probably due mainly to thermodynamic factors, whereas in the ferriin and ferricytochrome c systems mechanistic factors must play the major role. The reluctance of coenzyme Q to react directly with cytochrome c is of particular interest, since both are known to be electron carriers in the mitochondrial respiratory chain (Section 1-3). In the mitochondrial membrane electron transfer from the coenzyme to the cytochrome occurs by way of the enzyme complex III (cytochrome b-c₁). The present results indicate that mediation by this enzyme is indeed necessary if the electron transfer process is to operate efficiently. Several attempts were in fact made in this research to incorporate the b-c₁ complex into the coenzyme Q/ cytochrome c interface in the hope of stimulating the reaction, but

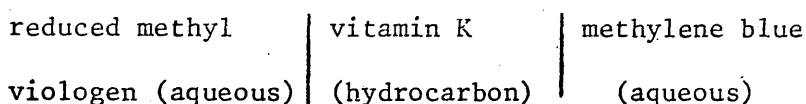
these failed due to inactivity of the enzyme preparation supplied. However, an assay procedure, based on the method of Rieske⁽⁹⁷⁾ has been devised, and further progress awaits the supply of an active enzyme preparation. Considering the experimental difficulties involved in the measurement of very slow reactions, it was decided to abandon further direct study of coenzyme Q as an electron transfer agent in these model membrane systems, at least until a ready supply of active b-c₁ complex could be obtained.

3-3.2 Vitamin K as an electron carrier

After the problems encountered with coenzyme Q, attention was focussed on vitamin K in the hope that it would prove to be a more useful reagent. Fortunately this was the case. Bulk redox membrane systems utilising vitamin K as the electron carrier were developed by Anderson⁽⁵⁷⁾ in work which paralleled that described above on coenzyme Q. Since the remainder of this Thesis is based on the use of vitamin K in model membrane systems, it is necessary here to summarise the results of this initial work, which was also extended in the present research. DCIP, methylene blue and cytochrome c were the relevant substrates studied. The experimental methods employed were identical to those described above (Section 3-2.4).

The redox indicator methylene blue is readily reduced by dihydrovitamin K in a biphasic reaction (Table 8), but the 'ideality' of the substrate had to be investigated before proceeding to more quantitative work. Two experiments were performed in the optical H-cell to test for solubility of the oxidised and reduced dyes in the membrane. In the first of these, aerated phosphate buffer in one limb was connected with a solution of the oxidised methylene blue via a membrane consisting of spectroscopic hexane; in the second

the methylene blue was initially reduced by the addition of excess sodium dithionite. A gradual increase in the absorbance of the buffer limb at 605 nm (an absorption maximum of methylene blue), which was observed only in the second case, indicated that the reduced dye was hexane soluble whereas the oxidised form was not. This was to be expected since the reduced (leuco) form of methylene blue is electrically neutral while the oxidised form is cationic. Thus methylene blue cannot be considered as a truly ideal substrate under the criteria laid down in Section 1-5. However the oxidised form does behave ideally with respect to membrane solubility, and this is sufficient to justify its use as a substrate in the following experiment. The general system studied may be represented:



The optical H-cell was filled, and reaction started, according to method A (Section 3-2.4).

When the initial concentrations of reduced vitamin K and oxidised methylene blue were kept constant at 4.57×10^{-4} M and 7.05×10^{-6} M respectively in a series of experiments, the reduction of the dye was found to follow first-order kinetics, with an average rate constant of $k_1 = (1.73 \pm 0.10) \times 10^{-2} \text{ min}^{-1}$. On varying only the initial concentration of methylene blue over the range 2-10 μM , whilst holding the vitamin K concentration constant at 4.57×10^{-4} M, the reaction rate was unaffected, the average first-order kinetic parameters calculated being $k_1 = (1.64 \pm 0.10) \times 10^{-2} \text{ min}^{-1}$ and $t_{\frac{1}{2}} = 42.3 \pm 2.7$ minutes. When the concentration of vitamin K in the membrane phase was varied within the range 3-1000 μM , while the initial methylene blue concentration was kept constant at 5.6×10^{-6} M,

it was found that the reaction rate did not change appreciably until the vitamin K concentration was reduced below about 10 μM , at which stage a marked retardation became apparent. Thus the reaction rate was insensitive even to fairly large variations in carrier concentration around the 'normal' value of 460 μM . It was also found to be insensitive to changes in the hydrocarbon solvent: hexane, heptane, octane, nonane, decane and dodecane all yielded the same rate of reduction of methylene blue by dihydrovitamin K dissolved in the membrane at a fixed concentration. Only tetradecane caused the rate to fall, presumably due to a much slower diffusion of vitamin K through the membrane layer in this case arising from the substantially higher viscosity of the C_{14} alkane⁽⁹⁸⁾. With the initial concentrations of all the reactants held constant and the carrier dissolved in hexane, the effects of varying the stirring speeds in both limbs of the H-cell were investigated. The rate of reduction of methylene blue was found to be unaffected by halving the stirring speed in the methyl viologen limb, but was decreased by about 40% when the stirring speed in the substrate limb was reduced from 1650 rpm (its normal value) to 850 rpm.

All these results pointed to the fact that the observed reaction rate was diffusion controlled on the substrate side of the interface. The insensitivity of the rate to changes in carrier concentration and membrane solvent was taken to indicate that, provided the vitamin K concentration was above 10 μM and a hydrocarbon solvent of chain length C_{10} or lower was chosen, the concentration of dihydrovitamin K at the substrate interface was always sufficient to ensure that every methylene blue molecule reaching the interface could be reduced. The general first-order

kinetic behaviour and marked dependence of the reaction rate on stirring efficiency in the substrate limb were obvious indications of a diffusion controlled mechanism, the observed reaction being dependent only upon the rate of diffusion of oxidised methylene blue from the bulk solution to the interface (Section 3-1.2). No information could therefore be obtained from these measurements regarding the actual rate of intermolecular electron transfer in these systems, although this is assumed to have been very fast. By substitution of the experimental values of $k_1 = 1.6 \times 10^{-2} \text{ min}^{-1}$, $A = 1 \text{ cm}^2$ and $V = 4 \text{ cm}^3$, together with the literature value of $D (22^\circ\text{C}) = 3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (99) for methylene blue, into equation (3-19), a value of $\delta = 28 \text{ }\mu\text{m}$ was obtained for the thickness of the unstirred layer on the substrate side of the interface. This is a very reasonable value⁽¹⁾.

In a separate series of experiments, the kinetics of reduction of vitamin K by reduced methyl viologen were investigated⁽⁵⁷⁾. Again the optical H-cell was used, but the system was simply

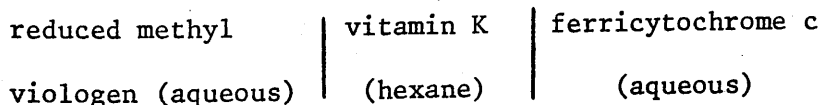
reduced methyl viologen (aqueous)/vitamin K (hexane) with vitamin K solution ($6.17 \times 10^{-6} \text{ M}$) both filling the 'substrate' limb and forming the membrane. The reduction of the quinone was followed by monitoring the increase in absorbance of the substrate limb at 248 nm. Analysis of the results showed that the reaction was first-order with respect to oxidised vitamin K, with a rate constant $k_1 = (1.11 \pm 0.08) \times 10^{-2} \text{ min}^{-1}$ and a half-life of about 63 minutes. This suggested that the biphasic reduction of vitamin K by reduced methyl viologen might be diffusion controlled on the hexane side of the interface. Using this assumption, along with the fact that its

reoxidation by methylene blue is diffusion controlled on the aqueous side of the opposite interface, and the measured rate constants for each process, it has been calculated⁽⁵⁷⁾ that, in the steady state during a typical H-cell run, 99% of the carrier should be present in the reduced form. However, a decrease in the total vitamin K concentration in the membrane from 460 μM (its normal value) to 10 μM causes the level of reduction of the carrier to fall to about 60%. This would explain the deviations from normal kinetic behaviour observed when the carrier concentration is very low.

The reduction of DCIP by dihydrovitamin K has also been examined kinetically⁽⁵⁷⁾, using the system reduced methyl viologen (aqueous)/vitamin K (hexane)/DCIP (aqueous). The non-ideality of DCIP as a substrate has already been discussed, with particular reference to its reduction by reduced coenzyme Q (Section 3-3.1). As in the coenzyme Q system, the reduction of the dye by dihydrovitamin K was found to be first-order with respect to oxidised DCIP: the appropriate parameters (corrected for the presence of the red protonated form) in this case were $k_1 = 4.4 \times 10^{-3} \text{ min}^{-1}$ and $t_{1/2} = 158$ minutes. These results may be interpreted in terms of diffusion control on the substrate side of the interface. The main reason for the apparently much slower reaction of the quinol with DCIP than with methylene blue is probably that stirring in the former case was much less efficient, since perspex stirrers were used in these initial experiments. A comparable methylene blue experiment carried out under the same stirring conditions yielded a rate which was about half the rate of a 'normal' methylene blue experiment. However, no strict comparison between the two substrates

was possible due to the apparent lack of a published diffusion coefficient for DCIP.

The third bulk redox membrane system incorporating vitamin K as electron carrier involved the use of ferricytochrome c as substrate. The ideality of this substrate had first to be demonstrated. The H-cell was filled according to method A (Section 3-2.4) with 20 μ M cytochrome c solution (containing about 10% of the reduced form) in 0.025 M phosphate buffer in one limb, and phosphate buffer alone in the other limb. The membrane layer consisted of spectroscopic hexane, and stirring and rocking were used as for a normal kinetics experiment. After overnight equilibration, ultraviolet/visible spectra of the aqueous buffer and hexane phases were recorded. The absence of any absorption in the range 225-700 nm indicated that neither of these phases contained a detectable level of cytochrome c, both redox forms of which absorb strongly in this region (Section 2-3.4). The SP 8000 spectrophotometer should be capable of detecting cytochrome c at a concentration of about 1.5×10^{-8} M. This result therefore proved that cytochrome c in both its redox forms is insoluble in the membrane and hence possesses all the characteristics of an 'ideal' aqueous substrate under the criteria laid down in Section 1-5. The kinetics of reduction of ferricytochrome c by dihydrovitamin K was investigated using the bulk membrane system:



The H-cell was filled according to method B (Section 3-2.4) in order to avoid contamination of its contents by traces of atmospheric oxygen. (In initial studies on this system using method A, it had

been discovered that the presence of even minute quantities of oxygen led to anomalous behaviour: an initial rapid, irreversible oxidation of the small reduced component in the starting material was followed by an induction period of variable length before reduction of the cytochrome c started⁽⁵⁷⁾. This behaviour was attributed to the formation, by reaction of vitamin K with oxygen, of hydrogen peroxide or vitamin K hydroperoxide which in turn could interact with ferrocytochrome c, causing rapid oxidation of the haem centre. Hydrogen peroxide and lipid hydroperoxides have been reported to react irreversibly with both ferro- and ferricytochrome c^(100,101). The fact that no similarly anomalous behaviour was observed in initial experiments using the much less oxygen-sensitive coenzyme Q as electron carrier further supported the proposed mechanism.) Normal stirring and rocking conditions (Section 3-2.4) were employed in these experiments, and after formation of the membrane the optical density of the cytochrome c solution (in 0.025 M phosphate buffer, pH 6.86) was measured (at 550 nm) at 20-30 minute time intervals. These optical densities were then converted to concentrations using equations (2-4) and (2-7), and the extinction coefficients listed in Table 8, and graphs of concentration of ferricytochrome c against time were drawn (Figure 3.3). It is very difficult to distinguish between zero- and first-order reactions when the concentration of the reactant is very low and the reaction itself is so slow that only a small percentage of the total possible reaction takes place during the time of observation. However, it was found in these and in all subsequent experiments involving the dihydrovitamin K/cytochrome c system (Section 3-3.4) that the results could reasonably be fitted to straight lines. It was therefore assumed that the kinetics of

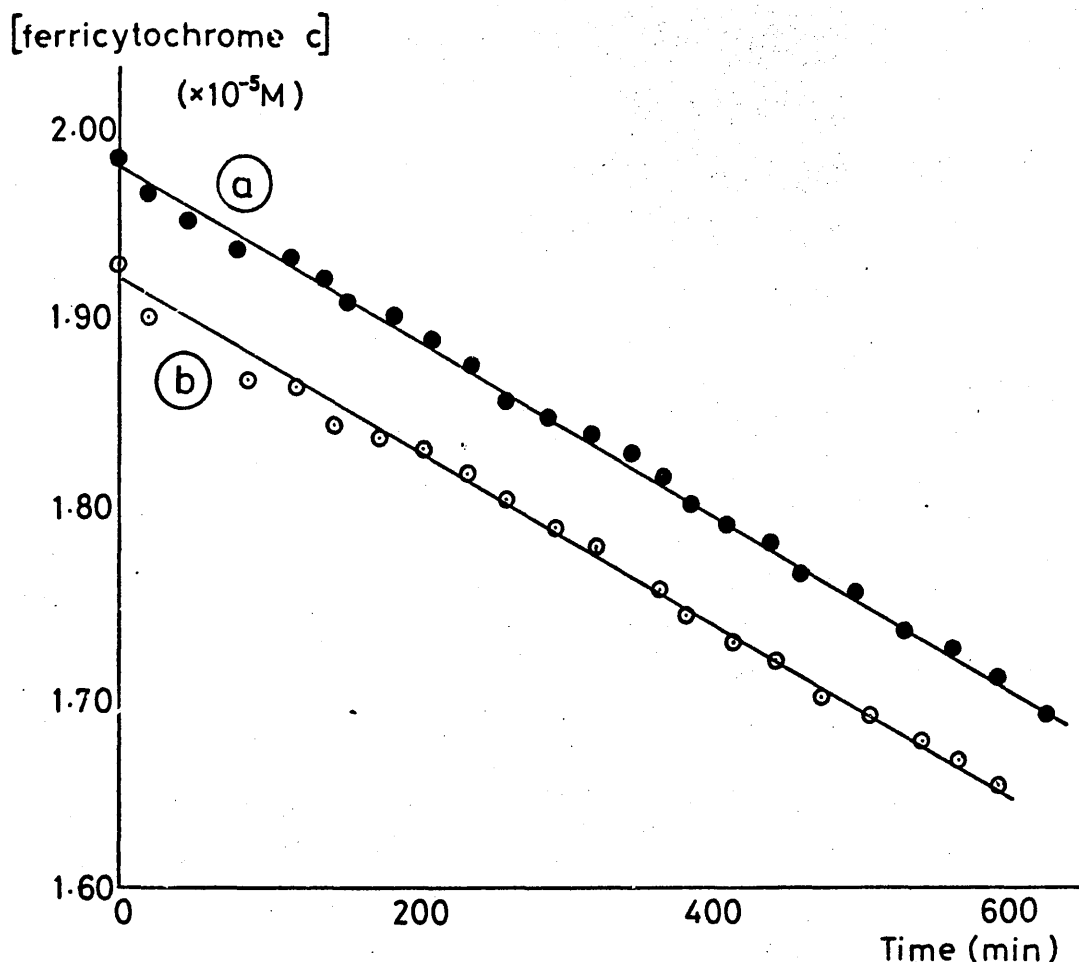


Figure 3.3 Kinetics of reduction of ferricytochrome c by dihydrovitamin K. Graphs of concentration of ferricytochrome c plotted against time for the system:

reduced methyl viologen (0.025M phosphate buffer, pH 6.86)	vitamin K (hexane)	ferricytochrome c (0.025M phosphate buffer, pH 6.86)
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The concentrations of the solutions were: methyl viologen — 2.7×10^{-2} M; vitamin K — 4×10^{-4} M; cytochrome c — (a) 1.98×10^{-5} M, (b) 1.93×10^{-5} M.

The experimental procedure followed is detailed under method B (Section 3-2.4). Standard stirring and rocking conditions were employed. Temperature $25.0 \pm 0.1^{\circ}\text{C}$.

reduction were zero-order with respect to ferricytochrome c concentration, although a first-order analysis might also have been possible. The average rate constant for the biphasic reduction of ferricytochrome c by dihydrovitamin K was calculated to be $k_o = 4.6 \times 10^{-9} \text{ mole l}^{-1} \text{ min}^{-1}$. Thus the reaction with vitamin K was faster than that found with coenzyme Q ($k_o = 3.3 \times 10^{-9} \text{ mole l}^{-1} \text{ min}^{-1}$) and was easily distinguishable from the spontaneous reduction process ($k_o = 2.7 \times 10^{-9} \text{ mole l}^{-1} \text{ min}^{-1}$) (Section 3-3.1), all of which were studied with the protein dissolved in 0.025 M phosphate buffer at pH 6.86. From these few results it is impossible to draw any conclusions concerning the reaction mechanism, but it can be stated quite categorically that the reaction in this case was not diffusion controlled, since a first-order rate constant of about $5 \times 10^{-3} \text{ min}^{-1}$ (equation (3-19)) and a half-life of about 140 minutes should have been expected for an unstirred layer of thickness 28 μm , (as calculated above) with $D = 9.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for cytochrome c⁽¹⁰²⁾.

3-3.3 Effects of phospholipids on reduction of methylene blue by dihydrovitamin K

The biphasic reaction between dihydrovitamin K and methylene blue has been discussed in the previous Section. The modifying influence of a number of biologically important phospholipids on the observed reaction kinetics has also been investigated⁽⁵⁷⁾. The phospholipids studied were phosphatidyl ethanolamine (PE), cardiolipin (CL), phosphatidyl inositol (PI) and lysolicithin (LL). Aliquots (0.03 ml) of stock solutions of these lipids (either about 10 mg ml^{-1} or about 1 mg ml^{-1} , in 10:1 (v/v) hexane:ethanol for CL, or in 9:1 (v/v) chloroform:methanol for the others) were added to the

membrane at the start of each H-cell kinetics run. Method A (Section 3-2.4) was employed to fill the cell, and standard stirring and rocking conditions were used throughout. The concentrations of vitamin K and methylene blue solutions used in each experiment were 4.07×10^{-4} M and $(6.5 \pm 0.5) \times 10^{-6}$ M respectively. The results obtained are summarised in Table 9.

Incorporation of phospholipids into the bulk membrane caused variable decreases in the first-order rate constants for reduction of methylene blue. Since the reaction kinetics were still diffusion controlled, the variations in k_1 must have reflected changes in the effective area A of the dihydrovitamin K/methylene blue interface (equation (3-19)). This was probably due to competition for sites on the interfacial monolayer between the redox inert phospholipid molecules and the redox active carrier. As phospholipids have much more strongly hydrophilic head groups than either reduced or oxidised vitamin K, the phospholipid molecules should tend to displace carrier molecules from the monolayer, hence causing a decrease in the surface coverage of quinol at the substrate interface. (A similar decrease in carrier adsorbed at the methyl viologen interface would also have been expected, but this should not have affected the observed reaction rates since reduced vitamin K was present in vast excess from the start of the reaction.) This would result in a corresponding decrease in the observed reaction rate: the stronger the adsorption of phospholipid, the slower the reaction. This hypothesis was borne out by the experimental results: in all cases the addition of phospholipid caused a lowering of the reaction rate which was more pronounced at the higher lipid concentration. The order of effectiveness, PE > CL > PI > LL, was difficult to rationalise since the actual

Table 9

Influence of phospholipid on the observed rate constants (at 25°C) for reduction of methylene blue by dihydrovitamin K₁. The weight of vitamin K in the membrane was 0.83 mg.

<u>Phospholipid</u>	<u>Weight added to membrane (mg)</u>	<u>*10³ x k₁ (min⁻¹)</u>	<u>t_{1/2} (min)</u>
PE	0.026	3.70	186
PE	0.26	1.04	665
CL	0.025	8.26	84
CL	0.28	4.88	142
PI	0.022	7.90	87.7
PI	0.25	5.72	121
LL	0.024	11.20	61.9
LL	0.24	9.07	76.5
None	-	13.58	51.0

* These results were calculated from the change in absorbance of methylene blue (measured at 655 nm) plotted against time for the system

RMV/Vit K (hexane) + phospholipid/Methylene blue

The concentrations of methylene blue (in 0.025 M phosphate buffer, pH 6.86) and vitamin K were 6.5 μM and 407 μM respectively.

These results are taken from reference (57).

concentration of lipid at the membrane/substrate interface was unknown. It is of interest, however, to note that lysolecithin had the least effect. This 'wedge-shaped' phospholipid is known to promote rupture of biological membranes by interacting with the lipid component and thereby increasing the permeability of the membrane to water⁽¹⁰³⁾. It would be expected, therefore, that LL in the model membrane system would not form such a closed, inert surface as the other phospholipids, and in consequence would not be as efficient in blocking quinol - substrate interaction as the other phospholipids. Phosphatidylethanolamine was found to be the most effective in blocking the reaction. It must therefore compete very strongly with dihydrovitamin K for sites on the interface, possibly forming a rather condensed monolayer from which the quinol molecules are substantially excluded. The PE used in these experiments was synthetic dipalmitoyl phosphatidyl ethanolamine, whose gel-to-liquid crystalline phase transition temperature of 105°C⁽¹⁹⁾ is unusually high. It seems likely then that at 25°C under the experimental conditions it tended to form a tightly packed 'solid' barrier at the interface. This would explain why PE had a more pronounced effect on the reaction rate than the other phospholipids, which were all biological extracts with relatively high side chain unsaturation and were therefore capable of forming more 'fluid' monolayers.

3-3.4 Further kinetic studies on the dihydrovitamin K/ ferricytochrome c system

Before entering into a more detailed discussion of the biphasic reaction between reduced vitamin K and ferricytochrome c, and the effects of phospholipids upon it, it is useful to summarise some

of the known facts concerning the structure and reactivity of the participants. Cytochrome c is a haem protein common to the mitochondrial respiratory chains of all eukaryotes⁽¹⁰⁴⁾, where it acts as a reversible electron carrier between complexes III (the cytochrome b-c₁ complex, or coenzyme Q-cytochrome c oxidoreductase) and IV (the cytochrome a-a₃ complex, or cytochrome c oxidase) as indicated in Figure 1.4. Its function is therefore somewhat similar to that of coenzyme Q which mediates electron transfer from complexes I and II to complex III⁽¹⁰⁵⁾. It is also similar to coenzyme Q in its ability to act as an interchain electron transfer agent, capable of equilibrating electrons among neighbouring respiratory assemblies⁽¹⁰⁶⁾. This could be due either to mobility of the protein molecule attached to the membrane⁽¹⁰⁷⁾ or to the ability of redox pairs of cytochrome c molecules to undergo an intermolecular electron transfer^(108,109). Cytochrome c, however, is distinct from coenzyme Q and all the other respiratory chain components in being readily extractable from the inner mitochondrial membrane by washing with aqueous salt solutions⁽⁷⁹⁾. It has therefore been classified as a 'peripheral' protein⁽¹¹⁰⁾, and has been shown to be bound specifically to the external surface of the inner membrane⁽¹¹¹⁾ (Figure 1.6).

The X-ray structure of horse heart ferricytochrome c has been determined to 2.8 Å resolution by Dickerson *et al.*⁽¹¹²⁾, and that of ferrocycytochrome c to 2.0 Å resolution by Takano *et al.*⁽¹¹³⁾. A stereoscopic α-carbon diagram of the oxidised form is given in Figure 3.4 (a). Cytochrome c is a small, globular protein of molecular weight about 12,400: the oxidised form is a prolate

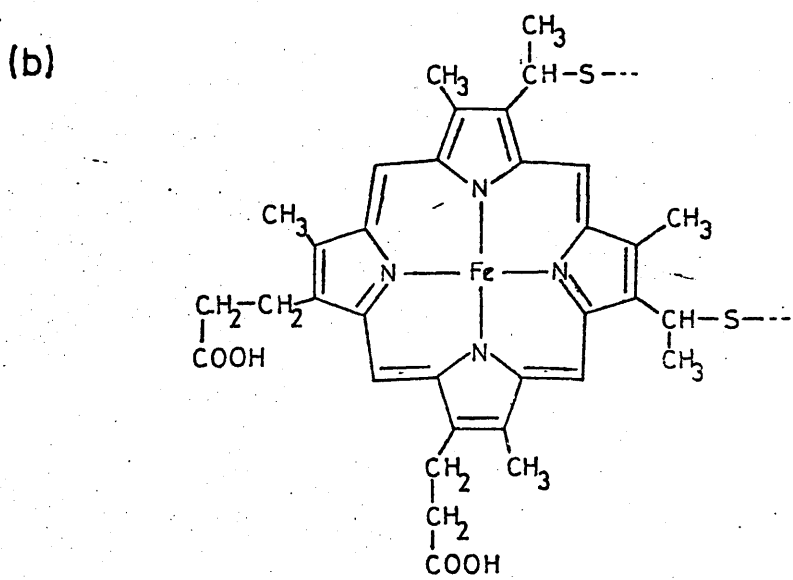
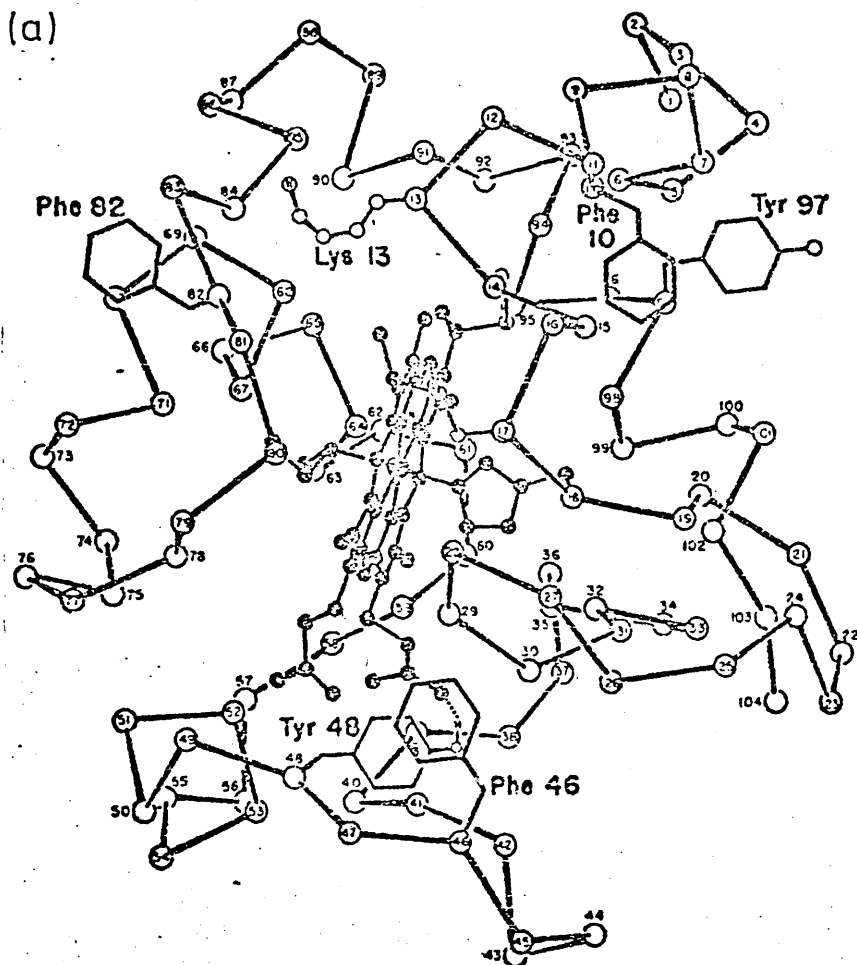


Figure 3.4 Cytochrome c. (a) Stereoscopic α -carbon diagram of the horse-heart ferricytochrome c molecule (reproduced from reference (79)).

(b) Structure of haem c, showing pyrrole substituents and sites of attachment to the protein.

spheroid, $30 \times 34 \times 34 \text{ \AA}$, including side chains. The prosthetic group, haem c (Figure 3.4 (b)) is covalently linked to a polypeptide chain of 104 amino acid residues. The unusual evolutionary invariance of the amino acid sequence in large sections of this chain has been noted following the examination of the cytochrome c of a wide range of organisms from man to yeast^(79,112) and the constant features are believed to mark the main functional regions of the molecule. A particularly important feature of the polypeptide chain in mammals is its high lysine content (19 residues in the horse protein) which makes the protein strongly basic, with an isoelectric point at $\text{pH } 10.04 \pm 0.04$ ⁽¹¹⁴⁾ and a resulting overall surface charge of + 8 to + 10 at $\text{pH } 7$ ⁽¹¹⁵⁾. Although all mammalian cytochromes c are basic, several bacterial cytochromes are known whose isoelectric points are well below $\text{pH } 7$, for example *Rhodospirillum rubrum* cytochrome c_2 with $\text{pI} = 6.11$ for the reduced form⁽⁷⁹⁾.

In both ferri- and ferrocytochrome c the iron lies in the plane of the four nitrogens of the porphyrin ring system, with the fifth and sixth co-ordination sites occupied by histidine 18 and methionine 80^(104,116). The change in oxidation state of the haem iron is however accompanied by considerable conformational changes affecting the tertiary structure of the protein. In the oxidised form the polypeptide chain is wrapped around the haem in two halves, to the right and left of the planar ring system, which sits in the resultant haem crevice with one edge exposed to the solvent⁽¹¹²⁾. The most striking change in chain folding on reduction is the closing off of the haem crevice by phenylalanine 82^(104,116), which results in the molecule generally becoming more compact, a factor thought to

be important in its stability to autoxidation⁽¹¹⁶⁾. In ferricytochrome c two 'channels' filled with hydrophobic side chains lead to the right and left from the haem to the surface of the molecule⁽¹¹²⁾. Each channel contains at least two aromatic rings in roughly parallel orientation, and is surrounded by a cluster of positively charged lysines where it meets the surface. The right channel, like the haem crevice, is blocked in the reduced form⁽¹¹⁶⁾. At the back of the molecule, between the two positive regions, is a cluster of nine negatively charged acidic groups. These surface features are proposed to play an important part in the interaction of the protein with the enzyme complexes of the respiratory chain. In particular, the haem crevice and right channel may be involved in binding to the oxidase while the left side and channel may bind to the reductase⁽¹¹⁶⁾.

Various mechanisms for the transfer of electrons to and from the iron centre have been suggested⁽¹¹⁷⁾, among them a 'stepwise' process involving consecutive transfers of the electron from the surface via parallel and neighbouring aromatic rings to the ring system of the haem⁽¹⁰⁴⁾, and an 'electron tunnelling' mechanism which is proposed to act between centres up to 30 Å apart⁽¹¹⁸⁾. Sutin⁽¹¹⁹⁾ has calculated a steric factor, based on the ratio of the exposed area of the haem crevice to the total protein surface area, which he has used successfully to calculate the rate of the electron exchange reaction between oxidised and reduced cytochrome c molecules in free aqueous solution. In fact all the proposed mechanisms involve some kind of steric requirement for reaction: this requirement may be satisfied at least partially by binding of the protein to the mitochondrial membrane. The lipid constituents of the membrane might be expected to play a critical role here, which explains the considerable

interest in phospholipid-cytochrome c interactions that has been shown by numerous investigators^(29,102,115,120-129). Cytochrome c has been found to form 'proteolipid' complexes with a number of purified and mixed phospholipids on shaking with the micelles in aqueous (30% alcohol) suspension^(29,115,120,121). Often these proteolipids contain a definite stoichiometric ratio of cytochrome c to lipid molecules: some also are soluble in iso-octane whereas others form a red precipitate at the iso-octane/water interface (Table 10). At pH 7 the basic protein will form complexes only with acidic phospholipids: cardiolipin (CL), phosphatidyl inositol (PI) and phosphatidyl ethanolamine (PE) all fall into this category. The highly acidic CL and PI form small, electrostatically bonded proteolipids with a lipid phosphorus:cytochrome c molar ratio of ten, while the more weakly acidic PE forms complexes with much larger P/cyt c ratios due to loose association of the outer lipid layers. Purified phosphatidyl choline (PC) being zwitterionic does not form a complex with cytochrome c by itself, but when mixed with a small amount of acidic phospholipid, an iso-octane soluble complex is readily formed (Table 10).

In addition to the work on proteolipid formation, many investigations into cytochrome c - phospholipid interactions have been carried out using model systems, namely phospholipid monolayers spread on the air/water interface^(102,122-125) and artificial bilayer membranes^(102,125-129). Quinn and Dawson^(122,123) have investigated the nature of interactions between cytochrome c (native and [¹⁴C] carboxymethylated) and monolayers of PE, PC, PA and CL by measurements of surface radioactivity, pressure, and electrical potential under

Table 10 Composition of various phospholipid - cytochrome c (horse heart) complexes formed at pH 7.

<u>Phospholipid</u>	<u>Iso-octane solubility</u>	<u>Atoms of lipid phosphorus per molecule of cyt c complexed</u>
mixed mitochondrial phospholipids	soluble	32
purified PC	-	no complex formed
purified CL	insoluble	10
purified PI	insoluble	10
purified unsaturated PE	slowly soluble	24
purified saturated PE	insoluble	~ 100
10:1 PC:acidic phospholipid	soluble	~ 86

Data from Das, Haak and Crane (120).

various conditions with the protein dissolved in the aqueous sub-phase. These methods allowed two distinct types of interaction to be distinguished: the first, termed 'adsorption', is an electrostatic association between the positively charged protein and a negatively charged monolayer surface; the second, termed 'penetration' involves the entry of all or part of the cytochrome c molecule into the lipid layer, resulting in expansion of the monolayer. Adsorption was observed in all monolayers apart from the uncharged PC surface, and penetration occurred to varying extents at low film pressures for all four phospholipids studied. Overall protein binding was strongest for the acidic phospholipids PA and CL and weakest for the zwitterionic PC, with the weakly acidic PE (pI = 3.1⁽¹³⁰⁾) in an intermediate position. The fact that adsorption was an electrostatic interaction was demonstrated by studying the binding as a function of pH and ionic strength of the aqueous sub-phase and observing the expected trends (Section 3-1.1). Adsorption could be prevented and substantially reversed by the addition of 1 M sodium chloride. Although low pressure PC films were penetrated by cytochrome c, the penetration was greatly facilitated in the case of the acidic phospholipids by the prior electrostatic binding, possibly due to a conformational change in the protein on binding. It was also noted that such electrostatically facilitated penetration was essentially irreversible, since the bound cytochrome c molecules were not released when the sub-phase was replaced by pure buffer.

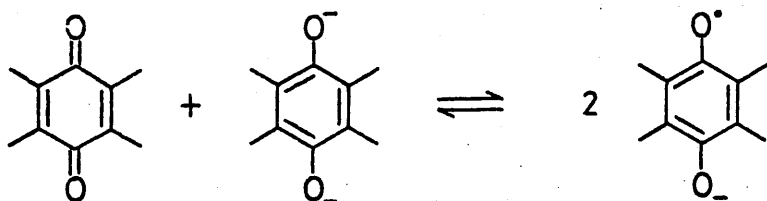
These observations of Quinn and Dawson^(122,123) have been extended by other investigators. Morse and Deamer⁽¹²⁴⁾, also working on monolayers, have demonstrated a requirement for headgroup charges

and chain unsaturation in the lipids for efficient adsorption and penetration of cytochrome c. Steinemann and Lauger⁽¹⁰²⁾ using monolayers spread on glass plates and planar bilayer membranes of PI, another acidic phospholipid, have shown that the adsorption of cytochrome c onto the charged surface is a rapid and reversible process which is diffusion controlled in the aqueous phase (Section 3-1.2) and leads to the build-up of a complete monolayer of protein over the surface. No information on the rate or extent of irreversible penetration was obtained, however. Kimelberg and Papahadjopoulos⁽¹²⁵⁾ have presented results which correlate increased ionic permeability of liposomal membranes induced by certain proteins, including cytochrome c, with the ability of these proteins to penetrate the bilayer. Two possible modes of penetration, either of which would account for the observed increase in monolayer surface pressure, were also described by these authors⁽¹²⁵⁾. Further observations on membrane-protein interactions based on studies of liposomal systems⁽¹²⁶⁻¹²⁹⁾ have been made and will be discussed in greater detail in Chapter 5.

Thus far the structure of cytochrome c and its interactions with charged lipid surfaces have been considered. The object of the present work was to study electron transfer from a membrane-bound carrier to cytochrome c at an oil/water interface and if possible to elucidate the role of mitochondrial phospholipids in this reaction. Ideally coenzyme Q, along with the cytochrome b-c₁ enzyme complex if necessary, might have seemed the best carrier to use, due to its proven involvement in the mitochondrial respiratory chain (Section 1-3). However, because of the adverse kinetic factors associated with coenzyme Q reoxidation (Section 3-3.1) its use in

the H-cell bulk membrane system was impracticable. Therefore only vitamin K was used as an electron carrier in these experiments. It has already been shown that dihydrovitamin K reduces ferricytochrome c in a biphasic reaction and that the reaction is probably zero-order and is much slower than can be explained in terms of a simple diffusion controlled mechanism (Section 3-3.2). The reaction is therefore kinetically controlled, a fact which indicated the possibility of gaining some insight into the molecular mechanism by appropriate changes in the conditions.

In a homogeneous aqueous solution, at neutral pH, the reduction of ferricytochrome c by benzoquinol is greatly accelerated by the addition of benzoquinone, an effect attributed to the formation of benzosemiquinone^(131,132). It is probable that a semiquinone intermediate plays a similar role in the reduction by dihydrovitamin K described here. Although no ESR studies were carried out at the vitamin K/cytochrome c interface, evidence has been presented for the presence of semiquinone radicals at the other (reduced methyl viologen/vitamin K) interface (Section 2-3.5). Formation of the semiquinone at pH 6.5 is thought to proceed mainly by interaction of the quinone with the doubly dissociated quinol, which exists in equilibrium with the undissociated and monodissociated forms⁽¹³²⁾:



Electron transfer then occurs from the semiquinone to the haem centre of the ferricytochrome c. The fact that a charged intermediate is involved is supported by the observed variation in the reaction rate with ionic strength of the homogeneous reaction medium⁽¹³¹⁾. In the

bulk membrane experiments described in the present research, the observation of cytochrome c reduction is dependent on at least three factors:

- (1) adsorption of the oxidised substrate molecule onto the interface
- (2) electron transfer from the carrier molecule to the substrate
- (3) desorption of the reduced substrate molecule from the interface.

The strength of binding of cytochrome c to the interface is therefore of great importance: if the binding is too weak, electron transfer may not occur; if, on the other hand, it is too strong, the reduced substrate molecule may remain irreversibly bound and any electron transfer which has occurred will not be detected.

3-3.5 Effects of phospholipids on the biphasic reduction of ferricytochrome c by dihydrovitamin K

Since the strength of binding of cytochrome c to the interface is considered to be important in these H-cell kinetics experiments, it was decided to investigate the effects of varying this strength by the addition to the bulk membrane of different purified mitochondrial phospholipids. The additions were made and the experiments carried out as detailed under method B, Section 3-2.4. Graphs of ferricytochrome c concentration against time were drawn, and from their gradients apparent zero-order rate constants were calculated. In all these experiments the initial concentrations of vitamin K and cytochrome c, and the pH and ionic strength of the aqueous solutions, were kept constant. The results are summarised in Table 11. From these a marked dependence of the observed reaction rate on the particular phospholipid added can be seen. Cardiolipin stimulated the reaction rate by a factor of about four times, while phosphatidyl inositol had

little effect and phosphatidyl ethanolamine blocked the reaction completely. By analogy with the methylene blue results discussed earlier (Section 3-3.3), it might have been expected that all of these phospholipids would decrease the reduction rate. Obviously in the present system CL does not function simply as an inert phospholipid, competing with carrier molecules for interfacial monolayer sites, but is involved in some catalytic way in the interaction with cytochrome c. Cardiolipin is an acidic phospholipid with a double negative charge at the pH of these experiments (pH \sim 7), so that its incorporation into the monolayer will cause the interface to become negatively charged. (Although the presence of reduced vitamin K will itself confer some negative charge on the interface, as mentioned above, this should be less significant since it exists mainly in the undissociated form at pH 7.) The observed rate enhancement is most probably due to improved binding of the basic protein to the interface in the presence of cardiolipin. Electron transfer may thus be eased by holding the ferricytochrome c at the interface for long enough to allow collision with the semiquinone reductant, and/or by causing conformational changes in the protein which open the haem crevice and provide a lower-energy path for reaction, and/or by binding the protein in a particular orientation which makes the haem more accessible to the carrier.

The reason for the apparent failure of phosphatidyl inositol, another acidic phospholipid, to enhance the rate of ferricytochrome c reduction in a similar manner to cardiolipin, is not clear. (In fact, considerable rate enhancements have been observed when PI was used in the presence of a trace of oxygen⁽⁵⁷⁾.) Monolayer and bilayer

Table 11 Influence of phospholipid on the observed rate constants (25°C) for reduction of ferricytochrome c by dihydrovitamin K. For reference, the weight of Vit K in the membrane was 0.83 mg in each case.

Expt. No.	Phospholipid	Weight added to membrane (mg)	Rate constant k_0 ($\times 10^{-8} \text{ mol l}^{-1} \text{ min}^{-1}$)	Rate relative to control
1	-	-	0.46	1.00
2	CL	0.28	1.92	4.17
Ref. (57)	CL	0.28	1.80	3.91
3	PI	0.24	0.59	1.28
Ref. (57)	PI	0.24	0.31	0.67
4	PE	0.14	0.00	0.00
5	PE	0.026	0.00	0.00

The concentration of cytochrome c in each experiment was about 24 μM , in 0.025 M phosphate buffer, pH 6.86, ionic strength 0.1 M.

studies on the binding of cytochrome c to PI surfaces have produced results which parallel those obtained for CL^(102,123). The inhibitory effects of phosphatidyl ethanolamine, however, are more easily explained. Although monolayers of egg PE do bind cytochrome c quite strongly at pH 7 (PE has its isoelectric point at pH 3.1⁽¹³⁰⁾), the binding is significantly decreased by hydrogenation of the hydrocarbon chains^(122,123). In these experiments a fully saturated PE was used. This will result in a condensed monolayer at 25°C (well below the phase transition temperature for the saturated side-chains⁽¹⁹⁾) which would tend to act as an impermeable barrier between the electron carrier in the bulk membrane and the aqueous substrate.

If the binding of cytochrome c to the interface in these experiments was electrostatic in origin, then it should have been affected by varying the ionic strength of the aqueous medium. A series of experiments was therefore performed in which the ionic strength of the cytochrome c solution was varied in the range 0.01-0.60 M, with and without cardiolipin in the membrane. The experimental procedure adopted in most cases was method B, Section 3-2.4, although in a few instances method C was used, for reasons discussed below. Both phosphate and tris (hydroxymethyl) aminomethane — hydrochloric acid ('Tris-HCl') buffers were used in order to reveal any specific anion effects on the reaction rate. The kinetics curves obtained are given in Figures 3.5, 3.6 and 3.7, and the results are summarised in Tables 12 and 13.

In the absence of added phospholipids, the rate of biphasic reduction of ferricytochrome c by dihydrovitamin K was dependent on the ionic strength of the aqueous phase (experiments 1-4, Table 12, Figures 3.5 and 3.8). In every case the reaction followed simple

Figure 3.5 Kinetics of reduction of ferricytochrome c by dihydro-
vitamin K at different substrate ionic strengths. Graphs of
concentration of ferricytochrome c plotted against time for the system:

reduced methyl viologen (0.025M phosphate buffer, pH 6.86)	vitamin K (hexane)	ferricytochrome c (phosphate buffer, NaCl)
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The concentrations of the solutions were: methyl viologen — $2.7 \times 10^{-2}M$;
vitamin K — $4 \times 10^{-4}M$; cytochrome c $\sim 21 - 25 \mu M$. (The graphs are
shown spaced out along the concentration axis for clarity of
presentation: all experiments used approximately the same initial
concentration of ferricytochrome c.)

