ELECTRON TRANSFER ACROSS LIQUID MEMBRANES

by

STEPHEN ANDERSON

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For Diane

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This thesis is dedicated to my wife, whose help and encouragement aided most in the completion of this research.

DECLARATION

Much of the work described in Chapter 1 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 1-3.7) and Dr. L.D. Barron assisted during the recording of Resonance Raman spectra (section 4-2.2).

SUMMARY

The creation of chemically well-defined electron-transfer membranes was the overall objective of the research undertaken. Each membrane would form an intermediate phase between a primary aqueous reductant and a reducible aqueous substrate and would mediate electron transfer between the aqueous phases without allowing them to come into physical contact. Such membranes could be of a solid or liquid nature. Preliminary research, however, showed that liquid membranes, composed of an inert solvent in which redox active carrier molecules were dissolved, had the greatest potential for development. The existence of carrier transport in biological membranes is now well established and, indeed, the final membrane systems, evolved in these studies, employed the naturally occurring quinone vitamin K_1 as the electron (and proton) carrier. A hydrocarbon, normally hexane, was used as the inert membrane solvent.

A series of investigative studies were carried out to determine which aqueous reductants and oxidants could be used in these redox systems. Control experiments were conducted to ensure that those compounds, found to react with vitamin K, were totally membrane insoluble. In addition, similar control experiments were undertaken to

verify that the carrier was completely immiscible in water. Only if both the carrier and the aqueous reagents were immiscible in the adjacent phase could reaction be considered as occurring solely by carrier mediation. Once suitable redox membrane systems were established, the kinetics of electron transfer could then be investigated. The kinetics of reduction of a number of common oxidation-reduction indicators were first studied. Subsequently the heme protein, cytochrome c, was used as the substrate.

The electron-transfer membranes developed here operate by a carrier mechanism which is conceptually similar to those postulated for biological processes. In consequence the experiments conducted, using the membrane systems developed during the course of this research, can be regarded as modelling these natural processes. Certain biological reductants and reducible substrates, that are components of the mitochondrial respiratory chain, were incorporated into the synthetic redox membrane systems and the degree to which these membranes acted as viable models for biological systems has been assessed. The validity of these systems as biological models would be further enhanced if the membranes could be reduced to the thickness of biological membranes. Although redox reactions across a membrane of bilayer dimensions has not been attempted, electron transfer across a thin 'lens' membrane has been demonstrated.

Even though the redox systems developed used a naturally occurring carrier, a number of non-biological uses for such systems has been demonstrated. These systems provide new sensitive methods for the preparation and characterisation of solutions of reduced or oxygen-sensitive compounds, without the added complication of chemical contamination (other than protons required to ensure electroneutrality).

ABBREVIATIONS

A	Area Thickness in Angstroms
a	Isotropic hyperfine coupling constant (gauss)
AC	Alternating current
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BLM	Bilayer lipid membranes
C	Capacitance
c	Concentration (mole litre^{-1})
$\bar{c}$	Speed of light (cms^{-1})
CL	Cardiolipin
cp	Viscosity (centipoise)
D	Diffusion coefficient (cm^{2}s^{-1})
d	Thickness of the hydrocarbon region of BLM
DC	Direct current
DCIP	2,6-Dichlorophenol indophenol
DMPC	Dimyristoyl phosphatidyl choline
DPPC	Dipalmitoyl phosphatidyl choline
E^o	Standard reduction potential
E_m	Membrane potential
E_{mx}	Midpoint potential at pH X
$\bar{e}$	Dielectric constant
emf	Electro-motive force
EPR	Electron paramagnetic resonance

ABBREVIATIONS (Contd.)

F	Faraday (coulombs equivalent^{-1})
f	Activity coefficient
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FMN	Flavin mononucleotide
$FMNH_2$	Reduced flavin mononucleotide
F_p	Flavoprotein
g	Electronic g factor
H	Magnetic field (gauss)
h	Planck's constant (erg.s)
I	Intensity of light
K	Vitamin K_1 Equilibrium constant
k	Rate constant
k_o	Zero-order rate constant (mole litre^{-1}time^{-1})
k_1	First-order rate constant (time^{-1})
KH_2	Reduced vitamin K_1
ℓ	Path length (cm)
LL	Lysolecithin
ln	Natural logarithm (base e)
log	Logarithm (base 10)
M	Molarity
m	metre
MB	Methylene blue

ABBREVIATIONS (Contd.)

M_I	Nuclear spin quantum number in Z-direction
min.	minutes
M.phlei	Mycobacterium phlei
MV	Methyl viologen
n	Number of moles Number of electrons transferred
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NHI	Nonheme iron
NMR	Nuclear magnetic resonance
OD	Optical density
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PI	Phosphatidyl inositol
ppm	Parts per million
R	Gas constant (erg $deg^{-1} mole^{-1}$) Correlation coefficient Resistance (ohms)
RMV	Reduced methyl viologen
rpm	Revolutions per minute
T	Absolute temperature
t	Time variable

ABBREVIATIONS (Contd.)

$t_{\frac{1}{2}}$	Half-life (minutes)
V	Volts Volume
β	Electronic Bohr magneton . (erg $gauss^{-1}$)
δ	Thickness of the diffusion layer
ϵ	Molar extinction coefficient (litre $mole^{-1}cm^{-1}$)
λ	Wavelength (nanometers)
λmax	Wavelength of maximum absorption
ν	Frequency (hertz)
Σ	Summation sign

INTRODUCTION

The study of membranes possessing the ability to continuously transfer electrons across their continuum has been a neglected topic within the general area of membrane phenomena. At the outset of this research no satisfactory electron-transfer membranes of this type had been described or characterised. Development of electron-transfer membranes of the liquid/carrier type during these studies has permitted the evolution of such systems. The term system, used throughout this thesis, is used to denote an experimental arrangement which may be conveniently represented as:

aqueous solution/membrane/aqueous solution.

It is of interest to summarise briefly the history of membrane science to show why our particular redox systems were chosen, to describe their position within the general classification of membrane phenomena and to discuss their similarity to the biological membranes involved in the electron transport processes of respiration and photosynthesis.

The origins of the study of the physical chemistry of membranes may be traced back to the first half of the last century when Dutrecht, Jolly, Graham and others studied the diffusion of electrolytes and concomitant osmotic flow of

water across porous membranes made from either unglazed earthenware or animal tissue (1). These workers failed to observe however, the electromotive effects which ordinarily arise on the diffusion of electrolytes across membranes and it was not until the end of the nineteenth century that a theory was developed by Ostwald, to cover the electromotive properties of semipermeable membranes. The possibility that a membrane, made of a non-metallic material and immersed in an aqueous environment, could act as an electron conductor was first shown by Becquerel and later by Braun. On passing a direct current through a porous glass membrane interposed between two aqueous electrolyte solutions, a coupled redox reaction occurred. Reduction took place on the side of the membrane facing the anode and oxidation on the side facing the cathode. This process was termed electrostenolysis (2). However, spontaneous redox reactions could also occur across the membrane if substances with appropriate redox potentials were present at opposite sides (3). This process was termed spontaneous electrostenolysis. (Electrostenolysis is taken to mean electrolysis in a narrow passage.) The experiments of Becquerel and Braun were the first reported observations of electron transfer across a membrane (although not recognised as such at that time.)

From these early beginnings research into the physical chemistry, especially electrochemistry, of membranes

proceeded along two different lines according to whether solid "porous" (also called "structural" or "sieve") membranes or the "solvent-type" (also called "liquid", "oil" or "dissolution") membranes were used for study.

The vast majority of papers on the physical chemistry of synthetic membranes is concerned with membranes of the porous type. In the case of non-electrolyte permeants perm-selectivity is determined by solute size or the adsorption characteristics at the membrane-solution interface. With electrolyte permeants the size factor becomes secondary and functionality is now conferred by the membrane-fixed charge. Membranes cf fixed positive or negative charge are preferentially permeable to anions or cations respectively. These latter ion-exchange membranes function by virtue of their ability to reversibly interchange counterions electrostatically bonded to the fixed charges, with ions of similar charge in the bulk solution (4).

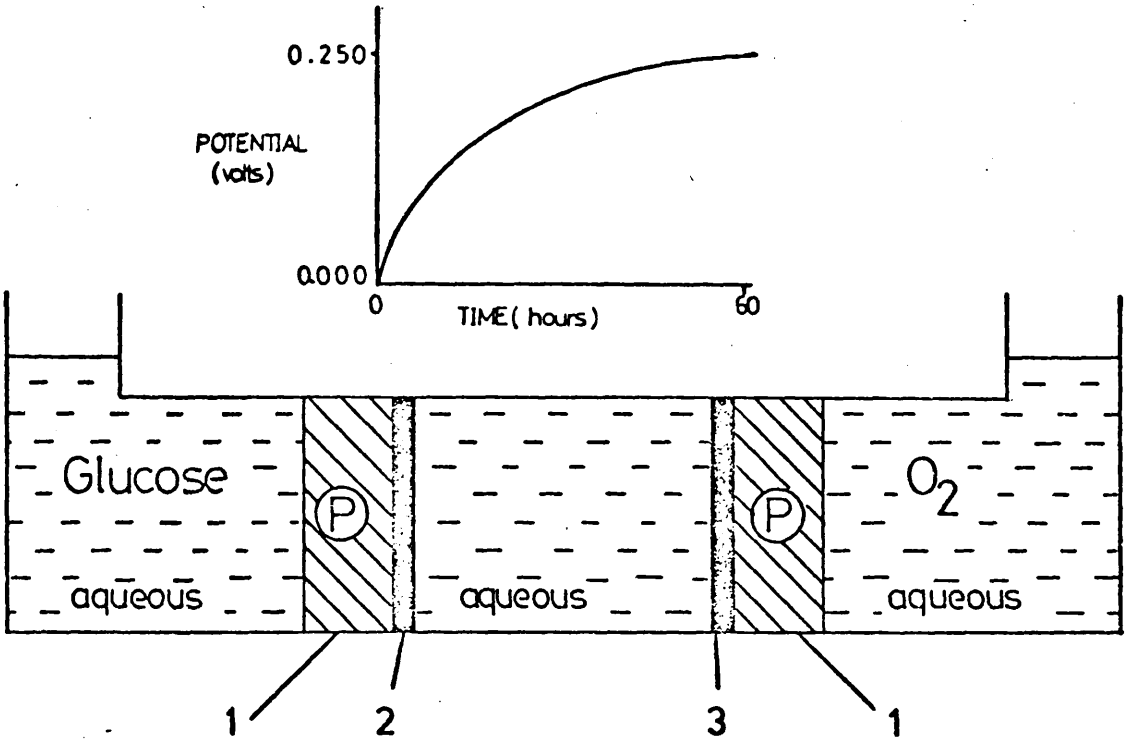
Soils were the first ion-exchange materials examined. Studies undertaken during the middle of the last century quickly showed that silicates and aluminosilicates (zeolites) were responsible for most of their exchange properties. By the turn of this century synthetic zeolites had been developed commercially for use as water softeners and as molecular sieves. The first organic ion-exchangers, synthesised by condensing formaldehyde with aromatic phenols and amines, appeared in 1935. In 1944 radical-induced

addition polymerisation was first applied to the synthesis of ion-exchange resins and the majority of present day resins are now prepared by this technique (5). It is only over the last few decades that ion-exchange polymers have been cast in sheet form and used as solid membranes. The main uses of these ion-exchange membranes are as permselective membranes allowing the electrochemical transport of ions of one charge type only. By virtue of this property these membranes have found widespread use in the general field of saline water and effluent treatment.

Since it was possible to reversibly exchange protons at the surface of some resins then, it was argued (6), it should also be possible to reversibly exchange other fundamental particles, namely electrons. In 1949 Cassidy succeeded in synthesising the first such material which he named an oxidation-reduction (or redox) polymer. Redox polymers (7), (8), are solid, insoluble, non-porous, high molecular weight substances that can reversibly transfer electrons when in contact with reactive ions or molecules. Morphologically these polymers may be classified according to whether the active redox group is part of the backbone of the polymer chain (Class I) or a substituent on the polymer chain (Class II.) The main studies employing redox polymers have been carried out using the quinone/quinol moiety as the functional group. Class I polymers were prepared by the copolymerisation of formaldehyde with

diphenols and Class II polymers by the addition polymerisation of vinylhydroquinones. Thiazine, ferrocene, pyridinium and viologen based redox active compounds are examples of some of the other types of molecules incorporated into redox polymers in this way. An alternative and little used method of generating oxidation-reduction polymers is to employ conventional strong acid and strong base resins to irreversibly absorb redox active cations and anions.

Manecke was the first to suggest that films of polyquinone polymers could act as solid state mediators if they separated two solutions, one containing an oxidant, the other a reductant (9). It was suggested that in the presence of for example a reductant, the initially reduced interfacial layer would interact with the adjacent sublayer of oxidised polymer to generate two layers of semiquinone. The surface layer would be further reduced and reduction would then proceed into the interior of the film by repetition of this process. An exactly analogous situation would apply for aqueous oxidant-reduced polymer interactions. This would be the only possible mechanism for electron conduction through such membranes for, unlike the liquid membranes to be discussed later, the functional groups are firmly held within the rigid polymer matrix and therefore lack the necessary mobility of a conventional carrier. Support for this theory came from the work of Beltzer and Batzold. Using the experimental system shown in figure 0-1 and



1. redox polymer, P.
2. Pt. electrode (became positive).
3. Pt. electrode (became negative).

Figure 0-1 The experimental system used by Batzold and Beltzer (10) to show the redox mediator capability of solid oxidation-reduction polymers.

employing quinone redox polymers, P, of either Class I or II, they were able to show that a potential difference developed between the two platinum electrodes fused to the redox membranes as shown. From this they inferred that solid state electrochemical mediation had occurred. They also obtained evidence to suggest that mediation was slow compared to the interfacial redox reactions (10).

The transfer of electrons through these redox polymers is totally dependent on the ability of pairs of adjacent redox groups within the material to move into an orientation which allows electron transfer to occur. Due to the rigid nature of the polymer matrix however, the required rotational motion is greatly restricted and, in consequence, electron-transfer processes will be exceptionally slow. For this reason liquid as opposed to solid redox exchangers were considered to be potentially more viable. This kinetic factor is also one of the principal reasons why redox polymers have not found widespread use. They have been shown, however, to have potential roles as antioxidants, as 'clean' oxidants or reductants and as chemotheraputic agents. Column or batchwise use of the polymer, in bead form, being the method of utilisation.

The literature on the physical chemistry of liquid membranes, though voluminous, is far less extensive than that on solid membranes. This, to a certain degree, is a reflection upon the fact that in practice liquid membranes

show a number of discouraging factors; they are not as easily handled as sheets of porous membrane, ion transport across them is very limited, their extremely high resistances make potentiometric measurements difficult and industry prefers the faster and more convenient method of serial extraction to the use of bulk liquid membranes (4). These disadvantages are offset by the fact that liquid membranes also display a number of highly desirable properties: membranes of infinitely variable but well-defined composition can be readily formed and studied since these membranes are easily generated, modified and analysed. Additionally, because of the fluid nature of the membrane continuum, the functional molecules and groups within the membrane possess a high degree of rotational and translational mobility. In consequence not only are reactions between adjacent pairs of molecules faster but the possibility of molecules moving from one side of the membrane to the other before reaction (that is acting as carriers) now exists. Liquid membranes should therefore exhibit greater rates of reaction than their solid counterparts.

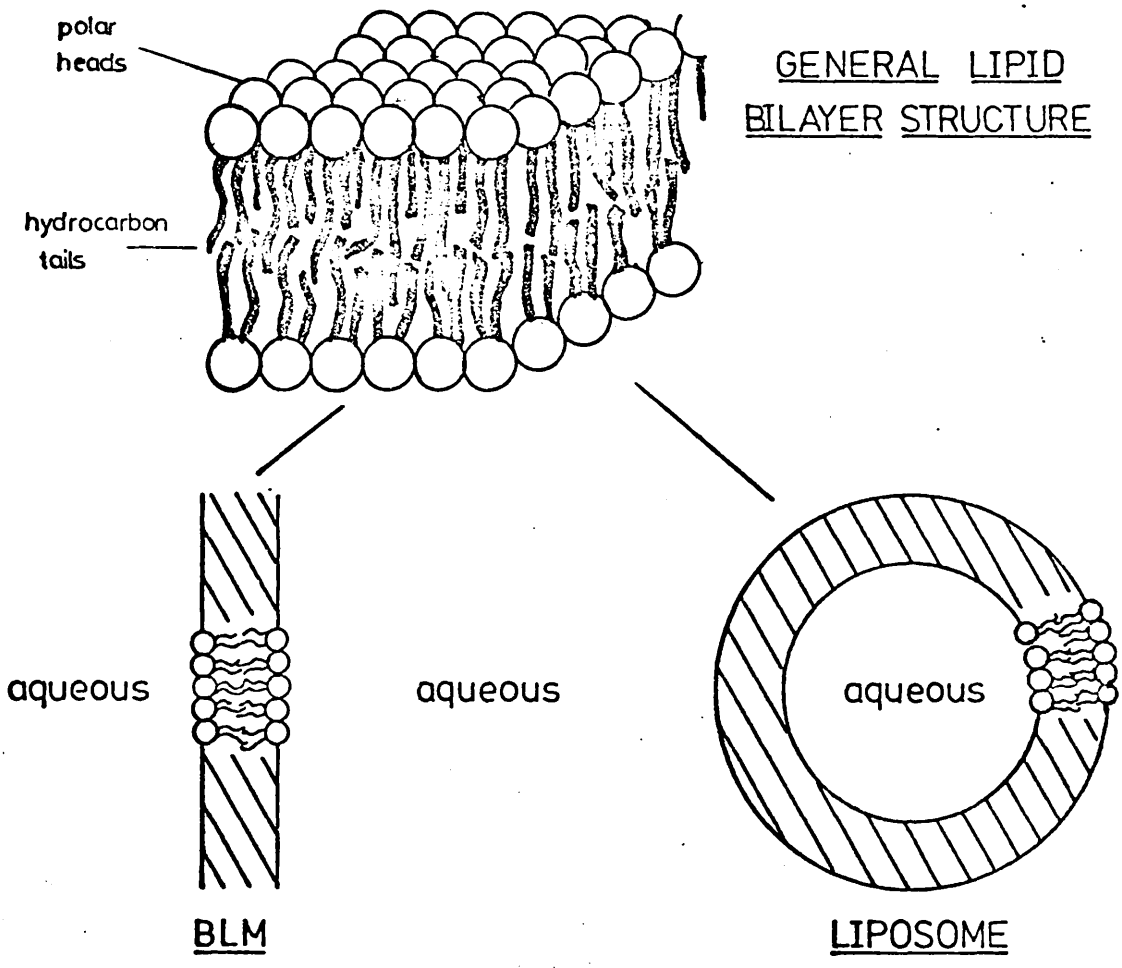
Membranes of the "dissolution" type were the first kind of liquid membrane studied (11). Membrane concentration cells in which a strong binary electrolyte was soluble in an "immiscible" liquid membrane phase in a highly dissociated state, were employed. The solvents

used were all hydroxylic compounds which actually contained considerable quantities of water (taken up from the aqueous phases) and were, therefore, not truly water-immiscible. The liquid membrane phase acted merely as a solvent for the electrolyte under consideration which, once dissolved in the membrane, could diffuse through it.

Membranes of the Nernst-Riesenfeld type would be unsuitable as electron-transfer membranes since they would allow aqueous redox reagents to dissolve in the membrane and therefore to react directly with the other redox reagent contained in the opposite aqueous phase. These hypothetical membranes may be considered as being the liquid analogue of the electrostenolysis membranes discussed earlier. Since in both of these cases reagent molecules would have to permeate the membrane before reaction could occur, reaction via membrane intervention offers no obvious advantage. Haber-Beutner type membranes, on the other hand, possess many of the requirements desired of a liquid redox membrane. The original studies with this type of membrane were contemporary with those of Nernst and Riesenfield (12), (13) but it took a long time before the molecular mechanism underlying the properties exhibited by these membranes could be attributed to the phenomenon of ion exchange. Over the last decade a new group of Haber-Beutner type membranes has been developed. These

liquid ion-exchange membranes show the characteristic electromotive properties of the original Haber-Beutner type membranes to a very high degree (14). Liquid ion-exchangers are solutions, in organic solvents (preferably of minimal water solubility and typically benzene or higher alcohols), of substances consisting of an ionogenic group attached to an organic molecule of a suitable nature to make these compounds and their salt forms very sparingly soluble in aqueous electrolyte solutions. In comparison to the solid exchangers, liquid ion exchangers are constrained to the membrane phase by solubility characteristics and not by chemical bonds. The neutral ion-pairs formed between the exchangers and their counter ions are able, therefore, to diffuse freely within the membrane continuum. With ideal Haber-Beutner membranes the only substantial process which can occur is the 'one-to-one' cross membrane exchange of anions or cations. This transport of ions across liquid ion exchange membranes is an example of the cross-membrane movement of a solute by a carrier process, a mechanism extensively utilised in nature. Commercially, liquid ion exchangers have found use in liquid-liquid extraction processes and 'ideal' membranes, by virtue of their property of acting electromotively in concentration cells like double sided reversible electrodes specific for the nominally permeable ion, have found use as ion-specific electrodes.

Liquid membranes of a more biological nature have been introduced relatively recently and may be considered as Haber-Beutner membranes of a very special type. These bilayer lipid membranes, introduced in the early 1960's are ultrathin structures possessing thicknesses of natural membranes. The membranes are formed from two dimensional sheets of amphipathic (polar) lipid material (structurally similar to liquid ion-exchangers). It is visualised that in these bilayers the hydrophobic hydrocarbon tails of the lipid extend inward to form a continuous hydrocarbon interior and that the hydrophilic heads face outward, extending into the aqueous phases. Planar bilayer lipid membranes (also known as bimolecular or black lipid membranes or simply BLM) are formed underwater by spreading the lipid, dissolved in a suitable hydrocarbon, across a small orifice (15). Closed bilayer sheets of lipid, called vesicles (also known as microvesicles, liposomes, spherules or smectic mesophases), are formed (16) by mechanically agitating or sonicating a suspension of hydrated lipid to give vesicles of around 50 and 0.05 microns diameter respectively. The structures of these two types of bilayer together with the structure of a typical lipid, are given in figure 0-2. Vesicles have the advantage that they do not require the presence of an organic solvent and that they present a much larger surface area for study. Their main disadvantage is in the fact



 denotes a lipid molecule, a typical example being :

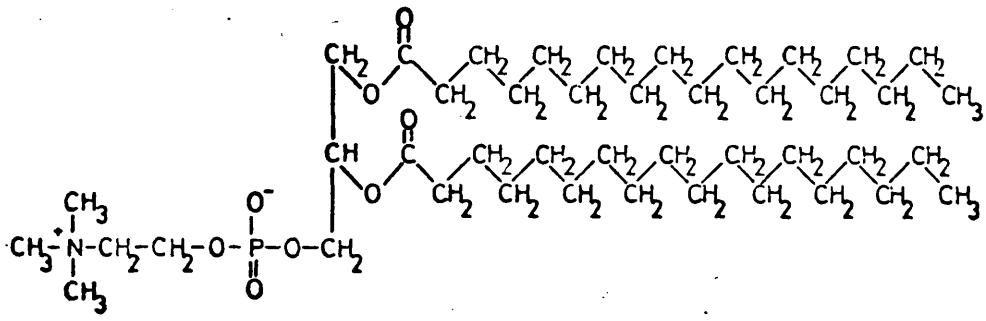


Figure 0-2 The structure of bilayer membranes. Also included is the structure of a typical lipid, phosphatidylcholine.

that, owing to their minute size, the powerful electrical methods used so successfully with BLM, cannot be applied.

The permeability properties of unmodified bilayers are very similar to those expected from a layer of liquid hydrocarbon of equivalent thickness. The insulating interior of the bilayer makes it virtually impermeable to ions. Water and non-electrolytes, on the other hand, show a much higher permeability, being translocated across the membrane by passive or hydrodynamic flow (17). The resistances of these BLM are very high ($> 10^{10}$ Ω .cm^{-2}) and the conductivity observed can be satisfactorily explained in terms of the dissociation products of water. For these membranes to exhibit ion-exchange or electron-transfer properties, the basic bilayer composition must be modified.

Introduction of specific lipid soluble ion-carriers into the bilayer can impose selective ion permeability eg. valinomycin, a cyclic oligopeptide, has been shown to render the membrane permeable to potassium ions (18). Planar bilayers so modified may be considered in all but scale to be ideal Haber-Beutner membranes where lipid has taken place of the organic solvent.

The ability of bilayer membranes to act as electron conductors has been demonstrated in a number of experiments. Common to all these studies was the involvement of a charge separation process which resulted in the creation of a

membrane potential. The nature of this potential is such that it opposes further electron transfer and therefore electron conduction will only be transient, stopping after only an extremely small number of charges have been translocated. Such a process cannot therefore sustain continuous electron transfer.

Certain modified BLM have been found to behave like redox electrodes. These membranes have been found to act either reversibly to an ion in solution, as for example planar bilayers in the presence of iodine (reversible to iodide) (17) or as an inert electrode serving merely as a conductor facilitating electron transfer. This latter property was demonstrated using a chlorophyll modified BLM as the indicator electrode during a potentiometric titration of Fe(II) by permanganate (19).

Lipid bilayers with a photo-electric response can be made by incorporating light absorbing molecules into either the membrane forming solution (eg. chlorophyll and carotenoid pigments) or into one of the two electrolyte compartments surrounding the bilayer (eg. cyanine dyes). In addition certain inorganic ions and organic dyes (eg. Fe(III), thionine, methylene blue and methyl viologen) can sensitise an otherwise photo-inactive bilayer membrane. All photo-electric effects may be thought of as arising from the production and separation of charge carriers in

the material under illumination. A redox electrode mechanism and a dipole mechanism have been postulated as two possible ways of creating charge separation, figure 0-3. For photo-electric effects to be seen, certain asymmetrical conditions must exist across the membrane. This could be the result of the presence of added modifiers, a difference in light intensity, or a gradient of temperature, pressure or electrical potential across the membrane. The generation of an electric field across the photo-active BLM, whether externally applied or generated by adding an electron acceptor, greatly enhances the photoresponse. This field acts by assisting the light induced charge separation (17). Although it is impossible to measure photopotentials in liposomes, photoreduction of cytochrome c by chlorophyll containing vesicles has been demonstrated (20). Photoredox reactions across BLM have also been studied (17). Using Fe(III) as the electron acceptor in one compartment, the effect of adding various modifiers to the other aqueous compartment was studied. It was found that a number of inorganic and organic electron donors enhanced the photopotential. The BLM is visualised as acting as two coupled redox electrodes with one side reducing and the other oxidising. Conversely, compounds known to act as electron uncouplers or proton carriers suppressed this emf. These latter compounds are thought to act by preventing charge separation

	REDOX ELECTRODE HYPOTHESIS	DIPOLE MODEL
EXCITATION	$Chl + h\nu \rightarrow Chl^*$	$P + h\nu \rightarrow P^*$
CHARGE SEPARATION	$Chl^* \rightarrow e^- + Chl^+$	$P^* + D \rightarrow P^- \cdots D^+$

Chl and P are photo-active molecules added to the membrane forming and aqueous solutions respectively. D is a donor molecule.

Figure 0-3 Probable mechanisms by which a photovoltage can be generated in a BLM.

in the membrane. A list of the modifiers which enhanced the photopotential is given in table 1.

All electron transfer reactions across bilayer membranes discussed up to now have required some kind of external impetus for electrons to be exchanged between membrane and bathing solution. In this context therefore they are analogous to Braun's electrostenolysis membranes. Studies involving bilayers analogous to the Becquerel type in which the redox agents themselves, by virtue of their favourable redox potentials, react spontaneously with the membrane have been of only limited number. One such experiment (21) used an inert BLM modified with coenzyme Q_6 (a quinone) to separate two aerated aqueous chambers one of which contained the non-autoxidisable reducing agent reduced nicotinamide adenine dinucleotide (NADH).

$$NADH + Q_6 \longrightarrow NAD^+ + Q_6H^- \qquad (0\text{-}1)$$

$$Q_6H^- + O_2 + H_2O \longrightarrow Q_6 + H_2O_2 + HO^- \qquad (0\text{-}2)$$

$$Q_6H^- + H^+ \rightleftharpoons Q_6H_2 \qquad (0\text{-}3)$$

The behaviour of the membrane potential, generated in solutions of low buffering capacity, may be interpreted in terms of an initial fast separation of charge, equation (0-1), followed by a slower quenching reaction, equation (0-2). However, when solutions of high buffering capacity

Table 1 Effect of modifiers on the Chℓ-BLM photo-emf.

The cell arrangement was:

Salt bridge / Aqueous solution $FeCℓ_3$ / Chℓ-BLM / Aqueous solution modifier / Salt bridge

	MODIFIERS
Inorganic	NaI, $Na_2S_2O_4$, $K_4Fe(CN)_6$ $FeCℓ_2$, TℓCℓ, CuCℓ
Organic	Ascorbic acid, hydroquinone, FMN, NAD, Cytochrome c, vitamin K

were employed no membrane potential was detectible. This was explained by the involvement of an additional process in which the quinol becomes protonated, equation (0-3). Under these conditions, since no charge separation now occurs, the quinone, in addition to acting as a proton carrier, can now act as an electron carrier capable of sustaining a continuous redox reaction between aqueous phases. This latter possibility was not appreciated by the authors, apparently.

Sustained transmembrane redox reactions using lipid bilayers have only been demonstrated experimentally in liposomes. Dibutyl ferrocene, N-methyl phenazinium methyl sulphate (PMS), both requiring the presence of tetraphenyl boron, and hydroquinone have all been successfully incorporated into liposomes which have the ability to mediate the reduction of internally trapped aqueous ferricyanide by ascorbate, present in the external solution (22). Reaction was found only to occur if the membrane potential created by electron transfer into the vesicle, shown to exist by studying its BLM analogue (23), was discharged by adding ionophorous compounds. Since however the redox modifiers used to generate the redox liposomes were membrane permeable, these vesicles exhibit the same disadvantage as the electrostenolysis and (hypothetical) redox Nernst-Riesenfeld membranes discussed

earlier. Cytochrome oxidase (24) is one of the many redox proteins (25), (26) which on incorporation into liposomes has been shown to function in a similar manner to the simpler redox mediators described above. Although these redox protein modifiers in general possess no water solubility and are therefore an improvement upon the simpler compounds just described, they suffer from the disadvantage of being chemically complex and frequently of uncertain composition. They are therefore also unsuitable as model systems for studying electron transfer across a membrane. The concomitant transport of protons and other ions through both types of vesicle were also studied.

The overall structure of bilayer membranes, figure 0-2, is identical to that proposed some forty years earlier (27) for the morphology of the plasma membrane and is now the generally accepted structure for the lipid component in most natural membranes (discussed subsequently). It is for this reason that these ultrathin membranes have found a widespread use throughout the biological sciences and they are, perhaps, the best studied type of liquid membrane.

Tracing the development of ion-exchange and electron-transfer membranes and materials, in addition to being interesting historically, is very informative. It indicates that one potentially fertile field of investigation

has been left fallow until now. This is more clearly illustrated by consulting table 2. The physical state and nature of the exchange properties of the membrane and membrane materials determine into which of the four quadrants it should be placed. Within each of the four subsections a chronological order applies. Examination of the table indicates that bulk liquid electron-transfer membranes have never been studied and, from the previous discussion, it is apparent that such studies carried out with bilayer membranes were mainly confined to membrane potential measurements.

Redox reactions are of crucial importance to biological cells by virtue of their involvement in electron-transfer and photosynthetic pathways. These processes are the cell's primary sources of adenosine triphosphate (ATP) which is a universal biological fuel. The energy released as complex organic molecules are successively broken down and oxidised along catabolic pathways is partially conserved by synthesising this high energy compound. The energy released during the subsequent hydrolysis of ATP (thermodynamically a very favourable reaction) is used by the cell for active transport, anabolism and many other endergonic processes. The major site of ATP synthesis in all aerobic eucaryotic cells (and hence animal cells) is the mitochondrion. This cellular organelle, of granular shape, is bounded on the outside by a double membrane. The inner membrane

Table 2 The development of synthetic ion-exchange and electron-transfer membranes and materials.

	ION-EXCHANGE	ELECTRON-TRANSFER
S O L I D	Soils (mid 1800) Molecular sieves (~1900) Permutits (~1900) Condensation polymers (1935) Addition polymers (1944) Ion-exchange membranes (1960's)	Electrostenolysis membranes (1861) Redox addition polymers (1949) Chemical modification of preformed polymers (Redox) (1952) Redox condensation polymers (1953) Polymer redox membrane (1971)
L I Q U I D	Nernst-Riesenfeld membranes (1902) Haber-Beutner membranes (1908) Modified lipid bilayer membranes (1962) Liquid ion-exchangers (1968)	Modified lipid bilayer membranes (1970)

is folded inwards at a number of places so as to project into the matrix as lamellae, termed cristae. Attached to these cristae are the enzymes of electron transport and oxidative phosphorylation. Electron transport is the process by which pairs of electrons derived from the intermediates of the tricarboxylic acid cycle flow down a multi-membered chain of electron carrier (redox) enzymes of successively lower energy level until they eventually reduce molecular oxygen. During this process ATP is synthesised from adenosine diphosphate (ADP) and inorganic phosphate by means of enzymatically coupled reactions, the process being termed oxidative phosphorylation (28). Metabolic pathways in the mitochondrion involve, therefore, redox reactions between aqueous solutes and membrane (lipid) phases.

The structural organisation of the lipid and protein molecules within chloroplast, mitochondrial and other functional membranes is widely, but by no means unanimously accepted as being that of a fluid mosaic (36), (37). The amphipathic lipids of the membrane (mostly phospholipids) are supposed to be arranged predominantly as an interrupted bilayer with their polar head groups in contact with the aqueous phases. Globular integral proteins are thought to alternate with sections of lipid bilayer and to penetrate the latter hydrophobic structure to varying degrees. Additionally, certain of these

proteins, if of a suitable nature and size, may transverse the entire bilayer structure, figure 0-4. Peripheral protein is suggested to be bound to the membrane by only rather weak electrostatic interactions. The point which distinguishes this model from the 'unit' membrane hypothesis (38)-(43), is that the lipid bilayer is envisaged as being in a fluid state thus allowing the two-dimensional translational diffusion of integral protein and phospholipid to occur freely within the plane of the membrane (lateral diffusion). The molecular tumbling or 'flip-flop' of these molecules across the membrane (transverse diffusion) would not however occur at any significant rate. It should be noted that transverse diffusion of lipid between the two dimensional sheets of phospholipid with the membrane is only slow for charged lipid. Neutral lipid, as for example coenzyme Q and vitamin K used in this research, exhibit fast rates of 'flip-flop' (44) due to the small free energy required to desorb them from one lamella and through the hydrophobic matrix. Lateral mobility of protein and lipid within both natural and artificial bilayer membranes has been amply verified experimentally (36), (45)-(48). The ingenious electron paramagnetic resonance experiment of Kornberg and McConnell (49) has shown that transverse diffusion

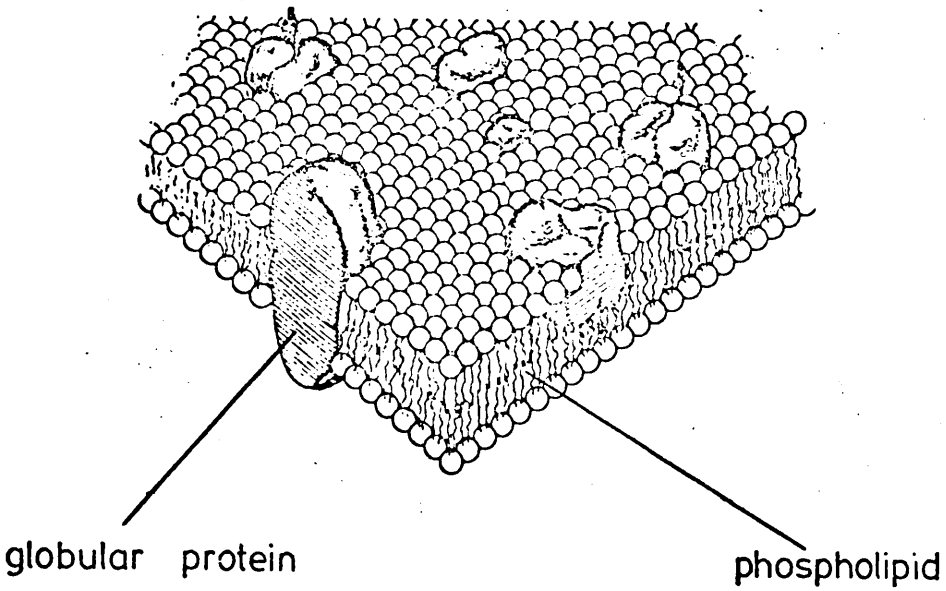


Figure 0-4 Schematic representation of the three dimensional organisation of functional membranes according to the fluid mosaic model.

of phospholipid in synthetic vesicles is indeed very slow.

Thus provided with a knowledge of the previous research into, and the biological importance of, electron transfer membranes, it was decided that the aims of this research would be the generation and study of chemically well-defined electron transfer membranes, capable of sustaining continuous redox reactions between aqueous substrates. In addition to filling an apparent gap in the membrane literature it was hoped that such membranes, by virtue of their ability to mimic the biological process of electron transport, could be used as models to study the role of lipid in biological energy transduction processes. Since bulk liquid membranes are experimentally much easier to handle than lipid bilayers but are intrinsically less useful as natural membrane models, it was decided to form the redox analogues of Haber-Beutner membranes initially, before trying to undertake similar experiments using ultrathin membranes.

Throughout the preceding discussion a number of requirements for a suitable electron-transfer system has been discussed. It is appropriate, at this stage, to gather together and expand upon the chemical and physical properties which must necessarily be upheld

by each of the system's components. The membrane solvent must not react with any of the reagents used (i.e. it must be chemically inert) and it must not act as a solvent for any of the aqueous reagents used. In addition, to ensure that the membrane cannot function in a manner analogous to Nernst-Riesenfeld membranes, it must be water immiscible. Chemical inertness, inability to solvate aqueous reagents and water immiscibility are the necessary prerequisites demanded of the solvent. Fulfilment of these 'a priori' conditions ensures that no reaction will be capable of taking place across the membrane in the absence of carrier. In addition it is experimentally desirable that the solvent used should have a minimal tendency to form micelles. Simple unbranched alkanes from hexane upward are found to exhibit all the desired criteria. Although paraffins are not completely water immiscible the amount of water which they do dissolve, between 40-150 p.p.m. (by weight) at 25°C (50), (51), for these purposes may be considered negligible. The function of the membrane solvent is, therefore, merely to provide a chemically inert hydrophobic barrier between the aqueous phases. This membrane is made functional only when the redox carriers are added. The aqueous oxidising and reducing agents must be totally confined to their respective aqueous phases and have no solubility

in the membrane, in any form whatsoever. In addition, it is desirable that once reacted, their redox products are also membrane insoluble. The carrier molecules, in both their oxidised and reduced state, must be totally confined to, and soluble in the hydrophobic membrane matrix. This requirement dictates that any redox reaction which occurs between aqueous and membrane-bound reactants must necessarily occur only at the interfaces. It is again worthwhile noting that the non-biological mediators used by Hinkle (22) did not conform with this criterion since they possessed appreciable water solubility. These membranes cannot therefore be considered as 'ideal' since reaction between mediator and aqueous reagents could also occur in the bulk aqueous phases. The carriers must also be capable of undergoing reversible oxidation and reduction if they are to behave as true carriers and allow continuous reaction to occur. A point of underlying importance to the whole scheme is that it must be thermodynamically favourable for the carrier to be both reduced and oxidised by the aqueous reagents. In other words, the standard reduction potential of the carrier must lie between those of the oxidant and reductant. A thermodynamic approach can only show whether a proposed scheme is energetically favourable. It cannot, of course, indicate how quickly

a 'favourable' reaction will actually occur.

Redox carrier molecules may be grouped conveniently into two generic types according to whether the carrier changes or maintains its overall charge after conversions between reduced and oxidised states. For convenience, they are denoted as type 1 and type 2 carriers respectively. Consider a liquid redox membrane containing a type 1 redox carrier. To be more specific, imagine that the carrier is some polychelated iron atom e.g. heme or ferrocene type molecules. The redox cycle of such a carrier would involve; generation of the Fe(II) form by donation of an electron from the aqueous reducing agent to the oxidised form of the carrier, translocation of the reduced form across the membrane, regeneration of the Fe(III) form by donation of an electron from the carrier to the aqueous oxidising agent and finally translocation of this oxidised form back across the membrane to the reducing interface. Completion of one such cycle has the overall effect of transferring one electron from the aqueous reductant to the aqueous oxidant with the concomitant development of an electrical potential gradient across the membrane. This membrane potential, created by the uncompensated transfer of the electron across the membrane, is of appropriate sign to oppose further electron flow. After only a very few

cycles, in the absence of other carriers, the large membrane potential developed would inhibit further reaction. From this a point of fundamental significance arises; any electron-transfer membrane having as its only carrier a redox molecule which undergoes an overall change of charge during its redox cycle, will not allow continuous electron flow between the aqueous oxidising and reducing agents which it separates. To allow the membrane to function in a continuous manner, any tendency to generate a membrane potential would have to be eliminated. As discussed earlier in connection with liposomes, this may be achieved by adding an ionophore as an additional carrier to the membrane phase to allow either the transport of cations in parallel to, symport, or anions in opposition to, antiport, the direction of electron flow (52), thereby maintaining electroneutrality. For a redox carrier to retain the same charge in both redox states an intrinsic ability to reversibly exchange both electrons and ions is required. Therefore, liquid membranes containing type 2 as opposed to type 1 carriers do not require the presence of additional ionophorous agents for continuous redox mediation to occur. Charged type 2 carriers would, however, require the presence of additional hydrophobic counterions for them to display the desired membrane solubility (this was the function

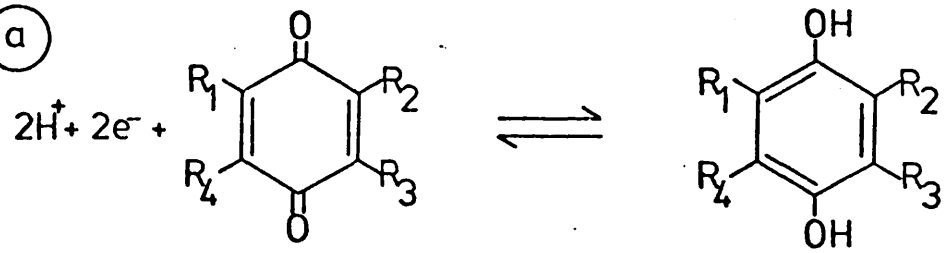
of the tetraphenylborate anion used in the redox lipid bilayer work discussed earlier). Neutral type 2 carriers do not require the addition of such counterions. The simplest type of liquid redox membrane capable of allowing electron flow is, therefore, one composed of solvent and type 2 carrier molecules only and it was to this kind of membrane that all subsequent attention was focussed.

Quinone molecules are by far the most commom example of neutral type 2 carriers. On reduction, the quinone molecule gains two protons and two electrons to form the corresponding dihydroquinone (quinol), figure 0-5(a). Quinone mediators are, therefore, bimodal functioning as both electron and ion (proton) carriers. Completion of each redox cycle results in the translocation of two protons and two electrons across the membrane. Quinones also have the added advantages that they can be reversibly reduced and are, in both redox states, generally more soluble in hydrophobic as opposed to aqueous solvents. For these reasons, the quinone molecule seemed ideally suited to the role of an electron carrier and it was from within this family of compounds that suitable carriers were sought.

The functioning of a liquid redox membrane of the type described requires that electrons (and protons)

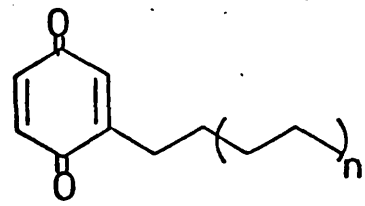
Figure 0-5 Quinones in electron transport. (a) Redox behaviour of quinones. (b) Structure required of an 'ideal' quinone carrier. (c) Naturally occurring quinones.

(a)

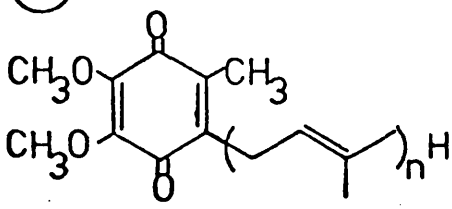


oxidised reduced

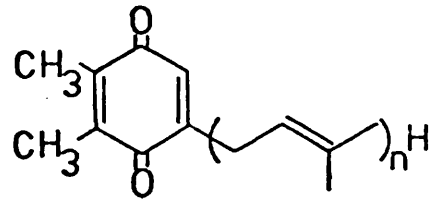
(b)



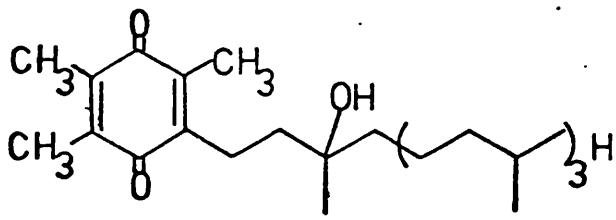
(c)



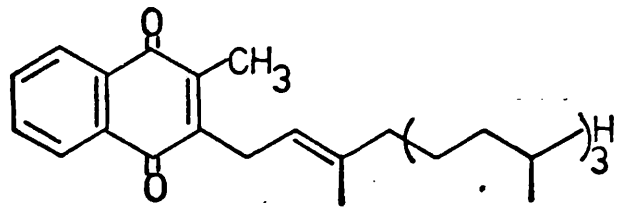
coenzyme Q_n (n=6-10)



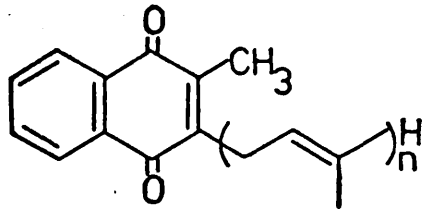
plastoquinone (n=3,4,9,10)



α-tocopherolquinone



vitamin K_1



vitamin K_2 (n=6,7,9)

be exchanged between aqueous reagents, and the quinone dissolved in an immiscible organic layer. Such liquid biphasic redox reactions have been studied using tetrachlorobenzoquinone (53). Because of its solubility in water (54) this quinone could not be considered for use in the liquid membrane system desired. To ensure that the quinone is totally confined to the hydrophobic membrane matrix it is advantageous for the molecule to possess a long hydrocarbon tail, figure 0-5(b). Synthetic quinones of this type are not readily available. Fortunately several naturally occurring and commercially available neutral lipids have exactly the structure desired. These lipophilic (lipid amphiphiles) quinones, ubiquitous throughout the bacterial, plant and animal kingdoms, may conveniently be divided into four classes; the ubiquinones (or coenzymes Q), the vitamins K, the plastoquinones and the tocopherolquinones, figure 0-5(c). All have been found localised in the various subcellular structures involved in the biological processes of oxidation and reduction i.e. in chloroplasts and mitochondria. Coenzymes Q are involved in both mitochondrial and bacterial photosynthetic electron transport; vitamins K are involved in photosynthesis, bacterial electron transport and possibly mitochondrial electron transport; the plastoquinones and tocopherol-

quinones are involved in purely photosynthetic electron transport (55)-(58). A critical examination of all the types of electron porters available eventually showed that the most attractive type of molecule would be a neutral quinone which possessed a long hydrophobic side chain. The ubiquitous occurrence of such quinones throughout the biological membranes involved in electron transfer processes would seem to indicate that Nature herself had previously come to a similar conclusion. Vitamin K_1 and, to a lesser extent, coenzyme Q_{10} were chosen as the quinones to function as the liquid membrane redox carriers. They will be referred to throughout as vitamin K and coenzyme Q (or ubiquinone) respectively.

The general form of the systems studied throughout this research is that of a liquid membrane, composed of a hydrocarbon in which were dissolved the vitamin K or coenzyme Q molecules, bridging two otherwise separate aqueous phases, one containing a reducing agent and the other an oxidising agent. Such a membrane may be visualised as being composed of three parts: two interfacial monolayers where the redox molecules are assumed to be in a highly orientated state and an intervening bulk (solution) phase in which the molecules are randomly orientated and freely mobile, figure 0-6(a).

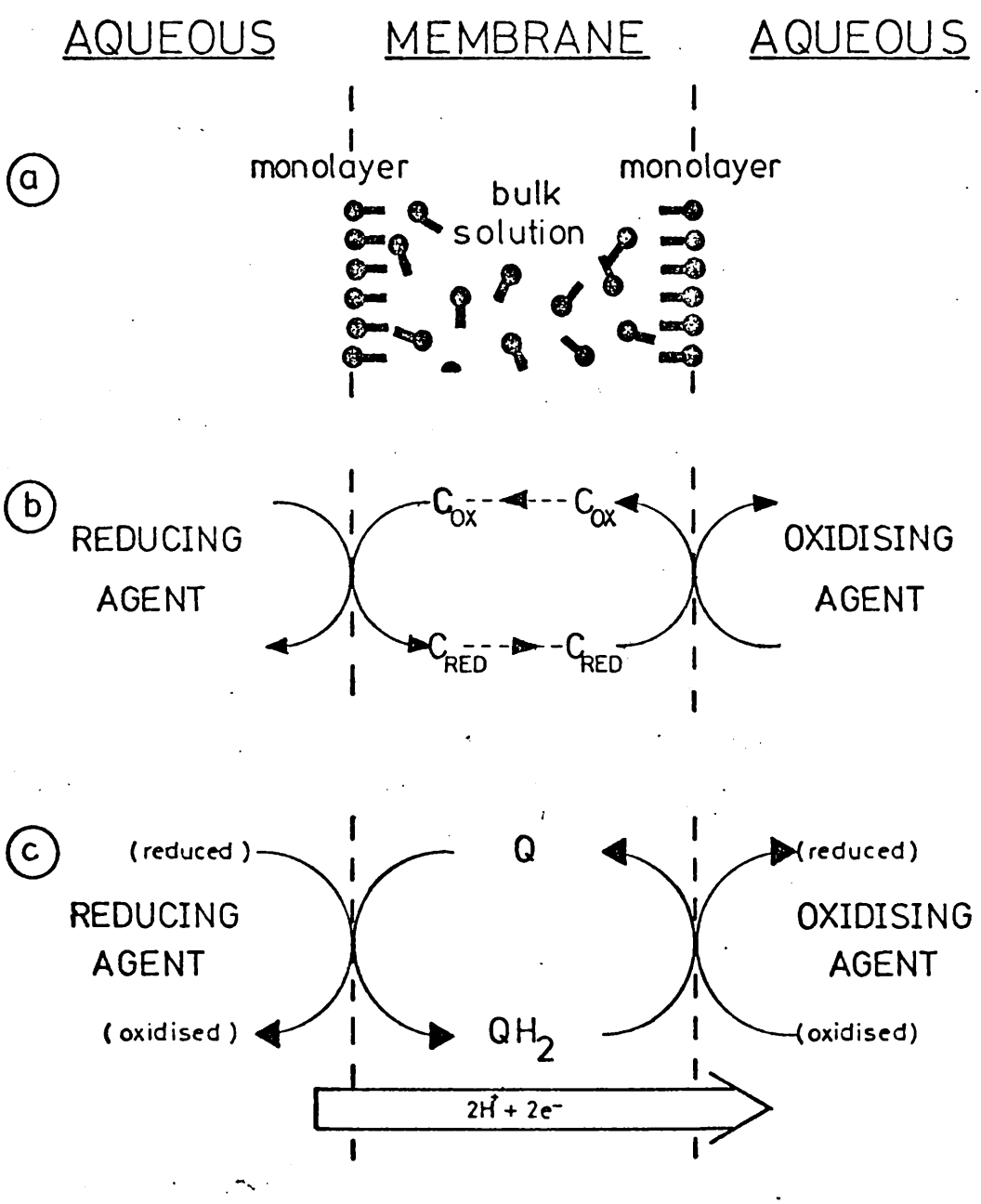


Figure 0-6 General representation of the system used to study liquid membrane mediated redox reactions. (a) The general three phase structure proposed to occur within the membrane. (b) The depiction of a general redox carrier cycle. C_{OX} and C_{RED} denotes the oxidised and reduced carrier respectively. (c) A general redox cycle involving reduced, QH_2, and oxidised, Q, quinone. The direction and stoichiometry of electron and proton transport is also indicated.

