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STUDIES ON THE INTERACTION OF GLUTAMATE DEHYDROGENASE WITH PHOSPHOLIPIDS AND WITH MITOCHONDRIAL MEMBRANES

A THESIS

presented in partial fulfilment of
the requirements for the degree
of
DOCTOR OF PHILOSOPHY
of the
University of Warwick
by

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Department of Molecular Sciences,
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To my parents ......

ACKNOWLEDGMENTS

I wish to express my gratitude to my supervisor, Dr G. H. Dodd for his help and encouragement throughout the course of this work.

I also wish to thank Mrs J. M. Cole for typing, Dr P. Moore for his assistance with the stopped-flow experiment and Messrs B. Menco and A. Menevse for performing the electron microscopy.

I am also grateful to many of my colleagues for their helpful discussions.

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
l-ala	l-alanine
CETAB	Cetyltrimethylammonium bromide
CL	Cardiolipin
DBDA	Dodecyl benzyl dimethyl ammonium bromide
EDTA	Ethylenediamino tetra-acetic acid
En	Enzyme
ESR	Electron spin resonance
GDH	Glutamate dehydrogenase
l-glu	l-glutamate
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
Hepes	N-2-hydroxyethylpiperizine-N'-2-ethanesulphonic acid
K	Equilibrium constant
Kd	Dissociation constant
Ki	Dissociation constant of enzyme-inhibitor complex
Km	Michaelis constant
α-Kg	α-Ketoglutaric acid
l-leu	L-leucine
LL	Lysolecithin
m.w.	Molecular weight
MLP	Monolauryl phosphate
NAD^+	Oxidised β-nicotinamide adenine dinucleotide

NADH	Reduced β-nicotinamide adenine dinucleotide
NADPH	Reduced β-nicotinamide adenine dinucleotide phosphate
N.M.R.	Nuclear magnetic resonance
O.D.	Optical density
pipes	Piperazine-N, N'-bis(2-ethanesulphonic acid)
pI	Isoelectric point
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
SDS	Sodium dodecyl sulphate
tris	Tris(hydroxymethyl)aminomethane
u.v.	Ultra violet
λ	Wavelength

Other abbreviations are explained in the text.

SUMMARY

The object of this work was to characterise the interaction between becf liver glutamate dehydrogenase (EC 1.4.1.3), the inner mitochondrial membrane and its constituent phospholipids.

Because of the similarities in the structure and properties of detergents with phospholipids, interaction of the enzyme with both types of amphiphiles was investigated. Interaction with phospholipid membranes was found to be a reversible process while detergents brought about irreversible denaturation at high concentrations.

Association between the enzyme and the amphiphiles showed that the nature of the head group determines the extent of complex formation. Zwitterionic lysolecithin and phosphatidylcholine showed no interaction, while the anionic sodium dodecyl sulphate, phosphatidylserine and cardiolipin showed high affinity for binding to the enzyme. The apparent Ki values in the case of the two phospholipids were found to be 1 - 2 μM and 3 - 5 μM respectively in the direction of reductive amination.

The possibility of different conformations of the enzyme binding specifically to these charged surfaces and conformational changes brought about as a result of complex formation was investigated.

The extent of binding decreased with increasing pH and ionic strength, suggesting contributions from electrostatic interactions. Evidence for hydrophobic interaction was indicated by the observation that the extent of complex formation increases with increasing temperature.

Binding of the enzyme to mitochondrial membranes also indicated similar types of specificities. The enzyme showed a much higher affinity for binding to the inner surface of the inner mitochondrial membrane than to the outer surfaces of the inner and outer membranes. The extent of binding was also shown to depend on the presence of metabolites such as NADH and ADP. Binding decreased with increasing pH and ionic strength as was found for pure phospholipids.

It is suggested that, in mitochondria, the reversible association between the enzyme and the inner mitochondrial membrane is controlled in a manner dependent upon the local pH, ionic strength and metabolite concentrations, and this may have important physiological significance in the control of metabolic activities of the enzyme. It is also suggested that the system exhibits the allosteric phenomenon which may be important for its regulation.

INDEX

		Page
ACKNOWLEDGMENTS		
ABBREVIATIONS		
SUMMARY		
CHAPTER I	INTRODUCTION	
	General Introduction	1
	Conformation of Hydrocarbon Chains - Lecithin-Water System	4
	Conformational States of Phospholipids	8
	Physical Properties of Phospholipids which are Important in Lipid-Protein Interaction	10
	Behaviour of Phospholipids	13
	Evidence for the Presence of Bilayer Structure in natural and Artificial Membranes	14
	Forces involved in Lipid-Protein Interaction	16
	Some Properties of Membrane Proteins	24
	Amino Acid Composition and Distribution of Membrane Proteins	26
	Conformation of Membrane Proteins	27
	Motional Freedom of Membrane Lipids	28
	Movement of Membrane Proteins	29
	Fluid State of Phospholipids	31
	The Fluid Mosaic Model	33
	Structural Changes in Proteins and Lipids brought about by their Interaction	34
	Experimental Models for Membranes	36
	Sonication - Preparation of Unilamellar Vesicles	39
	Some Lipid-Dependent Enzyme Systems	41
	Micelles	46
	Shape of Micelles	49
	Cardiolipin	50
	Glutamate Dehydrogenase	52

CHAPTER II MATERIALS 58

METHODS:

Kinetic Experiments 59

Sonication of Phospholipids 60

Fluorescence Measurements 61

Extraction of GDH-Phospholipid Complex into Isooctane 63

Stopped-Flow Experiments 63

Preparation of Beef Liver Mitochondria 63

Chromatography of Mitochondrial Phospholipids 64

Florosil Column Chromatography 65

Assays of Mitochondrial Enzymes 69

Phospholipase C Effect 69

Digitonin Effect 70

Binding of Purified GDH to Mitochondrial Membranes 70

Binding of Endogenous GDH to the Inner Mitochondrial Membrane 71

Electron Microscopy 72

CHAPTER III KINETIC EXPERIMENTS

Interaction of Glutamate Dehydrogenase with Detergents 74

Interaction of Glutamate Dehydrogenase with Phospholipids 82

CHAPTER IV FLUORESCENCE STUDIES

Intrinsic Fluorescence Studies 93

Extrinsic Fluorescence Studies 100

CHAPTER V a) EXTRACTION OF GDH-PHOSPHOLIPID COMPLEX INTO ISOOCTANE 108

b) EFFECT OF PHOSPHOLIPIDS ON THE THE STATE OF POLYMERISATION OF GLUTAMATE DEHYDROGENASE - STOPPED-FLOW EXPERIMENTS 113

CHAPTER VI a) REMOVAL OF THE OUTER MITOCHONDRIAL MEMBRANE (PREPARATION OF MITOPLASTS) OF BEEF LIVER MITOCHONDRIA 117

b) BINDING OF GLUTAMATE DEHYDROGENASE TO MITOCHONDRIAL MEMBRANES 121

CHAPTER VII GENERAL DISCUSSION 130

BIBLIOGRAPHY 142

KEY TO FIGURES AND TABLES

Figure 1	Various aggregated forms of amphipaths
Figure 2	The binary phase diagram of egg phosphatidylcholine and water
Figures 3 - 7	Conformation of Hydrocarbon Chains - Lecithin-Water System
Figures 8 and 9	Different conformational states of phospholipids
Figure 10	The electrical double layer
Figure 11	The effect of cholesterol on the thickness of the phospholipid bilayer
Figure 12 & 13	The fluid mosaic model of membrane structure
Figure 14	The two lamellar phases formed between lysozyme and phospholipids
Figure 15	Sonication of phospholipids
Figure 16	A spherical micelle
Figure 17 a)	Structures of some phospholipids
Figure 17	The effect of different reagents on conformation and association of glutamate dehydrogenase
Figure 18 & 19	The six ellipsoid subunits of a glutamate dehydrogenase oligomer and direction of polymerisation
Figure 20	The effect of SDS and lysolecithin on the glutamate dehydrogenase activity of GDH
Figure 20 a)	TLC of phospholipid fractions from Florosil column
Figure 20 b)	The fluorescence spectra of GDH in aqueous and hydrocarbon environments
c)	Photodecomposition of GDH
Figure 21 & 22	The effect of the preliminary incubation of glutamate dehydrogenase with SDS at different concentrations of the detergent
Figure 23 & 24	The effect of the preliminary incubation of glutamate dehydrogenase with SDS and MLP at different detergent concentrations
Figure 25 & 26	The effect of the preliminary incubation of glutamate dehydrogenase with MLP at different detergent concentrations
Figure 27	The dependency of the initial rate of loss of activity of glutamate dehydrogenase on detergent concentration
Figure 28	The dependency of the time taken for 50% denaturation of glutamate dehydrogenase on detergent concentration
Figure 29	The effect of α-Kg and NADH on the rate of denaturation of glutamate dehydrogenase

Figure 30	The effect of l-glu and NAD^+ on the rate of denaturation of glutamate dehydrogenase
Figure 31 & 32	First order plots of MLP and SDS effects on GDH activity (preliminary incubation)
Figure 33	The effect of NH_4Cl on the extent of inhibition of glutamate dehydrogenase activity by SDS
Figure 34	The effect of cardiolipin on alanine dehydrogenase activity of GDH
Figure 35 & 36	Plots of activity of SDS and MLP treated enzyme against different detergent concentrations at different times of detergent treatment
Figure 37 & 38	The effect of cardiolipin, phosphatidylserine and phosphatidylcholine on glutamate dehydrogenase activity of GDH
Figure 39	The time dependency effect of phosphatidylserine sonicated in phosphate buffer
Figure 40	The time dependency effect of phosphatidylserine
Figure 41 to 44	Stability of glutamate dehydrogenase in hepetri buffer of different molarities (pH 7. 7) at 3°C and at 25°C
Figure 45	The dependency of the extent of inhibition of GDH by cardiolipin on the time of incubation
Figure 46	Lineweaver-Burk plots of glutamate dehydrogenase inhibition by cardiolipin at different NAD^+ concentrations
Figure 47	Lineweaver-Burk plots of glutamate dehydrogenase inhibition by cardiolipin at different α-Kg concentrations
Figure 48	Lineweaver-Burk plots of glutamate dehydrogenase inhibition by cardiolipin at different l-glu concentrations
Figure 49	The dependency of the extent of inhibition of GDH activity on PC/CL ratio
Figure 50	The dependency of Ki_{app} on PC/CL ratio
Figure 51	The effect of NaCl on the extent of inhibntion of GDH by CL
Figure 52	The effect of NH_4Cl on the extent of inhibition of GDH activity by CL
Figure 53	The effect of Ca^{++} on the extent of inhibition of GDH activity by CL
Figure 54	The effect of pH on the extent of inhibition of GDH activity by CL
Figure 55	The effect of PS on thermal stability of GDH at 37°C
Figure 56	The effect of a cosonicate of PC-PE-CL (40%, 40% and 20% on thermal stability of glutamate dehydrogenase at 40°C
Figure 57	The effect of lysolecithin and SDS on the intrinsic fluorescence of GDH

Figure 58	The effect of CETAB and hexane sulphonate on the intrinsic fluorescence of GDH
Figure 59	The effect of phospholipids on the intrinsic fluorescence of GDH
Figure 60	The effect of phosphatidylserine on the intrinsic fluorescence of GDH at different enzyme concentrations
Figure 61	The dependency of the quenching of fluorescence intensity of GDH by CL on enzyme concentration
Figure 62	The effect of NH_4Cl on fluorescence quenching of GDH by CL
Figure 63	The effect of pH on quenching of intrinsic fluorescence of GDH by CL
Figure 64	The effect of ribonuclease on GDH fluorescence quenching by PS
Figure 65	The dependency of GDH fluorescence quenching by phospholipids on temperature
Figure 66 & 67	The effect of PS on NADH fluorescence when partially or fully bound to GDH
Figure 68	The effect of SDS on NADH fluorescence in the presence of GDH
Figure 69 & 70	Further enhancement of ANS flourescence (in the presence of GDH) by PS and CL
Figure 71 & 72	GDH fluorescence quenching by ANS - double reciprocal plot of percentage fluorescence quenching against ANS concentration
Figure 73	GDH fluorescence quenching by ANS
Figure 74 - 78	The stopped-flow experiments - turbidity changes of GDH polymers at 310 nm on interaction with phospholipids
Figure 79 & 80	Electron micrographs of intact bovine liver mitochondrial preparation
Figure 81 & 82	Electron micrographs of bovine liver mitochondria stripped of outer membranes
Figure 83 & 84	The effect of ammonium chloride on binding of GDH to the inner mitochondrial membrane
Figure 85	The effect of pH on binding of GDH to the inner mitochondrial membrane
Figure 86	The extent of GDH penetration into a phospholipid bilayer
Figure 87	Two different types of arrangement of a GDH oligomer on a phospholipid membrane

Table 1	Effect of α-Kg and l-glu on GDH fluorescence quenching by PS
Table 2	Effect of l-glu and NAD^+ on GDH fluorescence quenching byPS
Table 3	Effect of ADP on further enhancement of ANS fluorescence partially bound to GDH by phosphatidylserine.
Table 4	Effect of GTP and NADH on further enhancement of ANS fluorescence partially bound to GDH

Table 5 Effect of NAD^+ ($\pm$ α-Kg) on further enhancement of ANS fluorescence partially bound to GDH

Table 6 Extraction of GDH into isooctane

Table 7 Effect of phospholipase C on mitochondrial membranes - specific activities of mitochondrial enzymes

Table 8 Effect of digitonin on mitochondrial membranes - specific activities of mitochondrial enzymes

Table 9 & 10 Effect of digitonin on mitochondrial membranes - specific activities of mitochondrial enzymes

Table 11 Binding of GDH to mitochondrial membranes - primary data

Table 12 Binding of GDH to mitochondrial membranes - secondary data

Table 13 Effect of NH_4Cl on binding of endogenous GDH to the inner mitochondrial membrane

Table 14 Effect of NH_4Cl on binding of endogenous GDH to the inner mitochondrial membrane

Table 15 Effect of NH_4Cl on binding of endogenous GDH to the inner mitochondrial membrane

Table 16 Effect of pH on binding of endogenous GDH to the inner mitochondrial membrane

Table 17 Effect of metabolites on binding of endogenous GDH to the inner mitochondrial membrane

Table 18 Effect of ADP and GTP on binding of endogenous GDH to the inner mitochondrial membrane

Table 19 Effect of metabolites on binding of endogenous GDH to the inner mitochondrial membrane

Table 20 Effect of metabolites on binding of endogenous GDH to the inner mitochondrial membrane

Chapter I

INTRODUCTION

The interaction between lipids and proteins is of fundamental importance in Biology. The way in which lipids and proteins interact (a) in cell membrane; (b) in various enzyme reactions, e.g. with β. hydroxybutyrate dehydrogenase; (c) in blood coagulation processes; (d) in various lipase reactions; and (e) in the structure of the serum lipoproteins, are some of the many important areas under active consideration and research at the present time.

The Danielli-Davson bimolecular leaflet model for the structure of cell membranes (Danielli and Davson 1934, Davson and Danielli 1943) suggest that lipids provide a permeability barrier while the membrane proteins possess the essential responsibility for the various enzymic, transport, regulatory and ligand recognition properties known to reside in cell membranes.

Recent developments have begun to emphasise the potential role of lipids particularly phospholipids, in these latter processes and have contributed to the interpretations of the relative roles of lipids as structural and functional determinants of cell membranes.

The mitochondrion contains some 25% by weight of lipids (Fleischer et al, 1961). Approximately 95% of the lipid is in the form of phospholipids; the rest may be classified as neutral lipids. Among the components of the latter are coenzyme,Q, α- tocopherol, cholesterol, carotenoids and neutral fats (Brasford, 1959). In the case of the inner mitochondrial membranes the major lipids are phosphatidycholine, (lecithin) $\simeq$ 40%, phosphatidylethanolamine $\simeq$ 40% and cardiolipin $\simeq$ 20% (Colbeau et al, 1971).

Regarding the proportion of lipid to protein, it is found that membranes which behave mainly as barriers like the myelin sheath and erythrocyte

membranes contain relatively high proportions of lipid. By contrast, the inner mitochondrial membrane which is primarily a functional rather than a "barrier"type of membrane, contains more protein than lipid.

The phospholipids of beef heart mitochondria have the following characteristics (Green and Fleischer, 1964):

1) A high degree of unsaturation in the fatty acyl residues,

2) A high proportion of plasmalogen,

3) Fatty acyl residues with (predominantly) 16 or 18 carbon atoms.

In general, animal phospholipids contain mostly fatty acids with carbon chain lengths between 16 and 20: palmitic, stearic, oleic, linoleic and arachidonic acids predominate.

Lysophospholipids are reported to comprise a significant percentage of the total phospholipids of some subcellular organells, e.g. 5 - 6% lysolecithin in mitochondria and microsomes (Bergelson et al, 1970).

The phospholipids in mitochondria of animal and plant tissues generally show a high degree of unsaturation (Richardson et al, 1962) whereas the phospholipids of micro-organisms appear to be characterised by mono-unsaturated fatty acid residues. The degree of unsaturation of fatty acids has a profound influence on the physical properties of phospholipids.

In some cell membranes, such as occur in bacteria, acyl groups containing branched chains are present. The reason for this distribution of fatty acid residues associated with a given phospholipid is not fully understood; nor is it clear why, corresponding to occurrence of certain diseases or effects

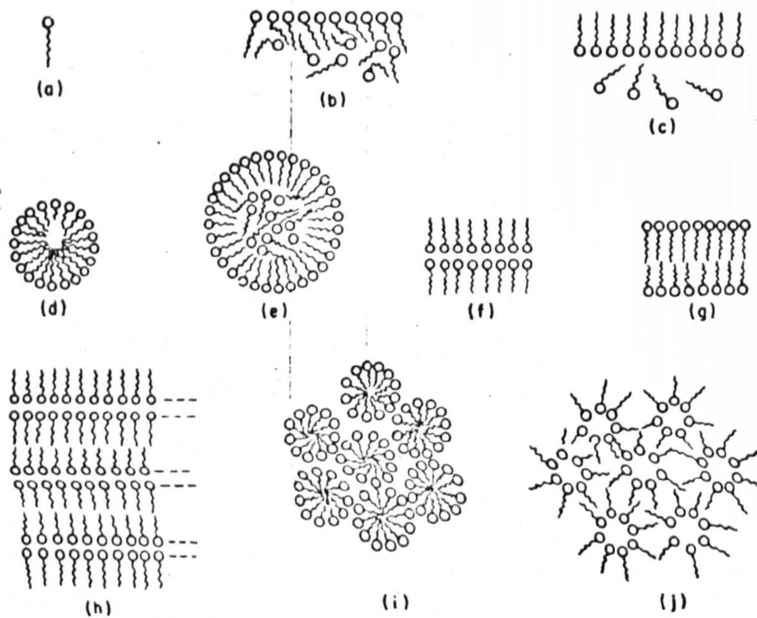


Fig. 1 Various aggregated forms of amphipaths: (a) lipid molecule; (b) bulk lipid; (c) lipid solution in water; (d) micelle; (f) lipid bilayer at air interfaces; (g) lipid bilayer at water interfaces; (h) myelinic; (i) hexagonal I, (j) hexagonal II phases of phospholipid dispersion.

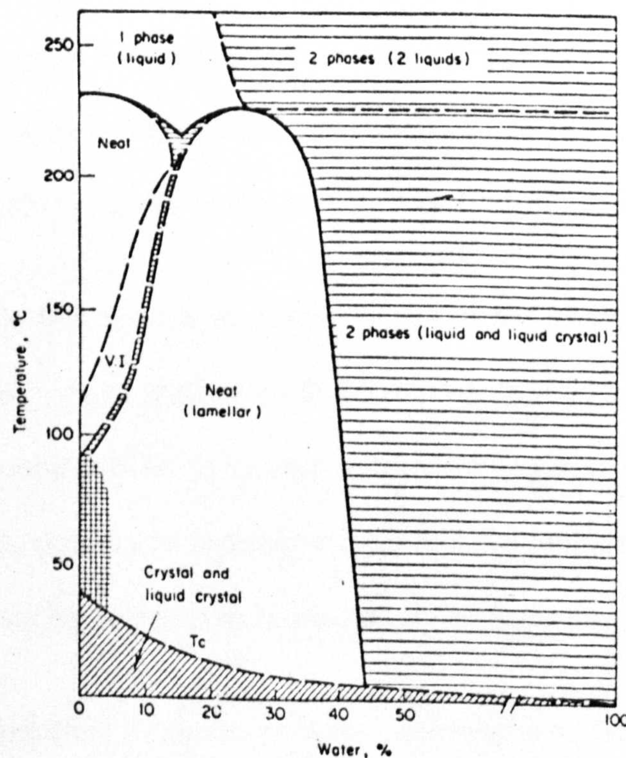


Fig. 2 The binary phase diagram of egg phosphatidylcholine and water as a function of temperature.

due to variation of temperature, these characteristic fatty acid patterns sometimes alter.

Highly developed polymorphism is a common feature of lipid-water system. A small variation in lipid composition, temperature, and other external conditions may induce dramatic changes in the properties of various forms of aggregates (phases) at equilibrium.

On increasing the temperature of lipid-water mixtures, the hydrocarbon chains melt over a narrow temperature range (Kraft temperature) allowing the water to enter the crystalline lattice and disperse the lipid. Various forms of amphiphiles are shown in Figure 1.

As regards the phospholipids of biological membranes, the most important arrangement is the liquid-crystalline phase. This phase shows the high degree of order usually associated with crystals but a mobility like that of a liquid. It is obtained when phospholipids are brought in contact with water at a temperature close to or above the melting point for the acyl chains. This transition temperature (T_c) is characteristic for each phospholipid species, and is defined mostly by the configuration of the acyl chain, but also by the degree of hydration of the head groups (Chapman et al, 1967). Thus, the presence of cis-double bonds, branching and decreasing chain length tend to reduce the T_c. This phenomenon is in accord with the diminishing Van der Walls interactions obtained under these conditions (Salem, 1962). For example, dipalmitoyl(C16) PC has a T_c of 41 while egg PC shows this thermal transition at -15/-7. The difference being, presumably, due to the presence of double bonds in egg phosphatidylcholine. Phospholipid membranes composed of mixed fatty acyl

chains, or containing more than one head group, exhibit very complex, but characteristic transitions. Differential scanning calorimetry (Chapman et al, 1969), X-ray diffraction studies (Luzzati, 1964, Engleman, 1970) N.M.R. and E.S.R. studies (Lee et al, 1972, Traube and Sackman, 1969) are some of the techniques which have been used to study the changes in molecular motion associated with the gel-to liquid crystalline phase transition. The binary phase diagram of egg phosphatidylcholine and water as a function of temperature is shown in Figure 2.

CONFORMATION OF HYDROCARBON CHAINS - LECITHIN - WATER SYSTEM

The phase behaviour of a homologous series of lecithin-dioctanoyl to distearoyl and a preparation from hen eggs have been studied (Tardieu et al, 1973). The hydrocarbon chains were found to adopt a variety of conformations which were characterised from their X-ray diagrams.

(a) α - conformation

α the liquid-like organisation was found to be the predominant conformation, common to most lipids in the presence of water and at sufficiently high temperatures and, the one more relevant to membranes. The X-ray diagrams of the α - conformation are characterised by a broad band around $(4.6A^{o})^{-1}$, very similar to a band observed with liquid paraffins (Luzzati et al, 1960).

It has been shown that all the phases with chains in the α - conformation display a peculiar temperature effect: the short dimension of the structure elements such as the thickness of the lipid in the lamellar phases, decrease as the temperature is raised, with an unusually large linear thermal

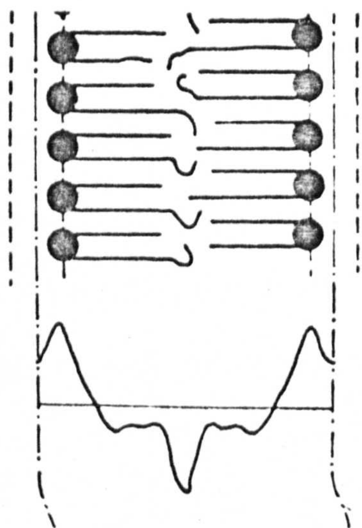


Fig. 3 Structure of the phase L β

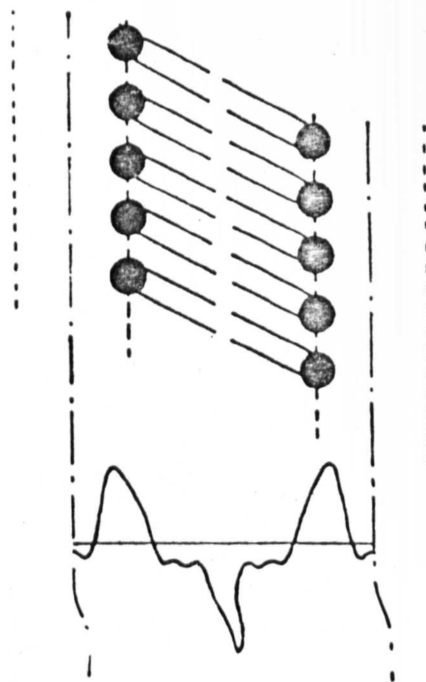


Fig. 4 Structure of the phase L β '

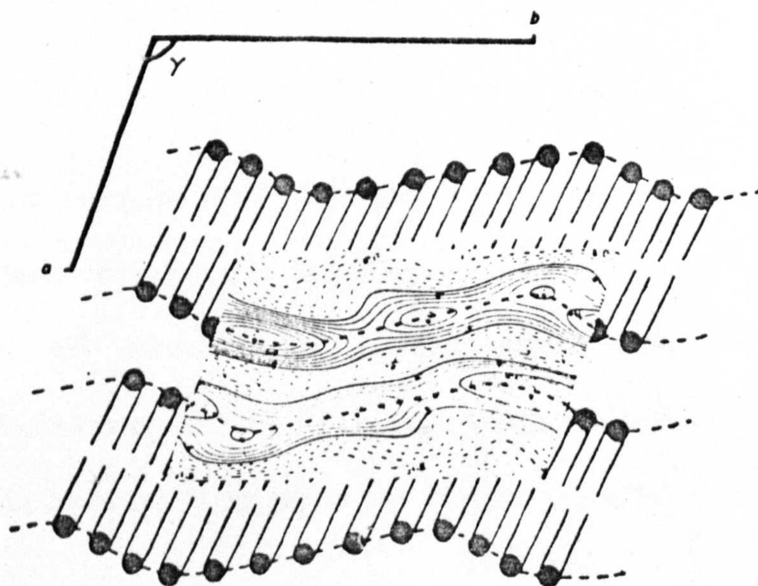


Fig. 5 Structure of the phase P β '

coefficient (of the order of $10^{-3}/^{o}C$). This phenomenon well known in rubber, is typical of a polymer with a highly disordered conformation.

Thus, the α- conformation can be visualised as highly disordered, like that of a liquid paraffin, still with the average of the chain orientation perpendicular to the lipid-water interface.

(b) β and β^1 conformations: phases $L\beta$, $L\beta^1$, $P\beta^1$

β is observed at lower temperatures in lipids whose chains are heterogeneous and in the presence of very small amounts of water. β^1 is found in synthetic leathers with identical chains, in the presence of variable amounts of water.

The β conformation has been observed in a variety of lipids including mitochondrial lipids (Gulik - Krzywicki et al, 1967). This conformation is characterised by a strong and sharp band at S_1 $(4.\ A^{o})^{-1}$, followed in some cases by faint and sharp reflections at $\sqrt{3}\ S_1$ and at $2S_1$; these reflections correspond to a two-dimensional lattice of cylindrical rods, and have been interpreted by the presence of fully extended chains organised with rotational disorder (Gulik - Krzymicki et al, 1967).

This type of conformation is observed in a lamellar phase, $L\beta$ which contains a very small amount of water (less than 10%). (See Figure 3).

The β^1 conformation is similar to the β conformation except that the chains are tilted with respect to the normal to the plane of the lamellae (Chapman et al, 1967), (Figure 4).

Another difference is that the amount of water incorporated in the phases

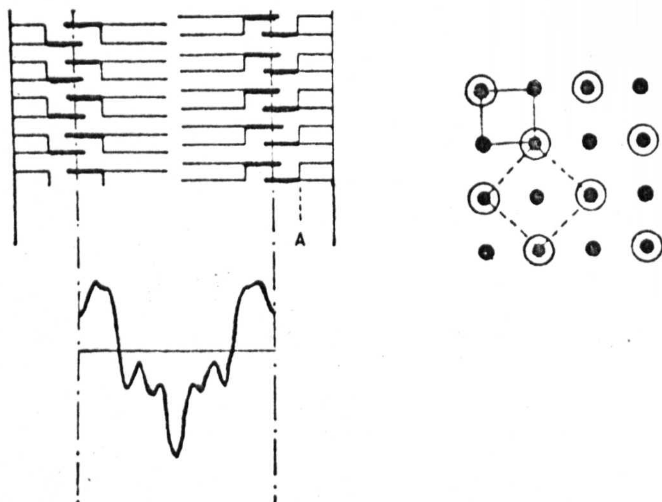


Fig. 6 Structure of the phase L δ

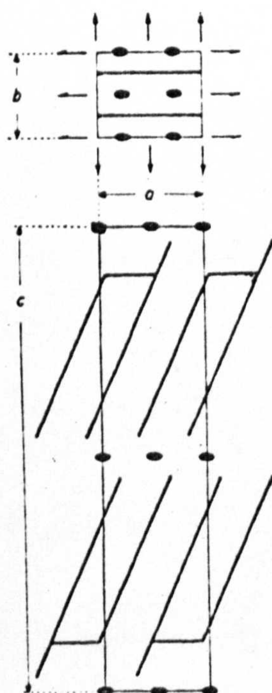


Fig. 7 Structure of the phase C of diC14-PC

of type β. In the X-ray diagrams, the β^1 conformation is characterised by a sharp reflection at $(4.2\ \mathring{A})^{-1}$ followed by a more diffuse reflection.

The phase $P\beta^1$ is similar to that of $L\beta^1$, namely that $P\beta^1$ contains slightly distorted lamellae of the β^1 type. (See Figure 5).

(c) δ conformation: phases $L\delta$ and $P\delta$

This conformation is characterised by its chains coiled into helices whose axes are perpendicular to the plane of the polar groups and are packed with rotational disorder in a two-dimensional square lattice. It is observed in dry lecithins and is characterised by two sharp reflections (Luzzati et al, 1968), one strong at $(4.8\ \mathring{A})^{-1}$ and one weak at $(3.4)^{-1}$. These reflections are ascribed to stiff rods organised with rotational disorder in a two-dimensional square lattice (Figure 6).

The lamellar phase $L\delta$ is observed at the very anhydrous end of the phase diagram, and over a smaller temperature range.

(d) Phase C, crystalline

This is a highly order crystalline phase, yet displaying rotational disorder of the chains. It is observed in all the lecithins anhydrous in di C18 - PC, slightly hydrated (2 to 4%) in the other compounds. The presence in all the X-ray diagrams of a few strong reflections near $(4.2\ \mathring{A})^{-1}$ suggest that the chains are fully extended as in the β and β^1 conformations (Figure 7).

In summary, the liquid-like nature of the α- conformation must be emphasised. This is the most widespread of all the conformations and probably the most relevant to biological membranes.

The other conformations are, at least partly ordered, namely the chain axes are organised according to two or three-dimensional lattices; the chains are fully elongated in the β and β^1 conformations as well as in phase C, and coiled into helices in the δ conformation. In the case of other lipids, similar studies would probably uncover other phases and other conformations of the chains.

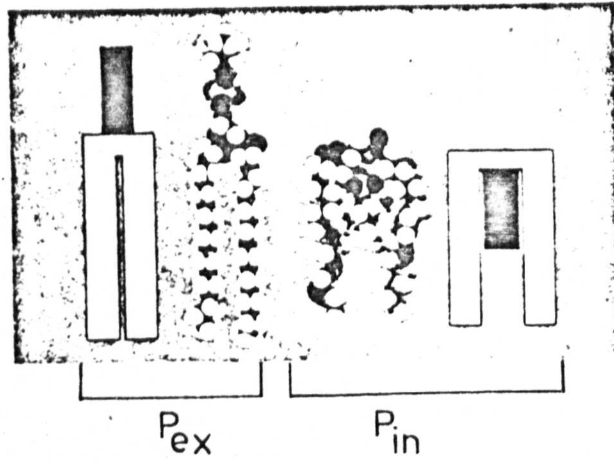


Fig. 8 Two different conformational states of phospholipids

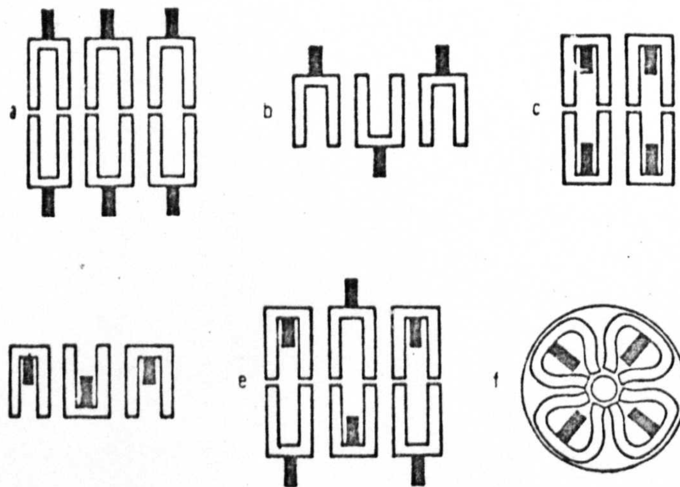


Fig. 9 a), b), c), d), e) conceivable laminar structures of lipids in the Pin- and Pex-conformation, f) cross section of a tubular lipid superstructure formed by four lipid molecules in the Pin-conformation

CONFORMATIONAL STATES OF PHOSPHOLIPIDS

Essentially two different conformational states of phospholipids are possible (Kreutz, 1972). In one case the polar group is located outside and in the other it is incorporated in the fatty acid domains (Figure 8). These are referred to as the Pex-conformation (polar group external conformation) and the Pin-conformation (polar group internal conformation).

These two conformational states permit the formm tion of lamellar and tubular-shaped structures as shown in (Figure 9). According to this model, lamellar structures can be formed both by lipids in the Pex-conformation and by lipids in the Pin-conformation, whereas the tubular structures which are always averaged in hexagonal lattices, can only be formed by lipids in the Pin-conformation.

According to this model, all saturated phospholipids occur at room temperature exclusively in the Pex-conformation and form lamellar structure. Accordingly, they reveal periods corresponding to twice the length of a fatty acid plus the polar groups in X-ray experiments, (Kreutz, 1970).

In his model, Kreutz (1972) claims that some phospholipids may be converted into the Pin-conformation by binding to specific ligands. Thus, according to Kreutz, the binding of Na^{+} and Ca^{++} stabilises the Pex-conformation in the case of phosphotidic acid while in the case of PS this conformation is totally converted into the Pin-conformation by the addition of alanine. The evidence for these latter findings do not seem to have been published by the author. It is interesting in this context, to refer to the results obtained by Guarnier et al (1971) who found only a few per cent of the

cardiolipin in intact mitochondria from rat liver and none of the cardiolipin in intact beef heart mitochondria is available for binding of anticardiolipin antibody. However, some recent reports (Schiefer, 1973 a, Schiefer, 1973 b) have shown that antisera against both cardiolipin and phosphatidylinositol combine specifically with the polar head groups of the respective phospholipids. The antibodies directed against the two phospholipids were detected in rabbits intravenously immunised with rat liver mitochondria, inner mitochondrial membranes and a membranous cytochrome oxidose preparation. From the results it was concluded that the hydrophilic groups of the phospholipids are, at least partially located at the mitochondrial membrane surface.

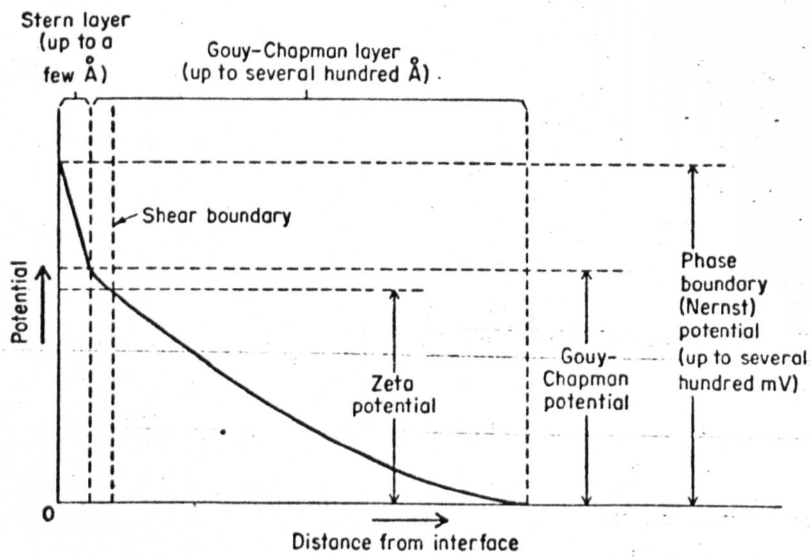


Fig. 10 A graphic representation of an electrical double layer.

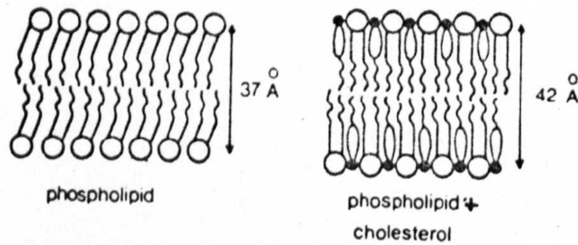


Fig. 11 The effect of cholesterol on the thickness of the phospholipid bilayer

PHYSICAL PROPERTIES OF PHOSPHOLIPIDS WHICH ARE IMPORTANT IN LIPID-PROTEIN INTERACTION

The electrostatic field at the interface between the phospholipid and the bulk aqueous phase is an important property which has to be considered. Phospholipid molecules orientate themselves so that their ionic head groups are directed towards the aqueous phase.

Because of the presence of fixed charges at a phospholipid surface, an electrical double layer is set up. This layer is composed of the innermost "stern" layer (the fixed layer) and the outer "Gouy-Chapman" layer (the diffuse layer) (Strigter, 1967). The potential at the plane of shear between these two layers is called ζ potential. The potential falls as a point is moved through the diffuse double layer to a position in the bulk phase far away from the surface. These are shown in Figure 10

On one side of this double layer, there will be charged groups derived from the material of the surface or adsorbed from the medium. This charge is balanced across the thickness of the double layer by charges of opposite sign derived from the surrounding medium. The thickness of the double layer is given approximately by:

$$\frac{I}{S} \quad \text{where } S = \frac{4\pi e^2 \; \Sigma n z^2}{\epsilon KT}$$

n is the ionic concentration of counter ions of valency z, e the electronic charge, ϵ the static dielectric constant, K Boltzmann's constant and T the absolute temperature.

Thus, the valency and concentration of counter ions and the static

dielectric constant of the surrounding medium will affect the thickness of the double layer.

Inclusion of materials such as proteins within the lipid layer of the surface or alteration in the lipid composition will alter the internal fields within the surface by altering the distribution of charges. The most probable structure will be that with the lowest free energy and electrostatic intra-molecular interactions will play a considerable role in determining this. The X-ray data of Levine and Wilkins (1971) with egg lecithin-cholesterol multi-bilayers have shown the effect of cholesterol in increasing the thickness of the bilayer from 37 A^{o} to 42 A^{o} (Figure 1) and also indicated a sharp localisation of a low electron density region in the middle of the membrane.

The pH near the charged phospholipid interface will be different from that in the bulk phase. Assuming a Boltzmann's distribution, the relationship is described by:

$$pH_s = pH_b + \frac{e\psi}{2.3\,kT}$$

where ψ is the surface potential, e is the charge, k is Boltzmann's constant and T is the absolute temperature.

Such effects will produce shifts in the apparent pKa values of functional groups. Thus, at a lipid-protein interface, the polar head groups of the lipids could produce substantial modifications in the apparent pKas of protein functional groups.

The adsorption of counter ions into the fixed part of the double layer has an enormous influence on the ζ potential and this effect increases greatly

with an increase in valency of the ion . If sufficient divalent or polyvalent counter ion are added to the bulk phase, a charge reversal occurs and the sign of the ζ potential changes.

Binding of calcium to phospholipid monolayers has been studied (Hauser and Dawson, 1967, Hauser et al, 1969). When $^{45}Ca^{++}$ is introduced into the subphase below monolayers of acidic phospholipids (e.g. phosphatidylserine), the surface radioactivity (calcium bound) initially increases with increasing concentrations of calcium added. At low concentrations, the relationship is not linear but initially follows a Langmuir-type adsorption isotherm: surface radioactivity = $k_1C/k_2 + C$, where C is the concentration and k_1 and k_2 are constants.

The adsorption data can be analysed in terms of the apparent association constant ka for the reaction

$$Ca^{++} + \text{Lipid} \rightleftharpoons \text{Ca surface}$$

with k_a being calculated from the mass equation:

$$K_a = \frac{(Ca)_s}{(Ca)_f \; (L)_f}$$

where $(Ca)_s$ is the Ca absorbed on the surface, $(Ca)_f$ the Ca in subphase, and $(L)_f$ the concentration of free binding sites on phospholipid surface.

It has been shown that (Papahadjopoulos and Bangham, 1966) at certain concentrations of calcium (eg. 1.0 M) there is a dramatic increase in the permeability of phosphatidylserine bilayers to univalent cations, and this implies that the calcium is causing profound physical changes in the membrane.

Behaviour of phospholipases

Reactions of phospholipases at the phospholipid-water interface have been studied by several workers. In such reactions, it must be remembered that the enzyme has approached the phospholipid-water interface where it forms the enzyme-substrate complex. Thus, interaction between the enzymes and their lipid substrates occurs in the primary sequence of events. These phospholipase systems clearly indicate the critical importance of the electrostatic field at the interface between the phospholipid and the bulk aqueous phase.

Phospholipase B catalyses the hydrolysis of lecithin to two fatty acids and glycerylphosphoryl choline. The hydrolysis only takes place when sufficient (long chain) anions are introduced to give the lipid-water interface a certain critical negative ζ potential (Dawson and Hauser, 1967, Bangham and Dawson, 1962). Conversely, phospholipase C (catalyses the hydrolysis of lecithin into digly ceride and phosphatidylcholine) is only active when the lecithin is given a positive ζ potential by mixing it with a long chain anion or on introducing metallic cations into the bulk phase (Basford, 1959).

EVIDENCE FOR THE PRESENCE OF BILAYER STRUCTURE IN NATURAL AND ARTIFICIAL MEMBRANES

Various workers have shown the presence of bilayer structure in multiple membrane structures such as myelin and retinal rods (Finean, 1969, Worthington and Blaurock, 1969). An account has been given for the X-ray intensities in terms of an electron density distribution within the basic repeat unit of these two membrane systems. Wilkins and associates (1971) have concluded that an X-ray bilayer pattern apparently exists in all of the membranes examined.

In general, X-ray diffraction studies of phospholipid-water mesophases have indicated that the predominant liquid crystalline phase is that of a lamellar arrangement (Luzzati, 1968).

The inclusion of cholesterol and the addition of salts also tend to stabilise lamellar bilayer structures. Only in the presence of additives that are more hydrophilic such as deoxycholate or lysolecithin is there any evidence for the possible existence of alternative mesophases in natural membranes (Husson and Luzzati, 1963, Bangham and Horne, 1964).

Additional support for the presence of bilayer structure comes from E.S.R. studies in artificial membranes (Butler at al, 1970), in nerve fibre and erythrocyte membranes (Hubbell and McConnell, 1969) and in Neurospora mitochondria (Dodge et al, 1963).

Forces affecting stability of the bilayer

The phospholipid bilayer structure illustrates the combined effects of

hydrophobic and hydrophilic interactions. The stability of lipid bilayer is thus affected by:

a) Electrostatic repulsion between the charged interfaces on either side of the membrane. This tends to prevent collapse of the membrane and ultimately determines the point at which any thinning process will stop.

b) Hydrophobic interaction between fatty acid chains of phospholipids. These are sequestered together away from contact with water, thereby maximising hydrophobic interactions.

c) Lateral dipole-dipole interactions between ion pairs at the surface of the bilayer. This interaction is especially significant in the case of zwitter-ionic lipids such as lecithin.

d) If there is an excess of charges of one sign, the instability may set in along the lines outlined above.

e) Lateral cohesion between membrane components due to London-Van der Waals forces.

Thus, it appears that in a membrane composed solely of lipids, the conditions most favouring a bilayer structure are (i) relatively small excess charge on the interfaces of the membrane (ii) a relatively large percentage of phosphatidylcholines which should have hydrocarbon chains of approximately equal lengths and full saturation. These conditions ensure that the lateral forces of cohesion are large and that dispersion forces will thin the membrane down to a bilayer.

The nature of these forces will be more fully discussed later in this chapter.

FORCES INVOLVED IN LIPID-PROTEIN INTERACTION

Some of the possible ways in which lipids and proteins can interact are by means of the following types of binding forces.

a) Covalent binding

Generally, lipids can be readily separated from proteins by simple solvent action so that covalent binding is not usually an important role in their interaction.

However, evidence has been presented recently that some intrinsic membrane proteins including the Folch-Lees proteolipid of myelin (Stoffyn and Folch-Pli, 1971) and a proteolipid from sarcoplasmic reticulum (MacLennon, 1972) have a considerable amount of fatty acid which is covalently bound to some of the polar residues of these molecules, thereby reducing the net charge on the protein and at the same time increasing the hydrophobic character of the molecule.

b) Electrostatic interaction

These forces are due to the mutual columbic attraction or repulsion of the net charges or electronic moments, carried by two interacting molecules. Salem (1962) has discussed the interaction of the negative phosphate ion of a phosphatidic acid molecule with a positively charged quaternary ammonium group of a lysine side chain.

The interaction between two charges obey the familiar Coulomb I/D Law; charge - dipole interactions vary as I/D^2, dipole-dipole interactions as I/D^3, etc. The interaction energy between two ions of net charge q and q^1 is given by the relation:

$$w = \frac{q q^1}{\epsilon D}$$

where ϵ is some effective value for the dielectric constant of the medium. When the two charges are very close to each other, they are no longer separated, and are only surrounded by an assembly of molecules belonging to the medium. Hence the effective value ϵ , or the "microscopic" dielectric constant, is much smaller than the familiar macroscopic value.

Pressman and associates (1946) obtained the relation:

$$\epsilon = 6D - 11$$

for the effective dielectric constant of water in the case of two ions separated by a distance of less than 10 argostroms; the expression is valid for $D \geq 5A^{o}$. This is, of course, an approximate estimation of the effective dielectric constant.

Every protein tested with an isoelectric point above pH9 has been found capable of forming complexes with anionic phospholipids (Green and Fleischer, 1964). However, when cytochrome c is acetylated or succinylated and the basic groups thus eliminated, the capacity to form complexes with phospholipids is lost.

The interaction of cytochrome c with phospholipid involves an additional parameter. At pH8 cytochrome c has 20 positive charges and 12 negative charges, giving a net positive charge of 8. The complex formed between an acidic phospholipid and cytochrome c contains exactly 8 molecules of phospholipid per molecule of protein. It must be presumed that 12 of the 20 positive

charges are part of a zwitterionic neutralisation (plus and minus charges juxtaposed) and only 8 positive charges are available for interaction with acidic phospholipids.

The interaction of cytochrome c or other basic proteins with phospholipid is suppressed when the ionic strength of the medium is increased to a sufficient degree. Also a pre-formed complex is dissociated completely when the ionic strength is raised to the same critical value at which interactions are involved in the fomation of complexes between acidic phospholipids and basic proteins. This interaction probably involves gross distortion of the flexible lipid particles to permit the exact neutralisation of charges by the two combining partners. Similar conclusions were made in studies on permeability variations of phospholipid membranes or interaction with basic proteins (Kimelberg H. K., Papahadjopoulos, D., 1971).

c) Polarisation interactions

These induction forces arise from the polarisation of one molecule by the charges or permanent electric moments of the other. A typical example of this type of interaction is that which could occur between the phosphate ion or the hydroxyl group of a cholesterol molecule with the CH_2 groups of a protein side-chain.

The polarisation energy varies as I/D^4 for a charge polarisation group interaction. The polarisation energy is given by:

$$W = - \frac{q^2 \alpha}{2\varepsilon^2 D^4}$$

where a charge q is interacting with a spherical group of isotropic

polarisability α. The energy involved in polarisation interactions is generally very small.

d) London-Van der Waals dispersion forces.

These forces arise between electrically neutral non-polar molecules and are due to the average interaction of an instantaneous electric moment on one molecule brought about by charge density fluctuations and the moment it induces on the other molecule. These forces will be particularly important in the interaction of non-polar groups. Thus, the CH_2 groups of protein side-chains will attract the CH_2 groups present in the phospholipids.

At distances which are large with respect to the size of the interaction systems, the dispersion energy varies as I/D^6. For two identical groups of polarisability α placed in vacuum, London's Law gives:

$$W\ disp = - \frac{3\ \overline{\gamma E}\ \alpha^2}{4D^6}$$

where $\overline{\gamma E}$ is the average electronic excitation energy which is often just equal to the ionisation potential of the interacting systems. For two CH_2 groups the energy variation is approximately

$$W = \frac{-1340}{D^6} \text{ Kilocalories per mole}$$

If the groups are $5A^o$ apart, there results an attraction energy of 0.1 Kilocalorie per mole. Although much smaller than electrostatic energies, such an energy may lead to large attractions if many similar interactions are involved simultaneously.

Salem (1962) has considered the attraction forces between parallel lipid chains and assumed that the dispersion energy is locally additive.

On this basis he calculates the total attraction energy between two parallel linear chains to be

$$W = A \frac{3 \pi L}{8 l^2 D^5}$$

where each chain is built out of units of length l, and for DL < L. He showed that in such cases the total energy of interaction can reach as much as 10 or 20 kilocalories per mole.

These dispersion forces are of great importance in determining the Kraft temperature for lipids - the temperature at which a polar lipid, in the presence of water, transforms from a crystalline to liquid crystalline condition (Chapman et al, 1967).

e) Hydrophobic interaction

When amphiphilic molecules are in an aqueous environment, hydrophobic interaction is a significant interaction. Nearly all proteins contain a relatively high proportion of amino acids with non-polar side-chains. The tendency of non-polar groups to associate in aqueous solutions, thereby reducing the extent of contact with neighbouring water molecules is referred to as hydrophobic "bonding". The formation of hydrophobic bonds is favoured only because of an entropy effect: the water molecules become more ordered around exposed non-polar solutes. When the hydrophobic bond is formed the order decreases, resulting in a favourable entropy and hence free energy of formation. The free energy change associated with this process is estimated to be about 3 Kal to 5 Kal per non-polar amino acid chain, or 1 Kal per CH_2 group.

The configuration adopted in water by phospholipids is also such that the molecules have their polar groups facing the aqueous environment and the hydrocarbon groups clustered together. Hydrophobic interaction is also considered to take place in this situation as well.

The addition of electrolytes to the aqueous medium is considered to strengthen hydrophobic bonds, whereas they weaken electrostatic linkages. Non-polar substances, on the other hand, weaken hydrophobic bonds, but strengthen electrostatic linkages.

Green and Fleischer (1963) concluded that hydrophobic binding has an important role in mitochondrial membrane structure and function. They studied the interactions of mitochondrial "structural protein", electron transport enzymes and phospholipids with each other and with detergents and bile salts.

Using nuclear magnetic resonance, Stein et al (1968) studied lysolecithin binding by serum albumin. They found that when lysolecithin was bound, the peaks attributed to fatty acid methylene and the terminal methyl groups were markedly broadened and reduced in height. They interpreted this to indicate that binding was by hydrophobic association between the fatty acid chains and non-polar regions of the proteins. The peak attributed to the quaternary amino group of the choline remains relatively unaffected, indicating that the polar portion of the lysolecithin was not detectably involved in the binding.

Klopfenstein (1969) studied the thermodynamics of binding lysolecithin to serum albumin. The lipid-protein binding in this case was shown to involve

a free energy change of -6.2 Kcal/mole and an enthalpy change of -18 Kcal/mole.

If the binding were hydrophobic as described by Kauzmann (1959), the reaction should not show the highly negative entropy charge observed. Klopfenstein (1969) from his thermodynamics results thus argues that since the binding is known to be apolar from the nuclear magnetic resonance measurements, but seems not to be hydrophobic, perhaps London Van der Waals forces are responsible for the association between lysolecithin and bovine serum albumin.

The demonstration of the free energy charge of -6.2 Kcal/mole, enthalpy change of -11.8 Kcal/mole for binding one molecule of lysolecithin per mole of bovine serum albumin (Klopfenstein, 1969) shows the enthalpy change to be the driving force for the reaction.

f) Other types of binding

In addition to the processes discussed above, there are other factors which may be involved. These include hydrogen binding, barriers to internal rotation, thermal motion, presence of inorganic ions, etc.

It has been suggested (McClare, 1967) that the binding of lipid to protein in the envelopes of Halobacterium Halobium may take place by a bridging divalent metal ion.

Kimelberg and Papahadjopoulos (1971) studied the interaction of a number of positively charged proteins (including cytochrome c) with negatively charged phosphatidylserine vesicles and correlated protein binding with its

effects on $^{22}Na^{+}$ permeability. From the data obtained in such studies they conluded that an initial electrostatic interaction takes place which is followed, to varying degrees, by other interactions, leading to changes in permeability.