Experimental method B (Section 3-2.4) was followed, and standard
stirring and rocking conditions were used.

Temperature $25.0 \pm 0.1^{\circ}C$.

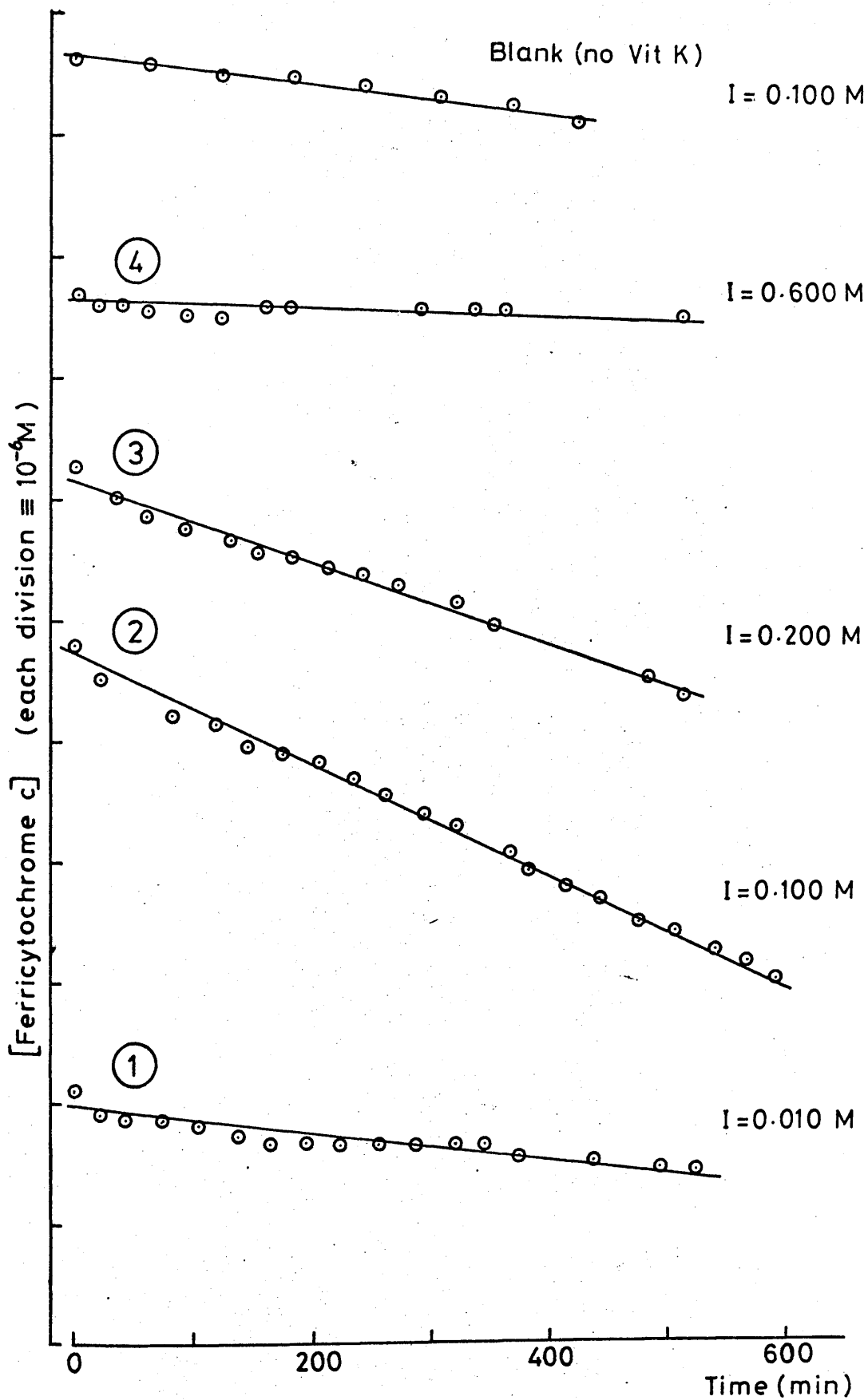


Figure 3.5

Figure 3.6 (a) and (b) Kinetics of reduction of ferricytochrome c by dihydrovitamin K, in the presence of added cardiolipin and at different substrate ionic strengths. Graphs of concentration of ferricytochrome c plotted against time for the system:

reduced methyl viologen (0.025M phosphate buffer, pH 6.86)	vitamin K, CL (hexane)	ferricytochrome c (phosphate buffer, NaCl)
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The concentrations of the solutions were: methyl viologen — $2.7 \times 10^{-2} \text{M}$; vitamin K — $4 \times 10^{-4} \text{M}$; cytochrome c $\sim 21\text{-}25 \mu\text{M}$. (The graphs are shown spaced out along the concentration axis for clarity of presentation : approximately the same initial concentration of ferricytochrome c was used in each experiment.)

In each case 0.03 ml of cardiolipin solution (in ethanol, 9.3 mg ml^{-1}) was added to the membrane. The procedure of method B (Section 3-2.4) was followed in all experiments except those marked with an asterisk (*) where method C was used.

Standard stirring and rocking conditions were employed throughout.

Temperature $25.0 \pm 0.1^\circ\text{C}$.

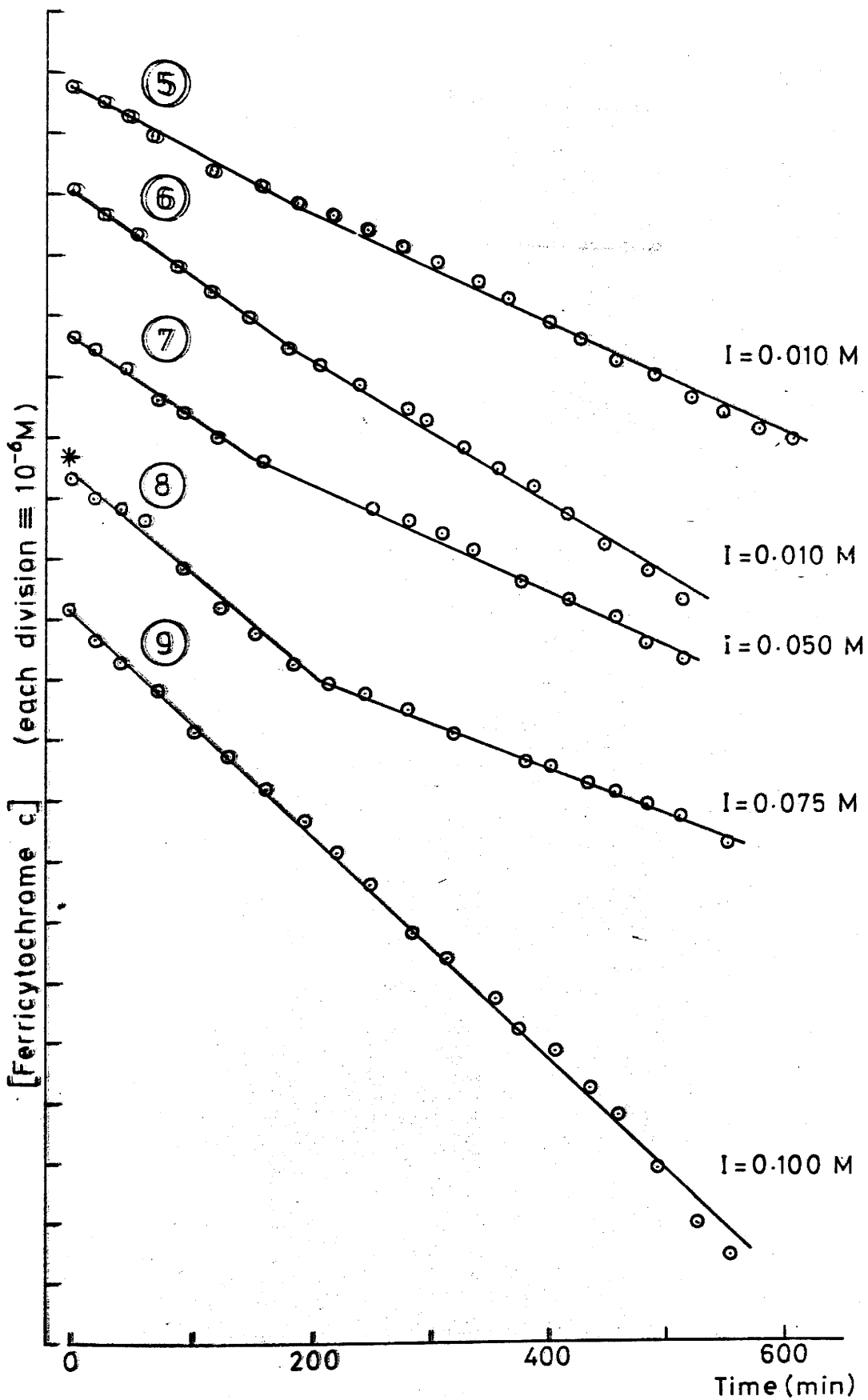


Figure 3.6 (a)

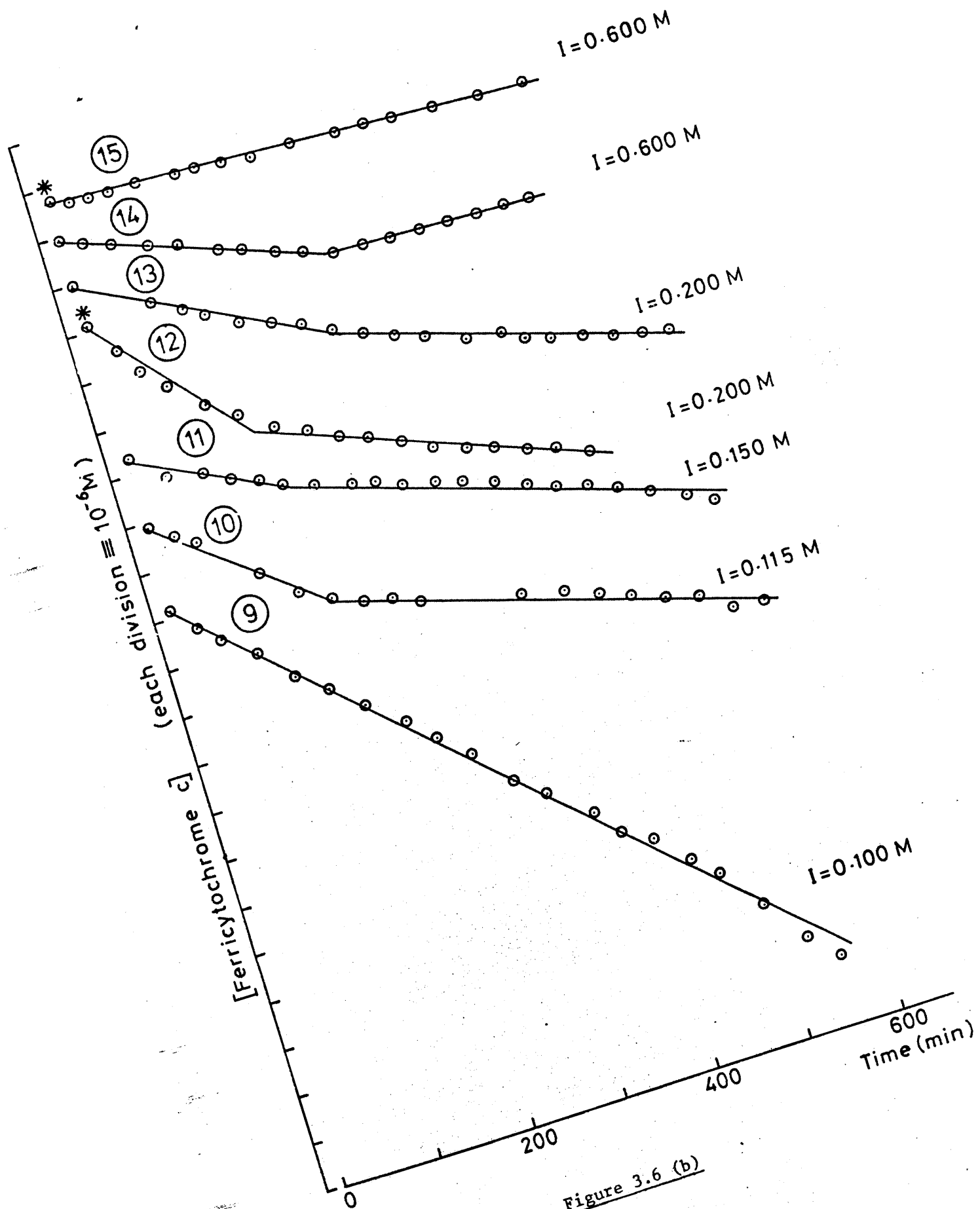


Figure 3.6 (b)

Figure 3.7 Kinetics of reduction of ferricytochrome c by dihydrovitamin K, in the presence of added cardiolipin and at different substrate ionic strengths. Graphs of concentration of ferricytochrome c plotted against time for the system :

reduced methyl viologen (0.025M phosphate buffer, pH 6.86)	vitamin K, CL (hexane)	ferricytochrome c (Tris buffer, NaCl)
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The concentrations of the solutions were: methyl viologen — $2.7 \times 10^{-2} \text{M}$; vitamin K — $4 \times 10^{-4} \text{M}$; cytochrome c $\sim 21\text{-}25 \mu\text{M}$. (The graphs are shown spaced out along the concentration axis for clarity of presentation : approximately the same initial concentration of ferricytochrome c was used in each experiment.)

In each case 0.03 ml of cardiolipin solution (in ethanol, 9.3 mg ml^{-1}) was added to the membrane. The procedure of method B (Section 3-2.4) was followed in all experiments except those marked with an asterisk (*) where method C was used.

† In Expt. 21, 0.10 ml of 0.025 M calcium chloride solution ($I = 0.075 \text{ M}$) was added to the cytochrome c, 85 min after the start of the reaction.

Standard stirring and rocking conditions were employed throughout.

Temperature $25.0 \pm 0.1^\circ\text{C}$.

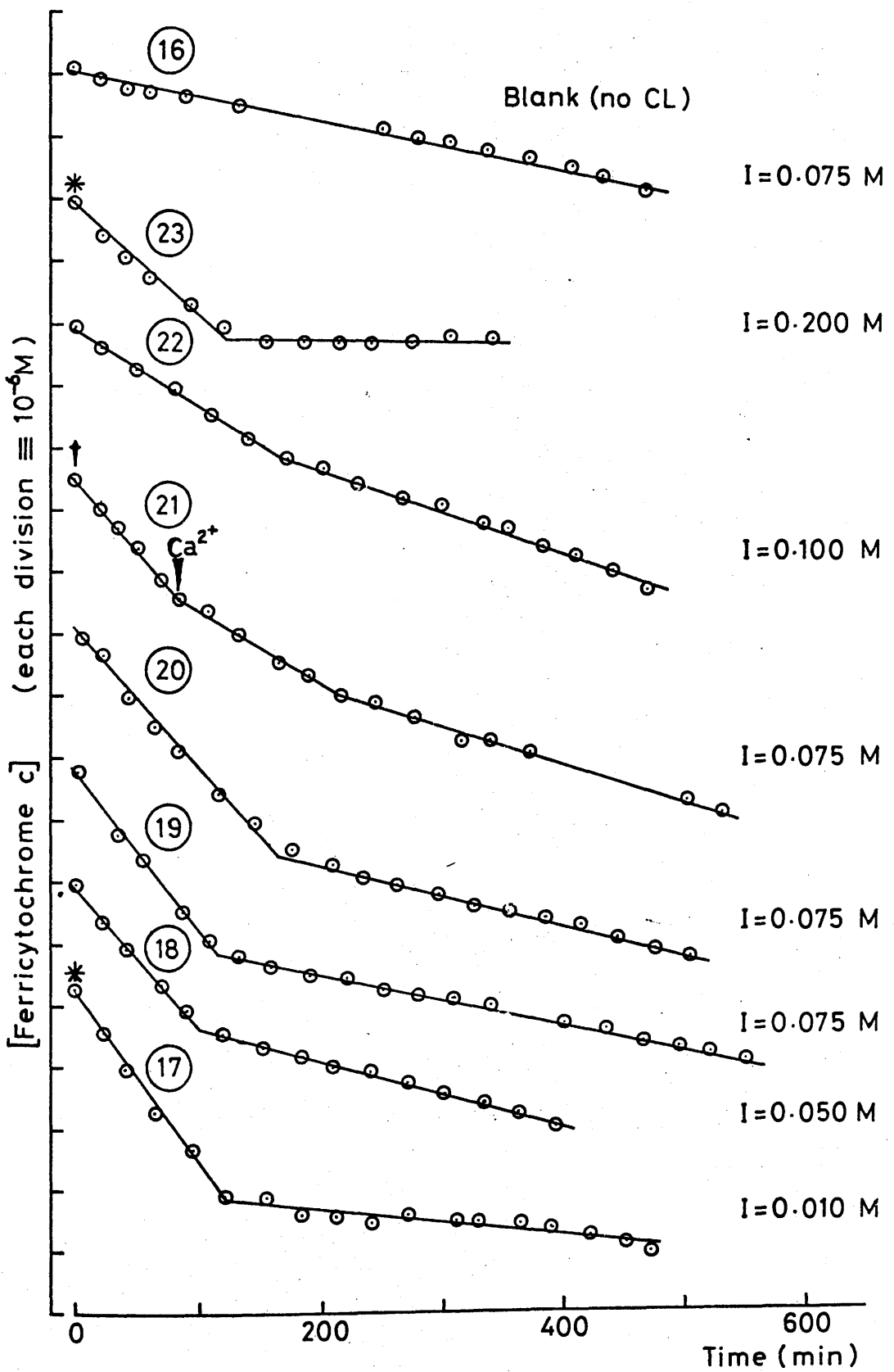


Figure 3.7

Figure 3.8 Influence of substrate ionic strength on the observed rate constants (at 25°C) for the biphasic reduction of ferricytochrome c, in phosphate buffer, by dihydrovitamin K, in the absence and presence of added cardiolipin.

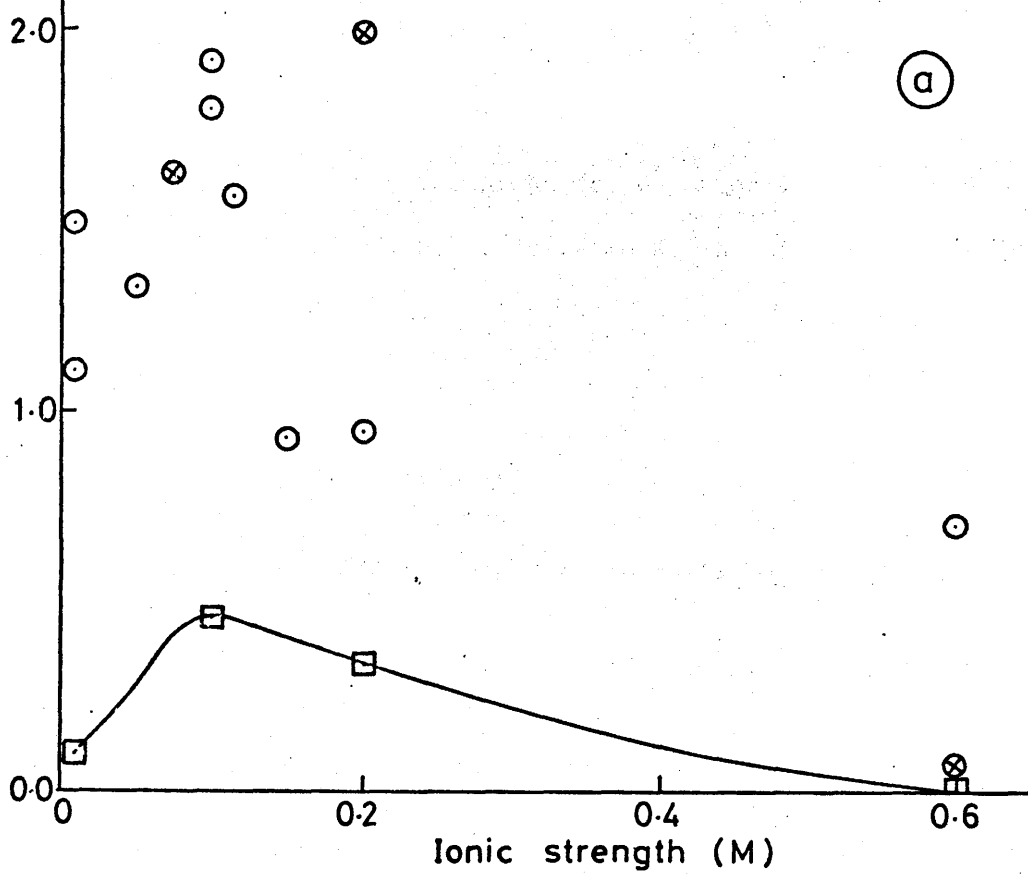
(a) Initial zero-order rate constant.

(b) Final zero-order rate constant.

Rate constants were calculated from the gradients of the graphs shown in Figures 3.5 and 3.6.

Symbols : \square No cardiolipin present.
 \odot Cardiolipin added (method B, Section 3-2.4)
 \otimes Cardiolipin added (method C, Section 3-2.4)

Initial rate k_o ($\times 10^{-8} \text{ mol l}^{-1} \text{ min}^{-1}$)



Final rate k_o ($\times 10^{-8} \text{ mol l}^{-1} \text{ min}^{-1}$)

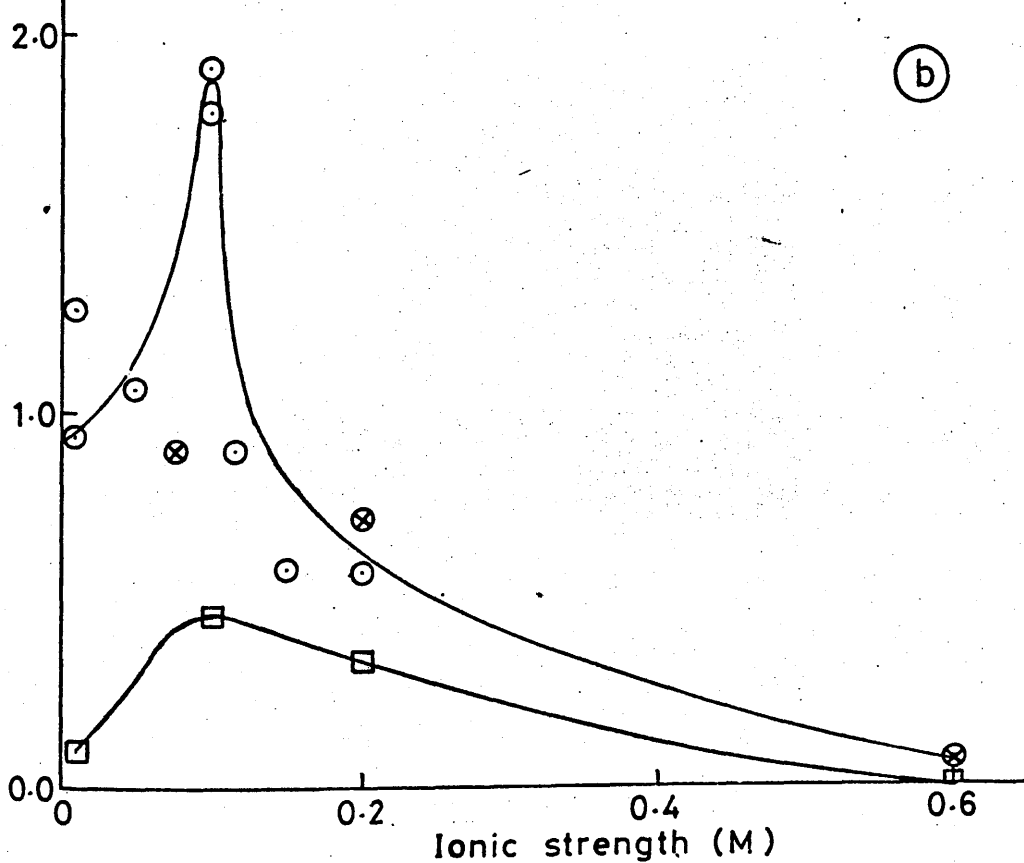


Figure 3.8

Figure 3.9 Influence of substrate ionic strength on the observed rate constants (at 25°C) for the biphasic reduction of ferricytochrome c, in Tris buffer, by dihydrovitamin K, in the absence and presence of added cardiolipin.

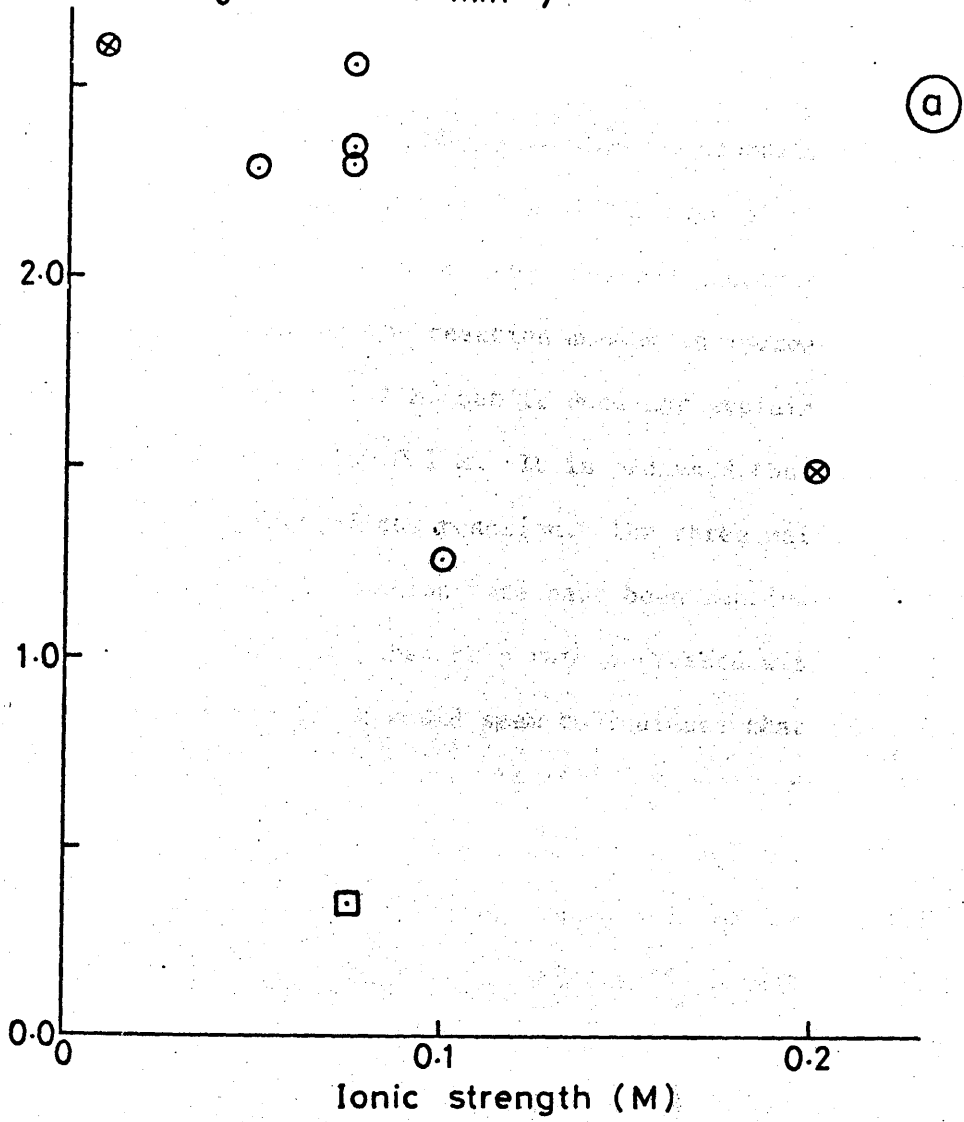
(a) Initial zero-order rate constant.

(b) Final zero-order rate constant.

Rate constants were calculated from the gradients of the graphs shown in Figure 3.7.

Symbols : □ No cardiolipin present.
 ⊙ Cardiolipin added (method B, Section 3-2.4).
 ⊗ Cardiolipin added (method C, Section 3-2.4).
 △ Calcium chloride added.

Initial rate $k_o (\times 10^{-8} \text{ mol l}^{-1} \text{ min}^{-1})$



Final rate $k_o (\times 10^{-8} \text{ mol l}^{-1} \text{ min}^{-1})$

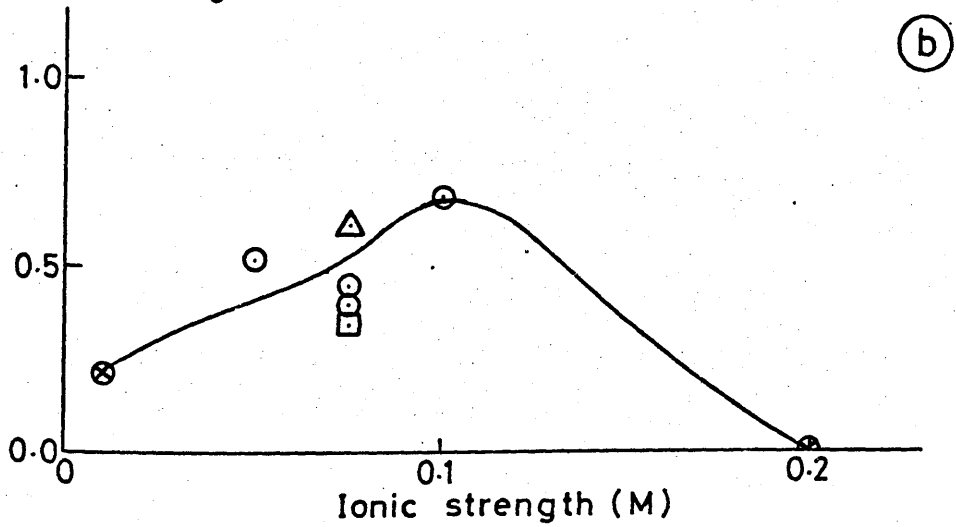


Figure 3.9

zero-order kinetics. The results show a maximum rate of reduction around 0.1 M, with the rate decreasing markedly at 0.01 M and dropping to zero at 0.6 M (Figure 3.8 (a)). According to the Brønsted-Bjerrum theory (Section 3-1.1) the rate of a homogeneous phase reaction between two oppositely charged species should decrease as the ionic strength of the reaction medium is increased. Clearly this was the case above 0.1 M, but it does not explain the decrease in the reaction rate below 0.1 M. It is proposed that this was due to the biphasic nature of the reaction. The three main factors governing the observed reaction rate have been mentioned above (Section 3-3.4): the fact that this rate decreased with decreasing ionic strength below 0.1 M would seem to indicate that under these conditions factor (3) became rate limiting, that is, binding became so strong that the cytochrome c molecules were not released into the bulk aqueous phase after reduction. These results are therefore consistent with an electron transfer process facilitated by electrostatic binding between the positively charged protein and the negatively charged vitamin K semiquinone radical at the interface. Some hydrophobic interaction may have occurred subsequently if the protein actually penetrated the lipid monolayer. In fact, Morrison *et al.*⁽¹³³⁾ have shown that benzoquinone readily forms a covalently bonded addition product with cytochrome c under near-physiological conditions.

Cardiolipin has already been shown to increase the rate of biphasic reduction of ferricytochrome c by dihydrovitamin K by a factor of about four times. As stated earlier, the reaction in 0.025 M phosphate buffer (pH = 6.86, I = 0.10 M) followed simple zero-order kinetics (experiment 9, Figure 3.6 (a)), as observed for

Table 12 Influence of ionic strength on the observed rate constants (at 25°C) for ferricytochrome c reduction in phosphate buffer, in the absence and presence of added cardiolipin.

Expt. No.	Membrane contents	Ionic strength (molar)	pH	Rate constants k_o ($\times 10^{-8}$ mol l^{-1} min $^{-1}$)	
				Initial rate	Final rate
1	Vit K	0.01	6.95	0.10	0.10
2	Vit K	0.10	6.86	0.46	0.46
3	Vit K	0.20	6.73	0.34	0.34
4	Vit K	0.60	6.49	0.00	0.00
5	Vit K, CL	0.01	6.95	1.11	0.94
6	Vit K, CL	0.01	6.95	1.50	1.29
7	Vit K, CL	0.05	6.88	1.33	1.07
*8	Vit K, CL	0.075	6.87	*1.63	*0.90
9	Vit K, CL	0.10	6.86	1.92	1.92
Ref. (57)	Vit K, CL	0.10	6.86	1.80	1.80
10	Vit K, CL	0.115	6.85	1.57	0.90
11	Vit K, CL	0.15	6.83	0.93	0.59

Table 12 (Continued)

Expt. No.	Membrane contents	Ionic strength (molar)	pH	Rate constants k_0 ($\times 10^{-8}$ mol l ⁻¹ min ⁻¹)
				<u>Initial rate</u> <u>Final rate</u>
*12	Vit K, CL	0.20	6.73	* 2.00 * 0.73
13	Vit K, CL	0.20	6.73	0.95 0.58
14	Vit K, CL	0.60	6.49	0.71 0.00
*15	Vit K, CL	0.60	6.49	* 0.07 * 0.07

The cytochrome c solutions used had concentrations in the range 21-25 μ M, and the buffers were prepared as indicated in Section 3-2.2 from 0.025 M phosphate buffer, by appropriate dilution or addition of sodium chloride.

Method B (Section 3-2.4) was adopted in all experiments except those marked with an asterisk (*) where method C was used.

Rate constants were calculated from the gradients of the graphs illustrated in Figures 3.5, 3.6 (a) and 3.6 (b).

Table 13 Influence of ionic strength on the observed rate constants (at 25°C) for ferricytochrome c reduction in Tris-HCl buffer, in the presence of added cardiolipin.

Expt. No.	Membrane contents	Ionic strength (molar)	pH	Rate constants k_0 ($\times 10^{-8}$ mol l ⁻¹ min ⁻¹)	
				Initial rate	Final rate
16	Vit K	0.075	7.37	0.35	0.35
*17	Vit K, CL	0.01	7.35	* 2.61	* 0.21
18	Vit K, CL	0.05	7.37	2.29	0.52
19	Vit K, CL	0.075	7.37	2.57	0.40
20	Vit K, CL	0.075	7.37	2.31	0.45
+21	Vit K, CL	0.075	7.37	2.35	+ 0.61
22	Vit K, CL	0.10	7.37	1.26	0.68
*23	Vit K, CL	0.20	7.39	* 1.51	* 0.00

The cytochrome c solutions used had concentrations in the range 21-25 μM, and the buffers were prepared as indicated in Section 3-2.2 from 0.001 M Tris-HCl, by appropriate addition of sodium chloride.

Method B (Section 3-2.4) was adopted in all experiments except those marked with an asterisk (*) where method C was used.

+ 0.10 ml of calcium chloride solution, 0.025 M (I = 0.075 M) was added to the cytochrome c, 85 min after the start of the reaction. Rate constants were calculated from the gradients of the graphs illustrated in

Figure 3.7.

the 'uncatalysed' reaction (experiment 2, Figure 3.5). For this reason it was assumed that the equilibration of cardiolipin between the two interfaces after its addition to the H-cell just below the methyl viologen/hexane interface (method B, Section 3-2.4) was rapid, leading to the formation of an essentially complete monolayer at the membrane/substrate interface almost from the start of the run. Most of these experiments were performed using method B in this assumption. However it became apparent from the results at different ionic strengths that the reaction went through two distinct stages, both of which were approximately zero-order (Figures 3.6 and 3.7). In general, for reactions in either phosphate or Tris buffer, an initial fast rate over the first 100-260 minutes was followed by a slower second rate which then remained constant overnight. This at first suggested that the rate of cardiolipin transfer through the bulk membrane might not be nearly as fast as had originally been supposed, resulting in a relatively slow build-up of the cardiolipin monolayer at the membrane/substrate interface and a consequent delay in the establishment of a steady reaction rate. This hypothesis was tested in the experiment described after method B in Section 3-2.4. The slow levelling of the meniscus observed at the membrane/substrate interface was taken to indicate that the cardiolipin monolayer was formed gradually, taking in that case about four hours to reach completion. The method of introduction of the phospholipid into the membrane was therefore modified in subsequent experiments, method C being adopted. Those results obtained using this later method are marked by asterisks in the Figures and Tables. When the kinetics experiment at 0.6 M ionic strength (experiment 14) was repeated using

method C, the initial fast rate was abolished (experiment 15) and the constant rate obtained (in this case practically zero) was equal to the original second rate. However this was not generally found to be the case, with all the other experiments still exhibiting two-part kinetics. It was concluded therefore that the observed two-stage behaviour was not an artefact produced by the experimental procedure but was in fact a real effect which would have to be accounted for in any proposed mechanism.

The initial and final rate constants were plotted separately as a function of ionic strength for phosphate (Figures 3.8 (a) and (b)) and Tris-HCl (Figures 3.9 (a) and (b)) buffers. The initial rates were rather irreproducible and lacked any obvious pattern, but the final rate in each case produced a reasonable curve with a pronounced maximum in the region of 0.1 M. (It should be noted that the ionic strength scale in Figures 3.9 (a) and (b) is expanded relative to that in Figures 3.8 (a) and (b).) Also, the final rates were independent of the experimental method used (B or C, as discussed above) whereas the initial rates were considerably altered by changing the method of adding the cardiolipin. This, together with the observation that experiments performed in the absence of cardiolipin always displayed simple zero-order kinetics with a single rate constant throughout (Figure 3.5), suggests that the two-stage behaviour was dependent on ionic strength and the presence of cardiolipin at the membrane/substrate interface. Thus it seems likely that the binding of cytochrome c to the interface was the controlling factor here.

It is probable that the cytochrome c used in these studies (Sigma Type III, from horse heart) contained more than one form of the protein, since considerable evidence for polymorphism has been

presented by numerous investigators^(96,114,134-136). In addition to the possible presence of small quantities of oligomers of cytochrome c⁽¹³⁴⁾, at least two forms of the monomer are known to exist, differing in their reactivity towards reducing agents such as ferrous ion, ascorbate and tetrachlorohydroquinone^(135,136). Taborsky⁽¹³⁶⁾ has noted in particular that different lots of Sigma Type III cytochrome c could vary widely in their reactivity towards ferrous ions in duplicated experiments at pH 7.5 in Tris or phosphate buffers, presumably due to different proportions of the various forms of the protein in each. It would appear that the monomeric forms differ in their tertiary structure⁽¹³⁵⁾, which is very sensitive to the precise conditions of isolation and purification of the protein, and that they are interconvertible by a slow, pH-dependent equilibrium^(96,135). At pH 7 the rate of interconversion must be extremely slow since the two forms are separable by physical methods⁽¹¹⁴⁾. Moreover, the relative proportions of the different conformations are not altered by variations in ionic strength at pH 7, as shown by the invariance of the optical rotatory dispersion spectrum of the protein when the ionic strength of the medium is changed⁽¹³⁷⁾.

Nevertheless the existence of two forms of cytochrome c cannot by itself explain the present results. Although it would readily account for the observation of two distinct rates — the first corresponding mainly to reaction of the more reactive species and the second to reaction of the less reactive form, with a changeover in rate when the reactive species was exhausted — both of these steps would be expected to show the same dependence on ionic strength, each passing through a distinct maximum (at the same value of I for a given buffer, since both conformations must have the same overall

charge) for reasons similar to those discussed above when the 'uncatalysed' reaction was considered. Clearly this was not the case, as comparison of Figures 3.8 (a) and (b), or of Figures 3.9 (a) and (b) shows, even taking into account the likely inconsistency in the initial rates due to the use of experimental method B. Some more subtle explanation must therefore be sought.

As mentioned earlier, two distinct modes of binding between cytochrome c and a cardiolipin monolayer are possible, the first involving a reversible electrostatic association (termed 'adsorption') and the second an irreversible hydrophobic interaction (termed 'penetration'). These two binding schemes should lead to two completely different mechanisms for the reduction of cytochrome c (Figure 3.10). Adsorption of the positively charged protein onto the negatively charged interface should facilitate the electron transfer from the vitamin K semiquinone anion (present at low concentration in the monolayer in dynamic equilibrium with cardiolipin) to the iron (III) centre (Figure 3.10 (a)). The observation of reduction would therefore depend exclusively upon positive-negative charge interactions in this case. Under favourable conditions, penetration might follow adsorption (Figure 3.10 (b)), the rate and final extent of which might reasonably be expected to be related to the strength of the original adsorption⁽¹²³⁾. A cytochrome c molecule, once penetrated, should not be readily released into the bulk aqueous phase since the penetration process is essentially irreversible⁽¹²³⁾. The penetrated ferricytochrome c should then be quite rapidly reduced by dihydrovitamin K (or vitamin K semiquinone) in the membrane phase, so that the interface would eventually become covered with hydrophobically bound ferrocytochrome c. If this

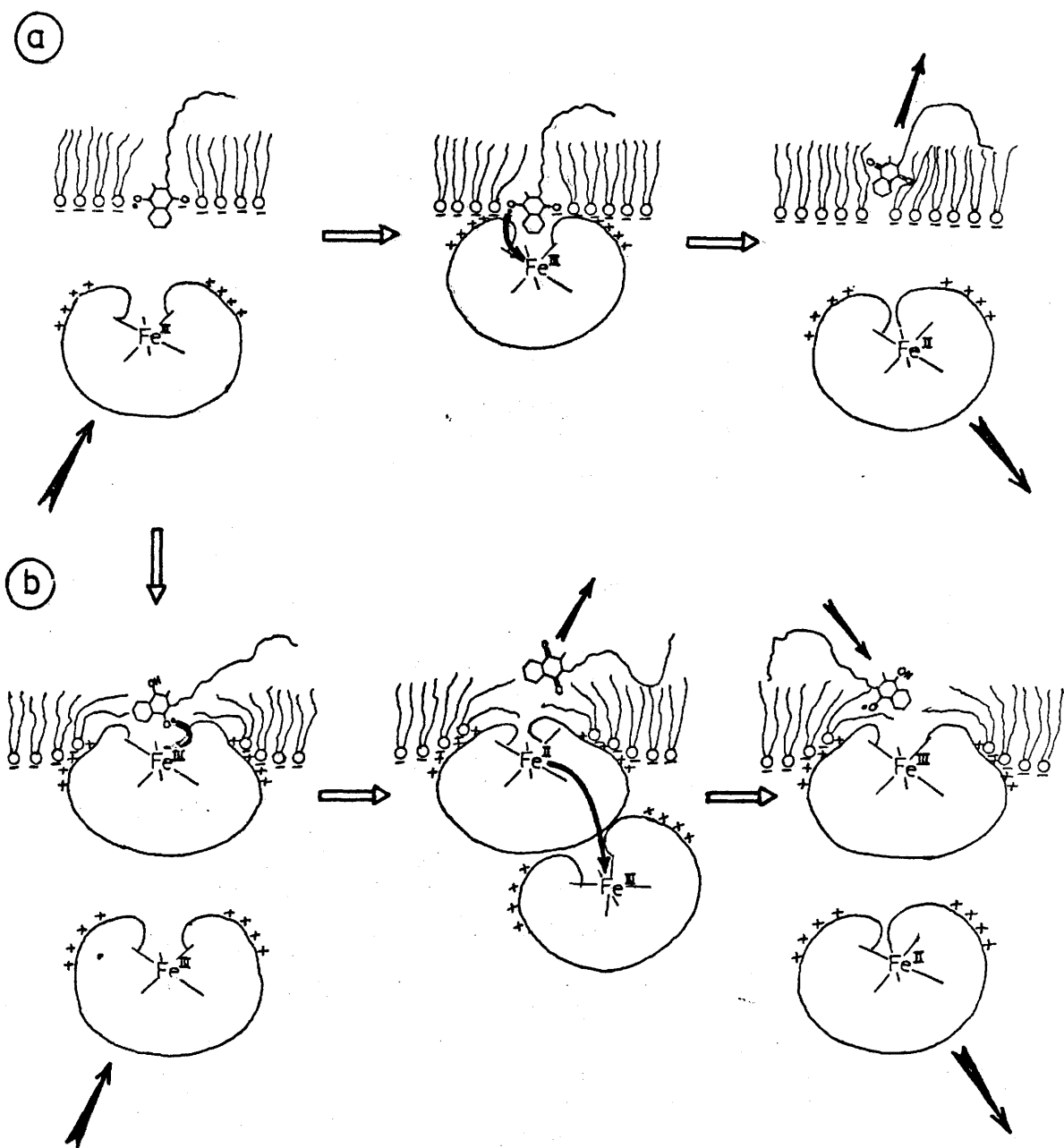


Figure 3.10 Suggested mechanisms for the biphasic reduction of ferricytochrome c by reduced vitamin K (semiquinone radical) at a negatively charged lipid/aqueous interface.

