

PHYSICAL STUDIES
OF THE CHROMAFFIN GRANULE
AND ITS MEMBRANE

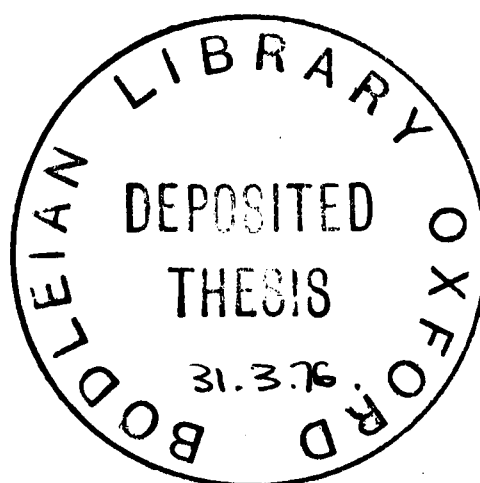
A Thesis
submitted in partial fulfilment
of the requirements for
the degree of
Doctor of Philosophy

-- by --

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Somerville College,
Oxford.

November, 1975.



To my Parents,

Lezli

and

John.

Some books are lies fra end to end
And some great lies were never penn'd....

But this that I am gaun to tell
Which lately on a night befell, .
Is just as true's the Diel's in Hell
Or Dublin City.

ROBERT BURNS (1759-1796)

ABSTRACT

Chromaffin granules, the catecholamine storage vesicles abundant in the chromaffin cells of the adrenal medulla, are immunologically identical to the adrenergic vesicles of the sympathetic nervous system. As a consequence of their common embryological origin, the chromaffin cell serves as a useful model system in which to investigate several aspects of nerve action. Chromaffin granules contain large concentrations of catecholamines and of ATP, acidic proteins termed the chromogranins, and the divalent metal ions, calcium and magnesium. The membrane of the chromaffin granule contains approximately 30% protein by weight. These proteins include a Mg^{2+} -activated ATPase, an electron transfer chain with an NADH:(acceptor) oxidoreductase activity and the enzyme dopamine- β -hydroxylase. The membrane has an unusually large content of lysolecithin, approximately 16% of the total phospholipid.

The adrenal medulla is innervated directly by a branch of the greater splanchnic nerve. Acetylcholine, released at the synapses, potentiates an influx of calcium ions into the chromaffin cell. This results in the migration of the granules through the cytoplasm to the cell plasma membrane where the two membranes fuse, expelling the total contents of the granules into the interstitial space. The lysolecithin present in the membrane has been implicated in this process of fusion.

There are many problems associated with the chromaffin granule system. It is not known how such large quantities of catecholamines, nucleotides and proteins can be assimilated and then maintained within the granule against the very large osmotic and concentration gradients. The roles of the membrane proteins, in vivo, have not been determined (apart from that of dopamine- β -hydroxylase). Roles have been proposed for the ATPase involving it in catecholamine synthesis and uptake, ATP synthesis, interaction with microtubules in the transcytoplasmic migration and also

interaction with the plasma membrane in exocytosis itself. Apart from the knowledge that the secretory stimulus is a calcium influx and that exocytosis involves membrane fusion, virtually nothing has been determined about the intermediate processes.

The aim of this thesis has been threefold; to determine the role of the ATPase, to discover the nature of the granule storage complex and to investigate the structure of the granule membrane in view of the proposed properties and the function of the membrane in exocytosis.

The studies on the ATPase are described in Chapter Two. It was found that the activity of the ATPase of both intact granules and granule membranes is uncoupler sensitive ie. the activity is greatly enhanced in the presence of conventional uncouplers of the mitochondrial ATPase activity and the electron transfer chain. The uncouplers which affected the chromaffin granule ATPase include 5-chloro,3-t-butyl, 2'-chloro, 4'-nitro-salicylanilide (S-13) and bis-hexafluoro-acetone (1799). The ATPase activity of intact granules was enhanced optimally four-fold, whilst that of the granule membrane was enhanced only 50%. The uncoupler sensitivity implies, according to the chemiosmotic hypothesis of oxidative phosphorylation, that the ATPase is able to generate a cation or proton gradient across the granule membrane. Neither the coupled nor the uncoupled ATPase activities were inhibited by oligomycin or aurovertin, but both were greatly inhibited by dicyclohexyl carbodiimide (DCCD) which is a potent inhibitor of the mitochondrial ATPase, and is thought to block proton channels. The inhibition by DCCD was only partially restored on solubilisation of the granule membrane although the uncoupling by S-13 was completely abolished. This indicates that the uncoupling phenomenon is dependent on the integrity of the membrane whilst DCCD inhibition is due, in part, to its direct interaction with the enzyme or tightly bound protein factor. It was also shown that the chromaffin granule membrane can generate an 'ANS Response' in the presence of ATP-Mg^{2+} ie. the fluorescence of the probe, 1-anilinonaphthalene-8-sulphonate (ANS)

interacting with the granule membrane was greatly enhanced on addition of ATP-Mg²⁺. This effect is also observed with submitochondrial particles, and is believed to be a reflection of the generation of a transmembrane potential. The enhancement was abolished when uncoupler molecules were added and was prevented by preincubation of the membrane with DCCD.

The pH dependence of the ATPase activity and the ANS response were investigated in the presence and absence of uncoupler. The pH optima of the coupled and uncoupled ATPase activities were very different. The coupled ATPase did not show an optimum; the activity remained constant between 6.0 and 8.0. The uncoupled activity was maximal at pH 6.4, as was the fluorescence enhancement of the ANS response. The 'reversal' of the ANS fluorescence enhancement by uncoupler had a linear dependence on pH at pHs greater than 6.0, but at pHs less than approximately 5.5, there was further enhancement.

The uptake of catecholamines by intact granules and by granule membrane vesicles, which is dependent on the hydrolysis of ATP, was abolished in the presence of uncouplers, even although the ATPase was up to four times as active. The uptake of these biogenic amines is, therefore, not a direct result of the ATPase activity, but is most probably a consequence of a potential energy gradient generated by the ATP hydrolysis. It is this gradient that is dissipated by uncouplers. The uptake is also inhibited by DCCD. These results indicate that the ATPase of the chromaffin granule membrane has similar properties to that of submitochondrial particles, where the ATP-dependent uptake of cations is abolished in the presence of uncouplers. It has not been possible to determine whether the electron transfer chain of the granule membrane is coupled to the ATPase, since the rates of NADH:(acceptor) oxidoreductase activity are very low.

Electron spin resonance (ESR) studies of the chromaffin granule membrane are described in Chapter Three. The structure of the membrane has been probed using a variety of spin labels. Both the effects

of temperature and high levels of calcium have been studied. The results from three positional isomers of the stearic acid spin label demonstrate that a substantial part of the membrane lipid is in a bilayer structure which undergoes a structural transition at 32°-38°C, characterised by an increase in the population of gauche isomers in the lipid chains. A possible mechanism for this transition would be preferential segregation of cholesterol. The covalently bound iodoacetamide spin label revealed a transition within the protein component of the membrane, or its immediate lipid environment, at approximately 32°C. This transition corresponds to an increased degree of motional freedom of the spin label. The lipid soluble TEMPO spin label exhibits a break at 34°C in the temperature dependence of its partitioning into the membrane. This could correspond to the onset of a lateral phase separation in the membrane lipid, again possibly involving a redistribution of cholesterol. Calcium abolishes, diminishes or shifts the transition observed by TEMPO, and decreases the amplitude of motion of the stearic acid spin labels. This also may be due to a redistribution of lysolecithin and cholesterol. The temperatures of the structural transitions agree well with those observed in the enzymic activity of the membrane ATPase and the NADH oxidase functions, and also with the results of fluorescent probe studies. It is possible that triggering of the transition either with calcium or some other stimulus, may play a role in catecholamine release and membrane fusion.

Nuclear magnetic resonance (NMR) studies have been made of intact adrenal glands, chromaffin granules and granule membranes (Chapter Four). ³¹Phosphorus NMR has been used as a sensitive non-destructive probe, permitting comparison of the structure of the contents of isolated granules with those of granules in intact glands, by measurement of the frequencies and line widths of the ATP resonances. Adrenal glands and chromaffin granules from pigs, sheep and cows have been examined, and it has been shown that the preparative procedure does not affect the contents of the

granules, as assessed by these criteria. The nature of the granule complex has been studied by making pH titrations of the ATP resonances in simulated contents at varying degrees of complexity. It has been concluded from these studies that the internal pH of the bovine chromaffin granule is 5.45 ± 0.2 . This is in excellent agreement with the findings described in Chapter Two. The simulation studies show that the reported contents themselves do not account for the broadening of the resonances observed in granule spectra, and that it is likely that the additional broadening is a result of the presence of low concentrations of paramagnetic ions. The total simulated complex, containing chromogranins and phospholipids in addition to ATP, catecholamines and divalent metal ions, is completely soluble at room temperature. This is a good indication that in vivo the storage complex does not exist as a crystalline or paracrystalline structure but as a very viscous solution. ^1H and ^{13}C NMR studies, in addition to ^{31}P , show that the chemical shifts present in spectra of intact granules and of ATP and adrenaline at 122mM and 550mM respectively are due to general ring current shifting, as a consequence of the large concentration of aromatic rings present in the solutions. There is no evidence for specific complex formation of more than a transitory nature. This is supported by preliminary relaxation studies.

The ^1H NMR spectrum of purified granule membranes shows no detail of lipid structure but resonances attributable to protein components, similar to the chromogranins, have been observed. ^{31}P NMR studies of the chromaffin granule membrane indicate that the spectrum is similar to that expected from a consideration of chemical shift anisotropy effects. A temperature study was made of this membrane. Unfortunately the spectra are rather poor, but changes in the spectrum can be observed between 30° and 40°C. This is in agreement with the ESR, fluorescence and enzyme activity studies.

Finally, the chromaffin granule membrane was examined by X-ray diffraction (Chapter Five). The intention was to obtain an electron density profile of the membrane, and to investigate the proposed existence of a protein lattice in the plane of the membrane. It was not possible to obtain this electron density profile since it was discovered that the membrane was not stable to the dehydration necessary to produce orientation. Electron microscopy of ultracentrifuged, partially dehydrated membrane pellets revealed the presence of large multilamellar structures which produce a sharp optical diffraction pattern, spacing approximately $46\text{\AA}$. X-ray diffraction of this pellet resulted in a strong lamellar diffraction, with a spacing of approximately $55\text{\AA}$, extending to the sixth or seventh order. No other diffracting species was present in the electron micrographs, but the spacings obtained from optical and X-ray diffraction are not in complete agreement. The reason for this has not been resolved. Preliminary studies at high humidity indicate that it may be possible to produce membrane vesicles that are sufficiently well oriented to give lamellar diffraction to the necessary resolution. At no time did the X-ray diffraction patterns show the presence of equatorial reflections which could be attributed to a protein lattice. This agrees with freeze-fracture studies carried out in this laboratory. The chromaffin granule membrane lipid extract was examined and the diffraction patterns at a range of humidities obtained. Although there were several minor diffraction series present, a major lamellar diffraction always occurred. From the spacings and the intensities of these lamellar diffractions, a one-dimensional Fourier was synthesised, and an electron density profile of the lipid bilayer was obtained. The electron density maxima of this profile are $45\text{\AA}$ apart, the same distance as that found with extracted mitochondrial lipids. This spacing is attributed to the presence of cholesterol in the bilayer. When chromaffin granules were irradiated there was no indication of an ordered diffraction of a crystalline nature, supporting the proposal that the storage complex is in a soluble,

disordered state.

In conclusion, this study has proposed that the ATPase of the chromaffin granule membrane is energy-coupled and may be similar to the mitochondrial and related ATPases. It is capable of generating a potential gradient across the membrane, which can drive other functions including catecholamine uptake. The internal pH of the chromaffin granule is around 5.5, which means that there should be a pH gradient across the membrane of greater than one pH unit. There also appears to be a phase transition in the granule membrane, at approximately 34°C, which may involve the partitioning of cholesterol and the consequent formation of lysolecithin-enriched areas.

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ACKNOWLEDGEMENTS

I have been very privileged during my three years in Oxford to have been a member of both the Laboratory of Molecular Biophysics and the Department of Biochemistry. I am most grateful to Professor D.C. Phillips F.R.S. and Professor R.R. Porter F.R.S. for the use of the facilities in their respective departments.

I owe much to my supervisors, Dr L.N. Johnson and Dr G.K. Radda, for their guidance and the constant interest they have shown in my work. I am very fortunate to have been able to carry out my research in such a stimulating and happy environment.

Probably the most valuable consequence of my dual existence has been the amount of help and the multiplicity of ideas so readily forthcoming from my many collaborators, associates and friends. The biochemical studies described in Chapter Two were made in collaboration with Lindsey Bashford and latterly also with Robert Casey. I am grateful to them both for their assistance and discussion during these experiments. I am also indebted to Dr Derek Marsh for his help with the detailed analysis of my ESR data.

The NMR studies would not have been possible without the help of John Seeley, whose expertise in operating the different instruments and whose thorough understanding of this complicated technique complimented my purely biochemical background. I am exceedingly grateful to him for his encouragement, criticism, ideas and neverending stamina throughout this work.

Dr Chris Rattew, Dr Barbara Doyle, Dr Chris Rodgers and Stephen White have at different times constructed, corrected and provided unfailing assistance with the operation of the rotating anode X-ray camera. Their patience in explaining its technicalities to me was most appreciated. I am also grateful to Dr Kevin Leonard for spending so much time helping me with the computing of my diffraction data and to Mrs Carol Van Horn for the many electron microscopic studies she made of my membrane specimens.

I must also thank the many other members of the Laboratory of Molecular Biophysics, in particular the members of Dr L.N. Johnson's and Dr A. Miller's research groups, and also my associates in the research groups of Dr G.K. Radda and Dr R.E. Richards F.R.S. for the support, encouragement and help, scientific or otherwise, that has been so generously given on innumerable occasions.

Without the assistance of Mr Richard Poynton at the FMC slaughterhouse in Thame much of this research would not have been possible. He remained undaunted by the most unusual requests and despite personal inconvenience always supplied the necessary tissues.

Finally, I should like to thank the Science Research Council for granting me a Research Studentship and Somerville College for providing me with such a pleasant background from which to work. The understanding of the College, especially of the Principal, Mrs Craig, the tolerance and kindness of the members of 17B, the generosity of Dr Peter Brunet and the photographic skills of Robin Gardner and Chris Blackmore have all greatly contributed towards the completion of this thesis.

ABBREVIATIONS

Å	Ångstrom unit
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-phosphate
ANS	1-anilinonaphthalene-8-sulphonate
ATP	adenosine-5'-triphosphate
ATPase	Adenosine triphosphatase
cf.	compared to
CG	Chromaffin Granule
CGM	Chromaffin Granule Membrane
DβH	Dopamine-β-hydroxylase
DCCD	dicyclohexyl carbodiimide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ESR	Electron Spin Resonance
FCCP	carbonyl cyanide-p-trifluoromethoxy-phenylhydrazine
HEPES	2-(N-2-hydroxyethylpiperazin-N'-yl)ethane sulphonic acid
GTP	guanosine-5'-triphosphate
h	Planck's constant
ħ	$h/2\pi$
Hz	Hertz
I(m,n)	stearic acid spin label
ITP	inosine-5'-triphosphate
MES	2-(N-morpholino)ethane sulphonic acid
NADH	reduced nicotinamide adenine dinucleotide
Nbf-Cl	4-chloro-7-nitrobenzofurazan
NMR	Nuclear Magnetic Resonance
ORD	Optical Rotatory Dispersion
PCMB	p-hydroxymercuribenzoate
Pi	inorganic phosphate
PNMT	Phenylethanolamine N-methyl transferase
p.p.m.	parts per million
RNA	ribonucleic acid
s	second
S	ESR order parameter
S-13	5-chloro, 3-t-butyl, 2'chloro, 4'nitrosalicylanilide
τ	correlation time
T ₁	spin-lattice relaxation time
T ₂	spin-spin relaxation time

TEMPO 2,2,6,6-tetramethyl-piperidine 1-oxyl
v frequency
w.r.t. with respect to
1799 bis-hexafluoroacetone

ENZYME CONVENTION NUMBERS

Adenosine triphosphatase	EC 3:6:1:4
Adenylate kinase	2:7:4:3
Dopamine- hydroxylase	1:14:17:1
NADH:(acceptor) oxidoreductase	1:6:99:3

CHAPTER ONE

Introduction

1.1 The Adrenal Gland

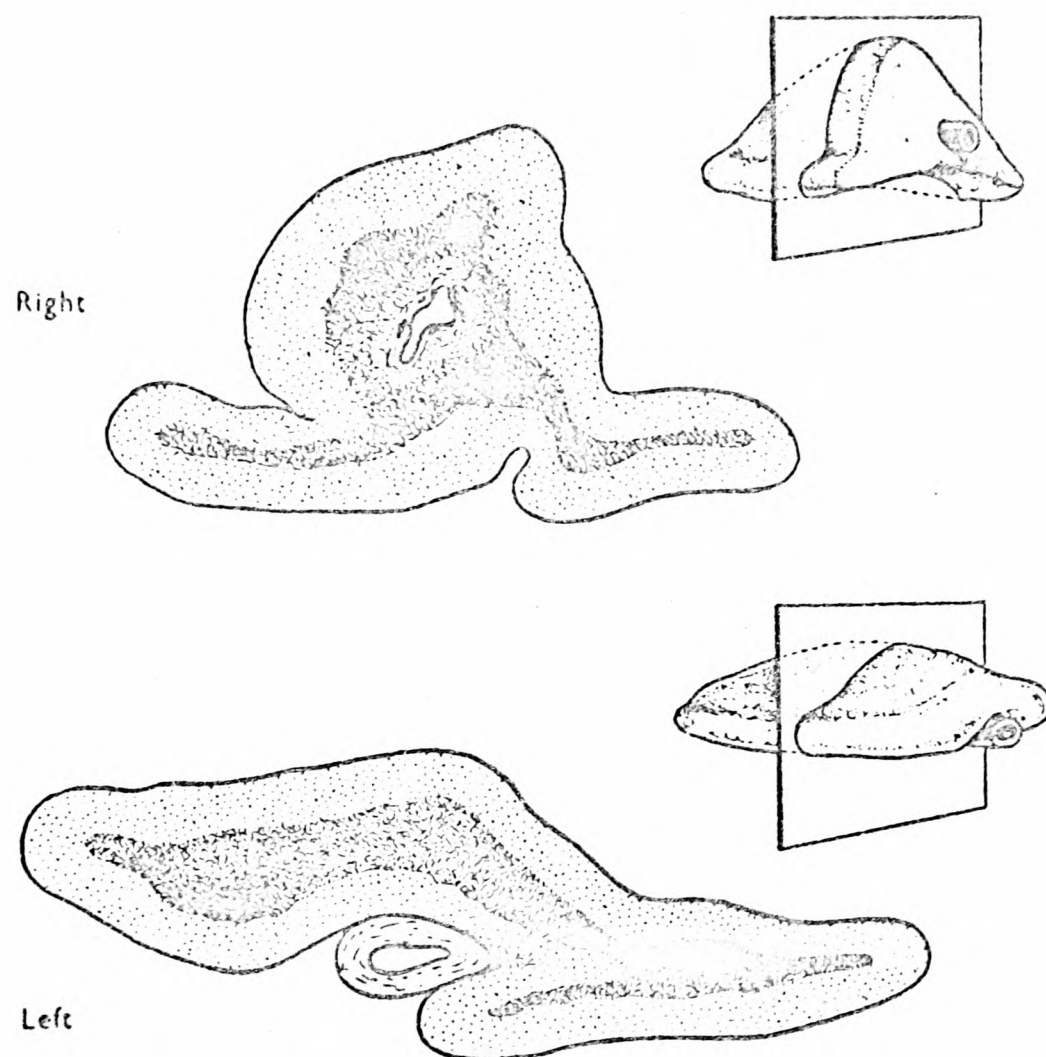
'The human adrenal bodies lie resting on the cranial poles of the kidneys like hats : that on the right is like a tricorne, the three cornered hat of the eighteenth century, and that on the left like an admiral's two-pointed cocked hat.' — A somewhat unorthodox, but very visual description of the adrenal glands, supported by Figure 1.1.1.(1). The size of the adrenal, however, relative to that of the kidney, varies markedly at different stages of development. In the eight week old foetus, the adrenal gland equals the kidney in size; at birth, it is one-third; whilst in the adult it is one-thirtieth. Associated with this change in relative size, great changes occur in the histological composition of the gland. The adrenal gland, in mammals, whilst having a definite anatomical unity, consists of two distinct endocrine organs, the cortex and the medulla. These tissues have quite different embryological origins.

A. The Adrenal Cortex

The adrenal cortex is of mesodermal origin. It can be divided into three distinct zones in the adult. There is the innermost zona glomerulosa, followed by a zona fasciculata and an internal zona reticularis surrounding the medullary tissue (Figure 1.1.2.). The adrenal cortex is primarily involved in the production and secretion of steroid hormones, seven of which are essential in maintaining the life of an adrenalectomised animal (2). The physiologically active steroids may be divided into three groups :

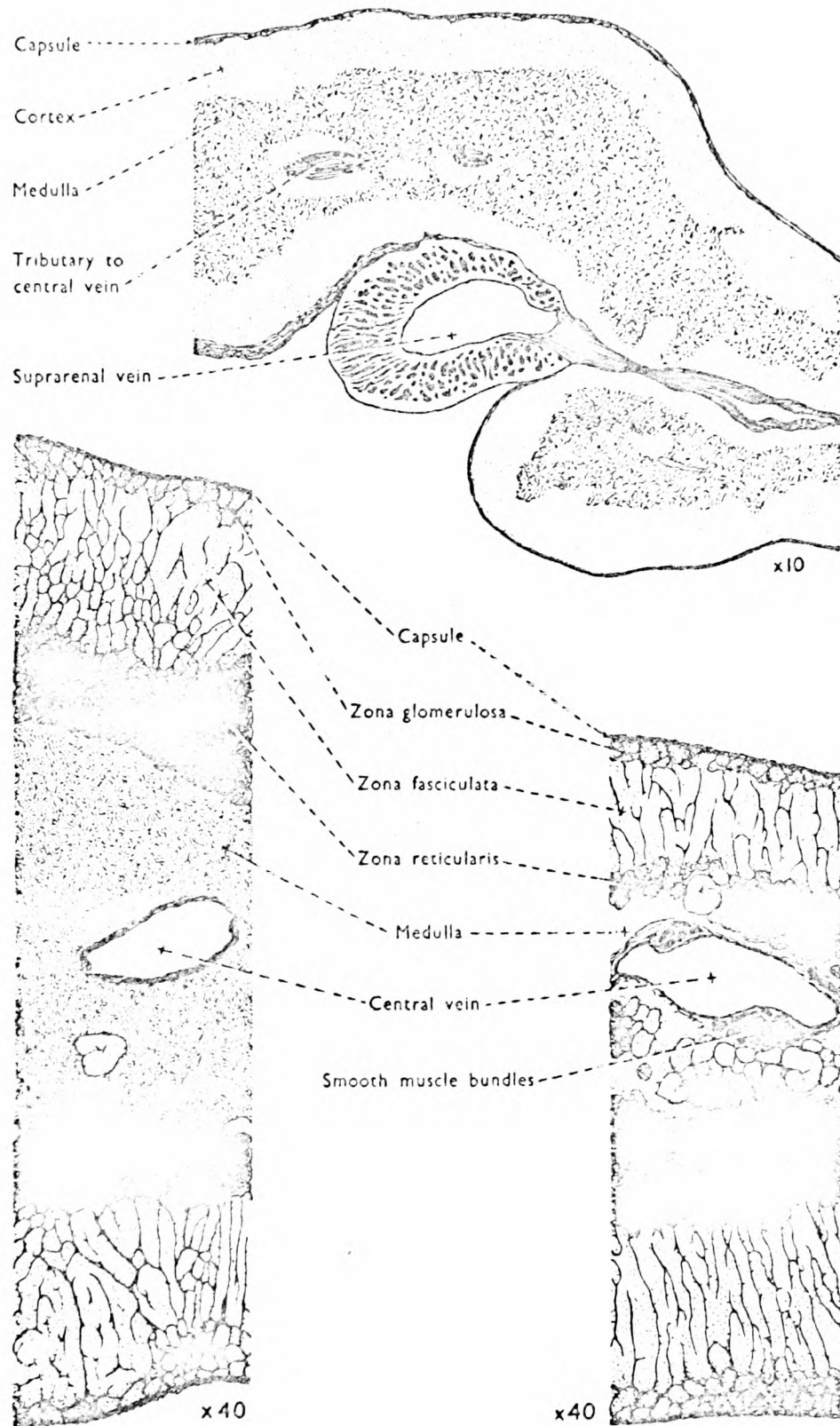
- (i) the Glucocorticoids eg. cortisol, corticosterone.
- (ii) the Mineralocorticoids eg. aldosterone, hydrocortisone.
- (iii) the Sex Hormones eg. androgens, oestrogens, progesterones.

FIGURE 1.1.1.



A diagrammatic representation of the human adrenal glands, indicating their shape and cross-sectional appearance.

FIGURE 1.1.2.



The detailed cross-sectional appearance of the adrenal cortex and medulla. The different tissue areas are indicated. (1)

The role of these hormones in metabolic control and differentiation and the regulation of their synthesis are described fully in many textbooks (1,2).

B. The Adrenal Medulla

The medulla is of ectodermal origin, and thus many of the cells in their early stages are similar to some of the neurones which leave the brain stem. The cells, however, do not continue the characteristic development of a neuron, but retain a simple epithelial character. The cells become capable of secretion and produce adrenaline and noradrenaline in large amounts. The cells of the adrenal medulla, therefore, correspond to the second motor neurons or post-ganglionic fibres of the sympathetic nervous system. The medulla is innervated directly by pre-ganglionic cholinergic neurons via the greater splanchnic nerve (Figure 1.1.3.) Functionally, the link is clear since the application of the secretion from medullary cells, which is mainly adrenaline, has similar, though not identical, effects as the stimulation of the post-ganglionic nerve fibres which have predominantly noradrenaline at their endings.

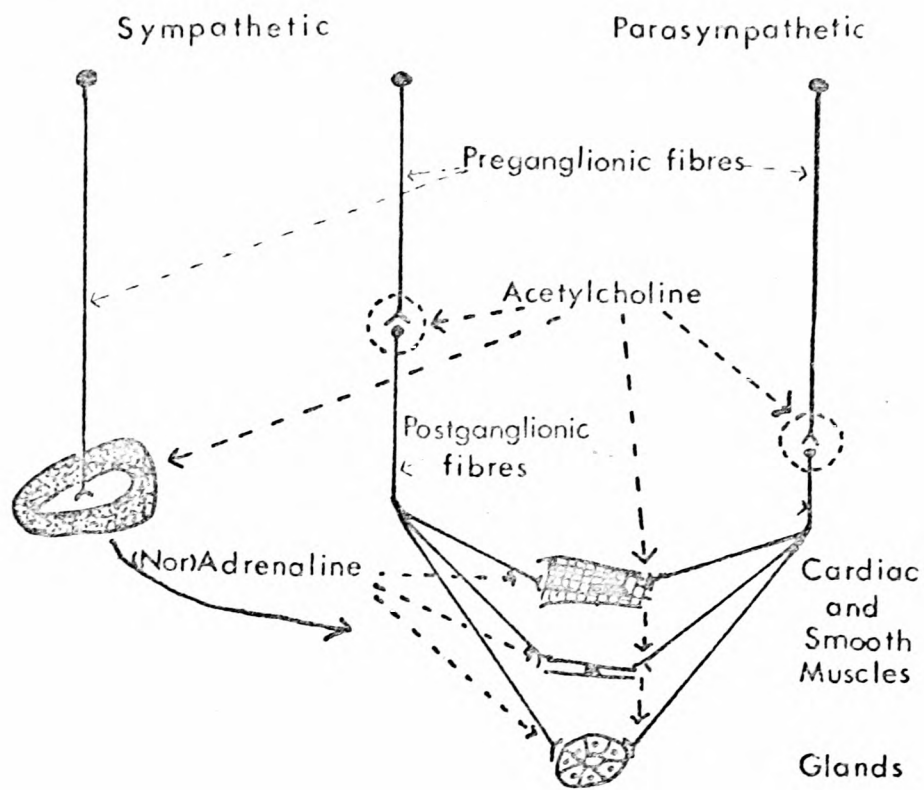
Early histological studies of the adrenal medulla showed the presence of tissue which stained brown with chromates (3). It is for this reason that it is referred to as 'Chromaffin' or 'Chromaphile' tissue. The chromaffin reaction due to the presence of catecholamines, in fact, led to the chemical identification, in 1904, of the first hormones, adrenaline and noradrenaline (4).

Approximately fifty years later, the subcellular organelles, responsible for the storage of the catecholamines within the adrenal medulla, were discovered independently by Blaschko and Welsh (5) and Hillarp et al (6). With reference to their staining with chromium salts, these catecholamines storage vesicles are commonly referred to as chromaffin granules.

Adrenaline and noradrenaline are now thought to be secreted

FIGURE 1.1.3.

AUTONOMIC NERVOUS SYSTEM



A comparison of the adrenal medulla with the enervation and action of the sympathetic and parasympathetic nervous systems. The transmitter molecules at each synapse are indicated.

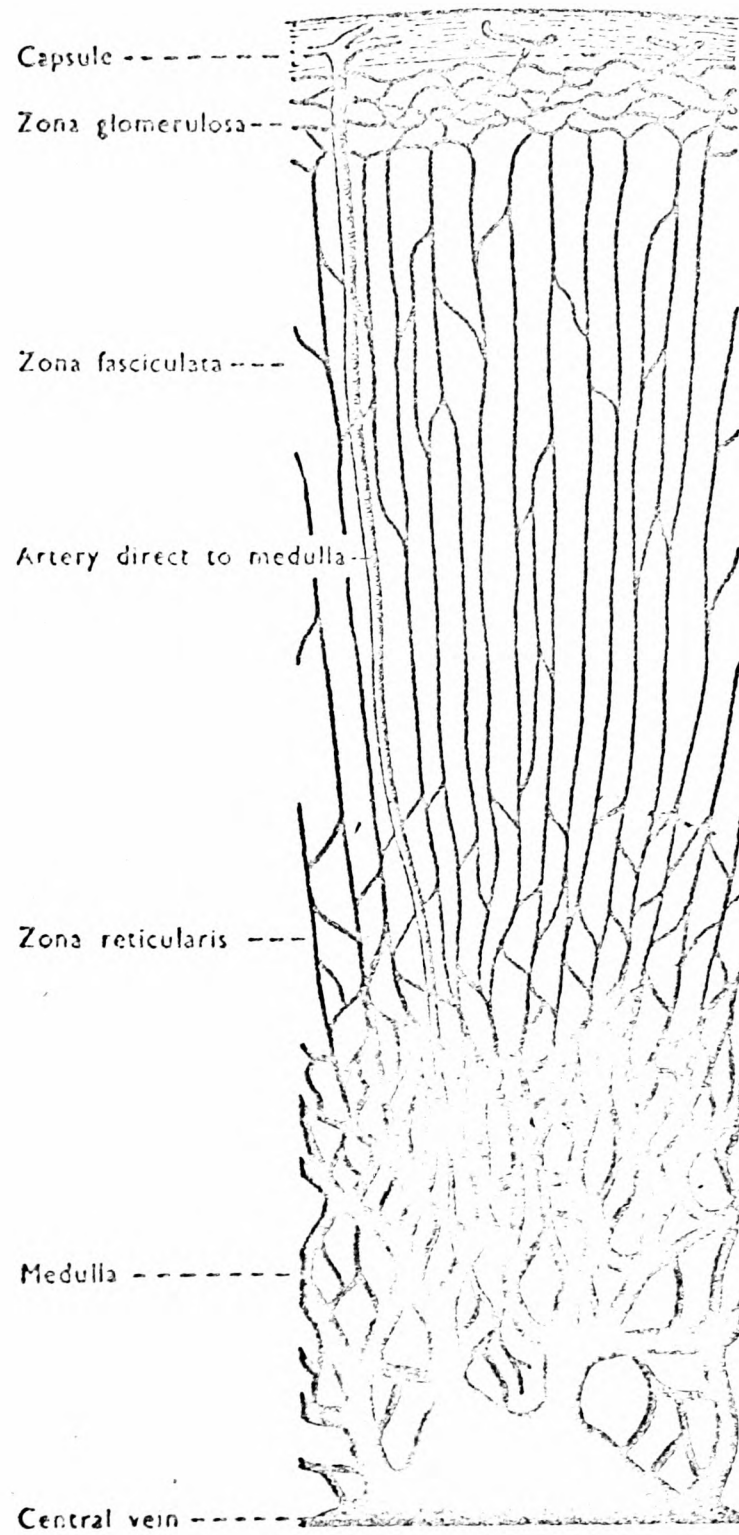
by different cell types (7). There is some evidence that adrenaline and noradrenaline containing cells are innervated separately and hence the hormones can be secreted separately. This appears to be dependent on the nature of the stimulus on the hypothalamus which, via a centre in the medulla oblongata, ultimately controls catecholamine secretion. Reflex stimulation of the sympathetic nervous system, and hence of the chromaffin cells, is produced under a variety of conditions including physical exercise, states of fear and exposure to cold. The secretion of catecholamines from sympathetic nerve endings and into the blood stream results in a mobilisation of those resources of the body involved in its response to an emergency, and an inhibition of those functions not of immediate importance in such a situation. This is often called ' the Fight or Flight response.'

C. The Blood Supply of the Adrenal Gland

The adrenal gland has a very rich blood supply. The adrenal arteries arise from several sources; the aorta, the phrenic artery and the renal artery. These large vessels divide into smaller branches in the capsule, (Figure 1.1.4.). Some of these branches form capillaries and pass into the cortex. Others pass as small arteries direct to the outer edge of the medulla via the cortex. Still others form the capillary network of the capsule, do not enter the gland and are drained by capsular veins. The capillaries that enter the cortex become sinusoids which surround the zona reticularis, branch and anastomise freely. They widen at the edge of the medulla and then form the much larger sinusoids of the medulla. The small arteries, as they penetrate the medulla, also branch to enter the network of sinusoids between chromaffin cells.

The medulla, therefore, not only receives blood which has been in close contact with cortical cells and contains their hormonal secretion, but also blood which has not been in exchange with cortical tissue.

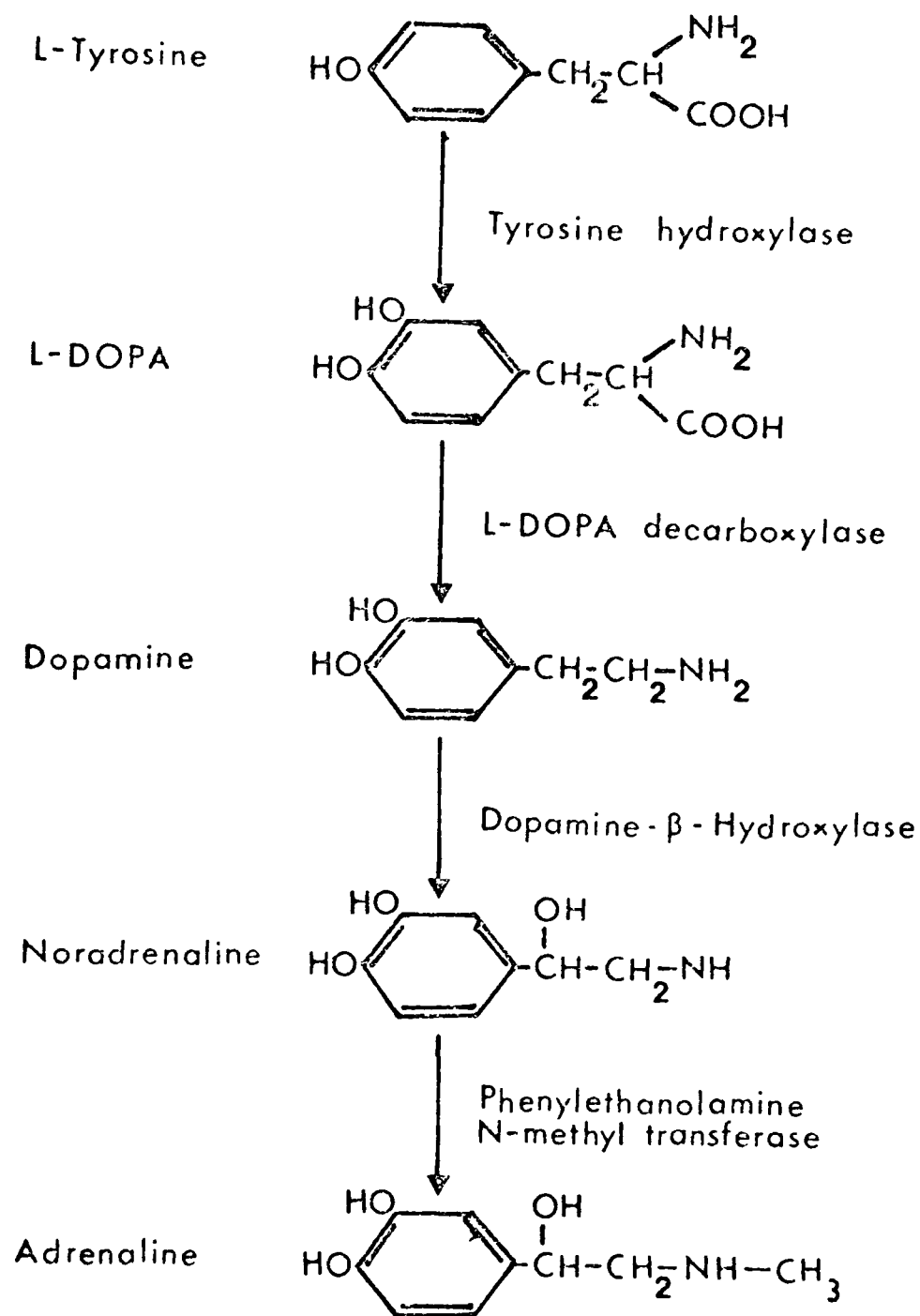
FIGURE 1.1.4.



The blood supply to the adrenal gland. (1)

FIGURE 1.1.5.

The Synthesis Of Adrenaline



The biosynthetic pathway for the production of adrenaline from tyrosine in the chromaffin cell.

All the blood from the parenchyma of the adrenal gland drains into a single central vein. The organ, therefore, has several arteries of supply, but only one outgoing venous channel.

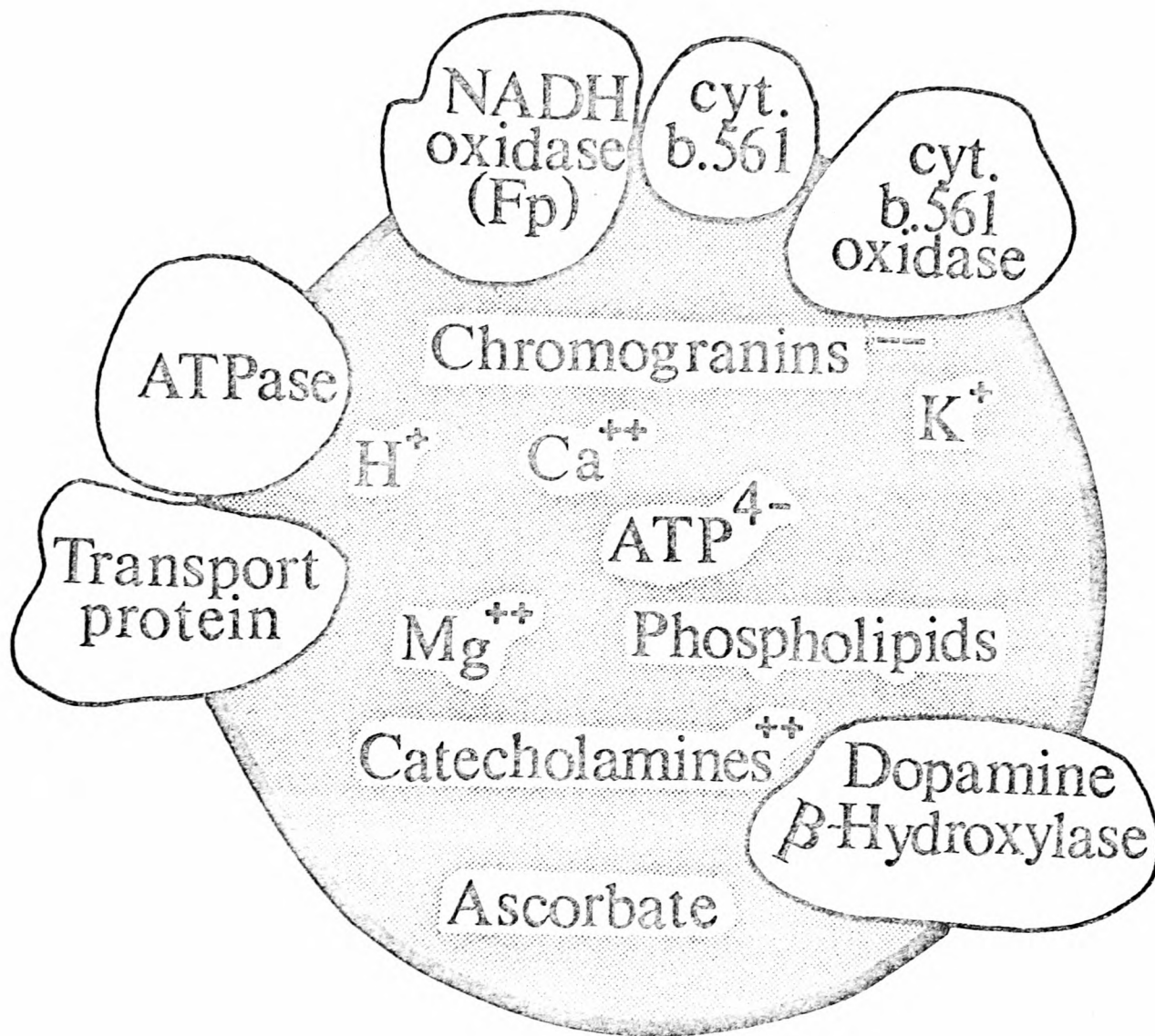
D. The Influence of the Cortex on the Medulla

Why have the adrenal medulla and cortex fused to form one organ, the adrenal gland? This is a question which has long puzzled researchers. One answer to this problem has recently been found.

The synthesis of adrenaline from tyrosine is shown in Figure 1.1.5. All these enzymes are present in the chromaffin cell, but only dopamine- β -hydroxylase is located in the chromaffin granules; the others are present in the soluble fraction of the cell (9,10,19). Fetal adrenal medulla is unable to methylate noradrenaline to adrenaline until it establishes anatomical and circulatory contact with cortical tissue (11,12,13,14). It has been shown that the development of phenylethanolamine N-methyl transferase, PNMT, is glucocorticoid dependent (15,16).

In view of this, and other evidence (17,8,18), it appears that the morphological and circulatory proximity of the adrenal cortex and medulla are essential for adrenaline, but not noradrenaline formation.

FIGURE 1.2.1.



A schematic representation of the Chromaffin Granule.

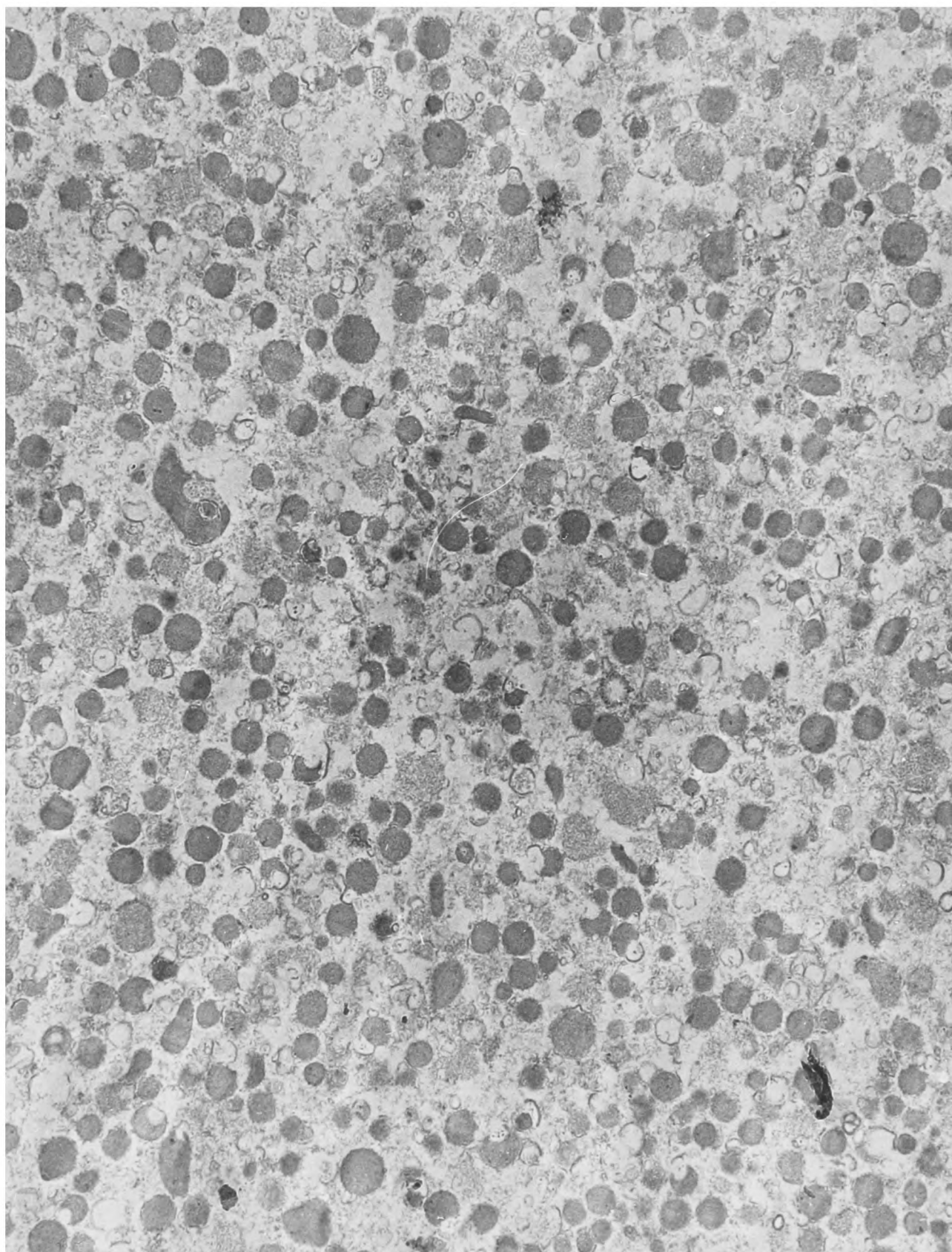
1.2 The Chromaffin Granule

A. Its Contents and the Internal Structure

The catecholamine storage vesicles of the adrenal medulla, the chromaffin granules, contain not only high concentrations of adrenaline and noradrenaline (0.5M), but also high levels of adenosine triphosphate (0.12M), of acidic proteins, the chromogranins, (120mg/ml ; effective carboxylate concentration, 0.2M), and of divalent cations, magnesium and calcium (20). A schematic representation of a chromaffin granule is shown in Figure 1.2.1. The presence of ascorbate in the granule has recently been reported by Flatmark (21). It was originally thought that RNA was present in chromaffin granules (22), but its presence is most likely to have been due to contamination of the 'granule fraction' with microsomes (23).

There has long been a controversy about the nature of the complex within the granule. The presence of such large concentrations of molecules must pose considerable osmotic stress on the granule membrane and introduce large concentration gradients across the membrane. In ultrastructural terms, two populations of granules can be detected in electron micrographs of chromaffin granules fixed with gluteraldehyde. Noradrenaline is precipitated in situ, while adrenaline remains soluble and so is washed out of fixed granules (24). Using this fixation method, chromaffin granules are depicted as membrane enclosed granular matrices; the noradrenaline granules appear as electron dense, whilst the adrenaline granules exhibit moderate electron density (Figure 1.2.2.). It has been suggested that since the fixed adrenaline containing granules contain the protein and lipid remaining after adrenaline depletion, the substructure of these matrices may reflect the macromolecular organisation of the catecholamine storage complex (25). However, this should be considered carefully, due to the introduction of artifacts by the technique. After certain treatments, bilayered configurations have been observed at the interface between the matrix and aqueous influx, and also concentric

FIGURE 1.2.2.



An electron micrograph of a pellet containing chromaffin granules. Adrenaline- and noradrenaline-containing granules may be distinguished. The granules were fixed with gluteraldehyde. Magnification: 15,000.

membranous structures within a single granule (26). Helle et al have proposed, on the basis of this evidence, that phospholipids may be present, not only in the outer membrane, but also in the matrix and so account for the liposomal configurations observed within chromaffin granules under certain conditions (26,27,28,29). From a comparison of the lipid phosphate and protein concentrations of various preparations, Helle and Serck-Hanssen reached a value of 0.23-0.26 μ moles of lipid phosphate per milligram of matrix protein (29). The true nature of the proposed complex within the chromaffin granule is unknown. Much conflicting evidence has been put forward (30,31). An aggregation between catecholamines and ATP has been shown to exist (32,33). Aggregates of high molecular weight, in aqueous solution, may be formed but only in the presence of large concentrations of divalent cations and at low temperature, neither of which conditions are physiological (34).

B. The Chromogranins and Dopamine- β -Hydroxylase

There has been considerable controversy about the distribution of proteins within the granule. Helle found over several preparations that an average value of 66% of the total granule protein could be rendered water soluble. The range was 57% minimum to 85% maximum (35). She concluded that the protein content was dependent on differences in the excited state of the animals before slaughter, rather than differences in the methodology of the lysis procedure. 'The chromogranins' is the term for all the protein components of the granule lysate, but is often taken to exclude the soluble dopamine- β -hydroxylase, (DBH). Most of the more recent studies of the chromogranins have involved amino acid analysis and immunological cross-reactivity studies (29).

The main component of the soluble proteins, chromogranin A, has been isolated and characterised (20). It has also been shown to be present in a membrane bound form, distinguishable with respect to electrophoretic mobility from the main water-soluble form (35,38). Chromogranin A,

approximately 40% of the soluble protein, appears to consist of two identical or almost identical polypeptide chains of $32-40 \times 10^3$ daltons in molecular weight (37,38). In buffers of low ionic strength which do not contain phosphate ions, the dimer is predominant. The amino acid composition of chromogranin A is very similar to that of the total granule lysate (29). It is a highly acidic protein with aspartate and glutamate accounting for 32% of the residues, lysine and arginine for 14% and proline for 8% (20). The effects of this highly unusual amino acid composition are reflected in its optical rotatory dispersion (ORD) properties which indicate that very little α -helical structure is present (38). Antisera, prepared by Hortnagl et al against purified chromogranin A, cross reacts with all the other chromogranins but not with dopamine- β -hydroxylase (39). On the basis of this finding in addition to the similarities in amino acid composition, it has been proposed that the other acidic chromogranins are accounted for as other molecular forms of chromogranin A than as totally unrelated acidic proteins (29).

Dopamine- β -hydroxylase, which catalyses the conversion of dopamine to noradrenaline (Figure 1.1.4.), was first purified from whole granules or granule membranes solubilized in Triton X-100 (40). It is a copper containing protein with a maximum of 7 μ moles of copper per mole of enzyme and an approximate molecular weight of 29×10^4 daltons (42). The enzyme is a component of the matrix as well as of the membrane phase, although the specific activity of the matrix enzyme is much less (35). Helle observed that the soluble dopamine- β -hydroxylase could be dissociated into a subunit of molecular weight, $33-39 \times 10^3$ daltons, which could not be distinguished from chromogranin A (37). Anti-chromogranin A antisera prepared by her, also reacted with her preparation of soluble dopamine- β -hydroxylase, in disagreement with the results of Hortnagl et al (37,39). She has proposed that dopamine- β -hydroxylase and chromogranin A have a common denominator polypeptide (29).

A number of 'purified' dopamine- β -hydroxylase preparations have now been described and evidence of the subunit composition of this enzyme is accumulating (40,41,42,43,44,45,46). A tetrameric composition, composed of subunits of molecular weight, 75×10^3 daltons, has been agreed upon, based on preparations of very varied specific activity (40-46). Aunis et al propose a molecular weight of 37.5×10^3 daltons for each polypeptide and conclude that dopamine- β -hydroxylase is composed of eight identical polypeptides (44). There is, however, a very large difference in the published amino acid compositions of many of these preparations. This may be due to contamination by small basic peptides, including the peptide of cytochrome b_{561} , which are solubilised by Triton X-100 (47). Present evidence indicates, but by no means conclusively, that chromogranin A is the inactive subunit of dopamine- β -hydroxylase. The actual organisation of the subunits of dopamine- β -hydroxylase remains a subject of speculation.

The question remains, however, 'What is the function of the chromogranins within the granule?' It was initially thought that chromogranin A is involved in the storage complex within the vesicle. Although it binds two or three times as much adrenaline as does bovine serum albumin, either in the presence or absence of Mg^{2+} and ATP, this binding could only account for 1 to 2% of the total catecholamine within the vesicle (36)

C. The Chromaffin Granule Membrane

(i) The Lipid Content

Lipids represent 22% of the dry weight of bovine chromaffin granules (52). The main lipids of the chromaffin granule membrane are cholesterol and phospholipids. The molar ratio of cholesterol to lipid phosphorus is 0.58 compared to 0.44 and 0.23 for the microsomal and mitochondrial fractions respectively (48). The phospholipid composition of bovine granule membranes is shown in Table 1.2.1. where it is compared to the phospholipid compositions of other vesicles membranes. What is most striking is the high content of lysolecithin in the membrane of the chromaffin

TABLE 1.2.1.

	Chromaffin Granules	Nerve Trunk Vesicles	Synaptosomes	Platelet Granules
Cholesterol:	0.56	0.45	0.41	0.61
Phospholipid				
PC	26.0%	36.6%	39.3%	33.8%
PE	36.1%	30.8%	33.6%	31.9%
PS	9.2%	7.1%	16.9%	14.4%
PA	0.6%	4.9%	2.3%	—
Lysolecithin	16.8%	3.1%	—	1.9%
Sphingomyelin	10.9%	17.6%	5.3%	17.1%
% Recovery	99.6%	100.1%	97.4%	99.1%
	(47)	(48)	(49)	(50)

The lipid compositions of several biological storage vesicles, including the chromaffin granule membrane.

- Key : PC Phosphatidyl choline
- PE Phosphatidyl ethanolamine + ethanolamine plasmalogen
- PS Phosphatidyl serine + Phosphatidyl inositol
- PA Phosphatidic acid + Cardiolipin.

granule. This would appear to be non-artifactual since when the adrenal medulla was homogenized in the presence of the phospholipase inhibitor, diethyl p-nitrophenyl phosphate, the membrane still contained $13.0 \pm 0.4\%$ lysolecithin, compared to $17.4 \pm 1\%$ in the non-inhibited preparation (51). Also, the mitochondria and microsomes from the adrenal medulla contain very little lysolecithin, less than 2% of the lipid phosphorus (48).

The presence of lysolecithin in the adrenal medulla was shown as long ago as 1957, when Hajau observed that ox adrenal medulla, not cortex, was rich in a substance which had a digitalis-like action on the heart. This substance was isolated by Hajau et al and shown to be lysolecithin (53). The cardiac action of lysolecithin is not due to a direct digitalis-like action, but is the result of the release of noradrenaline from sympathetic nerves, (54). In 1940, Feldberg had shown that lysolecithin caused the release of adrenaline from perfused adrenal glands and also from a particulate fraction of the adrenal homogenate (55). The lysolecithin contents of chromaffin granules from different species are shown in Table 1.2.2. Whilst there is variation in all systems studied, the lysolecithin content is significantly large.

A study of the fatty acid compositions of lysolecithin and lecithin from bovine chromaffin granules concluded that the lysolecithin is entirely the 1-acyl isomer. The main fatty acids present in lysolecithin were stearic acid (43%), palmitic acid (30%), and oleic acid (19%) (57).

Howell and Lucy were able to show that lysolecithin can cause biological membranes to fuse. Addition of lysolecithin to suspensions of hen erythrocytes not only caused haemolysis but also induced the formation of multinucleate cells (58,59). It has been proposed, therefore, that the lysolecithin present in the chromaffin granule membranes is involved in the process of membrane fusion, prior to the secretion of the granule contents.

TABLE 1.2.2.

<i>Species</i>	<i>Lysolecithin (% of Total Lipid-P)</i>
<i>Ox (English)</i>	<i>16.8</i>
<i>Ox (Austrian)</i>	<i>16.8</i>
<i>Ox (Canadian)</i>	<i>12.9</i>
<i>Horse</i>	<i>7.1</i>
<i>Pig</i>	<i>11.3</i>
<i>Pig (total chromaffin granules)</i>	<i>13.5</i>
<i>Pig (mainly NA containing chromaffin granules)</i>	<i>19.7</i>
<i>Rat</i>	<i>15.4</i>
<i>Human Phaetochromocytomas (3)</i>	<i>11.7</i>
	<i>17.8</i>
	<i>23.8</i>

The Lysolecithin content in Chromaffin Granules from different species.

References to the different values may be obtained from reference (56).

(ii) The Protein Content

The chromaffin granule membrane contains approximately 30% protein by weight (20). The proteins of the membrane which have known enzymic functions are shown in Figure 1.2.1. The in vivo functions of the proteins of the chromaffin granule membrane, with the possible exception of dopamine- β -hydroxylase, already discussed, have long been the subjects of speculation (56).

The membrane contains a Mg^{2+} -activated ATPase, first described by Hillarp (60), and later by Banks (61) and by Kirshner et al (62). It has been suggested that this enzyme is involved in two apparently opposed functions, in the uptake of catecholamines and in their release. The enhanced uptake of catecholamines in the presence of Mg^{2+} and ATP has been demonstrated not only with isolated chromaffin granules, incubated in isotonic sucrose at 37°C (63,64), but also with vesicles of the chromaffin granule membrane (65,66). Catecholamine uptake and the ATPase activity are both inhibited by sulphydryl reagents such as N-ethyl maleimide (67,68) and are both activated by Mg^{2+} indicating that the two processes might be connected. Von Euler and Lishajko (69) showed the effects of some metabolic co-factors and inhibitors on catecholamine uptake by isolated adrenergic nerve granules. These included a marked inhibition of the flux of noradrenaline across the membrane of storage vesicles from the splenic nerve by mitochondrial uncoupling agents. They suggest in this paper that the facilitated uptake process requires electron transport, and that the ability of the nerve granules to retain bound noradrenaline is dependent on the energised state of the system, with at least some of the steps of electron transport-coupled phosphorylation, similar to those present in mitochondria, being involved. However they did not investigate the effects of these drugs on the storage vesicle ATPase, except in the case of Desaspidin where the result was difficult to reconcile, with stimulation at low uncoupler concentration and inhibition at high. It is essential to

extend these findings to a detailed study of the chromaffin granule and its ATPase in order to obtain valuable information about the involvement of the ATPase in catecholamine uptake and possible energy coupling across the membrane of the storage vesicle.