The interaction between sodium dodecyl sulphate and ferricytochrome c has been studied by Burkhard and Stolzenberg (1972). They took their spectroscopic results to suggest that the interaction occurs in two phases. The first of these involves an electrostatic binding of the dodecyl sulphate anions to the cationic sites of the protein followed by binding of the hydrophobic portions of the dodecyl sulphate to the hydrophobic amino acid residues of the protein.

SOME PROPERTIES OF MEMBRANE PROTEINS

Membrane systems contain two broad categories of proteins, which differ in their position with respect to the lipid bilayer and therefore in their mode of interaction with the lipid.

Intrinsic or integral membrane proteins (Singer and Nicholson, 1972) penetrate into and sometimes completely through the interior of the bilayer and have, therefore, predominantly hydrophobic interaction with lipids.

Several examples of intrinsic proteins have been clearly defined. Evidence has been obtained from electron microscopy, and X-ray diffractionn studies, that rhodopsin penetrates deeply into the lipid bilayer in the rod outer segment (Vanderkooi and Sundaralingam, 1970). Electron microscopic evidence has been interpreted to indicate that cytochrome oxidase penetrates completely through the bilayer (Vanderkooi et al, 1972), and the observation that the major glycoprotein of the human red cell membrane can be chemically labelled from both sides of the membrane supports the concept that this protein also spans the lipid bilayer (Bretscher,1971). These, and indeed all, intrinsic membrane proteins can only be liberated from their respective membranes by reagents which disrupt hydrophobic interactions. They are insoluble in aqueous solutions in the absence of detergent or other solubilizing agents, although in some cases polymerisation occurs which yields a water soluble aggregate. Further, all intrinsic membrane proteins have the ability to recombine with lipid to form membranes.

Extrinsic or peripheral membrane proteins (Singer and Nicholson, 1972) do not penetrate the lipid bilayer, but are held at the surface of the membrane

by predominantly electrostatic interactions. Examples of extrinsic proteins include cytochrome c of the mitochondrial inner membrane (Green and Machennan, 1969), spectrum of the red cell membrane and the basic protein of myelin, all of which can be removed from their respective membranes by reagents which disrupt electrostatic interactions. These and all extrinsic proteins are characteristically soluble in aqueous solution, once liberated from the membrane.

AMINO ACID COMPOSITION AND DISTRIBUTION OF MEMBRANE PROTEINS

Intrinsic proteins have the property of being partially buried in the hydrocarbon interior of the lipid bilayer and partially exposed to the surrounding aqueous medium (Figure 13). For thermodynamic reasons discussed by Singer and his associates (Singer and Nicholson, 1972, Glazer et al, 1970) these "bimodal" or "amphipathic" proteins must have an asymmetric distribution of polar and non-polar groups about their surface, such that charged groups are exposed to the aqueous phase and not to the hydrophobic interior of the bilayer. In fact, it has been demonstrated (Hatch and Bruce, 1968) that the amino acid composition of the extrinsic and intrinsic proteins differ significantly. The soluble proteins contain more charged and hydrophilic amino acids than do the membrane intrinsic proteins. The high degree of hydrophobicity of the constituent amino acids of membrane proteins are essential for their adaptation to the apolar environment of membranes.

It has also been shown that both cytochrome b5 (Spatz and Strittmatter, 1971) and cytochrome b5 reductase (Strittmatter et al, 1972) which are considerably more polar than the majcrity of intrinsic proteins, have in fact an asymmetric distribution of polar and non-polar groups along their polypeptide chain. Thus, a very hydrophobic region is present which can penetrate the hydrophobic interior of the lipid bilayer. An asymmetric distribution of polar and non-polar amino acid residues has also been identified in the polypeptide chain of the major glycoprotein of the human red cell membrane (Segrest et al, 1972). In the case of cytochrome c, it has been shown that the lysine residues as well as the hydrophobic amino acids tend to occur in distinct clusters along the protein chain (Margoliash, 1962).

CONFORMATION OF MEMBRANE PROTEINS

The proteins of a variety of intact membranes, on the average, show appreciable amounts of α - helical conformation as shown by Wallach and Zahler (1966) and Lenard and Singer (1966). For example, circular dichroism measurements of aqueous suspensions of intact and mechanically fragmented erythrocyte membranes reveal that about 40% of the protein is in the right-handed α - helix conformation (Glaser and Singer, 1971). Most soluble globular proteins whose circular dichroism spectra have been obtained exhibit a smaller fraction of α - helix in their native structures. This suggests that integral proteins in intact membranes are largely globular in shape rather than spread out as monolayers.

Some studies indicate in some cases the near absence of β - structure (Maddy and Malcolm, 1965) while others show that as much as 40% of the proteins in the plasma membrane are stabilized by β - structure (Choules and Bjorklaund, 1970).

MOTIONAL FREEDOM OF MEMBRANE LIPIDS

When the liquid crystalline behaviour of some substances was discovered, it was pointed out that these phases must play an important role in living organisms, since they could exchange components while maintaining both plasticity and a high degree of order. It is known that the cholesterol of the erythrocyte membranes exchange with that of plasma lipoproteins (Bruckdorfer, K. R., Graham, J. M., 1968).

N.M.R. and E.S.R. evidence for the association of the fluidity of the bilayer with the distance from the interface has been discussed above.

Kornberg and McConnell (1971) have estimated that phospholipid molecules move very rapidly within the plane of the membrane (molecular frequency of the translational step for lateral diffusion $> 10^3$ sec^{-1} at 0^oC), while the exchange of molecules between the two sides of the bilayer (phospholipid "flip-flop") is very slow ($\leq 2 \times 10^{-4}$ sec^{-1} at 40^oC).

In another study, Kornberg and McConnell (1971), using a spin-labelled derivative of phosphatidylcholine incorporated into liposomes, have reported that the labelled molecules present in the inner monolayer were more immobilised than those in the outer monolayer.

MOVEMENT OF MEMBRANE PROTEINS

Frye and Edidin (1970) investigated the membrane properties of some cell fusion heterokaryons and have presented some experimental evidence which suggests the fluidity of membrane proteins. Human and mouse cells in culture were induced to fuse with one another, with Sendai virus as the fusing agent. The distribution of human and mouse antigenic components of the fused cell membranes was then determined by immunofluorescence, with the use of rabbit antibodies directed to the whole human cell, mouse antibodies directed against a specific antigen on the mouse cell membranes, and, as indirect stains, goat antiserum to rabbit γ-globulin and goat antiserum to mouse γ-globulin labelled with two different fluorescent dyes. Shortly after cell fusion, the mouse and human antigenic components were largely segregated in different halves of the fused cell membranes; but after about 40 minutes at $37^{o}C$ the components were essentially completely intermixed.

Frye and Edidin (1970) suggest that the intermixing of membrane components is due to diffusion of these components within the membrane, rather than to their removal and re-insertion. Thus, the cell surface of heterokaryons is not a rigid structure and is fluid enough to allow free diffusion of surface antigens.

Additional evidence of protein movement comes from the low-angle X-ray scattering measurements of the arrangement of rhodopsin molecules in frog retinal receptor disc membranes. Blasie and Worthington (1969) showed that the nearest neighbour frequency of the rhodopsin molecules was modified by changes in temperature as well as by the addition of antirhodopsin antibodies.

From these results, these authors concluded that the rhodopsin molecules are in a liquid environment.

The use of pyrenebutyric acid which, as developed by Knopp and Weber (1967), has a very long excited-state lifetime, could allow the determination of the tumbling rates of proteins in membranes. The motion of fluorescent probes bound to membrane proteins in the nanosecond range has been reported (Wahl et al, 1971).

FLUID STATE OF PHOSPHOLIPIDS

It has been shown that, under physiological conditions, the lipids of functional cell membranes with the exception of myelin are in a fluid, rather than a crystalline state. This evidence comes from a variety of sources, such as spin-labelling experiments (Hubbell and McConnell, 1968), X-ray diffraction studies (Engelman, 1970) and differential calorimetry (Melchoir et al, 1970). If a membrane consisted of proteins dispersed in a fluid lipid matrix, the membrane would in effect be a two-dimensional liquid-like solution of monomeric or aggregated integral proteins dissolved in the lipid bilayer. The mosaic structure would be a dynamic, rather than a static one. The integral proteins would be expected to undergo translational diffusion within the membrane, at rates determined in part by the effective (microscopic) viscosity of the lipid.

Recent ESR studies (Hubbell and McConnell, 1971) have shown that the fluidity of the bilayer varies with the distance from the interface, becoming more fluid toward the middle. Analysis of NMR spectra (Birdsall et al, 1971, Darke et al, 1972) have confirmed that the molecular motion within a fluid bilayer increases substantially along the length of the hydrocarbon chains and the picture that emerges from these studies with lecithin bilayers indicates a relatively rapid molecular motion of the choline groups, relatively hindered motion in the region of the glycerol backbone and the first few carbons into the interior, and an increasing mobile region extending into the middle of the bilayer.

The biological significance of these studies can be appreciated from

comparisons of the spectra of spin-labelled probes embedded in biological membranes, and the bilayer membranes composed of the extracted lipids. These studies indicate a considerable similarity in the motion and solubility of the probes in bilayers and membranes of nerve cells (Hubbell and McConnell, 1968) and mitochondria of neurospora (Keith et al, 1970). However, studies with erythrocyte (Hubbell and McConnell, 1969) and inner mitochondrial membranes (Hsia et al, 1972) have shown that the probes are slightly but significantly less mobile in the intact membranes compared to their motion in isolated lipid bilayers.

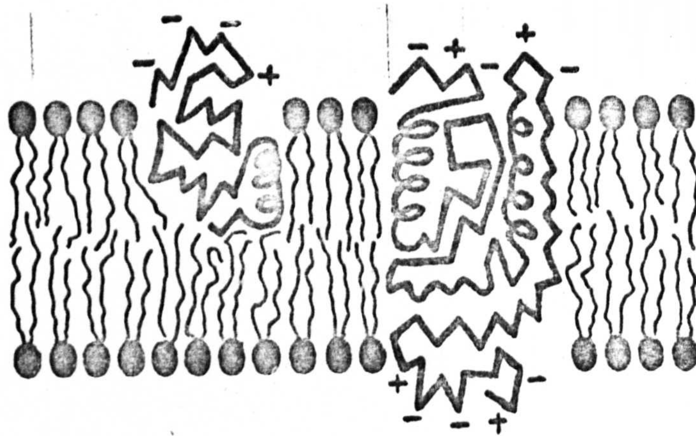


Fig. 12 A schematic representation of the cross section of the lipid-globular protein mosaic model of membrane structure.

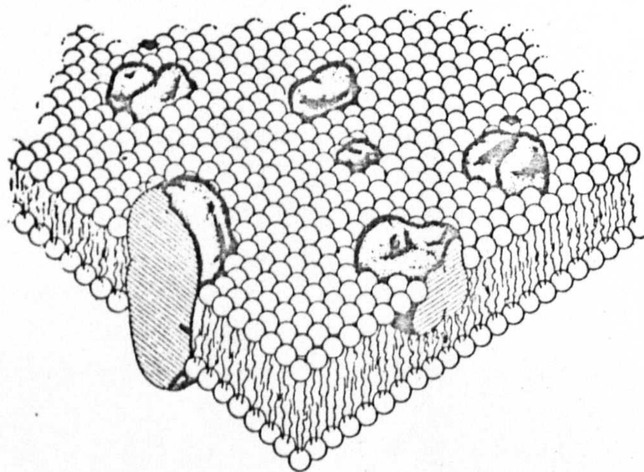


Fig. 13 A schematic representation of the three-dimensional organisation of the mosaic structure with the phospholipids forming the matrix of the membrane. The globular protein molecules are partially embedded in the membrane, and partially protrude from it, as in Fig. 12

THE FLUID MOSAIC MODEL

According to this model, (Wallach and Zahler, 1966, Singer and Nicholson, 1972), hydrophobic portions of integral proteins are immersed to varying degrees in the lipid matrix (Figures 12 and 13). An integral protein molecule with the appropriate size and structure may traverse the entire membrane.

The thermodynamic considerations and experimental results so far discussed fit in with the above model. It complies with the requirement of lowest free energy and maximum entropy allowing for optimum hydrophobic and electrostatic interaction.

Protein-protein interactions that are not explicitly considered in Figures 12 & 13 may be important in determining the properties of the membrane. Indeed, as discussed,above, some membranes such as the inner mitochondrial membrane have a high protein/lipid ratio in which protein-protein interaction may be the dominating factor.

Thus, the mosaic structure with a lipid matrix, which under physiological conditions is in a fluid rather than a crystalline state, would be a dynamic rather than a static one. The integral proteins would be expected to undergo translational diffusion. The rates of such diffusion would be, at least partly, determined by the local viscosity of the lipid environment. On the other hand, a mosaic membrane with a protein matrix should make for a relatively rigid structure with essentially no translational diffusion of its protein components within the membrane.

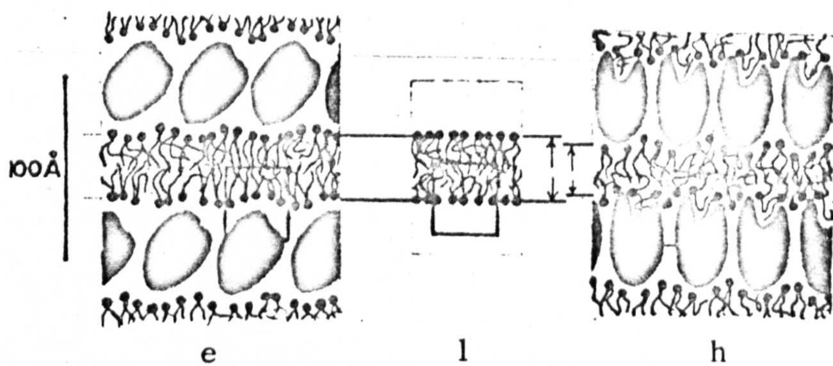


Fig. 14 Schematic representation of two lamellar phases formed between lysozyme and phospholipids. The thickness of the lipid leaflets is the same in phase e and in the lipid-water phase l, and it decreases in phase h

STRUCTURAL CHANGES IN PROTEINS AND LIPIDS BROUGHT ABOUT BY THEIR INTERACTION

Hammes and Schullery (1970) made the very interesting observation that polylysine changed from a random coil to an α- helix conformation upon interaction with phosphatidylserine vesicles and that the $-CH_2$ groups of the phospholipid fatty acyl chains were relatively immobilised, indicating penetration of the bilayer by the polypeptide molecules. Reduction in mobility is probably a result of tighter packing of the polar moieties in their crystalline array.

Gulik-Krzywicki et al (1969), using a combination of binding experiments and X-ray studies concluded that cytochrome c bound to the polar head-groups of aqueous dispersions of phospholipid membranes without perturbing the structure of the phospholipid bilayers. Lysozyme, however, appeared to show both polar and non-polar binding, with thinning of the bilayer membrane and a consequent increase in the area per lipid molecule.

Cardiolipin-lysozyme mixtures gave two lamellar phases (Shechter et al, 1971) for which the contributions of the components to the overall lattice thickness were computed. In one of the lamellar phases, the lipid layer thickness is the same as for the lamellar lipid-water phase above, whereas in the other case, this thickness is decreased. These are shown in Figure 14.

In essence, these studies (Gulik-Krzywicki et al, 1969, Shechter et al, 1971) suggest that for weak electrostatic interactions no decrease in the thickness of the lipid bilayer occurs, whereas when hydrophobic interactions occur, the observed shrinkage in the thickness results.

A number of proteins have been shown to increase the cation permeability of liposomes to values found for biological membranes. Thus, soluble basic proteins, such as lysozyme and cytochrome c have been shown to increase the $^{22}Na^{+}$ permeability of phosphatidylserine vesicles by several orders of magnitude, at neutral pH and low ionic strength (Kimelberg and Papahadjopoulos, 1971). These effects were later correlated with the ability of such proteins to penetrate or expand monolayers of the same phospholipid (kimelberg and Papahadjopoulos, 1971, b).

From their studies on the interaction of ferrocytochrome c with cardiolipin, Jori et al (1974) have come to the conclusion that the interaction provokes a perturbation of the protein conformation, which possibly involves the disruption of the hydrogen bonds linking the aromatic rings of tryptophan-59 and tyrosine-48 with one propionic side chain of the heme. Thus, the three-dimensional structure of cytochrome c bound to cardiolipin dispersion is different from that of the protein in aqueous solution.

EXPERIMENTAL MODELS FOR MEMBRANES

1) Monolayers

Most lipids, when placed in a water surface, disperse into a thin film. Under suitable conditions, such films can be dispersed to the thickness of a molecule.

In order to investigate the binding of proteins to lipid monolayers, the protein solution is injected into the subphase and the increase in film pressure, grossly referred to as film penetration, is measured as a function of time. From the analysis of some of the data reported (Colacicoo, 1970, Quinn and Dawson, 1970), it appears that the following successive interactions occur as gradual compression of the film: (a) Initially, whole molecules of protein penetrate the lipid film and occupy the same area as that of the protein spread at the air-water interface; (b) Above a given film pressure, a part of each protein molecule, probably the hydrophobic side chains, penetrate the film. The change in surface pressure per unit of bound protein is much smaller than in (a); (c) At higher film pressures adsorption without penetration occurs.

Compared with other models, monolayers are less satisfactory as structural models for biological membranes. A monolayer consists of only one layer of lipid molecules which has at the interface between water and air, or between oil and water, and it does not separate two aqueous phases. Hence, factors that affect the stability of the lipid film in relation to the arrangement of its constituent molecules are probably quite different in the monolayer from what they are in natural membranes.

2) Bilayers

A considerable amount of data has been accumulating since the finding

of Tsofina et al (1966) that protein can be bound to phospholipid bilayers (black lipid membrane, BLM) decreasing the electrical resistance of the lipid membrane to the biomembrane level.

The formation of BLM separating aqueous phases is conceptually simple: two compartments are separated by a thin partition and communicate through an aperture in this partition. The compartments are filled with an aqueous medium and a (thick) membrane of amphiphilic lipid is formed in the aperture separating the squeous phases. The thick membrane spontaneously thins by draining to form a ring or annulus of bulk phase lipid around the margin of the aperture. A large variety of purified and crude natural lipids, and a variety of synthetic amphiphiles have been used for the preparation of BLM. The composition of the membrane-forming lipid solution depends upon the nature of the lipid used.

Results obtained by Chevy et al (1971) illustrate the type of data elicited by the use of black membranes as model systems. Conductance and optical reflectance techniques were used to study the interaction of erythrocyte apoprotein with erythrocyte lipid bilayers. It was found that addition of the protein increases the conductance and reduces the stability of the bilayer.

3) Liposomes

This appears to be the most frequently used model system, presumably because liposomes are so easily prepared. Most of the work has been done with water-soluble non-membranous proteins, some of which, like lysozyme, polylysine, cytochrome c and ribonuclease are highly basic polypeptides. The conclusions drawn by Hammes and Schullery (1970) from the interaction of phospholipid liposomes with water-soluble polypeptides seems to be

representative of the outcome of the various other studies of this kind (Shipley et al, 1969, a, b, Kimelberg and Papahadjopoulos, 1971, Kimelberg et al, 1970, Sweet and Zull, 1970).

Their conclusions were: (1) electrostatic attraction is important for the initial interaction of the protein with the lipid; (2) the interaction is partially stabilised by hydrophobic bonding; (3) the interaction favours the formation of membrane-bound vesicles consisting of a single phospholipid bilayer coated with the polypeptide; (4) the helix content of the polypeptide tends to increase upon complexing with phospholipid as demonstrated by circular dichroism measurements; (5) the mobility of the fatty acid groups in the phospholipid bilayer is reduced.

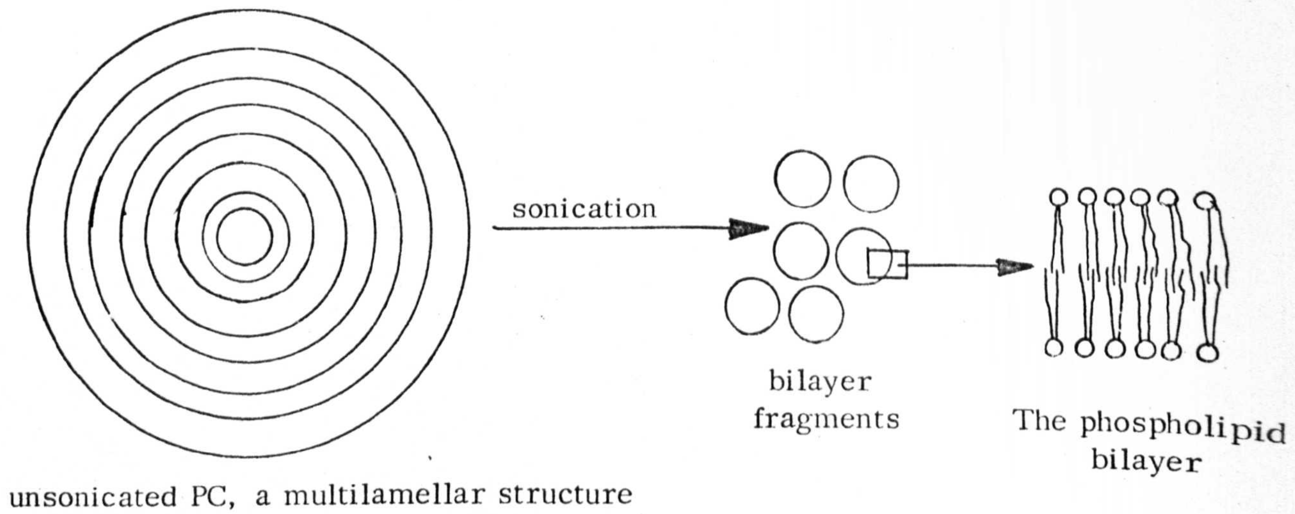


Fig. 15 Formation of small bilayer fragments from large phospholipid aggregates

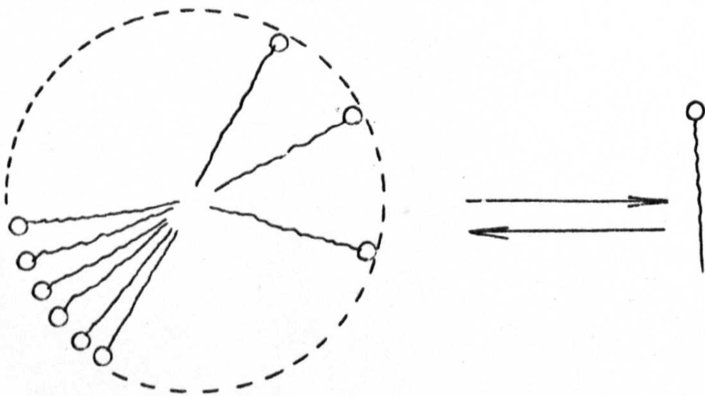


Fig. 16 Suggested structure of a cross-section of a spherical micelle in equilibrium with the monomer

SONICATION - PREPARATION OF UNILAMELLAR VESICLES

When dried preparations of naturally occurring phospholipids are allowed to come in contact with water, they swell and form large multilamellar particles.

In the process of sonication, lipid particles are given high kinetic energy by the sonicator and break down on colliding with each other. The collisions produce complete disruption of the sultilamellar particles into short-lived bilayer fragments which then reaggregate to form single-shelled bilayer vesicles of uniform size.

Unsonicated aqueous egg yolk lecithin dispersions contain particles of diameter from 0.5 to 20 μ consisting of concentrically arranged lamellae of lipid bilayers separated by layers of water. Such dispersions are generally found unsuitable for biochemical and biophysical studies. Instead, sonicated dispersions are used to increase the homogeneity and surface accessibility of the system (Figure 15).

The effect of ultrasonic irradiation on aqueous egg yolk lecithin has been studied intensively by several investigators (Papahadjopoulos and Miller, 1967, Hung, 1969, Johnson et al, 1971) and two basic effects have been reported. Firstly, prolonged exposure to sound can lead to significant chemical degradation, which is enhanced in the presence of oxygen. Secondly, sonication causes a reduction in particle size, with the formation of small vesicles. The particle size of these vesicles ranges between 190 and 300 A^{o} (average 230 A^{o}).

Other important factors related to the sonication of phospholipids are:

(a) The volume of the phospholipid dispersion.

(b) The size of the probe.

(c) Phospholipid concentration.

(d) pH.

(e) Presence or absence of organic solvents.

In a study of sonication of egg yolk lecithin (Patrick et al, 1972) the phospholipid particles were shown to consist of two fractions (as isolated by column chromatography), fraction I consisting of relatively large multi-lamellar structures (diameter 0.2 - 4 μ) and fraction II of smaller vesicles. (about 230 A^{o} in diameter). The sonication conditions can be adjusted to give only fraction II vesicles.

Phospholipids and lecithin in particular, may undergo two forms of degradation in an aqueous system (Klein, 1970). Hydrolysis may occur at the β - ester linkage with the production of lysophosphatides and free fatty acids, or the unsaturated hydrocarbon chains may become oxidised. The hydrolysis may be detected using thin layer chromatography on silica gel H plates (Skidmore and Entenman, 1962).

The methods available for determining the extent to which the sample has become oxidised could include the measurement of the peroxide value, the increase in absorption at 233 due to increasing diene conjugation (Klein, 1970). The change at this wavelength reflects the increase in conjugated diene hydroperoxides.

SOME LIPID - DEPENDENT ENZYME SYSTEMS

The molecular mechanisms that regulate the interaction of phospholipids with membrane-bound proteins are not clearly understood. That these interactions are important to the functional role of biological membranes have been made by a wealth of recent data, which demonstrates that the activity and stability of membrane enzymes may be altered by the action of phospholipases.

There is an absolute dependence on phospholipids, both for the process of membrane reconstitution and biochemical activity in the inner mitochondrial membrane (Triggle, 1970). One of the mitochondrial enzymes - β - hydroxybutyrate dehydrogenase has a specific and absolute requirement for lecithin (Green and Fleischer, 1963, Gazzotti et al, 1974). The higher the degree of unsaturation in the fatty acid residues, the more effective is the lecithin in restoring activity (Jurtshuck, 1968).

Another enzyme which has a specific requirement for a phospholipid is a galactosyl transferase present in the outer membrane of S. typhimurium. It catalyses the addition of galactose to a lipopolysaccharide and has a specific requirement for phosphatidylethanolamine (Romea et al, 1970). A specific association between mitochondrial ATPase and cardiolipin has been indicated (Santiago et al, 1973). It has been shown, however, in several cases, that there is not necessarily a strict requirement for the phoshpolipids but rather for specific properties of the environment that they provide (Fiehn and Hasselbach, 1970, Zakim, 1970, Vessey and Zakim, 1971).

There are two criteria for lipid-dependence: these are loss of activity following the removal of lipid, and reactivation on adding lipid to the inactive

enzyme. Once enzymic activity is abolished by lipid depletion, its reconstitution can be tested by the addition of various lipids. Recombination of the lipid with the lipid-depleted membranes is usually done by the method of Fleischer et al (1966). Some lipid-dependent enzyme systems are listed in Tables I and II.

In lipid-protein interaction, various consequences may occur, dependent upon the resultant free energy of the product and involving some, or all of the possible types of binding forces. Lipids and proteins may lose or largely retain their original conformation.

The following functions may be envisaged of lipids (Triggle, 1970):

(a) cofactor requirements

(b) provision of sites for hydrophobic interaction

(c) determination of (or contribution to) the tertiary structure of the protein

(d) generation of a lipid-protein matrix that may provide both substrate and cofactor binding opportunities not available in protein alone with added catalytic potential through interaction in the non-polar interior and the polar exterior of the lipid-protein complex.

There is no evidence of the phospholipid required for proper enzyme function participating as a cofactor or a reaction partner in any enzyme process. The amount needed seems too large for catalytic action and the specificity too low. The specificity is sometimes so low that detergents may serve as an adjacent substitute for phoshpolipids (Martonosi et al, 1968, Duttera et al, 1968).

It seems more likely that the lipids, by various interactions, keep the

enzyme protein in an enzymatically active form by imposing a certain conformation.

Levey (1970, 1971) has demonstrated that treatment of cat heart adenyl cyclase with the non-ionic detergent lubrol-PX results in loss of the enzyme's response to norepinephrine and glucagon, and that, depending on phospholipid used, hormonal response can selectively be restored. Thus, addition of phosphatidylinositol selectively and specifically restored the response to the catecholamine and addition of phosphatidylserine restored that to glucagon. These results may be taken as indications of interaction of the phospholipids with specific sites on the adenyl cyclase system followed by conformational changes in the enzyme.

I

SOME LIPID - DEPENDENT ENZYME SYSTEMS

Enzyme and Source	Lipid Specificity	Reference
β-Hydroxybutyrate Dehydrogenase (mitochondria)	Lecithin	Green & Fleischer, 1963 Gazzotti et al, 1974
Electron Transport chain (mitochondria)	No absolute specificity	Fleischer et al, 1962 Green et al, 1967
Glucose-6-phosphatase Rat liver microsomes	Phosphatidylethanolamine <u>or</u> lysolecithin	Duttera et al, 1968
Na^+/K^+ ATPase (rat brain)	Phosphatidylserine	Fenster et al, 1968
Galactosyl transferase (S. Typhimurium)	Phosphatidylethanolamine	Romeo et al, 1970
Cytochrome c oxidase (mitochondria)	Cardiolipin	Brierley & Merola, 1962 Amasthi et al, 1970

II

SOME LIPID - DEPENDENT ENZYME SYSTEMS (continued)

Enzyme and Source	Lipid Specificity	Reference
NADH-cytochrome c reductase (microsomes)	Lecithin & Lysolecithin	Jones & Wakil, 1967
Glycoprotein: glycosyl transferase (rat kidney)	Lysolecithin	Kirschbaum & Bosmann, 1973
Glucosyl synthetase (M. Laidlawii)	Fatty acid or detergent	Smith, 1969

MICELLES

Micelles are aggregates of colloidal dimensions. The monomers that can take part in the process of micelle formation are characterised by their amphipathic properties - they consist of one part that is sympathetic (hydrophilic) and another part that is antipathic (hydrophobic) to water. They are hydrated structures, spherical in very dilute solutions, and changing to asymmetrical structures at higher concentrations (Corkill and Herrmann, 1963). Structure of a spherical micelle is shown in Figure 16.

Expulsion of the hydrophobic groups on the surfactant molecules by water molecules, produces the required tendency for aggregation and micelle formation. Because of the reduction of surface area between the hydrophobic part and water, the total free energy of the molecules decreases. Thus, the most stable micelles are formed under conditions in which the free energy of the system is at its minimum (Skidmore and Entenman, 1962)

The principal types of compounds with these properties are (a) anionic, e.g. sodium dodecyl (lauryl) sulphate, SDS; (b) cationic, e.g. hexadecyl (cetyl) trimethylammonium bromide, (CETAB); (c) non ionic, e.g. polyoxyethylene monohexadecyl ether and (d) amphylolytic, e.g. N-dodecyl-N : N-dimethyl betaine.

(a) $CH_3(CH_2)_{11}\ SO_4^-\ Na^+$

(b) $CH_3(CH_2)_{15}\ N^+\ (CH_3)_3\ Br^-$

(c) $CH_3(CH_2)_{15}\ (OCH_2CH_2)_{21}\ OH$

(d) $C_{12}\ H_{25}\ N^+\ (CH_3)_2\ CH_2$

The concentration at which micelles begin to be formed is called the critical micelle concentration, CMC. Fatty acid salts with less than 8 c-atoms do not form micelles. This tendency commences with sodium capylate above a relatively high concentration. This is not a single sharp concentration, but a narrow range (Williams et al, 1955).

In the case of nonionic compounds, micelle formation is enhanced by the absence of electrostatic repulsions between similarly charged head groups.

In the law of mass action approach to the theoretical description of micelle formation, it is considered that there is an equilibrium between monomers and micelles (Mysels and Princen, 1959).

For nonionised detergents the equilibrium can be written:

$$K = Cmi \; / \; Cmon^{n}$$

where K is the equilibrium constant.

For ionised detergents:

$$K = Cmi \; / \; Cmon \;\; Cc^{(n - P)}$$

where Cc is the concentration of counterions, P of which are not bound to the micelle, e.g. the degree of ionisation of the micelle $\alpha = P/n$.

In dilute solutions and when the aggregation numbers exceed 20 - 30, the free energy of micellisation is:

$$8\ Gm = RT \; / \; n\ CMC.$$

The CMC decreases as the hydrocarbon chain length increases (Mysels

and Princen, 1959). For the same head groups, compounds containing bnger hydrocarbon chains form micelles at lower concentration than those containing short chains. The CMC is related to the number (n) of carbon atoms in a straight hydrocarbon chain by:

$$\log \text{CMC} = A - Bn$$

where A and B are constants for a homologous series.

The addition of salts decreases the CMC of ionised detergents (Mysels and Princen) presumably because the screening action of the electrolytes lowers the repulsive forces between the polar head groups, and less electrical work is required in micelle formation. The CMC's of mixtures of detergents of the same type lie between those of the pure separated components. For both ionic and nonionic detergents, at low temperatures, the CMC decreases with temperature.

The micelles of ionic compounds do not exceed a certain size. Debye (1949) explains this as being due to repulsion between the ionised terminal groups. This concept, naturally, cannot apply to nonionic agents.

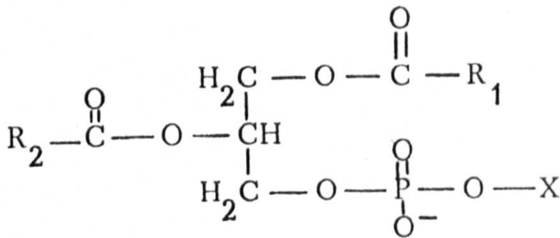
SHAPE OF MICELLES

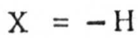
McBain (1944) assumed the existence of ionic micelles below the critical micellar concentration; above this, ionic and lamellar micelles are thought to be in equilibrium. The latter contain alternating layers of water and amphipathic molecules which are twice the length, because they are orientated so that their hydrophobic parts are directed towards each other and the hydrophilic parts point away outwards. The equilibrium is shifted at higher concentration to favour the formation of lamellar shaped micelles.

The nature of the molecular aggregates of lysolecithin and mixed dispersions in aqueous solution has been reviewed by Saunders (1966). Three types of micelle are apparent in these systems: spherical, helical and coiled laminar types. The main factor influencing the shape is the stereochemistry of the individual participating molecules.

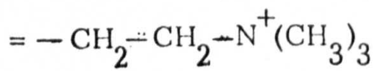
The light-scattering molecular weight of lysolecithin which forms spherical micelles was found to be $9.2 \pm 0.6 \times 10^4$, indicating 181 monomers per micelle.

Fig. 17 a) Structures of some phospholipids

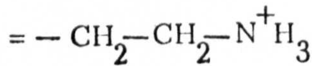




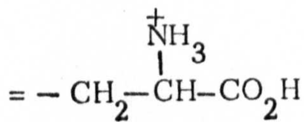
Phosphatidic acid



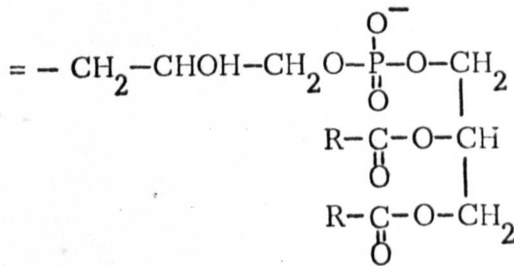
Phosphatidylcholine or **lecithin**



Phosphatidylethanolamine



Phosphatidylserine



Diphosphatidyl glycerol
(cardiolipin)

CARDIOLIPIN

Cardiolipin is a double unit in that it has two molecules of phosphatidic acid esterified to a central glycerol (Figure 17) and is more appropriately designated as diphosphatidylglycerol. It occurs in large amounts in bacterial, mitochondrial and chloroplast membranes.

Some earlier studies indicated that cardiolipin might be a unique component of mitochondria (Martinetti et al, 1958). Later results support this finding (Colbeau et al, 1971). Unlike other phospholipids, which exchange between cell organells, cardiolipin does not change its position within the cell and remains in the inner mitochondrial membrane (McMurray and Dawson, 1969). This indicates some specific function of cardiolipin for the inner mitochondrial membrane. A specific association between the phospholipid and mitochondrial ATPase has been demonstrated (Santiago et al, 1973). Also, a small amount of cardiolipin was found to be tightly bound to cytochrome oxidase (Amasthi et al, 1970).

Cardiolipin is the most highly unsaturated phospholipid and only 10% of its fatty acids are saturated (Colbeau et al, 1971). It contains more than 80% of C18 fatty acids : Linoleic acid representing more than 60% of the total. It is therefore responsible for the high degree of unsaturation of the inner mitochondrial membrane which contains 20% of cardiolipin (Fleischer et al, 1967 b, Colbeau et al, 1971). The outer mitochondrial membrane contains only about 4 to 5% of the phospholipid. In rats, the degree of unsaturation of the fatty acids of cardiolipin has been shown to vary with the diet (Tischer and Glenn, 1965).

Several facts suggest that cardiolipin may be found predominantly on the inner surface of the inner mitochondrial membrane (Writz and Zilversmit, 1968, Fleischer and Fleischer, 1967) with its hydrophilic groups at least partially located on the membrane surface (Schiefer, 1973 a, Schiefer, 1973 b). These points will be more fully discussed in the following chapters. It is probably associated with phosphatidylcholine which has been shown to be necessary for the immunogenicity of cardiolipin (Inoue et al, 1967).

Within the membrane, the fatty acid content of cardiolipin can change quite rapidly. It has been shown by labelling methods that whereas the glycerophosphate backbone turns over very slowly (a few days), the fatty acid turnover is very high (a few minutes) (McMurray and Dawson, 1969). The structures of some phospholipids are shown in Figure 17 a).

GLUTAMATE DEHYDROGENASE

In this work, a well-defined allosteric enzyme, glutamate dehydrogenase, has been chosen as a system for exploring the possibility of regulatory mechanisms associated with lipid-enzyme interactions and of possible involvement of the allotopic phenomenon. The latter, according to Racker's original definition (Racker, 1967) is a phenomenon of membrane-enzyme complexes manifest by the alteration of properties of both enzyme and membrane.

The enzyme catalyses the interconversion of glutamic and α- Keto-glutaric acids; it performs this function in the mitochondria of the liver. In common with some of the other dehydrogenase enzymes, it requires 1 mole of hydrogen from its substrate glutamic acid via the imino derivative. The reaction is subject to allosteric regulation.

The enzyme is present in high concentration and entirely in the mitochondrial matrix. Its activity is to some extent dependent upon the rodox state of nicotinamide nucleotides. In isolated mitochondria, added glutamate is metabolized, mainly through the transaminase pathway and relatively little is deaminated (Borst et al, 1962, De Haan et al, 1967). It occupies a central position in mammalian nitrogen metabolism since the reaction which it catalyses provides the major pathway for the interconversion of α- amino group, nitrogen and ammonia. In the deamination direction, the enzyme is thought to provide the NH_3 required for carbamoyl phosphate synthesis in the urea cycle. In the reverse direction it may catalyse the synthesis of glutamate from NH_3 produced by various deaminases. Other important enzymes of amino acid metabolism usually employ pyridoxal 5^1-phosphate in transamination or decarboxylation as the initial reaction step.

The enzyme has six identical protomers (peptide subunits). Each protomer has an active site, an ADP binding site, a GTP binding site and binding sites for thyroxine and oestrogens. The activity of the enyzme is subject to allosteric regulation by these molecules and a number of other nucleotides and hormones, e.g.GTPis a strong inhibitor and ADP is an activator (Frieden, 1963).

Glutamate dehydrogenases from all animal tissues are strongly and specifically affected by purine nucleotides. Since the enzyme occupies a key place in metabolic process, there is no question that it is under regulation in all animal tissues. It also seems clear that an important factor for the control of its activity are the purine nucleotides.

NADH,in contrast to NADPH, binds to a second nonactive site with enzymic activity being altered as a consequence of such binding. Thus, in the absence of purine nucleotides, high levels of NADH ($>10^{-4}$ M) inhibit the oxidation of NADH. This inhibition is potentiated by GTP, On the other hand, ADP relieves the NADH inhibition. Also, there is good evidence that ADP might compete for the second NADH binding site (Pantaloni and Dessen, 1969).

The dissociation constant for GTP and ADP have been found to be 0.43 x 10^{-6} M and 8 x 10^{-6} M respectively (Frieden and Colman, 1967). Thus, GTP shows a much higher affinity for the enzyme than ADP. NADH increases the affinity of GTP binding to the enzyme (Bayley and Radda, 1965).

Yielding and Tomkins (1961) observed that isolated glutamate dehydrogenase is activated by l-leucine and certain other amino acids. The effect of l-leucine on the isolated enzyme was further investigated by Kun and Achmatowicz (1965).

Recently, evidence for the formation of a complex between l-leucine and glutamate dehydrogenase with perturbation of enzyme structure has been obtained (Prough and Fisher, 1972, Prough, 1972).

The enzymatically active monomeric form of bovine liver glutamate dehydrogenase consists of six identical subunit polypeptide chains. The presently known sequence contains 500 residues in the subunit single peptide chain, each with a molecular weight of about 56,000 (Moon and Smith, 1973). Amongst these are 33 lysine residues and 3 tryptophan residues. A value of four tryptophan residues per polypeptide chain has also been suggested (Witzmann et al, 1974).

Of the 500 amino acid residues, four have been characterised with respect to their function in the active site, in the regulatory site, and in the association process: lysine - 126 (Moon et al, 1972), tyrosine - 406 (Smith et al, 1970, Moon et al, 1972) and lysine - 422 (Goldin and Frieden, 1971). Recently, (Rached et al, 1974) another lysine residue, lysine - 27 was found to be involved in the binding of α - ketoglutarate.

Regulation of enzyme activity isthought to achieve through conformational changes, and dissociation is only a secondary consequence of these changes (Bittensky et al, 1965).

Two conformationally different monomeric forms of the enzyme are suggested (Tomkins et al, 1965):

Monomer X is in equilibrium with monomer Y.

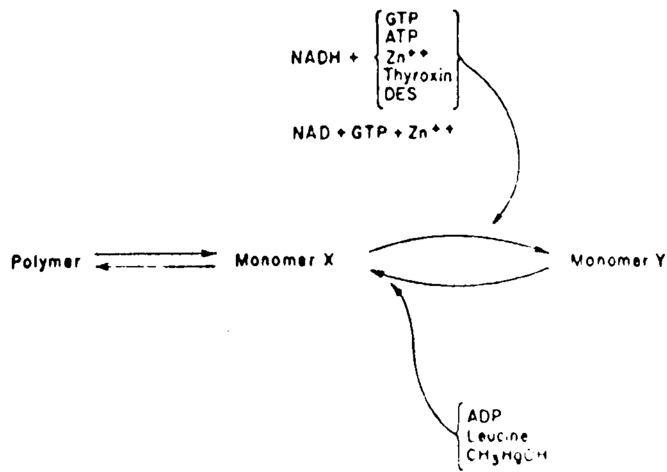


Fig. 17 The effect of different reagents on conformation and association of glutamate dehydrogenase

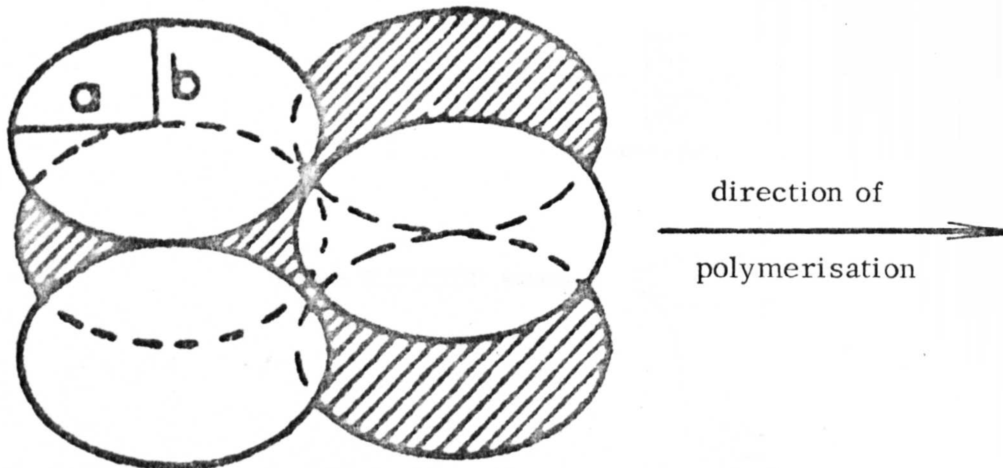


Fig. 18 A proposed structure for the monomer of bovine liver glutamate dehydrogenase composed of six subunits. Each subunit is approximated by an ellipsoid of revolution with a = 33 A^{o} and b = 22.5A^{o}. As indicated, polymerisation occurs as a lengthwise process

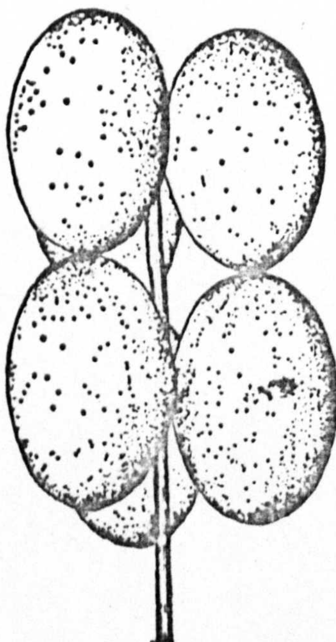


Fig. 19 The six ellipsoid subunits of a glutamate dehydrogenase oligomer

Monomer X is in equilibrium with monomer Y, has GDH activity and can associate to form the polymer which also has this activity. Monomer Y has alanine dehydrogenase activity and little or no glutamate dehydrogenase activity, and can associate to form the polymer. Thus, all reagents which promote association of the enzyme to a higher molecular weight also activate the glutamate dehydrogenase reaction (Figure 17). On the other hand, reagents which promote dissociation of the enzyme to oligomer inhibit this activity and promote alanine (and other monocarboxylic amino acid) dehydrogenase reactions (Tomkins, 1963). The associated polymeric form of the enzyme has a molecular weight of 1.6 to 2 x 10^6 and is formed as a result of a concentration-dependent association reaction. Below concentrations of 0.1 mg/ml, the predominant enzymatic species is the monomer comprised of six subunits. At higher levels, the enzyme polymerises to higher molecular weight forms and at 5 mg/ml, for example, the predominant molecular weight species is about 2 million.

From the dependence of weight average molecular weight as a function of protein concentration as measured by light scattering (Eisenberg and Tomkins, 1968), viscosity (Eisenberg, 1970) and other data (Chun et al, 1969), it has been concluded that the enzyme undergoes a stepwise association with increasing protein concentrations shifting the predominant species from the monomer to the dimer, trimer and so on. Thus, the polymer exists as a rod with the individual monomeric units associating along a single axis (Sund et al, 1967). The polymerisation is along the major axis of the ellipsoid subunits (Figure 18). The six ellipsoid subunits are also shown in Figure 19.

In the presence of coenzyme, GTP and GDP bind less tightly to the associated polymeric form than they do to the monomeric form of the enzyme.

Also the specific activity of the enzyme in the absence of purine nucleotides is essentially independent of enzyme concentration (Frieden and Colman, 1967).

It is interesting to note that although the amino acid composition and the peptide maps of rat liver glutamate dehydrogenase (King and Frieden, 1970) are quite similar to the beef liver enzyme, this enzyme lacks the ability to undergo a concentration-dependent reversible association reaction even in the presence of nucleotides such as ADP which promote the association of beef liver enzyme (Frieden and Colman, 1967). It has been suggested (Ifflaender and Sund, 1972) that the relatively small differences in the primary structure in the case of the rat liver enzyme have caused a loss of only some of the structural characteristics of the rat liver enzyme which, in the case of the beef liver enzyme, are responsible for the association reaction.;

The level of glutamate dehydrogenase differs widely in various mammalian tissues (Frieden, 1965). Liver, kidney and brain contain considerable amounts of the enzyme (e.g. in beef liver mitochondria, the concentration is at least 2 mg/ml, not allowing for compartmentation, while heart has about 10% of their amount).

Glutamate dehydrogenase from various animal sources utilise both NAD and NADP and are regulated by purine nucleotides. In contrast, the enzymes from non-animal sources are specific for either NAD or NADP and are not influenced by purine nucleotides.

There has been relatively little information acquired on the structure of glutamate dehydrogenase from non-animal sources. Barratt and Strickland (1963) found that the NADP-dependent enzyme of Neurospora crassa had a

molecular weight of about 280,000 and did not polymerise at high concentrations. Recently (Hooper et al, 1974), the enzyme has been purified 67-fold from *Tetrahymena pyriformis*.

Chapter II

MATERIALS and METHODS

MATERIALS

Glutamate dehydrogenase (as a glycerol solution) was obtained from Boehringer Mannheim, G.m.b.H., Germany, as were NAD^+ and NADH. The enzyme is pure and homogeneous.

Phosphatidylcholine, phosphatidylethanolamine and lysolecithin from egg yolk, phosphatidylserine from bovine spinal cord and cardiolipin from ox heart, all grade 1 and in the form of monosodium salt, were purchased from Lipid Products, Surrey, England.

L-glutamate, α-ketoglutaric acid, l-alanine, l-leucine, pyruvic acid and the adenine nucleotides were all purchased from the Sigma Chemical Co. Specially purified sodium dodecyl sulphate was obtained from British Drug Houses.

Hepes and pipes buffers were purchased from Hopkin and Williams Biochemicals Ltd. Bio-Rex 70 (200 - 400 mesh) in the form of its sodium salt was obtained from Bio-Rad Laboratories, Richmond, California, U.S.A.

MN silica gel G (100 mesh) was obtained from Macherey, Nagel and Co., Germany, and Florisil (mesh 60 to 100) from Hopkin and Williams Biochemicals Ltd. ANS, purchased as the sodium salt from
was purified by several recrystallisations from saturated $MgCl_2$ solutions.

Crystalline bovine serum albumin was obtained from Sigma Chemical Co. Ribonuclease and cytochrome c were purchased from Boehringer. All other chemicals were Reagent grade and solutions were routinely made up in double glass distilled water.

METHODS

Kinetic Experiments

A stock solution of the enzyme for kinetic studies was made by dilution of the glycerol solution. The enzyme solution containing 10 mg/ml of the enzyme was diluted 200 times in 0.1 M phosphate buffer, pH 8.0 containing EDTA(1×10^{-4} M) and 10 μl of the dilute solution was taken for glutamate dehydrogenase activity in the direction of NADH oxidation. The final concentrations in the assay mixture were α-Kg, 5 mM; NH_4Cl, 50 mM; NADH, 8×10^{-5} M; EDTA, 0.05 mM.

For NAD reduction assay, the enzyme was diluted 20 times in the same buffer and 10 μl of it was taken for activity measurement each time. The final l-glutamate and NAD^+ concentrations in the assay mixture were 10 mM and 2×10^{-4} M respectively. Both these assays were carried out either in 0.1 M phosphate buffer, pH 8.0 or in 0.06 M hepetri buffer (with 0.02 M hepes, 0.02 M tris and 0.02 M pipes) pH 7.7. A mixture of these buffers was used so that the effect of pH on lipid-enzyme complex formation could be studied without changing the buffer system.

Alanine dehydrogenase activity was carried out at pH 9.0 with the above buffer system using sodium pyruvate and ammonium chloride concentrations of 0.04 M and 0.05 M respectively. A final NADH concentration of 8×10^{-5} M was chosen.

NADH solutions were made up in 0.05 M tris-HCl buffer, pH 9.0 and their concentrations were determined by extinction at 340 nm (

6.22 = 1 mM NADH). They were kept in the cold and were made fresh every two to three days. NAD^+ solutions were made in the same way. Enzyme concentrations were determined using the extinction coefficient of 0.97 mg/ml at 279 (Olson and Anfinsen, 1952).

Enzyme activities were measured by following the decrease (NADH oxidation) or increase (NAD^+ reduction) of extinction at 340 nm. All assays were carried out using a Unicam SP 1800. The sample compartments were thermostatically controlled at 25°C. Normally, a 0 - 0.05 full-scale absorption was used. A linear O.D. change of one minute was regarded as satisfactory.

Sonication of Phospholipids

For the preparation of phospholipid dispersions, the required amount of the phospholipid dissolved in chloroform-methanol was taken into a glass test tube (internal diameter of 1.3 cm, 2.0 cm or 2.9 cm, length 5 cm) and the mixture was taken to complete dryness by evaporation under nitrogen at 35°C for phosphatidylserine and 50°C for egg yolk lecithin. The required volume of buffer (normally, 0.06 M hepetic, pH 7.7 + 1×10^{-4} M EDTA) was then added and sonication was carried out under nitrogen in the glass test tube surrounded by ice water with the tip of the soniprobe immersed to about half its height and the power supply tuned to maximum cavitation. Thus, special care was taken to prevent oxidation of phospholipids. This was checked by taking u.v. spectra of the phospholipids before and after sonication. No increase in absorption at 233 nm was noted in our conditions. An increase in absorption at this wavelength has been shown to indicate possible oxidation (Klein, 1970).

Also, no hydrolysis products were indicated when the phospholipid sonicates were tested by T. L. C. An MSE sonicator was used and fresh sonicates were prepared daily. Normally, sonication for 1 - 2 minutes in the case of PS and CL, and 10 - 15 minutes in the case of PC, were required to give clear dispersions. The time of sonication was dependent on phospholipid concentration and was found to vary slightly from experiment to experiment.

Fluorescence Measurements

Fluorescence studies were carried out on a Perkin-Elmer fluorescence spectrophotometer MPF-3. The enzyme in glycerol solution was dialysed in 0.1 M phosphate buffer, pH 8 (+ 1 x 10^{-4} M EDTA) overnight. For intrinsic fluorescence studies, 10 ml of the dialysed enzyme was taken each time giving final enzyme concentration of 5 - 6 mg/ml. Excitation and emission wavelengths of 290 - 292 nm and 333 - 334 nm were chosen. In these conditions there was very little photodecomposition of the enzyme. A filter at position 31 was used which minimised the scatter contribution from phospholipids to 2 - 8% (depending on phospholipid concentration) of the fluorescence due to free enzyme.