(a) Mechanism involving only surface adsorption of the protein.

(b) Mechanism in which penetration of cytochrome c into the 'membrane' follows adsorption.

See text for full description.

bound ferrocyanochrome c was inactive, no further reduction of the bulk aqueous solution would be observed. However, it has been shown that electron exchange occurs between ferro- and ferricytochrome c in aqueous solution^(108,109), and this should be enhanced in the present case both by the partial neutralisation of the positive charge on the bound protein and by the lowering of its redox potential (from + 273 mV to + 225 mV) on binding⁽¹²⁸⁾. It is therefore not unreasonable to suppose that electron transfer could occur from the bound ferrocyanochrome c to the bulk ferricytochrome c at the interface. The bound cytochrome would then be reduced again by dihydrovitamin K to allow continuation of the reaction. In this case the observation of reduction would depend upon both positive - negative (for the penetration process) and positive - positive (for the electron transfer step) charge interactions.

The experimental results may now be analysed in relation to these two postulated mechanisms, acting consecutively. Discussion of initial rates will be limited to those kinetics runs in which the cardiolipin monolayer at the membrane/substrate interface was complete from the start of the reaction (results marked with an asterisk) since in the majority of cases the slow build-up should have resulted in anomalous results. However it is assumed that the onset of a steady second rate signified that the cardiolipin monolayer was by that time complete, so all the final rates measured are taken to be significant. The point at which the changeover from initial to final rate occurred therefore corresponded to the time taken for penetration of the monolayer by cytochrome c to reach its equilibrium value: the observed wide variations in this time (between 100 and 260 minutes)

could be due to the combination of a number of factors, such as strength of adsorption, the final coverage level attained, the inhomogeneity of different cytochrome c solutions (discussed above) and the complicating effects of slow cardiolipin build-up (in the earlier experiments).

In the initial stages of the reaction, prior to the establishment of an equilibrium penetrated monolayer, mechanism (a) (Figure 3.10) should predominate. As discussed above, the rate of such a reaction, governed by a positive-negative charge interaction, might have been expected to pass through a maximum at some value of ionic strength, but this was not apparent from the results (Figures 3.8 (a) and 3.9 (a)). The reason for the randomness of the results was probably the large number of complicating factors involved, to which may be added the gradual changeover from mechanism (a) to mechanism (b). All that can really be taken from these results is that the initial rate, in phosphate or in Tris buffer, showed a marked decrease towards high ionic strength, as predicted by the Brønsted-Bjerrum equation (equation (3-15), Section 3-1.1). It is perhaps at first sight surprising that the initial rates should follow simple zero-order kinetics, but it should be remembered that all these reactions were, in absolute terms, extremely slow, so that an apparent lack of dependence of the observed rate on the bulk substrate concentration might be expected when the time of observation was limited.

In contrast to the initial rates, the final reaction rates varied in a characteristic way with ionic strength, with both the phosphate and Tris results passing through a pronounced maximum around 0.1 M (Figures 3.8 (b) and 3.9 (b)). On the basis of

mechanism (b), Figure 3.10), which involves a positive-positive charge interaction, the rate should be expected to increase with increasing ionic strength. This would explain the behaviour of these curves up to 0.1 M. Above 0.1 M, however, the observed rate dropped off markedly. This again was to be expected, since the level of penetration of the monolayer by cytochrome c (related to the strength of adsorption of the protein) should be much reduced at high ionic strength. The fact that the final rates measured in phosphate buffer were significantly higher than those found for Tris - HCl buffer of the same ionic strength is also interesting. A similar faster rate of reduction of ferricytochrome c by ferrous ions in phosphate than in Tris buffer has been observed by Taborsky⁽¹³⁶⁾, who attributed the effect to the ability of phosphate anions to bind with both reactants^(114,136). Comparison of the measured final rates in experiments in which cardiolipin was present in the membrane with those obtained when the membrane contained only vitamin K shows that, in many cases, the phospholipid was rather ineffective in enhancing the reaction rate. This could be explained if, in the 'uncatalysed' reaction, only mechanism (a) operated (that is, penetration of cytochrome c into monolayers of vitamin K was negligible) whereas the 'catalysed' reaction occurred primarily via the intrinsically slower mechanism (b). The observation that none of the 'uncatalysed' reactions (experiments 1-4, Figure 3.5 and experiment 16, Figure 3.7) exhibited two-stage kinetics supports this hypothesis. The equality, or near-equality, of the initial and final rates in several of the 'catalysed' kinetics runs (notably in experiment 9, Figure 3.6), which was quite reproducible in duplicated

experiments, was most probably coincidental.

The dual mechanism proposed above for the cardiolipin - catalysed biphasic reduction of ferricytochrome c by dihydrovitamin K, although somewhat speculative, is therefore able to explain all the experimental observations. One final test was applied: Calcium ions are known to complex very strongly with cardiolipin⁽¹³⁸⁾, so that calcium ions added to the aqueous substrate should compete effectively with cytochrome c for adsorption sites on the interfacial monolayer. This in turn should cause a reduction in the initial reaction rates whilst not markedly affecting the final rate (which is determined by a positive-positive charge interaction). This indeed was found to be the case when a small amount of calcium chloride (0.10 ml of 0.025 M solution) was added to the cytochrome c solution in experiment 21, when Tris-HCl (a non-complexing buffer) was used (Figure 3.7).

It is useful in conclusion to summarise the main results obtained from these bulk liquid membrane studies. Both coenzyme Q and vitamin K have been shown to function as reversible electron carriers through a bulk hydrocarbon membrane phase. Kinetics studies on a variety of systems have indicated that substrate reduction proceeds more rapidly with vitamin K as the electron transfer agent than with coenzyme Q. When the redox indicator methylene blue was used as aqueous substrate, its reduction was diffusion controlled at the membrane/substrate interface, and the reaction was inhibited to varying degrees by the addition of different amphipathic phospholipids to the membrane. With ferricytochrome c as the aqueous substrate, however, the biphasic reaction with dihydrovitamin K was no longer diffusion controlled, but was determined by mechanistic factors.

The very slow zero-order reaction was greatly stimulated by the addition of cardiolipin to the membrane, the maximum rate being attained at an ionic strength of 0.1 M, in phosphate or in Tris-HCl buffer. None of the other phospholipids tried was equally effective, so that it would appear from these results that cardiolipin fulfils a specific role in catalysing the reduction of cytochrome c. Such a property might also be of considerable importance in the mitochondrial electron transfer process. It is perhaps, therefore, not a coincidence that the cytochrome c and cardiolipin contents of mammalian tissue appear to be related⁽¹³⁹⁾.

Chapter Four

PLANAR BIMOLECULAR LIPID MEMBRANES

4-1 INTRODUCTION

The second model membrane system investigated was the planar bimolecular lipid membrane, or BLM as it is commonly known. The work described in Chapter 3 on bulk liquid membranes has shown that vitamin K and coenzyme Q are capable of transferring electrons between certain aqueous reductants and aqueous oxidants across a hydrocarbon barrier of macroscopic dimensions, where the diffusion path between the two interfaces was about five centimetres. Although such a system can sometimes provide useful information — for example on reaction mechanisms or on the role of phospholipids — its usefulness as a model for biological membranes of the type described in Section 1-2 is severely restricted because of the bulk of the 'membrane' phase. In these systems the amphipathic carrier adsorbed at the interfaces was considered to be in a state of rapid dynamic equilibrium with the free carrier in solution in the membrane. In most biological membranes, however, the main structural element is the lipid bilayer, which represents the limiting case of a bulk liquid membrane as the solvent is removed to bring the two interfacial monolayers into contact. Since such a membrane contains no reservoir of carrier solution, it is by no means certain that electron transfer, which would have to involve a 'flip-flop' motion of the carrier molecule, could occur across a bilayer.

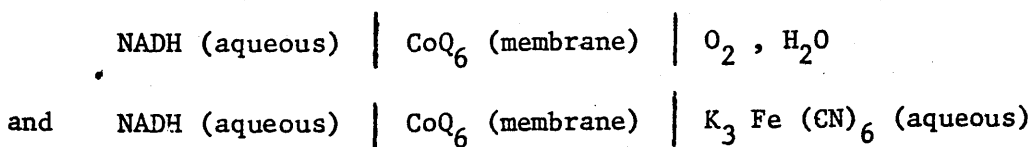
The movement of lipid molecules from one side of a bilayer to the other was first studied in films of stearate⁽¹⁴⁰⁾, which was found to transfer with a half-time of 25 minutes at 25°C. Phospholipid 'flip-flop' has been investigated by an EPR spin-labelling technique in liposomes of egg lecithin⁽¹⁴¹⁾: the half-time at 30°C was 6.5 hours, which is at least eight orders of magnitude slower than translational migration within the plane of the bilayer⁽¹⁴²⁾. More recently the rate of cholesterol transfer in egg lecithin-cholesterol liposomes has also been estimated⁽¹⁴³⁾, this time by a method involving fluorescence quenching, and the half-time at 30°C was found to be 72 minutes. The observation that sterol 'flip-flop' is much faster than that of phospholipids is readily explained since sterol molecules have only about half the mass of phospholipids and have a smaller hydrophilic group, so that much less energy is required for their transfer across the hydrophobic region of the lipid bilayer⁽¹⁴³⁾. Vitamin K and coenzyme Q are also weakly hydrophilic in both their oxidised and reduced forms, but their mobility in a bilayer might be seriously impeded by the length of their isoprenoid side-chains, which in the case of coenzyme Q₁₀, if in the stretched all-trans form, is almost exactly double the length of a typical phospholipid acyl chain.

It was therefore the main object of the work described in the present Chapter to discover whether these long-chain isoprenoid quinones could still function efficiently as electron transfer agents within the strict confines of a membrane of biological thickness. The BLM was chosen as a model system since it has a relatively well defined physical structure which allows its thickness and surface area

to be measured accurately by electrical and optical methods. The first stage in this work involved the formation and characterisation of BLM containing vitamin K₁ or coenzyme Q₁₀. Thereafter attempts were made to perform redox reactions across some of these membranes.

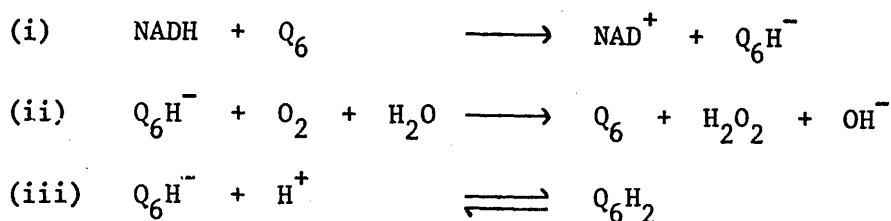
The possibility of redox reactions occurring across BLM has been considered by several workers, most of whom have been interested in the generation of photo-potentials across membranes containing, or in contact with, suitable pigment molecules⁽²⁰⁾. These photo-electric effects are considered to arise from the production and separation of charge carriers in the system under illumination, and require that some asymmetric condition exists across the membrane⁽¹⁴⁴⁾. It has been shown that unequal illumination of the two surfaces of a suitably modified BLM may be sufficient to generate a photo-emf⁽¹⁴⁵⁾.

Of more direct relevance to the present research is the work of Ismailov *et al.*^(78,146) They have demonstrated the spontaneous generation of transmembrane potentials in a number of systems, among them



in which the membranes were formed from mixtures of lecithin and cholesterol in decane and contained coenzyme Q₆ at a concentration of 10 mg ml⁻¹. No potential was detected in the absence of the membrane-bound carrier, but with coenzyme Q₆ present in either membrane, the potential generated was positive on the reductant (NADH) side and negative on the oxidant side. This observation of a membrane potential is contrary to the expected behaviour of quinone/quinol-mediated electron transfer, which, for the reasons given earlier (Section 1-1), should be non-electrogenic. However, the authors have

explained their results in terms of the formation of the Q_6H^- species (in reaction (i)) at the aqueous reductant/membrane interface, followed by its migration across the membrane to the oxidant side:



These experiments were carried out under aerobic conditions, so that the subsequent reoxidation of Q_6H^- by atmospheric oxygen (ii) could occur on both sides of the membrane. It was also found that the membrane potential was diminished by increasing the buffering capacity of the aqueous media: at buffer concentrations above 0.2 M it was completely abolished⁽⁷⁸⁾. This could be explained if the equilibrium (iii) were shifted to the right at the higher buffer concentrations, making the uncharged quinol species Q_6H_2 the only significant electron carrier. In fact it seems more likely that the quinol should have been the predominant carrier under any of the conditions investigated. This possibility was apparently not considered by the authors, which is perhaps not surprising since their experimental approach (namely, the measurement of membrane potentials) was incapable of detecting any non-electrogenic component of electron transfer.

The object of the present research was to investigate continuous, and therefore necessarily non-electrogenic, electron transfer processes mediated by membranes containing coenzyme Q_{10} or vitamin K_1 . The work of Ismailov *et al.* was however interesting in that it suggested the intrinsic ability of suitably modified BLM to act as electron transfer membranes. One further point worthy of note

was the observed increase in membrane potential in the above systems brought about by adding FMN to the NADH solution^(78,146). This was attributed to an increased rate of reduction of coenzyme Q₆, and is in agreement with the results obtained earlier (Section 2-3.2, Table 5), which showed that the rate of biphasic coenzyme Q₁₀ reduction (undetectable in the presence of NADH alone) was substantially increased when the FMN free radical species was used as reductant.

4-1.1 Formation and structure of planar BLM

Despite earlier progress towards the understanding of the basic structure of biological membranes (see Section 1-2), the first successful attempt to make membranes with a bimolecular lamellar arrangement of lipids in an aqueous medium was not reported until 1962, when Mueller, Rudin, Tien and Wescott described the formation of such membranes from solubilised lipids of cellular origin⁽¹⁴⁷⁾. Since then numerous investigations have been carried out by these and other workers into the properties of such structures, which have been variously termed 'bimolecular', or 'bilayer', or 'black' lipid membranes (all conveniently abbreviated to BLM)^(19,20,148). Although other methods have been used^(19,20), membranes are most commonly formed by a 'painting' technique which involves spreading the lipid, usually as a 1-2% (w/v) solution in a long-chain hydrocarbon solvent such as *n*-decane, over a small, smooth orifice, 1-10 mm in diameter, in a hydrophobic partition separating two aqueous solutions. Initially a thick lens is formed in the orifice, but under favourable conditions this spontaneously thins to form a BLM in the centre surrounded by an annulus (or torus) of solution known as the Plateau-

Gibbs border. The progress of thinning is conveniently monitored optically, by studying light reflected at a small angle from the membrane (Figure 4.1). The patterns observed have been likened to the behaviour of soap films in air: as the membrane thins, bright swirling patterns of interference colours are displayed; then black spots appear, either singly or several at once, and these gradually expand and coalesce until most of the aperture is covered⁽²⁰⁾. When viewed by reflected light the BLM finally appears as a faint grey sheen, across which coloured spots occasionally swirl, demonstrating the fluidity of the structure. Light is reflected from a thin membrane at both aqueous/lipid interfaces, but the reflected waves are 180° out of phase. When the thickness of the membrane, and hence the path difference between the two reflected beams, is much less than one quarter of the (average) wavelength of the incident radiation (which is true of a BLM illuminated with white light) complete destructive interference results, so that the membrane in this state appears black when viewed against a dark background. During the thinning process, when the membrane thickness is equal to or greater than one quarter of the illuminating wavelength, constructive and destructive interference both operate to produce a sequence of coloured fringes⁽¹⁴⁹⁾.

It has been suggested that thinning of lenses ('primary black films') to BLM ('secondary black films') occurs due to a combination of Plateau-Gibbs border suction, gravitational flow, and diffusion⁽¹⁵⁰⁾. Due to the curvature of the surface in the border region, this is always a seat of low pressure relative to the adjacent flat film, and hence there is a tendency for lipid solution to be sucked from the membrane into the Plateau-Gibbs border. Membranes are generally

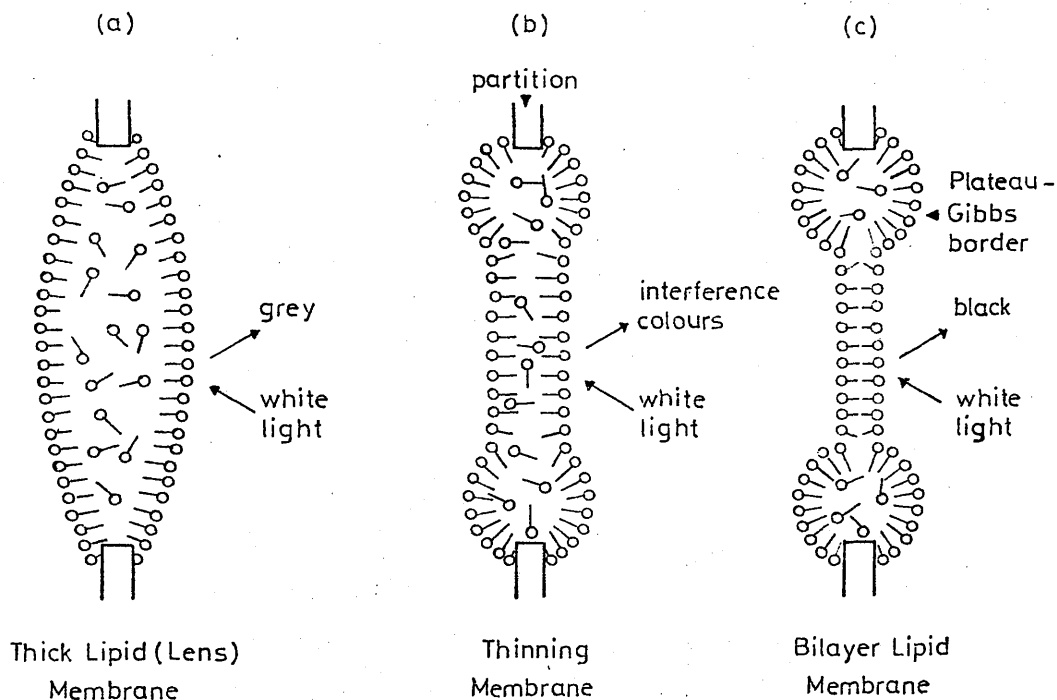


Figure 4.1 Diagram illustrating the three stages observed during the thinning of a lipid membrane formed in a circular orifice in a hydrophobic partition separating aqueous media. The nature of the light reflected by the membrane at each stage is indicated⁽¹⁴⁹⁾.

formed in a vertical plane, so there will also be a tendency for the lipid solution, which, if in *n*-decane, is less dense than the aqueous phase, to rise in the membrane. Thus the formation of black areas normally starts in the lower half of the membrane and spreads upwards. The final thinning step has been attributed⁽²⁰⁾ to chance contacts between the two interfaces, caused by thermal motion, mechanical vibration or trace impurities (such as dust particles). Once the two interfaces come close enough together, van der Waals attractions between the opposing hydrocarbon chains become sufficiently strong to produce a bilayer in a small region of the membrane. In the process, neighbouring apposing molecules are also brought close together, and growth of the BLM ensues by a so-called 'zipper effect'⁽²⁰⁾. The rate of thinning, and the stability of the resulting BLM, if formed, are dependent upon a number of experimental parameters, such as the composition of the lipid solution (the most important factor), the composition of the bathing aqueous phases, the material and geometry of the support, temperature, and differential pressures across the film (caused, for example, by vibration).

When a drop of lipid solution is introduced into the orifice, any of three distinctly different behaviours may be observed: (a) the solution remains as a thick lens; (b) the solution drains sufficiently to give a coloured film (1,000 - 10,000 Å in thickness) but remains in this state for a long period of time; (c) the membrane thins spontaneously to give a BLM in equilibrium with its Plateau-Gibbs border, or breaks. It is evident that the spontaneous formation of a BLM at the aqueous/oil/aqueous biface must be due to an overall decrease in the free energy of the system, and its continued stability

once formed, to a balancing of forces when thermodynamic equilibrium is achieved⁽²⁰⁾. The attractive forces between the interfacial monolayers would include van der Waals interactions and Plateau-Gibbs border suction, whilst the repulsive forces would include steric interactions and electrostatic repulsion between the charge double layers situated on both interfaces. The thermodynamics of BLM formation has been treated in detail by several authors^(19,20,151); it is sufficient to point out here that the stability of BLM formed from highly amphipathic lipids seems to be due at least in part to the low interfacial free energy (or 'interfacial tension') of the bilayer membrane.

To date an impressively wide variety of lipid materials have been used to form BLM⁽²⁰⁾, among them cell and organelle extracts, purified and synthetic phospholipids and some mono- and diglycerides. In addition, some substances outside this range, notably 'oxidised cholesterol'⁽¹⁵²⁾ have been successfully employed. Other molecules, although by themselves incapable of forming BLM, may be incorporated into BLM composed mainly of 'successful' lipids. Of particular relevance to the present research are the experiments of Leslie and Chapman⁽¹⁵³⁾, who were able to make BLM from egg lecithin containing vitamin K or β -carotene but found that the incorporation of coenzyme Q₆ gave membranes which were stable only in the thicker, silver-grey condition. Although for accurate physical characterisation of membranes the purity of the lipid materials used is obviously important, in general extreme purity does not appear to be a prerequisite for the formation of stable BLM: in fact in some cases deliberate maltreatment of lipid preparations (for example, exposure to air oxidation) is

positively beneficial⁽¹⁵²⁾. However the observation that BLM cannot be prepared from pure synthetic saturated phospholipids at 36°C⁽¹⁵⁴⁾ is more likely a consequence of the high phase transition temperatures of these lipids, since stable BLM have been formed at temperatures above the phase transition temperatures⁽²⁰⁾. Thus mobility of the hydrocarbon chains within the bilayer seems to be important for the stability of BLM: perhaps this degree of liquidity enables the membrane to cope with random mechanical shocks. Redwood et al.⁽¹⁵⁵⁾ have synthesised a pure, branched-chain saturated lecithin, 1,2-diphytanoyl-3-*sn*-phosphatidyl choline, which is reported to form very stable BLM at 24°C. This non-oxidizable phospholipid would appear to be an ideal choice for many BLM studies, including those to be described here, but unfortunately it is not yet commercially available. The addition of cholesterol to phospholipid solutions considerably enhances the stability of the resulting BLM⁽¹⁵⁶⁾, a property almost certainly related to the ability of cholesterol to condense phospholipid monolayers⁽¹⁵⁷⁾.

The choice of solvent for the preparation of membrane forming solutions is also worth consideration. In practice a number of organic solvents have been found suitable, among them chloroform-methanol mixtures, the *n*-alkanes from octane to hexadecane and various long-chain hydrocarbon oils. The rate of thinning of the membrane is very dependent upon the viscosity of the solvent: if it is too viscous then thinning will be very slow, or may not occur at all; if, on the other hand, it is too mobile, then thinning may occur so rapidly that the membrane ruptures. The importance of insolubility in the aqueous phase of both the lipid and solvent constituents of

the membrane has also been indicated⁽¹⁵⁸⁾. Clearly methanol does not meet this criterion, and so this solvent was avoided as far as possible in the present experiments (although small quantities of chloroform - methanol were sometimes necessary to solubilise the phospholipids). *n*-Decane was found to be the most suitable solvent for general use in these studies.

It should be noted that the precise chemical composition of even the simplest BLM is not known with certainty, since it is not simply related to the composition of the bulk membrane forming solution, as a result of differential adsorption on the interfaces. Direct chemical analysis of the membrane is difficult since the total number of molecules in a typical BLM is only of the order of 10^{14} ⁽²⁰⁾. However a few methods do now exist which allow reasonable estimates of BLM composition to be made⁽¹⁵⁸⁾. Henn and Thompson⁽¹⁵⁹⁾ have reported that the ratio of lecithin to *n*-decane in their BLM was approximately 1:10 both with and without added cholesterol, compared to 1:700 in the original solution, whilst the cholesterol : lecithin ratio was close to unity in both cases. From this result it would appear that the ratio of the lipid components in a BLM should be fairly similar to their relative proportions in the membrane forming solution, whilst the solvent is preferentially 'squeezed out' from the bilayer.

4-1.2 Measurement of BLM thickness

One of the most important characteristics of the BLM, which has gained it widespread acceptance as a viable model for biological membranes, is its bimolecular dimension, being generally less than 100 Å across (Section 1-2). Several techniques have been used to measure the thicknesses of BLM^(19,20), notably electron microscopy,

optical, and electrical methods. Of these the last is by far the simplest, and probably also the most accurate, so electrical methods were employed in the present investigations. Both AC and DC methods are available for the measurement of BLM electrical properties. Although AC measurements are reported to be more accurate⁽¹⁵⁸⁾ and are preferred by many workers, DC methods are relatively straightforward and simple to apply: both methods have been found to give very similar results⁽²⁰⁾. In any case the major source of error in the calculated membrane thicknesses arises from uncertainty in the BLM area (see below), so that any additional accuracy afforded by the use of AC methods would be minimal. For these reasons, and since the absolute value of the membrane thickness was not of primary importance in the present work (only the fact that a bilayer was formed) a 'DC transient' method was routinely used here.

The method finally adopted, after testing numerous experimental arrangements, was a modification of that described by Huemoeller and Tien⁽¹⁶⁰⁾, the main difference being that the direct measurement of BLM resistance — a rather inaccurate process — was here avoided by the determination of voltage decay curves at two different values of the external resistance. The method is based upon the assumption that a thin membrane may be treated as a parallel-plate capacitor, in which the hydrophobic region serves as the 'dielectric', and the hydrophilic head group regions (with their associated electric double layers) serve as the 'plates'. The membrane capacitor is first charged up to a small voltage, and then allowed to discharge through a known resistor in parallel. The capacitance is found from the decay time of the applied voltage, and from it the thickness of the 'dielectric' is calculated. The equivalent circuit used is shown in

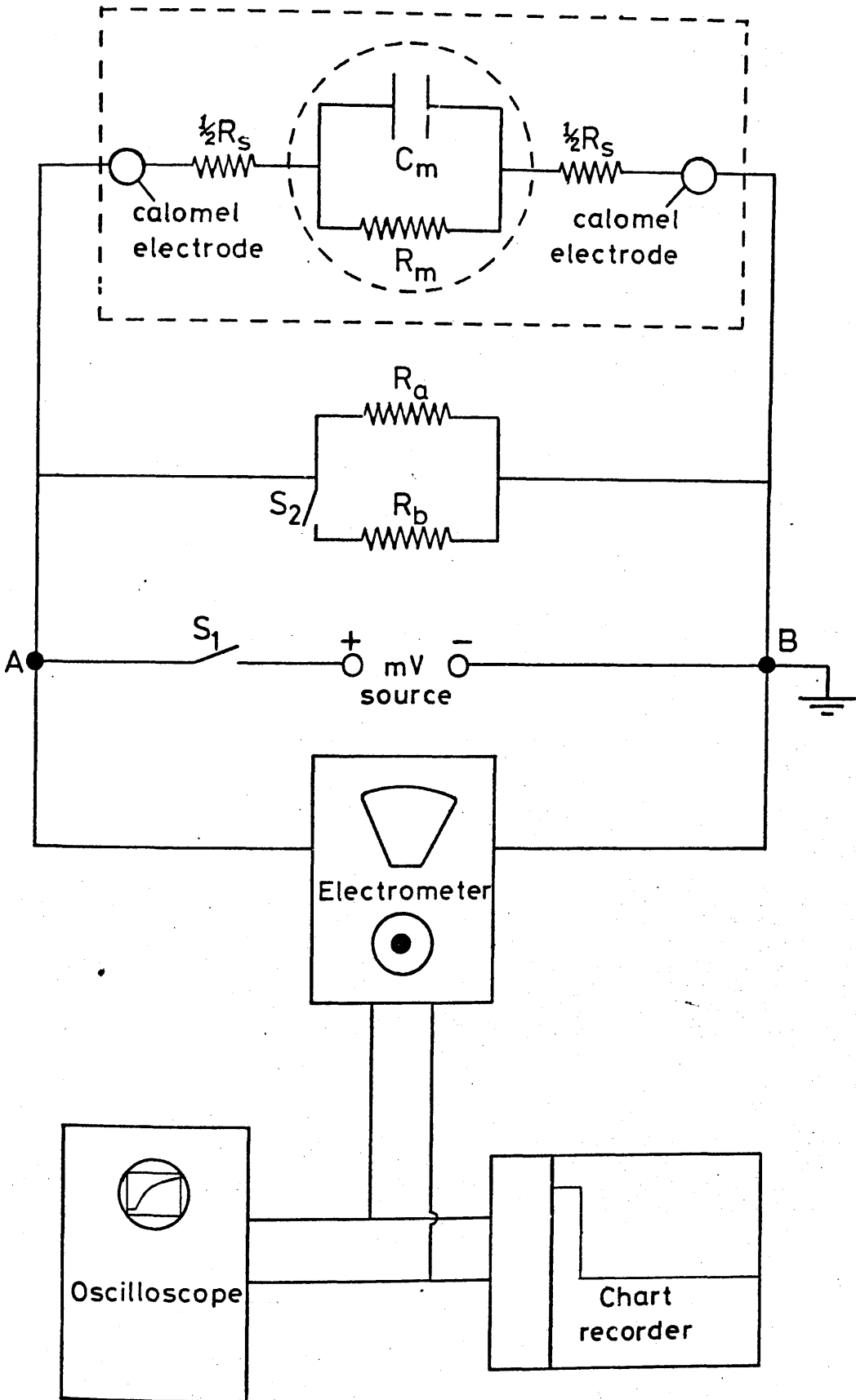


Figure 4.2 Circuit used for the measurement of resistances and capacitances of BLM by the DC transient method, as described in Section 4-1.2

Figure 4.2: the section inside the rectangular box (broken line) comprises the electrodes, aqueous solutions and membrane, contained in a glass vessel (Figure 4.3); the membrane itself is represented by a capacitor and resistor in parallel (circled with a broken line). After charging to a fixed potential (switch S_1 closed) in the range 40-100 mV (the exact value is not required in the calculations) the membrane is allowed to discharge, which it does both through its own intrinsic resistance ($R_m \sim 10^6$ ohms) and, via the bathing solutions and electrodes (combined resistance $R_s \sim 2 \times 10^4$ ohms), through the external standard resistor (R_a , or by closing switch S_2 , $R_c = R_a R_b / (R_a + R_b)$). When R_a alone is used, the total resistance of the external charge leakage pathway is given by

$$R_1 = R_a + R_s \quad (4-1)$$

When both R_a and R_b are used in parallel, the total resistance becomes

$$R_2 = R_c + R_s = R_a R_b / (R_a + R_b) + R_s \quad (4-2)$$

The discharge is monitored, through an electrometer of high input impedance ($\sim 10^{14}$ ohms), by an oscilloscope which records the decay curve. The voltage decay is given by the expression⁽¹⁶⁰⁾

$$E_t = E_o e^{-t/CR} \quad (4-3)$$

where E_o and E_t are the voltages measured initially and at time t (seconds) respectively, C (farads) is the overall capacitance, and R (ohms) the overall resistance of the circuit. In practice C is almost identical to the membrane capacitance since the capacitance of the circuit in the absence of a membrane is negligible. The overall resistance R is the resultant of combining the membrane resistance R_m with the total parallel resistance (R_1 or R_2). Thus for R_a alone,

$$R = \frac{R_n R_1}{R_n + R_1} \quad (4-4)$$

whilst for R_a and R_b used together,

$$R = \frac{R_n R_2}{R_n + R_2} \quad (4-5)$$

The time constant τ (seconds) of an R - C circuit is defined as the time taken for the voltage to fall to $1/e$ of its initial value, where $e = 2.718$ is the base of natural logarithms. When this condition is reached, equation (4-3) simplifies to

$$\tau = C R \quad (4-6)$$

The two alternative resistances R_1 and R_2 , defined above, will yield different time constants, τ_1 and τ_2 , for any given membrane.

Combination of equations (4-4) and (4-5) with (4-6) gives

$$\tau_1 R_1 = R_m (R_1 C - \tau_1) \quad (4-7)$$

and
$$\tau_2 R_2 = R_m (R_2 C - \tau_2) \quad (4-8)$$

Division of equation (4-7) by (4-8), followed by rearrangement, then yields

$$C = \frac{\tau_1 - \tau_2 x}{R_1 - R_2 x} \quad (4-9)$$

where $x = \tau_1 R_1 / \tau_2 R_2 \quad (4-10)$

The capacitance of the membrane, as calculated from equation (4-9), is not very useful as it stands: for the purposes of comparison, specific capacitances are normally quoted, that is, capacitance per unit area of membrane (C/A , in farads cm^{-2} , where A is the area of the thin film). The capacitance of a parallel-plate capacitor is proportional to the area A (cm^2) of the plates and inversely proportional to the distance d (cm) between them:

$$C = \frac{\epsilon A}{4\pi d} \quad (4-11)$$

where ϵ is the dielectric constant. For the purposes of equation (4-11) both C and ϵ should be expressed in electrostatic units (1 farad = 9×10^{11} esu). The value of ϵ may be estimated by taking the bulk dielectric constant of the hydrocarbon solvent. Although this assumption has been considered by several investigators to be a major source of error, recent theoretical calculations have shown that the dielectric constant of a bilayer differs from that of the bulk hydrocarbon solvent by only a few percent⁽¹⁶¹⁾. The value of d calculated from equation (4-11) therefore corresponds to the thickness of the hydrophobic region of the membrane. To convert this to an overall thickness, the contributions from the surface hydrophilic layers must also be taken into account. The amount to be added depends upon the sizes and orientations of the head groups of the particular amphipathic lipids used. For egg lecithin membranes the thickness of the polar group region is approximately 8 \AA ⁽¹⁵⁸⁾, so a correction of 16 \AA must be added to the calculated d values to give an estimated total thickness of the membrane.

4-2 EXPERIMENTAL

4-2.1 Chemicals

All the chemicals used in this work — lipids, solvents and other reagents — were of the highest purity available commercially. Their sources are listed in Appendix I. The purity of the lipids was checked chromatographically by thin layer chromatography (TLC) on silica gel GF254, the plates being developed with chloroform:methanol:water, 65:25:4 v/v/v, and visualised with iodine^(71,149). All showed

at most only trace contamination and were judged to be suitable for use without further purification.

Aqueous solutions were prepared in 0.025 M phosphate buffer, pH 6.86, with sodium chloride added as necessary to equalise the tonicities of solutions on either side of a membrane. 0.1 M sodium chloride was also added to the aqueous phase when capacitance measurements were to be made, in order to improve electrical contact with the membrane. (The addition of electrolyte has been found to stabilise BLM⁽¹⁴⁹⁾, but the membrane capacitance is rather insensitive to electrolyte concentration except at very high ionic strengths (above 1 M) when collapsing of the electrical double layers at the membrane/aqueous interfaces becomes important⁽¹⁹⁾.)

4-2.2 Preparation of membrane-forming solutions

A variety of pure lipids and lipid mixtures were tested for their ability to form BLM. Preliminary experiments⁽⁵⁷⁾ indicated that *n*-decane was the most generally suitable solvent, so it was always used in the preparation of membrane-forming solutions in this work. Stock solutions of the various lipids used were prepared as indicated below. The membrane-forming solutions were then made up from these by dilution and/or mixing as required. The concentrations of all the solutions tested are given in the Tables of results. Solution volumes were measured accurately by the use of Hamilton syringes, and weighings were made to ± 0.005 mg on a precision microbalance (Mettler M5, Mettler Instruments AG, Switzerland). Membrane-forming solutions were normally made up immediately prior to use, but when storage was necessary the solutions were kept under nitrogen in hermetically sealed glass ampoules at -20°C . Under these conditions,

any cholesterol present in the solutions tended to precipitate out, so slight warming (to about 30°C on top of a drying cupboard) was sometimes required to redissolve the lipid before use.

Coenzyme Q and vitamin K were supplied as a solid, and as a viscous oil, respectively. Standard solutions of each, at concentrations of about 50 mg ml⁻¹, were prepared in decane.

Cholesterol was supplied as a crystalline white solid. Stock solutions containing 15 mg ml⁻¹ in decane were prepared.

Egg lecithin was supplied as a solution, 100 mg ml⁻¹, in hexane.

0.15 ml of this solution was transferred to a small glass vial, and the hexane was removed under a stream of nitrogen. 0.98 ml of decane was then added, followed by 0.02 ml of a 2:1 chloroform:methanol mixture, which, with shaking and slight warming (to about 30°C) redissolved the yellow solid, giving a stock solution of concentration 15 mg ml⁻¹.

Dioleoyl lecithin was supplied as a solution, 20 mg ml⁻¹, in chloroform. Initial attempts to prepare membrane-forming solutions by methods analogous to that described above for egg lecithin were unsuccessful due to the apparent insolubility of the dioleoyl lecithin in decane:chloroform:methanol solvent systems. However it was discovered that similar difficulties had been met by other investigators⁽¹⁵⁸⁾ who had suggested that traces of water in the solution were responsible, causing the phospholipid to gel. The technique developed by Fettiplace et al.⁽¹⁵⁸⁾ was therefore tried and found to be successful: 1 ml of the stock dioleoyl lecithin solution was placed in a small round-bottomed flask and the chloroform solvent was removed under vacuum. The residue was suspended in 1 ml

of Analar acetone which was then also removed by slow evaporation under vacuum. This treatment was repeated with a second 1 ml aliquot of acetone, and then with two 1 ml aliquots of anhydrous diethyl ether. Finally, the dried residue was dispersed into 1 ml of decane, (which had just been passed through an alumina column (Woelm, W. Germany)) in which it then dissolved quite readily, giving a standard solution of 20 mg ml^{-1} .

Cardiolipin was supplied as a solution, 9.3 mg ml^{-1} , in ethanol.

Again there were difficulties in preparing a membrane-forming solution in decane, even when the above method, used successfully for dioleoyl lecithin, was tried. Eventually the following procedure was found to be suitable: 1.075 ml of the stock solution was placed in a small round-bottomed flask and the ethanol solvent was removed by evaporation under vacuum. The residue was treated with Analar acetone and anhydrous diethyl ether, as indicated above. It was then redissolved in a solvent mixture containing 0.5 ml of diethyl ether and 1.0 ml of decane. Finally the volume of the solution was reduced to 1.0 ml under vacuum. (As diethyl ether has a much higher vapour pressure than decane, it was expected that most of the ether would be removed, leaving a solution of cardiolipin in decane.) The cardiolipin then remained in solution, at a concentration of 10 mg ml^{-1} .

4-2.3 Apparatus for membrane formation studies

The formation of BLM separating aqueous phases is in principle quite simple: two compartments are separated by a thin solid partition so that the only contact between them is via a small orifice in the partition, in which the membrane is formed. However, the design of apparatus and choice of materials are limited in practice by a number of factors, particularly when physical characterisation of the

membranes is required. It is useful at this stage to note some of these requirements:

1. The membrane support must be made of a material which is chemically inert to the organic solvents used. It must also be a good electrical insulator and must provide a hydrophobic surface which will be wetted by the lipid solution but not by water (to allow secure attachment of the film border). Mechanical strength and good machine workability are also often important. Although a number of materials have been used, Teflon (polytetrafluoroethylene), which possesses all of these characteristics, has been preferred by most workers (20,149,158).
2. The edges of the orifice must be narrow but smooth in order to stabilise the Plateau-Gibbs border. A narrow groove around the periphery of the hole is also frequently effective in this respect (19). These precautions also minimise the chances of electrical conduction pathways becoming established at the edges of the membrane.
3. Provision for simultaneous optical and electrical monitoring of the membrane must be made. A window in one of the aqueous compartments is therefore required, while the other is kept in darkness, to allow for observation of the membrane by reflected light. If transmitted light is to be used (Section 4-2.5) then windows must be fitted to both compartments. Electrical contact to the aqueous solutions on either side of the membrane may be made either by direct immersion of suitable electrodes in these solutions, or indirectly to external electrodes via salt bridges.
4. For electrical measurements non-polarisable electrodes are required. For the DC transient method used in this research the response time of the circuit in the absence of a membrane had to be

less than 0.01 ms. Calomel (mercury/mercurous chloride) electrodes were found to be ideal for this purpose.

5. The whole assembly must be free from mechanical vibrations, since BLM are rather unstable to hydrostatic pressure differences across the membrane, due to their low interfacial tension. When the diameter of a flat circular membrane is 2 mm and the interfacial tension is 5 dyne cm^{-1} , a pressure difference of 10^{-4} atm is sufficient to cause the film to adopt the form of a hemisphere; a little more pressure ruptures the membrane⁽¹⁹⁾. For this reason it is desirable that one of the aqueous compartments should be completely closed and free of (compressible or expandable) air bubbles, especially if perfusion of the aqueous solutions is required (Section 4-2.5).

5. The aqueous solutions on either side of the membrane must be maintained at the same osmolarity; otherwise volume flow of water may cause membrane distension and rupture.

6. If stirring in the aqueous phases is required, the stirrers should be positioned so that their disrupting effect on the membrane is minimised, yet close enough to ensure adequate mixing near the membrane surfaces.

7. Careful electrical screening of the membrane cell and all associated leads and equipment is absolutely essential to exclude 'noise' from extraneous electromagnetic fields: mains frequency interference can be a particular problem. The electrical measurements also require that no ionic or electronic conductive pathway exists between the two aqueous compartments except via the membrane.