In this system, quinone molecules in the membrane reduced at one interface leave the monolayer and are translocated across the membrane to the other interface. On entering this second monolayer the aqueous oxidant is now able to react with and thereby reoxidise the membrane-bound quinol molecules. To complete a cycle the molecules, once reacted, leave the monolayer and are translocated back through the bulk membrane to the reducing side where they (once more) re-enter the monolayer. A redox cycle of this kind, figure 0-6(b) is typical of a carrier mediated process, each quinone molecule translocating two protons and two electrons (symport) across the membrane per cycle, figure 0-6(c). These (quinone) redox carriers are analogous to liquid ion-exchangers. Mobility within the membrane continuum is a necessary pre-requisite for any carrier mechanism to apply and for this reason liquid membranes are ideally suited to the study of carrier mediated transport. By decreasing the amount of bulk solution within the liquid membrane the surface to volume ratio increases whilst the distance between interfacial monolayers decreases. Eventually when all the bulk solution has been extruded the two monolayers will be in contact with each other. If neutral amphipathic lipid molecules are used as carriers, then the structure of such an

'ultrathinned' membrane will be that of a lipid bilayer. Lipid bilayer membranes, therefore, may be considered as the limiting structure of a liquid lipid membrane interposed between two aqueous solutions where charged lipid replaces the organic solvent and neutral lipid functions as the carrier.

Chapter One

BIPHASIC REACTIONS OF VITAMIN K AND COENZYME Q

The search for 'ideal' redox Haber-Beutner type membranes is the main theme of the research discussed in the present chapter. The many prerequisite properties required of the individual components of such membranes, discussed at some length in the introduction, impose severe limitations upon the chemicals used and frequently frustrate the experimenter. Despite this, the establishment of chemically well defined liquid redox membrane systems in the laboratory, that lay within these constraints, was sought.

Vitamin K and coenzyme Q are more stable in their oxidised forms under normal laboratory conditions. The first step towards the genesis of a liquid redox membrane system involved, therefore, a search for aqueous reagents which would biphasically reduce both quinones. Due to the extreme oxygen sensitivity of reduced vitamin K (59) and of a number of the aqueous reducing agents used, these experiments had to be carried out in the strict absence of

oxygen. Additionally, since both quinones are known to be degraded by sunlight or ultraviolet radiation (60), (61), it was advisable to carry out these experiments in the absence of light.

Once suitable reducing agents had been found it was then necessary to verify experimentally that the reagents displayed all the necessary requirements, before any complete system could be assembled. In addition to carrying out immiscibility experiments, the reversibility of carrier reduction was also investigated. Ultraviolet spectroscopy and nuclear magnetic resonance spectroscopy (NMR) were employed for this latter study. Both quinones contain unsaturation in their isoprenoid chains and therefore possess potential sites where unwanted side reactions with the reducing agent could occur. NMR provided a useful method of investigating this possibility. Because these isoprenoid chains are not conjugated to the ring system, the absorption spectra of these quinones are not affected by the double bonds present in the side chain. Ultraviolet spectroscopy could not, therefore, be used to probe unwanted reactions in this region of the molecule. It was, however, used to investigate, both qualitatively and quantitatively, the reversibility of the redox reaction with respect to the conjugated ring systems.

The ability to reversibly reduce the carrier at the aqueous/hexane interface using an 'ideal' aqueous reductant

allowed the search for suitable aqueous reagents (frequently referred to as substrates), which would oxidise the reduced carrier, to begin. These experiments were carried out under anaerobic conditions as before.

Mechanistically it was of interest to study the biphasic transfer of electrons across the aqueous/hexane interface. Electron paramagnetic resonance spectroscopy (EPR) was used to elucidate the nature of the electron transfer processes known to occur.

1-1 MIDPOINT POTENTIALS OF VITAMIN K AND COENZYME Q.

Values for the midpoint potentials at pH7, E_{m7} (62), of vitamin K and coenzyme Q, the quinones used throughout this study, were required at the outset of these experiments. Such a knowledge would prevent thermodynamically unfavourable reactions from being attempted. Various values have been reported in the literature and these, together with their sources, are given in table 3. Measurements using isolated coenzyme Q and vitamin K have all been performed in alcohol-HCl-water solutions and require that the values found be extrapolated to pH7. In certain cases this is an arbitrary procedure due to the temperature being unknown. Examination of the coenzyme Q results shows that the values of E_{m7}, found from the isolated quinone studies, are some

Table 3 Midpoint potential values, adjusted to pH 7.0, of vitamin K_1 and coenzyme Q_{10} as reported in the literature. Values for 1,4-naphthoquinone and 2,6-dimethoxybenzoquinone are also included for comparison. Temperature was 25°C unless otherwise indicated.

Quinone	E_{m7} (volts)	Medium	Method	Reference
1,4-Naphthoquinone	+ 0.084	0.15M Tris HCℓ (1.0M NaCℓ), pH 7.3 and propanol (1:2 v/v)	PL	(63)
	+ 0.056*	0.1M HCℓ	PT	(64)
Vitamin K	- 0.138*	80% ethanol, 0.02M in sodium acetate and acetic acid		(65)
	- 0.044*(20°C)	95% ethanol, 0.2M in HCℓ and LiCℓ	PT	(66)
	- 0.090*(30°C ?)	alcohol solution		(67)
	- 0.075	0.2M LiCℓ, 0.2M HCℓ and propanol (1:1 v/v)		(68)
	- 0.078*	0.15M Tris HCℓ (1.0M in NaCℓ), pH 7.3 and propanol (1:2 v/v)	PL	(63)
2,6-Dimethoxy-benzoquinone	+ 0.100*	0.1M HCℓ	PT	(64)
	+ 0.115*	0.5-1.0M HCℓ, 50-95% ethanol	PT	(69)

Table 3 (Contd.)

Quinone	E_{m7} (volts)	Medium	Method	Reference
Coenzyme Q	+ 0.121 (30°C ?)	95% ethanol, 2M HCℓ, 2M LiCℓ		(70)
	+ 0.112	0.2M LiCℓ, 0.2M HCℓ and propanol (1:1 v/v)	PT	(73)
	+ 0.066	50 mM phosphate	SMP	(71)
	+ 0.082*	0.3M mannitol, 10 mM Tris, pH 7.2	MP	(72)

* Results have been extrapolated from authors work assuming that $\Delta E_m/\Delta pH = RT/F\ell n[H^+]$.

PT denotes a potentiometric and PL a polarographic study. SMP and MP represent studies using submitochondrial and mitochondrial particles respectively.

40-60mV more positive than those obtained from studies where the quinone was membrane-bound (71). This difference, interpreted as a solvent effect, may indicate that the coenzyme Q incorporated into the membrane is in a more aqueous environment than the isolated quinone dissolved in aqueous ethanol, since the simpler benzoquinone derivatives also exhibit a 10-40mV more negative potential when dissolved in water, table 3. It seems, therefore, that the benzoquinone ring of the ubiquinone is protruding out of the lipid phase of the (mitochondrial) membrane, in which the isoprenoid chain of the coenzyme is immersed, and into the aqueous phase (71). As the quinone is visualised as being similarly orientated at the liquid interface of a liquid redox membrane, figure 0-6(a), a value of $E_{m7} \simeq 75$mV (25°C) for ubiquinone would seem to be most appropriate for this study. As far as the author is aware only the midpoint potentials of isolated vitamin K have been determined. Recent studies, judged to be the most reliable, give a value of $E_{m7} = -75$mV (25°C) for vitamin K. However, 1,4-naphthoquinone itself shows an $E_{m7} \sim 30$mV more negative in aqueous, as opposed to alcoholic solution, table 3, and therefore, reasoning as above, this value may well be several tens of millivolts too positive for vitamin K molecules located within the interfacial monolayers of the liquid redox membrane.

Although the precise midpoint potentials for each quinone are uncertain, it is clear that vitamin K has a midpoint potential at least 100mV more negative than coenzyme Q, suggesting that oxidised vitamin K will be more difficult to reduce and the corresponding quinol easier to oxidise than its benzoquinone counterpart. This was borne out during subsequent experiments.

1-2 EPR THEORY

The technique of electron paramagnetic resonance spectroscopy is only applicable to chemical species that possess magnetic moments: an unpaired electron possesses intrinsic angular momentum due to the spin and orbital closed loop type motions which it undergoes. Since it is also charged, this spinning electron also possesses a magnetic moment. This vector takes up one of two possible orientations in the presence of an externally applied magnetic field, H_o. The magnetic field therefore establishes two magnetic energy levels (Zeeman effect). Transitions between levels may be made to occur if a second, suitably polarised, magnetic field is applied which has a frequency given by,

$$\nu = g\beta H/h \qquad (1\text{-}1)$$

where ν is the resonant frequency, β is the electronic

Bohr magneton, h is Planck's constant and H is the magnetic field. The electronic factor, g, is a tensor which, for organic radicals generated in liquids, has a value approaching 2.00232. The field H is really the field experienced by the electron whose resonance characteristics are being examined and is, therefore, the vector sum of the applied magnetic field and of the internal magnetic fields exerted by neighbouring magnetic (spinning) nuclei. In liquids the resultant field, H, is given by,

$$H = Ho + \sum_i a_i (M_I)_i \qquad (1\text{-}2)$$

where a_i is the isotropic hyperfine coupling constant for magnetically identical nuclei, i, and M_I is the quantum number which specifies the z- component of their nuclear spin angular momentum. These additional magnetic fields cause further smaller splittings of the magnetic energy levels and result in fine structure being superimposed upon the major absorption spectrum. The EPR spectra of organic free radicals in solution commonly display fine structure due to the hyperfine interaction between the unpaired electron and hydrogen or nitrogen magnetic nuclei.

An electromagnet with an available field range of 500-5000 gauss and with a field uniformity of $1:10^6$ is required for accurate EPR studies. The magnitude of the fields employed dictates that microwave radiation be used to generate the circularly polarised magnetic field.

Since, however, amplifiers do not function directly at high microwave frequencies the DC signal (obtained from the crystal detector when the resonance condition is reached, equation (1-1)) is converted to an AC one by modulation of the magnetic field. Provided that the modulation amplitude of this additional alternating magnetic field is small, the derivative of the absorption is recorded. This detection method has the added advantage of increasing signal-to-noise ratio.

The absolute number of free radicals required for detection is typically 10^{12} electrons, but for a detailed analysis a concentration of 10^{-6} - 10^{-5} M is normally necessary. It is important to realise, however, that failure to record an EPR spectrum cannot be taken as proof that a free radical is absent; it may be due to the sensitivity limitations of the spectrometer or to the extreme instability of the radical (74). None the less, electron paramagnetic resonance spectroscopy permits the investigator to detect and, in favourable cases, to characterise very small concentrations of molecules with unpaired electrons.

1-3 EXPERIMENTAL

1-3.1 Materials

Chemicals: Unless otherwise stated the source and purity (the highest readily available) of every chemical used in this research is that given in appendix I.

Solutions: Solutions of 0.1M hydrochloric acid and 0.1M sodium hydroxide were made using 'CONVOL' CVS vials (Hopkin and Williams, England).

Buffer solutions of pH4 (phthalate), pH7 (phosphate) and pH9 (borate) were made from buffer tablets (BDH, England).

0.025M phosphate buffer pH 6.86 (25°C) was made according to the method of Vogel (104) using potassium dihydrogen phosphate and disodium hydrogen phosphate.

Freshly distilled water was used throughout to prepare the aqueous solutions.

Solutions of vitamin K and coenzyme Q were prepared using hexane as the hydrocarbon solvent.

Nitrogen: Commercially 'pure' nitrogen gas (British Oxygen Co. Ltd., Scotland) was further deoxygenated by passing it through a gas train containing a mixture of sodium anthraquinone-2-sulphonate, sodium dithionite and potassium hydroxide dissolved in water (75). This solution readily removes the last traces of oxygen. The presence of the red anthraquinol aids rapid oxygen absorption and acts as an indicator, becoming colourless when the solution

is exhausted. All glass-to-glass connections were made using butyl rubber tubing (Watson & Marlow, England) since it is relatively impervious to oxygen (62).

The nitrogen used in conjunction with vacuum line work was deoxygenated by passing the above commercial source slowly over copper filings, Cuprin reagent (Coleman Instruments, USA), heated to between 400-450°C, (62).

Rubber septa: One of the major experimental difficulties encountered during this research was the maintenance of an oxygen-free environment within reaction vessels. Frequently the most suitable method of achieving such a condition was by employing rubber septa, commercially called 'suba-seals' (Freeman & Co. Ltd., England). These closures, made of red natural rubber, possess the very important property of reforming an airtight seal, after having been punctured by hypodermic syringe needles. Using needle inlets and outlets, the former connected to a nitrogen line, apparatus fitted with these seals could be easily flushed free of oxygen. These septa also provided a facile method of adding reagents to or extracting them from the apparatus, under conditions which minimised contamination by oxygen. Additionally, since they could be obtained in a number of different sizes, a large variety of glassware could be modified to accept one of the sizes available.

1-3.2 Biphasic reductions of vitamin K and coenzyme Q.

(a) The vial method: This method involved the use of glass tubes which could be hermetically sealed after the addition of reagents. The tubes were constructed from an 18 cm section of pyrex tubing (10 mm internal diameter) closed at one end by a 2.5 cm diameter bulb. The neck of the tube was constricted to ~0.2 cm internal diameter.

To fill each of these vials the solid reductant, or a 3 ml solution of the oxidised form of the reductant, was added. The tubes were isolated from the atmosphere by fitting No. 17 'suba-seals' to their open ends and their contents deoxygenated by passing a gentle flow of nitrogen through each of the vials. Pairs of syringe needles with B7 (female) glass sockets araldited into their luer ends were used as gas inlets and outlets. Groups of six vials were connected in series using sections of butyl rubber tubing fitted with B7 (male) glass sockets to form a gas train, the last outlet being connected to a small gas bubbler. The tubes were deoxygenated for a minimum period of 30 min. After this period 3 ml aliquots of either aqueous solvent or, if the reductant was to be generated 'in situ', 0.5 ml aliquots of sodium dithionite solution were added. This was followed by the addition of 3 ml of coenzyme Q solution or 1 ml of vitamin K solution. All needles were removed, the vials cooled in liquid nitrogen

and then hermetically sealed. The tubes, containing the two-phase systems, were covered with aluminium foil, placed on a vibro-mixer (Chemie-Apparatebay, Zürich) and gently agitated for 0.5-5 days at 25°C.

Concentrations of 3 mM vitamin K and 50 μM coenzyme Q were used throughout these experiments together with molar excesses of aqueous reductants in the range 10-500X and 50-1000X respectively.

(b) Vacuum line method: To allow both qualitative and quantitative ultraviolet spectroscopy to be used when investigating the reduction of vitamin K, a second method of study was devised. This method had the added advantage that it permitted the use of greater molar excesses of reductant.

This second procedure again involved the use of a specially designed piece of apparatus, shown in figure 1-1(a). The apparatus consisted of a 15 ml glass reaction vessel fitted with a short sidearm, into which the potentially reacting two-phase system was placed. A much longer sidearm led to an ultraviolet/visible optical cell of 10 mm path length (Thermal Syndicate Ltd., England). A third sidearm was used to connect the apparatus to a vacuum line. The vessel was sealed by a 'Rotaflo' tap (Quickfit and Quartz Ltd., England) which, when open, allowed the cell to be evacuated.

Figure 1-1 Apparatus designed to carry out two-phase reactions under oxygen-free conditions.

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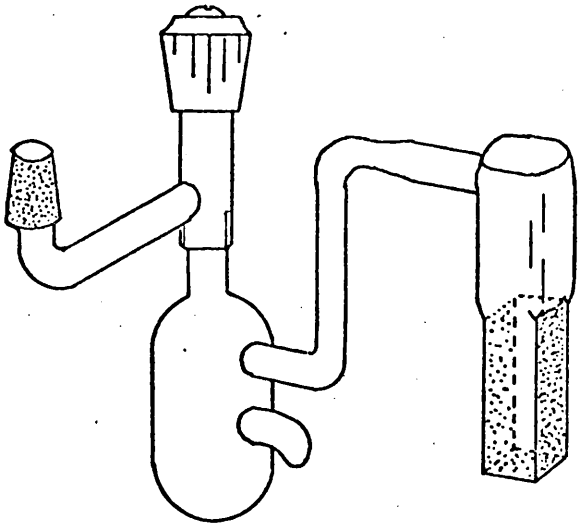

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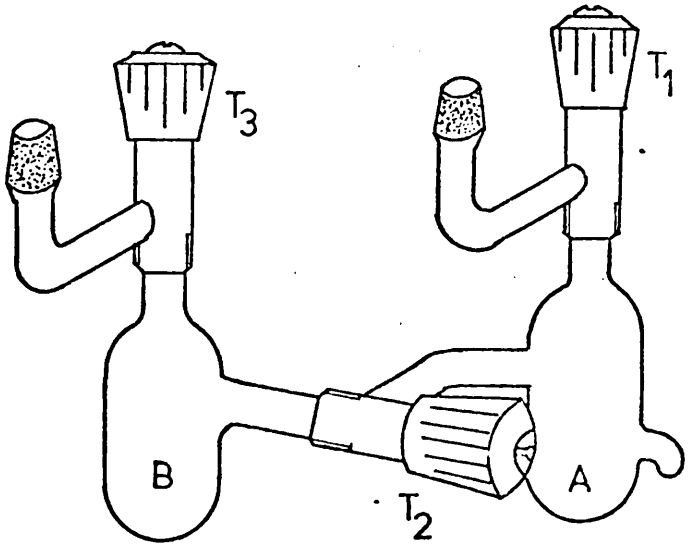
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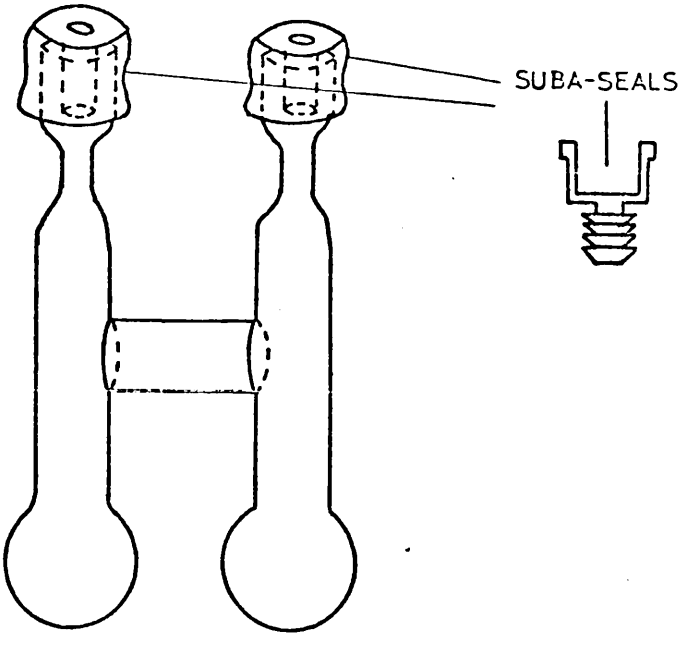

SUBA-SEALS

To fill the apparatus the teflon section of the tap was completely removed from the apparatus and the solid reductant manipulated into the short sidearm. A small teflon 'flea', together with the appropriate volume of aqueous solvent, (4 ml) was added so that the level of the aqueous phase was just below the opening of the sidearm. At this stage, therefore, the aqueous solvent and the solid reductant did not come into contact with each other. An aliquot (5 ml) of a hexane solution of the quinone was added, the teflon tap replaced in position and the apparatus attached to a vacuum line. The solutions were then degassed by freezing with liquid nitrogen and evacuating. At least four freeze-pump cycles were carried out before the inside of the vessel was brought back to atmospheric pressure using pure, oxygen-free, nitrogen. With the teflon tap tightly closed the apparatus was removed from the vacuum line, wrapped in aluminium foil and allowed to once again reach room temperature. The aqueous solvent and solid reductant were now mixed and the two-phase system left stirring for a period of 0.5-5 days at 25°C. Careful tilting of the apparatus allowed the transfer of the hexane solution into the optical cell. In this way the spectrum of the hexane layer could be easily recorded.

Concentrations of vitamin K and coenzyme Q used were 5 μM and 50 μM respectively. A 50-500X excess of reductant was employed.

Detection of reaction: A solution of oxidised vitamin K in hexane is yellow in colour whereas the corresponding quinol solution is colourless. Hence, if the vitamin K layer in the vial experiments became colourless, reduction was assumed to have taken place. The very different ultraviolet spectra exhibited by the quinone and quinol forms of both vitamin K and coenzyme Q (Chapter 2), enabled any quinone reduction to be readily detected when the vacuum line technique was employed. Since ubiquinol is only slowly reoxidised in air (76) the hermetically sealed vials containing coenzyme Q could be opened to the atmosphere and the hexane layer examined spectroscopically, according to standard procedure.

At the end of each experiment the aqueous phases were tested for the continued presence of reducing agent. This ensured that interpretation of negative results was not misleading.

1-3.3 Immiscibility tests.

Experiment A. Equal volumes (4 ml) of methyl viologen, 25 mM, and pH 6.86 buffer (control) were both equilibrated with an equal volume of spectroscopic hexane (section 2-2.2) for a period of 12 hours using the vial method (section 1-3.2). Spectra of the top hexane layers were recorded on a Pye-Unicam SP 8000 ultraviolet/visible spectrometer.

Experiment B. Vials containing 4 ml aliquots of either vitamin K dissolved in spectroscopic hexane, 2.5 mM, or

spectroscopic hexane (control) were equilibrated over equal volumes of pH 6.86 buffer and spectra of the bottom aqueous layers recorded, as above.

Experiment C. Equal volumes (4 ml) of spectroscopic hexane and reduced methyl viologen (excess dithionite), 25 mM, were equilibrated with each other according to the vacuum line method (section 1-3.2), but with the viologen solution replacing the aqueous solvent. The ultraviolet/visible spectrum of the hexane layer was recorded after 6 hours.

Experiment D. The insolubility of reduced vitamin K in water was tested using a 'V-shaped' reaction vessel. The inside of the apparatus and its contents were kept oxygen-free by fitting 'suba-seals' to the two open ends and then flushing with nitrogen. Various reagents were injected into the cell through these caps. A 5 ml aliquot of methyl viologen, 25 mM, (dissolved in pH 6.86 buffer) already contained in the vessel was reduced by injecting 1 ml of dithionite solution (60 mg ml^{-1}). This volume of aqueous solution was carefully chosen to ensure that the upper section of the vessel was divided into two otherwise isolated compartments. Spectroscopic hexane and vitamin K in spectroscopic hexane respectively, were added to opposite compartments to form the system,

vitamin K solution/reduced methyl viologen/hexane.

The aqueous phase was left gently stirring. After two days the seals were carefully removed and the contents left to reoxidise in air. An ultraviolet spectrum of the 'pure' hexane solvent layer was recorded.

1-3.4 NMR studies

A tube containing 100 mg of sodium dithionite was capped with a 'suba-seal' and flushed with nitrogen. Aliquots (5 ml) of hexane, containing 25 mg of either vitamin K or coenzyme Q, and of an aqueous solution 0.1M in both sodium hydroxide and methyl viologen, were added and the contents gently shaken until the quinone in the hexane layer became fully reduced (as indicated by the decolourisation of the quinone layer). The contents were then opened to the atmosphere and the hexane layer extracted, washed and dried (anhydrous sodium sulphate). Reoxidation of vitamin K and coenzyme Q was effected by oxygen (air) and silver oxide respectively. Finally the hexane was evaporated off, the quinones dissolved in deuterochloroform (C.E.A., France) and their NMR spectra recorded using a Varian T60, 60 MHz spectrometer. Spectra of untreated samples of quinone were recorded for comparison.

1-3.5 Ultraviolet spectroscopy studies

The apparatus shown in figure 1-1(b) was specially designed for use in these experiments: two reaction vessels fitted with 'Rotoflo' taps were interconnected by a section of glass tubing, containing another such tap.

The tap, when open, allowed the transfer of liquid between the limbs but eliminated unwanted mixing when closed. One reaction bulb was fitted with a short sidearm and could be connected to a vacuum line by means of a limb fitted with a B14 cone.

A small teflon 'flea' and an aliquot (4 ml) of previously oxygen saturated buffer (pH 6.86) were added to bulb B and taps T2 and T3 then closed, figure 1-1(b). A solution of vitamin K (10 ml) in spectroscopic hexane was reduced in bulb A by a solution of reduced methyl viologen (25 mM) in pH 6.86 buffer, using the method outlined in section 1-3.3, experiment C. Once reduced, tap T2 was opened and approximately half of the quinol solution transferred into bulb B. T2 was then shut once more and T1 opened to allow air to enter bulb A. All taps were then securely closed (to prevent hexane evaporation), the bulbs covered with aluminium foil and their contents left to reoxidise overnight. A small sample of untreated vitamin K solution was also left at room temperature, similarly protected from light, as a control. The optical densities of the control and the vitamin K solution in bulbs A and B were then determined at the wavelengths of maximum absorption exhibited by vitamin K (Chapter 2), using a Hilger H700 'UVISPEK' spectrometer.

A similar procedure to that outlined for vitamin K was used for coenzyme Q. However, the fact that ubiquinol

is only slowly oxidised by oxygen necessitated the use of silver oxide as the oxidant (in bulb B) and that only the quinone solution in this bulb be analysed spectrophotometrically at the absorption maximum of ubiquinone (Chapter 2).

1-3.6 Genesis of Haber-Beutner type redox membranes

The cell designed to carry out these experiments, figure 1-1(c), may be considered to be formed by the bridging together of two of the previously described pyrex vials (section 1-3.2) by means of a horizontal section (5 cm) of pyrex glass tubing (1 cm diameter) fused onto the stem of each vial at a point midway between the reaction bulb and the constriction. The cell therefore appeared 'H-shaped'.

The procedure adopted to fill each cell was as follows: 4.5 ml of 25 mM methyl viologen solution and 5.0 ml of the test oxidant solution, both dissolved in phosphate buffer (pH 6.86), were added to opposite bulbs of the same cell. The two openings of the cell were sealed using rubber septa and the apparatus flushed with nitrogen for one hour. After this period, the methyl viologen was reduced by the addition of 0.5 ml of sodium dithionite solution (20 mg ml^{-1}). The solvent membrane was generated by the addition of hexane, of sufficient volume to ensure that the two aqueous phases were connected via a hexane layer and that the horizontal limb was almost completely filled by this membrane phase.

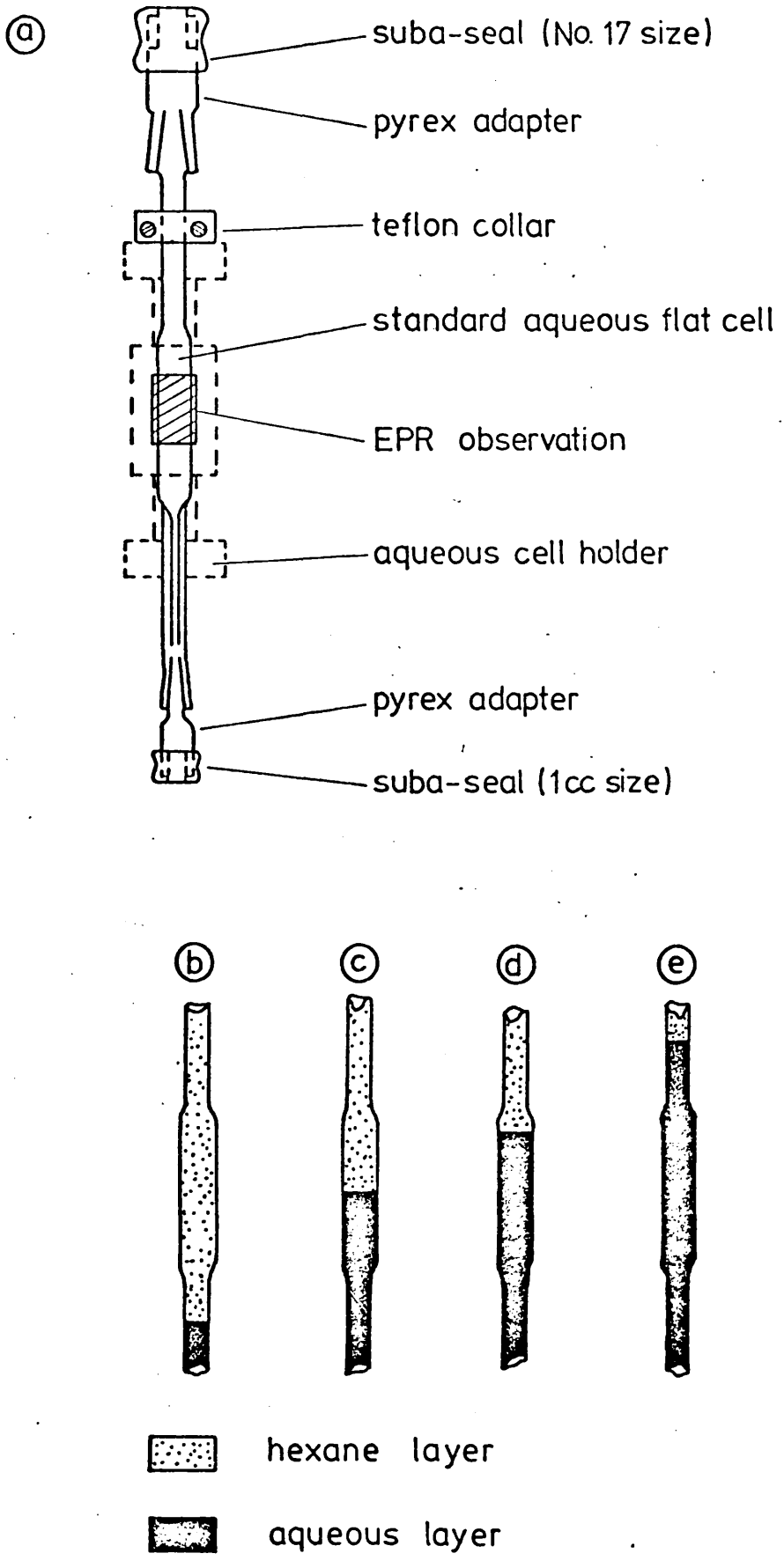
The liquid membrane systems were then placed on the vibro-mixer and left reacting for 12-48 hours at 25°C. Control experiments were carried out using quinone-free, but otherwise identical systems. Reduction was judged to have occurred if the originally coloured test solution became colourless or vice-versa.

1-3.7 EPR studies

EPR experiments were carried out using an aqueous flat sample cell (Optiglass Ltd., England) of 0.2 mm path length. Glass adaptors, designed to accept No. 9 and 1 cc-type 'suba-seals', were fitted with female and male sockets respectively, of appropriate size to couple with those already present on the cell, figure 1-2(a). The joints were made airtight with Kel-F wax (Minnesota Mining and Manufacturing Co., USA) cement. This, together with the use of rubber septa, allowed the maintenance of an oxygen-free environment within the cell during the period of study. The position of the cell within the cavity of the spectrometer was adjusted, by means of a movable teflon collar, until the flattened section of the cell alone was in the cavity. This ensured that only the spectra of radicals present in this section of the cell would be detected.

The concentration of aqueous redox agents was 10^{-2} M, the solvent being pH 6.86 phosphate buffer. The oxidised

Figure 1-2 Apparatus for EPR studies. (a) The modified aqueous flat sample cell used. (b), (c), (d) & (e): The positions of the hexane/water interface when the bulk hexane, the interface, the aqueous phase just below the interface, or the bulk aqueous phase, respectively, were being investigated.


ⓐ
suba-seal (No. 17 size)
pyrex adapter
teflon collar
standard aqueous flat cell
EPR observation
aqueous cell holder
pyrex adapter
suba-seal (1cc size)
ⓑ
ⓒ
ⓓ
ⓔ
hexane layer
aqueous layer

and reduced vitamin K solutions, dissolved in spectroscopic hexane, were 10^{-2} M and ~2.10^{-3} M (saturated solution) respectively. The method of filling the cell with oxygen--free solutions was as follows: the aqueous solutions were placed in small tubes which were then capped with 'suba-seals'. The solutions were purged of oxygen by a stream of pure nitrogen for a period of one hour. At the end of this period, if methyl viologen or FMN samples were being used, a solution of sodium dithionite was added to generate reduced forms. Reduced vitamin K in hexane was generated from the corresponding oxidised solution by reacting it with reduced methyl viologen, in a rubber capped vial, for a similar period. The EPR cell, fitted with 'suba-seals', was flushed with nitrogen for at least 15 minutes before being filled. To minimise oxygen diffusion, gas-tight Hamilton syringes (V.A. Howe, England) were used to transfer the solutions from the preparation tubes into the EPR cell, by way of the lower seal. A small volume of aqueous solution was first added. This was immediately followed by the addition of the hexane solution (0.5 ml), allowing it to pass slowly through the aqueous (reducing) layer. In this way the hexane was prevented from coming into contact with the rubber seal and, if used, the oxidised vitamin K solution was at least partially deoxygenated since the aqueous reductant layer

should function as an oxygen scavanger. Additional aqueous solution was injected until the (top) hexane phase filled the flattened section of the cell and the interface was situated ~1 cm below the beginning of this region. Further addition of aqueous solution allowed the position of the interface to be adjusted to the centre, to the top and finally to totally 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, figure 1-2(b), (c), (d) and (e) respectively.

All spectra were recorded on a Decca X3 spectrometer combined with a Newport instruments 11-inch magnet system using 9270 MHz radiation, modulated at 100 k Hz.

Reduced methyl viologen/vitamin K, $FMNH_2$/vitamin K and methylene blue/reduced vitamin K were the three biphasic systems investigated.

1-4 RESULTS AND DISCUSSION

1-4.1 Biphasic reductions of vitamin K and coenzyme Q.

The results of the attempted biphasic reductions of hexane solutions of both vitamin K and coenzyme Q are summarised in table 4. Also included in this table are the midpoint potentials or E° values for the various aqueous reductants, the number of electrons transferred during

Table 4 Test of the reducing abilities of a number of two-electron (n=2) and one-electron (n=1) reducing agents towards vitamin K_1 and coenzyme Q_{10} with the half system aqueous reductant/quinone (hexane).

Reductant	n	pH or nature of aqueous solution	$E_m^*, (E^o)^{\phi}$ (volts)	Results: vitamin K	Results: coenzyme Q	Verification of the presence of excess reductant	Comments
sodium dithionite	2	4	(-0.08)	-		decolourised	unstable at pH<7.
		7		-	+	methylene	
		9	(-1.12)	slight	+	blue	
sodium borohydride	2	7		-	+	"	at pH<10, vigorous evolution of hydrogen.
		9			+	"	
		0.1M NaOH		-		"	
hydrazine	2	7	(-0.023)	-	-	generation of I_2 and N_2 on addition IO_3^-	
		9	(-1.16)		-		
		0.1M NaOH	(-1.16)	-			
chromium (II)	1	4	(-0.41)	+		retention of blue colour	
		0.1M HCℓ	(-0.41)	+			
†Leuco phenosafranine	2	4	-0.073	-		remained colourless	precipitation at all pH's used.
		7	-0.252	-			
		9	-0.303	-			
†RuV	1	4		-		remained blue	at pH<7 a cream precipitate formed
		7	-0.446(30°C)	+	+	"	
		9	-0.446(30°C)	+	+	"	

Table 4 (Contd.)

Reductant	n	pH or nature of aqueous solution	E_m,$(E^o)^{\emptyset}$ (volts)*	Results		Verification of the presence of excess reductant	Comments
				vitamin K	coenzyme Q		
†Reduced benzyl viologen	1	4		–		remained violet	precipitation at all pH's used.
		7	-0.359(30°C)	+	+	"	
		9	-0.359(30°C)	+	+	"	
L-cysteine	2	0.1M HCℓ	-0.139	–		ultraviolet spectroscopy	
		0.1M NaOH	-0.848	–			
NADH	2	4	-0.222	–	–	"	
		7	-0.311	–	–	"	
		9	-0.370	–	–	"	
NADPH	2	7	-0.313(27°C)	–	–	"	
†$FMNH_2$	1/2	4	-0.049	+	+	"	green solution at all pH's
		7	-0.213	+	+	"	
		9	-0.435	+	+	"	
†$FADH_2$	1/2	7	-0.213	+	+	"	green solution

* Reference (62), temperature 25°C unless otherwise indicated.

$\emptyset$ Reference (77), but values are converted to reduction potentials. Temperature 25°C.

† Generated by adding dithionite to corresponding oxidised form.

____ Where compound is underlined, vacuum line method (section 1-3.2) was used.

their reduction, n, and a summary of the experimental conditions used.

An examination of table 4 reveals that vitamin K displays poor reactivity towards inorganic reductants, but reacts with a number of organic ones. Coenzyme Q, on the other hand, displays a more general reactivity towards both inorganic and organic reagents. Although all the reactions attempted were thermodynamically favourable it can be seen that a number of potentially very favourable reactions did not occur, presumably because of kinetic limitations. Sodium dithionite and borohydride, common strong inorganic reductants, were unable to reduce vitamin K biphasically even though both compounds can reduce the quinone in a homogeneous solution (78), (79). The inability to react must, therefore, be attributed to an additional kinetic factor imposed by the biphasic nature of the reaction. In contrast, sodium dithionite and borohydride, known to reduce coenzyme Q in a homogeneous medium (80), also biphasically reduce this quinone. The difference in reactivity between the two quinones may be due to the fact that the quinone ring of vitamin K at the interface is shielded from the aqueous reductants by the aromatic ring of this naphthoquinone, the ring tending to form a hydrophobic barrier that would repel the charged aqueous species. The bulky aromatic residue may also

impede reaction because of a steric effect.

Vitamin K can be seen to prefer to react biphasically with one-electron agents whereas coenzyme Q is less selective and reacts with both one- and two-electron reductants. This difference in reactivity is considered to be due not to their relatively small differences in midpoint potential but rather to some difference in their mode of reaction.

Chromium(II), reduced methyl viologen (RMV), reduced benzyl viologen, $FMNH_2$ and $FADH_2$ were all found to be successful aqueous reductants. However, the necessity of an acidic solvent for chromium(II), the unfavourable solubility characteristics of reduced benzyl viologen and the unavailability of a ready supply of $FADH_2$ permitted only RMV and $FMNH_2$ to be considered as general reductants for subsequent experiments which, in analogy with biological systems, were to be carried out at neutral pH.

The inability of NADH to reduce coenzyme Q biphasically to any significant extent apparently contradicts the results of Ismailov et al. (21) which were explained in terms of the existence of such a reaction (see Introduction). These different findings may be reconciled by the fact that only a very small number of molecules is required to react to generate a membrane potential, whereas for a net chemical reaction to be detected a much larger number must react.

1-4.2 Ideality of carriers and aqueous reductants.

The ultraviolet spectra obtained from the hexane layers, which had previously been equilibrated over methyl viologen (MV) and RMV (section 1-3.3, experiments A and C), were found to show no absorption over a 225-700 nm range, within which both redox states of the viologen exhibit strong maxima. The ultraviolet spectra recorded from the aqueous buffer layers, previously equilibrated with vitamin K solutions, showed no trace of the quinone. Both the control and test solutions, however, showed a minor, broad absorption (optical density <0.05) with a λmax ~265 nm. This was assumed to be due to some common contaminant present in the hexane or released by the 'suba-seal'. The ultraviolet spectrum of the 'pure' hexane layer, from the experiment designed to test the insolubility of reduced vitamin K in the aqueous phase (section 1-3.3, experiment D), showed no trace of vitamin K (optical density <0.05 over the range 225-275 nm). Should the quinol, generated by reaction between vitamin K and RMV, have even an extremely small solubility in the aqueous phase then, it was assumed, the quinol would be continuously re-extracted into the formerly pure hexane layer to yield a detectible concentration of the vitamin. It should be pointed out, however, that on a number of occasions the spectra recorded were contaminated with

aromatic compounds leached out of the rubber septa used. This effect could be minimised, but never totally eliminated. A similar experiment carried out with coenzyme Q replacing vitamin K, was found to give the same negative result (81).

The NMR spectra of vitamin K and coenzyme Q which had been put through a reduction-oxidation cycle are shown in figure 1-3(a) and (b) respectively. Both quinones were reduced by RMV; vitamin K and coenzyme Q were reoxidised using atmospheric oxygen and silver oxide respectively (section 1-3.4). The NMR spectra of treated and untreated samples of vitamin K were found to be identical to each other and to that reported in the literature (78). The chemical shifts obtained from this research and those previously published, were found to agree to within $\pm$0.05 ppm. From the integral trace the theoretical relative ratios of protons per signal were obtained. The NMR spectrum of treated coenzyme Q was, however, found to differ from those of the untreated and published (80) ones in that two additional resonances were observed with chemical shifts of 1.25 and 0.88. These resonances correspond to R–CH_2–R and R–CH_3 proton resonances respectively. The chemical shifts for the other resonances agreed to within $\pm$0.02 ppm of the literature values. The integral trace indicated that it was only that section of the spectrum in the range 0.5-2.5 ppm which was anomalous.

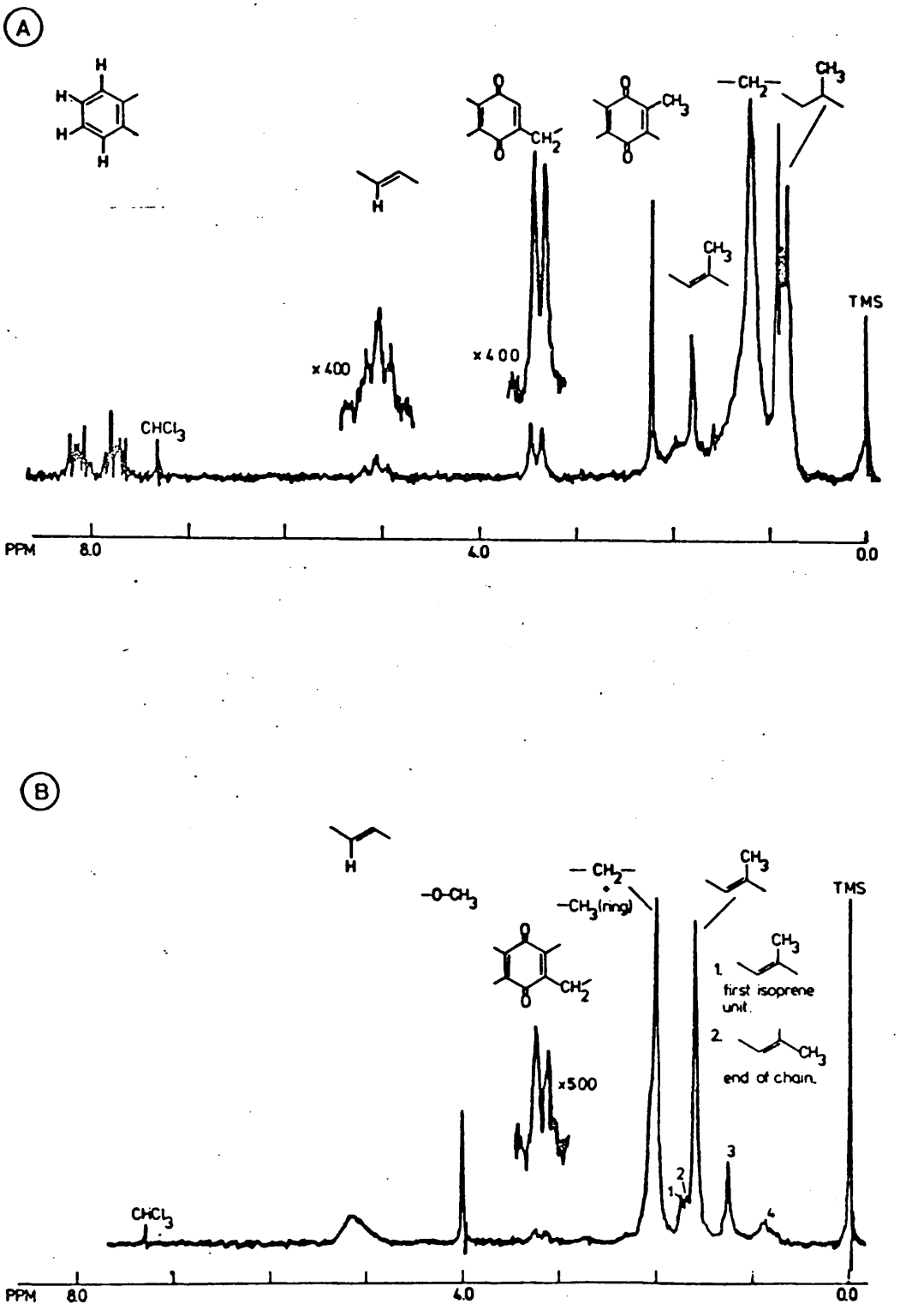


Figure 1-3 NMR spectra of (a) vitamin K and (b) coenzyme Q recorded after the quinones had undergone a reduction--oxidation cycle. For details see text.

This finding throws doubt upon the reversibility of coenzyme Q reduction by RMV.

The absorbances obtained from the more quantitative ultraviolet studies carried out using both vitamin K and coenzyme Q (section 1-3.5) are recorded in tables 5 and 6 respectively.

The first two columns of table 5 show that when a solution of vitamin K is left at room temperature, unprotected from air, for a period of ~12 hours, small changes in optical densities result. For this reason all further comparisons were made relative to the control. It can be seen that the absorbances obtained from bulbs A and B are very similar to each other and to the control. The slightly higher readings obtained at lower wavelengths in column four are attributable to oxygen, for it had been shown previously that prolonged contact of vitamin K solutions in air caused a shoulder to appear at 240nm in the ultraviolet spectrum. Control and treated solutions of vitamin K yielded identical and normal spectra. From an analysis of these results it may be concluded that RMV has reversibly reduced a minimum of 96% of the vitamin K present. These results further demonstrate the water insolubility of reduced vitamin K, since the solutions in both bulbs A and B, after reoxidation of the quinone, contained exactly the same concentration of vitamin K

Table 5 Absorbance of oxidised vitamin K_1 solutions before and after a reduction-oxidation cycle.

λ (nm)	Absorbance of vitamin K solutions			
	UNTREATED		TREATED	
	FRESH	CONTROL	Bulb A	Bulb B
243	0.473	0.464	0.461	0.487
248	0.487	0.482	0.474	0.490
261	0.481	0.464	0.451	0.459
269	0.479	0.456	0.441	0.447
325	0.086	0.082	0.083	0.083

Table 6 Absorbance of oxidised coenzyme Q_{10} before and after a reduction-oxidation cycle.

Equilibration time with Ag_2O (hours)	OD of Coenzyme Q at 270 nm	
	UNTREATED	TREATED
0	0.771	-
2	-	0.715
16	-	0.626

(because they exhibited similar absorbances). No quinol could, therefore, have dissolved in the RMV phase prior to the transfer of solution into bulb B.

The results obtained for coenzyme Q, table 6, show that 93% of the original quinone could be regenerated after two hours equilibration of the quinol with silver oxide. However, as the reaction time was increased the per cent recovery decreased and was accompanied by the concomitant development of an inflexion point at 252nm in the ultraviolet spectrum.

The NMR and ultraviolet spectroscopy results obtained for vitamin K show that RMV radicals react at least 96% reversibly with the quinone centre of vitamin K and remain chemically inert towards the remainder of the molecule. Results from the NMR and ultraviolet studies using coenzyme Q are much more difficult to interpret. The NMR results clearly indicate the presence of a contaminant. An obvious explanation would be that RMV irreversibly interacts with this quinone in some way. However, since strong base is also known to destroy ubiquinone, (76) an alternative explanation would be that this effect was caused, not by the reductant, but by base. Results from the ultraviolet studies suggest yet another explanation. Although it has been reported in the literature (76) that silver oxide regenerates the oxidised form of the coenzyme

cleanly from the corresponding quinol, the results obtained from this research, table 6, do not corroborate this claim. The anomalous NMR spectrum may therefore originate from a side reaction occurring between the silver oxide and the quinone or quinol. The effect is small amounting to <7% after a period of 2 hours and would be difficult to detect for experiments conducted over short periods of time.

The experimental results obtained from immiscibility tests, NMR studies and ultraviolet studies show that RMV and vitamin K possess all the necessary properties required of the aqueous reductant and redox carrier components respectively, of an 'ideal' redox membrane system. In addition, $FMNH_2$ and coenzyme Q may be suitable substitutes for RMV and vitamin K respectively, although additional control experiments would have to be carried out for this to be verified. In view of the proven ideality of RMV and vitamin K, these compounds were incorporated into all future redox systems that were to be subjected to detailed study.

1-4.3 Genesis of Haber-Beutner type redox membranes.

The results of the experiments carried out to find suitable aqueous oxidants for both reduced quinones (section 1-3.6) are given in table 7. Further, since the experimental arrangement used was

RMV (aqueous)/quinone (hexane)/substrate (aqueous)

every system that contained a reacting substrate should be

Table 7 Tests of the reducing abilities of reduced vitamin K_1 and coenzyme Q_{10} with a variety of common substrates dissolved in phosphate buffer (pH 6.86) at 25°C. The system used was,

RMV/quinone(hexane)/substrate

Substrate	E^*_{m7} volts	Experimental Results		Control	Comments
		Reduced vitamin K	Reduced coenzyme Q		
indophenol	0.228	+	+	noticeable colour loss	
DCIP	0.217	+	+	noticeable colour loss	Back transfer to protonated form.
thionine	0.063	+	-	no change	
methylene blue	0.011	+	slight	no change	
$KMnO_4$		+		slight colour loss	Brown precipitate formed. Irreversible oxidation.
$[Fe(o\text{-phen})_3]Cl_3$	1.12	+	(+)	slight colour gain	Back transfer of uncomplex o-phenanthroline.
cytochrome c N.crassa	0.255†	+	(+)		
horse heart	0.255ø	+	(+)		

* Reference (62) except for: † reference (82) and ø reference (83). Temperature 25°C.

( ) Results from reference (81).

considered as a Haber-Beutner type redox membrane, the ideality of the substrate, at this stage, being open to question.

Whereas coenzyme Q was easier to reduce than vitamin K, the reverse order applies when the reducing abilities of the corresponding quinol forms are considered, table 7. In all reactions where vitamin K and coenzyme Q reacted with a common substrate, the former was always found to react at an appreciably greater rate than the latter. In addition, vitamin K was found to react with thiazine dyes (methylene blue and thionine) whereas coenzyme Q displayed little reactivity towards this group of dyes, table 7. This could reflect thermodynamic, as opposed to kinetic, control of the reaction since the midpoint potentials of the two thiazines and that of coenzyme Q are very similar. However, the observations that methylene blue reacts (slowly) with coenzyme Q but thionine does not argue against this since methylene blue has the more negative midpoint potential.

The iron(III) o-phenanthroline complex, a potential model compound for redox heme-type molecules, was reduced by both quinones. However, the hexane solubility of the uncomplexed chelate precluded detailed investigation of this compound. No simple heme-type analogue was found which would be suitable for study and, therefore, the biological heme, cytochrome c, was used in subsequent research.