Extrinsic fluorescence studies were carried out using ANS (magnesium salt) as the fluorescence probe.

In the case of the zwitterionic and cationic amphiphiles, LL, PC and CETAB, the negatively charged probe was incorporated into their structures. These amphiphiles showed good affinity for ANS binding and gave rise to maximum fluorescence at low concentrations. An excitation wavelength of 370 nm was chosen and λ max of emission of ANS fluorescence incorporated into the amphiphiles was found to be 466 nm, 473 nm and 476 nm for PC, LL

and CETAB respectively. The possibility of complex formation was followed by looking at fluorescence changes on addition of the enzyme (0.2 - 0.3 mg/ml). The ANS concentrations used in these experiments were 1 mM in the case of PC and CETAB and 10 mM in the case of LL. The enzyme was previously dialysed in 0.1 M phosphate buffer , pH 8.0.

To follow the interaction between the enzyme and the anionic phospholipids, PS and CL, ANS was first added to the enzyme and fluorescence changed on addition of the phospholipids were then followed. The slits were always left at 6 nm and the filter at position 43 for these studies. Excitation and emission wavelengths were normally at 370 nm and 470 nm respectively. ANS and enzyme concentrations were 20 - 25 mM and 0.6 - 0.7 mg/ml. The phospholipid concentration was varied from experiment to experiment. All these studies were carried out in 0.06 M hepetin with 6 - 8 mM phosphate buffer at a final pH of 7 - 7.3. In all cases, the required quantity of the enzyme (usually 0.2 ml) dialysed and left in 0.1 M phosphate buffer (+ EDTA), was added to an ANS solution in hepetri buffer (0.06 M, pH 6.0) in the absence or presence of additional metabolites. The phospholipids were sonicated and left in the hepetri buffer and were added last. Control measurements were taken and the true enhancement of ANS on binding to the enzyme and further fluorescence increase on addition of the anionic phosholipids were recorded.

When the effect of NADH on lipid-enzyme complex formation was explored, excitation and emission wavelengths of 410 nm and 550 nm were chosen. No NADH fluorescence could be detected in these conditions and the slight fluorescence due to bound ANS was detected at high sensitivities. The final volume of the mixtures in the 1 cm cell was normally between 1.2 - 1.5 ml.

Extraction of GDH-Phospholipid Complex into Isooctane

PC - PS cosonicates were prepared at 0.1% concentration. Sonication was carried out in 0.06 M tris buffer, pH 6.0 (+ 1 x 10^{-4} M EDTA) in a manner described above. 5 ml of the sonicates were prepared each time with the zwitterionic and anionic lipids at different ratios (PC : PS of 3 : 2, 4 : 1, 2 : 3 and 1 : 4 . 1 ml of each phospholipid sonicate was added to 1 ml of the enzyme (0.86 mg/ml) in a 50 ml volumetric flask and the mixture was shaken for a few minutes. To this 0.6 ml of ethanol was added which made it 30% with respect to ethanol. 5 ml of isooctane were then added and the flasks were shaken on a flask shaker at a low speed for 30 minutes. After shaking, the mixtures were centrifuged in a bench centrifuge for 5 minutes and the isooctane layer was removed.

Stopped-flow Experiments

A stopped-flow apparatus made in the Department (Benton, 1972) was used. The enzyme was previously dialysed in 0.1 M phosphate buffer, pH 8.0 (+ EDTA). Phospholipid sonicates were prepared in 0.02 M hepetri buffer, pH 7.7. Transmission changes were then followed at 310 nm and 15°C with a 2 cm cell path.

Preparation of Beef Liver Mitochondria

The procedure described by Brosnan et al, 1973, was followed. All manipulations were carried out at 4°C. Beef liver was freed of connective tissue capsule and cut into small pieces. These pieces were then ground in a cooled meat grinder and suspended in a homogenisation medium (Medium A),

consisting of mannitol (220 mM), sucrose (70 mM), hepes (2 mM), bovine serum albumin (1 mg/ml) and EDTA (2 x 10^{-4} M) at pH 7.4. For each 100 g of liver, 300 ml of the medium were used. The suspension was homogenised with three passes of a motor-driven homogeniser in a Potter-Elvehjem homogeniser of large clearance. A preliminary centrifugation at 800 g for 10 minutes removed unbroken cells, nuclei, cell debris, etc. and mitochondria were isolated from the supernatant fraction by centrifugation at 8000 g for 10 minutes. The mitochondria were subsequently washed twice by resuspension in the homogenisation medium and recentrifugation at 8000 g for 10 minutes. All these operations were done at 4°C, using a Sorvall RC2 - B superspeed centrifuge. Protein concentrations were determined by the Biuret method using bovine serum albumin as standard.

P/O ratios of the mitochondrial suspensions were measured using a reaction mixture containing 20 mM tris-HCl buffer, 210 mM mannitol, 70 mM sucrose, 2 mM $MgCl_2$, 10 mM KCl and 5 mM Na_2HPO_4 - $KHPO_4$, pH 7.4. Final succinate and ADP concentrations were 8 mM and 0.32 mM respectively. 4 - 5 mg of mitochondrial protein was used and the process was carried out at 25°C on an oxygen electrode. The P/O ratios of two different mitochondrial preparations were found to be 1.75 and 1.9 respectively.

Chromatography of Mitochondrial Phospholipids

Mitochondria were prepared from fresh beef liver as described above. 20 ml of the mitochondrial suspension containing 480 mg of protein were added dropwise into 260 ml of swirling methanol (13 ml per ml of mitochondrial suspension). 520 ml of chloroform (26 mg/ml of mitochondrial suspension) were

then added and the mixture was homogenised in a Potter-Elvehjem homogeniser. The homogenate was then filtered through a sintered glass filter of coarse porosity under vacuum. To this, BHT was added to the final concentration of 0.05%.

The extract was then transferred to a separating funnel to which 800 ml of 0.034% $MgCl_2$ was added. The mixture was then shaken thoroughly and was left overnight for separation of the layers. The lower phase was then removed and the upper phase discarded.

To the lower phase, 600 ml of a mixture prepared by mixing in a separatory funnel chloroform, methanol and 0.034% $MgCl_2$ in the ratio of 8 : 4 : 3 (vol/vol/vol). This partition was repeated three times. After each partition the upper layer was removed and discarded. The filtrate was then transferred to a round-bottomed flask and dried in vacuo by attaching the flask to an aspirator and then leaving it under vacuum overnight.

Florosil Column Chromatography

Florosil (60 - 100 mesh) was first acid-treated. 100 g of florosil was mixed with 350 ml of conc. HCl in a 1 litre flask and heated on a steam bath for 3 hours. The supernatant was decanted carefully. The residue was washed with 50 ml of conc. HCl and heated overnight with another 350 ml of conc. HCl. The supernatant was decanted and the residue was washed with water on a Buchner funnel until washings were neutral. The residue was then dried and transferred to a glass dish and then heated overnight at 120°C. The acid treatment was repeated and the residue was washed with 150 ml each of methanol, methanol-chloroform (1 : 1, v/v), chloroform and finally, ether. The residue

was then transferred to a glass dish, allowed to dry and finally activated by heating overnight at 120°C.

A column with an internal diameter of 2 cm and a height of 35 cm was packed with 30 g of acid-treated florosil slurried in chloroform. The total lipid extract (about 400 mg net weight) was dissolved in 5 ml of chloroform and then applied onto the column. Elution of the column with chloroform and chloroform-methanol was then carried out in the cold at the rate of about 3 ml/min. Freshly distilled solvents were used. The following fractions were obtained.

Fraction 1, chloroform, which elutes neutral lipids, such as hydrocarbons, sterols and fatty acids.

Fractions 2 & 3, chloroform-methanol (95 : 5, v/v) and chloroform-methanol (90 : 10, v/v), which elute phosphatidic acid and cardiolipin.

Fraction 4, chloroform-methanol (3 : 1, v/v), which elute phosphatidylethanolamine and phosphatidylglycerol.

Fractions 5 & 6, chloroform-methanol (1 : 1) and pure methanol which elute lecithin and lysolecithin.

Progess in fractionation was followed by TLC on small slides. The following solvent systems were used:

For neutral lipids:

Petroleum ether-ethyl ether, acetic acid (90 : 10 : 1);

For phospholipids:

I) Chloroform-methanol-H_2O (65 : 35 : 5);

II) Chloroform-methanol-NH_3 (65 : 25 : 5).

For phospholipids, solvent system II was found to be more appropriate as the spots were nearly of a compact shape.

Volumes of solvents for fractions 1 - 6 were: 200 ml for fraction 1, 100 ml for fraction II, 200 ml fraction III, 340 ml for fraction IV, 100 ml for fraction V and 300 ml for fraction VI.

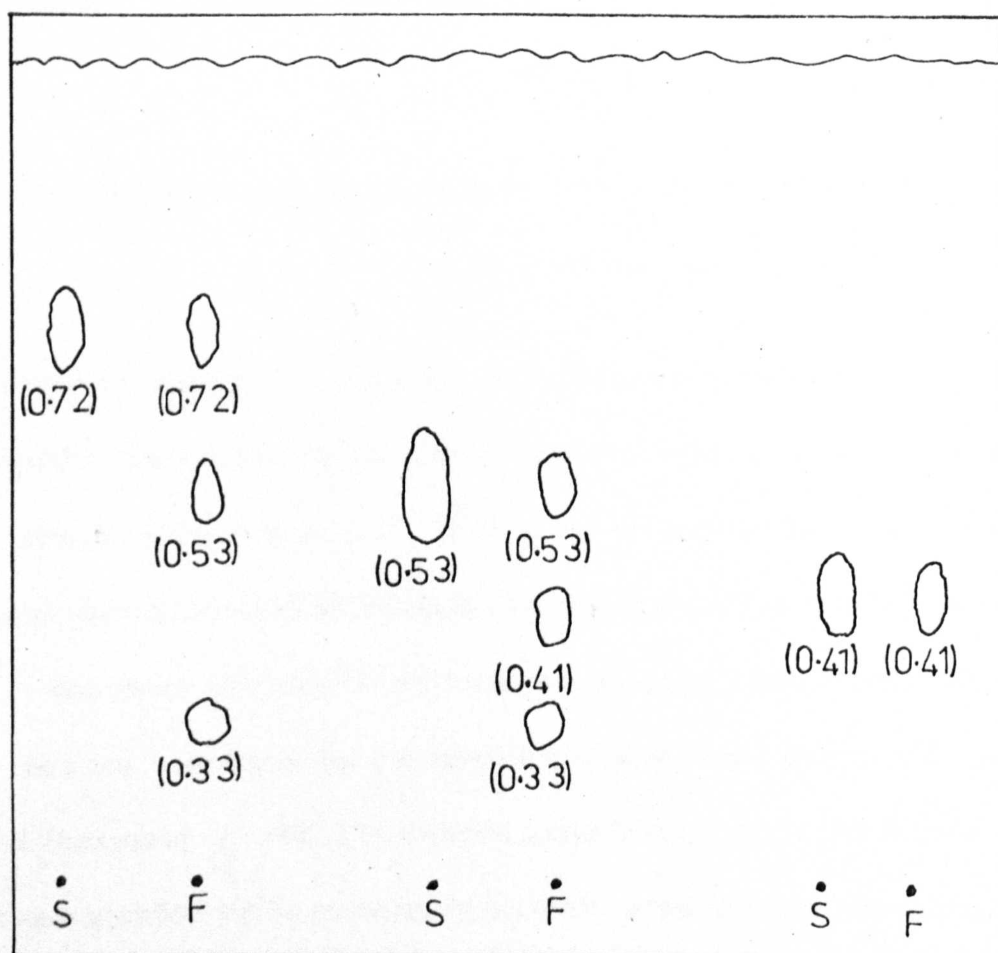
Fractions II and III (containing cardiolipin) were pooled together, so were fractions V and VI (containing lecithin).

Total phosphorus estimation was then carried out. An aliquot of each lipid solution was put in a large test tube (Pyrex) and the solvent was evaporated to complete dryness in a stream of air at 35°C. 2.0 ml of perchloric acid (72%) were then added and the strength was digested be leaving the tube in hot sand, heated electrically. This was continued until digest was clear and colourless. The cooled digest was then diluted to 6 ml with water and was mixed on Vortlex mixer. 2 ml of Amidol solution (1%, freshly prepared) and 1.0 ml of molybdate solution were added and absorbance of the solution was read at 680 nm. A solution of KH_2PO_4 was used as standard phosphate solution.

The total lipid obtained in the five fraction was found to contain 3909 mg of phosphorus corresponding to 94 mg of phospholipids. This gave a value of 8.1 mg phosphorus/mg of mitochondrial protein.

The presence of cardiolipin, phosphatidylethanolamine and lecithin were

Fig. 20 a) TLC of phospholipid fractions from Florosil column



demonstrated by TLC on a large plate. These samples were applied to a plate with CL, PE and PC standards. The plate was then run in a solvent system of chloroform-methanol-ammonia 65 : 25 : 5 (vol/vol/vol), let to dry and charred with conc. H_2SO_4 with slight heating. The positions of the samples are shown in Fig. 20a) and their R. f. values indicated in the brackets.

It can be seen that the fraction containing cardiolipin showed two additional spots, one corresponding to PE (R. f. 0.41) and one of a lower r. f. value (0.33) which was probably a decomposition product of cardiolipin or phosphatidylethanolamine (Fleischer et al, 1967). This spot may also correspond to phosphatidylinositol or lysolecithin (Amasthi et al, 1971).

Final purification of the samples was then carried out by TLC. The plates were prepared as described in the previous chapter. A layer thickness of 0.75 mm was chosen. An automatic streaker was used for application of the samples to the plates. About 20 mg of the lipid was applied to a plate each time. This streak occupied about $\frac{2}{3}$rd of the plate. A sample of the appropriate standard was also applied onto the plate. The plates were then immediately put in a tank saturated with solvent system chloroform-methanol-NH_3 (65 : 25 : 5) in the cold ($\simeq 4^oC$).

The solvent was allowed to ascend to within 2 - 3 cm of the top of the plates. The plates were then allowed to evaporate for a short period. They were then partly covered with a clean glass plate so that the spot corresponding to the standard and a small section of the streak corresponding to the sample were exposed. The phospholipids were then detected by the use of Zinzade's reagent (sodium molybdate, hydrazime sulphate, and conc. H_2SO_4). The part of the plate corresponding to the blue spots were then scraped off and dissolved

in chloroform-methanol (2 : 1) with 0.05% BHT and left in the deep freeze.

The purity of these lipids was then checked by TLC on a large plate. Each phospholipid showed only one spot corresponding to the standard.

Assays of Mitochondrial Enzymes

Glutamate dehydrogenase activity was carried out in 0.06 M hepetri buffer, pH 7.7 (+ 1 x 10^{-4} M EDTA) in the presence of 0.1% Triton X-100 and 0.4 mM sodium cyanide.

Rotenone-insensitive NADH-cytochrome c reductase was assayed by following the reduction of cytochrome c at 550 nm. The assay mixture contained 0.1 mM NADH; 0.1 mM cytochrome c; 0.3 mM KCN; 1.5 mM rotenone and 50 mM phosphate buffer, pH 7.5.

For succinate-cytochrome c reductase, the assay mixture contained 3 mM succinate, 0.1 mM cytochrome c, 0.3 mM KCN in 50 mM phosphate buffer, pH 7.5.

Phospholipase C Effect

Beef liver mitochondria were prepared as described above. They were resuspended in a medium containing 0.25 M sucrose and 50 mM tris (+ 1 x 10^{-4} M EDTA), the pH of which was adjusted to 7.4 with acetic acid. $MgSO_4$ was then added to give a final concentration of 1 mM, and incubation with various amounts of phospholipase c was carried out with stirring for 15 minutes at room temperature. The magnesium salt was necessary for the requirement of a positive zeta potential for phospholipase c action. After incubation, the

mitochondrial suspension was centrifuged at 44,000 g for 10 minutes using a Bechman model L-2 analytical ultracentrifuge. The pellet from this centrifugation was resuspended in the sucrose-tris-acetate medium.

The effect of different concentrations of phospholipase c was examined by the use of the marker enzymes.

Digitonin Effect

To study the effect of digitonin on beef liver mitochondria, a 1% stock solution was prepared by adding 0.25 M sucrose in 50 mM tris-acetate buffer, pH 7.4 (medium B), mixing briefly and sonicating for 2 min in ice. All digitonin solutions were prepared immediately before use. Aliquot of mitochondrial suspensions in medium A were placed in an ice bath and different amounts of cold digitonin solutions were added with continuous stirring. After 20 minutes, the mitochondrial suspensions were centrifuged at 44,000 g for 10 min, using the Bechman model L-2 analytical ultracentrifuge. The pellets from this centrifugation were resuspended in the sucrose-tris-acetate medium and protein estimation of the fractions was carried out. The fractions were then assayed for the matrix, inner and outer membrane enzymes.

Binding of Purified GDH to Mitochondrial Membranes

Binding affinity of glutamate dehydrogenase to different sides of mitochondrial membranes was explored by incubating the purified enzyme (the enzyme in glycerol solution was dialysed in 0.1 M phosphate buffer, pH 8.0 + EDTA) with different mitochondrial suspensions.

For binding of the enzyme to the outer surfaces of the outer and the inner membranes, whole mitochondria and mitoplasts were used. These were previously washed and pelleted in medium B (0.25 M sucrose in 50 mM tris-acetate buffer, pH 7.4).

For binding of the enzyme to the inner surface of the inner membrane, the mitoplasts were either sonicated in medium B or transferred to a hypotonic environment (0.06 M hepetri buffer, pH 7.4). These were then washed and recentrifuged at 44,000 g for 20 minutes. GDH at the final concentration of 0.018 mg/ml was used and the mitochondrial suspensions had protein concentrations in the range of 0.15 to 0.4 mg/ml. Control experiments were carried out with the enzyme alone and mitochondrial suspensions alone. The suspensions were then made to 5.0 ml with medium B and were then centrifuged at 44,000 g for 20 minutes. All these procedures were carried out in ice or at $4^{o}C$ and the supernatants were assayed for glutamate dehydrogenase activity.

Binding of Endogeneous GDH to the Inner Mitochondrial Membrane

For these experiments, mitoplasts prepared by digitonin treatment were used. Sonication (for 30 seconds) or transfer to a hypotonic environment (0.06 M, hepetri) were used for preparation of mixtures of right-side and inside-out vesicles. NH_4Cl and pH effects were studied by using solutions made at different NH_4Cl concentrations in medium B or at different pHs in 0.06 M hepetri buffer. Normally, 0.5 ml of the mitoplast suspension was made up to 5 ml with final protein concentration of 1.2 - 1.4 mg/ml. GDH activity of the supernatant fractions was determined with 10 - 20 ml of each sample.

The effect of metabolites on binding of GDH to the inner mitochondrial

membrane was explored by incubating the mitoplasts with the metabolites in medium B or 0.06 M hepetri buffer. Normally, the final protein concentration was in the range of 0.3 - 0.5 mg/ml. In some cases, it was higher. Sonication of the mitoplast suspensions in medium B was carried out for 30 seconds and the samples were centrifuged at 44, 000 g for 20 minutes.

The supernatant fractions were then assayed for their glutamate dehydrogenase activities using 10 or 20 ml of each fraction.

Electron Microscopy

Mitochondria or mitoplasts were prefixed in 4% glutaraldehyde which was made up in 0.08 M sodium acetate buffer, pH 7.4 for a period of 24 hours. They were then washed with the same buffer. 1% OsO_4 was used as a post-fixative. The preparations were left in this solution for a period of 4 hours. They were then washed and left overnight in the acetate buffer.

Dehydration was then carried out with the following concentrations of ethanol:

30%	1 hour
50%	3 hours
70%	overnight
90%	1 hour
absolute ethanol	15 minutes
absolute ethanol	10 minutes

The preparations were then embedded first in a mixture of 30% araldite and 70% ethanol for six hours and then 50% araldite and 50% ethanol overnight. The second step was repeated with a mixture of 70% araldite and 30% ethanol.

These were then transferred into capsules which contained pure araldite and were left in an oven at 60°C for three days. They were then sectioned.

Staining was done with 2% Uranyl acetate for 30 min and 1% lead citrate for 10 min.

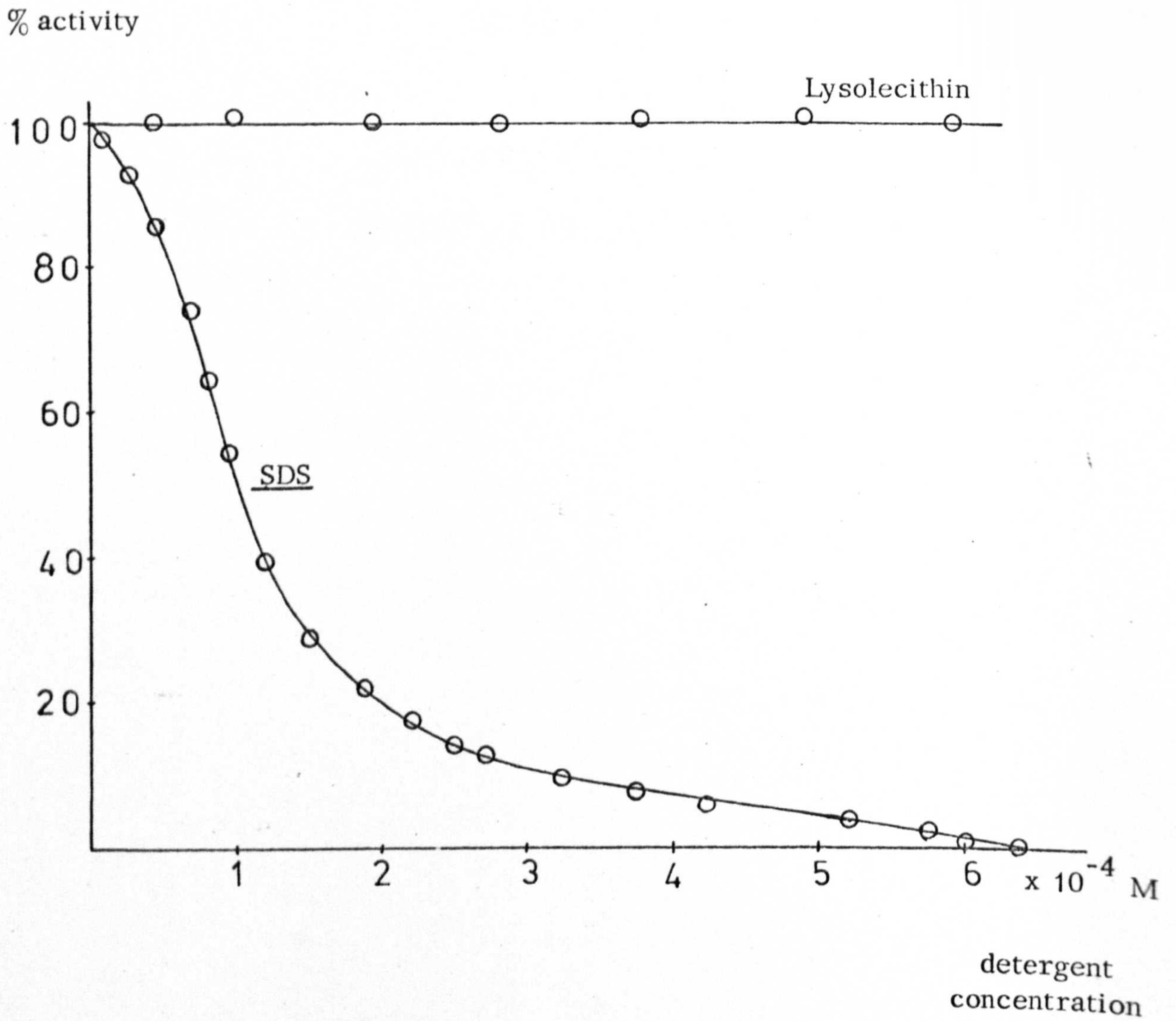
Chapter III

KINETIC EXPERIMENTS :

RESULTS and DISCUSSION

(Reproduced from the M. Sc. Thesis , M. Nemat-Gorgani, 1972)

Fig. 20 The effect of SDS and lysolecithin on the glutamate dehydrogenase activity of GDH.



Initial experiments were carried out to study the influence of the charge type of simple amphiphiles on their interactions with the enzyme.

It has previously been shown that the charge type of the phospholipid head group determines the extent of complex formation with the enzyme (Dodd, 1972, Dodd, 1973, Nemat-Gorgani and Dodd, 1974). The same rule applied to the binding of single amphiphiles such as the ionic detergents, despite the differences in the phase behaviour of the two types of amphiphiles.

The utility of the detergents lies in the fact that their phase behaviour is simpler than the phospholipids under the conditions chosen for this study. Micelles are in equilibrium with high concentrations of the monomer, which means that the monomer-detergent interaction can play a significant role in the binding of the amphiphile to the protein. In contrast with the detergents, the phospholipids used in this investigation were in the form of single-shelled bilayer vesicles for which no significant concentration of monomer is found (Robinson, 1966).

Figure 20 shows the effect of these compounds on the glutamate dehydrogenase activity of the enzyme. Different amounts of the detergents were added to the assay mixture each time and the activity was measured. The anionic detergent SDS inhibits the enzyme, but the zwitterionic amphiphile lysolecithin does not affect the activity up to a concentration of 1×10^{-3}M. A short-chain amphiphile, n-hexane sulphonate, which did not form a micelle at the range of concentrations used in our studies (up to 5×10^{-3}M) did not cause any inhibition of the enzyme.

The most significant difference between the effect of the simple single

(Reproduced from the M.Sc. Thesis, M. Nemat-Gorgani, 1972)

Fig.21/2 The effect of the preliminary incubation of glutamate dehydrogenase with SDS at different concentrations of the detergent

% activity

100

80

60

40

20

2×10^{-4} M SDS

4×10^{-4} M SDS

20 40 60 80 100 120 140 160

minutes

% activity

100

80

60

40

20

1.5×10^{-4} M SDS

3.5×10^{-4} M SDS

20 40 60 80 100 120 140 160

minutes

(Reproduced from the M. Sc. Thesis, M. Nemat-Gorgani, 1972)

Fig. 23/4 The effect of the preliminary incubation of glutamate dehydrogenase with SDS and MLP at different detergent concentrations

% ACTIVITY

enzyme alone

2.5×10^{-4} M SDS

3×10^{-4} M SDS

MINUTES

% ACTIVITY

enzyme alone

1.25×10^{-3} M MLP

2.25×10^{-3} M MLP

MINUTES

(Reproduced from the M. Sc. Thesis, M. Nemat-Gorgani, 1972)

Fig. 25/6 The effect of the preliminary incubation of glutamate dehydrogenase with MLP at different detergent concentrations

% ACTIVITY

100

80

60

40

20

enzyme alone

1×10^{-3} M MLP

2×10^{-3} M MLP

40 80 120 160

MINUTES

% ACTIVITY

100

80

60

40

20

enzyme alone

1.5×10^{-3} M MLP

40 80 120 160

MINUTES

Fig. 27 The dependency of the initial rate of loss of activity of glutamate dehydrogenase on detergent concentration

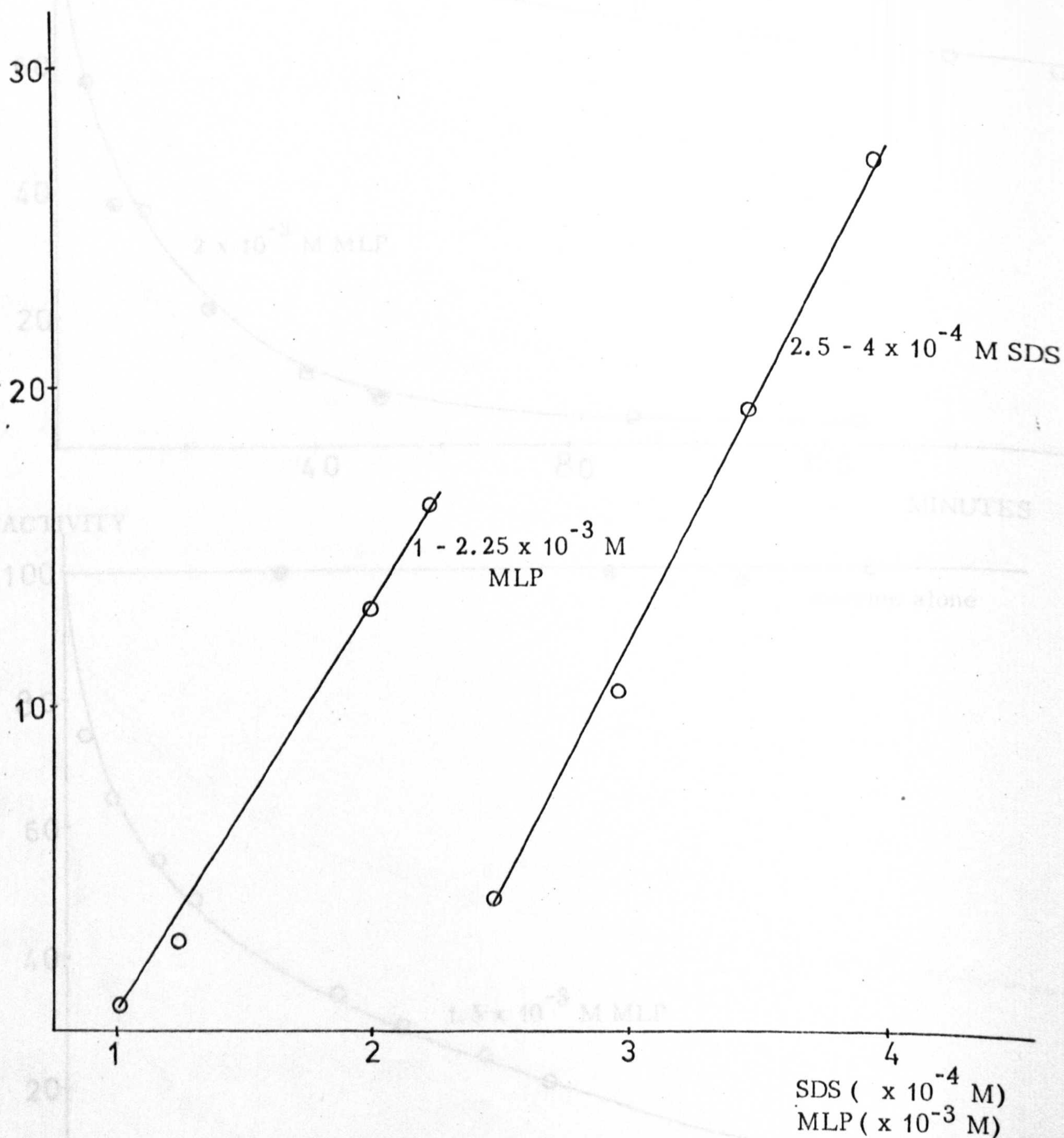
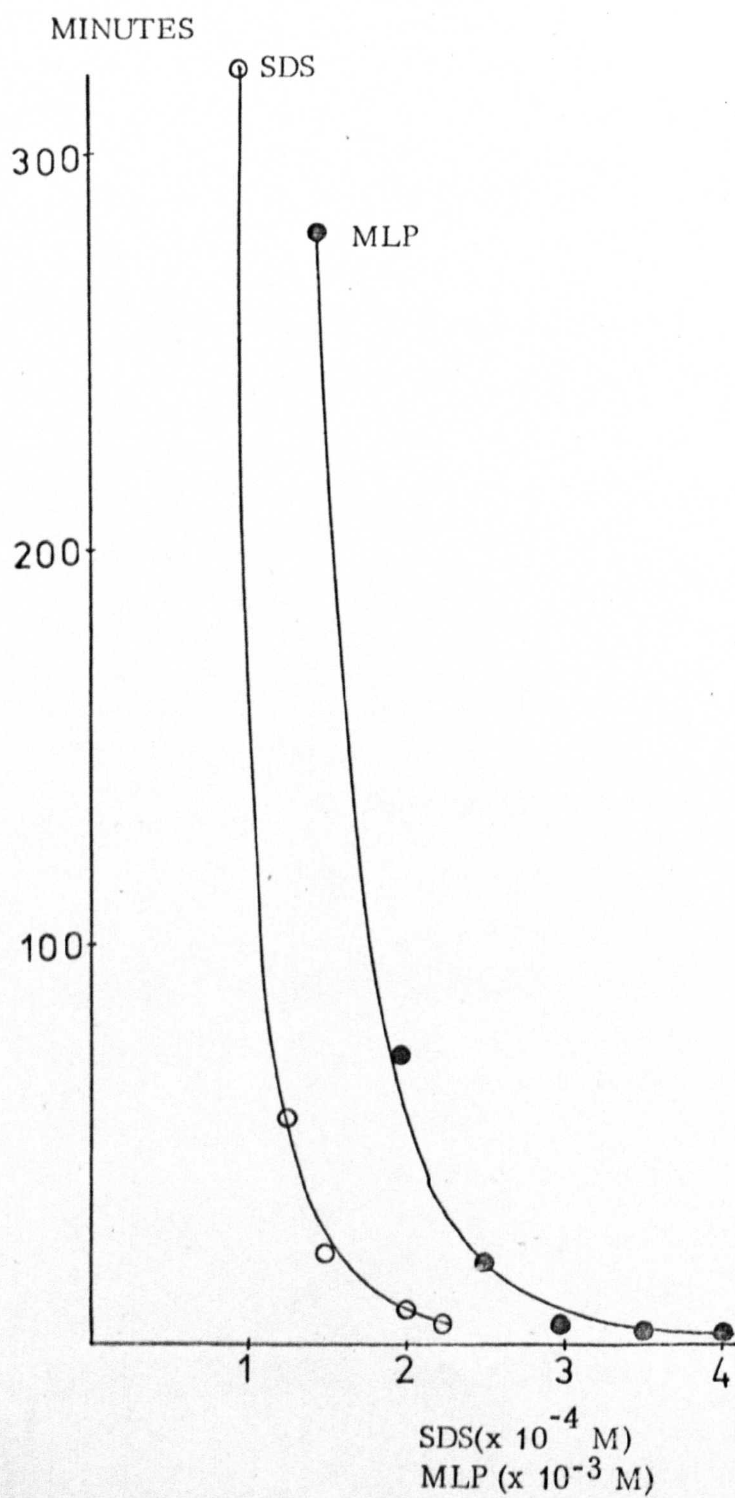


Fig. 28 The dependency of the time taken for 50% denaturation of glutamate dehydrogenase on detergent concentration



chain amphiphiles and the anionic phospholipids is that the former class of compounds can bring about an irreversible inhibition of the enzyme. In the case of SDS and MLP, it was found that the effect of the preliminary incubation of the detergent with the enzyme was concentration dependent. The same was found for the cationic detergent DBDA.

The enzyme-detergent complex formed at SDS concentrations up to 0.1 mM was dissociated on dilution and the enzyme activity was not altered. Higher concentrations of the detergent brought about time-dependent inhibition (Figures 21 - 26) and dialysis (in 0.1 M phosphate buffer, pH 8.0 +EDTA in the cold) showed that this was an irreversible process. The reversibility of the detergent inhibition under these conditions has been previously studied (Rogers and Stanley, 1969). Double reciprocal plots of enzyme velocity versus glutamate concentrations for glutamate dehydrogenase treated with low concentrations of SDS ($< 10^{-4}$ M) were made. The results indicated an uncompetitive inhibition mechanism.

MLP was found to be less effective (about one order of magnitude less) than SDS in bringing about this effect (Fig.21-6). CETAB and DBDA showed similar behaviour.

The initial rate of loss of activity of the enzyme increased sharply with increasing detergent concentration and was found to be a linear function of the detergent concentration over a limited range. This was $1 - 2.25 \times 10^{-3}$ M in the case of MLP and $2.5 - 4 \times 10^{-4}$ M in the case of SDS (Figure 27). The times taken for 50% denaturation vary with detergent concentration as shown in Figure 28.

Fig. 29 The effect of α-Kg and NADH on the rate of denaturation of glutamate dehydrogenase

[En] = 0.05 mg/ml

[SDS] = 1.5×10^{-4} M

[α-Kg] = 10 mM

[NADH] = 1×10^{-3} M

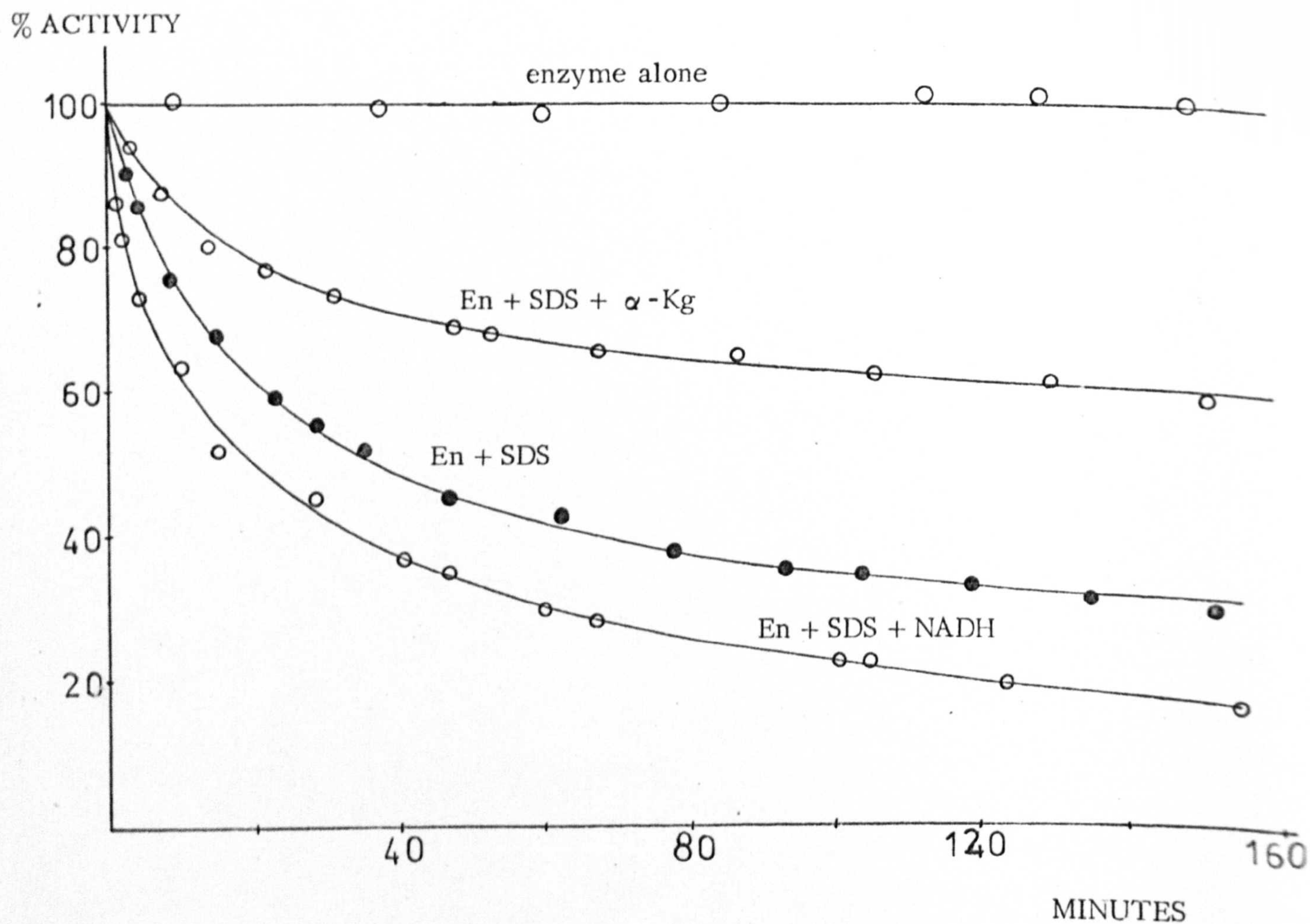
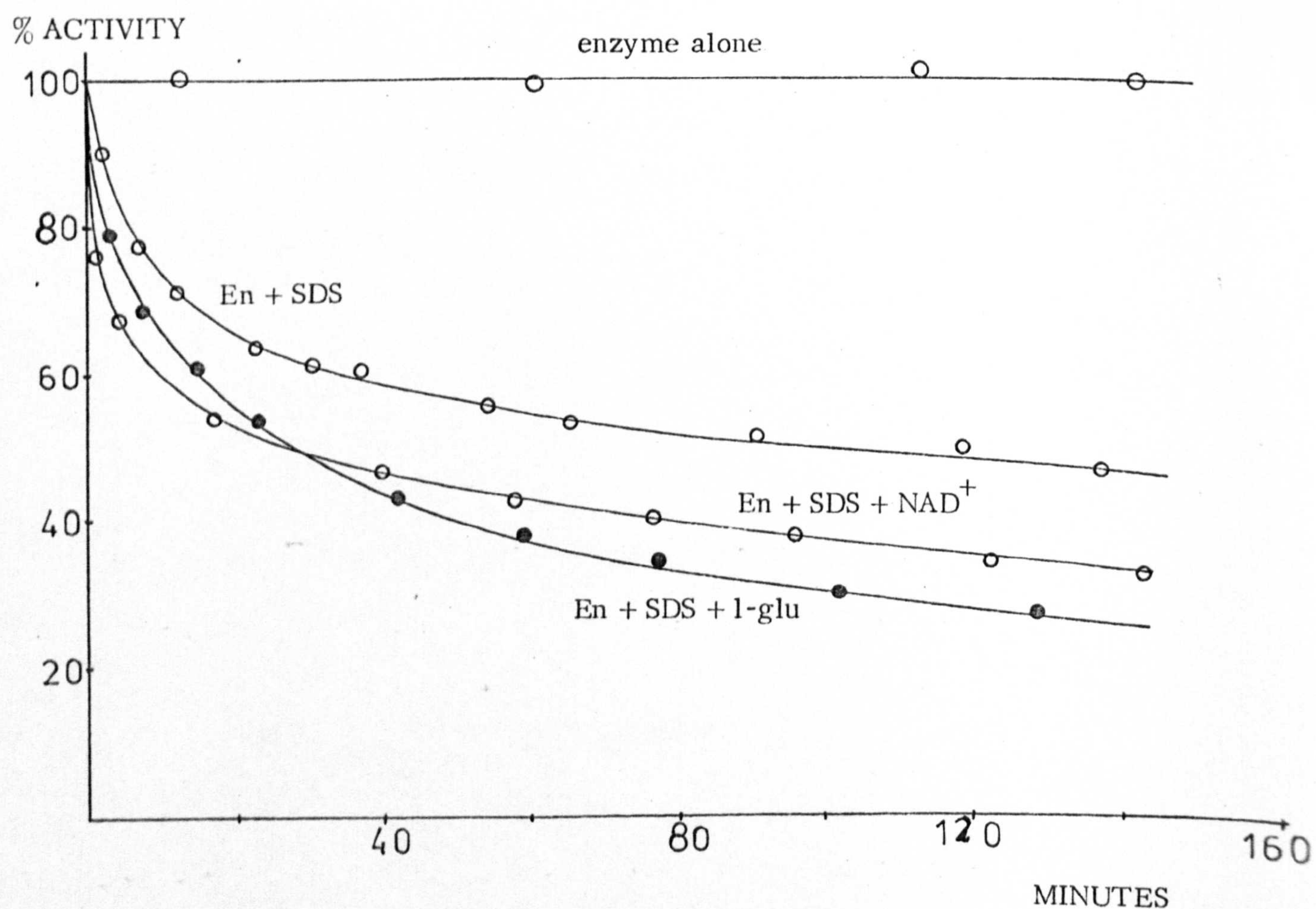


Fig. 30 The effect of l-glu and NAD^+ on the rate of denaturation of glutamate dehydrogenase

[En]	=	0.05 mg/ml
[SDS]	=	1.5×10^{-4} M
[l-glu]	=	20 mM
[NAD^+]	=	2 mM



Dialysis experiments (in 0.1 M phosphate buffer, pH 8.0) with detergent concentrations of 0.2 mM and 0.5 mM showed that the effect was an irreversible process. At the higher detergent concentration the enzyme lost all its activity after incubating the mixture for 5 minutes and failed to regain any activity after dialysis. At detergent concentration of 0.2 mM, 37% inhibition was obtained after incubation for a period of 30 minutes. When the mixture was rapidly dialysed at this point, no further loss of activity was found.

The cationic detergents also induced a time-dependent irreversible loss of activity of the enzyme when the two were incubated together and the kinetic curves resembled those obtained for the anionic detergents. Generally, in this type of study, SDS is chosen as a model system since its properties in aqueous solutions are well understood and the variation of monomer and aggregate concentration with such external parameters as ionic strength (Mysels and Princen, 1959) and temperature (Goddard and Benson, 1957) is known precisely.

The substrate α-Kg slowed down the rate of denaturation, but did not abolish it (Figure 29). The time taken for the enzyme to reach 50% denaturation was found to increase from 30 minutes to 370 in the presence of 10 mM α-Kg at SDS concentration of 1.5×10^{-4} M. NADH, l-glutamate and NAD^+ increased the rate slightly (Figure 30).

It has previously been shown that α-Kg protects glutamate dehydrogenase against inactivation by 4-Iodoacetamidosalicylic acid (Malcolm and Radda, 1968) and 2, 4, 6-Trinitrobenzenesulphonic acid (Freedman and Radda, 1968). In this case, the authors concluded that modification was probably occurring at the active site. In our case, however, it is difficult to make such conclusions

Fig. 31 and 32 First order plots of MLP and SDS effects on GDH activity (preliminary incubation)

% loss of activity

GDH at final concentration of 0.05 mg/ml

$+1.5 \times 10^{-3}$ M MLP

$+1.25 \times 10^{-3}$ M MLP

1
9
8
7
6
5
4
3
2

40 80 120 160

MINUTES

% loss of activity

$+2.5 \times 10^{-4}$ M SDS

$+2 \times 10^{-4}$ M SDS

1
9
8
7
6
5
4
3
2

40 80 120

MINUTES

as the effect of these metobolites could well occur through conformational changes in the enzyme, which are denatured at different rates by SDS. Thus, the type of conformation may determine the rate (extent) of denaturation. It is also interesting to note that high NAD^+ concentrations have been shown to stabilize the enzyme against urea inactivation, whereas low NAD^+ concentrations increase the rate of urea inactivation (Di Franco, 1971). Aleo Friden (1963) found that low concentrations of NADH and NADPH caused an increase in the rate of denaturation in tris-acetate buffer, while higher concentrations of these coenzymes stabilized the enzyme.

Frequently, it has been observed in the literature that the heat stability of particular enzymes is influenced by the presence of the substrate for the enzymic reaction. In most cases, the substrate causes increased stability of the enzyme, but cases of substrate-induced instability have also been observed (Caravaca and Grisolia, 1959). For beef liver glutamate dehydrogenase, it was first shown by Inagaki (1959) that the enzyme was inactivated more rapidly in the presence of NADH than in its absence.

At certain detergent concentrations, the process of denaturation showed first order kinetics with a number of clearly defined steps (Figures 31 and 32). In other cases, attempts to fit the data to equations for a first or second order process proved unsuccessful. The process was hence more complex.

The first portions of these curves indicate rapid denaturation caused by interaction between detergents and the enzyme followed by slower charges. These charges were proportional to the detergent concentration. Similar findings have been reported on the interaction between SDS and carboxypeptidases A and B (Nakagama and Jirgensons, 1973).

Fig. 33 The effect of NH_4Cl on the extent of inhibition of glutamate dehydrogenase activity by SDS

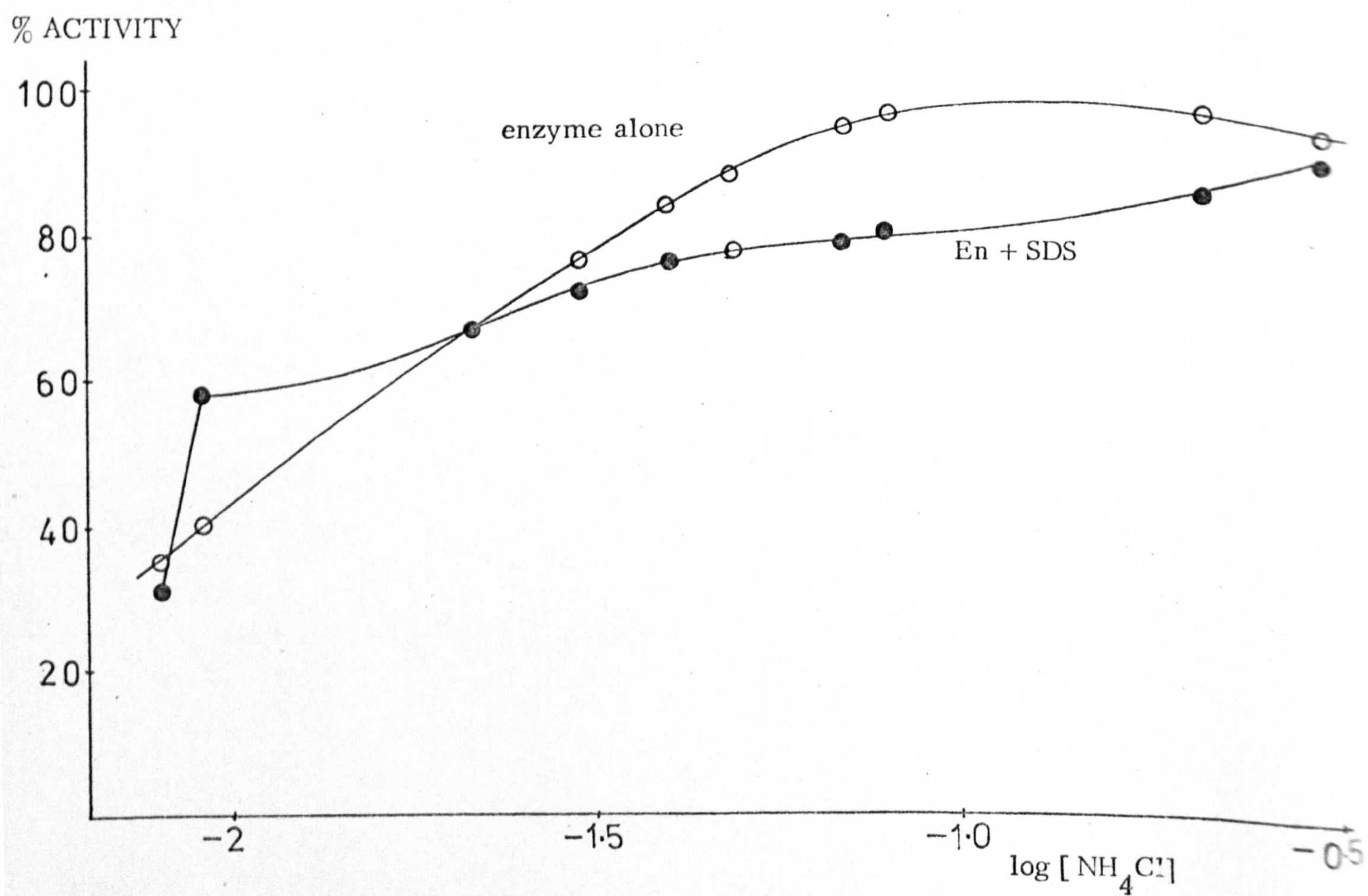
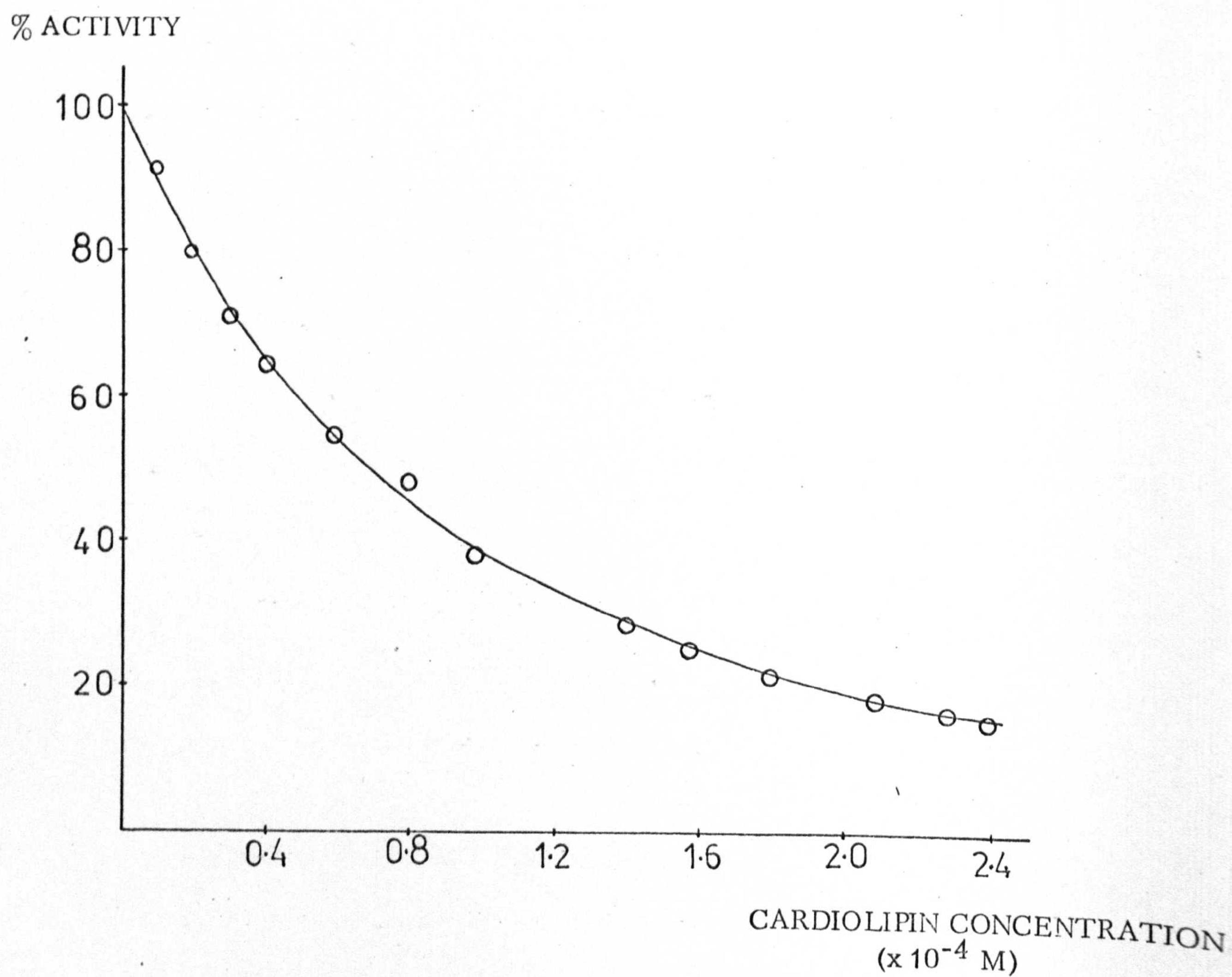


Fig. 34 The effect of cardiolipin on alanine dehydrogenase activity of GDH



Inhibition of glutamate dehydrogenase activity diminished with increasing ionic strength, suggesting a strong electrostatic component in the association (Figure 33).