The classical and very potent inhibitor of the ATP-Mg²⁺ dependent uptake of exogenous catecholamines into the chromaffin granule storage vesicles is reserpine (63,64,70). The inhibitory effects are irreversible in as much as they cannot be removed by washing (70). However, the degree of inhibition is highly dependent not only on the concentration of the drug, but on the concentration of amines in the medium. This suggests competition at some level between reserpine and the amines (70,71,72). Stjarne has observed that reserpine causes 50% inhibition of the amine uptake at a molar amine:reserpine ratio as high as 10³:1. He suggests that reserpine does not act at amine binding sites but at some strategic transport step (72). Taugner and Hasselbach propose that there is more than one ATPase present in the membrane because they found moderate inhibition of what they call the 'transport ATPase' by reserpine (73). They calculate that the degree of enzyme inhibition correlates well with the blocking of catecholamine uptake, and suggest that at least part of the mechanism of action of reserpine on amine uptake consists of inhibition of their proposed specific transport ATPase. However other workers, including Ferris et al (74), failed to find any inhibitory effects of reserpine on the ATPase in these amine storage vesicles.

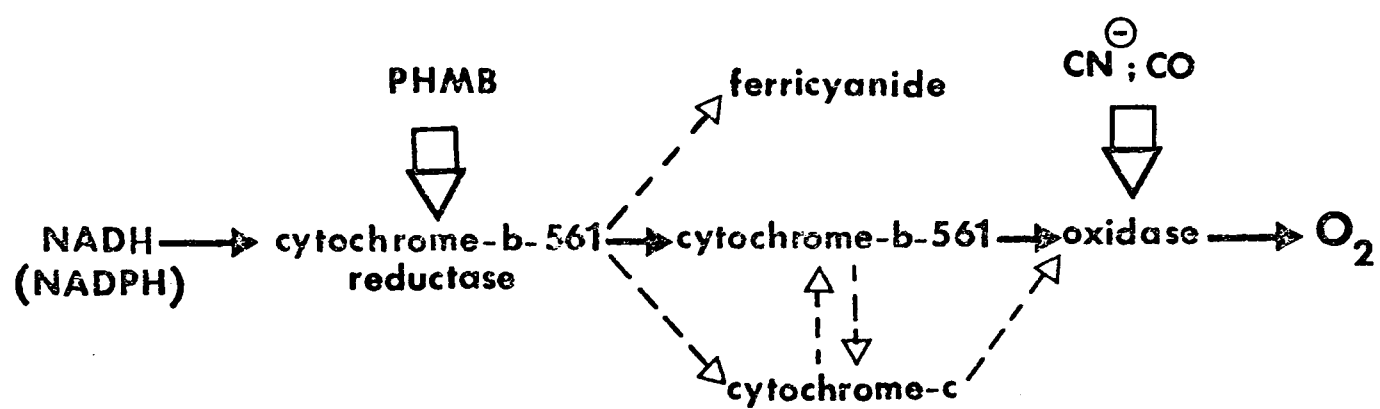
The second proposal, as to the function of the ATPase, is in the mechanism of catecholamine release. When isolated chromaffin granules are incubated in an electrolyte solution containing ATP, Mg²⁺ and Cl⁻, they release their total soluble contents (75,76,77). It has been suggested that this release is triggered off in vitro by an activation of the ATPase, and that the secretion of catecholamines in vivo involves similar activation. In vivo, the catecholamines are released by exocytosis. This process

involves an interaction between the granule membrane and the chromaffin cell membrane which results in a fusion of these two membranes, with the concomitant expulsion of the total soluble contents of the granule (78,79,80,81,82). It is difficult, therefore, to extrapolate the in vitro activation to the in vivo system with any confidence.

The presence of a unique b-type cytochrome in the bovine adrenal medulla has been reported (61,83,84,85). It has been termed cytochrome-b₅₅₉, and more recently cytochrome-b₅₆₁, after the wavelength maximum of its α -band (86). This cytochrome is common to both the chromaffin granule membrane and the microsomal membrane of the adrenal medulla (61,84). A heme-protein, with identical spectral properties, has also been found in the membranes of noradrenaline storage vesicles from bovine splenic nerve (87). Recently, Sisland and Flatmark solubilised this heme-protein from chromaffin granule membranes. They isolated it in a highly purified and water-soluble form using the non-ionic detergent, Triton X-100 (88). The prosthetic group of this cytochrome is protoheme IX, consistent with other b-type cytochromes. Gel electrophoresis of this isolated cytochrome, in sodium dodecyl sulphate (SDS), revealed a band of a low molecular weight polypeptide computed, on the basis of its amino acid composition, to have a minimum molecular weight of 4380 daltons. The estimated average equivalent of protein per heme group of 35,915 daltons suggests that there are 8.0 of these polypeptide chains per heme. The amino acid analysis, also, shows the protein to be a typical integral membrane protein, with 36% hydrophobic amino acids and with a high average hydrophobicity of 1173 calories per residue (88).

Cytochrome b₅₆₁ is thought to be a component of an electron transport chain in the chromaffin granule membrane (86,89). The NADH (NADPH):acceptor oxidoreductase activity of the granule membrane is believed to be attributable to a p-hydroxymercuribenzoate (PCMB)-sensitive flavoprotein, a cyanide sensitive oxidase and cytochrome b₅₆₁ (86). The

FIGURE 1.2.3.



cytochrome-b-561 reductase is also known as NADH dehydrogenase or NADH oxidase.

The electron transfer chain of the chromaffin granule membrane.

electron transport chain is represented schematically in Figure 1.2.3. Determination of the oxidoreductase activities of intact chromaffin granule preparations is almost impossible due to the very high concentration of redox-active compounds, the catecholamines, contained within the organelles. Hence all studies have had to be made on thoroughly washed membrane preparations. Another problem is that of the stability of the various oxidoreductase activities during the isolation, storage and the lysis of the chromaffin granules, as well as their partitioning between the membrane and matrix phases (89). Terland and Flatmark have found that the functional properties of the membrane, as measured by the NADH:ferricyanide oxidoreductase activity, are sensitive to storage at 4°C when the protein concentration is low. Addition of the matrix protein, however, stabilised the activity (89). Most recent studies have, therefore, used the unfractionated dialysed membrane plus the matrix phase.

The use of additional electron acceptors and of inhibitors has made it possible to investigate separately the different activities of the electron transport chain. The overall oxidation of NADH was inhibited to some degree by low concentrations of PCMB and cyanide, which suggests that part of the reducing equivalent is transferred to a cyanide-sensitive electron transfer component via a PCMB-sensitive dehydrogenase. The NADH:cytochrome b_{561} oxidoreductase activity is inhibited by PCMB and slightly increased by cyanide. These and other findings suggest the role of the cytochrome as an electron mediator between the reductase and the cyanide-sensitive component, as indicated in Figure 1.2.3. (86,87,88,89). It was previously suggested (86) that the cyanide-sensitive and carbon monoxide-sensitive cytochrome b_{561} oxidase activity, not due to cytochrome $a+a_3$, contamination, could be explained by the copper-containing enzyme, dopamine- β -hydroxylase. It was demonstrated that cyanide and carbon monoxide bind to the copper of this enzyme, inhibiting its catalytic activity. It was suggested, therefore, that the electron transfer chain

was involved in the synthesis of catecholamines. More recent studies, however, indicate that this is probably not the case since the copper-containing enzyme is inhibited approximately 98% by fusaric acid (90), which has no effect on the oxidation of cytochrome b_{561} or the cytochrome c-stimulated NADH oxidation (89). An additional oxygen-activated enzyme has therefore been postulated. The role of the electron transfer chain is still unknown.

A phosphatidyl inositol kinase activity in the granule membrane has also been reported by Phillips (110). Phosphorylation of bovine chromaffin granules by ATP leads to the formation of diphosphoinositide in the granule membrane. Both the enzyme and the substrate are present in the granule membrane. The reaction is Mg^{2+} -dependent, with a broad pH profile and a 'transition' temperature in the activation energy at 27°C. The sites of phosphorylation appear to be on the cytoplasmic surface of the granule membrane.

1.3 Catecholamine Secretion

It is now generally accepted that the mechanism of catecholamine secretion from the adrenal medulla is an exocytosis process, in which the membrane of the chromaffin granule fuses with the chromaffin cell membrane. The evidence for this is that the total contents of the granule, including chromogranin A and dopamine- β -hydroxylase, can be detected in the perfusate from a stimulated gland (78). The idea that the granule constituents were released directly to the exterior of the cell, and not by diffusion through the cytoplasm, was supported by the finding that no release of cytoplasmic proteins took place from stimulated glands (81). There is also negligible release of phospholipids and cholesterol from glands after stimulation (91). An all-or-none release mechanism suggested by in vivo experiments reported by Kirshner et al (92,93) and by in vitro studies of Serck-Hanssen (94) proposes that the total matrix phase is extruded in the process of secretion. Douglas and his collaborators proposed that as a mechanism of preserving the integrity of the plasma membrane, the emptied granule vesicles are retained by the cell after secretion (91,82).

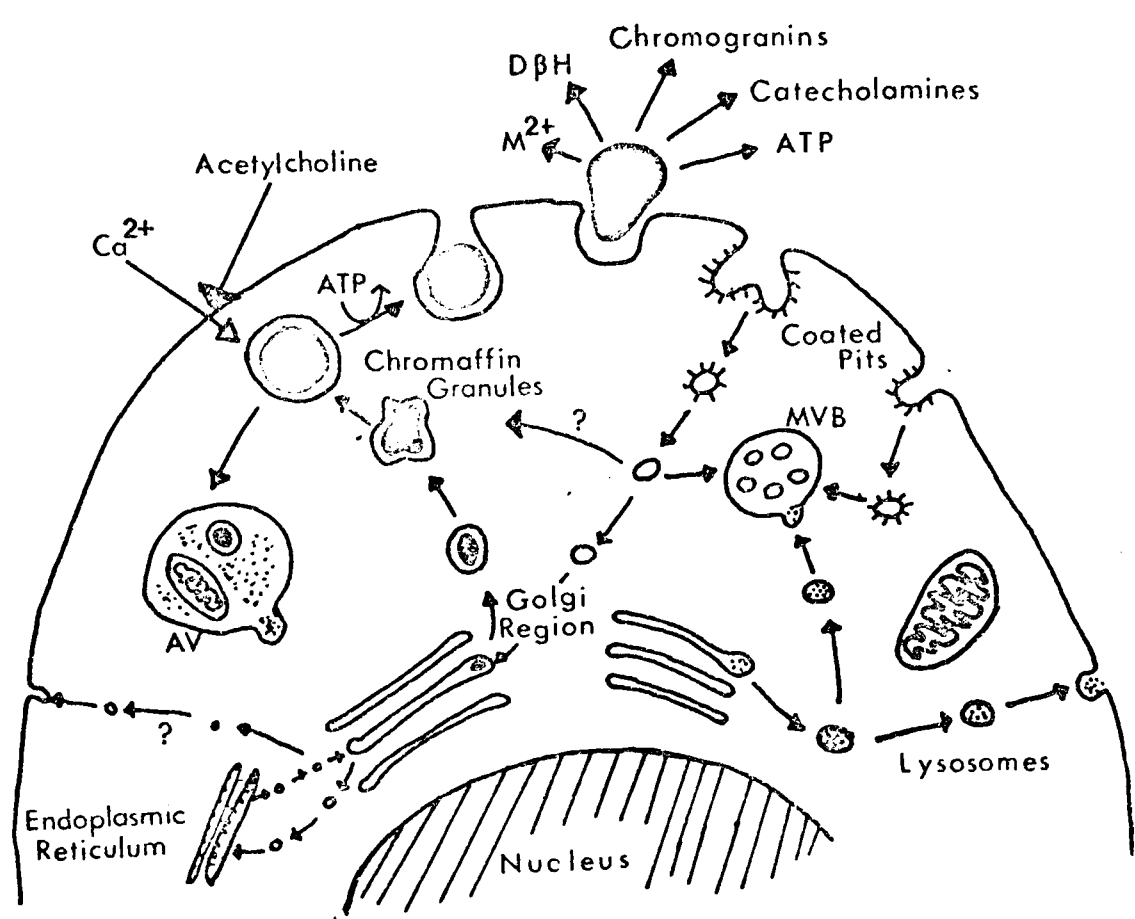
Initial electron microscopy studies indicated, but not very conclusively, that this was the case (95). However, unequivocal and abundant evidence was obtained by Diner from a detailed study of hamster adrenals (79,96). Exocytosis in the adrenal medulla has also been demonstrated using the technique of freeze-fracture electron microscopy (112).

The secretion process is stimulated by acetylcholine released at the nerve terminals, adjacent to the plasma cell membranes. This is the primary stimulus. The secondary stimulus resulting from this is an influx of calcium into the chromaffin cell. There is considerable evidence for this (97). Catecholamine secretion from the adrenal medulla can be induced using the ionophore, A-23187, in the presence of calcium (98). Whilst there is evidence of a calcium influx acting as an effector and also of granules fusing with the cell membrane, there is very little evidence for processes

which may occur between the two.

The adrenal gland contains both actin and myosin (99,100). Roles for actomyosin in the process of exocytosis have been proposed by Berl et al (101). Their model suggests a distribution of an actin-like protein and a myosin-like protein in the plasma and vesicle membranes respectively, with Ca^{2+} promoting the interaction of the two proteins, thus forming a contractile complex. Burridge and Phillips have demonstrated the interaction of myosin and filamentous actin with the chromaffin granule membrane, but have not been able to specify the nature of these interactions (100). Banks has suggested that Ca^{2+} may act as a bridge between the cell membrane and the granule membrane, thus overcoming the electrical repulsion between these membranes (102). In analogy to muscle contraction, Ca^{2+} has to be completely removed to complete the secretory process; therefore, a function of mitochondria and microsomes in regulating the cellular concentration of free Ca^{2+} has been proposed (103). The role of Ca^{2+} in exocytosis has been thoroughly dealt with elsewhere (29,97,106). It is possible that the Mg^{2+} -activated ATPase is involved in excitation-secretion coupling since it has been shown that the process requires metabolic energy (104,105). A very simplified model of the subcellular dynamics of the chromaffin cell is shown in Figure 1.3.1.

FIGURE 1.3.1.



A schematic representation of the subcellular dynamics of the chromaffin cell.

1.4 The Biogenesis and Ultimate Fate of the Chromaffin Granule

It has been suggested that that chromaffin granules originate in the Golgi Apparatus (107). It is probable, however, that the chromogranins are synthesised in the rough endoplasmic reticulum. It is not known how the newly synthesised chromogranins are transferred to the cisternae of the Golgi region (106). The chromaffin granules round the Golgi region are smaller than the mature granules in the periphery of the cell. It is possible, as is shown in Figure 1.3.1., that several small granules fuse to form a large one. Biochemical analysis shows that chromaffin granule membrane is chemically distinct from the membranes of other chromaffin cell organelles (108); only cytochrome b_{561} is known to be common to both chromaffin granules and the microsomal fraction (61). It has not been possible to examine the Golgi Apparatus in any detail, although an ATPase characteristic of the Golgi Apparatus of the adrenal medulla has been described (111).

In insulin-treated, amine-depleted glands, the synthesis of ATP and dopamine- β -hydroxylase occurs several hours before the resynthesis of catecholamines (109). This is possibly the normal sequence of events in the cell. After exocytosis has occurred, the vesicle membrane forms a bristle coating on the cytoplasmic-facing side (Figure 1.3.1.). It is thought that the vesicle membrane is then drawn back into a sphere, leading to the fission of the vesicle membrane from the cell membrane. A coated vesicle is formed. This may then be split into smaller vesicles. These vesicles may be recycled in any of the ways shown in Figure 1.3.1.

1.5 What are the Problems?

This introduction has shown that whilst there have been tremendous advances in our knowledge of the chromaffin granule and the chromaffin cell systems in the past twenty years, there are still many gaps in our understanding of catecholamine synthesis, storage and secretion. This applies not only to the chromaffin cell system itself, but also to the immunologically identical, noradrenaline storage vesicles of sympathetic neurons. Since it is difficult to prepare these nerve vesicles in sufficiently large quantities to study them using many techniques, the chromaffin cell system has been investigated as a model of adrenergic nerve action.

The problems which have still to be solved are numerous, as has been indicated in the previous discussion. The actual biogenesis of the chromaffin granule is not fully understood. How do the chromogranins, ATP and adrenaline get inside the granule, and once there how does the granule maintain such a large concentration of metabolites against the osmotic pressure and concentration gradients which must exist across the membrane? The secretory process is not explained at a mechanistic level, let alone at a detailed molecular level. After the calcium enters the cell, what happens? Is the ATPase involved? Is the chromaffin granule membrane poised, so that a conformational change can occur in the presence of calcium permitting migration of the granules to the plasma membrane, by means of microtubules or otherwise, allowing exocytosis to take place? Is the lysolecithin present in the granule membrane involved in the exocytosis-fusion process? These are only a few of the most obvious questions that must be asked about the system.

1.6 The Aim of the Work

As with all detailed problems, those of the chromaffin granule system must be solved by first answering some of the more basic but contributory ones. This work has, therefore, attempted to provide answers to several fundamental problems of catecholamine storage and secretion, in the hope that these may help towards an understanding of the more complex facets of the chromaffin cell system.

The questions to which an answer has been sought are primarily:

- (a) What is the role of the ATPase? Can it be likened to any other ATPase?
Is it linked to the electron transport chain present in the membrane?
- (b) What is the nature of the 'granule complex'? Is it partially crystalline, in a gel state or quite fluid? What interactions, if any, exist within the complex?
- (c) What is the internal pH of the chromaffin granule?
- (d) What is the structure of the chromaffin granule membrane?

In the past, most of the studies of the chromaffin granule have involved the use of more classical biochemical and pharmacological techniques. Whilst certain problems can best be studied using these methods, some of them are more suited to study using biophysical techniques. The only biophysical approaches used so far, in any depth, apart from electron microscopy, are light scattering (113,114,115), X-ray diffraction (116) and proton magnetic resonance (30,31). The light scattering studies have not been aimed particularly at solving the questions delineated above, and which are of immediate interest in my studies. They investigate the condition of the granules after various treatments, by measuring their shrinkage, swelling and interactions. The X-ray diffraction and proton magnetic resonance studies reported so far have been of a preliminary nature, and have not as yet been followed up by their authors.

The studies on the chromaffin granule ATPase are described in Chapter 2. These studies have been based on methods used in investigations of the ATPase of submitochondrial particles, and have not been used in previous studies of the granule ATPase.

The structure of the chromaffin granule membrane has been investigated by means of three different biophysical techniques: electron spin resonance (ESR), nuclear magnetic resonance (NMR) and X-ray diffraction. A study of the membrane using fluorescent probes was made concurrently by Mr C.L.Bashford, in this laboratory. The information obtained using these techniques may be correlated, thus hopefully building up a fairly complete description of the granule membrane. The ESR, NMR and X-ray diffraction studies are described in Chapters 3,4, and 5 respectively.

The granule complex has been investigated using, primarily, ^{31}P NMR, since the granule contains such large quantities of ATP. The frequencies of the ATP resonances are sensitive to their environment, and provide an excellent probe of the intact system. ^1H and ^{13}C NMR have also been used to form a more complete picture of the granule complex. The internal pH of the granule has also been determined using ^{31}P NMR, in addition to biochemical methods. The magnetic resonance studies are described in Chapter 4.

These techniques and their applicability are discussed more comprehensively in the introductions to each chapter.

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CHAPTER TWO

Biochemical Studies of Enzymic Activities of the Chromaffin Granule Membrane, in particular of the Mg^{2+} -activated ATPase.

2.1 Introduction : A Working Model for the ATPase

Despite the fact that the presence in the chromaffin granule membrane of a Mg^{2+} -activated ATPase has been known for many years (1,2), very little is known about its bioenergetics. ATP and divalent metal ions, particularly Mg^{2+} , are essential to the enhanced catecholamine uptake by intact purified granules and by granule membrane vesicles (3,4). This has been discussed in the previous chapter. A catecholamine transport protein has not been isolated. It is necessary to postulate such a protein, or protein subunit, since reserpine does not inhibit the ATPase or only to a limited extent (5,6), yet effectively inhibits the catecholamine uptake at very low concentrations (3).

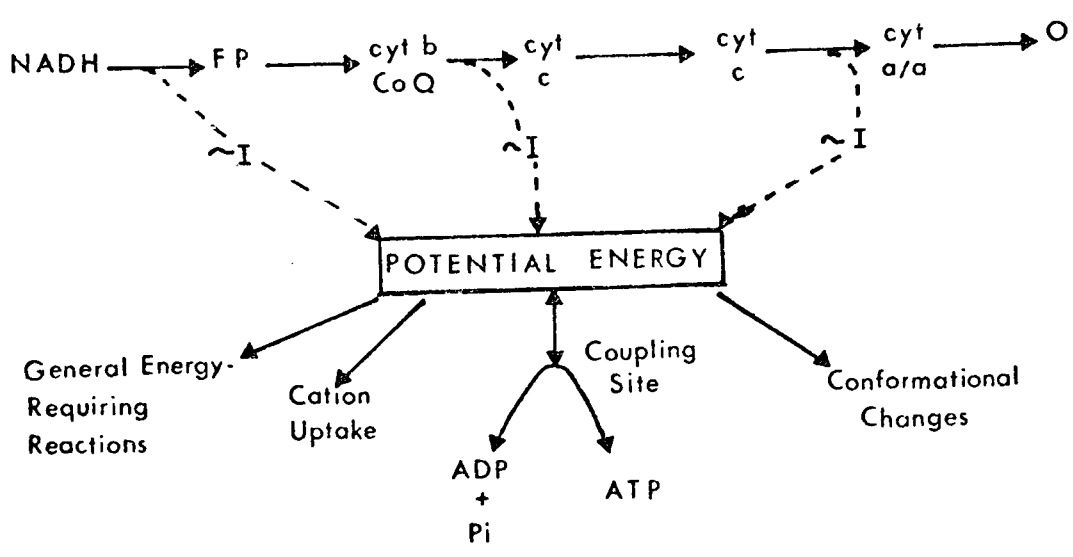
How then is the potential energy, released when the 'high energy' bond of ATP is cleaved, transformed into 'useful work' ie. catecholamine uptake?

The properties of the chromaffin granule ATPase were compared to those of other characterised ATPases in order to devise the most realistic working model, on which preliminary experiments could be framed. The properties of the ATPase, that are of the most obvious importance in such a comparison are :

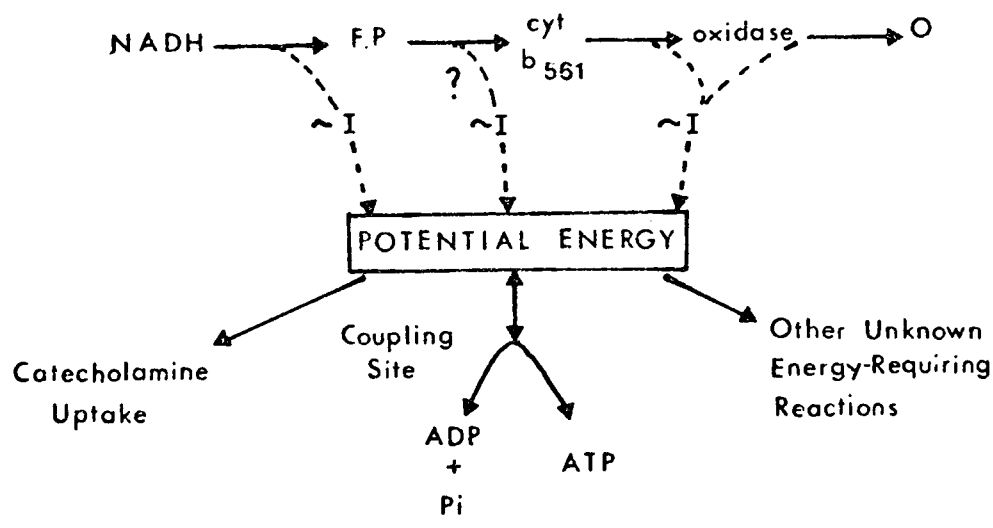
- (a) It has a divalent metal ion requirement ie. it is Mg^{2+} -activated.
- (b) Its proposed molecular weight is approximately <500,000 daltons (7).
- (c) It is apparently involved in energising catecholamine uptake.
- (d) An NADH : (acceptor) oxidoreductase activity in the form of an electron transfer chain is present in the membrane.
- (e) It is not ouabain sensitive (Personal observation).

FIGURE 2.1.1.

Mitochondria



Chromaffin Granules



Simplified models of the mitochondrial ATPase and its relationship to the electron tranfer chain and other energy requiring processes with in the mitochondrion. and for the chromaffin granule ATPase and its relationship to similar processes within the chromaffin granule.

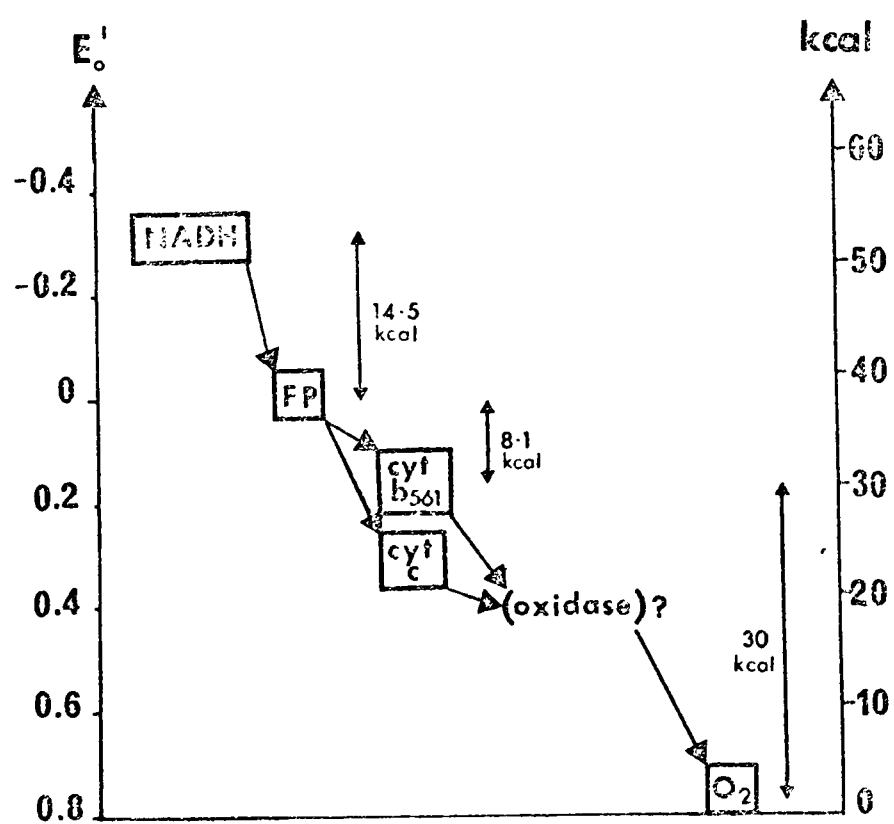
In view of these properties, the $\text{Na}^+ - \text{K}^+$ ATPase and sarcoplasmic reticulum ATPase can be eliminated as useful models since they do not comply with several of them. The best model was thought to be the mitochondrial ATPase, F_1 , and the related bacterial ATPases, which have several properties in common. The mitochondrial ATPase fulfils all but property (c), but it equivalently energises cation uptake and other mitochondrial functions.

A simplified model of the mitochondrial ATPase, and its relationship to the other biochemical processes which occur in the mitochondrion and its membrane are shown in Figure 2.1.1. The actual mechanism of 'energy coupling' between the electron transfer chain and ATP formation (and other energy requiring processes) is not known although several theories have been proposed : the Chemical Intermediate hypothesis (8), the Chemiosmotic theory (9) and the Conformational hypothesis (10). The experiments devised will be independent of the mechanism of energy coupling.

A comparable model for the chromaffin granule ATPase is also shown in Figure 2.1.1. The hypothesis is that energy from the oxidation of NADH may be transferred into potential energy which may be used, if the system is tightly coupled, to form ATP or to promote catecholamine uptake. Energy may also enter the potential energy pool from the breakdown of ATP by the ATPase, and this may also be used to enhance the catecholamine uptake and possibly other unknown energy requiring processes. If the coupling of the ATPase to the potential energy pool is destroyed, then even if ATP is broken down very fast, catecholamine uptake will not occur.

A free energy diagram for electron flow down the transport chain to oxygen is shown in Figure 2.1.2. The redox potentials for the different components are those described in the literature or else are based on comparable molecules in the mitochondrial electron transfer chain. The position of the oxidase is not known since the nature of the oxidase itself has yet to be discovered. On the basis of these approximate values

FIGURE 2.1.2.



A free energy diagram for electron flow in the electron transfer chain of the chromaffin granule membrane.

for the redox potentials of the various components, there is sufficient energy difference between NADH and the flavoprotein, and between cytochrome b_{561} and oxygen for ATP molecules to be formed. The difference between the flavoprotein and cytochrome b_{561} is just greater than the energy required, therefore it is unlikely that the reaction would be sufficiently efficient for an ATP molecule to be synthesised. On a crude estimation, therefore, two molecules of ATP could be formed for every NADH molecule oxidised.

Unfortunately, due to the presence of catecholamines in suspensions of chromaffin granules, the electron transfer chain of the granule membrane can only be examined in well washed preparations of the membrane. In addition, the rate of its NADH : (acceptor) oxidoreductase activity is very low (11). This means that it is not feasible to study the ATPase by means of the state of its electron transfer chain, and vice versa. The properties of the ATPase must be investigated through examination of its activity in the presence of drugs and metabolites known to effect the mitochondrial ATPase. The most important of these are the 'uncouplers'. Classical uncouplers are the substituted phenols, unsaturated fatty acids, arsenate and dicoumarol. The recent development of herbicides, pesticides and other inhibitory compounds has revealed much more potent uncouplers, such as substituted carbonylcyanides, benzimidazoles and salicylanilides (12). In the chemical intermediate hypothesis, the energy of this intermediate is irreversibly dissipated by the presence of uncouplers. In the chemiosmotic hypothesis, uncouplers are assumed to dissipate the proton gradient across the membrane by increasing the permeability of it to protons. The uncouplers can be accommodated into the conformational hypothesis by assuming that they induce a low energy conformation in the mitochondrial proteins.

Thus in mitochondria, uncoupler molecules enhance the electron transport system because the energised state is irreversibly converted into

the low energy components necessary for the redox reactions to proceed, and also because it is no longer coupled to the rate limiting phosphorylation reaction. At the same time, the hydrolysis of ATP is stimulated and all energy utilising reactions are inhibited (12).

Another drug which may provide information about the granule ATPase is NN' -dicyclohexylcarbodiimide (DCCD), a known inhibitor of the mitochondrial ATPase. It has been shown to cause its inhibition, not by binding directly to the ATPase, but to a proteolipid of molecular weight 10,000 daltons, which is tightly bound to the membrane (13). The inhibitory effects of DCCD can be accounted for by assuming that it blocks the transfer of protons across the membrane. The effects of DCCD and uncoupler molecules on the mitochondrial ATPase are discussed in detail elsewhere (12,13).

The catecholamine uptake of the chromaffin granules and chromaffin granule membrane vesicles will also be investigated in the presence of uncoupler molecules and DCCD. Unfortunately, due to the nature of these studies, the results may appear to be presented in a rather disjointed manner. They will, however, be interrelated and discussed thoroughly later in the chapter.

2.2 Methods

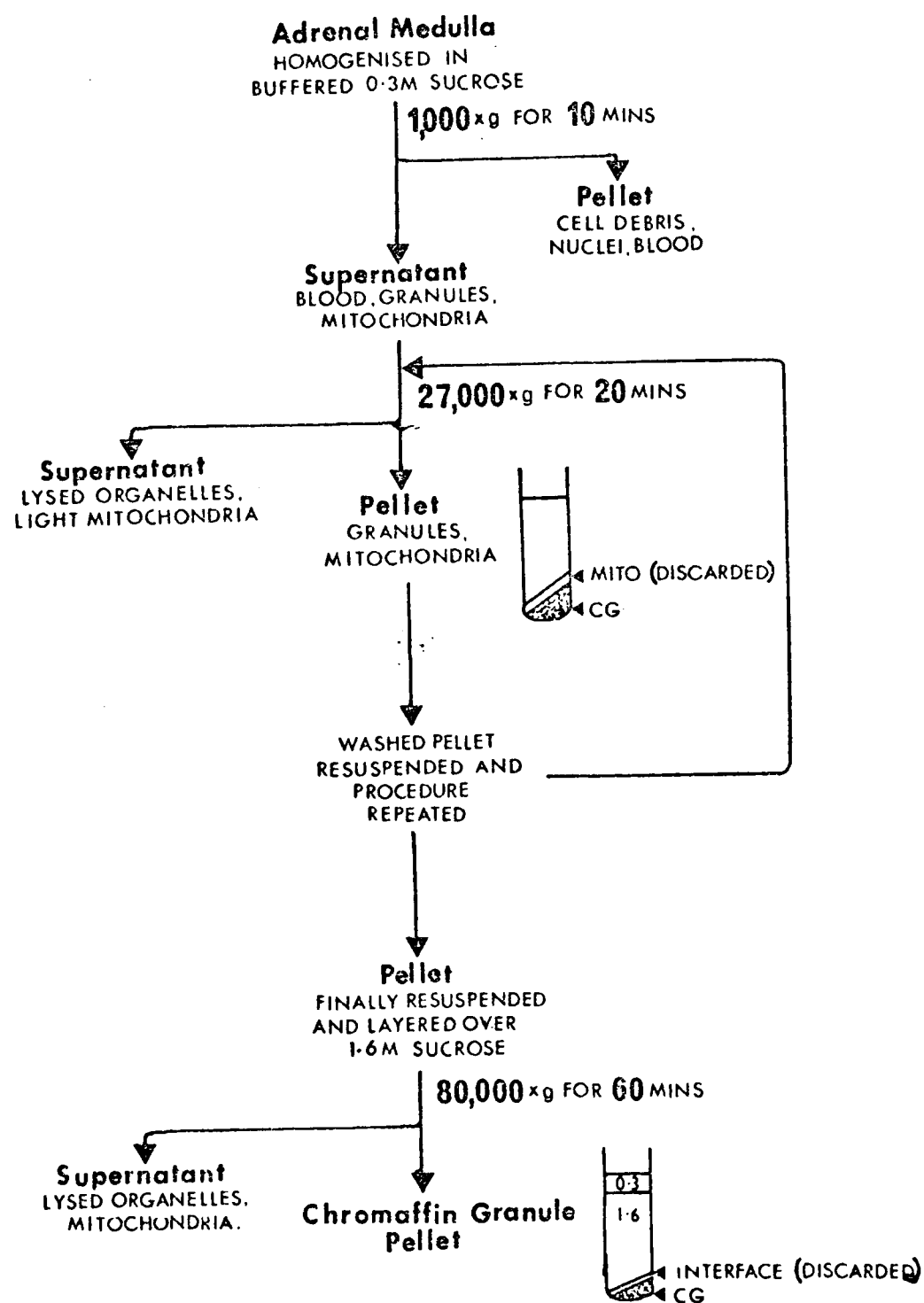
A The Preparation of Chromaffin Granules

Chromaffin granules were prepared from bovine adrenal medullae as described by Kirshner (3). The bovine adrenal glands were cooled on ice and used within one hour of slaughter. The medullae were minced and homogenised in iso-osmotic sucrose (0.3 M) buffered with 10 mM HEPES, pH adjusted to 7.4 with 2 M KOH. This solution is henceforth referred to as 'buffered sucrose'. Following centrifugation at 1000xg for 10 mins, the supernatant was centrifuged at 27000xg for 20 mins. Centrifugation carried out at 4 °C. The upper layer of the resulting pellet, mainly mitochondria, was removed and the remainder was resuspended and recentrifuged. This procedure was carried out three times and the resultant pink pellet of crude chromaffin granules was resuspended in buffered sucrose. The final pellet of such a preparation is contaminated with lysosomes and mitochondria (14), but further purification by density gradient centrifugation gives rise to preparations of greater fragility and impaired catecholamine transport activity (4).

Purified chromaffin granules, when required, were prepared by a modification of the method of Smith and Winkler (14). A small volume, approximately 2 mls, of the resuspended pellet of the crude chromaffin granules in buffered sucrose (0.3 M) was layered on approximately 25 mls of 1.6 M sucrose, buffered with 10 mM HEPES, pH 7.4. Helle and Serck-Hanssen have observed that the purity of the final pellet of chromaffin granules is greater, the larger the volume of 1.6 M sucrose used as a barrier (15). It was then centrifuged at 80000xg for 60 mins at 4°C. The pellet was washed with 0.8 M sucrose to remove any interfacial contamination and was then taken up in 0.3 M buffered sucrose.

The purity of these preparations with respect to mitochondrial contamination was assessed by monoamine oxidase assay. Monoamine oxidase activity cannot be detected in purified granules and mitochondria are very

FIGURE 2.2.1.



The preparation of chromaffin granules : a flow chart.

· rarely observed in electron micrographs of this fraction. It has been reported that this final pellet of chromaffin granules is also devoid of lysosomal enzymatic contamination, but has a constant, but low, glucose-6-phosphatase activity, indicative of slight microsomal contamination (16).

An outline of this preparation is shown in figure 2.2.1.

B Preparation of Chromaffin Granule Membranes

Lysis of chromaffin granules, prepared as described above, was obtained by successive hypo- and hyper-osmotic shocks, by dialysis or by column chromatography.

(i) Hypo- and Hyper-osmotic Shock

Granules resuspended in buffered sucrose (0.3, 0.8 or 1.6 M) were diluted into between 20 and 100 volumes of hypotonic 10 mM HEPES buffer, pH 7.4, and subsequently centrifuged at 48000xg for 20 mins at 4 °C. This resulted in almost complete lysis of the granules as assessed by analysis of the catecholamine content of this first and subsequent lysates. The membrane pellet was resuspended in buffered, 0.3 M sucrose and the procedure repeated twice. The lysate contained the water soluble contents of the granules, including the chromogranins, the catecholamines and ATP.

(ii) Dialysis

Membranes were prepared by dialysis, in the cold, of the granules against several changes of 500 volumes of 10 mM HEPES buffer, pH 7.4 which initially contained 10 mM ascorbic acid. The dialysis tubing used was previously boiled in 20 mM EDTA, then thoroughly washed in distilled water. The advantage of this method (cf. 2.2.B.a) is that the small molecules including adrenaline and ATP are removed, leaving a suspension of membranes and water soluble macromolecules which can be separated by centrifugation at 48000xg for 10 mins at 4 °C. The membrane pellet was then washed twice in 20 volumes of 10 mM HEPES buffer, pH 7.4.

(iii) Column Chromatography

This method is based on that described by Phillips (4).

Granules, suspended in a small volume of sucrose, were applied to a column of Sephadex G-50, equilibrated with 10 mM HEPES, pH 7.0, at approximately 15 °C. They were washed down the column with a volume of the HEPES buffer slightly greater than the void volume of the column. The resultant material was pelleted by centrifugation at 48000xg for 10 mins. This column could also be used to partially purify the chromogranins, as they were eluted after the membrane vesicles. The smaller molecules, including the catecholamines and ATP, were eluted off the column with a very much larger volume of buffer.

This method was seldom used. Routine preparation was by the first two methods.

C Measurement of Enzyme Activities

(i) Adenosine Triphosphatase

The ATPase activities were determined using a Technicon Autoanalyser system which permits the continuous determination of the production of inorganic phosphate by a modified method of Hurst (17). The incubation medium based on that of Banks (2), contained MgCl₂ (2.5 mM), ATP (2.5 mM), approximately 0.1 to 0.3 mg of sample protein per ml and a suitable buffering system, dependent on the pH at which the activity is being determined. It was generally incubated at 30 °C or 37 °C. The Autoanalyser was calibrated to measure inorganic phosphate in the concentration range of 0 to 20 mM and was most accurate between 0 and 3 mM as it plots the phosphate concentration on a logarithmic scale.

(ii) Dopamine-β-Hydroxylase

Dopamine-β-hydroxylase was assayed by a method based on that of Friedman et al (18), using ³H-tyramine as substrate and converting the octopamine produced to β-hydroxybenzaldehyde, which was extracted in toluene.

(iii) Monoamine Oxidase

Monoamine oxidase activity was used as a specific mitochondrial marker when the preparation of the chromaffin granules was being developed. The method employed was that of McCaman et al (19), based on the conversion of ^{14}C -tyramine to p-hydroxyphenylacetic acid and p-hydroxyphenylacetaldehyde by the enzyme.

(iv) NADH(NADPH):(acceptor) Oxidoreductase

The NADH:(acceptor) oxidoreductase activity was measured by following the fluorescence of NADH (excitation at 340 nm, emission at 460 nm). The reaction was started by the addition of the enzyme preparation. This is based on the procedure of Terland and Flatmark (17).

D Measurement of ATP Synthesis

ATP synthesis was measured by two different methods.

(i) The luciferin-luciferase method

The method used was based on that of Stanley and Williams (22). The light emitted was measured using a liquid scintillation counter operated manually. This method does not discriminate between myokinase activity and ATP synthesis from ADP and P_i .

(ii) ^{32}P -Phosphate transfer method

The transfer of ^{32}P labelled inorganic phosphate to ADP was measured using the method of Avron (23). This method does discriminate between the synthesis of ATP from ADP and P_i and its synthesis by myokinase from ADP.

E Fluorescence Measurements

Fluorescence measurements were made with an Hitachi-Perkin-Elmer MPF-2A spectrofluorimeter equipped with a thermostatted cell block.

The excitation wavelength for ANS was 380 nm and fluorescence was followed at 480nm.

F Determination of Protein and Catecholamines.

Protein was determined by the Biuret method (20). After precipitation with 10% trichloroacetic acid, the protein was spun down in an MSE bench centrifuge. The pellet was washed with ethanol and was redissolved in 3%(w/v) NaOH:2%(w/v) sodium deoxycholate for assay. The standard used was crystalline bovine serum albumin from the Sigma Chemical Company.

G Incorporation of Catecholamines by Chromaffin Granules and by Granule Membrane Vesicles.

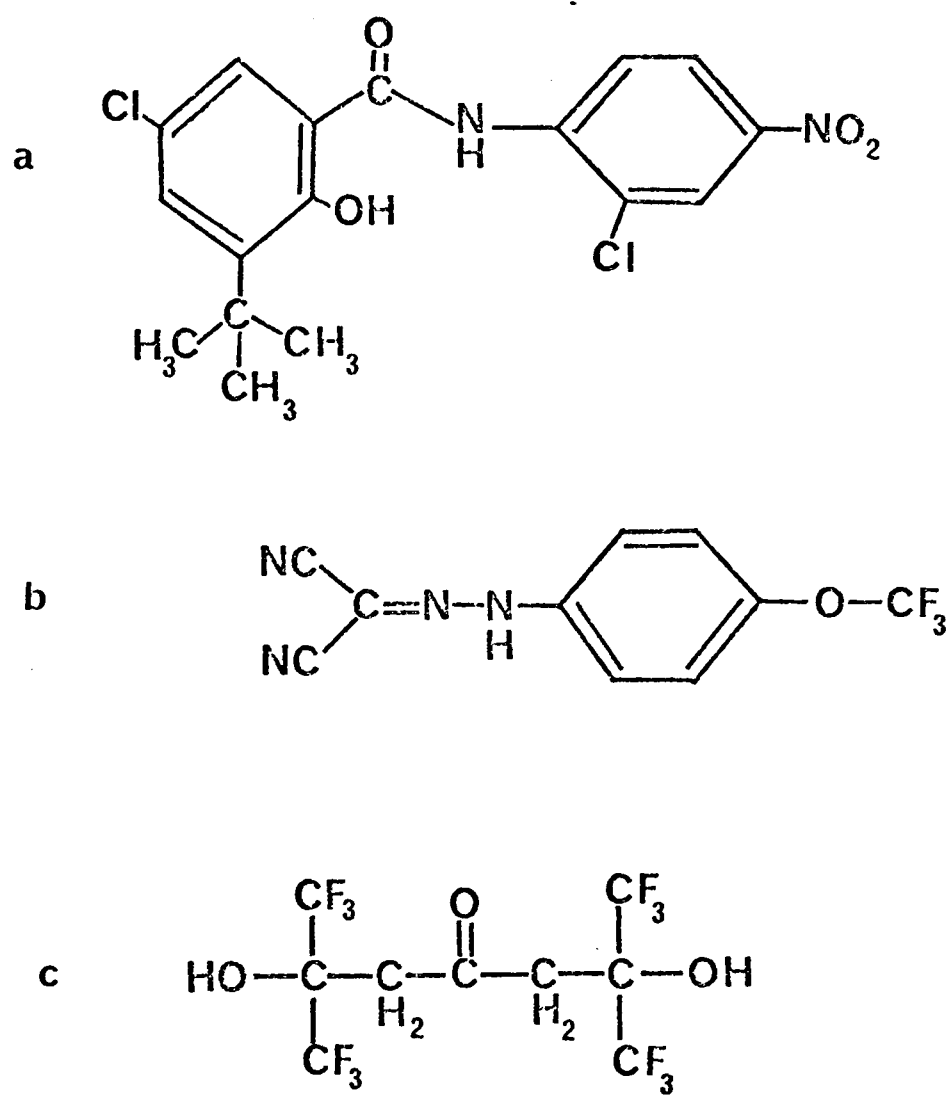
The transport experiments were performed at 37°C in 0.3M sucrose buffered with 10mM HEPES, pH 7.4, using 100µM DL-[7-¹⁴C]-adrenaline (specific activity: 8.25mCi/mMole), 5mM ATP, 2.5mM MgCl₂ and other reagents at the concentrations specified. The total volume of incubation was 0.5ml. Reactions were started by the addition of the membrane prepared by successive hypo- and hyperosmotic shocks (final concentration, approximately 500µg protein/ml) or of granules, prepared in either 0.3M or 1.6M sucrose (final concentration, approximately 1.5mg protein/ml). Reactions were terminated by the addition of 1ml ice-cold buffered sucrose containing 1.5µM reserpine. The membrane vesicles or granules were immediately pelleted by centrifugation at 48,000g for 10 minutes at 4°C as before. The pellets were washed with buffered sucrose, containing reserpine, and finally taken up in 1ml of water. Radioactivity was determined by liquid scintillation counting in toluene scintillation fluid, containing 2,5 diphenyloxazole (5g/litre), 1,4-bis-(5-phenoxazole-2-yl)benzene (0.1g/litre) and 25%(v/v) Triton-X-100, using a Beckman liquid scintillation counter.

The uptake experiments using noradrenaline, dopamine and methylamine, at similar specific radioactivities, were carried out using the same procedure.

H Methods.

The uncoupler molecules, 5-chloro, 3-t-butyl, 2'-chloro, 4'-nitrosalicylanilide (S-13), carbonylcyanide-p-trifluoromethoxy-phenylhydrazine (FCCP) and bis-hexafluoroacetone (1799), whose structures are given in Figure 2.2.2., were a gift from Dr P.G. Heytler, Du Pont Chemicals, U.S.A. The fluorescent probe, 1-anilinonaphthalene-8-sulphonic acid (ANS), was obtained from K and K Laboratories, and was recrystallised as the magnesium salt before use. Antimycin A, rotenone, oligomycin, reserpine, HEPES and MES were obtained from the Sigma Chemical Company; atractylate and ATP were purchased from Boehringer; N,N' dicyclohexyl carbodiimide (DCCD) from British Drug Houses. All other reagents were of the highest grade commercially available.

FIGURE 2.2.2.



The 'uncouplers' used in this study:

(a)	S-13	pK_a	=7.4
(b)	FCCP		=6.1
(c)	1799		—

2.3 Results

A. Studies on the ATPase

(i) The Effects of Uncouplers

One of the fundamental aspects of the model, proposed in the introduction to this chapter to account for the role of the ATPase in the granule membrane, is that the ATPase is 'coupled' to the 'energised state' of the system. This proposal was tested by investigating the ATPase activity of both intact chromaffin granules and chromaffin granule membrane vesicles in the presence of various uncoupler molecules.

(a) The Chromaffin Granule ATPase Activity

Whilst in many of the subsequent experiments, the ATPase activity, coupled and uncoupled, was studied in granules prepared through 0.3M sucrose, the uncoupling was initially looked at in pure granules, prepared through 1.6M sucrose but resuspended in 0.3M sucrose. The ATPase activity of the pure bovine granules was measured in the presence of several uncouplers, at different concentrations (Table 2.3.1.). The effectiveness of the different uncouplers at a given concentration, 0.5 μ M, decreases in the order : S-13 > FCCP $\geq$ 1799. This is in agreement with results using the uncouplers in mitochondrial systems (12). S-13 is a very efficient uncoupler of the chromaffin granule ATPase at low concentrations. The concentration dependence of the uncoupling of the ATPase by S-13 was looked at in greater detail (Figure 2.3.1.). Considerable uncoupling is achieved by very small concentrations of S-13, but optimum uncoupling requires 200nM S-13 or greater. In subsequent experiments, to ensure maximum uncoupling, S-13 at a concentration of 0.5 μ M was normally used.

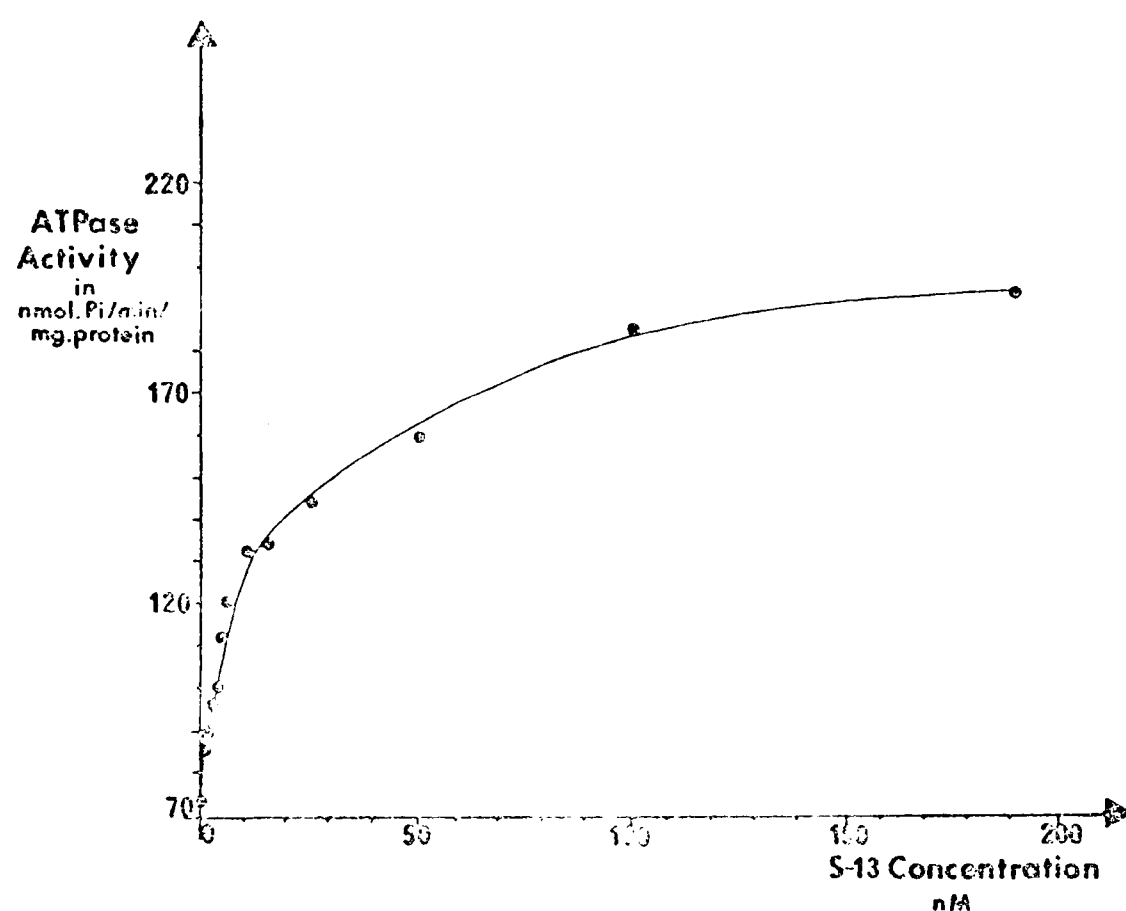
A comparison of the ATPase activities, coupled and uncoupled, of granules prepared through 0.3M sucrose (Table 2.3.2.) and through 1.6M sucrose (Table 2.3.1.) shows that those prepared through 1.6M sucrose have (in the absence of uncoupler) a higher coupled₁ rate, although the uncoupled rates are similar. This means that the preparative procedure through the high density sucrose has probably disrupted the granule membrane, reducing the coupling of the ATPase by

TABLE 2.3.1.

	Rate	Percentage of Coupled Rate
<i>Control</i>	68.6	100
<i>+ 5nM S13</i>	94.0	137
<i>+ 200nM S13</i>	152.0	222
<i>+ 200nM FCCP</i>	101.9	148
<i>+ 1000nM FCCP</i>	132.4	193
<i>+ 200nM 1799</i>	90.1	131
<i>+ 1000nM 1799</i>	130.1	190

The effect of different uncouplers on the ATPase activity of chromaffin granules, prepared through 1.6M sucrose but resuspended in 0.3M sucrose. Reactions were performed at 30°C, in a buffer containing 0.3M sucrose, 15mM HEPES and 2.5mM ATP-Mg²⁺ at pH 6.6. Rates are expressed in nmoles of phosphate produced per minute per mg of protein.

FIGURE 2.3.1.



The concentration dependence of the uncoupling of the ATPase of chromaffin granules, prepared through 1.6M sucrose, by S-13. Conditions as described in the text. The temperature of the assay was 30°C.

increasing the leakiness of the membrane.

(b) The Chromaffin Granule Membrane ATPase Activity

The ATPase activity of isolated chromaffin granule membranes was also enhanced on the addition of uncoupler molecules (Table 2.3.4.). This increase in activity was considerably less than that found with the granule system. At pH 6.4, it was maximally a two fold increase over the control rate. The average percentage uncoupling was around 50%, as in Table 2.3.4. The lower percentage increase was thought to be due to the membranes being damaged during their isolation, or failing to form tightly sealed vesicles. This may have rendered the membrane vesicles leaky, and unable to maintain the 'energised state'. This, according to the chemiosmotic hypothesis, could be interpreted in terms of their inability to maintain a proton gradient efficiently. That is to say, the control rate is already partially uncoupled. Unfortunately, because the protein content of granules varies (15) and the exact percentage of the membrane protein attributable to the ATPase molecule is not known (7), it is not possible to accurately compare the ATPase activities of granule and membrane preparations in terms of the turnover of each ATPase molecule.

It is not known if some of the membrane vesicles have inverted during preparation, as submitochondrial particles do (13), resulting in the cytoplasmic side of the membrane being on the inside of the membrane vesicle. This is obviously important in studies of the uncoupling of the membrane vesicles, since it would be ideal to have two homogeneous populations of vesicles with opposite sidedness. Eagles et al (24) have proposed that the carbohydrate molecules attached to the chromaffin granule membrane are only found on the inner face of the membrane. They found that intact granules were not retained on Concanavalin A Sepharose columns, but that a large proportion of their membrane preparation was. It is conceivable, therefore, that such a column would retain the 'inside-out' vesicles, as these should have their glycoprotein

TABLE 2.3.2.

	Coupled Rate	Uncoupled Rate
Control	41 ± 10%	164 ± 10%
+ Atractylate (8μM)	44	158
+ Rotenone (10μM)	38	167
+ Oligomycin (5μM)	42	160
+ Antimycin A (10μM)	46	152
+ Aurovertin (2μM)	41	171
+ Ethanol (2%)	45	151

The effect of mitochondrial inhibitors on the activity of the chromaffin granule ATPase. The chromaffin granules were prepared in 0.3M sucrose and the reactions carried out in 0.3M sucrose buffered with 20mM MES, pH 6.5. The uncoupled rate is that in the presence of 200μM S13. The rates of activity are expressed in nmoles phosphate produced per minute per mg of protein.

and glycolipid molecules, which have Concanavalin A binding sites, on the outside. The vesicles retaining the native polarity should be eluted in the void volume of the column. Preliminary experiments were carried to test the validity of this proposal. A large percentage of the membrane vesicles were applied to the Concanavalin A: Sepharose column were eluted in the void volume, and only a small percentage were eluted off in the presence of α -methyl glucoside. However, neither the vesicles eluted in the void volume nor those eluted off by α -methyl glucoside had any measurable ATPase activity, even in the presence of uncoupler. Accordingly, the effect of Concanavalin A on the ATPase activity of isolated granule membranes was examined, since there may have been a small amount of free Concanavalin A present in the column. The ATPase activity was examined in the presence of increasing concentrations of the lectin. There was no apparent agglutination, although light scattering measurements were not made to demonstrate this conclusively. However, the ATPase activity was completely inhibited in the presence of 0.5 mg Concanavalin A/mg membrane protein. This is a very interesting observation, since it has been assumed that the ATPase is on the outside of the membrane, and should not, therefore, possess Concanavalin A binding sites. It is not known whether this inhibition is specific or non-specific. The effect of Concanavalin A on the ATPase activity of granules prepared free of sucrose must be investigated and the reversibility of the inhibition of the membrane ATPase by α -methyl glucoside ascertained before any conclusions can be made.

(ii) The Effect of Mitochondrial Inhibitors

The ATPase activity, coupled and uncoupled, of chromaffin granules prepared in 0.3M sucrose was investigated in the presence of several inhibitors of the mitochondrial ATPase (Table 2.3.2.). In all cases, the inhibitors, at concentrations which cause substantial inhibition of the mitochondrial ATPase of beef heart and rat liver, have no significant effect on the activity of the chromaffin granule ATPase. The ATPase

TABLE 2.3.3.

	Activity
Mitochondrial Rich Fraction	65.6 ± 7.5%
+ Atractylate (4 M)	42.7
+ Atractylate (8 M)	36.9
+ Atractylate (16 M)	33.5
+ Oligomycin (5 M)	36.9
+ Ethanol (1%)	54.7
+ S-13 (0.5µM)	77.7

The effect of uncouplers and mitochondrial inhibitors on the ATPase activity of the mitochondrial rich fraction. The mitochondrial rich fraction was present in the assays at a concentration of 0.25mg/ml and the activities measured under the same conditions as in Table 2.3.2.

activities of pure granules (1.6M sucrose) and of granule membranes were not inhibited either. It was noted also that ethanol, in the concentrations used in the experiments, had no effect on the activity.