The noticeable loss in colour intensity of the indophenols during the control experiments is of interest and deserves further discussion. Immiscibility tests, similar to those described in section 1-3.3 were carried out using 2,6-dichlorophenol-indophenol, DCIP. At low concentrations of dye the hexane layer, which had been equilibrated above aqueous solutions of the dye, appeared free of DCIP. However, if very strong solutions of the dye were used (10^{-3}M) the visible spectrum of an orange coloured compound could be obtained. This spectrum was found to be identical to that obtained for the fully protonated (red) oxidised form of DCIP, figure 1-4, dissolved in hexane (λmax = 465nm). This form, being neutral, is very hexane soluble and is continuously extracted into the organic layer. This results in the continuous production of more protonated form in the aqueous layer thus preserving thermodynamic equilibrium (pKa = 5,7). In this way, even though at any given time only 5% (pH 7) of the total DCIP is in the red form, a continuous loss of dye from the aqueous phase results. An approximate value of 20 was obtained for the distribution coefficient of the fully protonated form of the oxidised dye. It is worth noting that DCIP (and indophenol), in both its oxidised and reduced forms, can behave in this fashion, figure 1-4.

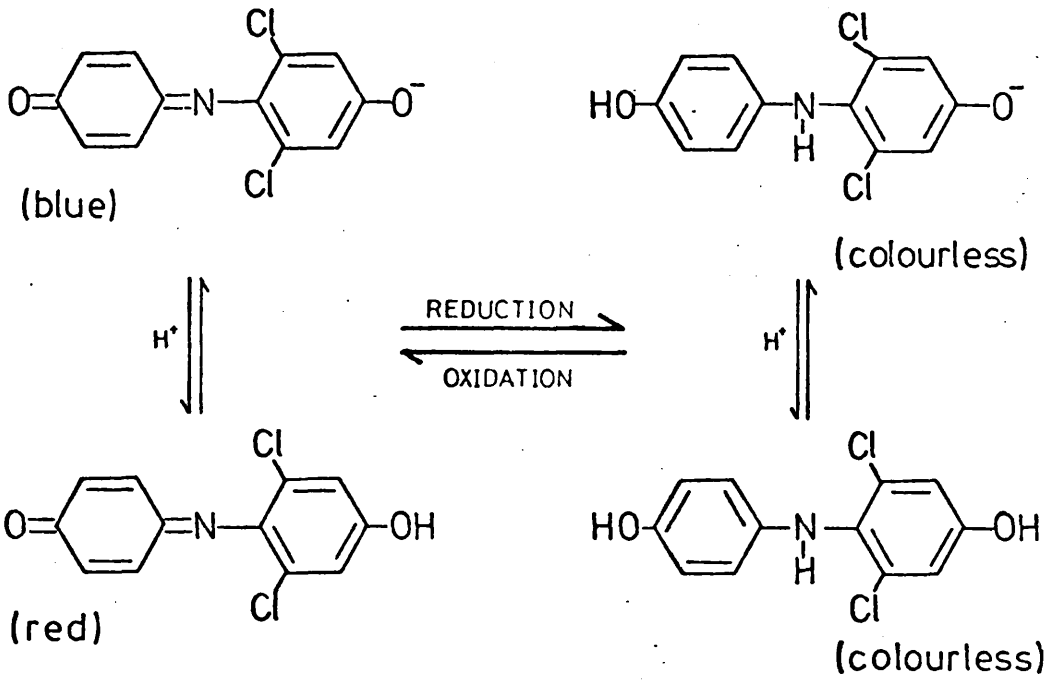


Figure 1-4 Redox states of 2,6-Dichlorophenol indophenol (DCIP) showing the bimodal properties of the molecule in both its redox states.

The bimodal nature of DCIP explains why the compound can function as a lipid soluble electron mediator (84). The observation that succinate dehydrogenase activity via DCIP as acceptor is not affected by the extraction of the lipid (79) could also possibly be explained by the fact that DCIP is capable of penetrating both hydrophobic and hydrophilic environments. Thus, despite the change in polarity around the enzyme's active site, resulting from lipid extraction, DCIP would always manage to reach the site. The behaviour exhibited by the indophenols is exactly analogous to the mode of action of the simple mediators used to generate the redox liposomes discussed earlier.

1-4.4 EPR studies

> "All oxidations of organic molecules, although they are bivalent, proceed in two successive univalent steps, the intermediate state being a free radical"
>
> Michaelis (85)

The EPR spectra recorded from the experiments using RMV/vitamin K, $FMNH_2$/vitamin K and reduced vitamin K/ methylene blue are recorded in figures 1-5, 1-6 and 1-7 respectively. The g factors quoted were calculated using equation (1-1).

As both the oxidised and reduced forms of vitamin K are insoluble in water, a logical assumption would be that their intermediate radicals, if formed, would also behave

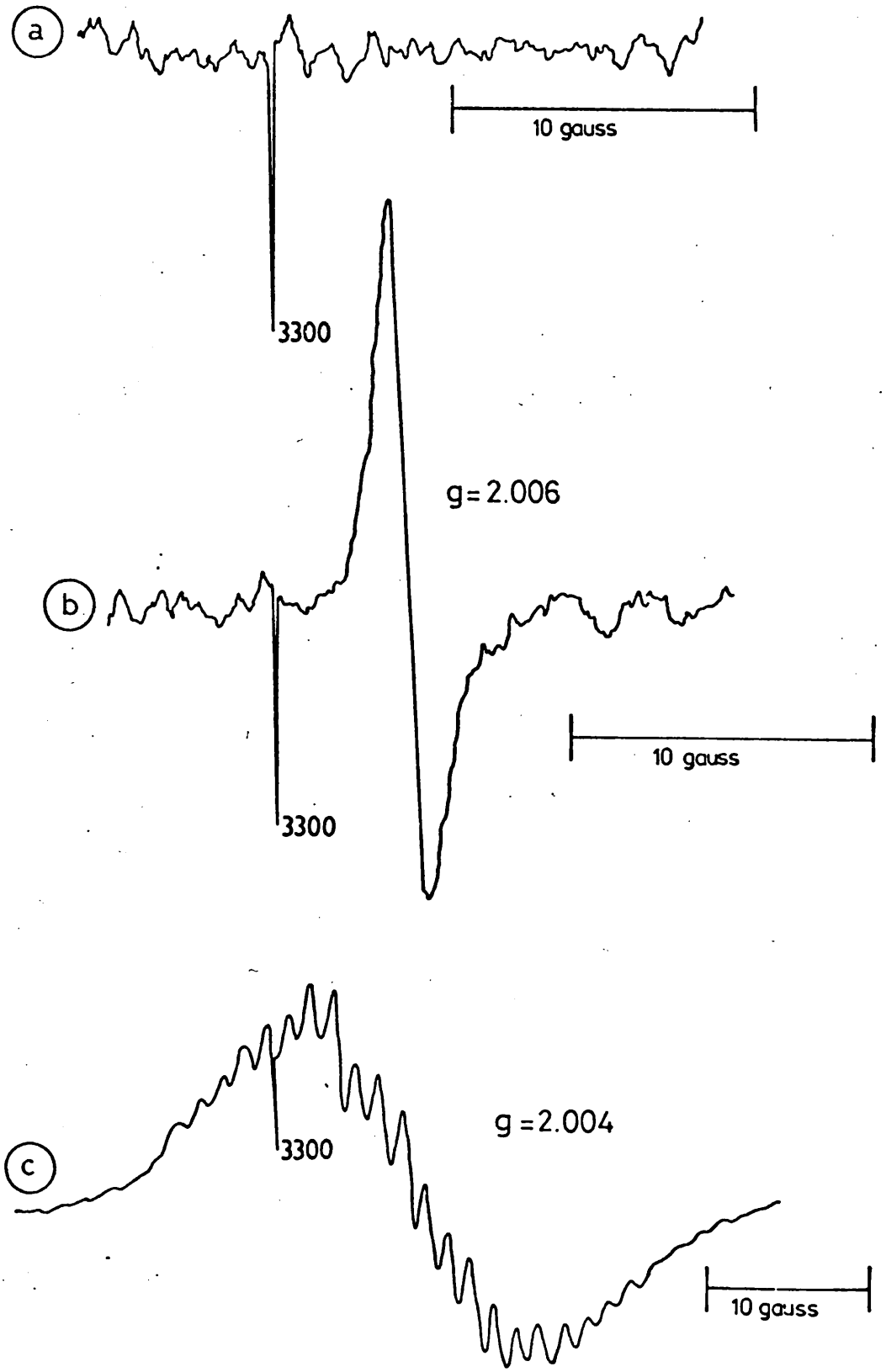


Figure 1-5 EPR spectra of the vitamin K/RMV two-phase system obtained from (a) the bulk hexane phase, (b) the interface and (c) the bulk aqueous phase.

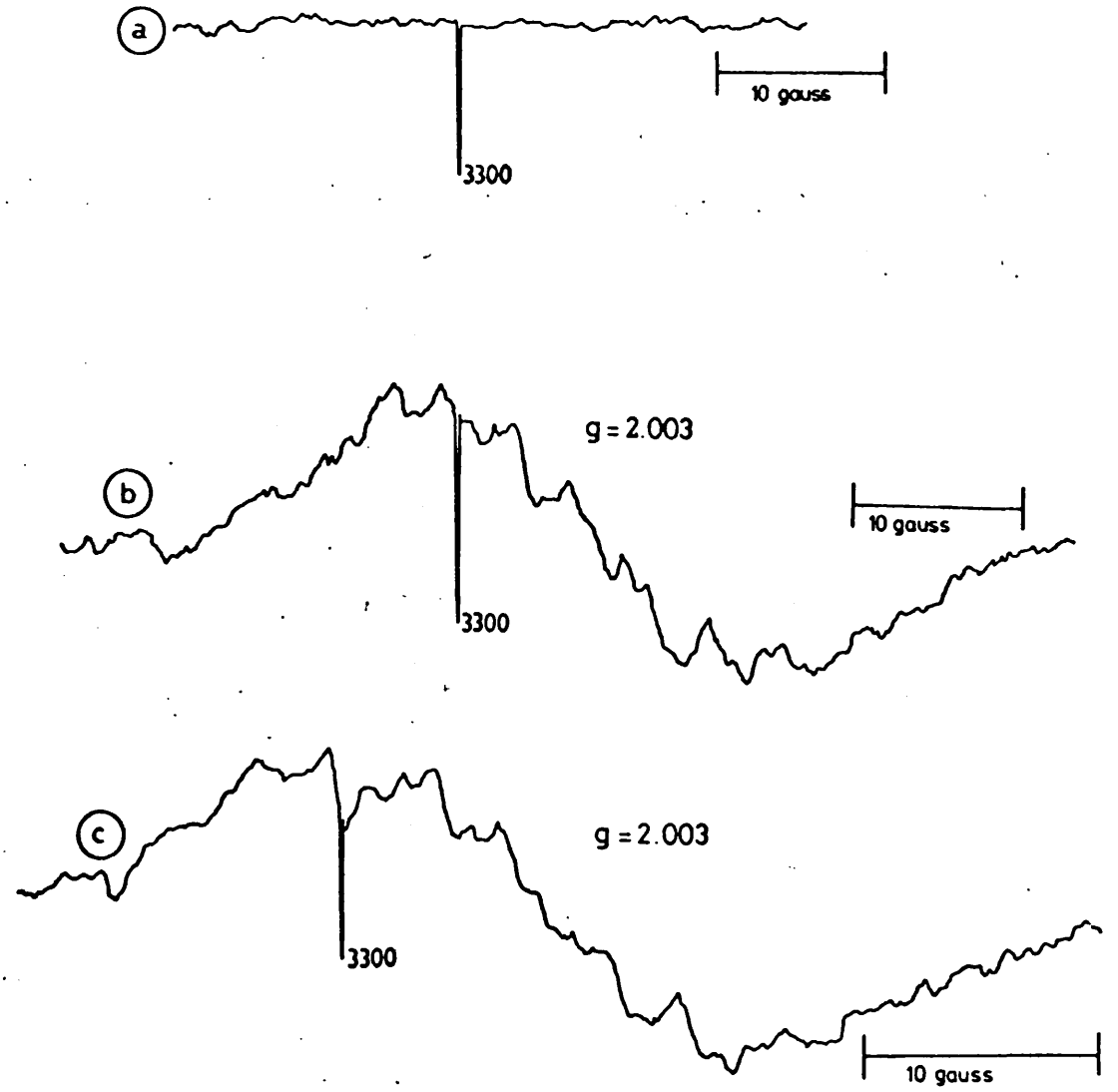


Figure 1-6 EPR spectra of the vitamin K/$FMNH_2$ two-phase system obtained from (a) the bulk hexane phase, (b) the interface and (c) the bulk aqueous phase.

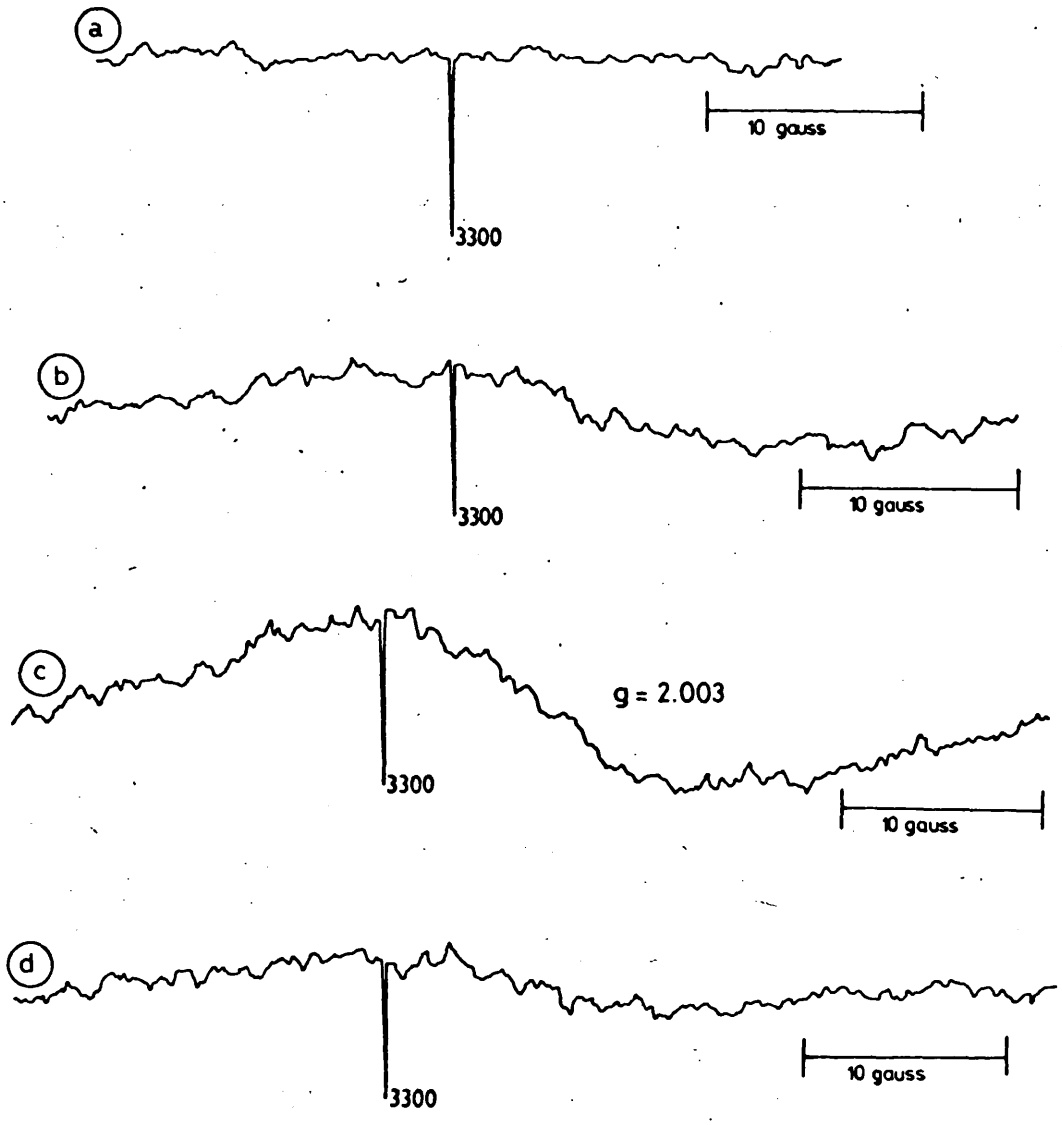


Figure 1-7 EPR spectra of the reduced vitamin K/methylene blue system obtained from (a) the bulk hexane phase, (b) the interface, (c) the aqueous phase just below the interface and (d) the bulk aqueous phase.

similarly. Radicals detected in the bulk aqueous phase were therefore considered as resulting from paramagnetic species derived from the aqueous reactant. Hence, the spectra obtained from the bulk aqueous phases of the RMV/vitamin K, $FMNH_2$/vitamin K and methylene blue/reduced vitamin K experiments were attributable to the methyl viologen cation radical, figure 1-8(a), FMN semiquinone radicals, figure 1-8(b) and to the methylene blue radical, figure 1-8(c). The methyl viologen radical EPR spectrum, figure 1-5(c), was the same as that previously obtained for this radical dissolved in aqueous ethanol (86) with the exception that the broad spectrum obtained in this study (due either to direct dipole or solvent effects), masked the weaker hyperfine interactions observed in the ethanolic solution. The spectra obtained from the FMN radicals figure 1-6(b) and (c) are closely similar to those previously reported (87), (88) for these radicals (at pH 7). No published EPR spectrum of methylene blue radicals could be obtained for comparison. However, potentiometric (89) and (ultraviolet) spectroscopic (90) studies have verified their existence in solution.

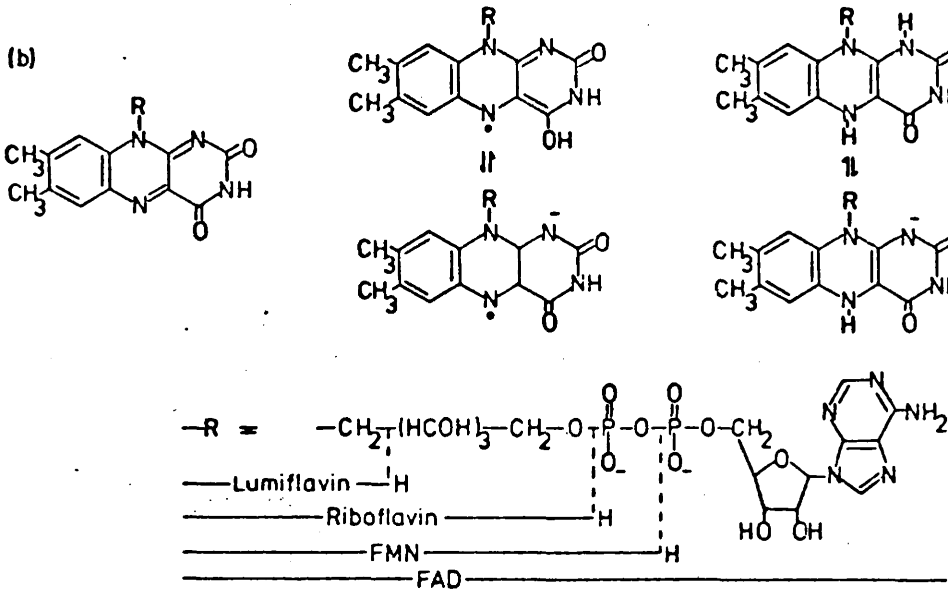
The sharp signal recorded at the RMV/vitamin K interface, figure 1-5(b) indicated that a second radical, different from RMV, was present. The signal for this radical was much weaker than that of RMV and hence the

Figure 1-8 Redox states of (a) methyl viologen (b) flavocoenzymes (c) thiazines and (d) vitamin K.

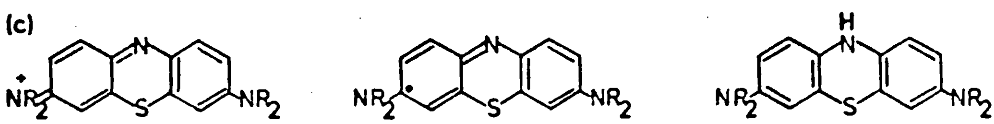
FULLY OXIDISED | INTERMEDIATE RADICAL | FULLY REDUCED

(a)

(b)



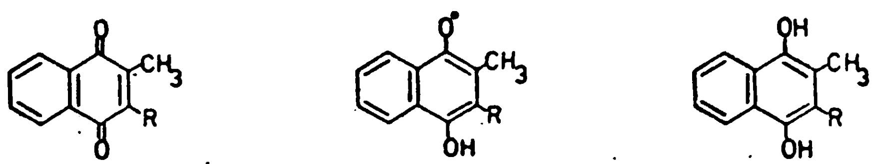
(c)

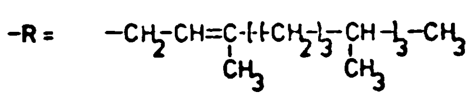


R = -H, Thionine.

R = $-CH_3$, Methylene Blue.

(d)





concentration must, correspondingly, be much less. Reduced methyl viologen and vitamin K were the only two compounds present in the EPR cell which could generate paramagnetic species and, therefore, this new signal must correspond to vitamin K semiquinone radicals, figure 1-8(d). Published spectra of this radical, recorded in a variety of solvents (91), (92), all show a broad signal, exhibiting multiple hyperfine structure, the smallest recorded splittings being in the order of one gauss. Since splittings of this order are detectible with the methyl viologen radical, these hyperfine interactions should also be detectible with the semiquinone. The explanation proposed for the sharpening of the spectrum is that a charge transfer complex was formed between either vitamin K and RMV molecules or between pairs of vitamin K molecules of differing redox states, one of which being the semiquinone. The unpaired electron was considered to be rapidly exchanged between the conjugated rings of the pairs of molecules constituting the charge transfer complex. The observation that no semiquinone radicals are detectible at the interfaces during the other two experiments, figures 1-6(b) and 1-7(b), suggests that either the lifetime of the uncomplexed semiquinone radical is very short, or that its concentration at any given time is much less than that obtained when using RMV as the reactant. Indeed both factors may be important.

The negative result obtained when the bulk hexane layer of the RMV/vitamin K experiment was studied, figure 1-5(a) is consistent with the semiquinone radical having only a short lifetime. The absence of detectible concentrations of radicals in this layer further demonstrates the hexane immiscibility of the RMV radical.

Analogous EPR experiments using coenzyme Q/RMV and coenzyme Q/$FMNH_2$ biphasic systems have also been studied (81). These systems were found to yield spectra similar to those obtained from the corresponding vitamin K experiments described in this research.

These EPR experiments clearly demonstrate the presence of radicals in the aqueous phase and therefore support the hypothesis that vitamin K prefers to react biphasically with one-electron agents. The fact that methylene blue, figure 1-7(c), (d), thionine (89) and indophenol (93) radicals have all been shown to be present in aqueous solutions of the partially reduced dyes, indicates that reduced vitamin K may also display a similar preference to one-electron agents. The flavin mononucleotide solutions used in the EPR studies contained 80% reduced FMN. For this reason the presence of radicals in solution does not necessarily imply that they are involved in the reaction with vitamin K, since mixtures of FMN and $FMNH_2$ are known to generate radicals spontaneously by the formation of a charge transfer complex between the reduced

and oxidised forms (94), (95). The fact that FMN radicals are detected in the bulk aqueous phase supports this argument, figure 1-6(c). Similar caution must be applied to the methylene blue results, figure 1-7(c), (d).

The possible involvement of a charge transfer intermediate between vitamin K and RMV is of interest since charge transfer complexes have been proposed to have important roles in nature (96). Although no such complex could be detected when $FMNH_2$ replaced RMV, figure 1-6(b), the fact that flavins are avid complex formers (95) and that riboflavin forms a charge transfer complex with p-hydroquinone (96) and lumiflavin forms one with bis-naphthalene-2,3diol (98) suggests that a charge transfer complex between $FMNH_2$ and vitamin K might well be formed. It is of interest that charge transfer complexes between vitamins K and flavins have previously been postulated as being possible intermediates in biologically important electron-transfer reactions (99). As discussed in section 1-2, the fact that no vitamin K semiquinone resonances were detectible in figures 1-6(b) and 1-7(b) does not necessarily preclude the involvement of these radicals during reaction. It is of further interest to note that symmetrical EPR signals similar to that shown in figure 1-5(b), having a g value of 2.003 and a line width of ~10 gauss, have been obtained from frozen respiratory membranes. These results have been attributed to the presence of small

quantities of ubisemiquinone within the membranes (100), (101). It may be, therefore, that isoprenoid quinones located at either a RMV (aqueous)/hexane interface or an aqueous/respiratory membrane (lipid) interface, behave in an analogous manner, transferring as Michaelis' general postulate predicts, electrons according to two successive univalent steps.

1-4.5 Further aspects of biological relevance.

In addition to those biological aspects discussed earlier it is convenient to conclude this chapter by comparing the general scheme of electron transport found in these studies with that occurring in respiratory membranes.

The widely accepted scheme for the electron-transport system in mammalian mitochondria (79), showing the proposed site of coenzyme Q, is given in figure 1-9(a). As shown in this figure, ubiquinone appears to transfer electrons from the flavoprotein dehydrogenases, F_p (and their associated nonheme iron) to cytochrome b. These flavoproteins contain either FMN or FAD as their prosthetic groups. A similar scheme, involving one of the K vitamins (actually vitamin K_2, figure 0-5 (n = 9), in which one of the double bonds in the side chain is saturated), for the electron transport system of Mycobacterium phlei (M. phlei) is given in figure 1-9(b) (102). Since vitamin K_1 could act

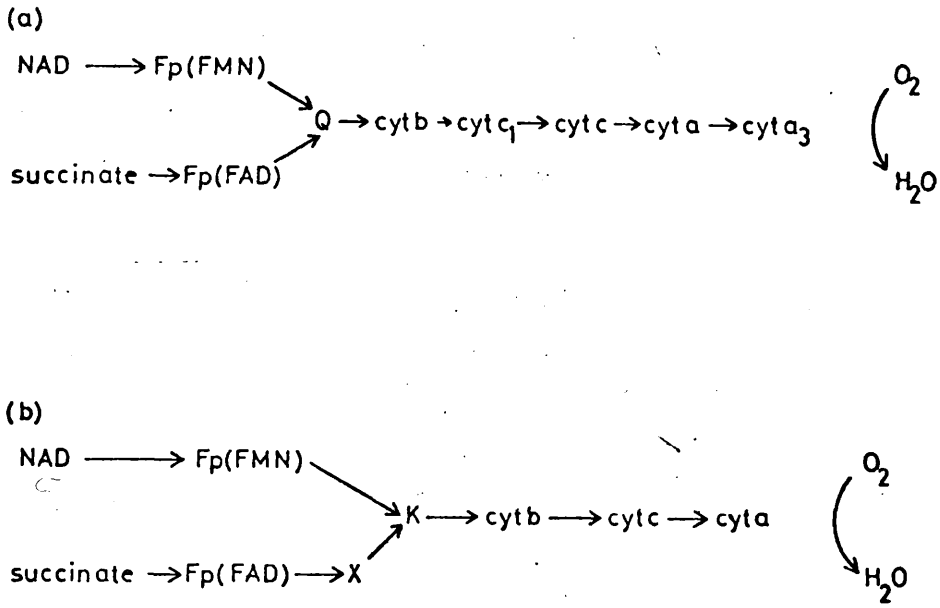


Figure 1-9 Electron transport sequences (a) in beef-heart mitochondria showing the proposed site of coenzyme Q and (b) in M. phlei showing the site of vitamin K (X is a factor destroyed by ultraviolet light).

as a substitute for the endogenous vitamin (103), this scheme may be considered as applying directly to vitamin K.

Of particular relevance to the role of coenzyme Q and vitamin K in natural membranes were the observations that, although aqueous solutions of NADH and NADPH were unable to reduce the quinone directly, they may do so indirectly by a stepwise reduction involving FMN (or FAD) as an essential intermediate, table 4. These results, therefore, indicate that the limiting kinetic factors intrinsic to the electron-transfer process within these biological systems are already present in this simple analogue even though the enzymes, necessary to carry out these reactions at more favourable rates, are absent. This may be a fortuitous coincidence however, since at the substrate interface ubiquinol and dihydrovitamin K were both found to react directly with soluble cytochrome c, table 7.

Chapter Two

QUANTITATIVE ULTRAVIOLET/VISIBLE SPECTROSCOPY

2-1 THEORY

2-1.1 General description

If electromagnetic energy is allowed to impinge upon a transparent medium, some is reflected, some absorbed and the remainder transmitted. When the transmitted radiation is passed through a device (prism or grating) capable of resolving the beam into its constituent wavelengths, an absorption spectrum is obtained. This spectrum indicates those portions of the incident beam that have been absorbed by the medium. Electronic spectra arise from transitions between electronic energy levels (of outer shell electrons) accompanied by changes in both vibrational and rotational states. However, when solutions are examined, the fine structure, due to the accompanying vibrational and rotational transitions, is generally not resolved due to solvent-solute interactions which tend to smooth out the spectrum into

bands. The difference in energy between electronic energy levels is relatively large (typically 400,000 $Jmole^{-1}$). Virtually all electronic transitions, therefore, occur in the visible (400-700 nm) and ultraviolet (10-400 nm) regions of the electromagnetic spectrum. Only in those collisions between quanta of radiant energy and molecules, where the energy of the photon exactly equals the difference in energy, ΔE, between the ground and excited states, will absorption of energy occur:

$$\Delta E = h\bar{c}/\lambda \qquad (2\text{-}1)$$

where $\bar{c}$ is the speed of light. While it is not always possible to interpret the nature of the energy levels of the electronic states between which these transitions occur, it has been recognised since the early days of spectroscopy that organic compounds containing certain groups of atoms within their molecules showed distinctive absorption bands in the near ultraviolet and visible regions of the spectrum. These groups, or chromophores, are mostly of a polar or unsaturated nature. The wavelengths and intensities of maximum absorption are quite characteristic of the chromophoric group concerned.

The principle characteristic of radiation affecting an electronic transition is the energy content, equation (2-1). In addition, the symmetry of the molecule being irradiated introduces a further selectivity. Thus, although a particular transition may be energetically

favourable, it is not always observed, or else the intensity of such a band is much less than might be anticipated. Theoretically forbidden transitions are sometimes exhibited because the molecular symmetry is distorted by molecular vibrations. In such cases the molar extinction coefficient is normally less than 1000.

Electronic spectra of organic substances in solution commonly obey the Beer-Lambert Law, equation (2-2). This law mathematically expresses the relationship between the amount of light absorbed, the concentration of the solution and the length of solution through which the light passes:

$$\log (I_o/I) = \epsilon c \ell \qquad (2\text{-}2)$$

where I is the intensity of transmitted light, I_o is the intensity of the incident beam, ϵ is the molar extinction coefficient (a property of the compound under investigation at a given wavelength), c is the concentration in moles litre^{-1} and ℓ is the path length expressed in centimetres (105). The value of $\log (I_o/I)$ is referred to as the absorbance or optical density (OD). The absorbance is normally measured at a wavelength where ϵ has a maximum value since the change in absorbance for a given change in concentration, (which determines the analytical sensitivity), is greatest at this point. Additionally, measurement on the side of a band, where the absorbance changes rapidly as a function of wavelength,

renders the measurement very sensitive to errors in the wavelength scale of the instrument.

The graph of absorbance of a simple solution against its concentration is normally a straight line, in accordance with Beer's Law. In practice, the graph may deviate, positively or negatively, from a straight line at higher concentrations. Deviations from ideal behaviour may be due to complex formation with the solvent, polymerisation, or change of pH as the concentration increases.

Since the absorbance of a substance is proportional to its concentration, spectrophotometry can be used as an analytical tool or as a method of monitoring the kinetics of a reaction: if a material has a characteristic absorption band within the spectral range of the instrument, or can produce, in a quantitatively reproducible chemical reaction, a species which does absorb in this range, it can be analytically determined, using equation (2-2). Similarly, the monitoring of the change in optical density of a reactant or product during a chemical reaction may be used to measure the corresponding change in concentration occurring during the reaction. A single analytical wavelength is chosen to be one at which absorption of the component is reasonably free from interference by other reactants. A prior knowledge of ϵ is, of course, essential. Both of these applications of ultraviolet/

visible spectrophotometry have been used extensively throughout this research.

2-1.2 Binary conjugate systems

The analysis of multicomponent mixtures is more complex since the absorptions of the individual components are superimposed and a change in the concentration of one component may affect the absorbance value at the wavelength where another component has an absorption maximum. Fortunately, binary mixtures were the only type of multi-component system used in this study. An additional simplification arose because these two components were different redox or protonated states of the one common substance. They may therefore be considered as conjugate pairs. Since the absorbance of the mixture is the arithmetic sum of the absorbancies of the two individual components, providing there is no interaction between them, the Beer-Lambert Law can be written:

$$A_l = (\epsilon_l{}^x c^x + \epsilon_l{}^y c^y)\ell \qquad (2\text{-}3)$$

where A_l is the observed absorbance at wavelength l, $\epsilon_l{}^x$ is the extinction coefficient of component x at this wavelength ....etc. If the total concentration of the substance, c, remains constant through the experiment, equation (2-4) must always be valid:

$$c = c^x + c^y \qquad (2\text{-}4).$$

Combination of equations (2-3) and (2-4), together with the assumption that the path length is 1 centimetre, gives equation (2-5),

$$c^x = \frac{\epsilon_1^y c - A_1}{\epsilon_1^y - \epsilon_1^x} \qquad (2\text{-}5)$$

If the total concentration of the compound in solution is known, together with the extinction coefficients of the two conjugate forms, then, from the observed optical density, the concentration of the individual components in solution can be calculated using equations (2-5) and (2-4).

2-2 GENERAL EXPERIMENTAL ASPECTS

2-2.1 Instrumentation

All fixed wavelength measurements carried out throughout this study, unless otherwise indicated, were determined using a Hilger H700 'Uvispek' spectrophotometer. The optical system contained in this single wavelength instrument is outlined in figure 2-1. Light from either side of the stabilised lamps enters the monochromator slit and is dispersed into a spectrum, a selected portion of which passes through a second slit. Selection of the spectral region is made by rotating the dispersing prism through a small angle. The light from the exit

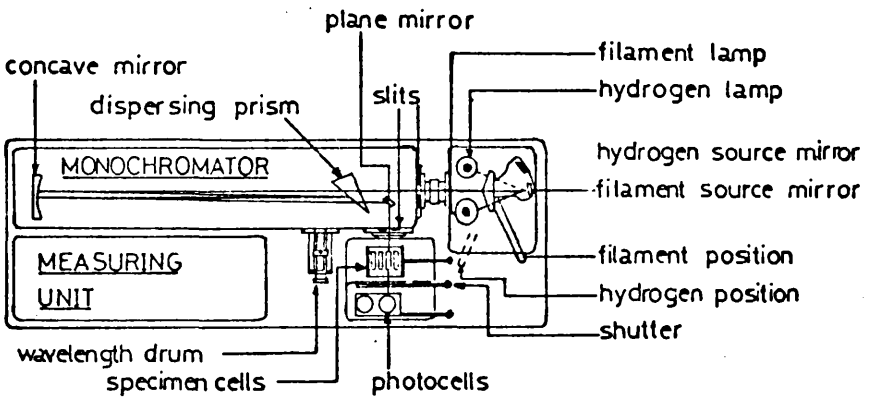


Figure 2-1 The optical system of a Hilger H700 'Uvispek' spectrometer.

slit of the monochromator then passes through a cell of solution on its way to a photocell. The output of the latter (proportional to the radiation it receives) passes to an electronic measuring unit where it can be balanced by an opposing electro-motive force from a calibrated potentiometer. A galvanometer indicates when the opposed potentials are equal. Optical densities in the range 0-3 can be measured to an accuracy of $\pm$0.001. In this study, however, solutions with absorbances below 1.0 were always employed.

Ultraviolet/visible spectra of all the compounds studied were obtained using Unicam SP8000 and SP800 dual wavelength recording spectrophotometers. For all wavelength maxima determinations the former instrument was employed and the wavelength readings calibrated using holmium (200-640 nm) and didymium (570-800 nm) filters.

2-2.2 Solvents

The aqueous solvents used throughout all the ultraviolet/visible studies have been previously described (section 1-3.1).

The hexane employed as a solvent was either commercially available spectroscopic hexane or hexane purified to give an absorbance low enough for use in spectroscopic work. The latter was achieved by passing hexane through a column of chromatographic silica gel (Silica Gel Ltd.,

England), previously heated to 350°C (106). For consistency, the same type of spectroscopic hexane was used throughout any one set of measurements.

2-2.3 Sample cells

The sample cells used throughout this research were 'Spectrosil' (synthetic silica) ultraviolet/visible cells (Thermal Syndicate Ltd., England) of 1 cm path length.

Glass surfaces can rapidly adsorb contaminants and thereby exhibit a decrease in optical transmission. It was important, therefore, that cells were cleaned immediately prior to use. The following cleaning procedure, recommended by the manufacturer, was adopted: Each cell was left immersed overnight in freshly prepared chromic acid (potassium dichromate dissolved in concentrated nitric acid). The cells were copiously rinsed with first tap and then distilled water, given a final rinse in acetone and dried in an oven at 40-50°C.

During measurements, care was taken to ensure that the optical faces of the cells were kept free from contamination and that the radiation was always incident upon a given face. After having determined the cell bias at the start of an experiment, care was always taken to ensure that the same reference and sample cells were used during subsequent measurements.

2-3 EXPERIMENTAL DETERMINATION OF EXTINCTION COEFFICIENTS

2-3.1 2,6-Dichlorophenol Indophenol

An approximately 10 mM solution of DCIP was made by dissolving the solid in pH 6.86 phosphate buffer. The solution was filtered and the resulting stock solution standardised against a standard ascorbic acid solution (107), equation (2-8). Due to the autoxidisable nature of the leuco form of the dye, it was found necessary to carry out the titration under a nitrogen atmosphere, a precaution reported as being unnecessary. The ascorbic acid solution was standardised against iodine, generated 'in situ' by the addition of excess potassium iodide to a known volume of a standard potassium iodate solution in an acidic (~0.5M HCℓ) medium, equations (2-6) and (2-7). No indicator was used since starch has an inhibitory effect on the reaction (108). The standard iodate solution was prepared by dissolving a weighed amount of the pure compound in distilled water and diluting to a definite volume (104).

$$IO_3^- + 5I^- + 6H^+ \rightleftharpoons 3I_2 + 3H_2O \qquad (2\text{-}6)$$

$$I_2 + C_6H_8O_6 \rightleftharpoons C_6H_6O_6 + 2H^+ + 2I^- \qquad (2\text{-}7)$$

$$C_6H_8O_6 + \text{DCIP} \rightleftharpoons C_6H_6O_2 + \text{leuco DCIP} \qquad (2\text{-}8)$$

Accurate volume dilutions of the stock solution were made using pH 6.86 buffer and the optical density

of each of these solutions measured at the wavelength where the compound displayed maximum absorption.

2-3.2 Methylene blue

The commercially available sample of the dye was recrystallised from water before use. An approximately 10 mM stock solution of methylene blue (MB) was made by first dissolving the dye in distilled water and then filtering the resultant solution.

The stock solution was standardised by carrying out a gravimetric analysis (109), based on the formation of an insoluble complex between methylene blue and dichromate, equation (2-9). A 50 ml aliquot of the stock and a 25 ml aliquot of a 0.02M potassium dichromate solution were pipetted into a 100 ml volumetric flask and the volume adjusted to the mark with distilled water. After 5 minutes of occasional shaking, the resultant mixture, composed of the solid methylene blue complex and excess dichromate in solution, was carefully transferred to a 250 ml beaker, using more dichromate solution to transfer precipitate adhering to the flask and stopper. The reaction mixture was heated to 75°C for a further 5 minutes, allowed to cool and the precipitate quantitatively transferred to a previously weighed, porosity 3, Gooch crucible, again using dichromate solution to ensure quantitative transfer of material. The precipitate was

washed with 10 ml of cold water, dried at 100°C for 1 hour and for further half-hour periods until constant weight readings were obtained.

$$2MB^{+} + \text{excess } Cr_2O_7^{2-} \longrightarrow (MB)_2\, Cr_2O_7 + Cr_2O_7^{2-} \qquad (2\text{-}9)$$

Solutions of methylene blue in pH 6.86 buffer were obtained by volume dilution of the stock using phosphate buffer. The optical densities of these solutions were determined at the two wavelengths where absorption maxima are found.

2-3.3 p-Nitrophenol

A stock solution of p-nitrophenol, 85.4 mM, was prepared by dissolving a known weight of the compound in 0.1M NaOH. Volume dilutions of this solution were carried out using 0.1M HCℓ and 0.1M NaOH as solvents to give standard solutions of the fully protonated and fully dissociated forms of the compound respectively. The optical densities of all the standard solutions were measured at the wavelengths where the dissociated and undissociated forms of the acid displayed individual absorption maxima.

2-3.4 Oxidised vitamin K

A quantity of vitamin K was left overnight in a desiccator, containing phosphorus pentoxide, to remove traces of water. A known weight of the oil was then transferred into a volumetric flask and diluted to the

mark with hexane. Solutions of suitable optical densities were obtained by volume dilution. The absorbance of each solution was determined at the five wavelengths at which the quinone exhibits an absorption maximum.

2-3.5 Reduced vitamin K

Solutions of oxidised vitamin K in hexane, of concentration suitable for ultraviolet studies, were prepared by taking approximate dilutions of a stock solution. The concentration of each of these solutions was determined by firstly measuring its optical densities at the four ultraviolet wavelengths where the quinone exhibits absorption maxima and then by applying equation (2-2), using the previously determined ε values.

Aliquots of the standardised vitamin K solutions were reduced in the special vacuum line cell shown in figure 1-1(a), employing the experimental technique outlined in section 1-3.2. Reduced methyl viologen, 25 mM, was used as the aqueous reductant. Spectra of the fully reduced quinone were recorded on a Unicam SP8000 spectrometer. Due to the time taken to generate the quinol form, only one spectrum could be recorded per day. To allow correction to be made for the day-to-day absorbance accuracy deviations of the spectrometer the spectrum of a dichromate solution, 60 mg per litre of 0.005M sulphuric acid, was additionally recorded. A baseline was recorded

on each spectrum using matched cells containing pure solvent. The bias of these cells towards the vacuum line cell was determined experimentally. The difference between the absorbance readings from the spectrum and the baseline at any given wavelength gave the uncorrected absorbance of the sample at that wavelength.

Absorbance accuracy corrections were easily calculated from an analysis of the dichromate spectrum. The absorbance readings obtained at the two maxima, 257 and 350 nm, and the two minima, 235 and 313 nm, on each spectrum were compared with the expected ones of 0.869, 0.644, 0.748 and 0.293 respectively. The observed deviations were plotted as a function of wavelength and from the resulting calibration graph the appropriate correction at any wavelength within this range, could be obtained. The deviations measured never amounted to more than 5% of the observed optical density.

The true optical density readings for reduced vitamin K at each wavelength under consideration were found by sequentially applying two corrections to the uncorrected absorbances. Firstly absorption accuracy corrections were determined and applied and, secondly, cell bias corrections, which took account of the bias existing between the vacuum line cell and the two matched cells used to record the baseline, were applied to these readings.

2-3.6 Experimental results

The wavelengths at which each compound was found to exhibit an absorption maximum, together with the extinction coefficients of the compounds at these maxima, are recorded in tables 8 and 9. Also included in these two tables are previously reported ε values. The graphs of optical density against concentration obtained with methylene blue and reduced vitamin K are shown in figures 2-2 and 2-4 respectively. The absorption spectra of p-Nitrophenol, obtained using acidic, neutral and basic solutions, are given in figure 2-3. The spectrum obtained from a solution of vitamin K, before and after its reduction, is given in figure 2-5.

2-4 EXTINCTION COEFFICIENTS USED DIRECTLY FROM LITERATURE SOURCES

2-4.1 Oxidised and reduced coenzyme Q

Values for the extinction coefficients of coenzyme Q in hexane could not be found. However, they have been determined for the quinone dissolved in light petroleum (80). Since the wavelength maxima quoted agreed with those experimentally obtained in this research when hexane was used as solvent, the extinction coefficient values of 15100 at 270 nm for the oxidised form and 4470 at 291 nm

Table 8 Quantitative ultraviolet/visible data for aqueous solution of DCIP, methylene blue and p-nitrophenol.

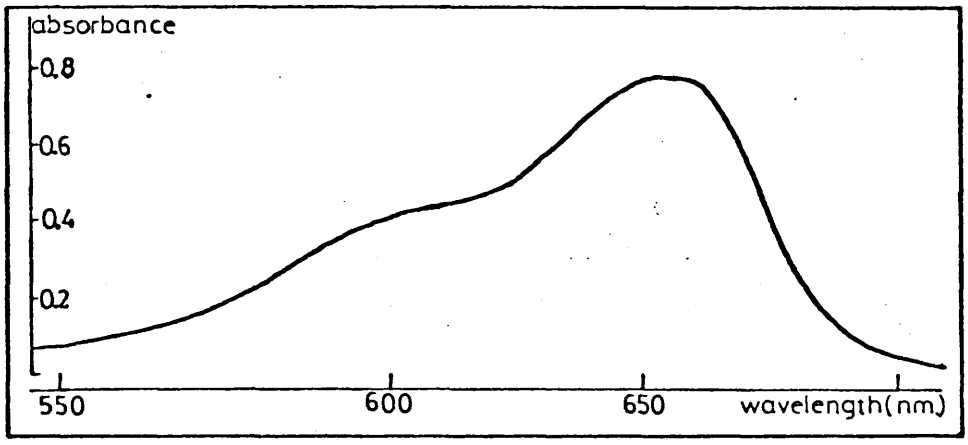
Compound	Solvent	λ^{*}_{max} (nm)	ε	log ε	Literature λ^{*}_{max}(log ε)	Source
DCIP	phosphate buffer, pH 6.86	605	21,000	4.32	600(4.32), pH 7.6 600(3.8), pH 6.1	(110) (111)
Methylene blue	phosphate buffer, pH 6.86	655 605	69,800 40,800	4.84	660(4.7), 605(4.7), H_2O	(112)
p-Nitrophenol	0.1M HCℓ 0.1M HCℓ	318 (403)	10,300 80	4.01 1.90	318(3.99), 0.1M HCℓ 317(3.99),(407(~0)), pH 4	(113) (114)
	0.1M NaOH 0.1M NaOH	(318) 403	1,460 19,200	3.16 4.28	(317(3.14)),407(4.26), pH 9 402(4.23), 0.1M NaOH	(114) (113)

* Values in parentheses do not correspond to absorption maxima.

Table 9 Quantitative ultraviolet data for hexane solutions of vitamin K_1 and dihydrovitamin K_1

Redox state vitamin K	λmax/(λ)	ε	log ε	Literature* λmax (ε)
oxidised	243	18,800	4.27	243 (18,700)
	248	19,600	4.29	249 (20,000)
	261	18,600	4.27	260 (18,000)
	269	18,600	4.27	270 (18,000)
	325	3,400	3.53	325 (3,200)
reduced	(243)	43,500	4.64	
	245	44,500	4.65	
	(248)	40,400	4.61	
	325	5,400	3.73	

* From reference (115)



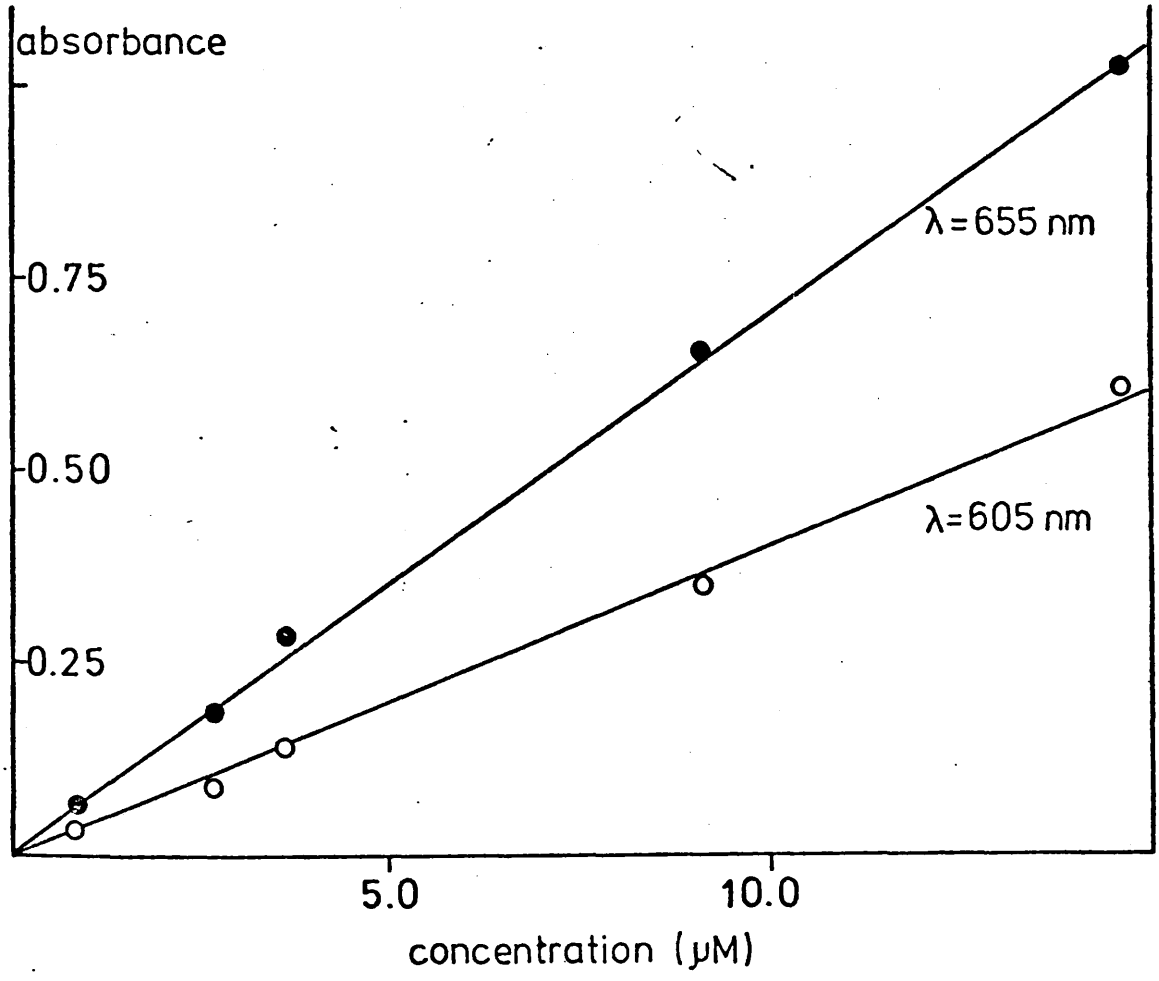


Figure 2-2 Optical density of methylene blue, dissolved in 0.025M phosphate buffer (pH 6.86), as a function of concentration. Insert shows the visible absorption spectrum of a 10μM solution.