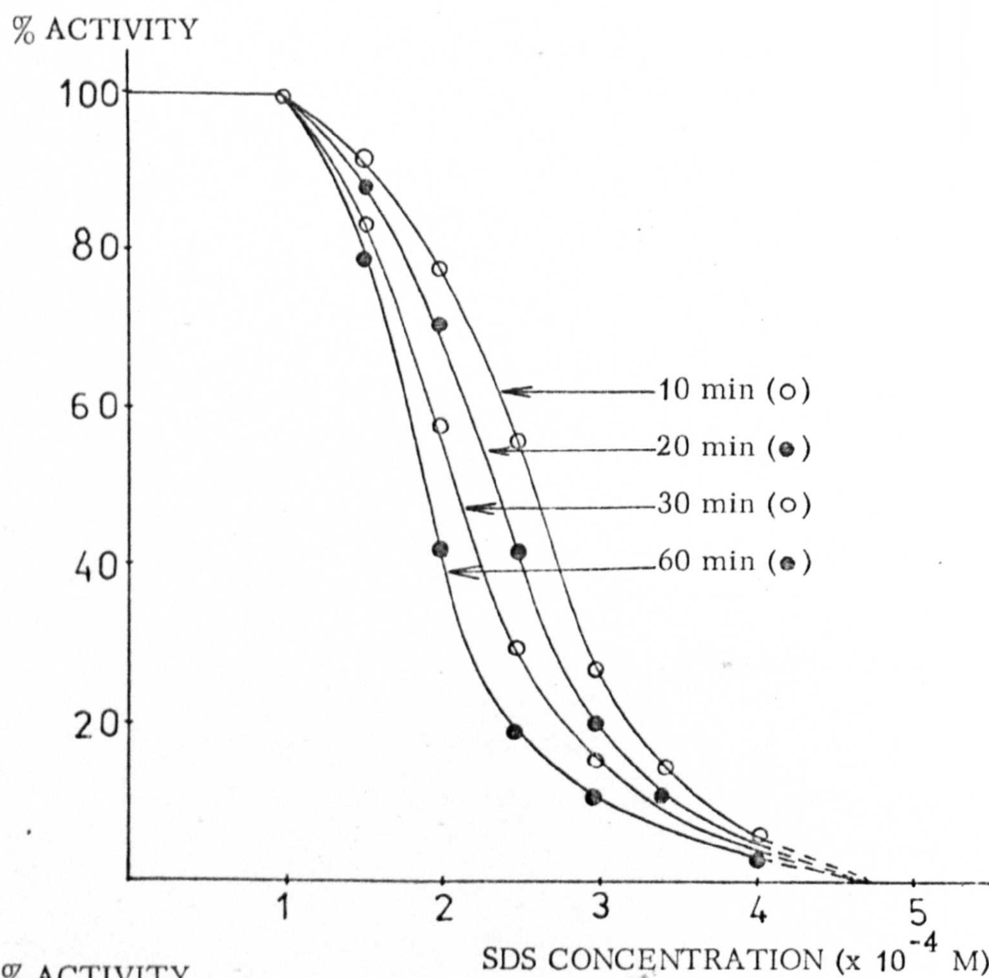
The alanine dehydrogenase activity of the enzyme was affected by CL in the same way (Figure34). Also, both SDS and MLP inhibited this activity of the enzyme. SDS at concentration of 5×10^{-5} M caused 41% inhibition while MLP brought about 49% inhibition at 2×10^{-4} M. Neither n-hexane sulphonate nor lysolecithin affected enzymic activity at concentrations up to 10^{-3}M.

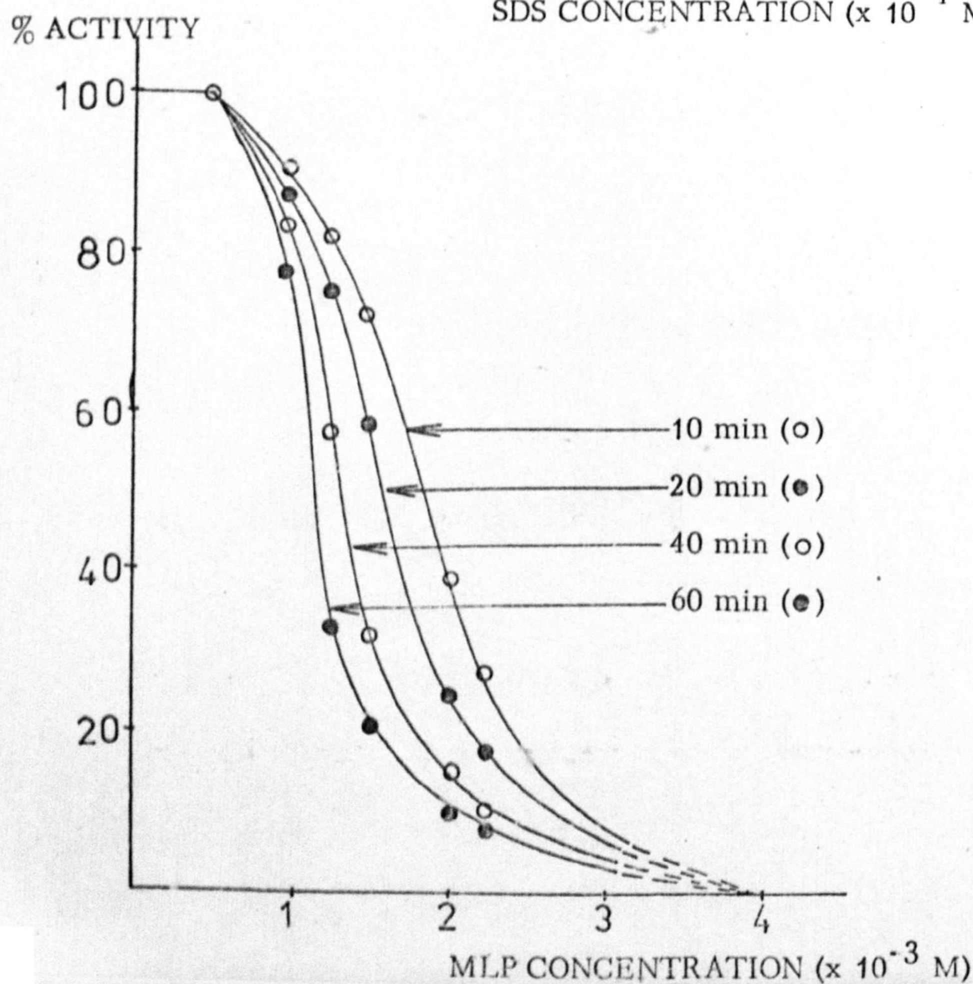
In aqueous solutions, SDS can exist as monomer and as micellar aggregates, the concentration of each depending upon the total SDS concentration, the ionic strength, and the temperature. An important property of the system is that increasing the total concentration at a given ionic strength will not result in a measurable increase in monomer concentration above a specific critical value, the critical micelle concentration. Above this concentration, each new SDS molecule added to the system is incorporatec into a micelle. The critical micelle concentration decreases with increasing ionic strength at constant temperature. Thus, it is possible to vary the monomer and micellar composition by simply altering the ionic strength. Thus, the interaction of both SDS species with a protein can be studied.

Reynolds and Tanford (1970) studied the binding of SDS to a variety of proteins at high binding ratios. The ionic strength was varied from 0.05 to 0.52 and the total concentration of SDS was in the range of 0.5 to 4.6 mM. The equilibrium monomer concentration (cf., is below the critical micelle concentration) based on the ionic strength dependence of the critical micelle concentration of SDS was in the range between 0.5 and 3.58 mM.

(Reproduced from the M. Sc. Thesis, M. Nemat-Gorgani, 1972)

Fig. 35 and 36 Plots of activity of SDS and MLP treated enzyme against different detergent concentrations at different times of detergent treatment





It was found that increasing the total concentration or the micellar concentration did not result in an increase in the binding ratio (of bovine serum albumin, chymotrypsinogen, ovalbumin and lysozyme). However, an increase in the equilibrium monomer concentration led to a larger value of g SDS/g protein.

The plot of the binding ratio as a function of the equilibrium monomer concentration was found to have two plateau regions resulted from a sudden drop in the binding ratio with the equilibrium monomer concentration.

Figures 35 & 36 show plots of activity of SDS and MLP treated enzyme against different detergent concentrations at different times of detergent treatment. They resemble those obtained by Reynolds and Tanford in that the curves have all two plateau regions. Figure 27 shows that at least over a limited range, the initial loss of activity is a linear function of the detergent concentration. Hence, it is reasonable to compare the two systems (equilibrium binding and kinetic) and suggest that the concentration at which this sudden drop in activity is occurring (Figures 35 - 36) is in fact a value related to the equilibrium monomer concentration.

Comparison of Figures 35 & 36 indicates that the concentration at which the sharp drop in activity occurs is about one order of magnitude lower in the case of SDS than it is in the case of MLP. The reason for this is that the charge density on the sulphate group would be expected to be lower than that on the phosphate group. The electrostatic repulsive forces in the latter case would be higher and this would cause a higher value for the critical micelle concentration. The charge density in both cases, of course, depends on the

pH and the ionic strength of the medium.

Reynolds and Tanford made the following conclusions from their studies on binding of SDS with various proteins:

a) The binding of large amounts of SDS to protein is primarily hydrophobic in nature.

b) The effect of ionic strength is entirely on the concentration of free SDS monomer.

c) Micelles do not bind to the proteins investigated.

As mentioned above, n-hexane sulphonate does not form micelles. The reason for this is that micelle formation requires the existence of two opposing forces, the hydrophobic force favouring aggregation, and a repulsive force that prevents growth of the aggregates to larger size. In hexane sulphonate, because of its short hydrocarbon chain, the hydrophobic force is not sufficient to cause micelle formation.

In general, association between proteins and ligands can only occur if the free energy gained by association with the protein exceeds the free energy gained by other processes to the ligand molecule (Tanford, 1972). In this case, therefore, micelle formation and association with the protein represent competitive phenomenon.

The standard free energy charge of micellization is given by:

$$\mu^{o}\text{mic.} - \mu^{o}\text{w} = RT\ 1n^{(1)}\ CMC$$

where μ^{o}mic. and μ^{o}w are standard free energy of the

amphiphilic molecule in a micellar structure and free in aqueous solution, respectively.

The standard free energy change for the binding of the amphiphilic molecule to the protein is given by $\mu^o p - \mu^o w$ where $\mu^o p$ is the free energy of the molecule when bound to the protein.

Thus, the binding of an amphiphilic molecule to a protein takes place if $\mu^o p - \mu^o w$ is smaller than $\mu^o mic - \mu^o w$. The former values have been found to be more negative than the latter ones.

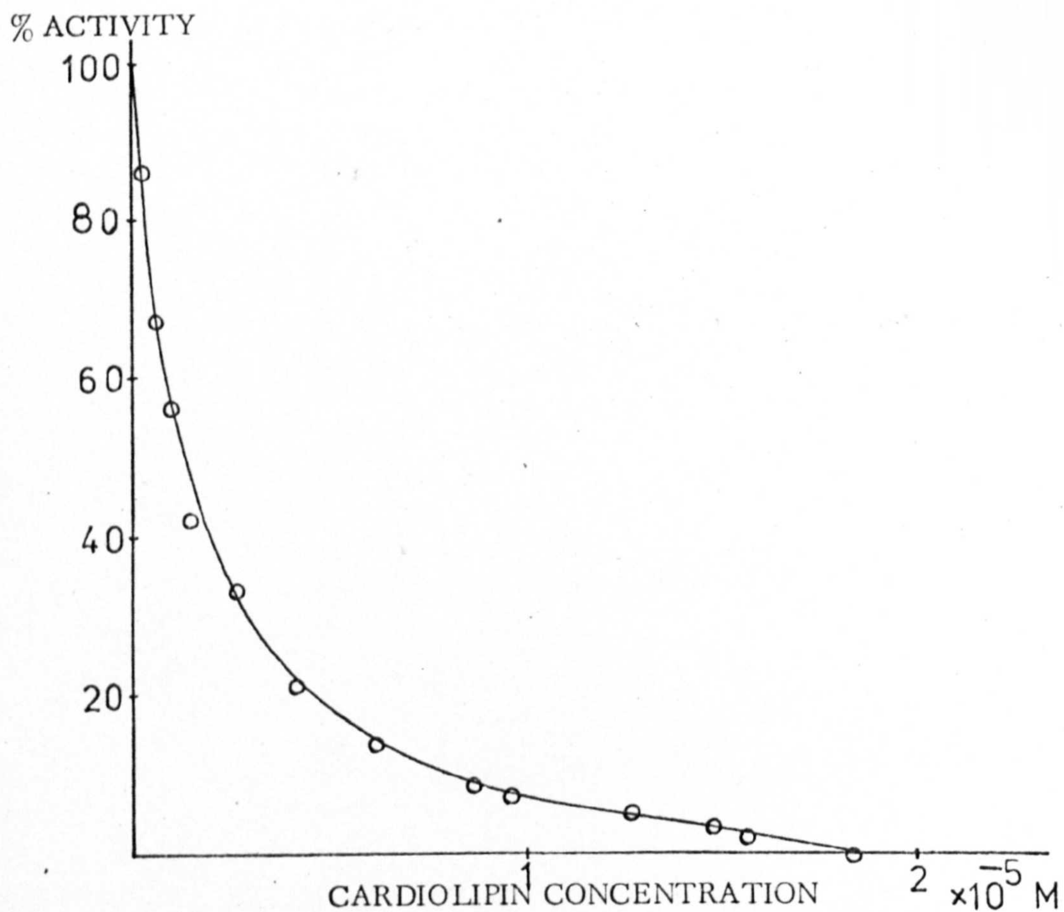
In our studies, the process of enzyme inhibition may be considered as taking place in two stages:

1) Complex formation between the enzyme and the detergent molecules.

2) Conformational changes occurring in the enzyme with loss of activity.

For the first step, it has been found that both anionic and cationic amphiphiles are effective but the zwitterionic amphiphiles such as lysolecithin are most effective. In this case, the process of complex formation may be firstly due to the interaction of the hydrophilic residues in the enzyme (such as the ϵ-amino groups of lysine residues) with the polar head groups of the amphiphiles. This may be followed then by hydrophobic or Van der Waals interaction between the hydrophobic residues in the enzyme and the hydrocarbon chains in the detergent molecules. The process may thus be considered to take place in two stages, and the high negative free energy charge for $\mu^o p - \mu^o w$ may have contributions from the two types of binding.

Fig. 37 and 38 The effect of cardiolipin, phosphatidylserine and phosphatidylcholine on glutamate dehydrogenase activity of GDH



The specific attraction for the hydrophilic head groups may thus be considered to be the primary and major factor with the hydrophobic binding being the secondary factor. Indeed, it is quite possible that the interaction of the head groups of the amphiphiles with the polar residues in the enzyme exposed to the aqueous medium cause some conformational changes in the enzyme with the hydrophobic residues being exposed as the result. In this type of situation, the hydrophobic binding could be an important secondary process and could make extensive contributions to the free energy change. It is quite possible that an amphiphile such as n-hexane sulphonate does bind to the enzyme, but because of its short hydrocarbon chain, the free energy change due to the hydrophilic interactions is not high enough.

The interaction of phospholipids with the enzyme was found to resemble that of the simpler amphiphiles in that the charge type of the head group determines the extent of complex formation. Since the phospholipids of the cellular membranes have either zwitterionic or anionic head groups, members of these groups were studied. Beef heart cardiolipin and beef brain phosphatidylserine inhibited the enzyme strongly (Figures 37 - 38) with apparent Ki in the range of 1-2 μm and 3-5 μm respectively in the direction of reductive amination.

Sonication of phospholipid dispersions were carried out as described in the previous chapter. 0.1 M phosphate buffer, pH 8.0 was used as the sonication medium. The effect of ionic strength and pH on sonication of phosphatidylserine was studied. It was found that the extent of inhibition of the enzyme varies with preparations of phosphatidylserine in phosphate buffers of different molarities but not of different pH values in the range of 6.5 - 9.0. Very small differences in the sonication conditions such as size of the probe,

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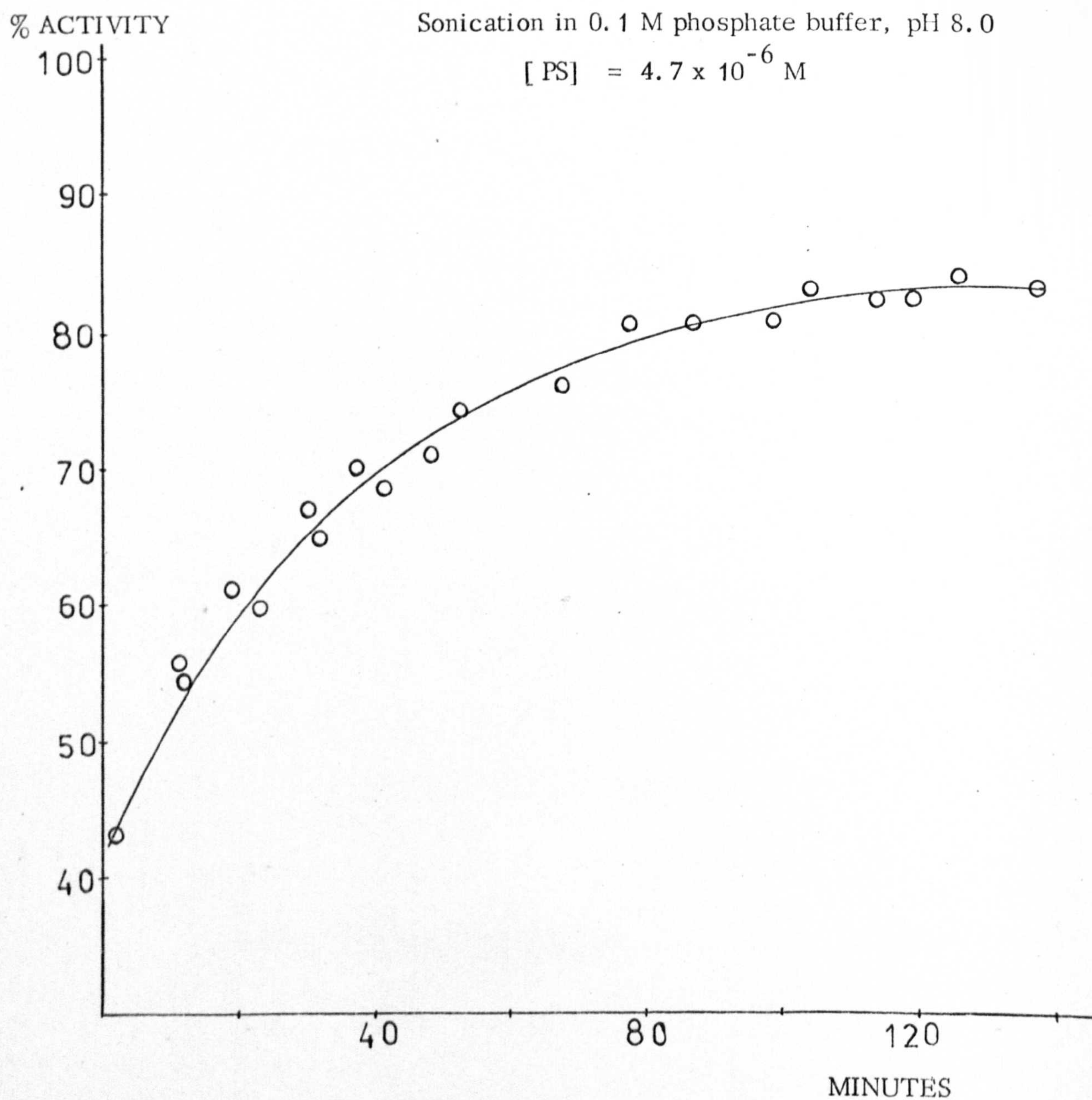
Fig. 39 The time dependency effect of phosphatidylserine sonicated in phosphate buffer



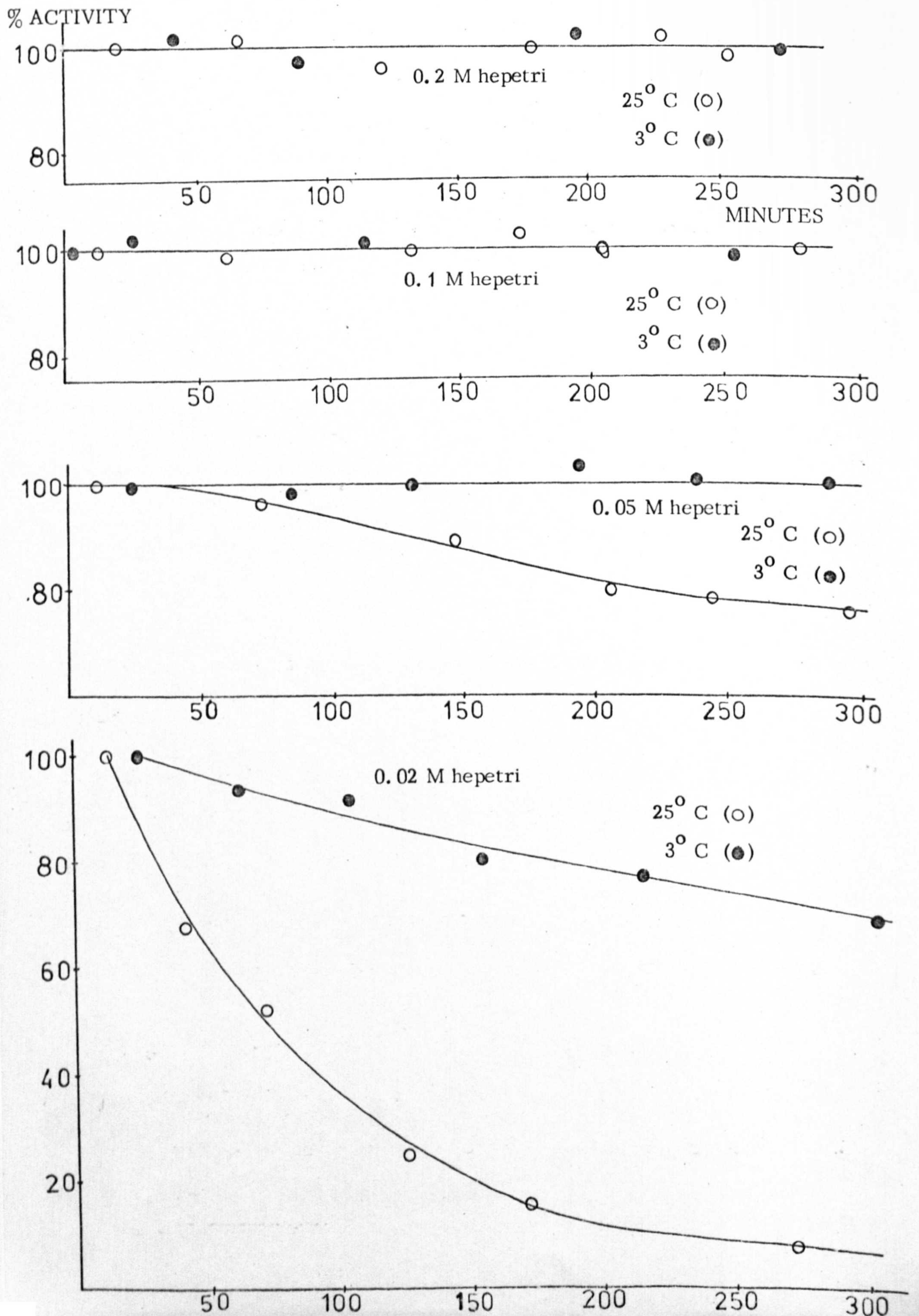
volume of the dispersion, lipid concentration presence of organic solvent, time of sonication and temperature were found to have profound effects on the nature of the sonicated product.

Phospholipids may undergo two forms of degradation in an aqueous system. Hydrolysis may occur at the β-ester linkage with the production of lysophosphatide and free fatty acids, or the unsaturated hydrocarbon chains may be oxidised. We checked for the possibility of degradation using thin layer chromatography, as suggested by Skidmore and Entenman (1962). No lipid decomposition products were formed under our sonication conditions.

To determine the extent to which the sample had been oxidised, the increase in absorption at 233 nm due to increasing diene conjugation was measured (Klein, 1970). Sen et al (1956) investigated the autoxidation of phosphatidylethanolamine and phosphatidylcholine and has shown that changes occur in the ultraviolet spectrum at 235 nm and at 270 - 280 nm on exposure to oxygen. The former charge reflecting the increase in conjugated diene hydroperoxides, we checked carefully for the production of hydroperoxides during the sonication process. The degradation of lipid hydroperoxides leads to the production of lipid dicarbonyl compounds, which are potent cross-linking agents and lead to irreversible enzyme inhibition. Our observed inhibition was not due to lipid decomposition products.

The inhibitory effect of phosphatidylserine sonicates (in 0.1 M phosphate buffer, pH 8.0) were studied at different time intervals and it was found that this effect greatly diminished with time (Figure 39). Sonication after diminution of inhibitory power failed to bring back the original property of the lipid. Subsequently, other buffers were tried as sonicating media and it was found

Fig. 41 to 44 Stability of glutamate dehydrogenase in hepetri buffer of different molarities (pH 7.7) at 3°C and at 25°C



that tris and hepes (N-2-hydroxyethylpiperazine N'-2-ethane sulphonic acid) buffers were much better media for sonication of the lipid. As indicated in Figure 40, the time dependency observed is much less when the sonicates are made in these buffers. A mixture of three buffers - hepes, pipes (piperazine-N, N'-bis(2-ethane sulphonic acid)) and tris, each at 0.02 M concentration, pH 7.7 (+ 1×10^{-4} M EDTA) was found to be a good sonication medium. A mixture of these three buffers which cover a pKa range of 6.8 (pipes) to 8.3 (tris) was chosen so that the effect of pH on enzyme-lipid complex formation would be studied without changing the buffer system. The time dependency effect was found to be very low in this system (Figure 40), which was used for all the subsequent sonications. Stability of the enzyme in hepetic buffer of different molarities at 0°C and 25°C was investigated and the results are shown in Figures 41 to 44.

This time-dependency effect may be because of the special property of hepes, tris and pipes in not binding any metal ions. The metal-buffer binding constants obtained for these buffers for Mg^{++}, Ca^{++}, Mn^{++} and Cu^{++} were found to be negligible in all cases (Good et al, 1966). However, binding of the metal ions and other cations in solution may take place by phosphate radicals in solution and this causes a different ionic environment for the phospholipid bilayer. As discussed in the previous chapter, the ionic environment of phospholipids is of fundamental importance to their physico-chemical properties.

According to Ohki and Aono (1970), the effect of change on the bilayer in extending the surface area of a lipid structure can be calculated from the relative free energy charge Grel over the extension, from the equation:

(see next page) .....

Fig. 40 The time dependency effect of phosphatidylserine

(Reproduced from the M. Sc. Thesis, M. Nemat-Gorgani, 1972)

Sonication in : Tris-HCl (0.02 M, pH 7.7) (I)
Hepes (0.02 M, pH 7.7) (III)
Hepetri (0.06 M, pH 7.7) (II)

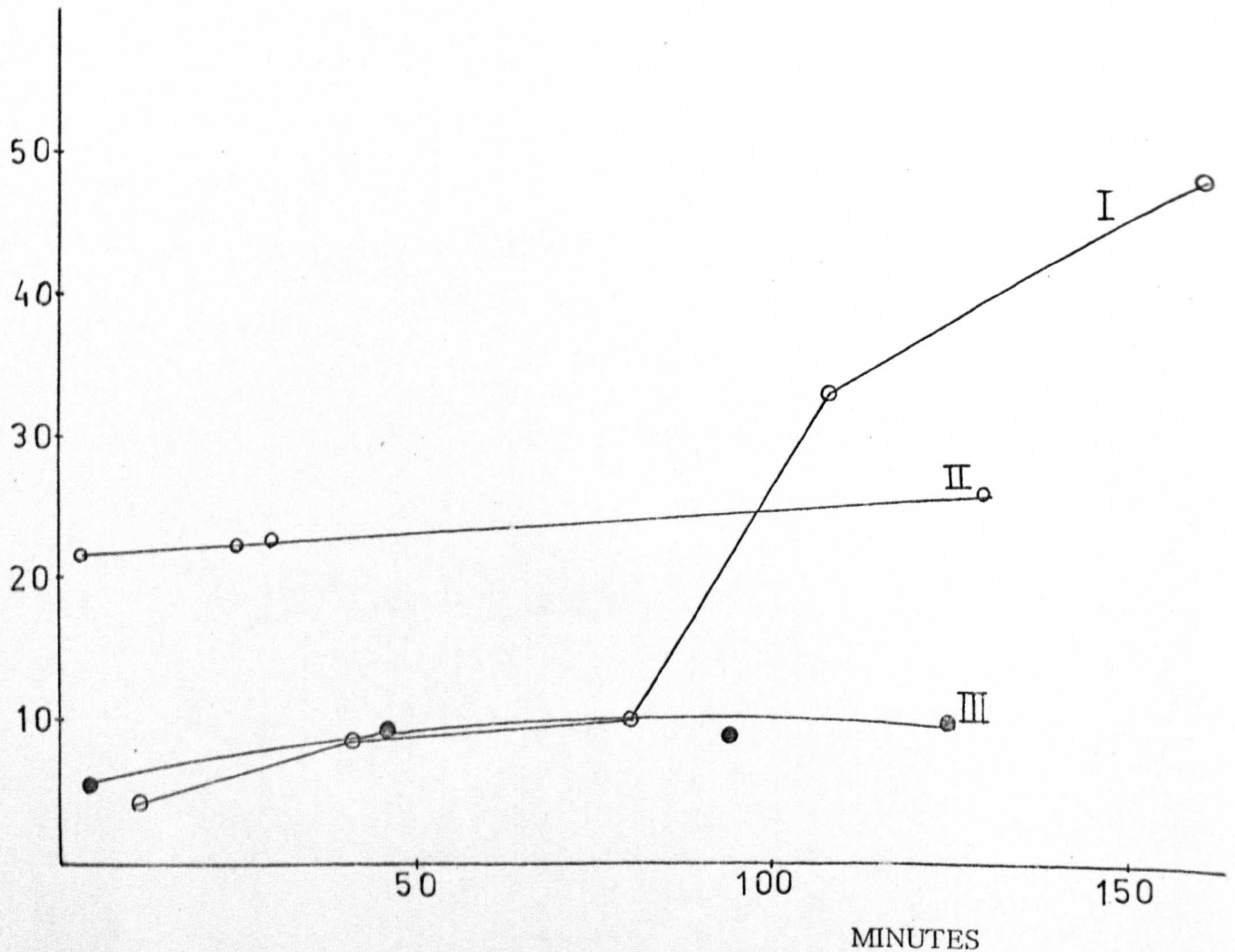
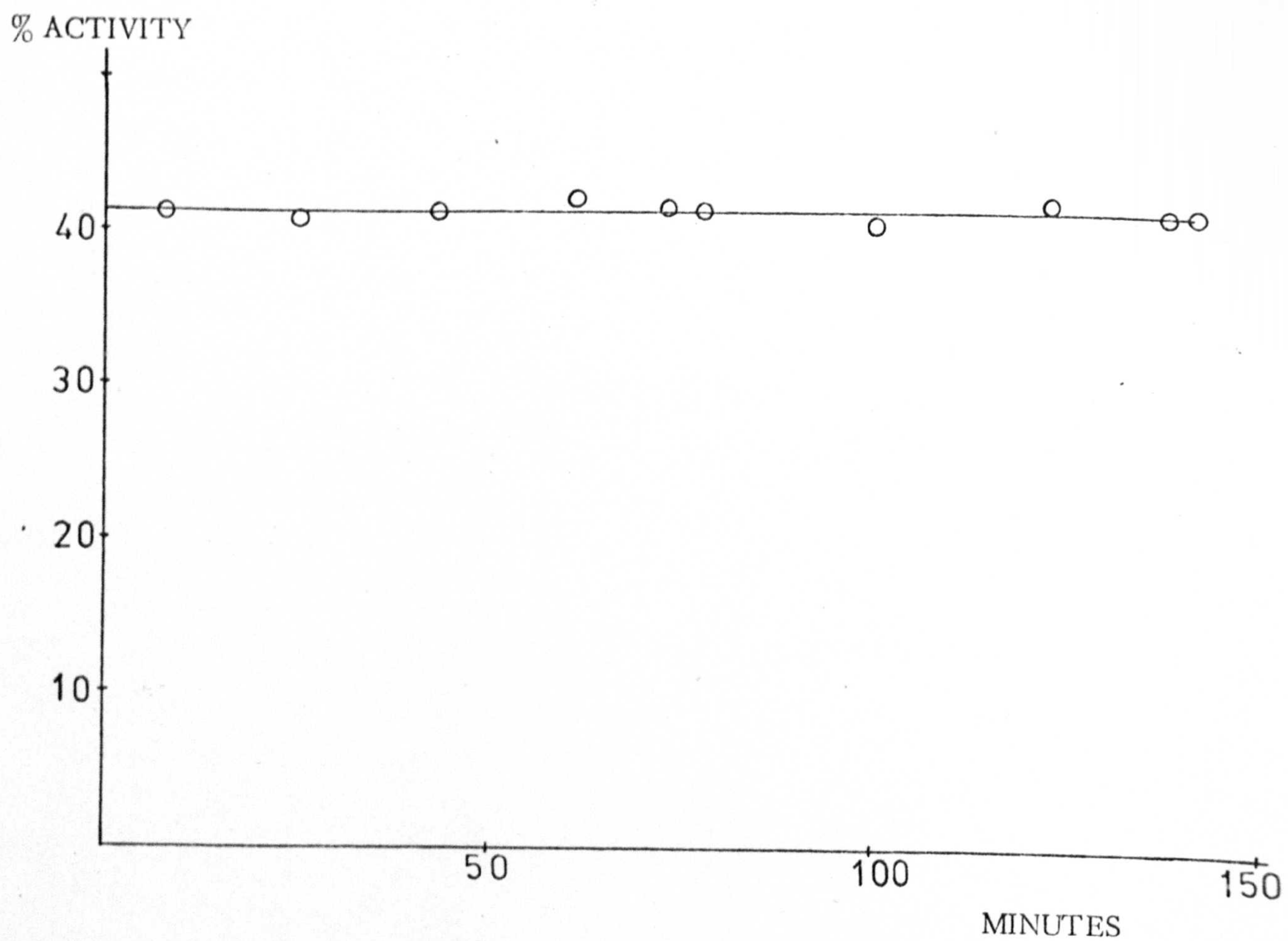


Fig. 45 The dependency of the extent of inhibition of GDH by cardiolipin on the time of incubation



$$Grel = 8 (A - Ao) + \frac{2 \pi e^2 \delta^2}{\varepsilon o K A}$$

where Ao is the molecular area per molecule before extension (in A^{o2}), and A is the area per molecule after extension (in A^{o2}), εo the static dielectric constant of the surrounding water, δ the degree of ionisation of the polar groups, K the Debeye-Huckel constant, e the electronic charge (kn e.s.u.) and 8 the surface tension (in dyne/cm) after expansion. As δ increases, in other words, as the net charge per molecule rises, the relative free energy of the bilayer rapidly increases. For the bilayer to be a stable structure, it is essential that the charge per molecule is below 1.25 e (Ohki and Aono, 1970). Thus, it may be argued that in phosphate buffer, this condition is not met.

The fact that sonication of the phospholipid vesicles did not restore the original inhibitory power of the lipid probably indicates that aggregation did not occur. Fusion would be one of the candidates for the process of aggregation. However, the possibility of a conformational change in the state of the lipid bilayer from a Pex to a Pin conformation (refer to the previous chapter) cannot be ruled out. The availability of the negatively charged head groups of the lipid is essential for complex formation.

When the anionic phospholipids (phosphatidylserine or cardiolipin) were incubated with the enzyme at 25^{0}C (Figure 45) and aliquots were assayed for enzyme activity at different periods of time, it was found that the extent of inhibition was not affected by the time of incubation. Thus, the equilibrium in the reversible formation of the complex at this temperature was formed rapidly.

Fig. 46 Lineweaver-Burk plots of glutamate dehydrogenase inhibition by cardiolipin at different NAD^+ concentrations

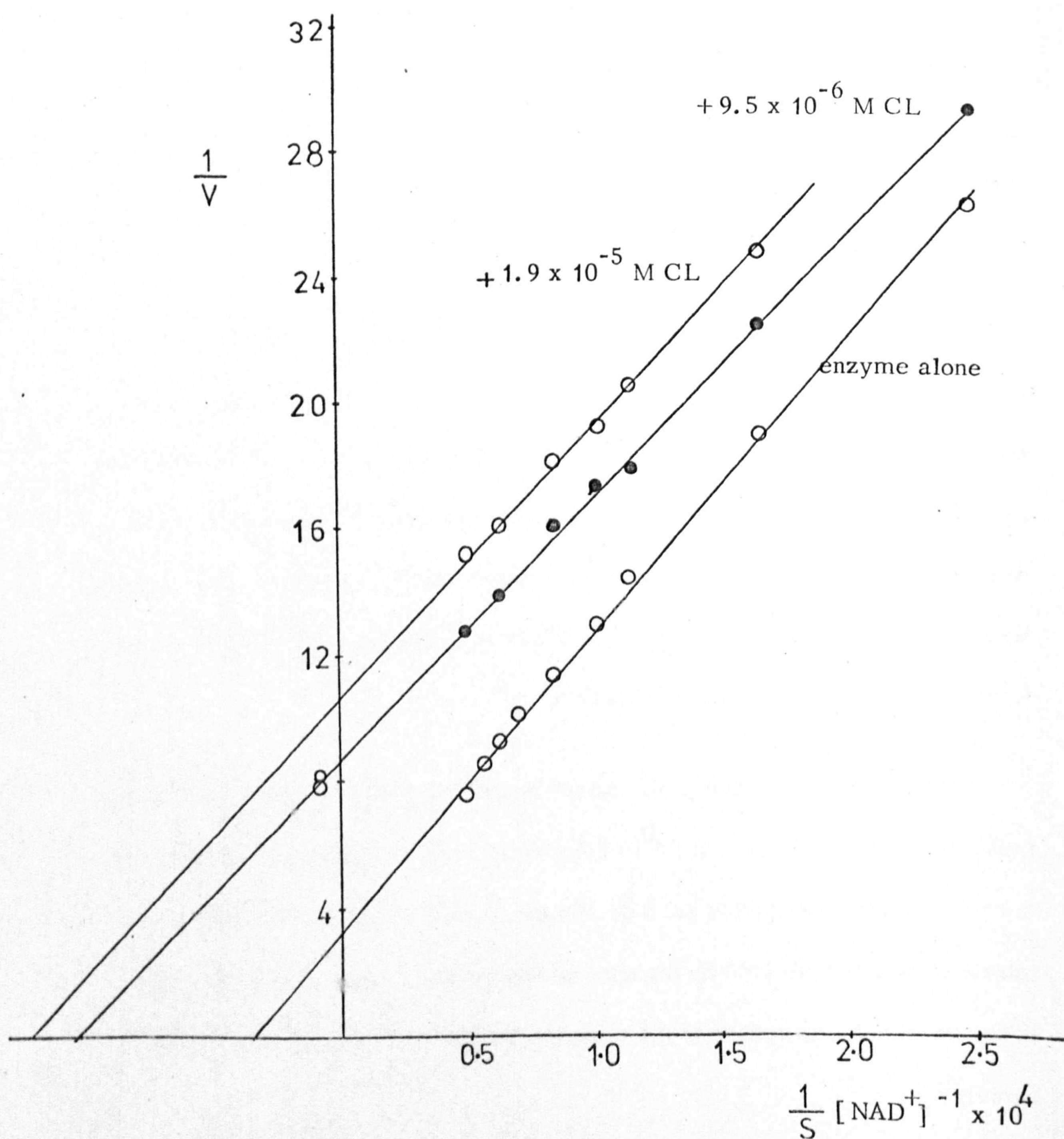


Fig. 47 Lineweaver-Burk plots of glutamate dehydrogenase inhibition by cardiolipin at different α-Kg concentrations

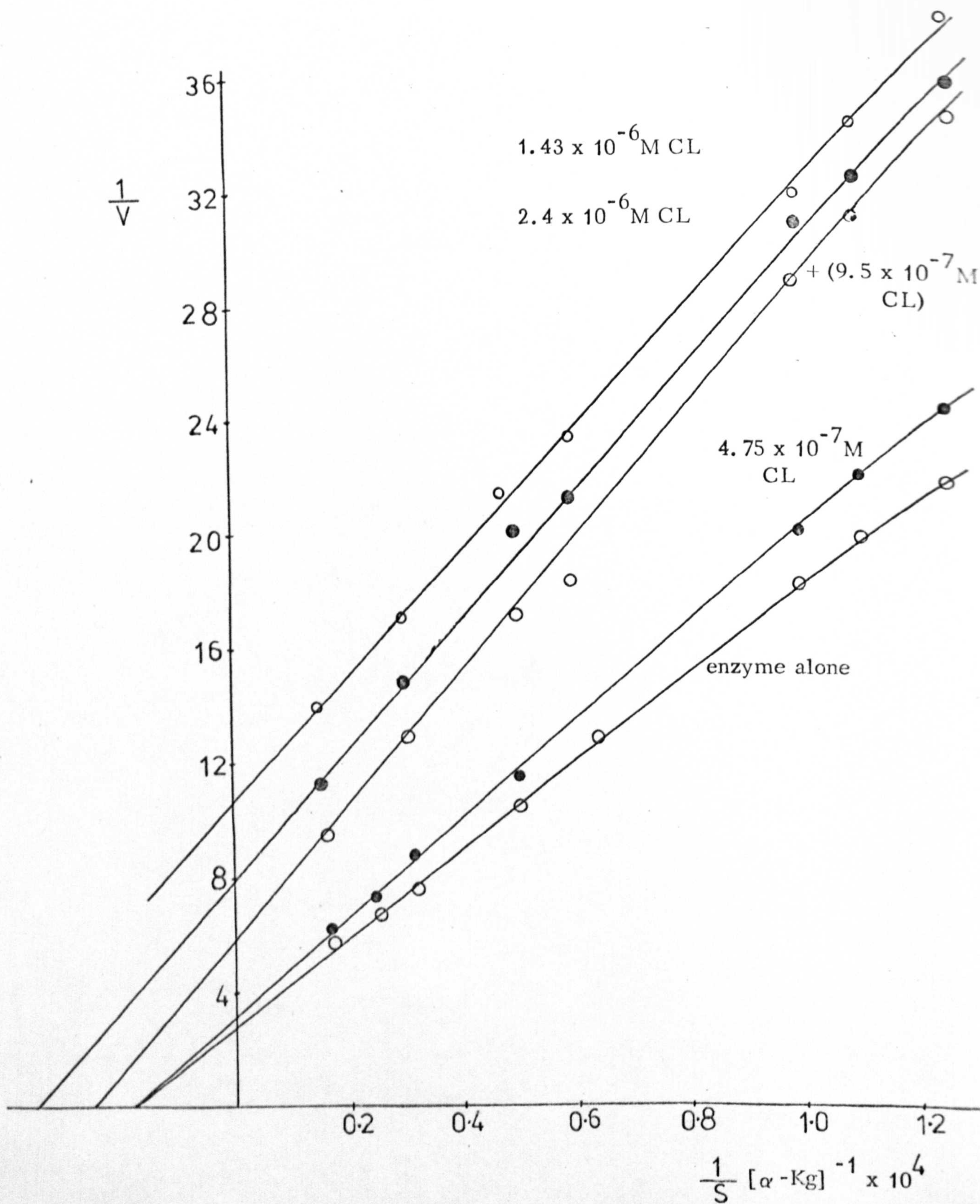


Fig. 48 Lineweaver-Burk plots of glutamate dehydrogenase inhibition by cardiolipin at different l-glu concentrations

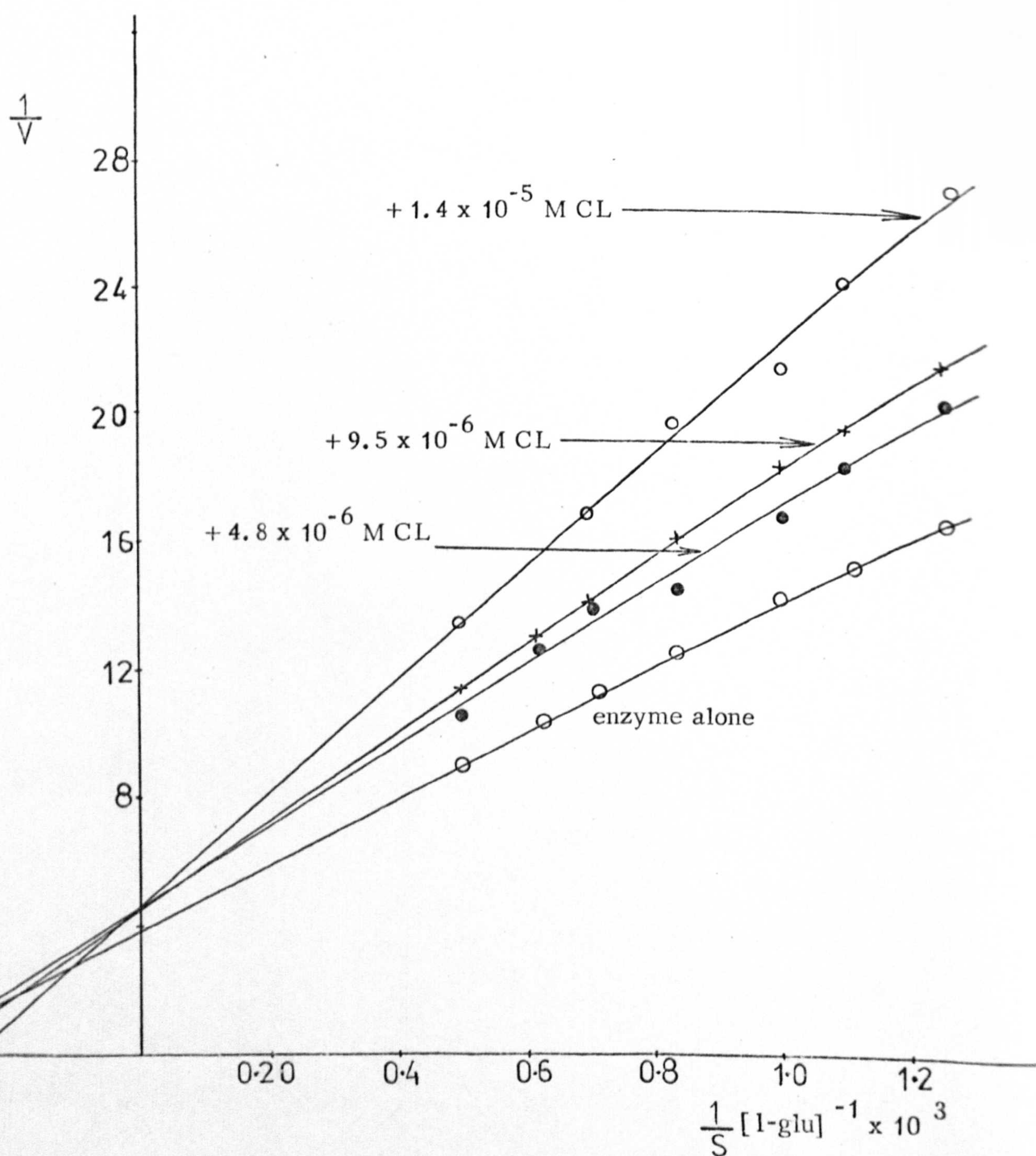


Fig. 49 The dependency of the extent of inhibition of GDH activity on PC/CL ratio

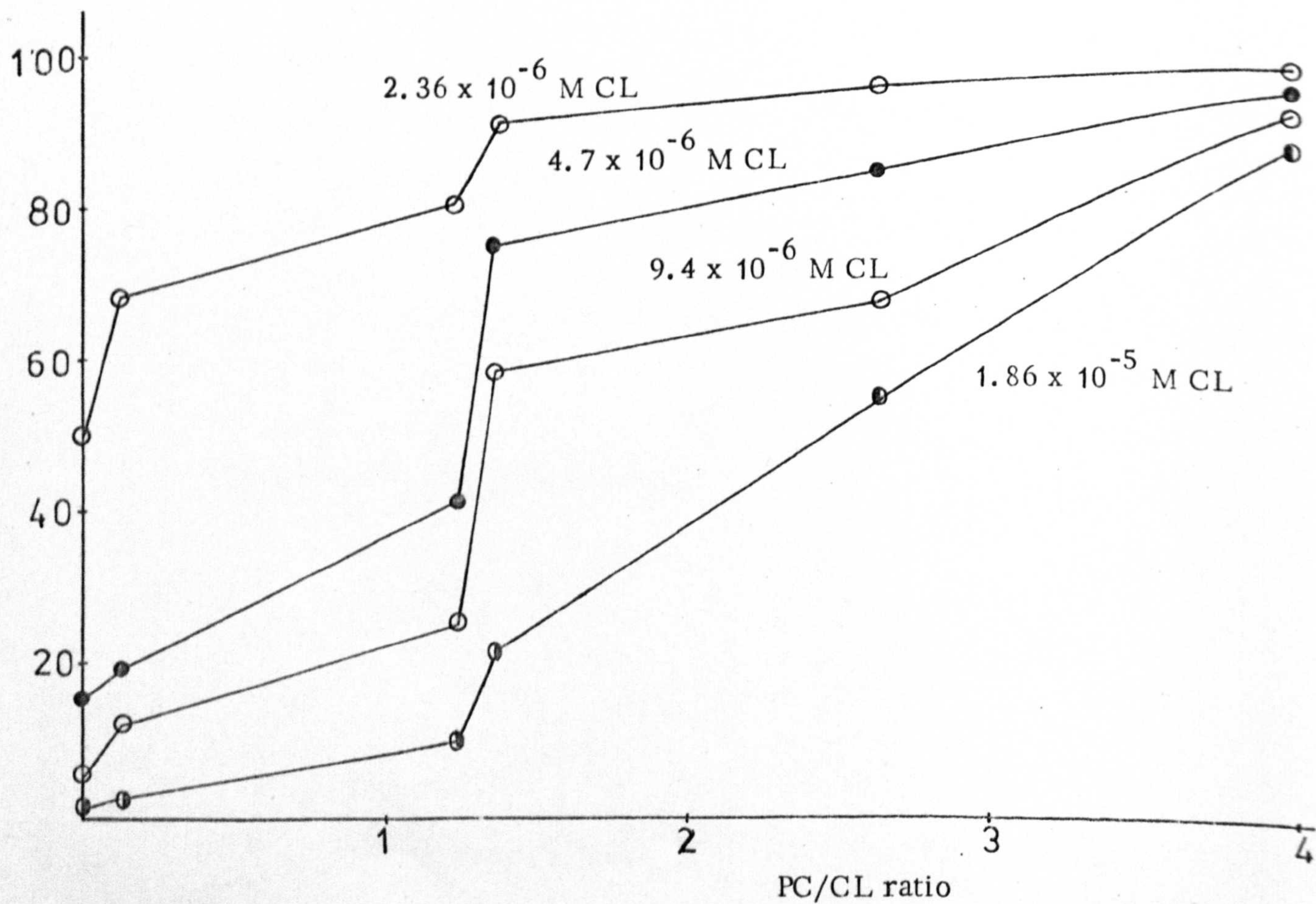
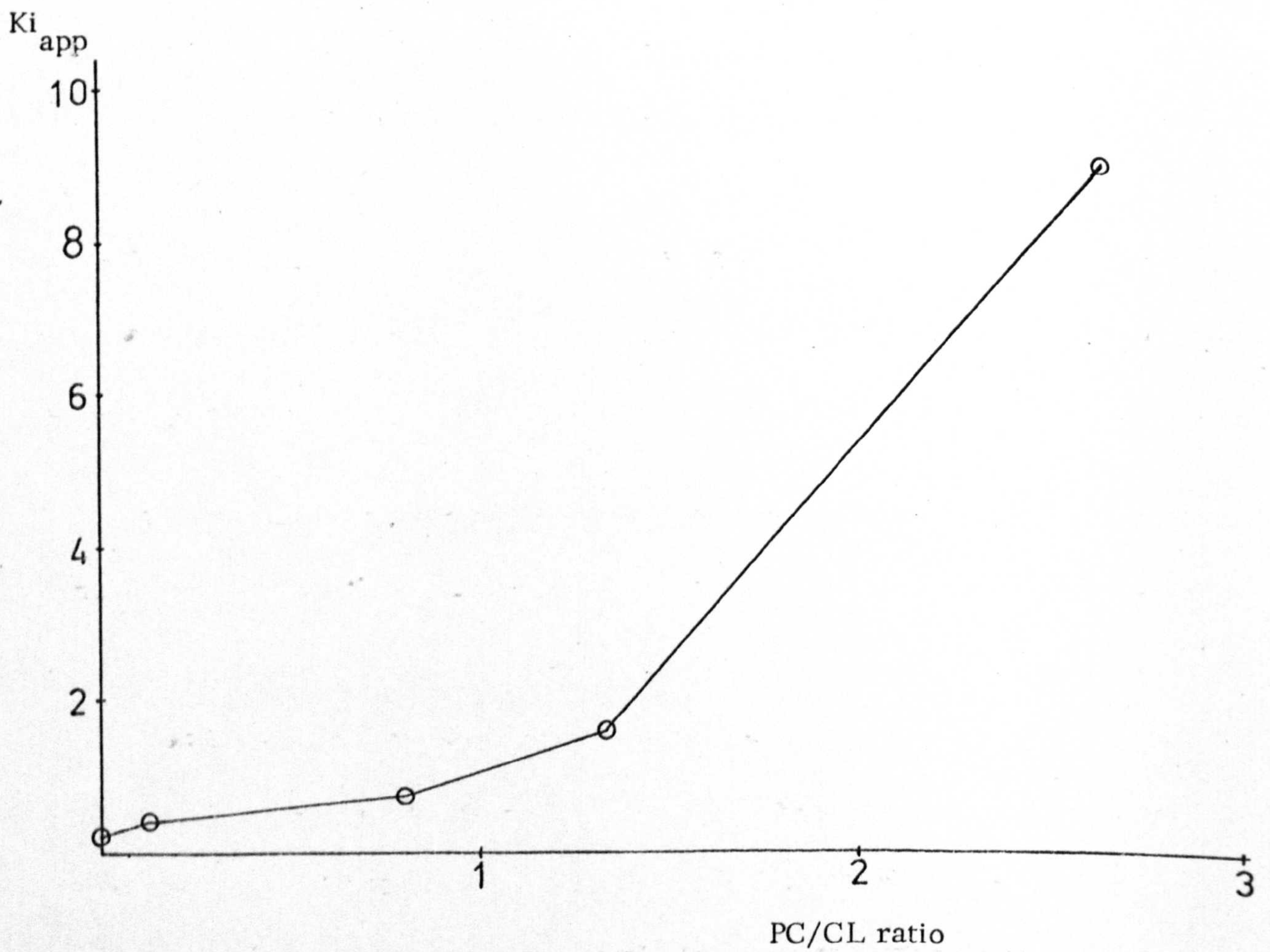


Fig. 50 The dependency of Ki_{app} on PC/CL ratio



Reduction of α-ketoglutarate and oxidation of l-glutamate by the enzyme were studied to characterise the type of reversible inhibition caused by the anionic phospholipids. In most cases, mixed types of inhibition were indicated (Figures 46 - 48 and Nemat-Gorgani, 1972).