The apparatus used in membrane formation studies in this research is shown in Figure 4.3. The inner compartment consisted of

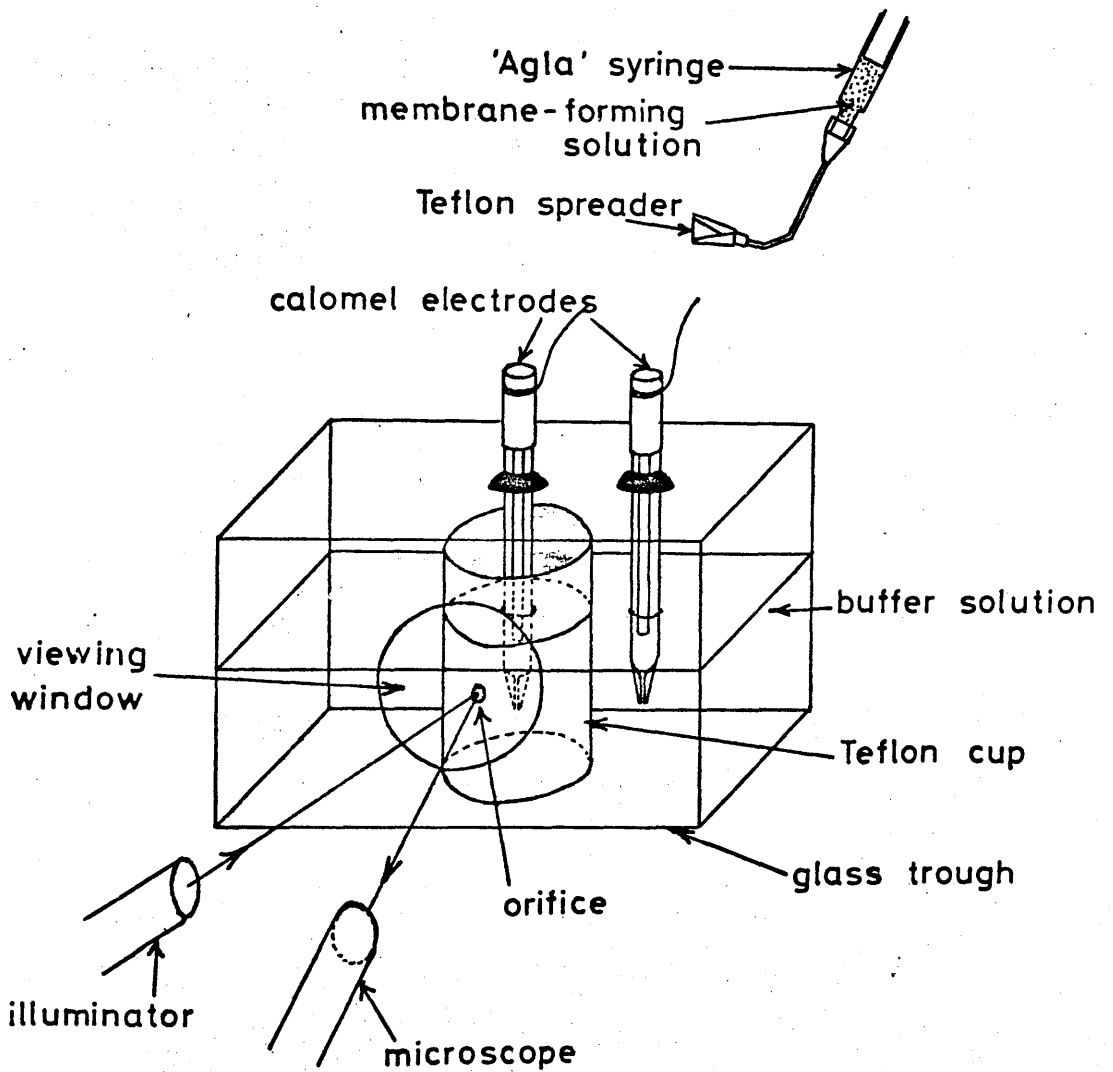


Figure 4.3 Apparatus used in initial membrane formation and characterisation studies.

a small Teflon cup, 5 cm high and 4 cm in diameter, machined from a solid rod. A small area of the wall was thinned to 0.3 mm and a hole was drilled through this section, at a point halfway up from the base. The periphery of this orifice, which was 2.061 mm in diameter (measured with a travelling projection microscope — Model 6C, Nikon Kogaku K.K., Japan) was carefully smoothed with a heated needle. The outer compartment was a small glass trough, 10 x 7 x 6 cm, with a 3 cm diameter hole in one face covered with a 4 cm diameter disc of optically clear glass which was fixed in position with 'Araldite'. The Teflon cup was fixed in position inside the trough, using a piece of 'Plasticine' to join the two above the level of the aqueous solutions, so that the orifice was directly opposite this window, through which the membrane was illuminated and viewed. Illumination was provided by a microscope illuminator (4V, 8W, Cambridge Instruments Ltd., England) and the membrane was viewed through a microscope of short focal length. The angles of light incidence and reflection from the membrane were about 20° . Both compartments were filled with aqueous solution until the orifice was submerged about 5 mm below the surface. The glass trough was placed inside a black cardboard box covered on the outside with aluminium foil, which acted as a Faraday cage, screening the membrane and electrodes from extraneous electrical fields. The top of the box had a closeable flap to allow access during membrane formation, and a circular hole was cut in the side opposite the window. A dip-type calomel electrode (Pye Unicam, No. 11161) was placed in each aqueous solution and was held in a fixed position in the lid of the box. The whole apparatus was securely mounted on a weighted stand which was placed on rubber supports on a vibration-damping table (Gallenkamp, England).

Membranes were formed in the orifice of the Teflon cup by a 'painting' method, using the specially designed Teflon spreader, shown in Figure 4.3, which was attached to a bent syringe needle fixed to an 'Aglar' syringe. A little of the membrane-forming solution in the syringe was ejected onto the spreader, which was then passed slowly down through the aqueous solution, over the orifice, and back up again, the edge being held in contact with the Teflon cup throughout the operation. Membranes were quite easily formed by this method when suitable solutions were used. It was also found, in agreement with the observations of other investigators^(20,149,158), that the stability of the membranes formed was markedly improved by 'preconditioning' the orifice. This was done by air-drying a small annular ring of the membrane-forming solution around the aperture both inside and outside the partition. The reasons for the effectiveness of this pretreatment are not fully understood, but are probably associated with the ease of spreading of the phospholipid into its monolayer at the hydrocarbon/water interface⁽¹⁵⁸⁾.

4-2.4 Estimation of membrane stabilities and thicknesses

A wide variety of lipid solutions were tested for their ability to form stable BLM, using the apparatus described above and illustrated in Figure 4.3. The stability of the membranes and their thinning characteristics were observed initially by visual inspection by noting the times taken for the transitions from a 'lens', through the various 'coloured' stages, to a black film in equilibrium with its Plateau-Gibbs border, and finally to rupture. However, for the more long-lived membranes it was more convenient to measure the stability

(that is, the lifetime in a particular state) by an electrical method. This was based on the fact that membrane rupture was accompanied by a huge decrease in the resistance measured across the calomel electrodes, from around 10^6 ohms to 2×10^4 ohms. The circuit used for monitoring the resistance is shown in Figure 4.2. Both switches S_1 and S_2 were kept open. With an external resistance R_a of 2.76×10^5 ohms, there was an approximately tenfold drop in the total resistance across AB when the membrane disappeared. The resistance was measured with a high impedance electrometer (Keithley Model 610C, Keithley Instruments, USA), operating on the 0.01×10^8 ohms scale setting. Under these conditions the measuring voltage applied across the electrodes was less than 0.4 mV, which was much too small to have any destabilising influence on the membrane. The amplified output from the electrometer was displayed on a chart recorder (Servoscribe Type RE511, Goerz Electro, Austria). The trace produced was therefore a graph of resistance as a function of time on which membrane rupture was clearly indicated by a sudden dramatic change following a period of relative invariance corresponding to the lifetime of the membrane.

The thicknesses of membranes at various stages during their lifetimes were calculated from their measured DC capacitances using equation (4-11). Again the circuit shown in Figure 4.2 was employed, but this time the Keithley electrometer was operated on the 0.1 volt scale and the output was displayed on a storage oscilloscope (Tektronix Type 564, Tektronix Ltd., Guernsey). This allowed the discharge curve to be photographed with a Polaroid camera. Various external resistors R_a and R_b were used, and their resistances were measured accurately using an AC bridge (Type B331, Wayne Kerr Comp.

Ltd., England). Typical values were $R_a = 2.763 \times 10^5$ ohms and $R_b = 1.584 \times 10^5$ ohms. The resistance of the aqueous solution plus electrodes in the absence of a membrane was also measured using the AC bridge and was found to be $R_s = 2.08 \times 10^4$ ohms. The membrane was first charged up to a small fixed voltage (in the range 40-100 mV) using a standard DC source (Voltage Calibrator Type 2003S, Time Electronics Ltd., England) with switch S_1 closed. It was then allowed to discharge (S_1 open) through the external resistor R_a (S_2 open), and the voltage decay curve was recorded on the oscilloscope screen along with lines corresponding to $E = E_0$ (the initial charging voltage) and $E = 0$ (the final voltage). The trace was photographed, and the charge-discharge cycle was repeated immediately with the second resistor R_b in parallel with R_a (S_2 closed). (This use of a second resistor added in parallel with the first to change the total external resistance was preferred to a direct replacement of one resistor by another, because experience had shown that the latter method frequently caused the membrane to rupture when the first resistor was disconnected.) This procedure therefore yielded two discharge curves, corresponding to two known external resistances and one single membrane resistance (assumed constant over the short time scale of the measurements — about one minute). From each photograph, knowing the appropriate time base in each case, a time constant τ was found. Figure 4.4 shows a typical exponential voltage decay curve, and the method of extraction of τ from this curve. The membrane capacitance was then calculated using equation (4-9). From visual inspection of the membrane during the capacitance measurements, the area of the (thin) membrane was estimated: the total measured area of the orifice was multiplied by the approximate fraction covered by the (thin) membrane. This

Figure 4.4 A typical voltage decay curve for a BLM, showing the method of extraction of the decay constant τ .

The membrane forming solution was composed of egg lecithin and cholesterol (7.5 mg ml^{-1} of each in decane) and the capacitance was measured 30 minutes after formation of the membrane. The membrane appeared black by reflected light and had an area of 3.33 mm^2 .

An external resistance of 1.584×10^5 ohms was used, and the resistance of the aqueous medium plus electrodes was 2.08×10^4 ohms, giving a total resistance R , in parallel with the membrane, of 1.792×10^5 ohms.

Using the approximation $\tau \sim C_m R$ (equation (4-6)) with $R = 1.792 \times 10^5$ ohms and $\tau = 1.43 \text{ ms}$, the specific capacitance of the membrane was $0.24 \text{ } \mu\text{F cm}^{-2}$, corresponding to a hydrocarbon layer thickness of about 74 \AA .

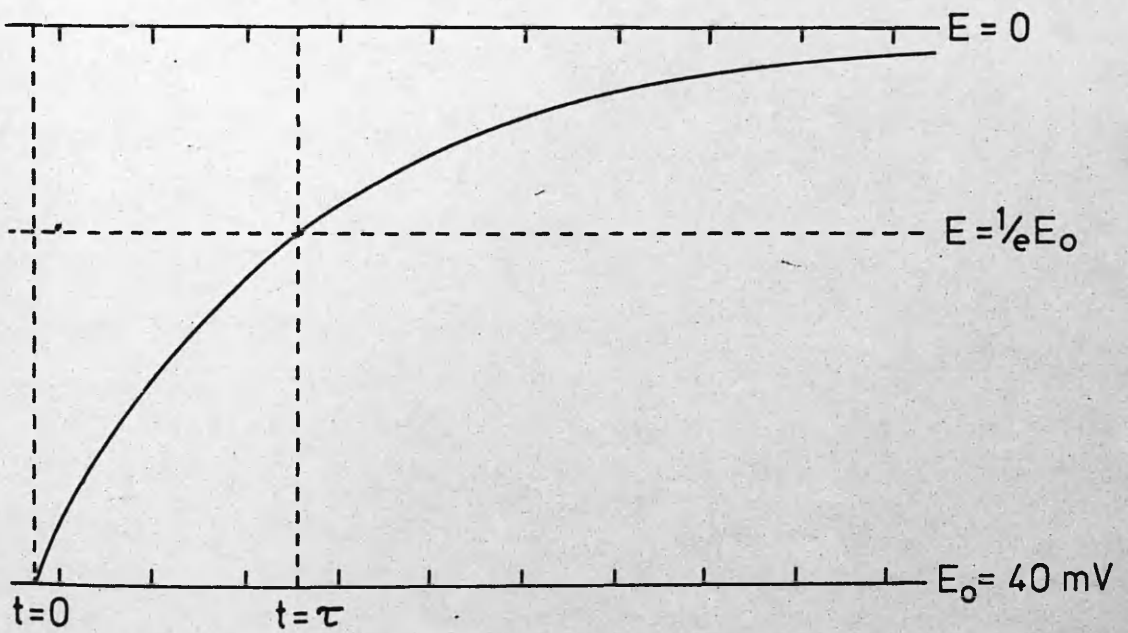
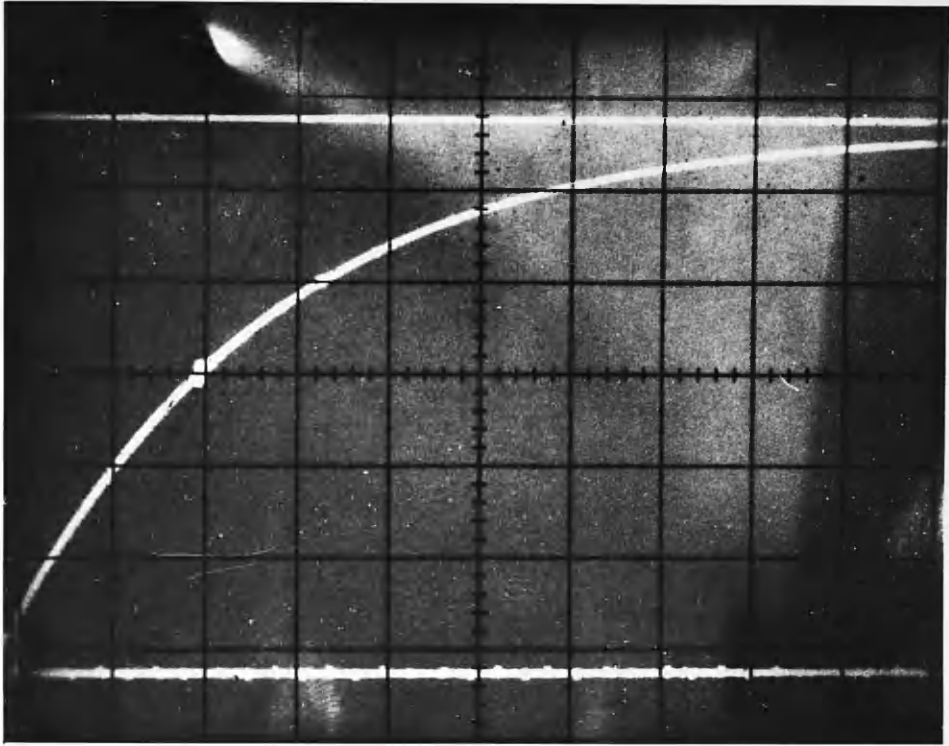


Figure 4.4 Oscilloscope time base : 0.5 ms per division

Decay constant $\tau = 2.86$ divisions = 1.43 ms

allowed the membrane capacitance to be expressed as a value per unit area, a much more meaningful quantity. Finally the membrane thickness was calculated using equation (4-11).

4-2.5 Design and use of membrane cells for kinetics studies

The main general structural requirements for any apparatus to be used in the study of BLM separating aqueous media have already been discussed in some detail (Section 4-2.3). These demands on the experimental set-up posed considerable design and engineering problems. The solution of some of these problems in the present research was a lengthy task, involving a number of design modifications to the apparatus, and the complete construction of several different cells. Only two of these cells — the first ('membrane cell A') and the last ('membrane cell B') — will be described here, since all the reactions of interest were performed using these cells. In addition to all the general requirements specified earlier, this research necessitated the imposition of further design restrictions. Basically the final object of this work was to perform carrier mediated redox reactions across BLM, and hence to achieve a miniaturisation of the bulk membrane systems described in Chapter 3. Here again it was intended to use reduced methyl viologen as the reductant, vitamin K as the membrane-bound carrier, and redox indicators such as methylene blue, DCIP or ferriin as substrates, and to examine the kinetics of reduction by monitoring the changing absorbance of the substrate at an appropriate wavelength.

Kinetics experiments on the bulk membrane systems have shown that these biphasic redox reactions were diffusion controlled at the membrane/substrate interface, with the observed rates being

principally determined by the apparatus itself: the ratio (area of interface)/(volume of substrate), and the efficiency of stirring in the substrate solution were the main controlling factors. Similar behaviour was expected in the miniaturised BLM systems. In order to maintain an observable reaction rate, therefore, the (membrane area)/(substrate volume) ratio had to be maximised. This was particularly important since observation times were severely limited by the relatively short lifetimes of BLM — a problem not encountered in the bulk membrane case. As the membrane area could not reasonably be increased beyond about 3 mm^2 without seriously destabilising the film, the only remaining solution to the problem seemed to be to decrease the substrate volume. However the substrate compartment had to be kept sufficiently large to allow absorbance measurements to be made in a conventional double-beam spectrophotometer. Clearly some kind of compromise was needed. Another specific requirement, dictated by the oxygen-sensitivity of the redox dyes and carriers used, was that the membrane cell had to be completely anaerobic. This in turn necessitated introduction of the membrane-forming solution into the orifice by some 'remote' method which avoided the opening of either compartment to the atmosphere. The cell design had also to allow for perfusion of at least one of the cell compartments — the reductant compartment — so that reaction could be initiated at a set time after the membrane had attained an equilibrium state. Finally, to prevent this perfusion from causing distension and rupture of the membrane, precautions had to be taken to ensure that no gas bubbles were present anywhere within the closed cell.

Membrane cell A: A scale diagram (twice actual size) of the original apparatus constructed for the study of membrane mediated redox

processes is given in Figure 4.5. The cell was constructed in three sections: two aqueous compartments made of black perspex, and an intervening gasket, of glass-impregnated Teflon (which has better machine characteristics than pure Teflon) in which the membrane was formed. The dimensions of the gasket were 25 x 12 x 4 mm, and it had a standard 'Luer' fitting, slightly below halfway up one side, to allow for attachment of a syringe needle. A smooth hole, 2 mm in diameter, was bored through the middle of the gasket, and the area surrounding the hole was thinned down in a funnel shape to about 0.5 mm around the periphery of the orifice. A second hole, 0.2 mm in diameter, was bored through the Luer adaptor to meet the membrane orifice tangentially at its base. The two compartments were made from black perspex blocks, 25 x 12 x 12 mm, with 7.5 mm diameter holes bored through each side. Glass windows, cut from a microscope slide, were recessed into three of the side walls of each compartment, covering the holes to form viewing windows. They were fixed in position with 'Tensol' perspex cement (I.C.I., England). When the cell was assembled, the sections were clamped together so that the uncovered holes were placed against the central gasket, allowing communication between the two compartments through the membrane orifice. Both compartments contained shallow wells to accommodate small glass-coated magnetic stirrers. An 8 mm diameter hole was bored from the top into the reductant compartment, and this was closed by means of a small, threaded stopper and a rubber 'O-ring'. Stainless steel tubing (1 mm outer diameter) provided inlets and outlets for the two chambers, allowing perfusion of the reductant compartment and ejection of air or solution from the substrate

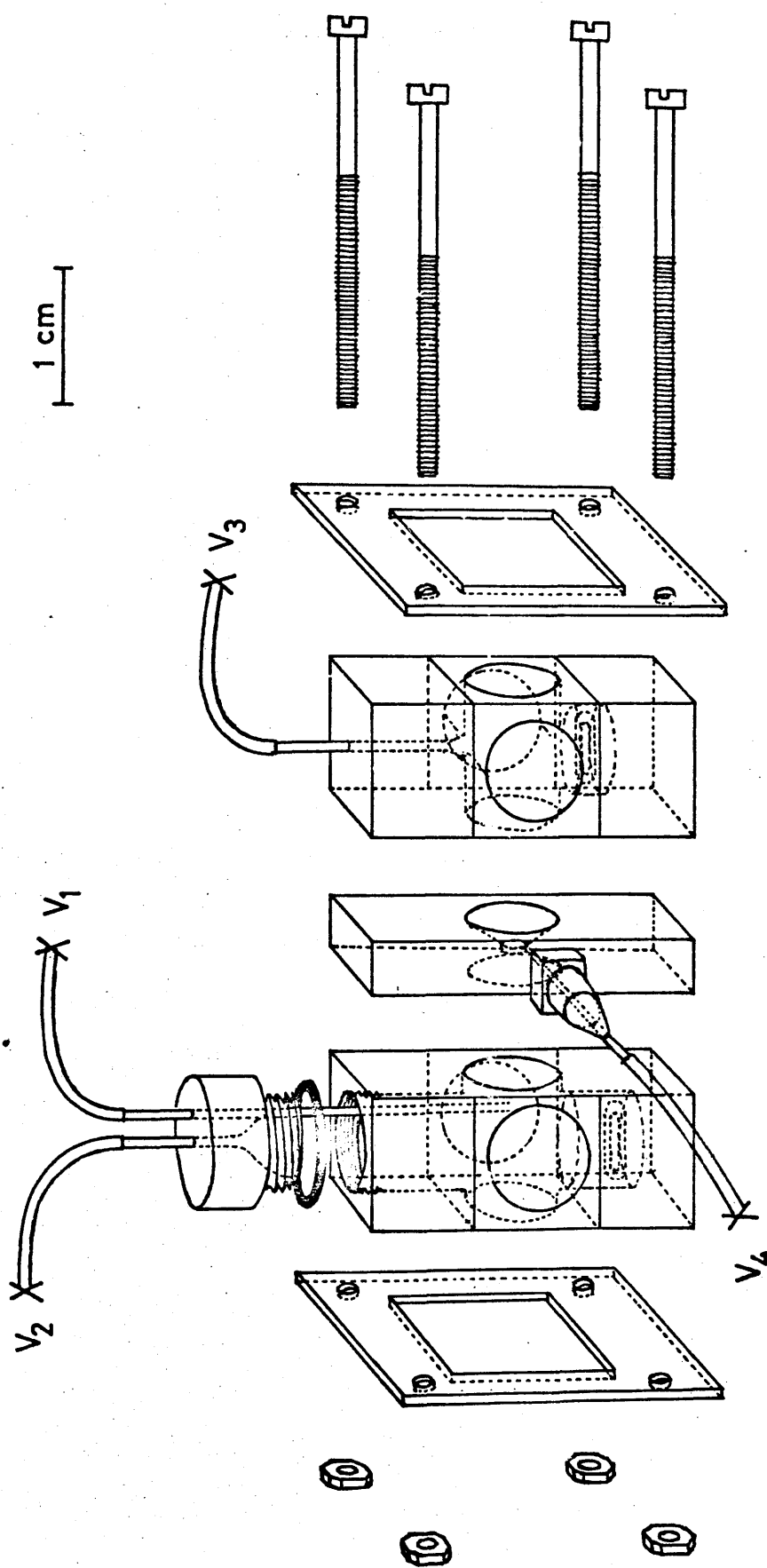


Figure 4.5 Membrane cell A. See text for description.

compartment. Teflon tubing (Biolab Ltd., England), 0.8 mm internal diameter, was used to connect the steel tubing through two-way 'Omnifit' Teflon valves (Biolab Ltd., England) to the ends of stainless steel needles fitted to glass syringes which contained the various reagents required in the experiment. The assembled cell was securely mounted on a magnetic stirrer unit which sat on a vibration-damping table. The flexible Teflon tubing minimised the transmission of vibrations to the cell during perfusion of aqueous solutions.

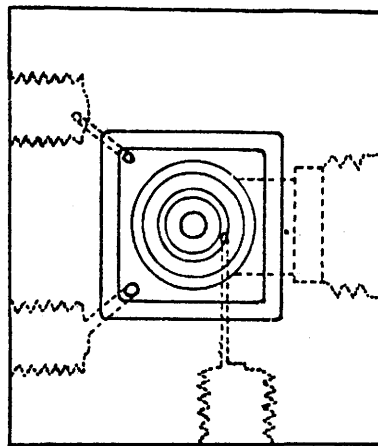
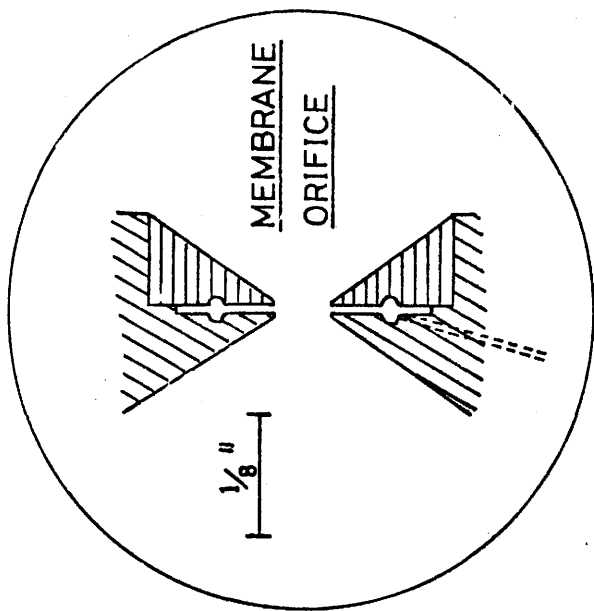
A little membrane-forming solution was first introduced into the preconditioned orifice to ensure that no air bubbles were left in the entry hole or tubing, and valve V_4 was closed. The cell was then filled completely with freshly degassed phosphate buffer, great care being taken to expel any air bubbles. The buffer was introduced through valve V_1 with V_2 and V_3 also open, and several perfusions were performed to scavenge residual traces of air. The buffer was then replaced by similar perfusion with the substrate solution, which was also thoroughly degassed (under vacuum) immediately prior to its introduction into the cell. With both compartments now full of substrate solution, a (thick) membrane was formed by injection of a small quantity of the lipid solution into the orifice. The thinning process could then be assisted by slowly withdrawing excess lipid solution. This method, known as 'marginal suction' has been used successfully by other workers^(19,20,149). During thinning the membrane was viewed by transmitted light, the illuminator and microscope being set up on opposite sides of the cell for this purpose. It was of course impossible to estimate the thickness of the membrane by this method, but at least its existence could be established. When the membrane appeared to be in a stable condition; valves V_1 and V_2 were

opened (V_3 and V_4 closed) and the reductant compartment was slowly perfused with reduced methyl viologen solution to initiate the reaction. Valves V_1 and V_2 were then closed and the cell was left stirring for several hours, until the membrane ruptured. The substrate solution was inspected visually during this time for any sign of reduction. Since accurate spectrophotometric measurements on this simple test cell were impossible, it was thought better to choose a substrate whose reduction could be easily followed visually. The iron-o-phenanthroline complex, which changes from colourless in the oxidised form (ferriin) to bright orange when reduced (ferroin), appeared to be the most suitable. It had already been shown that ferriin is reduced by dihydrovitamin K in a biphasic reaction (Table 7).

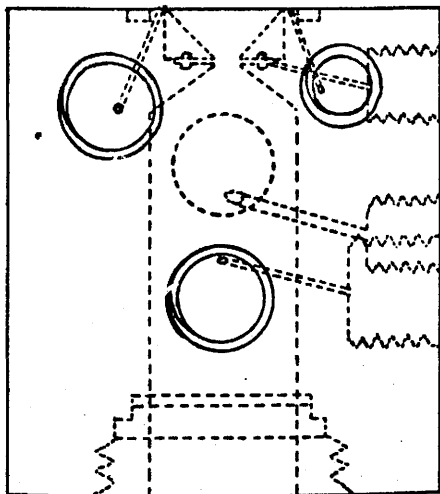
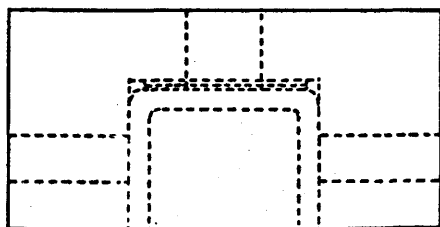
The methyl viologen used was a 2.72×10^{-2} M solution in 0.025 M phosphate buffer. This was 75% reduced to the blue radical species by addition of a measured volume of the degassed solution, from a syringe, to a small sealed bottle containing the appropriate amount of solid sodium dithionite under nitrogen (1.02×10^{-5} moles of dithionite was required per ml of methyl viologen solution). The osmolarity of the resulting reduced methyl viologen solution was calculated to be 0.1224 os M (see Table 14 for calculation). It was therefore necessary to ensure that all substrate solutions were made isotonic with this reductant solution to prevent a gradient of osmotic pressure developing across the membrane. This was achieved by the addition of sodium chloride to the substrate solutions as required (Table 14). The osmolarity of the phosphate buffer was ignored in these calculations since its concentration in the reductant

and substrate solutions was the same. In the present experiment the substrate was 1.0×10^{-4} M ferriin solution (prepared as indicated in Section 2-2.1 in 0.025 M phosphate buffer) and was 0.061 M in added sodium chloride. Reaction was detected by development of the orange colour of ferriin.

Membrane cell B: A scale drawing of the cell finally developed for BLM studies is given in Figure 4.6. The main advantages of this design over the original were; (a) it allowed the formation of bi-layer membranes, (b) it allowed electrical measurements to be made on the membrane, (c) it allowed spectrophotometric monitoring of the substrate, and (d) it was more impervious to oxygen. The cell this time was composed of only two main sections, the membrane orifice being situated in the same block as the reductant compartment. This necessarily rather complex chamber was machined from a block of glass-impregnated Teflon, $1\frac{1}{4}$ " x $1\frac{1}{8}$ " x 1". For the purposes of this description the terms 'front', 'back', 'side', 'top' and 'bottom' refer to the faces indicated on the diagram. A circular well $\frac{1}{8}$ " deep was cut out in the middle of the front face and the central area of the bottom of this well was further recessed by about 0.1 mm. A narrow, shallow circular channel was made in this recessed region. From the back a $\frac{3}{8}$ " diameter hole was bored through the block, using a tapered bit, until the point emerged through the front face, leaving a 1.5 mm diameter circular aperture at the bottom of the well. The back face was then recessed and the hole partially threaded as shown, to take a circular glass window, a Teflon pressure collar, and a hollow brass screw which held the window firmly in position. From the bottom a $\frac{1}{4}$ " diameter hole was bored upwards into the main chamber. This was then blocked off with a Teflon plug and brass screw, to form



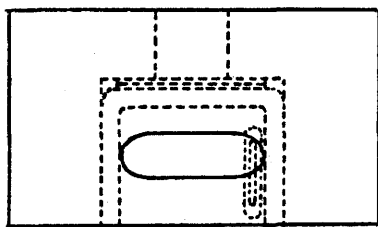
FRONT



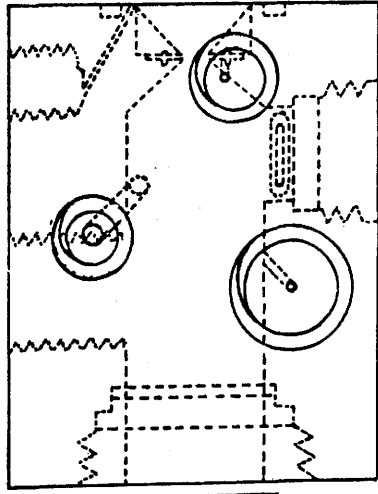
TOP

$\frac{1}{2}$ inch

BACK



BOTTOM



SIDE

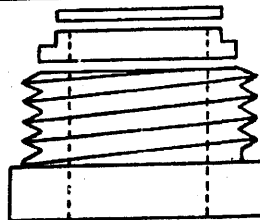


Figure 4.6 Membrane cell B. See text for description.

Table 14 Osmolarity calculations for aqueous reductant and substrate solutions in membrane cell experiments*

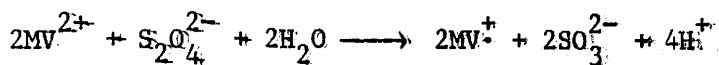
<u>Reagent</u>	<u>Species</u>	<u>Molarity (M)</u>	<u>Osmolarity (osM)</u>
Reduced methyl viologen [†]	MV ²⁺	0.68 x 10 ⁻²	0.0068
	MV ⁺	2.04 x 10 ⁻²	0.0204
	Cl ⁻	5.44 x 10 ⁻²	0.0544
	Na ⁺	2.04 x 10 ⁻²	0.0204
	SO ₃ ²⁻	2.04 x 10 ⁻²	0.0204
			<hr/> 0.1224 osM <hr/>
Ascorbic acid	C ₆ H ₈ O ₆	2.72 x 10 ⁻²	0.0272
	≠ added NaCl	4.76 x 10 ⁻²	0.0952
			<hr/> 0.1224 osM <hr/>
Ferriin	Fe(o-phen) ₃ ³⁺	1.0 x 10 ⁻⁴	0.0001
	Cl ⁻	3.0 x 10 ⁻⁴	0.0003
	Cl ⁻ (from HCl)	1.0 x 10 ⁻⁴	0.0001
	≠ added NaCl	6.095 x 10 ⁻²	0.1219
			<hr/> 0.1224 osM <hr/>
DCIP	DCIP	2.28 x 10 ⁻⁵	0.00002
	≠ added NaCl	6.12 x 10 ⁻²	0.1224
			<hr/> 0.1224 osM <hr/>
Methylene blue	methylene blue	1.16 x 10 ⁻⁵	0.00001
	≠ added NaCl	6.12 x 10 ⁻²	0.1224
			<hr/> 0.1224 osM <hr/>

* The contribution of phosphate buffer to the total osmolarities is neglected in these calculations since its concentration was always the same on both sides of the membrane.

(Continued over)

Table 14 (Continued)

† The reaction between dithionite and methyl viologen may be written :



The H^+ produced is removed by the buffer: $HPO_4^{2-} + H^+ \longrightarrow H_2PO_4^-$.

Therefore H^+ may be neglected in these calculations, and the osmolarity of the buffer remains unchanged.

‡ Sodium chloride was added to these solutions to bring their total osmolarities to 0.1224 osM (the osmolarity of the reduced methyl viologen solution).

a well in which a small magnetic stirrer could be positioned. A hole was also bored from the top of the cell to enter the main chamber, and this was threaded to allow an 'Omnifit' Teflon tubing connector to be screwed in. Two threaded wells were also made in the top of the cell to receive other connectors, and three such wells were made in the side of the cell, as shown. The small size of the apparatus required some of these Teflon connectors to be specially constructed, but their mode of operation was exactly the same as that of the commercially supplied connectors (Figure 4.7), and the same fitments (rubber and steel 'O-rings', and Teflon cones) were used with both. Two of the connectors required slightly wider holes to allow Teflon tubing for the salt bridges to be passed through them, while a third needed a very narrow capillary hole to carry the membrane-forming solution to the orifice without undue wastage. Each of the connectors was linked to a specific point on the inside of the cell by a precisely positioned capillary hole (about 0.2 mm in diameter), as indicated in Figure 4.6. A small pure Teflon plug, which was a tight press fit into the well at the front of the cell, was tapered down towards a 1.5 mm diameter hole in the centre, and a narrow, shallow circular channel in its planar face lay opposite that on the main block. It was found that membranes were further stabilised when a very thin Teflon gasket (cut from a 'Teflon sleeve') with a smooth hole punched in the centre was sandwiched between the bottom of the well and the push-in plug.

The substrate compartment was made from a standard UV/visible optical cell of 1 cm path length (Thermal Syndicate Ltd., England). The cell was cut cleanly in two at a height of 1 cm above its base.

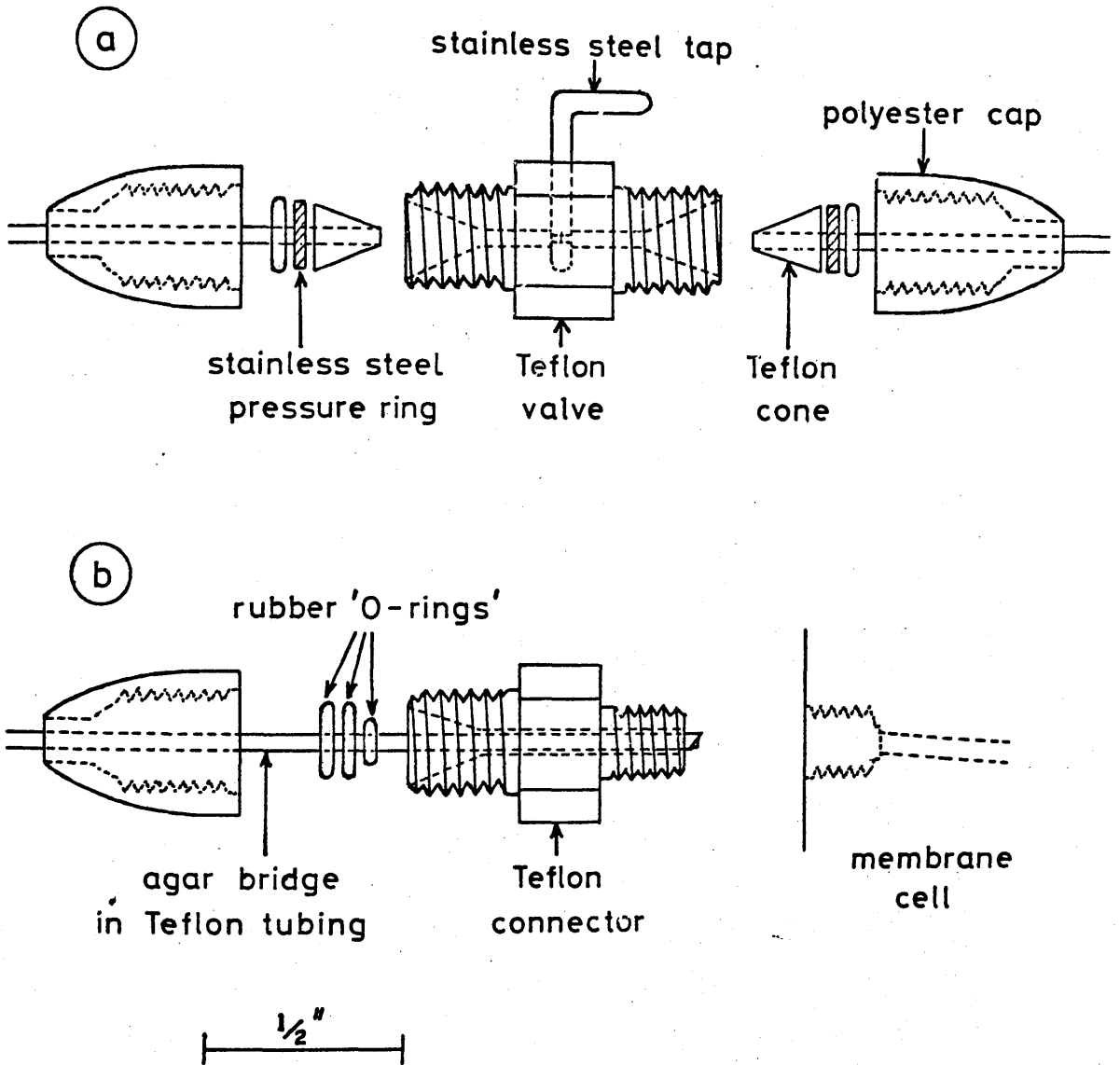
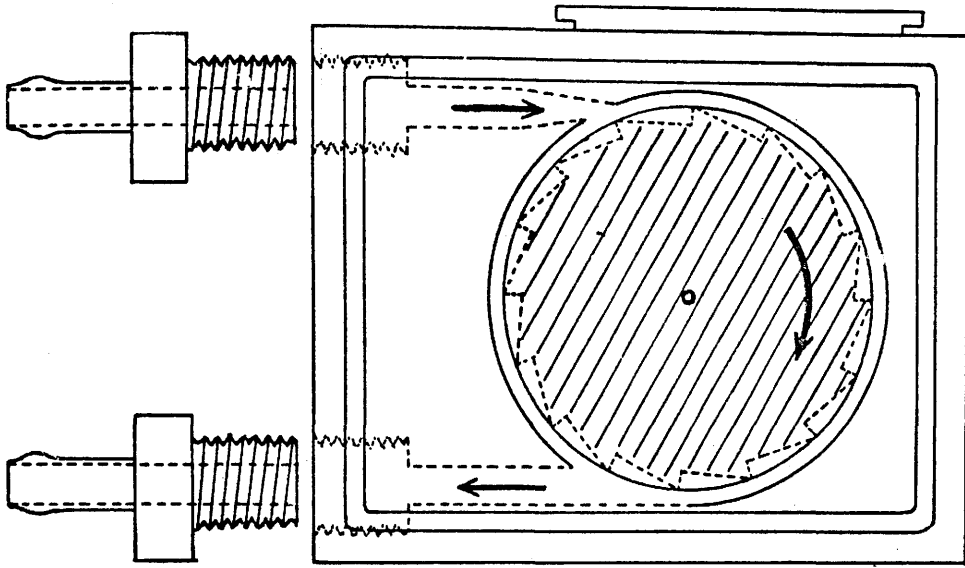


Figure 4.7 Valves and connectors used in conjunction with the membrane cells: (a) Biolab 'Omnifit' Teflon two-way valve. (b) Teflon tubing connector.

The lower portion was mounted with dental wax on a crystal polisher (Logitech PM2, Scotland) and the base was carefully polished with successively finer grades of diamond paste until it was completely transparent and optically flat. (This process took many hours.) The cell was then mounted the other way around and the cut edges were similarly polished to give a very smooth finish. This was essential if air leaks into the cell were to be avoided. A channel was cut in the front face of the Teflon block, into which the cell, a 1 cm cube, was then a tight fit. A square well was sunk in a $1\frac{1}{8}$ " x 1" x $\frac{9}{16}$ " black perspex block, the dimensions of the well being chosen so that the optical cell was held firmly inside, but with the open end protruding slightly above the level of the block. A hole was bored from the end of this well through the perspex block to provide a viewing window. Two slits, 1 cm long and $\frac{1}{8}$ " wide, were made in the sides of the block, leading in to the optical faces of the cell, to allow the passage of the spectrometer beam through the substrate solution. Before the two halves of the apparatus were clamped together, a little silicone rubber fluid ('Bathtub Caulk' - Dow Corning Corp., USA) was spread on the face of the perspex block around the optical cell. This helped to form an airtight seal between the two blocks when the brass clamp was tightened up. Thin rubber gaskets placed behind the optical cell prevented it from cracking during assembly. The silicone rubber gasket was left to dry for at least two hours before the membrane cell was filled.

Stirring of the aqueous solutions inside the membrane cell was accomplished by means of two small glass-coated magnetic stirrers, one in each compartment. These were driven by an external magnet

which was turned by water pressure and was contained in a stirrer unit below the cell (Figure 4.8). The soft iron magnet, $1\frac{3}{4}$ " in diameter and $\frac{1}{2}$ " deep, was completely encased in a perspex covering. Using a $\frac{3}{16}$ " diameter drill, indentations were cut around the perimeter of the perspex case, as shown, to allow more efficient energy transfer from the flowing water to the rotor. A stainless steel pin passing through the centre of the magnet and its perspex case acted as a pivot. The magnet was placed in a cylindrical well, $2\frac{1}{8}$ " in diameter and $\frac{3}{4}$ " deep, in a $3\frac{1}{2}$ " x $2\frac{3}{4}$ " x $1\frac{17}{32}$ " perspex block. An inlet jet and water outlet were bored from one side of the block to meet the stirrer well tangentially. The outer sections of these holes were threaded to accept screw-in brass connectors. A rectangular perspex lid, $\frac{1}{4}$ " thick, was screwed down onto the block, with a rubber tubing gasket providing a watertight seal between the two when compressed. Small Teflon plugs were inserted in the block and lid to provide bearings for the pivoted magnet. The top of the perspex lid was recessed to accept the membrane cell in a fixed position, and the cell was secured to the stirring unit by means of a small brass clamp. A brass plate was attached to the stirring unit, which allowed it to be positioned accurately in the SP800 spectrophotometer. This arrangement ensured that the membrane cell was automatically aligned in the light beam every time and that it was in the best position for optimum stirring efficiency in both compartments. Water at 25°C was pumped from an external thermostatted supply tank via PVC tubing to the stirring unit. The rate of water flow, and hence of stirring, was controllable by adjustment of a Teflon 'rotaflo' tap which allowed diversion of a proportion of the water.