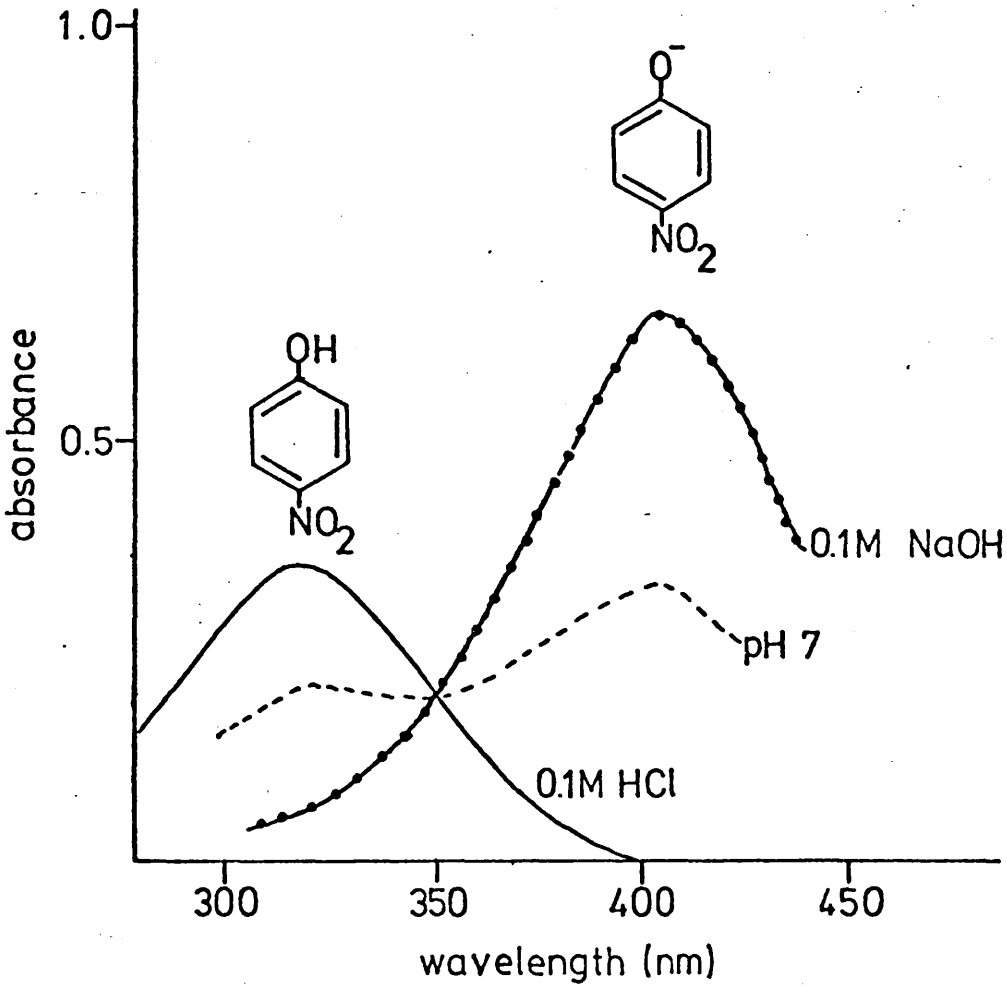


Figure 2-3 Spectra of a (36μM) p-nitrophenol in acid, base and phosphate buffer. The structure of the two conjugate forms of this weak acid is also included.

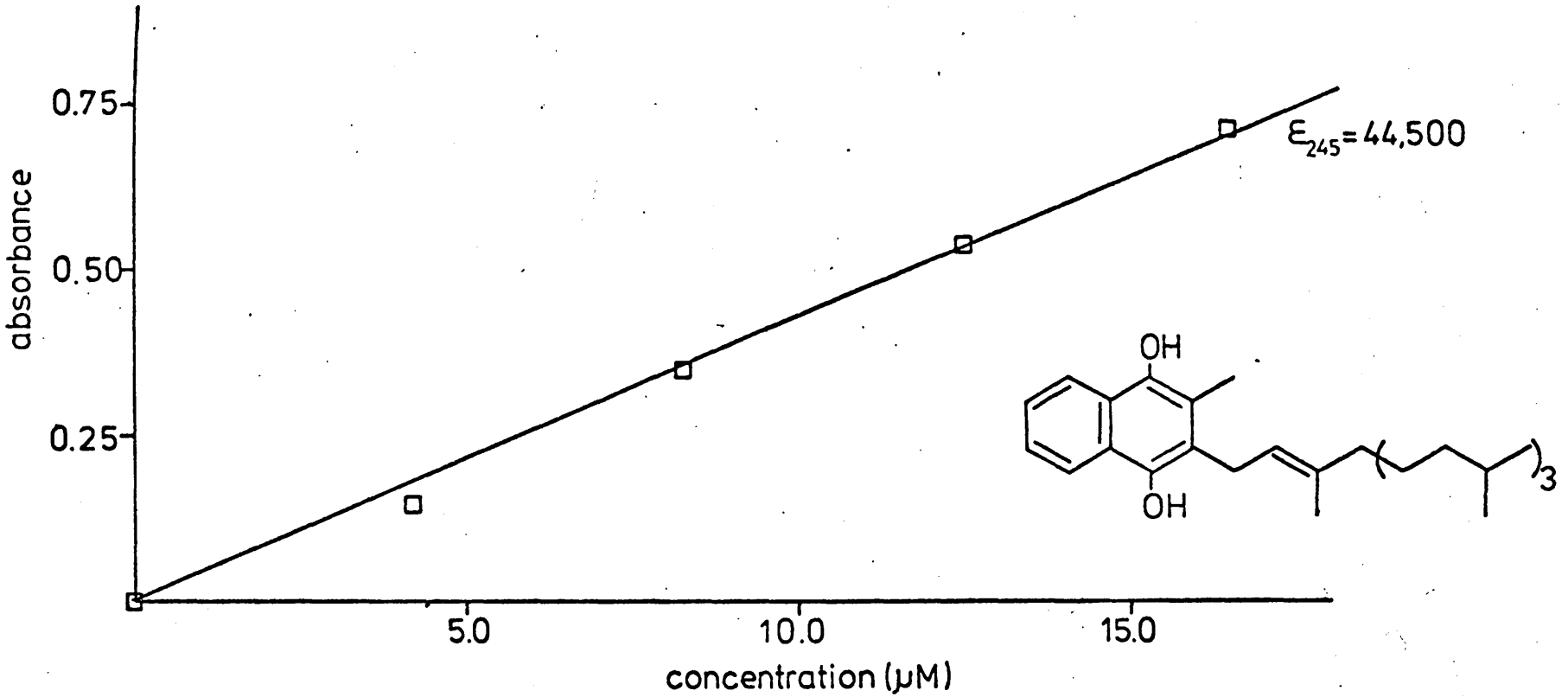


Figure 2-4 Optical density of reduced vitamin K_1 in hexane as a function of concentration.

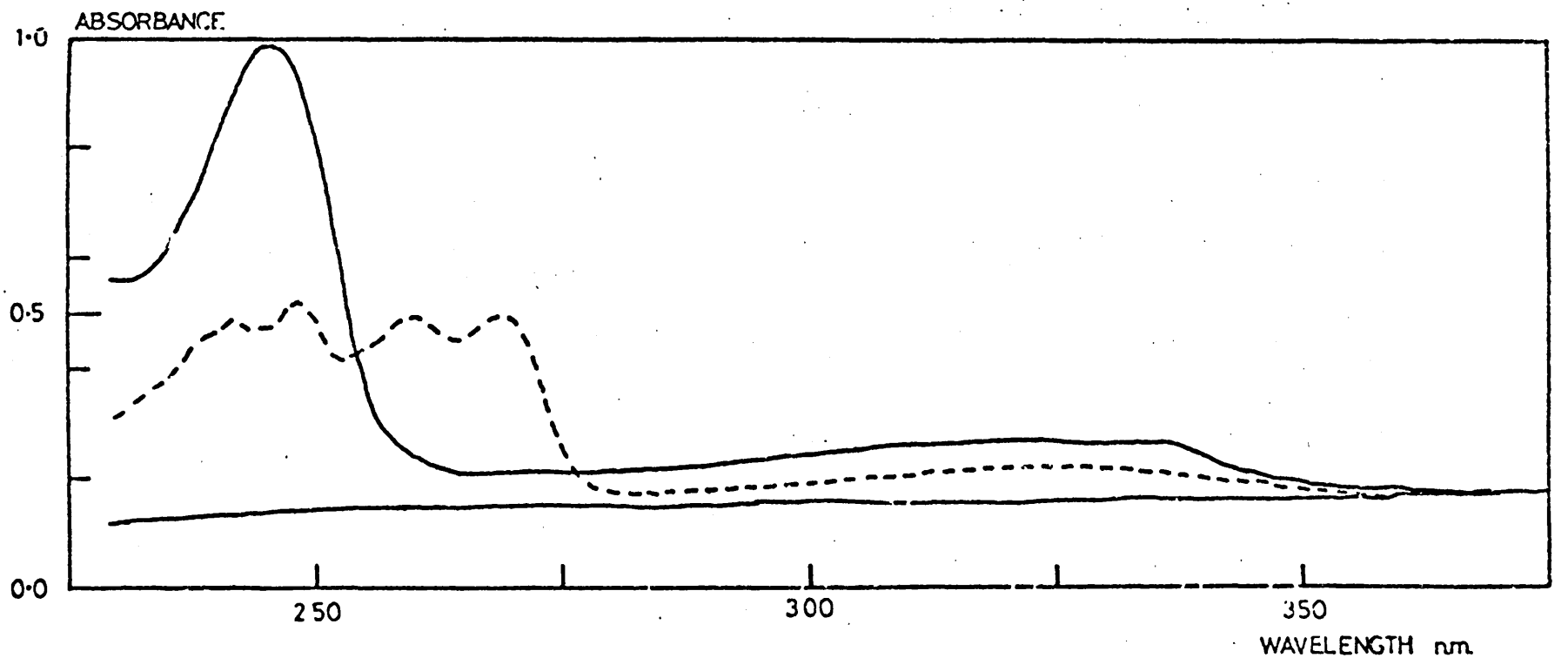


Figure 2-5 Ultraviolet absorption spectrum (in hexane) of reduced vitamin K_1 ——— and oxidised vitamin K_1 - - -.

for the reduced form, were considered suitable for use. The spectra of both the oxidised and reduced forms of coenzyme Q are shown in figure 2-6.

2-4.2 Oxidised and reduced cytochrome c

Reduced cytochrome c, ferrocytochrome c, exhibits a typical double banded hemochrome-type spectrum in the visible part of the spectrum with maxima at 550 nm (α-band) and 521 nm (β-band). In addition, a stronger absorption at 416 nm (Soret or γ-band) is also observed. Oxidised cytochrome c, ferricytochrome c, displays two maxima in the visible region of the spectrum, at 529 nm and 410 nm (Soret or γ-band), figure 2-7, (82).

The reduction of ferricytochrome c can be conveniently monitored at 550 nm. Provided that the extinction coefficients for both ferrocytochrome and ferricytochrome c are known at this wavelength, the concentration of each form may be obtained using equation (2-5). The most reliable values for the molar extinction coefficients of the reduced and oxidised forms at 550 nm (α-band) have been judged (82) to be 2.95×10^4 and 0.84×10^4 respectively, (116). These values were used in the current research.

2-4.3 Thionine

Thionine, when dissolved in phosphate buffer, exhibits a visible absorption spectrum which is similar to that observed for methylene blue, the presence of both monomer

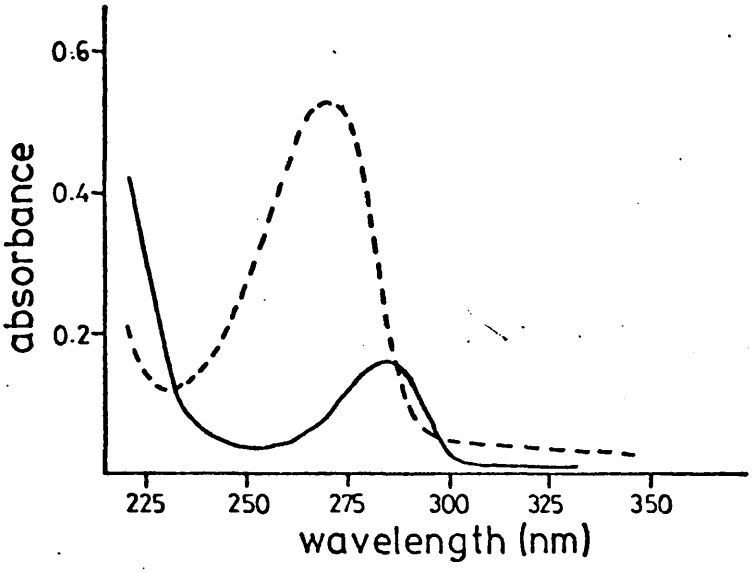


Figure 2-6 Spectra of oxidised ---- and reduced ——— coenzyme Q_{10} dissolved in hexane.

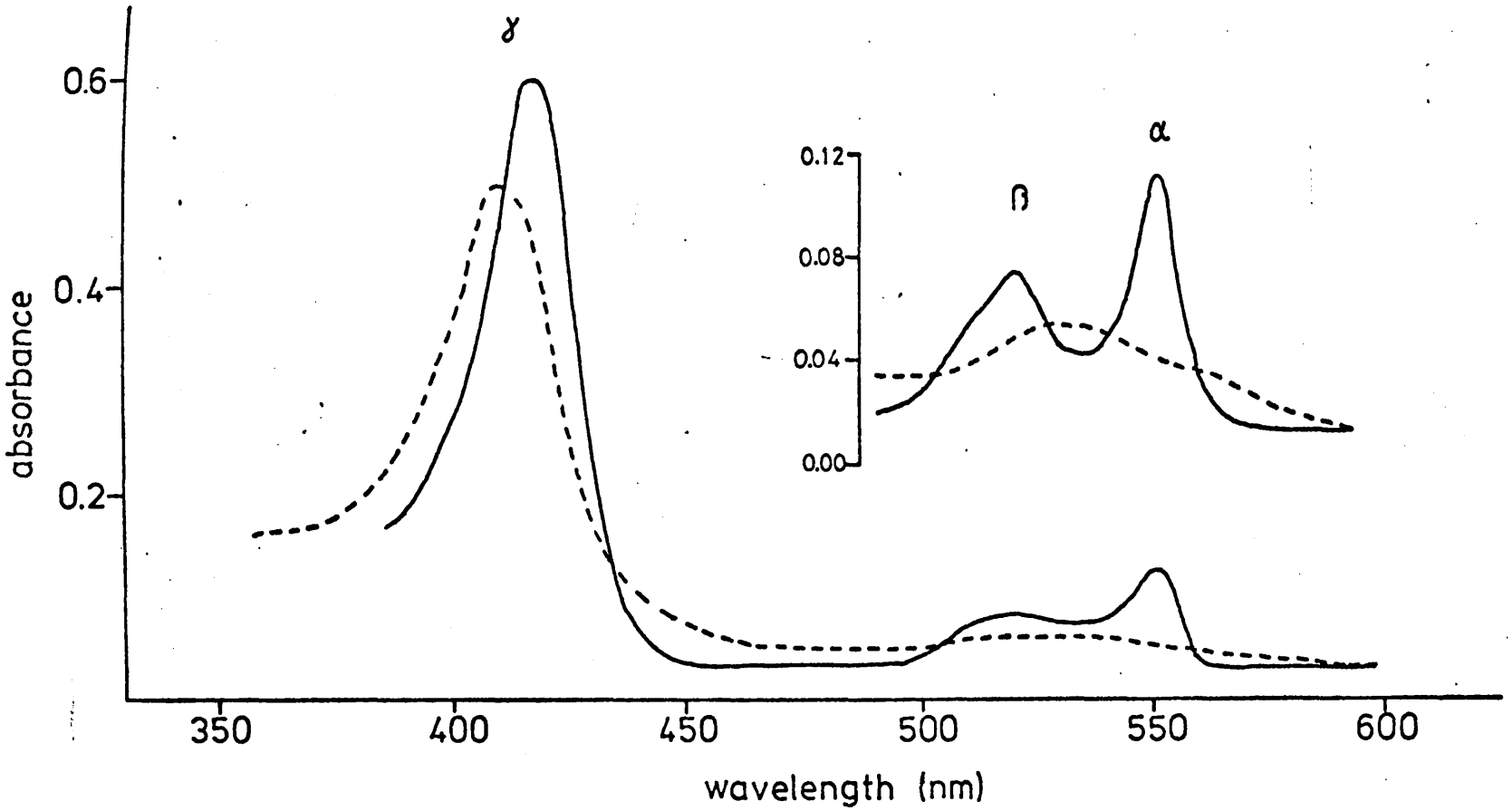


Figure 2-7 Visible absorption spectrum of horse-heart ferrocytochrome c ——— and ferricytochrome c ----. The solvent was 0.025M phosphate buffer, pH 6.86.

and dimer being indicated by an absorption maximum at 600 nm and a shoulder at 570 nm respectively. The literature value of ϵ = 52,000 at 600 nm (117) was used in this research.

2-5 DISCUSSION

Examination of tables 8 and 9 shows that the extinction coefficients determined experimentally during this research agree closely with those previously reported in the literature. In addition the wavelengths at which the absorption maxima occur all agree to within $\pm$5 nm.

The extinction coefficient obtained experimentally for DCIP will be a function of pH, since the undissociated and dissociated forms of this compound (a weak acid) contribute unevenly to the absorbance at 605 nm. At this wavelength the dissociated form of the dye absorbs strongly whereas the protonated (red) form shows negligible absorbance. Hence, if the absorbance of the dye is monitored at 605 nm, the extinction coefficient determined will decrease with decreasing pH. This in part explains the discrepancy between the current results and those of Euler et al. (111). Such behaviour has been used to determine the dissociation constant of p-nitrophenol (114) and other weak organic acids. The true extinction coefficient of the dissociated form of the dye at pH 6.86 will be ~5% higher than that quoted in table 8. Reduced

DCIP exhibits no absorption at 605 nm (110), and therefore, during kinetic experiments, the concentration of oxidised DCIP, at any given time, is readily found from the absorption at this wavelength.

Methylene blue, a classical electron acceptor, has been reported as having two properties which make it unsatisfactory for use in spectrophotometric experiments (110). Firstly, the dye has been found to exist in solution as an equilibrium mixture of monomer and dimer, the ratio changing with concentration (118). The monomer exhibits a strong band at 655 nm and the dimer a slightly weaker one at 605 nm. In consequence the absorbance at each wavelength is reported as not being proportional to concentration. The second disadvantage cited was that the reduced form of the dye is only sparingly soluble in water, resulting in an anomalous increase in the absorbance because of light scattering caused by the crystallising out of the reduced dye in the form of a scarcely visible cloud of microscopic needles.

The results obtained from this study, figure 2-2, indicate that, within the 0-15 μM concentration range, the deviations from Beer's Law, expected of methylene blue due to its metachromatic behaviour, are small and may, to a good first approximation, be neglected. Precipitation of leuco methylene blue from aqueous solution, found

when using homogeneous aqueous conditions, does not occur with the biphasic aqueous/hexane systems used in these studies since the reduced methylene blue is very soluble in the organic phase (section 3-3.2). The reduced form is therefore continually and rapidly extracted out of the aqueous phase and into the upper organic layer to leave the aqueous phase optically clear.

Having shown that the objections to the use of methylene blue were in fact not valid under the experimental conditions employed throughout this research, methylene blue was used in a number of spectrophotometrically monitored kinetic experiments, the reduced form of the dye showing no complicating absorption at either 605 nm or 655 nm.

The absorption spectra of p-nitrophenol (36 μM), hereafter referred to as nitrophenol, was investigated over the spectral range 300-440 nm using 0.1M HCℓ, pH7 buffer and 0.1M NaOH as the solvents, figure 2-3. In acid solution there is a maximum absorption at 318 nm (ε = 10,300); the absorption at this wavelength diminishes as the solution becomes more alkaline and the extinction coefficient approaches a minimum value (ε = 1460) in very alkaline solution. As the solution becomes more alkaline the absorption at 403 nm, which is negligible below pH 5 (ε = 80), is enhanced and becomes a pronounced

maximum in the absorption spectra at pH values above 7, the extinction coefficient reaching a maximum value (ϵ = 19,200) in very alkaline solution. The maxima at 318 nm and 403 nm are attributed to the acid and salt form respectively, of the nitrophenol (114). Measurement of the optical densities of an aqueous solution of nitrophenol at both 318 nm and 403 nm allows the pH of the solution to be obtained (section 3-2.6). The nitrophenol can therefore be used as a pH indicator.

Quantitative ultraviolet data for reduced vitamin K has, as far as the author is aware, never been determined up to now. The spectrum of the quinol, dissolved in hexane, found during this study is similar to that obtained using ethanol as solvent (79), the former spectrum displaying a strong, sharp absorption maximum at 245 nm and a much broader, weaker one at ~325 nm, figure 2-5. A more rigorous comparison cannot be carried out since no quantitative data was cited. The spectrum shown in figure 2-5 is also similar in shape to that obtained for the non-autoxidisable diacetate derivative of vitamin K (119), (120) although the latter (in petroleum ether) exhibits absorption maxima at lower wavelengths and molar extinction coefficients of higher values, (231 nm, ϵ = 82,400 and 285 nm, ϵ = 6000), as compared to the quinol.

Examination of figures 2-5 and 2-6 shows that reduction of vitamin K and coenzyme Q is accompanied in each case by dramatic changes in absorption characteristics. This property, as previously discussed (section 1-3.3), provided a useful method of investigating the reducing abilities of a number of aqueous reagents.

Chapter Three

BASIC KINETIC STUDIES

The nature of the chemical compounds which could be used as components of a liquid redox membrane system has been, up to now, the prime consideration. The discovery of suitable compounds that were capable of oxidising and reducing vitamin K now allowed the time parameter to be introduced and permitted the kinetics of reaction of a number of membrane components, both in the absence and presence of chemical modifiers, to be quantitatively investigated. The latter type of studies are discussed in the next chapter. The rate parameters obtained will be a function not only of the nature of the chemical species present but also of the hydrodynamic conditions existing within the membrane system. For this reason, it is necessary to have constant, reproducible hydrodynamic conditions so that when reagents are changed and the subsequent rates compared, this comparison is between otherwise physically identical systems.

3-1 THEORY

3-1.1 The reaction rate

The rate of a reaction is defined as the rate of disappearance of a reactant, or the rate of appearance of a product, divided by the stoichiometric coefficient of the reagent in the balanced equation describing the overall chemical reaction. Reaction rates are always positive. If the reaction system is one of constant or near constant volume, the change in the amount of reagent will correspond to a change in the concentration of that reagent. For liquid systems the rate of reaction is normally expressed in terms of the rate of change of the molar concentration of the reagent.

The principal experimental approach to the study of the reaction process is through the measurement of the rate with which a reaction proceeds and the dependence of this reaction rate on the concentrations of the reacting species at a given temperature. These factors are grouped together in the term reaction kinetics and the results summarised, for a given reaction, by a rate equation which is of the general form,

Rate = k(T) x function of reagent concentrations (3-1).

The quantity k(T) is the rate constant and, if the term involving the reagent concentrations correctly expresses the rate dependence on concentration, it will be a function

of only temperature. Since rates of reaction are very sensitive to temperature, (for reactions in solution the rate is frequently doubled by a rise in temperature of 10°C), it is necessary for the reaction to be carried out in a system that is carefully maintained at constant temperature. The overall order of a reaction is the sum of the concentration exponents (orders) of the individual species appearing in the rate law and, hence, involved in the reaction. It should be noted that the rate law has no necessary relation to the form of the equation of the overall reaction and that, whereas a postulated mechanism is of theoretical origin, the rate equation and the order of a reaction are experimentally derived quantities. Equation (3-1) gives the reaction rate of a single reaction step. Multistep processes, in general, exhibit more complicated rate equations, the rate being proportional to the slowest step. It must be emphasised that it is never possible to arrive at a firm conclusion about a reaction mechanism. If evidence points towards a particular mechanism, it is always possible to devise a more complicated one that is equally consistent with the facts. In view of this, the principle of Occam's razor is applied and the simplest mechanism, that is consistent with all the available facts, accepted (121).

3-1.2 First-order reactions

A first-order reaction is defined as one for which, at a given temperature, the rate of the reaction depends only on the first power of the concentration of a single reacting species, or mathematically

$$-dc/dt = kc \qquad (3\text{-}2).$$

The first-order rate constant, k, has the units of reciprocal time. The values of concentration determined experimentally at various times are best compared with the integrated form of the rate law. If the initial concentration at time t = o, is c_o and if at some later time t the concentration has fallen to c, it follows that

$$\int_{c_o}^{c} dc/c = -k \int_{o}^{t} dt \qquad (3\text{-}3).$$

Integration yields equation (3-4a) which rearranges to the more convenient form of equation (3-4b).

$$\log(c_o/c) = \frac{kt}{2.3026} \qquad (3\text{-}4a)$$

$$\log c = -\frac{kt}{2.3026} + \log c_o \qquad (3\text{-}4b).$$

A reaction can therefore be said to be first-order if a plot of $\log(c_o/c)$ or log c against time gives a straight line. From the magnitude of the gradient of such a line the value of the rate constant can be obtained using equations (3-4a) and (3-4b).

For first-order reactions it is customary to use not only the rate constant k for the reaction but also the related quantity, the half-life of the reaction. The half-life, denoted $t_{\frac{1}{2}}$, is defined as the time required for the concentration of the reagent to decrease to half its initial value. The relation of half-life to rate constant is easily found by inserting $t = t_{\frac{1}{2}}$, $c = c_o/2$ into equation (3-4a). In this way equation (3-5) is obtained.

$$t_{\frac{1}{2}} = \frac{0.6931}{k} \qquad (3\text{-}5).$$

For a first-order reaction, therefore, there is a simple reciprocal relation between k and $t_{\frac{1}{2}}$. Furthermore, since the expression involves no concentration term, the half-life is independent of the initial concentration of reactant.

3-1.3 Zero-order reactions

A number of reactions are known in which the rate is independent of the concentration of the reactant molecules. Such a reaction will follow zero-order kinetics, the rate of disappearance of reactant being given by

$$\frac{-dc}{dt} = k \qquad (3\text{-}6)$$

where k is the zero-order rate constant, having the units moles $\text{litre}^{-1}\text{time}^{-1}$. The rate of reaction is therefore constant throughout the time of the reaction. Integration of equation (3-6) over the same limits used in the first-order analysis, yields

$$c = c_o - kt \qquad (3\text{-}7).$$

Hence, in a zero-order reaction, the concentration of the reactant decreases linearly with time.

The half-life of reaction, easily obtained from equation (3-7), is given by the relationship

$$t_{\frac{1}{2}} = \frac{c_o}{2k} \qquad (3\text{-}8).$$

Again a simple reciprocal relationship between $t_{\frac{1}{2}}$ and k is obtained but, unlike first-order reactions, the half-life is directly dependent on the initial concentration of reactant.

3-1.4 Reactions in solution

The reaction between two molecules in solution can be thought of as occurring in three well-defined stages: (1) diffusion of the molecules to each other, (2) the actual chemical transformation and (3) diffusion of the products away from each other. When reaction occurs at a liquid-liquid interface the interfacial absorption of reactants and desorption of products, occurring before and after step (2) respectively, must also be considered (121). A reaction occurring at the liquid-liquid interface present in liquid redox membranes may, therefore, involve five consecutive steps.

Consider a reaction taking place in solution and assume that the reaction proceeds according to the scheme

$$A + B \rightleftharpoons X \longrightarrow A' + B' \qquad (3\text{-}9),$$

an activated complex, X, being involved. The general rate is given by

$$\text{Rate} = k'[X] \qquad (3\text{-}10).$$

If the reactants and complex are in thermodynamic equilibrium, the concentration of X may be expressed as

$$[X] = K[A][B]\, f_A f_B / f_X \qquad (3\text{-}11).$$

Here K is the true equilibrium constant and f denotes an activity coefficient. Substitution of equation (3-11) into equation (3-10) gives

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

$$k = k'K f_A f_B / f_X \qquad (3\text{-}13).$$

The form of equation (3-12) is identical to that obtained for a second-order reaction between two reactants in the gas phase. However, the rate constant experimentally obtained from the solution reaction is not only dependent upon temperature but also upon the nature and ionic strength of the medium, equation (3-13), since activity coefficients are functions of these variables. The foregoing analysis is strictly valid only for reactions in an inert solvent which has little effect on kinetic behaviour.

Equation (3-12) may be considered as the general rate equation applying to reaction at either aqueous/hexane interface present in the liquid redox membrane system. However, if the concentration of one of the reactants is

experimentally kept constant, then equation (3-12) simplifies to

$$\text{Rate} = k_1[A] \qquad (3\text{-}14)$$

where $k_1 = k[B]$ is a pseudo first-order rate constant, being constant because of the experimental constraints imposed on the system. If, in addition, the reaction rate is independent of the concentration of A, then equation (3-14) further simplifies to

$$\text{Rate} = k_o \qquad (3\text{-}15)$$

where $k_o = k[A][B] = k_1[A]$, is a pseudo zero-order rate constant. If k_1 and k_o are, in addition, independent of the concentration of B, then they are true first- and zero-order rate constants respectively.

3-1.5 Analysis of reactant concentration with time

The reaction rate, discussed above, is defined as the rate of disappearance or appearance of a reactant or product respectively. In the investigation of reactions in solution the most common procedure involves chemical analysis for one of these species at known times during the course of the reaction. These data lead to values for the reaction rate and to deductions of the rate equation. For a reaction that is slow, relative to the time it takes to perform an analysis, the data from which the rate equation may be deduced can be obtained by any analytic method, physical or chemical, that is applicable to the

particular system. Any physical property can be made use of so long as the property alters characteristically as the reaction mixture changes from reactants to products. If one species in the reaction system absorbs ultraviolet/visible radiation at a particular wavelength then, under favourable conditions, the value of the optical density at this wavelength can be used as a measure of the concentration of that species (section 2-1.1).

It is a characteristic of first-order reactions that, as equation (3-4a) shows, all that need be measured to judge whether or not a reaction is first-order is the ratio of concentrations of the reactant at various times to the concentration at some initial time. The measurement of any quantity that is proportional to the concentration of the reagent can, therefore, be used and actual concentrations need not be calculated. Furthermore, the slope of the straight line gives the same rate constant as would be obtained if the treatment had been in terms of concentration. For this reason, absorbance readings can be directly used when analysing a first-order reaction. For all other reactions, however, analysis must involve concentrations and, therefore, all optical density readings must be converted to concentrations using predetermined extinction coefficients.

3-1.6 Diffusion control

Diffusion in a liquid, like many other physical processes, has an activation energy. The magnitude of this energy

barrier is, however, small and generally not greater than 20 $kJmole^{-1}$. Since many chemical reactions have activation energies much greater than this, they cannot involve diffusion as the slow step. On the other hand there are processes occurring in solution in which diffusion is rate controlling; these are certain reactions which occur very rapidly (121). A number of heterogeneous reactions between solids and liquids are of this type.

According to hydrodynamic theory, the rate of convection of a fluid in a stirred system, relative to a (solid) interface, decreases continuously from the bulk solution to the interface, where this rate is zero. By virtue of the stirring, the concentration of a solute is uniform in the bulk solution. Near the phase boundary, however, where convection is slow, the equalisation of concentration differences by the stirring is much less effective and the transport of solute across this layer consequently occurs primarily by diffusion. Nernst (122) introduced the concept of a fictitious completely unstirred layer which, in the literature, is referred to variously as the 'diffusion', 'unstirred', or 'Nernst' layer. The transport of solute across these layers is assumed to occur solely by diffusion, from the bulk solution, where the solute concentration (in mole cm^{-3}) is c_2', to the interface, where the concentration is c_1', or vice-versa. The quantity of material,

dn, which is transported in the time dt is given by Fick's equation,

$$dn = DA \frac{(c_1' - c_2')}{\delta} dt \qquad (3\text{-}16).$$

Here D is the diffusion coefficient of the solute, A the area of the interface and δ the nominal thickness of the diffusion layer. The thickness of the diffusion layer as calculated from equation (3-1.6) varies according to the experimental conditions, mainly the intensity of the stirring. In solid-liquid systems with mechanically well stirred aqueous solutions at room temperature, diffusion layers of 20-30 microns are commonly assumed to persist. In liquid-liquid systems; due to the intensity of maximal stirring being severely restricted by the danger of emulsification, correspondingly thicker diffusion layers persist, a layer 300 microns thick being considered reasonable (4). Such diffusion layers will exist on both sides of a liquid-liquid interface, the δ values only displaying a common value if the efficiency of stirring on both sides of the interface is the same.

Consider a chemical reaction occurring at an interface. If the interfacial reaction is very fast then all the molecules arriving at the interface will react immediately and, therefore, the concentration of reactant will be zero at this surface. Under these conditions the overall

reaction rate is governed by the rate at which solute molecules can diffuse from the bulk solution, across the diffusion layer, to the interface. Thus, since $c_1' = 0$, equation (3-16) simplifies to

$$\frac{-dn}{dt} = \frac{DA}{\delta} \cdot c_2' \qquad (3\text{-}17).$$

Dividing the left-hand side of equation (3-17) by the volume, V, of the solvent in which the reactant is dissolved, gives the rate of change of solute concentration with time, equation (3-18).

$$\frac{-dc}{dt} = \frac{DA}{V\delta} \cdot c_2 \qquad (3\text{-}18).$$

Since $D(cm^2s^{-1})$, $A(cm^2)$, $V(cm^3)$ and $\delta(cm)$ are all (approximately) constant throughout the reaction, equation (3-18) has exactly the same form as equation (3-2). Thus a diffusion controlled reaction occurring at an interface will exhibit first-order kinetics, the rate constant k_1 being given by,

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

The parameters V, A and δ are apparatus dependent and, therefore, the absolute rate observed is of less importance than the rate relative to other comparable systems. For an interfacial reaction in which membrane translocation is the fastest possible, equation (3-19) dictates that the maximum rate obtainable could not have a first-order rate constant greater than $DA/V\delta$. Diffusion controlled rate

constants are, therefore, directly proportional to the diffusion coefficient of the aqueous species and to the area of the interface; they are inversely proportional to the volume of the aqueous phase and to the thickness of the diffusion layer.

3-2 EXPERIMENTAL

3-2.1 Apparatus

The H-cell format, described in section 1-3.6, was used once more but with the lower limbs containing the aqueous phases replaced by 'spectrosil' optical cells of 1 cm path length. As with the vibrator H-cell, this optical H-cell was sealed from the atmosphere using two 'suba-seals'. The aqueous phases were stirred magnetically and the rotational speed of the two individual external magnets was adjustable over a range of 0-2,000 revolutions per minute (rpm). Unless otherwise stated however, a stirring speed of 1650 $\pm$ 100 rpm was employed. Turbulence and mixing of the quinone membrane solution was effected by rocking a pivoted platform which held the cell and stirrer unit, figure 3-1. Rocking was achieved by placing an eccentric at the end of this platform, its edge tangential to the wheel. The eccentric was turned by means of a low-geared electric motor. Both rocker and magnetic stirrers were, unless otherwise indicated, always employed

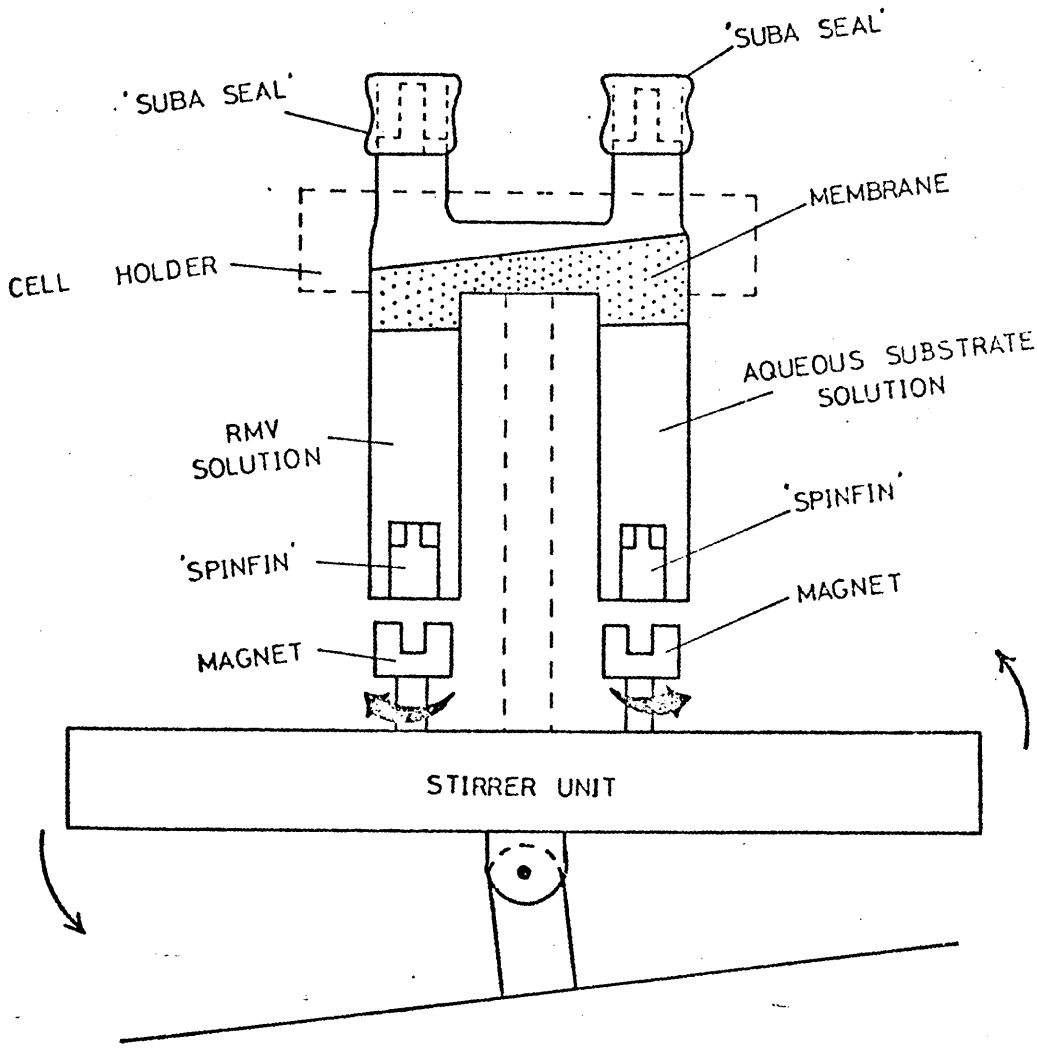


Figure 3-1 Apparatus for kinetic experiments.

during these studies. As discussed above, kinetic studies are very temperature dependent. For this reason, the whole apparatus was enclosed within an air thermostated box, kept at 25.0 ± 0.1°C.

The H-cell and spectrometer cells used throughout these studies were cleaned according to the method outlined in section 2-2.3.

3-2.2 Stirrers

Initial kinetic studies employed perspex 'spinfins' as the stirrers in the aqueous limbs. Control experiments, however, showed that they continually adsorbed redox dyes, displaying no tendency towards saturation. For this reason these stirrers were employed only until more suitable ones became available.

Commercially available teflon 'spinfins' (Bel-Art Products, USA), specially designed for use in spectrometer cells, were found to be far more suitable than their perspex analogues. Control experiments indicated that although redox dyes still adsorbed onto the surface of the teflon, the adsorption process was not continuous and after only a very short time (~30 min) no further dye was adsorbed, 5-10% of the total dye present initially in solution having become adsorbed.

Stirrer uptake correction: If it is assumed that the fractions of dye present in solution and bound on the teflon stirrer are in equilibrium, then the results obtained

during a kinetic experiment can be corrected for this adsorption in the following manner: the difference at time zero between the optical densities of the aliquot of solution that contains the stirrer and one in which no stirrer is present, CO, gives a direct measure of the amount of dye adsorbed onto the stirrer (in OD units). Addition of CO to the adsorbance found in solution gives the optical density which would have been obtained if no adsorption had occurred. As reduction occurs, the percentage decrease in oxidised dye concentration on the stirrer will be exactly the same as that in solution. Hence, if CO, measured at the beginning of a run, is multiplied by the fractional decrease in solution optical density, calculated after recording the first absorbance reading A1, then the value obtained will give the amount of adsorbed dye reduced (in OD units). When this value is subtracted from CO, the resultant value, C1, gives the amount of oxidised dye still adsorbed. Addition of C1 to A1 gives the optical density which would have been obtained had no adsorption occurred. C1 and A1 now replace CO and AO respectively, and the process is repeated for the next reading .... and so on. In this way a stirrer uptake correction may be applied. This analysis is only applicable to cases where the absorbance measured is directly proportional to the concentration of reactant.

3-2.3 Chemicals

The sources of the chemicals used in these studies have already been given (section 1-3.1).

All aqueous solutions, unless otherwise specified, were made up with 0.025M phosphate buffer, pH 6.86.

The membrane solvent used throughout all the experiments, unless specifically described, was spectroscopic hexane.

The concentrations of vitamin K, DCIP, methylene blue, thionine and cytochrome c solutions were determined from the observed optical densities of the solutions, or known dilutions of these solutions, at the appropriate wavelength, using the methods described in Chapter 2.

A stock solution of cardiolipin (CL) of concentration 9.3 mg ml^{-1} in ethanol, was obtained from the suppliers and used directly. Dilution of this stock with hexane was used to prepare a solution of concentration 0.84 mg ml^{-1}, the solvent being 10:1 (v/v) hexane/ethanol. Solutions of phosphatidyl ethanolamine (PE), of concentration 8.76, 4.55 and 0.87 mg ml^{-1}, lysolecithin (LL), of concentration 8.32 and 0.82 mg ml^{-1} and phosphatidyl inositol (PI), of concentration 8.30, 8.14 and 0.75 mg ml^{-1}, were prepared by dissolving predetermined weights of the phospholipids in known volumes of a 9:1 (v/v) $CHCl_3/CH_3OH$ solvent mixture. The solutions were stored in sealed ampoules below 0°C

in the absence of light. 0.03 ml samples of these solutions were added to the membrane, when required, by means of a Hamilton syringe.

3-2.4 Procedure for kinetic runs

The H-cell was filled according to one of three basic methods, developed during the period of this research, each method allowing a different kind of kinetic study to be investigated.

RMV, used as the vitamin K reductant throughout these kinetic studies, was generated by the addition of 0.5 ml of a freshly prepared sodium dithionite solution, 22 mg ml^{-1}, to a solution of MV, 25 mM. The volume of MV and substrate solutions used was 4 ml. The volume of the membrane was 4.5 ml. When all additions had been completed araldite was placed over the puncture holes in the 'suba-seal' septa.

Method A: Starting from fully oxidised carrier.

Both the MV and substrate solutions were degassed on a water pump for 15 minutes and, at the same time, the H-cell was flushed with nitrogen. The stirrers were added at this stage. Care was always taken to ensure that the MV and substrate limbs each received the same stirrer throughout. After this period the solutions were brought back to atmospheric pressure using nitrogen and aliquots of each solution quickly, but carefully, pipetted into opposite limbs. A 0.5 ml aliquot of the quinone solution

was then injected above the MV solution using a Hamilton syringe. 'Suba-seals' were placed in position and 2 ml aliquots of membrane solvent added with another Hamilton syringe. The volumes chosen were such that on injection of the membrane solvent the organic solution bridged the two aqueous limbs, forming a liquid membrane. The cell was placed on its holder and left stirring and rocking for 1 hour, to allow the substrate time to equilibrate with the stirrer, to allow the cell and its contents time to reach the desired temperature and to generate a homogeneous membrane solution. Reaction was started by adding the dithionite solution to the methyl viologen limb.

Method B: Starting from the fully reduced carrier.

The aqueous solutions were degassed, brought up to atmospheric pressure and the H-cell filled, as described in method A. Once the 'suba-seals' were placed in position, the dithionite solution was added to the viologen limb and the cell stirred and rocked for an equilibration period of two hours. The volumes of MV and vitamin K solution were carefully chosen to ensure that, even with rocking, the vitamin K solution was completely confined to the methyl viologen limb at this stage. During this period the vitamin K solution floating above the RMV became fully reduced. Because of the extreme oxygen sensitivity of reduced vitamin K, this procedure also ensured that, by

the time the reaction was started, any oxygen present in the cell immediately after filling was removed. The reaction was started by injecting two 2 ml aliquots of hexane into the cell. If lipid was also to be present (Chapter 4), then it was also added at this time.

The procedure outlined was by far the most common one adopted throughout this research and, in consequence, where no method of filling the cell is specified, method B is to be assumed used.

Method C: Rigorous exclusion of oxygen.

Investigation of the reduction of cytochrome c (Chapter 4) involved the development of a procedure which minimised still further the presence of oxygen throughout the filling stage. Method B was unsuitable since the membrane solvent added to start the reaction had not been deoxygenated. To satisfy these requirements a third method of filling the cell was devised.

Aliquots of MV and substrate solution were placed in the cell, both solutions having been previously degassed and then nitrogen saturated as before. The cell was sealed and nitrogen bubbled through the substrate solution for 30 minutes. The MV solution was then reduced and the cell flushed with nitrogen for a further 30 minutes. At the same time, aliquots of vitamin K and hexane were equilibrated, in containers sealed with rubber septa, over a solution of RMV to fully reduce and deoxygenate the

solutions. At the end of this period a 0.5 ml aliquot of reduced vitamin K solution, followed by two 2 ml aliquots of the deoxygenated hexane solution were injected into the cell. These solutions were transferred, through air, by means of gastight Hamilton syringes and were added to the viologen side of the cell. Care was taken to avoid the transfer of RMV into the horizontal section of the H-cell. If lipid was also to be used, it was added at this stage by injecting the solution through the RMV layer (Chapter 4).

3-2.5 Generation and treatment of results

Separate ultraviolet cells, filled with either buffer or substrate solution, acted as the blank and control respectively, for that experiment. The absorbances of the substrate in both the H-cell and control cell were measured during the equilibration period. The magnetic 'spinfin' present in the substrate limb of the H-cell was held out of the optical path of the light beam by means of a small magnet appropriately positioned on the cell carrier. If there was no significant difference in successive absorbance readings (i.e. < 0.003) from the substrate (test) limb, then an equilibrium state between the stirrer and substrate was assumed and the reaction started, otherwise the equilibration period was extended until this condition was satisfied. This procedure was

not applicable to the few experiments in which perspex stirrers were used. From the absorbance of the control, the starting concentration of the substrate solution could be calculated. The difference in absorbance between the control and test limbs at the start of the run was subsequently used to calculate stirrer uptake corrections (section 3-2.2).

Measurements of the optical density of the test limb were monitored with time, readings being recorded every 20-30 minutes on a Hilger H700 spectrometer. Since the time taken to effect a reading was ~1 minute and the half-lives of the reactions were found to be >40 minutes, it was considered that interruption of the reaction for these short periods would not adversely affect the overall result. Similarly the short periods of time during which the cell was not thermostated, as absorbances were being measured, was considered negligible, especially since the ambient temperature was close to 25°C. For consistency, however, readings were taken at approximately equal time intervals throughout sets of runs. To check instrument reproducibility during a run, the absorbance of the control was also measured with time. The control absorbances obtained always agreed to within $\pm$ 0.005 units of the value obtained initially from each experiment.

If the absorbances measured were directly proportional to substrate concentration and the results obeyed first-order kinetics then the rate constant and half-life for each run were obtained from the gradient of the best fitting straight line, when the logarithm of the optical density was analysed as a function of time using the 'least squares' program described in Appendix II. In all other cases the optical densities were first converted to concentration of reactant before being analysed for first- or zero-order behaviour by fitting the logarithm of concentration or concentration itself respectively, against time, using the same computer program.

3-2.6 Procedure to investigate the stoichiometry of accompanying proton transfer

Reduced vitamin K and oxidised methylene blue (MB) react according to the equation

$$MB^+ + \bar{Q}H_2 \longrightarrow MBH + \bar{Q} + H^+ \qquad (3\text{-}20)$$

(the bar above the quinone denotes that it is located within the membrane). The stoichiometry of this equation indicates that, as the reaction proceeds, protons will be ejected into the aqueous phase; one proton per two electrons transferred, or, equivalently, one proton per molecule of substrate reduced. The stoichiometric ratio of protons ejected to methylene blue reduced, $|\Delta H_o^+|/|\Delta MB|$, is therefore, expected to be unity. The characteristic change in the

absorption spectrum of nitrophenol with pH (Chapter 2) was used to study this effect experimentally. It was assumed that the neutral, reduced methylene blue (MBH), formed during the course of the reaction, did not remain in the aqueous substrate phase, but was removed into the upper hexane layer.

In order to be able to detect the changes in pH of the methylene blue limb, 0.05M sodium chloride was used as the aqueous solvent, replacing the previously used phosphate buffer. This solvent was also used to prepare an approximately 450 μM nitrophenol solution. Prior to the start of an experiment all ultraviolet cells were left soaking in the saline solution for 30 minutes, rinsed with distilled water and dried. The measurement and transfer of all aliquots of methylene blue and nitrophenol solutions were carried out using Hamilton syringes which delivered precisely known volumes. All apparatus was sealed to the atmosphere using 'suba-seals'.

To remove dissolved oxygen and carbon dioxide the stock solutions of methylene blue and nitrophenol, each in a sealed flask, were saturated with pure nitrogen. During this period one limb of the H-cell was filled with 4 ml of methyl viologen and 0.5 ml of vitamin K solution and 'suba-seals' fitted, as before. The methyl viologen was reduced and stirred to allow the vitamin K to become

reduced. After a period of 2 hours two 1.9943 ml aliquots of the stock methylene blue solution were transferred to the empty limb of the H-cell and a separate aliquot transferred into a sealed box-top 'spectrosil' cell, previously flushed with nitrogen. The aqueous solutions in the H-cell were stirred for a further hour to allow the teflon stirrer time to equilibrate with the dye. The optical density of the methylene blue limb was measured at 655 nm. As before, the reaction was started by the addition of hexane (4 ml) to form the membrane-bridge. The concentration of vitamin K in the membrane was 0.407 mM. At a convenient time the reaction was stopped and the absorbances of the H-cell limb, now containing a mixture of both reduced and oxidised methylene blue, measured at 655, 403 and 318 nm. A 1.9943 ml aliquot of this solution was then transferred into another box-top optical cell which had been previously sealed and nitrogen flushed. Into this cell and into the one containing the deoxygenated stock solution 0.5033 ml of nitrophenol solution was added. The optical densities of these two solutions, together with those of the undiluted stock, were again measured at 655, 403 and 318 nm.

To obtain the true optical densities of oxidised methylene blue (λmax = 655 nm) and of dissociated, A^- (λmax = 403 nm), and undissociated, HA (λmax = 318 nm), nitrophenol, corrections, over and above normal cell bias

adjustments, had to be taken: the absorbance readings for methylene blue were corrected for dye adsorption onto the stirrer (section 3-2.2) and those for nitrophenol were corrected for the small absorption exhibited by methylene blue. These latter corrections were calculated by multiplying the absorbance readings (at 403 and 318 nm) of methylene blue, recorded from the undiluted stock and H-cell limb after reaction, by the dilution factor of 0.7985. Subtraction of these values from those recorded at the corresponding wavelengths using the nitrophenol solutions, which contained unreacted and reacted dye respectively, yielded absorbances due solely to nitrophenol.

The concentration of species to which the corrected absorbances corresponded were calculated by applying equations (2-2) and (2-5) to the corrected methylene blue and nitrophenol absorbances respectively. The concentration of hydrogen ion in solution was calculated using equation (3-22)

$$[H^+] = K[HA]/[A^-] \qquad (3\text{-}22)$$

The value of $K = 1.023 \times 10^{-7}$ (114), used in these calculations, had been determined without the application of activity corrections. For this reason concentrations, and not activities, were used to calculate $[H^+]$. Since the concentration of nitrophenol in both solutions was constant, a loss of dissociated form must be accompanied

by a corresponding increase in undissociated acid. The average amount of nitrophenol protonated during the course of the reaction may be obtained, therefore, by averaging the unsigned changes observed for the conjugate forms of the indicator. Because an increase in acidity results in both the formation of more HA and free H^+ ions, the stoichiometric ratio is given by

$$|\Delta H_o^+|/|\Delta MB| = (|\Delta H^+| + |\Delta HA|)/|\Delta MB| \qquad (3\text{-}23)$$

3-3 RESULTS AND DISCUSSION

3-3.1 DCIP as the aqueous substrate

The membrane system used in this set of experiments may be represented as

RMV/K (in hexane) / DCIP,

and was the first type of solvent redox membrane to be kinetically investigated. Due to the non-ideality of DCIP as a substrate (section 1-4), only a relatively few experiments were carried out with this system. Nonetheless, such experiments gave a valuable insight into the type of rate processes to be considered in studies using bulk liquid redox membranes. The effects on the observed kinetics of membrane agitation and of starting the reaction with the carrier in either the fully oxidised or reduced state,

were investigated. The initial concentration of DCIP solution for all experiments was kept constant at 12 $\pm$ 1 μM and the change in absorbance of the dye at 605 nm used to investigate the reduction process. Throughout all the experiments the aqueous solutions were stirred using standard conditions (section 3-2.1).