A mitochondria-rich fraction was prepared at the same time. This was the fluffy, brown fraction pelleted above the chromaffin granules after centrifugation in 0.3M sucrose. It still contained a few, small chromaffin granules, as demonstrated by further centrifugation through 1.6M sucrose. The results obtained when this mitochondrial fraction was investigated are shown in Table 2.3.3. The uncoupling observed was less than 20% compared to the 400% uncoupling observed with the chromaffin granule-rich-fraction. There was slight inhibition of the ATPase activity of the mitochondria-rich fraction by ethanol, but a more substantial inhibition about 50% occurred in the presence of atractylate (16 μ M) and oligomycin (5 μ M). No inhibition of the chromaffin granule fraction was apparent. As there is little contamination of the chromaffin granules by other subcellular organelles and membranes (15), the conclusion is that the chromaffin granule ATPase is uncoupler sensitive and mitochondria inhibitor, including oligomycin, insensitive.

(iii) The Effect of NADH, Catecholamines and Methylamine.

It had been observed in this laboratory that the NADH : (acceptor) oxidoreductase activity of the chromaffin granule membrane was stimulated in the presence of dopamine (25). The ATPase activities of both chromaffin granules and chromaffin granule membranes were examined in the presence of dopamine and NADH. These metabolites had no effect on the coupled or uncoupled activities of either system (Table 2.3.4.). The activity of the granules was also examined in the presence of large concentrations of adrenaline and methylamine. Adrenaline was found to inhibit the ATPase at high concentrations. However, the 35% inhibition, at an adrenaline concentration of 100mM is probably not of physiological significance, since it is far in excess of the cytoplasmic adrenaline

TABLE 2.3.4.

	Coupled Rate	Uncoupled Rate
Chromaffin Granules	42 ± 10%	152 ± 10%
+ Adrenaline, 10mM	40	
+ Adrenaline, 20mM	31	
+ Adrenaline, 100mM	25.8	
+ Dopamine, 2.5mM	40	141
+ NADH, 2.5mM	39	142
+ NADH, 2.5mM + Dopamine, 2.5mM	41	148
+ Methylamine, 10mM	49-74	177
+ Methylamine, 100mM	52-94	
Preincubated 15mins with Methylamine, 10mM	72	
Preincubated 15mins with Methylamine, 100mM	72-99	
Chromaffin Granule Membranes	210	343
+ Dopamine, 2.5mM	206	317
+ Noradrenaline, 2.5mM	205	317
+ Adrenaline, 2.5mM	194	288
+ NADH, 2.5mM	194	318

The ATPase activity of chromaffin granules and chromaffin granule membranes in the presence of catecholamines, NADH and methylamine. The adrenaline and methylamine solutions were made up in 0.3M sucrose:20mM MES, pH adjusted to 6.5. Other conditions as in Table 2.3.2.

concentration (Table 2.3.4.).

Methylamine has been used to determine the internal pH of chloroplasts and other subcellular organelles, since it can equilibrate according to the proton gradient across a membrane (26). Methylamine was found to have a concentration and time dependent activation of the chromaffin granule ATPase, but under the conditions used, this did not exceed 150% (Table 2.3.4.). The uncoupled rate, ie. in the presence of S-13, was unaffected by the presence of methylamine in the concentrations used. The activation of the 'coupled' activity by methylamine is most probably an uncoupling due to its interfering with the charge gradient across the membrane.

The chromaffin granule membrane ATPase was also examined in the presence of noradrenaline and adrenaline, both 2.5mM. These had no significant effect on either the coupled or uncoupled activity, although the adrenaline may cause a slight inhibition.

(iv) The Effect of Reserpine

As mentioned in Chapter One, there has been controversy about whether reserpine, which inhibits the ATP-Mg²⁺-dependent uptake of catecholamines by granules and by membrane vesicles, causes inhibition of the ATPase. Taugner and Hasselbach (6) reported that reserpine caused a slight inhibition of the ATPase activity and proposed that this was due to inhibition of a specific 'transport ATPase'. Ferris et al (5) failed to find any inhibition. The effects of reserpine on the coupled and uncoupled ATPase activity of membrane vesicles was, therefore, examined (Table 2.3.5.). Contrary to the results of Taugner and Hasselbach, and in agreement with Ferris et al, there was no inhibition of the ATPase in the presence of 1μM reserpine. If anything there was a slight enhancement of the activity, both in the presence and absence of uncoupler.

TABLE 2.3.5.

	Coupled Rate	Uncoupled Rate
Chromaffin Granules	41	150
+ Reserpine (1 μ M)	42	153
Chromaffin Granule Membranes	205	324
+ Reserpine (1 μ M)	222	358

The effect of reserpine on the ATPase activity of both chromaffin granules and chromaffin granule membranes, in the presence and absence of 0.5 μ M S-13. Conditions of preparation and assay as in Table 2.3.4. Activity is expressed in nmoles of phosphate produced per minute per mg of protein.

(v) The Purine Triphosphatase Activity of Chromaffin Granules and the Metal Ion Dependence of the ATPase.

The purine triphosphatase activity of the ATPase of granules prepared through 1.6M sucrose was examined in both the coupled and uncoupled systems (Table 2.3.6.). Within experimental error, the ratio of the ATP : ITP : GTP activities was the same for the coupled and uncoupled enzyme. This infers that the control exerted by the coupling effect on the purine triphosphatase activity decreases the rates by a constant percentage. The nucleotide, itself, is the rate limiting factor, but the turnover is increased by the same factor in all three cases when the system is uncoupled.

With the coupled system at pH 6.6, use of other divalent metal ions, namely Mn^{2+} and Co^{2+} in place of Mg^{2+} , had little significant effect on the ATPase activity (Table 2.3.7.). However, in the uncoupled system, the different ions had differing efficiencies : $Mg^{2+} > Mn^{2+} > Co^{2+}$. The ANS response (Chapter 2.3.B.) to energisation using magnesium and manganese was identical, but was diminished when cobalt was used. In this case, however, there was the complication that cobalt is known to be a good quencher of fluorescence.

(vi) The pH Dependence of the ATPase Activity

If the uncoupling of the ATPase is, as the chemiosmotic hypothesis proposes (9), due to an equilibration of a proton gradient, then the pH of the external medium might be expected to exert some effect on this activity, apart from the usual pH dependence due to substrate and enzyme charge. The pH dependence of the ATPase activity of both coupled and uncoupled granules was investigated (Figure 2.3.2a.). What is most striking is the constant activity, within experimental error, of the coupled ATPase activity across the pH range, 6-8. In contrast, the uncoupled ATPase activity shows a marked pH dependence, with a pH maximum around pH 6.5. The activity falls off much more steeply towards low pH than towards high. At pH <5.5, the uncoupled activity is less than the

TABLE 2.3.6.

<i>Nucleotide</i>	<i>Coupled Activity</i>	<i>% ATP</i>	<i>Uncoupled Activity</i>	<i>% ATP</i>	<i>% Uncoupling</i>
<i>ATP</i>	50.2	100	100.5	100	200
<i>ITP</i>	24.7	49	55.3	55	224
<i>GTP</i>	35.5	71	68.1	68	193

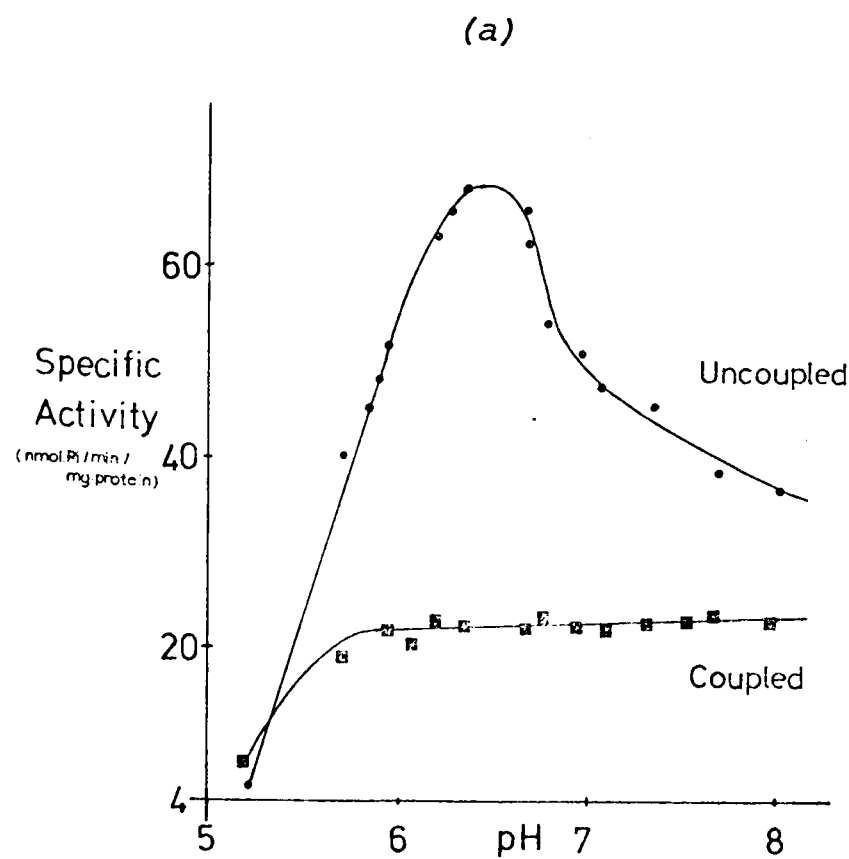
The purine triphosphatase activities of the ATPase of intact chromaffin granules, prepared through 1.6M sucrose but resuspended in 0.3M sucrose:20mM MES,pH 6.6. Reactions were performed at 30°C; all nucleotide concentrations were 2.5mM. Uncoupled rates were in the presence of 0.5μM S-13. Rates are in nmoles of phosphate produced per minute per mg of protein.

TABLE 2.3.7.

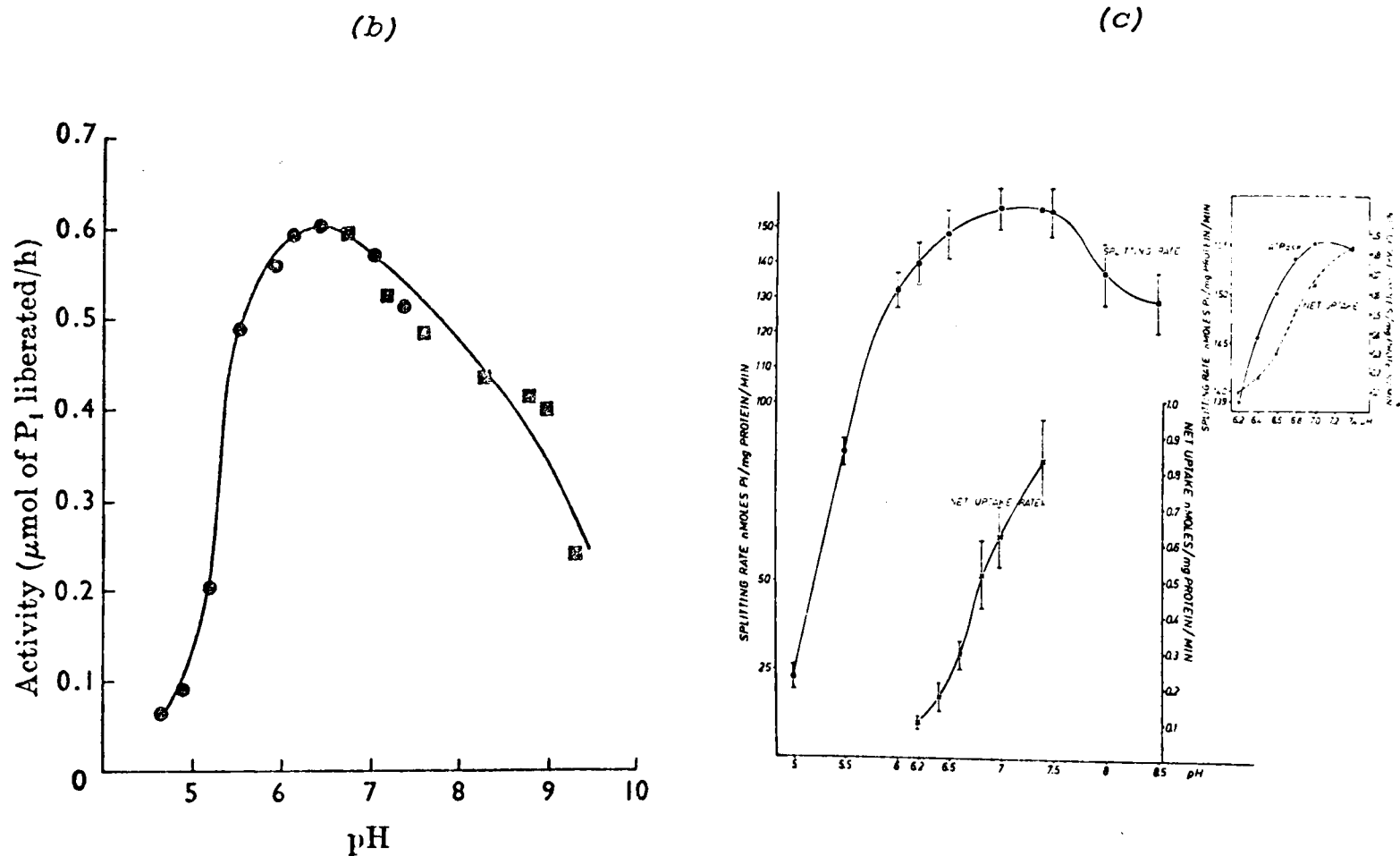
<i>Metal</i>	<i>Coupled Activity</i>	<i>% Mg²⁺</i>	<i>Uncoupled Activity</i>	<i>% Mg²⁺</i>	<i>% Uncoupling</i>
<i>Mg²⁺</i>	41	100	164	100	400
<i>Mn²⁺</i>	40	98	146	89	365
<i>Co²⁺</i>	37	90	108	66	292

The ATPase activity of chromaffin granules, prepared in 0.3M sucrose, when activated by different divalent metal ions, all at 2.5mM. All other reaction conditions as in Table 2.3.6.

FIGURE 2.3.2.



The pH dependence of the ATPase activity of chromaffin granules, prepared through 0.3M sucrose, in the presence and the absence of the uncoupler, S-13, at a concentration of 500 μ M. The assays were carried out as is described in the text, the pH being measured before and after each assay. The temperature of assay was 30°C.



The pH dependence of the ATPase activity of chromaffin granule membrane vesicles from published work.

(b) Winkler *et al* (27)

(c) Taugner (28).

coupled, but the experimental error at these low rates is large, so that this difference may not be significant. The pH dependent maximal activity was not a reflection of the ionic state of the uncoupler molecule used. The experiment was repeated using FCCP instead of S-13. FCCP is a very different molecule, with a different pK (Figure 2.2.2.). The pH dependence using FCCP was, within experimental error, identical to that using S-13, shown in Figure 2.3.2a. Thus, the pH dependence is a function of the system, not of the method of uncoupling.

The pH dependence of the ATPase activity of isolated chromaffin granule membranes has been reported by Winkler et al (27) and by Taugner (28). Their data is shown in Figure 2.3.2b and 2c respectively. There is a close similarity between the pH dependence of the membrane ATPase activity and of the uncoupled granule ATPase activity. In all three cases, the pH maxima at approximately pH 6.5 are observed. These observations support the view that in the membrane preparations studied by Winkler et al and by Taugner, the ATPase was largely uncoupled. The rate limiting steps in the hydrolysis of ATP by the coupled and uncoupled enzymes are not the same.

(ii) The Effects of DCCD on the ATPase Activity

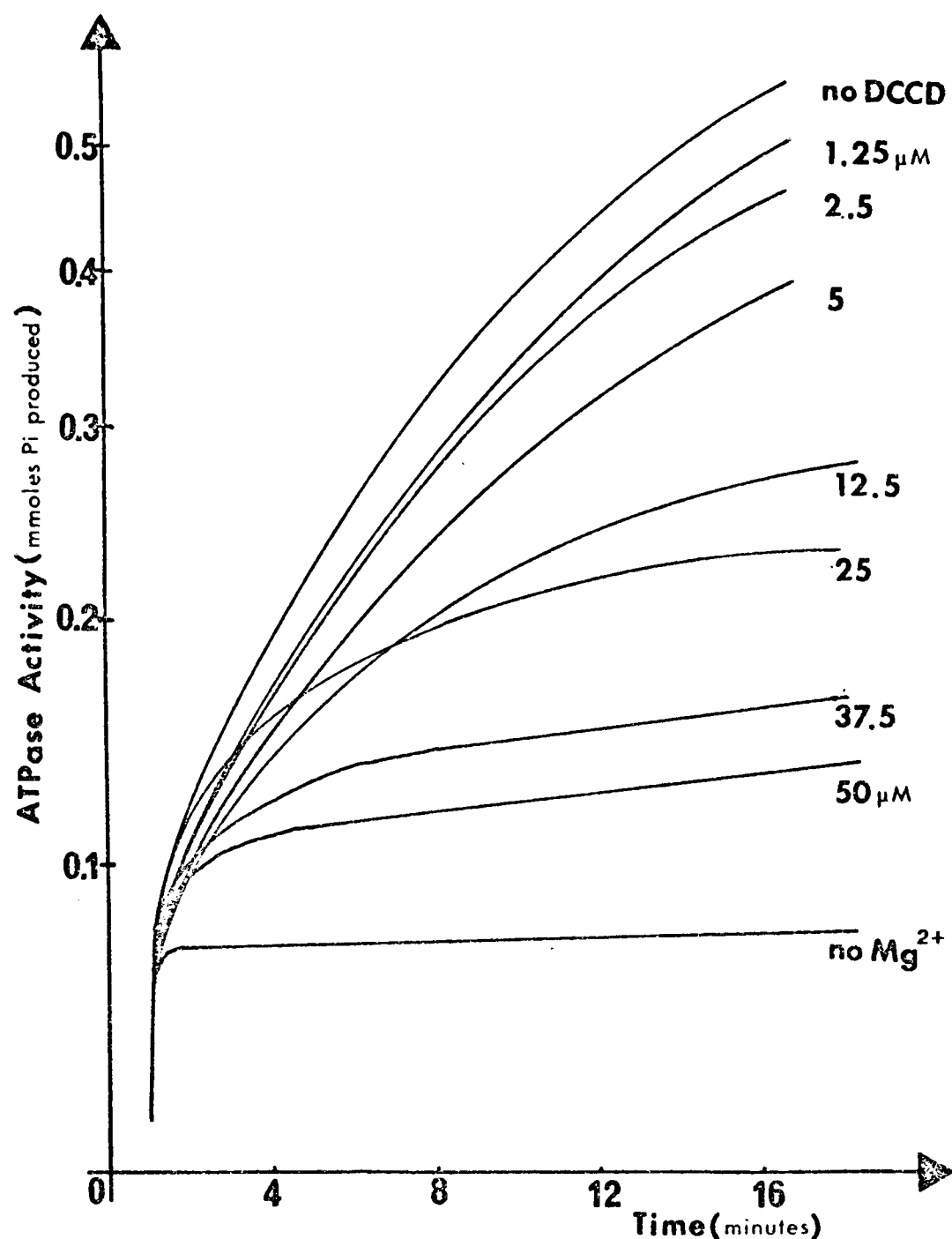
The effects of DCCD on the ATPase activity of mitochondria was described in the introduction to this chapter. Its effects on the chromaffin granule ATPase were investigated (Table 2.3.8.). The ATPase activity of coupled chromaffin granules was greatly inhibited by 25 μ M DCCD. The inhibition was time dependent. The uncoupled activity was also inhibited gradually (Table 2.3.8.). This initial study indicated that complete inhibition could only be achieved if the chromaffin granules were preincubated with DCCD for relatively long periods of time before assaying. It also showed that the kinetics of the inhibition of the coupled and uncoupled systems were similar. Therefore, all further experiments were made using the uncoupled system since the ATPase activity

TABLE 2.3.8.

	Coupled Activity	Uncoupled Activity
Chromaffin Granules	45	181
+ DCCD, 25 μ M	gradual inhibition	gradual inhibition
+ DCCD, 50 μ M	$\rightarrow$ 10	
30min preincubation with 50 μ M DCCD, then addition of S-13 and ATP-Mg ²⁺	complete inhibition	
+ Methanol (1%)	43	183

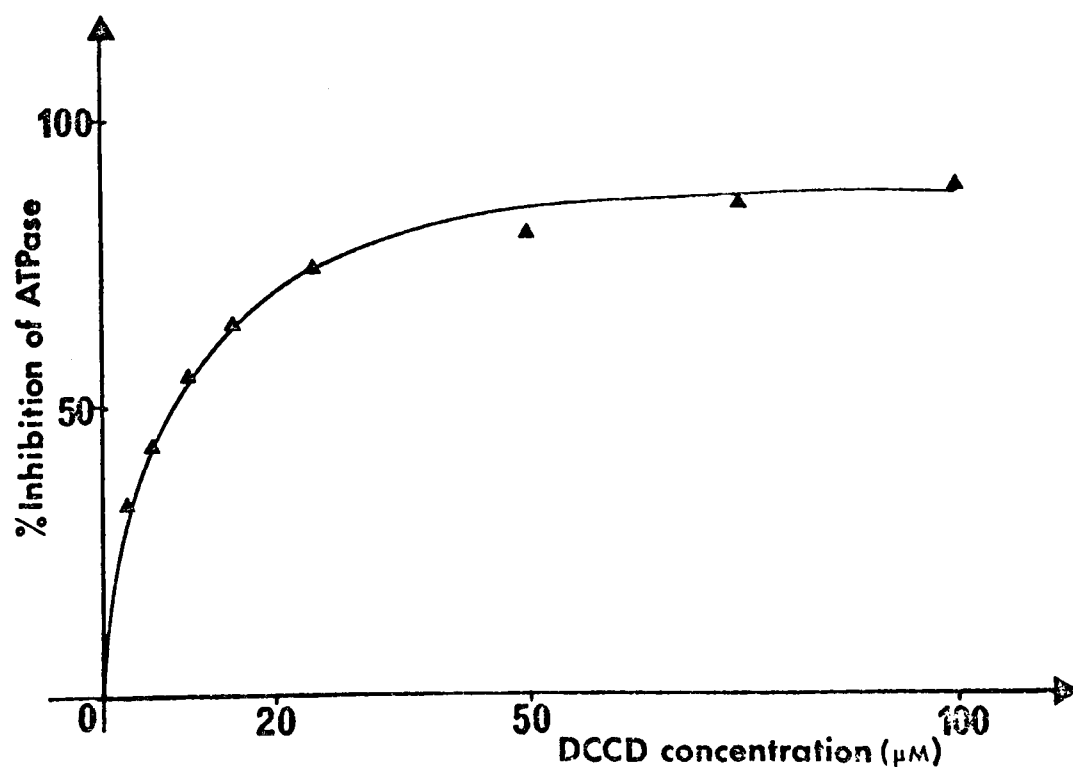
The effect of DCCD on the ATPase activity of chromaffin granules prepared through 0.3M sucrose. Reactions were carried out in 0.3Msucrose:20mM MES, pH 6.5 at 30°C.

FIGURE 2.3.3.



The time courses of the inhibition of the uncoupled ATPase activity of chromaffin granules by various concentrations of DCCD. The curves do not pass through zero due to the lag-time of the autoanalyser. The reaction was carried out at 30°C in 0.3M sucrose buffered with 20mM MES, pH 6.4. Other conditions as in text.

FIGURE 2.3.4.



The concentration dependence of the DCCD inhibition of the uncoupled ATPase activity of chromaffin granules. The granules were preincubated with DCCD for 30 minutes prior to assay. Other conditions as Figure 2.3.3.

in the presence of S-13 is four times greater. This reduced the experimental error considerably. DCCD was dissolved in methanol which was shown to have no effect on either the coupled or uncoupled rate. The time courses of the inhibition of the uncoupled ATPase by various concentrations of DCCD are shown in Figure 2.3.3. The gradual inhibition is clearly shown, with a considerable difference between the initial rates and the rates after 15 minutes. The rates did, however, appear to stabilise towards the end of each time course. The concentration dependence of the DCCD inhibition of the ATPase activity after incubation for a fixed time was also determined. Granules, prepared through 1.6M sucrose but taken up, spun down and resuspended in 0.3M sucrose, were used in this study. These granules were not so tightly coupled as the granules prepared in 0.3M sucrose, therefore, the activity in the absence of uncoupler was sufficiently large to permit the accurate determination of the concentration dependence. The ATPase activities, determined in this manner after incubation for 30 minutes, were relatively constant for the duration of the assays. There is a considerable concentration dependence with almost complete inhibition at a DCCD concentration of 100 μ M (Figure 2.3.4.). The concentration dependence of the ATPase inhibition is compared to those of the inhibition of catecholamine uptake and the inhibition of the ANS response by DCCD in the discussion to this chapter.

(vii) The Effect of the Solubilisation of the Chromaffin Granule Membrane on the Uncoupling and DCCD inhibition of the ATPase Activity

Chromaffin granules, prepared in 0.3M sucrose, were solubilised using the non-ionic detergent, Lubrol-PX. The effect of the solubilisation on the ATPase activity was highly dependent on the concentration of Lubrol used (Table 2.3.9.). The ATPase activity was almost completely inhibited by 0.2% (v/v) Lubrol, but at the lower concentration of 0.005% (v/v), the activity was slightly enhanced. Solubilisation was assumed to have occurred in all cases since the suspensions were completely

TABLE 2.3.9.

	Activity
1. Chromaffin Granules	41
2. + S-13, 0.5 μ M	173
3. Incubated with Lubrol, 0.2% (v/v)	5.5
4. 0.01%	41
5. 0.005%	69.2
6. as 4, but no ATP-Mg ⁺ present	3
7. as 4, but incubated with 0.5 μ M S-13	37.5
8. Preincubated at 30°C for 20mins, then solubilised in Lubrol, 0.01%	40
9. Preincubated at 30°C for 20mins with 0.5 μ M S-13, then solubilised with Lubrol, 0.01%	43

10. Preincubated with DCCD, 50 M for 20mins.	0
11. as 10, then solubilised with Lubrol, 0.01	0 $\rightarrow$ 13.3
12. as 10, then S-13, 0.5 M added	0
13. Preincubated with DCCD, 50 M, and S-13, 0.5 M for 20mins	7.5
14. as 13, then solubilised with Lubrol, 0.01	7 $\rightarrow$ 14.3
15. Preincubated with DCCD, 50 M, and Lubrol, 0.01 for 20mins	8.5
16. Preincubated with DCCD, 50 M, Lubrol, 0.01, and S-13, 0.5 M for 20mins	9.2
17. Preincubated with Lubrol, 0.01, then with 50 M DCCD; total incubation 40mins	8.6

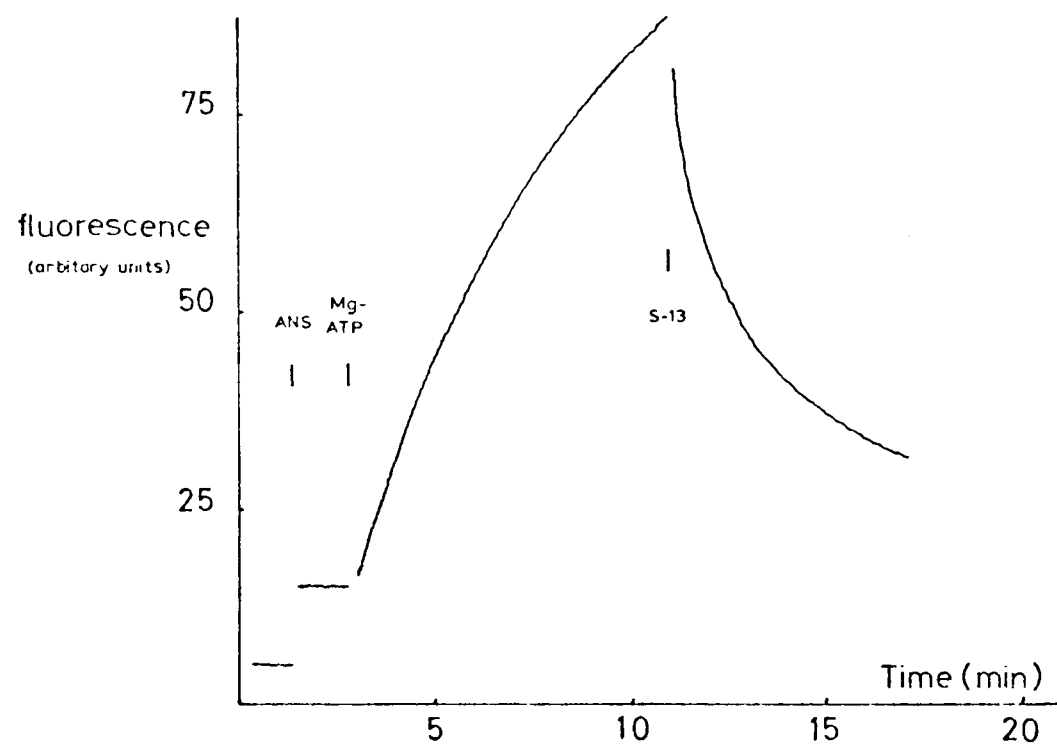
The effects of solubilisation on the uncoupling and DCCD inhibition of chromaffin granules prepared through 0.3M sucrose. Conditions of preparation and assay as in Table 2.3... All incubations are for 20mins at 30°C.

clear. The intermediate concentration of 0.01% (v/v) Lubrol was used in the remainder of the experiments. There is no uncoupling of the ATPase activity of solubilised granules when S-13 is added, either before or after solubilisation. Trifaró and Wagner (7) used Lubrol-PX to solubilise the ATPase prior to purification. They used 0.2% (w/v) Lubrol. However, they only measured the total ATPase activity present in the supernatant after solubilisation, and did not test the solubilisation and ATPase activity in the presence of the detergent directly. The danger of accounting units of enzyme recovered in terms of activity of the enzyme per milligram of protein should be emphasised. It is not a valid measurement of the recovery if the activity is affected, whether positively or negatively, by the solubilisation or the presence of the detergent.

The inhibition of the ATPase by DCCD was gradually, but only partially reversed by solubilisation, both in the presence and the absence of the uncoupler S-13. The final activity is the same in both cases (Table 2.3.9). The degree of inhibition was the same, whether the ATPase was incubated with DCCD before or after solubilisation. These results indicate that the DCCD inhibition is probably not due to a general interaction of DCCD with the granule membrane, but is due to direct inhibition of the ATPase, itself, or via a component closely attached to it. The loss of complete inhibition on solubilisation may be explained by a lowering of the binding constant of the enzyme (or attached component) for DCCD on solubilisation, since the activity was the same irrespective of whether the granules were solubilised before or after treatment with DCCD. Other explanations are, of course, possible. For instance, the binding of an inhibitor protein may be removed or 'loosened' by the solubilisation, thus restoring partial activity.

The solubilisation process could be described as an uncoupling since the membrane integrity is disrupted, and the enzyme activity increased. Lubrol, itself, would appear to be inhibitory.

FIGURE 2.3.5.



The response of the fluorescence of ANS to 'energisation' by ATP-Mg^{2+} . ANS, $5\mu\text{M}$, was added to chromaffin granules (0.25mg protein/ml) in 0.3M sucrose; 15mM HEPES, pH 6.6. ATP-Mg^{2+} (2.5mM) was then added to start the reaction. Uncoupling by $0.3\mu\text{M}$ S-13.

B. The ANS Response

(i) The Nature of the Response and the Effect of Uncouplers on it

The fluorescent probe, 1-anilino-naphthalene-8-sulphonate (ANS), binds to bovine heart submitochondrial particles. This interaction can be followed by a blue shift and the enhancement of the fluorescence emission spectrum (29,30,31). Addition of respiratory substrates or ATP-Mg^{2+} to the particles results in an increase in the probe fluorescence, termed the ANS response. This reflects an energised state of the membrane, since the addition of uncouplers reverses this effect (29,30,31). The origin of these fluorescence changes, when the system is energised by the hydrolysis of ATP, has been shown to be an increase in the amount of probe bound to the membrane (32). When respiratory chain substrates are present, the effect can be attributed in part to an increased quantum yield of the bound probe molecules. Ferguson et al discuss the nature of the ANS response in submitochondrial particles and liposomes, and its dependence on the method of its generation, very thoroughly in their paper (33).

When equimolar quantities of MgCl_2 and ATP were added to a suspension of chromaffin granules in the presence of ANS, there was a marked time dependent enhancement of the fluorescence (Figure 2.3.5.); a similar effect to that found with submitochondrial particles. At pH 6.6, the half time for this increase in fluorescence is approximately 5 minutes. Addition of an uncoupler of mitochondrial oxidative phosphorylation, S-13, $0.5\mu\text{M}$, causes this response to collapse with a half time of approximately 1.5 minutes. Addition of the uncoupler prior to the addition of ATP and Mg^{2+} completely removes the initial fluorescence enhancement.

Bashford (25) has investigated the nature of the ANS response of chromaffin granules, in detail. He finds that, as with submitochondrial particles, the ANS response in the presence of ATP-Mg^{2+} is due to an increase in the amount of probe bound to the membrane, not to an increased quantum yield of the bound probe molecules. In view of these and other

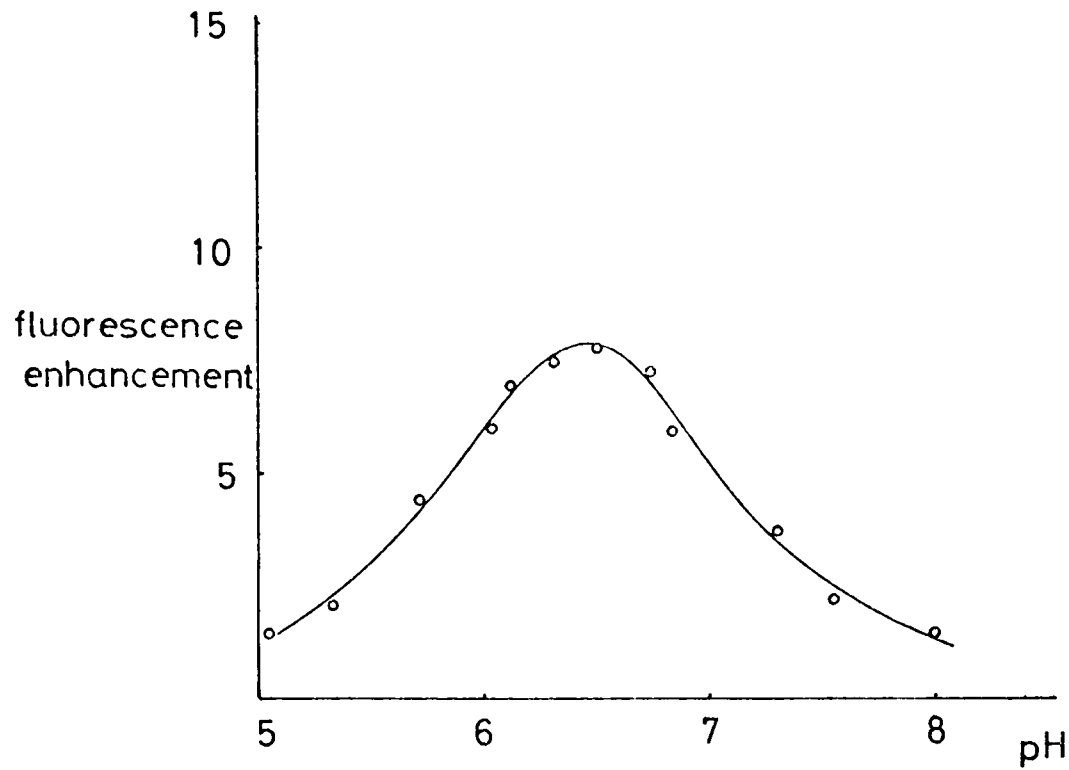
kinetic properties (25), the nature of the ANS response in chromaffin granules appears to be very similar to that of submitochondrial particles.

A valinomycin/KCl-induced ANS response can also be developed with chromaffin granules and chromaffin granule membranes. The nature of this response is not the same as that induced by ATP-Mg²⁺. This response is very rapid. It subsequently decays, probably paralleling the decrease of the potassium diffusion potential. This is a similar finding to that with submitochondrial particles, in the same conditions (33). In both cases, it was concluded that a quantum yield change is the main, if not the sole, cause of the ANS fluorescence enhancement. It is of interest that neither the chromaffin granule ATPase (Personal observation) nor the submitochondrial particle ATPase are uncoupled by valinomycin/KCl (34).

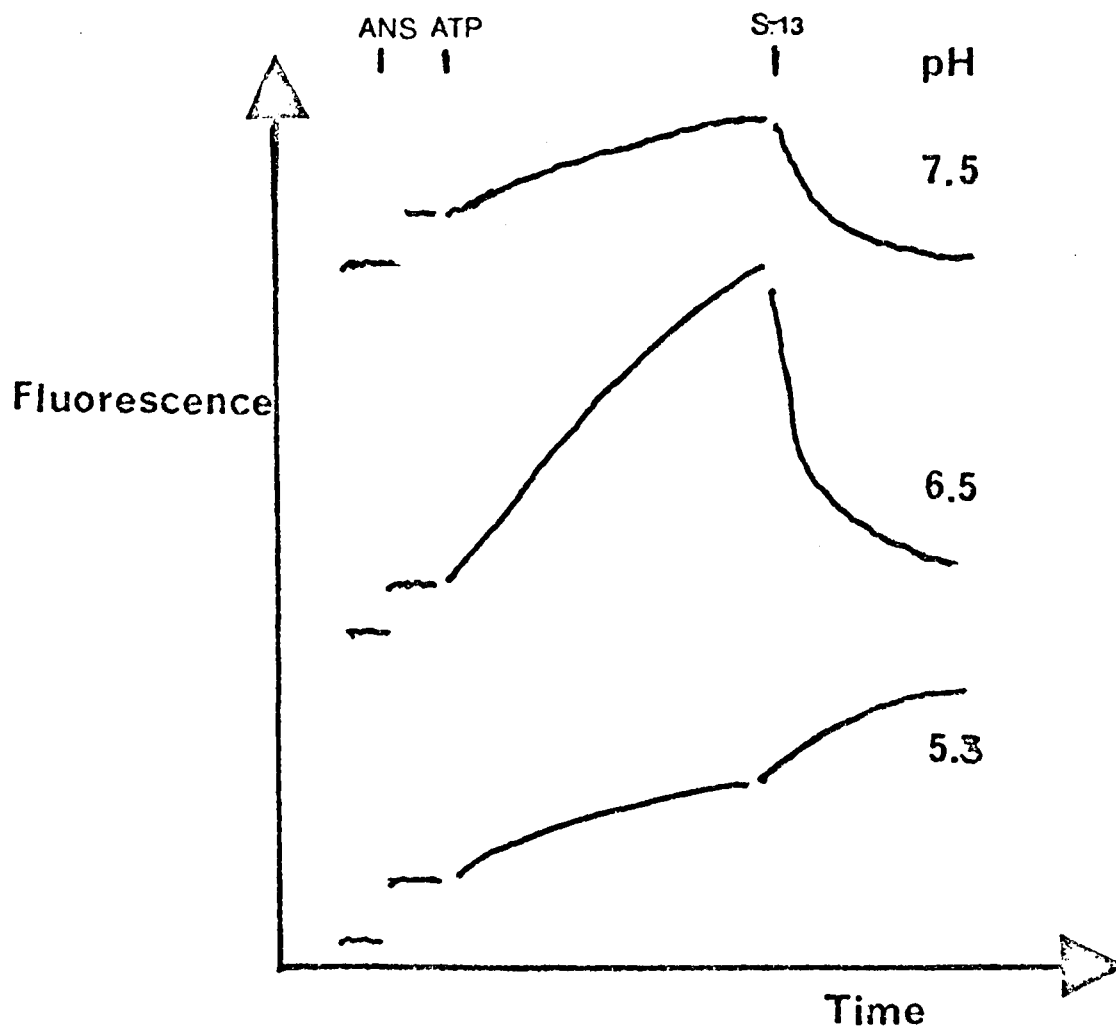
Despite detailed research, the precise nature of the ANS response in both chromaffin granules and submitochondrial particles is not known. It does mirror some alteration of the membrane, probably of its charge state. Ferguson *et al*, however, suggest that, in view of the differences in the valinomycin/KCl- and ATP-Mg²⁺-induced ANS responses, the fluorescence of ANS should not be used as an indicator of membrane potential in submitochondrial particles but as an indicator of a related phenomenon. It is probable that the ANS response of the chromaffin granule membrane, in the presence of ATP-Mg²⁺, is a reflection of this same, but undefined, phenomenon.

(ii) The pH Dependence of the ANS Response and of its Reversal by Uncouplers

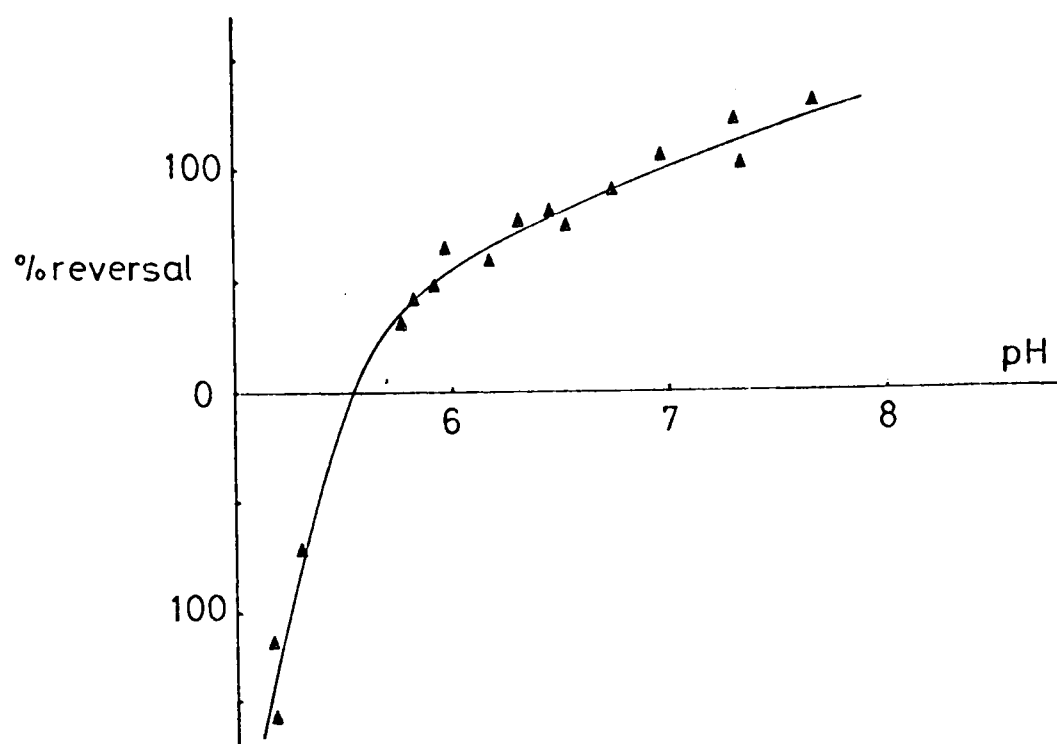
The pH dependence of the fluorescence enhancement of the ANS response of chromaffin granules was measured over a wide range of pH. In each case, the pH was measured before and after the experiment lest the buffering capacity of the buffered sucrose was inadequate to maintain the pH on the breakdown of ATP. There was an insignificant variation of pH, in every case, with a maximal deviation of 0.01 pH units. The fluorescence enhancement shows a considerable pH dependence, with the optimal response

FIGURE 2.3.6.

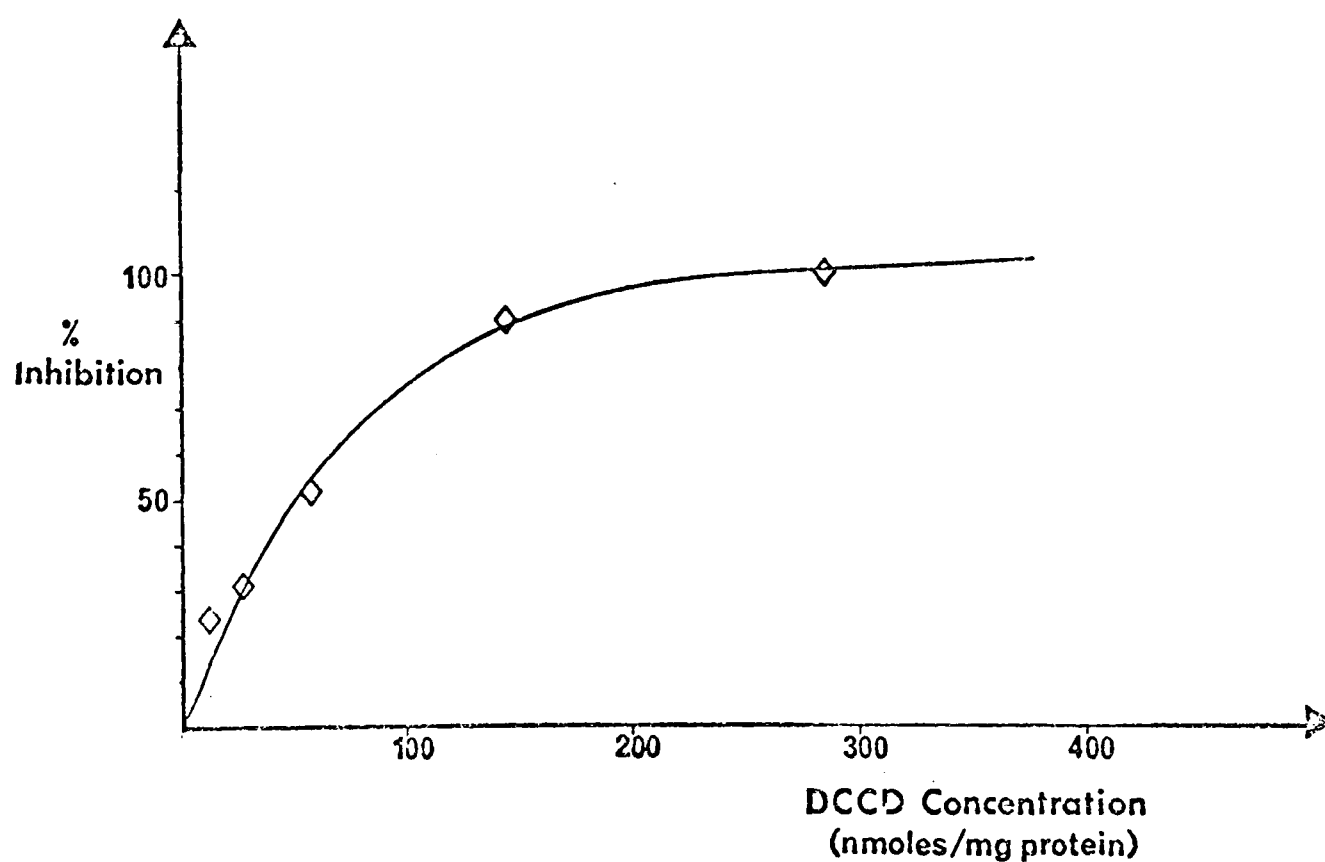
The pH dependence of the fluorescence enhancement of ANS ($0.5\mu\text{M}$) bound to chromaffin granules. Conditions as in Figure 2.3.5.

FIGURE 2.3.7.

A representation of the ANS response of chromaffin granules to 'energisation' and of its 'reversal' by the uncoupler, S-13, at three different pH values: 5.3, 6.5, 7.5. This is not the actual data, but is taken from Figures 2.3.6. & 8. It is intended to act as an aid to the visualisation of the effect.

FIGURE 2.3.8.

The pH dependence of the 'reversal' of the ANS response, to 'energisation' by ATP-Mg^{2+} of the chromaffin granule, which occurred in the presence of the uncoupler, *S*-13 ($0.5\mu\text{M}$) Conditions as in Figure 2.3.5.

FIGURE 2.3.9.

The concentration dependence of the inhibition of the ANS response to 'energisation' of chromaffin granule by DCCD. The granules were preincubated for 30 minutes with DCCD before assay. Conditions as in Figure 2.3.5. and text.

of the probe occurring around pH 6.4 (Figure 2.3.6.). The enhancement of the fluorescence at pH 6.4, is approximately six fold greater than at pH 5.0 and pH 8.0.

The reversal of the fluorescence enhancement by the uncoupler, 0.5 μ M S-13, at three pHs, is shown in Figure 2.3.7. It can be seen that at pH 5.0, there is, in fact, a further enhancement of the fluorescence. At higher pHs, the fluorescence is reduced to different extents. The percentage 'reversal' of the enhancement was examined over a wide pH range (Figure 2.3.8.). The pH range shown is pH 5.0 to 8.0, since the enhancements outside this range are very small, and the stability of the granules, themselves, would be uncertain. It can be seen that at pHs greater than 5.5, there is a true 'reversal', the extent of which has a linear dependence at pHs greater than 6.5. At pHs less than 5.5, there is a further enhancement of the fluorescence, on the addition of the uncoupler. The significance of this result will be described later in this chapter.

(iii) The Effect of DCCD

The ANS response of submitochondrial particles is almost completely abolished when the membrane is preincubated with large concentrations of DCCD, but the degree of inhibition of the ANS response and of the ATPase activity do not correspond over a range of DCCD concentrations (33). The degree of the ANS response of chromaffin granules and membrane vesicles was investigated in the presence of various concentrations of DCCD, to correlate with the ATPase studies described earlier (Ch.2.3.A) on the same preparation (Figure 2.3.9.). The ANS response is inhibited by about 90% in the presence of 50 μ M DCCD ie. 200nmoles of DCCD per milligram of protein. In fact, the DCCD inhibition of the ANS response and the ATPase activity do appear to correlate. This is shown in Figure 2.4.1. in the discussion of this chapter.

C. Studies on the Enhanced Catecholamine Uptake

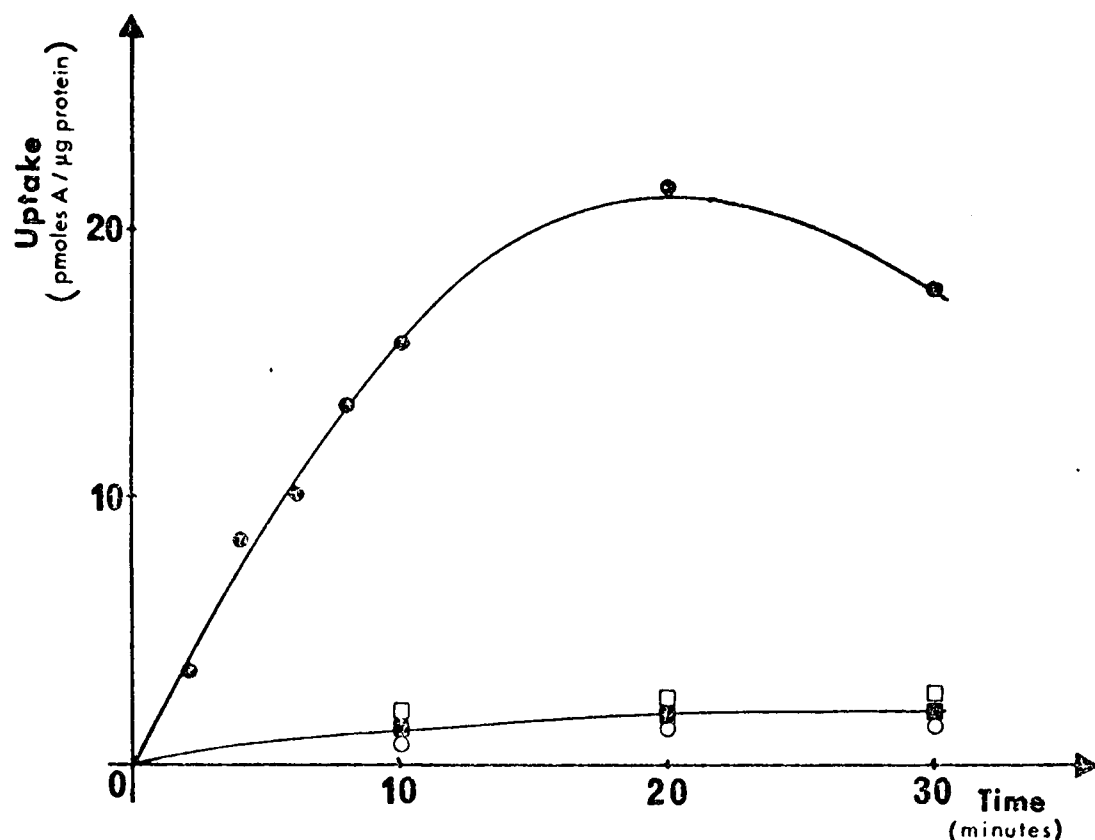
(i) The Effects of Uncouplers

According to the model proposed in the introduction to this chapter, the ATP-Mg²⁺-enhanced uptake of catecholamines should be abolished in the presence of uncouplers, since the 'potential energy pool' which drives this uptake should be dissipated by the uncouplers. The uptake of catecholamines by both intact chromaffin granules and chromaffin granule membrane vesicles was investigated.

The pattern of adrenaline incorporation by membrane vesicles is essentially the same as that observed by Phillips (36), although he observed maximal incorporation after incubation for one hour (Figure 2.3.10.). The earlier appearance of maximal incorporation in these experiments was probably due to the more rigorous conditions of the membrane preparative procedure used, which may render the membrane vesicles more leaky. The uptake of the other catecholamines, dopamine and noradrenaline, was also studied (Figures 2.3.11. and 12.). In these experiments, the vesicles were not incubated for longer than 15 minutes, which appears from Figure 2.3.10. to be the optimum incubation time for adrenaline incorporation. It is apparent that for the membrane system used in these studies, the efficiency of the incorporation of the different catecholamines is : noradrenaline > adrenaline > dopamine. This does not agree with the results of other workers (37). Da Prada et al (37) used the technique of equilibrium dialysis and find that the maximum dopamine uptake is greater than that of noradrenaline, but is slower to develop, which may be the reason that it is lower in this set of experiments. It was not the aim of this work, however, to compare the actual extent of uptake of the different catecholamines in the presence of ATP-Mg²⁺, but to determine the effects of uncouplers and other such drugs on this uptake.

The effects of the presence of S-13, 1μM, on the incorporation of the different catecholamines are also shown in Figures 2.3.10-12. It can

FIGURE 2.3.10.

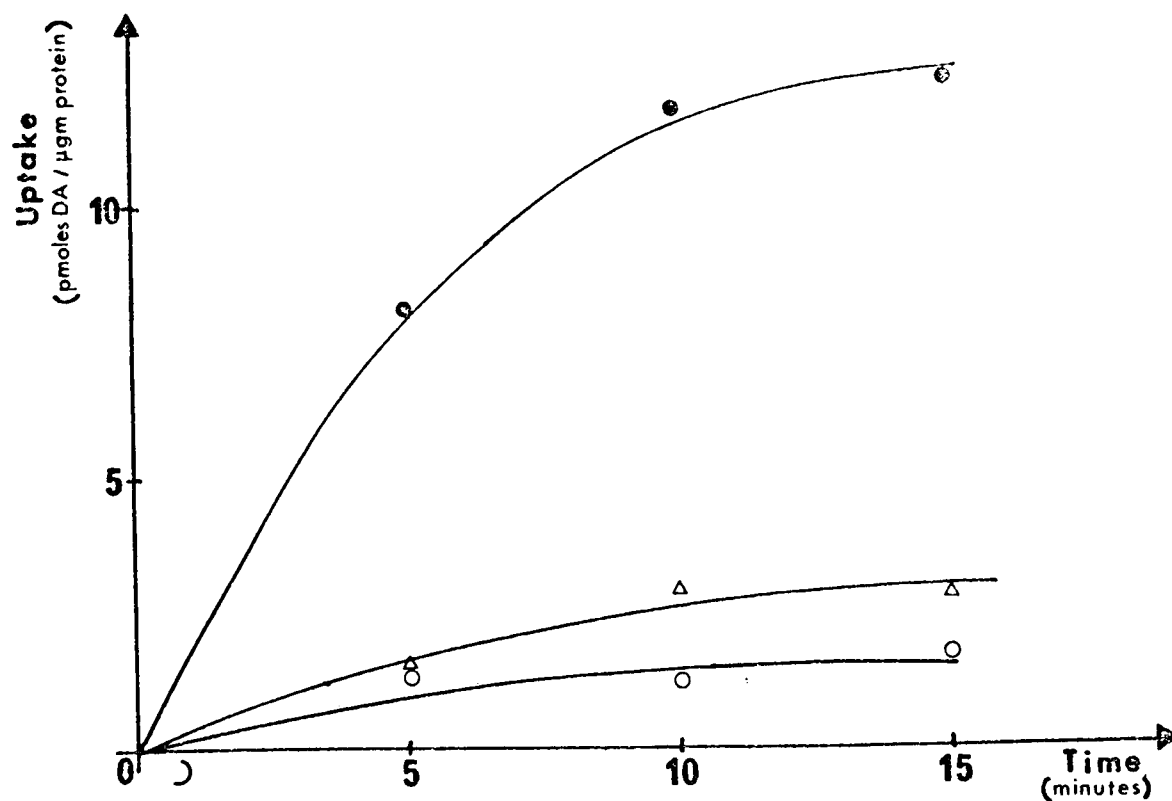


The incorporation of DL-[7-¹⁴C]-adrenaline by chromaffin granule membrane vesicles. The incorporation was carried out as described in the text.

Key:

- + ATP-Mg²⁺ (2.5mM)
- - ATP-Mg²⁺
- + ATP-Mg²⁺ + S-13 (5μM)
- - ATP-Mg²⁺ + S-13.

FIGURE 2.3.11.