1"

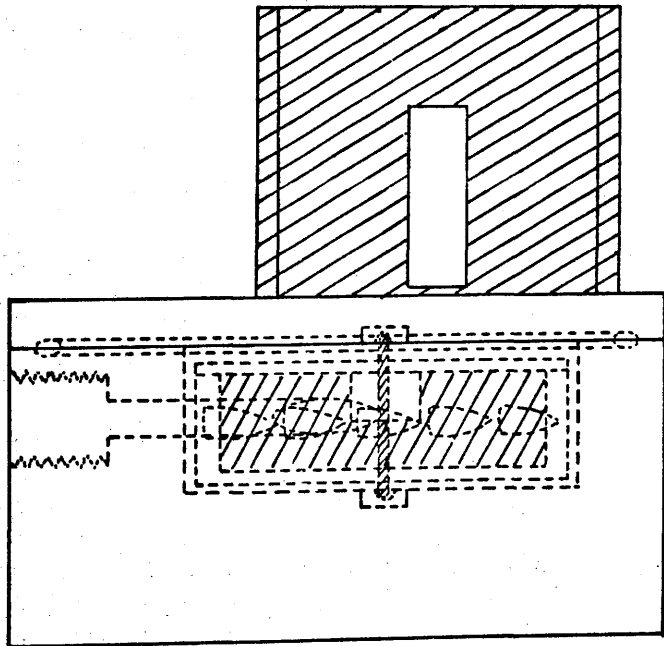


Figure 4.8 Water-driven stirrer unit for membrane cell B.

See text for description.

The membrane cell, mounted on the stirring unit, was positioned in the Pye Unicam SP800 spectrophotometer as shown in Figure 4.9. The water inlet and outlet tubes were of matt black PVC and followed the routes provided through the spectrometer casing. A small bulb, 3V, 0.5 A, housed in a black cardboard box and connected to an external power supply (Solartron PSU AS1410.2, Schlumberger, USA) provided illumination of the membrane when required. The membrane was viewed by transmitted light using a microscope of short focal length. Two stoppered small glass bottles were filled with saturated potassium chloride solution and contained the external calomel electrodes (Pye Unicam, No. 11161). Each compartment of the membrane cell was connected to one of these external electrodes by means of an agar-potassium chloride bridge⁽¹⁶²⁾ formed in a piece of flexible Teflon tubing of 0.8 mm internal diameter. The remaining inlets and outlets of the membrane cell were connected via Teflon tubing and two-way valves to external glass syringes which contained the membrane-forming solution and aqueous solutions. To minimise the transmission of ambient mechanical vibrations to the membrane, the spectrometer was mounted on four vibration pads (Mettler Instruments, Switzerland) on top of the vibration-damping table.

The procedure used to assemble and fill the membrane cell was rather complicated because of the need to free the interior completely of air bubbles. The membrane orifice was first preconditioned in the usual way with the lipid solution to be used (Section 4-2.3). With a small stirrer in each compartment and a silicone rubber gasket between them, the Teflon and perspex blocks were tightly clamped together. Valves V_3 and V_4 were opened, and lipid solution was

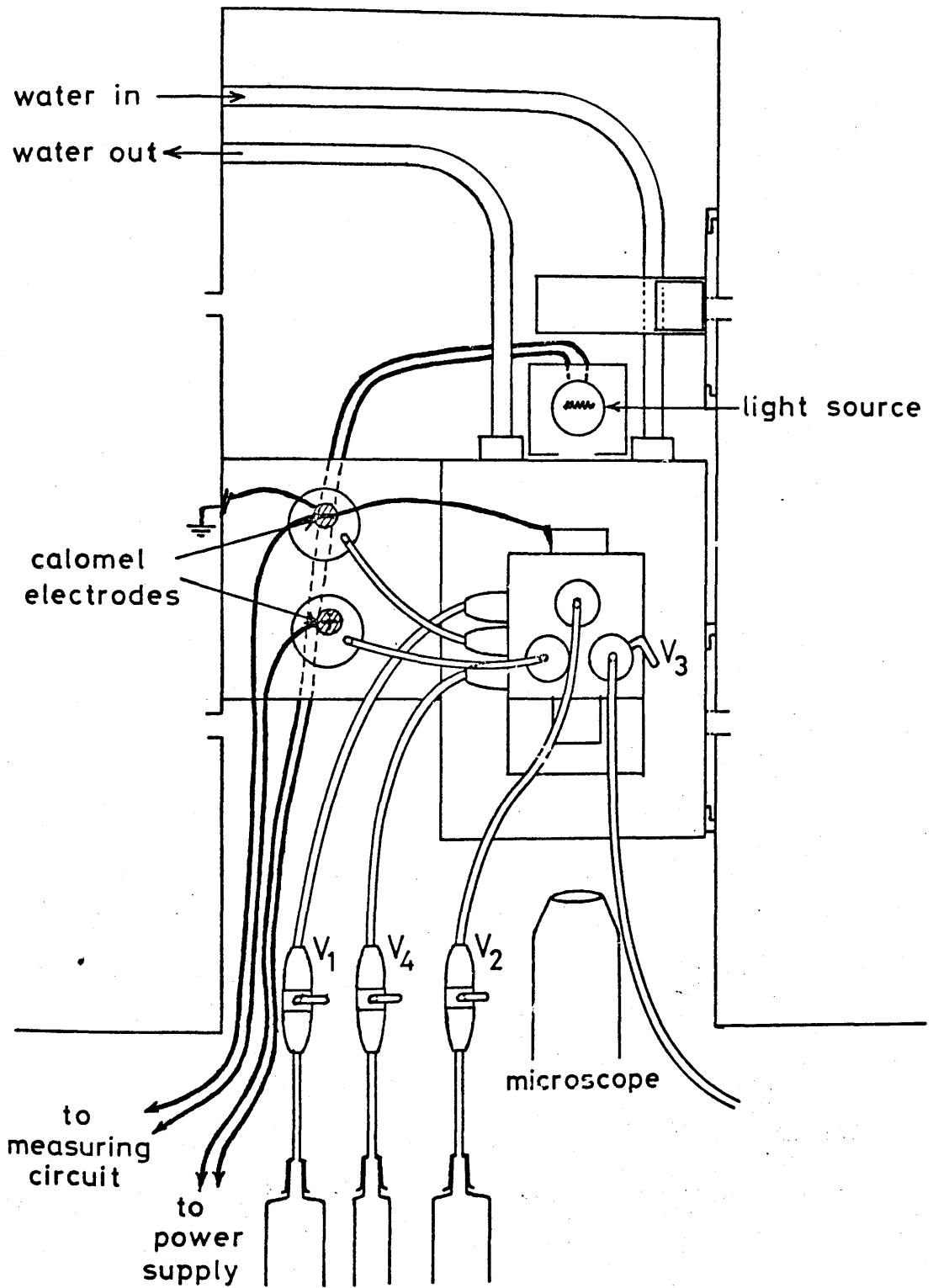


Figure 4.9 Diagram showing the arrangement of membrane cell B, along with the associated stirrer unit, external electrodes and solution reservoirs, in the Unicam SP800 spectrophotometer during a kinetics experiment.

introduced from the 'Agla' syringe via V_4 until it emerged from the membrane orifice with no sign of air bubbles. V_4 was then closed, V_1 , V_2 and V_3 opened, and the two tubing connectors which were to take the agar bridges were firmly blocked with glass rods. Freshly degassed buffer was slowly added to the cell via V_1 until it emerged, free of air bubbles, from both V_2 and V_3 . The cell was tilted and shaken vigorously during this operation, to dislodge and flush out as many air bubbles as possible. In order to remove tiny air bubbles still trapped inside the cell, its contents were then degassed under reduced pressure. Valves V_2 , V_3 and V_4 were closed, and V_1 was opened. The 5 ml syringe attached to V_1 was full of freshly degassed buffer, and its barrel was greased to prevent air from leaking in. The glass rod blocking one of the agar bridge connectors was replaced by a wide bore stainless steel syringe needle which was connected via PVC tubing and a two-way tap to the side-arm of a 1 litre filter flask. The flask was then evacuated and the tap was opened. Air bubbles immediately expanded inside the cell and these were flushed out by adding more buffer through V_1 . When all the bubbles had been removed in this way the vacuum was released. This procedure was found to be adequate to remove even the most stubborn of air bubbles (for example those trapped in the lipid solution around the orifice). Subsequently great care had to be taken to avoid the accidental introduction of air bubbles in the solutions injected into the cell, since a bubble getting past any of the valves could require the degassing process to be repeated. The agar bridges were carefully fitted into the cell which was then secured in its position on the stirring unit inside the spectrometer (Figure 4.9). The calomel electrodes were connected via screened leads to the external measuring circuit shown in Figure 4.2.

With the stirrers on, the whole cell was thoroughly perfused with the substrate solution to be used in the experiment (prepared as indicated in Table 14). This was introduced through valve V_1 , with V_2 and V_3 also open. Next V_1 was closed and V_4 opened to allow the introduction of a small amount of the membrane-forming solution into the orifice. Subsequent withdrawal of excess lipid solution caused the membrane to thin slightly, but it was found that forcing the membrane into the bilayer state by this 'marginal suction' method resulted in its premature disappearance. All the valves were then closed and the membrane was observed. It was possible by transmitted light to distinguish each of the three main conditions of the membrane — lens, thin, and bilayer — although no coloured interference patterns could be seen. In the case of the final BLM the bilayer region could be easily distinguished from the Plateau-Gibbs border, the two being separated by a distinct rim. This allowed an estimate of the BLM area to be made, knowing the total area of the orifice ($1.77 \times 10^{-2} \text{ cm}^2$). Membrane capacitance measurements could also be made, and thicknesses calculated, using the procedure described in Section 4-2.4. (The total resistance of the electrodes plus agar bridges plus aqueous solutions, measured in the absence of a membrane, was found to be 4.54×10^4 ohms.) Thus it was possible to characterise the membrane and to ensure that it was in a reasonably stable condition in the substrate solution before proceeding to the perfusion of one of the compartments with the reducing agent. Perfusion was achieved by slowly injecting the freshly prepared and degassed reductant solution through valve V_1 , with V_2 open but V_3 and V_4 closed. Finally all the valves were closed and spectrophotometric monitoring of the substrate was begun.

For the purposes of these kinetics experiments, the SP800 spectrophotometer was operated on a fixed wavelength mode, the wavelength chosen being a λ_{max} value of the substrate (Table 8). The absorbance was displayed as a function of time on a chart recorder connected to the spectrophotometer through an SP250 scale expansion accessory (Pye Unicam Ltd., England). In all these experiments 0.025 M phosphate buffer was used as reference in the spectrophotometer, and it was necessary to multiply the measured absorbances by a factor of 1.12 to take account of a small loss in sensitivity resulting from interruption of the sampling light beam by the rotating stirrer in the bottom of the cell. Knowing the extinction coefficients of the oxidised and reduced forms of the dye at the measuring wavelength (Table 8), and the chart speed, the kinetics of reduction of the substrate were analysed and a rate constant for the membrane-mediated process was evaluated.

4-2.6 Cleaning of apparatus

The formation of stable BLM may be seriously impaired by the presence of surfactant-type impurities in the lipid or aqueous phases⁽¹⁴⁹⁾. Therefore scrupulous cleaning of all pieces of apparatus used in this work was absolutely essential. The methods used were those recommended by Howard and Burton⁽¹⁴⁹⁾. New pieces of perspex apparatus were washed in hot aqueous detergent in an ultrasonic cleaning bath. They were then rinsed for several hours in hot running tap water, and copiously in distilled water, before being dried in the oven. New pieces of Teflon apparatus, after washing as above, were left in chloroform:methanol (2:1, v/v) overnight. They were finally boiled in distilled water and dried.

Subsequently all Teflon, perspex and glass apparatus was cleaned by one standard procedure: the pieces were left in aqueous detergent solution (about 2% 'Decon') overnight. They were then rinsed for at least two hours in hot running tap water, followed by copious amounts of distilled water. Finally they were dried in the oven.

4-3 RESULTS AND DISCUSSION

4-3.1 Membrane formation studies

The first part of this work was directed towards the formation and physical characterisation of BLM containing vitamin K or co-enzyme Q, as a preliminary to the use of these membranes in electron transfer studies. The apparatus and methods used have been described in Sections 4-2.3 and 4-2.4, and the test lipid solutions were prepared as indicated in Section 4-2.2. All experiments were carried out at 25°C in an aqueous medium comprising 0.025 M phosphate buffer, pH 6.86, and 0.1 M sodium chloride. A wide range of lipids and lipid mixtures were tested for their abilities to form stable BLM under these conditions: the results obtained are shown in Table 15. The hydrocarbon solvent used in all these experiments was *n*-decane, since preliminary investigations had shown this to be the most generally suitable⁽⁵⁷⁾. It was found that very few of the lipid solutions tried were capable of forming stable membranes in the Teflon cup on their first application to the orifice, even when the surrounding area had been preconditioned with the lipid (Section 4-2.3). Often three or four applications were required to produce a membrane of sufficient stability to allow electrical measurements to be made. Also, the stability of successive membranes seemed to improve with

time, at least over the first few hours. This may have been due to a continuous conditioning of the orifice, or perhaps to the generation of some stabilising impurity (for example, an oxidation or hydrolysis product) in the membrane-forming solution. In general the results quoted in Table 15 apply to the most stable membranes formed from the given lipid solutions: many others were formed which did not survive for long enough to allow their complete characterisation. In many cases the measurement of capacitance itself destabilised the membrane, with rupture frequently occurring during the discharge process. This could be avoided to some extent by lowering the charging voltage or the external resistance, but lower resistances yielded shorter, and hence less accurate, time constants. 'Compromise' values of around 10^5 ohms were normally chosen.

It seems reasonable to assume that under constant conditions a given lipid solution, if it forms BLM at all, should eventually form a bilayer of a well-defined thickness, depending on the chain lengths, level of unsaturation, polarities and other physical properties of the molecules of which it is composed. It might also be expected that the time taken to reach this limiting bilayer condition should be similar for all membranes formed from the same solution. However neither of these expectations was borne out in practice (Table 15): compare, for example, membranes 12, 13 and 14. The maximum limit of error in the calculated thicknesses is about $\pm 15\%$ on each result, by combination of the individual errors in the evaluation of time constants from the discharge curves and in the estimation of membrane areas (the more important factor): below 100 μ^2

Table 15 Physical characteristics of membranes formed from a variety of lipid solutions at 25°C

Ref. No.	Membrane composition (concentration of constituents in mg ml ⁻¹ in decane)	Time since formation (min)	Specific capacitance (μF cm ⁻²)	Thickness d (Å)	Appearance/Comments
1	{ egg lecithin	20	0.19	93	black
2	{ cholesterol	30	0.22	80	black
3	{ egg lecithin	45	0.39	46	black; stable > 24 hours
	{ cholesterol				
4	coenzyme Q				stable lens; no thin membranes formed
5	vitamin K				thick lens; stable > 48 hours; no thin membranes formed
6	vitamin K	60	0.006	2800	stable lens
7	{ vitamin K				stable lens;
	{ cholesterol				no thin membranes formed
8	{ vitamin K				stable lens;
	{ cholesterol				no thin membranes formed
9	{ egg lecithin	7	0.015	1149	
	{ vitamin K	14	0.041	430	
		18	0.041	430	coloured
		31	0.042	420	
		50	0.095	186	partially black; lasted ~ 1 hour
10	{ egg lecithin	12	0.036	490	coloured
	{ vitamin K	35	0.20	88	black

Table 15 (Continued)

Ref. No.	Membrane composition (concentration of constituents in mg ml ⁻¹ in decane)	Time since formation (min)	Specific capacitance (μF cm ⁻²)	Thickness d (Å)	Appearance/Comments
11	{ egg lecithin } { coenzyme Q } 7.5 } 7.5 }	7 14	0.024 0.027	737 664	membranes unstable; none lasted > 15 min
12	{ egg lecithin } { cholesterol } { vitamin K } 7.5 } 3.75 } 3.75 }	5 13 29 62 100	0.032 0.038 0.051 0.053 0.099	545 467 348 331 178	membrane lasted > 2 hours
13	"	10 20	0.081 0.208	218 85	black; lasted ~ 25 min
14	"	32 45	0.059 0.295	300 60	black; lasted ~ 55 min
15	{ egg lecithin } { cholesterol } { vitamin K } 7.4 } 3.7 } 4.0 }	65 95 145	0.17 0.23 0.33	100 77 53	black black black
16	{ egg lecithin } { cholesterol } { coenzyme Q } 6.75 } 6.75 } 1.0 }	35 60	0.17 0.23	100 77	black black; lasted ~ 65 min
17	dioleoyl lecithin	60	0.042	420	membrane lasted > 90 min
18	"	55	0.29	60	black; lasted > 60 min
19	{ dioleoyl lecithin } { cholesterol } { vitamin K } 7.7 } 4.2 } 3.1 }	5 6 8	0.061 0.156 0.155	290 113 114	coloured membranes quickly formed black; lasted 15 min black; lasted > 3½ hours

Table 15 (continued)

Ref. No.	Membrane composition (concentration of constituents in mg ml ⁻¹ in decane)	Time since formation (min)	Specific capacitance ($\mu\text{F cm}^{-2}$)	Thickness d (\AA)	Appearance/Comments	
22	{ dioleoyl lecithin { vitamin K { cholesterol	7.7 } 5.0 } 2.3 }	11	0.108	163	coloured; lasted 27 min
23	{ cholesterol	16 44	0.114 0.295	155 60	coloured black; lasted 110 min	
24	{ cardiolipin { egg lecithin { cholesterol { vitamin k	3.0 } 3.75 } 1.88 } 1.88 }	20 35 65	0.059 0.087 0.148	300 204 119	coloured coloured black with spot of colour in centre; lasted 265 min
25	"	15	0.46	39	black; lasted 250 min	
26	"	20 60			coloured black; very stable; lasted > 7 hours	

thickness maximum errors are less than $\pm 12\%$, since the longer time constants obtained could be measured more accurately. Therefore experimental error cannot by itself adequately account for the observed differences. Although both membranes 13 and 14 thinned to the 'black' condition, their final thicknesses were found to be rather different, being 85 \AA for the former and only 60 \AA for the latter. In each case immediate repetition of the measurements gave decay curves identical to the original curves, so that the measured capacitances were individually reproducible. The most likely explanation for the difference is that membrane 13 had not thinned down completely to a bilayer when the measurements were made, twenty minutes after its formation. Rupture of the membrane prevented further thickness determinations. Thus it is evident that 'blackness' of a thin film does not prove that it has reached an equilibrium bilayer condition, and that membrane capacitance is a much more sensitive indicator. The time taken to reach the bilayer state can vary widely, even among membranes of identical composition:

membrane 14 was a bilayer within 45 minutes whereas membrane 12 did not reach the bilayer condition even after 100 minutes. This is not surprising when the number of uncontrolled factors governing the rate of thinning is considered (Section 4-1.1). For example, the amount of lipid solution originally applied to the orifice may be expected to alter the size and shape of the Plateau-Gibbs border region, hence varying the effectiveness of border suction, and any chance vibrations are certain to effect the drainage rate of the film, possibly even promoting the transition to the final bilayer state by bringing the two opposing monolayers into contact.

For the purposes of the present research, it was sufficient to determine which lipid solutions were capable of forming stable bilayer membranes ('stable' in this context meaning invariant for a period of at least two hours). The apparatus and measuring techniques were initially tested using solutions containing both egg lecithin and cholesterol in decane. Such solutions had been reported to produce bilayers of high stability, with specific capacitances in the range $0.38 - 0.56 \mu\text{F cm}^{-2}$ (156). Membranes 1, 2 and 3 (Table 15) were typical of many formed from this lipid system: the membranes were very easily formed and thinned quite rapidly through coloured stages to the black condition, which was then stable for many hours. Egg lecithin - cholesterol BLM stable for more than 24 hours were obtained on several occasions. The highest capacitance recorded was $0.39 \mu\text{F cm}^{-2}$, corresponding to a thickness of 46 \AA for the hydrophobic region. This gives an overall membrane thickness of about 62 \AA , when the head group regions are taken into consideration (Section 4-1.2), which is a very reasonable value for a bilayer membrane(19).

Several attempts were made to form membranes containing only coenzyme Q (membrane 4) or vitamin K (membranes 5 and 6) in decane, but these solutions failed to produce bilayer, or even coloured, membranes. Instead stable 'lenses' about 3000 \AA in thickness were formed. Possibly the quinone head groups are not sufficiently hydrophilic to allow formation of the stable interfacial monolayers required. It was therefore necessary to incorporate other, more hydrophilic, lipids into the membrane-forming solutions in order to produce BLM containing the electron carrier molecules. Mixtures of vitamin K with cholesterol also failed to produce thin films

(membranes 7 and 8). Egg lecithin - vitamin K mixtures did form membranes which thinned down gradually to the black state, (membranes 9 and 10) but none of these were stable for more than one hour. An egg lecithin - coenzyme Q solution produced only thick membranes, none of which were stable for more than fifteen minutes (membrane 11). When the original highly successful 1:1 w/w egg lecithin - cholesterol mixture was modified by replacing half of the cholesterol with vitamin K, the resulting solution produced stable bilayers, some of which lasted for more than two hours (membranes 12, 13, 14 and 15). The thickness of the final bilayer was about 53 \AA (membrane 15) which is only slightly greater than that of an egg lecithin - cholesterol BLM with no added vitamin K (membrane 3). Coenzyme Q added at low concentration to the basic egg lecithin - cholesterol system did not prevent it from forming BLM (membrane 16), but none of the membranes formed was stable for much more than one hour.

Egg lecithin is a complex mixture of molecular species differing in the nature of their hydrocarbon chains^(154,163): C_{16} and C_{18} saturated, and C_{18} mono- and di-unsaturated fatty acid residues predominate. In order to produce a membrane system which was chemically well defined, it was therefore desirable to replace egg lecithin by a single, purified phospholipid species. None of the highly purified saturated synthetic lecithins available were suitable since they all have phase transition temperatures near or above 25°C . However, synthetic L- α -dioleoyl lecithin (a C_{18} mono-unsaturated lecithin) seemed to offer reasonable prospects for success, since its phase transition occurs at -22°C ⁽¹⁶⁴⁾. In

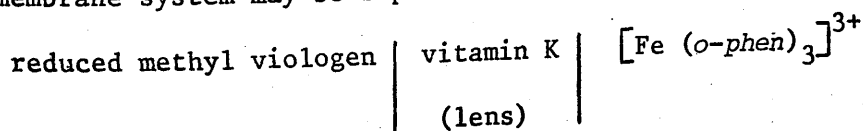
preliminary experiments (membranes 17 and 18) a solution of dioleoyl lecithin in decane was found to be capable of forming BLM of limited stability. Solutions containing mixtures of dioleoyl lecithin, cholesterol and vitamin K were then tested for their ability to form stable BLM (membranes 19-23). Coloured membranes were formed almost immediately after application of the solution to the orifice, and these thinned quickly to the black state. One of the BLM produced (membrane 21) lasted for three and a half hours.

The specific role of cardiolipin in catalysing the reduction of cytochrome c by dihydrovitamin K at the membrane/substrate interface has been discussed in some detail in the last Chapter (Section 3-3.5). It was hoped eventually to study this reaction in a BLM system, so with this in mind attempts were made to produce BLM incorporating both vitamin K and cardiolipin (membranes 24-26). The cardiolipin-egg lecithin-cholesterol-vitamin K solution used formed coloured membranes very easily, and these thinned quite rapidly to the black state. A BLM only 39 Å thick was produced, and this was stable for more than four hours. On one occasion, when electrical disturbance of the membrane was avoided, a BLM stable for more than seven hours was obtained. Thus the results of these preliminary studies showed that vitamin K and coenzyme Q could be incorporated into stable BLM formed from a variety of lipid mixtures. Although BLM containing vitamin K have been reported before, by Leslie and Chapman⁽¹⁵³⁾, these investigators were unable to form BLM containing coenzyme Q₁₀: it is thought that the present research contains the first successful attempt to do so.

4-3.2 Electron transfer across ultrathin membranes containing vitamin K

Many attempts were made to form BLM in the orifice of membrane cell A (Figure 4.5); using the egg lecithin - cholesterol solutions which had given the most stable membranes in the Teflon cup. However all of these failed: although stable 'lens' membranes were formed, these did not thin spontaneously to BLM, and attempts to promote thinning by the marginal suction method led only to rupture of the membrane. The problem was probably caused by the unfavourable geometry of the orifice: perhaps the breadth of the rim (0.5 mm) was too great to allow formation of a suitable Plateau-Gibbs border (Figure 4.1). Considerable attention was paid to this particular feature in subsequent cell designs, and substantial improvements were in fact made (see below). Nevertheless, very stable 'lens' membranes containing vitamin K were readily formed in membrane cell A, so it was decided at this stage to test the ability of such membranes to mediate an electron transfer process.

The experimental procedure employed is outlined in Section 4-2.5 under 'membrane cell A'. On this occasion ferriin was chosen as substrate, for the reasons mentioned earlier (Section 4-2.5). The redox membrane system may be represented:



The membranes were formed from a solution of vitamin K in decane, at a concentration of 8.0 mg ml⁻¹. The compositions of the reductant and isotonic substrate solutions were as given in Table 14: the reduced methyl viologen was 2.04 x 10⁻² M and the ferriin was 1.0 x 10⁻⁴ M. Initially there was no stirring in the aqueous phases,

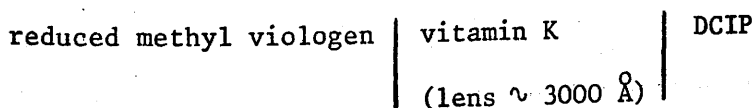
and after a few hours the substrate solution, originally colourless, was distinctly orange in the vicinity of the membrane. Stirring in both compartments was then started, and the cell left overnight at 25°C. After a period of 24 hours the entire substrate solution was strongly orange in colour, due to reduction of ferriin to ferroin. Similar results were obtained in a repeat experiment. Therefore it may be deduced that vitamin K is capable of transferring electrons across an 'ultrathin' membrane, only a few thousand Angstroms in thickness. Moreover the reaction, although slow in this miniaturised system (due to the small (membrane area)/(substrate volume) ratio), does proceed at a rate which should be measurable by spectrophotometric techniques. These observations encouraged further development of ultrathin membrane systems.

In the light of experience gained from several unsuccessful design variants, membrane cell B (Figure 4.6) was eventually developed. The orifice was a 1.5 mm diameter hole punched in a thin (0.05 mm) disc of pure Teflon. This disc was sandwiched between the main cell block and a press-in Teflon plug, so that on introduction of the membrane-forming solution, the solution filled the capillary spaces between the Teflon surfaces, providing a reservoir of lipid around the orifice. The marginal suction technique could then be used to withdraw excess solution from the orifice, the suction now being effective all round the membrane border rather than only at the bottom of the membrane (as in cell A described above). The cell was assembled and filled with 0.025 M phosphate buffer, 0.1 M in sodium chloride, as indicated in Section 4-2.5 under 'membrane cell B', and was clamped in position on the stirrer unit

inside the sample compartment of the spectrometer (Figure 4.9). Attempts were then made to form and characterise membranes produced from a solution containing 3.75 mg egg lecithin, 1.88 mg cholesterol, 3.00 mg cardiolipin and 1.88 mg vitamin K per ml of *n*-decane). This was the solution which had previously been found to give the most stable membranes containing vitamin K (membranes 24-26, Table 15). BLM were formed very quickly after injection of a little of this solution into the orifice; capacitance measurements indicated that bilayers were produced within five to ten minutes. However these membranes were rather unstable, and none persisted for more than nineteen minutes. When this is compared with a lifetime of seven hours in the Teflon cup it is clear that some apparatus-dependent factor must have been decreasing the stability of membranes formed in the membrane cell. This may have been associated in some way with the construction of the orifice region, or may alternatively have arisen from mechanical vibrations transmitted to the membrane cell from the stirrer unit or from the spectrometer itself. (Both were in operation throughout the formation and measurement procedures.) With the spectrometer and stirrer switched off, egg lecithin - cholesterol solutions yielded BLM which lasted for five hours; with the spectrometer and stirrer on, however, similar membranes lasted no more than forty minutes. Membranes which had been allowed to thin to the bilayer condition in the absence of vibration tended to burst as soon as the stirrer or spectrometer was switched on. Therefore it would appear that vibration was the major factor responsible for membrane rupture.

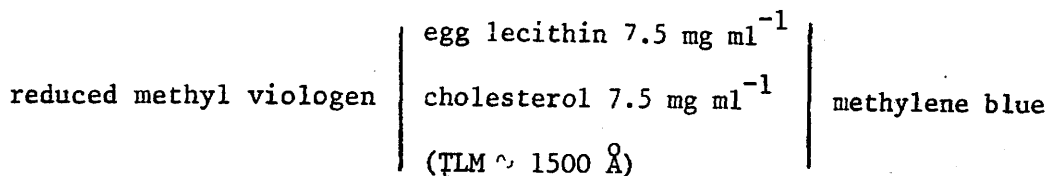
Unfortunately it was not possible in the period of the present research to eliminate these vibrations so as to allow the

study of electron transfer reactions across BLM. Nevertheless several experiments were carried out using slightly thicker membranes ('TLM' - 'thin lipid membranes'), the results of which were encouraging. In the absence of the central Teflon disc, highly stable TLM could be formed in the orifice of membrane cell B. The compositions of the isotonic reductant and substrate solutions used are given in Table 14, and the experimental procedure is detailed in Section 4-2.5. Two consecutive experiments were performed using DCIP as substrate, and the reduction was followed visually, by observing the disappearance of the blue form of the dye. The same 'lens' membrane, about 3000 \AA in thickness, and formed from a solution of vitamin K (10 mg ml^{-1}) in decane, was used in both experiments. In the first, the reductant compartment contained ascorbate ($E_{m7} \sim +0.058 \text{ V}$), a reagent which reacts rapidly with DCIP ($E_{m7} = +0.217 \text{ V}$) but does not reduce vitamin K ($E_{m7} \sim -75\text{mV}$). This served as a convenient 'control' system, since no carrier-mediated electron transfer could occur, and since the rate of back-transfer of the protonated oxidised DCIP (Section 3-3.1) should be the same in this as in the 'active' methyl viologen system. No significant reduction of the dye was observed over a seventeen hour period. The reductant compartment was then carefully perfused with reduced methyl viologen, to form the system:

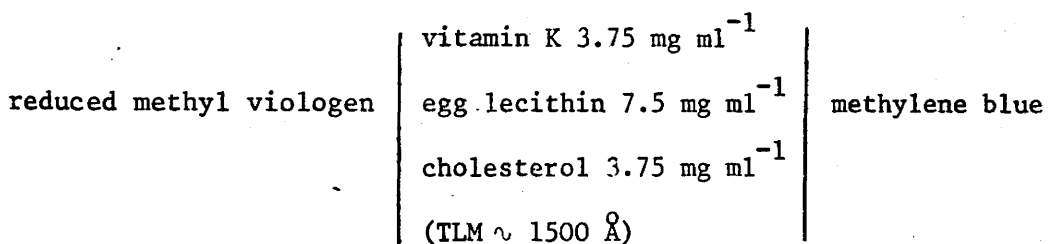


Thereafter reduction of the dye commenced, and the substrate was completely colourless within ten hours. Electrical measurements showed that the lens membrane was still present at the end of the reaction.

Experiments were also performed in which methylene blue was used as substrate, its reduction being followed spectrophotometrically, by monitoring the decrease in absorbance at 655 nm as a function of time. Thin membranes, about 1500 Å in thickness, were formed from solutions containing egg lecithin and cholesterol, with and without vitamin K. The 'control' system may be represented:



and the 'test' system was:



The compositions of the reductant and isotonic substrate solutions used were as given in Table 14: the reduced methyl viologen was 2.04×10^{-2} M and the methylene blue was 1.16×10^{-5} M. The forms of the absorbance - time curves produced by a chart recorder linked to the spectrophotometer are shown in Figure 4.10. Rupture of the membrane was clearly indicated by a sudden drop in the absorbance to the base line followed by an equally sharp rise to a value off scale. This was due to reduced methyl viologen flooding through the orifice, causing first reduction of the methylene blue (which produces two colourless species) and then a rapid buildup in concentration of the blue methyl viologen radical in the substrate compartment. The steepness of these transitions is a good indication of the efficiency of stirring in the cell. From the results of these experiments it can be seen that reduction of the methylene blue took place only when vitamin K was present in the membrane. The graph of absorbance

Figure 4.10 Kinetics of reduction of methylene blue by reduced methyl viologen, mediated by a thin lipid membrane.

The reactions were performed at 25°C in membrane cell B, using the methods described in Section 4-2.5. The compositions of the reductant, substrate, and membrane forming solutions are given in the text.

The 'CONTROL' system contained no electron carrier in the membrane.

The 'TEST' system contained vitamin K in the membrane.

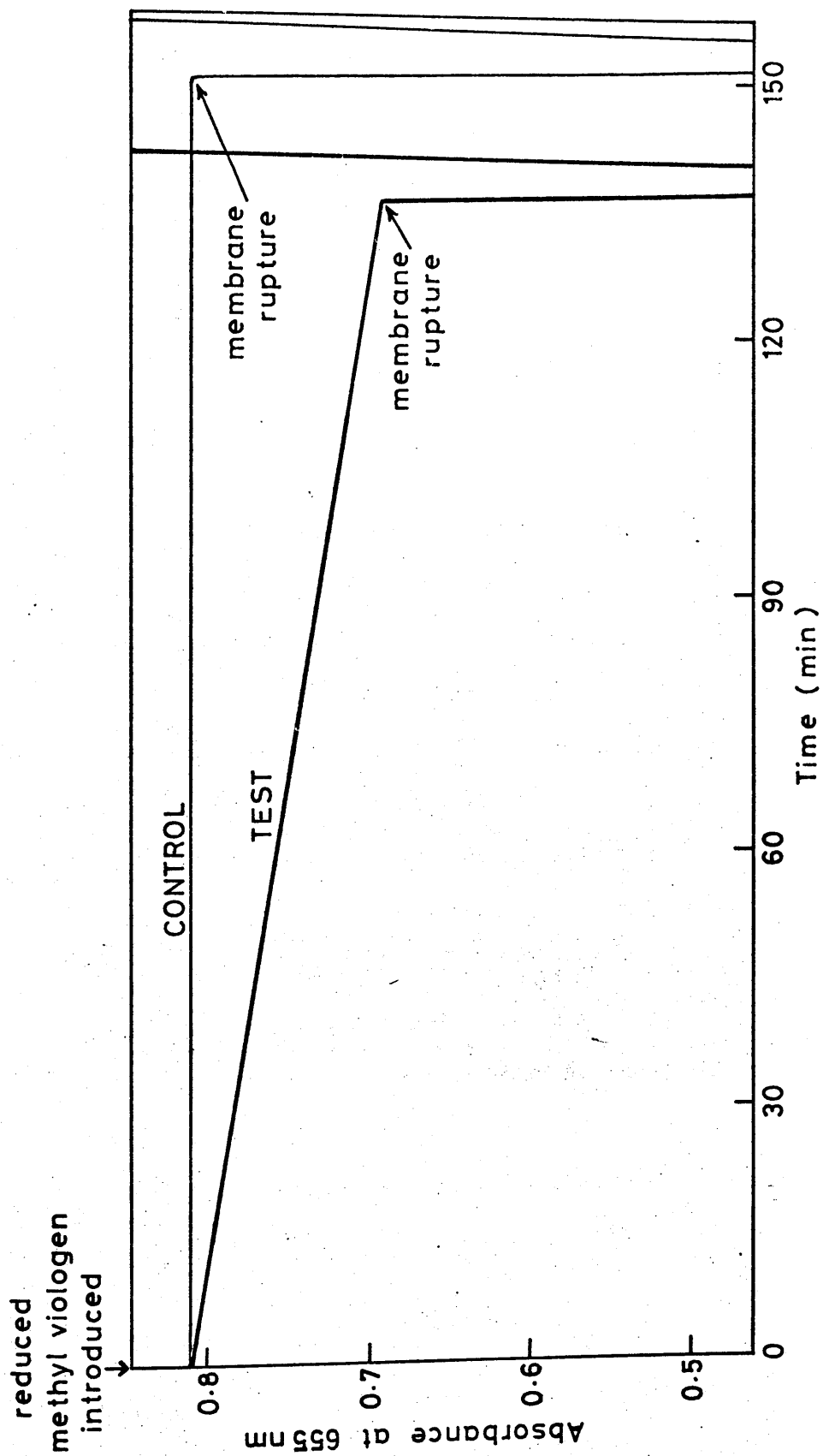


Figure 4.10

against time was very nearly a straight line, but a plot of log (absorbance) against time gave an equally good straight line. Since bulk membrane studies on the biphasic dihydrovitamin K/methylene blue system had indicated that all these reactions were first-order with respect to the oxidised substrate (Sections 3-3.2 and 3-3.3), a first-order kinetic analysis was applied in the present case. This gave a rate constant $k_1 = 1.14 \times 10^{-3} \text{ min}^{-1}$, with a corresponding half-life of 608 minutes.

It is interesting to compare this result with those obtained from bulk membrane studies. As mentioned earlier (Section 4-2.5) the ratio (substrate volume)/(membrane area) is a critical factor in determining the observed rates of reactions which are diffusion controlled at the membrane/substrate interface. In the H-cell used for bulk membrane studies, the area of the interface was approximately 1 cm^2 , and the substrate volume was 4 cm^3 , giving a ratio of 4. In membrane cell B the area of the interface was about 0.0177 cm^2 while the volume of the substrate compartment was roughly 1 cm^3 , giving a ratio of 56.5. Thus the (substrate volume)/(membrane area) ratio for the membrane cell was about fourteen times that for the H-cell. From this it might be expected that the half-life for a diffusion controlled reaction in the membrane cell should be about fourteen times that measured for the same reaction in the H-cell, using equations (3-8) and (3-19) and assuming that the thickness δ of the unstirred layer at the interface is the same in both pieces of apparatus. Division of 608 minutes, the half-life for the dihydrovitamin K/methylene blue reaction measured in the membrane cell, by 14 gives 43.4 minutes for the half-life of an identical reaction in the H-cell. This figure is in remarkably good agreement with the

value of 42.3 ± 2.7 minutes obtained in bulk membrane studies (Section 3-3.2). In fact, even if stirring in the membrane cell was equally efficient as stirring in the H-cell, the result is still surprising, since the presence of large amounts of highly amphiphilic phospholipid in the TLM might have been expected to lower the reaction rate, as observed in bulk membrane studies (Section 3-3.3). The reasons for the apparent failure of egg lecithin to displace dihydrovitamin K from the interfacial monolayer are not altogether clear. However it should be noted that egg lecithin is neither charged, like cardiolipin and phosphatidyl inositol, nor fully saturated, like the phosphatidyl ethanolamine used earlier, and so should have less effect than these other phospholipids in blocking the reaction.

It is unfortunate that the instability of planar bimolecular lipid membranes has thus far prevented their use in the study of continuous carrier-mediated electron transfer reactions, and in this respect the main objective of this Chapter has not been realised. However 'recipes' for stable BLM containing the carrier molecules vitamin K and coenzyme Q have been produced, and it is felt that, with some further development and a little luck, the membrane cell described here could be used to study electron transfer across these BLM.

Chapter Five

LIPOSOMES

5-1 INTRODUCTION

In Chapters three and four, two very different redox membrane systems have been described, and the advantages and disadvantages of each as a model for biological electron transfer membranes have been discussed. Bulk liquid membranes, although mechanically stable and therefore comparatively easy to handle and characterise, can hardly be regarded as truly viable models for the biomembranes described in Chapter one. Planar bilayer lipid membranes (BLM), on the other hand, are more readily identifiable with naturally occurring structures, but are at the same time much more difficult to study due to their inherent fragility. A major disadvantage shared by both bulk liquid membranes and BLM is the smallness of the membrane surface area in relation to the volume of substrate solution, a factor which dictates that the observed reaction kinetics must always be very slow. For these reasons, and particularly since all attempts to demonstrate carrier-mediated electron transfer across lipid bilayers had been frustrated by the instability of BLM, it was decided to investigate liposomes as a third model system. These are in principle the most viable of all the experimental model systems developed in this work, and the large surface areas afforded by spherical membranes make them particularly suited to the study of slow reactions and reactions involving labile molecules.