In the experiment where no membrane agitation was employed, the H-cell was filled according to method A (section 3-2.4) and the concentration of vitamin K in the membrane was 0.2 mM. The graph of the absorbance of DCIP against time, obtained from this experiment, is shown in figure 3-2. The intermediate section of this graph obeyed zero-order kinetics and gave values of $k_o = 1.46 \times 10^{-8}$ moles litre^{-1} min^{-1} and $t_{\frac{1}{2}} = 400$ minutes. The initial induction period observed is interpreted as the finite time necessary for the reduced carrier, generated at the RMV interface, to diffuse across the membrane and into the substrate interface. The subsequent zero-order behaviour with respect to DCIP, is interpreted as signifying that the diffusion of DCIP across the aqueous Nernst layer is not the rate-determining step, but rather that the rate of transfer of quinol across the membrane is rate limiting. Under these conditions the concentration of quinol at the interface will be zero. As the concentration of oxidised DCIP decreases with time, a stage will eventually

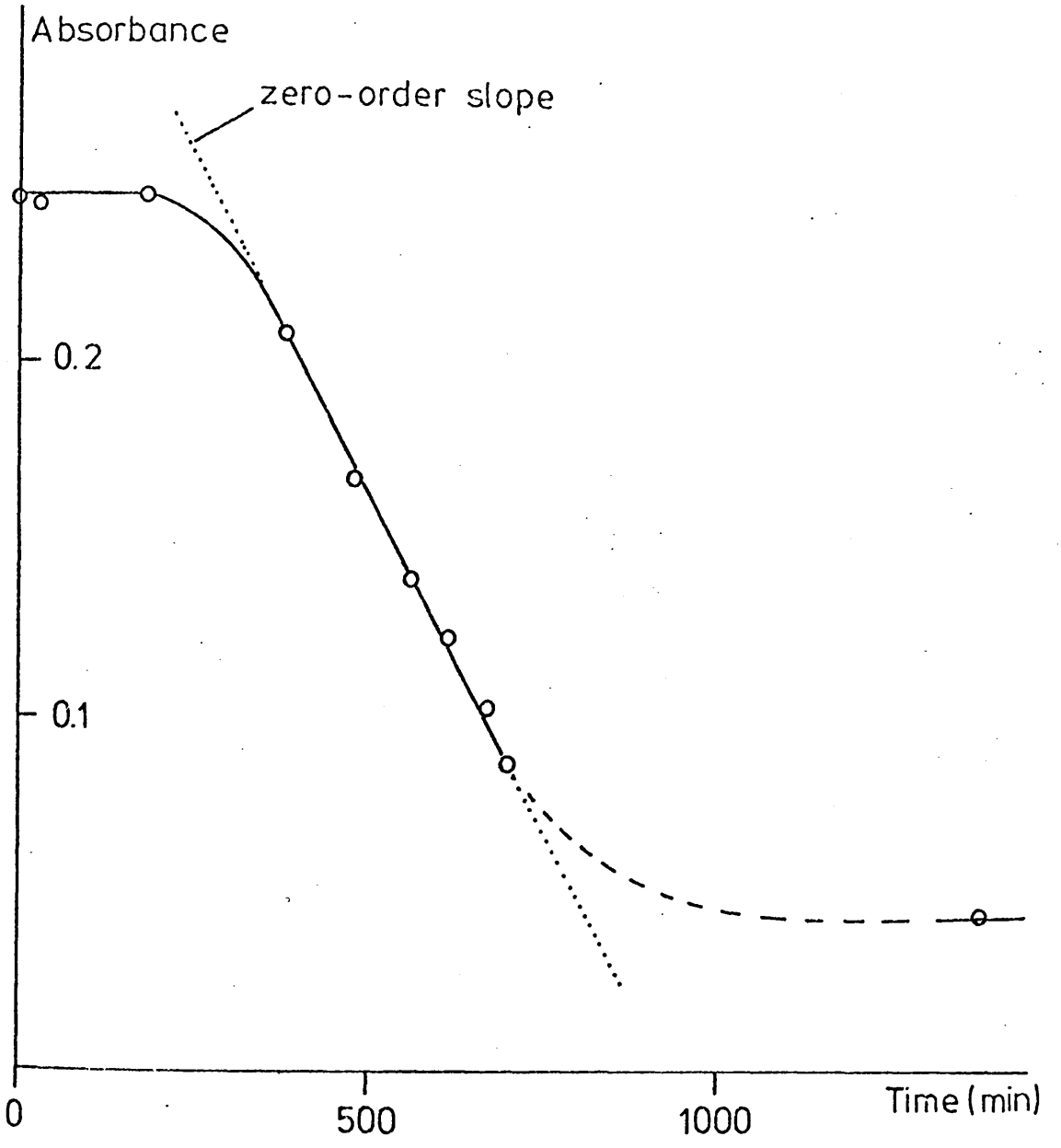


Figure 3-2 The absorbance of DCIP, measured at 605 nm, plotted against time for the system:

RMV/Vitamin K/DCIP.

The kinetic run (25°C) was started with the carrier in the oxidised form and was carried out in the absence of membrane agitation. The concentrations of DCIP (dissolved in 0.025M phosphate buffer, pH 6.86) and vitamin K_1 (dissolved in hexane) were 12 μM and 0.2 mM respectively. Standard stirring conditions (section 3-2.1) in the aqueous limbs were used.

be reached in which the rate of diffusion of the dye towards the interface becomes important and a change in reaction order results. Hence the deviation from zero-order observed.

A similar experiment to the one described above was carried out, but the membrane was agitated. The cell was filled as before but the concentration of vitamin K used was increased to 2.2 mM. The graph of the absorbance of DCIP against time for one of many such experiments is shown in figure 3-3(a). The results obeyed first-order kinetics and gave an average rate constant of $k_1 = (4.4 \pm 0.9) \times 10^{-3}$ min^{-1} and an average half-life of $t_{\frac{1}{2}} = 160 \pm 25$ minutes. Comparison of figures 3-2 and 3-3(a) shows that the introduction of membrane agitation results in the abolition of the initial induction period found in the absence of agitation. This finding supports the postulate that the induction period is a result of the finite time required for the reduced carrier to diffuse across the membrane and also indicates that the rate of reduction of the quinone by RMV is fast compared to the diffusional process. The change in order and the faster reaction rate found when the membrane contents are now mixed by turbulence, indicate the genesis of a new rate-determining step. As will be discussed subsequently, diffusion control within the aqueous phase would account

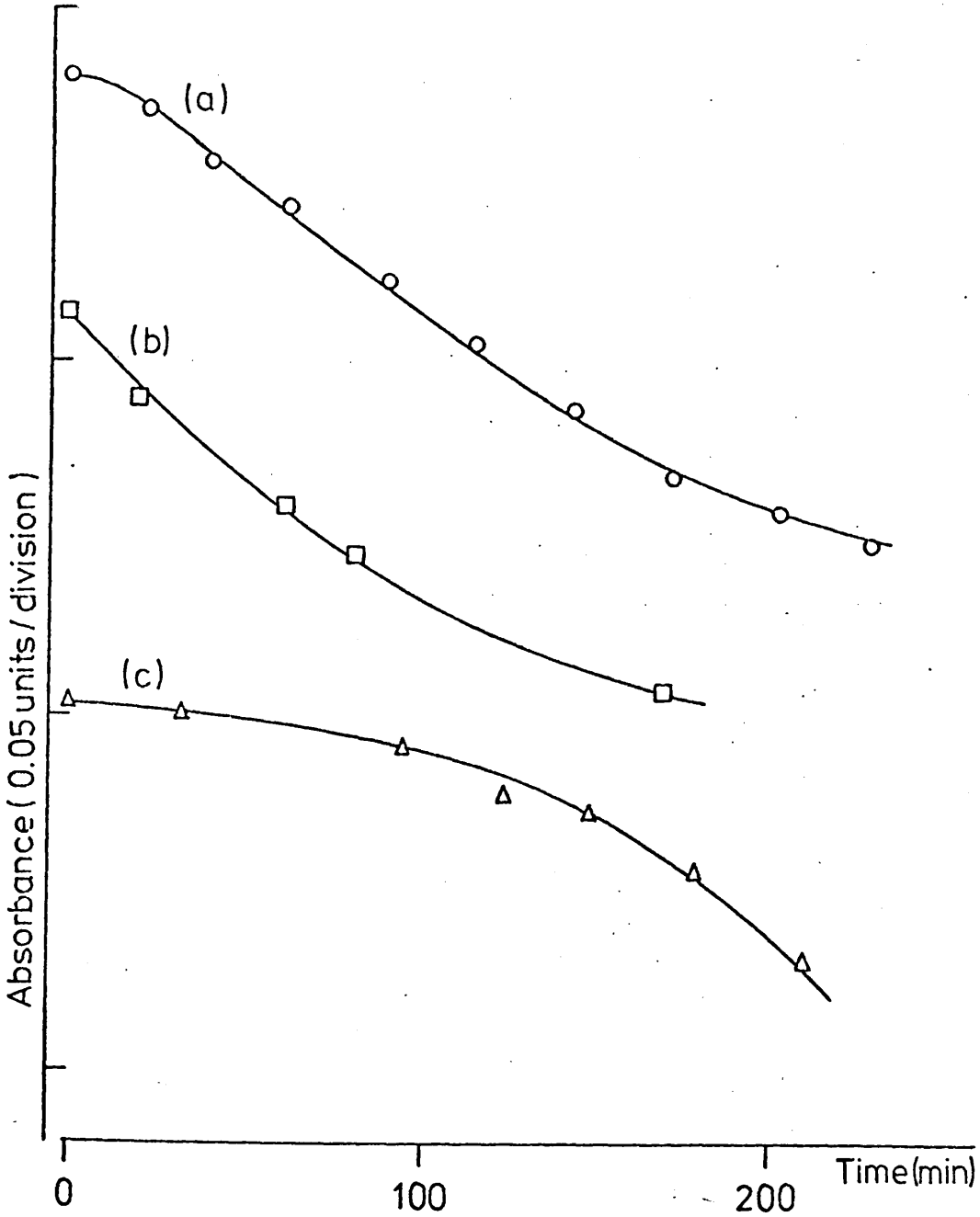


Figure 3-3 The absorbance of DCIP plotted against time for the system:

RMV/Vitamin K/DCIP.

The kinetic experiments (25°C) were started with the carrier both in the oxidised state, (a) and in the reduced state (b). The membrane was agitated in all experiments. The concentrations of vitamin K_1 kinetic runs (a), (b) and (c) were 2.2 mM, 0.39 mM and 0.0 respectively. All the experiments used the same initial concentration of DCIP solution (12 μM) and hence all the experiments started at a common optical density. For clarity of presentation, these zero-time absorbances have been set at arbitrary points on the ordinate of this figure. Standard stirring conditions were used.

for both the order and the magnitude of the rate constants observed.

The previous studies were carried out with the carrier present in the oxidised form at the start of each experiment. It was of interest to investigate whether the conversion of the carrier to the reduced form, prior to the commencement of each experiment, had any effect on the observed kinetics. In consequence, the H-cell was filled according to method B (section 3-2.4) and a similar series of experiments carried out, membrane agitation again being employed. The concentration of vitamin K in the membrane was lowered to 0.39 mM. The graph of the absorbance of DCIP against time for a typical experiment is given in figure 3-3(b). Figure 3-3(c) records the results obtained from a control run carried out in the absence of carrier but using an otherwise identical procedure. The results obtained from experiments in which carrier was present obeyed a first-order rate law, the average kinetic parameters obtained being $k_1 = (4.2 \pm 0.2) \times 10^{-3}$ min^{-1} and $t_{\frac{1}{2}} = 170 \pm 10$ minutes.

Whereas the graphs obtained from kinetic runs started with vitamin K in the fully oxidised form, figure 3-3(a), showed initial minor deviations from first-order kinetics, those obtained from runs in which the quinone had been fully reduced prior to the generation of the membrane

showed no such deviations, figure 3-3(b). These deviations, attributable to either incomplete elimination of the induction period or traces of oxygen, did not, however, affect the rate constant since both kinds of experiment yielded first-order rate constants of $k_1 \simeq 4 \times 10^{-3}$ min^{-1} ($t_{\frac{1}{2}} \simeq 170$ minutes). It must be remembered, however, that oxidised DCIP is lost not only through reaction but also, though to a much lesser degree, by dissolution in the membrane and adsorption onto the stirrer, hence the results of the control experiment, figure 3-3(c). The true rate constants will therefore be somewhat smaller than those quoted. If approximate corrections for this loss are applied (calculated using the control experiment results) a true rate constant of $k_1 \simeq 3.5 \times 10^{-3}$ min^{-1} ($t_{\frac{1}{2}} \simeq 200$ minutes) is obtained. The carrier concentration in experiments started in the presence of fully reduced carrier was approximately five times less than that used in comparable experiments initiated with fully oxidised quinone. The identical rate constants obtained from both types of experiment argues that the interfacial reduction of DCIP is largely independent of the concentration of carrier in the membrane and probably signifies that the reduction is proceeding according to saturation kinetics and is diffusion controlled. This will be discussed more fully in the following section dealing with methylene blue as a substrate.

3-3.2 Methylene blue as the aqueous substrate

The previously described biphasic studies involving methylene blue (Chapter 1) only demonstrated the potential of the dye as a dihydrovitamin K oxidant. The adherence of the dye to the criteria required of an aqueous oxidant (Introduction) has not been, however, sufficiently demonstrated. To establish the degree of immiscibility of the substrate two further control experiments were undertaken. Aliquots of aerated 0.025M phosphate buffer (pH 6.86) and either oxidised or reduced methylene blue solution, both dissolved in buffer, were pipetted into opposite limbs of the H-cell. Reduced methylene blue was generated by adding excess dithionite to a solution of the oxidised dye. The two solutions were bridged by a membrane of pure hexane and the cell sealed. All additions were carried out under normal atmospheric conditions. The aqueous phases were stirred and the membrane agitated for a period of 24 hours. During this time absorbances of the aqueous solutions were measured at 605 nm, one of the wavelengths where oxidised methylene blue shows a maximum in absorption.

The absorbance of the initially pure buffer limb during experiments using reduced methylene blue, figure 1-8(c), was found to increase with time. A spectrum of oxidised methylene blue (see insert, figure 2-2) was

obtained from these limbs at the end of the experiments. This showed that leuco methylene blue was membrane soluble; the neutral reduced dye, by virtue of its membrane solubility, was transferred across to the opposite interface where it was oxidised by the oxygen present in the buffer, to yield oxidised methylene blue which dissolved in the buffer solution. In contrast, the absorbance of the pure buffer limb from the analogous experiment with oxidised dye, remained constant. This proved that oxidised methylene blue, which is a cation, possessed no membrane solubility whatsoever since even a trace solubility in the membrane phase would quickly lead to a detectable amount of dye. The solubility characteristics of oxidised and reduced methylene blue in the hydrocarbon membrane are just those expected of a charged and neutral species respectively.

Methylene blue can, therefore, be considered as an 'ideal' oxidant in the sense that the oxidised substrate is membrane impermeable. Although the compound has the disadvantage of having a membrane-soluble leuco form, in the absence of a more attractive alternative, extensive studies were carried out using this dye as the substrate.

The system used in the set of kinetic investigations outlined in this section may conveniently be represented by,

RMV/Vitamin K(hydrocarbon)/Methylene blue.

The influence of individual variables on the observed

kinetics was determined by varying this parameter whilst all others were kept fixed. The concentration of RMV was fixed in the manner described previously (section 3-2.4). Teflon spinfins and membrane agitation were used to mix aqueous and membrane solutions respectively, throughout all the experiments to be discussed. The change in absorbance of the dye at 655 nm was used to investigate the reduction process occurring at the membrane/methylene blue interface. Where no method of filling the H-cell is indicated, method B is to be assumed used. Similarly standard stirring conditions in the aqueous limbs (section 3-2.1) are to be assumed, unless otherwise specified.

The reproducibility of results and the influence of stirrer uptake corrections on the rate constants determined from these results were investigated by repeating the same experiment a number of times. The concentration of vitamin K present in the membrane was 0.457 mM and that of methylene blue, present in the test limb, was 7.05 μM. The graphs obtained when the absorbances of methylene blue were plotted against time, are shown in figure 3-4, the absorbance readings having been corrected for the dye uptake of the stirrer (section 3-2.2). If the logarithms of these absorbances are plotted against time, a straight line is obtained, figure 3-5. This indicates that the

Figure 3-4 The absorbance of methylene blue, measured at 655 nm, plotted against time for the system:

RMV/Vitamin K (hexane)/Methylene blue.

Kinetic runs (25°C) were started with the carrier in fully oxidised form. The membranes were agitated and standard stirring conditions were used. The concentrations of methylene blue (dissolved in 0.025M phosphate buffer, pH 6.86) and vitamin K_1 were 7.05 μM and 0.457 mM respectively. ●, O and x are the results from duplicate experiments.

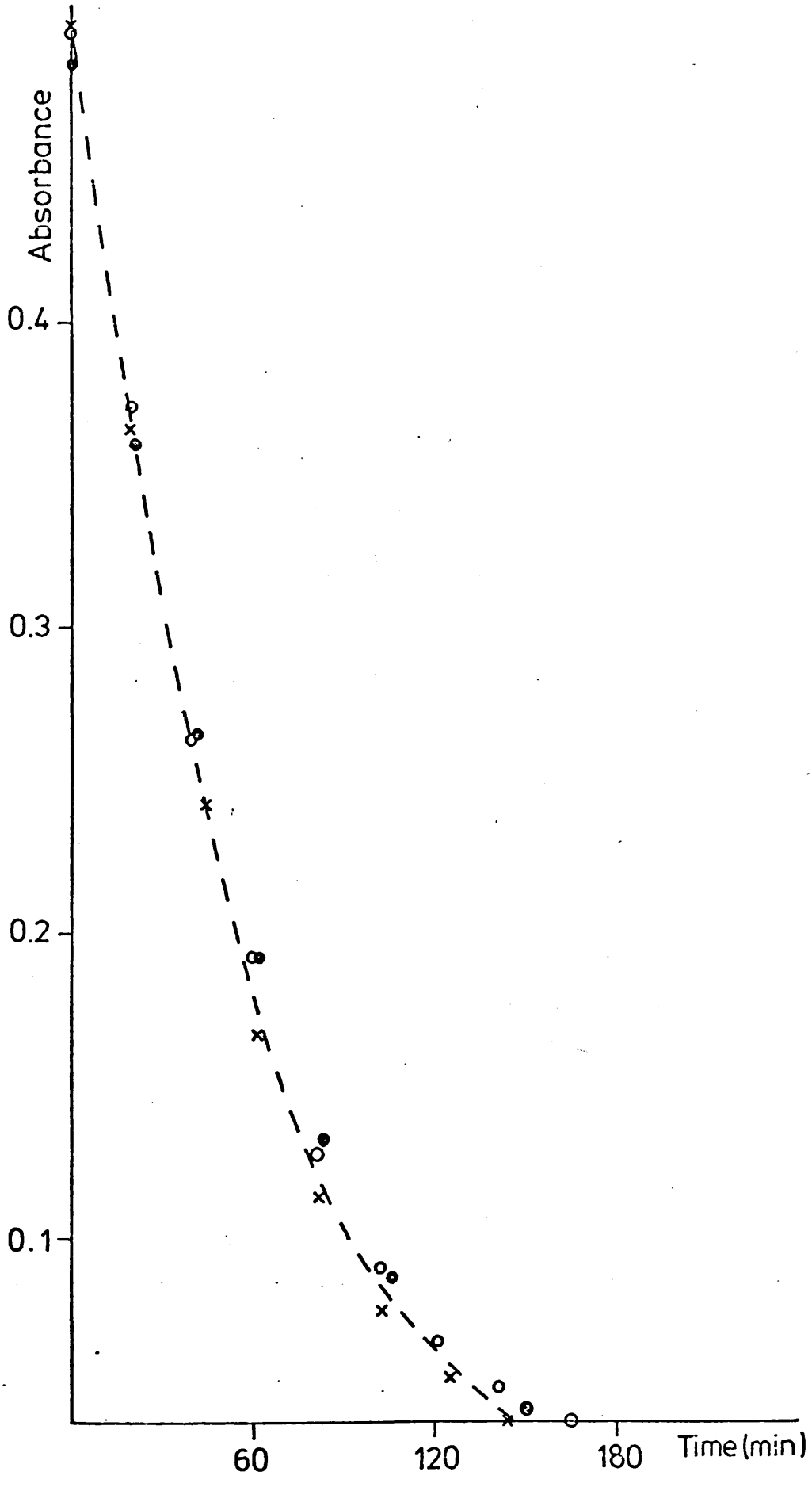
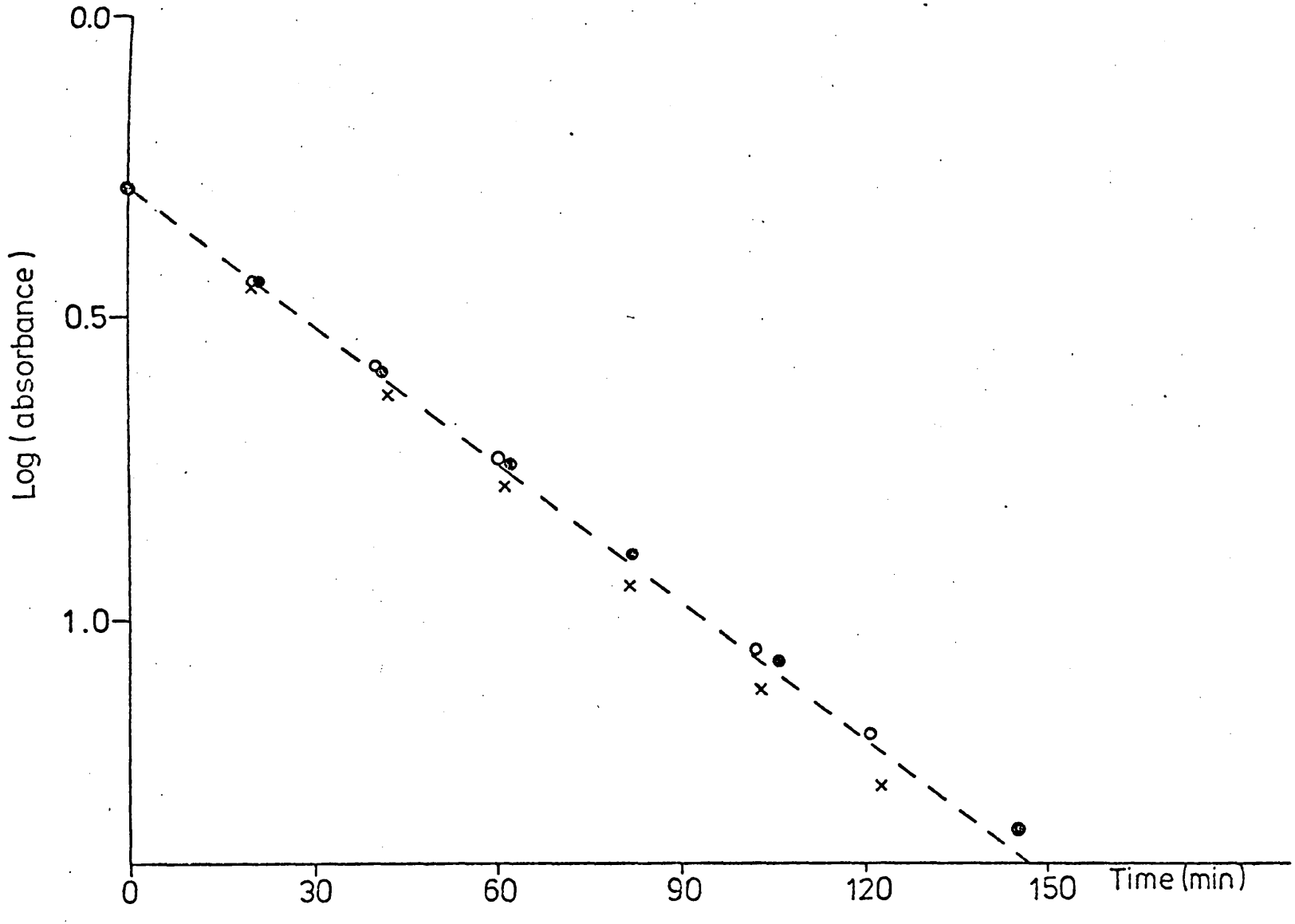

Absorbance
0.4
0.3
0.2
0.1
60
120
180
Time (min)

Figure 3-5 The logarithm of the absorbance of methylene blue plotted as a function of time. Experimental conditions are given in the legend of figure 3-4.


0.0
0.5
1.0
Log (absorbance)
0
30
60
90
120
150
Time (min)

reaction is first-order with respect to methylene blue concentration. The first-order rate constants and the correlation coefficients (R) obtained from these results, both before and after stirrer uptake corrections were applied, are recorded in table 10. From this table it can be seen that these corrections cause only very small alterations to the final results. Despite this, stirrer uptake corrections were always applied, where appropriate, throughout this research. These results also indicate high reproducibility between duplicate kinetic runs, deviations being of the order of $\pm$ 5%.

The influence of membrane agitation on the observed rate constant was investigated using an identical method to that described for DCIP (previous section). The concentration of vitamin K and methylene blue were kept constant at 0.407 mM and 6.5 μM respectively. In contrast to DCIP, the results obtained, when the reaction was started using vitamin K in either the fully oxidised or reduced state, were identical and no initial deviation from first-order kinetics was observed. As before, both types of experiment gave similar rate constants. The average first-order rate constant obtained having the value $k_1 = (12.7 \pm 0.8) \times 10^{-3}$ minutes. This finding again supports the idea that the rate of reduction of the carrier by RMV and the rate of transport of carrier across the membrane are not rate limiting.

Table 10 Reproducibility of and the influence of stirrer uptake corrections on the first-order rate constants (25°C), k_1, determined using methylene blue as the substrate. Standard stirring conditions (section 3-2.1) were used.

RUN	NO CORRECTION		WITH STIRRER UPTAKE CORRECTION	
	$10^3 \times k_1$ (min^{-1})	R	$10^3 \times k_1$ (min^{-1})	R
1	16.86	-0.9996	16.88	-0.9996
2	18.03	-0.9997	18.30	-0.9997
3	16.68	-0.9990	16.65	-0.9990

The dependence of the observed reaction rate on the concentration of methylene blue in solution was investigated by carrying out a series of experiments in which only the initial concentration of methylene blue was varied. The carrier concentration in the hexane membrane was kept constant at 0.457 mM. Each of these kinetic runs was found to obey first-order kinetics and yielded, on analysis, a rate constant and a reaction half-life which were independent of initial substrate concentration, table 11. From the results collected average values of $k_1 = (16.4 \pm 0.9) \times 10^{-3}$ min^{-1} and $t_{\frac{1}{2}} = 42.5 \pm 2.4$ minutes are obtained. The independence of the rate constants on the initial dye concentration proves that the interfacial redox reaction occurring between reduced vitamin K and oxidised methylene blue is first-order with respect to the concentration of the latter.

A series of experiments, designed to investigate the dependence of the observed reaction rate on the concentration of vitamin K in the membrane phase, was conducted by keeping the concentration of methylene blue constant at 5.6 ± 0.3 μM and varying only the carrier concentration. Typical results obtained from experiments using different carrier concentrations are shown in figure 3-6, where the absorbances of methylene blue are graphed against time. The concentration of vitamin K used in each of these kinetic

Table 11 Influence of the initial concentration of methylene blue on the observed rate constants (25°C) for the system:

RMV/Vitamin K (hexane)/Methylene blue

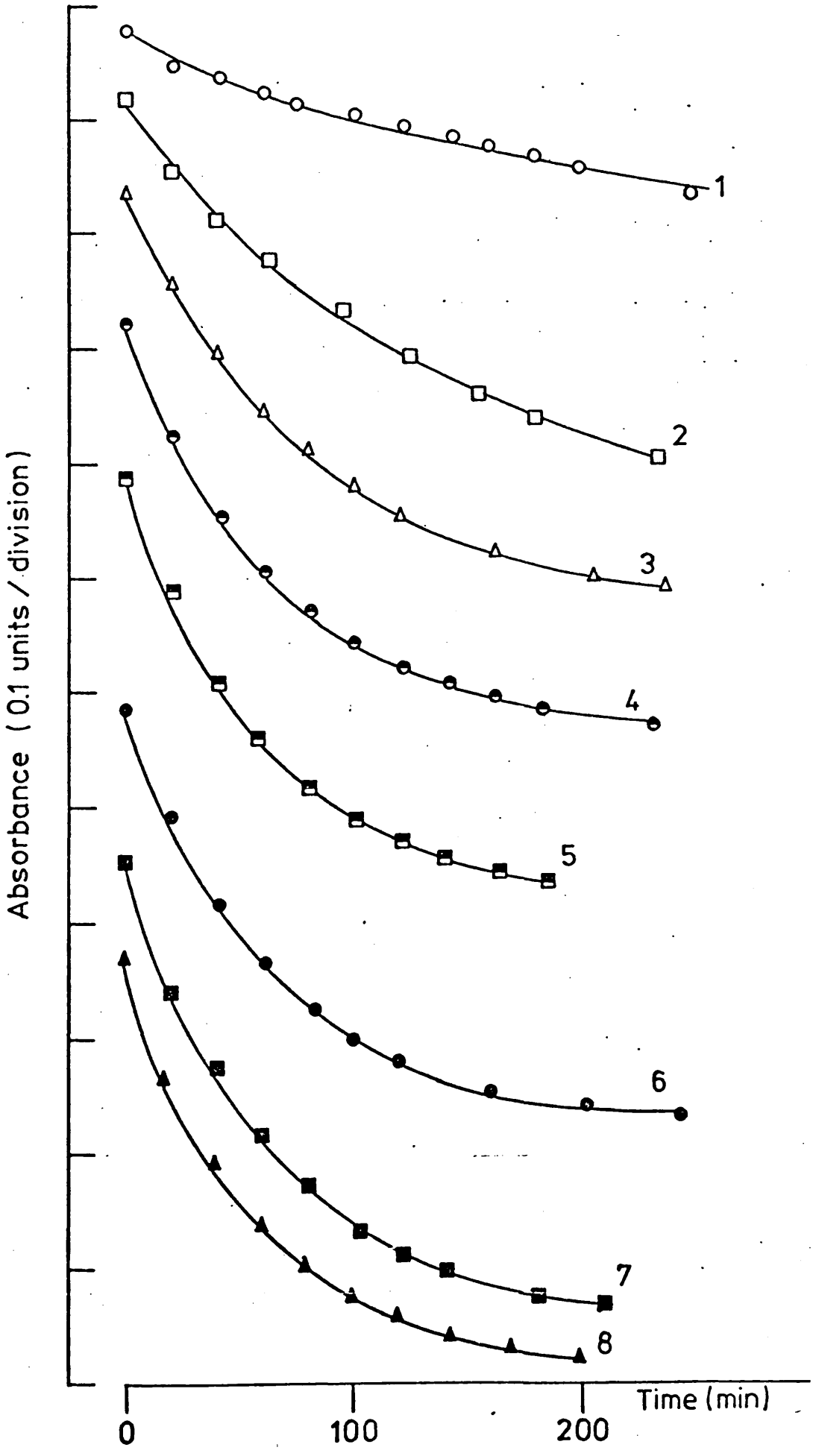
The concentration of vitamin K_1 was constant at 0.457 mM and the concentration of methylene blue varied over the range 2-10 μM. Standard stirring conditions were used.

RUN	Initial [Methylene blue] (μM)	$10^3 \times k_1$ (min^{-1})	$t_{\frac{1}{2}}$ (min)
1	2.92	15.9	43.5
2	2.92	16.5	42.1
3	4.77	17.0	40.7
4	4.77	16.9	40.9
5	5.63	14.8	46.8
6	5.63	16.1	43.1
7	7.05	16.9	41.1
8	7.05	18.3	37.9
9	7.05	16.7	41.6
10	9.00	14.8	44.2
11	9.00	16.1	45.5

Figure 3-6 The absorbance of methylene blue plotted against time for the system:

RMV/Vitamin K (hexane)/Methylene blue.

The concentration of vitamin K_1 was varied over the range 1-1000 μM and progressively increased from run 1 through to 8 (see table 12). All the experiments used the same initial concentration of methylene blue solution (5.6 μM) and hence all the experiments started at a common optical density. For clarity of presentation these zero-time absorbances have been set at arbitrary points on the ordinate of this figure. Standard stirring conditions were used.


Absorbance (0.1 units / division)
1
2
3
4
5
6
7
8
Time (min)
0
100
200

runs is recorded in table 12. All results were again found to obey a first-order kinetic analysis. The results obtained from duplicate experiments were averaged and the resultant mean rate constants and half-lives, together with the mean deviations observed, are recorded in table 12. A graphical summary of the results obtained is given in figure 3-7 where the half-lives found are plotted as a function of the vitamin K concentration used.

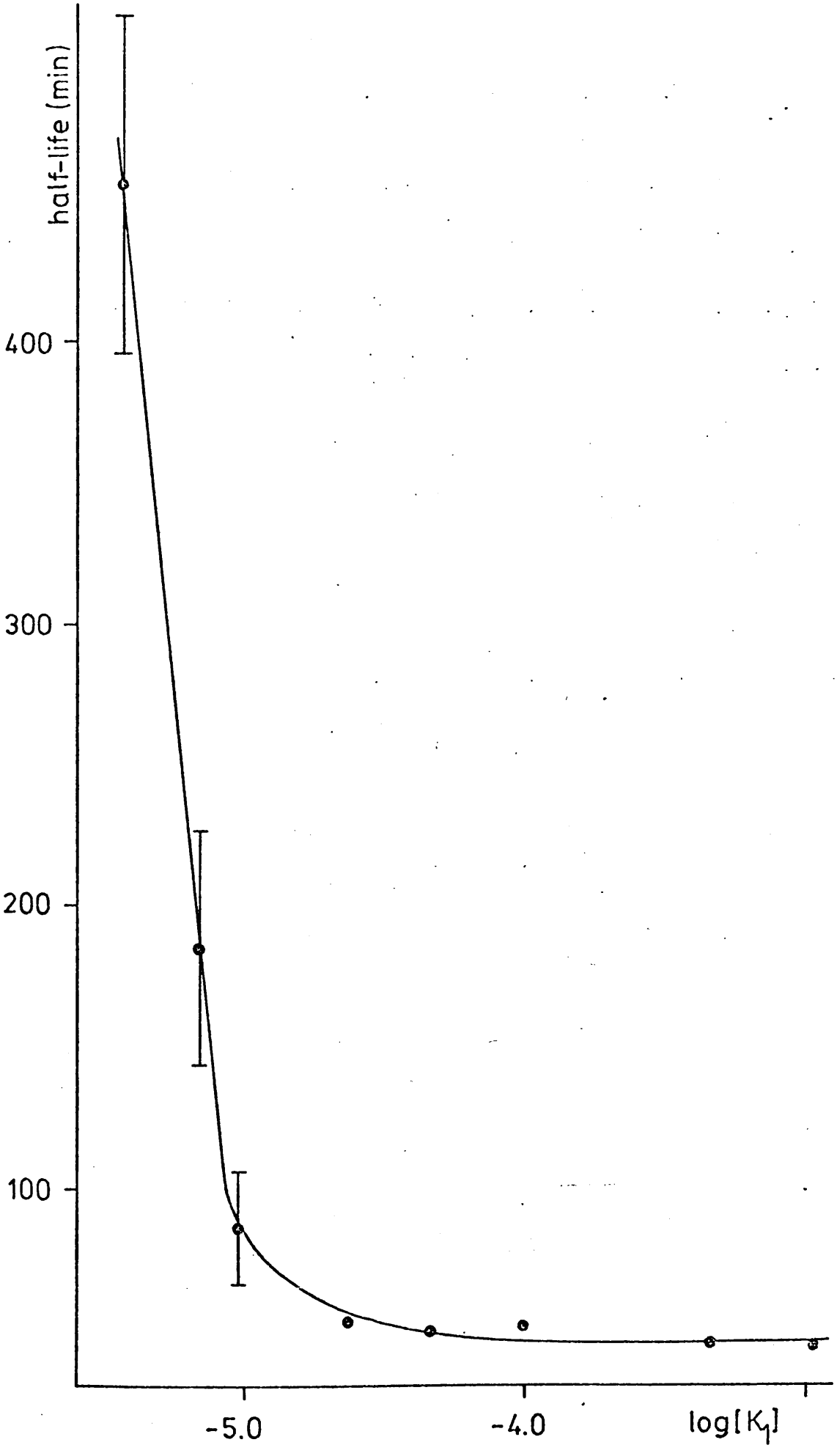
The reproducibility of the results from the methylene blue experiments is very high over a wide range of carrier concentration, deviations between duplicate experiments being less than 5% provided the vitamin K concentration does not decrease below ~10 μM, tables 10, 11 and 12. At lower carrier concentrations however, reproducibility decreases markedly, table 12, presumably because of the increasing influence of small, unavoidable oxygen leaks. In the range of vitamin K concentration 10-1000 μM the reaction half-life is effectively constant but below ~10 μM the half-life becomes a function of carrier concentration, figure 3-7.

The insensitivity of the reaction rate toward a hundredfold decrease in vitamin K concentration, table 12, is typical of carrier mediated transport and indicates that, provided a quinone concentration of greater than ~10 μM is employed, the rate of transfer of carrier across

Table 12 Dependence of the observed rate constants (25°C) on the concentration of vitamin K_1 in the membrane.

RUN	[vitamin K] (μM)	$10^3 \times k_1$ (min^{-1})	$t_{\frac{1}{2}}$ (min)
1	3.76	1.55 ± 0.18	455 ± 59
2	6.84	3.94 ± 0.88	185 ± 41
3	9.49	8.42 ± 1.67	86.2 ± 19.1
4	23.6	13.08 ± 0.17	53.0 ± 0.7
5	45.7	14.28 ± 0.10	48.6 ± 0.4
6	92.9	13.60 ± 0.15	51.0 ± 0.6
7	457	15.45 ± 0.64	44.9 ± 1.7
8	1060	15.84 ± 0.02	43.8 ± 0.2

Figure 3-7 The variation in the half-life of reduction of methylene blue (25°C) as a function of vitamin K_1 concentration.


half-life (min)
400
300
200
100
-5.0
-4.0
log[K_I]

the membrane is not rate determining. The reaction is therefore displaying saturation kinetics. Only when the total concentration of the carrier becomes comparable to that of the substrate does the rate become markedly affected. As the carrier concentration decreases so also will the amount of reduced carrier reaching the substrate interface and therefore a point will eventually be reached where the rate will become dependent on the rate of transfer of the carrier across the membrane in addition to the concentration of substrate in solution. From these results this crossover point is reached at a carrier concentration of ~10 μM, figure 3-7. Since the reaction order always remains first-order with respect to dye concentration it would appear that the translocation rate of the carrier never becomes the sole rate determining factor since under these conditions the rate would become independent of dye concentration (zero-order) as seen with DCIP, figure 3-2.

Comparison between the rates of reaction of methylene blue and DCIP is difficult since different stirrers were used in each set of experiments. Preliminary experiments with methylene blue in which perspex stirrers were employed, gave an average rate constant of 8.33×10^{-3} min^{-1} at comparable vitamin K concentrations (4 mM), almost half the value obtained using the teflon 'spinfins'. This finding allows a correction factor of x2 to be used to

generate a rate constant of $k_1 \simeq 7 \times 10^{-3}$ min^{-1} for DCIP. This artificial value can now be directly compared with the value of $k_1 \simeq 16 \times 10^{-3}$ min^{-1} obtained with methylene blue. The latter redox dye, therefore, reacts with dihydrovitamin K at over twice the rate found for DCIP.

If the experimental values of $k_1 = 16 \times 10^{-3}$ min^{-1}, $A = 1$ cm^2 and $V = 4$ ml, together with the literature value of D (22°C) $= 3 \times 10^{-6}$ cm^2 s^{-1} (123) for methylene blue, are substituted into equation (3-19) a value of $\delta = 28\mu$ is obtained for the thickness of the Nernst layer present in the methylene blue side of the interface. The approximate independence of reaction rate with quinone concentrations above ~10 μM, table 12, verifies that the reaction rate is solely dependent on the concentration of aqueous substrate, and therefore supports the above analysis. DCIP, as discussed earlier, also showed this insensitivity toward vitamin K concentration. By substituting $\delta = 28\mu$ into equation (3-19) an estimate for the diffusion coefficient of DCIP, of $D = 1.3 \times 10^{-6}$ cm^2 s^{-1}, is obtained. This is a very reasonable value. Additionally the doubling of the reaction rate observed on changing the stirrers suggests that the thickness of the diffusion layer was halved on changing to the teflon spinfins. Both the methylene blue and DCIP results, therefore, are consistent with the reaction being diffusion controlled in the aqueous

phase. The applicability of such a mechanism allows a number of important deductions to be made regarding the methylene blue system. Firstly, it implies that the shuttling of carrier across the membrane is relatively efficient. Secondly, the actual chemical (redox) reaction occurring at the interface must be very fast, each dye molecule reaching the vitamin K monolayer rapidly reacting with the excess of reduced carrier present. It is worth emphasising that the slow reaction rates displayed by the system are due to the physical limitations imposed by the experimental apparatus employed. Finally the rate constants determined with this system will be truly first-order ones.

Diffusion controlled experiments are very sensitive to changes in stirring speeds (section 3-1.6). For this reason experiments were carried out in which the concentration of vitamin K and methylene blue were kept constant at 1.06 mM and 7.2 μM respectively, and only the stirring speed in the aqueous limbs varied. The results (from duplicate sets of runs) were analysed according to first-order kinetic principles and are summarised in table 13. The marked decrease in the observed reaction rate, when the stirring speed of the 'spinfin' in the methylene blue solution was lowered, further supports that the methylene blue reduction is diffusion controlled. The ~40% drop

Table 13 Influence of stirring rate on the observed rate constants (25°C) for the system:

RMV/Vitamin K (hexane)/Methylene blue

The concentration of vitamin K_1 and methylene blue were kept constant at 1.06 mM and 7.2 μM respectively

STIRRER SPEED		$10^3 \times k_1$	$t_{\frac{1}{2}}$
RMV limb (rpm)	MB limb (rpm)	(min^{-1})	(min)
1650	850	9.73 ± 0.07	71.3 ± 0.8
* 1650	* 1650	15.84 ± 0.02	43.8 ± 0.1
850	1650	15.49 ± 0.08	44.8 ± 0.2

* Standard stirring speeds.

in reaction rate observed on halving this stirrer's speed, table 13, is completely consistent with such an analysis and would correspond to the increase in the apparent thickness of the diffusion layer to the new value of 46μ. These experiments also indicate that the reaction rate was relatively independent of the stirring speed in the RMV limb. In view of the observation that the rate was insensitive to a 50-fold decrease in carrier concentration, table 12, the small decrease in the rate of reduction of the quinone expected to be caused by a decrease in stirrer speed (~40% if reaction is diffusion controlled) would not, therefore, be anticipated to cause any significant change in the observed rate.

The influence of membrane (hydrocarbon) solvent on the observed reaction rate was next investigated. The concentration of methylene blue was kept constant at 7 ± 1 μM. The vitamin K solutions were prepared from a stock hexane solution of the quinone: equal aliquots of this solution were injected into separate flasks, the hexane blown off with nitrogen and finally a constant volume of various hydrocarbons pipetted into each flask. In this way the membrane, although generated using a variety of alkanes, was always 0.105 mM in vitamin K. This procedure ensured that the membrane solvent was the only parameter varied. Experiments, repeated at least

once, gave results which obeyed first-order kinetics. The averaged results together with the viscosity values of the membrane hydrocarbons (124) are recorded in table 14. As may be seen from this table the overall rate measured was insensitive toward the hydrocarbon used, tetradecane being the only alkane to cause any significant alteration to the rate. The increase in half-life obtained with tetradecane is interpreted as a viscosity effect, the very high viscosity of this solvent causing the rate of transfer of the carrier to be slowed down sufficiently for it to now become involved in the rate determining step of the reaction. However, increasing the viscosity of the solvent up to a value of ~1.4 cp appears to have no effect on the measured rates, a fact which is of importance since artificial planar bilayer membranes normally use decane as the lipid solvent.

Stoichiometry of accompanying proton transfer

Redox membrane systems that use quinones as the carrier transfer both protons and electrons across the membrane (figure 0-6(c) according to the equation

$$\overline{QH}_2 \longrightarrow \overline{Q} + 2H^+ + 2e^- \qquad (3\text{-}24)$$

Practical demonstration of this effect involves substrate reduction. The most suitable system studied to date used methylene blue as the substrate (section 3-2.6). Under

Table 14 Influence of membrane (hydrocarbon) solvent on the observed rate constants (25°C) for the system:

RMV/Vitamin K (hydrocarbon)/Methylene blue

The concentration of vitamin K_1 in each hydrocarbon and the concentration of aqueous methylene blue were both kept constant at 0.105 mM and 7 μM respectively. Standard stirring conditions were used.

Hydrocarbon	Viscosity* (cp)	$10^3 \times k_1$ (min^{-1})	$t_{\frac{1}{2}}$ (min)
hexane	0.292	13.12 ± 0.32	52.9 ± 1.3
heptane	0.390	14.12 ± 0.29	49.1 ± 1.0
octane	0.514	13.19 ± 0.45	52.6 ± 1.8
nonane	0.667	13.56 ± 0.58	51.3 ± 2.2
decane	0.854	13.13 ± 0.82	53.0 ± 3.3
dodecane	1.358	13.89 ± 0.32	50.0 ± 1.2
tetradecane	2.081	8.36 ± 0.42	83.1 ± 4.2

* From reference (124).

ideal conditions one proton will be released into the substrate solution for every molecule of methylene blue reduced, equation (3-20).

Measurement of the accompanying proton transfer proved to be experimentally very difficult because of the required use of short reaction times and low substrate concentrations. Direct measurement of pH changes was found to be impossible due to the low buffering capacity of the system and to the adsorption of methylene blue onto the glass electrode. The accompanying acidity changes were indirectly measured, however, using nitrophenol as a pH indicator. The method is described in section 3-2.6. The results from a typical experiment are given in table 15. From this experiment a value of $|\Delta H_o^+|/|\Delta MB| = 1.45$ was obtained. This result clearly demonstrates that protons are being ejected into the aqueous phase on reduction of methylene blue by dihydrovitamin K and, therefore, supports the idea that vitamin K molecules are functioning as both electron and proton carriers, shuttling both species across the membrane.

The discrepancy of the observed stoichiometric ratio from unity deserves further comment. Small atmospheric leaks of oxygen, both during and after the completion of the reaction would be expected to have no effect on the acidity change, since the net effect of such leaks would be the reduced methylene blue catalysed autoxidation of vitamin K, equation (3-25).

Table 15 Stoichiometry of accompanying proton transfer across the redox system: RMV/Vitamin K (hexane)/Methylene blue

The methylene blue was dissolved in 0.05M NaCl to a concentration of 6.6 μM. The concentration of vitamin K_1 was 0.407 mM. The number of moles of each species present refers to a volume of 2.4976 ml of solution. The appropriate dilution factor has been applied in the case of methylene blue.

Time	CORRECTED OD			NANOMOLES OF EACH SPECIES			
	MB (655 nm)	* A^- (403 nm)	* HA (318 nm)	MB	* A^-	* HA	H^+
Start	0.461	0.142	0.914	13.17	17.59	218.89	3.18
Finish	0.107	0.066	0.951	3.06	7.65	229.27	7.65
Finish -Start				-10.11	-9.94	10.38	4.47

* HA and A^- denote the acid and salt forms of p-nitrophenol.

$$\overline{QH}_2 + \tfrac{1}{2}O_2 \xrightarrow{MBH} \overline{Q} + H_2O \qquad (3\text{-}25)$$

There is no possibility of reduced methylene blue acting as an acid (since it is already in its base form). Indeed, if any reduced substrate were present, it would tend to take up H^+ ions from solution as its amine groups became protonated. This would result in a stoichiometry of <1 being observed. A stoichiometric ratio of >1 can only be attributed to the effect of atmospheric carbon dioxide. Although all due care was taken, contamination of the solutions by traces of carbon dioxide during the transfer and dilution of solutions, probably occurred. Only an extremely small quantity of carbon dioxide (~ few nanomoles) would be required to markedly affect the result. In conclusion, there can be no serious doubt that the stoichiometric ratio is unity and that the deviation found is a result of experimental difficulties.

Kinetics of vitamin K reduction by RMV

All the kinetic experiments previously described involved an investigation of the kinetics of reaction at the dihydrovitamin K/substrate interface. The set of experiments to be described, using the system

RMV/K (hexane)

was designed to investigate the kinetics of reduction, with respect to the concentration of vitamin K, at the RMV/membrane interface.

The procedure adopted was similar to that described in section 3-3.2 except that no vitamin K solution was added initially and hexane replaced the aqueous substrate solution. Once the H-cell was sealed and the methyl viologen reduced, 4 ml of hexane was added. One limb of the cell together with the horizontal membrane layer were therefore filled with hexane. The reaction was started by the injection of a 0.5 ml aliquot of vitamin K solution. Measurement of the absorbance of a similarly diluted vitamin K solution allowed the starting optical density, and therefore the concentration of the quinone, to be determined. All optical densities were measured at 248 nm. The stirrer speed in the hexane limb had to be reduced to 650 rpm to effect magnetic coupling. Since, however, this stirrer was well removed from the interface and since the stirrer in the RMV limb was maintained at its normal value (1650 rpm), only a minor effect on the reaction rate was expected to result from this necessary adjustment.

Three experiments using a 6.17 μM concentration of vitamin K and one using a 3.51 μM concentration were conducted. From the absorbance readings measured, the concentrations of oxidised vitamin K at each time could be calculated using equation (2-5) and the previously determined extinction coefficients for oxidised and reduced vitamin K (section 2-3.6). The graphs obtained when the logarithms of these concentrations were plotted against

time are shown in figure 3-8. The insert in this diagram records the characteristic changes which took place in the spectrum as the quinone became progressively more reduced. The concentration of vitamin K to which each run corresponds is given in table 16. Kinetic runs 1 and 4 obey first-order kinetics over the total time period covered. Deviations from ideal first-order behaviour were found to occur, however, with runs 2 and 3 after a time period of ~80 minutes. These deviations are considered to be due either to the effect of plasticiser or some other compound leached out of the rubber septa, or to the presence of small oxygen leaks. Because of these possibilities rate constants from these runs were determined using only data obtained over the 0-80 minute time period. The rate constants, half-lives and correlation coefficients found are recorded in table 16.

Investigations on the kinetics of reduction of vitamin K, although limited, point to the reaction being first-order with respect to vitamin K since a linear relationship between the logarithm of quinone concentration and time and an independence of rate constant to quinone concentration, are both observed, table 16. A pseudo first-order rate constant of $(11.07 \pm 0.76) \times 10^{-3}$ min^{-1} and a half-life of 62.9 ± 4.6 minutes were obtained on averaging the results.