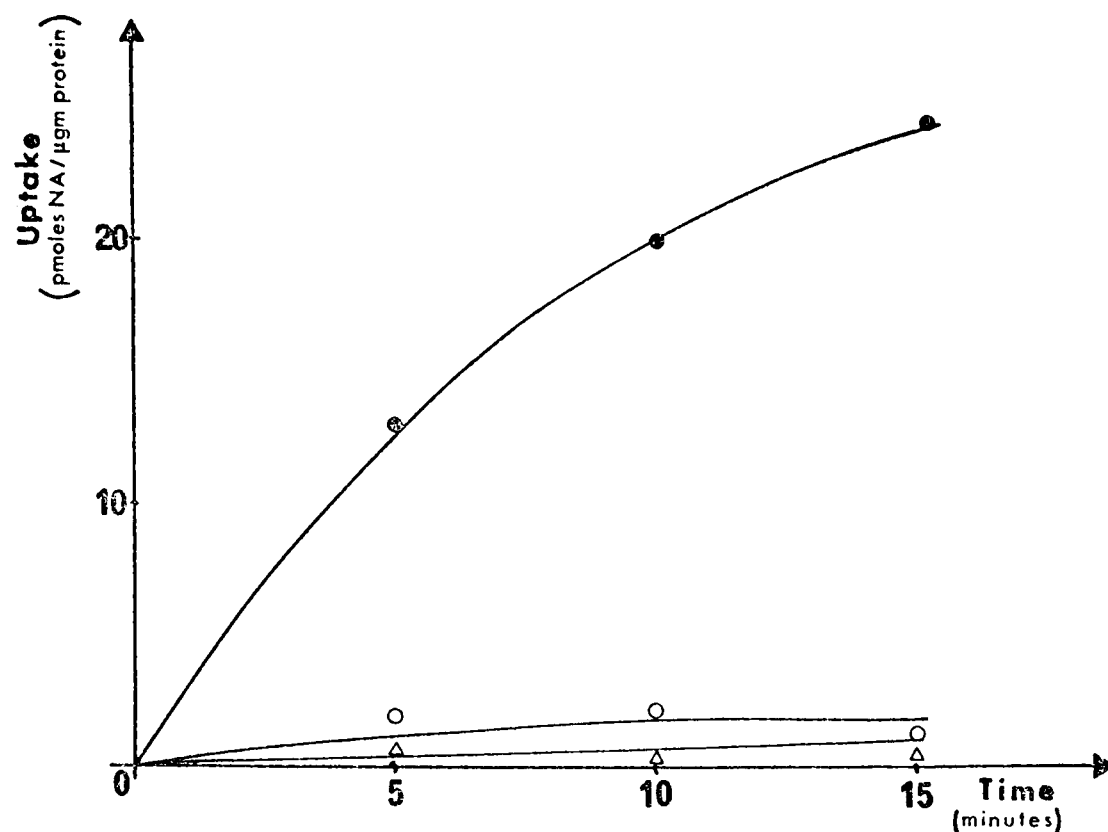


The incorporation of ¹⁴C-dopamine by chromaffin granule membrane vesicles. The incorporation was carried out as described in the text.

Key:

- + ATP-Mg²⁺ (2.5mM)
- Δ + ATP-Mg²⁺ + S-13 (1μM)
- - ATP-Mg²⁺

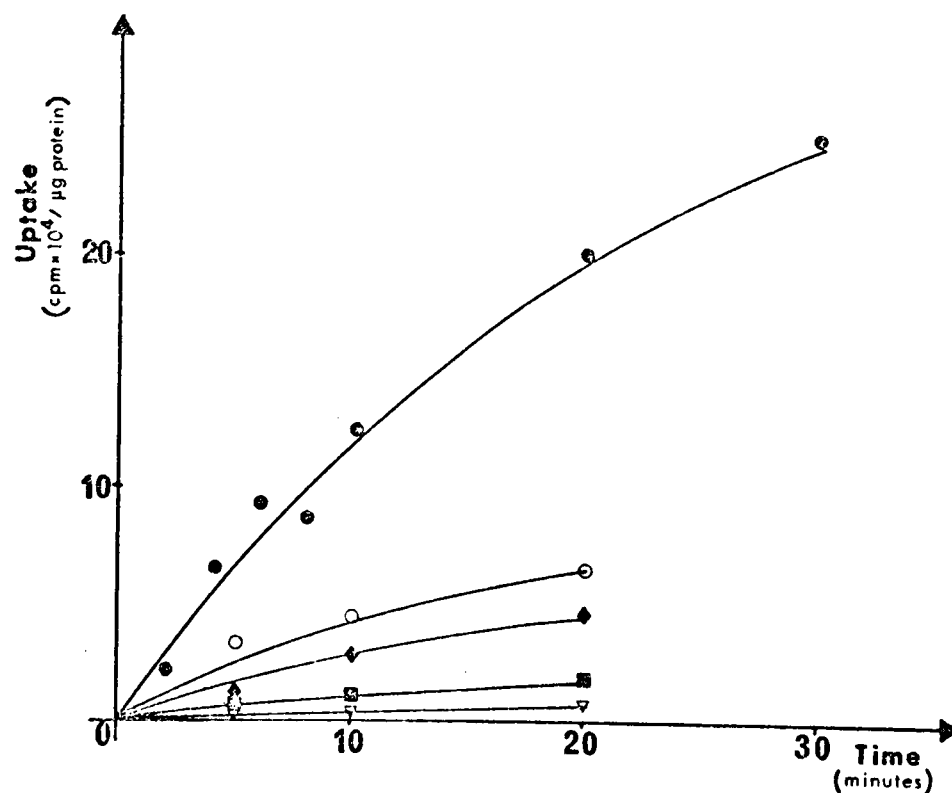
FIGURE 2.3.12.



The incorporation of DL[7-¹⁴C]-noradrenaline by chromaffin granule membrane vesicles. The incorporation was carried out as described in the text.

Key: ● + ATP-Mg²⁺ (2.5mM)
 Δ + ATP-Mg²⁺ + S-13 (1μM)
 ○ - ATP-Mg²⁺

FIGURE 2.3.13.



The incorporation of DL-[7-¹⁴C]-adrenaline by bovine chromaffin granules. Incorporation was carried out as described in the text.

Key: ● + ATP-Mg²⁺ (2.5mM)
 ○ - ATP-Mg²⁺
 ◆ + ATP-Mg²⁺ + 1799 (1μM)
 ■ + ATP-Mg²⁺ + S-13 (1μM)
 ▽ + ATP-Mg²⁺ + NH₄ CNS (5mM)

be seen, from Figures 2.3.10. that S-13 completely abolishes the enhanced adrenaline uptake. The incorporation, when both S-13 and ATP-Mg^{2+} were present, was identical, within experimental error, to the rate in the absence of ATP-Mg^{2+} . The uptake of dopamine and noradrenaline were also greatly inhibited by the presence of uncoupler (Figures 2.3.11 and 12.) The uptake of dopamine was also looked at in the presence of reserpine, which has long been known to inhibit catecholamine uptake (38,39). However, in one case, it was found that the rate of incorporation in the presence of reserpine was less than, and in the other case, greater than the rate of incorporation in the presence of S-13. Both rates, however, were very low compared to the energised rate.

The uptake of adrenaline by intact chromaffin granules was also studied (Figure 2.3.13). Since the specific radioactivity of the adrenaline present in the assay was not known due to its dilution by endogenous catecholamine, the results are expressed in terms of counts per minute per microgram of protein. The results with intact granules were qualitatively similar to those with membrane vesicles (Figure 2.3.10.). The uptake was abolished in the presence of S-13, $1\mu\text{M}$.

The efficiencies with which different uncouplers inhibit the uptake of catecholamine was examined for both membrane vesicle and intact granule incorporation. S-13, 1799 and FCCP all inhibit the uptake (Table 2.3.10.). At equivalent concentrations, their efficiencies in inhibiting the incorporation were not the same as their effectiveness in enhancing the ATPase activity and in reversing the ANS response. In this case, 1799 appears to be more efficient than FCCP, which is the reversal of their efficiencies in activating the ATPase (Table 2.3.1.). In neither case, however, is the difference between the relative efficiencies very large, and they are always much less efficient than S-13.

Another procedure which perturbs membrane permeability, incubation with ammonium thiocyanate, also inhibits the ability of the

TABLE 2.3.10.

Adrenaline incorporation by chromaffin granule membrane vesicles		Adrenaline incorporation by chromaffin granules	
Uptake	% of control	Uptake	% of control
Control	13.2	1248	100
+ S-13, 1μM	1.0	101	8.1
+ 1799, 1μM	1.7	297	23.8
+ FCCP, 1μM	2.3	-	-
+ NH ₄ CNS, 1mM	10.1	-	-
+ NH ₄ CNS, 5mM	2.9	55.6	4.5
ATP blank	1.2	442	35.4
+ S-13, 1μM	1.1	-	-

The effect of uncouplers on adrenaline incorporation by chromaffin granules and their membrane vesicles. The incorporation was determined as described in the text. The incorporation in the absence of ATP-Mg²⁺ was termed the ATP blank. The uptake by membrane vesicles is expressed in pmol adrenaline per 10mins per μg of protein. The uptake by granules is expressed as c.p.m. found in the pellet after a 10 minute incubation in the assay medium.

membrane vesicles and granules to incorporate adrenaline, despite their enhanced ATPase activities (not shown).

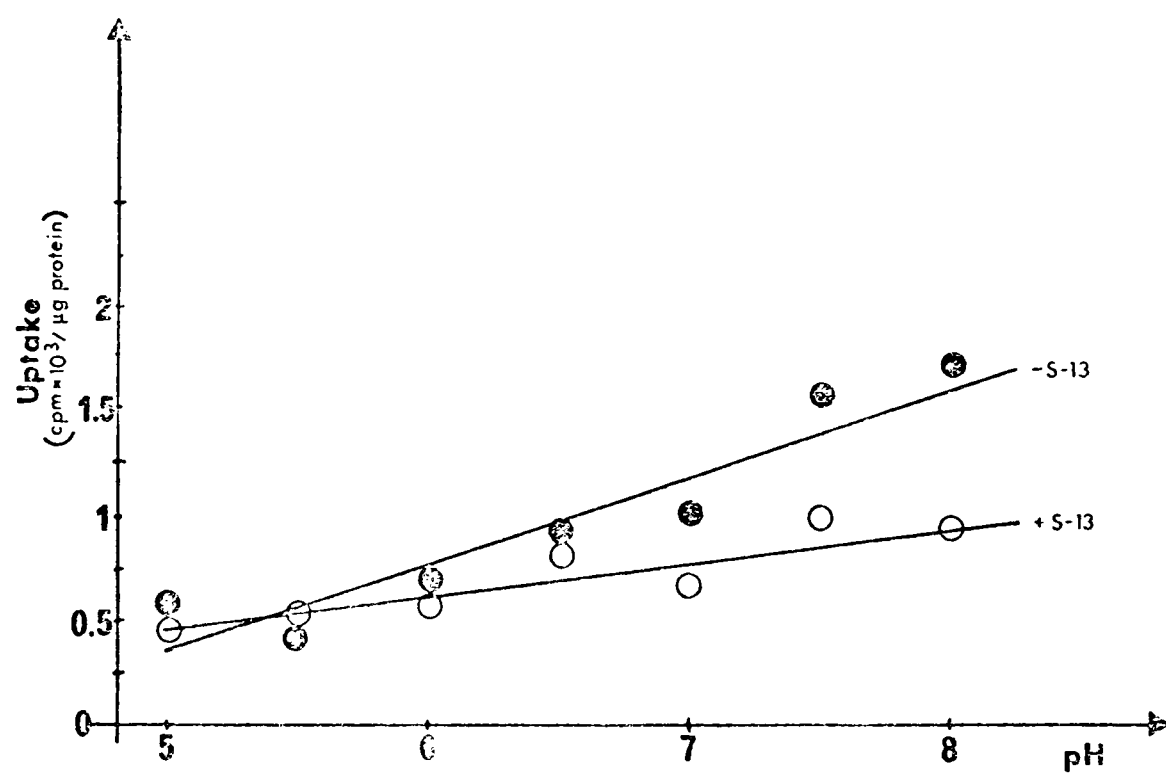
The uptake of methylamine by membrane vesicles was also studied. This was found to have no enhanced uptake with increasing incubation time. Methylamine was shown to enhance the ATPase activity at high concentrations, 10 and 100mM (Table 2.3.4.), but it does not uncouple the activity fully, even at concentrations as high as 100mM. It is unlikely, therefore, that the lack of uptake was due to uncoupling, since the concentration of methylamine used in the uptake experiments was 100 μ M.

(ii) The pH Dependence of Catecholamine Uptake in the Absence of ATP-Mg²⁺

The dependence of the adrenaline distribution with pH across the unenergised chromaffin granule membrane was examined in the presence and absence of uncoupler. Fresh chromaffin granules were prepared in 0.3M sucrose, but the uptake buffer used was 20mM NaH₂PO₄.12H₂O, 20mM citric acid and 0.3M sucrose, pH as indicated, not MES or HEPES buffer as before. The granules were incubated for 20minutes. No ATP or MgCl₂ were present. The results are shown in Figure 2.3.14. The value of the residual activity in the supernatant was 155,500 $\pm$ 3.5% c.p.m. which shows very high reproducibility. The results are the means of duplicates.

The uptake has very little pH dependence in the presence of S-13. In the absence of S-13, however, there is a marked pH dependence. When a least squares fit is applied to the data, the gradient of the uptake in the absence of S-13 is 458 $\pm$ 78cpm/pH unit, whilst that in the presence of S-13 is 169.5 $\pm$ 44cpm/pH unit. The two slopes intersect at pH 5.5. This is the pH where the ANS response uncoupling changes from a positive to a negative effect, and where the uncoupled and coupled ATPase activities intersect. The importance and implication of this and other related results are discussed later in this thesis.

FIGURE 2.3.14.



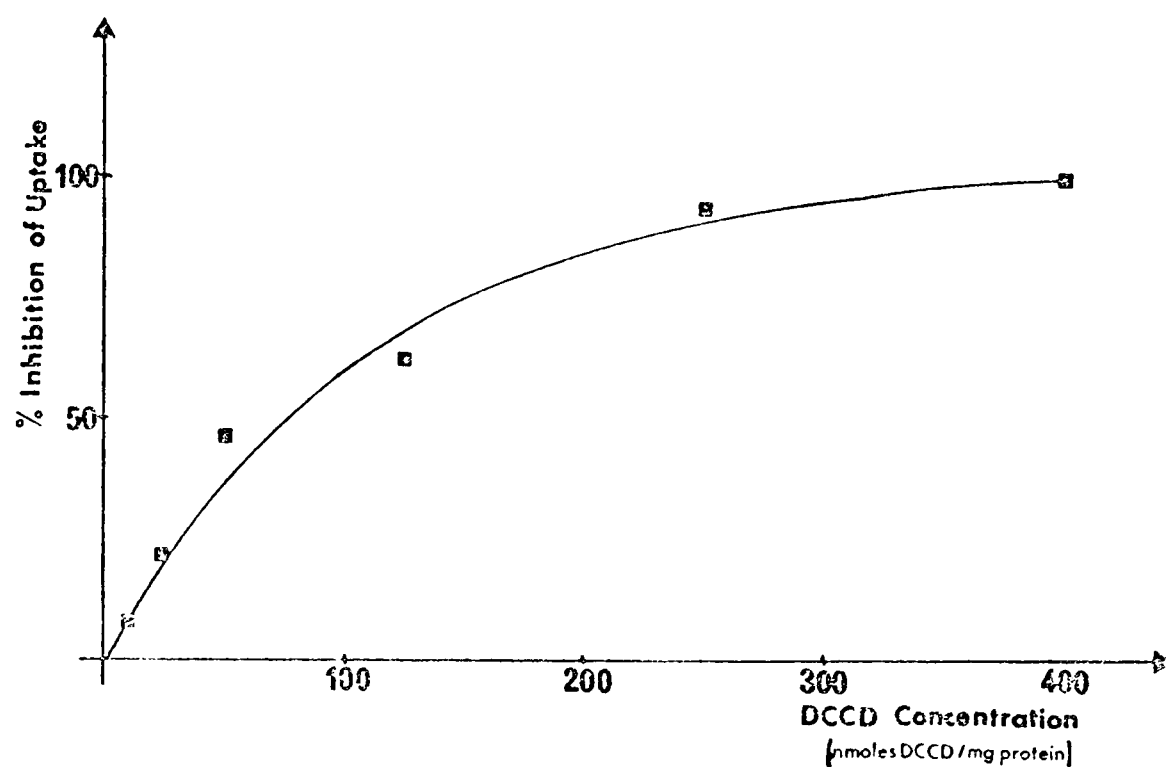
The distribution of LL-[7-¹⁴C]-adrenaline across the membrane of unenergised chromaffin granules, as a function of the pH of the medium, and in the presence and absence of S-13 (1μM).

(iii) The Effects of DCCD

The effect of DCCD, at various concentrations, on the uptake of ^{14}C - noradrenaline by chromaffin granules was studied (Figure 2.3.15.). There is a concentration dependent inhibition of the noradrenaline uptake, with complete inhibition at a DCCD concentration of $200\mu\text{M}$. This inhibition occurs at an approximately two hundred fold greater concentration than the inhibition caused by S-13. The two drugs are reported to exert their effects through very different mechanisms (12,13). This is epitomised by the opposite effects which they have on the chromaffin granule ATPase activity ; S-13 activates it, DCCD inhibits it.

The concentration dependence of S-13 uptake was also investigated. The results of this titration are shown in Table 2.3.11. It is very difficult to titrate S-13, but it is clear that complete inhibition of uptake occurs at a concentration of 200nM S-13.

FIGURE 2.3.15.



The concentration dependence of the inhibition by DCCD of DL-[7-¹⁴C]-noradrenaline uptake by chromaffin granules. Conditions as in text and Figure 2.3.4.

TABLE 2.3.11.

	<i>Uptake</i>	<i>% Inhibition</i>
<i>Control</i>	9.75	0
<i>+ 0.02μM S-13</i>	4.15	57.4
<i>+ 0.04μM S-13</i>	1.61	83.5
<i>+ 0.10μM S-13</i>	1.56	84.0
<i>+ 0.20μM S-13</i>	0	100
<i>+ 0.50μM S-13</i>	0.38	96.1
<i>ATP blank</i>	2.30	76.4

The concentration dependence of S-13 inhibition of nor-adrenaline uptake by chromaffin granule membrane vesicles
Conditions as in Table 2.3.10. Uptake is expressed in
pmoles of noradrenaline per 10 minutes per μg of protein.

D. Can Chromaffin Granules make ATP?

In view of the large concentration of ATP within the chromaffin granule, and the presence of an electron transfer chain and ATPase in the granule membrane, it was decided to test whether, under apparently favourable conditions, the ATPase could reverse its activity and synthesis ATP from ADP and Pi.

Two methods of assay were used. The first, based on the luciferase : luciferin system extracted from firefly tails, measured the presence of 'indiscriminately formed ATP'. This system of assay was only applicable to study of membrane vesicles, since such large quantities of ATP are present in the granules. These results are shown in Table 2.3.12. There is considerable ATP in the ADP, 0.4%. The ADP used was the free acid since the disodium salt contained so much ATP, >5%, that it was not possible to use it in the assay. Previous experiments showed the absence of ATP in NADH. ATP was formed by the membrane vesicles in the presence of ADP and Pi. The amount formed, however, was not altered significantly by the presence of Pi or uncoupler molecules. The presence of NADH did not alter the rate of ATP production either. It was concluded that the ATP formed was probably the result of myokinase activity, which is a contaminant of most preparations. There were indications that ATP remained bound to the membrane, even after careful washing. This amount was not very large however.

The second method, using radioactive ^{32}P i, only measures ATP formed from ADP and ^{32}P i. Thus, myokinase activity will not affect the results. It was also possible to assay the ATP synthetase activity of intact chromaffin granules using this method. Despite the sensitivity of this method, the amount of ^{32}P incorporated into ATP was very small in all cases. With the membrane vesicles, the amount of radioactivity incorporated into ATP in the presence of NADH and by exchange in the presence of excess ATP, are not significantly greater than the control level. The incorporation in the presence of ferricyanide, in addition to NADH, was slightly higher. The

TABLE 2.3.12.

Conditions	ATP formed (Arbitrary units)
1. Particles alone	425
2. + ADP, 2mM	21,000
3. + ADP, 2mM, MgCl ₂ , 4mM	75,000
4. + ADP, 2mM, MgCl ₂ , 4mM, K ₃ PO ₄ , 2mM	110,000
5. + ADP, 2mM, MgCl ₂ , 4mM, K ₃ PO ₄ , 2mM, S-13, 5μM	150,000
6. + ADP, 2mM, MgCl ₂ , 4mM, K ₃ PO ₄ , 2mM, NADH, 25μM	100,000
7. + ADP, 2mM, MgCl ₂ , 4mM, K ₃ PO ₄ , 2mM, NADH, 25μM, S-13, 5μM	160,000
8. ADP, 2mM, alone	23,000

The measurement of ATP formed by Chromaffin Granule Membranes using the Luciferase:Luciferin assay. The determination was carried out as described in the text. The protein concentration of the particles is approx. 0.15μg per assay, in a total volume of 1ml.

TABLE 2.3.13.

Conditions	ATP formed (Arbitrary units)
1. Membranes + NADH,ascorbate,O ₂	116
2. Membranes + NADH,ascorbate,O ₂ and ferricyanide.	216
3. Membranes: ATP exchange	95
4. Blank: no membranes	50

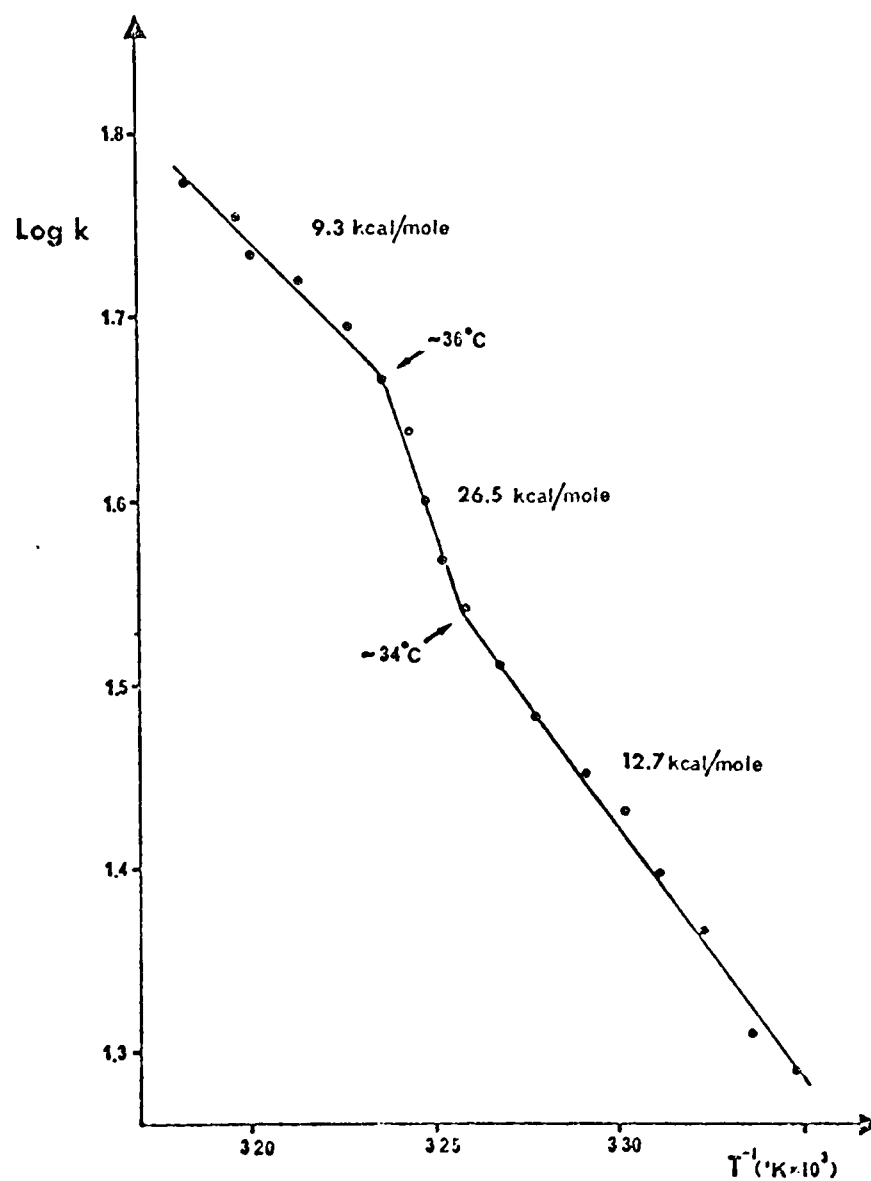
5. Granules + NADH,ascorbate,O ₂	107
6. Granules + NADH,ascorbate,O ₂	20
7. Granules: ATP exchange	160

The measurement of ATP formed by Chromaffin Granules and by Chromaffin Granule Membrane Vesicles using a radioactivity transfer assay. The assays were carried out as described in the text. Each assay mixture contained ADP, 1mM, Pi, 0.5mM, and ³²Pi (equivalent to approximately 660,000 cpm). NADH was 20μM; Fe(CN)₆³⁻ was 50μM. The membrane particles were at a final concentration of 0.3mg/ml and the granules, 0.25mg/ml. ATP when present was 1mM. Reactions were carried out in MES, 10mM, MgCl₂, 1mM, pH 6.6.

incorporation of ^{32}P into ATP by intact granules followed a different pattern. In this case, the incorporation by exchange was greater than that in the presence of NADH and this in turn was greater than that in the presence of NADH and ferricyanide. With both intact granules and granule membranes, there was the same degree of incorporation after 15 minute and 30 minute incubations, in all conditions. The presence of S-13, oligomycin, dopamine or antimycin A, at various concentrations had no effect; neither did an alteration in the pH from 6.6 to 7.9. (Table 2.3.13.)

It was concluded, therefore, that the chromaffin granule ATPase cannot make ATP under the in vitro conditions used, even when the electron transport chain is primed with NADH, ferricyanide and oxygen. This may be due to the very low activity of the electron transfer chain of the chromaffin granule compared to those of other systems (11).

FIGURE 2.3.16.



The temperature dependence of the ATPase activity of chromaffin granule membranes. Conditions as described in text. $\log k$ is the logarithm of the activity expressed in $\mu\text{moles phosphate/minute/mg protein}$. The activation energies of the different linear portions are also given.

E. The Temperature Dependence of Membrane-Bound Enzyme Activities.

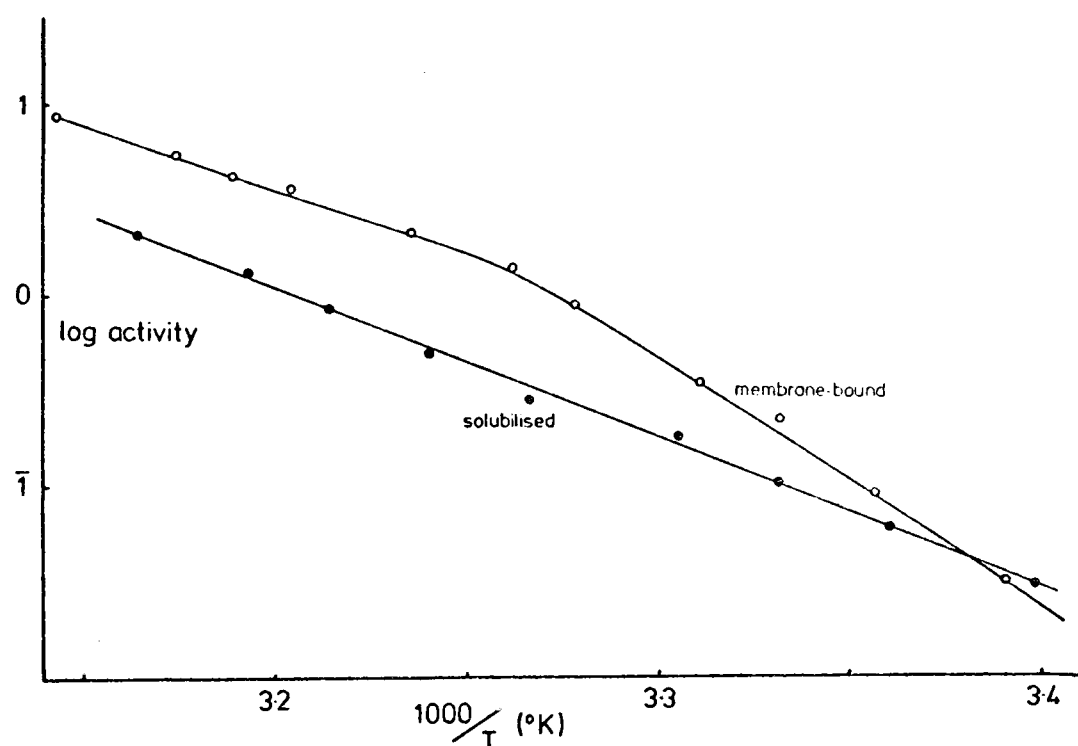
In view of the unusual lipid composition of the chromaffin granule membrane (40), a study was initiated, using magnetic resonance techniques and fluorescence (Chapters Three and Four), of the structural and dynamic properties of the membrane. Many such properties often exhibit a marked temperature dependence. In order to correlate any such changes in these properties of the lipids with the biochemical activities of the system, the temperature dependence of the ATPase and of the NADH:(acceptor) oxidoreductase activities were also examined.

(i) The ATPase Activity

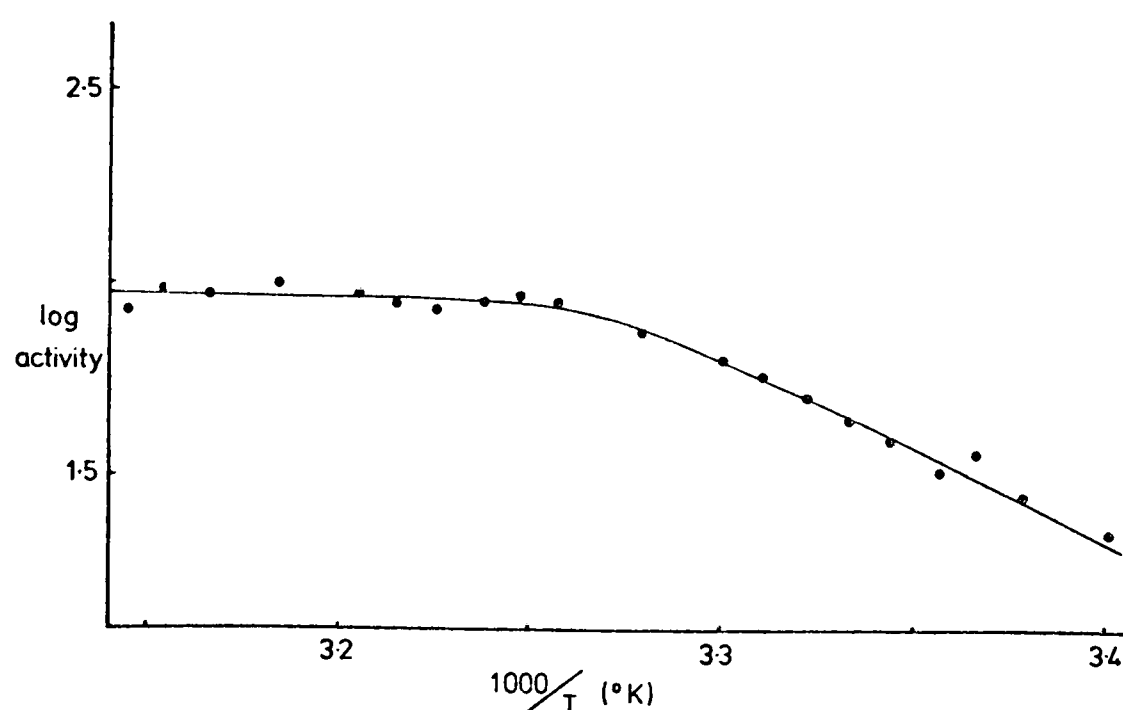
Small temperature increments were used in this study since, very often, the breaks observed in the Arrhenius plots of enzyme activities are small and within experimental error insignificant if there are insufficient points in the graph. In these experiments, every point is an average of three separate assays. Repeats at low temperatures were made once the full temperature range had been completed since the experiment extended over more than eight hours. The Arrhenius plot of the enzyme activity versus temperature is presented in Figure 2.3.16. The plot has two breaks, not the normally observed single break. These discontinuities occur at approximately 34°C and 36°C which are very close indeed to the physiological range. The activation energies over the three approximately linear portions are also shown.

(ii) The NADH:(Acceptor) Oxidoreductase Activities.

The temperature dependence of this enzyme activity in intact granules, using an endogenous acceptor was investigated. The rate of activity was fairly low. In the Arrhenius plot of this activity, only one break was observed, in this case at approximately 33°C (Figure 2.3.17.). Under conditions where the lipid-protein interactions were disrupted ie. in the presence of the detergent Lubrol PX, the NADH:(acceptor) oxidoreductase activity no longer retained the same temperature dependence.

FIGURE 2.3.17.

The temperature dependence of the activity of the NADH;(acceptor) oxidoreductase of the chromaffin granule membrane, in the absence and the presence of Lubrol-PX. Conditions of assay as (17).

FIGURE 2.3.18.

The temperature dependence of the activity of the NADH:(ferricyanide) oxidoreductase of the chromaffin granule membrane. Conditions as described in text and (17).

The rate is linear with no apparent discontinuities (Figure 2.3.17.).

The temperature dependence of the NADH:ferricyanide oxidoreductase activity of granule membranes was also examined. The Arrhenius plot, in this case, was also biphasic with a break at approximately 34°C (Figure 2.3.18.). What is unusual in this plot is the apparent lack of temperature dependence of this activity above 34°C. This may mean that the activity is diffusion limited above this temperature.

The discontinuities in the Arrhenius plots of the ATPase, NADH:(acceptor) oxidoreductase and the NADH:ferricyanide oxidoreductase activities all occur at around 34°C, very close to the physiological temperature. These results will be discussed in the context of the other studies in Chapter 3.4.

2.4. Discussion

How realistic is the proposed model?

The finding that the ATPase activity of the chromaffin granule, and to a lesser extent that of the chromaffin granule membrane, are sensitive to uncouplers has far reaching and important implications. It is known that the energy linked ATPase activities of mitochondria, submitochondrial particles and the plasma membranes of some microorganisms are sensitive to these uncoupling agents (8). Changes in ANS fluorescence have also been correlated with 'energisation' in submitochondrial particles (22). It is believed that this is the first report of an uncoupler-sensitive ATPase activity not of this general type.

Both the uncoupled ATPase activity and the ANS response were highly sensitive to pH, with a pH optimum for the uncoupled ATPase activity and for the fluorescence enhancement at around pH 6.4. No significance is attached to this finding, except that this pH is also close to the pH optimum of the enzyme dopamine- β -hydroxylase, (41). What is considered significant is the finding that at pHs less than 5.5, the presence of uncoupler molecules actually enhanced the fluorescence of the ANS instead of collapsing it. The coupled and uncoupled ATPase activities also crossover at around this pH, as do the coupled and uncoupled adrenaline uptakes in the absence of ATP-Mg²⁺. Two points should be noted, however. Firstly, the ANS response itself is not very large at pHs less than 5.5, therefore the increase in absolute fluorescence on the addition of uncoupler is fairly small although it corresponds to a large percentage increment. Secondly, the activity of the ATPase at low pH is also small, hence difficult to measure with the necessary accuracy, if a definite statement of a crossover in the coupled and uncoupled activities is to be made.

One interpretation of these results is that the ATPase activity of the chromaffin granule is associated with the movement of cations,

probably protons, across or into the membrane. Such a movement of protons would lead to the development of both electrical and chemical potentials across the membrane. The ANS response to energisation may be to either of these components. However, the uncoupling observed under conditions where the membrane is 'leaky' with respect to the charged species eg. in the presence of the permeant anion, thiocyanate, indicates that the element due to electrical potential is important both in terms of the ATPase activity and the ANS response to energisation. It was also noted that the degree of uncoupler sensitivity found with membrane vesicles was much less than that found with intact granules. This may be interpreted in terms of a leakiness of the membrane ie. a permeability of the membrane to cations and protons as a result of the preparative procedure. Other interpretations were discussed, previously, in the text.

If the role of the uncoupler is to equilibrate protons across the membrane, as the chemiosmotic hypothesis proposes, removing the restraint on the ATPase, then it can be inferred that the pH of the chromaffin granule matrix is around pH 5.5. An independent series of experiments using ^{31}P NMR have been carried out to confirm this. These are described in Chapter Four.

The coupling of the ATPase activity to a 'Potential Energy Pool' was one of the fundamental characteristics of the proposed model. According to this model, when the ATPase activity is uncoupled, it cannot supply energy to the system, as the proposed electrochemical potential is dissipated. Thus when uncoupled, the ATPase should not drive catecholamine uptake. This is precisely what was found. The uptake by both intact granules and membrane vesicles was inhibited when uncoupler was present. In some cases the uptake in the presence of uncouplers was even less than in the diffusion limited case. The inhibition of the uptake was dependent on the concentration of the uncoupler used, and also on the efficiency of the uncoupler, as was the case with the enhancement of the ATPase activity. The inhibition of uptake is independent of the catecholamine present; adrenaline, noradrenaline and

dopamine uptake are all inhibited. Reserpine has no effect on the inhibition. It is interesting that the permeant anion, thiocyanate, which enhanced the ATPase activity also inhibited the catecholamine uptake.

This is excellent evidence for the proposal that the catecholamine uptake is not a direct result of the ATPase activity, but is a consequence of the development of a cation or proton gradient across the membrane. It is believed that this has not previously been shown for the chromaffin granule or related secretory systems.

The uptake by this method appears to be catecholamine specific since the uptake of methylamine is not significantly greater than the control value, is not enhanced by the presence of ATP-Mg²⁺ and is not abolished by the presence of uncouplers. Thus it can be postulated that a transport protein specific for the catecholamines and 'energised' by the electrochemical potential may be present in the membrane of the chromaffin granule.

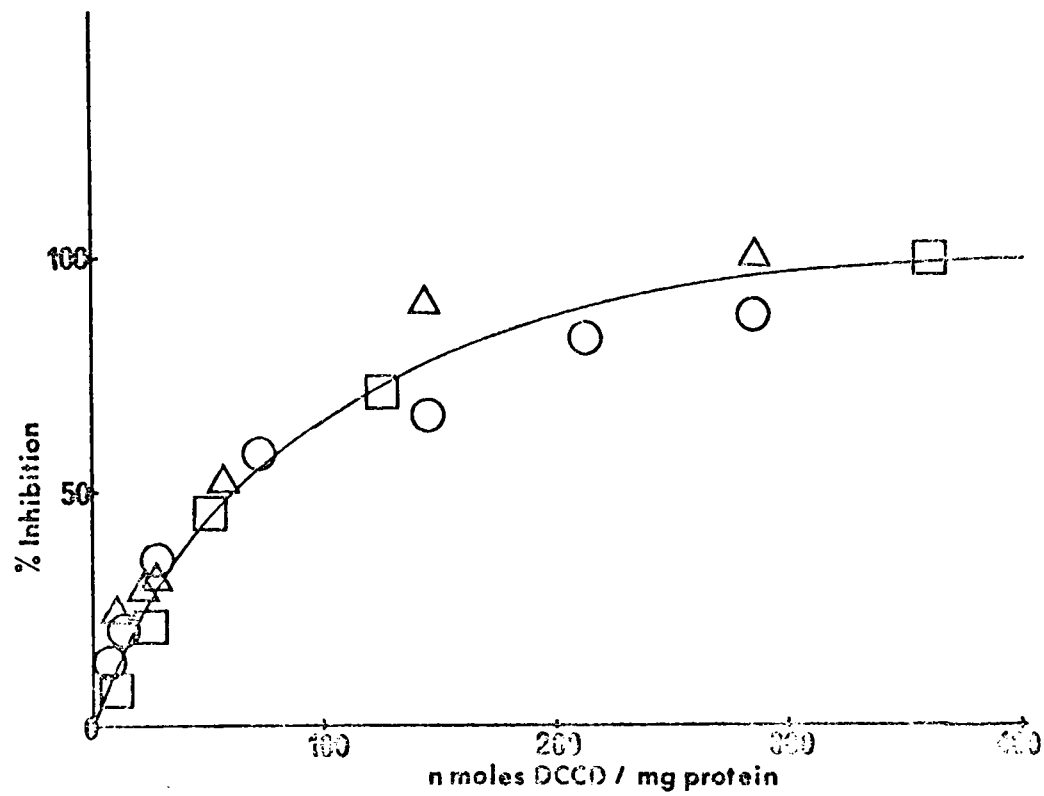
Thus part of the proposed model would appear to be relatively accurate. Unfortunately none of the experiments designed to confirm the role of the electron transfer chain in this system produced positive results. The activity of the NADH:(acceptor) oxidoreductase activity of the granule membrane is low, as is the NADH:ferricyanide oxidoreductase activity. In the experiments carried out in this laboratory, following the methods of Terland and Flatmark (17), it was not possible to reproduce their quoted activities (25). The activity quoted by Terland and Flatmark is very much less than that reported for the mitochondrial electron transfer system.

From the model, the electron transfer chain should be capable of driving catecholamine uptake in the absence of ATP, inhibiting the coupled or the uncoupled ATPase activity by effectively increasing the coupling of the ATPase, and under favourable conditions, promoting the synthesis of ATP from ADP and Pi. The NADH:(acceptor) oxidoreductase activity should also be uncoupler sensitive. In practice, none of these

functions could be demonstrated. The effect of uncouplers on the NADH:(acceptor) oxidoreductase activity was inconclusive. On some occasions there was demonstrable activation of the activity in the presence of S-13, but on other occasions, no activation was observed. It is not possible to assay this activity in whole granules due to the presence of the catecholamines. This is unfortunate since the uncoupling, if present, would probably be more marked, as in the case of the ATPase, in the whole granule system. The presence of NADH, ascorbate and oxygen in the absence of ATP-Mg^{2+} did not produce significant uptake of adrenaline, and this uptake was not affected by uncouplers. Similarly, the ATPase activity of both intact granules and membrane vesicles were not affected by the presence of NADH (and ascorbate, in the case of the membrane vesicles). It was not possible to demonstrate ATP synthesis by either granules or membrane vesicles under a variety of conditions. Thus it has not been possible to prove or disprove the upper portion of the proposed model. No role for the electron transfer chain in the granule membrane has yet been found.

However, in view of the very low activity of this respiratory chain, these results are not altogether surprising. It is probably essential if a function is to be conclusively demonstrated for the electron transfer chain in the chromaffin granule membrane that an increased NADH:(acceptor) oxidoreductase activity is obtained in vitro. It is possible that components of the electron transfer chain are lost during the isolation of the chromaffin granules and the membrane vesicles prepared from them. Whilst there is no evidence that this is the case, a comparison of the electron transfer chain of the chromaffin granule with that of the mitochondrion shows that the former contains three fewer components. These all occur between cytochrome b_{561} and the cytochrome oxidase. It is known that cytochrome c can be incorporated into the membrane, and via it the flow of electrons can bypass cytochrome b_{561} (Figure 1.2.3.). It would be interesting to investigate whether other biological electron carriers could also be incorporated into

FIGURE 2.4.1.



A comparison of the concentration dependence of the DCCD inhibitions of the ATPase activity, the ANS response and catecholamine uptake by chromaffin granule membrane vesicles. Conditions for all assays were as similar as possible. The conditions are described in the text in section 2.3. of this chapter.

Key: ○ ATPase activity,
 △ ANS response,
 □ Catecholamine uptake.

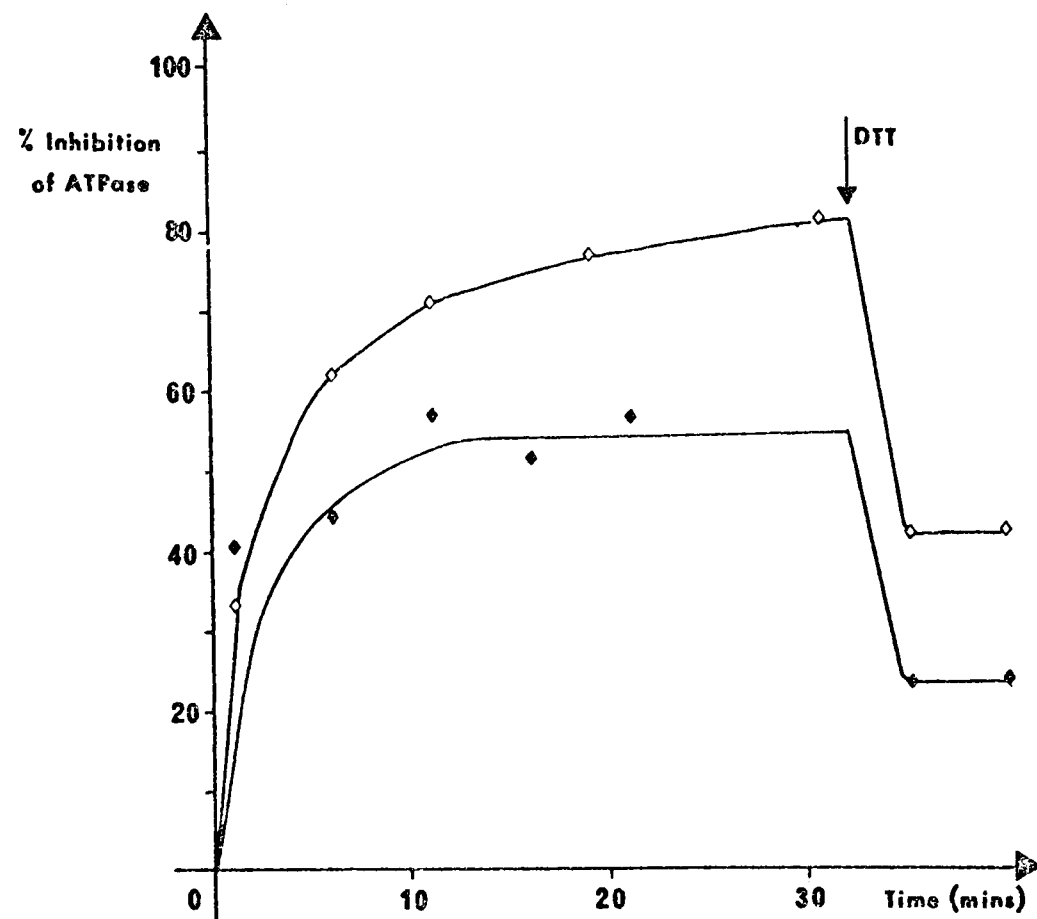
the chromaffin granule membrane, and enhanced activity of the respiratory chain promoted.

This model for the electron transfer chain : ATPase system should not be discarded until it can be shown that none of the proposed properties of the electron transfer chain are correct when a considerably enhanced NADH:(acceptor) oxidoreductase activity is present.

Other experiments reported in this chapter were designed to examine the similarity of the mitochondrial and chromaffin granule ATPases in view of the uncoupler sensitivity of the chromaffin granule ATPase. DCCD has been shown to inhibit the mitochondrial ATPase, F_1 , in submitochondrial particles and also the plasma membrane ATPases of various microorganisms (42). It does not inhibit soluble F_1 except in very high concentrations (43). DCCD was found to inhibit the chromaffin granule ATPase, the ANS response and the uptake of adrenaline by chromaffin granule membrane vesicles. The concentration dependences of these inhibitions are shown in Figure 2.4.1. The similarity of the dependence of the degrees of inhibition on the concentration of DCCD is striking. This is not in agreement with the results from the submitochondrial particles, where the magnitude of the ANS response is not affected even although the ATPase is substantially inhibited by certain concentrations of DCCD (33). DCCD has been shown to bind specifically to a proteolipid component, M.Wt. 10,000 daltons, associated with F_1 in submitochondrial particles. In view of the solubilisation results (Table 2.3.9.) which show that solubilisation of the DCCD inhibited chromaffin granule ATPase does not reverse the inhibition by greater than 20%, it is not unreasonable to propose that DCCD either binds directly to the chromaffin granule ATPase or to a minor component closely associated with it. The DCCD inhibition shows very clearly the dependence of the ANS response and the catecholamine uptake on an active ATPase.

Earlier experiments, using the sulphydryl reagents N-ethyl maleimide and bis-(N-maleimide ethyl) ether whilst showing that they caused

FIGURE 2.4.2.



The inhibition of the chromaffin granule membrane ATPase by the reagent *Nbf-Cl* (0.2mM). The conditions used for labelling were similar to those used by Ferguson *et al* (44). The upper curve (open symbols) was at pH 7.9 and the lower curve (closed symbols) was at pH 6.4. The partial reversal of the inhibition by an undefined but excess amount of dithiothreitol is also shown.

inhibition not only of the ATPase but also of the catecholamine uptake, could demonstrate no specificity of the binding apart from the fact that the presence of ATP sometimes partially protected the ATPase activity from inhibition (6). The proposed unique labelling of DCCD could be demonstrated using radioactive DCCD. Unfortunately this is not commercially available, and it was not possible to obtain any before this study was curtailed.

The membrane was also labelled with Nbf-Cl which under certain conditions binds to a unique tyrosine residue on the purified mitochondrial ATPase (44). Nbf-Cl can also bind to sulphhydryl residues. The inhibition of the chromaffin granule ATPase by this reagent was clearly shown (Figure 2.4.2.). This inhibition was reversed by dithiothreitol, DTT. However, until the ATPase has been purified completely or the equivalent of sub-mitochondrial particles formed, it will not be possible to determine the nature of the binding of Nbf-Cl to the chromaffin granule membrane ATPase, since this is normally done spectrophotometrically and hence requires almost unique binding. One major difference between the chromaffin granule ATPase and F_1 is the failure of the former to show oligomycin sensitivity. This sensitivity is conferred on the soluble F_1 by an oligomycin-sensitivity conferring protein and is not a function of the ATPase itself (45).

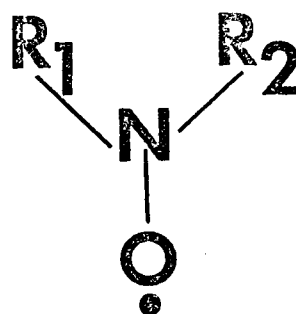
It will be interesting when the chromaffin granule ATPase is finally purified and its structure and subunit composition investigated thoroughly, to see if it bears any relationship to the mitochondrial ATPase, the chloroplast ATPase and the ATPases in the plasma membranes of certain microorganisms. It has been reported to have a large molecular weight of less than 500,000 daltons on the basis of fairly crude analysis (7). All these other ATPases have molecular weights of around 350,000 daltons (46,47). In view of the results reported in this chapter, it is not inconceivable that the ATPase of the chromaffin granule, and by inference those of related secretory vesicles, in fact belong to the class of coupled ATPases typified by the mitochondrial ATPase, F_1 .

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FIGURE 3.1.1.



The general formula of a nitroxide spin label molecule.

CHAPTER THREE

Electron Spin Resonance Studies of the Chromaffin Granule and the Chromaffin Granule Membrane.

3.1 Introduction

One of the most interesting and potentially important spectroscopic methods used to investigate the molecular structure of membranes is Electron Spin Resonance, ESR, used in conjunction with paramagnetic reporter molecules, the 'Spin Labels'. Spin labels are analogous to the optically absorbing or fluorescent molecules that are so useful in other spectroscopic techniques. The spin label is either covalently attached to specific functional groups in the system under investigation or else is diffused into the region of interest eg. hydrophobic regions.

The spin label probes used to investigate membrane structure are the organic free radicals, the nitroxides, which have the general formula shown in Figure 3.1.1. The nitroxide moiety contains the unpaired electron that renders the molecule paramagnetic, and hence suitable for detection by ESR. The alkyl side chain confers stability on the free radical, whilst the remainder of the molecule can contain various chemically reactive groups to permit covalent labelling. The nitroxide moiety, itself, is relatively unreactive under the majority of conditions.

The spin labels normally used to investigate membrane structure are fatty acid and cholesterol derivatives. These spin labels, when introduced into biomembranes, model membranes and membrane component systems reflect the motion and orientation of the membrane lipids, and the polarity of their environment. Since the parameters of the ESR spectrum are sensitive not only to these variables, but also to the proximity of other spin labels, there is a diverse range of applications of this technique to biological

problems.

In view of the unusual phospholipid composition of the chromaffin granule membrane and the proposed involvement of the lysolecithin present in the membrane in the mechanism of exocytosis, both discussed in Chapter One, the membrane was studied using both lipophilic and covalently binding spin labels. The aim was to determine whether the properties of the membrane as detected by spin labels could account for their proposed involvement in the secretory process, including fusion with the cell plasma membrane

3.2 The Theory of Electron Spin Resonance

The technique of ESR detects and characterises the presence of unpaired electrons. The essential property detected is the magnetic dipole moment, $\underline{\mu}$, associated with the electron spin. Although a rigorous description of the phenomenon of ESR must come from quantum mechanics (1,2), useful analogies may be drawn from classical physics.

Magnetic moments tend to be oriented by a magnetic field, $\underline{H}$. Work must be done to disorient a magnetic moment, hence it can be inferred that there is a potential energy difference between a condition of arbitrary orientation of $\underline{\mu}$ and $\underline{H}$, and one in which $\underline{\mu}$ and $\underline{H}$ are parallel. The amount of work, E , required to disalign $\underline{\mu}$ and $\underline{H}$ is given by :

$$\begin{aligned} E &= -\underline{\mu} \cdot \underline{H} = -\mu H \cos(\underline{\mu} \cdot \underline{H}) \\ &= \mu_z H \end{aligned} \quad 3.1$$

where μ is the magnitude of the magnetic moment, $\underline{\mu}$,

μ_z is the projection of $\underline{\mu}$ along the z-axis,

H is the magnitude of the magnetic field, $\underline{H}$,

$\cos(\underline{\mu} \cdot \underline{H})$ is the cosine of the angle between $\underline{\mu}$ and $\underline{H}$.

The electron magnetic moment can be considered as arising from the spinning of an electron about its axis. All electrons have an intrinsic spin, characterised by a spin angular momentum, $\underline{P}$. $\underline{P}$ and $\underline{\mu}$ are always proportional;

$$\underline{\mu} = \gamma \underline{P} \quad 3.2$$

where γ is the magnetogyric ratio.

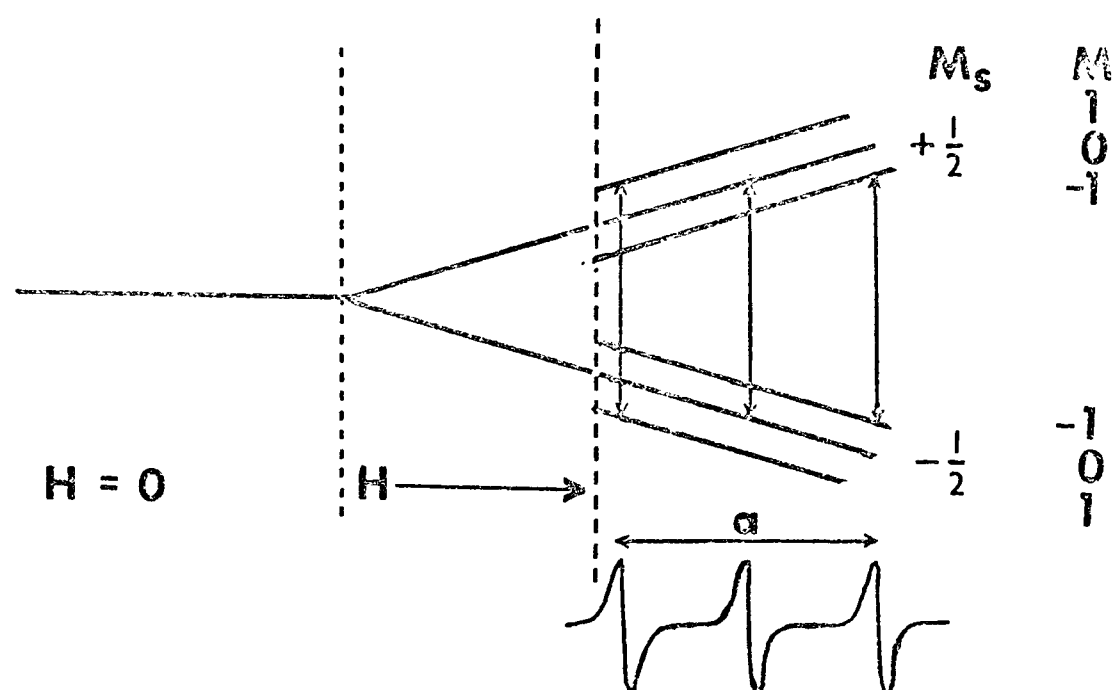
From quantum mechanics, the component of $\underline{P}$, and hence of $\underline{\mu}$, along a given direction can only have two values. If P_z is this component then:

$$P_z = M_s \frac{h}{2\pi} = M_s \hbar \quad 3.3$$

where $\hbar$ is Planck's constant,

M_s is the spin quantum number, equivalent to $\pm \frac{1}{2}$ for an electron spin.

FIGURE 3.2.1.



The energy levels of the spin moments of an electron as a function of the magnetic field

For classical motion,

$$\gamma = - \frac{e}{2mc} \quad 3.4$$

where e is the charge of the electron,

m is the mass of the electron,

c is the speed of light.

By substitution of Eqn. 3.4. in Eqn. 3.2., and by consideration of the components of μ in the z direction,

$$\mu_z = - \frac{eh}{4mc} M_s \quad 3.5$$

Eqn. 3.5. applies to the classical rotational motion of a charge about a fixed centre, and hence applies to the orbital motion of an electron about a nucleus. Spin angular momentum, P , is, however, a purely quantum mechanical effect and cannot be described accurately by classical methods. Hence, for electron spin, Eqn. 3.5. must be rewritten as:

$$\mu_z = -g \frac{eh}{4mc} M_s = -g\beta M_s \quad 3.6$$

where β is the Bohr magneton,

g is 2.00232 for a free electron.

By substitution of Eqn. 3.6. into Eqn. 3.1., the energies of electron spin moments in a magnetic field are given by :

$$E = g\beta H M_s = \pm \frac{1}{2} g\beta H \quad 3.7$$

These energies are expressed as a function of magnetic field in Figure 3.2.1.

Now, if the sample is irradiated with electromagnetic radiation at a certain magnetic field, H_r , absorption will occur. It is apparent that at H_r , the photon energy, $h\nu$, must match the separation of energy levels.