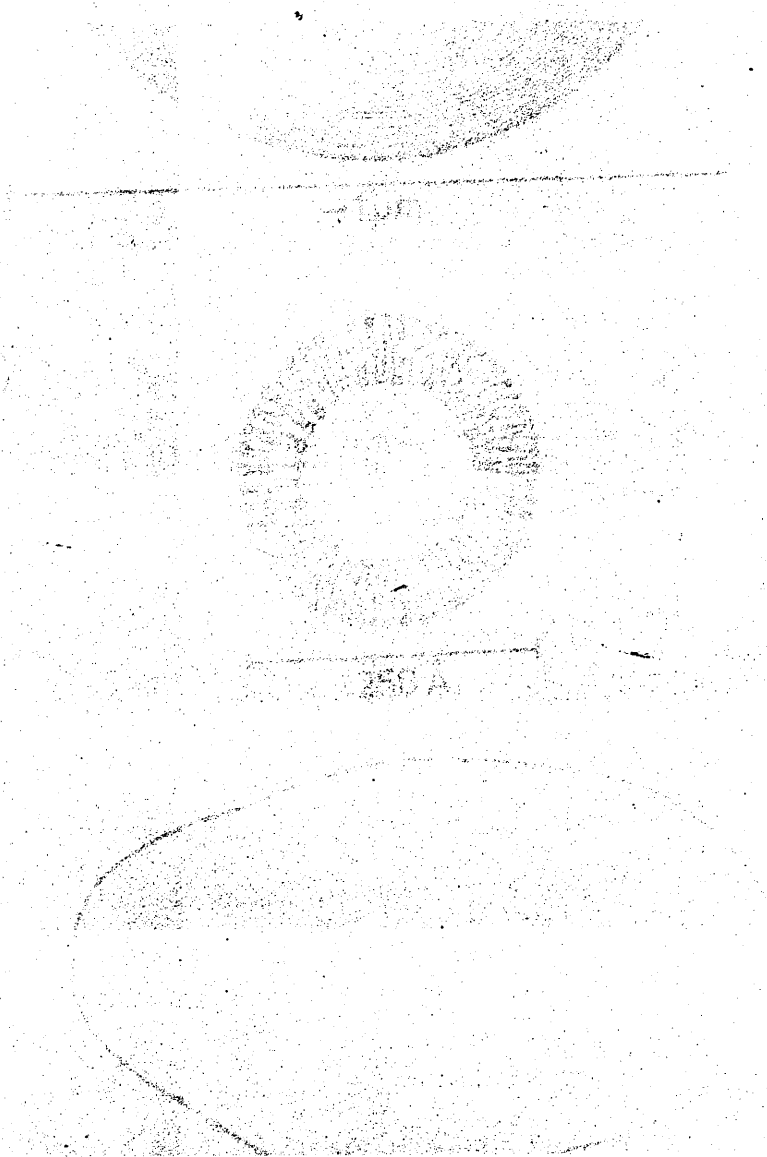
5-1.1 Preparation and properties of liposomes

Before describing the assembly of a suitable liposomal system for studying electron transfer reactions, it is useful to summarise the preparation and properties of liposomes. The recent upsurge of interest

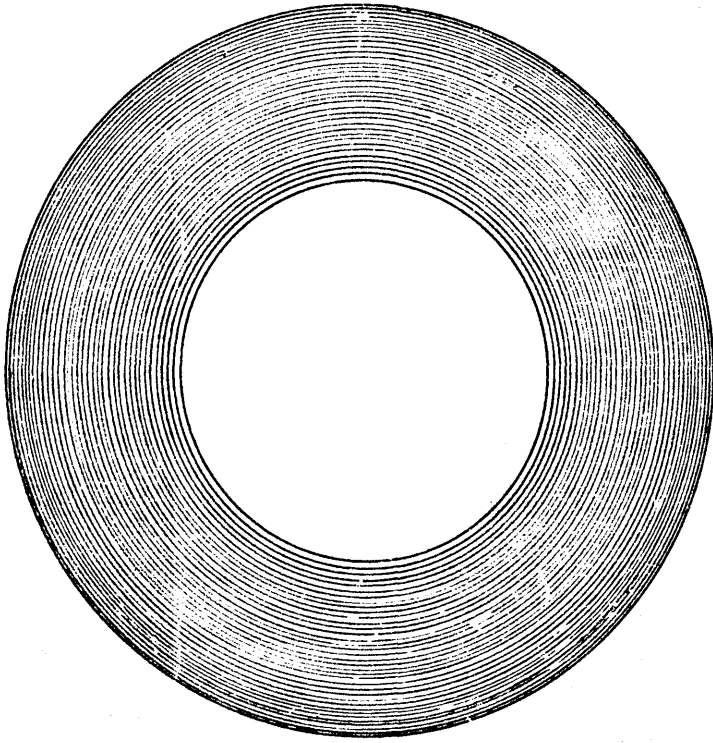
in liposomes was sparked off in the mid-1960's by Bangham's observation that the structures present in aqueous lecithin suspensions altered shape in response to changes in solute concentration in the external aqueous phase⁽¹⁶⁵⁾. A number of excellent reviews have been published, covering all aspects of research involving liposomes (also referred to as 'lamellar smectic mesophases', 'lyotropic liquid crystals', 'bilayer vesicles' or 'spherules') over the past decade⁽¹⁶⁶⁻¹⁷¹⁾.

Figure 5.1(a) shows the type of structure formed spontaneously when almost any pure phospholipid or mixture of phospholipids above the phase transition temperature is shaken with excess aqueous solution. The multilamellar liposome consists of a series of closed concentric bilayer vesicles, with the lipid membranes separated by layers of aqueous medium. These structures are typically spheroidal (rather than spherical, as illustrated for convenience) in shape, and their dimensions vary from about 0.5 μm to 10 μm . Their morphology has been studied using various techniques, notably light microscopy with crossed polaroids⁽¹⁷²⁾, electron microscopy by negative staining^(172,173), freeze-fracture electron microscopy⁽¹⁷⁴⁾, and X-ray diffraction⁽¹⁷²⁾. Under the light microscope with crossed polaroids, lamellar smectic mesophases exhibit birefringence, the net sign and magnitude of which depend on the summation of the positive 'intrinsic' double refraction of the ordered molecules of the bilayer and the negative 'form' double refraction of the alternate layers of lipid and aqueous phase⁽¹⁶⁷⁾. From studies of multilamellar liposomes prepared from phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl inositol (PI) and phosphatidic acid (PA), and various mixtures of these in aqueous salt solutions, it has been found that the sizes and shapes of the vesicles, as well as the degree of birefringence and the extent to which negative stain penetrates between the lamellae

Figure 5.1 Structures of different types of liposomes : (a) a large multilamellar liposome, or 'smectic mesophase'; (b) a small unilamellar liposome, or 'microvesicle'; (c) a large unilamellar liposome, 'LUV', or 'macrovesicle'.

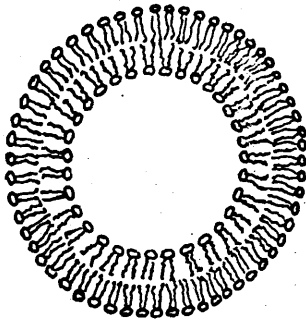


a



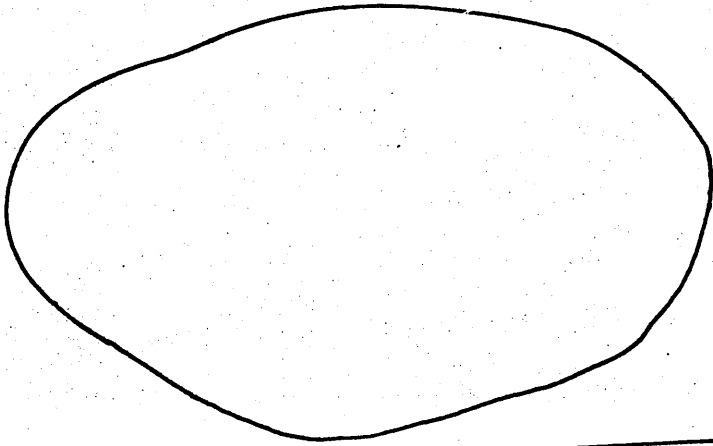
~1 μm

b



250 Å

c



~1 μm

Figure 5.1

during electron microscopy, are characteristic of the particular phospholipid species used⁽¹⁷²⁾. X-ray diffraction shows the presence of multilamellar structures with repeat distances varying from 54 Å for PC, to 75 Å for PS, and 125 Å for a PS-cytochrome c complex⁽¹⁷²⁾. Phosphatidyl ethanolamine is exceptional in its tendency to form aggregated structures of high 'porosity' in aqueous salt solutions⁽¹⁷²⁾.

Despite their ease of formation, large multilamellar liposomes present a number of problems in the interpretation of experimental results, due to the unavoidable polydispersity of the suspensions. For this reason, much effort has been directed towards the preparation of monodisperse populations of unilamellar structures. The most successful of these have involved small unilamellar liposomes, or 'microvesicles', which can be prepared simply by subjecting the large hand-shaken liposomes to ultrasonic irradiation. The formation of small vesicles by sonication appears to be a two-step process in which disintegration of the large particles to small unstable fragments is followed by re-aggregation of the fragments into microvesicles⁽¹⁷⁵⁾. Prolonged sonication results in a homogeneous population of single-shelled vesicles with a particle weight (for egg lecithin) of about 2.1×10^6 daltons and an external diameter of about 250 Å⁽¹⁷⁶⁾. This indicates the presence of only 2,700 phospholipid molecules per vesicle, arranged in a closed bilayer: the limiting size of the vesicles could be a reflection of strain in molecular packing as the radius of curvature is reduced⁽¹⁶⁹⁾. It has been calculated, assuming that the outer and inner layers of the bilayer retain similar molecular packing (about 66 Å² of surface per molecule), that the proportion of molecules participating in the inner layer of a microvesicle should be only 26-30% of the total⁽¹⁷⁷⁾. Although sonication is by far the most common method for generating microvesicles, a few other methods have been documented.

Batzri and Korn⁽¹⁷⁸⁾ have described a method in which a solution of phospholipid in ethanol is injected rapidly into an aqueous solution through a fine hypodermic needle. Kagawa and Racker⁽¹⁷⁹⁾ have formed liposomes incorporating proteins by mixing aqueous solutions of protein and lipid in detergent, and then removing the detergent by slow dialysis. More recently, Brunner, Skrabal and Hauser⁽¹⁸⁰⁾ have prepared unilamellar vesicles of uniform size (300 Å in diameter) by solubilising unsonicated egg lecithin dispersions with sodium cholate and removing the detergent from the mixed lecithin-cholate micelles by gel filtration.

Although reasonably monodisperse suspensions of microvesicles can be prepared quite easily by any of the above methods, these too have their disadvantages in certain types of experiment. The very small volume of the trapped aqueous phase means that each liposome can hold only a small number of solute molecules (of the order of one hundred for a 0.1 M solution), so that the feasibility of any experiment which relies on statistical compartmentation of, for example, a diffusing or reacting species (as in the present work) must be considered carefully. A secondary problem is that the high curvature of the liposomal membrane might affect its permeability properties (see below). Ideally a large, unilamellar liposome would represent the best model for most biological systems. So far only three methods have been documented for the preparation of such 'LUV's' (large unilamellar vesicles) or 'macrovesicles': all are reported to yield unilamellar liposomes with diameters around 1 μm⁽¹⁸¹⁻¹⁸³⁾. Reeves and Dowben⁽¹⁸¹⁾ have prepared 'thin-walled vesicles, one to a few bilayers thick' by allowing a thinly spread layer of hydrated phospholipids to swell slowly in distilled water or in an aqueous solution of non-electrolytes. The most serious restriction of this method is that electrolyte solutions cannot be

encapsulated. Deamer and Bangham⁽¹⁸²⁾ have prepared LUV's by slowly injecting ethereal lipid solution through a fine needle into the aqueous phase, which is held at 60°C. The ether immediately evaporates, leaving the lipid to aggregate into liposomes. The third method is that of Papahadjopoulos *et al.*⁽¹⁸³⁾. Sonicated phosphatidyl serine liposomes are first disrupted and fused into large sheets by the addition of calcium ions, which neutralise the negative surface charge. The resulting spiral 'Swiss roll' type structures have been termed 'cochleate cylinders' by these authors. When further incubated with EDTA, which removes the calcium by complexing, the sheets unroll and close to form large unilamellar vesicles. A series of remarkable freeze-fracture electron micrographs in the original publication shows clearly the changes in morphology at each stage in the preparation⁽¹⁸³⁾. Unfortunately this method does not appear to work for any negatively charged phospholipid other than phosphatidyl serine⁽¹⁷⁰⁾.

Before the liposome could be accepted as a valid model for a biological cell or organelle membrane, it had to be shown to exhibit similar permeability properties to a natural membrane. This requires a permeability for water some eight or nine orders of magnitude higher than that for cations or for uncharged solutes. Turbidity measurements on suspensions of multilamellar liposomes have shown that these are osmotically active, the gains or losses of encapsulated water in response to changes in concentration of the external solution being in accordance with the Boyle-Van't Hoff law⁽¹⁸⁴⁾. Closely related work on large unilamellar liposomes has produced water permeability coefficients and activation energies consistent with the view that water permeates by dissolution and diffusion in the hydrocarbon membrane interior⁽¹⁸⁵⁾. The permeability of liposomal membranes to ions and non-electrolytes has been studied mainly by monitoring the release of radioactively-labelled

species from the suspension contained in a dialysis bag, after first removing all traces of the label from the extraparticulate solution⁽¹⁸⁶⁾. The observed faster rate of diffusion of chloride than sodium or potassium ions, and the selective stimulation of potassium ion diffusion on addition of the ionophore valinomycin⁽¹⁸⁷⁾, established conclusively the fact that liposomal membranes are closed permeability barriers. In general, positively charged liposomes tend to be impermeable to cations, whilst negatively charged liposomes are permeable to cations : small anions diffuse relatively rapidly through negatively and positively charged lipid membranes, and also through uncharged membranes⁽¹⁸⁸⁾. By increasing the degree of saturation or the length of the phospholipid fatty acyl chains, the permeability of liposomes to all solutes is reduced : a similar effect can also be produced by addition of cholesterol to the lipid mixture⁽¹⁸⁹⁾. The permeability of liposomes would therefore appear to be influenced by the fluidity of the membrane. The importance of membrane fluidity in relation to mitochondrial structure and function, and to the action of a carrier mediated electron transfer membrane as envisaged here, has already been stressed (Section 1-5).

5-1.2 Liposomes as model membrane systems

Liposomes of various types, prepared by the techniques outlined above, have been used by many investigators as simple models for biological membranes⁽¹⁶⁸⁾. Perhaps the work most relevant to the present research concerns the interaction of proteins, particularly those known to be involved in mitochondrial electron transport and energy coupling, with liposomes^(126-128,169,190-195), and redox reactions occurring across liposomal membranes^(126,128,191-195). The structures of several protein-lipid-water systems, including cytochrome c-PI, cytochrome c-CL, and cytochrome c-mitochondrial lipids, have been

examined by X-ray diffraction⁽¹⁹⁰⁾. Kimelberg et al.⁽¹²⁷⁾ have studied in some detail the binding of cytochrome c to multilamellar liposomes containing acidic phospholipids. They found that cytochrome c added during the formation of lecithin-cardiolipin liposomes in 15 mM potassium chloride was readily bound, and that about 50% of the bound protein could not be removed by repeated washing with 150 mM potassium chloride. In keeping with results from monolayer and BLM studies^(102,122-125), they concluded that the binding was electrostatic, since it was dependent on pH and on the presence of cardiolipin, and was completely inhibited by raising the initial concentration of potassium chloride to 0.1 M. On adding ascorbate to the external solution, only some 10-20% of the bound cytochrome c was reduced. The remainder was, however, rapidly reduced if a lipid soluble redox mediator was also added: N-methylphenazinium methyl sulphate (PMS), dichlorophenol indophenol (DCIP) and tetramethyl-*p*-phenylenediamine (TMPD) were all found to be effective⁽¹²⁶⁾. Alternatively, the addition of detergents such as Triton X-100, which cause membrane lysis, also promoted reduction of the bound cytochrome c by ascorbate⁽¹²⁸⁾. These observations indicated that the bound protein was electrostatically associated with the surfaces of individual lamellae and was sequestered within the permeability barrier of the outermost liposomal membrane. Kimelberg and Lee⁽¹²⁸⁾ also noted a reduction in the standard redox potential (E_o') of the cytochrome c from + 273 mV in free solution to + 225 mV for the membrane-bound protein, which they attributed to some minor conformational change on binding.

Jasaitis et al.⁽¹⁹¹⁾ demonstrated the formation of a membrane potential in two types of liposomes, one inlaid with cytochrome c plus cytochrome oxidase, and the other with oligomycin-sensitive ATPase. To detect the potential generated during the oxidation of ascorbate

(in the presence of PMS or TMPD) or during the hydrolysis of ATP, they used the membrane-penetrating anionic probe phenyldicarbonyl-decaborane (PCB^-), which changes its fluorescence properties in a potential gradient. In a series of very interesting and relevant papers, Hinkle and co-workers have described several liposomal systems in which redox processes were established⁽¹⁹²⁻¹⁹⁴⁾. Some of these are outlined in Table 16. Ferricyanide contained within phospholipid vesicles could be reduced by external ascorbate when dibutylferrocene, PMS, or hydroquinone was added⁽¹⁹²⁾. However, for continuous reaction several other additions were also required. In the case of dibutylferrocene and PMS (both charged carriers), addition of CCCP (carbonyl cyanide *m*-chlorophenylhydrazone — an uncoupler which acts as a proton carrier) or valinomycin (an ionophore which carries only potassium ions) or a mixture of valinomycin plus nigericin (an ionophore which acts as an exchange-diffusion carrier for potassium ions and protons by an electrically neutral mechanism) was necessary to equilibrate the electrical potential gradient generated. A small amount of tetraphenylboride (TPB^-) was also required for optimum reaction rates : this was thought to increase the permeability of the membrane to the cationic carriers. In the case of hydroquinone, only nigericin stimulated the reaction, presumably by neutralising the unfavourably acidic internal pH without disturbing the electroneutrality of the overall process⁽¹⁹²⁾.

Turning to model systems more directly relevant to mitochondrial electron transfer, Hinkle and co-workers have succeeded in incorporating three of the respiratory chain complexes, known to be sites of energy coupling, into liposomes, using the cholate dialysis method mentioned above⁽¹⁹²⁻¹⁹⁴⁾ (Table 16). More recently, Eytan et al.⁽¹⁹⁵⁾ have incorporated similar enzymes into liposomes formed from PC, PE and

Table 16 Electron transfer reactions in liposomal systems. (Data of Hinkle et al. (192-194).)

Carriers mediating electron transport through the membranes are underlined. Further additions required for optimum reaction conditions are indicated in parentheses. (TPB⁻ - tetraphenylboride; PMS - N-methylphenazinium methyl sulphate; CCCP - carbonyl cyanide *m*-chlorophenylhydrazone; val - valinomycin; nig - nigericin.) Note that all these molecules, except the respiratory complexes, are substantially water-soluble and were added to the external aqueous solutions after formation of the liposomes.

Internal aqueous solution	Membrane composition	External aqueous solution	Observations	Ref. No.
potassium chloride + ferricyanide	phospholipids + <u>dibutylferrocene</u> (+ TPB ⁻ + CCCP or val) or <u>PMS</u> (+ TPB ⁻ + CCCP or val or val/nig) or <u>hydroquinone</u> (+ nig)	potassium chloride + ascorbate	Reduction of encapsulated ferricyanide (OD ₄₂₀ decreases)	(192)
potassium chloride + oxygen	soybean phospholipids + <u>cytochrome oxidase</u> (+ CCCP or val/nig)	potassium chloride + ferricytochrome c + oxygen	Oxidation of ferricytochrome c by pulses of oxygen, catalysed by cytochrome oxidase. pH inside liposomes increases as electrons transferred to internal oxygen (shown by ionisation of encapsulated phenol red indicator)	(192)
potassium chloride + phenol red + oxygen	soybean phospholipids + <u>cytochrome oxidase</u> (+ val)	potassium chloride + ferricytochrome c + ascorbate + oxygen		
potassium phosphate buffer	soybean phospholipids or PC/PE/CL mixtures + <u>CoQH₂-cyt c reductase</u> (+ CCCP or val/nig)	potassium phosphate + ferricytochrome c + CoQ ₂ H ₂	Reduction of ferricytochrome c by CoQ ₂ H ₂ , catalysed by CoQH ₂ - cyt c reductase. External pH decreases if CoQ ₂ H ₂ oxidised in absence of buffer and ionophores.	(193)
potassium chloride	PC/PE mixtures + <u>NADH - CoQ reductase</u> (+ CCCP or val/nig)	potassium chloride + CoQ ₁ + NADH	Oxidation of NADH by CoQ ₁ , catalysed by NADH-CoQ reductase. External pH increases if CoQ ₁ reduced in absence of ionophores.	(194)

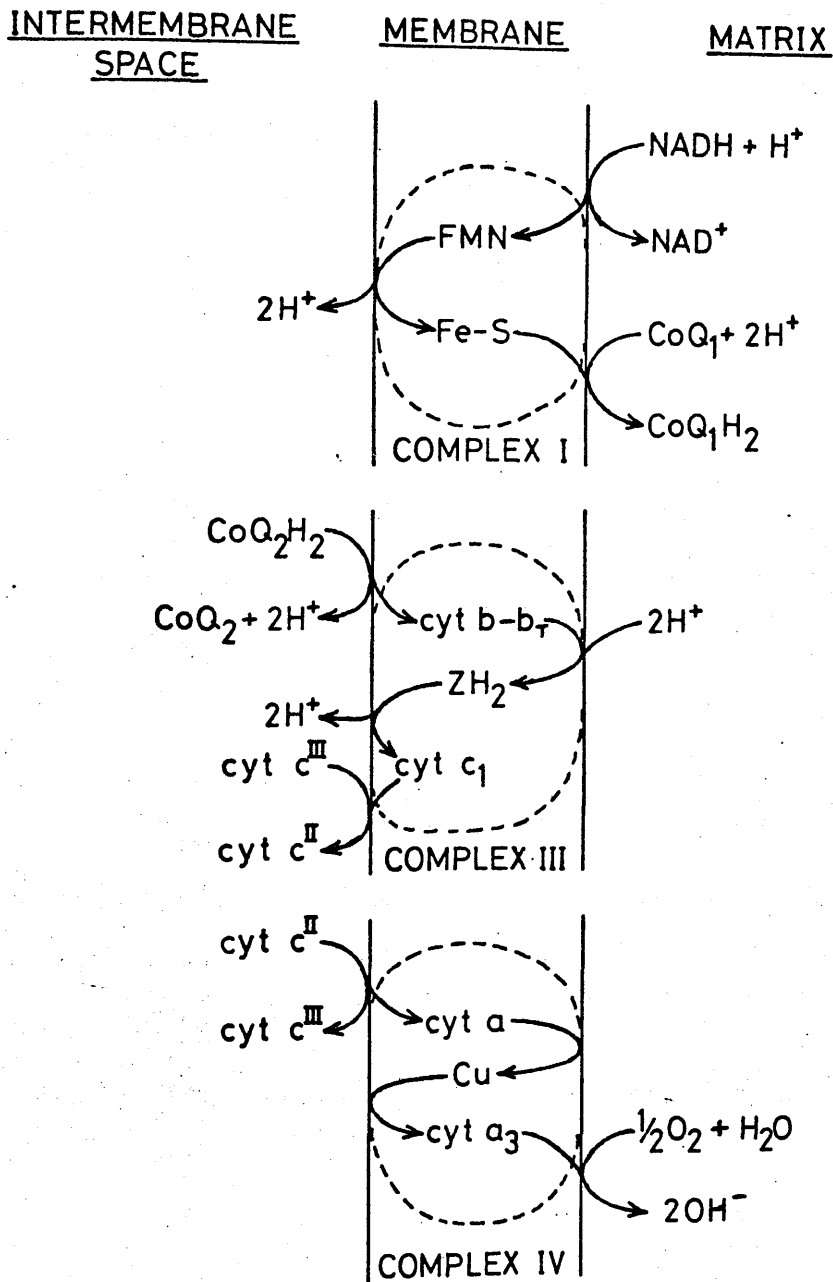


Figure 5.2 Schematic representation of electron transport at the three energy coupling sites in the mitochondrial respiratory chain, as deduced from the work of Hinkle *et al.* (192-194) on liposomal model systems. See text (Section 5-1.2) and Table 16 for details.

an acidic phospholipid, by an equilibration procedure which avoids sonication and addition of detergents, and yields unidirectional orientation of the proteins, as found in the mitochondrion. Cytochrome oxidase (complex IV) catalysed the oxidation of external ferrocytochrome c by molecular oxygen⁽¹⁹²⁾, CoQH_2 - cyt c reductase (complex III) catalysed the reduction of ferricytochrome c by reduced coenzyme Q_2 ⁽¹⁹³⁾, and NADH-CoQ reductase (complex I) catalysed the oxidation of NADH by coenzyme Q_1 ⁽¹⁹⁴⁾. In each case addition of CCCP, or valinomycin plus nigericin, was found to increase the reaction rate : this stimulation of electron transport by uncouplers has been termed 'respiratory control' and is a property exhibited by mitochondria⁽¹⁹²⁾. A schematic representation of these three stages in the electron transport process, deduced from Hinkle's results, is shown in Figure 5.2. On comparing this with Ernster's representation (Figure 1.7), which was derived from biochemical investigations on the inner mitochondrial membrane itself, it is clear that these liposomal systems provide useful models for the natural processes. However one significant difference between the two is evident; namely the location of the coenzyme Q species. In the model systems investigated so far, the quinones used — the short-chain homologues coenzymes Q_1 and Q_2 — were substantially water soluble and so are shown in Figure 5.2 located in the aqueous phase : in the mitochondrion, coenzyme Q_{10} is completely restricted to the membrane phase. It was the aim of the present research to find out whether such a membrane-bound quinone is capable of acting as an electron carrier across a lipid bilayer.

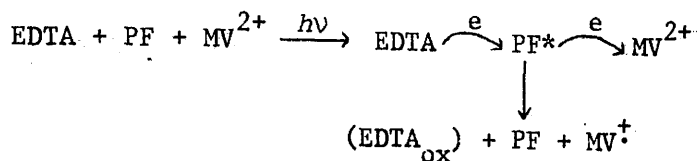
5-1.3 Generation of a liposomal redox system

In order to establish a continuous electron transfer process across a liposomal membrane separating aqueous oxidising and reducing agents, it was necessary to encapsulate one or other of the aqueous

reactants inside the liposome and to incorporate a suitable redox mediator in the membrane during its formation. Because of its superior reaction kinetics (Section 3-3.2), vitamin K₁ was again chosen in preference to coenzyme Q₁₀ for use as the membrane-bound carrier in these initial experiments. It was decided to encapsulate the reductant rather than the oxidant, to allow easy isolation of the reduced reaction product after removal of the liposomes from the suspension by centrifugation. Also, since it was proposed to use cytochrome c as a substrate, its encapsulation within the liposomes at spectrophotometrically detectable levels would have required the use of prohibitively large amounts of the protein at the liposome preparation stage. However, the encapsulation of the reductant presented several experimental problems. All the reductants capable of reducing vitamin K (Table 5), as well as the reduced vitamin K itself, were extremely sensitive to oxidation by atmospheric oxygen. Thus it would have been virtually impossible to have maintained the liposomes in a reduced, 'active' state throughout the lengthy preparation procedure. A method of generating the reducing species *in situ*, after encapsulating it in the liposomes in an inactive form, was therefore required.

Grimaldi *et al.* (196) have used a photochemical system to produce reduced methyl viologen in bulk solution and hence to induce electron transport across artificial membranes, made of diphenyl ether supported on a Millipore filter, and containing menadione or dibutylferrocene (both substantially water soluble molecules) as carrier species. Methyl viologen is photoreduced in aqueous solution by a reaction involving visible light, a sensitising dye — proflavine ($\lambda_{\text{max}} = 445 \text{ nm}$) — and the electron donor ethylenediamine tetra-acetic acid (EDTA) (197). The triplet excited state of proflavine (PF*) accepts an electron from EDTA to form the singly-reduced species of

proflavine, which then donates an electron to the oxidised form of the bipyridyl to form the blue radical cation $MV^{\cdot+}$. The reaction occurring may be considered to be:



The fate of the EDTA is not known but the oxidised form does not interfere with the subsequent redox reactions. In this way reduced methyl viologen can be continuously regenerated by irradiation of the system for as long as EDTA — the 'fuel' of the reaction — remains⁽¹⁹⁸⁾. This reducing system was used here in the initial experiments on liposomes prepared from egg lecithin, but was found to be unsuitable for later experiments involving acidic phospholipids, due to 'leakage' of the cationic proflavine from the liposomes (Section 5-3.2). An alternative photosystem was therefore sought.

Flavin mononucleotide (FMN), a prosthetic group of the flavo-protein enzymes (such as complex I of the mitochondrial respiratory chain) is a fluorescent species having a chromophore rather similar in structure to proflavine, but bearing a net negative charge due to the attachment of a phosphate group. Although a preliminary experiment showed that FMN could not replace proflavine as photosensitiser in the above reaction scheme, it was discovered that FMN was photoreduced in the presence of EDTA by visible light. A description of this photosystem was subsequently found in the literature⁽¹⁹⁹⁾. Earlier studies had shown that the free radical reduced form of FMN produced in this reaction acts as an efficient reductant for vitamin K and coenzyme Q at the hexane/aqueous solution interface (Sections 2-3.2 and 2-3.5). This simpler EDTA/FMN photosystem was therefore adopted in later work, as it showed little tendency to 'leak' spontaneously from the liposomes (Section 5-3.2).

The choice of a suitable type of liposome in which to encapsulate the photosystem was governed by the requirements for activity of the trapped photosystem and for a continuous electron transfer process. Clearly the large multilamellar type was unsuitable, since only the reductant present in the outermost aqueous compartment would be capable of transferring its electrons through the membrane to the external substrate. The microvesicular type of liposome was also ruled out for reasons made clear by consideration of Table 17. From this it can be seen that, at the concentrations required in these experiments, the probability of any microvesicular liposome containing even only one of each of the photosystem's components is very small. The much larger macrovesicular type of liposome, on the other hand, should contain sufficient photosolution to allow normal reaction to take place. Attention was therefore focussed on these large unilamellar liposomes in the present work.

5-2 EXPERIMENTAL

5-2.1 Light source and filter

The light source used in all these experiments was a 'Wotan' halogen lamp (No. 64640, 24 V, 150W, Osram GMBH, Germany) housed in a 35 mm slide projector. In a preliminary experiment, when a solution of methylene blue under nitrogen was irradiated with light from this source, it was found to bleach rapidly. Therefore before methylene blue could be used as a substrate, the offending wavelengths had to be removed from the incident light. The most obvious method might have been to have used a filter containing a strong solution of methylene blue itself, but this would also have tended to become transparent to all wavelengths after prolonged exposure to light. In practice an aqueous solution of 2,6 - dichlorophenol indophenol (DCIP) was found to be more satisfactory. After ensuring that aqueous DCIP was unaffected

Table 17. Comparison of microvesicular and macrovesicular unilamellar liposomes and their abilities to encapsulate an EDTA/PF/MV photosystem.

	External radius (Å)	Internal radius (Å)	Enclosed volume (cm ³)	Concentration of photosolution components (moles l ⁻¹)			Number of molecules of each component encapsulated per liposome		
				EDTA	PF	MV	EDTA	PF	MV
Microvesicles	125	75	1.77x10 ⁻¹⁸	6.0x10 ⁻²	1.6x10 ⁻⁴	3.0x10 ⁻³	64	0.17	3.2
Macrovesicles	5000	4950	5.08x10 ⁻¹³	6.0x10 ⁻²	1.6x10 ⁻⁴	3.0x10 ⁻³	1.84x10 ⁷	4.90x10 ⁴	9.18x10 ⁵

	Area of inner surface (Å ²)	No. of molecules facing inward*	Area of outer surface (Å ²)	No. of molecules facing outward*	Total no. of molecules per liposome	No. of liposomes per μ mole lipid	Volume encapsulated per μ mole lipid (cm ³)
Microvesicles	7.069x10 ⁴	1071	1.964x10 ⁵	2976	4047	1.488x10 ¹⁴	2.63x10 ⁻⁴
Macrovesicles	3.142x10 ⁸	4.76x10 ⁶	3.079x10 ⁸	4.67x10 ⁶	9.43x10 ⁶	6.387x10 ¹⁰	3.24x10 ⁻²

* These calculations assume a surface area per phospholipid molecule of 66 Å²(177).

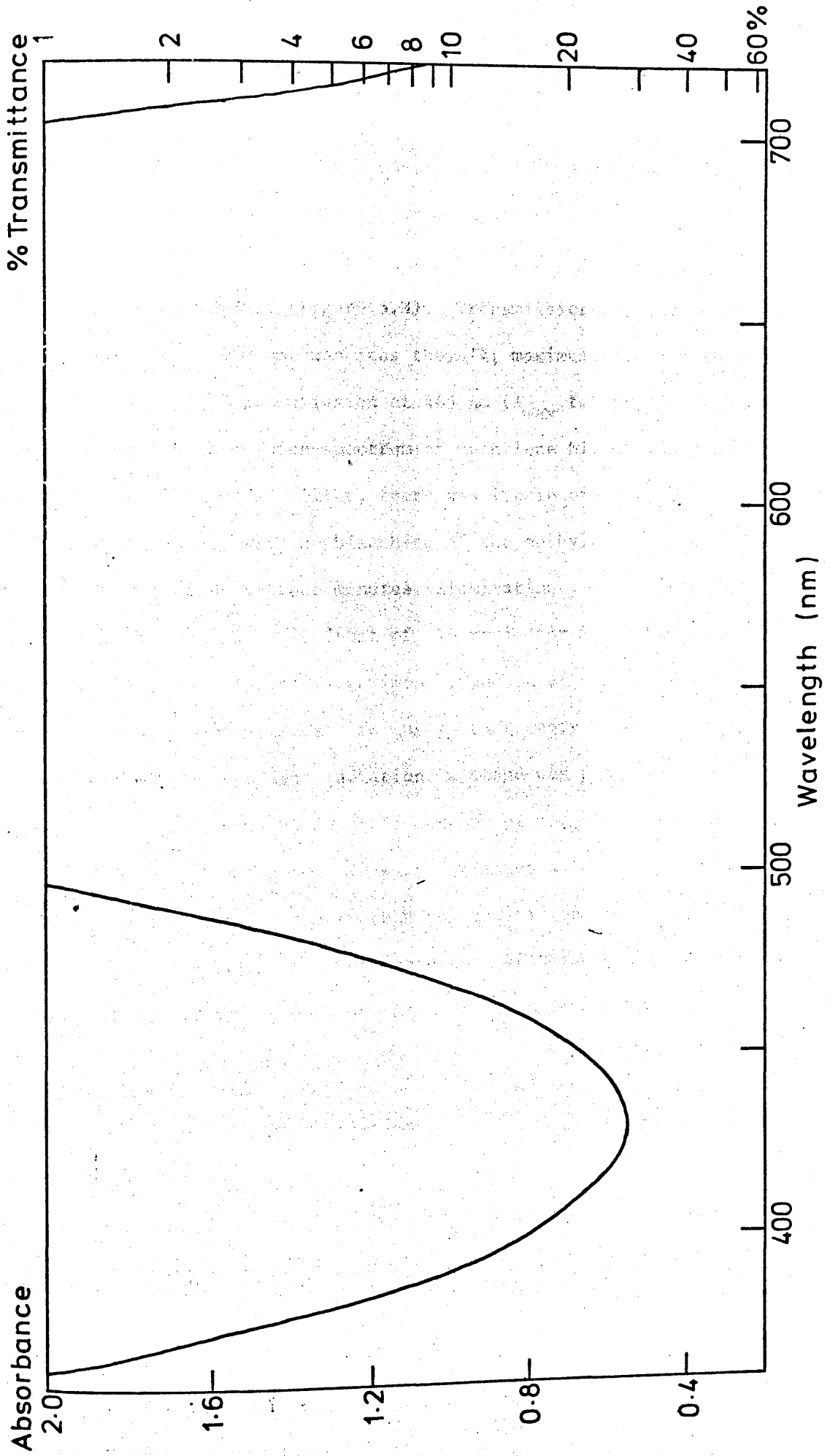


Figure 5.3 Visible absorption spectrum of the DCIP light filter described in Section 5-2.1.

by light (there being no perceptible change in the absorption spectrum after fifteen minutes irradiation), a filter was made by placing a concentrated DCIP solution in a small clear perspex box (2" broad x 3" high x $\frac{1}{2}$ " light path length) with a tight-fitting lid. The absorption spectrum of this filter was measured against air in the SP800 spectrophotometer (Figure 5.3). Transmission between 500 and 700 nm, and below 360 nm, was less than 1%; maximum transmission was 28%, at 430 nm; and transmission at 445 nm (λ_{max} for proflavine) was 24%. Since the absorption spectrum of methylene blue fell completely within that of the DCIP filter, there was little or no absorption of the filtered light, with no bleaching of the methylene blue being detected even after fifteen minutes illumination. This blue light filter, placed directly in front of the projector lens, was used in most of the later liposome experiments, and in all those involving methylene blue as substrate. To ensure that every sample received the same intensity of incident radiation, a stand was placed in front of the projector to hold the optical cell containing the solution in a fixed position in the light beam. This was arranged with the cell about 1 cm in front of the filter, and so that the entire contents of the cell were exposed to light. The heating effect of the light beam on the sample was minimal, most of the infrared component being absorbed by the water in the filter.

5-2.2 Preparation of photosolutions

(a) EDTA/PF/MV photosolutions

The chemicals used were from the sources, and of the purities, indicated in Appendix I. A solution containing 1.6×10^{-4} M proflavine (hemi-sulphate), 3.0×10^{-3} M methyl viologen (dichloride), 6.0×10^{-2} M EDTA (disodium salt) and 4.0×10^{-2} M phosphate buffer, was prepared in freshly distilled water. The osmolarity of the solution

was about 0.39 osM (photosolution 1, Table 18). A sample of this solution, in a box-top UV cell fitted with a suba-seal, was deoxygenated by bubbling nitrogen through it for fifteen minutes. The solution was then illuminated with the unfiltered intense light of the halogen lamp. After only a few seconds illumination the characteristic blue colour of the methyl viologen radical ion appeared, indicating that photoreduction had been effected. This was confirmed by recording the visible absorption spectra of the solution before and after illumination (Figure 5.4). The spectrum of MV^+ shows a broad absorption centred on 605 nm and a very sharp maximum at 396 nm, while proflavine shows a single peak at 445 nm. On opening the cell to oxygen the blue colour disappeared rapidly and the spectrum reverted to the original. Further degassing and illumination regenerated the characteristic blue colour of reduced methyl viologen. Similar results were achieved using blue filtered light, although in this case photoreduction was slightly slower, due to the lower intensity of the incident radiation. (It was a happy coincidence that the transmission spectrum of the DCIP filter corresponded almost exactly with the absorption spectrum of proflavine.)

One final test on the photosolution was necessary before it could be incorporated into liposomes : it had to be shown that none of the photosystem's components were membrane soluble and could therefore leak out of the liposomes, deactivating the photosystem. Methyl viologen has previously been shown to be insoluble in a hydrocarbon membrane (Section 2-3.3); EDTA, due to its relatively large size and double negative charge at pH 7, was unlikely to cross the membrane. In order to check that proflavine (a cationic species) was also insoluble in a hydrocarbon phase, a bulk membrane experiment, using the H-cell as detailed in Chapter 3, was performed. The system set up was :

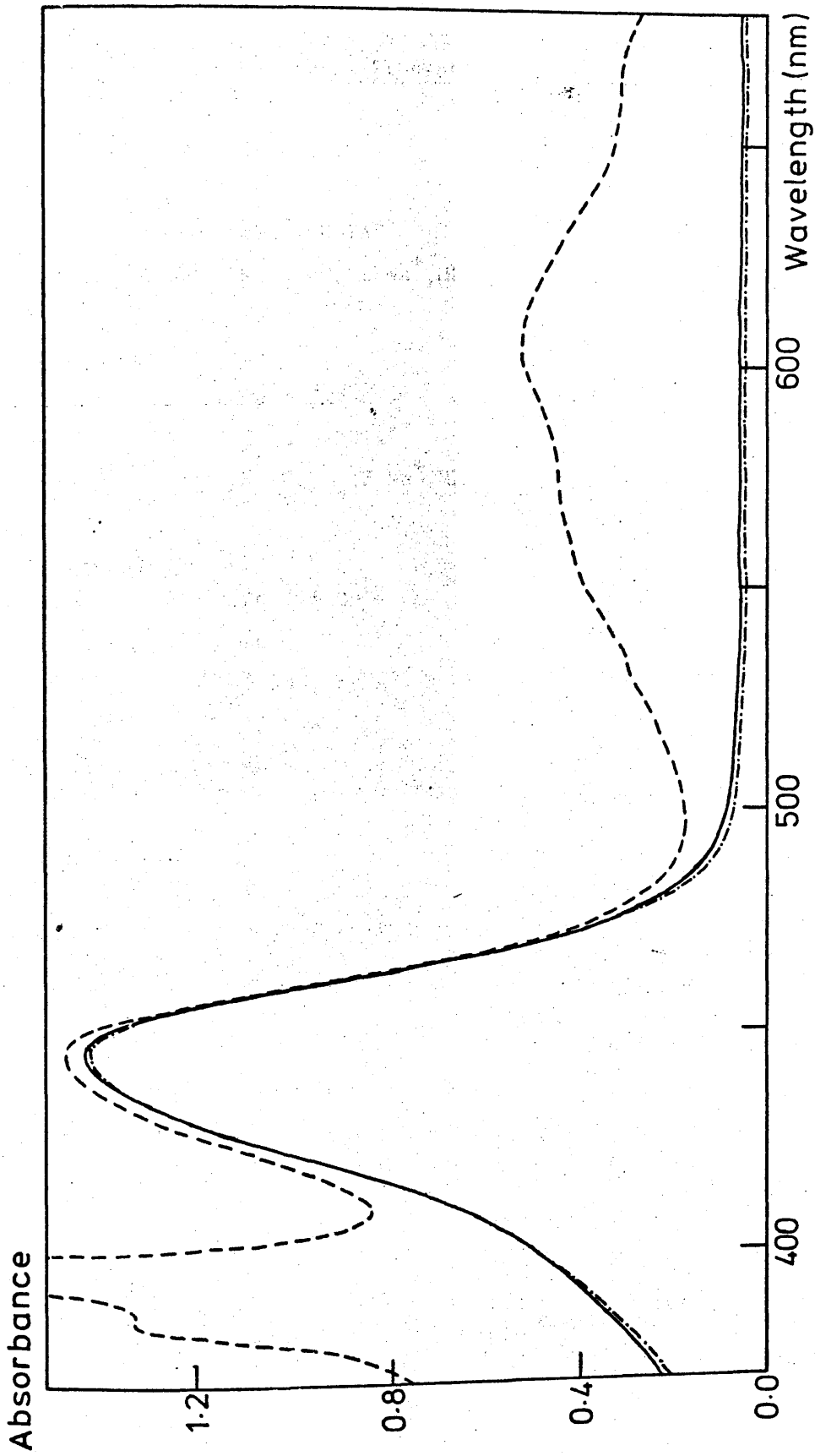


Figure 5.4 Visible absorption spectra of the EDTA/PF/MV photosystem (photosolution 1, Table 18) before illumination (—), after 10s illumination (---), and after opening the illuminated solution to the atmosphere (-.-.-).

Table 18. Compositions of photosolutions and buffers used in liposome preparations.

<u>Component</u>	<u>Particles Present*</u>	<u>Molarity (M)</u>	<u>Osmolarity (osM)</u>
<u>Photosolution 1</u>			
Proflavine	PF ⁺ , $\frac{1}{2}$ SO ₄ ²⁻	1.6 x 10 ⁻⁴	0.00024
Methyl viologen	MV ²⁺ , 2 Cl ⁻	3.0 x 10 ⁻³	0.00900
EDTA	2Na ⁺ , EDTA ²⁻	6.0 x 10 ⁻²	0.18000
Phosphate buffer	K ⁺ , H ₂ PO ₄ ⁻ , 2 Na ⁺ , HPO ₄ ²⁻	4.0 x 10 ⁻²	<u>0.20000</u>
			Total osmolarity = 0.38924 osM
<u>Photosolution 2</u>			
FMN	Na ⁺ , FMN ⁻	1.0 x 10 ⁻²	0.020
EDTA	2 Na ⁺ , EDTA ²⁻	6.0 x 10 ⁻²	0.180
Phosphate buffer	K ⁺ , H ₂ PO ₄ ⁻ , 2 Na ⁺ , HPO ₄ ²⁻	4.0 x 10 ⁻²	<u>0.200</u>
			Total osmolarity = 0.400 osM
<u>Photosolution 3[†]</u>			
Proflavine	PF ⁺ , $\frac{1}{2}$ SO ₄ ²⁻	3.2 x 10 ⁻⁴	0.00048
Methyl viologen	MV ²⁺ , 2 Cl ⁻	6.0 x 10 ⁻³	0.01800
EDTA	2 Na ⁺ , EDTA ²⁻	1.2 x 10 ⁻¹	0.36000
Tris	Tris	1.0 x 10 ⁻²	0.01000
NaOH	Na ⁺	9.7 x 10 ⁻²	<u>0.09700</u>
			Total osmolarity [†] = 0.48548 osM
<u>Photosolution 4[†]</u>			
FMN	Na ⁺ , FMN ⁻	1.0 x 10 ⁻²	0.020
EDTA	2 Na ⁺ , EDTA ²⁻	1.2 x 10 ⁻¹	0.360
Tris	Tris	1.0 x 10 ⁻²	0.010
NaOH	Na ⁺	1.12 x 10 ⁻¹	<u>0.112</u>
			Total osmolarity [†] = 0.502 osM

(continued over)

[†]Note that the compositions of all the photosolutions listed here refer to the initial stock solutions. In the case of liposomes prepared by the 'cochleate cylinders' method (Section 5-2.3(b)), the final concentrations of photosolutions 3 or 4 in the encapsulated solutions were equal to half those given here. See Section 5-2.3(b) for osmolarity calculations.