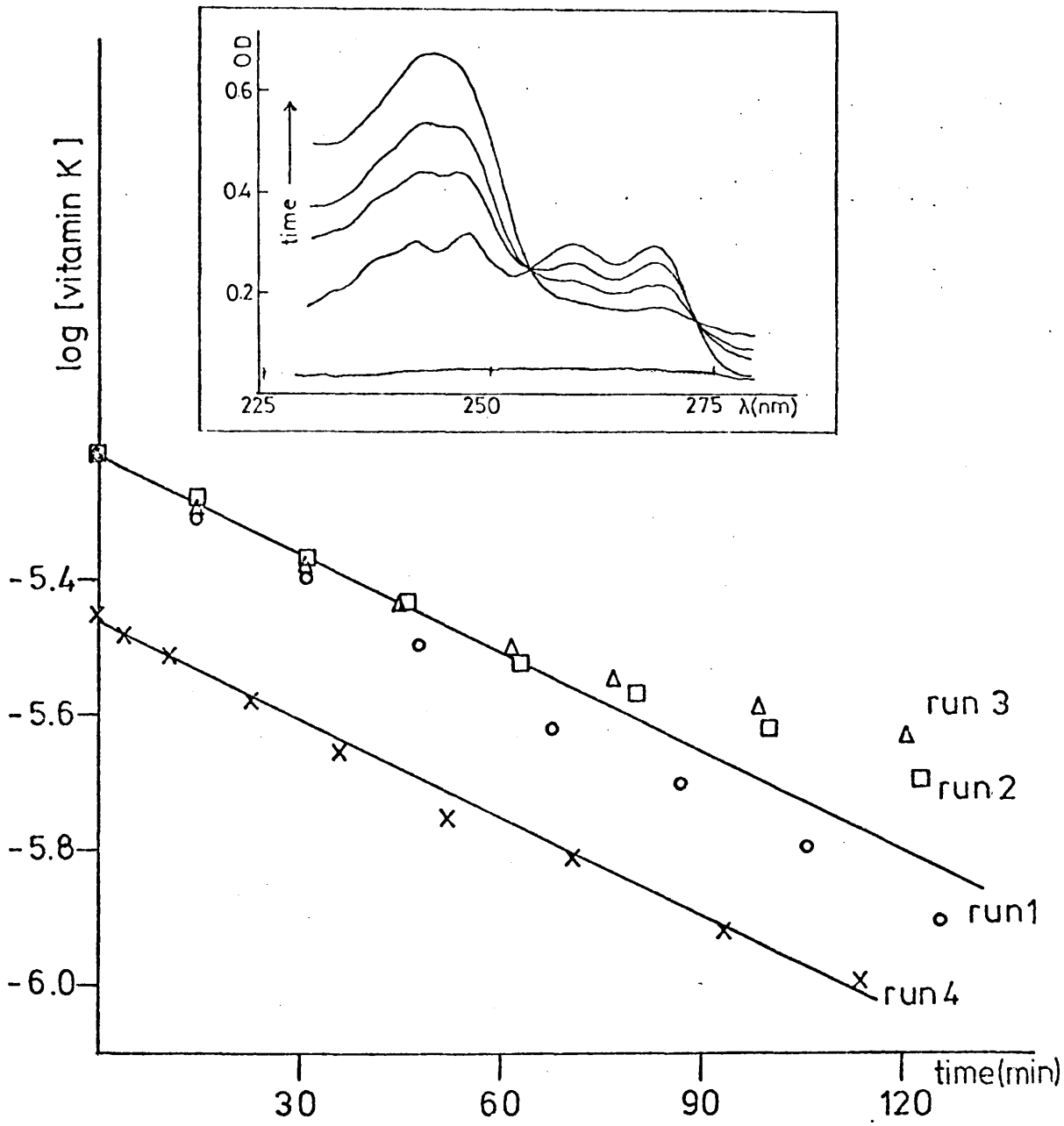


Figure 3-8 The logarithm of the concentration of oxidised vitamin K plotted against time for the system:

RMV/Vitamin K (hexane)

The temperature was 25°C. The insert shows the characteristic changes occurring in the ultraviolet spectrum of the quinone as reduction proceeds.

Table 16 Investigation of the rate of reduction (25°C) of vitamin K_1 by RMV for the system:

RMV (aqueous)/Vitamin K (hexane)

RMV was generated as previously described (section 3-2.4). Stirring speeds of 1650 and 650 rpm were used in the RMV and vitamin K limbs respectively. The total volume of vitamin K solution was 8.5 ml.

RUN	[vitamin K] (μM)	$10^3 \times k_1$ (min^{-1})	$t_{\frac{1}{2}}$ (min)	R
1	6.17	12.39	55.6	-0.9985
2	6.17	10.64	65.2	-0.9951
3	6.17	10.18	68.1	-0.9923
4	3.51	11.05	62.7	-0.9960

The above analysis of the RMV/vitamin K biphasic system involved the use of equation (2-5) which is only valid if the quinone and quinol in no way interact with each other. Quinone-quinol charge transfer complexes, quinhydrones, are well documented in the literature (67) and therefore it is worth questioning why such an approach is valid. A visual examination of the spectra obtained from incompletely reduced vitamin K solutions, figure 3-8 showed no trace of any charge transfer bands in both the ultraviolet and visible regions of the spectrum. This, together with the finding that the related benzoquinone, coenzyme Q, shows no evidence of quinhydrone formation (76), has been taken as justification for the validity of this analysis.

The first-order behaviour displayed by vitamin K during these reactions indicates that the interfacial reduction of quinone by RMV may be diffusion controlled within the hexane layer, the rate determining step being the rate of diffusion of the quinone from the bulk solution up to the interface. If it is assumed that vitamin K reduction by RMV and oxidation by methylene blue are diffusion controlled in the membrane and substrate sides respectively of the H-cell, the fraction of carrier reduced during an H-cell experiment may be estimated for any particular concentration of carrier used.

The rate of production of vitamin K (K) by RMV is given by

$$d[K]/dt = k_1[K] \qquad (3\text{-}26)$$

The value of $k_1 = 11 \times 10^{-3}$ was experimentally determined when the volume of the vitamin K solution was 8.5 ml. For a normal membrane, the volume of hexane solution was 4.5 ml and, in consequence, a rate constant of

$$k_1 = (8.5/4.5)(11 \times 10^{-3}) \simeq 20 \times 10^{-3} \qquad (3\text{-}27)$$

would be expected (equation 3-19). The rate of dihydrovitamin K (KH_2) oxidation at the opposite interface will be equal to the rate of methylene blue reduction

$$-d[KH_2]/dt = k_1'[MB] \qquad (3\text{-}28)$$

k_1' for methylene blue is $\sim 16 \times 10^{-3}$ (table 11). It should be noted that all the concentrations quoted refer to those in the bulk solution. In the steady state

$$k_1[K] = k_1'[MB] \qquad (3\text{-}29)$$

The total vitamin K (K_t) concentration remains constant throughout the run and, therefore,

$$[K_t] = [K] + [KH_2] \qquad (3\text{-}30)$$

Equations (3-29) and (3-30) together yield

$$[KH_2]/[K_t] = 1 - (k_1'/k_1)([MB]/[K_t]) \qquad (3\text{-}31)$$

The concentration of methylene blue used during a typical kinetic run was ~ 5 μM. Substitution of this value together

with the values of 20×10^{-3} and 16×10^{-3} for k_1 and k_1' respectively, into equation (3-31) gives

$$[KH_2]/[K_t] = 1 - 4 \times 10^{-6}[K_t]^{-1} \qquad (3\text{-}32)$$

For normal experiments where $[K_t] \sim 460$ μM, equation (3-32) predicts that $[KH_2]/[K_t] = 0.99$, that is the membrane carrier would be 99% reduced in the steady state. For concentrations of carrier ~10 μM, however, a value of $[KH_2]/[K_t] = 0.60$ is obtained and so the carrier is estimated to be only 60% reduced. These estimates refer only to bulk carrier concentrations and it is likely that, due to the greater hydrophilicity of reduced vitamin K, the fraction of vitamin K in the fully reduced form may be higher in the interfacial monolayers.

The above calculations indicate that, under normal experimental conditions, the membrane contains virtually only reduced carrier. Since the concentration of quinol far exceeds that of the substrate, the substrate reduction would be expected to be independent of carrier concentration and to exhibit saturation kinetics. This is exactly what was found experimentally. As the concentration of carrier is reduced, however, the amount of quinol present in the membrane also decreases. At a concentration of ~10 μM of vitamin K, just over half the carrier is predicted to be in the reduced state. Below this concentration the half-lives of methylene blue reduction were found to increase markedly, figure 3-7. From the foregoing analysis it

would appear that, when the carrier concentration falls below the value of 10 μM, the rate of quinol production is now not of sufficient magnitude to maintain a high concentration of dihydrovitamin K in the membrane. The steady state fraction of reduced vitamin K falls and the methylene blue reduction, in consequence, becomes additionally dependent on the amount of carrier reaching the interface. The resultant change in kinetics causes the reaction rate to decrease markedly.

3-3.3 Thionine as the aqueous substrate

It was of interest to study the kinetics of reduction of thionine, a thiazine homologue of methylene blue, to investigate whether small differences in structure, figure 1-8(c), effect changes in the observed kinetics. The system used in this experiment may be represented as

RMV/Vitamin K (hexane)/Thionine

The concentration of vitamin K in the membrane was 0.407 mM and the concentration of thionine was 9 μM. The absorbance of the oxidised form of the dye was monitored at 600 nm over a period of three hours. In analogy to methylene blue, the teflon stirrers adsorbed ~10% of the dye and therefore stirrer corrections were applied to all the recorded absorbances (section 3-2.2). The H-cell was filled according to method B (section 3-2.4). Membrane agitation and standard stirring conditions were again used.

The results from this experiment, together with those from a methylene blue run carried out under similar conditions, are given in figure 3-9. The thionine results were found to obey zero-order kinetics yielding a rate constant of 7.195×10^{-9} mole litre^{-1} min^{-1} and a half-life of 625 minutes. The first-order methylene blue reaction gave a rate constant and half-life of 14.00×10^{-3} min^{-1} and 49.5 minutes respectively.

The marked differences in behaviour exhibited by the structurally similar thiazines, methylene blue and thionine, figure 3-9, clearly demonstrate the potential dangers assuming similar chemical behaviour purely on the basis of structural similarity. Not only does thionine react more than ten times slower than methylene blue, but it also appears to exhibit a different reaction order. Great caution must be applied, therefore, when trying to generalise from any of the specific results obtained. This is true not only of substrate, but also of carrier behaviour since coenzyme Q, which is structurally similar to vitamin K, does not react with either thiazine to any significant extent (81). The change in order and much slower half-life found when thionine was substituted for methylene blue, indicates a new rate determining step. Substrate reduction may now be reaction as oppossed to diffusion controlled.

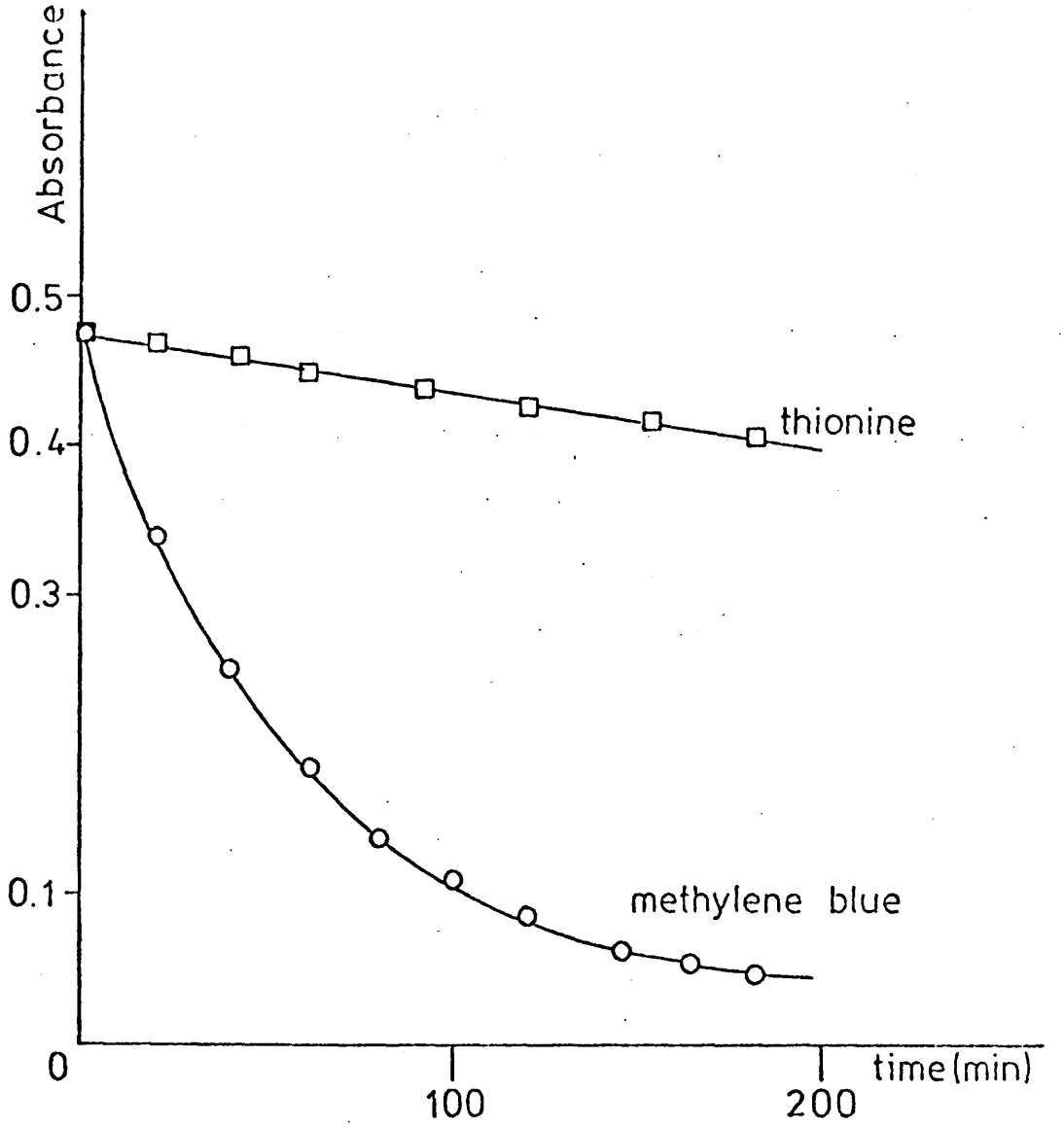


Figure 3-9 The absorbance of thionine (measured at 600 nm) and methylene blue (measured at 655 nm) plotted against time for the system:

RMV/Vitamin K (hexane)/thiazine

The concentrations of thionine, methylene blue (both dissolved in phosphate buffer, pH 6.86) and vitamin K_1 were 9 μM, 7 μM and 0.407 mM respectively. The temperature was 25°C. Standard stirring conditions were used.

Chapter Four

KINETIC STUDIES OF BIOLOGICAL RELEVANCE

The biological implications of the results obtained during this study, using bulk liquid redox membranes containing vitamin K as the carrier, are given in this chapter. Before such a discussion can be made, however, it is necessary to give a more detailed description of biological electron transport and energy transduction processes.

4-1 RESPIRATORY MEMBRANES

In eucaryotic cells the enzymes catalysing electron transport and oxidative phosphorylation are located in the inner mitochondrial membrane, whereas in procaryotic cells they are found in the cell membrane. In spite of this difference, there is a striking similarity in composition

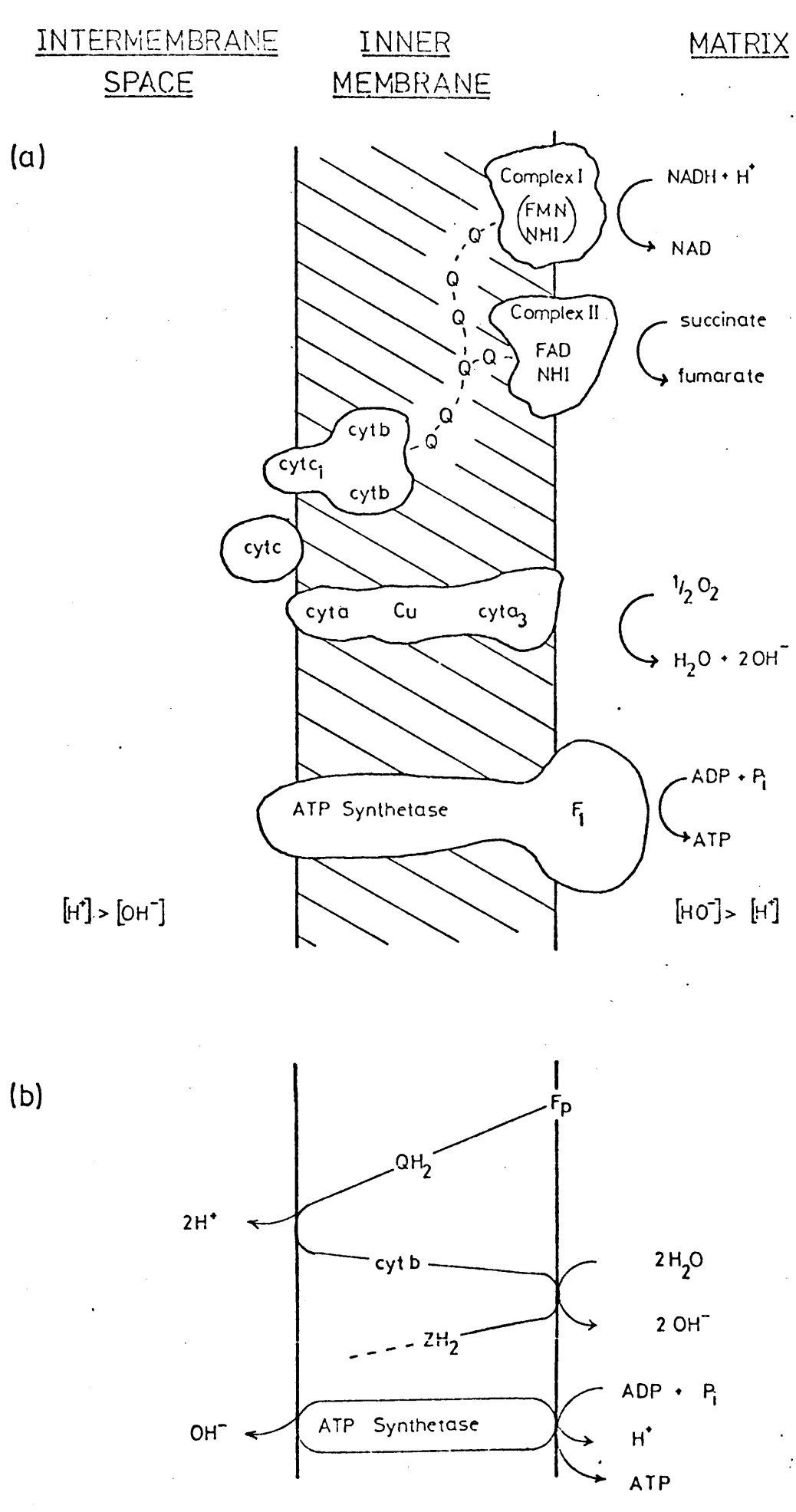
and reaction mechanism between mitochondrial and bacterial respiratory and phosphorylating systems. Indeed the same is true for the light induced electron transport and phosphorylating systems of plants and micro-organisms (125). For this reason, although the following discussion is limited mainly to mitochondrial systems (since they are the best understood cell organelles in terms of ultrastructure, molecular organisation and functional role in cell metabolism), the principles discussed should be considered as being typical of the general processes occurring during electron transport and oxidative phosphorylation. It should be noted, however, that even with these systems very little is known of the ultimate molecular mechanism by which the energy of electron flow is conserved as ATP.

Available evidence indicates that the electron carrier molecules constituting the mitochondrial electron transport chain, figure 1-9(a), are arranged into supramolecular clusters, called respiratory assemblies, which contain a fixed number of molecules of each carrier and which are embedded in the structure of the membrane. Four lipoprotein aggregates have been isolated: Complex I (NADH-Q reductase) which contains FMN, nonheme iron (NHI) and coenzyme Q, Complex II (succinate-Q reductase) which contains cytochrome b, NHI and cytochrome c_1, Complex III (QH_2-cytochrome c reductase) and Complex IV (cytochrome oxidase) which contains

cytochromes a and a_3 together with copper. Each complex contains multiple prosthetic groups and a number of polypeptide subunits (125). An additional protein aggregate, ATP synthetase, has been identified as the enzyme catalysing energy coupling, the coupling factor F_1 of this complex actually carrying out the ATP synthesis. These large proteins are thought to perturb the bilayer structure by penetrating the membrane (126). The proposed transverse topology of these inner mitochondrial membrane catalysts is depicted in figure 4-1(a), (125), (126). As can be seen from the diagram the oxidation of NADH, succinate and cytochrome oxidase, together with the synthesis of ATP, all occur in the inside, or matrix, of the mitochondrion. This vectorial dispersal of components is thought to be crucial to the energy coupling process (126).

It is now generally accepted that the respiratory chain contains three ATP coupling sites, located in Complexes I, II and IV, all of which use a common ATP synthetase (125). Oxidative phosphorylation is a very labile process that requires membrane integrity if it is to be observed. Careful studies have shown that for each pair of electrons passing down the chain, two protons are pumped out of the mitochondrion at each energy-conserving site. These observations prompted Mitchell to propose the Chemiosmotic Hypothesis as the mechanism of oxidative phosphorylation (127), (128). The hypothesis assumes that the membrane

Figure 4-1 Mitochondrial electron transport. (a) Illustration of the possible orientation of inner mitochondrial membrane components. Components which interact with the membrane predominantly by electrostatic forces (viz. cytochrome c) are surface components and those organised in the centre of the membrane are predominantly bound by strong hydrophobic forces. The large ATP synthetase complex is thought to be bound by both types of forces. (b) Scheme for the coupling between redox reactions and proton transport in Complex III. Z is an unknown hydrogen carrier, possibly cytochrome b or an iron-sulphur protein (26), (129).


INTERMEMBRANE SPACE
INNER MEMBRANE
MATRIX
(a)
Complex I
(FMN NHI)
NADH + H+
NAD
Q
Complex II
FAD NHI
succinate
fumarate
cytb
cytc1
cytb
cytc
cyta Cu cyta3
1/2 O2
H2O + 2OH−
ATP Synthetase
F1
ADP + Pi
ATP
[H+] > [OH−]
[HO−] > [H+]
(b)
FP
QH2
2H+
cyt b
2H2O
2 OH−
ZH2
ADP + Pi
OH−
ATP Synthetase
H+
ATP

is an essential element in the mechanism of oxidative phosphorylation and that the membrane is passively impermeable to protons and ions in general. It is further suggested that transport of electrons along the respiratory carriers generates a proton gradient across the membrane and that it is the formation of this 'protonmotive' force which conserves the free energy decline, occurring in electron transport, by providing the driving force for ATP synthesis. The proton gradient is envisaged as arising through the action of alternating hydrogen and electron carriers which form loops across the membrane in such a manner that protons are taken from the matrix and are released into the intermembrane space. Three such loops were proposed, each translocating two protons across the membrane per two electrons transferred through the chain. The process was considered to be vectorial in nature, the enzymes' active sites allowing proton flow in only the direction discussed. The energy rich gradient generated by electron transport is postulated to cause the formation of ATP, formally a dehydration reaction, by promoting the catalytic removal of water, as H^+ and OH^-, from ADP and phosphate. These ions are ejected, by the F_1 factor, into the matrix and intermembrane aqueous phases respectively, where they are effectively trapped by the excess of their conjugate ions with the formation of water.

A typical redox loop which involves coenzyme Q as the proton carrier, is shown in figure 4-1(b) (26), (129). The uncoupling of oxidative phosphorylation from electron transport, effected by uncouplers, is thought to be caused by the ability of these molecules to discharge the proton gradient.

The Chemiosmotic Hypothesis requires no direct physical or chemical contact between redox chain assemblies and ATP synthetase. These enzymes are linked via a common 'protonmotive' force. The Chemiosmotic Hypothesis is also thought to apply to bacterial and photosynthetic phosphorylation (127), (128), (130). It should be pointed out however, that though the hypothesis has been widely accepted as being basically correct (131), (132), other mechanisms (133), (134), have been postulated.

The apparent roles of lipid and cytochrome c in the mitochondrion are of particular relevance to this research and, therefore, attention will be focussed briefly on the function of these components in this organelle.

The presence of phospholipids as the predominant form of mitochondrial lipid is well recognised (79). In excess of 90% of the total lipid is phospholipid, the balance being a neutral lipid (mainly coenzyme Q) fraction, table 17. The basic pattern of lipid occurring within the respiratory assemblies is not significantly different from the original

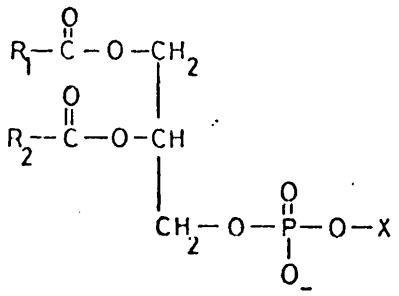
Table 17 Phospholipid composition of mitochondrial inner and outer membranes (79).

Phospholipid	% Phospholipid*	
	inner membrane	outer membrane
total phospholipid	21.4	45.1
cardiolipin	21.5	3.2
phosphatidyl inositol	4.2	13.5
phosphatidyl ethanolamine	27.7	25.3
phosphatidyl choline	44.5	55.2
unidentified	2.2	2.5

* % phospholipid relative to protein + phospholipid.

mitochondrion, the lipid being an absolute requirement for the functioning of each complex. Mitochondrial lipids are rich in unsaturated fatty acids and have a net negative total charge due to phosphatidyl inositol (PI) and cardiolipin (CL) having one and two negative charges respectively. Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) are zwitterionic and neutral overall (135). The general structures of these lipids are given in figure 4-2. The functions of phospholipid in electron transport processes are thought to be diverse. Lipid may act by holding the enzymes in specific intermolecular alignments. The phospholipid matrix may form a suitable non-aqueous environment in which electron transport and energy coupling can occur. Finally, certain phospholipids, especially CL, may have a role in binding the electron transport particles together. The phospholipid bilayer in addition, of course, would still function as a permeability barrier to the unrestricted flow of solutes (79), (135).

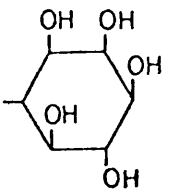
The neutral lipid coenzyme Q, of cristae membranes, has a concentration far in excess of the other membrane-bound carriers, table 18, and is not covalently bound to the membrane (79). It is now well established that ubiquinone is a functional member of the electron transfer chain, apparently transferring electrons from the flavoprotein dehydrogenases and their associated nonheme iron, to the cytochromes (28), (79), (125), (135), (136). Ubiquinone



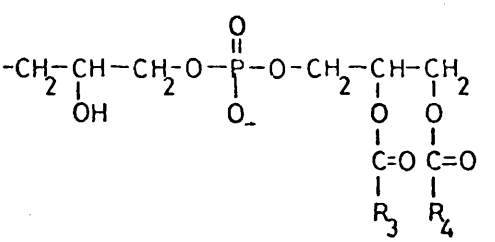
Phosphatidyl ethanolamine X = $-CH_2-CH_2-NH_2$

Phosphatidyl choline X = $-CH_2-CH_2-\overset{+}{N}(CH_3)_3$

Phosphatidyl inositol X =



Cardiolipin X =



Lysolecithin

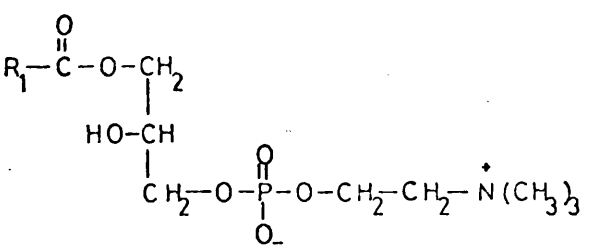


Figure 4-2 General structures of the phospholipids used during this research.

Table 18 Molar ratio of electron carriers in beef-heart mitochondria, (relative to cytochrome a + a_3), (28).

Carrier	Ratio
Cytochrome a + a_3	1.0
Cytochrome c + c_1	1.5
Cytochrome b	1.0
Total flavins	~1.0
Nonheme iron	6
Coenzyme Q	8
NAD	12
NADP	2

is visualised as being a mobile carrier, present in the lipid phase, which is able to transfer electrons from Complexes I, II and III by virtue of its ability to move freely through the lipid phase (28), (79), (136)-(138). The quinone acts, therefore, not as a member of the respiratory chain but as an inter-chain electron-transfer agent, mediating between fixed respiratory chain assemblies (139). Kinetic studies (79), (137), (140), indicate that ubiquinone operates according to saturation kinetics, the stoichiometry dictating that only a small portion of the quinone is allowed to react at any one time. It has also been suggested that some of the large pool of ubiquinone has an antioxidant function, preventing the peroxidation of both unsaturated fatty acids and heme compounds (79).

Cytochrome c, unlike other respiratory proteins, is easily extracted from the respiratory chain, apparently being bound to the mitochondrial membrane by electrostatic forces (141). Mammalian cytochromes c are highly basic due to the large numbers of lysine residues which they contain, horse-heart cytochrome c having an isoelectric point at pH ~10.5 (82). These proteins are again unusual in that the prosthetic group, heme c, figure 4-3(b), is attached covalently to the peptide chain. The fifth and sixth coordination positions of the iron are occupied by histidine and methionine respectively (28). In the intact

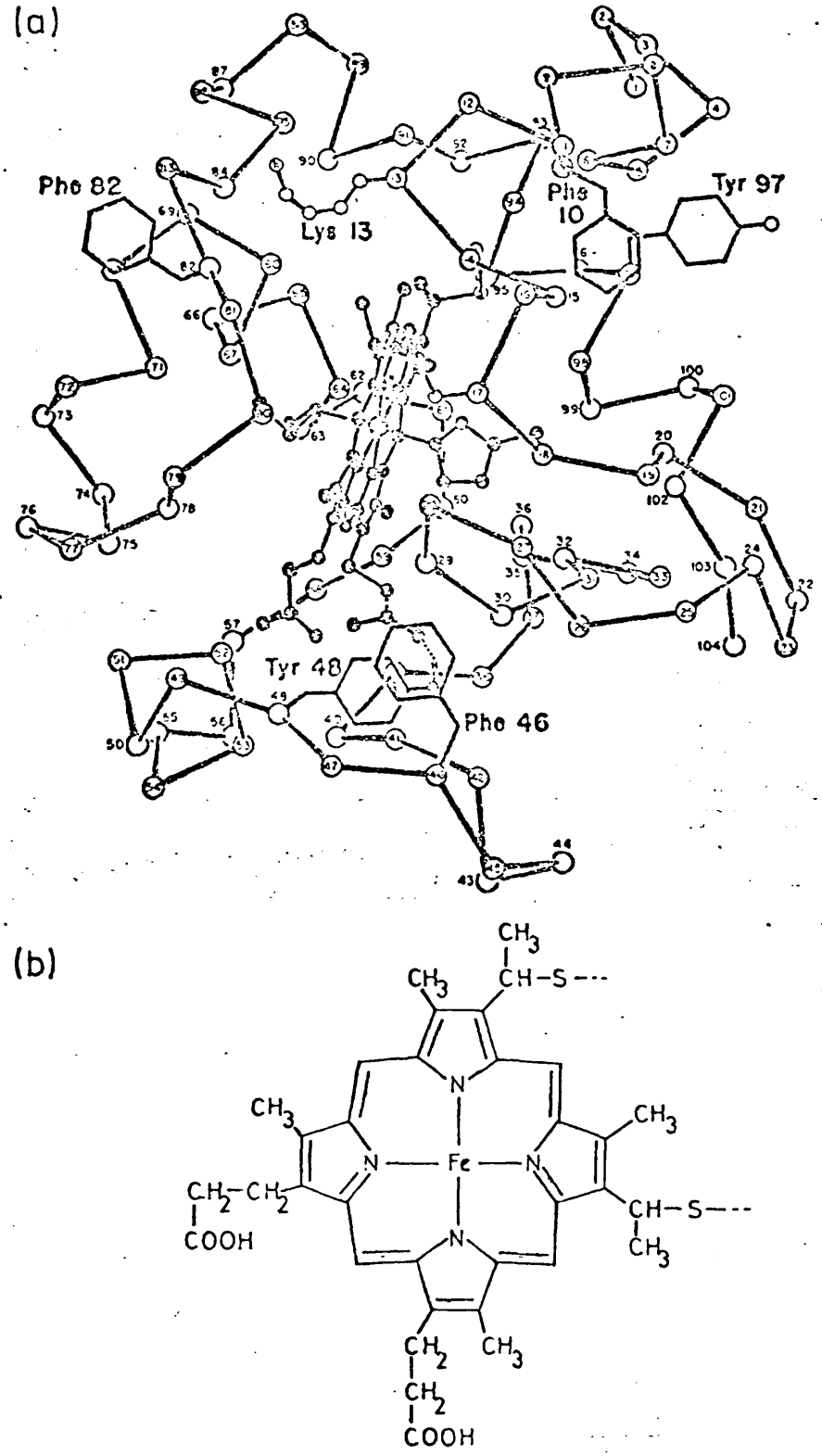


Figure 4-3 Cytochrome c. (a) Stereoscopic α-carbon diagram of the horse-heart cytochrome c molecule (reproduced from reference (82)). (b) Structure of heme c indicating pyrrole substituents and sites of attachment to the protein.

protein, the prosthetic group is 'buried' in a crevice. The heme is surrounded by tightly packed hydrophobic groups but one edge is exposed to the solvent at the heme crevice, figure 4-3(a). On reduction, although the overall structure remains the same, there is a general refolding of parts of the polypeptide chain which results in a closing of the heme crevice and the molecule becoming more compact (142). This is a major factor in the resistance of ferrocytochrome c to autoxidation at physiological pH (143).

Complexes formed between mammalian cytochrome c and phospholipids have been studied extensively (79), (135), (144)-(147). The binding within these phospholipids, synthesised by shaking phospholipid micelles with cytochrome c, is primarily electrostatic, with the external basic sites of the cytochrome, 8-10 sites in horse-heart cytochrome c (148), neutralised by the acidic lipids. These complexes, as expected, are dissociated, or their formation prevented, by high ionic strengths. Cytochrome c proteolipids normally have a definite stoichiometric ratio which changes as different phospholipids are used, table 19. All these complexes were found to be isooctane insoluble. Phosphatidyl choline does not complex with cytochrome c at all whereas PE, the salt of a strong acid and a weaker base, interacts to a limited extent. Saturated PE is, however, unable to form a complex with a definite stoichiometric

Table 19 Composition of various phospholipid-cytochrome c (horse-heart) complexes, (pH 7).

Phospholipid	Atoms P per molecule of cytochrome c complexed.
phosphatidyl choline	no complex formed
saturated phosphatidyl ethanolamine	~100
phosphatidyl inositol	10
cardiolipin	10

ratio but forms a loosely bound aggregate with a high PE: cytochrome c ratio, table 19.

The formation of cytochrome c proteolipids has suggested that cytochrome c may actually function as a lipid complex in the native electron transport chain, functioning as a mobile electron 'shuttle' in a similar way to coenzyme Q (79), (148). This however awaits further clarification. Bacterial and plant cytochromes c are neutral or acidic proteins and are therefore incapable of forming these proteolipid complexes (135). Hence these lipid cytochrome c complexes should not be involved in bacterial electron transport.

It is useful at this stage to recall the previously discussed experimental results that relate to the electron transport scheme depicted in figure 4-1(a). NADH was found not to react (biphasically) with coenzyme Q (section 1-4.1). Coenzyme Q is mobile within biological membranes. Since the molecule is also amphiphilic, there is no reason to doubt that some of the quinone molecules are in direct contact with NADH at the inner membrane/matrix interface. The inability of NADH to react directly with coenzyme Q verifies that the electron transport chain would not be short-circuited if these two types of redox molecules came into contact with each other. Coenzyme Q can be reduced by $FMNH_2$ however (section 1-4.1) and, therefore, the quinone

could be reduced indirectly by NADH if the latter could reduce FMN. This is exactly the function of Complex I. This complex catalyses both the reduction of FMN by NADH and the reduction of ubiquinone by $FMNH_2$. The reactivity of vitamin K toward NADH and $FMNH_2$ exactly paralleled that of coenzyme Q (section 1-4.1). The oxidation of ubiquinol by cytochrome c was found to be extremely slow (section 1-4.3). This again is in agreement with the proposed site of cytochrome c in the electron transport chain, since these two components are considered not to react directly with each other. The extreme slowness of the reaction implies that a link in the chain is missing. The cytochrome b-c_1 section of the respiratory chain is thought to be the enzyme link that catalyses cytochrome c reduction by ubiquinol. In contrast to ubiquinone, vitamin K was found to react directly with cytochrome c (section 1-4.3). The role of vitamin K in biological electron-transfer membranes is still rather speculative. The close structural similarity of vitamin K to coenzyme Q, however, allows it to be used to investigate electron-transfer steps which are typical of those naturally occurring.

4-2 RESULTS AND DISCUSSION

The general redox membrane system again used in these studies may be depicted as

$$\text{RMV/Vitamin K (hexane)/}\begin{matrix}\text{Methylene blue}\\ \text{or}\\ \text{Cytochrome c.}\end{matrix}$$

When methylene blue was used as the substrate, vitamin K was found (section 3-3.2) to behave in a kinetically similar manner to that observed for membrane-bound ubiquinone (section 4-1), both quinones displaying saturation kinetics and both functioning as mobile electron carriers. In addition, vitamin K can accept electrons from $FADH_2$ or $FMNH_2$ and can donate electrons to cytochrome c (section 1-4). This resembles closely the behaviour of ubiquinone save that this quinone may reduce cytochrome c only by way of the cytochrome b-c_1 complex. The similarity in behaviour between vitamin K in the redox Haber-Beutner type redox membranes, used in this research, and ubiquinone in respiratory membranes enables the former membranes to be used as simple models for the complex biological systems. In consequence, a number of more biological studies were carried out with the above model systems, using both methylene blue and cytochrome c as substrates. In particular the effects of adding various modifiers to the systems were investigated.

4-2.1 Methylene blue as aqueous substrate

Skelton et al (149) have suggested that the antimalarial agent chloroquine (a 4-aminoquinoline), which inhibits electron transfer at the coenzyme Q section of the respiratory chain, figure 1-9(a), may function by inhibiting either the synthesis of coenzyme Q dependent enzymes or the oxidation of ubiquinol. This latter possibility was investigated with the model membrane system developed, using methylene blue as the substrate.

RMV was generated as previously described (section 3-2.4) and the H-cell was filled according to method B. The aqueous phases were stirred using standard conditions and membrane agitation was again employed (section 3-2.1). Three different solutions of chloroquine diphosphate were prepared, each 7 μM in methylene blue. The concentration of vitamin K was 0.407 mM. The first-order rate constants obtained from this set of kinetic runs, which includes one obtained from a chloroquine-free, but otherwise identical system, are recorded in table 20.

An examination of the results, table 20, clearly shows that the presence of chloroquine in the substrate limb had no effect on the reaction rate observed and, therefore, cannot be inhibiting dihydrovitamin K oxidation. If it is assumed that reduced vitamin K and ubiquinol behave similarly in the presence of this antimalarial agent, then the negative

Table 20 Influence of chloroquine on the rate of reduction of methylene blue (25°C) for the system:

RMV/Vitamin K (hexane)/Methylene blue + Chloroquine

The concentration of vitamin K_1 and methylene blue, the latter dissolved in 0.025M phosphate buffer (pH 6.86), were constant at 0.407 mM and 7 μM respectively. The concentration of chloroquine was varied over a 0-550 μM range. Standard stirring conditions were used (section 3-2.1).

[Chloroquine] (μM)	$10^3 \times k_1$ (min^{-1})
-	13.58
4.40	13.11
46.3	12.46
543	13.41

result observed implies that inhibition of electron transport by chloroquine is not caused by inhibition of quinol oxidation. In view of the differences in vitamin K and coenzyme Q behaviour described earlier (section 3-3.3) such an assumption may, however, be incorrect.

The modifying influence of a number of common phospholipids on the observed reaction rate was investigated using the four lipids; phosphatidyl ethanolamine, cardiolipin, phosphatidyl inositol, and lysolecithin. Aliquots (0.03 ml) of stock solutions of these lipids (either ~10 mg ml^{-1} or ~1 mg ml^{-1}) were added to the membrane. The concentrations of vitamin K and methylene blue solutions used in each experiment were 0.407 mM and 6.5 $\pm$ 0.5 μM respectively. The procedure used was identical to that described for the previous set of runs. During each run the test limb was carefully checked visually to ensure that no micelles were formed by the surfactant-like lipids. Fortunately this problem never arose. A summary of the results obtained is given in figure 4-4, where the absorbance changes with time are graphed. The types and concentrations of the lipids used in each of these runs are recorded in table 21. The order of reaction exhibited by the PE and CL modified membranes was unclear, for they could have been analysed as obeying either zero- or first-order kinetics. Since, however, correlation coefficients nearer to -1.0 and

Figure 4-4 The absorbance of methylene blue (measured at 655 nm) plotted against time for the system:

RMV/Vitamin K (hexane) $\pm$ Phospholipid/Methylene blue

The concentration of methylene blue (dissolved in 0.025M phosphate buffer, pH 6.86) and vitamin K_1 were 6.5 μM and 0.407 mM respectively. The concentrations of phospholipid used are given in table 21. All experiments (25°C) started at the same absorbance reading. For clarity of presentation, however, these zero-time absorbances have been set at arbitrary points on the ordinate. The H-cell was filled according to method B (section 3-2.4) and standard stirring conditions were used.

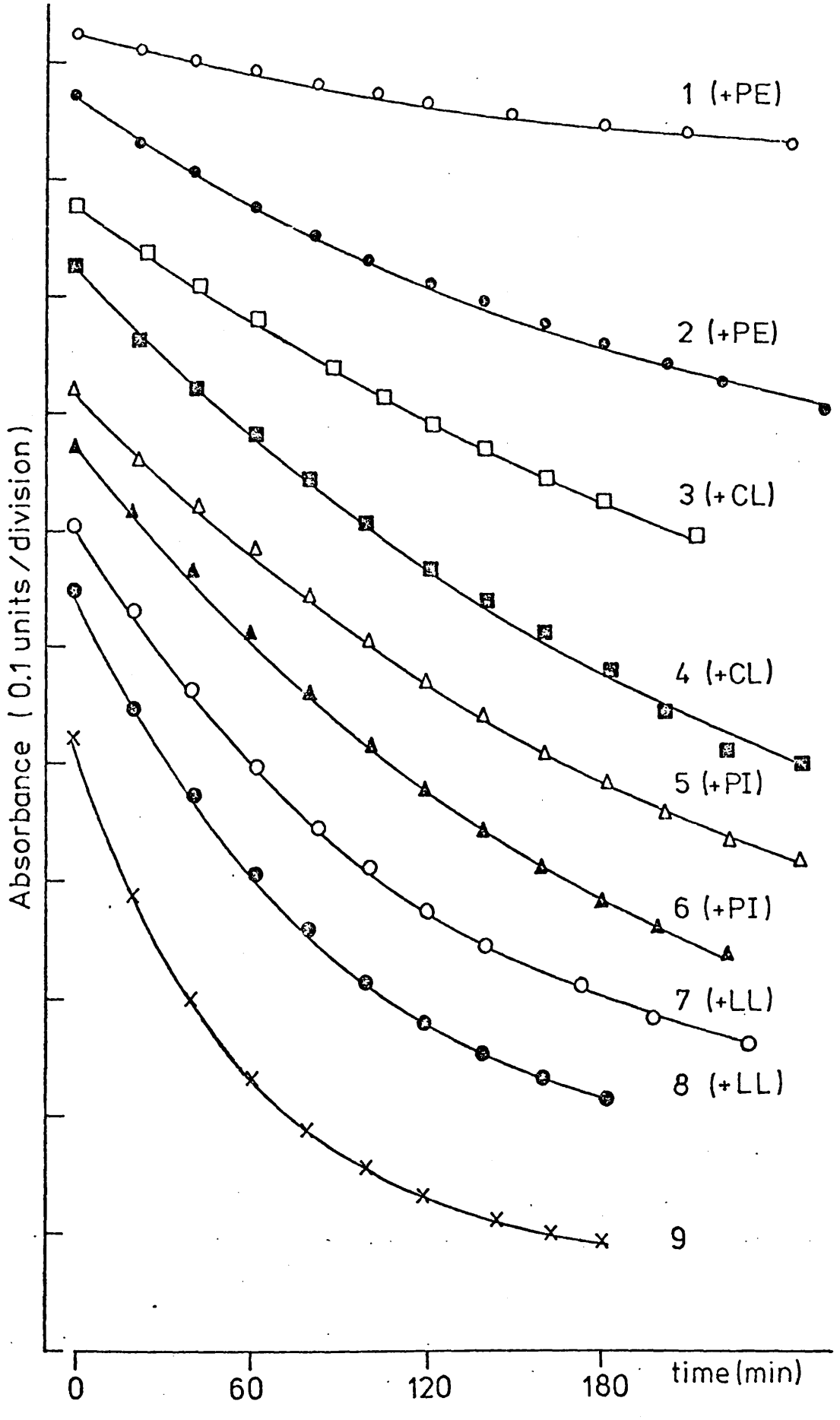

1 (+PE)
2 (+PE)
3 (+CL)
4 (+CL)
5 (+PI)
6 (+PI)
7 (+LL)
8 (+LL)
9
Absorbance (0.1 units / division)
0
60
120
180
time (min)

Table 21 Influence of phospholipid on the observed rate constants (25°C) for methylene blue reduction. For reference, the weight of vitamin K_1 in the membrane was 0.83 mg.

Run	Phospholipid	weight added to membrane (mg)	$10^3 \times k_1$ (min^{-1})	$t_{\frac{1}{2}}$ (min)	R
1	PE	0.26	1.042	665	-0.9953
2	PE	0.026	3.696	186	-0.9994
3	CL	0.28	4.875	142	-0.9988
4	CL	0.025	8.256	84.0	-0.9962
5	PI	0.25	5.724	121	-0.9988
6	PI	0.022	7.901	87.7	-0.9972
7	LL	0.24	9.066	76.5	-0.9997
8	LL	0.024	11.20	61.9	-0.9998
9	none	-	13.58	51.0	-0.9977

standard errors of estimate of the ordinate were smaller when a first-order analysis was applied to these two cases and since, in addition, both PI and lysolecithin (LL) results exhibited orders of unity, all the results obtained were analysed as arising from a reaction first-order with respect to methylene blue concentration. The rate constants found, together with one obtained from a similar run carried out in the absence of lipid, are also included in table 21.

The redox inert molecules of phospholipid, present in the membrane, are expected to compete with the redox active carrier molecules for sites on the monolayer. Since phospholipid is a much stronger amphiphile than either reduced or oxidised vitamin K, the phospholipid molecules will displace carrier molecules from the monolayers and cause a decrease in the surface coverage of quinol at the substrate interface. (There will also be a similar decrease in carrier adsorbed at the RMV interface but since the reaction is started with the carrier already previously reduced, this should not affect the initial reaction rates observed). This would result in a corresponding decrease of reaction rate, the stronger the adsorption of phospholipid, the slower the reaction observed. This hypothesis is supported by the experimental findings, table 21. In all cases the presence of lipid results in a decrease of reaction rate. The reduction in rate is more pronounced

with the larger concentration of phospholipid used, a ten times increase in lipid causing 20-400% decrease in the observed rate. The order of increasing effectiveness, LL<PI<CL<PE, is difficult to rationalise since the actual molar concentration of lipid in the membrane was unknown. It is of interest, however, to note that lysolecithin has the least effect. This lipid is known to cause membrane rupture by interacting with the lipid component of biological membranes, thereby causing the membrane to become more permeable to water (150). It would be expected, therefore, that LL in the model membrane system would not form such a closed, inert surface as the other lipids and, in consequence, would not be as efficient at blocking quinol reaction as the other phospholipids. The observed results are consistent with this postulate.

Coenzyme Q has been postulated to function as a mobile proton carrier in addition to acting as an electron carrier (section 4-1). The behaviour of vitamin K in the model membrane system described (section 3-3.2) verifies that isoprenoid quinones can indeed function as proton carriers and can, in addition, establish a 'proton motive' force across the membrane. As discussed above (section 4-1), this electrochemical potential gradient for protons is the primary driving force for ATP synthesis, according to the widely accepted Chemiosmotic Hypothesis.

4-2.2 Cytochrome c as the aqueous substrate

In view of the importance of lipids and cytochromes in respiratory membranes, it was of particular interest to investigate the system;

RMV/Vitamin K (hexane)/Cytochrome c.

The kinetics of reduction of cytochrome c (horse-heart), both with and without phospholipid present in the membrane, was studied. In analogy to methylene blue (section 3-3.2), the ideality of cytochrome c as a substrate was first demonstrated.

The immiscibility of cytochrome c in hexane was verified by equilibrating aliquots of spectroscopic hexane with similar volumes of oxidised cytochrome c, (dissolved in phosphate buffer to a concentration of 30 μM), overnight in hermetically sealed vials (section 1-3.1). The ultraviolet/visible spectra recorded from the hexane layers were found to show no absorption over a 200-700 nm range. Since ferricytochrome c absorbs strongly in this region (section 2-4.2) it was concluded that oxidised cytochrome c was insoluble in the membrane solvent and behaved, therefore, as an ideal substrate. Additionally, because cytochrome c is obtained from respiratory membranes and since membrane phospholipid can markedly influence observed reaction rates (previous section), it was important to ensure that the particular cytochrome c preparation used contained no

phospholipid contaminant. Thin layer chromatography on silica gel (GF 254, Merck) was used to analyse the hexane layers for the presence of phospholipid: a chloroform-methanol-water (65:25:4 v/v) solvent mixture was used as the moving phase and the plate was exposed to iodine vapour to detect any lipid present (151), (152). No phospholipid was detected by this means.

Verification of the suitability of cytochrome c as a reducible substrate allowed the kinetics of its reduction and the influence of phospholipid on this reduction, to be investigated. RMV was generated as before (section 3-2.4) and the H-cell was filled according to method B. The aqueous phases were stirred using standard conditions and the membrane was agitated as before (section 3-2.1). The concentration of vitamin K and cytochrome c solutions were 0.407 mM and 24.2 $\pm$ 2.5 μM respectively. The effect of adding 0.03 ml aliquots of CL, PI and PE solutions (either~10 mg ml^{-1} or ~1 mg ml^{-1}) on the reaction was determined. An experiment in which no vitamin K was present in the membrane served as a control. The results of these experiments are shown in figures 4-5, 4-6 and 4-7, where the absorbance of the cytochrome c solution is recorded as a function of time: the rate of change of absorbance is directly proportional to the rate of change of concentration of ferrocytochrome c. The nature and

Figure 4-5 The absorbance of cytochrome c (measured at 550 nm) plotted against time for the system:

RMV/Vitamin K (hexane)/Cytochrome c

The concentration of cytochrome c (dissolved in 0.025M phosphate buffer, pH 6.86) and vitamin K_1 were 24.2 μM and 0.407 mM respectively. All experiments (25°C) started at the same absorbance. For clarity of presentation, however, these zero-time absorbances have been set at arbitrary points on the ordinate. The H-cell was filled according to method B (section 3-2.4) and standard stirring conditions (section 3-2.1) were used.