Detergents were also incorporated into the phosphatidylcholine vesicles at low concentrations so that the bilayer structure of the phospholipid was kept intact. Such preparations up to phosphatidylcholine : SDS ratios of 2 : 1 did not cause any inhibition. These detergents in the absence of the phospholipid caused strong inhibition (Figures 20 - 26).

Co-sonicate phosphatidylcholine/phosphatidylserine and phosphatidylcholine/cardiolipin were examined and the extent of inhibition was found to decrease with increasing phosphatidylcholine proportion (Figure 49). Very little interaction takes place at phosphatidylcholine to cardiolipin ratios greater than 4. The lower extent of interaction is due to "dilution" of the negatively-charged heads of the anionic phospholipids on the surface of the bilayers by phosphatidylcholine. The dependency of Ki_{app} on PC/CL ratio is shown in Fig. 50.

In some ways, mixtures of the anionic phospholipids, cardiolipin or phosphatidylcholine with the zwitterionic phospholipid, lecithin, may be better models for the interaction. On the other hand, it is possible that cardiolipin molecules in the inner mitochondrial membranes are grouped into specific patches and the enzyme molecules are to various degrees organised by binding to cardiolipin of the membrane. In this context, it is interesting to refer to the asymmetrical nature of the lipid bilayer in erythrocyte membranes (Bretscher, 1972). In this system, the zwitterionic phosphatidylcholine was

found to be located chiefly in the outer half of the lipid bilayer and phosphatidylserine on the inner (cytoplasmic) half of the bilayer. Though the erythrocyte membranes are different from the mitochondrial membranes in terms of both lipid and protein composition, it is tempting to speculate that such arrangement may also exist in the inner mitochondrial membrane with cardiolipin mainly in the inner half of the membrane and phosphatidylcholine in the outer half. Some experimental observations support our speculation. Phospholipase A removes phosphatidylethanolamine and phosphatidylcholine from rat liver mitochondria quite rapidly and cardiolipin only very slowly (Fleischer, Fleischer, 1967). The former phospholipids exchange with ^{32}Pi or ^{14}C glycerol quite readily, whereas cardiolipin does not (Writz and Zilversmit, 1968).

Thus, bilayers of pure phosphatidylserine or phosphatidylcholine may indeed be reasonable model systems. The physiological relevance of these findings is limited, however, by the fact that it is not known how well the structure of the phospholipid membranes correspond to that of the mitochondrial membranes. Mitochondrial membranes have been shown to consist of fluid bilayer regions (Jost et al, 1973). The phosphatidylcholine and phosphatidylserine used in these studies were non-mitochondrial, but contain unsaturated residues, are above their transition temperatures and form a lamellar phase in our conditions. They are therefore suitable for this type of study.

Phosphatidylcholine and cardiolipin prepared from beef liver mitochondria showed the same behaviour. Phosphatidylcholine had no effect on the activity while cardiolipin brought about extensive inhibition.

Inhibition by the anionic phospholipids was found to decrease with increasing ionic strength, suggesting a significant electrostatic component in the

Fig. 51 The effect of NaCl on the extent of inhibition of GDH by CL

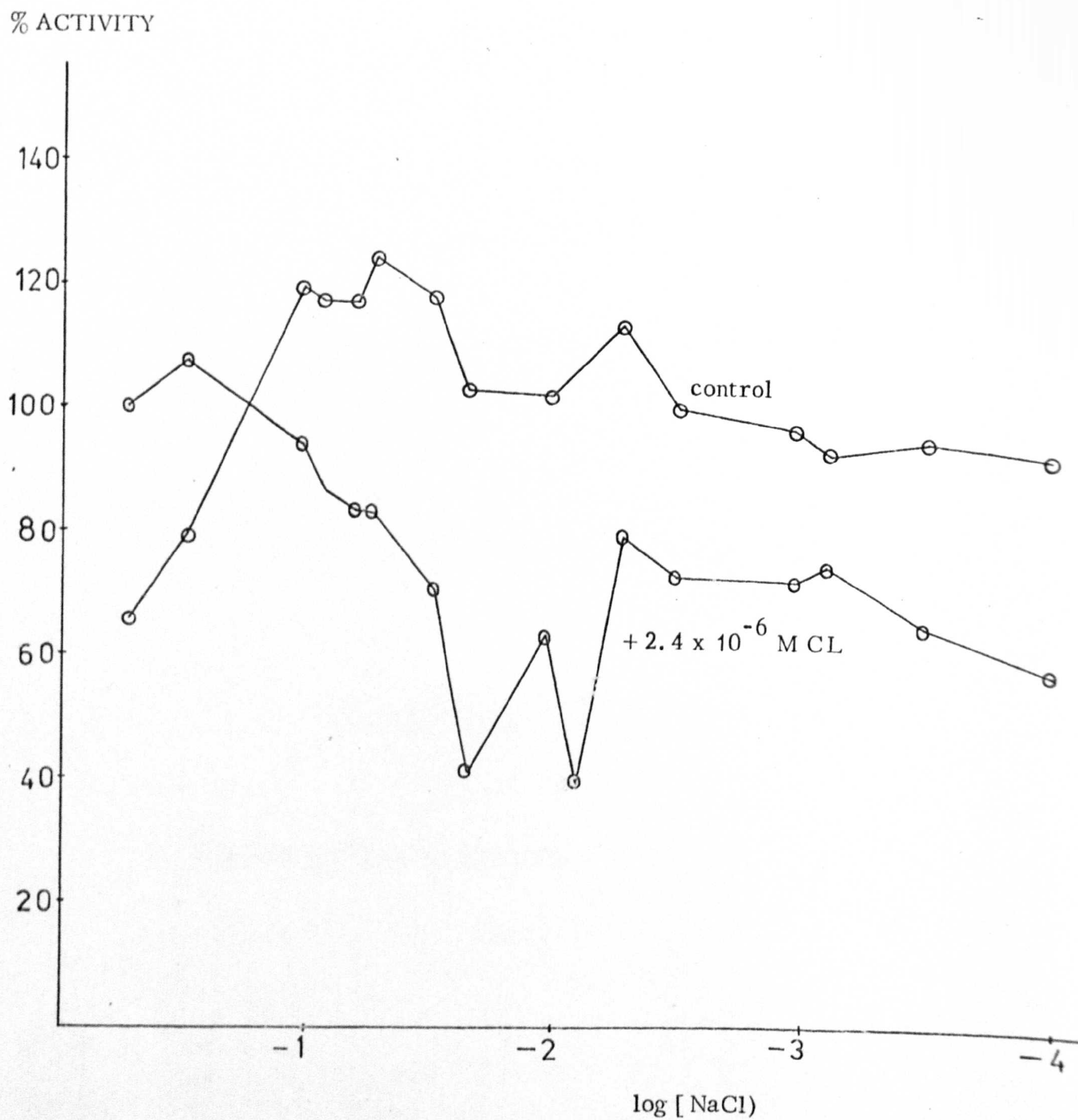
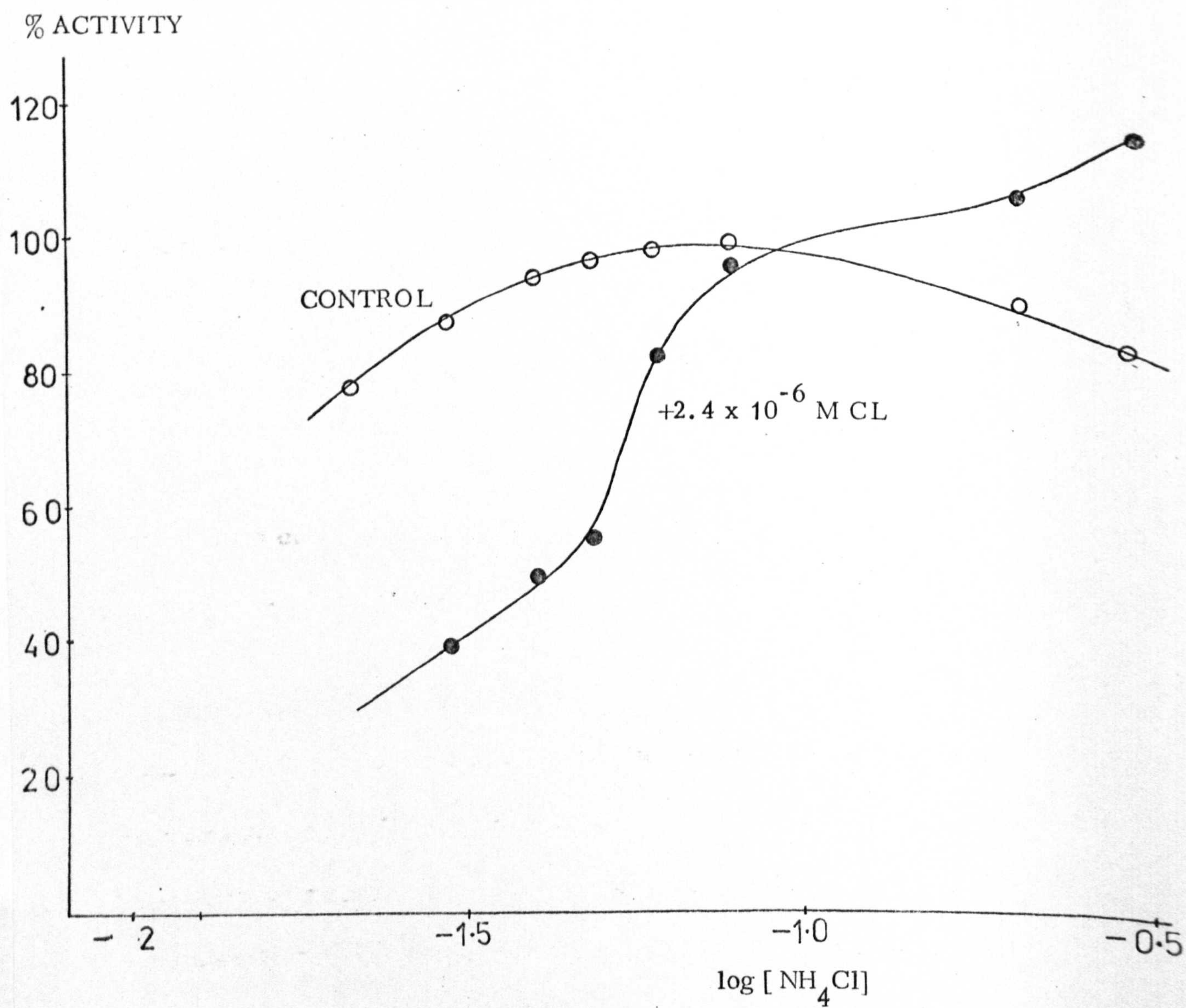


Fig. 52 The effect of NH_4Cl on the extent of inhibition of GDH activity by CL



association (Figures 51 & 52). In this type of study, it should be realised that the conformation of the enzyme and the behaviour of the phospholipid bilayers may be affected by the salt concentration. Therefore, the situation may be quite different from the behaviour of two simple charged entities and the dependence of their electrostatic interaction on ionic strength.

The enzyme has a complex structure and a change in ionic strength may have profound influence on the type of conformation it adopts. Evidence for such behaviour comes from the results obtained by Richter and Rotzsch (1970) who studied the effect of inorganic ions on kinetic properties of the enzyme. They found that increased concentrations of inorganic ions decrease the responsiveness of glutamate dehydrogenase to ADP and GTP.

NaCl showed a complex behaviour, (Figure 51) while NH_4Cl, which is a natural substrate for the enzyme (Km = 3.2 mM) was found to be a more favourable choice of salt for this purpose. In all subsequent studies, NH_4Cl was used for this type of investigation.

The effect of Ca^{++} on binding of GDH to anionic phospholipid membranes was also studied. To do this, it was necessary to assay the enzyme in the absence of EDTA.

A column of Bio-Rex 70 (200 - 400 mesh) was used to free the routinely used doubly distilled water from cation contaminants. GDH assay mixture was then prepared in hepetic buffer containing different Ca^{++} concentrations. The results indicate that Ca^{++} decreases the ability of cardiolipin to bring about enzyme inhibition. The effect of Ca^{++} may be taken to be through neutralisation of the negative charges on the anionic phospholipid. However, as discussed in

Fig. 53 The effect of Ca^{++} on the extent of inhibition of GDH activity by CL

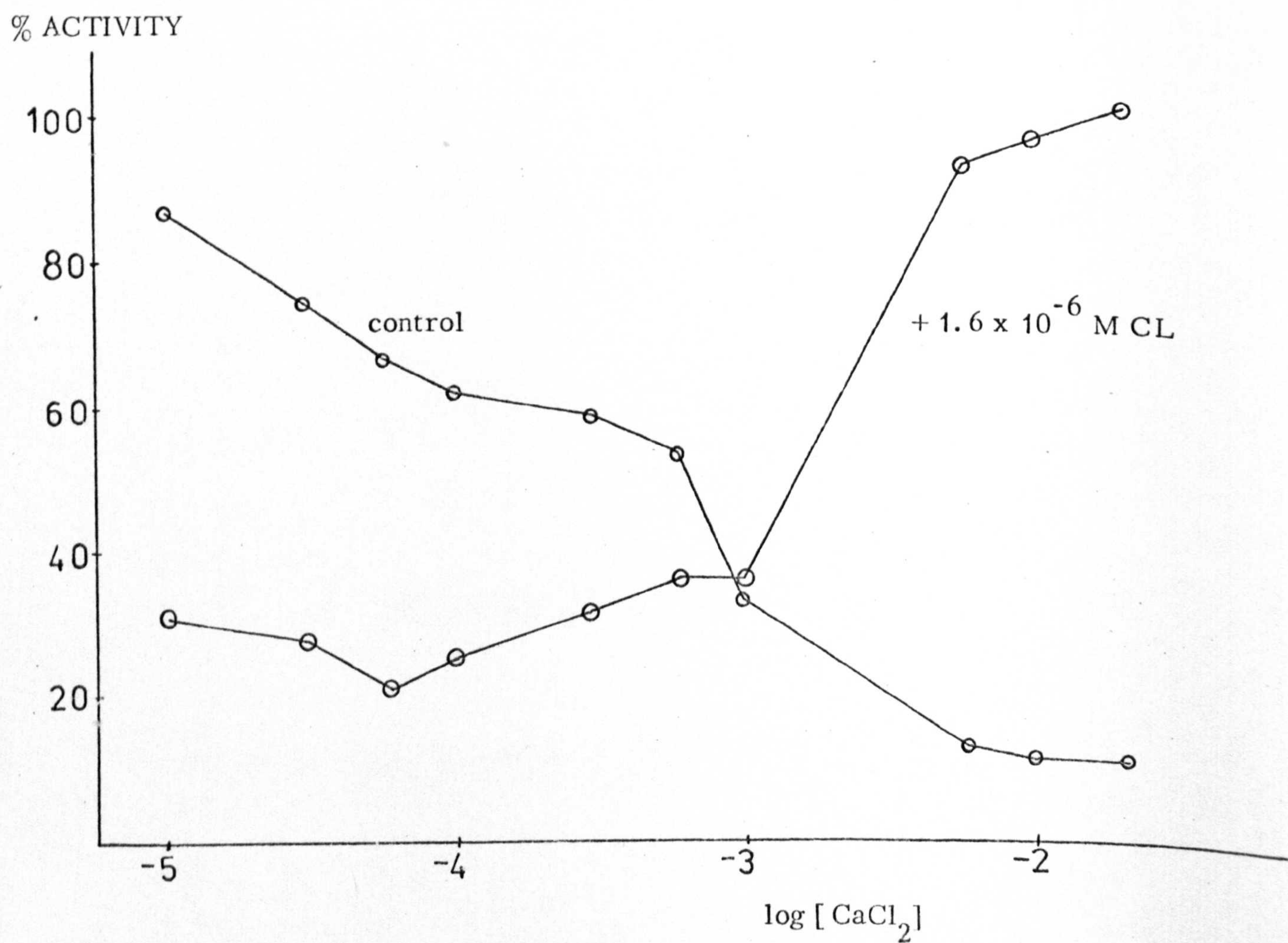
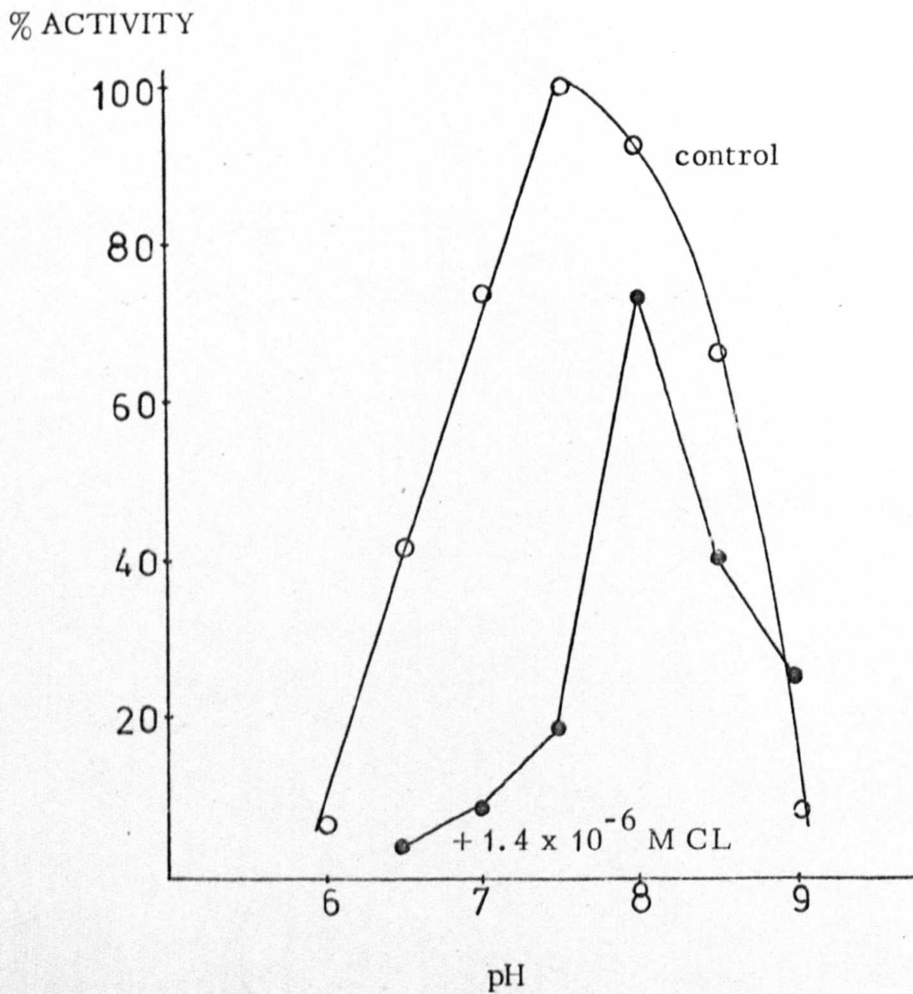


Fig. 54 The effect of pH on the extent of inhibition of GDH activity by CL



the previous chapter, Ca^{++} may bring about profound changes in the physical properties of the phospholipid. At high Ca^{++} concentrations (> 1 mM), conversion of the lamellar phase into a hexagonal phase is possible (Rand and Sengupta, 1972). The effect of Ca^{++} is shown in Figure 53.

The effect of pH on the extent of inhibition of the enzyme by cardiolipin is shown in Figure 54. It can be seen that the extent of inhibition decreases with increasing pH up to pH 8.0, above which it decreases. All our subsequent studies (other methods) on binding of the enzyme to phospholipids and mitochondrial membranes, showed a steady decrease in the extent of complex formation with increasing pH.

The pH dependence of the rates of hydrolysis of various substrates of papain and alkaline phosphatase embedded in collodion membranes has been found to deviate considerably from that observed with the corresponding native enzyme (Goldman et al, 1965, 1971). The rate of hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) by a papain membrane, when assayed in the absence of buffer, showed a monotonic increase with pH in contrast to the bell-shaped pH activity profile of the native enzyme. The theoretical aspects of such an effect have recently been reviewed by Goldman (1973).

Although the enzyme was found to be inhibited by the phospholipid at all pH values tested, in view of the above facts, it is difficult, and indeed, improper to make any conclusive suggestions on the dependence of the extent of complex formation on pH from such a study only.

A preformed complex between phosphatidylserine and the enzyme was partially dissociated by increasing the ionic strength of the solution. 79%

inhibition of the enzyme in the presence of 1.9×10^{-5} M phosphatidylserine was decreased to 59% when NH_4Cl concentration was increased from 0.06 M to 0.17 M. The increase in NH_4Cl concentration increased the activity of the enzyme in the absence of phosphatidylserine by 4%.

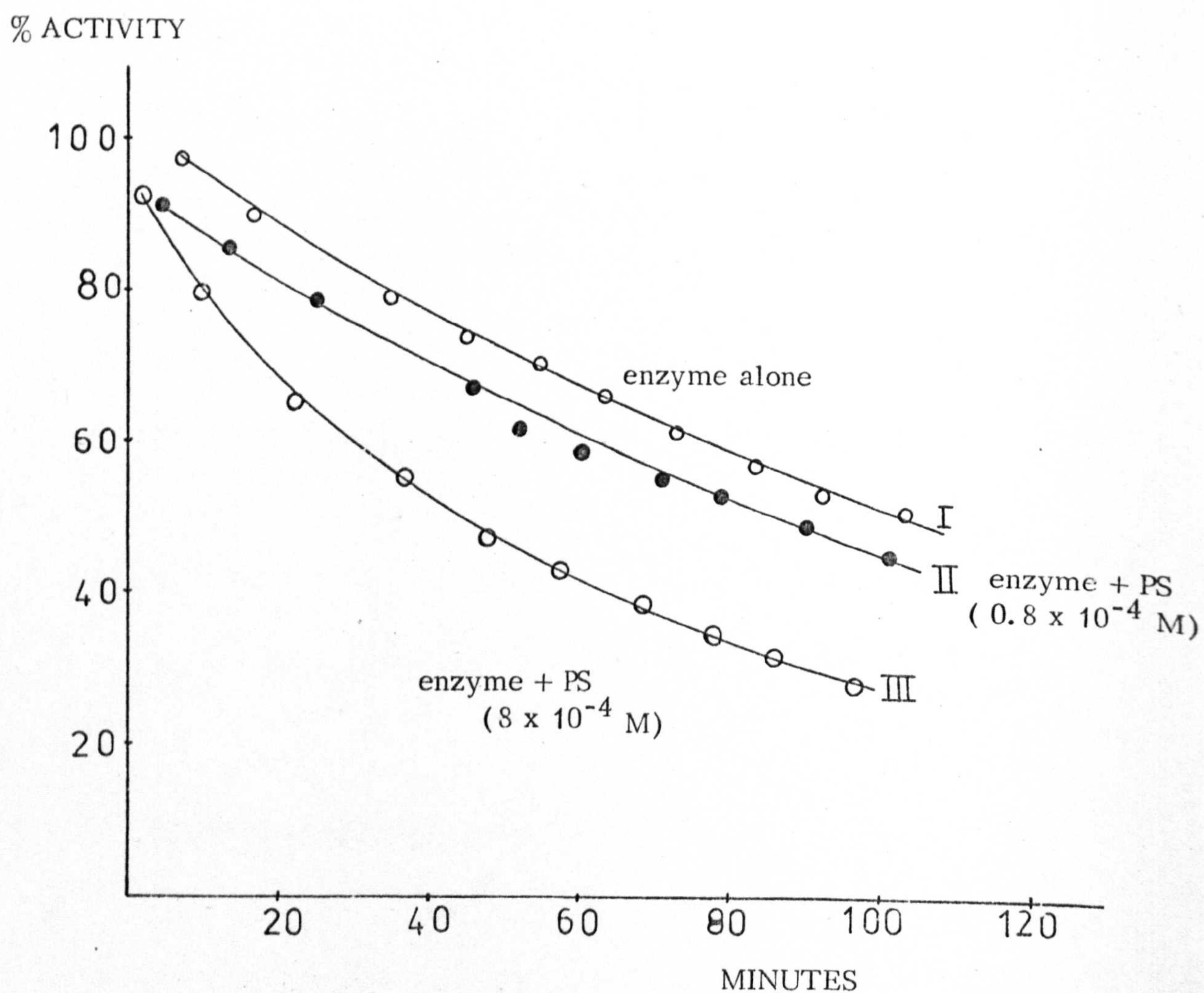
The fact that the complex was not totally dissociated by such a high NH_4Cl concentration suggests that a part of the enzyme, probably a ' hydrophobic tail' can penetrate into the hydrophobic region of the bilayer. Such a situation would make the occurrance of hydrophobic interaction possible in the formation of the complex between the enzyme and phospholipid. Our fluorescence studies (discussed in ChapterIV) do confirm the possibility of such an interaction. This will be more fully discussed later.

The presence of cytochrome c was shown to diminish the extent of interaction between the enzyme and cardiolipin. In the presence of 2×10^{-5} M cytochrome c, cardiolipin showed no binding to the enzyme up to a cardiolipin concentration of 1.7×10^{-5} M. The activity of the enzyme was not affected by cytochrome c at this concentration. On the other hand, the enzyme inhibited by cardiolipin (7% activity at CL concentration of 1.9×10^{-5} M) regained more activity when cytochrome c was added (16% and 30% activity on addition of 1×10^{-6} M and 2×10^{-6} M cytochrome c respectively). The interaction of this basic protein (pI = 10.6) with negatively charged phospholipids has already been discussed in the previous chapter.

The fact that a preformed complex between the enzyme and the anionic phospholipids can be partially dissociated with the addition of NH_4Cl or cytochrome c confirms our previous conslusion that the process of complex formation is a reversible one.

Fig. 55 The effect of PS on thermal stability of GDH at $37^{o}C$

$[En] = 0.05$ mg/ml



Reference to the results on the effect of cytochrome c or NH_4Cl before and after complex formation indicates that:

1) If sufficient concentration of the salt or protein is added to the phospholipid suspension before the addition of the enzyme, no complex formation can take place.

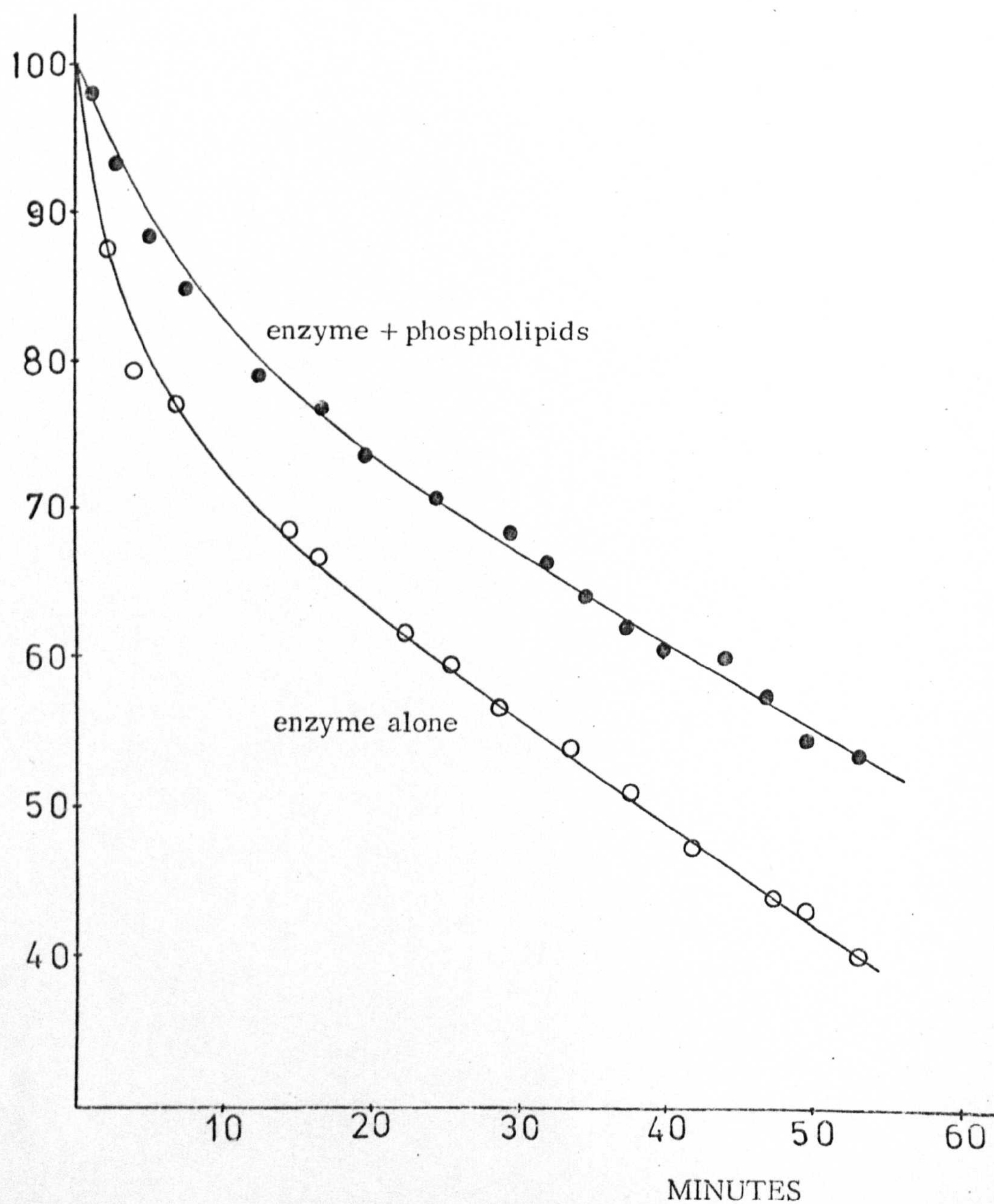
2) After formation of the complex, only partial dissociation takes place.

Thus, a basic protein, such as cytochrome c, shows a better affinity for complexing with an acidic phospholipid. Also, in the presence of a fairly high concentration of NH_4Cl (> 0.1 M) no complex formation may take place. The addition of a preformed complex between the enzyme and an anionic phospholipid is only partially dissociated because of the possible involvement of hydrophobic interaction between the hydrophobic amino acid residues in the enzyme and the hydrophobic region of the phospholipid bilayer.

The thermostability of bovine liver glutamate dehydrogenase was shown to be considerably higher in the cell system than in the isolated state (Yakovleva and Gubnitski, 1973). Thermal inactivation was found to accompanied by retention of allosteric activation by ADP.

The possibility of thermal stabilisation of the enzyme by the anionic phosphatidylserine was investigated. Though the diluted enzyme is stable over periods of a few days at 25°C in our conditions, it was found to lose activity at 37°C. This rate of loss of activity was found to increase in the presence of increasing amounts of phosphatidylserine (Figure 55). However, the rate of loss of activity at elevated temperatures was slightly less in the

Fig. 56 The effect of a cosonicate of PC-PE-CL (40%, 40% and 20%) on thermal stability of glutamate dehydrogenase at 40^oC.



presence of cosonicates of phospholipids of the same composition as the inner mitochondrial membrane (i.e. 40% PC, 40% PE and 20% CL) (Figure 56).

Chapter IV

INTRINSIC AND EXTRINSIC FLUORESCENCE STUDIES: RESULTS and DISCUSSION

Fig. 57 The effect of lysolecithin and SDS on the intrinsic fluorescence of GDH

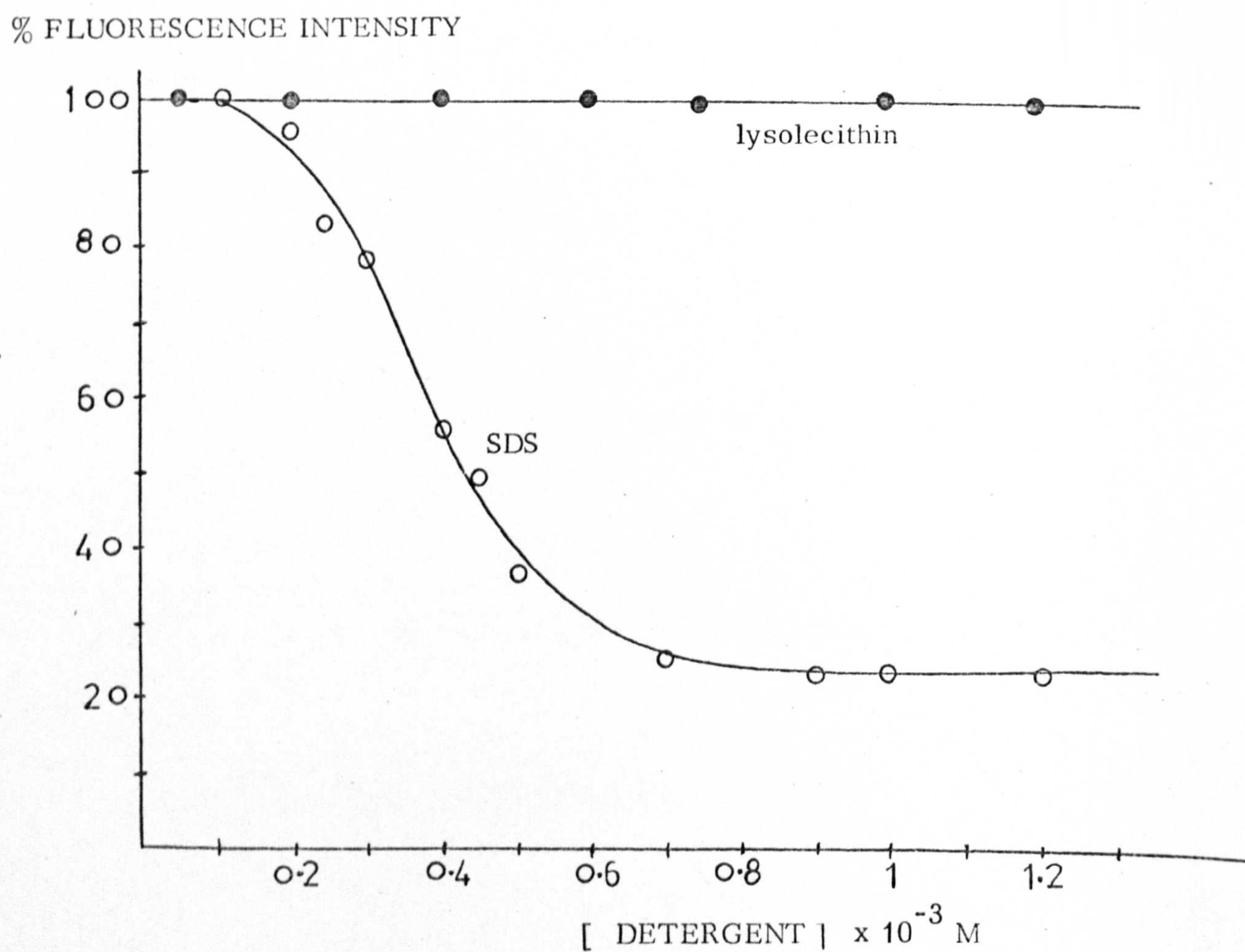


Fig. 58 The effect of CETAB and hexane sulphonate on the intrinsic fluorescence of GDH

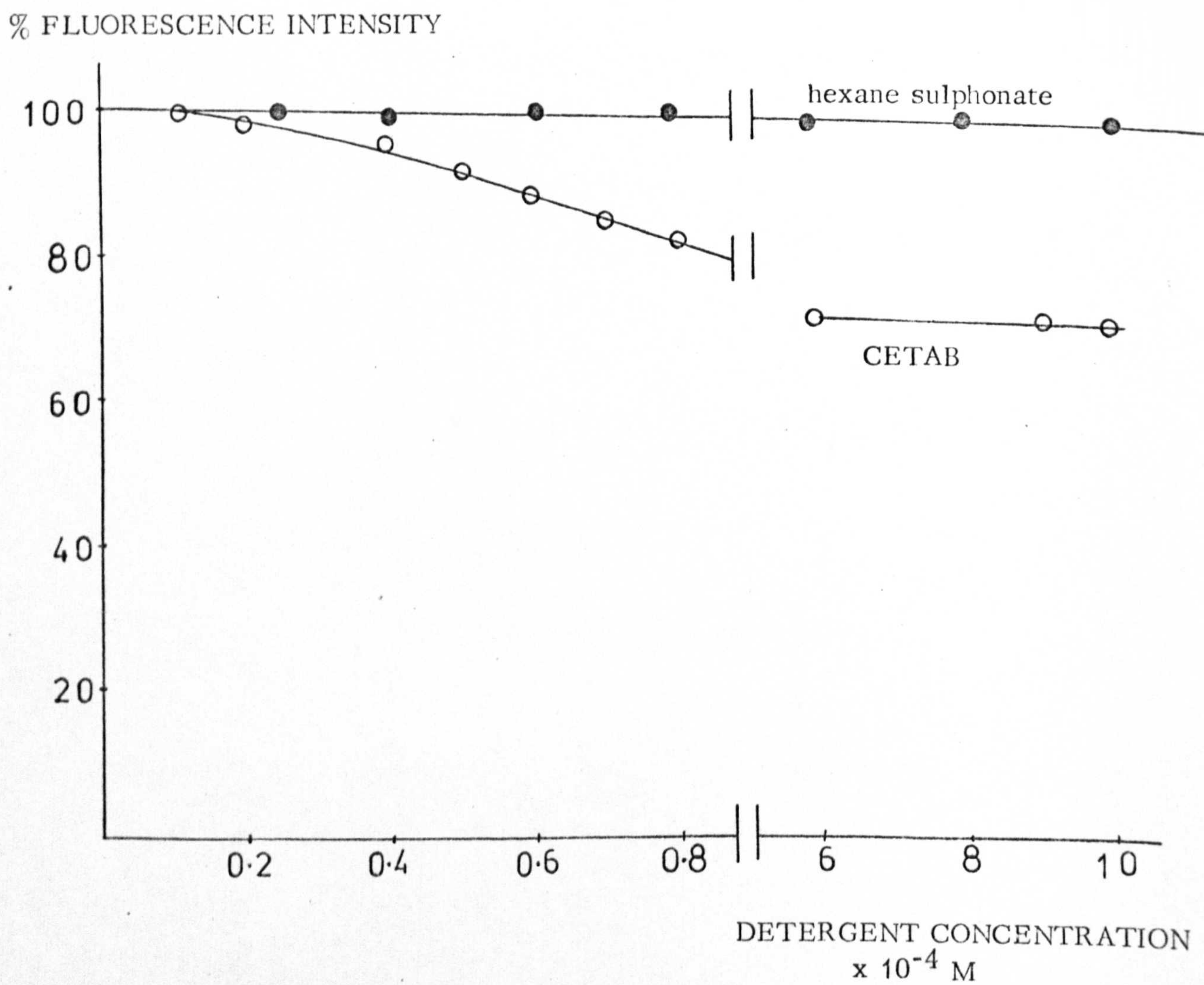


Fig. 59 The effect of phospholipids on the intrinsic fluorescence of GDH

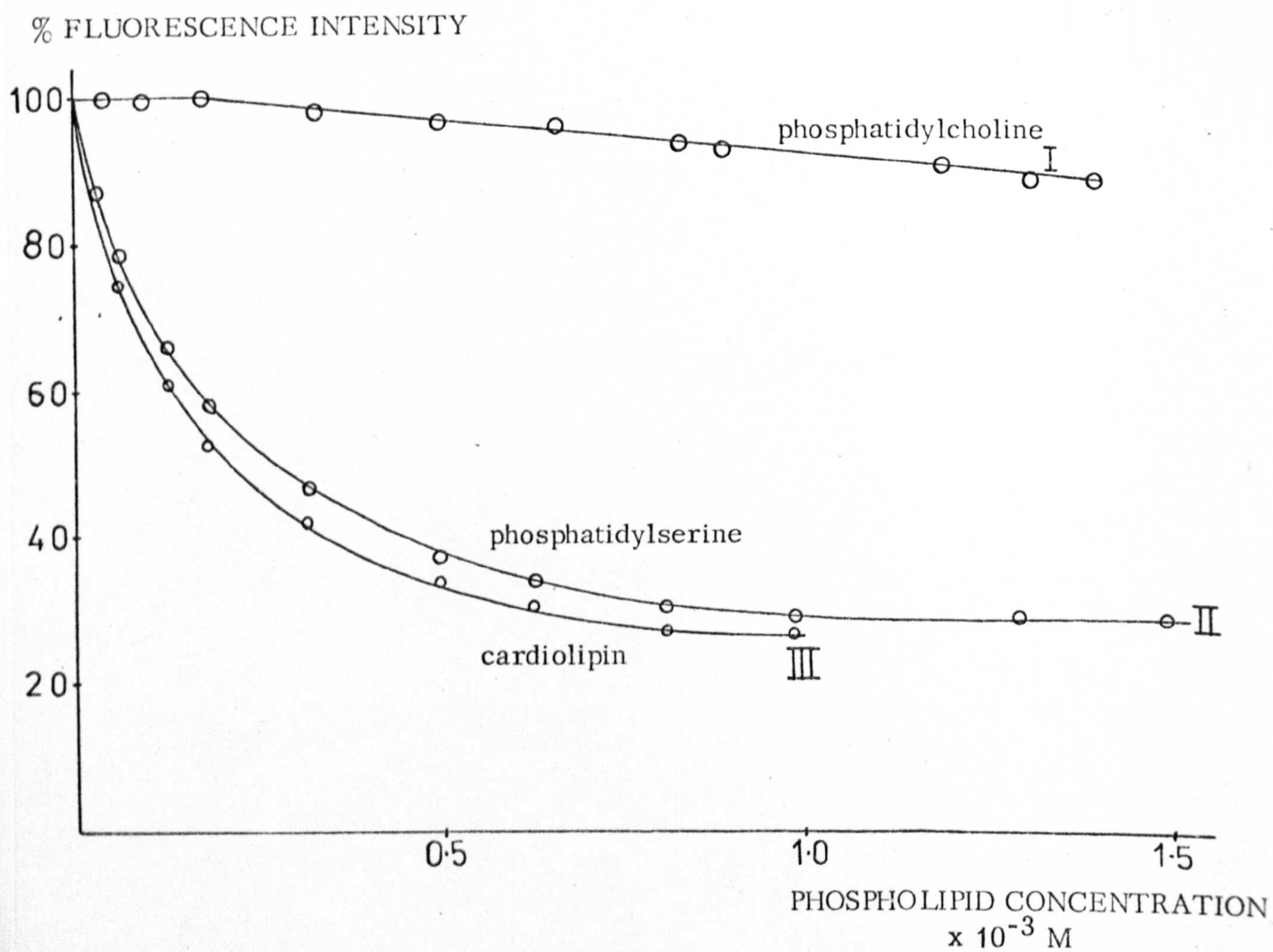


Fig. 60 The effect of phosphatidylserine on the intrinsic fluorescence of GDH at different enzyme concentrations

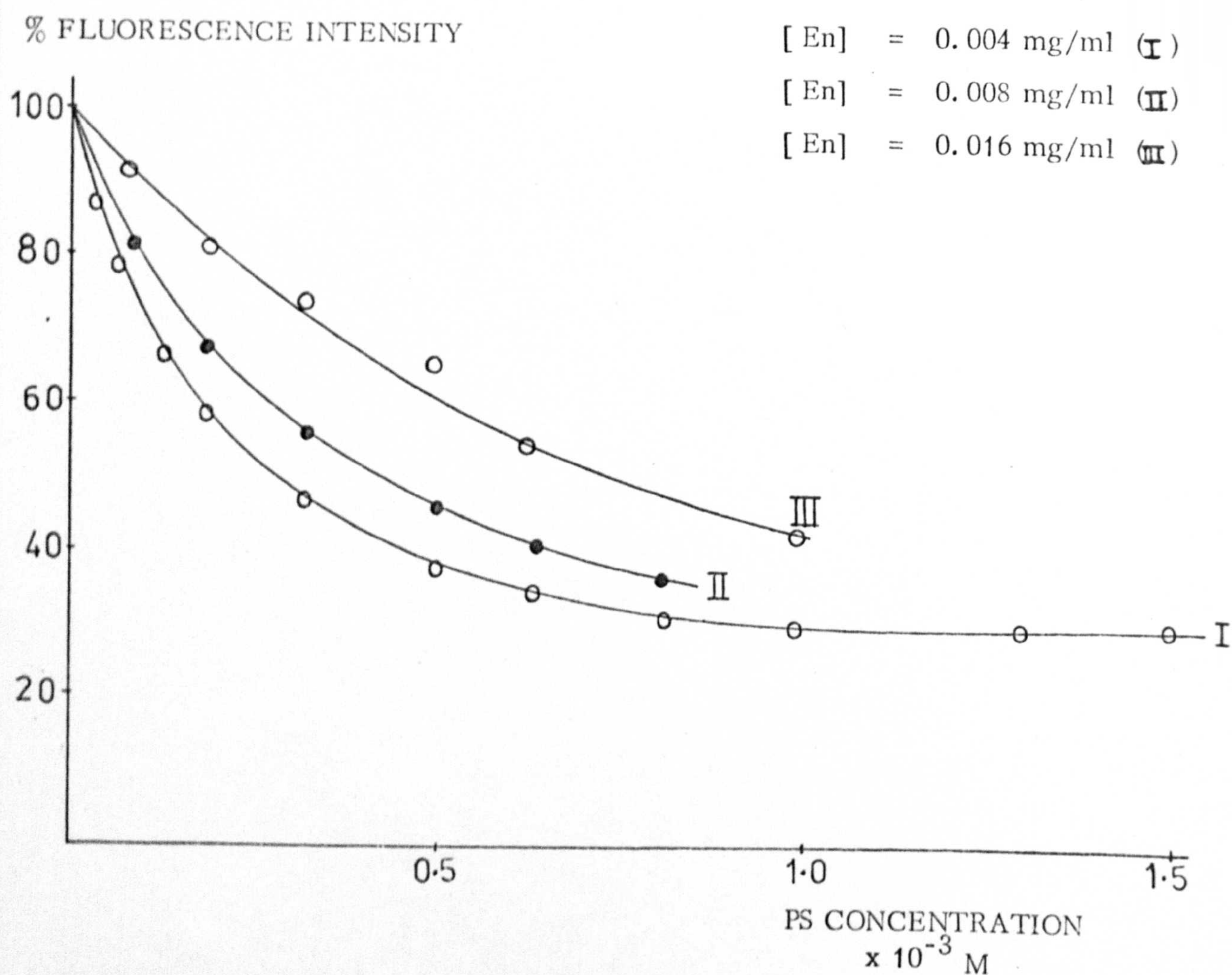
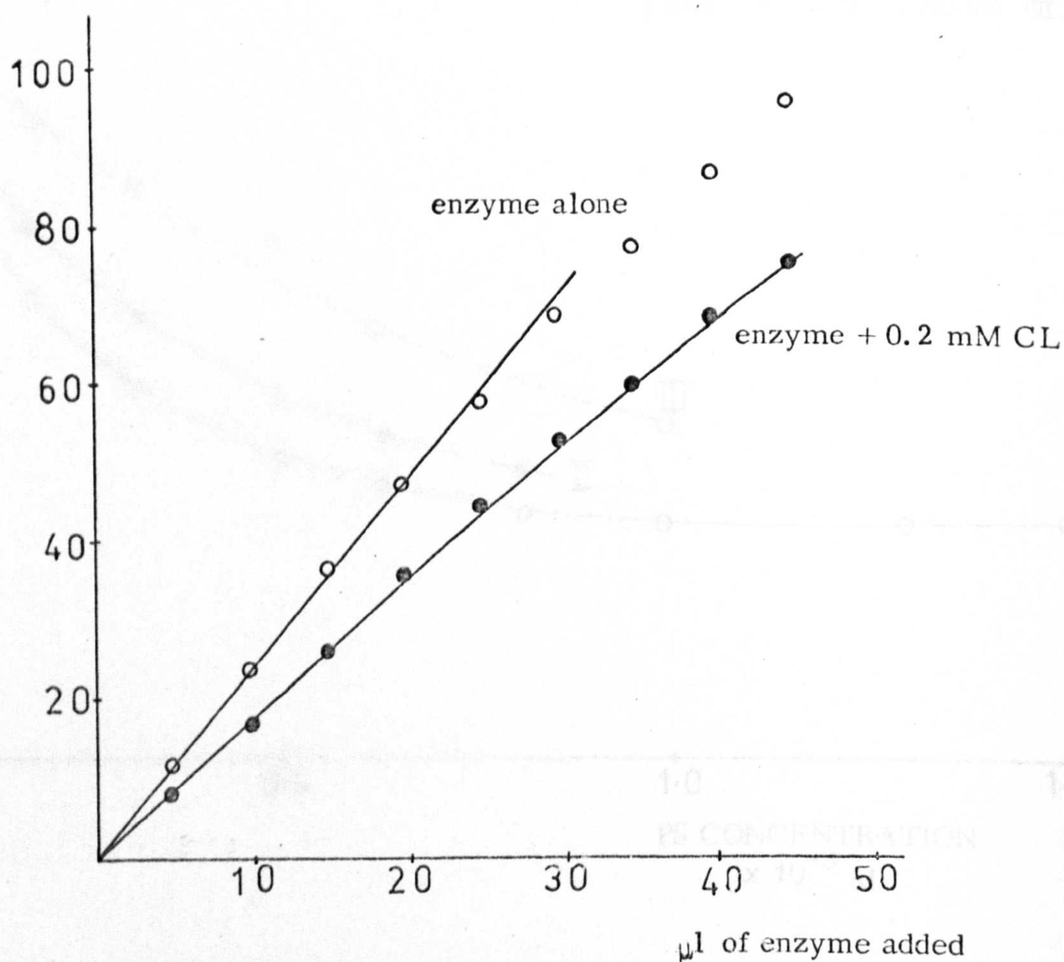


Fig. 61 The dependency of the quenching of fluorescence intensity of GDH by CL on enzyme concentration



Studies on the intrinsic fluorescence of the enzyme showed that those amphiphiles which caused inhibition of enzyme activity also quenched the enzyme fluorescence. Thus, SDS and CETAB caused strong quenching while lysolecithin and hexane sulphonate showed no effect (Fig.57,58). The anionic phospholipids cardiolipin and phosphatidylserine also quenched the intrinsic fluorescence of the enzyme (Figure59). Phosphatidylcholine, which does not affect the catalytic activities of the enzyme showed only a very small effect (Figure59).