Table 18 (cont'd)

<u>Component</u>	<u>Particles present*</u>	<u>Molarity (M)</u>	<u>Osmolarity (osM)</u>
<u>Sodium chloride buffer</u>			
NaCl	Na ⁺ , Cl ⁻	1.0 x 10 ⁻¹	0.2000
Histidine	Hist H ⁺ , Cl ⁻	2.0 x 10 ⁻³	0.0040
Tris	Tris	2.0 x 10 ⁻³	0.0020
EDTA	2 Na ⁺ , EDTA ²⁻	1.0 x 10 ⁻⁴	<u>0.0003</u>
			Total osmolarity = 0.2063 osM
<u>EDTA solution</u>			
EDTA	2 Na ⁺ , EDTA ²⁻	1.2 x 10 ⁻¹	0.360
NaCl	Na ⁺ , Cl ⁻	5.0 x 10 ⁻²	0.100
Histidine	Hist H ⁺ , Cl ⁻	1.0 x 10 ⁻³	0.002
Tris	Tris	1.0 x 10 ⁻³	0.001
NaOH	Na ⁺	4.0 x 10 ⁻²	<u>0.040</u>
			Total osmolarity = 0.503 osM
<u>Hypertonic sodium chloride buffer</u>			
NaCl	Na ⁺ , Cl ⁻	1.6 x 10 ⁻¹	0.320
Tris	Tris	6.0 x 10 ⁻³	0.006
Histidine	Hist H ⁺ , Cl ⁻	6.0 x 10 ⁻³	<u>0.012</u>
			Total osmolarity = 0.338 osM
<u>Hypertonic sodium chloride buffer (I ~ 0.3 M)</u>			
NaCl	Na ⁺ , Cl ⁻	3.0 x 10 ⁻¹	0.600
Tris	Tris	6.0 x 10 ⁻³	0.006
Histidine	Hist H ⁺ , Cl ⁻	6.0 x 10 ⁻³	<u>0.012</u>
			Total osmolarity = 0.618 osM

* Since all solutions were adjusted to near neutrality by titration with NaOH (Section 5-2.2), contributions from H⁺ or OH⁻ ions have been ignored here.

Photosolution containing proflavine	vitamin K in hexane	photosolution containing no proflavine
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After stirring and rocking the H-cell for a 72-hour period, the right-hand limb was examined spectrophotometrically. No absorption was detected at 445 nm, and even after several minutes illumination there was no appearance of reduced methyl viologen.

For use in experiments involving phosphatidylserine liposomes (Section 5-2.3(b)) an EDTA/PF/MV photosolution was required which did not contain phosphate, since this would have caused precipitation of the calcium ions added during the liposome preparation. Instead the solution was made up in Tris buffer (photosolution 3, Table 18) and the pH was adjusted, by titration with 1.0 M sodium hydroxide from a Hamilton syringe, to 7.4, which was the pH of the solution in which the PS liposomes were to be formed (Section 5-2.3). The photoactivity of this solution was established by subjecting a deoxygenated sample to a few minutes' illumination with blue light, when the blue colour of the $MV^{\cdot+}$ radical quickly appeared.

(b) EDTA/FMN photosolutions

A solution containing 10^{-2} M FMN (sodium salt), 6.0×10^{-2} M EDTA (disodium salt) and 4.0×10^{-2} M phosphate buffer, was prepared in distilled water, the pH being adjusted to 6.72 by titration with 1.0 M sodium hydroxide. The amount of alkali required was negligible, and the resulting solution had a tonicity of 0.40 osM (photosolution 2, Table 18). The photoactivity of this solution was tested by illuminating a deoxygenated sample (a one hundred times dilution of photosolution 2 in 0.086 M phosphate buffer) with blue filtered light. Figure 5.5 shows the changes which occurred in the visible absorption spectrum as the radical reducing species of FMN was generated. The solution reverted to its original yellow colour when the cell was opened to oxygen.

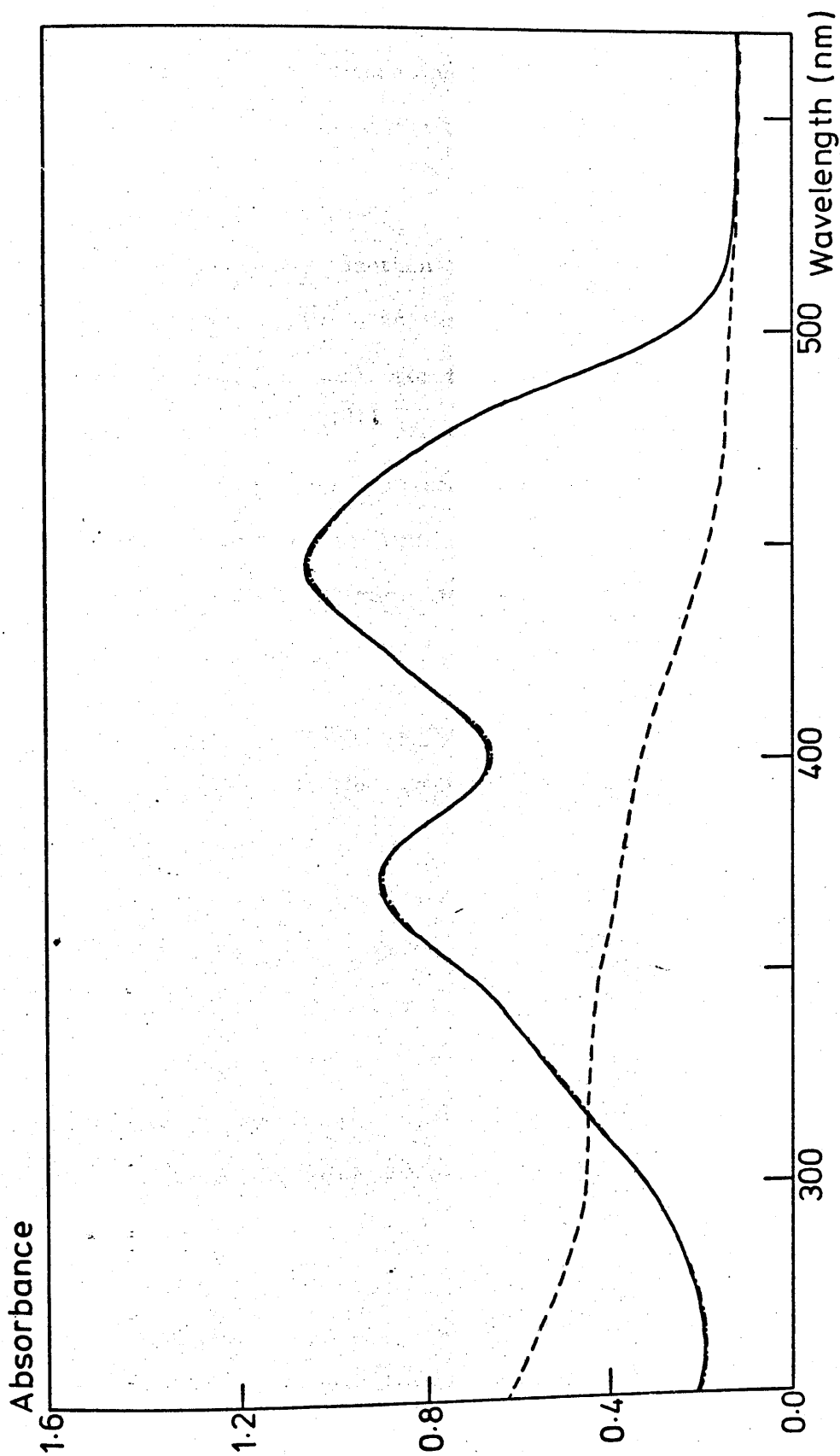


Figure 5.5 UV/visible absorption spectra of the EDTA/FMN photosystem (photosolution 2, Table 18, diluted 100 times) before illumination (—), after 1 min illumination (----), and after opening the illuminated solution to the atmosphere (-.-.-).

For use in experiments involving phosphatidylserine liposomes (Section 5-2.3(b)) an EDTA/FMN photosolution was made up in Tris buffer and adjusted to pH 7.4 as before (photosolution 4, Table 18). This solution was photoactive and had a tonicity of about 0.50 osM.

5-2.3 Preparation of liposomes

As indicated above (Section 5-1.3), only the large, unilamellar type of liposome was suitable for use in the present investigations. Of the three methods available for their preparation (Section 5-1.1) that of Reeves and Dowben⁽¹⁸¹⁾ had to be discounted because it could not accommodate electrolyte solutions, as required here. The 'choleate cylinders' method of Papahadjopoulos et al.⁽¹⁸³⁾ could only be applied to phosphatidylserine liposomes. It seemed therefore that the most promising general preparation method was that of Deamer and Bangham⁽¹⁸²⁾.

(a) The 'ether vaporization' method

This was basically the method described recently by Deamer and Bangham⁽¹⁸²⁾, except that it was slightly simplified and modified to allow for the preparation of liposomes under anaerobic conditions. The following procedure was adopted: A total of 20 μ moles of the required lipid or lipid mixture was measured out accurately into a small Quickfit test-tube, by volume from stock solutions of known concentrations, using Hamilton syringes. (The sources, purities and concentrations of the stock phospholipid solutions are given in Appendix I: these were all used directly, without further purification.) The following molecular weights were used: egg lecithin \sim 766⁽¹²⁷⁾; cardiolipin \sim 1382⁽¹²⁷⁾; dioleoyl lecithin = 806; vitamin K₁ = 451. The volatile solvents were then removed under vacuum, and the lipids were re-dissolved in about 2 ml of anhydrous diethyl ether. This was again slowly evaporated off, taking with it the last traces of the original solvents, and the dry residue was finally dissolved in 2.5 ml of

anhydrous diethyl ether. 2.5 ml of the solution to be encapsulated (phosphate buffer, photosolution 1 or photosolution 2, Table 18) was placed in a test-tube, 15 cm long and 2.2 cm in diameter. This was closed by a suba-seal and fitted with two stainless steel syringe needles; one long needle which reached to the bottom of the tube and was attached to a nitrogen inlet, and one short needle attached to a small gas bubbler. The test-tube was then immersed in a water bath which was stirred and held at $60 \pm 1^{\circ}\text{C}$ on an electric hot-plate. A fast flow of oxygen-free nitrogen was bubbled through the solution as it came to thermal equilibrium. The nitrogen was pre-warmed by passing it through a long coil of narrow gauge polythene tubing which was also held in the water bath (Figure 5.6). After about thirty minutes, during which the photosolution was carefully protected from light, addition of the ethereal lipid solution was commenced. This was done slowly, in 0.5 ml aliquots, using a 0.5 ml gas-tight Hamilton syringe fitted with a long, fine, stainless steel needle which reached to the bottom of the test-tube. During each addition, vigorous bubbling was observed in the solution as the ether rapidly evaporated and considerable frothing took place, with bubbles occasionally rising to the top of the tube. When the last traces of ether vapour had disappeared (detected by smell) a second aliquot of lipid solution was injected, and this was repeated until all the lipid had been added. (This generally took about twenty minutes.) The test-tube was then removed from the water bath and allowed to cool slowly to room temperature in the dark. Nitrogen flushing was continued during this period, although the inlet syringe needle was removed from the solution. The product of this preparation was a homogeneous but very turbid suspension, which was bright yellow when photosolution was encapsulated. (In preliminary experiments when nitrogen bubbling was not employed, large aggregates

of lipid had resulted. The function of nitrogen bubbling seemed to be two-fold : firstly, to create turbulence and hence more efficient mixing in the solution, and secondly, to remove, by frothing, any excess lipid which did not immediately form liposomes.) The cool suspension was then filtered through a 5.0 μm pore size 'Mitex' (pure Teflon) Millipore filter using a stainless steel 'Swinnex' filter holder attached to a 5 ml glass syringe. The solution passed through quite readily, leaving very little residue. However, the solution would not pass through 'MF-Millipore' filters of 0.22 μm or 1.2 μm pore sizes, indicating that the vesicles present were rather large.

Several attempts were made to separate the liposomes from the external solution by centrifugation, but these were unsuccessful, as the lipid always tended to spin upwards rather than forming a pellet. Instead the gel filtration technique was routinely used. Columns were prepared as follows : 5.0 g of Sephadex C-50-80, particle size 20-80 μm , was first expanded by boiling for one hour in about 150 ml of the same hypertonic buffer (see below) as was intended to form the external medium for the separated liposomes. After cooling, most of the excess buffer was decanted and the remaining white slurry was poured into a glass column, 36 cm long and 2.5 cm in diameter, and fitted with a porous glass frit and a Teflon stopcock. The stopcock was opened to let the gel settle more quickly, and the liquid level was allowed to fall until the bottom of the meniscus just touched the gel surface. The resulting gel column was 17 cm in length. To measure its void volume, that is, the volume in which a totally excluded particle should be eluted from the column, a 25 ml graduated cylinder was placed below the column, and 2 ml of blue dextran solution ($\text{MW} \sim 2 \times 10^6$) was very carefully layered on the surface of the gel. The solution was then allowed to penetrate the column until its meniscus just touched the surface, at which stage more of the hypertonic buffer was carefully

added. This procedure was continued as the narrow blue band moved down the column. The entire fraction was collected in about 5 ml of the eluate, representing a dilution of only two to three times, and the void volume of the column was found to be 20 ml. All the columns used in these experiments (a fresh column was used for each) were found to have similar characteristics.

Since liposomes are osmotically active⁽¹⁸⁴⁾, the relative osmolarity of the internal and external solutions is of critical importance : in a hypotonic medium liposomes swell and rupture, while in a hypertonic solution they tend to distort and shrink. From the point of view of the present studies, the latter was by far the lesser of the two evils. It was therefore decided to allow for a 'safety margin' by always using slightly hypertonic external media. Pre-treatment of the Sephadex gels with hypertonic buffer essentially saturated the gels with ions from the buffer; otherwise the gels would have had a desalting effect which would have led to bursting of the liposomes during their passage down the column.

After filtration, the liposome suspension was passed through a Sephadex column, prepared and used as described above. If the liposomes contained photosolution, two yellow bands separated out : the first, containing the liposomes, passed through in the void volume; the second, a much broader and more intense band, passed down the column rather slowly. The effectiveness of the separation could be judged by analysing successive 4 ml fractions of the eluate spectrophotometrically for lipid (by measurement of turbidity, which increases towards lower wavelengths) and for photosolution (by detection of the absorption peak of proflavine at 445 nm, or of the double peak of FMN at 370 and 445 nm). For a given separation to be considered as acceptable, at least one fraction between the two bands had to be free

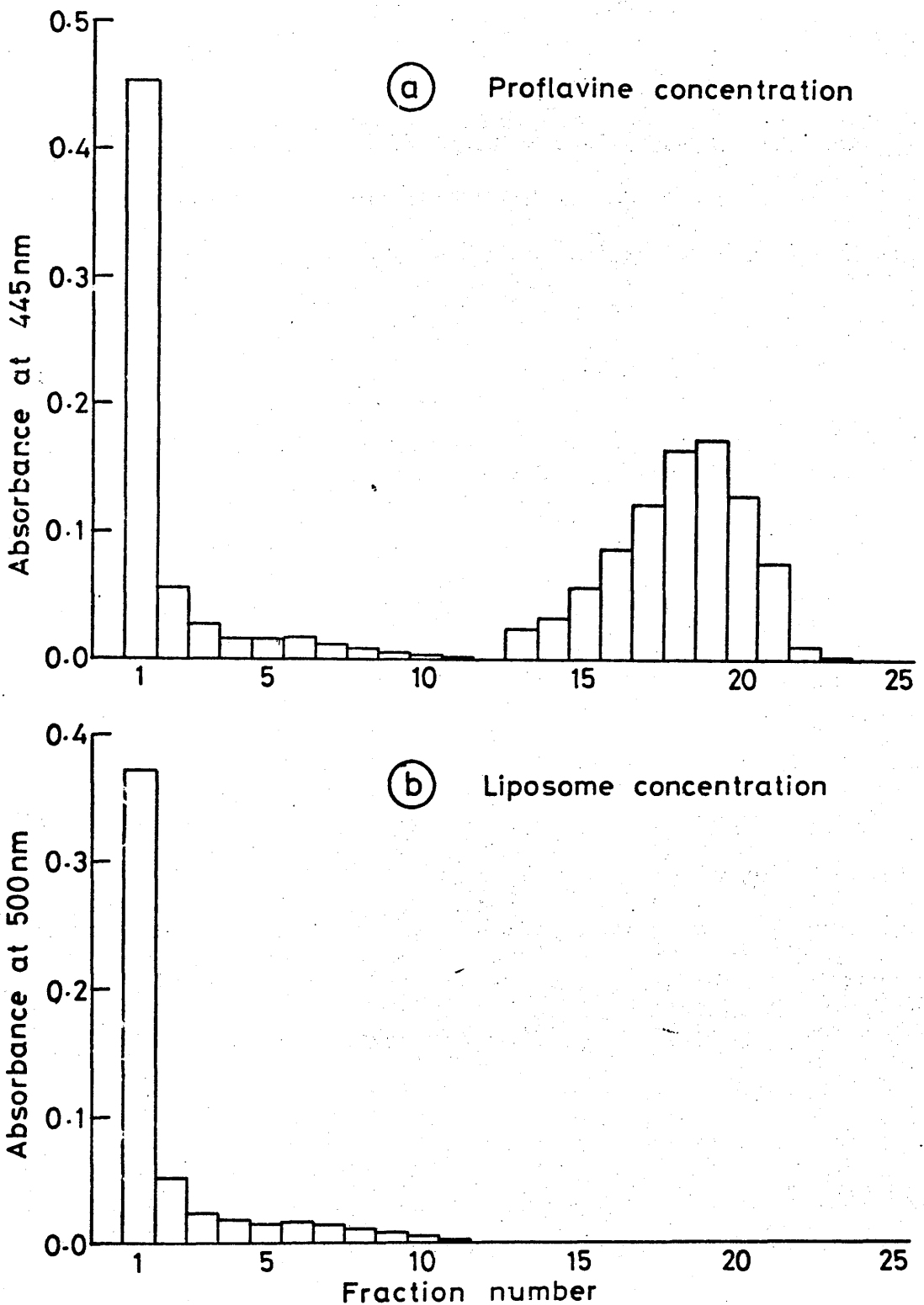


Figure 5.7 Analysis of column separations of liposomes from external photosystem. The histograms show the relative concentrations of (a) proflavine, and (b) liposomes, eluted in successive 4 ml fractions (following the main liposomal band) from a Sephadex G-50 column initially charged with 2.5 ml of a suspension of liposomes, prepared by the 'ether vaporization' technique, in photosolution 1 (Table 18).

of both lipid and photosolution. The results of a typical fraction analysis are shown in Figure 5.7. In general, separations on the column described above were always good unless it was overloaded by attempting to pass through more than about 2.5 ml of liposomal suspension. The liposomes, suspended in pure hypertonic buffer after their passage through the column, were then ready for use.

(b) The 'cochleate cylinders' method

This was basically the method of Papahadjopoulos *et al.* (183).

A total of 10 μ moles of the required pure phosphatidylserine, or mixture of PS with vitamin K or cholesterol, was measured out accurately by volume using Hamilton syringes (or, in the case of cholesterol, by weight) into a large round-bottomed tube with a Quickfit neck. (The sources, purities and concentrations of the stock phospholipid solutions are given in Appendix I : these were all used directly, without further purification. The following molecular weights were used : phosphatidylserine ~ 788 ⁽²⁰⁰⁾; vitamin K₁ = 451; cholesterol = 387.) The volatile solvents were removed under vacuum and the lipids were redissolved in about 2 ml of Analar chloroform. This also was evaporated off and a second 2 ml portion of chloroform was added. The lipid was redissolved in this, aided by brief sonication (a few seconds), if required. The tube was then placed on a vortex mixer (Vortex-Genie, Model K-550-GE, Scientific Industries Inc, Springfield, Mass.) as the chloroform was slowly removed under vacuum. This procedure coated the walls of the tube with a thin film of lipid.

In accordance with the method of Papahadjopoulos *et al.* (183), a 'sodium chloride buffer' was prepared, containing 100 mM sodium chloride, 2 mM histidine (hydrochloride), 2 mM Tris, and 0.1 mM EDTA (disodium salt), adjusted to pH 7.4 by the addition of a very small volume of 1.0 M sodium hydroxide. After filtering through a 0.22 μ m

Millipore filter to remove any dust particles or fibres which might have destabilised a membrane, 2.0 ml of the buffer was added to the dried lipid in the test-tube. On swirling the solution for a few seconds on the vortex mixer, the lipid whitened and fell from the walls of the tube to form a very turbid suspension of large multilamellar vesicles. The tube was then fitted with a suba-seal and flushed with nitrogen for thirty minutes. It was next transferred to an ultrasonic cleaning tank (Model ME 1.5, Mettler Electronics, Germany) containing about three litres of water and a few millilitres of 'Decon'.

(Bangham *et al.*⁽¹⁶⁶⁾ have noted that addition of detergent, by reducing the surface tension of the water, minimises the dissipation of ultrasonic energy by cavitation of the water in the bath itself.) The suspension was then sonicated, in fifteen minute bursts, allowing the tank sufficient time to cool between each, for a total time of up to 2½ hours, or at least until the solution became transparent (an indication that the large multilamellar structures had been broken down into microvesicles). The resultant solution was passed through a 0.22 µm Millipore filter to remove any remaining large particles, and was then allowed to come to thermal equilibrium in a test-tube immersed in a water bath at 37°C, and flushed with nitrogen. After thirty minutes equilibration, a volume of 30 mM calcium chloride solution, equal to half the volume of the liposome suspension, was injected through the suba-seal. (The calcium chloride solution had also been cleaned immediately prior to use by passing through a 0.22 µm Millipore filter.) A dense white flocculate was immediately formed, and slowly settled to the bottom of the test-tube : these particles were the 'cochleate cylinders', formed by the action of calcium ions on the acidic phosphatidylserine⁽¹⁸³⁾. The mixture was left to incubate at 37°C for one hour under nitrogen.

About 5 ml of the solution to be encapsulated (EDTA in sodium chloride buffer, photosolution 3 or photosolution 4, Table 18) was cleaned by Millipore filtration, placed in a small glass bottle fitted with a suba-seal, and flushed with nitrogen for thirty minutes prior to its requirement. A volume of this solution equal to that of the 'cochleate cylinders' suspension was then added rapidly via a syringe needle to the suspension. The flocculate quickly cleared as excess EDTA in the added solution complexed with the calcium ions. After a further thirty minutes incubation at 37°C, the suspension, still under nitrogen, was removed from the bath and left to cool slowly to room temperature in the dark. The resulting suspension, containing the large unilamellar liposomes, was generally quite transparent.

The liposomes had then to be separated from external solution, and in this case centrifugation was found to give satisfactory results. Since centrifugation has an advantage over gel filtration in allowing more concentrated liposome suspensions to be prepared, this technique was adopted for the separation of all liposomes formed by the 'cochleate cylinders' method. Again it was important to ensure that the solutions in which the separated liposomes were to be re-suspended, were slightly hypertonic. The osmolarities of the various solutions encapsulated were calculated from the concentrations and volumes of the solutions added at each step in the preparation of the liposomes. The osmolarity of the original sodium chloride buffer was 0.2063 osM (Table 18); the added calcium chloride was 0.090 osM. Since these were mixed in a 2:1 ratio by volume, the osmolarity of the resulting solution was $\frac{2}{3} \times 0.2063 + \frac{1}{3} \times 0.090 = 0.1675$ osM. On adding an equal volume of photosolution 3 (0.4855 osM, Table 18), the osmolarity of the solution finally encapsulated was the average of the two, that is, 0.3265 osM. Similarly, photosolution 4 gave a final internal osmolarity of 0.3348 osM, and EDTA solution (0.503 osM, Table 18)

gave an internal osmolarity of 0.3353 osM. A 'hypertonic sodium chloride buffer' was therefore prepared, containing 160 mM NaCl, 6 mM Tris and 6 mM histidine hydrochloride, adjusted to pH 7.4 by addition of a very small amount of 1.0 M sodium hydroxide. This buffer had an osmolarity of 0.338 osM (Table 18) and was therefore suitable for use in any of the above cases.

The liposomal suspension, after cooling, (about 6 ml in volume) was divided equally between two glass centrifuge tubes which were placed in a bench-top centrifuge and spun at 4000 rpm for thirty minutes. By this time the liposomes in each tube had sedimented out to give a compact pellet (volume 0.2 - 0.25 ml) and a clear supernatant solution. When photosolution was used in the preparation, both pellet and supernatant were strongly yellow. Using a Pasteur pipette, as much of the supernatant as possible was removed without disturbing the pellet. 5* ml of the hypertonic sodium chloride buffer was then added and the liposomes dispersed into this by holding the tube on a vortex mixer for a few seconds. After another fifteen minutes centrifugation the supernatant (pale yellow, if photosolution 4 was used) was again carefully removed and replaced by another 5* ml of fresh buffer. Finally, after twenty minutes centrifugation, the supernatant (now colourless) was removed, and the liposomes (yellow) were re-suspended in 3.0* ml of the hypertonic sodium chloride buffer. The contents of the two tubes were then mixed to give 6.5* ml of a homogeneous liposome suspension. (*The volumes of buffer used in washing and in final suspension of the liposomes were scaled down if the original volume of the liposome suspension was less than 6 ml. This ensured that the final suspensions obtained were of equivalent compositions.) The efficiency of the separation could be checked by spectrophotometric analysis of the final supernatant. The absence of light scattering

and of peaks in the visible absorption spectrum were taken to indicate that a satisfactory separation of the liposomes from external photo-system had been achieved.

5-2.4 Standardisation of liposome concentrations

When the results of a series of experiments were to be compared quantitatively, it was necessary to ensure that the liposome concentration remained constant throughout the series. This was done in the following way : The first liposomal suspension of the series was diluted slightly with the same hypertonic buffer as that in which it was prepared, and its visible spectrum was recorded in the range 750-400 nm, against the pure buffer as reference. A base line (buffer against buffer) was also recorded. The liposomal suspensions always showed high absorbances, increasing towards lower wavelengths, due to the light scattering effects of the dispersed particles. Successive liposomal suspensions were then similarly diluted with the appropriate buffer until their absorption spectra became exactly superimposable upon the first, at which stage it was assumed that the suspensions must be of equal concentration. This standardisation procedure could only be applied with confidence in cases where all the liposomal suspensions concerned had been prepared by the same technique. For example, no attempt was made to compare quantitatively results obtained using liposomes prepared by the two distinct methods described above: since it was unlikely that the distribution of vesicle sizes and shapes would be the same by both methods, their light scattering properties were also liable to be different.

5-2.5 Use of liposomes in photochemically initiated redox processes

The two reducible substrates used in these experiments were ferricytochrome c and methylene blue, both of which had already been

found suitable for bulk membrane studies (Chapter 3). The sources of these compounds were as indicated in Appendix I: the cytochrome c was used as supplied, without further purification; the methylene blue was recrystallised from water before use. Stock solutions of these were prepared in hypertonic buffers as indicated in the individual experiments. The substrate concentrations in these solutions were arranged to be twice those required in the final mixtures. After preparation, separation and standardisation, 2.5 ml of the liposomal suspension was transferred by Hamilton syringe to a test-tube and mixed with an equal volume of the stock substrate solution. The resulting homogeneous suspension was divided equally between two matched box-top optical cells ('Spectrosil' ultraviolet/visible cells of 1 cm path length, Thermal Syndicate Ltd., England) which were then fitted with suba-seals and deoxygenated by gently bubbling oxygen-purged nitrogen through the solutions for fifteen minutes. During these procedures the liposomes were carefully protected from light.

The cells were then placed in the sample and reference positions of a Pye Unicam SP800 ultraviolet/visible spectrophotometer, and a difference spectrum was recorded, in the range 750-400 nm. This provided a base line relative to which subsequent measurements could be made. One of the cells was removed and irradiation begun by placing it in a set position in the beam from the halogen lamp, using either unfiltered white light or blue light transmitted through the DCIP filter (Section 5-2.1). When cytochrome c was used as substrate, the 'sample' cell was illuminated, since reduction was monitored via the increase in optical density at 550 nm; when methylene blue was used, the 'reference' cell was illuminated, since in this case reduction was accompanied by a decrease in optical density at 655 nm. After ten seconds in the beam, the cell was returned to the spectrometer and the important part of the spectrum was run (600-500 nm for cytochrome c and 750-600 nm for

methylene blue). This normally took about thirty to forty seconds with the spectrometer set on the fast scan mode. Recording a section of the spectrum rather than only measuring the optical density at a set wavelength allowed a fairly sensitive check to be kept on any sedimentation which occurred. On immediately re-running the spectrum of the illuminated liposomes, no changes were observed. This showed that an equilibrium situation had been established within the time taken to record the first spectrum. Although not routine practice, this check was repeated in various different experiments and after different illumination periods, to ensure that the redox reactions had always attained equilibrium before absorbances were noted. The cell was placed in the light beam for another ten second period before re-running the spectrum, which then corresponded to a total irradiation time of twenty seconds. This procedure was repeated to give spectra corresponding to total irradiation times of thirty seconds, one, five and fifteen minutes. In practice some of these periods were often omitted, especially if the rate of photoreduction was either particularly fast or particularly slow. Finally, an excess of solid sodium dithionite was added to the solution in the cell, to yield the completely reduced substrate, whose spectrum was also recorded.

A third matched optical cell, containing pure hypertonic buffer, was placed in the reference beam of the spectrometer, and the spectrum of the unilluminated liposomal suspension was recorded. The procedures of illumination, recording of spectra, and final dithionite treatment, were then repeated as above for this second sample. The two sets of spectra were analysed to find the total amount of reduction after each illumination period. This was calculated as a percentage of complete reduction using the following formula:

$$\text{Percentage reduction in time } t = \frac{A_t - A_o}{A_d - A_o} \times 100\%$$

where A_t is the absorbance at time t

A_o is the absorbance before the start of illumination

and A_d is the absorbance after the addition of dithionite

Absorbances were measured at 550 nm for cytochrome c and at 655 nm for methylene blue, to an accuracy of ± 0.005 , which corresponded to about $\pm 1.7\%$ at the substrate concentrations used. Where both sets were obtained, the difference and normal spectra gave the same results : this served as a check on the accuracy and reproducibility of the results.

5-3 RESULTS AND DISCUSSION

In the course of establishing a liposomal electron transfer system in this research, two distinct methods of preparation of the liposomes, and two different encapsulated photoreduction systems were employed. Ultimately only one of the possible combinations proved to be successful, but many of the less successful experiments are included in the following account, for two main reasons. The first is to indicate the overall strategy of the work and to underline the crucial importance of control experiments in these studies. The second and equally important reason is to outline the course of development of the experimental techniques which were finally adopted. The work is presented here in chronological sequence, so that the unsuccessful experiments are discussed first, the 'ether vaporization' method described in the following Section being ultimately rejected in favour of the 'cochleate cylinders' method (Section 5-3.2).

5-3.1 Liposomes prepared by the 'ether vaporization' method

All the liposomal systems described in this Section were formed

by the 'ether vaporization' technique and were prepared for reaction as indicated in Section 5-2.3(a), 5-2.4 and 5-2.5. A suspension of liposomes composed of 90% egg lecithin and 10% cardiolipin, and encapsulating photosolution 1 (Table 18) was first prepared in a hypertonic medium containing 0.05 M phosphate buffer and 0.06 M EDTA, of osmolarity 0.43 osM. (In the early experiments EDTA was included in the external solution to sequester any trace metal ion contaminants which might have destabilised the membranes.) A deoxygenated sample of this suspension was illuminated with white light to test for activity of the encapsulated EDTA/PF/MV photosystem. Periodic spectrophotometric examination showed that this did not promote the appearance of the characteristic absorption peaks of the methyl viologen radical, as had been observed on illumination of the free photosolution (Figure 5.4), but instead caused gradual bleaching of the proflavine. From this it seemed that the encapsulated photosystem must be inactive, but in subsequent experiments where a reducible substrate was included it was found to be in fact quite active (see later results), despite the fact that the blue methyl viologen radical could never be detected.

The results obtained when liposomes, composed of 70% egg lecithin, 10% cardiolipin and 20% vitamin K, and encapsulating either photosolution 1 or an EDTA-phosphate buffer mixture, were irradiated with white light in the presence of external ferricytochrome c, are shown in Table 19. (Figure 5.8 shows the spectra, both normal and difference types, from which the results for Expt. 2 were derived : they are included here as typical examples of spectra obtained in the experiments described in this Section.) When the photosystem was present, 98% reduction was obtained in one minute (Expt. 1). However, even in the absence of photosystem, 19% reduction was observed in the same time (Expt. 2). Obviously some other component of the system was

Figure 5.8 (a) and (b) Typical absorption spectra, recorded during the photochemically initiated reduction of external ferricytochrome c in the presence of liposomes composed of 70% egg lecithin, 10% cardiolipin and 20% vitamin K, and encapsulating an aqueous solution 0.06 M in EDTA and 0.05 M in phosphate buffer. The substrate solution was 2.3×10^{-5} M in cytochrome c, 0.06 M in EDTA and 0.05 M in phosphate buffer. The liposomes were prepared by the 'ether vaporization' technique.

- (a) Normal spectra, recorded with an aqueous solution, 0.06 M in EDTA and 0.05 M in phosphate buffer, in the reference cell.
- (b) Difference spectra, recorded with an unilluminated liposomal suspension, otherwise identical to the sample, in the reference cell.

The time course of the photoreduction process was the same in both cases, and the results are given in Table 19 (Expt. 2). The spectrum of fully reduced cytochrome c was generated by adding excess solid dithionite to the sample cell at the end of the experiment.

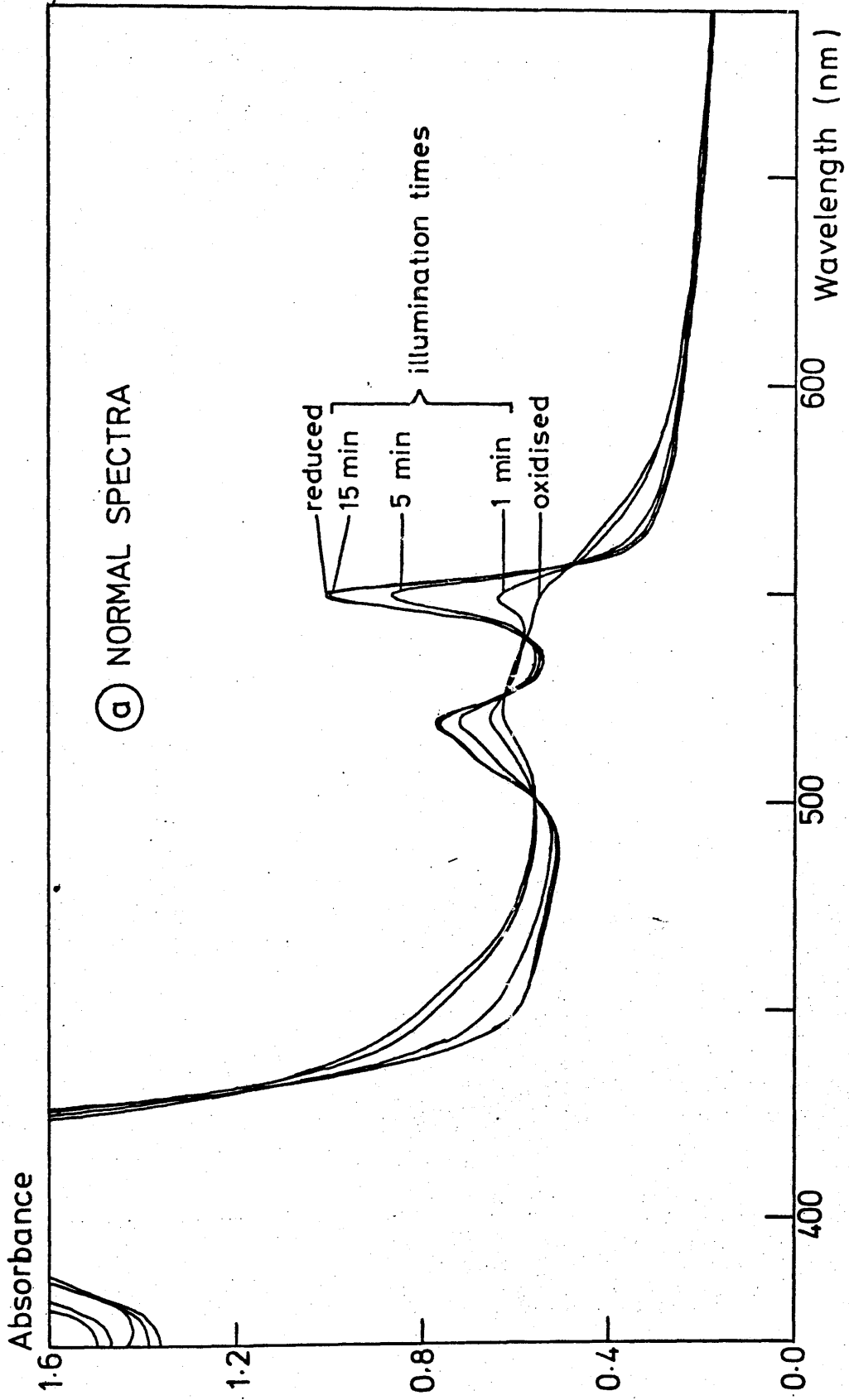


Figure 5.8 (a)

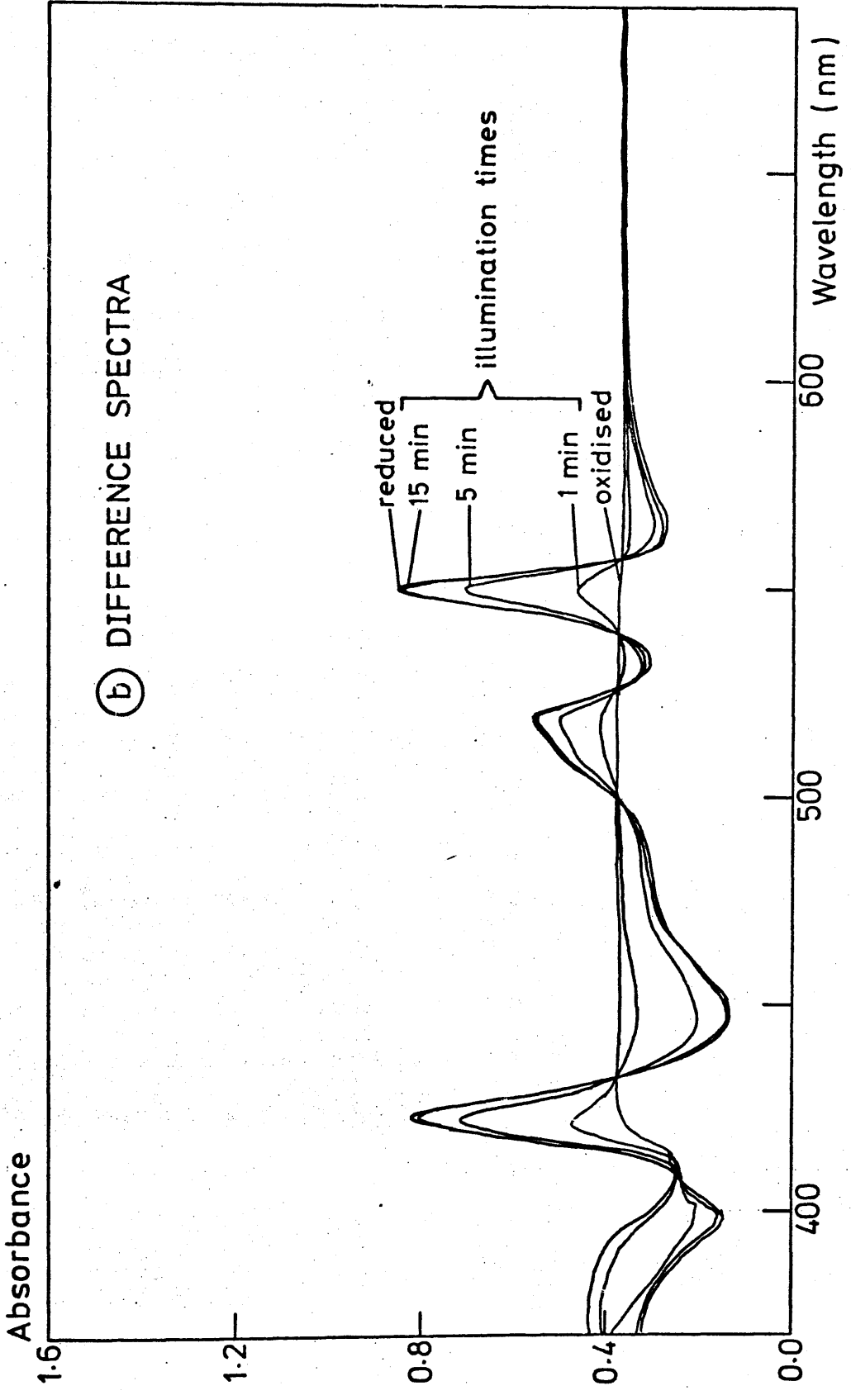


Figure 5.8 (b)

also capable of promoting photoreduction of cytochrome c. A deoxygenated sample of cytochrome c solution in the EDTA - phosphate buffer mixture, with no liposomes present, showed 7% reduction in one minute (Expt. 3) whereas a solution of cytochrome c in hypertonic phosphate buffer alone was only about 0.6% reduced under the same conditions (Expt. 4). This showed that EDTA was effective in accelerating photoreduction of the cytochrome, but it could still not account for the 19% observed in Expt. 2. It seemed therefore that one, at least, of the lipid components was also involved. Due to its proven involvement, EDTA was omitted from the substrate solutions in all subsequent experiments.

With liposomes formed from 90% egg lecithin and 10% cardiolipin, and containing only phosphate buffer, the rate of photoreduction of external cytochrome c was negligible (Expt. 5). This suggested that it was the vitamin K alone which was involved in the photoreduction process, a conclusion supported by the results of Expt. 6, in which almost 100% reaction was observed after five minutes illumination of pure dioleoyl lecithin-vitamin K liposomes. This photoactivity of vitamin K is perhaps not surprising : Beyer⁽⁷⁴⁾ observed that isolated rat liver mitochondria, after irradiation with near ultraviolet light, partially lost their ability to carry out oxidative phosphorylation, and that normal activity could be restored by addition of cytochrome c plus vitamin K₁. From this he deduced that a molecule closely related to vitamin K₁ (possibly coenzyme Q₁₀?) was altered and rendered inactive by exposure to intense light. Also, Leary and Porter⁽⁷⁵⁾ have detected semiquinone radicals among transients formed during the flash photolysis of vitamin K₁ in polar solvents. In view of these observations, an attempt was made to minimise the amount of unnecessary light (that is, those wavelengths falling outside the absorption band of proflavine) transmitted to the liposomes, by use of the blue light

Table 19. Reactivities of liposomal redox systems at 25°C.