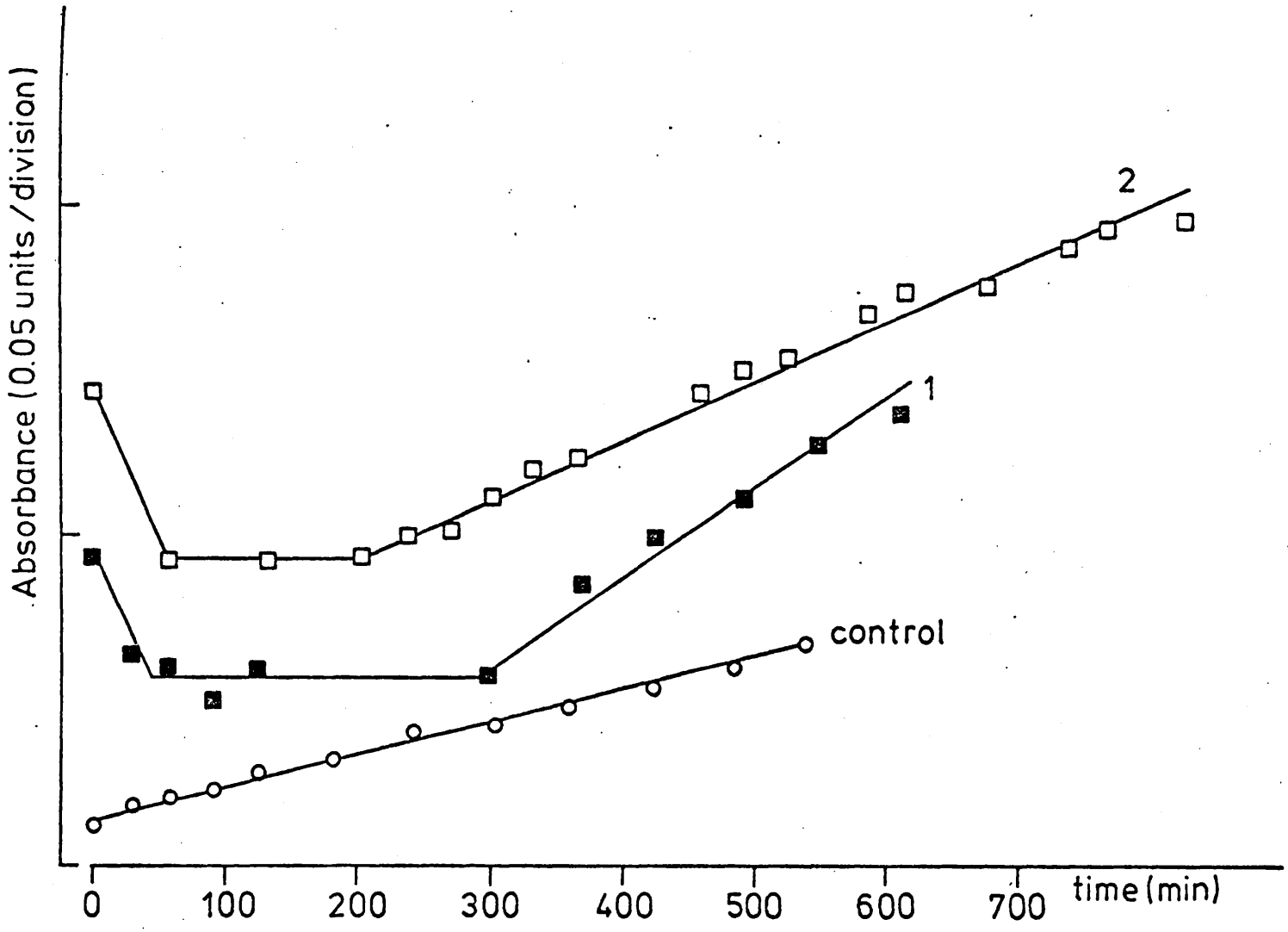
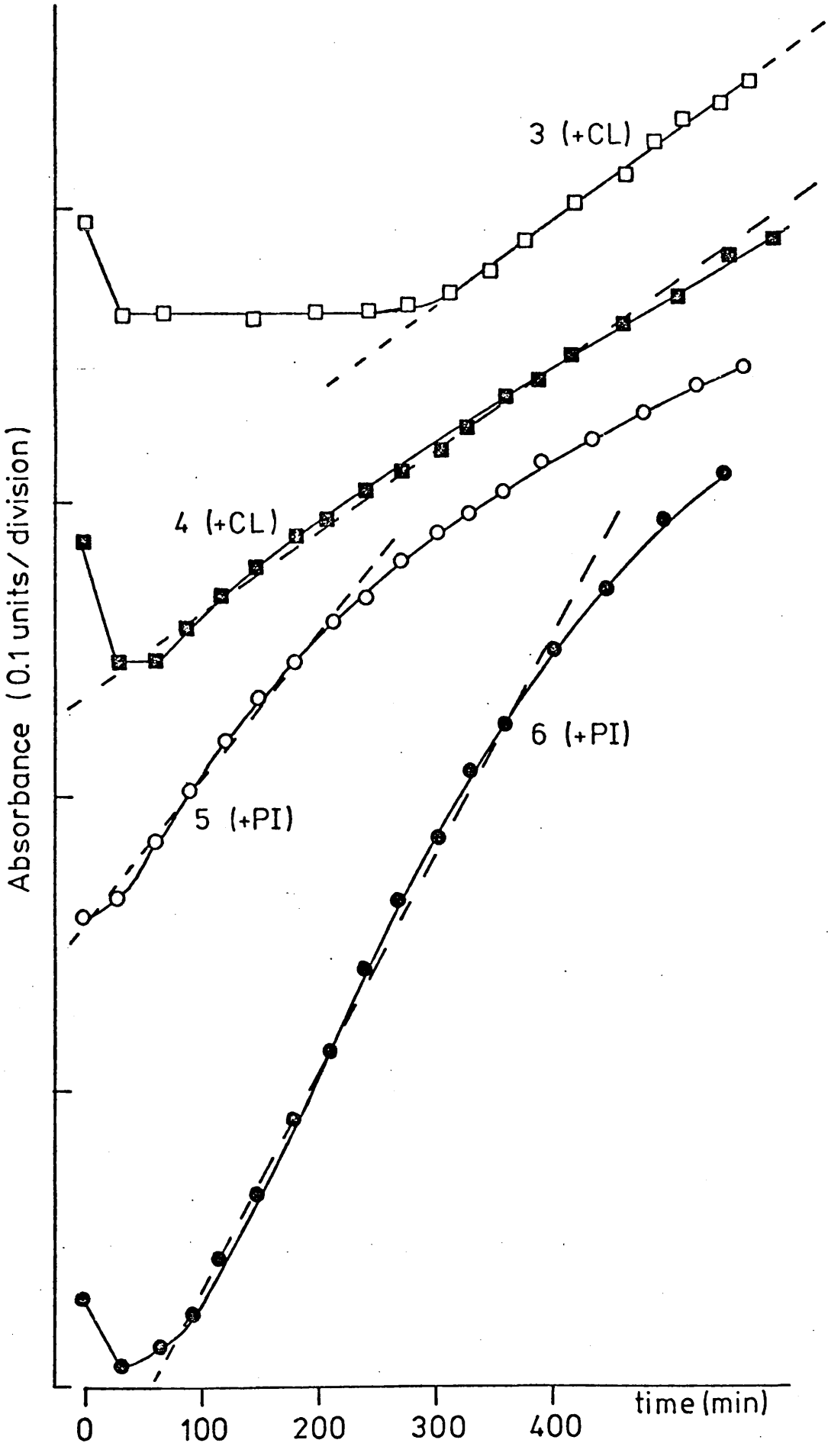

Absorbance (0.05 units / division)
2
1
control
0
100
200
300
400
500
600
700
time (min)

Figure 4-6 The absorbance of cytochrome c plotted against time for the system:

RMV/Vitamin K (hexane) + Phospholipid/Cytochrome c

The concentrations of PI and CL used are given in table 22(a). The broken lines were generated from 'least squares' analyses based upon zero-order kinetics. Other relevant information is given in the legend of figure 4-5.


3 (+CL)
4 (+CL)
5 (+PI)
6 (+PI)
Absorbance (0.1 units/division)
time (min)
0
100
200
300
400

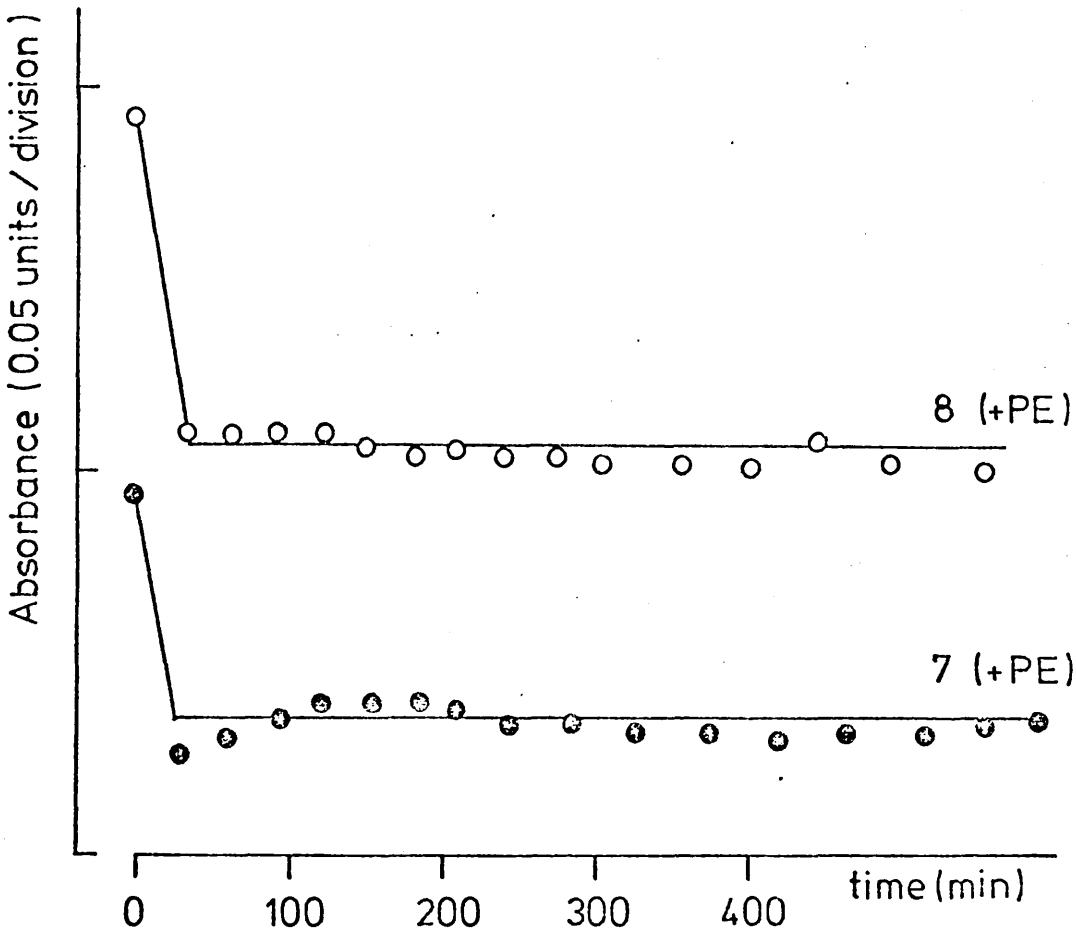


Figure 4-7 The absorbance of cytochrome c plotted against time for the system:

RMV/Vitamin K (hexane) + PE/Cytochrome c

The concentrations of PE used are given in table 22(a) and other relevant information is noted in the legend of figure 4-5.

concentration of the phospholipid used in each run is given in table 22(a).

The cytochrome c results, unlike those of DCIP and methylene blue, cannot be considered as obeying simple kinetics. The majority of the curves display a rapid initial drop in absorbance at the start of the reaction, the fall occurring within the time interval between first and second readings (usually 30 minutes). This fall was followed by an induction period, of variable length, during which the optical density remained constant and, finally, at the end of the induction period the reaction proper began, signified by an increase in absorbance. The reaction was found not to pass the induction period stage when PE was used as the modifier while negligible induction periods were observed with PI, figures 4-7 and 4-6 respectively. At the end of each experiment in which vitamin K was present in the membrane, the absorbance (550 nm) of the fully reduced (dithionite (153)) cytochrome c solution from the H-cell was always found to be less than that recorded from the original, similarly reduced ferricytochrome c stock solution from which the former was taken. No such discrepancy was obtained from the control run. This difference in absorption ranged between 5-10% of the value obtained from the fully reduced stock and was approximately four times greater than the fall

Table 22 Influence of phospholipid on the observed rate constants (25°C) for cytochrome c reduction both (a) without and (b) with stringent oxygen precautions being taken. For reference, the weight of vitamin K_1 in the membrane was 0.83 mg except for the control run where no carrier was present.

	Run	Phospholipid	weight added to membrane (mg)	k_o absolute (mole litre^{-1}min^{-1})	k_o relative	% reduction after 24 hours
(a)	control	-	-	2.701×10^{-9}	0.54	15
	1	-	-	5.913×10^{-9}	1.00	28
	2	-	-	4.119×10^{-9}		25
	3	CL	0.025	1.403×10^{-8}	2.80	54
	4	CL	0.28	1.299×10^{-8}	2.59	73
	5	PI	0.022	2.311×10^{-8}	4.61	69
	6	PI	0.25	3.532×10^{-8}	7.04	78
	7	PE	0.026	0.0	-	10
	8	PE	0.14	0.0	-	12

Table 22 (Contd.)

(b)

a	-	-	3.717×10^{-9}	0.74	29
b	CL	0.28	1.799×10^{-8}	3.59	94
c	PI	0.24	3.142×10^{-9}	0.63	30

in optical density observed at the beginning of the reaction. No rapid initial fall in absorbance and no induction period were observed during the control runs, figure 4-5. Instead a steady, but completely unexpected, rise in optical density was observed. The absence of vitamin K in the membrane was verified by recording an ultraviolet spectrum of the membrane layer. When coenzyme Q replaced vitamin K as the carrier, no initial decreases in absorbance and no induction periods were observed (81).

An increase in absorbance during cytochrome c runs can only be caused by ferricytochrome c being reduced to ferrocytochrome c. Consequently, the unexpected increase in optical density observed during the control experiments (figure 4-5), conducted in the absence of membrane carrier, must be the result of cytochrome c being anaerobically reduced. Spontaneous reduction of ferricytochrome c has been previously reported to occur during adsorption/elution off solid particles (154), (155), and it may well be that the hexane/aqueous interface behaves similarly. The mechanism of this reduction is unknown.

The observed losses in optical density in the kinetic runs proper, which were at first puzzling, were considered as being the result of one of (or a combination of) the following three processes:

(i) loss of cytochrome c

(ii) oxidation of cytochrome c

(iii) some other process.

Experiments were carried out to investigate these possibilities.

It was apparent from the outset that these effects could not be due to adsorption of the protein onto the stirrer since no drop in optical density was observed during the two hour period prior to membrane generation. Results from the immiscibility experiments, described previously, allowed consideration of a loss of cytochrome c due to an intrinsic membrane solubility to be similarly dismissed. Hence, losses of cytochrome c by a physical process could at once be eliminated from consideration.

Cytochrome c is known to form complexes with a number of lipids (144)-(147), which, under certain conditions, become soluble in hydrophobic solvents. The possibility existed, therefore, that a small amount of cytochrome c had formed a hexane soluble complex with vitamin K or the phospholipid modifiers. Cytochrome c-phospholipid complexes could not, however, account for the absorbance losses since these effects are observed in both unmodified and modified membranes, figure 4-5 and figures 4-6 and 4-7 respectively. If the losses in optical density were due to the formation of a hexane soluble vitamin K-cytochrome c complex, then

even allowing for the fact that only ~10% of the protein was lost, the characteristic absorption of cytochrome c should be detectible from the visible spectrum of the solvent membrane, especially in the region of the Soret peak. The solvent membranes from kinetic runs 3 and 6, table 22(a), gave spectra in which only the vitamin K absorption was apparent. Additional evidence against the extraction of a lipid-protein complex, proteolipid, came from experiments in which aliquots (3 ml) of a 30 μM cytochrome c solution were equilibrated overnight with equal aliquots of both 200 μM and 20 μM vitamin K (hexane) solutions in sealed vials (section 1-3.2). The optical densities of the vitamin K solutions, measured before and after equilibration, were found to be identical. This indicated that no vitamin K had become complexed with cytochrome c or extracted into the aqueous phase. It should be noted, however, that the absorption of the cytochrome c solution equilibrated below the quinone solution of higher concentration had decreased to 89% of its starting value. No such change was detected in the other cytochrome c solution. These experiments allowed the extraction of a proteolipid to be eliminated from further consideration. In general, alcohols cause a marked increase in autoxidation of ferrocytochrome c by promoting a conformational change in the protein that

renders the heme more accessible to oxygen in solution (143). The longer the hydrocarbon chain, the greater the rate enhancement. In addition, other simple organic molecules are known to promote conformational changes in the protein (156). For these reasons, and in view of the fact that stock ferricytochrome c solutions contained ~10% ferrocytochrome c, the decrease in absorbance observed is probably due to the autoxidation of the ferrocytochrome c component resulting from a conformational change induced by the prolonged interaction of oxidised vitamin K with this protein.

Chemical modification of cytochrome c could cause the protein to display altered absorption characteristics. To investigate whether the losses in optical density were due to a change in the wavelength of maximum absorption of cytochrome c, the optical densities of the fully reduced test and stock solutions from kinetic run 4, table 22(a), were recorded at the end of the experiment over the range 540-560 nm. The shape of the absorptions obtained were identical, both displaying an absorption maximum at 550 nm. A similar experiment was conducted using a protein solution of 5 μM, but this time the optical densities were recorded over 400-420 nm range (Soret region), both immediately before and a few hours after membrane generation. Phosphate buffer replaced methyl viologen in this experiment. As

before, although a decrease in optical density was recorded, no differences in absorption characteristics were detectible, both spectra exhibiting maxima at 410 nm. These experiments clearly demonstrated that no change in absorption characteristics, over 400-420 nm and 540-560 nm ranges, accompanies the loss of optical density. This finding is not surprising since absorption in the alpha and Soret regions of the cytochrome c spectrum is not very sensitive to structural modifications of the protein (82).

Once satisfied that the observed absorbance losses were not the result of a physical loss of cytochrome c from the substrate limb, the possibility of cytochrome c oxidation was next considered. As discussed previously, the stock solutions of ferricytochrome c used during these studies contained ~10% ferrocytochrome c. The (reversible) oxidation of the ferrocytochrome c fraction, to ferricytochrome c, by the addition of small amounts of solid potassium ferricyanide (153), caused the optical densities to fall to values similar to those obtained during the kinetic runs, after the initial fall in absorbance had taken place. This clearly demonstrated that the observed absorbance losses could be explained, at least in part, in terms of an irreversible oxidation of ferrocytochrome c. The oxidation would necessarily have to be irreversible if the optical densities of the fully reduced treated and

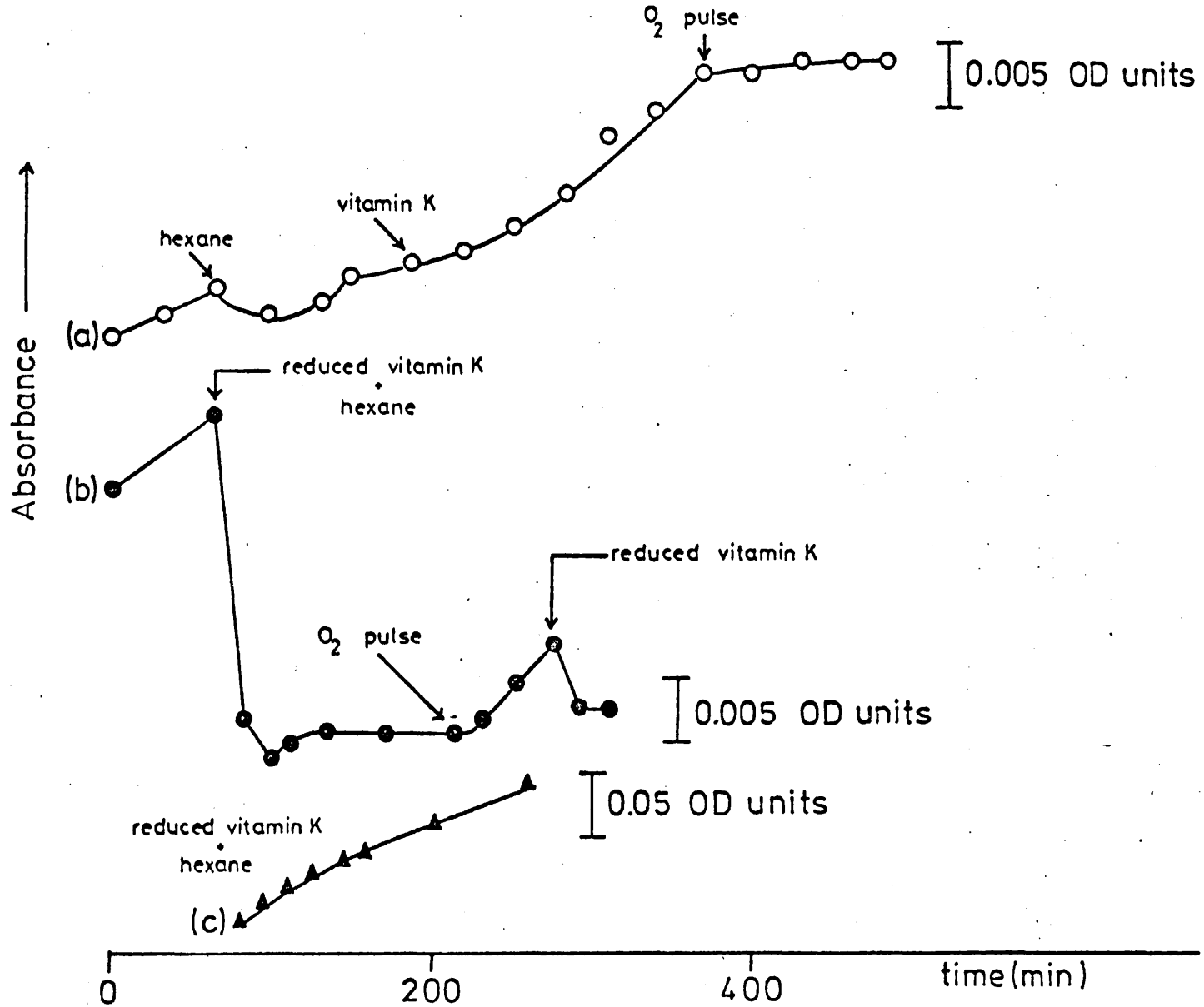
stock cytochrome c solutions were not to be the same at the end of the run. A series of experiments, using the H-cell, were carried out to further investigate this possibility.

Deoxygenated buffer and cytochrome c (25 μM) solutions were placed in opposite limbs of the H-cell and the apparatus sealed according to standard procedure. After a short period, hexane (4 ml) was added. Some time later an aliquot of (oxidised) vitamin K solution (0.5 ml) was added to generate a membrane 0.4 mM in quinone. Finally, after a few hours, a pulse of oxygen was injected into the test cytochrome c solution. The optical densities of both aqueous solutions were recorded (550 nm) as a function of time. A similar experiment in which the carrier solution added had been previously fully reduced (using RMV) was also carried out. The absorbances recorded from the cytochrome c limbs of each experiment are shown in figure 4-8(a), (b). The absorbance readings from the buffer limb of both experiments retained their initial values throughout the duration of the experiment, indicating that there was no net transport of cytochrome c solution from the one aqueous limb, through the hexane and into the other. Whereas before only bulk solubility of cytochrome c within the membrane could be dismissed, this additional finding

Figure 4-8 The absorption of cytochrome c plotted against time for the system:

Buffer/Vitamin K (hexane)/Cytochrome c

The initial concentrations of vitamin K_1 and cytochrome c were 0.4 mM and 25 μM respectively. All experiments started at the same absorbance reading. For clarity of presentation, as before, these zero-time absorbances have been set at arbitrary points on the ordinate. Only in figure (c) had the hexane been entirely deoxygenated. Further details are given in the text.


O_2 pulse
0.005 OD units
vitamin K
hexane
(a)
Absorbance
reduced vitamin K
hexane
(b)
reduced vitamin K
O_2 pulse
0.005 OD units
0.05 OD units
reduced vitamin K
hexane
(c)
0
200
400
time (min)

showed that the protein did not have even an undetectibly small solubility in the hexane layer. This would result in a continuous build up of concentration in the opposite aqueous phase, as experienced with DCIP (section 1-3.6). The results from the experiment using oxidised carrier, figure 4-8(a), demonstrated that in the absence of reduced carrier a small quantity of cytochrome c still became reduced. As discussed earlier, this is most likely due to an interfacial effect. The small increase in optical density observed before the generation of the membrane would suggest that spontaneous reduction also occurs at the nitrogen/aqueous solution phase boundary. Oxygen appears to inhibit spontaneous reduction, figure 4-8(a). Throughout this experiment no fall in optical density was recorded. When reduced vitamin K was added immediately after the addition of hexane, figure 4-8(b), a rapid decrease in optical density was recorded. Addition of oxygen caused an increase in absorbance which was reversed by the addition of a further aliquot of reduced vitamin K. These two experiments indicated that both oxygen and reduced vitamin K, but not oxidised vitamin K, were somehow involved in the effects being considered. To investigate this possibility further the H-cell was filled according to method C (section 3-2.4). Any oxygen originally present in the hexane would be removed by the previous equilibration

with RMV. The results obtained, figure 4-8(c), proved that the initial fall in optical density, previously observed, could be eliminated, provided oxygen was stringently excluded from the system. The magnitude of the fall in optical density, observed initially during the experiments where the hexane still contained traces of oxygen, was found to be the same as the difference between the absorbances of the fully reduced stock and test solutions, measured at the end of the experiment, provided that the duration of the experiment was less than about six hours and that no oxygen pulses were used. If either of these conditions were not obeyed, the initial drop in absorbance was always less than the difference in optical density found at the end of the experiment. Further, if the initial decrease in absorbance was eliminated by using the special technique developed (section 3-2.4), the optical density readings obtained from the fully reduced stock and test solutions, (again provided the experiment lasted less than about six hours) were now found to be identical. Thus, the optical density differences observed at the start of the kinetic experiments and at least part of the absorbance losses detected at the end of these experiments, were the result of a process which occurred at the start of each experiment.

The series of experiments just described show conclusively that ferrocytochrome c, dihydrovitamin K and oxygen are all intimately involved in the process which caused the noted losses in absorbance. To account for these experimental findings it is proposed that reduced vitamin K and oxygen react to generate either hydrogen peroxide or a vitamin K hydroperoxide and that one of these peroxides interacts with the heme of ferrocytochrome c, possibly through a free radical mechanism, causing the rapid, irreversible, oxidation of the protein and the rapid initial fall in optical density. The discrepancy between the absorbance loss at the start and end of each run can be explained by postulating the existence of a second, slow, denaturing reaction involving ferricytochrome c. In addition, small amounts of the ferrocytochrome c, generated during the course of the experiment, will be lost by reaction with small amounts of peroxide continually formed throughout the reaction due to the presence of minute oxygen leaks in the cell. The ability of vitamin K to expose the heme crevice to oxygen (discussed earlier) may similarly aid attack by peroxide.

The mechanism proposed requires that the carrier be readily oxidised by molecular oxygen. The autoxidisable nature of dihydrovitamin K is well known (59). The observation that no initial drop in absorbance occurs when

the non-autoxidisable quinone, coenzyme Q, replaced vitamin K as the carrier (81), provides strong support for the validity of the proposed mechanism. Additional support for this scheme comes from the fact that hydrogen peroxide and lipid hydroperoxides have both been found to react irreversibly with ferro- and ferricytochrome c and to cause similar falls in absorbance (157), (158).

The variable and complex behaviour of cytochrome c under the differing reaction conditions employed meant that a rigorous kinetic analysis of the results was impracticable. To aid comparisons to be made between the results obtained, the absorbance readings from the sections of the graph where the reaction proper occurred were used to calculate the concentrations of ferricytochrome c present, using equation (2-5) and the extinction values given in section 2-4.2. The absorbance values, measured at the end of these experiments by fully reducing the substrate solutions, were considered to be more accurate measures of the concentrations of total reducible cytochrome c present during the reactions than the absorbances obtained from the fully reduced stock solutions. The former values were, therefore, used in these calculations. For each experiment the concentrations of ferricytochrome c calculated were found to vary, at least initially, linearly with time. Zero-order rate constants were calculated, using these

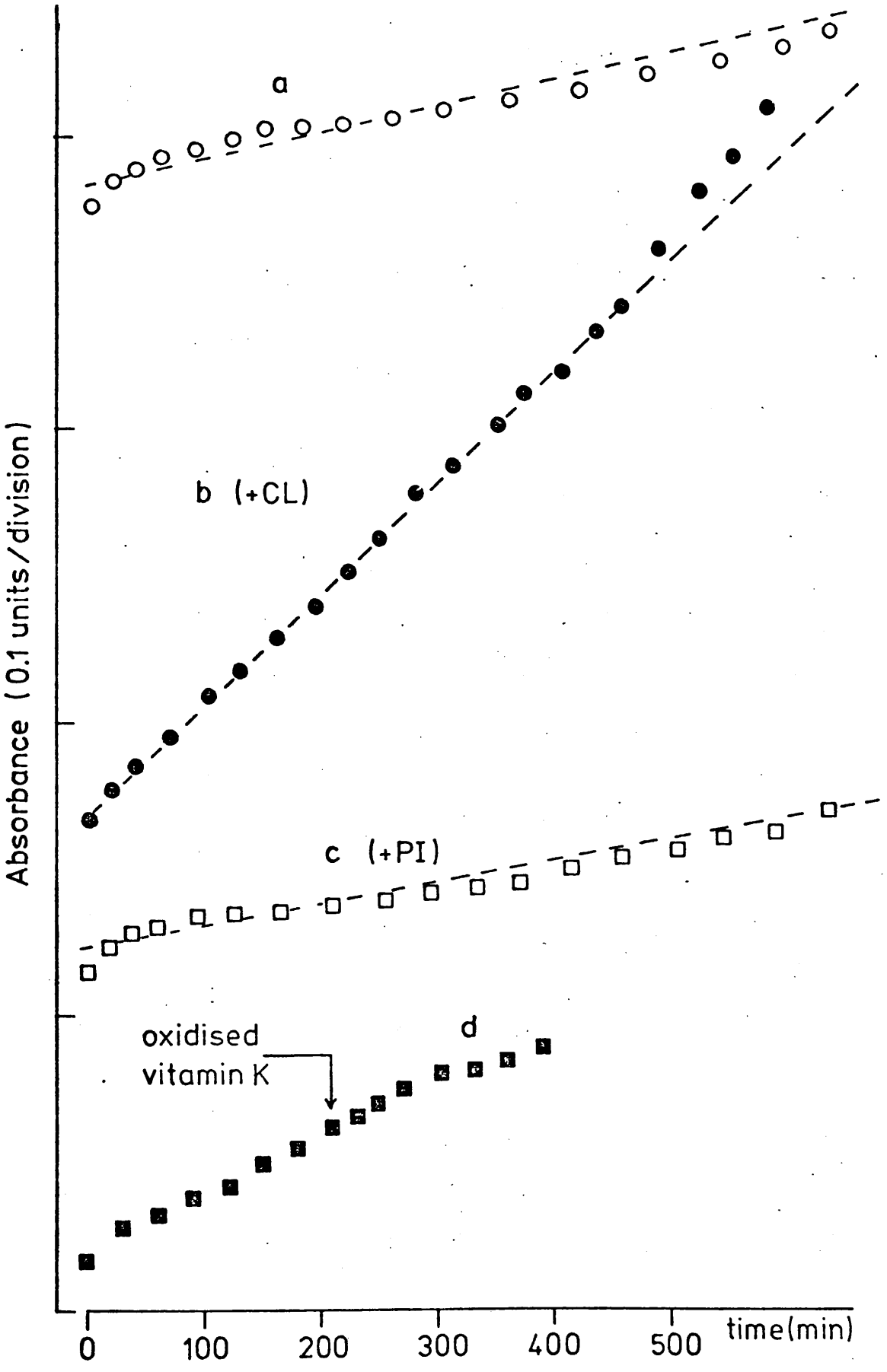
concentrations, and the rate constants used to provide the initial reaction rates. The first two sections of each graph were ignored when carrying out this analysis. Values for the 'initial' reaction rates, together with the ratio of these rates to the average rate obtained when no phospholipid was present, are given in table 22(a). The percent of the cytochrome c reduced after 24 hours reaction, is also included. In contrast to the behaviour exhibited by cytochrome c when carrier containing membranes were employed, the results from the control experiment obeyed a simple zero-order analysis for the reduction of cytochrome c. The rate constant obtained is also included in table 22(a).

To investigate whether the peroxide reaction had any effect on the observed kinetics of reaction, three of the previously described kinetic experiments were repeated, using conditions which rigorously excluded oxygen from the cell. This was achieved by filling the cell according to method C (section 3-2.4). The experiments were otherwise identical. Figure 4-9(a), (b) and (c) records the absorbances (550 nm) of cytochrome c obtained as a function of time for each of these runs. An additional kinetic run in which no phospholipid was present, but which was interrupted after 200 minutes by the addition of a second aliquot of oxygen-free carrier solution, this time in the

Figure 4-9 The absorbance of cytochrome c plotted against time for the system:

RMV/Vitamin K (hexane) $\pm$ Phospholipid/Cytochrome c.

These experiments were selected repeats of those shown in figures 4-5 and 4-6 except that method C (section 3-2.4) was used to fill the cell. The conditions were otherwise identical. The broken lines were generated from 'least squares' analyses based upon zero-order kinetics.


a
b (+CL)
c (+PI)
d
oxidised
vitamin K
Absorbance (0.1 units/division)
0
100
200
300
400
500
time(min)

oxidised form, was similarly carried out. The absorbances obtained, plotted as a function of time, are recorded in figure 4-9(d). The observed optical densities were converted to concentrations of ferricytochrome c, using equation (2-5), and these concentrations, which varied linearly with time, used to calculate zero-order rate constants. The values obtained are recorded in table 22(b). At the end of each of these experiments only small differences (1-5%) between the absorbances of the fully reduced test and stock solutions of cytochrome c were now found. This indicates that the large deviations previously obtained were, in fact, primarily due to the second and irreversible reaction.

Comparison of figure 4-9 with figures 4-5 and 4-6 clearly demonstrates that rigorous exclusion of oxygen from the system has a marked effect on the kinetic behaviour observed: both the initial losses in cytochrome absorption and the induction periods are eliminated. An accidental equality in the rate of substrate reduction by carrier and reoxidation by the secondary peroxide reaction would account for the induction period. Only when the latter reaction had almost ceased would net reduction begin to be observed. If this approach is correct, it follows that the reaction proper occurs at the end of the induction period and thus justifies the earlier analysis (table 22(a)).

The absence of induction periods in the first PI runs, figure 4-6, could be due to a quenching of the radical side-reaction by the hydroxyl groups of the inositol residue on the phospholipid, figure 4-2.

Comparison of systems lacking phospholipid modifiers shows that similar rate constants are determined, table 22, for runs conducted both with (run (a)) and without (runs 1, 2) rigorous oxygen precautions having been taken. The result from the control run, table 22, suggests that a correction of -2.7×10^{-9} should be applied to these rate constants to take account of the spontaneous (interfacial) reduction of cytochrome c. However, since the peroxide reaction causes an opposite, but undetermined, effect, no correction was applied to these, or to the other, rate constants. A mean value of $k_o = 4.58 \times 10^{-9}$ mole litre^{-1} min^{-1} was obtained on averaging the results and this corresponds (equation (3-8)) to a half-life of 2640 minutes.

The approximate zero-order behaviour exhibited by the unmodified cytochrome c systems and the long half-lives displayed contrast markedly with the fast, first-order, reactions displayed by both DCIP (section 3-3.1) and methylene blue (section 3-3.2) systems. If cytochrome c reduction was diffusion controlled, as was the case for methylene blue systems, a first-order rate constant of $k_1 \simeq 5 \times 10^{-3}$ min^{-1} and a half-life of $t_{\frac{1}{2}} \simeq 140$ min would be expected

(equation (3-19)) for an unstirred layer thickness of 28 μ; $D = 0.95 \times 10^{-6}$ cm^2 s^{-1} for cytochrome c (159). Thus diffusion control in the substrate solution cannot now account for the observed kinetics. As it has been verified that quinol translocation is fast relative to solution diffusion control (section 3-3.2) it seems likely that the actual chemical reaction between dihydrovitamin K and ferricytochrome c is now rate determining. In view of the large size of the cytochrome c molecule (roughly a sphere of 31A diameter (160)), steric effects may play an important part in this interfacial electron transfer.

In a homogeneous aqueous solution, at neutral pH, the reduction of cytochrome c by benzoquinol is greatly accelerated by the addition of benzoquinone (161), (162). The acceleration was ascribed to the formation of benzosemiquinone. In contrast, the addition of oxidised vitamin K to the membrane, during a cytochrome c run from this research, was found to have no effect on the observed kinetics, figure 4-9(d). This may reflect the non-involvement of vitamin K semiquinone radicals in the reaction. It may also, however, be a consequence of the short existence of oxidised carrier (and therefore additional semiquinone) in the membrane, since the rate of reduction of quinone by RMV ($t_{\frac{1}{2}} \simeq 60$ min) is much greater than the rate of dihydrovitamin K reoxidation by ferricytochrome c ($t_{\frac{1}{2}} \simeq 2500$ min). Tentative support

for the latter conclusion comes from the observation that during an experiment, figure 4-8(b), where no RMV was present, the injection of a small amount of oxygen into the reduced carrier phase caused an increase in the rate of protein reduction: reaction of oxygen with dihydrovitamin K is known to produce semiquinone radicals (91). As found earlier (section 1-4.4), vitamin K semiquinone radicals can be formed at the hexane/aqueous interface.

The effect of adding phospholipid to the cytochrome c systems being discussed would be expected to cause a slowing down of all the reaction rates, by analogy to methylene blue (previous section). This indeed was observed with PE modified membranes, figure 4-7, where the reaction was virtually totally inhibited. With PI and CL modified membranes, however, such inhibition was not observed, figures 4-6 and 4-9. Cytochrome c reductions by CL modified membranes, both with and without stringent oxygen precautions being taken, were noticeably faster than those displayed by unmodified membranes, table 22. When oxygen was not rigorously excluded PI modified membranes showed similar rate enhancements. On removal of oxygen, however, a rate close to that exhibited by unmodified membranes was obtained once more. Owing to the lack of additional information, no attempt will be made to rationalise this difference. The apparent acceleration by oxygen is, however, noted.

The kinetic results from phospholipid modified membranes, table 22, indicate that CL and, perhaps, PI do not function simply as inert phospholipids, which compete with carrier for interfacial monolayer sites, but that they are involved in a specific interaction with cytochrome c which results in the protein becoming more reactive towards carrier. Adsorption of CL and PI onto the hexane/aqueous interface will cause the interfacial monolayers to become negatively charged, since both are acidic phospholipids. To account for the observed acceleration of cytochrome c reduction by CL and, perhaps PI, it is proposed that the ferricytochrome c molecules (positively charged) are electrostatically bound to the monolayer where they react with the phospholipids to generate a protein-lipid complex. This complex is considered to penetrate the interfacial monolayer. The subsequent rate enhancement recorded may be due either to the ferricytochrome c being more favourably aligned in this proteolipid towards reaction with carrier or may be due to a conformational change, induced by proteolipid formation, that opens the heme crevice and thereby facilitates reaction with carrier. Proteolipid formation (section 4-1), binding of cytochrome c to monolayers and bilayers of PI (159) and to monolayers of CL (163), have all been previously reported. Evidence for the penetration of CL monolayers by cytochrome c and for subtle change in protein conformation, both a direct

result of complex formation, were also obtained. The observation from this research that PE modified membranes showed no rate enhancement, table 22(a), further supports this theory since saturated PE forms proteolipid complexes to only a limited extent (144).

The increase in the rate of cytochrome c reduction observed when the basic membrane system was modified by adding CL, suggests that this lipid may have, in addition to the more general functions of phospholipid outlined earlier (section 4-1), a specific role in catalysing the reduction of cytochrome c. PI may also have a similar role. It is perhaps not a coincidence that the cytochrome c content of mammalian tissue appears to be related to the CL content (164). It must be remembered, however, that isoprenoid quinones are generally thought to react directly with b and not c cytochromes, in respiratory membranes, figure 1-9.

Apart from any biological implications, liquid redox membrane systems provide a new, sensitive, method for the preparation and characterisation of solutions of reduced or oxygen-sensitive compounds, without the added complication of chemical contamination (other than protons required to ensure electroneutrality). In addition to the study of the very oxygen-sensitive reduced vitamin K described earlier (section 2-3.5), these systems have also been used to

generate a solution of ferrocytochrome c for use in laser Resonance Raman spectroscopy studies.

Resonance Raman spectroscopy (165) can selectively enhance the vibrations of a particular chromophore of a molecule if the energy of the incident light approaches that of the electronic absorption band. This property, coupled with the fact that water is a poor Raman scatterer, makes the technique an excellent one for probing biological structure. Resonance Raman spectra of ferro- and ferri-cytochrome c are dominated by the porphyrin vibrational modes (1000-1700 cm^{-1}) which are enhanced by resonances with the allowed electronic transitions in the visible (α- and β- bands) and near-ultraviolet (Soret-band) regions (165).

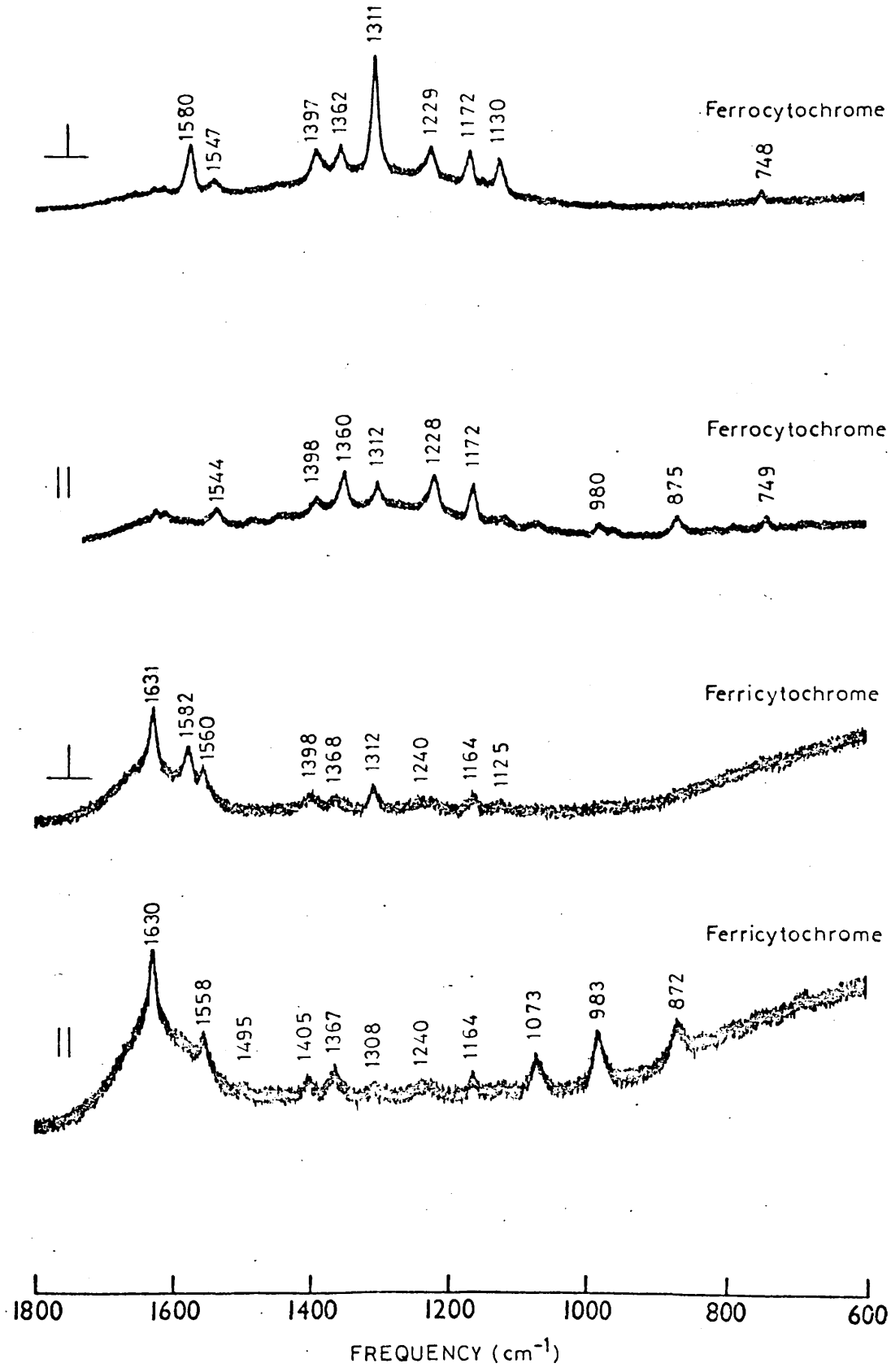
The cytochrome c was reduced in a vibrator H-cell according to the method described in section 1-3.6. To aid reaction 0.075 ml of a 9.3 mg ml^{-1} solution of CL (in ethanol) was added. The concentrations of vitamin K, dissolved in hexane, and cytochrome c, dissolved in phosphate buffer (pH 6.86), were 86 μM and 24 μM respectively. After a few days the cell was opened and a Resonance Raman spectrum of the solution recorded. An exciting frequency of 5145A was used. A detailed description of the experimental arrangement used can be obtained from reference (166). The optical density of a separate aliquot of solution showed

that the sample had been 90% reduced. The Resonance Raman spectra recorded from this reduced solution, together with those recorded from the original oxidised solution, are given in figure 4-10.

Both the depolarised ($\perp$) and polarised ($\parallel$) spectra of ferro- and ferricytochrome c experimentally obtained, figure 4-10, were almost identical to those previously reported in the literature (167)-(169), the wave number of each peak agreeing to within ± 10 cm^{-1}. The polarised spectrum of ferrocytochrome c exhibited one additional resonance in the region 800-875 nm. Since such an anomalous resonance was also found in the polarised spectrum of ferricytochrome c, the peak was dismissed as being the result of the presence of common contaminant, possibly an impurity or the phosphate buffer used. The spectra were otherwise identical to the literature ones. This agreement between literature and experimental spectra supports that the reduction of cytochrome c by the membrane system causes no irreversible changes to occur in the molecule, or at least to the heme portion. Additionally, since both solutions contained ~10% of the conjugate redox state, it also indicates that the spectra of ferro- and ferricytochrome c are rather insensitive to the presence of small amounts of ferri- and ferrocytochrome c respectively. The baseline in the spectrum from the ferrocytochrome c, prepared as discussed, was found

Figure 4-10 Polarised Raman spectra of ferrocytochrome c and ferricytochrome c using 5145 A incident laser light. $\perp$ and $\parallel$ indicate the linearly polarised perpendicular and parallel scattering components. Ferrocytochrome c was prepared from the corresponding ferricytochrome c solution by reduction of the latter, using the liquid redox membrane system:

RMV/Vitamin K_1 + CL/Ferricytochrome c.


Ferrocytochrome c
1580
1547
1397
1362
1311
1229
1172
1130
748
Ferrocytochrome c
1544
1398
1360
1312
1228
1172
980
875
749
Ferricytochrome c
1631
1582
1560
1398
1368
1312
1240
1164
1125
Ferricytochrome c
1630
1558
1495
1405
1367
1308
1240
1164
1073
983
872
1800
1600
1400
1200
1000
800
600
FREQUENCY (cm⁻¹)

to be far lower and much narrower than that obtained with ferrocytochrome c prepared by dithionite reduction (170). This latter method of preparation exhibits baselines similar to those experimentally observed with ferricytochrome c, figure 4-10. This experiment highlights the great advantages of using liquid redox membrane systems when carrying out reductions: only with such a system can the solution generated remain entirely free from contamination.

Chapter Five

'ULTRATHIN' MEMBRANES

The foregoing chapters have described, in detail, the genesis and study of chemically well-defined bulk liquid redox membranes of the Haber-Beutner type. Systems involving such membranes have been shown to display interfacial redox reactions similar to those occurring during electron transport in biological systems. This similarity allowed the synthetic systems developed to be used as simple models for selective steps known to be involved in biological electron transport. To improve these models it was desirable to reduce the thickness of the solvent membranes studied and, if possible, to form lipid bilayers containing vitamin K. As discussed in the introduction, bilayer lipid membranes may be considered as the limiting structure of a liquid membrane in which most (or all) of the bulk solution phase (figure 0-6(a)) has been removed. This chapter describes preliminary studies carried out to try to form and characterise vitamin K bilayers of well-defined composition

and to verify that the redox reactions mediated by the previously described 'macroscopic' liquid membranes are similarly mediated by 'ultrathin' ones.

5-1 BIMOLECULAR LIPID MEMBRANES

5-1.1 General aspects

Thin membranes can be formed in aqueous media from amphiphilic lipids and will spontaneously approach a limiting thickness of bimolecular dimensions (151). The limiting structure of these BLM, figure 0-2, is reminiscent of one of the mesomorphic structures of concentrated soap solutions (171): The polar groups of the lipids face outward in contact with the aqueous solution. The interior hydrocarbon chains in the bilayer are assumed to be in the liquid state (172). From the viewpoint of interfacial science, lipid bilayers are the limiting case of a three component liquid system;

aqueous phase/lipid phase/aqueous phase

in which the central lipid phase has been reduced to molecular dimensions (151). It is important to realise at the outset that the study of bimolecular lipid membranes has not acquired the stature of an 'exact science'. The subject is still in its infancy. This is particularly evident from the appreciable variability and conflicting

experimental observations reported by different investigators (171).

A wide variety of experimental cells have been described for black lipid film studies (17), (151), (173), (174). In most instances the type of experiment to be performed dictates the main features of the design. Membranes are always formed on a smooth annulus made of a suitable material. They may be formed, however, either vertically or horizontally. If the nature of the apparatus is such that, on formation of a membrane, the cell is divided into two separate aqueous chambers, these should be maintained at equal or near equal osmolarities. This requirement is necessary because osmotic pressure gradients can cause volume flow of water across the membrane resulting in membrane deformation and rupture (151). A marked limitation on materials suitable for the membrane support is imposed by the prerequisites of chemical inertness to both aqueous and organic solvents. In addition electrical non-conductance is important. Of the many materials tested as membrane supports, polytetrafluoroethylene(teflon), is preferred by most workers (151), (171), (175). This polymer is wetted by the hydrophobic lipid solutions, used to form the membranes, but not by water. It is not significantly swollen or distorted by either hydrophilic or hydrophobic solvents, is chemically inert, and is an

excellent electrical insulator. The thickness of the support around the hole is typically 0.2 mm and the diameter of the aperture is usually restricted to 1-2 mm (173). The lipid solution is commonly applied to the hole by either a 'Painting' or 'Marginal Suction' method (151). The former involves spreading or ejecting of membrane-forming solution over the hole with a trimmed sable hair brush or a teflon spatula or a hypodermic needle attached to a micrometer syringe. Sable hair brushes are little used now since they contaminate the lipid solutions. The 'Marginal Suction' method involves the ejection of membrane-forming solution from a micrometer syringe and through a small capillary hole drilled into the membrane support from the outside to lumen side, entering the lumen at the bottom of the orifice. Membranes are formed by ejecting solution from the syringe until the annulus is completely filled with a drop of liquid. Excess bulk phase lipid solution is then withdrawn back into the syringe. Since the areas of these BLM are usually small, a light source and low-power microscope are commonly used to observe the membrane during its formation. The whole cell assembly, with the exception of a porthole for the light path, is blackened to minimise stray reflections. Reflected light from the membrane is viewed.

Just as aqueous soap films are formed from a solution of at least one surfactant in water, so lipid films are formed from solutions of one or more lipids in nonpolar solvents. However, relatively few lipids and nonpolar solvents are suitable. The solvent used for bilayers must be water insoluble otherwise the membrane will not survive as the membrane solvent is leached out into the aqueous solution (175). In addition it must have an appropriate viscosity and dissolve the amphiphilic lipids (171). The most successful single solvents for lipid material are the normal aliphatic hydrocarbons from octane to hexadecane. The particular one used does not seem to be critical (171). The lipid amphiphiles themselves must be appreciably water insoluble (175). The lipids known to form stable black films include many naturally occurring phospholipids and some mono- and diglycerides (17), (173). In addition, some substances outside this range, notably 'oxidised cholesterol' (176), have been successfully employed. For physical characterisation of bilayer lipid membranes it is an obvious advantage to use chemically well-defined systems but, despite this, egg lecithin - decane mixtures (both with and without cholesterol) are commonly used. The concentration of lipid required to form BLM is typically 1-2% (w/v). Though bilayers can be formed in distilled water, they are markedly stabilised

by aqueous media containing ions (151) and are relatively insensitive to the concentration of salt in the bathing medium (171).