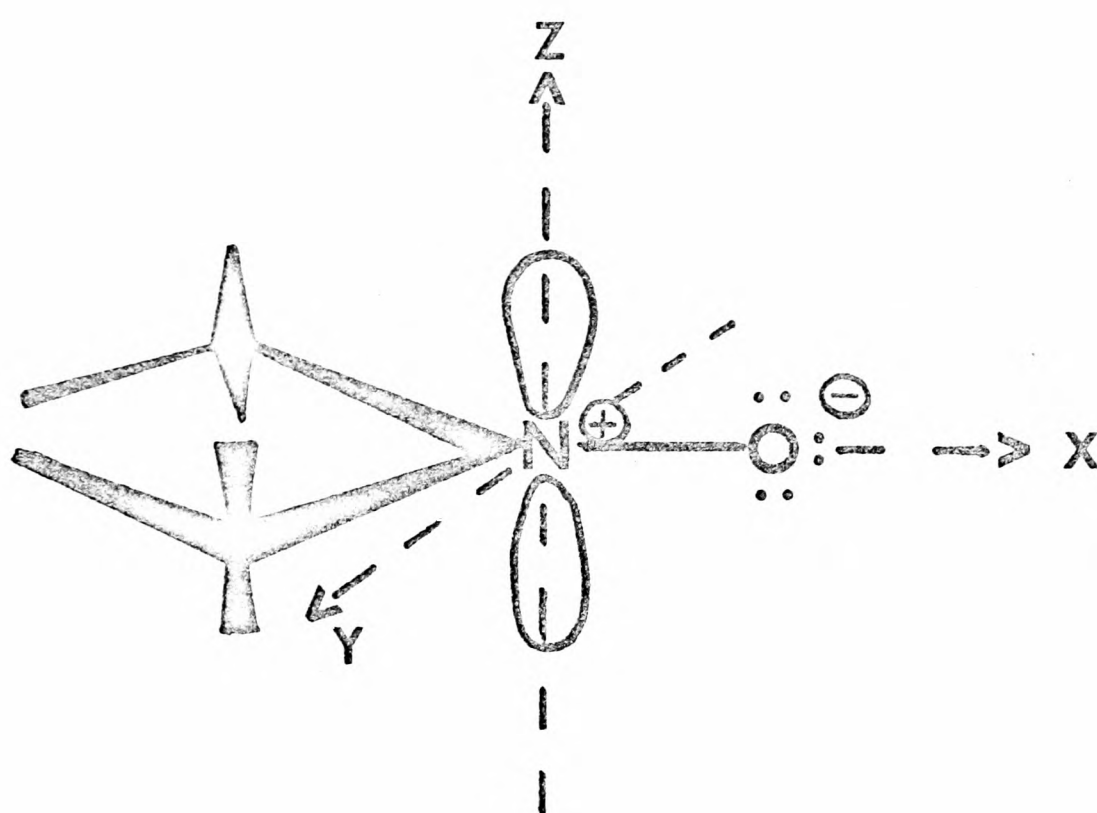
Thus the resonance condition is

$$h\nu = g\beta_e H_r \quad 3.8$$

where H_r is the resonance field.

The second condition for absorption is when the magnetic component of the microwaves is oriented perpendicular to the direction of the static magnetic field. This condition is easily met at microwave

FIGURE 3.2.2.



The conventional axis system for a nitroxide molecule.

frequency. Eqn. 3.8. is satisfied over a wide range of frequency and magnetic fields. For sensitivity reasons, frequencies in the microwave region were used. Typically for a frequency of 9.5GHz, the magnetic field, H_x , is 3400 Gauss.

More detailed ESR theory is adequately described elsewhere (1,2). Particularly important to an understanding and interpretation of spin label spectra are the nuclear hyperfine interaction and the g factor. These are discussed especially well in reference (1).

A. Information in a Nitroxide Spin Label Spectrum

There are three forms of useful parameters present in nitroxide spin label spectra. These are the g tensor, the nitrogen hyperfine coupling tensor and the width of the individual ESR lines. From their combination, information can be derived about the mobility, orientation and environment of the nitroxide.

(i) The Hyperfine Coupling Tensor and the g Tensor

The spin Hamiltonian which adequately describes the ESR spectrum of the nitroxide radicals, omitting the nuclear Zeeman factor, is:

$$\hat{H} = \beta_e \underline{\underline{H}} \cdot \underline{\underline{g}} \cdot \hat{\underline{\underline{S}}} + h \hat{\underline{\underline{S}}} \cdot \underline{\underline{A}} \cdot \hat{\underline{\underline{I}}} \quad 3.9$$

where $\underline{\underline{g}}$ is the g value tensor,

$\underline{\underline{A}}$ is the hyperfine coupling tensor,

β_e is the electron Bohr Magneton,

$\underline{\underline{H}}$ is the external magnetic field,

$\hat{\underline{\underline{S}}}$ is the electron spin operator,

$\hat{\underline{\underline{I}}}$ is the nuclear spin operator.

This equation is derived in reference (1).

The conventional axis system for nitroxides is defined in Figure 3.2.2. The unpaired electron is considered to occupy a molecular orbital composed of the $p\pi$ orbitals of the nitrogen and the oxygen. The observed values for the components of the hyperfine tensor indicate that a substantial fraction of the unpaired electron is localized on the

nitrogen atom, and, therefore, suggest that the nitrogen-oxygen, three electron π bond has polar character as shown in Figure 3.2.2.

Because the molecular orbital containing the unpaired electron is made up partially of a degenerate p_π orbital, the g tensor for the nitroxide moiety is expected to be anisotropic, ie. $g_{xx} \neq g_{yy} \neq g_{zz}$, (2). The hyperfine coupling tensor is also anisotropic and is often nearly axially symmetric, ie. $A_{xx} \approx A_{yy} \neq A_{zz}$, because of the occupation by the unpaired electron of a molecular orbital of π symmetry. Thus, the magnetic field value at which the ESR spectrum occurs and the separation between the hyperfine lines will depend very much on the orientation of the free radical with respect to the magnetic field. In order to obtain accurate values for the g tensor and the hyperfine coupling tensor, the dependence of the nitroxide ESR spectrum on the angles between the magnetic field and the symmetry axis of the radical must be determined. This is most easily done by doping a diamagnetic host crystal of known three-dimensional structure with the nitroxide of interest. Despite numerous attempts, it has proved very difficult to obtain dilute solid solutions of commonly used spin labels in appropriate hosts. Recently, a study of the cholestane spin label has been made (3). The resultant parameters, determined by Hubbell and McConnell, are, in gauss :

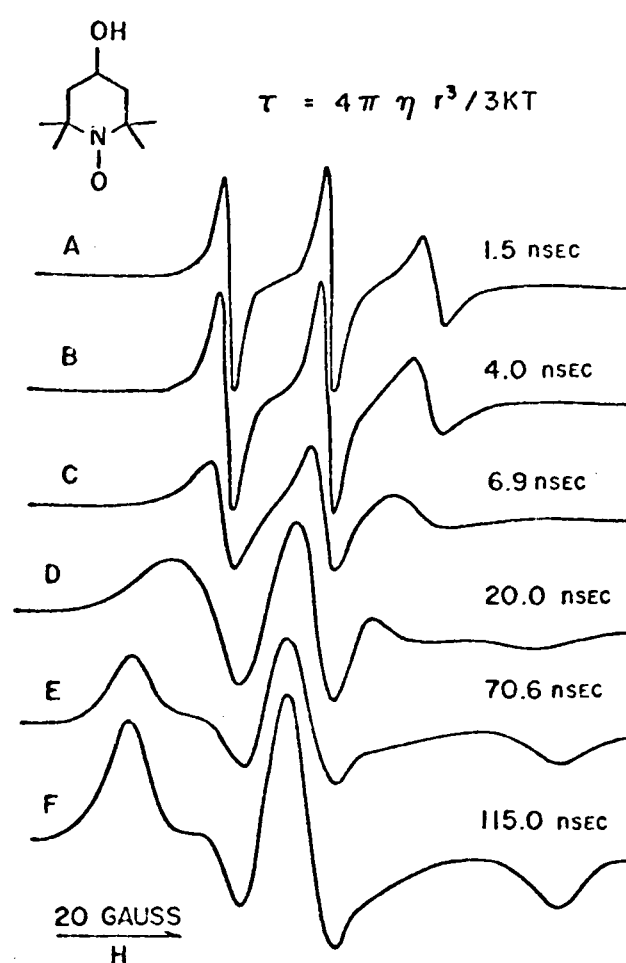
$$\begin{array}{lll} A_{xx} = 5.8 & A_{yy} = 5.8 & A_{zz} = 30.8 \pm 0.5 \\ g_{xx} = 2.0089 & g_{yy} = 2.0058 & g_{zz} = 2.0021 \pm 0.0001 \end{array}$$

Comparison of this and the data of Griffiths et al, (4) supports the generalisation :-

$$32G \approx A_{zz} \gg A_{xx} \gg A_{yy} \approx 5G ; \quad g_{xx} > g_{yy} > g_{zz} .$$

Due to the ionic nature of the nitroxide bond, the value of both the g and the hyperfine coupling tensor of a given spin label will vary with the polarity of the environment. In general, the isotropic g factor will decrease and the isotropic hyperfine splitting constant will

FIGURE 3.2.3.



ESR spectra of N-oxyl-2,2,6,6-tetramethyl-4-piperidinol in aqueous solutions of varying glycerol concentrations. (1). Spectra were measured at the following viscosities and temperatures :

A, 0.116 poise, 273°K	B, 0.367 poise, 293°K
C, 0.523 poise, 293°K	D, 1.58 poise, 283°K
E, 5.77 poise, 273°K	F, 9.4 poise, 273°K

increase as the polarity of the solvent increases (5). This sensitivity to environmental polarity makes the spin labels extremely valuable as probes in situations where perturbations are thought to cause rearrangement of the hydrophobic and hydrophilic elements in a biological system.

(ii) Isotropic Motion of Spin Labels

A spin label ESR spectrum is highly sensitive to both the nature and the rate of motion undergone by a probe molecule. If the nitroxide rotates symmetrically, at a rate greater than the frequency corresponding to the largest differences between the major components of the hyperfine coupling tensor, ie.

$$\text{the rate of tumbling} \gg |A_{ZZ} - A_{XX}| \approx 73 \text{ MHz,}$$

and of the g tensor, ie.

$$\text{the rate of tumbling} \gg |g_{XX} - g_{ZZ}| \beta H h^{-1} \approx 29 \text{ MHz,}$$

(for X band spectrometers operating at fields of approximately 3.3 KG)

then the isotropic parameters, A_0 and g_0 , are observed. The ESR lines are narrow and of equal width. When the rate of rotation about any of the axes becomes comparable to these differences, changes in the individual linewidths and overall positions occur.

The simplest case to consider is that of gradually decreasing rates of symmetrical rotation of a nitroxide radical. This is shown in Figure 3.2.3. (6). Until spectrum (c), the line position changes little, but the relative linewidths increase considerably with increasing correlation time. From (d) to (f), the high and low field lines move gradually to higher and lower frequency respectively. This is due to the manifestation of A_{ZZ} , the maximum component of the hyperfine coupling tensor. Spectrum (f) is that of a strongly immobilised spin label.

Nitroxide ESR spectra are, therefore, very sensitive to the rate of molecular motion, covering the range of correlation times of 10^{-10} to 10^{-7} seconds within which most biological molecules, including biomembranes, lie.

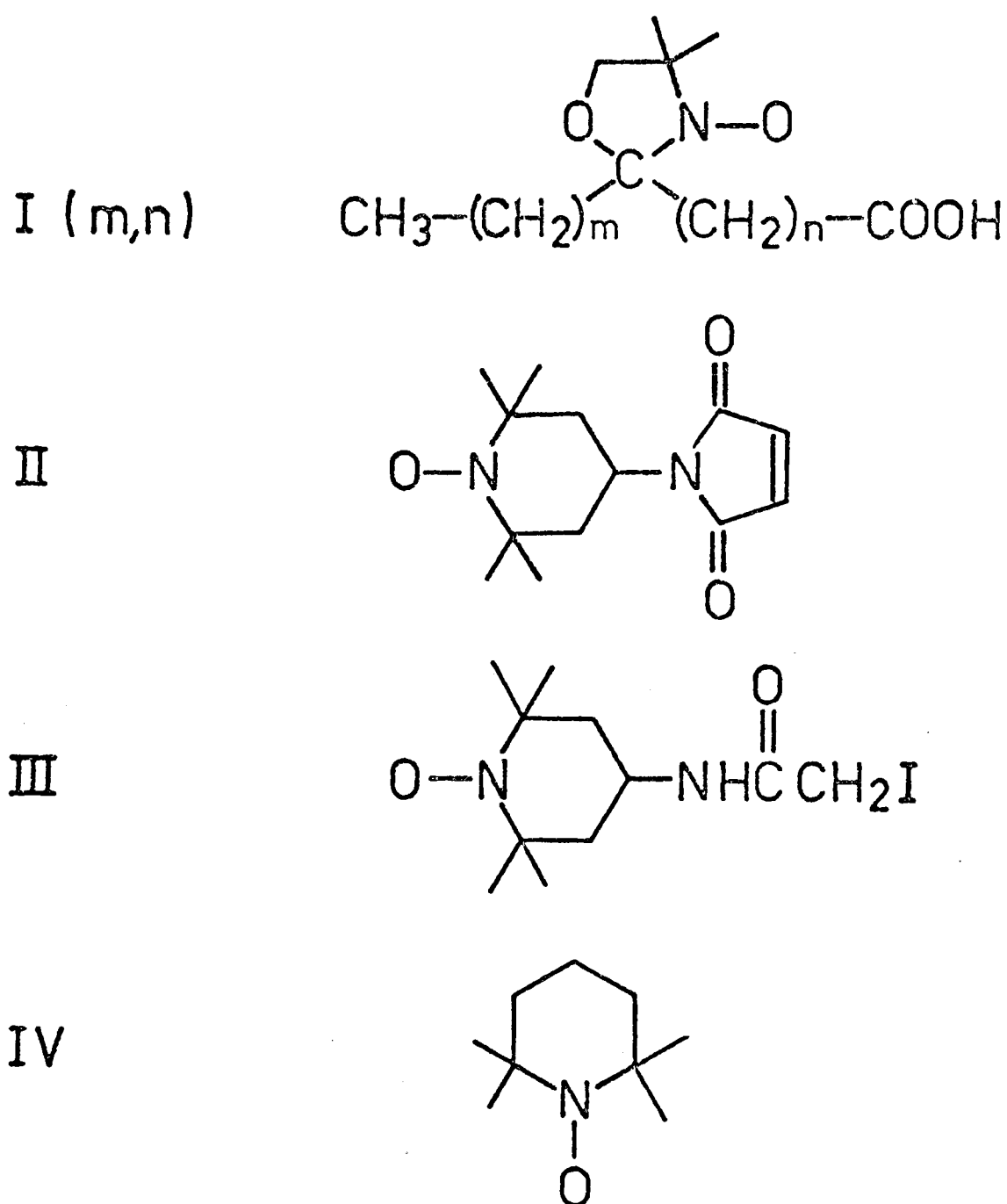
3.3 Materials and Methods

Chromaffin granules and their membranes were prepared as described in Chapter 2.2 A. The membrane pellet was then resuspended in 10mM HEPES buffer, pH 7.4, prior to spin labelling. Spin labelling was carried out by the addition of a concentrated solution of spin label in ethanol, such that the total ethanol concentration did not exceed 1%. The spin-labelled membranes were then centrifuged, and the membrane pellet homogenised with a small quantity of 10mM HEPES buffer, pH 7.4. In the case of the covalent spin labels, maleimide and iodoacetimide derivatives, the excess unreacted spin label remaining after a 30 minute to 2 hour incubation was removed by repeated washing of the membrane in 10mM HEPES, pH 7.4 , prior to the final centrifugation and homogenisation. With the stearic acid spin labels, the final step was performed immediately after the addition of spin label. In this case, the quantity of spin label added was far less than that which produces detectable interaction broadening of the spin label ESR spectrum (7). The homogenised spin-labelled membrane was sucked into thin walled glass capillaries of 1mm diameter and the ends sealed with high melting point wax. The capillaries were contained in standard 4mm quartz ESR tubes for examination in the ESR spectrometer. The sample temperature was measured by a copper-constantium thermocouple placed adjacent to the capillary in the 4mm quartz tube. The ESR spectra were recorded on a JEOLCO JES-PE-1X ESR spectrometer equipped with variable temperature facilities, and operating in the 9.5GHz frequency range.

Materials

The spin labels which were used are shown in Figure 3.3.1. They were all obtained from Sylva Corp., Palo Alto, California, USA. with the exception of the TEMPO spin label which was a gift from Prof. H.C. Berdenson of the University of Groningen, The Netherlands. All other reagents were of the highest grade commercially available.

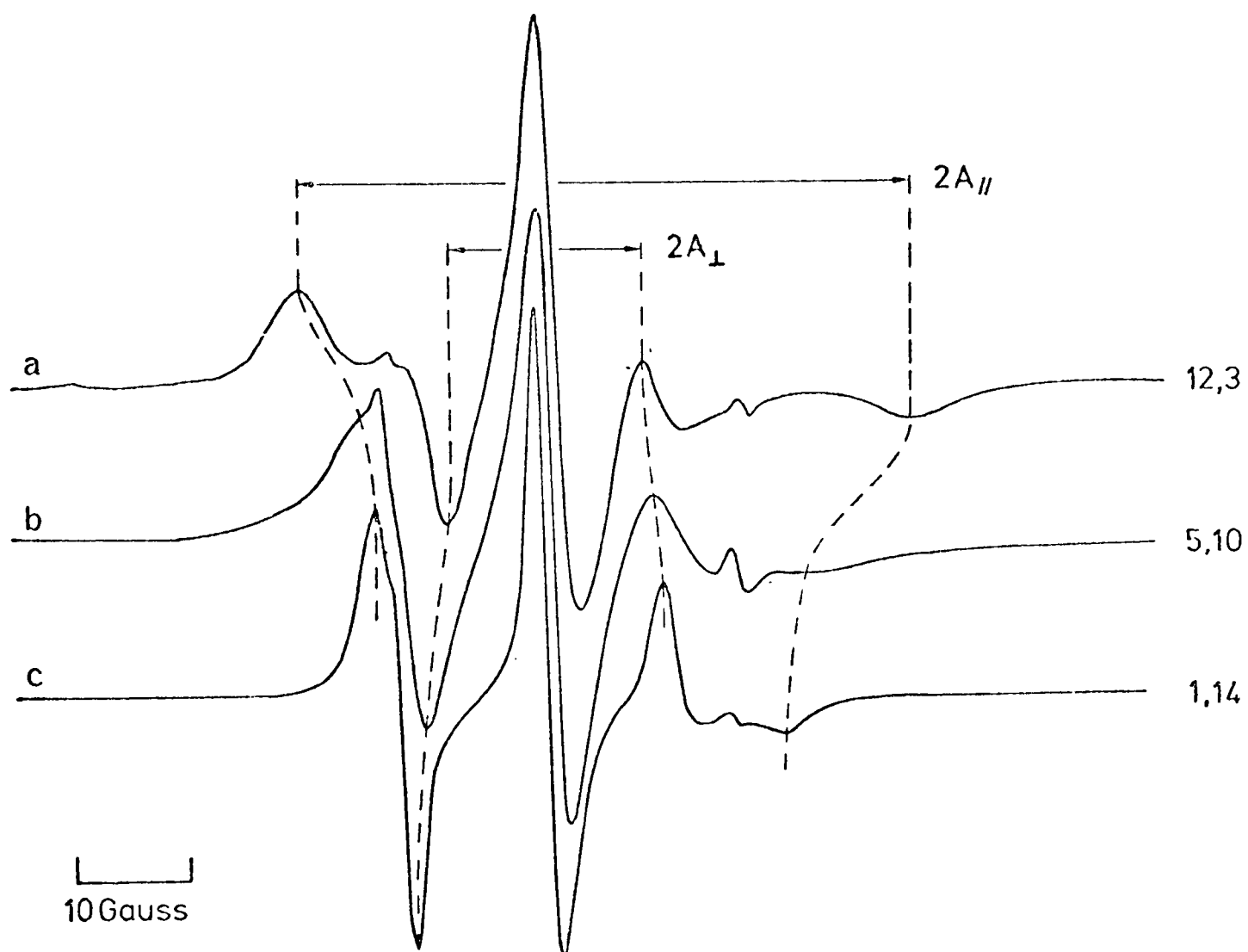
FIGURE 3.3.1.



The spin labels used in this study.

- I. (m,n) Stearic acids labelled on the (n+2) carbon atom. The isomers used were :
 - (a) 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3 oxazolidinyloxy.
 - (b) 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy
 - (c) 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy.
- II. Maleimide spin label derivative:
4-maleimido-2,2,6,6-tetramethyl-piperidinoxyl.
- III. Iodoacetimide spin label derivative:
4-(2-iodoacetimido)-2,2,6,6-tetramethyl-piperidinoxyl.
- IV. TEMPO spinlabel:
2,2,6,6-tetramethyl-piperidine-1-oxyl.

FIGURE 3.4.1.



ESR spectra of stearic acid spin labels intercalated into the lipid phase of chromaffin granule membranes, at 20°C.

*Key to spectra: (a) Label I(12,3),
 (b) Label I(5,10),
 (c) Label I(1,15).*

Modulation 10gauss; power 50mW ; frequency 9.1GHz.

3.4 Results

A. Studies on the Chromaffin Granule Membrane

The ESR spectra of the three positional isomers of the stearic acid spin label, I (Figure 3.3.1.), intercalated in the lipid phase of the chromaffin granule membrane are shown in Figure 3.4.1. The spectral splittings indicated in Figure 3.4.1. can be analysed in terms of an order parameter, S , which is a measure of the amplitude of motion of the stearic acid chain segment to which the spin label moiety is attached, and an isotropic hyperfine splitting constant, a_o , which is a measure of the effective polarity of the environment of the nitroxide moiety (7,8). The two parameters are defined by the following equations :

$$S = \frac{A_{||el} - A_{\perp r}}{A_{ZZ} - A_{XX}} \quad 3.10$$

$$a = \frac{1}{3}(A_{||el} - A_{\perp r}) \quad 3.11$$

where A_{ZZ} and A_{XX} are the values of $A_{||el}$ and $A_{\perp r}$ respectively obtained when the stearic acid chain is rigidly oriented. The values are taken from a single crystal spectrum of 2-doxyl-propane (9).

The isotropic splitting constants and the order parameters of the three stearic acid labels, along with those from red blood cell ghosts included for comparison, are given in Table 3.4.1. On proceeding down the hydrocarbon chain, there is a systematic decrease in both a_o and S which is characteristic of fatty acid spin labels in lipid bilayer structures (9,10). In the calculations for Table 3.4.1. and in all subsequent calculations, a correction of +0.8 gauss, based on spectral simulation for random membrane dispersions (11), has been added to $A_{\perp r}$ values as measured from Figure 3.4.1. A polarity correction has been made to the S parameters (12).

The temperature variation of the order parameters of the stearic acid spin labels in the membrane is shown in Figure 3.4.2. The

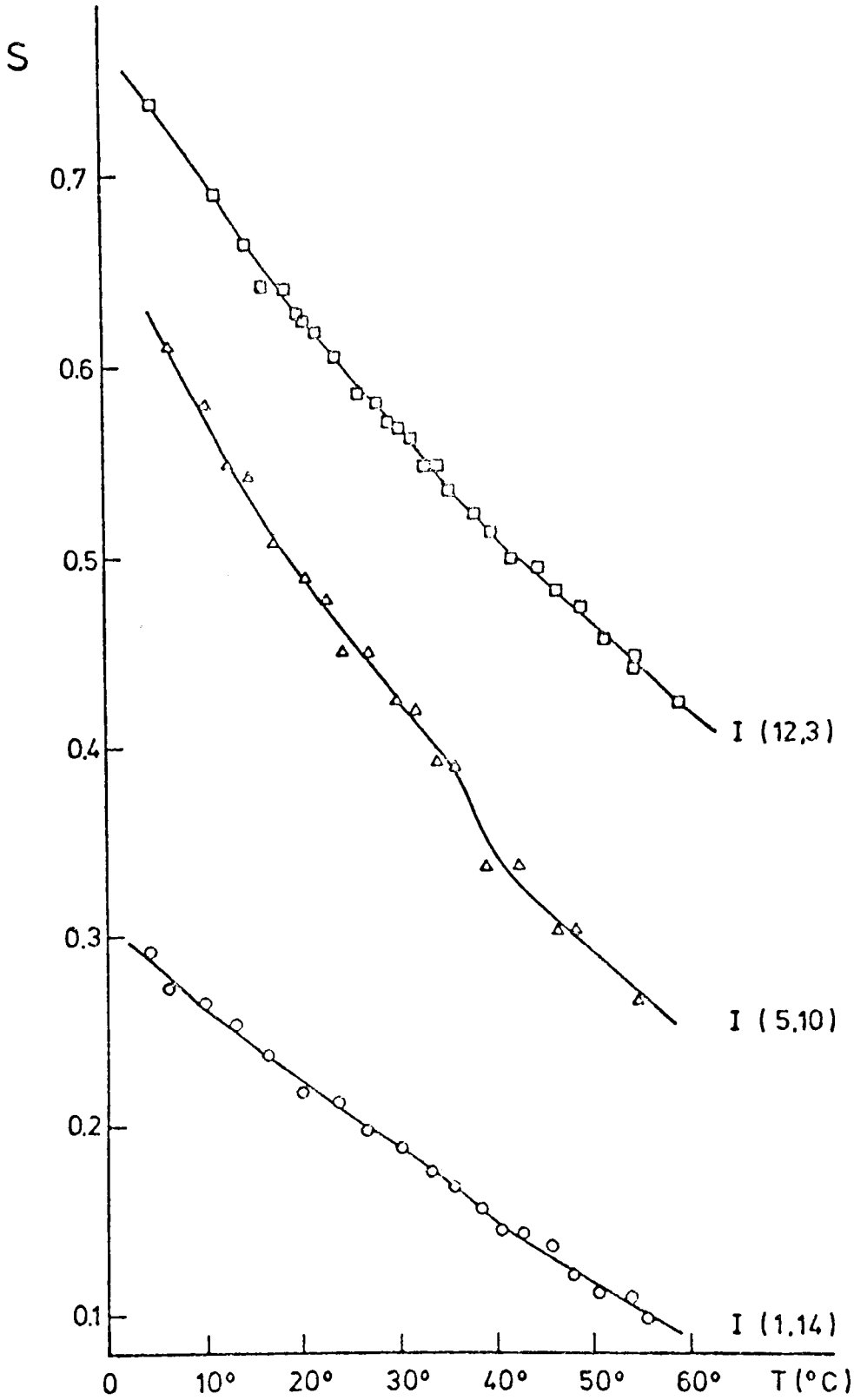
TABLE 3.4.1.

	Spin label	a_0	S
Chromaffin Granule Membranes	$I(12,3)$	15.5	0.629
	$I(5,10)$	14.5	0.489
	$I(1,14)$	13.9	0.217
Red Blood Cell Membranes	$I(12,3)$	15.8	0.693
	$I(5,10)$	14.8	0.567
	$I(1,14)$	14.4	0.282

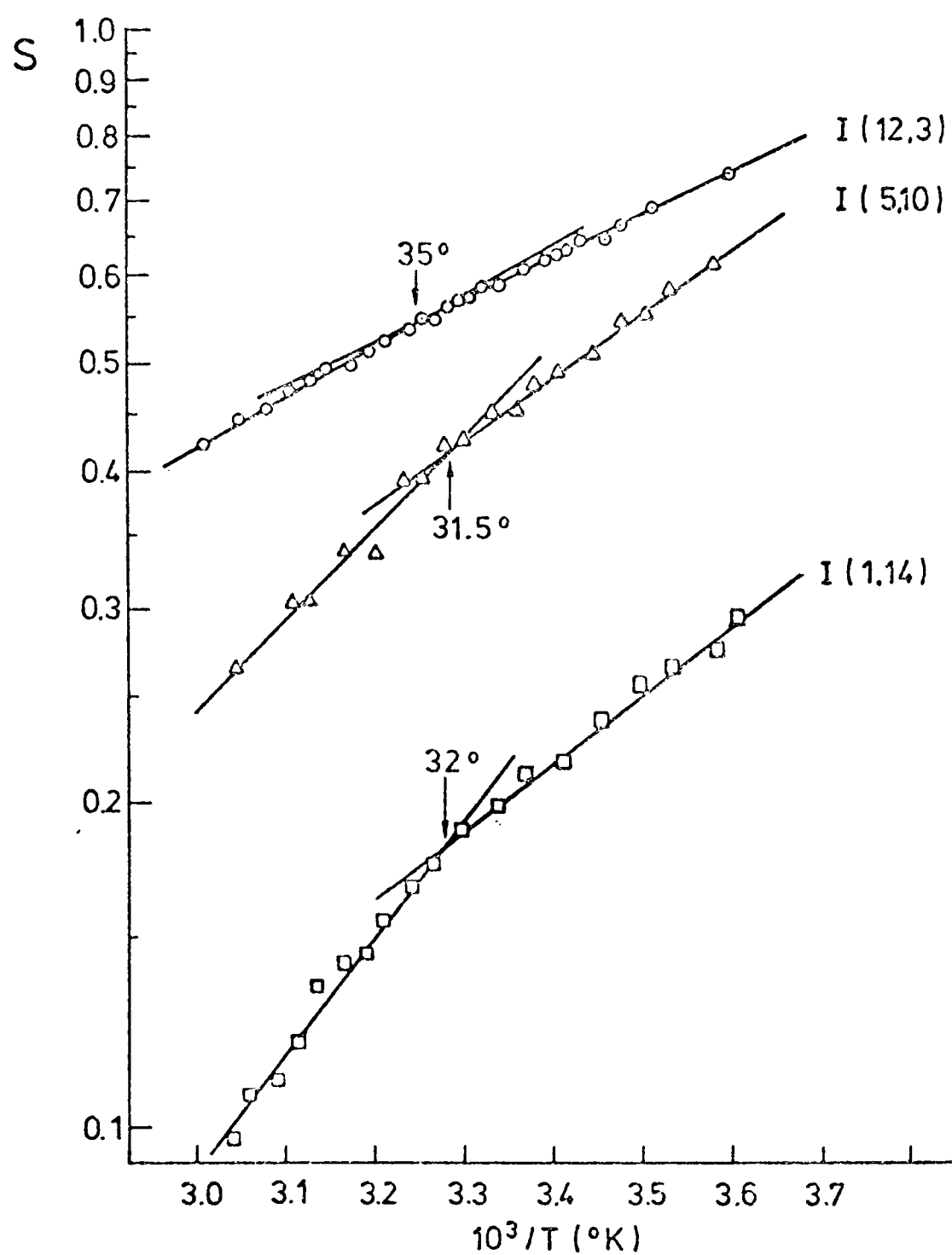
The isotropic hyperfine splittings (expressed in gauss) and the order parameters of stearic acid spin labels, $I(m,n)$, incorporated into both chromaffin granule membranes and into red blood cell membranes. Temperature, 20°C.

FIGURE 3.4.2

(a)



(b)



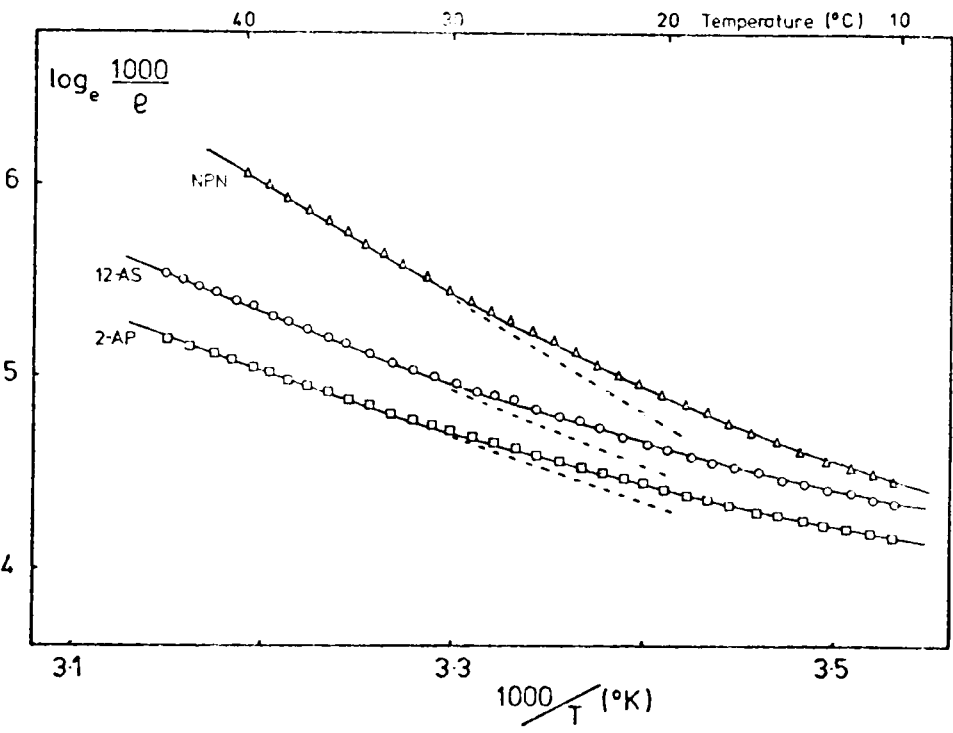
The temperature dependence of the order parameters of the $I(m,n)$ stearic acid spin labels in the chromaffin granule membrane.

(a) The order parameters plotted against temperature,

(b) The logarithm of the order parameters plotted against reciprocal temperature (°K).

Conditions as in Figure 3.4.1.

FIGURE 3.4.3:



The temperature dependence of the probe rotation rate ($10^3/\ell$) for probes bound to chromaffin granules. Data were calculated from the Perrin Equation (12).

semilogarithmic plots (Figure 3.4.2b.) reveal a biphasic dependence of the S parameters of all three labels on the reciprocal absolute temperature, with discontinuities in the region of 32° to 35°C. This is the same temperature range as the breaks in the Arrhenius plots of the ATPase and NADH : acceptor oxidoreductase activities of the membrane, described in Chapter 2.3.E. and also in the same range as the 'transition' temperatures detected using various fluorescent probes, including ANS. Results of these comparable fluorescence studies are shown in Figure 3.4.3. This work by Mr C.L.Bashford is described in detail elsewhere (12,13).

The effect of calcium on the chromaffin granule membranes as reflected by the stearic acid spin label order parameters is given in Table 3.4.2. It is seen that for all three labels, the order parameters increase on the addition of Ca^{2+} .

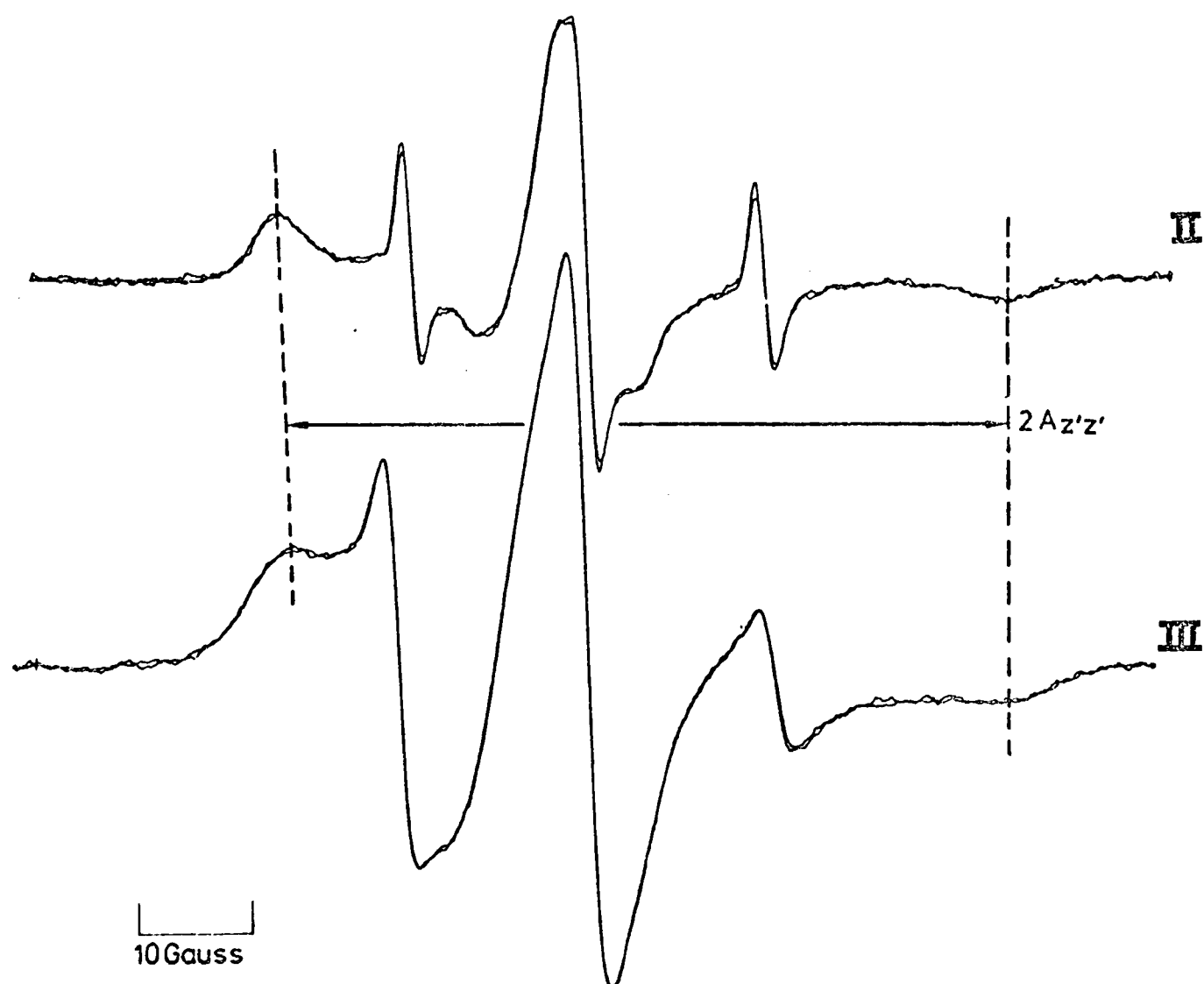
The ESR spectra of the maleimide, II, and the iodoacetimide, III, spin labels covalently bound to the protein of the granule membrane are given in Figure 3.4.4. In both cases, the spectra are seen to consist of bound outer components, indicated by the $2A_{z',z'}$ splitting in Figure 3.4.4. which arise from strongly immobilised spin label molecules, and two narrow inner components which arise from very mobile spin label molecules (7,8). The highly mobile components occur in the same region of the spectrum as would the unreacted spin label, but these components could not be removed by repeated washing or dialysis. The integrated intensity under the narrow, mobile peak is much smaller than that under the broad, immobile peak indicating that most of the bound spin label is of the strongly immobilised type.

The temperature variation of the maximum splitting, $A_{z',z'}$, of the strongly immobilised spin labels were investigated (Figure 3.4.5.). The temperature variation of the splitting of the maleimide spin label spectra is very slight. The magnitude of $A_{z',z'}$ approaches the maximum obtainable, derived from the maximum splitting value, $A_{zz} = 32\text{-}33$ gauss, which is found

TABLE 3.4.2.

Spin Label	S	
	0mM Ca ²⁺	150mM Ca ²⁺
I(12,3)	0.713	0.732
I(5,10)	0.487	0.523
I(1,14)	0.251	0.267

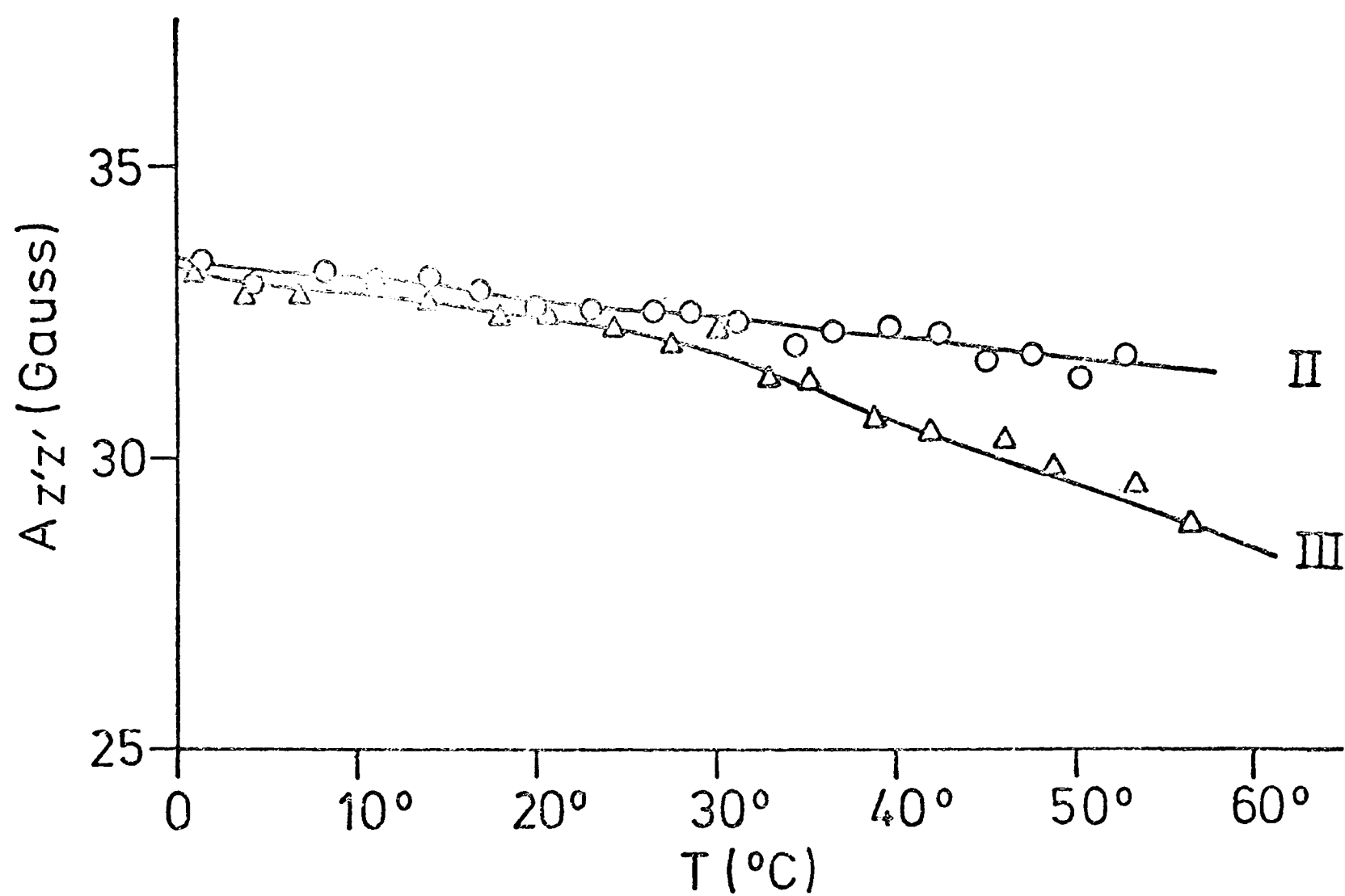
The effect of calcium on the order parameters of the stearic acid spin labels, I(m,n), incorporated into chromaffin granule membranes at 18°C.

FIGURE 3.4.4.

The ESR spectra of the maleimide, II, and iodoacetimide, III, spin labels covalently bound to protein in the chromaffin granule membrane.

Modulation 1.6 gauss ; power 50mW ; frequency 9.1GHz.

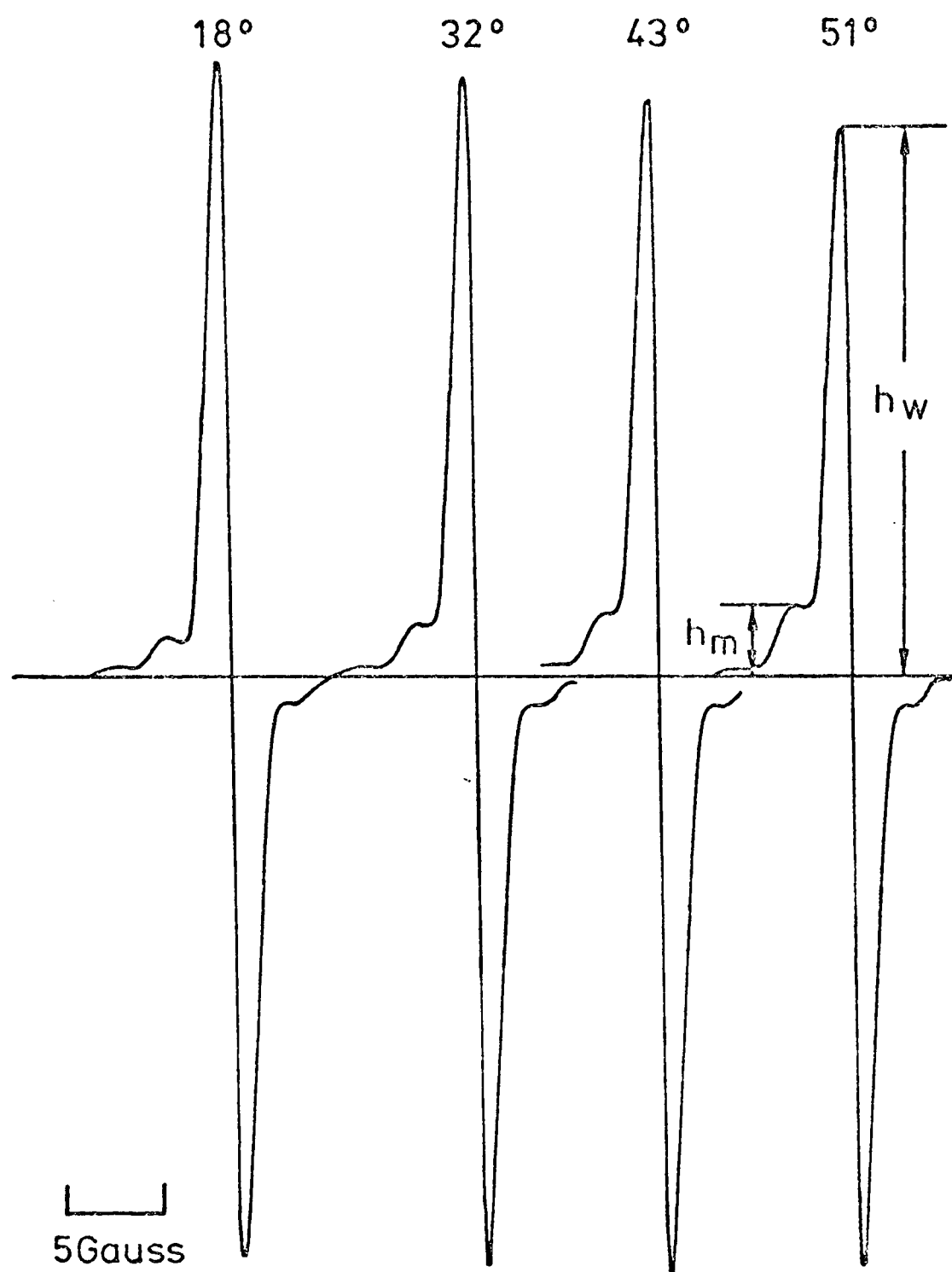
FIGURE 3.4.5



The temperature variation of the maximum splitting, $A_{z'z'}$, of the strongly immobilised spin label covalently bound to the chromaffin granule membrane.

Key: II, maleimide spin label,
III, iodoacetamide spin label.

FIGURE 3.4.6.



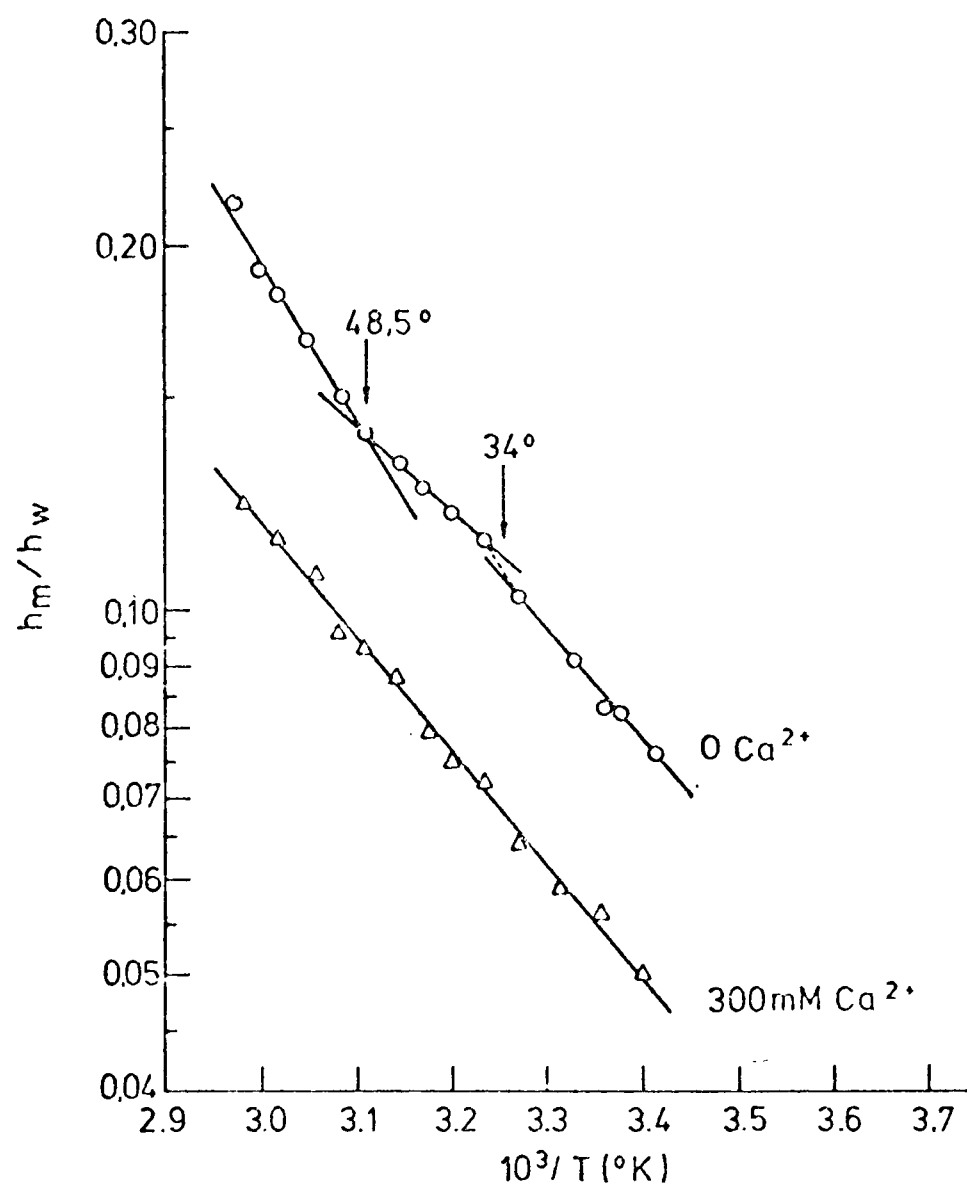
The ESR spectra of the high field hyperfine line of the TEMPO spin label, IV, in a chromaffin granule membrane suspension at various temperatures.
Modulation 5gauss : power 50mW : frequency 9.1GHz.

for a spin label rigidly oriented in a hydrophobic, single-crystalline environment (9). Assuming that the spin label is totally immobilised, the polarity of its environment may be estimated from the empirical relationship, established by Griffiths et al (14), that a limiting value of $A_{z,z'}$ of 33.5 gauss corresponds to an isotropic splitting factor of 14.5-14.9 gauss. The temperature variation of the iodoacetamide spin label is slightly different. At low temperatures, the $A_{z,z'}$ maximum splitting of the immobilised label is very similar to that of the maleimide label, implying that it also is rigidly held in an environment with an a_o value in the region of 14.7 gauss. However, at higher temperatures, the $A_{z,z'}$ splitting of the iodoacetamide label becomes progressively smaller than that of the maleimide label. The point of departure of the two plots is at a temperature of approximately 32°C, which also corresponds to a discontinuity in the gradient of the $A_{z,z'}$ splitting of the iodoacetamide label.

The ESR spectra of the partitioning of the TEMPO spin label, IV, (Figure 3.3.1.) between the aqueous and membrane phases of a chromaffin granule membrane suspension are presented in Figure 3.4.6. The high field hyperfine line consists of two peaks; one, designated by the height, h_m , arises from the spin label tumbling isotropically in the membrane, and the other, designated, h_w , arises from the spin label present in the aqueous phase (9,10). The line height ratio, h_m/h_w is thus a measure of the degree of TEMPO partitioning into the fluid phase of the membrane.

The temperature variation of the lineheight ratio representing the TEMPO partitioning into the chromaffin granule membranes in the presence and absence of calcium is shown in Figure 3.4.7. The extent of partitioning clearly depends on the membrane concentration present. Due to the difficulty in obtaining accurately reproducible concentrations at the high membrane concentrations required for adequate resolution of the membrane peak throughout the whole temperature range, only the relative

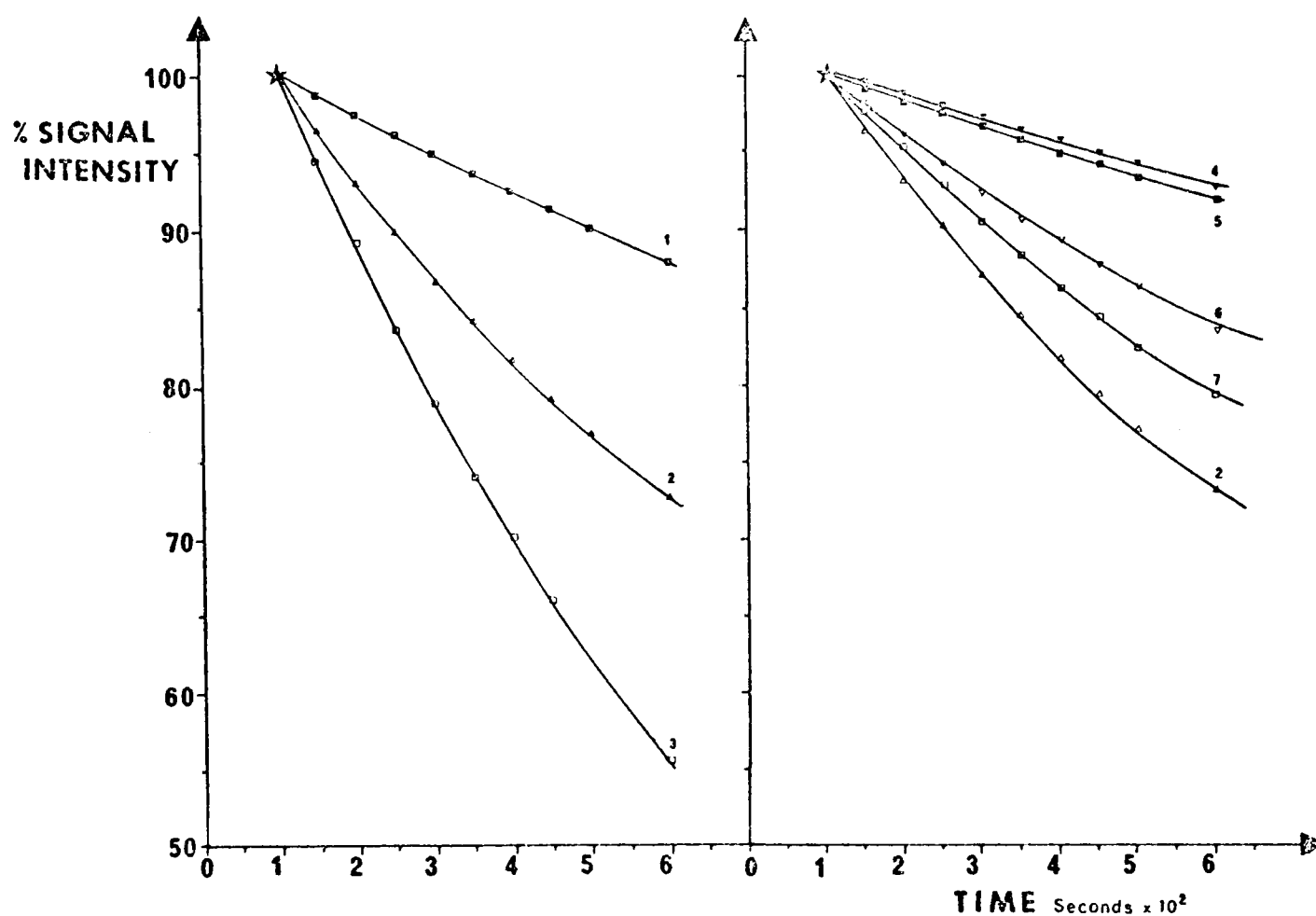
FIGURE 3.4.7.



The temperature dependence of the partitioning of the TEMPO spin label, IV, into chromaffin granule membranes, in the presence and absence of calcium.

The dependence is plotted as the logarithm of the ratio of the ESR high field peak heights of TEMPO in the membrane to that in the aqueous phase against the reciprocal absolute temperature.

FIGURE 3.4.8.



- (a) The dependence of the reduction of the spin label, TEMPO (0.5mM), on the concentration of chromaffin granules.
1. 16mg granule protein/ml of assay medium (0.3M buffered sucrose, pH 7.4)
 2. 8mg/ml.
 3. 4mg/ml.
- (b) The effect of various sulphydryl reagents on the rate of reduction of the spin label, TEMPO (0.5mM) by chromaffin granules (8mg protein/ml).
4. after preincubation for 120 minutes in the presence of 13mM NEM, 13mM IAM and 13mM IAA.
 5. after 40minute incubation in the presence of 13mM PHMB.
 6. after preincubation with sulphydryl reagents in 4 for 65 minutes.
 7. after preincubation with the sulphydryl reagents in 4 for 35 minutes.

temperature variations of the samples with and without calcium may be compared (Figure 3.4.7.) It is seen that in the absence of calcium, there is a discontinuity in the partitioning at approximately 34°C , and a further discontinuity in the gradient of the temperature dependence at around 49°C . In contrast, no discontinuities were observed in the presence of calcium.

B. The Reduction of Spin Labels by Chromaffin Granules

Spin labels, including the lipid directed, stearic acid labels and the water soluble TEMPO, are rapidly reduced when added to a suspension of purified, bovine chromaffin granules in 1.6M sucrose:10mM HEPES, pH 7.4. The time dependence of the reduction of the TEMPO spin label by various concentrations of chromaffin granules was examined (Figure 3.4.8.). The parameter monitored is the height of the water peak, h_w , as described previously. In each case the initial height of h_w is dependent on the tuning of the cavity, but the proportional reduction is independent of the initial value. The reduction is proportional to the concentration of granules present. Chromaffin granules isolated from porcine adrenal glands also exhibit this property. Membranes, purified from the granules, do not reduce the spin label. Neither do adrenaline, ATP, dopamine or purified chromogranins, alone or in any combination, either in the presence or absence of the chromaffin granule membrane.