The liposomes were prepared by the 'ether vaporization' method and were illuminated with unfiltered white light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in	
				1 min	5 min 15 min
1	photosolution 1	egg lecithin 70% cardiolipin 10% vitamin K 20%	2.3x10 ⁻⁵ M cytochrome c 0.06M EDTA 0.05M phosphate buffer	98	100 100
2	0.06M EDTA 0.05M phosphate buffer	"	"	19	70 98
3	no liposomes present		"	7	20 35
4	no liposomes present		2.3x10 ⁻⁵ M cytochrome c 0.086M phosphate buffer	0.6	2 3
5	0.086M phosphate buffer	egg lecithin 90% cardiolipin 10%	"	0	1 2
6	"	dioleoyl lecithin 80% vitamin K 20%	"	21	99 100
7	photosolution 1	egg lecithin 90% cardiolipin 10%	"	26	70 96

filter described above (Section 5-2.1) in subsequent experiments. Despite this precaution, however, substantial photoreduction of cytochrome c by egg lecithin - vitamin K liposomes in the absence of encapsulated photosystem was still found (Table 20, Expt. 8). The reduction in reaction rate, from 21% (Expt. 6) to 9% in one minute could have been due simply to the lower light intensity in the second case. It is unfortunate that vitamin K, also yellow in colour, absorbs in the same region of the visible spectrum as proflavine. In the absence of any suitable alternative photosensitiser it was therefore necessary to accept this situation, albeit reluctantly, and to take the direct photochemical reaction between oxidised vitamin K and ferricytochrome c into account when analysing subsequent results.

When liposomes composed of egg lecithin and cardiolipin, without vitamin K but encapsulating the EDTA/PF/MV photosolution, were irradiated in the presence of external cytochrome c, substantial reduction of the substrate was observed (Expt. 9). Similar results were obtained when the same liposomes were irradiated with white light (Expt. 7, Table 19) and when dioleoyl lecithin - cardiolipin liposomes were irradiated with blue light (Expt. 10). Individual discrepancies in the rates observed were probably due to differences in light intensity (Expt. 7) and liposome concentration or morphology (Expt. 10). All three experiments (7, 9 and 10) involved phospholipids which were inactive in the direct photochemical reduction of cytochrome c. This led to the conclusion that components of the photosystem must have been present in the external solution in each case. Since all the column separations were judged to be good by the criterion laid down in Section 5-2.3(a), the appearance of photosystem in the external solution must have occurred after removal of the liposomes from the column. Several explanations for this leakage were possible:

Table 20. Reactivities of liposomal redox systems at 25°C.

The liposomes were prepared by the 'ether vaporization' method and were illuminated with blue filtered light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in	
				1 min	5 min 15 min
8	0.086M phosphate buffer	egg lecithin 80% vitamin K 20%	2.3x10 ⁻⁵ M cytochrome c 0.086M phosphate buffer	9	51 90
9	photosolution 1	egg lecithin 90% cardiolipin 10%	"	5	19 53
10	"	dioleoyl lecithin 83% cardiolipin 17%	"	11	51 91

- (a) The liposomes ruptured randomly and spontaneously, continuously releasing some of their contents.
- (b) The liposomes ruptured when they came into contact with cytochrome c.
- (c) The liposomes ruptured when nitrogen was bubbled through the suspension.
- (d) The liposomes ruptured on illumination.

Each of these possibilities was investigated. In view of (d) it was necessary to incorporate a non-photochemical reductant into the liposomes for some of these studies. Ascorbate was chosen because it is unaffected by light and does not react with vitamin K, but does reduce cytochrome c at an easily measurable rate. A preliminary experiment in free solution showed that, with ascorbate present in 66 times excess, the reduction of ferricytochrome c (25 μM solution in 0.086M phosphate buffer) followed first-order kinetics, with a half-life of about 4.4 minutes. Liposomes were prepared from egg lecithin in the presence of a solution containing 0.10 M sodium ascorbate in 0.04 M phosphate buffer, pH 6.83. After removal of the liposomes as a milky white suspension from the gel filtration column, successive fractions were examined for free ascorbate by titration with DCIP (Tillman's reagent). These examinations showed that a satisfactory separation had been achieved. 2.5 ml of the liposome fraction was then mixed with an equal volume of 50 μM cytochrome c solution in hypertonic phosphate buffer (0.086 M), and the resulting suspension was divided between two optical cells, A and B. Cell A was immediately transferred to the spectrophotometer and the optical density at 550 nm was monitored as a function of time, while the solution in cell B was deoxygenated by bubbling nitrogen through it in the usual manner. A slow but steady increase in the optical density of sample A was observed as the cytochrome c was reduced by ascorbate. After fifteen minutes

reaction the two cells were interchanged, and the graph of optical density against time for sample B was found to be a simple continuation of that for sample A. Since none of the species present was particularly oxygen sensitive, this result indicated that the procedure of bubbling nitrogen through liposomal suspensions had no effect on the rate of release of encapsulated solutions. To determine whether illumination affected this rate, sample B was irradiated with blue filtered light for one, five and fifteen minute periods. No effect on the steady course of cytochrome c reduction was observed, indicating that exposure to light did not promote liposome rupture.

These results then allowed possibilities (c) and (d) above to be discounted. Moreover, the zero-order kinetics of cytochrome c reduction in the liposomal system suggested that the release of encapsulated ascorbate was a continuous process, rather than a single event caused by the act of mixing the liposomes with cytochrome c. However, the possibility that cytochrome c aggravated liposome leakage could not be discounted. An attempt was therefore made to minimise any such effect by deoxygenating the liposomal suspensions and cytochrome c solutions separately, and commencing irradiation immediately after mixing. Table 21 shows the results of a series of experiments performed under these conditions with liposomes encapsulating the EDTA/FMN photosystem (photosolution 2, Table 18). Expt. 12 showed substantial reduction of external cytochrome c in the absence of a carrier molecule, which indicated that the liposomes were still leaking despite all the precautions taken. When this experiment was repeated using as nearly as possible the same procedure, the results obtained were significantly different (Expt. 13). Only when the same original liposomal suspension was used could the results be nearly duplicated (for example, in Expts. 13 a and b). An even greater irreproducibility was found when the liposomes contained vitamin K (Expts. 14 and 15). A comparable set

of results was obtained when samples from the same liposomal suspensions were used with methylene blue as substrate (Table 22). For example, the liposomes used in Expt. 16 were from the same batch as those used in Expt. 11; Expts. 17 and 12, 18 and 14, and 19 and 15 were similarly paired. From such random results it was impossible to draw any conclusion other than that the liposomal suspensions must have been very variable in composition. Variations in size or morphology of the vesicles might well have arisen during preparation if these characteristics were critically dependent on such experimental parameters as the rate of injection of lipid, or the relative positions of the needles admitting nitrogen and lipid into the solution (Section 5-2.3(a)).

In view of these problems with 'leakage' of the liposomes and irreproducibility of results, it was decided to abandon this work on liposomes prepared by the 'ether vaporization' method in favour of an alternative preparative technique. However, the work described above was valuable in that it had allowed a number of important conclusions to be drawn. Firstly, the activity of EDTA/PF/MV and EDTA/FMN photo-systems when encapsulated within liposomes had been established, and a light filter had been developed to allow methylene blue to be used as a substrate in these photochemically initiated reactions. In addition, a small photoactivity of vitamin K in the absence of any primary photoreducing system had been noted. The liposomes themselves had been shown to be mechanically stable to illumination and to physical agitation, but had been found to 'leak' significantly in the presence of external methylene blue or cytochrome c solutions. All these observations were utilised in designing rigorous control experiments for the subsequently successful work on liposomes prepared by the 'cochleate cylinders' method.

Table 21. Reactivities of liposomal redox systems at 25°C.

The liposomes were prepared by the 'ether vaporization' method and were illuminated with blue filtered light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in		
				10s	20s	30s
11	0.086M phosphate buffer	egg lecithin 80% cardiolipin 10% vitamin K 10%	2.3x10 ⁻⁵ M cytochrome c 0.086M phosphate buffer	2	4	9
12	photosolution 2	egg lecithin 90% cardiolipin 10%	"	16	34	50
13 a b	"	"	"	16	34	50
				27	50	72
14	"	egg lecithin 80% cardiolipin 10% vitamin K 10%	"	23	47	69
				27	50	72
15	"	"	"	6	12	18
				48	89	100

Table 22. Reactivities of liposomal redox systems at 25°C.

The liposomes were prepared by the 'ether vaporization' method and were illuminated with blue filtered light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in						
				10s	20s	30s	1 min	5 min	15 min	
16	0.086M phosphate buffer	egg lecithin 80% cardiolipin 10% vitamin K 10%	7.3x10 ⁻⁶ M methylene blue 0.086M phosphate buffer			1	2	9		
17	photosolution 2	egg lecithin 90% cardiolipin 10%	"	73	100	100	100	100	100	100
18	"	egg lecithin 80% cardiolipin 10% vitamin K 10%	"	51	78	100	100	100	100	100
19	"	"	"	98	100	100	100	100	100	100

5-3.2 Liposomes prepared by the 'cochleate cylinders' method

The second technique used for the preparation of liposomes was the 'cochleate cylinders' method of Papahadjopoulos *et al.* (183). Although this method could only be applied to liposomes composed of phosphatidyl serine, this was not regarded as a serious restriction because the inner mitochondrial membrane, which these liposomal systems were designed to model, itself contains a high proportion of acidic phospholipid, 21.5% of the total phospholipid being cardiolipin (Table 2). Furthermore, the presence of cardiolipin at the membrane/substrate interface has already been shown to increase the rate of reduction of cytochrome c by dihydrovitamin K (Section 3-3.5), and phosphatidylserine was expected to have a similar effect since it is also able to form a proteolipid complex with cytochrome c (201).

All the liposomal systems described in this Section were prepared for reaction by the procedures detailed in Sections 5-2.3(a), 5-2.4 and 5-2.5. Initially liposomes were prepared from phosphatidylserine (PS) (Koch-Light) in the presence of the EDTA/PF/MV photosystem (photo-solution 3, Table 18). The vesicles were sedimented out of the suspension by centrifugation at 4000 rpm, which yielded a yellow pellet and a clear yellow supernatant. However, even after repeated washings with hypertonic sodium chloride buffer (Table 18) the supernatants removed after consecutive centrifugations showed little decrease in the intensity of the yellow colour, indicating considerable 'leakage' of proflavine from the liposomes. After three such suspension - centrifugation cycles into fresh hypertonic buffer each time, both the liposomes and the supernatant were tested for their ability to photo-reduce methylene blue (8.5×10^{-6} M solution in hypertonic sodium chloride buffer). Neither was found to be very effective, giving respectively 2% and 0% reduction in 1 min, which suggested that the

components of the photosystem had become separated, with the proflavine leaking out of the liposomes while the EDTA remained inside. In an attempt to prevent loss of the photosensitiser from the liposomes, sufficient proflavine was added to the external hypertonic sodium chloride buffer to abolish its concentration gradient across the liposomal membrane. (Since proflavine alone was incapable of photo-reducing methylene blue, this should not have caused any complications in the interpretation of results.) However it was found that when the liposomes were washed with the proflavine-containing buffer, an orange precipitate formed, which gradually settled to the bottom of the tube on standing. This indicated some specific interaction between the cationic dye and the negatively charged PS membrane. Because of these findings an alternative photosystem was sought : the EDTA/FMN system described in Section 5-2.2 was found to be satisfactory, and was used in all subsequent experiments involving phosphatidylserine liposomes. When photosolution 4 (Table 18) was incorporated during formation, there was no detectable leakage of FMN (which has a negatively charged phosphate group) from the liposomes, the concentrations of FMN in successive washings with hypertonic sodium chloride buffer being in accordance with the dilutions made.

(a) Methylene blue as substrate

Blue light irradiation of PS liposomes encapsulating the EDTA/FMN photosystem caused gradual bleaching of the yellow suspension, accompanied by disappearance of the 445 nm absorption peak of oxidised FMN. This bleaching, which was reversible by opening the cell to oxygen, was evidence for the formation of reduced FMN (Figure 5.5) and indicated that the encapsulated solution was photo-active. A series of experiments was performed using liposomes composed of PS alone and of PS with added vitamin K : the results obtained with methylene blue

as substrate are summarised in Table 23. When liposomes composed of 95% PS and 5% vitamin K, and encapsulating the photosystem, were used, external methylene blue was very rapidly reduced (Expt. 20). A second aliquot from the same liposome suspension was centrifuged at 4000 rpm for thirty minutes to allow the external solution to be separated off. This supernatant showed a very much slower rate of reaction when mixed with methylene blue and irradiated under the same conditions (Expt. 21). The fact that reduction still proceeded, suggested that some liposomes or free photosystem remained in the supernatant after centrifugation. The amount of FMN present was below the limit of spectrophotometric detection: its concentration was estimated to be less than 5×10^{-7} M by extrapolating the sequential decrease in the measured concentrations of the preceding series of liposome washings. On independently adding this concentration of FMN plus the corresponding concentration of EDTA (6×10^{-6} M) to a suspension of liposomes which did not encapsulate photosystem, the rate of reduction of methylene blue was very slow (Expt. 22). It was however faster than the rates observed when neither of the photosystem's components were present (Expt. 23), or when EDTA alone was encapsulated (Expt. 24). Although low levels of free FMN and EDTA did cause some photoreduction of methylene blue, their effect was not sufficient to explain the results of Expt. 21. Liposomes remaining in the supernatant after centrifugation must therefore have been responsible for most of the observed reaction in that experiment.

When 100% PS liposomes encapsulating the photosystem were irradiated in the presence of external methylene blue, very rapid reduction of the substrate was obtained, the reaction reaching completion within twenty seconds (Expt. 25, Table 23). Comparing this result with that of Expt. 20, it is evident that the presence or absence of vitamin K in the liposomal membrane had no significant effect on the reaction rate. Two explanations for this were possible:

Table 23. Reactivities of liposomal redox systems at 25°C.

The liposomes were prepared by the 'cochleate cylinders' method and were illuminated with blue filtered light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in					
				10s	20s	30s	1 min	5 min	15 min
20	photosolution 4	PS vitamin K 5%	8.4x10 ⁻⁶ M methylene blue hypertonic NaCl buffer	55	98	100	100	100	100
21	supernatant from liposomal suspension used in Expt. 20	"	"	1	3	5	10	54	100
22	100 mM NaCl buffer	PS vitamin K 5%	8.4x10 ⁻⁶ M methylene blue hypertonic NaCl buffer 5x10 ⁻⁷ M FMN, 6x10 ⁻⁶ M EDTA				3	12	52
23	"	"	8.4x10 ⁻⁶ M methylene blue hypertonic NaCl buffer				1	3	7
24	60 mM EDTA	"	"				1	5	18
25	photosolution 4	PS 100%	"	59	100	100	100	100	100
26	supernatant from liposomal suspension used in Expt. 25, after 1 min pre-illumination	"	"	5	7	9	28	100	100
27	photosolution 4	PS vitamin K 5%	8.4x10 ⁻⁶ M methylene blue hypertonic NaCl buffer (I ~ 0.3 M)			4	14	96	100

- (a) the PS used contained some redox active contaminant which functioned as an electron carrier across the liposomal membrane, or
- (b) the reaction observed took place in free solution, with the liposomes releasing some of the trapped photosystem into the external medium.

Although thin layer chromatography of the stock PS solution used (from Koch-Light, nominally 95% pure) indicated the presence of a number of contaminants, no quinone-type molecule capable of acting as a redox mediator could be detected by this technique or by ultraviolet spectrophotometry. Considering the origin and preparation of the phospholipid, the major contaminant was most likely to be lysophosphatidyl-L-serine, which if anything should have stabilised the liposomal membranes⁽²⁰²⁾. Possibility (a) above was therefore discounted.

The anomalous result of Expt. 25 must then have been due to leakage of the liposomes. Since the centrifugation and resuspension processes did not cause marked release of photosystem, as evidenced by the efficient separations achieved by this method, liposome rupture had to be attributed to one, or a combination, of the following factors:

- (i) nitrogen bubbling through the suspension during deoxygenation,
- (ii) illumination of the suspension, or
- (iii) interaction with the methylene blue substrate.

Expt. 25 was repeated, but deoxygenation was achieved by flushing nitrogen over the liposome suspension rather than through it. The rate of photoreduction of external methylene blue was not affected by this change, with 100% reaction still occurring in only twenty seconds illumination. Factor (i) above could therefore be discounted. Another sample from the same liposome suspension in hypertonic sodium chloride buffer was irradiated with blue light for one minute prior to centrifugation. The separated supernatant was then mixed with

methylene blue, and the resulting solution deoxygenated and illuminated. Reaction this time was much slower, with only 7% reduction in twenty seconds (Expt. 26). Assuming that any photosystem released during the pre-illumination period was not immediately inactivated by destruction of a large proportion of the EDTA (which seems unlikely in the absence of a reducible substrate in addition to a very low concentration of FMN), this slow reaction rate was taken to indicate that the concentration of free photosystem was correspondingly low. Hence it was concluded that illumination itself did not cause leakage of the liposomes. This left only factor (iii) to explain the experimental observations, namely, that liposome rupture was stimulated by the presence of external methylene blue. Possible reasons for this are discussed more fully below, in connection with cytochrome c as substrate, but there was obviously some specific interaction between the cationic dye and the acidic phospholipid. If this interaction was electrostatic in origin, it should have been inhibited by increasing the ionic strength of the medium. In Expt. 27 (Table 23) the ionic strength of the external methylene blue solution was increased from 0.16 M to 0.30 M by the addition of sodium chloride. This had the effect of reducing the reaction rate enormously, from 100% in thirty seconds (in Expt. 20) to only 4% in the same illumination time. Although it seems likely that this change reflected a decrease in liposome leakage, it is impossible to draw any firm conclusions from the results, since increasing the ionic strength should also have affected the intrinsic reaction rates (Section 3-1.1).

(b) Cytochrome c as substrate

The second aqueous substrate investigated in these PS liposomal systems was cytochrome c. In an initial experiment, in which a suspension of PS liposomes was mixed with a solution of cytochrome c

in the hypertonic sodium chloride buffer (ionic strength 0.16M, Table 18), an orange precipitate quickly formed and settled to the bottom of the tube. This indicated the formation of a PS - cytochrome c complex similar to the cardiolipin - cytochrome c complexes described earlier (Section 3-3.4). On increasing the ionic strength of the medium to 0.30 M by addition of sodium chloride, this precipitation was prevented, so all subsequent experiments involving cytochrome c were performed at this higher ionic strength. No attempt was made to raise the ionic strength further, since this should have led to complete inhibition of the electron transfer reaction between the cytochrome and reduced vitamin K (Section 3-3.5).

The results of a series of experiments in which ferricytochrome c was used as substrate are shown in Table 24. When the liposomes were composed of PS and vitamin K, and encapsulated the EDTA/FMN photosystem, the photoreduction of external cytochrome c was quite rapid (Expt. 28). However, the reaction rate was also fast when the quinone was omitted from the liposomal membrane (Expt. 29). This behaviour, which paralleled that described above for PS liposomes in the presence of a methylene blue substrate, was again indicative of liposome leakage. On centrifugation of the liposomal suspension in ferrocytochrome c solution after reaction (in Expts. 28 and 29), a yellow pellet separated out beneath a transparent pink supernatant whose visible absorption spectrum was indistinguishable from that of dithionite - reduced cytochrome c. Therefore, although some photosystem was undoubtedly present in the external solution, the amount released must have been very small in comparison to the total encapsulated. In the case of methylene blue it was deduced that liposome rupture was stimulated by the presence of the cationic dye on one side of the membrane : it is not unreasonable to suppose that cytochrome c might have had a similar effect. Indeed there is evidence in the literature

which supports this conclusion. Addition of cytochrome c to a suspension of multilamellar PS liposomes produces smaller vesicles, about 0.5 μm in diameter, having only two or three lamellae with a repeat spacing of about 125 \AA ^(169,172). The formation of these structures must necessarily have involved rupture of the original membranes to admit the protein into the interlamellar spaces. Furthermore, the addition of cytochrome c to a suspension of sonicated PS liposomes greatly increases the rate of efflux of encapsulated $^{22}\text{Na}^+$ from the vesicles⁽²⁰¹⁾: the anomalously high 'diffusion rates' observed could only be satisfactorily accounted for in terms of leakage channels through the membranes. It has been proposed⁽²⁰¹⁾ that the initial electrostatic interaction between the basic protein and the acidic phospholipid is followed by penetration of a hydrophobic part of the protein molecule into the lipid bilayer, which in turn renders the membrane more 'leaky'. This suggestion is in keeping with the results of other investigators⁽¹²²⁻¹²⁵⁾ working on the monolayer systems discussed earlier (Section 3-3.4), but this mechanism does not seem likely in the present case, where any tendency towards complex formation should have been inhibited by the high ionic strength of the medium. Another possibility is that the negative charge on the outer surface of the liposomal membrane was partially neutralised in the presence of cationic species such as cytochrome c or methylene blue. This would have led to the generation of a transmembrane potential gradient which if sufficiently large might have destabilised the membrane.

In any case it was necessary to take steps to avoid or minimise liposome leakage in these experiments. Papahadjopoulos *et al.*⁽²⁰¹⁾ found that the rate of release of $^{22}\text{Na}^+$ from PS liposomes could be substantially reduced (by a factor of 38 times) by incorporation of 50% cholesterol into the membrane. An attempt was therefore made to

Table 24. Reactivities of liposomal redox systems at 25°C.
 The liposomes were prepared by the 'choleate cylinders' method and were illuminated with blue filtered light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in					
				10s	20s	30s	1 min	5 min	15 min
28	photosolution 4	PS vitamin K 5%	1.7x10 ⁻⁵ M cytochrome c hypertonic NaCl buffer (I ~ 0.3M)	8	18	27	41	98	100
29	"	PS 100%	"	6	10	14	22	75	100
30	"	PS 75% cholesterol 25%	"				4	15	53
31	supernatant from liposomal suspension used in Expt. 30, centrifuged after mixing with cytochrome c						12	34	64
32	photosolution 4	PS 100%	"		5	9	32	61	
33	"	PS 95% vitamin K 5%	"		5	10	37	75	

incorporate cholesterol at a similar level into the liposomes used here. Unfortunately the preparation had to be abandoned following the sonication stage when, even after two and a half hours of sonication, the suspension did not lose its turbidity and failed to pass through a 0.22 μm Millipore filter. The preparation was repeated with a 75:25 mole per cent mixture of PS and cholesterol, which did allow large unilamellar liposomes to be produced by the normal 'cochleate cylinders' route. When a sample of these liposomes, encapsulating the EDTA/FMN photosystem, was irradiated with blue light in the presence of external ferricytochrome c (Expt. 30, Table 24), a much slower rate of reduction was observed than had been found in Expts. 28 and 29. The remainder of the liposomal suspension was mixed with ferricytochrome c solution and was centrifuged at 4000 rpm for twenty minutes. The clear orange-red supernatant was then removed, deoxygenated, and irradiated. The rate of photoreduction of cytochrome c (Expt. 31) was then found to be faster than that for the whole liposomal suspension. This showed that, although much decreased by the inclusion of cholesterol, liposome rupture still occurred, and was stimulated by addition of the protein to the external medium. A major disadvantage in the use of cholesterol in these experiments arose from its property of reducing the mobility of the phospholipid tails. This would have increased the viscosity of the internal hydrocarbon phase and hence would have reduced the rate of 'flip-flop' of vitamin K molecules from one side of the bilayer to the other, thereby reducing the rate of electron transfer.

Since the interaction of liposomes with external cytochrome c caused release of photosystem, an attempt was made to minimise the relative importance of this effect by delaying the mixing until just before irradiation, the liposome suspension and cytochrome c solution

being deoxygenated independently (Expts. 32 and 33). Although the resulting reaction rates were much reduced in comparison to Expts. 28 and 29, the problem of leakage was still not satisfactorily overcome, there being no substantial difference between the rates with and without a carrier molecule present in the membrane. The efflux of photosystem from the liposomes in the presence of external cytochrome c could have been a continuous process, with either a steady rate of leakage from all the vesicles, or rupture of a set proportion of the vesicles in equal intervals of time. Alternatively it could have been a single event induced by the initial mixing of the liposomes with the protein, and affecting only a given proportion of the vesicles (presumably those which were for some reason less stable or whose membranes had different permeability properties from their neighbours). If the latter were the case, then it should have been possible to remove external photosystem by washing the liposomes with cytochrome c solution, without causing the release of more photosystem with each washing. This possibility was tested, and the results are shown in Table 25, Expts. 35 and 36.

To PS - vitamin K liposomes prepared in the EDTA/FMN photosolution was added an equal volume of cytochrome c solution (3.4×10^{-5} M, in hypertonic sodium chloride buffer, ionic strength 0.30 M). The liposomes were then left, at room temperature and under nitrogen, for four hours, bathed in this medium which contained both cytochrome c and the original photosolution. Thus it was hoped to convert the liposomes to a more uniformly stable condition without any net loss of encapsulated photosystem due to leakage during the initial protein addition. The suspension was then centrifuged at 4000 rpm for thirty minutes, and the supernatant replaced by isotonic 1.7×10^{-5} M cytochrome c solution, in which the liposomes were re-suspended. Further equilibrations were also carried out using this

cytochrome c solution, and the fourth and fifth supernatant solutions remaining after removal of the liposomes by centrifugation were tested for photoactivity (Expts. 35(a) and (b)). The same reaction rates were observed in each case, indicating that the concentration of free photosystem was the same in both. This could only be the case if leakage was a continuous process rather than a single event. On resuspending the liposomes in the remainder of the fifth supernatant, the rate of reduction of ferricytochrome c was not much enhanced, so clearly this method of pre-equilibration with the fully concentrated substrate gave no improvement over those described above.

In Expt. 36, liposomes, prepared in the normal way from PS and vitamin K, and encapsulating the photosystem, were first washed with hypertonic sodium chloride buffer (ionic strength 0.16 M). After three such washings, 10% by volume of a solution of cytochrome c, 1.7×10^{-5} M, in hypertonic sodium chloride buffer (ionic strength 0.30 M), was mixed with the original buffer, and the liposomes were resuspended in this mixed solution. Centrifugation was followed by resuspension in a second mixed medium, this time containing 20% of the cytochrome c solution. The sixth equilibration was carried out in the undiluted cytochrome c solution, and after centrifugation the supernatant was removed and irradiated with blue light under nitrogen as usual. The liposomes were resuspended in two further equal aliquots of cytochrome c solution, and the supernatants after centrifugation were similarly examined. The results are shown in Table 25, Expts. 36 (a), (b) and (c) : the seventh supernatant contained less photosystem than the sixth, as expected, but the amount present increased on going from the seventh to the eighth. This was surprising, but may have been due to some factor, such as air oxidation of the phospholipid, which resulted in destabilisation of the ageing liposomes. Nevertheless, on

Table 25. Reactivities of liposomal redox systems at 25°C.
 The liposomes were prepared by the 'cochleate cylinders' method and were illuminated with blue filtered light.

Expt. No.	Internal solution	Membrane composition	External solution	Percentage reduction in						
				10s	20s	30s	1 min	5 min	15 min	
34			1.7x10 ⁻⁵ M cytochrome c hypertonic NaCl buffer (I ~ 0.3 M)			1	3	5		
35 a	supernatant from 4th equilibration of liposomes (described in Expt. 35c), in 1.7 x 10 ⁻⁵ M cytochrome c solution			7	14	19	32	85	100	
b	supernatant from 5th equilibration of liposomes (described in Expt. 35c), in 1.7 x 10 ⁻⁵ M cytochrome c solution.			8	12	17	31	86	100	
c	photosolution 4	PS 90% vitamin K 10%	1.7x10 ⁻⁵ M cytochrome c hypertonic NaCl buffer (I ~ 0.3M) (supernatant no. 5)	10	16	23	42	98	100	
36 a	supernatant from 6th equilibration of liposomes (described in Expt. 36d), in 1.7 x 10 ⁻⁵ M cytochrome c solution			4	7	10	16	51	91	
b	supernatant from 7th equilibration of liposomes (described in Expt. 36d), in 1.7 x 10 ⁻⁵ M cytochrome c solution						3	9	38	
c	supernatant from 8th equilibration of liposomes (described in Expt. 36d), in 1.7 x 10 ⁻⁵ M cytochrome c solution.			2			11	41	81	
d	photosolution 4	PS 90% vitamin K 10%	1.7x10 ⁻⁵ M cytochrome c hypertonic NaCl buffer (I ~ 0.3M) (supernatant no. 8)	3	5	10	22	86	100	
37 a	supernatant from 6th equilibration of liposomes (described in Expt. 37d), in 1.7 x 10 ⁻⁵ M cytochrome c solution					2	4	13	28	
b	supernatant from 7th equilibration of liposomes (described in Expt. 37d), in 1.7 x 10 ⁻⁵ M cytochrome c solution						6	24	67	
c	supernatant from 8th equilibration of liposomes (described in Expt. 37d), in 1.7 x 10 ⁻⁵ M cytochrome c solution.						7	26	50	
d	photosolution 4	PS 90% vitamin K 10%	1.7x10 ⁻⁵ M cytochrome c hypertonic NaCl buffer (I ~ 0.3M) (supernatant no. 8)	9	19	27	45	100	100	
38	60 mM EDTA	PS 90% vitamin K 10%	1.7x10 ⁻⁵ M cytochrome c hypertonic NaCl buffer (I ~ 0.3 M)				1	3	7	

resuspending the liposomes in the remainder of the eighth supernatant, the rate of photoreduction of ferricytochrome c was doubled, from 11% to 22% in one minute. When the experiment was repeated, reducing handling times to a minimum and filtering the supernatants to remove any residual liposomes, a clearer result was obtained (Expts. 37 (a) - (d)), with 7% reduction in one minute for the final supernatant being increased to 45% when the liposomes were resuspended in the medium. In a separate experiment it was found that, when 0.0001 ml of photo-solution 4 (Table 18) was added to a 2.00 ml sample of the cytochrome c solution, the rate of photoreduction was very similar to those observed for the supernatants in Expt. 37, with 6% reduction in one minute and 21% in five minutes. This confirmed the suspicion that the actual amount of photosystem released by the liposomes was extremely small, amounting to less than 1% of the total encapsulated volume, and giving a free FMN concentration of only 10^{-6} M.

One final control experiment was necessary before any conclusions could be drawn from these results. It was shown earlier that vitamin K itself caused some photoreduction of cytochrome c (Section 5-3.1): the extent of this side reaction had therefore to be assessed in the present case. Liposomes composed of PS and vitamin K in the same proportions as used in Expts. 36 and 37, but encapsulating only EDTA in sodium chloride buffer, were irradiated in the presence of external cytochrome c (Expt. 38) : the concentrations of liposomes and protein in the final suspension were identical to those in Expts. 36 and 37. Under these conditions the cytochrome c was only 1% reduced in a one minute illumination period. The results of this experiment, along with those of Expt. 37, are presented graphically in Figure 5.9, from which it can be seen that the differences between the three reaction rates were very marked. Since it has already been shown that the liposomes did not

Figure 5.9 Percentage reduction of ferricytochrome c plotted against illumination time, for

- (a) external cytochrome c in the presence of liposomes composed of 90% PS and 10% vitamin K, and encapsulating an EDTA/FMN photosolution (Expt. 37d),
- (b) the separated supernatant from the liposomal suspension later used in (a), after removal of the liposomes by centrifugation (Expt. 37c),
- (c) external cytochrome c in the presence of liposomes composed of 90% PS and 10% vitamin K, but encapsulating only EDTA solution (Expt. 38).

The concentration of cytochrome c in each case was 1.7×10^{-5} M.

Equal concentrations of liposomes were used in (a) and (c).

% Reduction

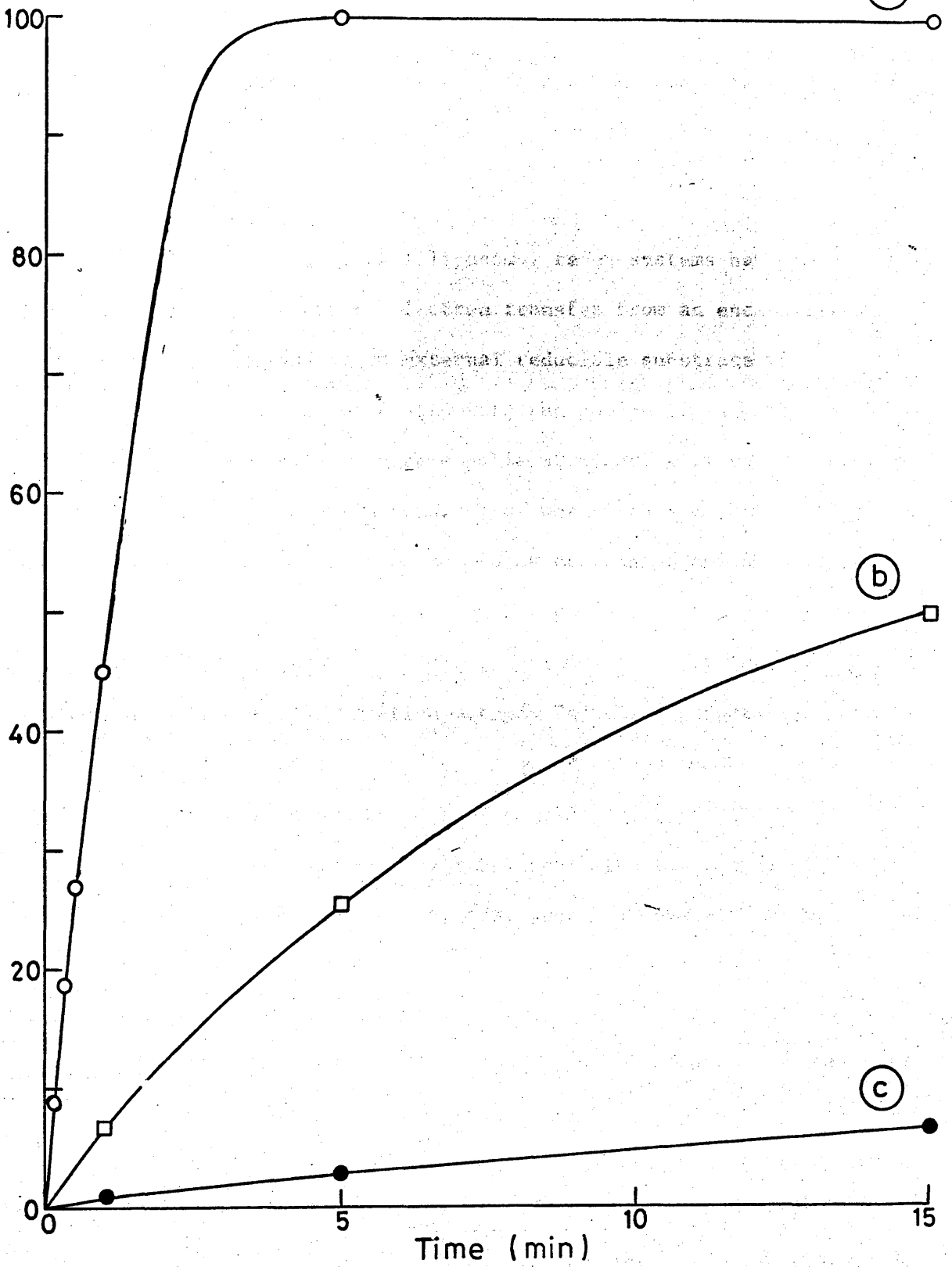


Figure 5.9

leak appreciably during the processes of centrifugation and re-suspension, deoxygenation, and irradiation, the result of Expt. 37 may be taken as conclusive proof that carrier mediated electron transfer occurred through these liposomal membranes.

5-3.3 Concluding remarks

In this Chapter, several liposomal redox systems have been discussed. In all of these, electron transfer from an encapsulated photochemical reductant to an external reducible substrate was mediated by vitamin K incorporated into the phospholipid membrane. Two different photosystems, both capable of reducing vitamin K, were investigated, but due to the tendency of one of the photosensitisers — proflavine — to escape through membranes containing acidic phospholipids, only the EDTA/FMN photosystem was found to be generally acceptable. A requirement for large, unilamellar liposomes was demonstrated, and two alternative methods for their preparation were investigated. The first — the 'ether vaporization' method — was found to be unsatisfactory because of non-uniformity of the resulting suspensions, leading to marked variability in the reaction rates measured in duplicate experiments. The second — the 'choleate cylinders' method — appeared to yield satisfactory suspensions, but was restricted in that only phosphatidylserine liposomes could be prepared. This led to complications when using the cationic substrates methylene blue and cytochrome c, which tended to associate with the acidic phospholipid, causing leakage of the encapsulated photosolution. Various attempts were made to minimise this effect, but none proved entirely successful. However, the experimental procedure finally adopted gave sufficiently low reaction rates in control experiments to allow the membrane mediated electron transfer component to be clearly distinguished from unavoidable side reactions. Thus it was possible

to conclude that vitamin K could indeed act as an efficient carrier of electrons (and protons) across a bilayer membrane separating aqueous FMN and cytochrome c solutions.

The liposomal system developed here may be considered as a simple but viable model for the biological electron transfer membranes discussed in Chapter one (Figure 1.9). Although coenzyme Q_{10} was not substituted for vitamin K in the present experiments, it seems reasonable to suppose that it should act similarly, although much less efficiently (Section 3-3.1) as a shuttle for electrons. Incorporation of the cytochrome b-c₁ enzyme complex into the liposomes, for which methods now exist^(193,195), might allow the present model to be successfully extended to coenzyme Q. This would be particularly interesting in that it would constitute the 'synthesis' of a sizeable section of the mitochondrial respiratory chain (Figure 1.9 (a)).

In a recent paper⁽²⁰³⁾, which appeared shortly after the completion of this work, Hauska has described several liposomal systems in which he has used plastoquinones and ubiquinones as translocators of electrons and protons through phospholipid membranes. The reduction of encapsulated ferricyanide by external dithionite was followed spectrophotometrically, and it was found that the reaction actually proceeded faster when plastoquinone-9 or coenzyme Q_{10} was incorporated into the membrane than when it contained the much smaller (and presumably more mobile) carriers trimethylbenzoquinone or coenzyme Q_1 . It should be noted that Hauska's liposomal systems were inverted relative to those developed in the present research, so that the reducible substrate was trapped within the (sonicated) liposomes and therefore could not be readily isolated in a pure form at the end of the reaction. Furthermore, neither the oxidant nor the reductant used by Hauska conformed to the criteria of ideality stipulated here :

the oxidation of reduced coenzyme Q by ferricyanide is not completely reversible (Table 7), and dithionite (or a reaction product thereof) is membrane permeable⁽²⁰³⁾. Nevertheless, these results are interesting in that they confirm the finding expressed above; namely, that long-chain quinones such as vitamin K₁ and coenzyme Q₁₀ are efficient translocators of electrons across membranes of biological thickness.

Appendix I

SOURCES OF MATERIALS

The forms, purities and sources of all the chemicals of particular importance in this work are listed here. All other reagents were of the highest purity readily available, normally 'Analar' grade.

The following abbreviations are used to indicate the suppliers :

BDH	BDH Chemicals Ltd., Poole, Dorset, England.
Coleman	Coleman Instruments Inc., U.S.A.
H & W	Hopkin and Williams Ltd., Chadwell Heath, Essex, England.
JM	Johnson Matthey & Co. Ltd., London, England.
KL	Koch-Light Laboratories Ltd., Colnbrook, England.
O	Organon Laboratories Ltd., Newhouse, Scotland.
Pharmacia	Pharmacia Fine Chemicals, Uppsala, Sweden.
S	Sigma London Chemical Company Ltd., Surrey, England.

<u>Name</u>	<u>Description</u>	<u>Source</u>
Ascorbic acid	grade > 99%	H & W
Benzyl viologen	-	BDH
Calcium chloride	dihydrate, 'Analar'	H & W
Cardiolipin	from bovine heart, sodium salt, ethanol solution, 9.3 mg ml ⁻¹	S
Chloroform	'Analar'	BDH
Cholesterol	grade 99+%, standard for chromatography	S
Coenzyme Q ₁₀	from bovine heart	S
Cuprin	-	Coleman
Cytochrome c	from horse heart, Type III, grade 95-100%	S
Cytochrome c	from <i>N. crassa</i>	A kind gift from Dr. I.D.A. Swan
n-Decane	grade 99+%	S
2,6-Dichlorophenol indophenol (DCIP)	-	BDH
Diethyl ether	'Analar'	BDH
Dioleoyl phosphatidyl choline	grade ~ 98%, synthetic, chloroform solution, 20 mg ml ⁻¹	S S
Disodium hydrogen orthophosphate	dodecahydrate, 'Analar' grade 99%	H & W
Egg lecithin	from egg yolk, Type III-E, hexane solution, 100 mg ml ⁻¹	S
Ethylenediamine tetraacetic acid (EDTA)	disodium salt, 'Analar'	H & W
Ferric chloride	hexahydrate, grade 98%	H & W

<u>Name</u>	<u>Description</u>	<u>Source</u>
Flavin adenine di-nucleotide (FAD)	disodium salt, grade III, 94-99%	S
Flavin mononucleotide (FMN)	sodium salt, grade 95-97%	S
n-Hexane	'Spectrosol' grade	H & W
Histidine	hydrochloride, 'Analar'	H & W
Indophenol	-	H & W
Lysolecithin (LL)	from soybean, Type IV, ~ 98%	S
Methanol	'Analar'	BDH
Methylene blue	-	BDH
Methyl viologen (MV)	-	BDH
NADH	disodium salt, grade 85%	BDH
NADPH	sodium salt, grade 78%	BDH
o-Phenanthroline	hydrate	S
Phosphatidyl choline (PC)	from egg yolk, Type III-E, hexane solution, 100 mg ml ⁻¹	S
Phosphatidyl ethanolamine (PE)	dipalmitoyl, synthetic grade I, ~ 98%	S
Phosphatidyl inositol (PI)	from soybean, ammonium salt, grade I, ~ 98%	S
Phosphatidyl serine (PS)	from bovine brain, grade 95%, chloroform: methanol (4:1) solution, 40 mg ml ⁻¹	KL
Phosphatidyl serine (PS)	from bovine brain, grade ~ 98%, chloroform:methanol (95:5) solution, 2 mg ml ⁻¹	S

<u>Name</u>	<u>Description</u>	<u>Source</u>
Potassium chloride	'Analar', 99.8%	H & W
Potassium dihydrogen orthophosphate	grade 99.2%	H & W
Potassium ferri- cyanide	'Analar', 99%	H & W
Potassium permanganate	'Analar', 99.5%	H & W
Proflavine (PF)	hemi-sulphate	S
Sephadex G-50-80	bead size 20-80 μ	Pharmacia
Silver oxide	grade 'Specpure'	JM
Sodium borohydride	-	O
Sodium chloride	'Analar', 99.9%	H & W
Sodium dithionite	-	H & W
Sodium sulphate	'Analar', 99.5%	H & W
Thionine	-	BDH
Tris (hydroxymethyl) aminomethane	crystalline, 99-99.5%	S
Vitamin K ₁	-	S

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