On application of the membrane-forming solution to the orifice a thick (lens) membrane is formed. This membrane spontaneously thins to form both a ring of bulk phase lipid solution around the aperture (Plateau-Gibbs border) and, under favourable conditions, a lipid bilayer in the central region of the orifice. The thinning process, identical to that exhibited by soap films, is thought to be caused by stretching, evaporation, gravity convection and marginal regeneration (171). The last arises because the Plateau-Gibbs border, a region of sharp curvature, has a pressure less than that in the film. This results in the suction of the film solution into the border. The final formation of the bilayer from the thin film so formed, is attributed (171) to chance contacts (caused by thermal motion and mechanical vibration) between the two interfaces. As the membrane gradually thins the probability of chance contacts increases and once the two interfaces come close enough together, van der Waals attractions between opposing hydrocarbon chains are strong enough to produce a bilayer in a small region. In forming this bilayer, adjacent molecules are drawn close enough to be attracted to the opposite interface ('zipper

effect'). The rate of thinning is a characteristic of the membrane forming solution (151). The gross optical behaviour of these thinning membranes, when viewed by reflected light, is similar to that of soap films in air (17). The thick membrane is devoid of colour. As the membrane thins, bright swirling patterns of interference colours are observed and in a short time a black spot (or spots) appears, which grows continuously until it covers the entire opening. At random intervals, coloured spots, which swirl in the black membrane and then disappear, demonstrate the fluidity of the structure. White light is reflected from a thin lipid membrane at both aqueous/lipid interfaces and these reflected beams are 180° out of phase. Only if the membrane thickness is greater than or equal to a quarter of the average wavelength of the incident light, will significant additional phase differences occur between these two beams: this results in constructive (as well as destructive) interference being observed. The above condition is not met when the membrane is very thin and the film, therefore, appears black because of the complete destructive interference between reflected light wavefronts, 180° out of phase (151). The thinning behaviour is summarised in figure 5-1.

The formation of BLM must take place within a given temperature range (151),(171). At low temperatures the

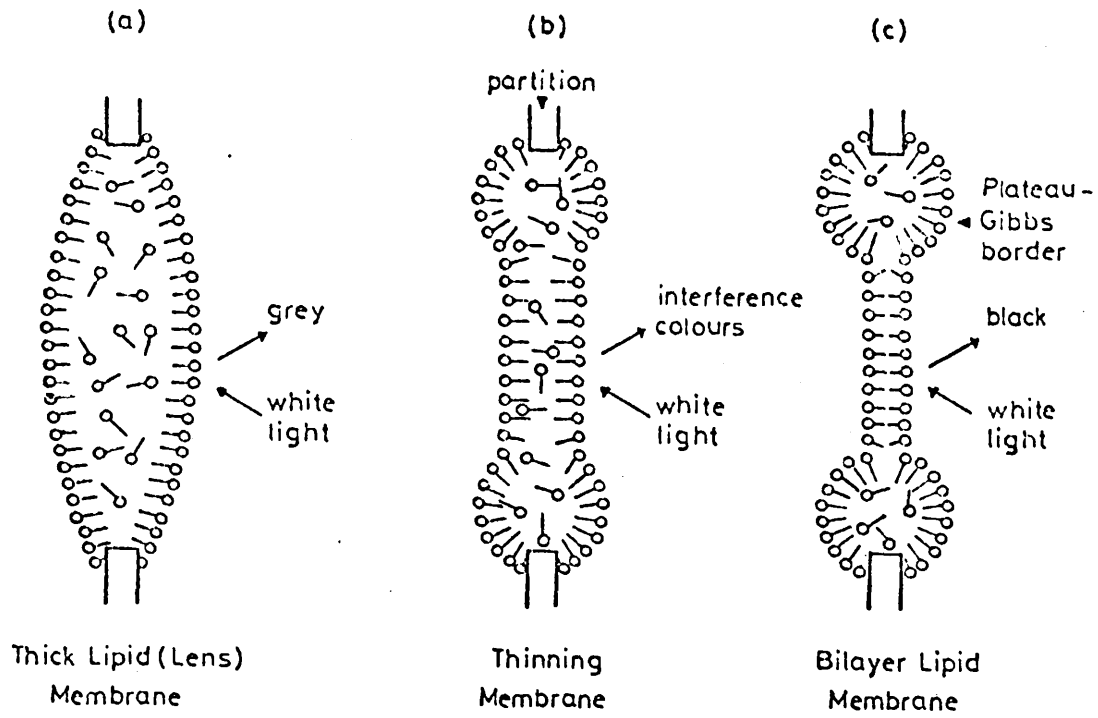


Figure 5-1 Diagram illustrating the three stages observed during the thinning of a lipid membrane in aqueous media, and indicating the patterns of reflected light.

rate of thinning may be so slow that a bilayer cannot be obtained. At high temperatures the thinning may occur so rapidly that localised turbulence develops and the film ruptures. These effects are a direct result of temperature-induced changes in the viscosities of the lipid solutions. In addition, temperature affects the liquid crystalline phase of the lipid. Membranes have been successfully formed throughout the temperature range 6-50°C.

The black lipid film is not precisely a lipid bilayer since, by the nature of the solution from which it is formed, it must, in general, contain some lipid solvent (175). A black film is stable as a consequence of the very strong adsorption or spreading of the polar lipids at its interfaces. This adsorption renders the composition of the black film quite different from the solution from which it is formed (175). Thus, in spite of the fact that BLM can be generated from various lipids, there is little direct evidence to indicate the precise composition of the actual bilayer (171). Available evidence, however, indicates that the concentration of lipid in the bilayer is some two orders of magnitude greater than in the membrane-forming solution (177).

Currently there is no known method which will predict what the stability of a particular membrane will be, or even whether stable BLM can be formed from given lipid

solutions. It has been suggested, however, that the stability of bilayers is due to a balance among van der Waals forces, electrical repulsion across the membrane and border effects (171). Thick membranes and bilayers can be formed on clean, untreated membrane orifices, but their formation and stability, especially during the early stages of thinning, are improved by 'preconditioning' the partitions. This is achieved by air-drying a small ring of the membrane forming solution around both sides of the orifice (151). The precise reasons for this precaution are not fully understood but are probably associated with the ease of spreading of the phospholipid into its monolayer at each hydrocarbon/water interface (175). The addition of cholesterol to phospholipid solutions considerably enhances the stability of the resulting BLM (178), (179). This is almost certainly related to the ability of the sterol to condense phospholipid monolayers (173). Trace impurities, especially detergents, markedly decrease the membrane stability as well as other physical properties. The forces responsible for the final rupture of the membrane are unknown. The lifetime of a bilayer, however, may be controlled by the thickness of the Plateau-Gibbs border (171).

Bilayer membranes are remarkably stable once formed, occasionally lasting overnight, but generally one to three hours. They are much more stable to vibration and transmembrane hydrostatic pressure changes than thicker membranes (151). Electron microscopy, optical reflectance and electrical capacitance studies have all shown that BLM possess thicknesses of 60-90 A (171), (173).

5-1.2 Capacitance and thickness determinations

The electrical properties of BLM are usually studied using an experimental arrangement which may be represented as follows:

KCℓ salt bridge	aqueous solution	BLM on teflon support	aqueous solution	KCℓ salt bridge
	1		2	

Aqueous solutions 1 and 2 are electrically isolated from each other by the membrane and teflon support. Saturated calomel, silver-silver chloride and platinum electrodes have all been used as the nonpolarizable electrodes. Because of the very high resistances of unmodified BLM great care is needed in the insulation of switches and connections to avoid current leakage (171).

The capacitances of BLM may be measured by a DC transient (charge leakage) method, using a standard high resistor (17). A typical circuit is shown in figure 5-2. The membrane is represented by a capacitor and resistance in parallel. The membrane is charged through a known

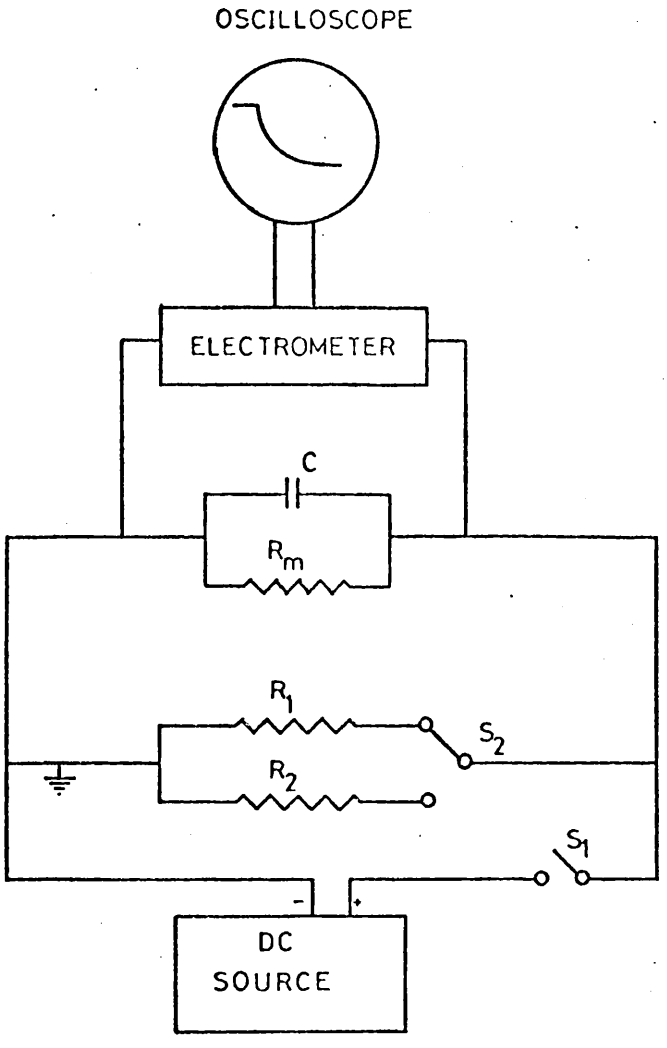


Figure 5-2 Equivalent circuit for DC measurement of electrical capacitances of BLM. C and R_m are the membrane capacitance and resistance respectively. Switch S_2 allowed either standard resistance R_1 or R_2 to be connected in parallel to the membrane. The membrane was discharged by opening Switch S_1.

high resistance, R_x (x = 1 or 2, depending on the position of switch S_2), by applying a small DC voltage (<100mV). After reaching a steady state, as indicated on the oscilloscope, switch S_1 is opened and the membrane discharged through the same known resistor. The decay of the membrane voltage, E_m, with time, is recorded using the oscilloscope.

The capacitance of the membrane, C, is obtained from the relationship,

$$E_m(t) = E_m(0)\ e^{-t/RC} \qquad (5\text{-}1)$$

where $E_m(0)$ and $E_m(t)$ are the membrane potentials at time zero and t respectively, and R is the total resistance (180). The total resistance in parallel with the membrane, R_{Tx} is given (81) by,

$$R_{Tx} = R_x + R_s \qquad (5\text{-}2)$$

where R_s is the resistance of the aqueous solution and electrodes. Thus the total resistance is given by,

$$R = \frac{R_m R_{Tx}}{R_m + R_{Tx}} \qquad (5\text{-}3)$$

If τ (the time constant) is defined as the time taken for E_m to fall to $^1/e$ of its original value ($E_m(0)$), (e is the base of natural logarithms), then equation (5-1) simplifies to,

$$\tau = RC \qquad (5\text{-}4)$$

Resistances R_1 and R_2 will yield two different time constants, τ_1 and τ_2, for a given membrane. Combination of equations (5-3) and (5-4) for each resistance yields equations (5-5) and (5-6).

$$\tau_1 R_{T1} = R_m(R_{T1}C - \tau_1) \qquad (5\text{-}5)$$

$$\tau_2 R_{T2} = R_m(R_{T2}C - \tau_2) \qquad (5\text{-}6)$$

Division of equation (5-5) by equation (5-6) gives the more useful expression,

$$C = \frac{(\tau_1 - \tau_2\alpha)}{(R_{T1} - R_{T2}\alpha)} \qquad (5\text{-}7)$$

where $$\alpha = \tau_1 R_{T1}/\tau_2 R_{T2} \qquad (5\text{-}8)$$

This method, therefore, allows the determination of membrane capacitances without having to measure the membrane resistance. Capacitances of BLM, usually expressed as capacitance/unit area of membrane, are typically 0.3-1.3 $\mu F\ cm^{-2}$ (17), (173).

Only the hydrocarbon region of any bilayer film contributes to the capacitance. The thickness of this region, d, can be calculated, assuming that the film behaves as a parallel plate condenser, using the formula,

$$C = \frac{\bar{e}A}{4\pi d} \qquad (5\text{-}9)$$

where A is the area of the film and $\bar{e}$ is the dielectric constant. The value of $\bar{e}$ may be estimated by taking the

bulk dielectric constant of the hydrocarbon used as solvent (171). To estimate the total bilayer thickness the thickness of both hydrophilic layers must be added to the thickness of the hydrocarbon layer, which is assumed to be given by d. For lecithin membranes the thickness of the polar group region is approximately 10A (17) and therefore a correction of 20A must be added to d to give an estimated total thickness of the membrane.

This method is subject to a number of sources of error. The estimation of membrane area and the assumption that the dielectric constant of the bilayer is equal to that of the bulk liquid phase hydrocarbon are probably two of the largest sources of error (151), (171). The simplicity of the method, however, makes it very convenient and useful.

5-2 EXPERIMENTAL

5-2.1 Chemicals

The lipids and solvents used in this work were all of the highest purity and were used as obtained. Their sources are given in Appendix I.

All aqueous solutions were maintained at pH 6.86 using phosphate buffer (section 1-3.1). In membrane formation and capacitance studies sodium chloride (0.1M) was also present.

5-2.2 Cleaning

Cleaning was carried out according to the methods recommended by Howard and Burton (151). Surface active agents (e.g. plasticizer and catalysts) were completely removed from new pieces of perspex equipment by washing and exposing the pieces to ultrasound in hot aqueous detergent, rinsing them for several hours in hot running tap water, and then in distilled water. Finally, they were oven dried. New pieces of teflon equipment were washed as above and then left in chloroform-methanol (2:1 v/v) overnight. They were finally rinsed in distilled water and dried. Subsequently, the perspex and teflon pieces were cleaned according to a standard procedure: They were left in detergent overnight, thoroughly rinsed with tap and then distilled water, and finally oven dried. Glassware and hypodermic syringes and needles were also cleaned by this method.

5-2.3 Membrane-forming solutions

Membrane-forming solutions were prepared from a variety of lipids and solvents. Aliquots (0.075 ml) of commercially available egg lecithin (100 mg ml^{-1}) were used directly when the solvent was tetradecane but, when dissolved in hexane, the solvent was blown off with nitrogen, a small aliquot (0.025-0.06 ml) of a chloroform-methanol mixture added (to redissolve the solid) and

decane then added. Membrane-forming solutions containing the synthetic lecithins dipalmitoyl phosphatidyl choline (DPPC) and dimyristoyl phosphatidyl choline (DMPC), were prepared by dissolving weighed amounts of the solid in small aliquots of a chloroform-methanol mixture and then adding the appropriate volume of decane. In some cases cholesterol and/or vitamin K were added to these solutions by injecting measured volumes of, previously prepared, decane solutions of the neutral lipids into known volumes of the above phospholipid solutions. Vitamin K solutions, in various hydrocarbon solvents, and cholesterol solutions, in decane, were prepared by accurately weighing out the lipids in volumetric flasks and adjusting the volume to the mark with the appropriate solvent. Vitamin K - cholesterol solutions were made by adding measured volumes of vitamin K solutions to either known weights of solid cholesterol or to known volumes of the sterol solutions. When the solutions, thus prepared, were not to be used immediately, they were transferred into small glass ampoules, the ampoules hermetically sealed and stored below 0°C in the dark.

5-2.4 Membrane formation studies

The arrangement used in the attempts to form ultra-thin membranes consisted of two chambers connected by a small hole in which the membrane was formed, figure 5-3.

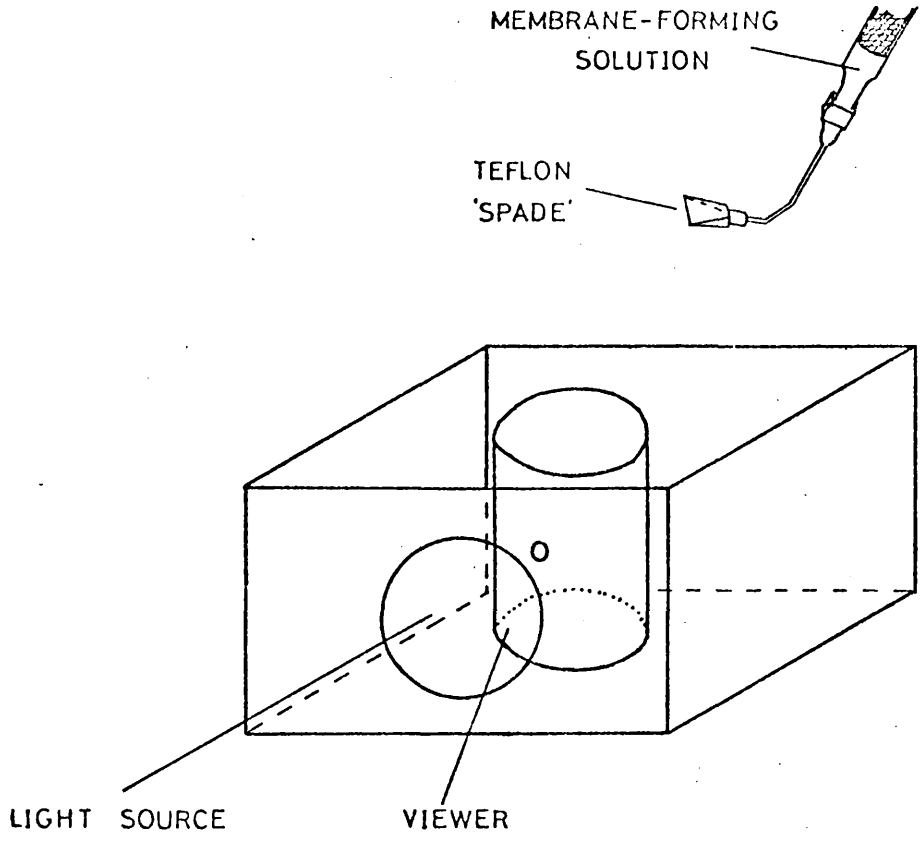


Figure 5-3 Apparatus for investigating 'ultrathin' membranes.

The inner chamber was made of a small teflon beaker, machined from a solid rod. A small area of the wall of this beaker was thinned to 0.5 mm and a hole drilled through this section, at a point halfway up from the base. The hole, ~2 mm diameter, was smoothed by a heated needle. The outer chamber was a 10 x 7 x 6 cm glass dish. A 3 cm diameter disc of glass had been removed from the centre of the front face of this chamber and a 4 cm diameter optically clear glass disc araldited over the hole. This window was used to view the membrane with the aid of a microscope.

After the membrane orifice had been preconditioned, the beaker and dish were filled with electrolyte solution until the level of the aqueous phase was ~1 cm below the top of the beaker. The lipid solution was spread across the hole using the 'Painting' method. A syringe needle, which had been fitted with a teflon 'spade', figure 5-3, and which was connected to a small micrometer syringe, was found to be the most successful method of forming membranes. On ejection of small quantities of lipid solution the teflon 'spade' caused the solution to fan out. The membrane was then easily formed by drawing the end of the 'spade' over the orifice.

All experiments were carried out in the atmosphere, at a temperature of 25 $\pm$ 1°C. To aid viewing of the

membrane, the apparatus, shown in figure 5-3, was covered with black cardboard except for the window. Since ambient vibrations tend to rupture thinning membranes (151), the whole assembly was mounted on a vibration-damping table (Gallenkamp, England).

5-2.5 Capacitance and thickness measurements

The cell used to form membranes for capacitance studies and the method of membrane formation, were identical to those just described. In addition, however, a dip-type saturated calomel electrode (Pye-Unicam, No. 11161) was placed in each compartment to allow potential measurements to be made. The electrical circuit shown in figure 5-2 was used. All conducting leads (made from coaxial cable) and instruments were electrically shielded from extraneous electromagnetic fields and from each other, by a common ground. The rapid fall in membrane potential, with time, was monitored on a Tektronix storage oscilloscope (Type 564, Tektronix Guernsey Ltd, USA) which was connected to a Keithley electrometer (Model 610C, Keithley Instruments, USA) as shown, figure 5-2. A DC Voltage Calibrator (Type 2003S, 0.02% Grade, Time Electronics Ltd., England) was used as the DC source.

Standard resistors (R_x) of 1.079 MΩ and 2.431 MΩ were used when thick membranes (>1000A) were being

studied whilst 100.6KΩ and 276.3KΩ resistors were used for thinner membranes. The resistance of the electrodes and solution (R_s) was found to be 20.8KΩ. All these resistances were determined using a Wayne Kerr bridge (Type B331, Wayne Kerr Comp. Ltd., England). The diameter of the membrane orifice was determined using a Nikon Profile Projector (Model 6C, Nippon Kogaku K.K., Japan), and was found to be 2.061 mm. The area of the hole was, therefore, $3.33 \times 10^{-2} cm^2$.

The method described in section 5-2.2 was used to determine membrane capacitances and thicknesses. For a given standard resistance, $E_m = 0$ and $E_m = E_m(0)$ lines were stored on the oscilloscope screen. The value of $E_m(0)$ was kept within the range 30-100mV. The membrane potential was discharged and its exponential decay from $E_m(0)$ to $E_m = 0$ also stored on the screen. The traces were then photographed using a Polaroid camera. This procedure was repeated immediately with the second resistance. From each photograph a time constant could be readily obtained, provided the oscilloscope's time base was known. Equations (5-2) and (5-7) were then used to calculate the membrane capacitance. To obtain an estimate for the thickness of the membrane, the capacitance calculated was first converted into e.s.u. units (by multiplying the capacitance in Farads by the scaling

factor of 9×10^{11}) before being used in equation (5-9). The dielectric constant of decane, $\bar{e} = 2.0$ (151), was used in these calculations. The approximate area of the membrane was obtained by multiplying the area of the orifice by the estimated fraction of the orifice not forming the Plateau-Gibbs border.

5-2.6 Electron-transfer across ultrathin membranes of vitamin K

In view of the oxygen sensitivity of both RMV and reduced vitamin K, the cell used in this experiment, as before, had to be airtight. A diagram of the cell designed for these experiments is given in figure 5-4. Although not completely impervious to air, this cell minimised oxygen leaks sufficiently for meaningful results to be obtained. The cell was composed of three sections; two black perspex sections and a thinner, intervening teflon one. A smooth hole (~2 mm diameter) bored in this gasket connected the two compartments A and B. The gasket was so constructed that the membrane could be formed by the 'Marginal Suction' method. Holes, drilled (from the outside) through the centre of each of the four sides of each perspex section, formed circular openings into the inner compartments. Glass, placed over three of the four holes from each section and araldited into position, formed viewing windows. When the sections

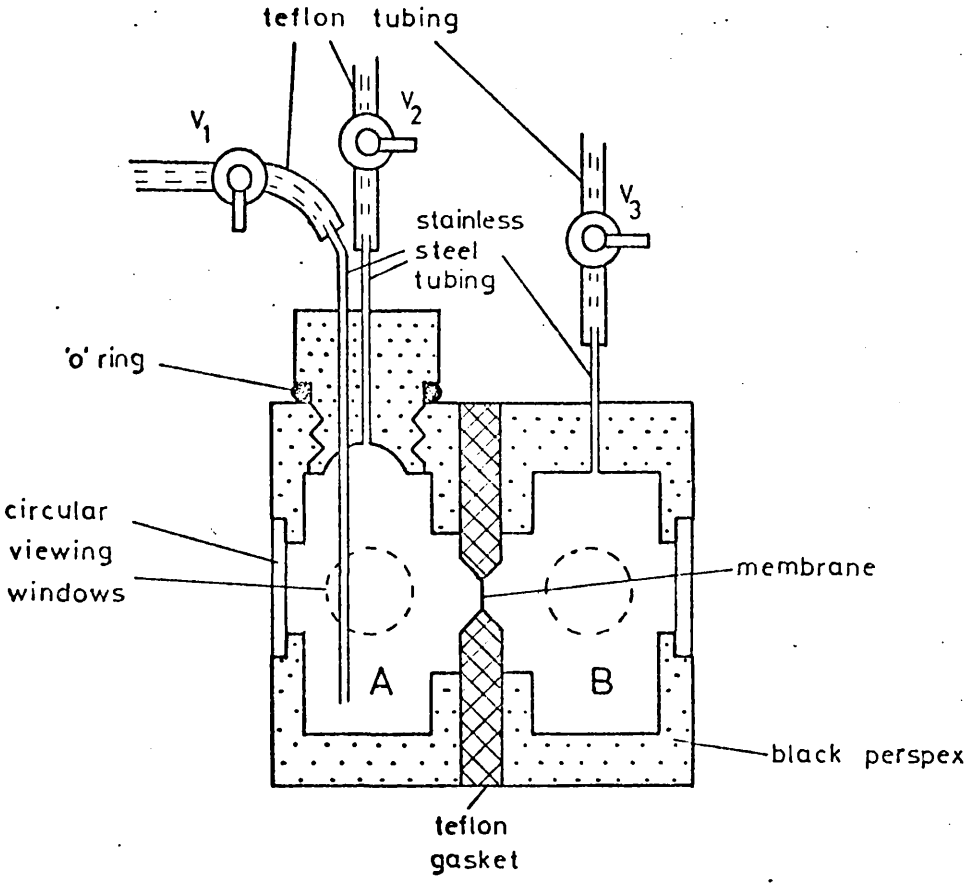


Figure 5-4 Cross-section of the cell used to investigate the electron-transfer properties of 'ultrathin' membranes. V_1, V_2 and V_3 represent two-way teflon valves. The actual cell was approximately half the size shown.

were assembled, the wall of each compartment, which had been left without a window, was placed against the teflon gasket. The membrane orifice lay at the centre of the holes in these faces, figure 5-4. A threaded plug formed the top of compartment A and an 'O' ring, placed as shown in figure 5-4, was used to form a gastight seal between the two pieces. Once assembled the three sections of the cell were held together by a brass clamp. Stainless steel capillary tubing (0.5 mm ID) inlets and outlets, figure 5-4, allowed perfusion of compartment A and ejection of air or solution from compartment B. Teflon tubing (Biolab Ltd., England), 0.8 mm ID, was used both to connect the steel tubing to two-way 'Omnifit' teflon valves (Biolab Ltd., England) and to connect the other ends of these valves to the syringes that contained the various reagents to be used. A similar assembly (not shown in figure 5-4) was used to connect the membrane orifice with the micrometer syringe that contained the membrane-forming solution.

Once the cell had been assembled, compartments A and B, and all sections of steel and teflon tubing, were filled with buffer solution. To achieve this, buffer was introduced through valve V_1 and the other valves opened or closed as necessary. This removed air from the system and, hence, avoided the formation of unwanted air bubbles.

By an identical procedure, the buffer was replaced by substrate solution. The membrane was then formed by the 'Marginal Suction' method. Compartments A and B were now separated by the membrane. All valves were then shut. Next, valves V_1 and V_2 were opened and compartment A slowly and carefully perfused with RMV solution. Finally V_1 and V_2 were closed once more. The cell, therefore, contained RMV in compartment A and substrate solution in compartment B. Reaction between these two solutions could only occur by way of the membrane.

The aqueous solvent, as before, was 0.025M phosphate buffer. RMV was generated by adding sufficient solid sodium dithionite to reduce a 20 mM MV solution by 50%. The substrate solution consisted of buffer, 0.1 mM in $[Fe(o\text{-phen})_3]^{3+}$, 0.3 mM in excess o-phenanthroline and 40 mM in sodium chloride. Excess o-phenanthroline was used to keep the Fe(III) fully complexed and the inert sodium chloride was necessary to make the substrate solution iso-osmotic with the RMV solution. This solution had been degassed and then saturated with nitrogen, prior to its introduction into the cell.

The $[Fe(o\text{-phen})_3]^{3+}$ complex is colourless whereas the corresponding reduced complex, $[Fe(o\text{-phen})_3]^{2+}$ (ferroin) is orange. Reaction could therefore be detected by the accompanying colour change in the substrate limb.

This change was monitored visually, with time.

5-3 RESULTS AND DISCUSSION

The apparatus shown in figure 5-3 was tested using membrane-forming solutions composed of egg lecithin and cholesterol, dissolved in either decane or tetradecane. Such solutions were known to give lipid bilayers of high stability (179), (181). As can be seen from table 23, stable bilayers were obtained. (For the purpose of this study, a stable membrane is defined as one which lasts for more than 10 minutes). The ability to form BLM showed that the apparatus was suitable to test the membrane-forming abilities of other lipid solutions. It is interesting to note that egg lecithin-cholesterol solutions using decane as the solvent gave bilayers of greater stability than those using tetradecane as the solvent. This may be a direct consequence of the fact that the latter membranes are known to be thinner and to contain much less solvent than the former ones (175). A black lipid membrane was judged to have been formed if the originally colourless membrane first became highly coloured and then became colourless (black) once more.

Natural egg lecithin is a complex mixture of molecular species differing in the nature of the fatty acid residues

Table 23 Abilities of various lecithin and lecithin-cholesterol, decane and tetradecane solutions to form stable BLM. The weights quoted are per ml of hydrocarbon. 0.05 ml of a 2:1 $CHC\ell_3/CH_3OH$ solution (v/v) was added (per ml of hydrocarbon) to each solution. The aqueous medium was 0.025M phosphate buffer (pH 6.86), 0.1M in $NaC\ell$. The temperature was 25°C.

Lecithin		Cholesterol	Hydrocarbon	Nature of Membrane
type	(mg)	(mg)		
egg	6.9	6.9	tetradecane	stable
egg	7.5	7.9	decane	stable
DPPC	9.3	-	decane	unstable
DPPC	4.6	-	decane	unstable
DPPC	7.2	7.2	decane	unstable
DPPC	3.6	7.2	decane	unstable
DMPC	7.6	-	decane	unstable

(152), (178): C-16 and C-18 saturated fatty acids and C-18 mono- and di-unsaturated fatty acids being the predominant residues (figure 4-2). It seemed desirable, therefore, to use lecithins of chemically uniform composition. In the search for lipid solutions of simple composition egg lecithin was substituted by the saturated lecithins DPPC ($R = -(CH_2)_{14}-CH_3$, figure 4-2) and DMPC ($R = -(CH_2)_{12}-CH_3$, figure 4-2). As can be seen, table 23, however, no stable membranes could be formed from either lecithin. Their inability to form bilayers had been previously attributed to the saturated nature of their fatty acid side chains (152). It now appears more likely that the negative results obtained are a direct result of the experiments being carried out at temperatures close to or below the phase transition temperature of these lipids. The term 'phase transition' is used to define the course of melting of the laterally contiguous hydrocarbon phase (48). DPPC and DMPC display phase transitions at 41°C and 23°C respectively (182). At or below these temperatures lipids are in a 'solid' crystalline phase and probably do not possess sufficient fluidity to form stable membranes. It should be appreciated that the 'solid' phase is 'solid' in the sense that the fatty acid residues have stiffer and more expanded chains; the rate of lateral diffusion may, however, be quite high (182).

The ability of decane solutions of DPPC to form membranes at a temperature of 50°C (183) shows that saturated lipids can indeed form stable membranes, provided the temperature is kept well above the transition temperature of the lipid being used. The presence of cholesterol in the membrane-forming solution, in addition to increasing the stability of the film, is also known to cause a lowering of the phase transition temperature (184). At equimolar cholesterol and lecithin ratios, the phase transition was eliminated altogether. Cholesterol was envisaged as controlling the fluidity of the hydrocarbon chains of the phospholipid by disrupting the crystalline lattice in the 'solid' phase and by inhibiting the flexing of the chains in the dispersed liquid crystalline phase. For this reason it was expected that lipid mixtures of DPPC and cholesterol, dissolved in decane, would form stable membranes. This, however, was not verified experimentally, table 23.

The ability to form stable bilayers from egg lecithin-cholesterol mixtures allowed studies to be carried out in which these membranes were 'doped' with vitamin K. Similar membrane-forming solutions, but which lacked cholesterol, were also prepared. In addition a few lipid solutions were prepared using DPPC or DMPC in place of egg lecithin. Results are recorded in table 24. As

Table 24 Abilities of various vitamin K_1-lecithin and vitamin K_1-lecithin-cholesterol decane solutions to form stable BLM. The weights quoted are per ml of decane. 0.025 ml and 0.33 ml of a 2:1 $CHCl_3/CH_3OH$ solution (v/v) was added (per ml of hydrocarbon) to lipid solutions containing DPPC and DMPC respectively. The aqueous medium was 0.025M phosphate buffer (pH 6.86), 0.1M in NaCl. The temperature was 25°C.

Lecithin		Cholesterol	Vitamin K	Nature of Membrane
type	(mg)	(mg)	(mg)	
egg	7.5	-	8.2	stable
egg	7.0	6.9	35.1	stable
egg	7.0	6.9	7.0	stable
egg	7.0	7.0	1.4	stable
DPPC	9.2	-	9.7	unstable
DPPC	11.0	-	60.1	unstable
DPPC	10.2	4.8	9.7	unstable
DPPC	8.9	8.4	9.7	unstable
DMPC	3.5	-	40.2	unstable
DMPC	10.0	-	30.6	unstable

expected, membrane-forming solutions containing the synthetic lecithins, both in the absence and presence of cholesterol, would not form stable membranes. Vitamin K-egg lecithin membranes, both in the presence and absence of cholesterol, formed stable BLM. These membranes were found to remain stable even when the weight of vitamin K far exceeded the weight of phospholipid present, table 24.

In any membrane containing vitamin K, only the quinone is necessary for the membrane to display redox properties. Any additional lipid present must be totally inert towards the aqueous redox agents (see Introduction). For these reasons, attempts were made to form thin membranes from membrane-forming solutions which contained vitamin K as the only lipid component. To vary the viscosities of the various lipid solutions both pure and mixed hydrocarbon solvents were employed. In all cases, however, no stable, thin, membranes could be formed, table 25. When decane, nonane or 1:1 mixtures of decane/octane or nonane/octane were employed as solvent, stable thick (lens) membranes were formed, that did not thin down to the black condition. The vitamin K-decane membranes were found to be stable over a period of several days. The formation of thick membranes by vitamin K has been reported previously (185), although no details of the membrane-forming solutions were given. As can be seen from table 26, the addition of cholesterol

Table 25 Abilities of various vitamin K_1 hydrocarbon solutions to form stable BLM. The weights of vitamin K quoted are per ml of solvent. The aqueous medium was 0.025M phosphate buffer (pH 6.86), 0.1M in NaCℓ. The temperature was 25°C.

Vitamin K (mg)	Solvent	Nature of Membrane
60.9	decane	stable but thick
30.4	decane	stable but thick
9.7	decane	stable but thick
10.7	nonane	stable but thick
9.0	octane	unstable
11.6	1:1 octane/decane (v/v)	stable but thick
10.0	3:1 octane/decane (v/v)	unstable
11.6	5:1 octane/decane (v/v)	unstable
10.2	10:1 octane/decane (v/v)	unstable
9.6	1:1 octane/nonane (v/v)	stable but thick

Table 26 Abilities of various vitamin K_1-cholesterol decane solutions to form stable BLM. The weights of lipid quoted are per ml of decane. The aqueous medium was 0.025M phosphate buffer (pH 6.86), 0.1M in NaCℓ. The temperature was 25°C.

Vitamin K (mg)	Cholesterol (mg)	Nature of Membrane
10.1	2.7	stable but thick
10.1	3.6	stable but thick
9.7	8.4	stable but thick
30.1	4.9	stable but thick

to decane solutions of vitamin K had no effect on the ability of the solutions to form stable, thick membranes. Cholesterol is known to cause an increase in the viscosity of the lipid solvent (186) and it would, therefore, be expected to aid the formation of thick membranes. This, however, was not tested.

The thicknesses of selective lipid membranes were determined using the capacitance method previously described (section 5-2.5). The composition, capacitance and thickness of the hydrocarbon layer for each of these membranes, at various times after their formation, are recorded in table 27. Previously reported capacitances of egg lecithin-cholesterol membranes lie within a 0.38-0.56 $\mu F\ cm^{-2}$ range (179). Thus, the value of 0.39 $\mu F\ cm^{-2}$, obtained during this research, table 27, agrees closely with those previously found and shows the usefulness of the simple DC transient method in determining membrane capacitances. The thickness of this membrane, calculated using equation (5-9), is typical of that obtained from the hydrocarbon region of lipid bilayers (17). Addition of vitamin K to the egg lecithin-cholesterol membrane results in a slowing down of the thinning rate but has no effect on the final thickness observed, table 27. Vitamin K-egg lecithin membranes were found to be less stable than egg lecithin-cholesterol membranes both with and without added vitamin K. Their

Table 27 Capacitance measurements obtained from lipid membranes. The weights of lipid quoted are per ml of decane (the hydrocarbon solvent). 0.06 ml of a 5:1 $CHC\ell_3/CH_3OH$ solution (v/v) was added (per ml of hydrocarbon) to each of the lecithin containing membranes. The aqueous medium was 0.025M phosphate buffer (pH 6.86), 0.1M in $NaC\ell$. The temperature was 25°C.

Vitamin K (mg)	Egg lecithin (mg)	Cholesterol (mg)	Time (min)	C $\mu F\ cm^{-2}$	d A
7.6	-	-	60	0.006	2800
8.0	7.4	-	12	0.036	490
			35	0.20	88
4.0	7.4	3.7	65	0.17	100
			95	0.23	77
			145	0.33	53
-	7.4	3.6	45	0.39	46

capacitance behaviour, however, was similar to that displayed by egg lecithin-cholesterol-vitamin K membranes, table 27. The general decrease in thinning rate displayed by vitamin K-containing egg lecithin membranes is considered to be a viscosity effect. Vitamin K, when dissolved in decane, is seen to give a membrane which is some fifty times thicker than that of a typical bilayer, table 27. This lens is envisaged as having the structure shown in figure 5-1(a). The membrane thicknesses quoted in table 27 must be considered as being only approximate since a dielectric constant of 2 has been used in the calculations. As vitamin K and cholesterol are more polar than hydrocarbons, they would be expected to increase the dielectric constant of decane. The thicknesses calculated must, therefore, be considered as minimum values.

Attempts to form vitamin K containing bilayer membranes from a variety of lipid solutions, showed clearly that vitamin K, in the absence of other lipid, is incapable of forming lipid bilayers. Additional lipid capable of forming a bilayer matrix and into which the functional quinone molecules can be added, is required. From these studies only egg lecithin or egg lecithin-cholesterol solutions were found to be suitable. Pure synthetic lipids with low transition temperatures e.g. dioleoyl phosphatidyl choline (transition temperature = -22°C (187)),

though not tried during this research, should also be suitable.

The investigation of redox reactions across a lipid bilayer, doped with a suitable membrane-bound carrier, was, as discussed earlier, one of the aims of this research. Although the lipid bilayer studies just described allowed such experiments to be attempted, studies using 'ultrathin' membranes of bilayer dimensions have not been possible as yet. Redox reactions across a vitamin K lens membrane ($d \simeq 2800A$) have, however, been carried out using the method outlined in section 5-2.6. The redox membrane system may be represented as,

$$\text{RMV} \left| \begin{matrix} \text{Vitamin K} \\ \text{(lens)} \end{matrix} \right| [\text{Fe(o-phen)}_3]^{3+}$$

The membranes were formed from a solution of vitamin K, in decane, of concentration 8.0 mg ml^{-1}. Six hours after the system was established the substrate solution close to the membrane had become distinctly orange in colour. After a period of 24 hours the whole substrate solution (in compartment B, figure 5-4) was dark orange. These findings were completely reproducible. Formation of the orange $[\text{Fe(o-phen)}_3]^{2+}$ complex proved that electrons were being transferred through the vitamin K membranes. This indicates that the vitamin K carrier molecules are still sufficiently mobile within the thin membrane to allow the redox reaction to occur at a measurable rate. The small membrane area and absence of stirring within the

aqueous phases account for the slow rate of reaction observed. The results from these preliminary experiments are encouraging since they verify that thin membranes of vitamin K can function in an analogous way to their bulk solvent membrane analogues, even in the absence of stirring, and, in addition, they indicate the feasibility of carrying out such reactions across vitamin K-doped lipid bilayers.

Appendix I

SOURCES OF CHEMICALS

The form, purity and source of all the chemicals of special importance to this work are given below. All other reagents were of the highest purity available, normally 'ANALAR'.

The following abbreviations are used to indicate the manufacturer from which each compound was obtained:

BDH	BDH Chemicals Ltd., Poole, Dorset, England.
H & W	Hopkin and Williams Ltd., Chadwell Heath, Essex, England.
JM	Johnson Matthey & Co., London, England.
O	Organon Laboratories Ltd., Newhouse, Scotland.
S	Sigma London Chemical Company Ltd., Surrey, England.

Name	Description	Source
Ascorbic acid	grade > 99%	H & W
Benzyl viologen	-	BDH
Borohydride	sodium salt	O
Cardiolipin	from bovine heart, sodium salt, ethanol solution	S
Chloroquine	diphosphate salt	S
Cholesterol	grade 99 + %, standard for chromatography	S
Coenzyme Q_{10}	-	S
Cytochrome c	horse-heart, Type III, grade 95-100%	S
Cytochrome c	from neurospora crassa	A kind gift from Dr.I.D.A Swan
n-Decane	grade 99 + %	S
2,6-Dichlorophenol indophenol	-	BDH
Dimyristoyl phosphatidyl choline	synthetic, grade ~98%, crystalline	S
Dipalmitoyl phosphatidyl choline	synthetic, grade I, 99% crystalline	S
Dithionite	sodium salt, grade > 85%	BDH
Dodecane	grade I, ~99%	S
Egg lecithin	from egg yolk, Type III-E, hexane solution	S
Egg lecithin	from egg yolk, Type IV-E, tetradecane solution	S
Flavin adenine dinucleotide	disodium salt, grade III, 94-99%	S

(Contd.)

Name	Description	Source
Flavin mono-nucleotide	sodium salt, grade 95-97%	S
n-Heptane	grade ~99%	S
Hexane	low in aromatics (0.1%)	H & W
Hexane (spectro-scopic)	'Spectrosol', for UV spectroscopy	H & W
Indophenol	-	H & W
Lysolecithin	from soybeans, Type IV, ~98%, contains primarily C-18 unsaturated fatty acids	S
Methylene blue	-	BDH
Methyl Viologen	-	BDH
p-Nitrophenol	spectrophotometer grade	S
n-Nonane	grade ~99%	S
n-Octane	grade ~99%	S
o-Phenanthroline	as hydrate	H & W
Phenosafranine	-	H & W
Phosphatidyl ethanolamine	dipalmitoyl, synthetic, grade I ~98%	S
Phosphatidyl inositol	from soybean, ammonium salt, grade I, ~98%	S
Reduced nicotin-amide adenine dinucleotide	disodium salt, grade 85%	BDH
Reduced nicotin-amide adenine dinucleotide phosphate	sodium salt, grade 78%	BDH

(Contd.)

Name	Description	Source
Silver oxide	grade 'Specpure'	JM
n-Tetradecane	grade ~99%	S
Thionine	-	BDH
Vitamin K_1	-	S

Appendix II

LINEAR 'LEAST SQUARES' PROGRAM

To speed analysis of the results obtained from the many kinetic runs carried out, a short computer program was written which would calculate the gradient of the best fitting straight line through each set of data pairs. The program, written in FORTRAN IV programming language, was run on an IBM 360 computer. The linear 'least squares' method of Moroney (188) was used.

The computer program written, followed by a description of the input parameters, together with a specimen input, and finally a specimen output are given in this appendix.

```
C      GENERAL 'LEAST SQUARES' PROGRAM .  DERIVES THE BEST
C      STRAIGHT LINE FIT FOR UP TO 100 PAIRS OF DATA POINTS.
C
       DIMENSION  NAME(20),X(100),Y(100),YY(100),PDEV(100)
C
  500 FORMAT (I3)
  510 FORMAT (20A4)
  520 FORMAT ( 2F10.4 )
  530 FORMAT (E15.4)
  540 FORMAT (2I3)
C
C
       KOUNT = 0
       READ (5,500) NSETS
       WRITE (6,600) NSETS
    5  READ (5,510) ( NAME(I),I=1,20)
       WRITE (6,610) ( NAME(I),I=1,20)
       READ (5,500)IFIT
C
       GO TO (12,13,14,15,16,17,18),IFIT
   12  WRITE (6,720)
       GO TO 19
   13  WRITE (6,730)
       GO TO 19
   14  WRITE (6,740)
       GO TO 19
   15  WRITE (6,750)
       GO TO 19
   16  WRITE (6,760)
       GO TO 19
   17  WRITE (6,770)
       GO TO 19
   18  WRITE (6,780)
   19  CONTINUE
C
       READ (5,540) NPTS,MINPTS
       READ (5,530) FACTOR
C
       DO 1000 I=1,NPTS
       READ (5,520) X(I),Y(I)
       Y(I) = Y(I)*FACTOR
 1000  CONTINUE
C
       WRITE (6,620) ( X(I),Y(I),I=1,NPTS )
       WRITE (6,605) NPTS
C
       I=1
       GO TO (20,30,40,50,60,70,80),IFIT
   20  X(I) = SQRT( X(I) )
       GO TO 90
   30  X(I) = X(I)*X(I)
       GO TO 90
   40  X(I) = ALOG10 (X(I))
       GO TO 90
   50  X(I) = 1.0/ X(I)
```

```
      GO TO 90
   60 Y(I) = ALOG10 ( Y(I) )
      GO TO 90
   70 X(I) = ALOG10 ( X(I) )
      Y(I) = ALOG10 ( Y(I) )
   90 I=I+1
      IF (I .LE. NPTS ) GO TO (20,30,40,50,60,70,80),IFIT
C
   80 SIGMAX = 0.0
      SUMX2  = 0.0
      SIGMAY = 0.0
      SUMY2  = 0.0
      SDEV2  = 0.0
      SPROD  = 0.0
C
      DO 2000 I=1,NPTS
      SIGMAX = SIGMAX + X(I)
      SIGMAY = SIGMAY + Y(I)
      SUMX2 = SUMX2 + X(I)*X(I)
      SUMY2 = SUMY2 + Y(I)*Y(I)
      PRODXY =  X(I)*Y(I)
      SPROD  = SPROD + PRODXY
 2000 CONTINUE
C
      SLOPE = (SPROD - ( SIGMAX*SIGMAY )/ FLOAT(NPTS) ) /
     1        (SUMX2 - (SIGMAX*SIGMAX / FLOAT(NPTS) ))
      CEPT  = (SIGMAX*SPROD - SIGMAY*SUMX2) /
     1        (SIGMAX*SIGMAX -  FLOAT(NPTS)*SUMX2)
      WRITE (6,640) SLOPE,CEPT
C
      DO 3000 I=1,NPTS
      YY(I) = SLOPE*X(I) + CEPT
      DEV   = YY(I) - Y(I)
      SDEV2 = SDEV2 + DEV*DEV
      IF (Y(I) .EQ. 0.0) GO TO 100
      PDEV(I) = 100.0*DEV / Y(I)
      GO TO 110
  100 PDEV(I) = 9.9999E  2
  110 CONTINUE
 3000 CONTINUE
C
      WRITE (6,630) ( X(I),YY(I),Y(I),PDEV(I), I=1,NPTS )
C
      RMEANX = SIGMAX / FLOAT(NPTS)
      RMEANY = SIGMAY / FLOAT(NPTS)
      PRODMS = RMEANX*RMEANY
      AVERP  = SPROD / FLOAT(NPTS)
      REAL = (SUMX2 / FLOAT(NPTS)) - (RMEANX*RMEANX)
      SDX  = SQRT(REAL)
      REAL = (SUMY2 / FLOAT(NPTS)) - (RMEANY*RMEANY)
      SDY  = SQRT(REAL)
      CORR = (AVERP - PRODMS) / ( SDX*SDY )
C
      REAL = 1-CORR*CORR
      IF ( REAL.LT.0.0E-04  .AND.  REAL.GT.-0.0E-04 )
```

```
      1REAL=0.0
       REAL = SQRT(REAL)
       SEEY = REAL*SDY
       SEEX = REAL*SDX
       WRITE (6,650) SEEX,SEEY,CORR
C
       IF (MINPTS .GE. NPTS) GO TO 25
       NPTS = NPTS-1
       GO TO 80
   25  KOUNT = KOUNT + 1
       IF (KOUNT .NE. NSETS) GO TO 5
C
  600 FORMAT ('1',1X,'NUMBER DATA SETS = ',I3 )
  605 FORMAT ( '0',1X,'NUMBER POINTS = ',I3 )
  610 FORMAT (///2X,20A4)
  620 FORMAT ('0',9X,'INPUT DATA'//9X,'X',18X,'Y'//
     1(4X,1PE11.4,7X,1PE11.4))
  630 FORMAT ('0',5X,'XOBS',9X,'YCALC',9X,'YOBS',5X,
     1'PERCENT DEV.'// 4(2X,1PE11.4))
  640 FORMAT (///7X,'GRADIENT = ',1PE11.4,5X,
     1'INTERCEPT = ',E11.4)
  650 FORMAT (///7X,'STANDARD ERROR OF ESTIMATE X= ',
     11PE11.4,//7X,'STANDARD ERROR OF ESTIMATE Y= ',
     1E11.4,//
     27X,'CORRELATION COEFFICIENT = ',0PF10.4)
  720 FORMAT ('0',5X,'CURVE FIT OF ROOT X VS Y')
  730 FORMAT ('0',5X,'CURVE FIT OF X SQUARED VS Y')
  740 FORMAT ('0',5X,'CURVE FIT OF LOG(10) X VS Y')
  750 FORMAT ('0',5X,'CURVE FIT OF RECIPROCAL X VS Y')
  760 FORMAT ('0',5X,'CURVE FIT OF X VS LOG(10) Y')
  770 FORMAT ('0',5X,'CURVE FIT OF LOG(10) X VS LOG(10) Y')
  780 FORMAT ('0',5X,'CURVE FIT OF X VS Y')
C
       STOP
       END
```

INPUT

Card 1.	NSETS	number of sets of data
Card 2.	NAME(I)	verbal description of data set following
Card 3.	IFIT	type of fit required:

f(x)	f(y)	IFIT
$\sqrt{x}$	y	ØØ1
x^2	y	ØØ2
log x	y	ØØ3
1/x	y	ØØ4
x	log y	ØØ5
log x	log y	ØØ6
x	y	ØØ7

Card 4.	NPTS, MINPTS	number of points in data set following, minimum number of points to be curve fitted.
Card 5.	FACTOR	factor by which input y values are to be multiplied.
Succeeding cards	Data pairs	

Specimen Input:

IBM

PROGRAM GENERAL 'LEAST SQUARES'.

PROGRAMMER S. ANDERSON. DATE

COMM.	STATEMENT NUMBER	CONT.	
	1		
MV-K1-THIONINE RUN 6-3-76			
	7		
	6 6		
			1.9054E-05
0.0			0.474
20.0			0 469
42.0			0.459
60.0			0.450
92.0			0.437
120.0			0.426

Specimen Output:

```
NUMBER DATA SETS =    1


MV-K1-THIONINE RUN 6-3-76 + S.U.C.

     CURVE FIT OF X VS Y

          INPUT DATA

        X                    Y

    0.0                 9.0316E-06
    2.0000E 01          8.9363E-06
    4.2000E 01          8.7458E-06
    6.0000E 01          8.5743E-06
    9.2000E 01          8.3266E-06
    1.2000E 02          8.1170E-06

NUMBER POINTS =    6

      GRADIENT = -7.8851E-09      INTERCEPT =  9.0608E-06

    XOBS          YCALC          YOBS      PERCENT DEV.

 0.0           9.0608E-06   9.0316E-06    3.2392E-01
 2.0000E 01    8.9031E-06   8.9363E-06   -3.7128E-01
 4.2000E 01    8.7297E-06   8.7458E-06   -1.8420E-01
 6.0000E 01    8.5877E-06   8.5743E-06    1.5680E-01
 9.2000E 01    8.3354E-06   8.3266E-06    1.0596E-01
 1.2000E 02    8.1146E-06   8.1170E-06   -2.9155E-02


      STANDARD ERROR OF ESTIMATE X=  2.6662E 00

      STANDARD ERROR OF ESTIMATE Y=  2.1068E-08

      CORRELATION COEFFICIENT =    -0.9979
```

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