Dependence of enzyme fluorescence quenching on relative amounts of the lipid and enzyme was measured either by looking at the effect of increasing the lipid concentration (Figures 59, 60) on that of the enzyme (Figure61) on this process.

Two main types of quenching of enzyme fluorescence can occur: static and dynamic quenching. Only the latter type of quenching is dependent on the viscosity of the solution.

Static quenching of fluorescence is described by the relation:

$$\frac{I}{I_o} = e^{-k[Q]}$$

where I and I_o are the fluorescence intensities in the presence and absence of the quencher respectively, k^1 is the quenching constant and [Q], the quencher concentration. In contrast, dynamic quenching is described by the Stern-Volmer relation:

$$\frac{I}{I_o} = \frac{1}{1 + k[Q]}$$

Quenching of enzyme fluorescence can take place either by an energy transfer mechanism (in which case the enzyme -lipid complex fluoresces to a smaller extent than the free enzyme) or through conformational changes in the enzyme structure accompanied by the accessibility of fluorescent residues to the surrounding aqueous medium.

A glutamate dehydrogenase oligomer consists of 6 identical polypeptide chains. There are 500 amino acid residues in each polypeptide chain with 18 tyrosine, 23 phenylalanine and 3 tryptophan residues (Piskiemicz et al, 1973, Moon and Smith, 1973). Values between 3 and 5.5 tryptophan residues have been reported in some earlier studies (London et al, 1971, Sund and Arkeson, 1964, Cross and Fisher, 1966, and Appella and Tomkins, 1966).

The maximum fluorescence emission is in the region of 335nm which is due to tryptophan residues. Studies on the spatial location of the residues (Cross and Fisher, 1966) have shown that 4 tyrosine residues (23% of the total), 2 phenylalanine residues (9% of the total) and 1 tryptophan residue (23% of the total) are located on the surface of the enzyme. Direct interaction between these residues and lipids is therefore a possibility.

To explore this possibility, the effect of these amphiphiles on fluorescence behaviour of Acetyltryptophanamide was studied. This compound is used as a model for tryptophan residues incorporated in a polypeptide chain (Radda and Dodd, 1968). Generally, additives which decrease the dielectric constant of water result in enhancement of fluorescence, while increase in dielectric constant has the opposite effect.

Fluorescence studies on N-acetyltryptophanamide showed that none of the

detergents affected its fluorescence (In the presence of CETAB, 2 - 4% increase was resulted with the detergent at 1 mM and N-acetyltryptophanamide at 0.4 mM concentrations respectively). On the other hand, the anionic phosphatidylserine and cardiolipin quenched N-acetyltryptophanamide (0.4 mM) fluorescence by 12% and 8% at 0.5 mM and 0.25 mM respectively. Lecithin, at a concentration of 0.5 mM caused only 2 - 3% quenching. The extent of quenching by the anionic phospholipids was independent of pH.

In the case of quenching of the enzyme fluorescence by detergents, the process takes place at high concentrations of the detergent (at and above 1×10^{-4} M in the case of SDS) at which irreversible denaturation takes place. Thus, direct interaction of the tryptophan residues exposed on the surface of the enzyme does not bring about any quenching of fluorescence. At high concentrations of the detergent (higher than 0.1 mM in the case of SDS) at which extensive unfolding in the structure of the polypeptides can take place, the tryptophan residues present in the interior of the enzyme structure may be displaced and positioned in a new environment. This new environment may have a higher dielectric constant in which the residues fluoresce to a smaller extent.

In the case of enzyme-phospholipid interactions which is a reversible process, the resulted fluorescence quenching can be partly due to direct interaction with the tryptophan residues possibly exposed at the surface of the enzyme and partly due to changes in the accessibility of the tryptophan residues buried in the anhydrous interior to the surrounding aqueous medium. Thus, conditions of low pH and ionic strength which are favourable for complex formation increase the accessibility of the tryptophan residues to the aqueous environment with the result that the fluorescence quenching of the enzyme is

Fig. 62 The effect of NH_4Cl on fluorescence quenching of GDH by CL

[En] = 40 μ g/ml

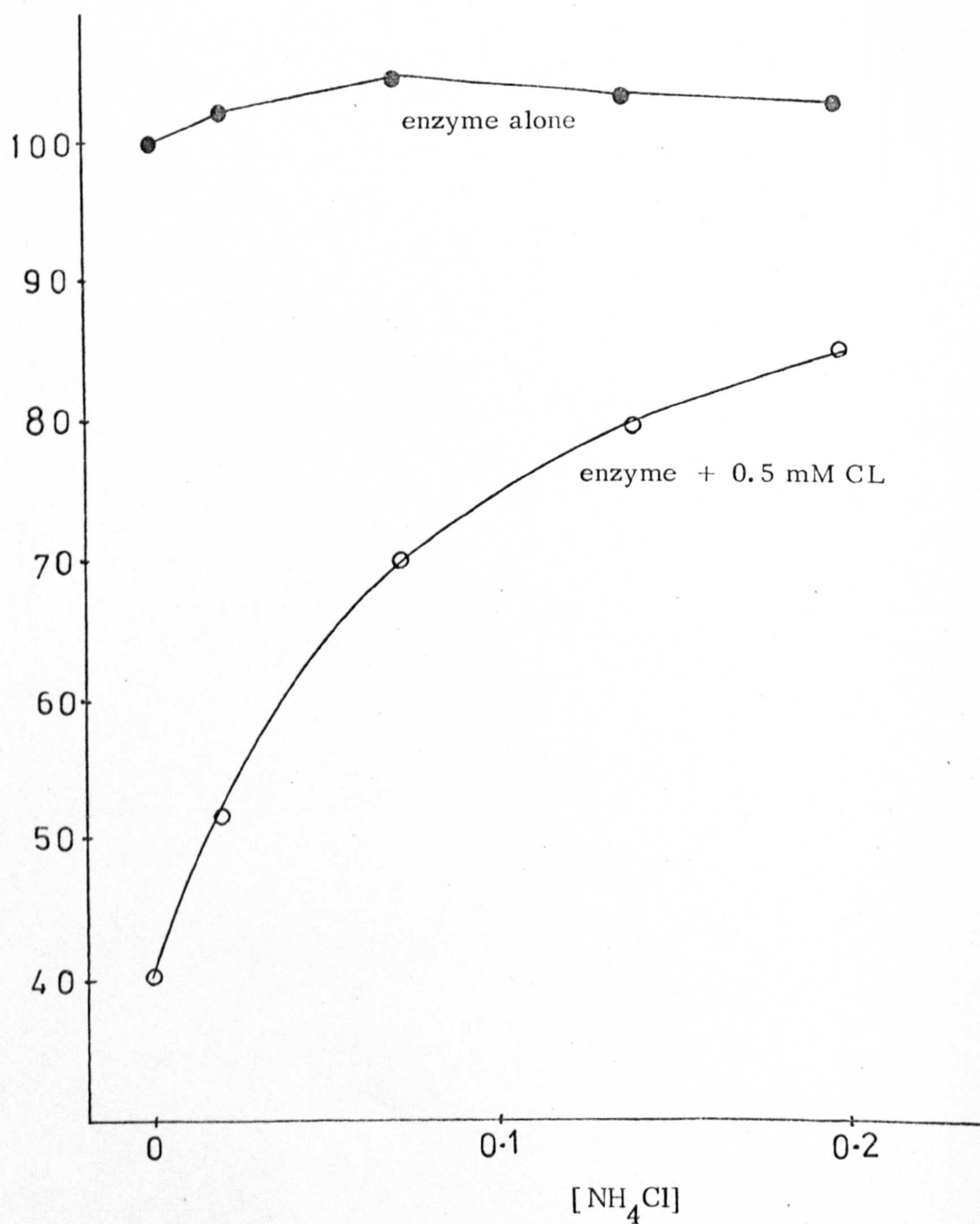


Fig. 63 The effect of pH on quenching of intrinsic fluorescence of GDH by CL

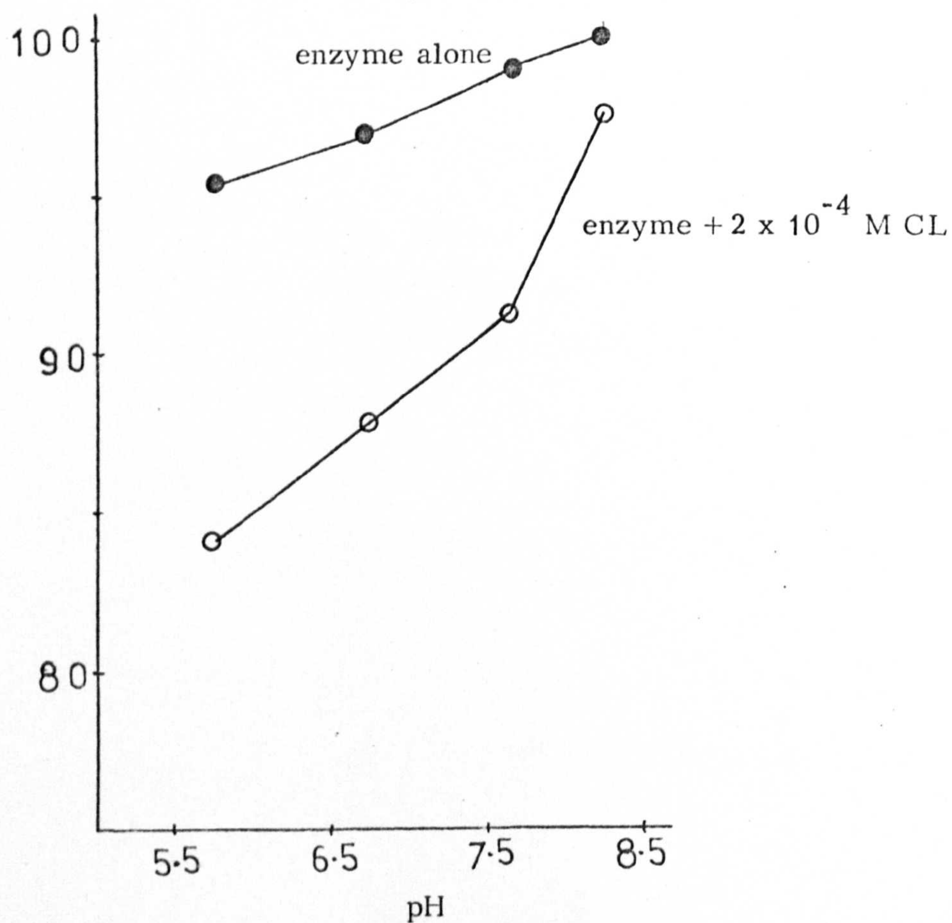


Fig. 64 The effect of ribonuclease on GDH fluorescence quenching by PS

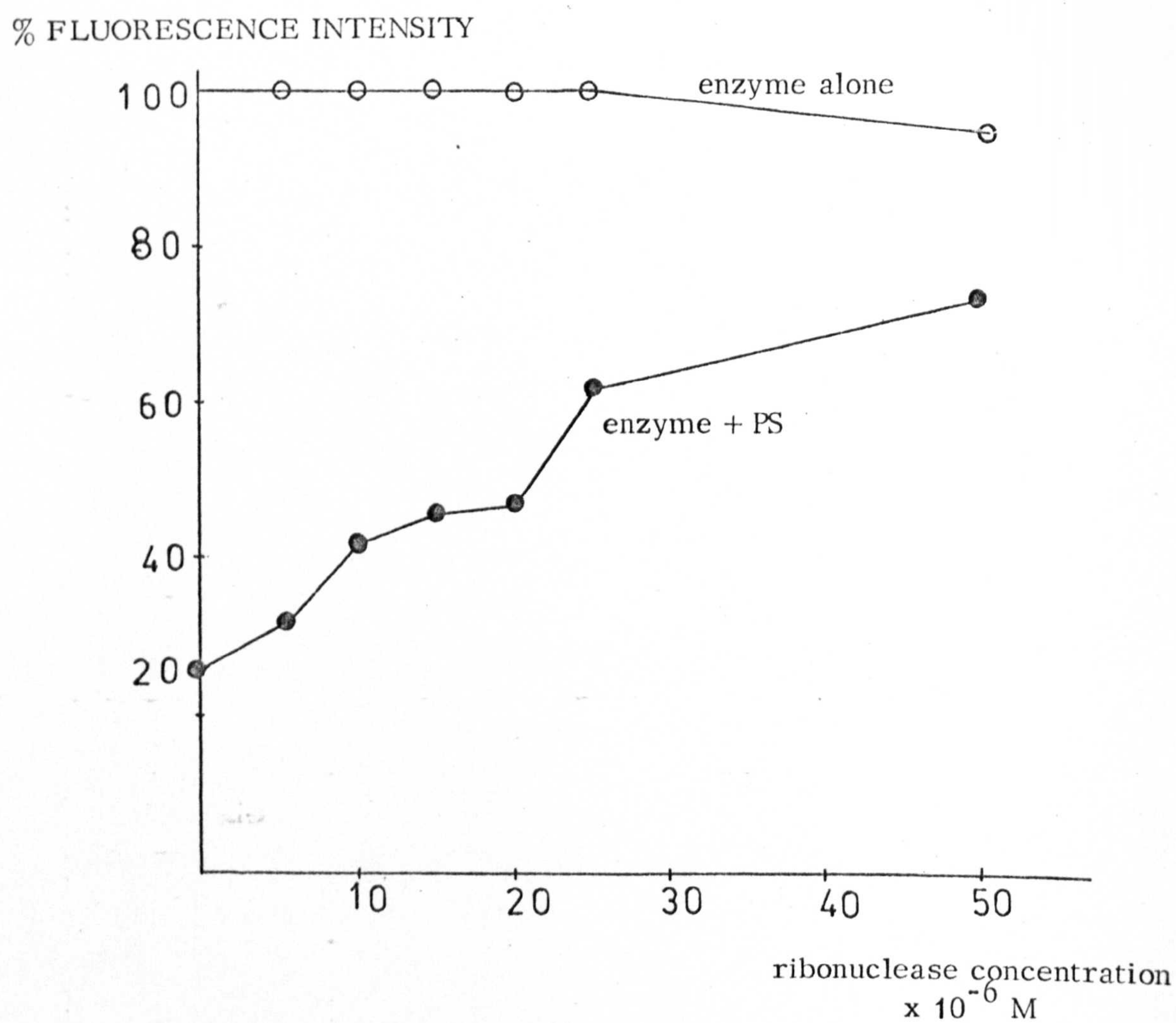


TABLE 1

EFFECT OF α-Kg AND l-glu ON GDH FLUORESCENCE QUENCHING BY PS. ENZYME AND PHOSPHOLIPID AT FINAL CONCENTRATIONS OF 40 μg/ml AND 0.5 mM RESPECTIVELY.

condition	fluorescence intensity		% fluorescence intensity (GDH + PS)
	GDH alone	GDH + PS	
0.06 M hepetri + 0.8 mM phosphate, pH 6.2	64	23	36
+ 0.14 mM α-Kg	60	22	37
+0.3 mM α-Kg	59	21.5	37
+ 0.7 mM α-Kg	57	21	37
+ 3 mM α-Kg	46.5	20	43
1.4 mM l-glu	63	23	36.5
4.3 mM l-glu	58.5	22	38
10 mM l-glu	56	23.5	56

TABLE 2

EFFECT OF l-glu AND NAD^+ ON GDH FLUORESCENCE QUENCHING BY PS. ENZYME AND PHOSPHOLIPID AT FINAL CONCENTRATIONS OF 40 μg/ml AND 0.6 mM RESPECTIVELY.

condition	fluorescence intensity		% fluorescence intensity (GDH + PS)
	GDH alone	GDH + PS	
0.06 M hepetri + 0.8 mM phosphate, pH 6.2	47.5	13	27.5
+ 10 mM l-glu	45.0	15	33.0
0.3 mM NAD^+	40.5	10	24
10 mM l-glu + 0.3 mM NAD^+	21.5	11	51.1

increased.

The extent of fluorescence quenching of the enzyme by the anionic phospholipids diminished with increasing ionic strength (Figure 62) and increasing pH (Figure 63), thus supporting the kinetic results.

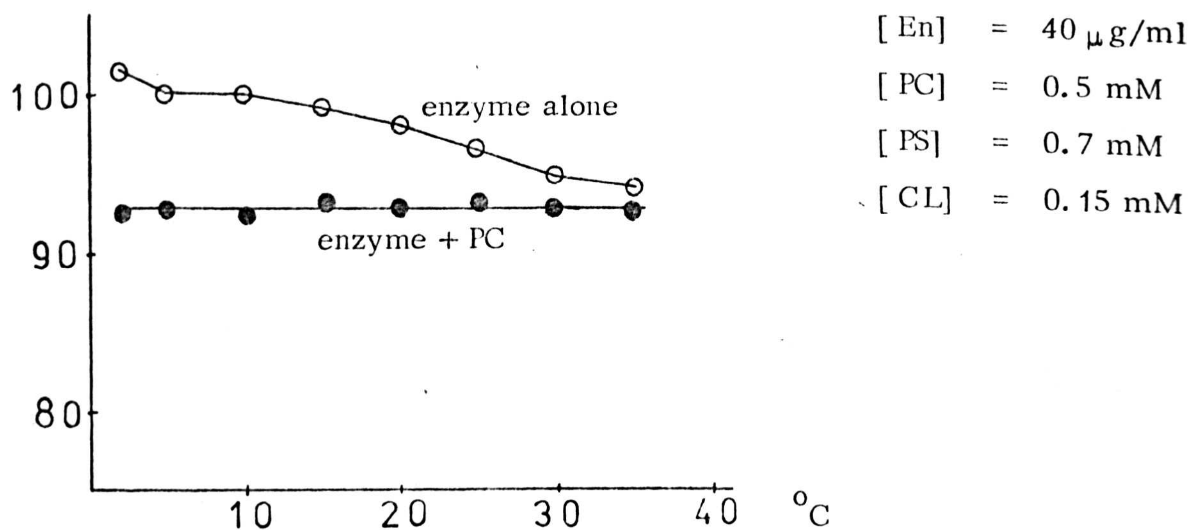
The extent of fluorescence quenching was also affected by the presence of basic proteins such as cytochrome c and ribonaclease. These proteins have been shown to interact with phosphatidylserine vesicles and to neutralise or even reverse the zeta potential of these vesicles (Kimelberg and Papahadjopoulos, 1971). Ribonuclease at concentrations up to 2.5×10^{-5} M did not show any effect on the intrinsic fluorescence of the enzyme but greatly diminished the quenching of enzyme fluorescence by phosphatidylserine (Figure 64). Cytochrome c had a similar effect. Ribonuclease was especially useful in this respect because of absence of any tryptophan residues in its structure.

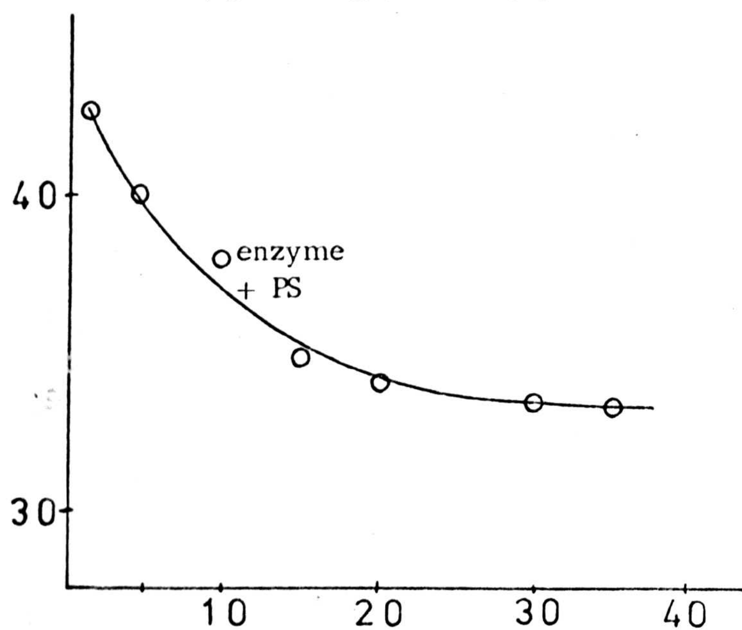
Both l-glutamate and α-ketoglutarate diminished the quenching of enzyme fluorescence by phosphatidylserine. These are summarised in table 1 The effect of NAD^+ and NAD^+ + l-glutamate are shown in table 2. However, NAD^+ alone caused a considerable quenching of the fluorescence of the enzyme and this makes it difficult to make conclusions of its effect on binding of the enzyme to the phospholipid membrane. These results support the findings of Godinot and Lardy (1973) who obtained 34.3% solubilisation of the enzyme activity from microsomal membranes in the presence of 20 mM l-glutamate and 2 mM NAD^+. Phosphatidylserine has been shown to be present in microsomal membranes (Colbeau et al, 1971).

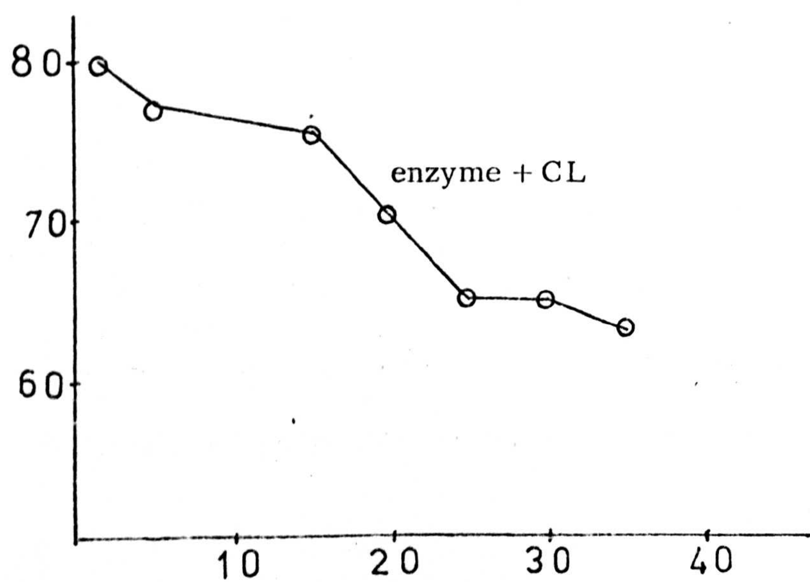
GTP, ADP and NADH quenched the intrinsic fluorescence of the enzyme

Fig. 65 The dependency of GDH fluorescence quenching by phospholipids on temperature

% FLUORESCENCE INTENSITY







strongly and hence, it was not possible to study their effects on the binding of the enzyme to phospholipid membranes.

Quenching of fluorescence by the anionic phospholipids also increased with increasing temperature, thus indicating the possible involvement of hydrophobic interaction (Figure 65). The small decrease of fluorescence intensity in the presence of high concentrations of PC (> 0.2 mM) was not affected by temperature. From this, two conclusions may be drawn:

i) that the decrease in fluorescence of the enzyme by the zwitterionic phospholipid at high concentrations is not due to lipid-protein complex formation;

ii) that the enzyme does not interact with the lipid over a wide range of temperatures.

In general, at low temperatures, hydrophobic interaction becomes stronger as the temperature increases. The maximum strength is reached at a certain temperature which has been estimated (Nemethy and Sheraga, 1962) to be near 58°C for aliphatic side chains and near 42°C for aromatic side chains.

The increase in the fluorescence quenching with increasing temperature can be taken as an indication of hydrophobic interaction, assuming that both the enzyme and the phospholipid are essentially unchanged by temperature changes.

Changes in temperature may bring about slight changes in the conformation of the enzyme due to variations in the hydrophobic and other interactions, which are temperature-dependent and which are of importance for the stability of enzyme structure. All the phospholipids used in this study are natural

Fig.66/7 The effect of PS on NADH fluorescence when partially or fully bound to GDH

FLUORESCENCE INTENSITY

100
80
60
40
20

NADH + GTP

NADH

[NADH] = 2 μM
[GTP] = 0.2 mM
[PS] = 0.53 mM

0·1 0·2 0·3 0·4 0·5 0·6

GDH CONCENTRATION
mg/ml

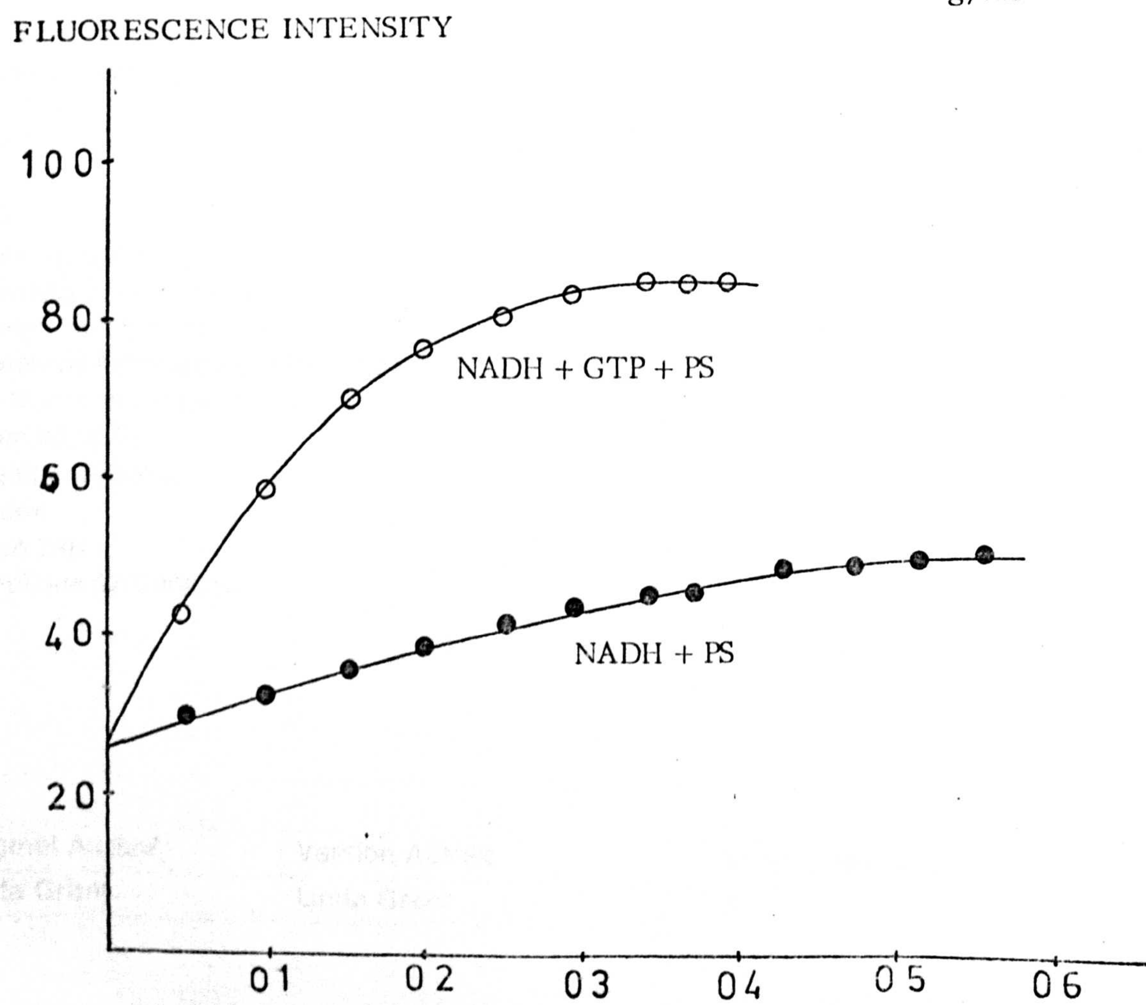
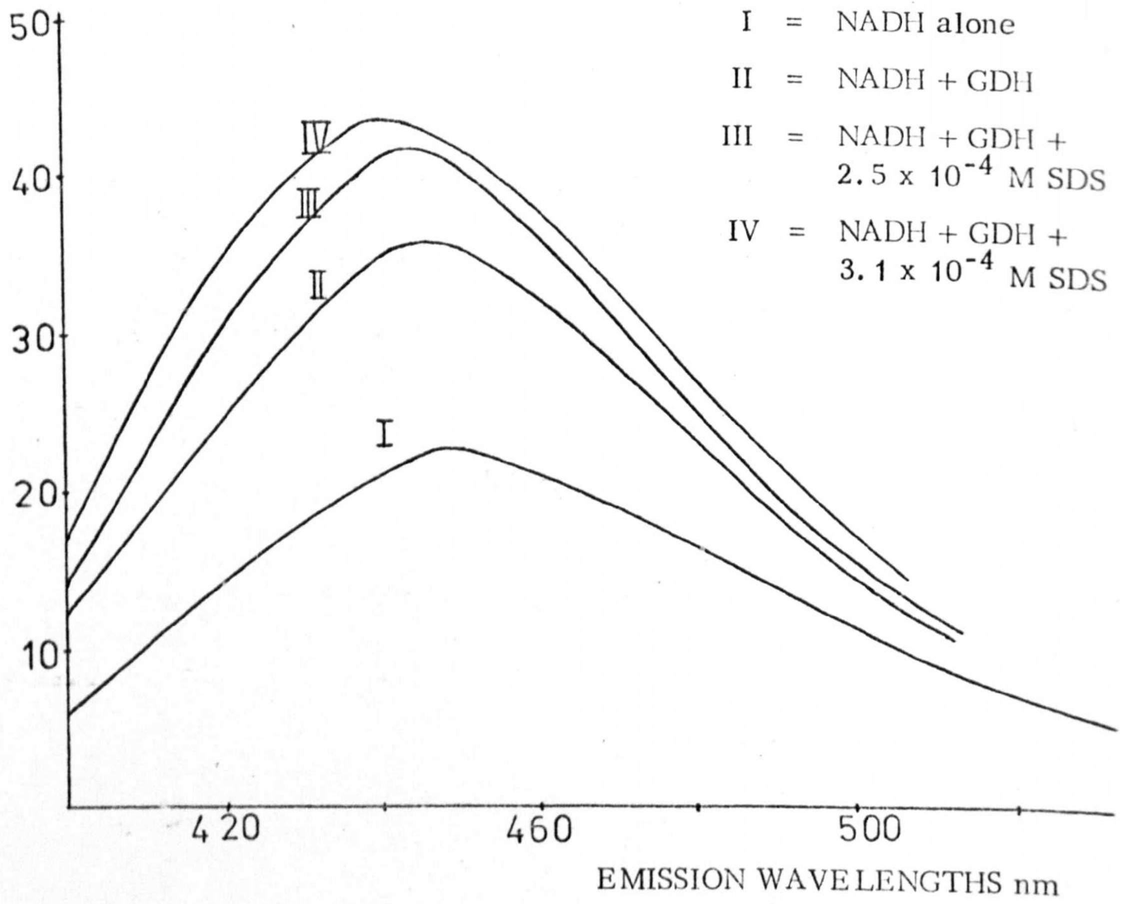


Fig. 68 The effect of SDS on NADH fluorescence in the presence of GDH



phospholipids which, due to the presence of double bonds in their structures, have transition temperatures well below $0^{o}C$ (Papahadjopoulos and Kimelberg, 1974). Thus, in the range of temperatures covered in these experiments, they are in their liquid crystalline state, with the hydrocarbon chains in an α - conformation and a lamellar phase (Tardieu et al, 1973).

It is a property of the enzyme to undergo allosteric conformational changes in the presence of NADH and GTP (Bayley and Radda, 1965). Thus, GTP further increases the NADH fluorescence enhancement in the presence of the enzyme (Figure 66).

Phosphatidylserine (0.53 mM) did not affect NADH (2 mM) fluorescence when partially or fully bound to the enzyme, both in the absence and presence of GTP (0.2 mM) (Figure 67). Thus, in the presence of the phospholipid membranes, the enzyme can bind NADH and can undergo the heterotropic allosteric conformational changes induced by GTP.

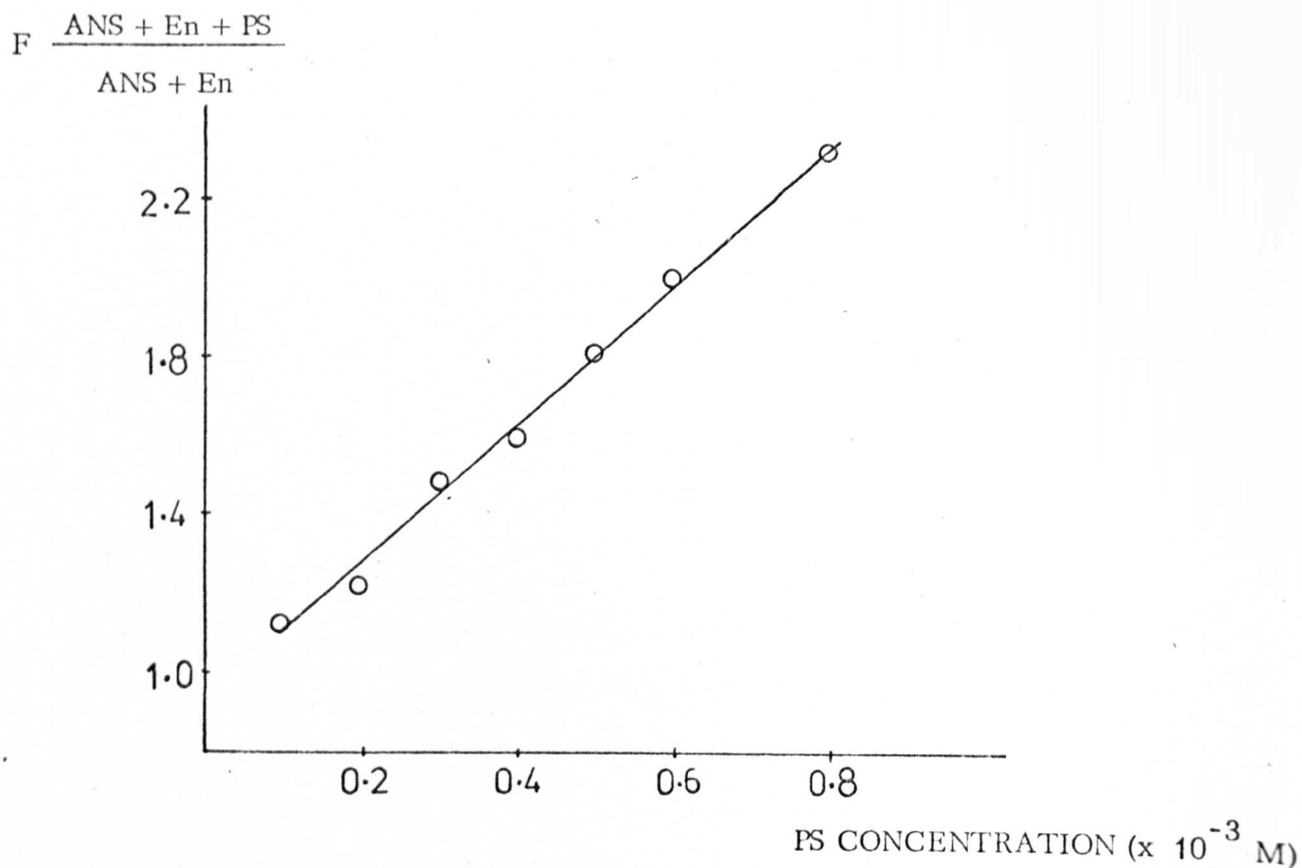
The anionic detergent, SDS, did not affect NADH fluorescence at concentrations up to 0.1 mM. Above this concentration at which irreversible complex formation occurs, NADH fluorescence (10 μM) is increased with increasing SDS concentration with λ max of fluorescence emission being slightly blue-shifted (Figure 68). Above a concentration of 0.3 mM, the system showed a time-dependency effect with NADH fluorescence rapidly decreasing with time.

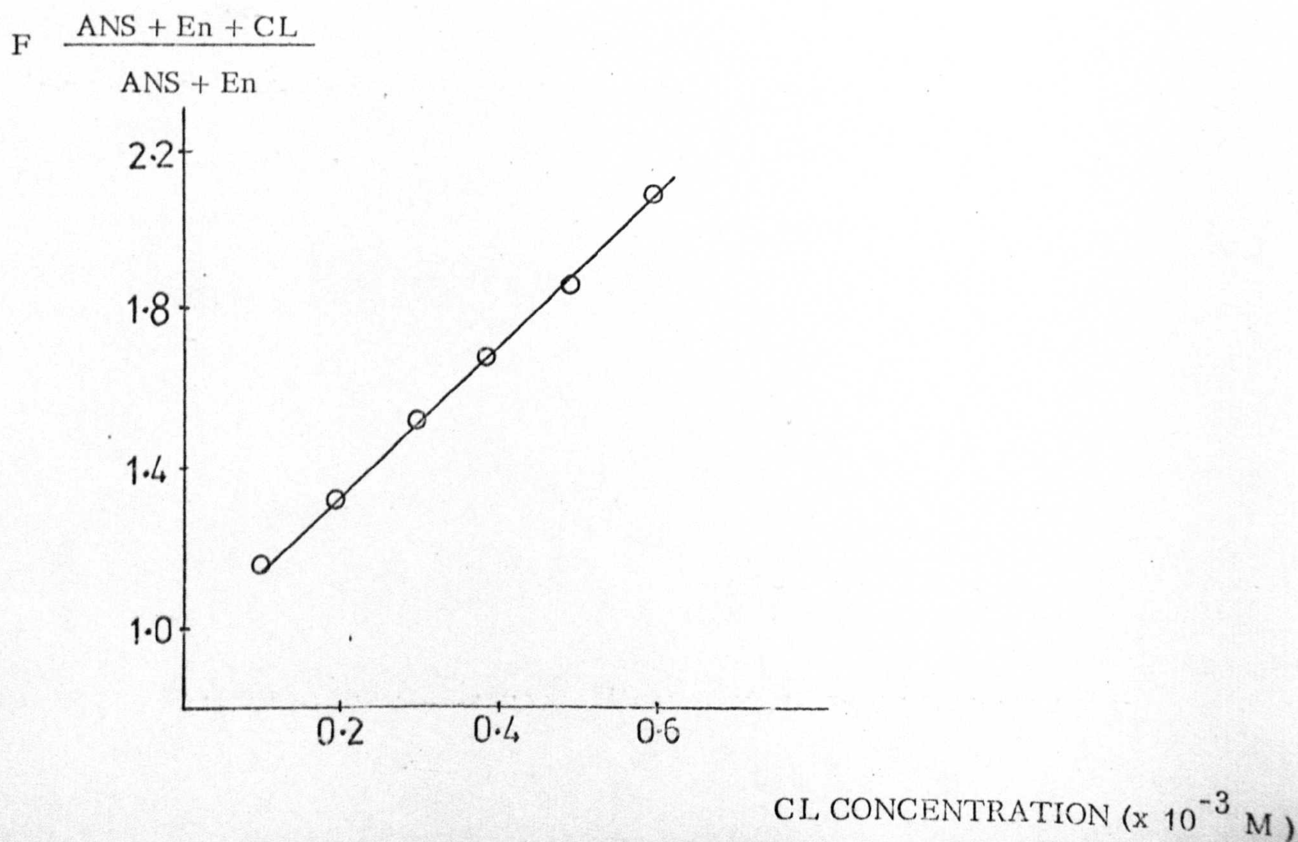
This effect of SDS clearly indicates strong dependency of structural changes brought about in the enzyme molecules on concentration of the detergent. At concentrations below 0.1 mM at which reversible complex

formation takes place, NADH binding to the enzyme is not at all affected. At concentrations above 0.1 mM, the detergent brings about unfolding of the enzyme structure, presumably creating new binding sites for the coenzyme. Above a concentration of 0.3 mM, total disruption of the polypeptide regions with respect to NADH binding sites takes place and the time-dependency effect is observed.

Thus, neither the intrinsic fluorescence of the enzyme (Figure 57) nor the NADH binding to the enzyme (Figure 68) is affected by SDS at concentrations lower than 0.1 mM.

Fig. 69/70 Further enhancement of ANS fluorescence (in the presence of GDH) by PS and CL





Extrinsic fluorescence studies were carried out using ANS as a fluorescent probe. ANS is probably the most widely used fluorescent probe. It is a type of molecule for which the dipole moments of its different electronic states are in the order $S_1 >, T_1 > S_0$ where S_1 is the excited singlet state, T_1 is the excited triplet state and S_0 is the ground state. Thus, the excited state is more polar than the ground state and this difference has been shown to be in the region of 10 - 12 D (Weber, 1961). Thus, polar solvents interact more strongly with the probe in the excited state than in the ground state. Therefore, there is an inverse relationship between its fluorescence quantum yield and solvent polarity and this makes it very sensitive for hydrophobic sites.

The probe showed a weak affinity for the enzyme and strong binding to the zwitterionic lipids, lysolecithin and phosphatidylcholine and the cationic amphiphile CETAB. ANS at a concentration of 1 μM showed maximum enhancement with the phospholipid and the cationic detergent at concentrations of 5.3×10^{-4} M and 5×10^{-5} M respectively. It showed no fluorescence enhancement with the anionic amphiphiles. The probe is essentially composed of two parts, the hydrophobic anilino-naphthalene ring and the polar sulphonate part. In binding to a micellar structure or a bilayer, the non-polar fluorescent moiety of the probe penetrates to a short distance between the fatty acid chains of the hydrocarbon core (Lesslauer et al, 1972).

ANS fluorescence when the probe was bound to lysolecithin (0.54 mM lysolecithin, 10 μM ANS) or phosphatidylcholine (0.53 mM PC, 1 μM ANS) was not affected by the enzyme confirming that no interaction between these lipids and the enzyme was occurring.

It has been shown by X-ray diffraction (Lesslauer et al, 1971) and

nuclear magnetic resonance (Colley and Metcalf, 1972) as well as from considerations of the quantum yield of the bound form (Haynes, 1972, Trauble, 1971) that the probe is located in the polar head group region of the membrane, probably in a manner described above (Lesslauer et al, 1972). The fact that ANS binding to these membranes increases with increasing lipid concentration, saturating at high concentrations suggest that ANS reacts with "binding sites" in these membranes.

ANS fluorescence in the presence of the enzyme was, however, affected by phosphatidylserine and cardiolipin. These phospholipids were shown to increase ANS fluorescence considerably (Figures 69 and 70).

This further enhancement of ANS fluorescence can occur in three ways:

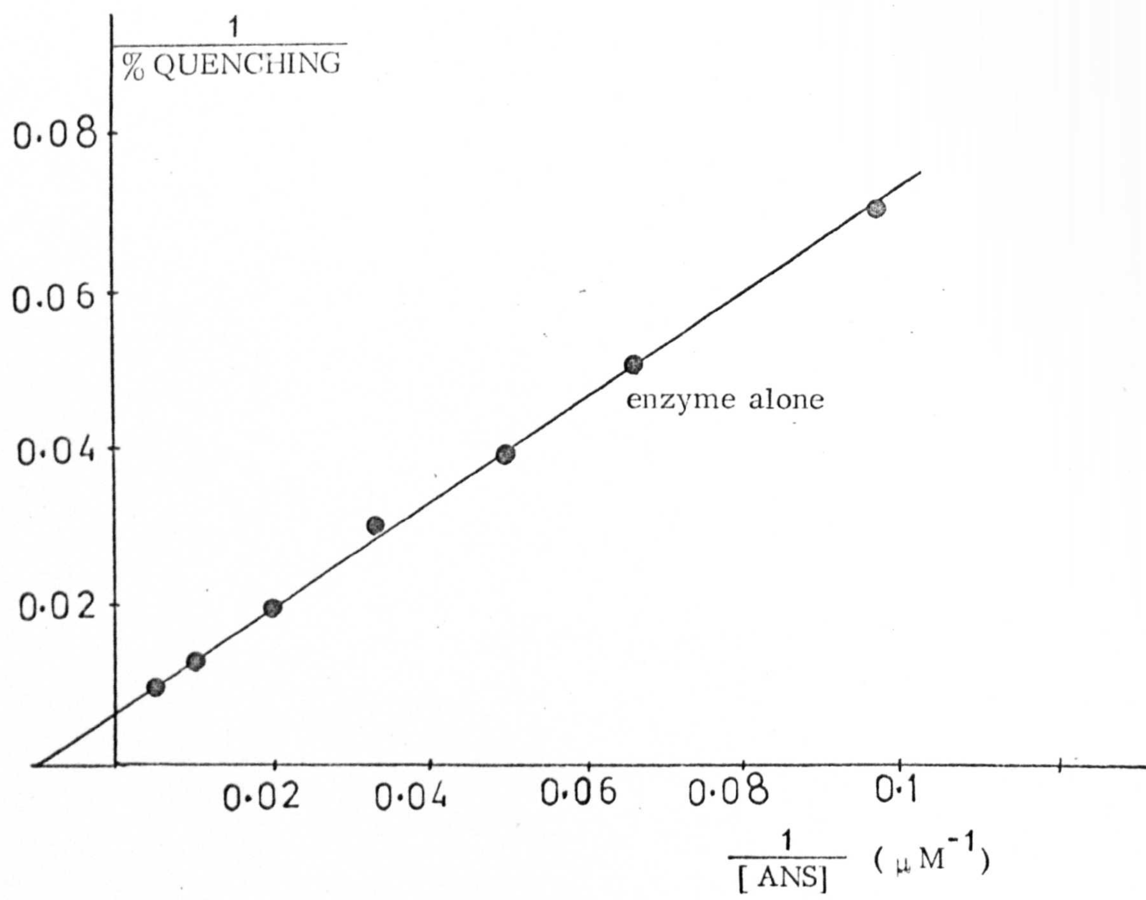
1) Anionic phospholipids can bring about conformational changes in the enzyme with new or more accessible hydrophobic binding sites in the enzyme structure for ANS binding.

2) In the process of complex formation, neutralisation of some of the negatively charged head groups in the phospholipid bilayer structure may take place. In this situation, penetration of the negatively charged ANS molecules into the hydrohpobic region of the bilayer is possible.

3) At positions in the bilayer where slight insertion of some of the hydrophobic residues of the enzyme into the phospholipid bilayer occurs, ANS penetration into the hydrophobic bilayer region may take place.

To investigate this further, binding of ANS to the enzyme was studied by looking at the quenching of intrinsic fluorescence of the enzyme brought

Fig. 71/2 GDH fluorescence quenching by ANS - double reciprocal plot of percentage fluorescence quenching against ANS concentration



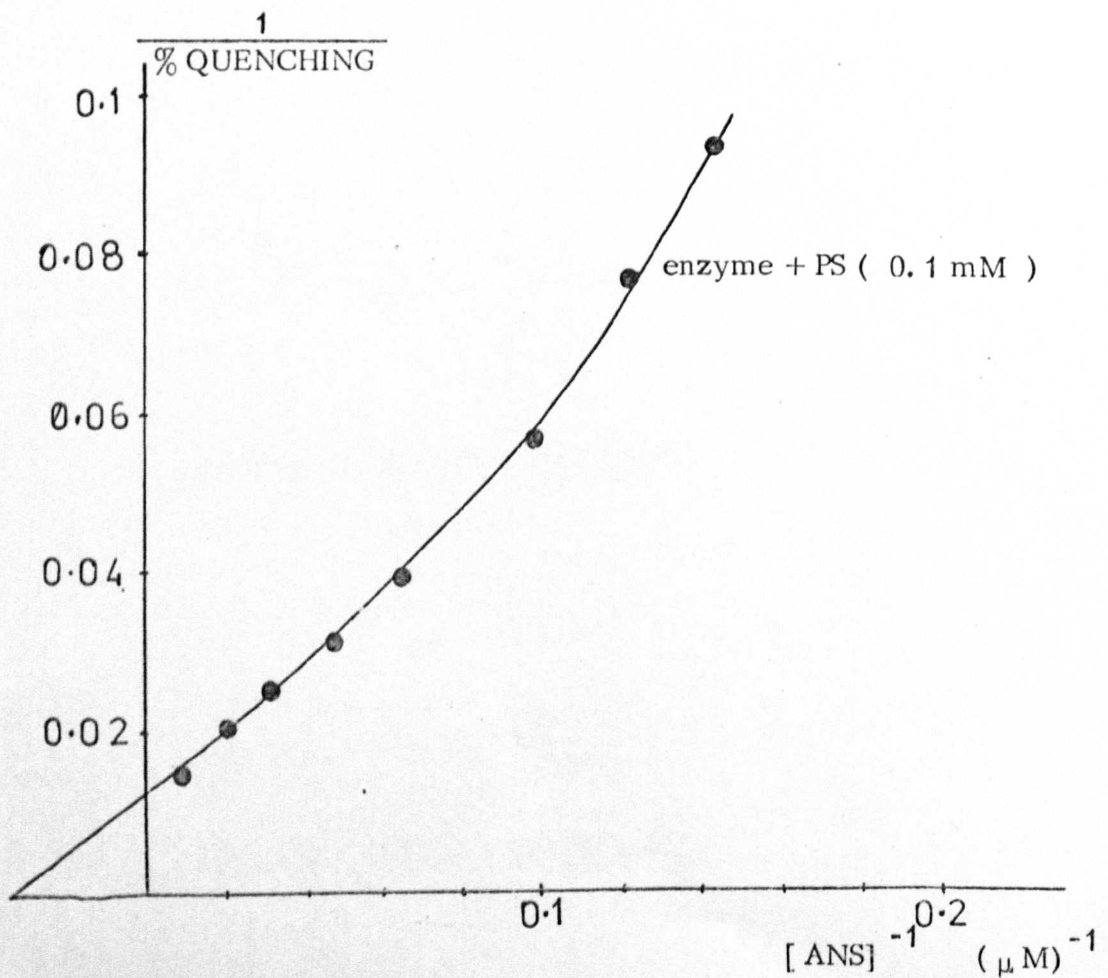
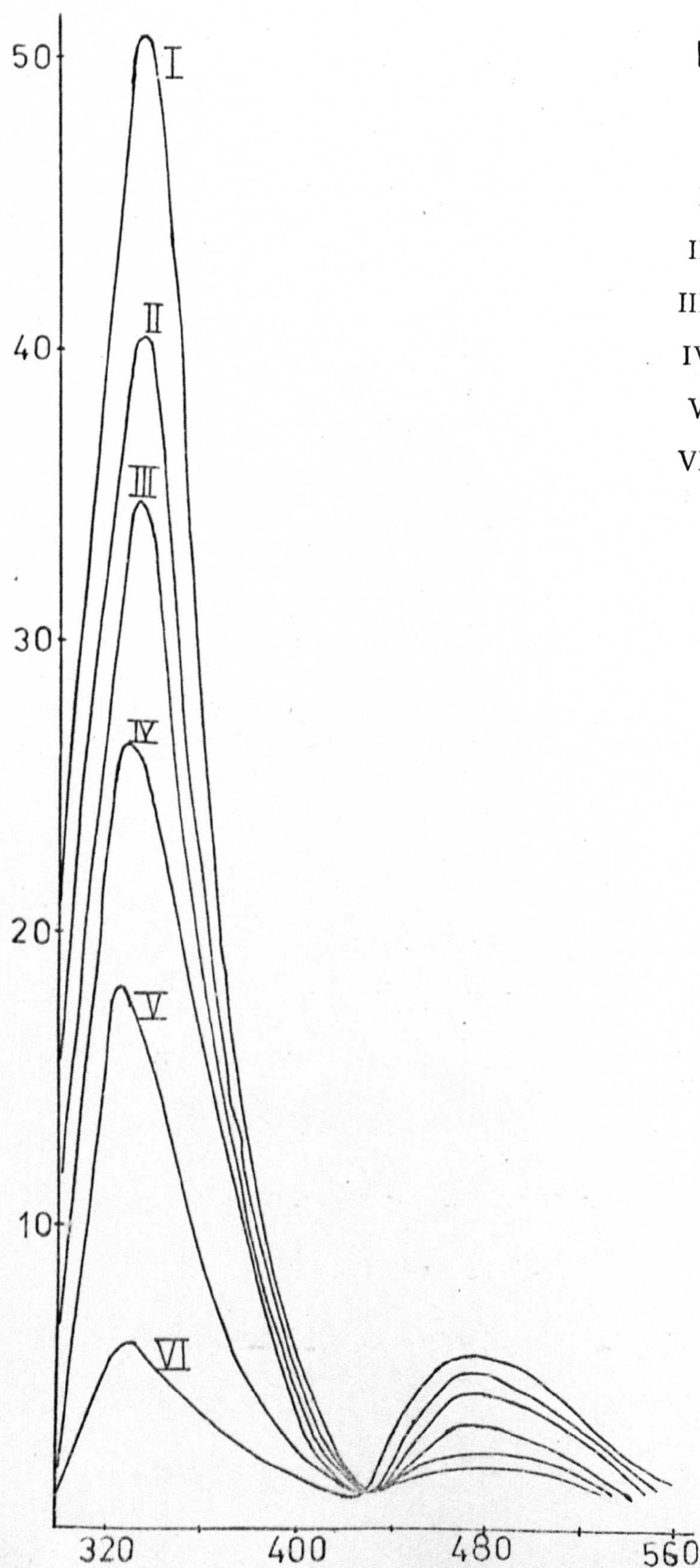


Fig. 73 GDH fluorescence quenching by ANS



[En] = 50 µg/ml

I = enzyme alone

II = enzyme + 10 µM ANS

III = enzyme + 20 µM ANS

IV = enzyme + 30 µM ANS

V = enzyme + 50 µM ANS

VI = enzyme + 100 µM ANS

EMISSION WAVE LENGTHS (nm)

about by ANS.

An average dissociation constant of 1.2×10^{-4} M was obtained from a reciprocal plot of the data obtained from a study of the interaction between ANS and the enzyme from the quenching data (Figure 71). A straight line from such a treatment in the absence of any phospholipid was converted to a curve thus showing the appearance of new binding sites for ANS in the lipid-enzyme complex. (See Figure 72).