The integrity of the granule, however, is not essential for this reduction. When the granules were completely solubilised with 0.02% Lubrol-PX, there was virtually no alteration in the rate of reduction of the spin label. The presence of ATP-Mg^{2+} , S-13, Antimycin A and NADH on the reduction was also investigated. The results are shown in Table 3.4.3. It can be seen that the rate of reduction is unaffected by the presence of these metabolites. The production of a transmembrane potential is not involved in the reduction, which appears to be due to the presence of

TABLE 3.4.3.

Conditions	%Reduction after 500 seconds
Chromaffin granules (16mg protein/ml)	45.2
+ ATP-Mg ²⁺ (5mM)	41.7
+ S-13 (1.5μM)	44.0
+ ATP-Mg ²⁺ (5mM) + S-13 (0.5μM)	40.1
+ Antimycin A (18mM)	44.1
+ Antimycin A (18mM) + ATP-Mg ²⁺ (5mM)	43.2

The percentage reduction of the ESR signal of 0.5mM TEMPO by chromaffin granules, prepared through 0.3M sucrose, in the presence of different metabolites.

an unknown reductant in the soluble fraction.

The reduction of the spin label was, however, inhibited when the granule suspension was preincubated with sulphydryl group - directed reagents for various periods of time (Figure 3.4.8.) The inhibition of the reduction is highly time dependent, and even after 120 minutes preincubation with a solution containing 13mM N-ethyl maleimide, 13mM iodoacetamide and 13mM iodoacetic acid, 30% of the uninhibited reduction still occurred. Reduction was completely inhibited if the chromaffin granule suspension was boiled for one to two minutes prior to the addition of the spin label, indicating that the reducing moiety is heat labile. Flatmark has recently shown that ascorbic acid, 10mM, is present in the bovine chromaffin granule (39). Ascorbate is capable of reducing spin labels, as a control experiment showed. However, in a suspension of chromaffin granules, as used in these studies, the total concentration of ascorbate is very much less than the concentration of TEMPO spin label which the suspension is capable of reducing. A model, which may explain the reduction, can be proposed. The spin label may be reduced by ascorbate, which in turn may be reduced by a sulphydryl group containing protein, thus recycling ascorbate. This model is quite feasible on the basis of oxido-reduction potentials of the different reactive groups. This model can explain why the reduction is sulphydryl reagent sensitive, and also why the chromogranins themselves did not reduce the spin label. It should be possible to set up this model system in vitro and test its practicability.

3.5 Discussion

The study of the chromaffin granule membrane, labelled with several different types of spin labels, shows that discontinuities occur at around 32° to 35°C in the temperature dependences of motionally sensitive parameters of all the spin labels used, except the covalently bound maleimide derivative. These breaks are apparent with structurally intercalated lipid labels, with the iodoacetimide label which is covalently attached to protein molecules, and with the small lipid soluble label, TEMPO. The characteristic temperature of these breaks is in the physiological range, or close to it. This correlates very well with the breaks observed in the Arrhenius plots of the enzymic activities of both the Mg²⁺-activated ATPase and the NADH : (acceptor) oxidoreductase present in the membrane (Chapter 2.3.E) and also with the anomalies in the temperature dependences of the reciprocal polarisation and rotational relaxation time of the fluorescent probes intercalated in the membrane (Figure 3.4.3). This evidence strongly implies that a structural change takes place in the lipid phase of the membrane, which may modulate the enzymic activities of the membrane proteins. The results from the various sets of spin labels must be considered in greater detail, in an attempt to discover more about the structure of the membrane and in particular about structural changes occurring in the 32° to 36° temperature range.

The results in Table 3.4.1. show that the stearic acid spin labels record both a profile of gradually decreasing polarity and a fluidity gradient of gradually increasing motional amplitude as the spin label proceeds away from the carboxyl end of the stearic acid molecule. Clearly, these amphiphilic probes are located in a precise structural manner in the chromaffin granule membrane. Such polarity profiles and fluidity gradients, in particular, are characteristic of fatty acid labels in lipid bilayers (7,10,11). It is therefore likely that the substantial portion of the lipid sensed by the spin labels in the chromaffin granule membrane is organised

in a lipid bilayer structure.

The comparison of the order parameters of the stearic acid labels in the chromaffin granule membrane with those in red cell ghosts (Table 3.4.1.) reveals that they are consistently greater in the latter membrane. That is the amplitude of motion of the lipid chains is greater in the chromaffin granule membrane. This result is not surprising in view of the lower cholesterol content, 36-41 mole% of total lipid (15,16), of the chromaffin granule membrane compared to that of that of the human red blood cell membrane, 50 mole% of total lipid (17), and also the unusually high content of lysolecithin present in the chromaffin granule membrane, 17% of the total lipid phosphorus (16,18). Cholesterol is well known to have an ordering and rigidifying effect on lipid chains in their fluid state (19,20) but it has recently been demonstrated that lysolecithin can form 1:1 complexes with cholesterol (21,22) which would reduce the ordering and rigidifying produced by the cholesterol.

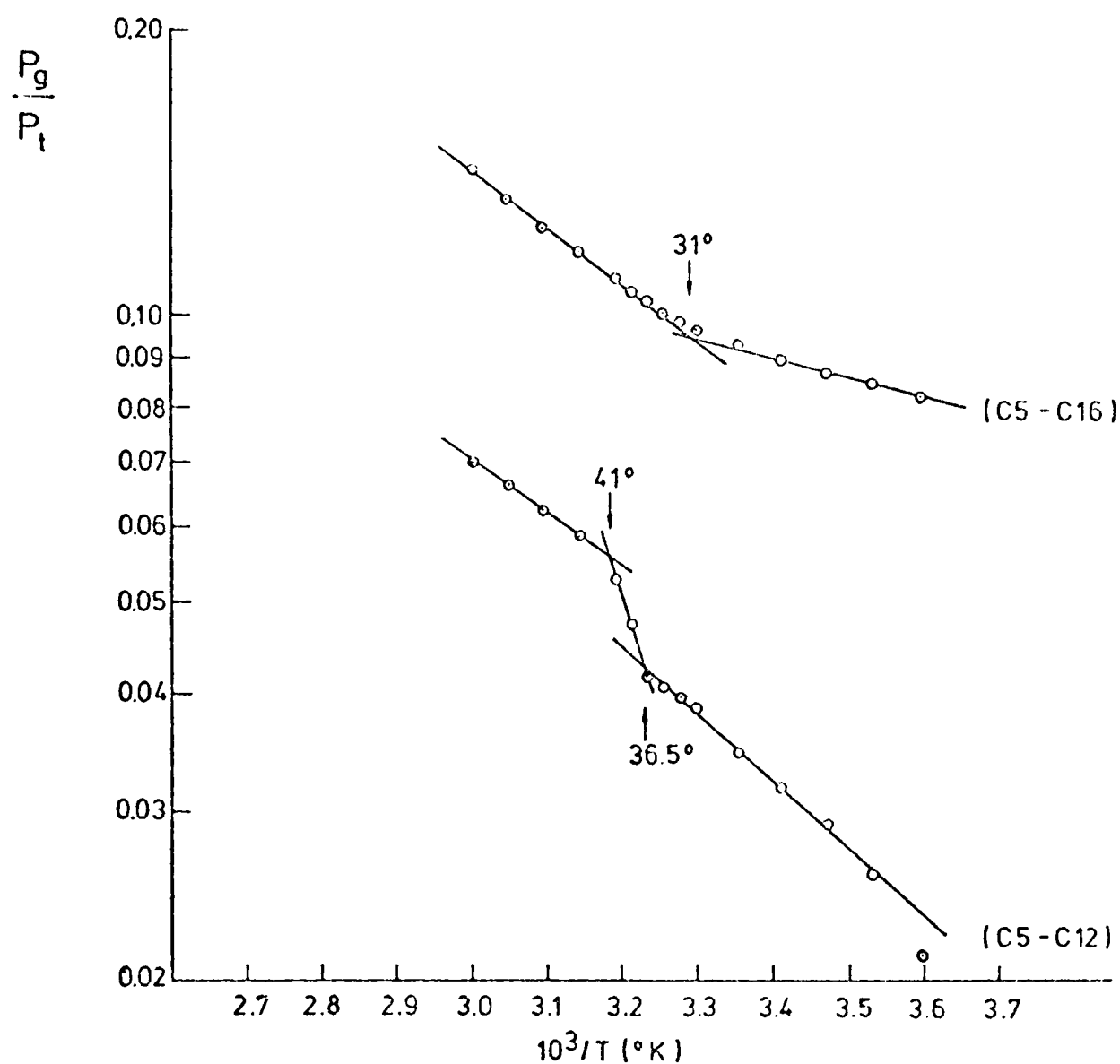
Comparison of the order parameters of the membranes in Table 3.4.1. with those of phospholipid bilayer vesicle model systems of similar cholesterol content (23) indicates that the chromaffin granule membrane order parameters are quite close to those of egg lecithin + 35-40 mole% cholesterol bilayers, whilst those for the red blood cell membranes are significantly higher than those of egg lecithin + 50mole% cholesterol. Because of the different phospholipid compositions, not only of the phosphatidylheadgroups but also of the degree of saturation of the fatty acid side chains of these various systems, it is impossible to draw detailed conclusions from these correlations, but it is significant to note the unusually low protein to lipid ratio, 0.45 : 1, of the chromaffin granule membrane (16) compared to the red blood cell ghost, for example, with its much higher ratio of 1 : 1 (24). Further, since egg lecithin has a higher proportion of unsaturated fatty acid chains, it forms very fluid bilayers in the absence of cholesterol. The comparison with the chromaffin

granule membrane suggests that if some preferential segregation of cholesterol could be induced, the cholesterol-depleted patches induced would be extremely fluid. It might be expected that the spin labels would preferentially partition into this more fluid phase. This phase separation may conceivably have a bearing not only on the enhanced enzymic activities observed above 33°C (Chapter 2.3.E.), but also on possible mechanisms of membrane fusion.

The exponential temperature variation of the stearic acid order parameters, shown in Figure 3.4.2., suggests a Boltzmann-type distribution of available motional amplitudes, rather than a continuous distribution, with a transition around 34°C to a state with different characteristic energies for the motional amplitudes. Similar plots for egg lecithin vesicles with varying cholesterol content produce either straight lines or discontinuities which show no correlation between the different spin labels (23). It is interesting to note that the trend in the temperature dependence of the spin label order parameters is in the same direction as that observed for the rotational relaxation time of the fluorescent probes also investigated (Figure 3.4.3.), (13). In both cases the fluidity below 33°C is higher than that which would be predicted by extrapolation from the higher temperature range, although the fluidity measurements are recorded in completely different ways. It is measured as an amplitude of lipid chain motion for the spin labels, but as a rate of motion for the fluorescent probes. These results also indicate that there are different lipid segregation and mixing patterns below and above the transition region.

The rotational energy levels of the trans and gauche isomers of the stearic acid chains (25) would give rise to a spin label temperature dependence similar to that indicated in Figure 3.4.3., but this fails to take account of the 'rigid stick' motion of the chain as a whole. Hubbell and McConnell (12) have shown that the 'rigid stick' motion can be

FIGURE 3.5.1.



The temperature dependence of the average probability of a gauche conformation about a single C-C bond, normalised with respect to the probability of trans conformation.

The dependence is plotted as the logarithm of probability versus the reciprocal absolute temperature.

Key : C -C ; P_g/P_t obtained by combining the data for I(12,3) and I(5,10),

C -C ; P_g/P_t obtained by combining the data for I(12,3) and I(1,14).

eliminated by combining the data from two positional isomers of the stearic acid label to give the mean probabilities, P_g and P_t , for the gauche and trans isomers respectively, about a single C-C bond in the hydrocarbon chain. If P_g is small, so that the probability of g-g conformations about adjacent bonds can be ignored, the order parameters of the I(m,n) labels are given by:

$$S_n = n \log P_t + \log S_0 + C \quad 3.12$$

$$P_g + P_t = 1 \quad 3.13$$

where S_0 is the 'rigid stick' order parameter

C is a coefficient only weakly dependent on n

n is the number of carbon atoms along the hydrocarbon chain from the carboxyl end to which the nitroxide moiety is bound.

Within the limits of this treatment, the temperature dependence of the mean probability of the gauche conformation about a single C-C bond is then given by:

$$\frac{P_g}{P_t} = 2e^{-E_g/RT} \quad 3.14$$

When the results of Figure 3.4.2. are analysed in this way, the plots shown in Figure 3.5.1. are produced. These again show breaks, in contrast to a similar analysis made for egg lecithin and egg lecithin + 30 mole% cholesterol bilayers, which do not show any breaks in the P_g/P_t range considered here (26). These breaks, at 31°C for the I(12,3) and I(1,14) data and 36°-41°C for the I(12,3) and I(5,10) data, are close to those already found for the order parameters, and again lie close to or within the physiological range.

The temperature induced transition is thus characterised by an increase in the population of the gauche isomers in at least some of the lipid chains, and a resultant alteration in the temperature dependence of the order parameter of the spin labels. Another feature of the temperature plots in Figure 3.5.1. is the large discontinuity in the P_g/P_t ratio for

the C_5-C_{12} region of the chain. One possible mechanism for such a change is the preferential segregation of cholesterol, since this is precisely the region of the hydrocarbon chain over which the rigid cholesterol molecule would have its maximum effect.

Another interesting implication from Figure 3.5.1. is that the average probability of gauche conformations is greater in the C_5-C_{16} region of the hydrocarbon chain than for the shorter C_5-C_{12} region, which is closer to the carboxyl headgroup. This infers that the probability of gauche conformations about a given C-C bond is greater towards the end of the chain, again mirroring the results of the spin labels in lipid bilayers (27) and the much more detailed results obtained from deuterium magnetic resonance studies using specifically deuterated lipid molecules (28). The deuterium NMR studies of dipalmitoyl lecithin bilayers were interpreted in terms of limited $g^\pm$ & $g^\mp$ 'kink' type structures in the upper C_1-C_{10} region of the phospholipid chains, and much greater rotational flexibility at the terminal methyl ends of the chains. It would appear that at least qualitatively, similar results hold for the lipid bilayer regions of the chromaffin granule membrane. This effect may be enhanced by the presence of cholesterol, since the rigid cholesterol molecules are expected to penetrate only to about C_{12} of the lipid chains.

The absence of a temperature dependence of the hyperfine splitting of the covalently bound maleimide spin label (Figure 3.4.4.) reflects not only the very small number of bonds from the nitroxide moiety to the point of covalent attachment, which results in limited rotational freedom, but also the lack of flexibility of the group to which it is attached or the environment by which it is constrained. The covalent labelling was non-specific, but the membrane ATPase and NADH oxidase are known to have highly reactive sulphydryl groups (29,30), so that these must at least account for part of the labelling. The a_0 value of 14.7 gauss for the nitroxide group suggests that the label must be situated in a

relatively apolar environment. This would imply that the label moieties were situated either in a moderately hydrophobic pocket in the protein or on a surface penetrating part way into the membrane lipid. The former possibility is probably more likely. However, it must be stated emphatically that since the a_o value was calculated assuming that the probe is totally immobilised and therefore must represent the lower limit, the site could, in fact, be considerably more polar.

The iodoacetamide spin label has considerably more single bonds linking the nitroxide moiety to the group which bonds to the protein, and thus, even if it were attached to an inflexible group, it can be expected to be more sensitive to environmental and fluidity changes, since it protrudes further from its point of attachment. This, in fact, may be observed in Figure 3.4.5. At approximately 32°C, the flexibility of the label changes from being virtually identical to, to being greater than that of the maleimide label, and exhibiting a considerably greater temperature sensitivity. Although a well defined inner splitting cannot be resolved in the spectra of Figure 3.4.4., as is the case with the stearic acid spin label spectra, the outer splitting, $A_{z,z'}$, is still a measure of the motional amplitude (31,32). The iodoacetamide labelling is also non-specific but it is probable that, whilst there will some differences in the specificity of the maleimide and the iodoacetamide labelling, there will be considerable overlap in the sites labelled, and in the case of the ATPase, its activity is abolished in the presence of both spin labels after the incubation time employed. The arguments used above regarding the location of the maleimide probe apply equally well to the iodoacetamide label. The transition observed at approximately 32°C must either represent a conformational change in the protein to a state in which the amplitude of motion of the label is less restricted, or else it may reflect a change in the lipid phase in the immediate environment of the proteins, which results in enhanced motion of the protein itself. It is possible that either

of these changes is driven by the structural transition observed in the lipid bilayer region of the membranes with the stearic acid labels, and may account for the changes observed in the temperature dependence of the enzymic activities of the membrane proteins.

The extent of TEMPO partitioning into membranes and bilayers is greater, the greater the fluidity of the lipid chains (33). Thus the break in the temperature dependence of the TEMPO partitioning (Figure 3.4.7.) must represent a change in the structure of the membrane which results in an alteration of the lipid chain fluidity. This would appear to be the same change as that sensed by the other labels, and also reflected by the enzymic activities of the granule membrane. The discontinuity in the gradient at 48.5°C , which is not detected by the stearic acid labels may reflect denaturation, partial or otherwise, of the membrane proteins. Such alterations in the fluidity of the membrane, detected by TEMPO, have been implicated with the onset (or possibly completion) of lateral phase separation of membrane lipids (34,35). This may be the case with the chromaffin granule membrane, since it has a very heterogeneous lipid composition and contains a considerable cholesterol content. The possibility of lateral phase separation of lipids, involving preferential segregation of cholesterol, has already been suggested from consideration of the stearic acid data.

When TEMPO partitioning was studied in the presence of calcium, the discontinuity at physiological temperature is broadened out or shifted to much higher temperature, as is the case for the phase transition in pure negatively charged phospholipids (36,37). The increase in the stearic acid order parameters on the addition of calcium (Table 3.4.2.) may also be explained in terms of an increase in the characteristic temperature of the structural transition. Lateral phase separation, induced by calcium ions, has been observed in a variety of mixtures containing neutral lipids (40,41,42). The chromaffin granule

negatively

membrane contains 9%₁ charged lipid (15), so it is possible that calcium may alter the lateral phase separation pattern of the lipids. If this change were to involve disruption of lysolecithin-cholesterol complexes (21,22) and the resultant creation of lysolecithin-rich regions and a higher cholesterol concentration in the remainder of the lipid, then this would account for the higher order parameters of the stearic acid labels if they were localised in the regions of increased cholesterol concentration. Such a hypothesis is attractive since it involves calcium as a trigger for the creation of lysolecithin-rich regions which could facilitate fusion (38). However, the levels of calcium used were very high since the probes used are non-specific and sense only changes in the bulk lipid. The composition of the membrane is so complex that such a mechanism must remain hypothetical.

It is unfortunate that it was not possible to spin label intact granules, since this would have permitted the study of , for example, catecholamine uptake or the generation of a transmembrane potential. It would also have been possible to specifically label the outer face of the membrane with covalent labels, hence reducing the number of binding sites.

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CHAPTER FOUR

Nuclear Magnetic Resonance Studies of the Chromaffin Granule and its Membrane.

4.1 Introduction

A The Applicability of the Different Magnetic Nuclei to Studies of Biological Systems.

The chromaffin granule contains very large concentrations of catecholamines (550mM), ATP (122mM) and chromogranins (Chapter One). It is, therefore, a system ideally suited to study using nuclear magnetic resonance spectroscopy (NMR). High resolution Fourier Transform NMR, in contrast to the technique of ESR using spin label molecules (Chapter Three), is non-perturbing and studies of systems more akin to those in vivo can be made.

The relative sensitivities of several different magnetic nuclei present in biological systems are shown in Table 4.1.1. It can be seen that the sensitivities of the most common organic, biological nuclei are in the order: $^1\text{H} \gg ^{31}\text{P} \gg ^{14}\text{N} > ^{13}\text{C} > ^{17}\text{O} \gg ^2\text{H}$ in non-enriched samples, at constant field (1).

The nucleus most widely used in the study of biological systems is that of hydrogen, ^1H . Since many biological molecules eg. enzymes, cannot be prepared in large quantities or else have limited solubility, it is essential to optimise the sensitivity of the system in order to obtain the maximum amount of information about structural and motional aspects of the molecules. Proton NMR has a sensitivity more than an order of magnitude greater than any other nucleus, and is, therefore, the first to be examined when studies of most biological molecules are undertaken.

An obvious disadvantage of proton NMR studies of macromolecules is the complexity of the spectra produced and the resultant difficulty encountered in the assignment of the resonances. Many additional devices have

TABLE 4.1.1.

<i>Nucleus</i>	<i>Nuclear Spin Quantum Number</i>	<i>Natural Abundance (%)</i>	<i>Sensitivity of Nucleus at constant field</i>	<i>Relative Sensitivity at Natural Abundance</i>
^1H	$\frac{1}{2}$	100	1.0	100
^2H	1	0.015	0.01	0.00015
^{13}C	$\frac{1}{2}$	1.1	0.016	0.018
^{14}N	1	99.6	0.001	0.1
^{17}O	$\frac{5}{2}$	0.036	0.027	0.001
^{19}F	$\frac{1}{2}$	100	0.833	83.3
^{23}Na	$\frac{3}{2}$	100	0.0925	9.25
^{25}Mg	$\frac{5}{2}$	10	0.0028	0.028
^{31}P	$\frac{1}{2}$	100	0.066	6.63
^{35}Cl	$\frac{3}{2}$	75.5	0.0046	0.35
^{39}K	$\frac{3}{2}$	93	0.00005	0.047

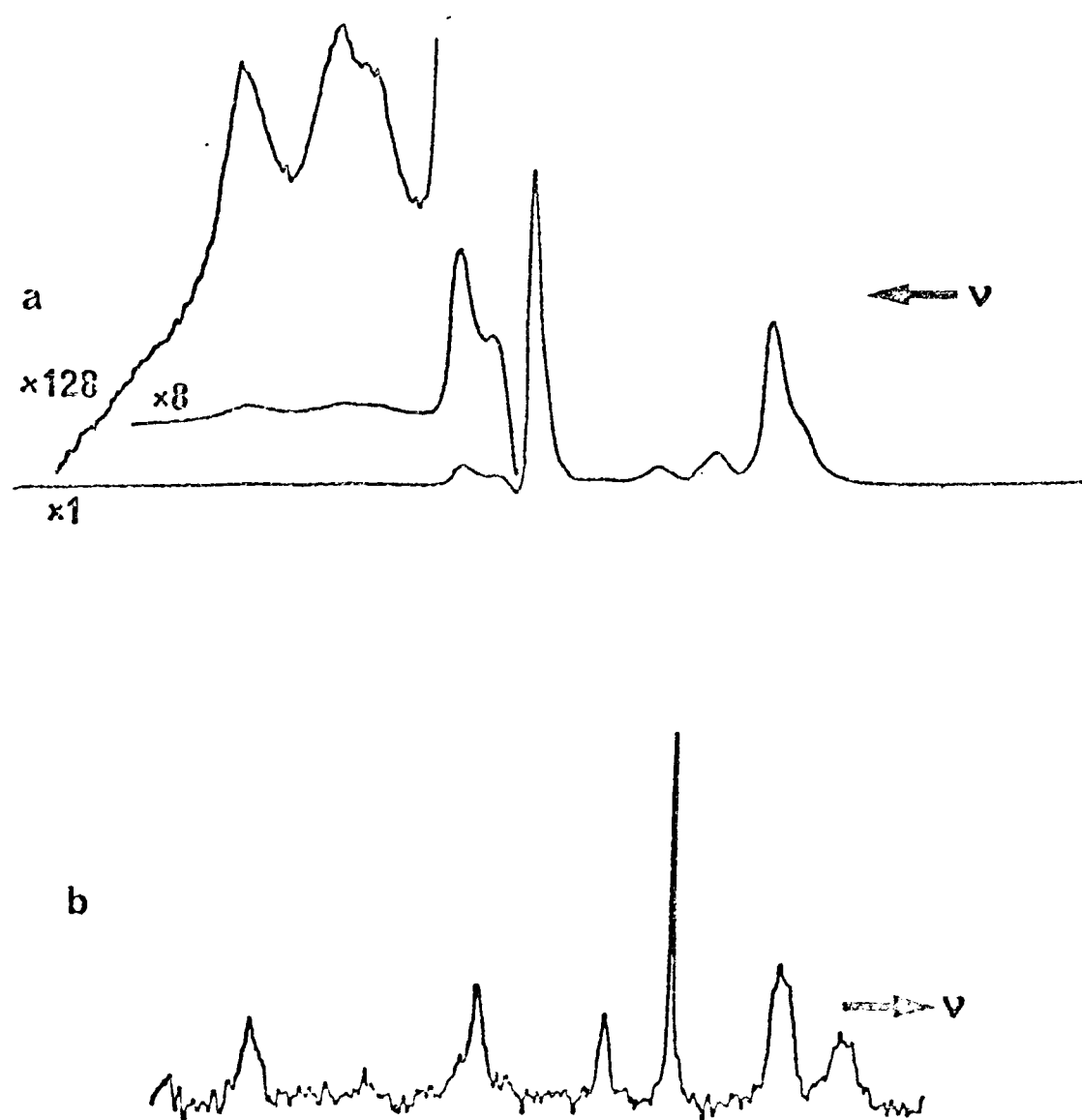
The magnetic properties of the most common biological nuclei.

to be employed in order to obtain enhanced resolution and to make assignments unambiguously. These include the use of extrinsic shift probes eg. lanthanide ions (2,3), convolution difference spectroscopy (4), and the pH dependence of titratable groups (5). Nonetheless, many of the resonances from macromolecules occur very close together and are very difficult to resolve even using the devices. Even now, much dependence has to be placed on the interpretation of the behaviour of the chemically shifted, aromatic proton resonances and on the development of more powerful spectrometers to provide increased resolution.

^{31}P Phosphorus NMR has only recently been applied to biological systems (6,7), and very few studies have been made in any great detail (8,9). In many systems, ^{31}P NMR offers multiple advantages over other magnetic nuclei. One very practical but considerable advantage is that since at constant field, H_0 , lower frequencies are used (cf. ^1H), it is less essential to maintain perfect field homogeneity across the sample. This means that sample tubes of larger diameter than those used with high resolution proton NMR may be used. These tubes, up to 10mm in diameter (outer), permit the study of intact tissues, many of which are too large to be examined with proton NMR. Further, the concentration of phosphorus-containing metabolites present in many tissues is often very high, and since these are usually small molecules eg. glucose-1-phosphate, with relatively unhindered motion in the cell, line widths are usually narrow. The decreased sensitivity of the nucleus compared to protons is, therefore, not too significant and only results in a slightly longer accumulation time. It has been found that concentrations of phosphate as low as 1mM may be detected after 450 scans with a signal:noise ratio of 20 (10). ^{31}P NMR spectra are very much simpler than those of ^1H and ^{13}C NMR. The ^1H and ^{31}P spectra of freshly incised rat soleus muscle are compared in Figure 4.1.1. The advantages of ^{31}P NMR in this respect are clearly shown.

The resonance frequency of the phosphorus nucleus is highly sensitive to its environment, including the bonding of adjacent phosphorus

FIGURE 4.1.1.



A comparison of (a) the proton (270MHz) and (b) the phosphorus (129MHz) spectra of excised rat soleus muscle immediately after removal from animal (49).

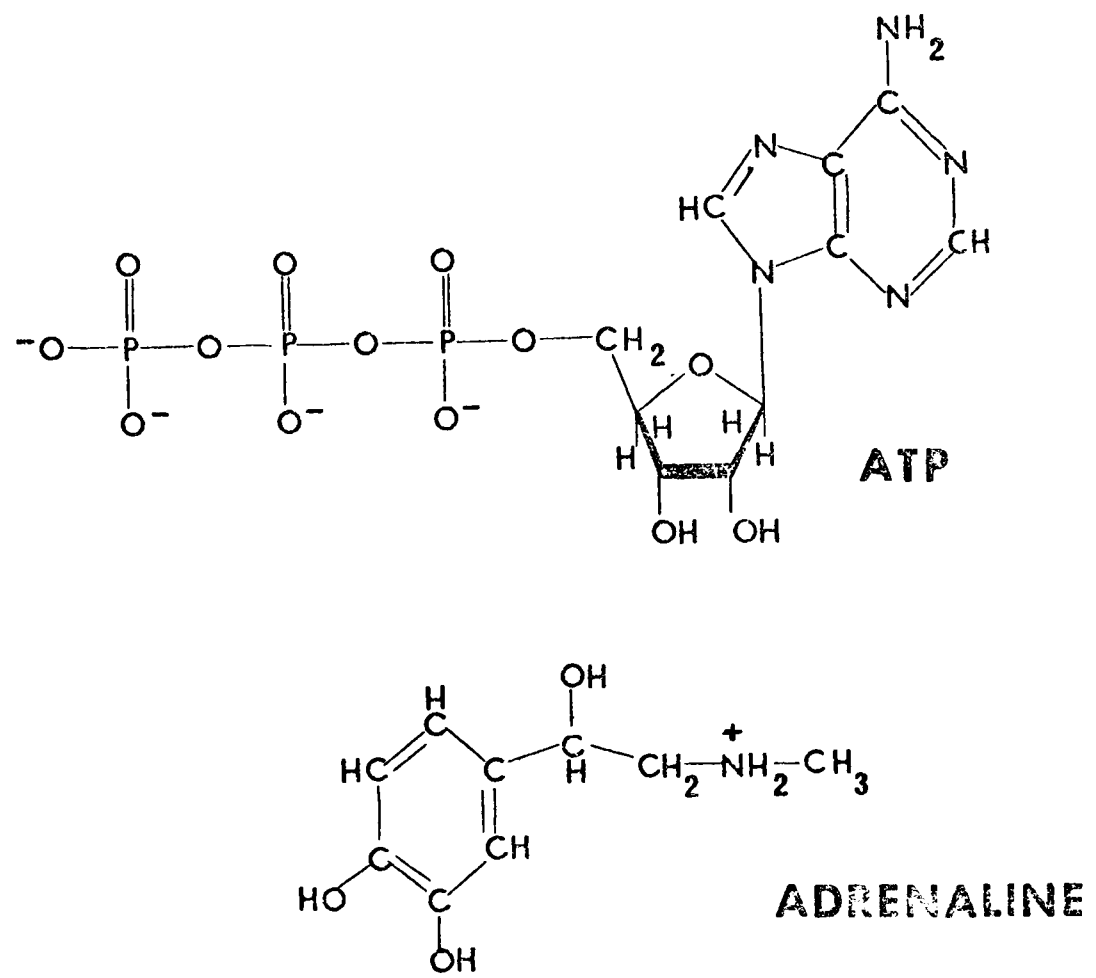
nuclei, the viscosity of the solution, pH, liganding of metal ions and complexing to other molecules (11). Also, the pH dependence of chemical shifts of phosphate molecules is larger than those found with protons (5,11). ^{31}P nuclei have been shown to exhibit chemical shift anisotropy (6). This results in increased line-width at increased frequency of the applied magnetic field, if the molecule is not in rapid motion. Information about motional rates may, in favourable circumstances, be derived from the relationship between line-width and the intensity of the magnetic field (6,12,13). However, it is apparent that when studying systems where lower rates of motion occur, the advantages of increased resolution obtained at increased magnetic field may be outweighed by the broadening of the resonances due to the chemical shift anisotropy.

The sensitivity of the ^{13}C nucleus is 1.6% of that of ^1H for equimolar amounts at the same magnetic field. However, its low natural abundance, 1.1%, necessitates signal enhancement if ^{13}C studies are to be carried out on biological systems. Several techniques have been devised in order to obtain this signal enhancement. Apart from Fourier transformation of the computer averaged accumulations (CAA), the two most important of these are hydrogen noise decoupling, which removes all the ^1H - ^{13}C spin-spin splittings, and ^{13}C isotope enrichment. Isotope enrichment is expensive and not always possible, but when it can be used, especially when selective enrichment of, for example, non-metabolised enzyme substrates is practicable, considerable information about the molecule can be derived (14). In non-enriched molecules, the spectra are simplified as ^{13}C - ^{13}C spin-spin couplings are not normally detected since, due to the very low natural abundance of ^{13}C , neighbouring nuclei are invariably the non-magnetic ^{12}C . Thus despite the sensitivity disadvantage, ^{13}C spectroscopy does result in simpler, more resolved spectra than those from proton NMR, and is widely used.

Although ^1H , ^{13}C and ^{31}P are the nuclei most frequently studied in biological systems, but ^2H , ^{14}N and ^{17}O are also investigated where suitable.

Recently deuterium magnetic resonance has emerged as a powerful method for studying biological membranes and associated model systems (15). Its low sensitivity, expressed in Table 4.1.1., is mainly due to its very low natural abundance. However, like ^{13}C NMR, considerable improvement in sensitivity can be obtained by deuterium enrichment, selective or otherwise, of the molecules to be investigated. Seelig has demonstrated this very beautifully in his systematic study of the freedom of motion and the resultant order parameters, equivalent to those measured by ESR and described in Chapter Three, at different distances along the hydrocarbon chains of phospholipids in bilayer systems. In order to achieve this, a series of phospholipids were prepared in which hydrogen atoms, attached to one carbon atom of the hydrocarbon chain, were selectively replaced by deuterium atoms. Both fatty acid chains were investigated in this manner. This, unlike ESR and fluorescence studies carried out on similar systems (16,17), permitted a highly specific and non-perturbing probe to be used. Interestingly, whilst the results for the motion at the methyl end of the chain were similar to those found using the other techniques, considerable differences in the motions at the carboxyl end were detected (18). Deuterium NMR may be expected to be applied to many other systems in the near future.

Both ^{14}N and ^{17}O are quadrupolar nuclei so their resulting resonances are often broad. Since ^{14}N NMR is also very insensitive and the spectral resonances broad, very high concentrations, 0.2M or thereabouts, may have to be used if studies are to be made. It is, therefore, not a very useful probe of biological molecules. Some studies of oxygen exchange and the liganding of water molecules to metal ions have been made using ^{17}O NMR (19), but its natural abundance is so very low that enrichment is essential

FIGURE 4.1.2.

The structural formulae of ATP and adrenaline.

if studies on any biological system are to be made. This isotope, like ^{13}C , is expensive, and since the signals are also broad, studies using selective labelling are infrequent.

B The Applicability of the Technique to the Study of Chromaffin Granules and their Membranes.

It is apparent from the above discussion that the advantages and the disadvantages of the magnetic resonance properties of the various magnetic nuclei in their application to biological problems are diverse. Careful consideration must, therefore, be made, not only of the system to be studied but of the problems to be solved and of the contributions that studies using the different nuclear resonances can make to an understanding of these problems.

Consider the chromaffin granule; as previously stated, this is a convenient system to study using nuclear magnetic resonance. Since it contains such large concentrations of ATP and catecholamines, in addition to the acidic proteins, the chromogranins, there should be little problem in achieving a good signal:noise ratio even with insensitive nuclei like ^{13}C . A glance at the formula of adrenaline and ATP (Figure 4.1.2.) shows that both molecules contain aromatic rings. Thus with ^1H and ^{13}C NMR, these aromatic resonances will be chemically shifted from the bulk of the aliphatic ones and hence easily resolved. The N-methyl group of adrenaline should also be distinct since with proton NMR it will lie upfield from the water peak. It will also be fairly large since three protons will contribute to the one resonance, which will not be split since the hydroxyl and amino protons are exchanged with solvent deuterium atoms and make little or no contribution. (In intact glands and samples with limited deuteration, the splitting will be present.) ATP contains three phosphate groups which are termed the α -, β - and γ -phosphate respectively. Their ^{31}P resonances are clearly shifted from each other at pHs greater than 4. The γ -phosphate, in particular, is highly sensitive to pH and to complexing with other

molecules, whether they be metal ions or proteins (11).

Thus not only ^1H but ^{13}C and ^{31}P spectra may be readily obtained from chromaffin granules, and even intact glands in the case of ^{31}P . It is apparent, therefore, that the system suits the technique, but what can the technique tell us about the system?

There are several problems to which NMR can help provide an answer. One of the most fundamental problems faced by workers studying any subcellular system is that its preparation may have disturbed its integrity, and hence in vitro results do not reflect the in vivo situation. This uncertainty has worried many research groups working on chromaffin granules and other neurosecretory vesicles. Traditional work on such systems was carried out by pharmacologists who worked on glands perfused in situ with various drugs (20,21). Most of the present research, however, is performed on granule preparations, purified using density gradient centrifugation through buffered sucrose and in the absence of isotonic salts, which simulate the intracellular medium, since these greatly reduce the stability of the preparation (22). Whilst NMR cannot facilitate comparative studies of the chromaffin granule membrane and its associated functions, it does permit detailed examination of the chromaffin granule storage complex, in vivo and in vitro. The relationship between the granules in the chromaffin cells of intact adrenal glands and the granules purified from these glands may be studied using ^{31}P NMR since the concentration of phosphates in the granule is so high. It has been possible to make this comparison in several species. Simulated biological processes can be followed without destruction of the tissue; 'before' and 'after' comparisons may be made on the same gland, rather than by averaging the results with several glands to account for the natural variation, a necessity of traditional methods.

Apart from such general problems, the chromaffin granule system poses many specific and as yet unanswered questions. Whilst some of these concern solely functional aspects, others also involve structural ones.

What is the nature of the storage complex? Is it crystalline or fluid?

How does the granule remain osmotically stable? There have been many theories put forward to account for both the appearance of the granule contents in the electron micrographs and its relatively inert osmolarity. Most of these suggest a rigid complex, possibly crystalline, which will have little osmotic activity and which involves ionic and hydrophobic interaction between the ATP, adrenaline and chromogranin molecules (23). There is, however, very little direct evidence for such a structure (Chapter Five).

An answer to the first two of these questions has been sought using ^{31}P NMR, in conjunction with ^1H and ^{13}C NMR. The ^{31}P spectrum of chromaffin granules may, naively, be considered as a simplified fingerprint since the frequency and the line-shape of a resonance are functions of the composition, pH and mobility of the whole system. With a knowledge of the components of the complex, in addition to the catecholamines, ATP and proteins, the system has been simulated at different degrees of complexity and over a practical pH range, in an attempt to learn more about the complex. In view of the results described in Chapter Two, ie. the pH dependences of the ANS response and the uncoupling of the ATPase activity, it is very important to determine the internal pH of the bovine chromaffin granule. This can most accurately be done using the positions of the β - and γ -phosphate resonances of ATP, once the exact nature of the complex is known. The state of fluidity and the internal motions available to the granule contents may be mapped by measuring the relaxation times, T_1 and T_2 , derived from studies involving all three nuclei. Preliminary studies using these approaches are described. NMR can tell us much about the system.

Another recent application of nuclear magnetic resonance is to the study of biological membranes. The composition and properties of the chromaffin granule membrane, with its large content of lysolecithin and cholesterol and low protein:lipid ratio, have already been described (Chapters Two and Three). It was thought that it might be a relatively

fluid membrane, and hence suitable for study using magnetic resonance techniques. However, the results of the ESR studies indicate that the cholesterol present compensates for the lysolecithin, resulting in a membrane which at room temperature is unremarkable.

Whilst many interesting results regarding the structure of model membranes in aqueous dispersions have been obtained using ^1H , ^{13}C and more recently ^{31}P NMR (24,25,26), the major conclusion to be drawn from the literature is that most membranes are too complex and too large to be studied directly by NMR (27,28). No high resolution proton spectra can be obtained from most biological membranes, and severe treatments eg. heating to 70°C or higher, are necessary before the membranes are sufficiently damaged for a spectrum to appear (28). Sonicated dispersions of extracted lipids, however, give well resolved high resolution spectra comparable to those from phospholipid:cholesterol model systems (29). Reasonably well resolved lipid resonances can also be seen in the spectra of erythrocyte lipoproteins, but it is not known what proportion of the lipids are contributing to the signal (30). ^{13}C spectra, however, have been obtained from intact erythrocyte membranes (25,31). The resonances due to the $-\text{N}^+-\text{Me}_3$ carbon atoms of lecithin in the membrane are resolved, though resonances due to fatty acid carbons are very broad (25). The ^{13}C spectrum of rat liver mitochondrial membranes is better resolved than that of erythrocytes, which has been attributed to the absence of cholesterol in the mitochondrial membrane (31). The problem of ^{13}C sensitivity is a large one as far as membrane studies are concerned but has been overcome in bacterial systems by growing the bacteria in a ^{13}C enriched medium (32). Unfortunately, apart from incorporating specifically enriched lipids, there is no way of enriching the chromaffin granule membrane.

Little work has been done so far on biological membranes using ^{31}P NMR, though the literature describing such studies is expanding (13,33). Although detailed structural information may not be forthcoming, the chromaffin granule membrane was studied using ^{31}P NMR to determine

whether the temperature dependent transition, detected by ESR (Chapter Three) was reflected in the properties of the phospholipid resonances. The proton NMR spectrum of the membrane was also examined.

4.2. The Theory of Nuclear Magnetic Resonance.

The fundamental theory of nuclear magnetic resonance (NMR) is similar to that of Electron Spin Resonance (ESR), described in Chapter 3.2. The same principles may be used to derive relationships which describe the resonance of a nucleus in a magnetic field. The possession of both spin and charge confers on a nucleus a magnetic moment, $\underline{\mu}_n$, which is proportional to the magnitude of the spin; that is

$$\underline{\mu}_n = \gamma_n \hbar \hat{\underline{I}} = g_n \beta_n \hat{\underline{I}} \quad 4.1$$

where γ_n is the nuclear magnetogyric ratio,

$\hat{\underline{I}}$ is the spin angular momentum vector,

g_n is the nuclear g factor,

β_n is the Bohr Nuclear Magneton.

The interaction of the nucleus with an external magnetic field, $\underline{H}$, can be described in terms of a Hamiltonian :

$$\hat{H} = -\underline{\mu}_n \cdot \underline{H} \quad 4.2$$

If the direction of the magnetic field is defined as the z direction, the interaction may be rewritten as :

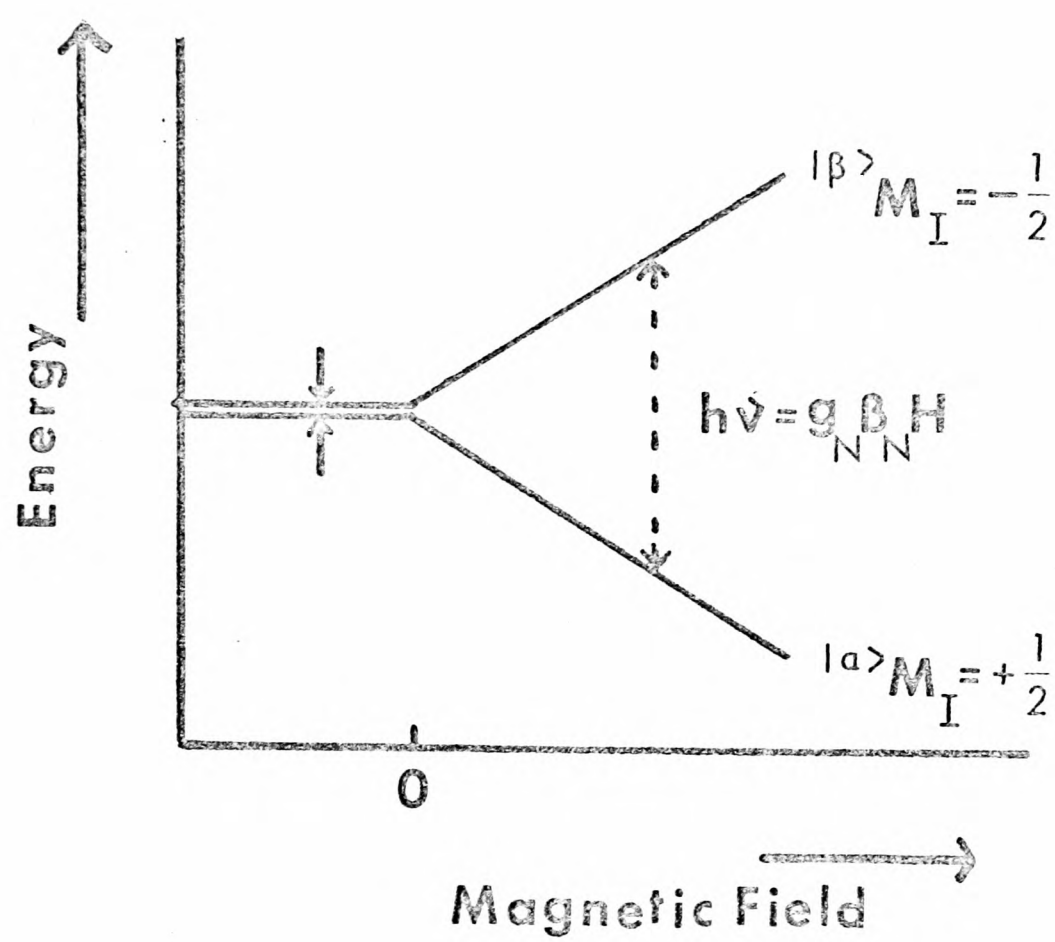
$$\hat{H} = -\gamma_n \hbar H \hat{I}_z = -g_n \beta_n H \hat{I}_z \quad 4.3$$

where $\hat{I}_z$ is the component of the nuclear spin in the z direction.

For the proton with $I = \frac{1}{2}$, M_I , the nuclear spin quantum number may only take the values $\pm \frac{1}{2}$. The lower energy level with $M_I = \frac{1}{2}$, in which the magnetic field and the nuclear moment are parallel, is denoted by $|\alpha\rangle$. The upper energy level with $M_I = -\frac{1}{2}$, in which they are antiparallel, is denoted by $|\beta\rangle$. This is illustrated in Figure 4.2.1. In order to induce transitions between the two nuclear spin levels, an oscillating magnetic field, with its magnetic vector perpendicular to the steady field, is applied. Absorption of energy occurs provided the frequency, ν , of the oscillating field satisfies the resonance condition :

$$\hbar \nu \approx g_n \beta_n H \quad 4.4$$

FIGURE 4.2.1.



Nuclear energy level diagram (25)

Although transitions from $|\alpha\rangle$ to $|\beta\rangle$ or $|\beta\rangle$ to $|\alpha\rangle$ are equally likely energy absorption from the radiation field can only be detected if there are more α spins than β spins.

Due to variations in the nuclear g factor and in the spin, different nuclei require widely different values of H or ν for resonance to occur. An excellent account of the more detailed NMR theory, with particular reference to the Fourier transform method, has been written by Birdsall, Lee and Metcalfe (34). This contains all the theory necessary for an understanding of the results described in this chapter, and is not complicated by advanced, highly mathematical descriptions of the various phenomena.

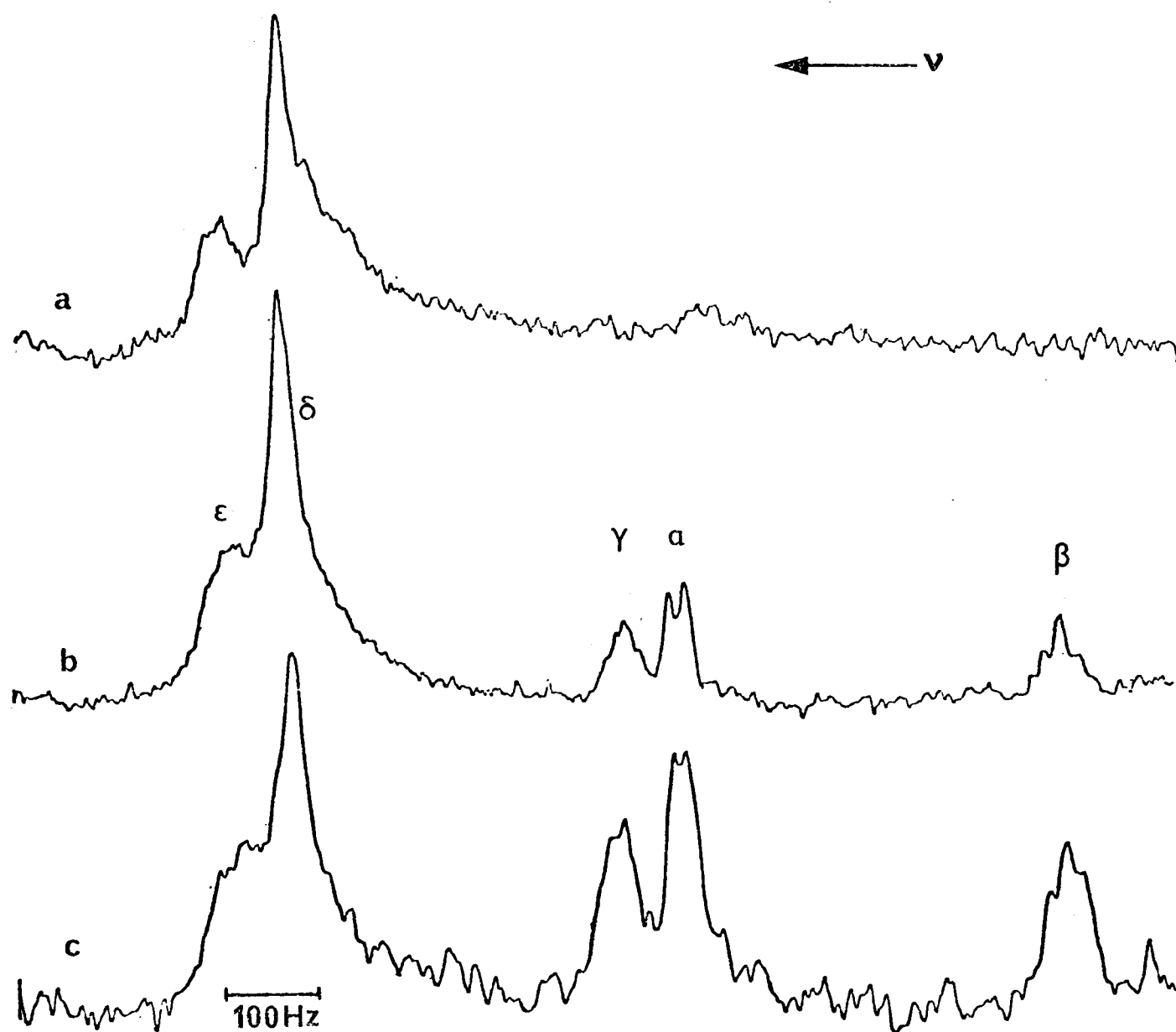
4.3. Materials and Methods.

All glands used in this study were obtained from freshly slaughtered animals, and were kept on ice until use. This period was kept as short as possible, although ^{31}P NMR examination of glands stored on ice for longer than 12 hours showed that they still contained ATP in comparable concentrations. Granules were prepared from the porcine and ovine glands by the method described in Chapter 2.2.A. for bovine granules. All chemicals used were of analytical grade. X-537A was obtained from Hoffman La Roche, Basel.

^{31}P NMR spectra, at 129 MHz (7.5Tesla) were obtained on an instrument constructed by Dr D.I.Hoult and Dr R.E.Richards F.R.S. (10) using Quadrature Fourier Transformation with a Cyclically Ordered Phase Sequence (CYCLOPS) of transmitter pulses, and the appropriate hardware routing in the computer store in order to orthonormalise the two free-induction decays. A Bruker WH-90 operating in the Fourier Transform mode was used to record ^{31}P spectra at 36.43MHz (2.1Tesla), ^1H spectra at 90MHz and ^{13}C spectra at 22.63MHz. Further ^1H spectra were obtained using a modified Bruker HFX fitted with an Oxford Instruments 6.3Tesla superconducting magnet and operating at 270MHz in the Fourier Transform mode. External deuterium oxide was used for the field frequency lock in the case of the ^{31}P studies.

The lipid extract of the chromaffin granules was made using the method of Bertsch et al, with the modification that the aqueous phase contained 5mM EDTA to remove the divalent metal ions, and hence the paramagnetic ions from the lipids.(66).

FIGURE 4.4.1.



^{31}P NMR spectra of (a) porcine adrenal cortex, (b) intact porcine adrenal gland and (c) porcine adrenal medulla.

Tissues were immersed in Tyrode's solution, pH 7.0. Spectra are accumulations of between 1000- 2000 pulses without proton decoupling, (Pulse interval 2s, Pulse angle 70°). Temp. = 16° . $\nu = 36.43$ MHz.

Peak assignments: α , β , γ are the corresponding phosphates of ATP,
 δ is inorganic phosphate,
 ϵ is AMP.

4.4 Results

A Studies of Intact Adrenal Glands.

^{31}P Phosphorus NMR at 36.43 MHz was used to study intact adrenal glands from pigs and sheep. These are systems infrequently studied using biochemical and pharmacological techniques, but studies of the composition of the granules derived from these glands have been made by various workers (22,35,36,37,38,39). The catecholamine contents of bovine, ovine and porcine glands are shown in Table 4.4.1. These systems were studied for the very practical reason that the glands were a suitable size to fit in the NMR tubes, but were also sufficiently large that only one gland was necessary to fill the cavity. If the glands from the small animals used in pharmacological research, namely rat, mouse and hamster, had been used, not only would several animals, six or more, have had to be killed for each experiment, but it would have been highly impracticable to prepare granules from these glands in sufficient quantities to permit study and comparison with the intact system.

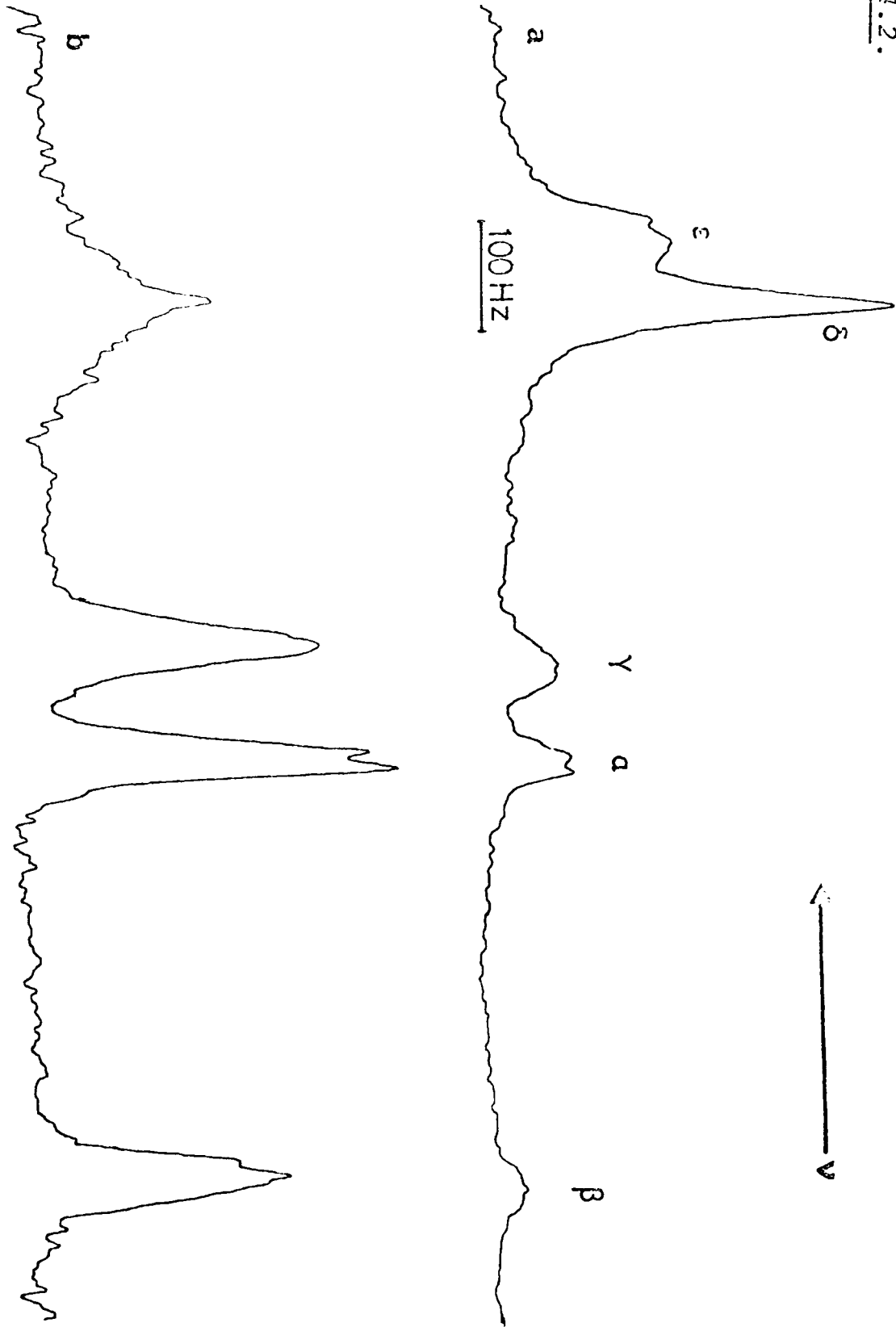
The spectra of a porcine adrenal gland and the adrenal medulla and the adrenal cortex carefully dissected from it are shown in Figure 4.4.1. The resonances are assigned as stated in the legend to the figure. The ATP resonances present in the intact gland are derived from the adrenal medulla not from the adrenal cortex. A large proportion of the remaining phosphate resonances, including those of inorganic phosphate and the monophosphates eg. AMP, glucose-1-phosphate, phospholipids, are derived from the adrenal cortex. It is interesting that so little ATP should be present in the cortex of the adrenal gland which is responsible for the synthesis of the mineralo- and glucocorticoids. Since chromaffin tissue is the major cell system present in the adrenal medulla, it is expected that the ATP resonances which can be seen in the spectra of the intact gland and the dissected medulla are derived from the ATP present in such large quantities in the chromaffin granules. A comparison of the spectra of the

TABLE 4.4.1.