As shown in Figure 73 when ANS is added to the enzyme, the enzyme fluorescence is quenched with increasing concentration of the probe and a band is progressively developed in the region of 465 - 475 nm indicating energy transfer from excited aromatic amino acid residues to ANS. Fluorescence emission spectra of the enzyme were recorded by exciting the system at 296 nm and high scan speed so that photoinactivation of the enzyme which occurs at lower wavelengths did not take place.

Thus, whatever, the mechanism of ANS fluorescence enhancement by phospholipids is, the process takes place because of complex formation between the enzyme and the lipids.

The effect of some of the metabolites involved in the activity and its regulation were subsequently studied. As discussed previously, some of these metabolites such as ADP, GTP and NADH were shown to quench the intrinsic fluorescence of the enzyme extensively and it was not found possible to look at their effects on the lipid-enzyme complex formation by intrinsic fluorescence studies. On the other hand, extrinsic fluorescence studies made this possible.

TABLE 3

EFFECT OF ADP ON FURTHER ENHANCEMENT OF ANS FLUORESCENCE PARTIALLY BOUND TO GDH BY PHOSPHATIDYLSERINE. ANS, GDH AND PS AT FINAL CONCENTRATIONS OF 25 μM, 0.67 mg/ml AND 0.83 mM.

condition	ANS + GDH	ANS + GDH + PS	ratio of enhancement
0.02 M hepetri + 1.6 mM phosphate, pH 7.3	23.5	53.5	2.27
1×10^{-5} M ADP	15	47.5	3.16
2×10^{-5} M ADP	14	46	3.28
1×10^{-4} M ADP	13.5	43.5	3.22
0.3 mM ADP	13	41	3.15
1 mM ADP	13	42	3.23
2 mM ADP	13	41.5	3.19

TABLE 4

EFFECT OF GTP AND NADH ON FURTHER ENHANCEMENT OF ANS FLUORESCENCE PARTIALLY BOUND TO GDH. ANS, GDH AND PS AT FINAL CONCENTRATIONS OF 25 μM, 0.67 mg/ml AND 0.7 mM.

condition	ANS + GDH	ANS + GDH + PS	ratio of enhancement
0.02 M hepetri + 1.6 mM phosphate, pH 7.3	8	15	1.88
0.1 mM GTP	8	15	1.88
1.0 mM GTP	8.5	15	1.81
0.1 mM NADH	10	16.5	1.65
0.2 mM NADH	11.5	18.0	1.57
0.5 mM NADH	14	21.5	1.53
1 mM NADH	15.5	23	1.48
2 mM NADH	22.5	31	1.38
0.1 mM GTP + 0.1 mM NADH	20.5	26	1.27
0.5 mM GTP + 0.5 mM NADH	24.5	31.5	1.28
1 mM GTP + 1 mM NADH	26.5	33	1.24

TABLE 5

EFFECT OF NAD^+ ($\pm$ α-Kg) ON FURTHER ENHANCEMENT OF ANS FLUORESCENCE PARTIALLY BOUND TO GDH. ANS, GDH AND PS AT FINAL CONCENTRATIONS OF 25 μM, 0.5 mg/ml AND 0.65 mM.

condition	ANS + GDH	ANS + GDH + PS	ratio of enhancement
0.02 M hepetri + 1.6 mM phosphate, pH 7.3	12.5	23.3	1.86
2 mM NAD^+	7.5	19.0	2.54
1 mM NAD^+	7.5	19.3	2.58
0.5 mM NAD^+	8.5	19.5	2.30
0.1 mM NAD^+	10.7	22.5	2.10
0.5×10^{-4} M NAD^+	11.6	22.5	1.94
0.5 mM NAD^+ + 1 mM α-Kg	7.5	22	2.93
0.5 mM NAD^+ + 5 mM α-Kg	9.3	27	2.90
5 mM α-Kg	13.0	24.0	1.85

ADP further increased ANS fluorescence enhancement by phosphatidylserine. NAD^+ also caused an increase. NADH, especially in the presence of GTP, caused a lowering of ANS fluorescence enhancement by phosphatidylserine. GTP showed no appreciable effect. These results are summarised in Tables 3-5.

The influence of the nucleotides and substrates on the degree of binding of the enzyme to phospholipid membranes may be explained by their effect on enzyme conformation.

The complexes formed between the enzyme and its substrates (α-Kg and l-glutamate) and its coenzymes (NADH and NAD^+) have been characterised (Cross, 1972, Pantaloni and Lecuyer, 1973). Also a number of monocarboxylic acids were shown to complex with the enzyme and to cause structural changes (Prough et al, 1972, Prough and Fisher, 1972). The activity of the enzyme is subject to allosteric regulation by ADP and GTP. GTP is a strong inhibitor and ADP is an activator (Frieden, 1963). Both these effectors cause conformational changes in the enzyme (Dodd and Radda, 1968, 1969).

There is therefore a high degree of specificity involved in the interaction between glutamate dehydrogenase and phospholipid membranes. The same type of specificity has been shown in the association of glyceraldehyde-3-phosphate dehydrogenase with erythrocyte membranes (Kant and Steck, 1973, Letko and Bohnensack, 1974). There is a loose association between this enzyme and erythrocyte membranes (Mitchell et al, 1965). Interaction was profoundly influenced by low concentration of certain metabolites including glyceraldehyde-3-phosphate and NAD^+. These have been shown to cause structural changes upon binding to the enzyme (Bolotina et al, 1967). The enzyme bound reversibly to

the inner (but not outer) erythrocyte membrane surface. In contrast, the basic protein cytochrome c lacked specificity in its association with erythrocyte membranes. Binding occurred at both membrane surfaces and was not affected by any metabolites tested. Thus, in the case of the association between the enzymes glutamate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase with membranes, the process represents specific binding rather than non-specific adsorption.

It has been shown that rat liver mitochondria respond to variations of the extramitochondrial environment by releasing proteins and enzymes from the inner membranal compartment towards the intermembranal space. Rebinding of released proteins was observed when the "releasing" effectors were withdrawn from the extramitochondrial medium (Waksman and Rendon, 1971 a, 1971 b). In particular, the extent of binding of mitochondrial aspartate aminotransferase and malate dehydrogenase was found to depend on succinate concentration. Further studies (Rendon and Waksman, 1973) showed that the process also occurs with isolated inner mitochondrial membrane and is not directly dependent upon the variation of ionic strength of the system.

In all these cases, it is possible that binding of the metabolites to the enzymes is followed by conformational changes in the protein molecules, and that different conformations of the enzyme show different affinities for binding to the membranes. As discussed above, ADP increased GDH binding to phospholipid membranes while NADH especially in the presence of GTP caused a decrease.

The effect of these metabolites on ANS fluorescence enhancement in the

presence of GDH and phospholipids resemble the findings of Azzi et al, (1969) on the dependence of ANS fluorescence enhancement on conformation of cytochrome c in cytochrome c-phospholipid complex. While no changes of ANS fluorescence were observed in the transition of ferri-cytochrome c in the absence of lipids, changes in fluorescence characteristics of bound ANS in the presence of the phospholipids were observed. These were a 2.5% increase in fluorescence at 470 nm and approximately a 5 nm blue shift of the peak, these changes being due to changes in the structure of the protein during the transition from an oxidised to a reduced state. These changes are much smaller than those observed in the case of glutamate dehydrogenase-phospholipid interaction. The enzyme is a much more flexible structure and can undergo large conformational changes.

Recently, (Jori et al, 1974), conformational changes in cytochrome c by its interaction with cardiolipin were indicated by an increase of its tryptophan fluorescence emission and appreciable perturbation of its circular dichroism spectrum. The results were taken to suggest that the interaction between cardiolipin and cytochrome c provokes a perturbation of the protein conformation which possibly involves the disruption of the hydrogen bonds linking the aromatic rings of tryptophan - 59 and tyrosine - 48 with one propionic side chain of the heme.

McGivan et al (1973) have reported the effect of l-leucine and some other monocarboxylic acids to stimulate glutamate dehydrogenase activity in intact mitochondria. Previous to these findings, it has been shown that these amino acids stimulate the isolated enzyme (Yielding and Tomkins, 1961, Kun and Achmatowicz, 1965) and bring about conformational changes in the enzyme

structure (Prough and Fisher, 1972).

In view of our findings, it is possible that these amino acids, by complexing with the enzyme, bring about conformational changes in its structure and this affects its binding to the mitochondrial membrane.

The fact that this stimulation only occurred in the direction of l-glutamate synthesis may be explained in terms of the conformation specificity of association of the enzyme to the mitochondrial membrane when in its quaternary complex E - NADH - α Kg - NH_4^+.

The affinity of the enzyme for binding to phospholipid membranes in the presence of its substrates, coenzymes and allosteric effectors has been explored as discussed above. However, it was not possible to explore such possibilities when the enzyme is in its final quaternary (E - NADH - α Kg - NH_4^+) or tertiary (E - NAD^+ - l-glu) complex because of formation of the products. Some attempts in the latter case have been made at a low pH as discussed above.

The other problem associated with such studies is that NH_4^+ decreases the binding of the enzyme to phospholipid membranes because of the involvement of an electrostatic interaction. Thus, the binding of the enzyme, solely due to the type of conformation that the enzyme will adopt in the presence of NH_4^+ (in the absence or presence of α - Kg and NADH), is complicated.

The above results also indicate that NADH especially in the presence of GTP reduced the binding. NAD^+ caused a slight increase. Thus, the state of oxidation/reduction of pyridine nucleotides in mitochondria may affect binding of the enzyme to the inner mitochondrial membrane. The mean [NAD^+] [NADH]

ratio within the rat liver mitochondria was found to be 8 to 1 in well fed rats and 5 to 1 in starved rats (Williamson et al, 1967). Thus, with all other conditions being equal, oxidised state of these nucleotides would be expected to cause a higher extent of binding. Also in vivo, metabolic activity can cause local changes in the state of reduction of the pyridine nucleotides and concentration of other metabolites and thus affect binding of the enzyme to the mitochondrial membrane.

Chapter V

PART a) EXTRACTION OF GDH-PHOSPHOLIPID COMPLEX INTO ISOOCTANE

PART b) EFFECT OF PHOSPHOLIPIDS ON THE STATE OF POLYMERISATION OF GLUTAMATE DEHYDROGENASE - STOPPED-FLOW EXPERIMENTS

EXTRACTION OF GLUTAMATE DEHYDROGENASE - PHOSPHOLIPID COMPLEX INTO ISOOCTANE

The formation of isooctane-soluble complexes between the basic protein cytochrome c and phospholipids has been described (Das and Crane, 1964, Das et al, 1965). Optical rotatory dispersion (Ulmer, 1965) and ESR spin-labelling technique (Barratt et al, 1968) and small-angle X-ray scattering studies (Shipley et al, 1969) have been used to further characterise these lipoproteins.

During these studies (Das and Crane, 1964), it has been shown that the complexes approach certain stoichiometric properties depending upon the conditions of formation. The major complex formed from mixed phospholipids (PS - PC) was found to contain 22 phosphorus per mole of cytochrome c when the original cytochrome c was in excess of the phospholipid. When more of the phospholipid was added, the ratio was found to be 32 : 1. Monovalent, divalent and trivalent cations inhibited complex formation in increasing order.

In the studies on the complex formation with highly purified lecithin and cytochrome c (Das et al, 1965) it was found that lecithin alone cannot form complex with cytochrome c but when it is included with the acidic phosphatidyl-serine, it increases the stability of the lipid-protein complex in the hydrocarbon solvent. In one such experiment, it was found that when the ratio of lecithin to phosphatidylserine in the aqueous phase reached the value of 1.43, there was maximum extraction of cytochrome c into the isooctane phase. The ratio of the extracted lecithin to acidic lipid in the complex was also found to be 1.43. The corresponding molar phosphorus to cytochrome c ratio in the complex was about 23.

TABLE 6

EXTRACTION OF GDH INTO ISOOCTANE

fraction number	PC/PS ratio	O.D. at 280 nm	% extraction
I	3 ; 2	0.155	90
II	4 : 1	0.030	17
III	2 : 3	0.103	60
IV	1 : 4	0.108	63

Lamellar structures were indicated from X-ray diffraction studies (Shipley et al, 1969). The structures were of two basic types with dimensions of 87 A^{o} and 116 A^{o} depending on the lipid : cytochrome c ratio. These dimensions were consistent with the incorporation of either one or two layers of cytochrome c molecules, respectively, between the phospholipid bilayer. Occasionally a hexagonal phase with a cylinder to cylinder axia of 85 A^{o} was obtained.

In an attempt to extract a GDH-phospholipid complex, four different 0.1% cosonicates of PS and PC were prepared in 5 mls in the following ratios:

1) PC : PS - 3 : 2

2) PC : PS - 4 : 1

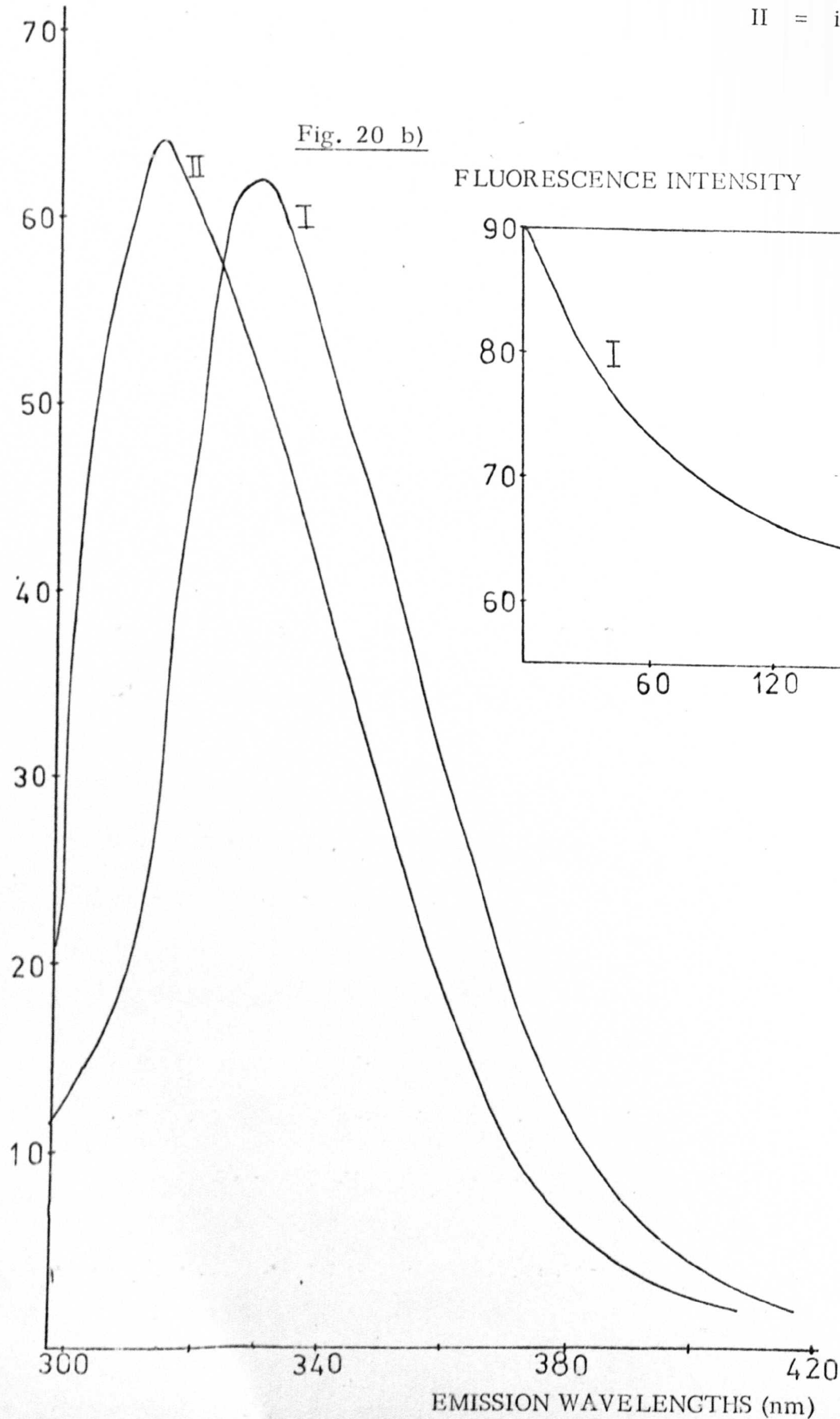
3) PC : PS - 2 : 3

4) PC : PS - 1 : 4

The phospholipids were sonicated in 0.06 M tris buffer + EDTA, pH 6.5. 1.0 ml of the phospholipid sonicate was added to 1 ml of the enzyme (0.86 mg/ml) in 0.06 M tris buffer in a 25 ml volumetric flask, and the mixture was shaken for a few minutes. The aqueous phase was then made 30% with respect to ethanol (0.6 ml of ethanol) and the flasks were shaken by a flask shaker for 30 minutes. The mixtures were then centrifuged on a bench centrifuge for 5 minutes.

The presence of the protein in the isooctane phase was shown by taking u.v. and fluorescence spectra of the fractions. The O.D. at 280 of the isooctane layer and the percentage of extraction of the protein corresponding to different PC : PS ratios are shown in table 6.

Figure 20 b) The fluorescence spectra of GDH in aqueous and hydrocarbon environments; c) Photodecomposition of GDH



The intrinsic fluorescence properties of the extracted enzyme were found to be different from that in an aqueous environment. These were a shift of about 15 nm of λmax of fluorescence emission and the ability of the enzyme to resist photodecomposition. These are shown in Figure 20 b) & c). When excited at 280 nm, there is a sharp initial decrease in the fluorescence intensity of the enzyme in an aqueous phase. This indicates that the conformational states of the enzyme in the two phases are probably different.

However, the optical rotatory dispersion of cytochrome c phospholipid complex in isooctane were found to be unaltered in going from an aqueous phase to an organic phase (Ulmer et al, 1965, Ulmer, 1965). This is in contrast to the results obtained by Jori et al (1974) who found that the protein undergoes conformational changes on complexing with phospholipids in an aqueous phase.

In these cases, charge neutralisation must take place and the following arrangement of phospholipid and protein in the complex extracted in isooctane have been suggested (Shipley et al, 1969).

1) A close-packed core containing all the protein molecules surrounded by a lipid shell. This seems an unlikely structure unless the sites of interaction are very asymmetrically arranged.

2) A structure composed of an inner lipid core, a protein shell, and an outer lipid envelope. In this structure, all the lipid polar groups would be able to interact with charged sites anywhere on the protein surface.

In both these structures, the hydrophobic shell required for solubility in the isooctane would be present.

The fact that the λ max of emission in the case of glutamate dehydrogenase extracted in isooctane is lowered by about 15 nm is probably due to:

1) conformational changes in the enzyme molecule, positioning its chromophore residues in a more hydrophobic environment.

2) The presence of isooctane in the system may decrease the overall dielectric constant of the protein environment (with a decrease in microscopic dielectric constant) and this may result in a more favourable condition for the intrinsic fluorescence of the enzyme.

The fact that photodecomposition of the enzyme does not take place when extracted in the hydrocarbon phase, can also be due to either of these effects.

It is, however, interesting to note that some of the tyrosine, phenylalanine and tryptophan residues in the enzyme are believed to be located on the surface of the enzyme (Cross and Fisher, 1966). Although no explanation seems to have been given for photodecomposition of the enzyme when in an aqueous phase, it is possible that in this condition, the chromophores when excited to the singlet state acquire a higher dipole moment and can strongly interact with the surrounding water molecules of a high dielectric constant (D = 78.5). On the other hand, such interactions would not occur in isooctane which has a dipole moment of zero. This type of mechanism would not demand a conformational change in the enzyme.

Several subsequent attempts failed to repeat the extraction of the enzyme-phospholipid complex into isooctane. Different PC/PS and lipid-protein ratios were tried. Variations in temperature, volume of ethanol added and substitution

of ethanol by Dioxan and dimethyl sulphoxide were tried. Also it was not possible to re-extract the enzyme back to the aqueous phase.

However, extraction of cytochrome c-phospholipid complex into iso-octane was found to be an easy and reproducible process.

EFFECT OF PHOSPHOLIPIDS ON THE STATE OF POLYMERISATION OF GLUTAMATE DEHYDROGENASE

Glutamate dehydrogenase exhibits an association-dissociation equilibrium between its oligomers (each consisting of six identical polypeptide chains) and polymers which are all enzymatically active (Reisler et al, 1970). The process of polymerisation occurs at high enzyme levels (> 0.2 mg per ml).

The enzyme has been shown to undergo a stepwise association with increasing protein concentration (Eisenberg and Tomkins, 1968) shifting the predominant species from the monomer to dimer, trimer and so on. The process of polymerisation occurs along the axis of its ellipsoid subunits as shown in Figure 18. Thus, the enzyme possess two association sites for the formation of the elongated particles. The specific activity of the enzyme in the absence of purine nucleotides is essentially independent of enzyme concentration (Frieden and Colman, 1967).

It has been shown (Dessen and Pantaloni, 1969) that the enzymes from the pig and bovine liver are essentially identical with respect to polymerisation. However, the rat enzyme does not polymerise at high protein concentrations (Sedgwick and Frieden, 1969).

It has been shown that in aqueous solutions saturated with toluene, association of GDH is considerably enhanced (Eisenberg and Reisler, 1970). Thus, in the presence of toluene, high molecular weight aggregates are formed at considerably lower enzyme concentrations than in the absence of toluene. Substitution of one or two amino groups with acetic anhydride (Colman and Frieden, 1966) or with pyridoxal phosphate (Anderson et al, 1966) affects the dissociation-association eqillibrium and influences the enzymatic properties. Recently (Hucho et al, 1973) a histidine residue has been shown to be essential for the association of glutamate dehydrogenase. It was found that after

photooxidation of one histidine residue per polypeptide chain with pyridoxal 5'-phosphate, the enzyme sediments with sedimentation coefficients significantly lower than the 25 s of the native enzyme. The effect of a number of metabolites on the state of polymerisation of the enzyme has been discussed in the previous chapter (pages 55 - 56). Solvent perturbation difference spectroscopy (Cross and Fisher, 1966) indicated the same degree of perturbation of the chromophoric groups independent of protein concentration. Thus, no appreciable changes occur in environment of the chromophoric groups of the enzyme upon association of the monomeric enzyme to higher molecular weight forms.

Huang and Frieden (1969) followed the rate of depolymerisation of the enzyme induced by its reduced coenzymes and the purine nucleotides GDP and GTP. This was done by looking at absorbency (turbidity) changes at 310 nm by stop-flow. The rate of depolymerisation was found to be dependent on the relative concentrations of these metabolites.

Some preliminary experiments were carried out to investigate the possible effect of phospholipids on the state of polymerisation of the enzyme. The procedure of Huang and Frieden (1969) described above was followed.

A final enzyme concentration above 1 mg/ml was used at which the enzyme is known to be mainly in a polymeric form. It was found that on mixing the enzyme (in 0.1 M phosphate buffer) with phosphatidylserine (in Hepetri buffer), there was a very rapid (< 1 sec) increase in turbidity followed by a slow decrease. The time taken for the second phase of the reaction was found to be about 250 m sec with final concentrations of the enzyme and phospholipid at 1.1 mg/ml and 0.75 mM respectively. The time taken for completion of the

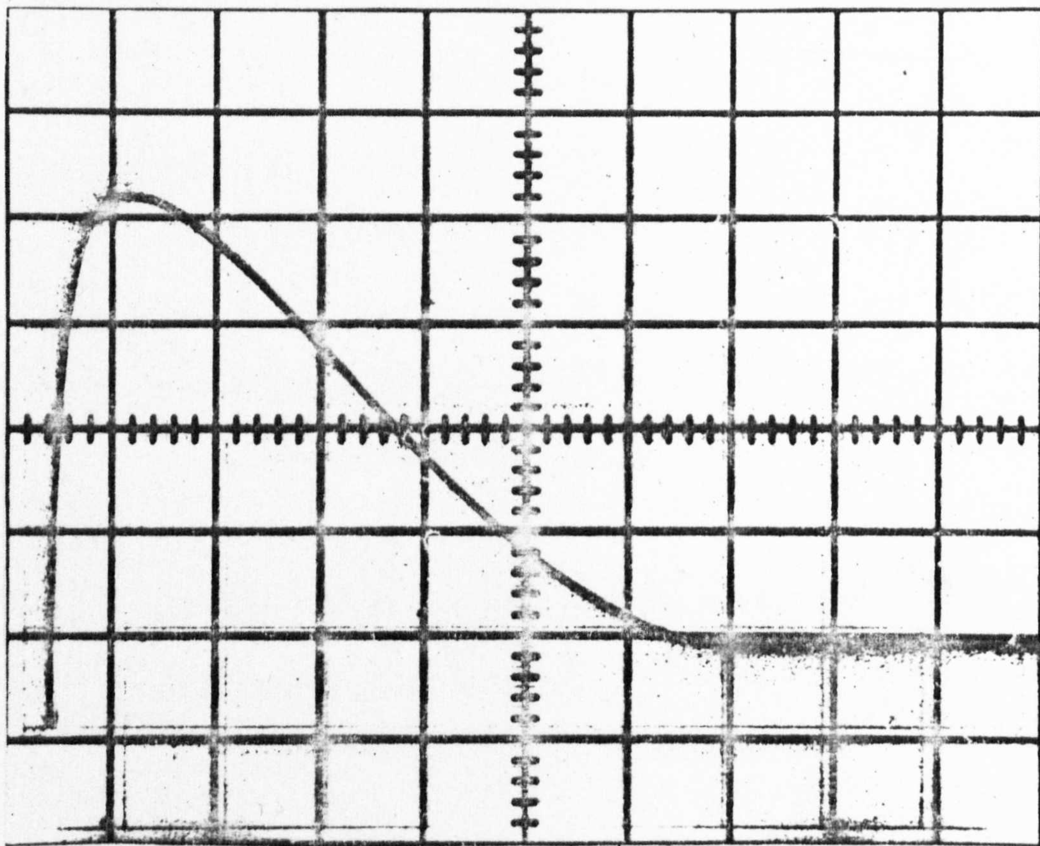


Fig. 74

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Fig. 74 Turbidity changes at 310 nm (50 m sec per division)

C_I) 1.1 mg/ml GDH

C_{II}) 0.75 mM PS

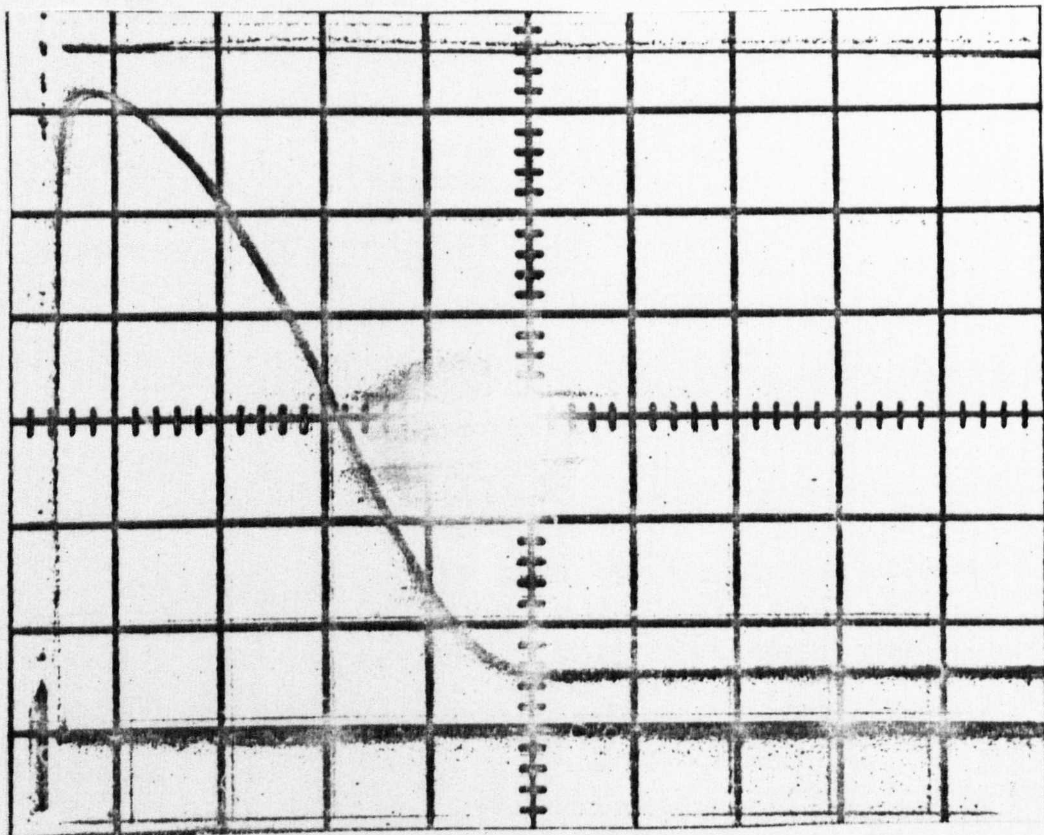

Fig. 75

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Fig. 75 Turbidity changes at 310 nm (15 sec per division)

C_I) 1.1 mg/ml GDH

C_{II}) 0.25 mM PS

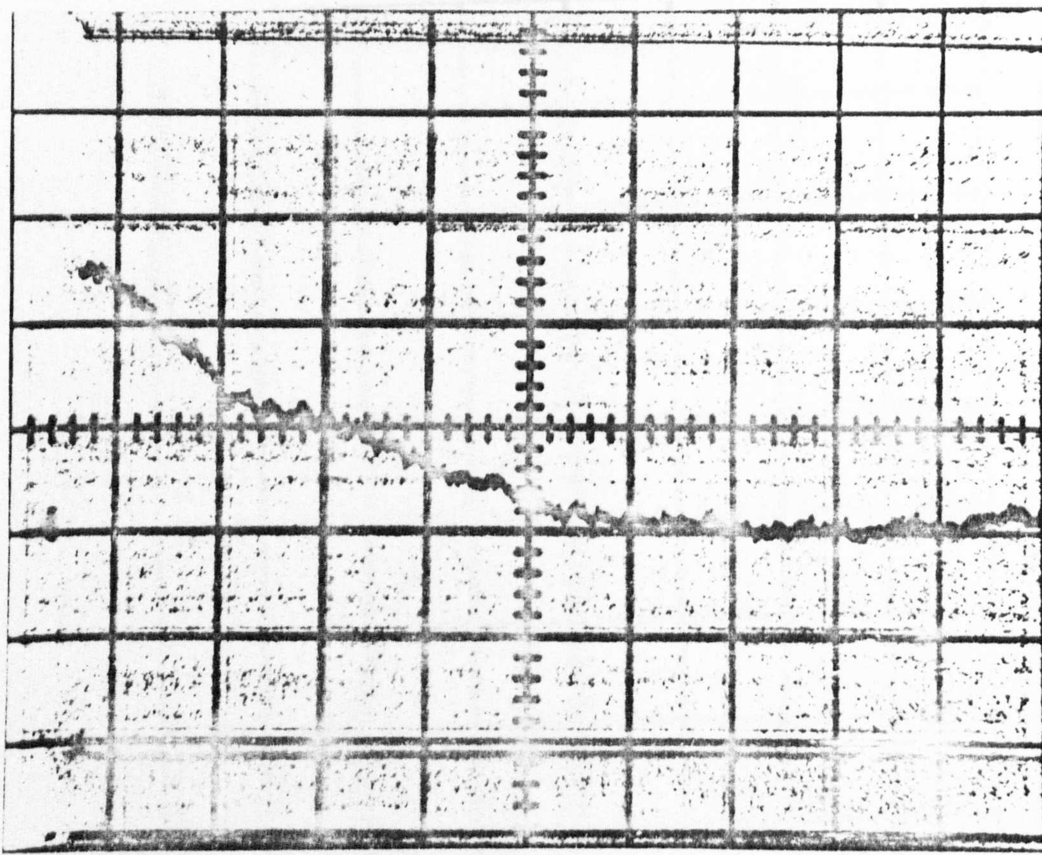

Fig 76

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Fig. 76 Turbidity changes at 310 nm (20 m sec per division)

C_I) 1. 15 mg/ml GDH

C_{II}) 30 μ M NADH + 50 μ M GTP

Fig 77

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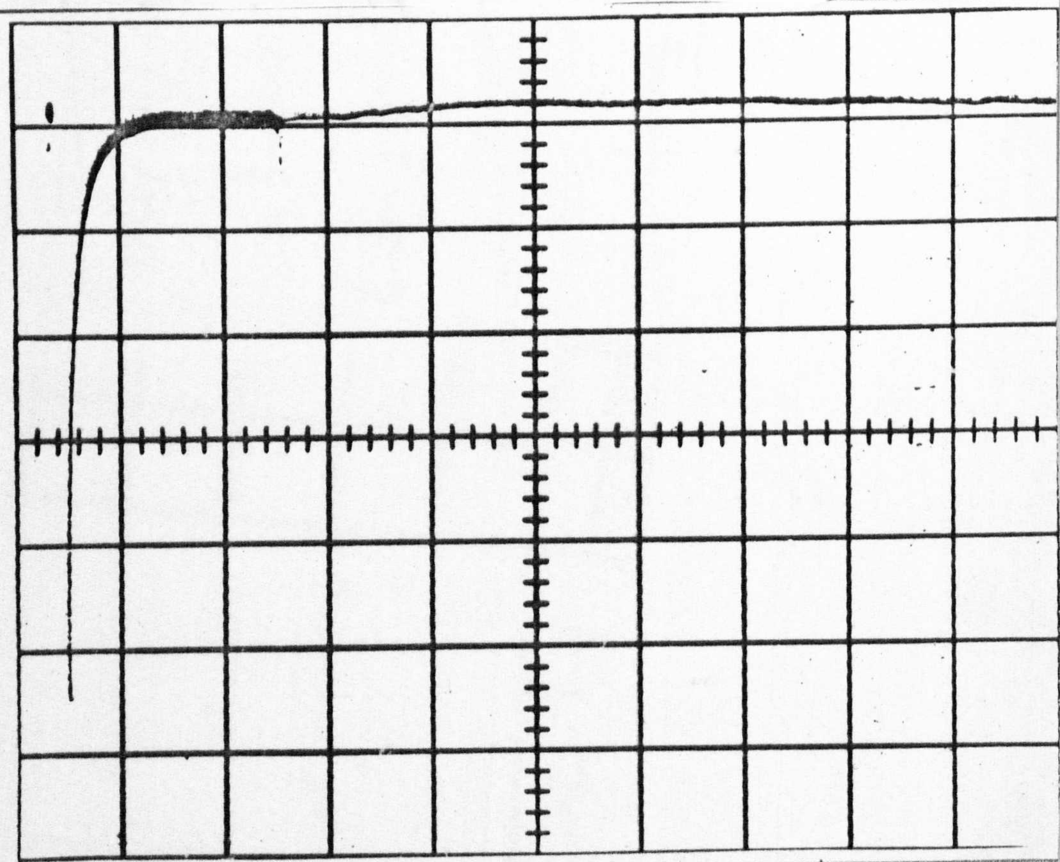

Fig. 77 Turbidity changes at 310 nm (15 sec per division)

C_I) 1. 15 mg/ml GDH + 30 μ M NADH + 50 μ M GTP

C_{II}) 0. 5 mM PS

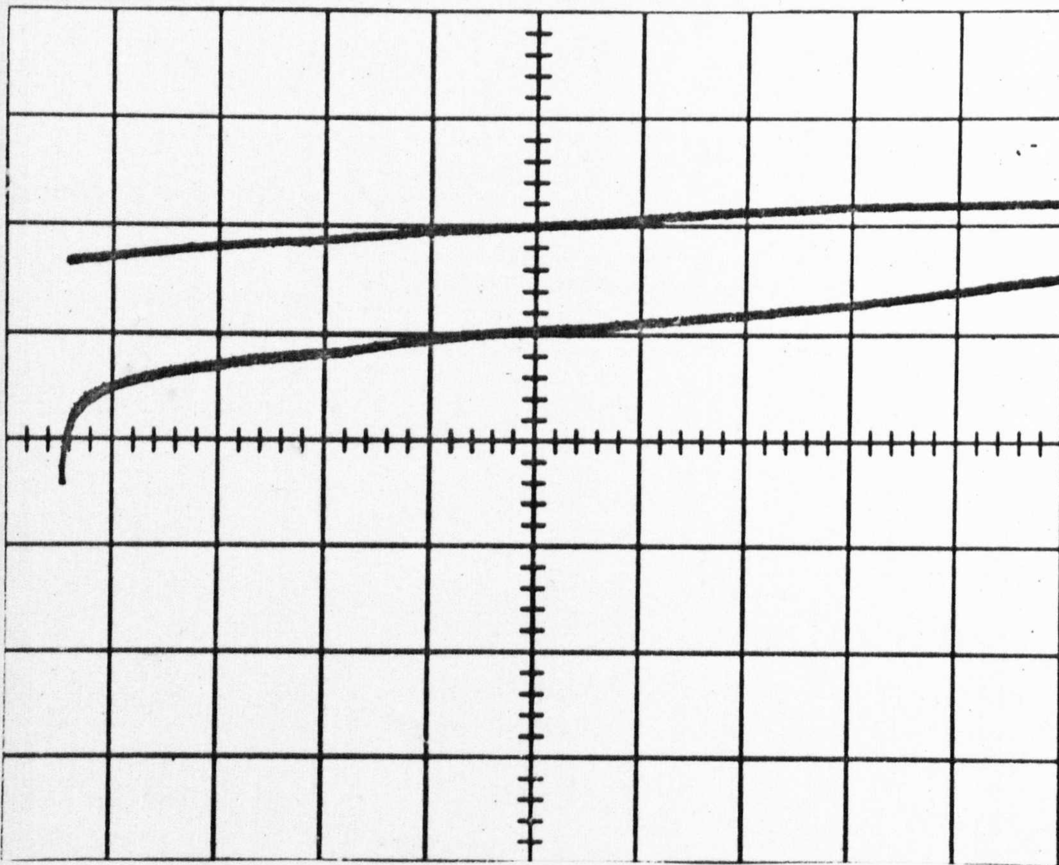

Fig 78

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Fig. 78 Turbidity changes at 310 nm (15 sec per division)

C_I) 1.15 mg/ml GDH

C_{II}) 0.5 mM PC

second phase was found to increase to 45 seconds with the same enzyme concentration but at PS concentration of 0.25 mM (Figure 74 & 75).

The fact that the enzyme was in a polymeric form was indicated by the decrease in turbidity of the enzyme at 310 nm in the presence of GTP (500 mM) and NADH (30 mM). The time taken for this process was found to be about 100 m sec (Figure 76). Also when the enzyme was incubated with these effectors at the above concentrations and its interaction with PS was repeated, no decrease in turbidity was resulted after the initial phase (Figure 77). Thus, the decrease in turbidity indicated in the second part of the diagram only occurred with a polymeric form of the enzyme and not with a depolymerised form. Also phosphatidylcholine which does not interact with the enzyme, does not cause any turbidity decrease (Figure 78).

The initial rise in turbidity was found to be, at least partly, due to interaction of the phospholipids with phosphate buffer. Though the phospholipid sonicates were prepared in hepetri buffer, the enzyme was left in 0.1 M phosphate buffer to make sure that no denaturation would take place. As discussed in the previous chapter, PS, when sonciated and left in phosphate buffer, lost its capacity to complex with GDH. The process, however, was found to be very slow (Figure 39).

The above results may be taken as an indication that the state of polymerisation of the enzyme is affected by its interaction with the anionic phospholipids such as PS. As discussed in the previous chapter, interaction of the enzyme with anionic phospholipids is followed by conformational changes. Polymerisation has been shown to be the result of end-to-end association (Sund, 1968) and an indefinite type with a single equilibrium constant (Krause et al, 1970).

Thus, the process of polymerisation only involves two association sites and this would mean that 4 of the 6 polypeptides of each enzyme oligomer are not involved in this process. As polymerization does not induce appreciable conformational changes in the enzyme (Cross and Fisher, 1966), most of the residues in the enzyme oligomer which may be involved in binding to a negatively charged phospholipid surface, are not expected to change their spatial position. The availability of these residues for binding to a phospholipid membrane is not, in view of the above argument, altered on association of enzyme oligomers.

It is, however, possible that after interaction of the polymeric form of the enzyme with a negatively charged phospholipid membrane, the two association sites discussed above are no longer exposed at the surface of the enzyme because of conformational changes. Even slight distortion of the spatial positioning of the residues close to or at the association sites may affect their availability for binding. Thus, the equilibrium in the process of association may be shifted in favour of enzyme oligomers.

All the above experiments were carried out at 15°C and did not cause any loss of enzymatic activity. A more detailed study in buffers which are free from phosphate is suggested.

Chapter VI

PART a) REMOVAL OF THE OUTER MITOCHONDRIAL MEMBRANE (PREPARATION OF MITOPLASTS) OF BEEF LIVER MITOCHONDRIA

PART b) BINDING OF GLUTAMATE DEHYDROGENASE TO MITOCHONDRIAL MEMBRANES

REMOVAL OF THE OUTER MITOCHONDRIAL MEMBRANE

Several methods are available for removal of the outer membranes of liver mitochondria. These are the swelling-shrinking procedure (Parsons et al, 1966, Parsons and Williams, 1967), digitonin fractionation (Schnaitman et al, 1967, Hoppel and Cooper, 1969) and a combination of swelling-shrinking and sonication (Sottocasa et al, 1967, Werner and Neupert, 1972). Phospholipase C has also been used for this purpose (Brosnan et al, 1973).

i) Phospholipase C effect

The effect of different concentrations of phospholipase C was examined by the use of marker enzymes. These were glutamate dehydrogenase, succinate-cytochrome c reductase and rotenone-insensitive NADH-cytochrome c reductase for, respectively, the matrix, the inner membrane and the outer membrane of mitochondria.

Thus, mitochondria divested of their outer membranes as compared to whole mitochondria would be expected to have higher specific activities for their matrix and inner membrane enzymes and a lower specific activity for the outer membrane enzyme. This will be discussed more fully later in this chapter.

In two separate experiments, concentrations of up to 100 mg and 212 mg of phospholipase C per 10 mg of mitochondrial proteins were tried. The results (Table 7) indicated that no appreciable changes in the specific activities of the marker enzymes were produced.

Phospholipase C (from clostridium perfringens) at concentration of 15mg/10 mg of mitochondrial protein was found to be sufficient for the

TABLE 7 EFFECT OF PHOSPHOLIPASE C ON MITOCHONDRIAL MEMBRANES - SPECIFIC ACTIVITIES OF MITOCHONDRIAL ENZYMES

mg phospholipase C per 10 mg mitochondrial protein	specific activities - μ moles/min/mg protein		
	glutamate dehydrogenase	succinate cytochrome c reductase	rotenone-insensitive cytochrome c reductase
0	1.36	0.159	0.91
8.5	1.27	0.185	0.86
17	1.39	0.21	0.82
34	1.40	0.211	0.80
59	1.43	0.22	0.89
110	1.39	0.23	0.83
212	1.34	0.25	0.81

TABLE 8 EFFECT OF DIGITONIN ON MITOCHONDRIAL MEMBRANES - SPECIFIC ACTIVITIES OF MITOCHONDRIAL ENZYMES

mg digitonin per 10 mg mitochondrial protein	specific activities - μ moles/min/mg protein		
	glutamate dehydrogenase	succinate cytochrome c reductase	rotenone-insensitive cytochrome c reductase
0	1.12	0.157	0.764
1.23	1.526	0.198	0.805
3.7	1.100	0.208	0.819
5.6	0.792	0.212	0.810
6.1	0.604	0.221	0.826
7.4	0.410	0.220	0.807
8.0	0.314	0.230	0.818
8.6	0.242	0.241	0.809
9.3	0.238	0.249	0.791

preparation of the mitoplasts (inner membrane plus matrix) of beef liver mitochondria (Brosnan et al, 1973). At high phospholipase/mitochondrial protein ratios (e.g. 60 mg/10 mg of mitochondrial protein) severe damage to the inner membrane was caused. The specific activity of phospholipase C used in this study is not given by the authors. Similar observations were made by Racker (1970). This type of effect would be clearly indicated by loss of glutamate dehydrogenase activity and therefore a small recovery of this enzyme (see digitonin effect, discussed later).

The results obtained with phospholipase C used in these experiments showed that (Table 7) even at very high phospholipase C concentrations, the specific activity of glutamate dehydrogenase was essentially unaltered. Thus, phospholipase C from cl. welchii did not show the capacity of removing the outer mitochondrial membrane under the conditions described.

ii) Digitonin effect

Digitonin has been extensively used for the preparation of inner membrane plus matrix particles (Schnaitman et al, 1967, Schnaitman and Greenawatt, 1968, Colbeau et al, 1971) from rat liver mitochondria. No such use has been as yet reported in the case of beef liver mitochondria. Despite the extensive use of digitonin for the solubilisation of lipoproteins, little is known about how it acts. It has been suggested that it may combine with free cholesterol to form a digitonide and that this in turn leads to solubilisation of disruption (Schnaitman et al, 1967).

Thus, the method is dependent on the fact that digitonin preferentially disrupts the outer mitochondrial membrane and that the degree of fragmentation is markedly dependent upon the digitonin-protein ratio.

TABLE 9 EFFECT OF DIGITONIN ON MITOCHONDRIAL MEMBRANES - SPECIFIC ACTIVITES OF MITOCHONDRIAL ENZYMES

mg digitonin per 10 mg mitochondrial protein	specific activities - μ moles/min/mg protein		
	glutamate dehydrogenase	succinate cytochrome c reductase	rotenone-insensitive cytochrome c reductase
0	1.205	0.145	0.665
0.49	1.260	0.150	0.702
0.68	1.293	0.168	0.630
0.78	1.300	0.171	0.678
0.88	1.336	0.175	0.690
0.99	1.336	0.175	0.690

TABLE 10 EFFECT OF DIGITONIN ON MITOCHONDRIAL MEMBRANES - SPECIFIC ACTIVITIES OF MITOCHONDRIAL ENZYMES

mg digitonin per 10 mg mitochondrial protein	specific activities - μ moles/min/mg protein		
	glutamate dehydrogenase	succinate cytochrome c reductase	rotenone-insensitive cytochrome c reductase
0	1.57	0.11	0.69
0.58	1.83	0.141	0.70
0.86	1.73	0.152	0.74
1.15	1.64	0.165	0.86

To study the effect of digitonin on beef liver mitochondria, aliquots of mitochondrial suspensions in medium A were placed in an ice bath and different amounts of cold digitonin solution were added with continuous stirring. In one such experiment, the digitonin concentration in the final mixture was varied to give digitonin-protein ratios of 0 - 9.3 mg digitonin/10 mg of mitochondrial protein.

It can be seen from the results summarised in Table 8 that initially, increasing the digitonin concentration resulted in higher specific activities of glutamate dehydrogenase and succinate-cytochrome c reductase as would be expected from disruption and removal of the outer membranes. However, at high digitonin/protein ratios, the specific activity of glutamate dehydrogenase progressively decreases with increasing digitonin concentrations. The dramatic decrease in the specific activity of the enzyme is taken to reflect disruption of the inner mitochondrial membrane by direct interaction or extensive swelling of mitochondria. Swelling of mitoplasts and disruption of the inner membranes have been shown to take place in the case of rat liver mitochondria at digitonin/protein ratio (expressed as mg of digitonin/10 mg of mitochondrial protein) of 1.94 (Schnaitman, 1967).

Subsequent experiments showed that in the range of 0.6 - 1.0 mg digitonin /10 mg of mitochondrial protein, the final preparations of digitonin-treated mitochondria had the highest glutamate dehydrogenase activities. Above and below this range, lower specific activities were resulted for glutamate dehydrogenase. Digitonin treatment also caused a higher specific activity for succinate-cytochrome c reductase. (See Tables 9 and 10).

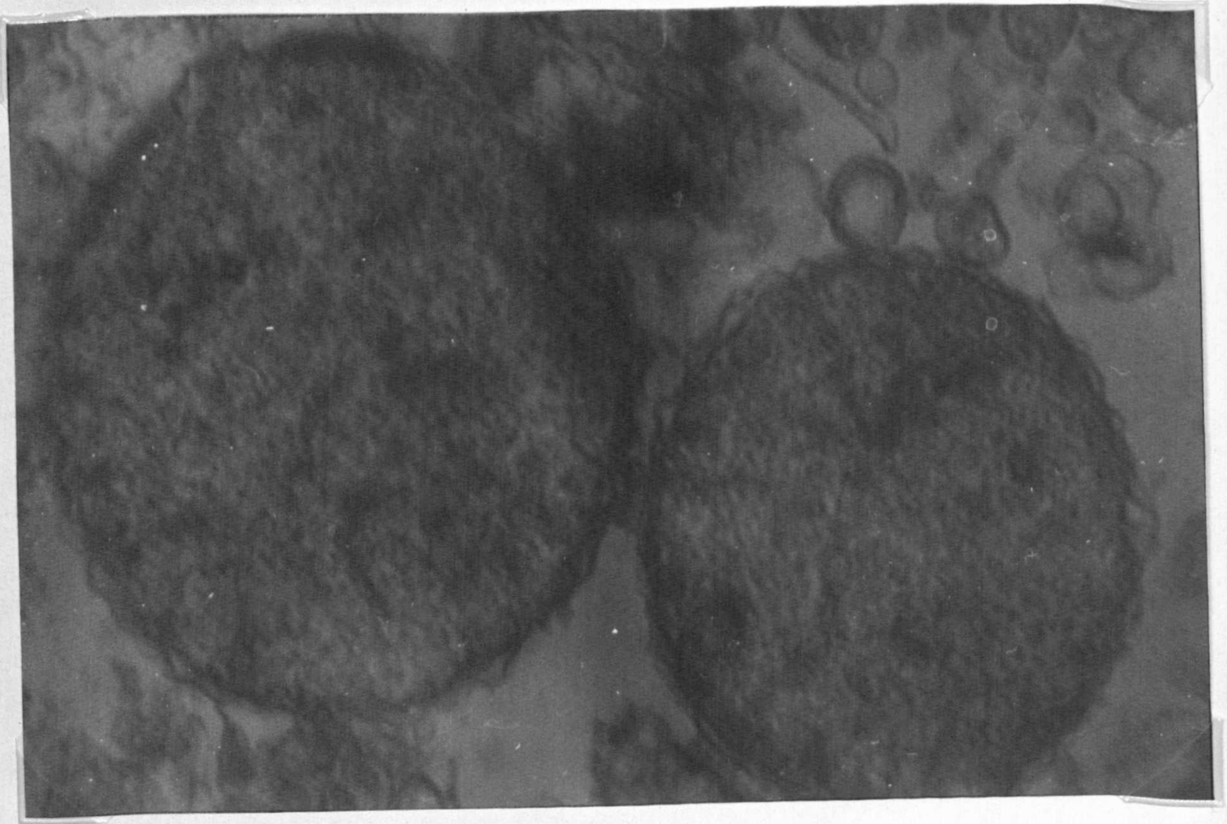


Fig. 79 Electron micrograph of intact bovine liver mitochondrial preparation. Original magnification x 25,000.

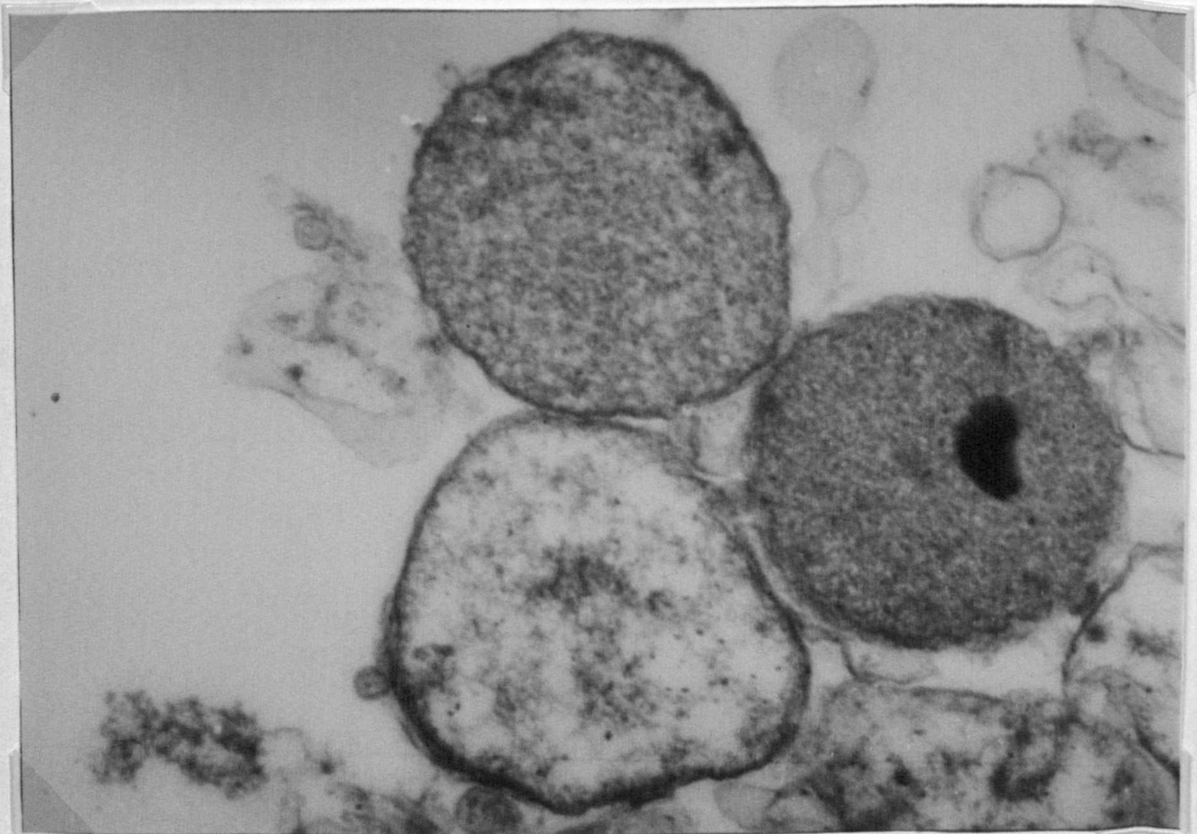


Fig. 80 Electron micrograph of intact bovine liver mitochondrial preparation. Original magnification x 16,000.

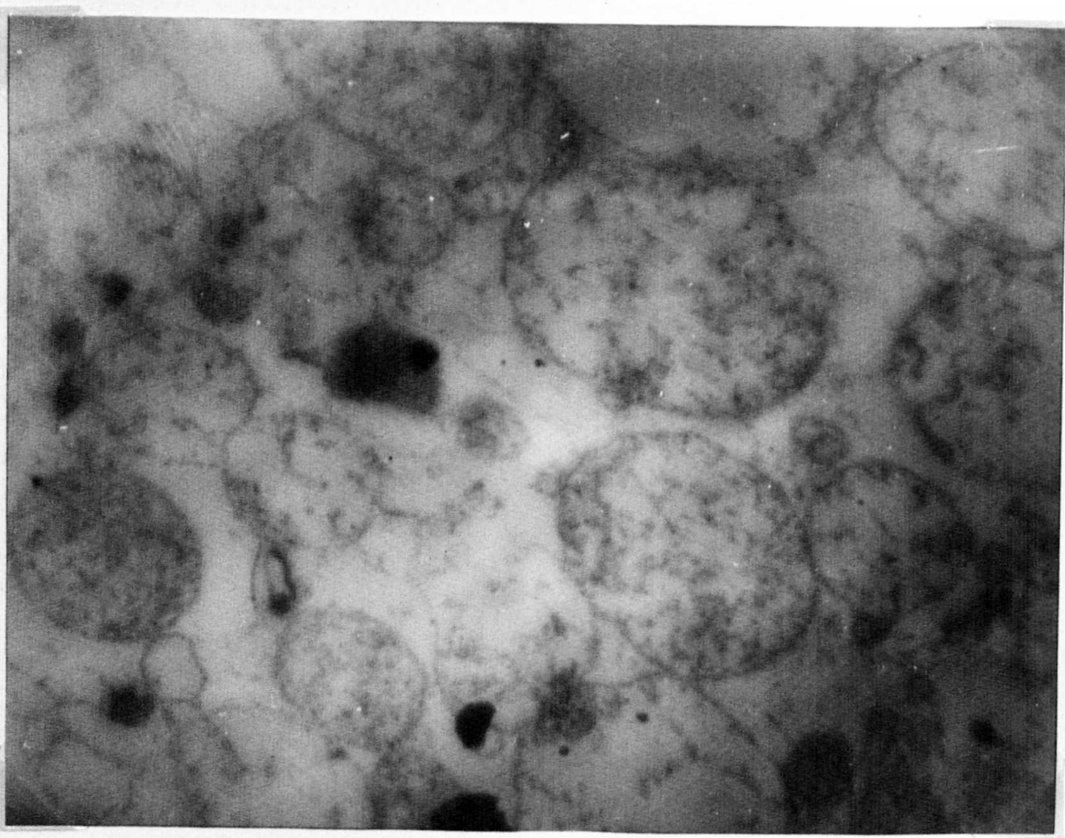


Fig. 81 Electron micrograph of bovine liver mitochondria stripped of outer membranes after treatment with digitonin (0. 86 mg digitonin per 10 mg mitochondrial protein). Original magnification x 8, 600.

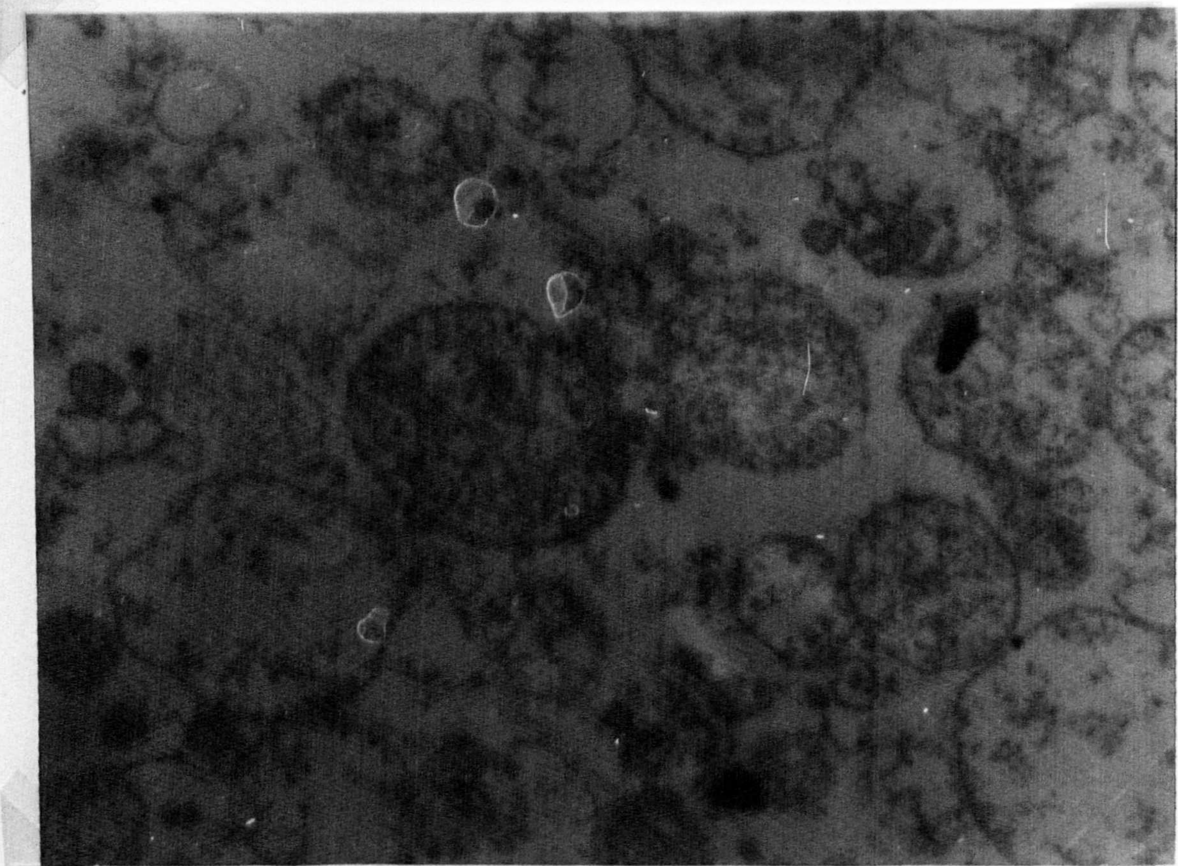


Fig. 82 Electron micrograph of bovine liver mitochondria stripped of outer membranes after treatment with digitonin (0. 86 mg digitonin per 10 mg mitochondrial protein). Original magnification x 7, 500.

Thus, digitonin, at high concentrations, does disrupt the inner membrane as well as the outer. This has also been observed in the case of rat liver mitochondria (Hoppel and Cooper, 1968, Morton et al, 1968). Disruption of the inner mitochondrial membrane was explained by the authors to be the result of a specific effect of digitonin on the inner membrane rather than the hypo-osmotic conditions prevailing during the digitonin treatment.

Thus, although the inner mitochondrial membrane is devoid of cholesterol, (Colbeau et al, 1971) all the cholesterol is present in the outer membrane, specific interaction between digitonin and the lipoproteins of the inner membrane may take place. It appears, therefore, that complex formation between digitonin and cholesterol is not the only mechanism of membrane disruption.

The intactness of mitochondria and the effect of digitonin were further investigated by electron microscopy. These are shown in Figures 79 to 82).

TABLE 11 BINDING OF GDH (FINAL CONCENTRATION OF 0.018 mg/ml) TO MITOCHONDRIAL MEMBRANES - PRIMARY DATA

GDH $\pm$ mitochondrial suspension	total activities in the supernatant (n moles/min)
GDH in medium B	38.7
whole mitochondria (0.4 mg/ml protein)	10.8
GDH + whole mitochondria	42.5
mitoplasts (0.35 mg/ml protein)	15.7
GDH + mitoplasts	50.5
mitoplasts (0.37 mg/ml protein)	16.2
GDH + mitoplasts	49.3
sonicated mitoplasts (0.15 mg/ml protein)	2.6 (2.6)
GDH + sonicated mitoplasts	20.1 (20.1)
mitoplasts previously transferred into buffer (0.15 mg/ml)	2.4
GDH + mitoplasts previously transferred into buffer	20.3

TABLE 12 BINDING OF GDH (FINAL CONCENTRATION OF 0.018 mg/ml) TO MITOCHONDRIAL MEMBRANES - SECONDARY DATA

type of mitochondrial suspension	% activities recovered in the supernatant	loss of activity due to binding /0.15 mg mito-chondrial protein
whole mitochondria (0.40 mg/ml protein)	86	5.2
mitoplasts (0.35 mg/ml protein)	93	3
mitoplasts (0.37 mg/ml protein)	90	4
mitoplasts sonicated for 30 sec.	50	50
mitoplasts transferred into 0.06 M hepetri buffer	50	50

BINDING OF GDH TO MITOCHONDRIAL MEMBRANES

Possible specificity in binding of GDH to mitochondrial membranes was investigated. This was done by incubating the purified enzyme with different mitochondrial suspensions.

To explore the possibility of binding of the enzyme to the outer surfaces of the outer and inner membranes, whole mitochondria and mitoplasts were used. In the case of the inner surface of the inner membrane, the mitoplasts were either sonicated for a period of 30 seconds or transferred into a hypotonic environment (0.06 M Hepetri). They were then centrifuged at 44,000 g for a period of 20 minutes, resuspended in medium B or buffer and centrifuged again. Sonication or transfer to the hypotonic environment were used for the preparation of particles with mixtures of right-side out and inside-out vesicles. The results obtained (secondary data) from such an experiment are summarised in Table 12. Here the activities recovered in the supernatant fraction are shown. The primary data are shown in Table 11. It can be seen from the results that the activity recovered in the supernatant in the case of the enzyme incubated with the mitoplasts which have been transferred to a hypotonic environment or were treated by sonication is about one fifth of the recovery with the untreated mitochondria or mitoplasts. Considering the difference in protein concentrations in the fractions, the difference is much higher (Table 12

The % error for assaying GDH in all these experiments was in the region of ± 3%. At least two control experiments were carried out each time. Different fractions were assayed for their glutamate dehydrogenase activities at least twice and more if there were large discrepancies. Average values are reported in the tables.

Thus, it appears that there is some specificity in the association of the enzyme with mitochondrial membranes. The most significant difference is that between the outer surface of the inner membrane (enzyme incubated with mitoplasts) and the inner surface of the inner membrane (enzyme incubated with sonicated or swelled mitoplasts). If this is a genuine effect, then the binding capacity of different surfaces of the mitochondrial membranes for the enzyme must be very different and this may explain the final localisation of the enzyme in the mitochondrion.

The results of Bretscher (1972) on erythrocyte membranes do indicate that the anionic phosphatidylserine is located chiefly in the inner surface of the membrane and the zwitterionic lecithin in the outer surface. Some experimental evidence on the asymmetrical distribution of different phospholipids on the mitochondrial membranes have been observed by Fleischer and Fleischer (1972) and Writz and Zilversmit and this has been more fully discussed in the previous chapter (page 87). Recently (Astle and Cooper, 1974), the sidedness of the mitochondrial inner membrane was characterised by specific labelling of the exposed surface of the membrane with ^{125}I by lactoperoxidase.

The sidedness of the mitochondrial membrane is, presumably, the result of asymmetric distribution of both membrane phospholipids and membrane proteins.

In mitochondria, all of the cytochrome c is released by gentle swelling in the presence of KCl which does not rupture the inner membrane (Jacobs and Sanadi, 1960). Ferricyanide which is impermeant to the inner membrane is also able to oxidise all the cytochrome c of mitochondria (Chance et al, 1970). On the other hand, F_1 (ATPase) is located on the side of the membrane opposite

to cytochrome c interval in mitochondria and external in submitochondrial particles. This location of F_1 is evidenced by the appearance of 90 A^o spheres internal in mitochondria and external in submitochondrial particles (Fernandez-Moran, 1962, Racker, 1972). In addition, antibody to F_1 inhibits the ATPase activity of submitochondrial particles but not of mitochondria as would be predicted, since the antibody is unable to penetrate the inner membrane (Fessenden and Racker, 1966).

The specificity in the binding of enzymes to biological membranes have been explored in a few cases. One of these is the binding of glyceraldehyde 3-P dehydrogenase to erythrocyte membranes. The enzyme was found to bind to the inner and not the outer erythrocyte membrane surface (Kant and Steck, 1973, Letko and Bohnensack, 1974). A loose association between this enzyme and erythrocyte membranes was indicated previous to these findings (Mitchell et al, 1965). Another enzyme which shows this type of specificity is monoamine oxidase (Racker and Proctor, 1970). The enzyme was detached from the outer mitochondrial membranes of kidney and heart mitochondria by sonication at pH 9.6. The purified enzyme recombined specifically with the resolved outer membranes. On the other hand, ATPase showed specific binding to the inner membrane. However, a small fraction of monoamine oxidase was found to bind to the inner mitochondrial membrane and a small fraction of ATPase to the outer membrane. These comparatively small amounts may represent nonspecific binding or may be due to cross-contamination of the two membrane types. The binding of these enzymes to membranes is probably controlled by the availability of specific binding sites on the membranes and some additional factors, such as pH and ionic strength.

Fig. 83/4 The effect of ammonium chloride on binding of GDH to the inner mitochondrial membrane

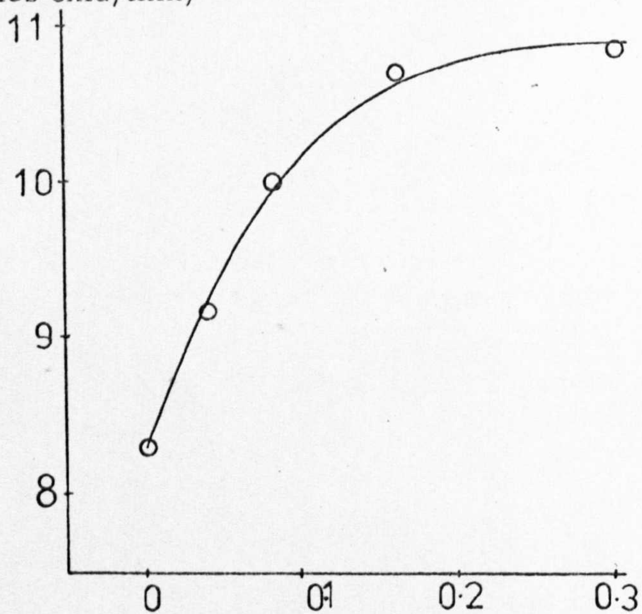


Fig. 84

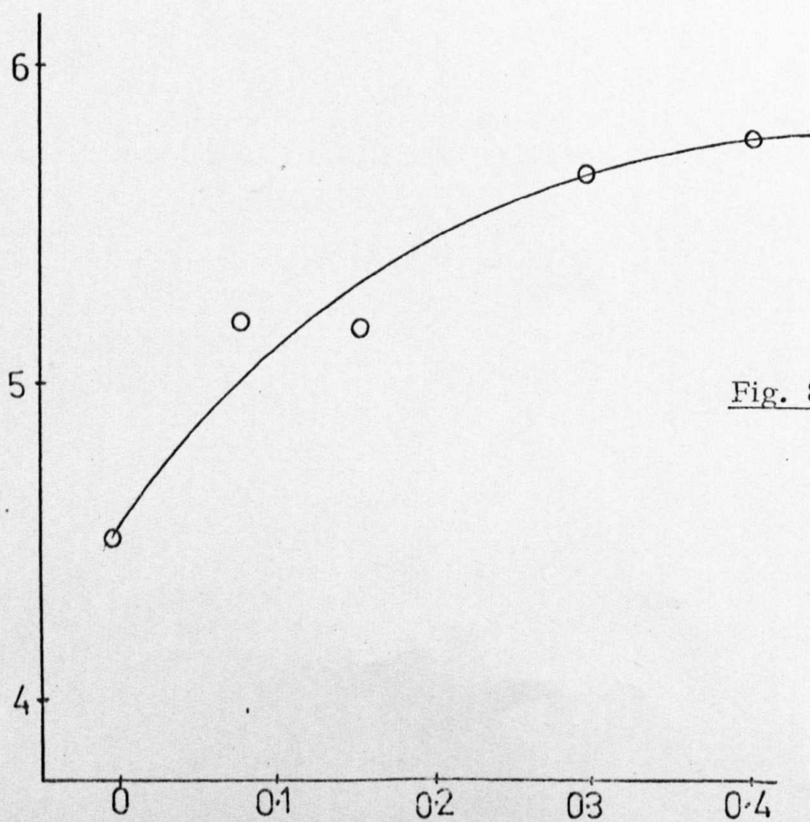


Fig. 83

EFFECT OF NH_4Cl ON BINDING OF ENDOGENOUS GDH TO THE INNER MITO. MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 0.31 mg/ml IN 0.06 M HEPETRI BUFFER (+ 0.025 M SUCROSE) pH 7.4

TABLE 13

total GDH activity in supernatant fractions n moles oxid/min	$[NH_4Cl]$ final
8.29	0
9.14	0.04
10.0	0.08
10.7	0.16
10.8	0.3

EFFECT OF NH_4Cl ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 3.2 mg/ml (IN 0.25 M SUCROSE, pH 7.4) SONICATED FOR 30 secs.

TABLE 14

total GDH activity in supernatant fractions (n moles of NADH oxid/min)	$[NH_4Cl]$ final
0.046	0
0.049	0.01
0.054	0.02
0.054	0.03
0.055	0.04
0.055	0.06
0.055	0.08
0.056	0.1
0.057	0.15

EFFECT OF NH_4Cl ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 0.31 mg/ml (IN 0.25 M SUCROSE, pH 7.4) SONICATED FOR 30 secs.

TABLE 15

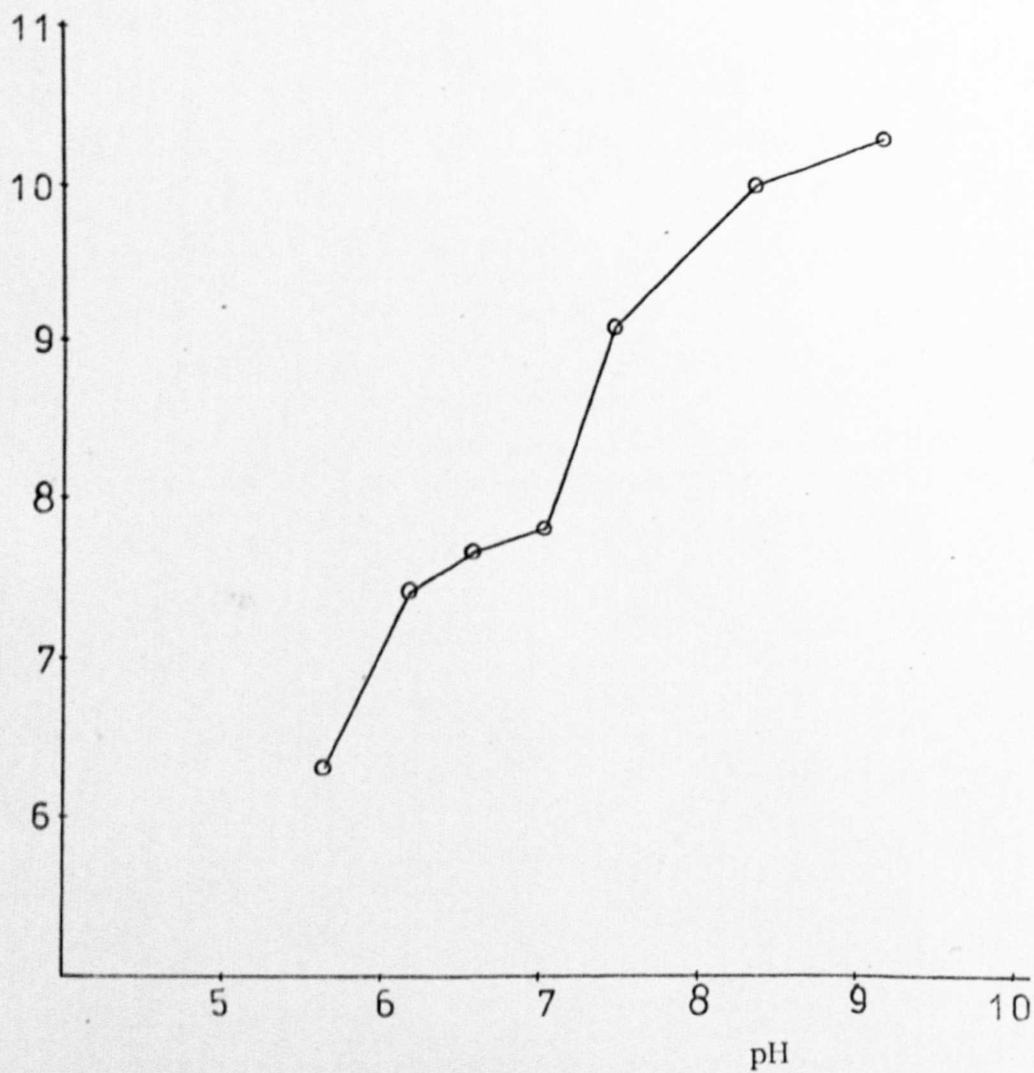
total GDH activity in supernatant fractions n moles oxid/min	$[NH_4Cl]$ final
4.53	0
4.82	0.04
5.19	0.08
5.14	0.16
5.63	0.3
5.75	0.4

TABLE 16

EFFECT OF pH ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 0.35 mg/ml, SUSPENDED IN HEPETRI BUFFER (0.025 M SUCROSE) OF DIFFERENT pH VALUES. TOTAL ACTIVITIES EXPRESSED (in n moles of NADH oxid/min).

pH	activity in the supernatant	activity in the pellet	total activity (supernatant + pellet)
5.65	6.29	6.86	13.15
6.2	7.38	6.04	13.42
6.6	7.64		
7.05	7.81	3.6	11.41
7.5	9.07	3.12	12.19
8.4	10.0	2.5	12.5
9.2	10.3	2.7	13.0

Fig. 85 The effect of pH on binding of GDH to the inner mitochondrial membrane



In contrast to these proteins, there are other proteins which do not show such specificity. For example, the basic protein cytochrome c was found to lack specificity in its association with erythrocyte membranes (Kant and Steck, 1973). Binding occurred at both membrane surfaces and was not affected by any metabolites tested. This might explain the dual localisation of some proteins. For example, cytochrome c_1, a and a_3, according to some experimental observations (Chance et al, 1970) are included on both sides of the inner mitochondrial membrane.

The effect of NH_4Cl on binding of the enzyme to the inner mitochondrial membrane was investigated. This was carried out by suspending the mitoplasts in medium B (0.25 M sucrose in tris-acetate, pH 7.4) containing different concentrations of NH_4Cl. They were then sonicated for a period of 30 seconds and centrifuged at 44,000 g for 20 minutes. The supernatant fractions were then assayed for their glutamate dehydrogenase activites. The results of two separate experiments using NH_4Cl concentrations in the range of 0 - 0.15 M and 0 -0.4 M are given in Tables 14 & 15 and shown in Figure 83. Transfer of the mitoplasts to hepetri buffer (o.06 M) containing different concentrations of NH_4Cl was also tried and the results are shown in Figure 84 and Table 13.

Thus, the binding of the enzyme to the inner mitochondrial membrane decreases with increasing NH_4Cl concentrations. Effect of NH_4Cl on binding of the enzyme to phospholipid membranes has been discussed in the previous chapters (pages 88 and 96).

Figure 85 and Table 16 show the effect of pH on binding of the enzyme to the inner mitochondrial membrane. The enzyme shows significantly higher binding capacity to the membrane at lower pH values.

Increase in binding of the enzyme (pI 4 - 5) to phospholipid and mitochondrial membranes with decreasing pH of the medium demands a closer analysis. At neutral pH, most membrane proteins have a net negative charge because of an excess of acidic residues (Rosenberg and Guidotti, 1968), and are repelled by acidic phospholipids. Several reconstitution experiments (Razin, 1972) indicate that divalent cations such as Mg^{++} are needed to overcome the electrostatic repulsion due to excess negative charges or membrane proteins and lipids at physiological values and enable the proteins and lipids to move up close enough for hydrophobic bond formation. The results of Zwaal and Van Deenen (1970) indicate that recombination of butanol-solubilised erythrocyte membrane proteins with membrane lipids only took place at a very low, unphysiological pH value. The process was spontaneous when the protein solution was mixed with the sonicated lipid suspension at pH 3.5 - 4.5. In this low range of pH, the erythrocyte proteins are positively charged and the phospholipids are negatively charged.

Glutamate dehydrogenase is strongly inhibited by divalent cations (Figure 53), such as Ca^{++} and Mg^{++} and all our experiments were carried out in the presence of EDTA ($1 - 2 \times 10^{-4}$ M). Each of the 6 polypeptide chains of a glutamate dehydrogenase oligomer consists of 500 amino acid residues. 33 of these are lysine residues (Piszkiewicz et al, 1973), some of which may be suitable for binding to negatively charged phospholipid surface.

Thus, the binding of the enzyme to these membranes probably involves specific positively charged residues (such as ε-amino groups of lysine residues). This primary electrostatic interaction may then bring the enzyme and the membranes close enough for hydrophobic bond formation. Indeed, conformational

TABLE 17

EFFECT OF METABOLITES ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 3.2 mg/ml, SONICATED FOR 30 sec. TOTAL ACTIVITIES EXPRESSED (in n moles of NADH oxid/min).

activity in the supernatant	metabolite concentration
50.3	0
50.7	0
54.6	1.5×10^{-3} M NADH
56.0	3×10^{-3} M NADH
53.7	1.5×10^{-4} M NADH
51.3	7.5×10^{-5} M NADH
50.1	3×10^{-5} M NADH
47.5	3×10^{-4} M ADP
47.8	1.5×10^{-4} M ADP
47.3	7.5×10^{-5} M ADP
48.9	3×10^{-5} M ADP

TABLE 18

EFFECT OF ADP AND GTP ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. SPECIFIC ACTIVITIES OF SUPERNATANT FRACTIONS AFTER DIALYSIS.

specific activity n moles/min/mg protein	metabolite concentration
0.201	0
0.215	3×10^{-4} M GTP
0.197	7.5×10^{-5} M GTP
0.168	3×10^{-4} M ADP
0.175	1.5×10^{-4} M ADP

changes in the enzyme structure may be followed soon after such electrostatic interactions and some of the hydrophobic amino acid residues may become more exposed to the surface of the protein structure and available for hydrophobic interaction with the hydrocarbon residues of the phospholipids. Evidence for the involvement of hydrophobic interaction in the complex formation between the enzyme and pure phospholipid membranes had already been discussed (page 97).

If the generally held assumption about the pH of 7 for mitochondria (Williamson et al, 1967) is a correct one, then according to these results, the binding affinity of the enzyme for the inner mitochondrial membrane is significant.

As described in the previous chapter, several metabolites, such as NADH and ADP, affected the binding of the enzyme to phospholipid membranes. To explore the effect of these metabolites on binding of the enzyme to the inner mitochondrial membranes, both sonication of the mitoplasts and their transfer to a hypotonic medium (0.06 M Hepetri) were tried.

Table 17 shows the effect of NADH, and ADP on binding of the enzyme to the mitochondrial membranes. In this experiment, the mitoplasts were incubated in ice in the presence of the effectors at different concentrations and then sonicated for 30 seconds. After 10 minutes, they were sedimented at 44,000 g for 20 minutes and the supernatant fractions were assayed for glutamate dehydrogenase activity. A higher activity in the supernatant in the case of NADH and a lower activity in the case of ADP indicated the effect of these metabolites in decreasing or increasing the binding affinity of the enzyme for the inner mitochondrial membrane.

TABLE 19

EFFECT OF METABOLITES ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 0.49 mg/ml (TRANSFERRED INTO 0.06 M HEPETRI BUFFER + 0.025 M SUCROSE, pH 7.4).

activity in the supernatant	metabolite concentration
7.25	0
7.20	0
8.07	5×10^{-4} M NADH
8.07	2.5×10^{-4} M NADH
8.46	1×10^{-4} M
7.78	1×10^{-2} M l-glu
7.67	1×10^{-2} M l-glu + 5×10^{-4} M NADH
7.61	1×10^{-2} M l-glu + 2.5×10^{-4} M NADH
7.78	5×10^{-4} M NAD^+
7.78	2.5×10^{-4} M NAD^+
8.07	1×10^{-4} M

TABLE 20

EFFECT OF METABOLITES ON BINDING OF ENDOGENOUS GDH TO THE INNER MITOCHONDRIAL MEMBRANE. MITOPLASTS WITH FINAL PROTEIN CONCENTRATION OF 0.63 mg/ml (TRANSFERRED INTO 0.06 M HEPETRI BUFFER). TOTAL ACTIVITIES EXPRESSED (in n moles of NADH oxid/min).

activity in the supernatant	metabolite concentration
9.51	0
9.96	5 mM l-leu
10.09	10 mM l-leu
9.98	1×10^{-4} M NADH
10.06	1 mM α-Kg
10.77	1 mM α-Kg + 5 mM l-leu
10.71	1 mM α-Kg + 5 mM l-leu + 1×10^{-4} M NADH

In the case of ADP effect, it must be realised that the metabolite is an allosteric effector of the enzyme and increases its activity. Therefore, any ADP present in the final assay medium would only increase the activity of the enzyme.

Both ADP and GTP show high affinities for binding to GDH. The association constant for GTP (in the presence of NADH) and ADP have been found to be 0.43×10^{-6} M and 8×10^{-6} respectively (Frieden and Colman, 1967). Thus, GTP shows a much higher affinity for the enzyme than ADP. NADH increases the affinity of GTP binding to the enzyme (Bayley and Radda, 1965).

Table 18 shows the effect of these effectors on binding of the enzyme to the inner mitochondrial membrane. After incubating the mitoplasts with these effectors (in ice for 10 minutes), sonication of the suspension was carried out for 30 seconds. They were sedimented at 44,000 g for twenty minutes and the supernatant fractions were then dialysed in 0.05 M phosphate buffer pH 8.0 (+ 1×10^{-4} M EDTA) at about 4°C. Extensive dialysis was found to be necessary especially in the case of GTP. Dialysis of some of the fractions was carried out over a period of 48 hours by two changes of buffer. The absence of the nucleotides was checked spectroscopically (280, 260 and 253 nm readings). The specific activities of the dialysates for glutamate dehydrogenase were then determined. The results are presented in Table 18. It can be seen that, except for the case of ADP, the effects are not appreciable.

Table 19 shows the effect of NADH alone and in the presence of l-glutamate on binding of the enzyme to the inner mitochondrial membrane. In this experiment, the mitoplasts were transferred to a hypotonic medium (0.06 Hepetri) and the metabolites were then added. Centrifugation at 44,000 g was

then carried out for a period of 20 minutes and the supernatant fractions were assayed for glutamate dehydrogenase activity.

The effect of l-leu, α-Kg and NADH are shown in Table 19. The experiment was carried out as described above.

The results indicate that both α-Kg and l-glutamate decrease the binding of the enzyme to the membrane. Results presented in Table 19 indicate that NAD^+ also slightly decreases the binding of the enzyme to the inner mitochondrial membranes.

An interesting effect found on the binding of the enzyme to the inner mitochondrial membrane is that of l-leucine. Table 20 shows the effect of l-leucine alone and in the presence of α-Kg and NADH on GDH binding to the membrane. The results indicate that l-leucine decreases the binding of the enzyme to the mitochondrial membrane.

Stimulation by l-leucine of glutamate dehydrogenase in intact mitochondria has been reported recently (McGivan et al, 1973). L-leucine and some other monocarboxylic amino acids have been shown to stimulate the isolated enzyme (Yielding and Tomkins, 1961, Kun and Achmatowicz, 1965) and form a complex with the enzyme with perturbation in its structure (Prough et al, 1972, Prough and Fischer, 1972).

Studies on the effect of l-leucine in intact mitochondria (McGivan et al, 1973) showed that stimulation by l-leucine only occurred in the direction of l-glutamate synthesis and not in the deamination direction. They explained their results by suggesting that l-glutamate deamination may be controlled by the rate of glutamate transport (Bradford and McGivan, 1973).

It may be suggested, in view of our experimental evidence on the specificity of binding of the enzyme to phospholipid and mitochondrial membranes that the effect of l-leucine and other monocarboxylic amino acids may be, at least partly, due to the same type of effect. The fact that l-leucine only affected glutamate synthesis would in view of the above arguments be expected, as the conformation of the enzyme in the presence of l-leucine, l-glutamate and NAD^+ would be different from that in the presence of l-leucine, α-Kg, NADH and NH_4^+.

It is also interesting that stimulation of l-leucine was only observed in disrupted and not in intact mitochondria. In this case, the enzyme is released from the mitochondrial membrane and no barriers between the enzyme and the effectors exist. Regulation of binding of the enzyme to the mitochondrial membrane would be expected to be a stronger possibility in the case of a released enzyme than in the case of a bound one.

Chapter VII

GENERAL DISCUSSION

GENERAL DISCUSSION

Previous studies on the interaction of glutamate dehydrogenase with detergents and phospholipid membranes showed that the head groups of the amphiphiles determine the extent of complex formation (Dodd, 1972 - 1973, Nemat-Gorgani and Dodd, 1974). Reports in a similar study were different in that no such specificity was observed and all phospholipids tested showed strong inhibition (Julliard and Gautheron, 1972). A more recent report from the same laboratory (Godinot, 1973) confirmed our conclusions. In these two separate studies, the enzyme from pig heart and rat liver mitochondria were looked at respectively.

There may be several reasons for the lack of specificity reported in the association of different phospholipids with the enzyme (Julliard and Gautheron, 1972). Poor characterisation of the phospholipids and the presence of oxidation products are two obvious possibilities. As discussed in the previous chapter, all our phospholipids were well characterised and were stored in the presence of the antioxidant, BHT. No oxidation or degradation products were detected in our experimental conditions.

The aim of this work was to explore possible specific interaction between glutamate dehydrogenase and the inner mitochondrial membrane. For characterisation of such interactions, amphiphiles of different head group types (anionic, cationic and zwitterionic) and different alkyl chains were chosen.

Interaction of the enzyme with phospholipid membranes and detergents was characterised mainly by kinetic and fluorescence studies. The possibility of different conformations of the enzyme binding specifically to these charged

surfaces and conformational changes brought about as a result of complex formation was investigated. Both types of amphiphiles indicated the head group specificity in their association with the enzyme. Thus, the cationic amphiphiles (e.g. CETAB) and the anionic amphiphiles (e.g. sodium dodecyl sulphate and cardiolipin) interacted with the enzyme while the zwitterionic amphiphiles (lysolecithin and phosphatidylcholine) did not interact.

Complex formation between the enzyme and the anionic phospholipids was found to be a reversible process. On the other hand, the anionic detergent, sodium dodecyl sulphate, showed the capacity of bringing about irreversible denaturation at high concentrations ($> 1 \times 10^{-4}$ M).

Detergents form micelles which are in equilibrium with high concentrations of monomers which can bring about irreversible inhibition of the enzyme by binding to specific sites. In contrast, the phospholipids form single-shelled bilayer vesicles for which no significant concentration of monomer is found (Robinson, 1960).

The six polypeptide chains of each enzyme oligomer appear to be arranged in two layers, each composed of three elongated ellipsoidal subunits (Horne et al, 1963, Sund et al, 1969). The oligomer has dimensions of about 140 A^{o} by 86 A^{o} by 90 A^{o} (Sund et al, 1969). The phospholipid vesicles are spherical in shape and have an average diameter of 230 A^{o} (Hauser, 1967). Thus, in the case of the interaction between glutamate dehydrogenase and phospholipids, the process takes place between two aggregates of similar sizes.

Ligand-induced conformational changes in enzymes is an important concept of molecular enzymology. The importance of such processes in the

regulation of enzymic activity has been discussed in detail by Monod et al (1963). The term "allosteric" effect defines a phenomenon in which a conformational change is induced in the enzyme by a molecule called "effector" bound at a site other than the active centre. This may then lead to either activation or inhibition. Glutamate dehydrogenase is an enzyme in this class. Some of the models of the regulatory mechanisms emphasize the role of co-operative protein-protein interactions (Koshland et al, 1966). The intracellular micro-environment of the allosteric enzymes might play an important role in modulating the co-operative responses.

The term allotopic phenomenon according to Racker's original definition (Racker, 1967) is a "phenomenon of membrane-enzyme complexes manifest by alterations in the properties of both enzyme and membrane". This definition was made with reference to the properties of the Mg^{++} dependent ATPase (F_1) which can be purified from the inner mitochondrial membrane. This soluble ATPase is cold labile and oligomycin insensitive (Pullman et al, 1960). Alternatively, the ATPase can be purified as a water insoluble lipid-protein complex which is both stable to cold and oligomycin sensitive (Kagawa and Racker, 1966). These changes in the properties of the protein brought about by the presence of lipid is coupled with changes in the properties of the membrane, as indicated by its increased resistance to trypsin.

Our results indicate that glutamate dehydrogenase bound to phospholipid membranes has different properties as compared with the free enzyme. These were indicated by loss of activity on binding to a phospholipid membrane together with diminution of intrinsic fluorescence and an increase in its capacity to bind ANS.

Formation of a complex between the enzyme and an anionic phospholipid membrane may also bring about changes in the properties of the lipid bilayer structure (such as charge neutralisation of the head groups) as discussed in previous chapters.

As discussed above, ANS does not give rise to fluorescence enhancement in the presence of anionic phospholipids. This is probably due to the fact that the negatively charged probe cannot penetrate into the hydrophobic regions of the bilayer. However, after formation of a lipid-enzyme complex, charge neutralisation of the phospholipid head groups (by interaction with the positively-charged groups in the enzyme structure) may take place and the lipid may acquire the capacity to "accommodate" ANS in its hydrophobic regions. A number of soluble basic proteins, such as cytochrome c and lysozyme, have been shown to increase permeability of phosphatidylserine vesicles to inorganic ions (Kimelberg and Papahadjopoulos, 1971) and showed the ability to penetrate or expand monolayers of the same phospholipid (Kimelberg and Papahadjopoulos, 1971, b).

At pH values which the kinetic and fluorescence experiments were carried out (pH 6 - 9) the anionic phospholipids and the enzyme (pI 4 - 5) have the same charge type. Formation of enzyme-lipid complexes increased with decreasing ionic strength and pH, thus showing the possibility of electrostatic interaction. However, the interaction must be different from that between basic protein such as cytochrome c and cardiolipin (Green and Fleischer, 1963) and between phosphatidylserine and ribonuclease (Kimelberg and Papahadjopoulos, 1971). The net charges of these basic proteins (pI's $>$ 10) are opposite to the anionic phospholipid bilayer surface. In the case of cytochrome c, it has been

shown that the lysine residues as well as the hydrophobic amino acid residues tend to occur in distinct clusters along the protein chain (Margoliash, 1962).

In the case of glutamate dehydrogenase, 33 out of the 500 amino acid residues of each polypeptide chain are lysine residues (Piszkiewicz et al, 1973), some of which may be suitable for binding to an anionic surface.

Involvement of hydrophobic interaction was indicated by increase in the extent of complex formation with temperature. It is possible that there is an asymmetrical distribution of the 500 amino acid residues in each polypeptide chain of the enzyme. Such asymmetric distributions have been shown in the case of cytochrome b5 (Spatz and Strittmatter, 1971), cytochrome b5 reductase (Strittmatter, 1972) and a glycoprotein of the human red cell membrane (Segrest et al, 1972).

Recently, (Gitel et al, 1973) the presence of a specific region in bovine prothrombin for binding to phospholipids has been demonstrated. A similar report was made by Lux et al (1972) who found a cyanogen bromide fragment from apo high density lipoprotein (Apo LP-Gln-II), to complex with phosphatidylcholine and to inhibit the reactivation of delipidated mitochondrial β-hydroxybutyrate dehydrogenase. The presence of a highly hydrophobic region in cytochrome b5 (Spatz and Strittmatter, 1971) and cytochrome b5 reductase (Strittmatter et al, 1972) has also been demonstrated.

Electrostatic interaction between a negatively charged membrane and positively charged residues in the enzyme may be followed by conformational changes in the enzyme and structural changes in phospholipid membranes. Hydrophobic interaction may then take place between the hydrophobic amino

acids of the enzyme exposed to the surface as a result of the primary electrostatic interaction, and the hydrophobic regions in the phospholipid bilayer. This is possible in view of the fluid state of phospholipids. Thus, after complex formation, some hydrophobic residues of the enzyme may penetrate into the bilayer structure of the phospholipid membranes as shown in Figure 86. Only a slight penetration would be expected in view of the low proportion of hydrophobic amino acid residues in the enzyme, its high solubility and the fact that an enzyme-phospholipid complex may be partially dissociated by increasing the ionic strength (page 89). Thus extensive penetration as shown in Figure 86b) and c) is not possible.

The kinetic and intrinsic fluorescence studies were carried out at very low protein concentration (<50 μg/ml) at which the enzymic species is the monomer comprised of six subunits. The subunits are ellipsoid in shape and their possible arrangement is shown in Figure 18. Polymerisation takes place along the major axis of the ellipsoid units (Sund et al, 1969) as shown in the figure.

Residues which are involved in formation of a complex between the enzyme and phospholipid membranes may be either present along the longitudinal axis of its structure or at the same sides at which polymerisation takes place. These two possibilities are indicated in Figure 87 (I and II).

Arrangement I would leave the sites involved in the process of polymerisation intact and any effect on such process would be due to secondary conformational changes in the enzyme. Arrangement II, however, would interfere with polymerisation directly and would predict competition between the two processes of polymerisation and lipid-protein complex formation.

Fig. 86 The extent of GDH penetration into a phospholipid bilayer

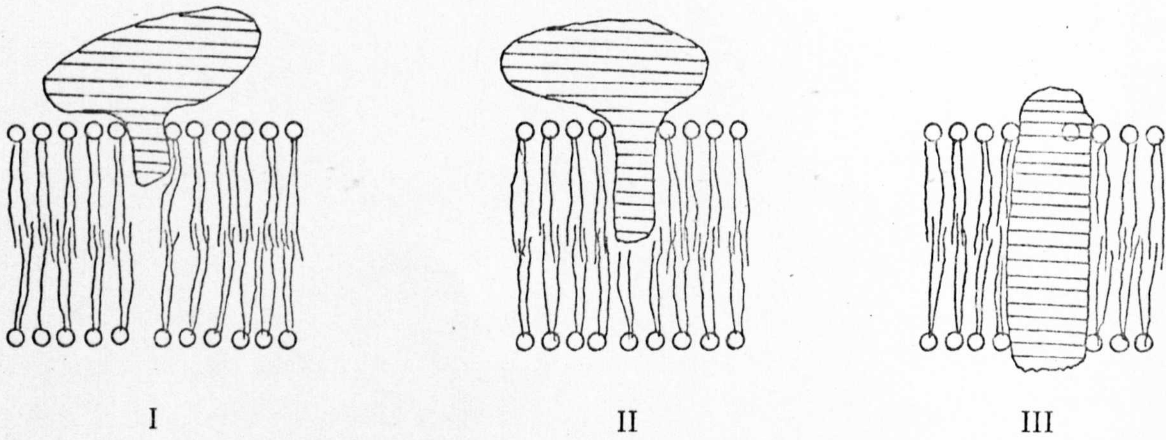
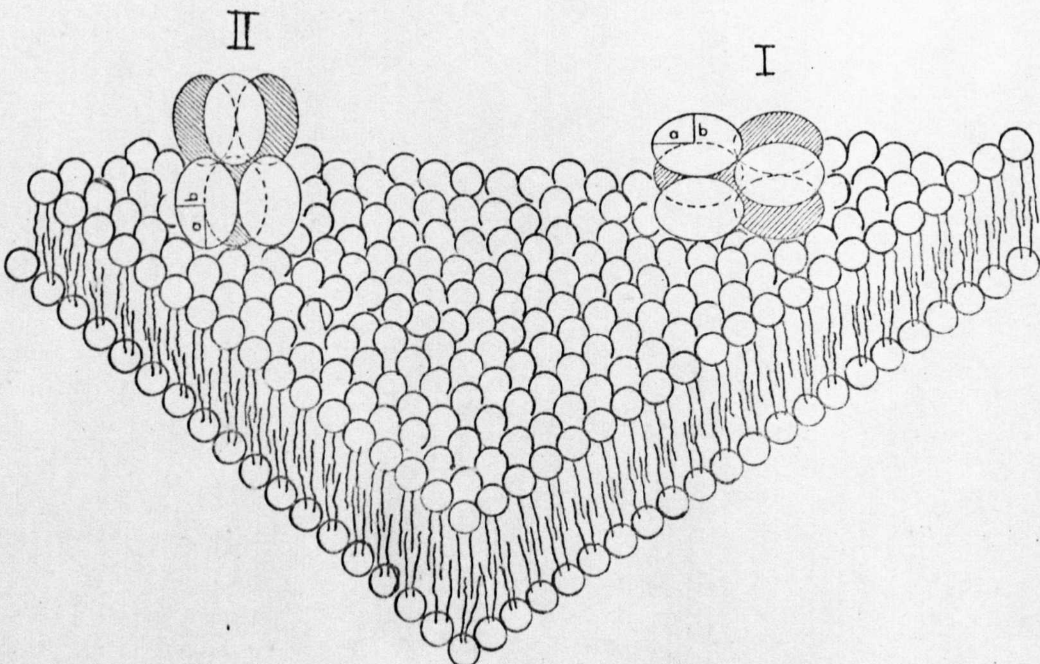


Fig. 87 Two different types of arrangement of a GDH oligomer on a phospholipid membrane



The stop-flow experiments indicate that the polymeric form of the enzyme also interacts with anionic phospholipids and enzyme oligomers are formed as a result. The enzyme polymer exists as a rod. Thus, it may be concluded that the enzyme oligomers are arranged with parts of their longitudinal surfaces in contact with anionic phospholipids. All six polypeptide chains in the enzyme are identical and may be involved in binding to anionic phospholipid membranes at the same time. After such complex formation, there may be extensive conformational changes with the result that the residues involved in oligomer-oligomer interaction are no longer available for such a process.

Each enzyme oligomer has dimensions of about 140 A^o by 90 A^o (Sund et al, 1969). The phospholipid vesicles are spherical in shape and have an average diameter of 230 A^o (Hauser, 1967). Thus, in the case of the interaction between glutamate dehydrogenase and phospholipids, the process takes place between two aggregates of similar sizes.

In the case of dipalmitoyl lecithin, the area of each phospholipid head group is about 48 $A^{o\,2}$ with a constant width for each head group layer of approximately 10 A^o (Engelman, 1970).

From the above data, it may be calculated that the maximum area involving 4 polypeptide chains in arrangement I (Figure 87) is roughly equal to 12,000 $A^{o\,2}$ which is equivalent to the surface area on a phospholipid vesicle occupied by 120 phospholipid molecules. In arrangement II, the total surface area is 7,700 $A^{o\,2}$ corresponding to 77 phospholipid molecules.

If the enzyme just touches the surface of the membrane, the total area of contact would be very small and would, as a rough estimation, be

equivalent to the area occupied by 6 or 4 phospholipid head groups for arrangements I and II respectively. With slight penetration of the polypeptide chain into the hydrophobic interior of phospholipid bilayers, these values increase to 36 and 11 phospholipid molecules.

Intrinsic and extrinsic fluorescence studies indicate that there is a high degree of specificity in the association of the enzyme with phospholipid membranes. Presence of different metabolites which are of importance to the metabolic activities of the enzyme and which bring about different conformational changes in its structure, affected the binding in opposite ways.

Similar type of specificity has been shown in the association of glyceraldehyde 3-phosphate dehydrogenase with erythrocyte membranes (Kant and Steck, 1973, Letko and Bohnensack, 1974). On the other hand, the basic protein cytochrome c lacked specificity in its association with erythrocyte membranes. Thus, in the case of the interaction between cytochrome c (pI = 10.6) and anionic membranes, the process is due to non-specific adsorption. Also, a preformed complex is dissociated completely when the ionic strength is raised sufficiently (Green and Fleischer, 1964). Thus, the process only involves electrostatic interaction.

Although glutamate dehydrogenase is not a structural component of the inner mitochondrial membrane, it may be nevertheless loosely bound to the membrane. This speculation was reached in view of the high affinity of binding of the enzyme to the anionic phospholipid membranes and the fact that the inner mitochondrial membrane consists of 20% cardiolipin (Colbeau, 1971).

The possibility of an asymmetrical distribution of the phospholipid with

its preferential localisation in the inner surface of the inner membrane supported the above speculation. Evidence for such asymmetrical nature of the mitochondrial membrane has been fully discussed in the previous chapter (pages 86 and 87).

Binding of the enzyme to mitochondrial membranes showed the two types of specificities (dependency on membrane composition and enzyme conformation) discussed above. The enzyme showed a much higher affinity for binding to the inner surface of the inner membrane than to the outer surfaces of the inner and outer membranes. Also, affinity of the enzyme for the inner mitochondrial membrane was found to be dependent upon the type of conformation it may adopt. Thus, different metabolites bound to the enzyme showed opposite effects - NADH decreased the binding while ADP caused an increase.

However, these effects were small and a full interpretation of such results in a mitochondrial system is difficult. These metabolites are added to a system which has already high concentrations of endogenous metabolites and active enzyme systems. Thus, any additional metabolite may affect the extent of binding of the enzyme to the inner mitochondrial membrane in the following ways:

i) Binding to glutamate dehydrogenase and causing conformational changes in the enzyme structure.

ii) Binding to proteins other than glutamate dehydrogenase, affecting their binding to the mitochondrial membrane and changing the availability of the binding sites on the mitochondrial membrane for glutamate dehydrogenase.

iii) Provision of the required metabolite(s) for the activity of other mitochondrial enzymes. This can result in the formation of new metabolites which can bring about changes through I and II.

iv) Binding to some of the mitochondrial proteins, changing their conformation and hence, the extent of protein-protein interactions.

In the case of glutamate dehydrogenase, the equilibrium constant for the enzyme may be defined as:

$$K = \frac{[\alpha\text{-Kg}]\,[NADH]\,[NH_4^+]}{[\text{l-glutamate}]\,[NAD^+]}$$

It has been shown that (Williamson et al, 1967) an equilibrium exists between the components of the glutamate and β-hydroxybutyrate dehydrogenase systems. An approximately three-fold increase of the NH_4^+ concentration in the rat liver brought about by intramuscular injection was found to bring about changes in the concentration of all the metabolites involved in these systems.

The glutamate concentration was increased while α-Kg concentration was diminished. Also the ratios $\frac{[\text{l-glutamate}]}{[\alpha\text{-Kg}]\,[NH_4^+]}$ and $\frac{[\beta\text{-hydroxybutyrate}]}{[\text{acetoacetate}]}$ changed in parallel.

In the case of glutamate dehydrogenase, an increase in l-glutamate or NAD^+ concentration may bring about increases in concentration of α-Kg NADH and NH_4^+. A reverse process can occur by increasing the concentration of any of the other metabolites (α-Kg, NADH and NH_4^+). Any such changes are controlled by the availability of the other required metabolites and by the fact that the equilibrium for the activity of the enzyme favours the formation of glutamate.

Concentrations of substrates of glutamate dehydrogenase system in well fed rats have been found to be 2.41 ± 0.29, 0.145 ± 0.013 and 0.47 ± 0.15 n moles/g fresh weight for l-glutamate, α-ketoglutarate and ammonia respectively (Williamson et al, 1967). In starved rats, lower levels for l-glutamate and NH_4^+ and a higher level for α-ketoglutarate were observed.

Binding of the enzyme to the inner surface of the inner membrane increased with decreasing NH_4Cl concentration and pH.

The reversible association of the enzyme with the inner mitochondrial membrane and the effect of pH, ionic strength and metabolite concentrations on such interaction may explain some important properties of the enzyme.

Glutamate dehydrogenase is present in the mitochondrial matrix of beef liver cells at concentrations as high as 2 mg/ml or even higher (Frieden and Colman, 1967). The isolated enzyme has a high specific activity, but under normal circumstances, its activity is strongly inhibited. The major pathway of glutamate metabolism is via transamination, rather than oxidative deamination by the enzyme (Greville, 1969). However, under abnormal conditions of high NH_4^+ concentrations, the excess ammonia is rapidly metabolised by the enzyme. This might be due to the release of the enzyme bound to the inner mitochondrial membrane to the matrix compartment.

Another interesting observation is stimulation by l-leucine of glutamate dehydrogenase in intact rat liver mitochondria (McGivan et al, 1973) and the fact that l-leucine decreases affinity of binding of the enzyme to the inner mitochondrial membrane. These findings have been fully discussed in previous chapters (pages 105 and 106).

In conclusion, data reported in this thesis are in support of the hypothesis that glutamate dehydrogenase may be specifically associated with the inner mitochondrial membrane and may partition between the membrane and the mitochondrial matrix in a manner responsive to local variations in pH, ionic strength and metabolite concentrations. The effect of metabolites includes the allosteric properties of the enzyme. Regulation of enzymatic activities may also take place through an allotopic mechanism on interaction with membranes.

It is suggested that the observations and conclusions discussed above have important physiological significance and may explain a number of findings reported previously on the properties of the enzyme in mitochondria. Also the system may throw some light towards characterisation of mechanisms of interaction (and organisation) of some mitochondrial enzymes which are not fully incorporated into the bilayer structure.

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