<i>Animal</i>	<i>Catecholamines</i>		
	<i>Adrenaline</i>	<i>Noradrenaline</i>	<i>Dopamine</i>
<i>Cow</i>	4000	1500	35
<i>Sheep</i>	2000	1600	350
<i>Pig</i>	1095	1055	—

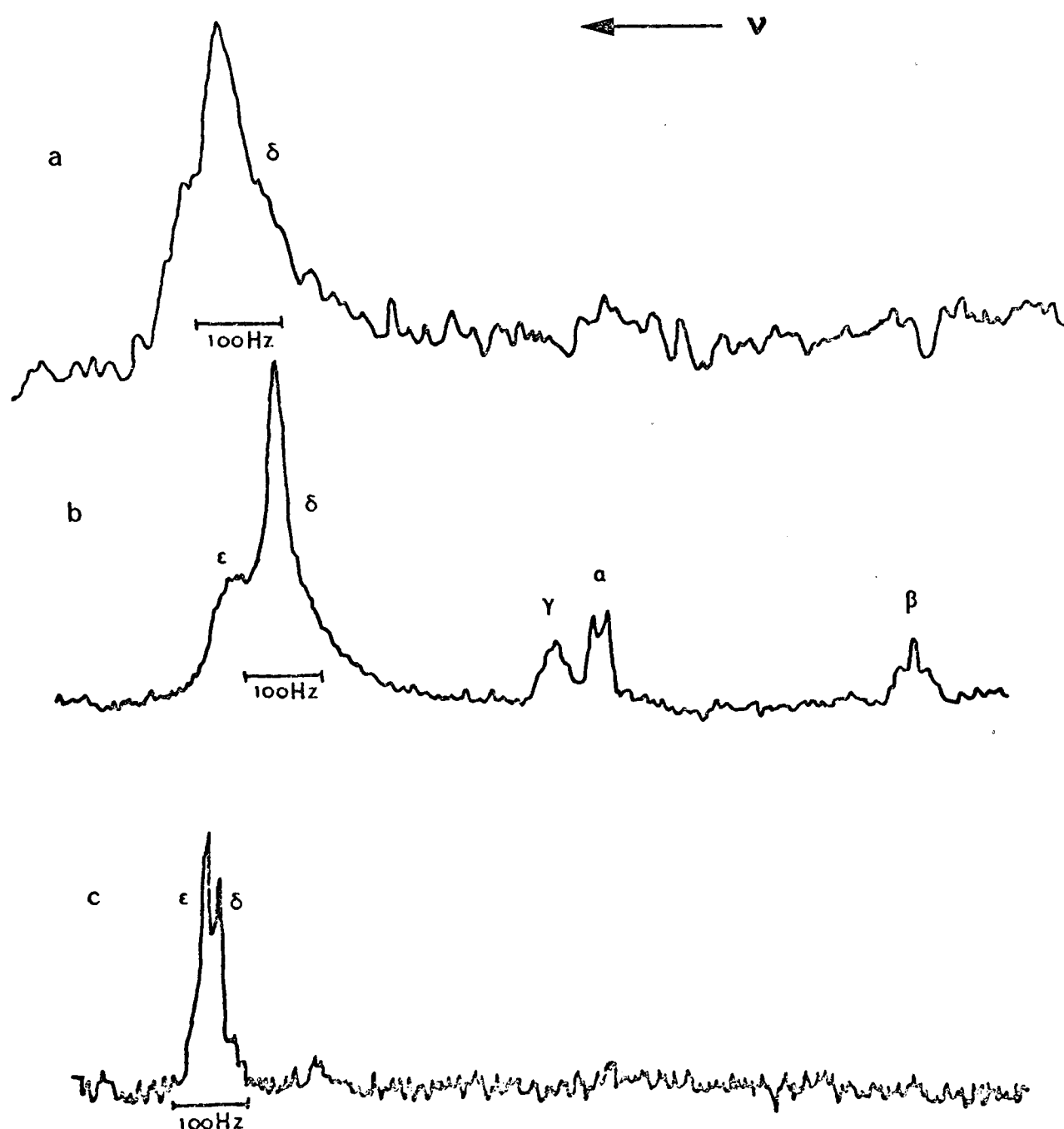
The catecholamine contents of the adrenal glands from three different species. The values are expressed in μg catecholamine/g medullary tissue and have been taken from reference (37).

FIGURE 4.4.2.



^{31}P NMR spectra of (a) a porcine adrenal gland, (b) porcine chromaffin granules suspended in 'buffered' 0.3M sucrose. Assignments and conditions as in Figure 4.4.1.

FIGURE 4.4.3.



^{31}P NMR spectra of (a) a porcine adrenal gland after stimulation with X537A/ Ca^{2+} , (b) a porcine adrenal gland before stimulation and (c) perfusate from two porcine glands (pH 7.4 in 10mM EDTA).

The glands were retrograde perfused with oxygenated Tyrode's soln. containing 0.5mM X537A and 10mM CaCl_2 , after the method of Banks (1977). Glands treated with solutions which lacked either Ca^{2+} or X537A gave spectra as in (b). The perfusate was centrifuged at 18,000g for 10mins to remove debris, treated with perchloric acid, 4%, to precipitate protein and returned to pH 6.0 using KOH before concentration by freeze-drying. Assignments and conditions as in Figure 4.4.1.

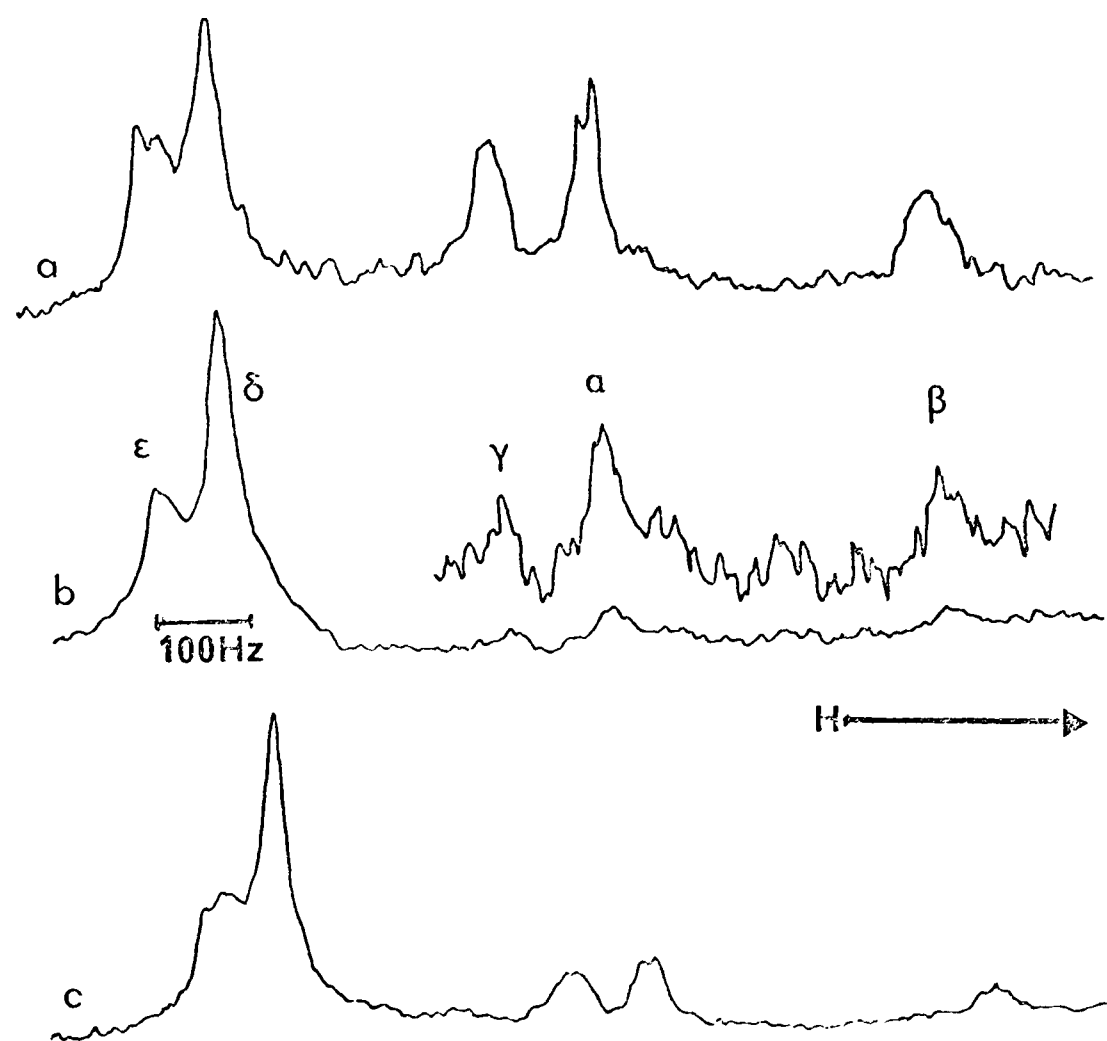
intact porcine adrenal gland and the chromaffin granules prepared from it (Figure 4.4.2.) shows that the resonances of ATP in both are virtually identical in frequency (to within 0.4ppm) and in lineshape. This, in itself, is a good indication that the resonances are derived from the same source. In order to show this identity conclusively, exocytosis of the contents of the chromaffin granules in the intact gland was produced in vitro. This is demonstrated in Figure 4.4.3. Retrograde perfusion of the gland with Ca^{2+} and X-537A results in the complete loss of the ATP signal from the gland and the appearance of AMP and inorganic phosphate in the perfusate. These results are in agreement with the classical analysis of the perfusate from bovine adrenal glands, retrograde perfused but stimulated with carbamylcholine (40). The spectrum of the gland after stimulation is almost identical to that of the cortex alone, though there is obviously a small contribution from the medullary tissue. The positions of the AMP and inorganic phosphate resonances do not coincide with those of the cortex due to the pH dependence of the resonance frequencies and the presence of EDTA in the perfusate which reduces the width of the resonances.

In view of the similarity between the ATP resonances of the intact gland and the purified granules, and the disappearance of these resonances after the gland is stimulated, a process which electron microscopy has shown removes the granules from the chromaffin cells (41), it may be concluded that the ATP resonances in the intact gland are due to the ATP present in the chromaffin granule complex.

B A Comparison of the Chromaffin Granules and the Adrenal Glands from Different Animals using ^{31}P NMR.

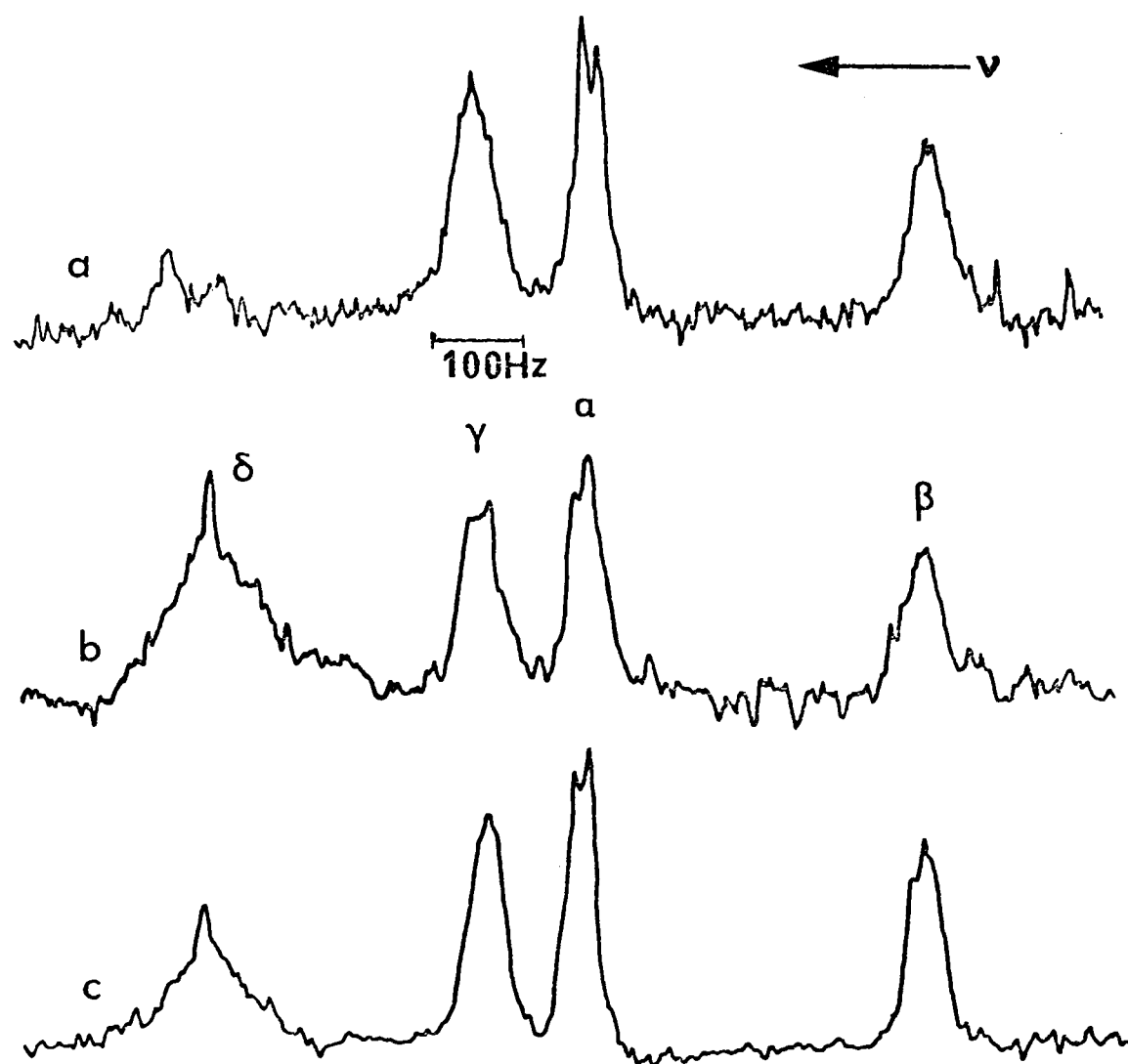
The ^{31}P spectra of porcine and ovine adrenal glands and bovine adrenal medulla and of the chromaffin granules prepared from these tissues are compared in Figures 4.4.4. and 5. The spectra in Figure 4.4.4. show that, although the ATP concentration present in each tissue is different, species differences are present. In the spectrum of the pig

FIGURE 4.4.4.



^{31}P NMR spectra of (a) a bovine adrenal medulla, (b) an intact ovine adrenal gland and (c) an intact porcine adrenal gland. Assignments and conditions as in Figure 4.4.1.

FIGURE 4.4.5.



^{31}P NMR spectra of (a) bovine chromaffin granules, (b) ovine chromaffin granules and (c) porcine chromaffin granules.

All are suspended in 'buffered' 0.3M sucrose.

Assignments and conditions as in Figure 4.4.1..

gland, the triplet structure of the β -resonance of ATP is clearly resolved. In contrast, although the spectrum of the bovine adrenal medulla has a better signal:noise ratio and the doublet of the α -resonance is apparent, there is no structure present in the β -resonance. The signal:noise ratio of the sheep gland spectrum is too low for a statement to be made, but there appears to be no splitting in the α -resonance though this splitting is present in a pig gland spectrum with a similar noise level. Many different glands from each species were examined in the spectrometer, and several trends were observed. Firstly, the pig gland always contained ATP at a fairly constant concentration, and the hyperfine splitting of the β -peak was nearly always observed. Secondly, the sheep glands did not always contain ATP. This was ascribed to the fact that sheep are more excitable animals than pigs and are killed in a different manner at the slaughter house. This generally lower level of ATP was also reflected in the much lower relative yield of granules from the sheep adrenal medullae compared to those from bovine and porcine sources. Finally, the spectra of the bovine adrenal medullae were very consistent, not only with respect to the presence and quantity of ATP, but to the resolution of the splittings in the resonances.

The line-shapes and the resolution of the splittings of the ATP resonances of the chromaffin granules are very similar to those in their respective glands; the resonances of the porcine gland are better resolved than those of the bovine which, in turn, are better than those of the ovine (Figure 4.4.5.). These differences are also reflected in the relaxation behaviour of the ATP resonances of the different systems. Briefly, the resonances of the bovine gland cannot be saturated by 50° pulses at 0.2s intervals, whilst those of the porcine granules can. This means that there is a mechanism or degree of relaxation available in the bovine granules which is not present in the porcine. This may be due to the presence of transition metal ions, but other explanations are equally

TABLE 4.4.2.

	α	β	γ
<i>Porcine Adrenal Gland</i>	8.56	11.17	22.45
<i>Porcine Chromaffin Granules</i>	8.13	11.22	22.01
<i>Ovine Adrenal Gland</i>	7.97	11.27	22.03
<i>Ovine Chromaffin Granules</i>	7.63	11.17	22.13
<i>Bovine Adrenal Medulla</i>	8.06	11.12	22.10
<i>Bovine Chromaffin Granules</i>	7.38	11.19	21.86

A comparison of the frequencies of the α , β and γ phosphate resonances of the ATP present in the Chromaffin Granules of three different animals. The frequencies are expressed in terms of the chemical shifts of the resonances from that of 88% Phosphoric Acid. The chemical shifts are in p.p.m.. The errors in the frequencies are approx. ± 0.4 p.p.m. due to the width of the resonances.

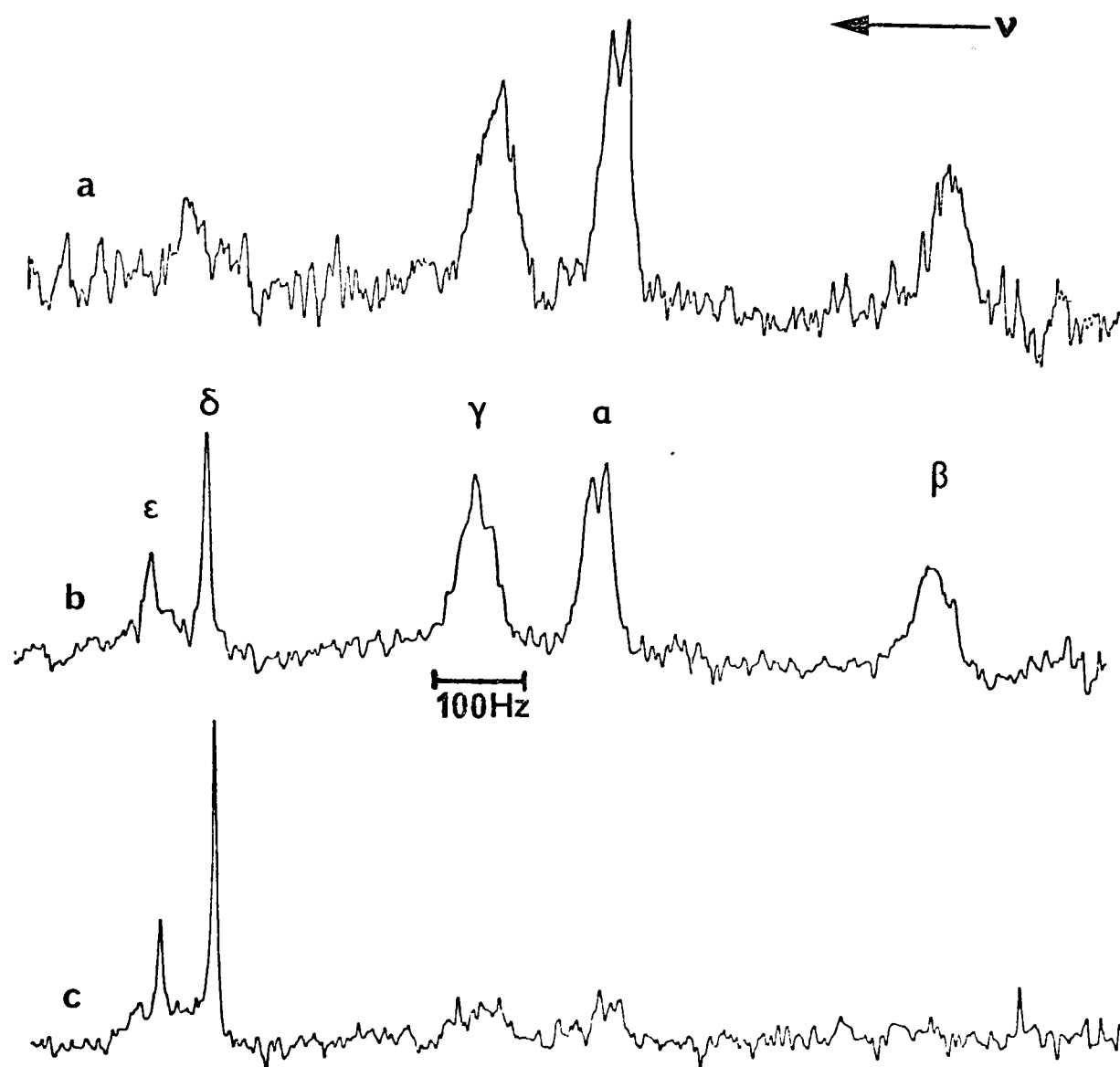
possible. This result is described more fully later in this chapter. The frequencies at which the resonances occur in the different systems are shown in Table 4.4.2. The positions of the resonances are virtually identical, within the error of measurement, in the bovine and ovine glands and granules but are slightly different in the porcine.

Thus the differences in the spectra derived from the various systems are not very large, but are of significance. A description of the nature of the complex must account for these differences.

C The Source of the Inorganic Phosphate and AMP in the Chromaffin Granule Spectra.

In all granule spectra, there are resonances present at around -1ppm (w.r.t. 88% phosphoric acid). Resonances at this frequency are from molecules containing a single phosphate moiety, or non-adjacent phosphates. In this case, these resonances have been assigned as AMP and inorganic phosphate in view of their absolute frequencies and their increasing concentration as the granules age. Phospholipids may contribute slightly, but the resonance from the membrane phospholipid is very broad and, therefore, will be insignificant in the presence of other mono-phosphates. If there is EDTA present in the buffer, which is unusual since the low concentration of divalent metal ions bound to the membrane helps to stabilise the isolated granules, these resonances sharpen up but they are normally broad and unresolved. The AMP and inorganic phosphate are the ultimate degradation products of the ATP which leaks out from the disrupted granules, since adenylate kinase is present as an extragranular contaminant (42). The resonances are not due to the presence of these molecules within the granules, themselves. This is shown in Figure 4.4.6. where the spectrum of granules suspended in 0.3M sucrose is compared to that of the supernatant obtained when the granules are pelleted and to that when cobalt ions are added to the suspended granules to broaden out the accessible supernatant resonances. Cobalt ions do not penetrate phospholipid bilayers (26) and

FIGURE 4.4.6.



^{31}P NMR spectra of (c) the supernatant in which the chromaffin granules in (b) were suspended, (b) bovine chromaffin granules and (a) bovine chromaffin granules in (b) in the presence of $10\mu\text{M}$ Cobalt ions.

Assignments and conditions as in Figure 4.4.1.

since there is no broadening of the ATP resonances, it is unlikely that they penetrate the chromaffin granule membrane. The supernatant spectrum contains two very sharp peaks at -2ppm and -0.3ppm (w.r.t. 88% phosphoric acid) which are assigned to AMP and Pi respectively. There is also an indication of the presence of ADP resonances at around 8ppm and 11ppm. There appears to be no ATP present since there is no resonance at 22ppm which would be attributable solely to the β -phosphate of ATP. When cobalt ions are added, the AMP and Pi resonances are broadened out in keeping with the other results.

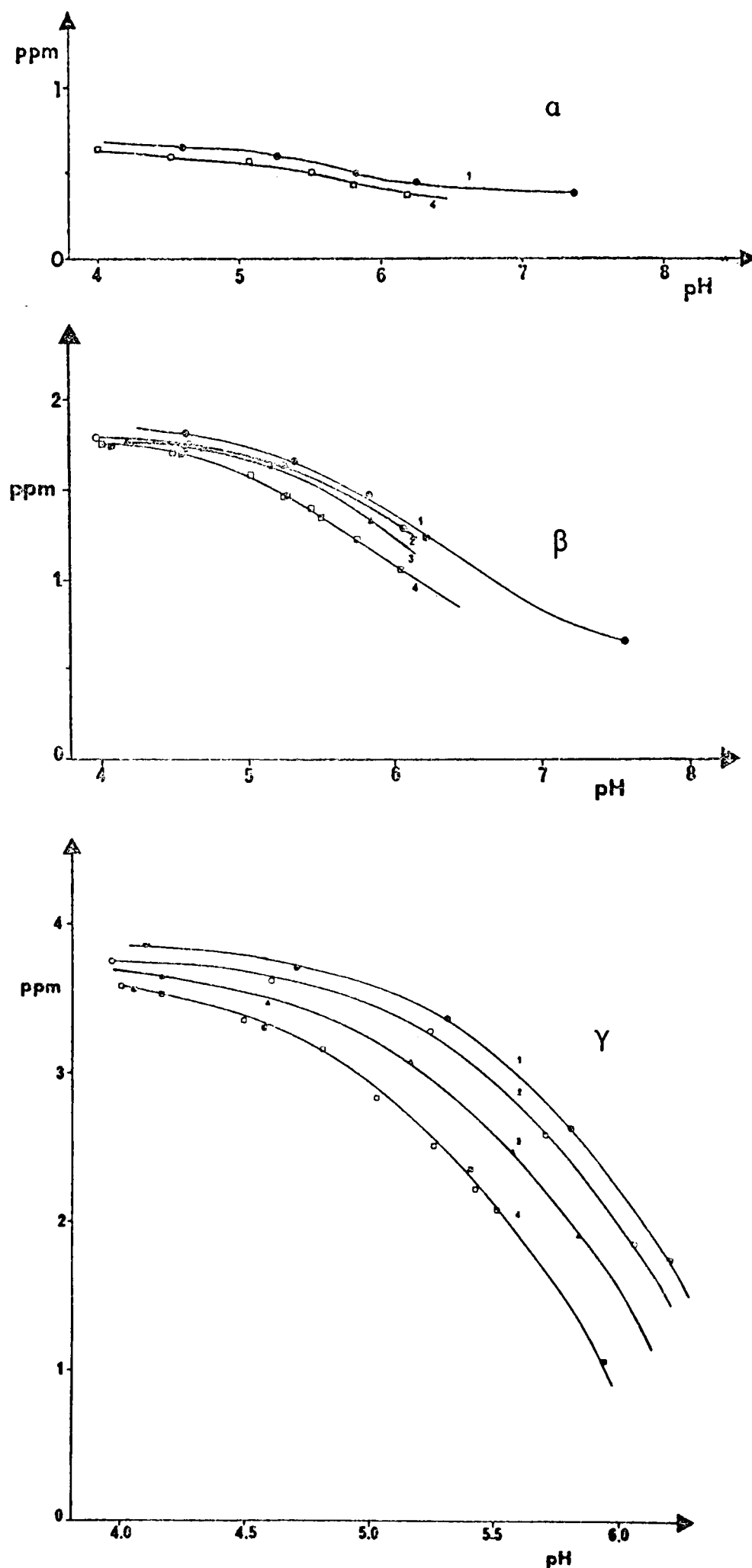
D The Determination of the Internal pH of the Bovine Chromaffin Granule.

Studies on the coupled ATPase and the ANS response (Chapter Two) indicate that the pH inside the bovine chromaffin granule, on which these studies were carried out, is considerably less than 6.8, the normal intracellular pH. The existence of such a proton gradient has considerable implication, since it may play a very critical role in the accumulation and maintenance of the large amounts of amines present in the granules.

The frequencies of the resonances of the β - and γ - phosphates of ATP are very sensitive to pH, in addition to being sensitive to other metabolites. That is to say, they are very sensitive, non-perturbing pH meters, once the system has been calibrated.

As related previously, the chromaffin granule is reported to contain, in addition to the large concentrations of catecholamines (550nM) and ATP (122mM), chromogranin proteins (120mgs/ml) (43), divalent metal ions (20mM Ca^{2+} and 8mM Mg^{2+}) (44) and possibly phospholipids (approx. 0.25 μ moles/mg of matrix protein) (45). The pH dependence of the resonances of 122mM ATP in the presence of other molecules in a variety of combinations and concentration was investigated.

FIGURE 4.4.7.



The pH titrations of the frequencies of the α , β , and γ phosphate resonances of 122mM ATP in the presence of different concentrations of adrenaline.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero.

Key : 1 122mM ATP alone
 2 + Adrenaline 100mM
 3 + Adrenaline 300mM
 4 $\square$ + Adrenaline 550mM
 $\blacksquare$ + Adrenaline 700mM.

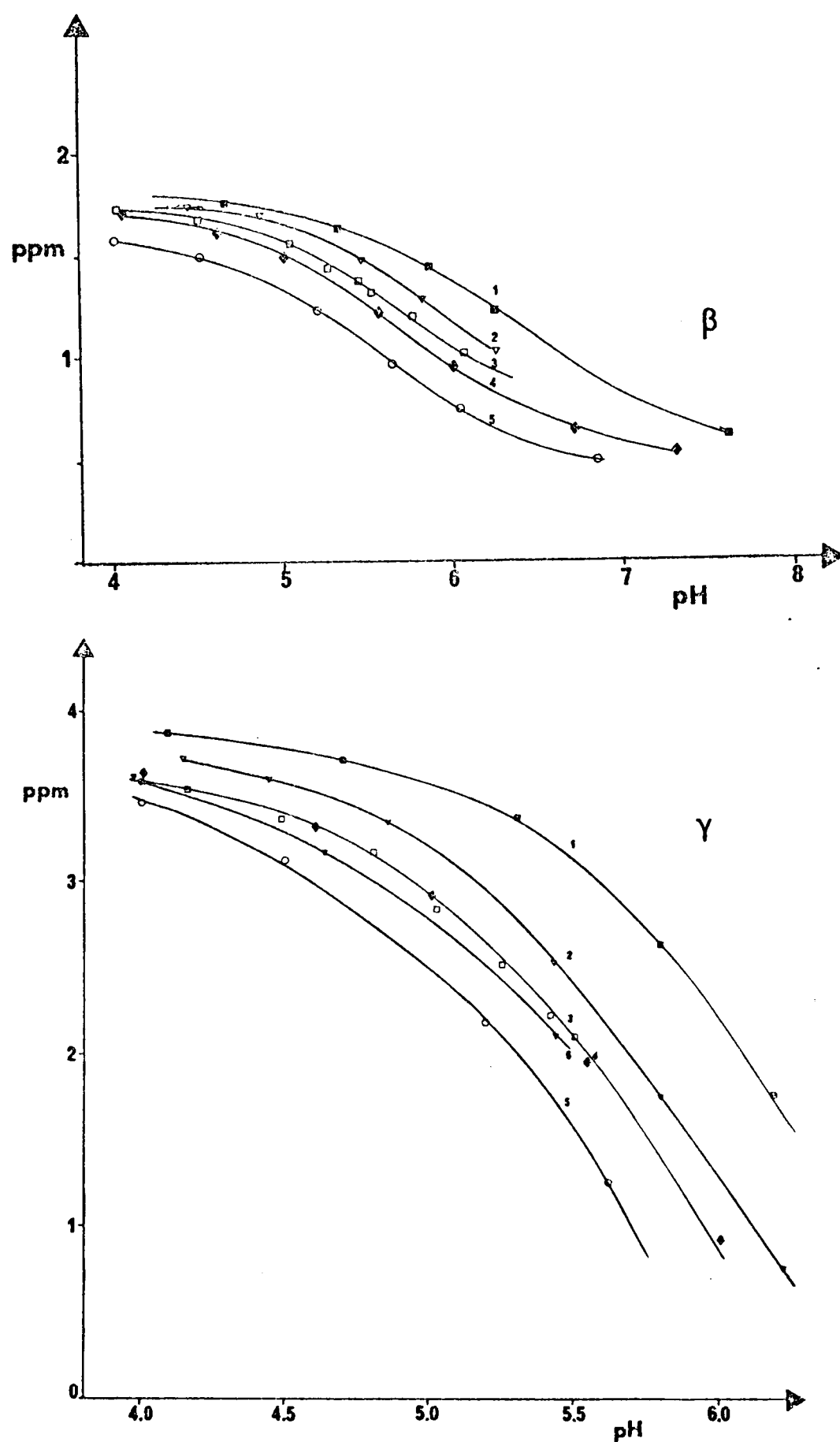
Conditions as in Figure 4.4.1.

(i) The Effect of Catecholamines.

The shifts in the phosphate resonances produced in the pH titration curves of disodium ATP, 122mM, on the addition of increasing concentrations of adrenaline are shown in Figure 4.4.7. The pH range studied was pH 4 to 6, since such large concentrations of ATP and catecholamines are only slightly soluble at pHs greater than 6. The shifts observed are dependent on the adrenaline concentration over the range 0 to 550mM, but the addition of catecholamines in excess of 550mM does not shift the resonances further. The pH titrations in the presence of 550 and 700mM adrenaline are very similar. The different sensitivities of the ATP phosphate resonances are apparent. Further pH titrations shown in this chapter will not show the α -resonance which has the least sensitivity.

The effect of several different amines on the pH titration curves of the β - and γ -phosphates of ATP are shown in Figure 4.4.8. In each case, the concentration of amine present is 550mM. Dopamine and adrenaline have similar effects on the titration curves, but noradrenaline shifts the curve further to lower field. Ethanolamine has much less effect than even adrenaline. It is important, therefore, to know precisely the relative contents of noradrenaline and adrenaline in the chromaffin granules. The total bovine granule preparation contains approximately 80% adrenaline (46,47). The titration curve in the presence of 440mM adrenaline and 110mM noradrenaline is also shown for the γ -phosphate. This curve lies, as might have been expected, between the noradrenaline and adrenaline curves, but closer to that of adrenaline. Individual granules, however, are not thought to contain mixed populations of catecholamines, since 'heavy' noradrenaline-containing granules can be purified by further centrifugation of the granules through 2.15M sucrose (39,48). In the case of the bovine granules, the resonance from the adrenaline-containing granules will be four times as large as that from the noradrenaline-containing granules and will hence dominate the signal. This heterogeneity may result in a

FIGURE 4.4.8.



The pH titrations of the frequencies of the β , and γ phosphate resonances of 122mM ATP in the presence of different catecholamines and ethanolamine, all 550mM.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions as in Figure 4.4.1..

Key : 1 ATP, 122mM, alone
 2 + Ethanolamine, 550mM
 3 + Adrenaline, 550mM
 4 + Dopamine, 550mM
 5 + Noradrenaline, 550mM
 6 + Adrenaline, 440mM, + Adrenaline, 110mM.

broadening of the resonances.

It is very interesting that the effect of dopamine is closer to that of adrenaline than to that of noradrenaline, since dopamine is structurally more similar to noradrenaline whose precursor it is. Thus the N-methyl and the β -hydroxyl groups must have antagonistic effects on the phosphate shifts. This would indicate at least a limited interaction between the side chain of adrenaline and the phosphate group of ATP. It is not possible to state from these results whether the shifts present in the pH titration curves are pK_a shifts or otherwise, since because of the insolubility of the molecules at pHs greater than 6 in the high concentrations studied here, the phosphate group is not being titrated across its full pK range.

(iii) The Effect of Divalent Metal Ions.

Divalent metal ions, including calcium and magnesium, are known to cause shifts in the resonance frequencies of the phosphates of ATP (11,49). The pH titration curves in the presence of these metal ions are moved to higher frequency. Unfortunately, there appears to be some uncertainty about the precise divalent metal ion content of chromaffin granules. Borowitz et al (44) initially reported the divalent metal ion concentrations present in bovine chromaffin granules prepared by unspecified differential and density gradient centrifugation to be: 20mM calcium, 8mM magnesium, 1.3mM iron, 0.7mM zinc and trace amounts of other metals including chromium, strontium, barium and manganese. In a subsequent paper, Borowitz examined the calcium and magnesium concentrations of granules at various stages of their preparation. The lysosome-contaminated granules prepared through 1.5M sucrose contained 22.3mM calcium and 7.0mM magnesium. The purer granules prepared through 1.75M sucrose contained 14.1mM calcium and 3.6mM magnesium whilst those through 2.0M sucrose contained 28.4mM calcium and 6.4mM magnesium (48). These discrepancies are due to the very large variation in the catecholamine:protein ratios of the various fractions

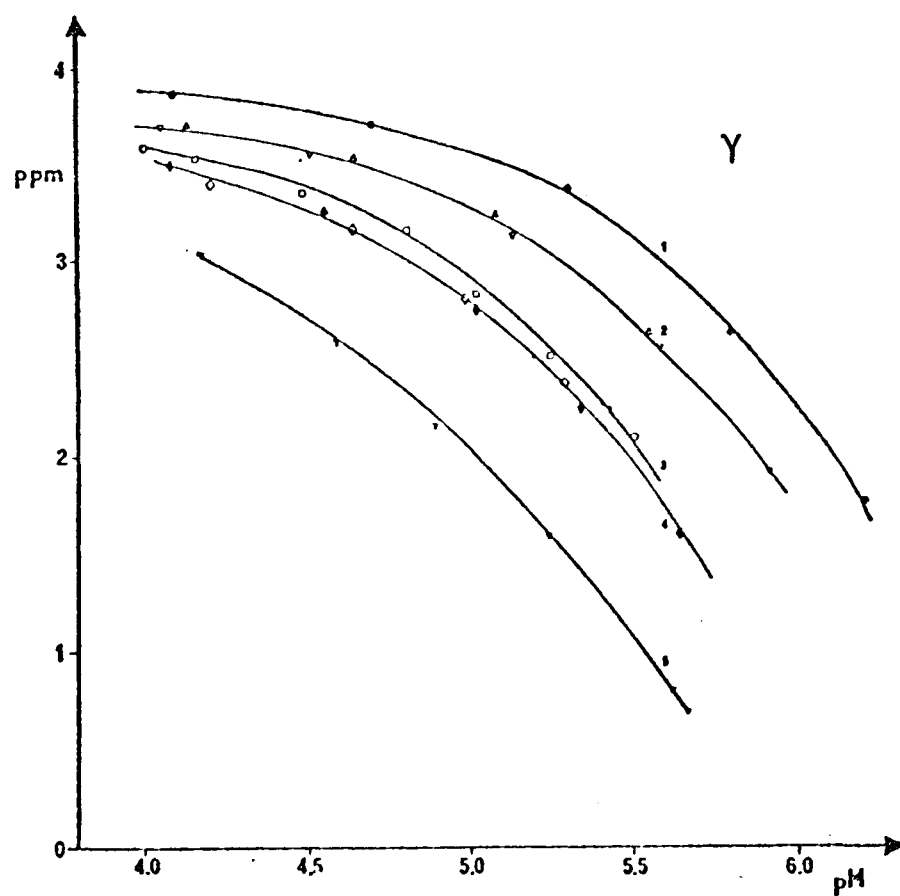
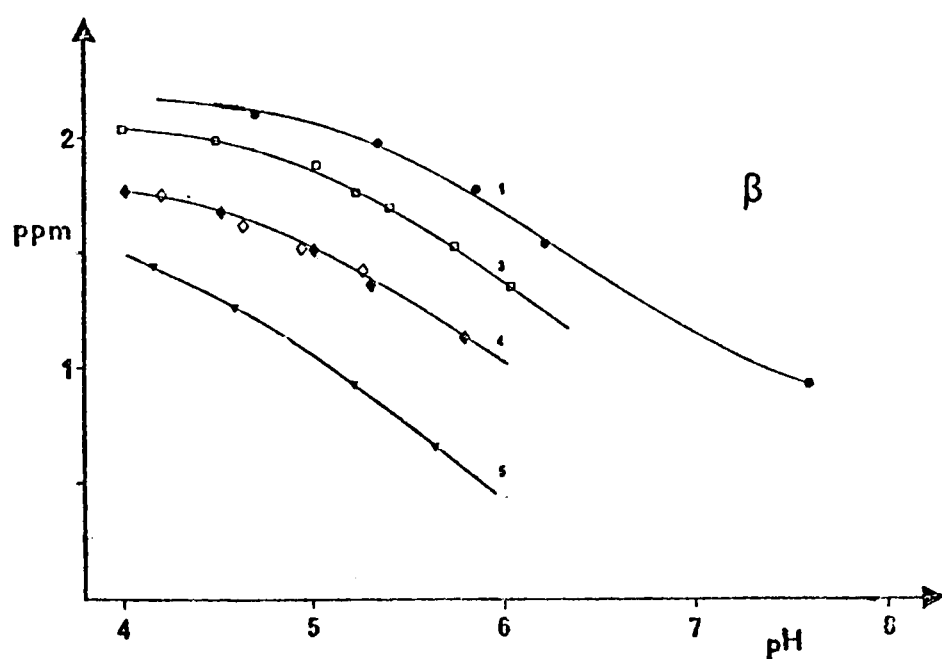
since the metal ion content is calculated assuming a catecholamine concentration of 550mM.

Phillippu and Schumann (50) report a relative $\text{ATP}:\text{Ca}^{2+}:\text{Mg}^{2+}$ concentration ratio of 3.65:2:1 in bovine granules also prepared by unspecified conditions through sucrose solutions. They do not quote catecholamine concentrations with this data. However, in another table they present different values, which when based on a 550mM catecholamine concentration, give values of 108mM ATP, 75mM calcium and 37mM magnesium. These are anomalously high values and cannot be compared to those found by Borowitz as no protein concentrations are shown.

Serck-Hanssen and Christiansen (51) have determined a calcium concentration of 27mM based on their determined catecholamine:protein ratio of 2.5 μ moles catecholamine / mg protein.

For these studies, the original values determined by Borowitz et al ie. 20mM calcium and 8mM magnesium have been used. It is unfortunate that the ATP concentration was not measured by all the workers, since it is the ATP:metal ion not the catecholamine:metal ion ratio that is important. The titration curves for disodium ATP in the presence of calcium and magnesium at various concentrations are shown in Figure 4.4.9. The effects of these ions on the ATP resonances when adrenaline is additionally present is also illustrated. The presence of 20mM calcium and 8mM magnesium results in a down field shift of 1.37ppm compared to ATP and adrenaline in the presence of EDTA at pH 5.0. A precise knowledge of the divalent metal content of the granules is essential if accurate values for the shifts due to these ions in the phosphorus spectra of chromaffin granules is to be ascertained. It should also be noted that there is mostly likely considerable heterogeneity in the metal ion contents of the different granules. This, like the variation in catecholamines, will accentuate the heterogeneity of the specimen, and contribute to a broadening of the line-widths of the resonances.

FIGURE 4.4.9.



The pH titrations of the frequencies of the β - and γ -phosphate resonances of 122mM ATP in the presence of different concentrations of divalent metal ions.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero.

- Key:
1. 122mM ATP
 2. + 8mM CaCl_2 ;
+ 8mM MgCl_2 .
 3. + 550mM adrenaline
 4. + 20mM CaCl_2 ;
+ 20mM MgCl_2 .
 5. + 550mM adrenaline + 20mM CaCl_2 + 8mM MgCl_2 .

Conditions as in Figure 4.4.1.

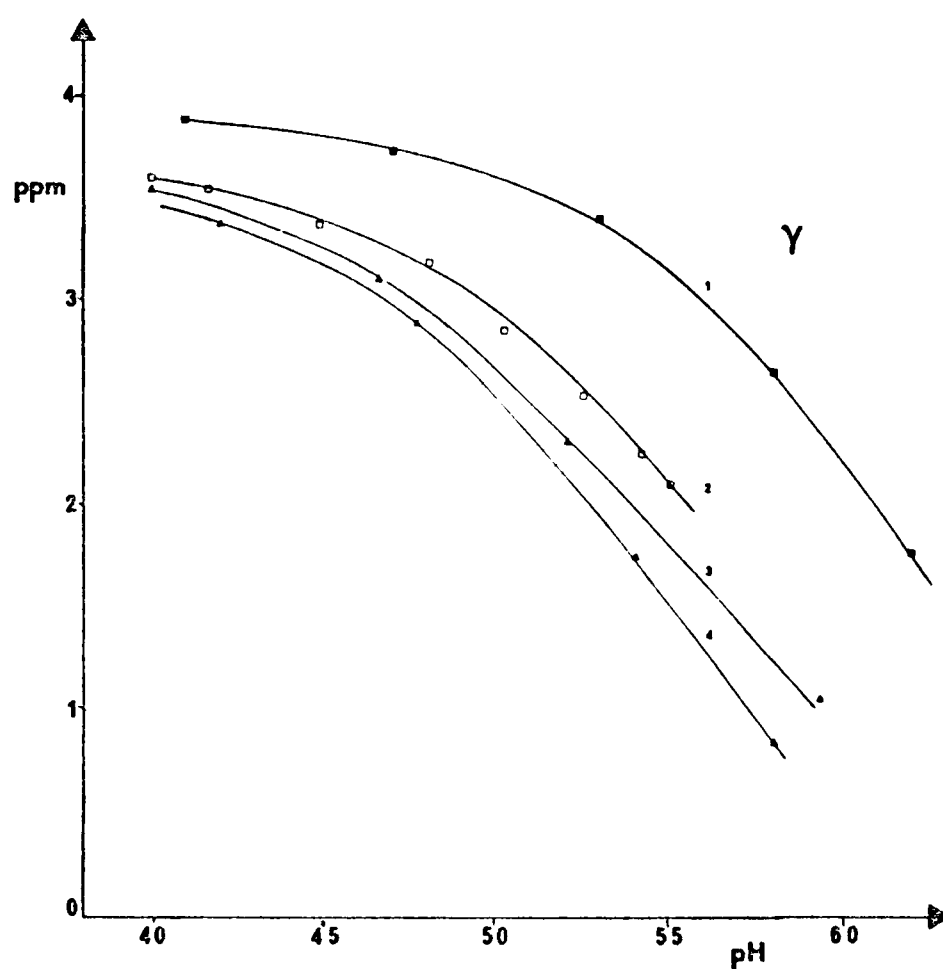
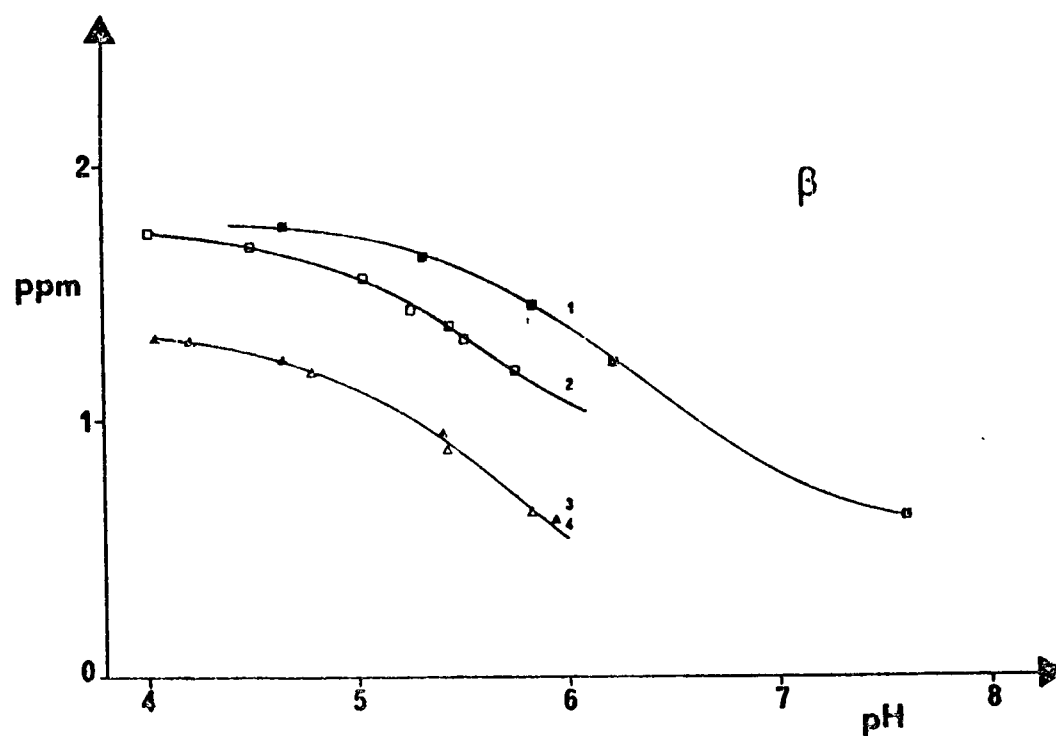
(iii) The Effects of Monovalent Ions.

One of the major problems encountered in trying to simulate the contents of the chromaffin granule is that the commercially obtained ATP is the disodium salt. The pH of the solutions examined was adjusted using hydrochloric acid and potassium chloride, thus introducing even more monovalent cations. This problem was tackled in two different ways.

Firstly, the effect of a large excess of potassium chloride on the pH titration of the phosphorus resonances of ATP, on its own, in the presence of adrenaline and in the presence of adrenaline and divalent metal ions was studied (Figure 4.4.10). Whilst the shifts produced by the salt are quite large in the absence of adrenaline, it is rather less when adrenaline is also present. The effects on the γ - and β -resonances are different. In both cases the salt causes a substantial shift in the presence of ATP alone. In the presence of adrenaline also, the γ -resonance is further shifted to high frequency, whilst there is no additional effect on the β -resonance. It is possible that in the granule, therefore, the effect of the monovalent cations may be reduced by the presence of other molecules.

Secondly, sodium-free ATP was prepared by column chromatography. The disodium ATP was passed down a carefully washed DOWEX ion-exchange column, collected and freeze-dried. Sodium-free ATP is not very stable, and must be used soon after preparation as degradation does occur, resulting in a mixture of AMP, ADP, ATP and inorganic phosphate. The sodium-free ATP was used in a pH titration in the presence of 550mM adrenaline. Even if AMP and ADP are present, they also interact with adrenaline. Hence, any shift observed in the ATP resonances should be comparable to those observed in a solution of pure ATP. The results of this pH titration are shown in Figure 4.4.12. One of the most irreconcilable anomalies observed in all previous pH titrations was that at no time did the resonances of both the β - and the γ -phosphates coincide in frequencies with those found in the spectra of the intact granules. For example, if the frequency of the γ -resonance in the

FIGURE 4.4.10.

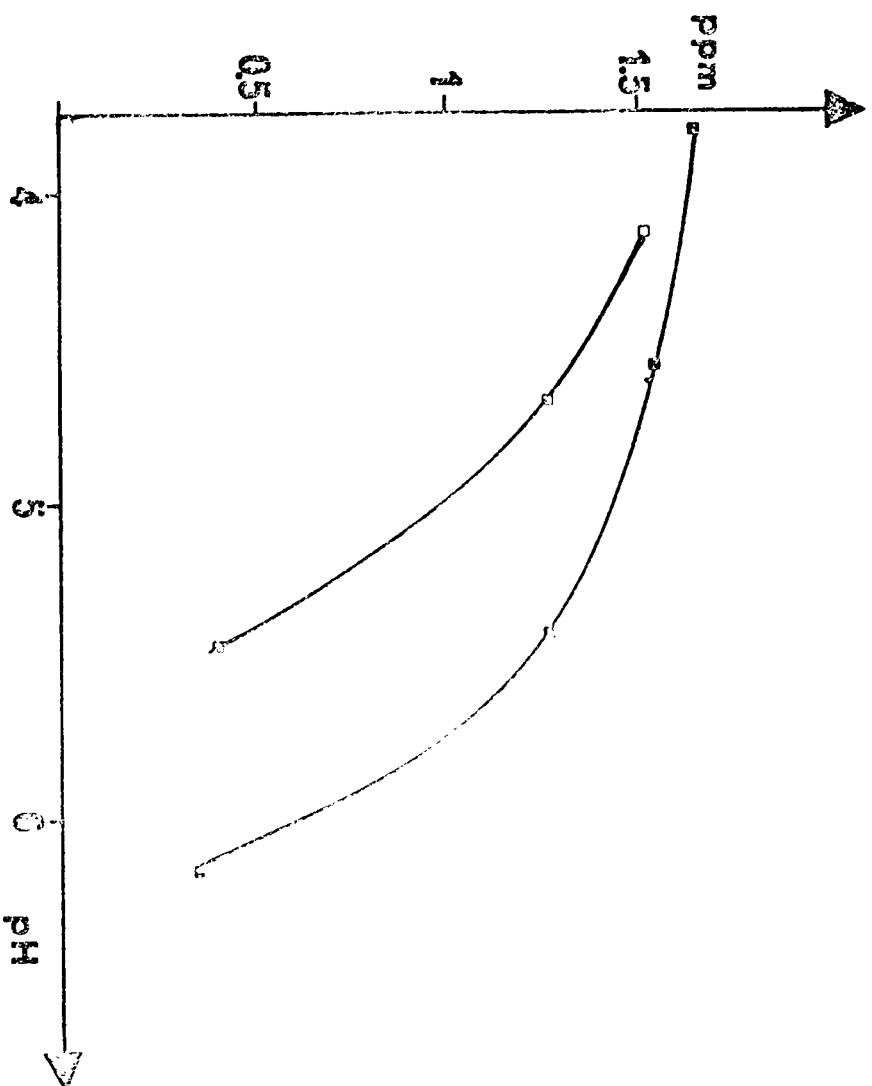


The pH titrations of the frequencies of the β , and γ phosphate resonances of 122mM ATP in the presence of Adrenaline, 550mM, and KCl, 1M.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions as in Figure 4.4.1.

Key : 1 ATP, 122mM, alone
 2 + Adrenaline, 550mM
 3 + KCl, 1M
 4 + Adrenaline, 550mM, + KCl, 1M.

FIGURE 4.4.11.

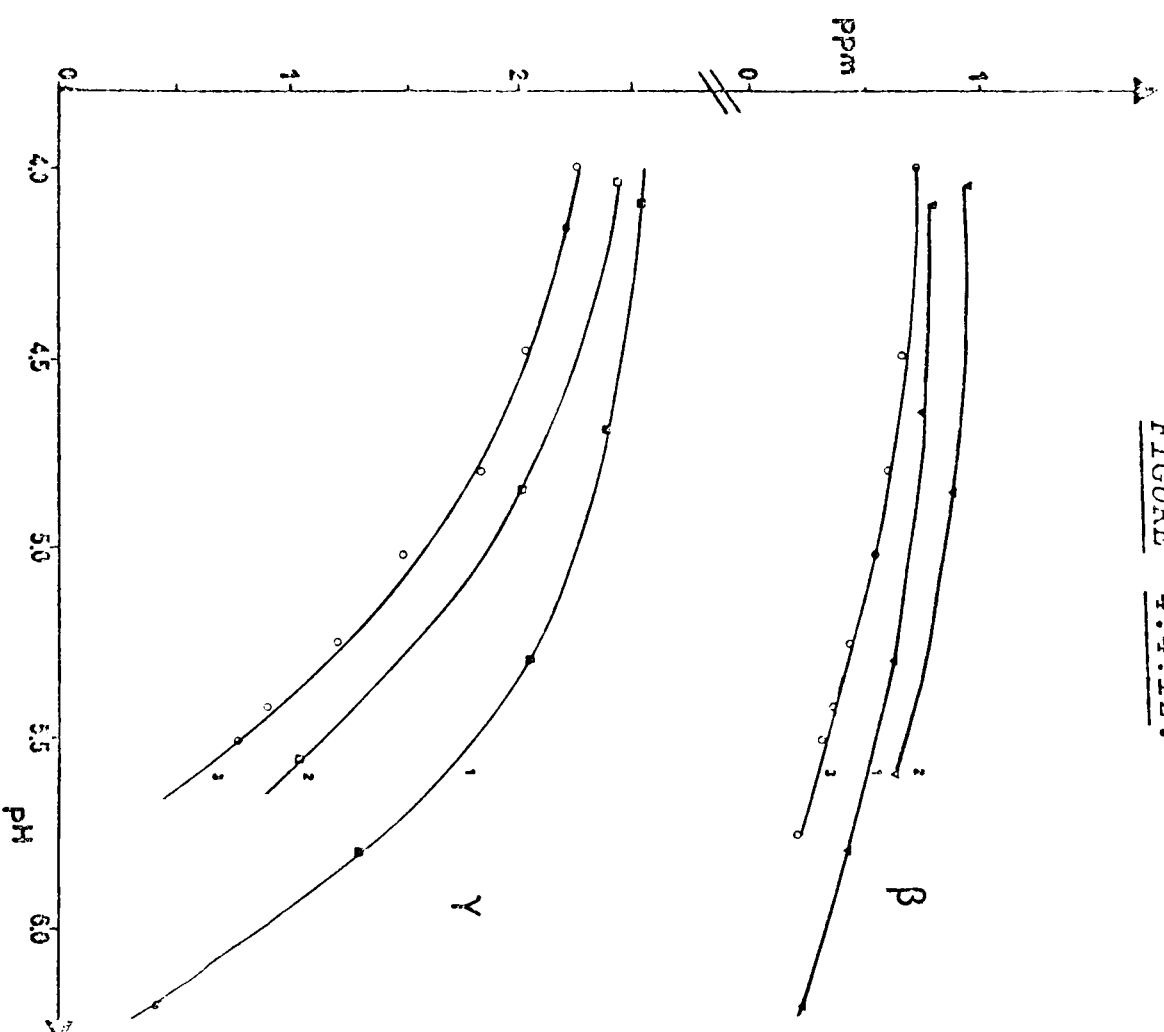


The pH titration of the frequency of the phosphate resonance of AMP, 122mM, alone and in the presence of Adrenaline, 550mM.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions as in Figure 4.4.1.

Key : 1 AMP, 122mM, alone
2 + Adrenaline, 550mM.

FIGURE 4.4.12.



The pH titrations of the frequencies of the β and γ phosphate resonances of disodium ATP, 122mM, and sodium-free ATP, 122mM, in the presence of Adrenaline, 550mM.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions as in Figure 4.4.1.

Key : 1 Disodium ATP, 122mM, alone
2 Sodium-free ATP, 122mM, + Adrenaline, 550mM
3 Disodium ATP, 122mM, + Adrenaline, 550mM.

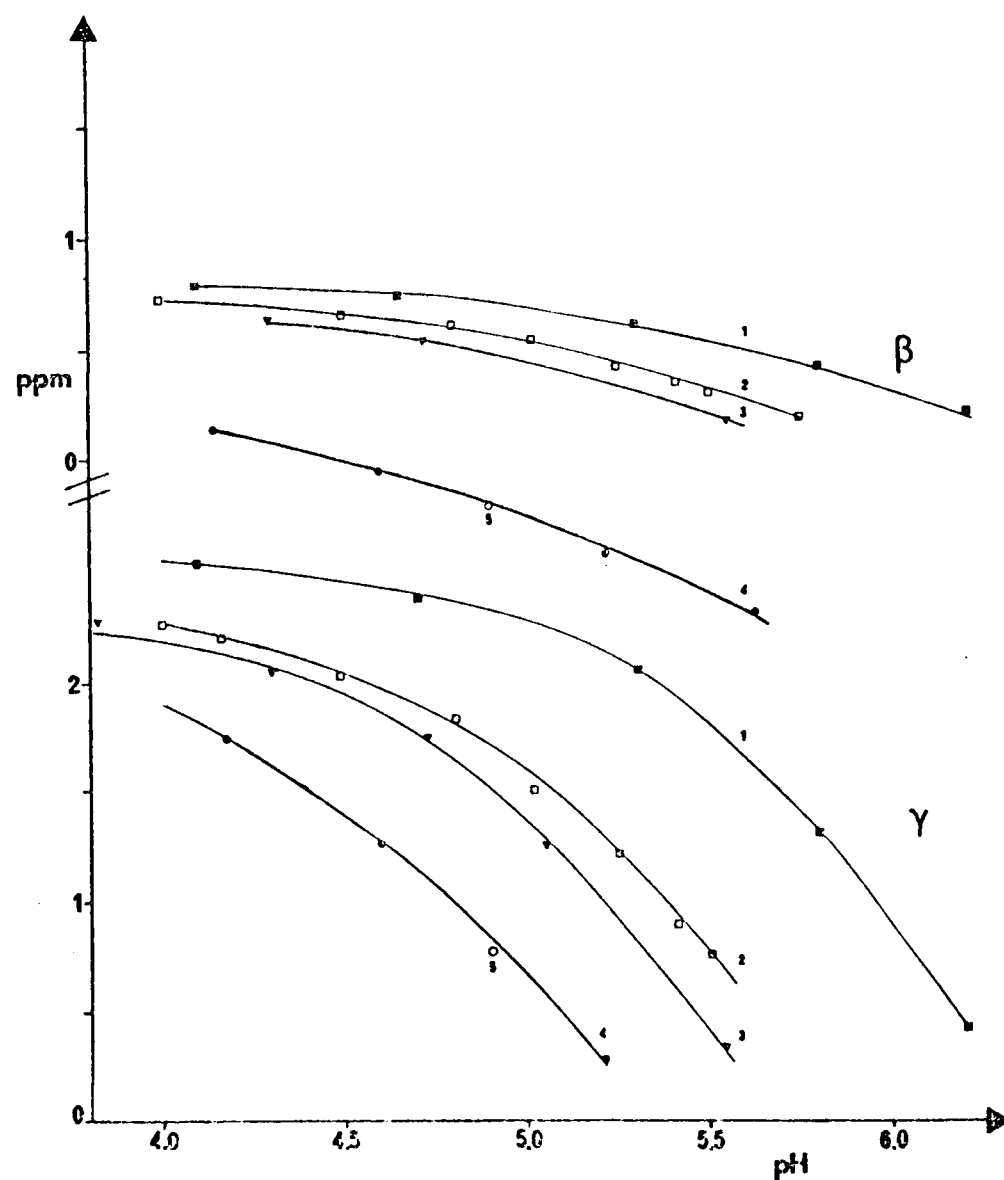
granule spectrum is considered to be 7.82ppm, this occurs at a pH of 5.46 on the titration curve of ATP in the presence of adrenaline and divalent metal ions in Figure 4.4.9. However, the corresponding β -resonance at that pH occurs at 21.34ppm, which is shifted 0.72ppm to a higher frequency than the resonance in the granule spectrum. On that particular titration curve, this difference is equivalent to greater than one pH unit. An explanation for this effect may be found on examination of Figure 4.4.11. Removal of sodium does not have a very large effect on the γ -resonance; the pH titration curve is shifted to a lower frequency, but lies intermediate to those of disodium ATP, alone and in the presence of adrenaline. The discrepancy is equivalent to 0.3ppm or 0.25 pHunits. The β -resonance, however, is much more greatly affected by the removal of sodium and the pH titration curve is shifted to a lower frequency than that of ATP(disodium) alone. The difference in this case is also 0.3ppm, but is equivalent to 0.8 to 1.0 pH units since the curves are much less steep.

This differential effect of the sodium ions, therefore, explains in part the fact that the γ - and β -resonances do not correspond in the pH titrations to the frequency values observed in the granule spectra. The concentration of sodium present in the granule is not known since it is difficult to obtain an accurate value for the concentration of sodium ions in a solution containing other metal ions, since its atomic absorption spectrum overlaps those of many other commonly occurring metals. The concentration of sodium present in the titrations, 244mM, is far in excess of the normal intracellular concentration, and can, therefore, be assumed to be far in excess of the concentration present in the chromaffin granule.

(iv) Simulation of the Complex.

Chromogranin proteins were prepared and their effect on the ^{31}P spectra of ATP (disodium) in the presence of other known constituent molecules was investigated. The pH titration of ATP in the presence of

FIGURE 4.4.13.



The pH titrations of the frequencies of the β and γ phosphate resonances of ATP, 122mM, in the presence of Adrenaline, 550mM, CaCl_2 , 20mM, MgCl_2 , 8mM, and Chromogranins in various combinations.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions as in Figure 4.4.1..

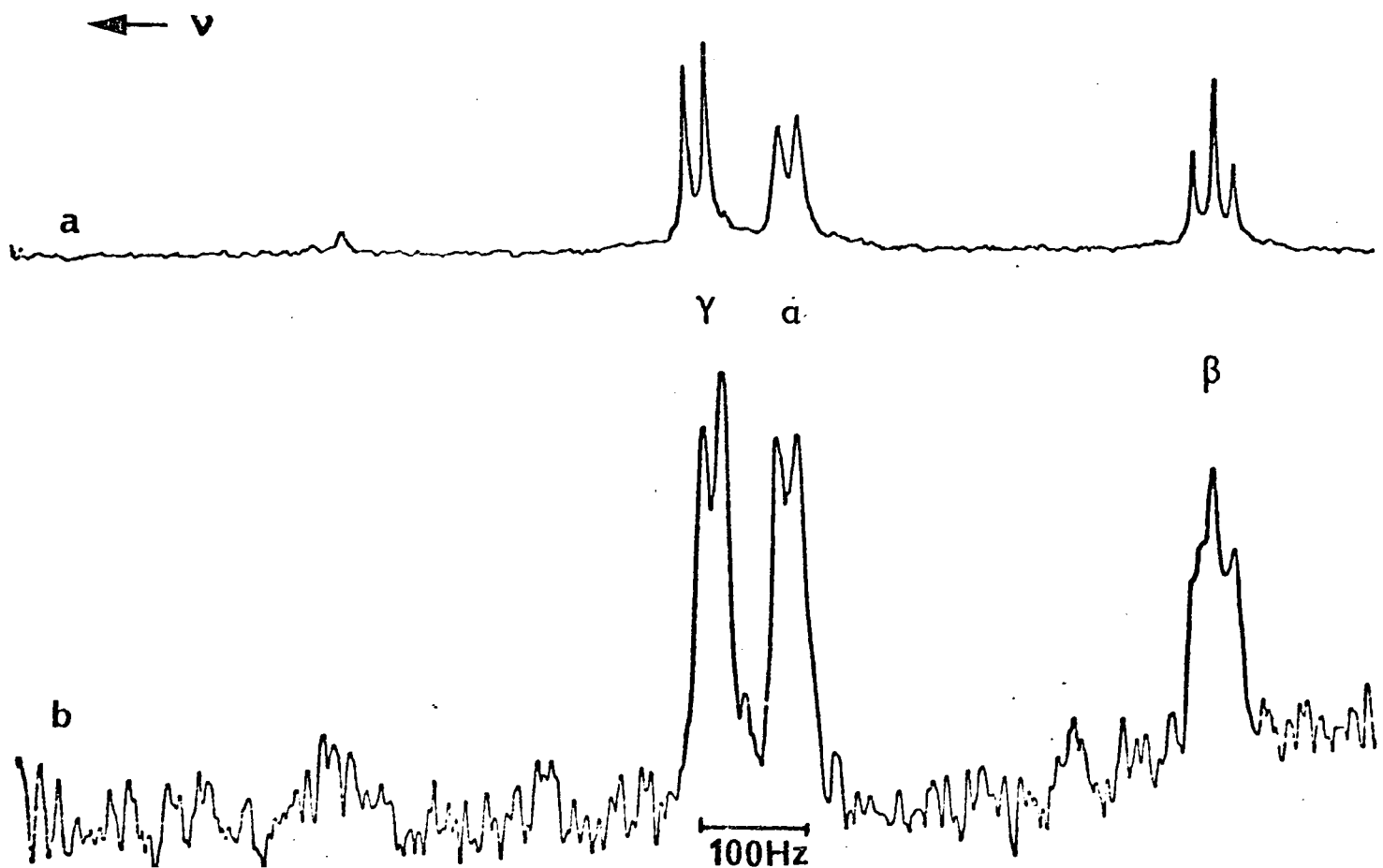
Key : 1 ATP, 122mM, alone
 2 + Adrenaline
 3 + Adrenaline + Chromogranins
 4 + Adrenaline + CaCl_2 + MgCl_2
 5 + Adrenaline + CaCl_2 + MgCl_2 + Chromogranins.

adrenaline (550mM) and the chromogranins (180mg/ml) is shown in Figure 4.4.13. There is a slight shift from the titration in the presence of adrenaline alone. When divalent metal ions, 20mM calcium and 8mM magnesium, were added to this complex, its titration curve followed that found in the absence of chromogranins. It can, therefore, be concluded that the chromogranins do not affect the resonance positions of the ATP phosphates in the complex to any large extent.

Helle and Serck-Hanssen have reported the presence of phospholipids in the matrix at a concentration of approximately 0.25µmoles lipid phosphate/mg matrix protein (45). Lipids were prepared from intact granules, the closest possible source of material, since no lipid composition is yet proposed for this matrix lipid. A complex containing the appropriate quantity of lipid, in addition to the chromogranins, ATP adrenaline and divalent metal ions, was constituted. It was noted that the chromogranin containing complexes, both in the absence and presence of phospholipids, though very viscous, were completely soluble at room temperature, if the pH was less than pH 5.9. There was no indication of precipitate or phase formation. If these reconstituted complexes were placed on ice, however, there was almost immediate precipitation of some of the contents. This observation may be of substantial importance in interpreting the differences in the results found in studies at 0-4°C and those at higher more physiological temperatures (22,52,53).

The spectrum of the total simulated complex containing ATP, adrenaline, chromogranins, phospholipids and divalent metal ions, at pH 4.75, is compared in Figure 4.4.14. to that of the complex in the absence of the phospholipids, at pH 4.9. The presence of the phospholipid has no effect on the pH titration of the ATP resonances, but it does appear to broaden the resonances, reducing the splitting of the β -resonance. It is possible that although the lipid extract was prepared with EDTA in the water phase, there were still some paramagnetic ions present in the very low concentrations

FIGURE 4.4.14.



^{31}P NMR spectra of (a) the 'simulated' granule complex without phospholipids and (b) the same complex with phospholipids present. The pH in each case is approximately 5.0.

Assignments and conditions as in Figure 4.4.1..

which would produce this effect. There is still a splitting of the γ -resonance, however, which is not present in any of the spectra of intact chromaffin granules. This broadening may, therefore, be due to the presence of some other component, possibly paramagnetic metal ions, which is not present to any large extent in these simulated solutions. It could also be due to the heterogeneity of the contents of the different chromaffin granules, since if the resonances from the different complexes are slightly shifted from each other, this will result in the overall splitting being lost.

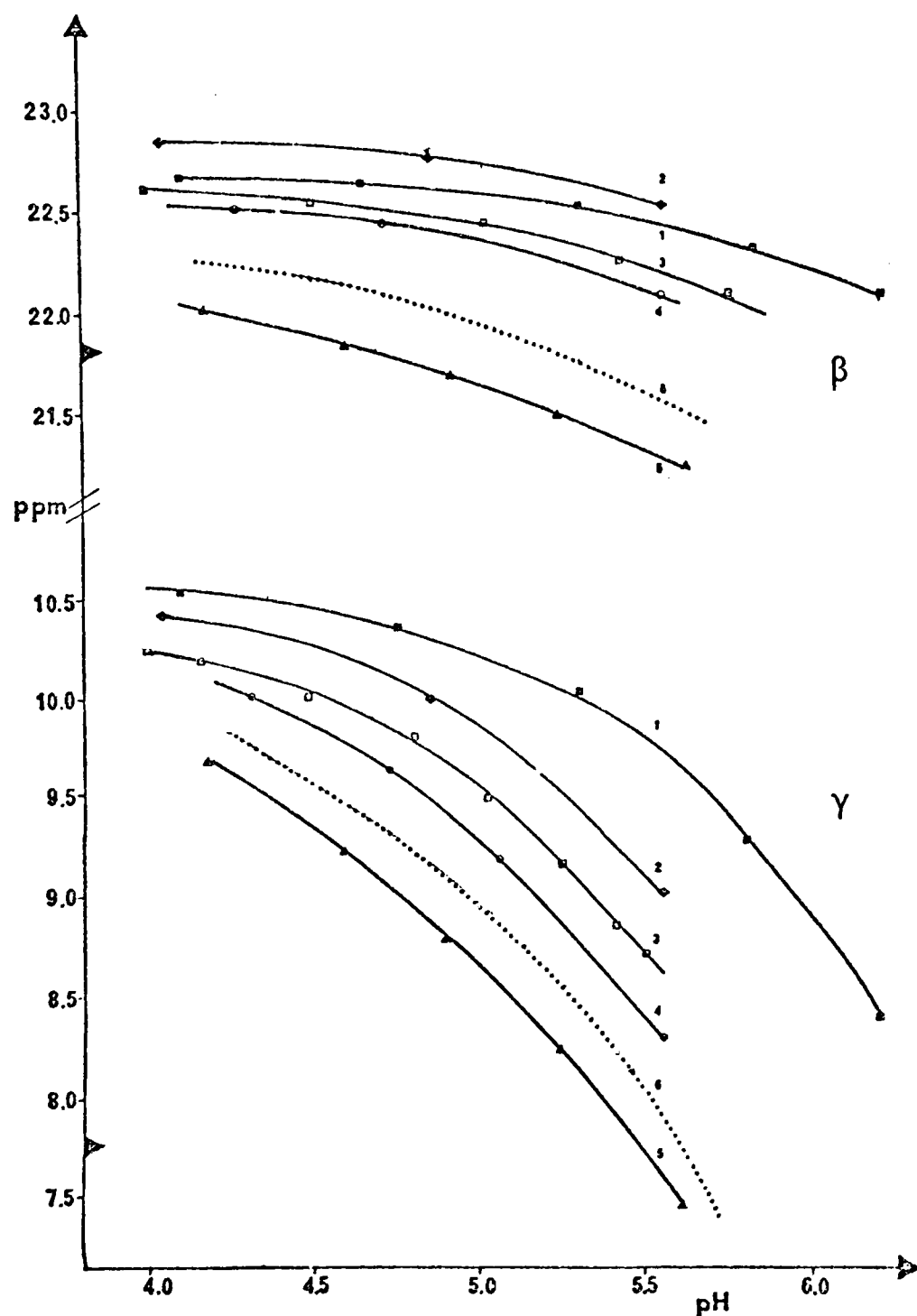
(v) What is the Internal pH of the Bovine Chromaffin Granule?

The ATP phosphate resonance most sensitive to pH, and, therefore, the most accurate probe of the pH is the γ -resonance. These studies show that the molecules which most perturb all the resonances of ATP, over the pH range 4 to 6, are the monovalent and divalent metal ions and the catecholamines. The chromogranin proteins and the phospholipids have very little effect on these resonances in the presence of the catecholamines and the metal ions.

The major difficulty in producing accurate simulations is, as already stated, the instability of the free-acid ATP. However, the preparation of sodium-free ATP showed that the γ -resonance is less sensitive than the β -resonance to sodium ions in the presence of adrenaline. Simulations using the disodium salt of ATP will, therefore, be more accurate with respect to the γ - than the β -resonance. The apparent inaccuracy introduced by sodium ions in the titration of the γ -resonance is equivalent to approximately 0.25 pH units, compared to the value of 0.8 to 1.0 pH units estimated for the β -resonance.

The catecholamine:ATP ratio of approximately 4:1 is well accepted, therefore, there should be little error in the assumption that the matrix concentrations of ATP and adrenaline are 122mM and 550mM respectively.

FIGURE 4.4.15.



The pH titrations of the frequencies of the β and γ phosphate resonances of ATP, 122mM, in the presence of the different metabolites most relevant to the reconstitution of the chromaffin granule storage complex.

The position of the resonances in the intact bovine chromaffin granules is indicated, and a calculated, but hypothetical, curve representing the true *in vivo* situation is also shown. The frequencies are expressed as chemical shifts in p.p.m. units w.r.t. 88% Phosphoric acid. Conditions as in Figure 4.4.1.

- Key :
- 1 Disodium ATP, 122mM, alone
 - 2 Sodium-free ATP, 122mM, + Adrenaline, 550mM
 - 3 Disodium ATP, 122mM, + Adrenaline, 550mM,
 - 4 Disodium ATP, 122mM, + Adrenaline, 550mM, + Chromogranins
 - 5 Disodium ATP, 122mM, + Adrenaline, 550mM, + CaCl_2 , 20mM, + MgCl_2 , 8mM, (+ Chromogranins).
 - 6 The Hypothetical Titration Curve.

The major source of error is the inaccuracy of the values for the total divalent metal ion concentration, since magnesium and calcium produce almost identical shift effects (11). The value used in these studies, 28mM, is the average of the values discussed, (apart from the very high values of Phillippu and Schumann(50) which have been ignored since they are so much in disagreement with other workers). The value of 28mM is heavily weighted by the intermediate granule preparation value of 17mM from the original paper of Borowitz (48). The other published values tend to be slightly larger. Thus if a value of 28 ± 8 mM total divalent metal ions is assumed, then this will result in a standard deviation of approximately ± 0.15 pH units at around pH 5.0, but the true value may have a tendency to be on the high frequency side ie. at lower pH. Thus the resultant effects of the divalent and monovalent ions will tend to cancel each other out, as the divalent will produce an error on the high frequency side and the monovalent on the low frequency. A comparison of the frequency positions of the phosphate resonances of the intact glands and the prepared granules with the most relevant pH titration curves is shown in Figure 4.4.15.

Consider the frequency positions of the β - and γ -phosphates of the ATP present in the bovine chromaffin granule. The frequency position of the γ -phosphate of ATP in the granules and in the adrenal medulla is 7.75 ± 0.25 ppm. The results indicate that the titration curve of the complete complex with the correction for sodium-free ATP is the most accurate available. The possible range of divalent metal concentrations imposes an additional error; thus it is most probable that from the frequency position of the γ -resonance, that the internal pH of the bovine chromaffin granule is 5.55 ± 0.2 .

Comparison of the resonance frequency of the β -phosphate of ATP in the intact glands with its titration curves indicates that, after the same corrections have been made, the most probable internal pH is 5.3 ± 0.25 . This value is in good agreement with that deduced for the γ -resonance

considering the variables involved. If both these semi-independent values are considered, then the most probable range in which the internal pH of the bovine chromaffin granule lies is 5.45 ± 0.25 .

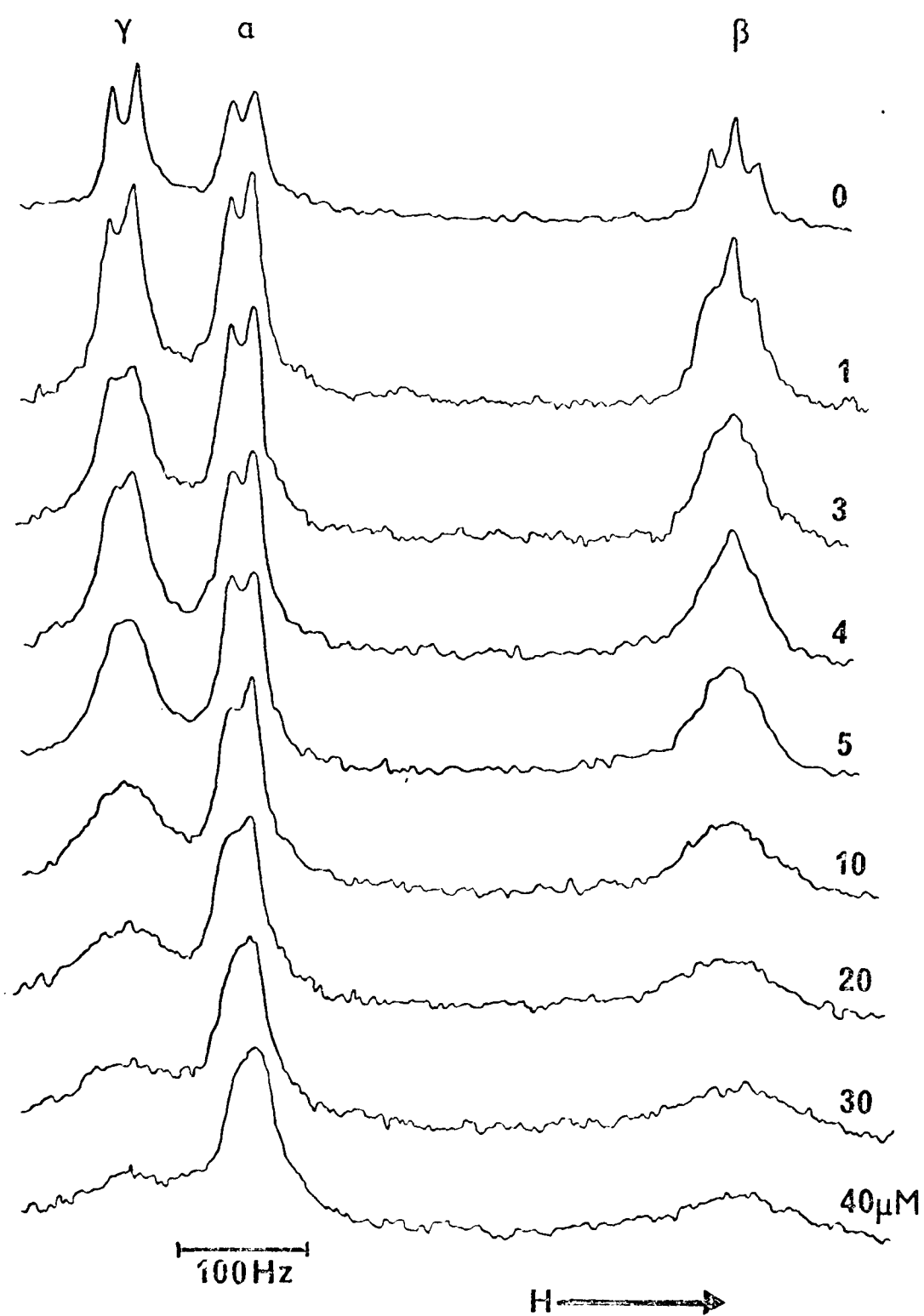
The studies of the uncoupler response of the ATPase and of the ANS response (Chapter Two) indicated an internal pH of around 5.5. The equilibration of adrenaline across the granule membrane, although a much less definitive result, also indicated a pH of between 5 and 6. The NMR results and the biochemical experiments are, therefore, in excellent agreement. The significance of this pH value to the processes of amine uptake and storage will be discussed in Chapter Six.

(vi) The Effects of Paramagnetic Ions.

Paramagnetic ions eg. Mn^{2+} , Cu^{2+} , Fe^{3+} present in very low concentrations in a solution of diamagnetic nuclei markedly reduce the nuclear relaxation times, especially the T_2 , of these nuclei. This enhanced relaxation results in a broadening of the resonance lines of the nuclei affected. There are little accurate data on the concentration of such metal ions present in the chromaffin granules of different species. Dopamine- β -hydroxylase is a copper containing enzyme with both Cu(I) and Cu(II) present in its prosthetic group. It is highly likely, therefore, that an equilibrium will exist and that a proportion of the copper will be present free in the granule matrix at any one time. There are 4-7 μ moles of copper per μ mole of pure dopamine- β -hydroxylase (54).

The effect of very small concentrations of copper on the resonances of ATP (122mM) is shown in Figure 4.4.16. The major broadening effect is initially on the γ - and β -phosphates. The splittings of these phosphate resonances are abolished if as little as 3 μ M Cu^{2+} is present. The splitting of the α -phosphate also becomes less defined and has virtually disappeared at a copper concentration of 30 μ M. The extreme sensitivity of the ATP phosphate resonances to the presence of copper ions is very clear. Manganese and other paramagnetic ions have similar effects (49).

Figure 4.4.16.



^{31}P NMR spectra of ATP, 122mM, in the presence of increasing concentrations of CuCl_2 .

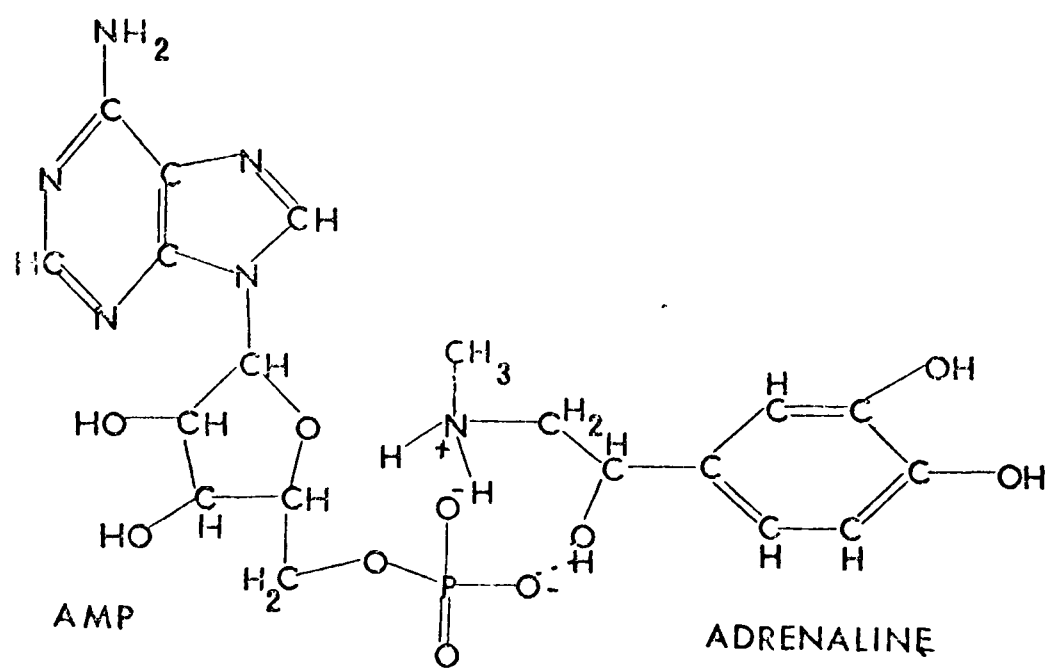
Assignments and conditions as in Figure 4.4.1.

Preliminary studies of the paramagnetic ion content of chromaffin granules have been made using atomic absorption spectroscopy. However, it has not been possible, as yet, to quote absolute values for the concentrations since, when a carefully prepared and calibrated sample was prepared, the copper and manganese lamps were not available. The preliminary results indicate, however, that copper and magnesium were present in concentrations proportionally similar to those determined by Borowitz et al and also that potassium, manganese and copper were also present.

E What is the Nature of the Storage Complex?

When the composition of the contents of the chromaffin granule was discovered, it was suggested by Blaschko et al (55) and by Falck et al (56) that a catecholamine:nucleotide complex is involved in the storage of amines in the granule. Several relevant studies of catecholamine:ATP interactions have been made using NMR.

Weiner and Jardetzky (57) made a study of catecholamine:nucleotide complexes using proton NMR. They studied the interaction of 300mM adrenaline with 100mM ATP and also with 100mM AMP. With the exception of a small concentration-dependent shift, 0.12ppm, of the catecholamine ring peaks, the spectrum of the mixture of adrenaline and ATP showed no changes in the chemical shifts of the resonances when compared with the spectra of the two separate components. Their experiments were carried out at pH 5.6 and 27°C. An intermolecular arrangement involving stacking of aromatic rings was ruled out. However, it was proposed that since there is a generalised broadening of all the resonances in the mixture that either some interaction takes place, or that the viscosity is greater in the mixture. The resonances of the side chain protons of adrenaline were broadened more than those from the aromatic ring or the methyl group protons, reflecting a larger change in the T_2 and T_1 of the former protons. Weiner and Jardetzky measured the spin lattice relaxation times, T_1 , using

FIGURE 4.4.17.

The Model of the AMP-Adrenaline interaction proposed by Weiner and Jardeitzky (57). Their Model for ATP- Adrenaline interaction is the same.

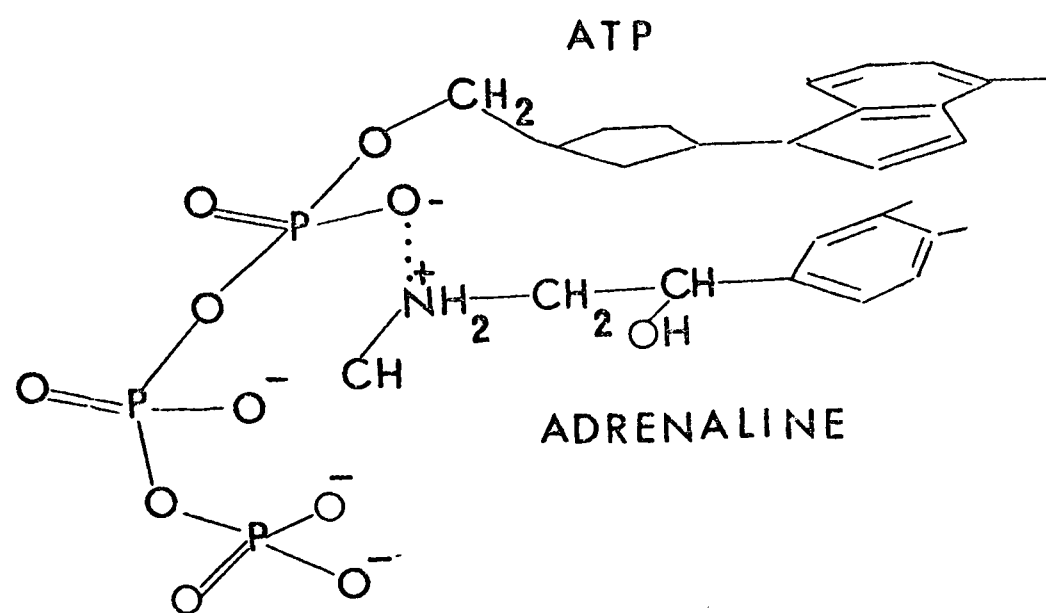
the direct saturation method but their spin-spin relaxation times, T_2 , were derived from measurements of the line-width. Their main findings were that:

- (i) the relaxation times of the adrenaline side chain protons decreased more than those of the catechol ring or methyl protons;
- (ii) the relaxation times of the adenine and ribose protons decreased to the same extent;
- (iii) T_1 and T_2 decreased proportionally in all cases. This indicated that the broadening of the side chain resonances reflects a change in the rotational freedom of these protons. The absence of selective changes in the relaxation times of the nucleotide protons suggests that the interaction occurs through the phosphate group alone.

The adrenaline:ATP ratio was varied and a comparison of the relaxation times showed that there is fast exchange between the free and complexed species. When adrenaline was replaced by dopamine, also 300mM, there were only small selective changes in the dopamine CH_2 resonance, indicating that the β -hydroxyl group makes a detectable difference to the stabilisation of the catecholamine side chain in its interaction with the nucleotide. The structure of the complex which can be deduced from this study is shown in Figure 4.4.17.

A more recent study of Muro et al (58) investigated the formation of a ternary complex between adrenaline, ATP and cobalt ions but a limited study was also made into the interaction of adrenaline and ATP in the absence of metal ions and also in the presence of some concentrations of magnesium ions. The concentrations of ATP and adrenaline used, however, in this study are not the same as those found in the granule complex. 100mM adrenaline was examined in the presence of 50, 100, 150 and 200mM ATP, and vice versa; thus the maximum adrenaline:ATP ratio examined was 2:1. Their study of adrenaline resonances in the presence of increasing ATP concentrations was interpreted in terms of a stacking of the aromatic

FIGURE 4.4.18



The Model of ATP-Adrenaline interaction proposed by Tuck and Baker (59).

rings of ATP and adrenaline. Shifts of 0.08 and 0.03 ppm were found in the frequencies of the resonances of the H₂ and H₈ protons of 100mM ATP (pH 2.5 $\pm$ 0.5) when the concentration of adrenaline was increased from zero to 200mM. These shifts are twice as large as those measured by Weiner and Jardetzky (57) in their complex of 100mM ATP and 300mM adrenaline (pH 5.6). In addition, Muro et al observe a differential broadening of the H₂ resonance of ATP compared to the H₈ but they do not state whether the aromatic ring protons are broadened by the same degree. It is proposed that at pH 2.5, and to a lesser extent at pH 5.5, a stacking exists between the ATP and adrenaline aromatic rings, the catechol ring being nearer to the H₂ proton of ATP. This is virtually the opposite conformation to that proposed by Weiner and Jardetzky (57).

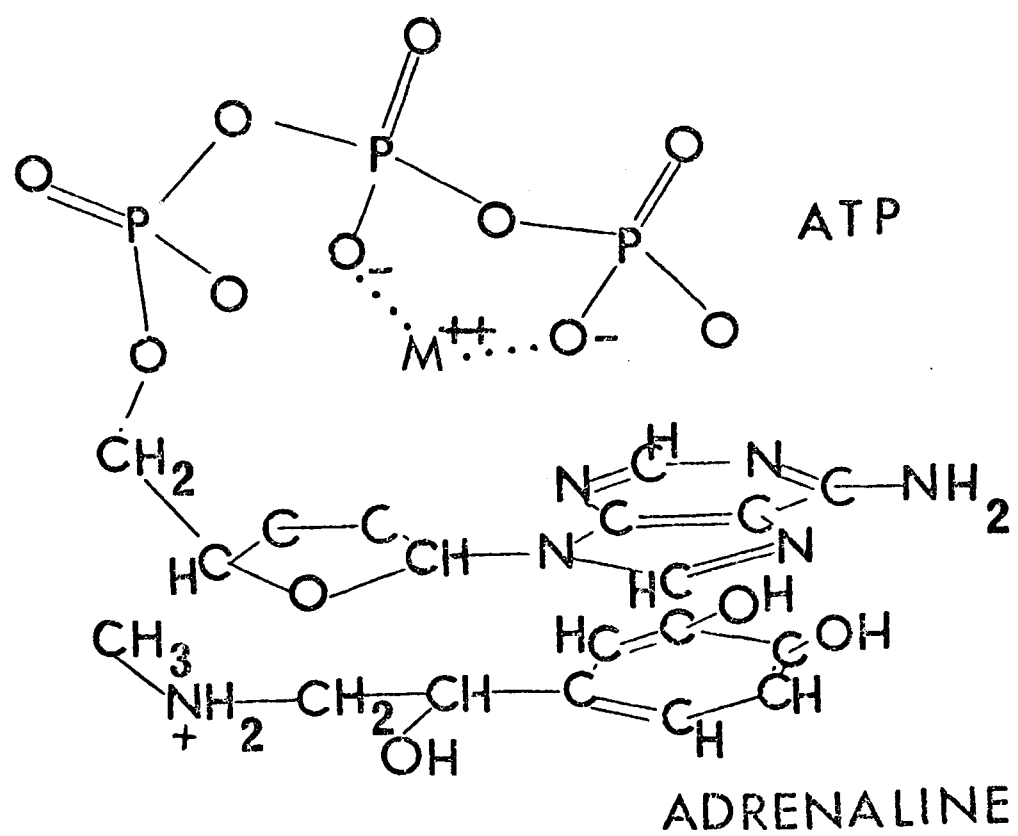
Tuck and Baker (59) examined the interactions between ATP, adrenaline and magnesium ions. Their work was not directed towards an investigation of the internal structure of the chromaffin granule, rather the possible configuration of the molecules in the active site of adenyl cyclase. Their data for chemical shifts as a result of complexing are relevant since they were looking at the complex of an equimolar ATP-Mg²⁺ chelate with adrenaline. The concentrations of adrenaline used were 20mM and 200mM in the presence of a range of ATP-Mg²⁺ concentrations of 20-400mM and 40-400mM respectively. The catechol protons of adrenaline showed upfield shifts which were interpreted in terms of an overlap of the purine and the catechol rings. Tuck and Baker agree with Weiner and Jardetzky that the addition of ATP-Mg²⁺ produced a broadening of all the proton resonances of adrenaline but differ as to the degree of broadening, finding that they are all broadened to about the same extent. This implicates both the catechol ring and the side chain of adrenaline in the ternary complex. This proposed ternary complex has a structure in which the aromatic rings overlap and the protonated amine of adrenaline interacts with the α -phosphate of ATP (Figure 4.4.18). This complex depends on an equimolar ATP:Mg²⁺ ratio,

however, and should not be considered to be an ideal model for the chromaffin granule complex.

Daniels et al (60) also carried out a proton and phosphorus NMR investigation into the storage complex. They show that the proton resonances are shifted upfield in the granules compared to those of the free components, and interpret this as a result of neighbouring ring current shifts due to stacking of the aromatic rings of adrenaline and ATP. The larger line widths are stated as indicating that either the complexes are of fairly large molecular weight or that there are multiple different types of complex present. The possible presence of paramagnetic ions is not considered as a possible cause of the broadening. A spectrum of the granule lysate is shown to demonstrate that the proton resonances shift back to those found in a solution of ATP and adrenaline when the integrity of the granule is disrupted. The spectrum which they show of the lysate is deconvoluted and EDTA is present (EDTA does not penetrate membranes) resulting in a much sharper and more resolved spectrum (easily seen by comparison of the sucrose and TSS peaks). The concentrations of ATP and adrenaline present in their simulated solution, 10mM and 40mM respectively and in their lysate where substantial dilution must have occurred are not comparable to those present in the granule. They completely fail to consider a concentration effect, as proposed by Weiner and Jardetzky (57), as a possible alternative and as a result, their substantial ring current shifts present in the spectrum of the granules are over those found in a solution 13.5 fold more dilute.

In their phosphorus NMR study, spectra of 50mM ATP (probably the disodium salt, but not stated) and of the intact granules and the granule lysate were compared. Neither the pH nor the temperature at which these spectra were run is stated. They find little or no shift in the α - and β -resonances of ATP in the spectra of the chromaffin granules from their positions in free ATP, but they do find the γ -resonance markedly shifted.

FIGURE 4.4.19.



The Model of the ATP-Adrenaline interaction within the Chromaffin Granule proposed by Daniels et al (60).

This observation was interpreted in terms of a ring current shift of the γ -phosphate, but not of the α - and β -phosphates. In addition, the broadness of the resonances was interpreted in terms of restricted motion, but the spectra are considered to show fast chemical exchange indicative of a solution complex. The model proposed by Daniels et al is shown in Figure 4.4.19. The evidence presented by Daniels et al to support this structure is not at all satisfactory in view of the original proton NMR studies by Weiner and Jardetzky and the phosphorus NMR results already described in this chapter. It was therefore necessary to investigate further these proposed structures, in a carefully controlled manner with conditions as close to the physiological ones as possible.

(i) Phosphorus NMR Studies.

The studies using ^{31}P NMR of the chromaffin granules and the simulated complexes, already described in this chapter, show that the pH dependent titration curves of the three ATP phosphate resonances are shifted to different degrees by the presence of adrenaline and metal ions. These studies indicate that the internal pH of the bovine chromaffin granule is around 5.45. A comparison of the resonance positions of the α -, β - and γ -phosphate resonances of ATP (w.r.t. 88% phosphoric acid) in the intact granule with those of ATP (122mM) and ATP (672mM), all at pH 5.45, is made in Table 4.4.3. It can clearly be seen that shifts are present in the resonances of all three phosphate groups, although the shifts in the α -phosphate resonance are quite small. The large difference in the overall extent of shift found with the three phosphate resonances is attributable to protonation of only the γ -phosphate in the pH range 6-7. The redistribution of electron density due to the terminal phosphate ionisation, and thus the range in phosphorus frequencies, attenuates with the distance along the polyphosphate chain.

These results are in disagreement with those of Daniels et al who did not observe shifts in the β -phosphate resonance of ATP in

FIGURE 4.4.3.

	α	β	γ
ATP, 122mM,	11.12	22.48	10.11
ATP, 672mM,	10.75	21.66	8.72
Bovine chromaffin granules	11.05	21.86	7.73

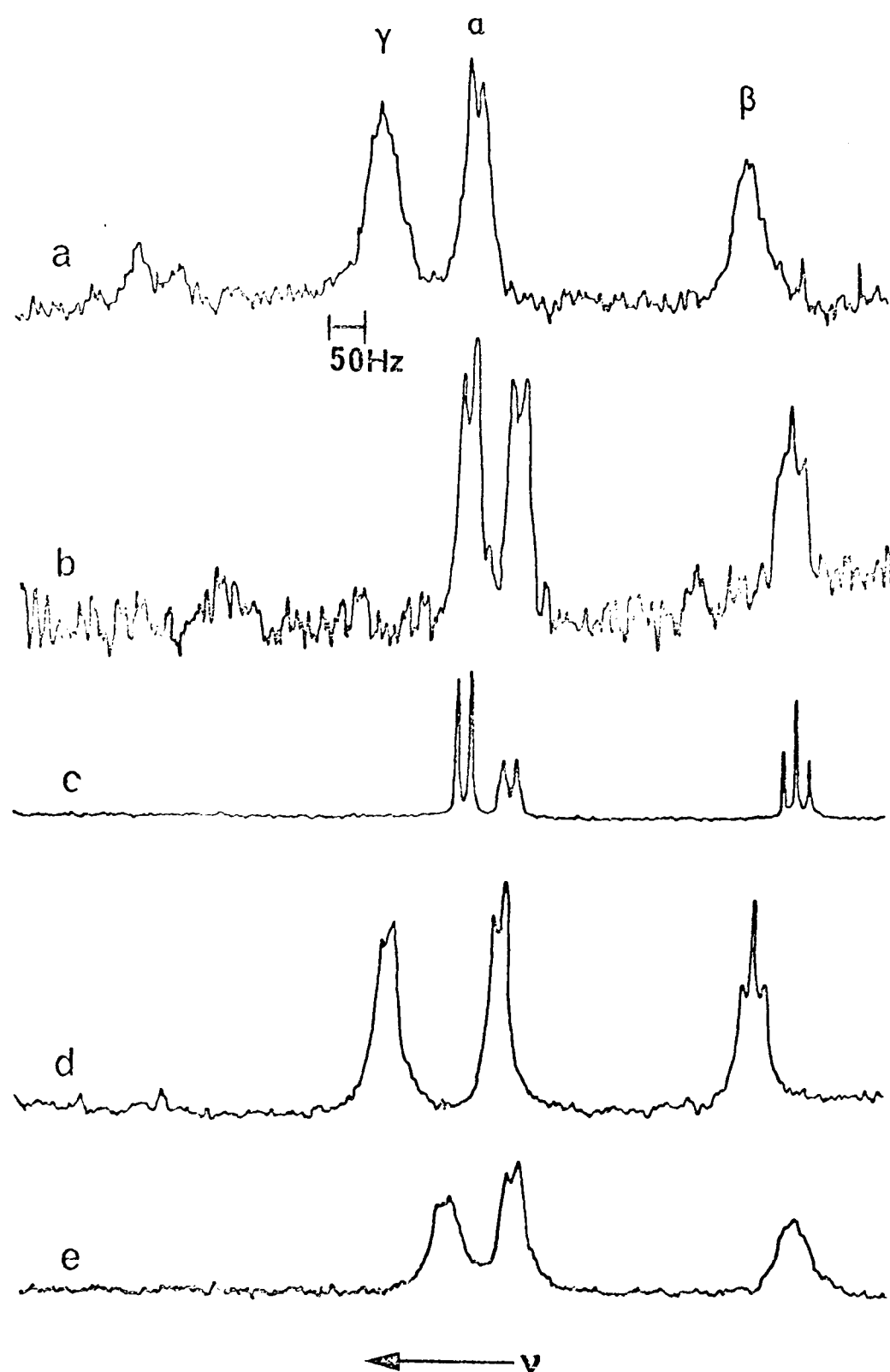
The frequency positions of the α -, β - and γ -phosphate resonances of ATP at two concentrations and in the storage complex of bovine chromaffin granules. The frequencies are expressed as chemical shifts in p.p.m. units with respect to 88% phosphoric acid. The values for 672mM ATP are slightly lower since the pH of the solution was 5.6, not 5.45. This obviously affects the γ -resonance most due to its greater pH sensitivity.

the granule when compared to the spectra of ATP and adrenaline alone.

The line widths at half height, $\Delta\nu_{1/2}$, of the phosphate resonances of the α -, β - and γ -phosphates of ATP in the intact bovine granules are 42.2, 53.6 and 48.7 Hz respectively (at 36.43MHz) and are 35.5, 48.4 and 37.55Hz respectively in the spectrum of the reconstituted complex. These two spectra, in addition to those of ATP (122mM) in the presence of EDTA, ATP (122mM) in the presence of 95% glycerol and ATP (122mM) in the presence of 3 μ M CuCl₂, are shown in Figure 4.4.20. The line widths at half height of the phosphate resonances in these spectra, in addition to those of pig and sheep granules, are compared in Table 4.4.4. The resonance lines in the spectra of bovine and sheep granules are, within experimental error, identical, whilst those of the porcine granule spectrum, especially the γ -phosphate resonance, are very much narrower. This observation was mentioned qualitatively earlier in this chapter. As far as is known, there is little difference in the composition of the complex present in bovine and porcine granules, apart from the catecholamine composition, and the pH results bear this out. It must mean, therefore, that the width of the resonances does not depend completely on the macromolecules present in the complex. The resonances of the total simulated complex are considerably broader than those of free ATP, but are slightly narrower than the resonances present in the pig granule.

The effects of viscosity and paramagnetic ions on the resonances of ATP are also shown in Table 4.4.4. The resonances are significantly broadened in 95% glycerol. The γ -resonance is most affected by this restriction of motion. It was observed that the line width of the γ -resonance in the simulated complex is narrower than that found in 95% glycerol whilst the α - and β -resonances are broader. The presence of paramagnetic metal ions in very small concentrations also broadens the resonances substantially. The value of 3 μ M CuCl₂ was chosen because the widths of the resonances in the presence of this small quantity of copper

FIGURE 4.4.20.



^{31}P NMR spectra of

- (a) bovine chromaffin granules in 0.3M sucrose,
- (b) the total reconstituted complex,
- (c) ATP, 122mM, in the presence of EDTA, 5mM,
- (d) ATP, 122mM, in the presence of 95%glycerol,
- (e) ATP, 122mM, in the presence of $3\mu\text{M}$ CuCl_2 .

Conditions and assignments as in Figure 4.4.1.

TABLE 4.4.4.

	α	β	γ
<i>Bovine Chromaffin Granules</i>	42.19	53.63	48.27
<i>Ovine Chromaffin Granules</i>	41.67	54.17	47.82
<i>Porcine Chromaffin Granules</i>	37.54	48.34	37.54
<i>'Simulated' Storage Complex</i>	35.42	45.83	33.33
<i>ATP, 122mM, + EDTA, 5mM.</i>	32.29	39.58	24.16
<i>ATP, 122mM, in 95% Glycerol</i>	33.33	42.72	39.58
<i>ATP, 122mM, + 3μM CuCl₂</i>	38.54	54.17	47.92
<i>Condensed phase from ATP, 122mM,</i>	35.42	42.17	32.30

A comparison of the $\Delta\nu_{1/2}$ values of the ATP resonances in different systems studied. The values are expressed in Hertz.

ions are similar to those observed in the bovine and ovine granule spectra. Thus if the resonances are already broadened by the viscosity effect of the high concentration of metabolites in the complex, the presence of even less than $3\mu\text{M}$ paramagnetic ions would be sufficient to broaden the resonances to the equivalent of the value found in the intact granule spectra. Since at room temperature, there is no indication from these studies that a more immobile, highly complexed gel state exists, it is more likely that the presence of a very low concentration of paramagnetic ions is the cause of a substantial part of the broadening. Broadening due to heterogeneity of the granule population, as discussed previously, may also make a sizeable contribution.

(ii) Proton NMR Studies.

In solutions where molecules the size of ATP and adrenaline are present in overall concentrations as large as 700mM , the molecules will be, on average, about $10\text{\AA}$ apart or less. Thus if all the molecules have aromatic or heterocyclic rings, there should be a resultant non-specific ring current shifting of all susceptible nuclei due to this proximity effect. This means that, if a specific conformation is proposed for an ATP:adrenaline complex, the resonances from this complex must be shown to differ specifically from those present in the spectra from concentrated solutions of single metabolites, where non-specific shifts occur.

A comparison was made of the spectra of ATP, AMP and adrenaline at various concentrations, and those of adrenaline (550mM) in the presence of AMP (122mM) and ATP (122mM). These results are shown in Table 4.4.5. There are significant differences between the resonance positions of all three molecules at high and low concentration. Within experimental error, the resonance positions of AMP at concentrations of 122mM and 672mM are the same as those of ATP at the same concentrations. In the presence of adrenaline, there are slight differences between the resonance positions of the AMP and ATP protons, but these do not exceed 0.10 ppm . The similarity

TABLE 4.4.5.

	ATP				Adrenaline	
	C ₈	C ₂	C ₁	φ _H	CH ₂	CH ₃
ATP, 122mM	2.45	2.80	4.83			
ATP, 672mM	2.62	3.08	5.00			
ATP, 122mM + Adrenaline, 550mM	2.64	2.98	5.03	4.20	7.85	8.24
Adrenaline, 672mM				4.11	7.77	8.22
Adrenaline, 550mM				4.05	7.72	8.18
Adrenaline, 122mM				3.92	7.57	8.07
AMP, 122mM + Adrenaline, 550mM	2.59	2.94	4.93	4.11	7.75	8.18
AMP, 672mM	2.66	3.08	5.00			
AMP, 122mM	2.48	2.81	4.83			

The frequencies of the ¹H resonances of Adrenaline, ATP, and AMP at different concentrations and in different combinations. The frequencies are expressed as chemical shifts, in p.p.m. units with respect to an arbitrary zero. The spectra were accumulations of 500 pulses (70°) at intervals of 0.6s. The temperature is 20°C; the frequency is 90 MHz.

between the resonance positions of ATP at 672mM and 122mM in the presence of 550mM adrenaline is striking. The chemical shifts of the H_2 , H_8 and H_1 protons of ATP when the concentration is increased from 122mM to 672mM are 0.17, 0.28 and 0.165 ppm respectively.

The adrenaline resonances also show substantial concentration dependent chemical shifts. The shifts experienced by the catechol ring, methylene and methyl protons are 0.19, 0.20 and 0.15 ppm respectively when the adrenaline concentration is increased from 122mM to 672mM. There are also significant shifts in these protons, 0.06, 0.06 and 0.045 ppm, when the adrenaline concentration is increased from 550mM to 672mM.

Whilst the adrenaline resonance positions in the presence of AMP agree well with those of 672mM adrenaline, those of adrenaline in the presence of ATP are further shifted eg. the methylene protons are 0.08 ppm upfield. This difference may be due to the formation of a weak complex between adrenaline and ATP, but it is surprising that equivalent shifts are not observed in the ATP resonances. There are two possible reasons for this. Firstly, the interaction of adrenaline may be with the phosphate chain of ATP. Secondly, it is possible that since the heterocyclic ring of ATP is larger than the catechol ring of adrenaline the ring current shifting potential of ATP molecules will be slightly greater than that of adrenaline molecules. This means that the general ring current shifts due to concentration in a solution of 672mM ATP and in a solution of 122mM ATP and 550mM adrenaline are not equivalent.

The results expressed in Table 4.4.5. clearly illustrate the dangers in assuming specific interactions in solutions where the concentration dependent shifts of the resonances are large,

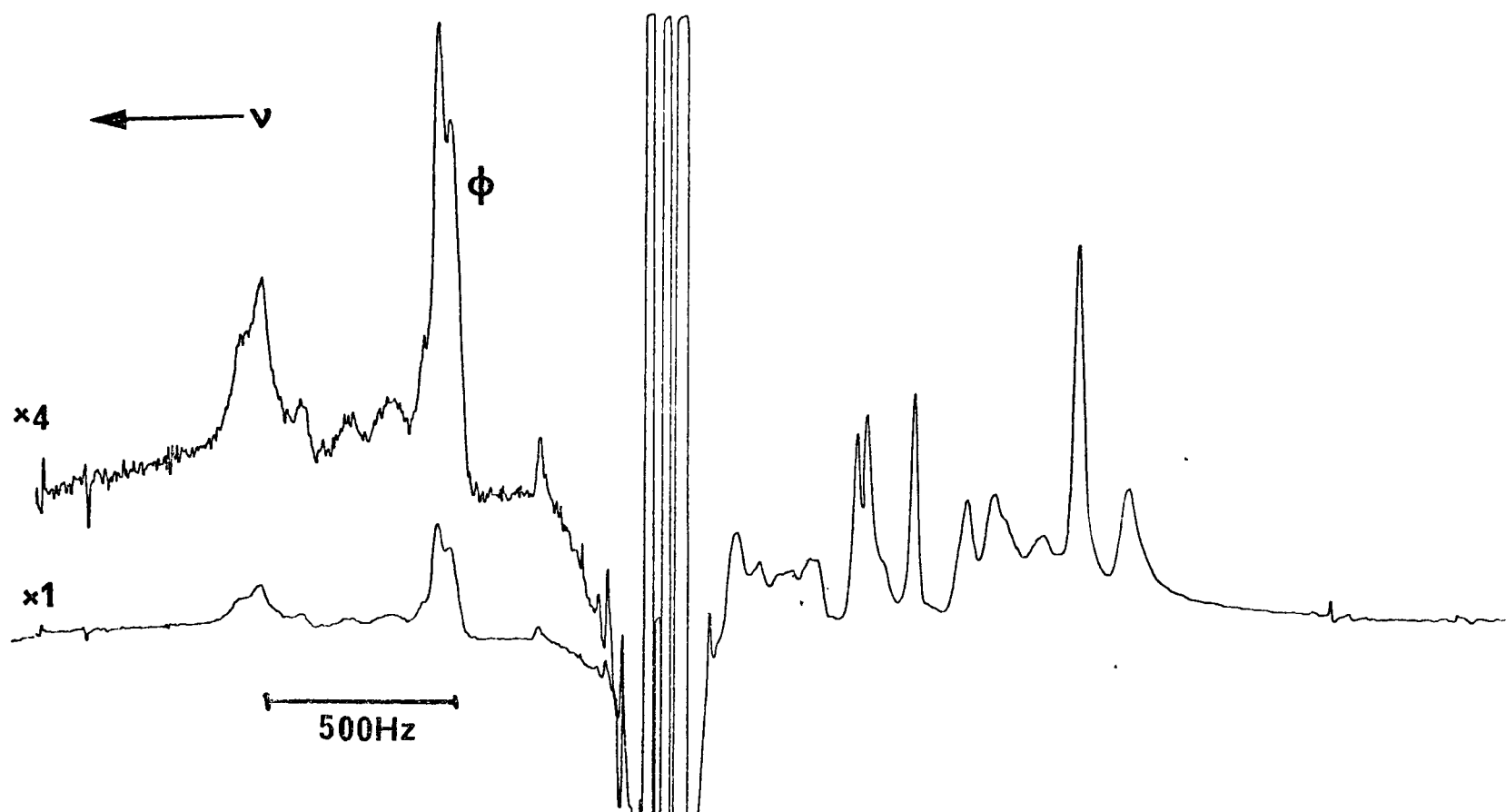
In accordance with the phosphorus NMR studies, proton spectra were obtained from porcine adrenal medulla and purified porcine adrenal medulla, in order to ascertain if any changes occurred during preparation. These spectra are complicated by the presence of chromogranins and other

unassigned resonances, including those of sucrose in the case of the granule spectra. These spectra are shown in Figure 4.4.21. along with a spectrum of 550mM adrenaline, 122mM ATP and an unspecified concentration of chromogranins, at pH 5.5 (cf. a spectrum of purified chromogranins, Fig. 4.4.23) There are several similarities and differences between the spectra.

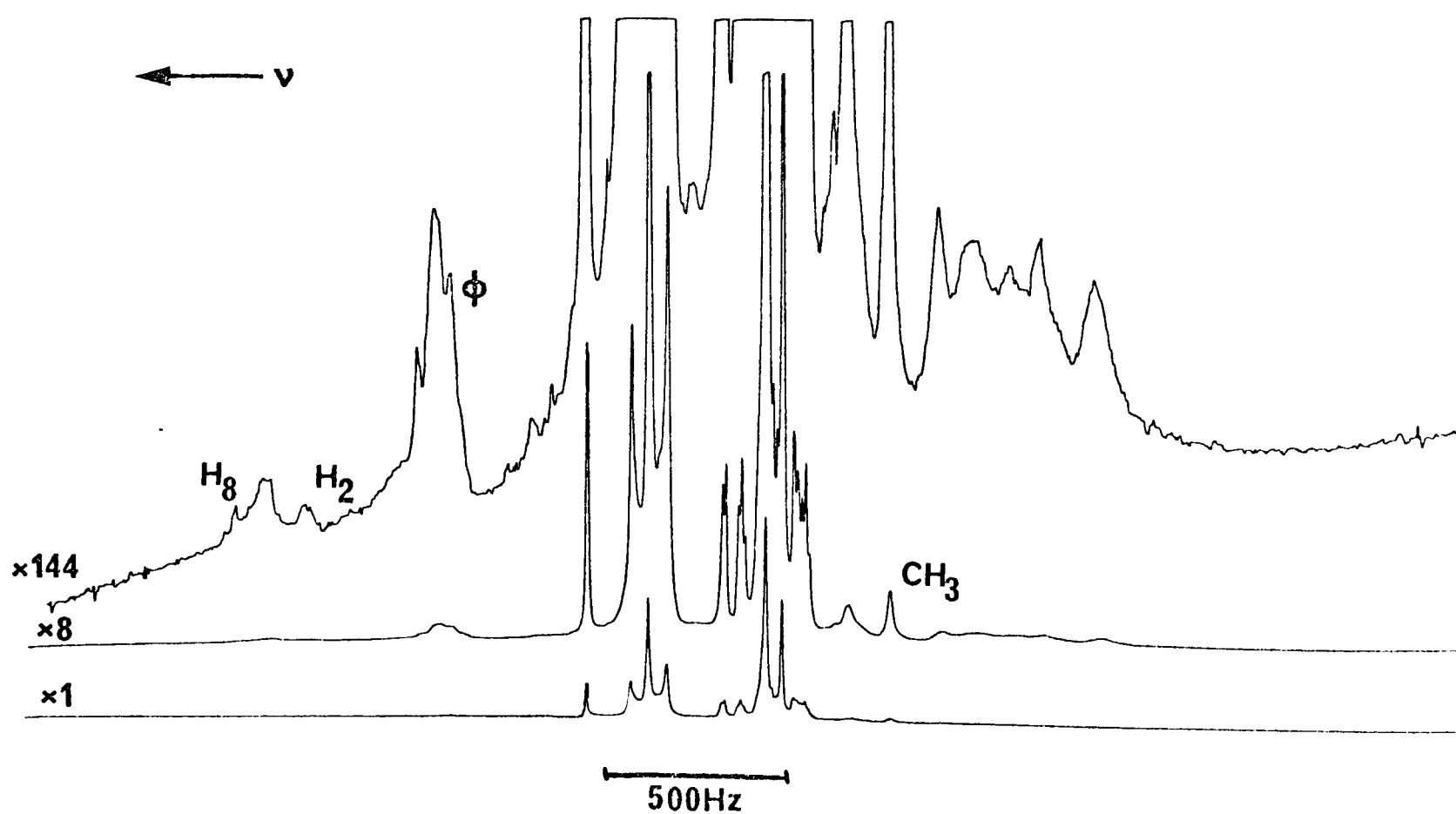
The H₈ and H₂ adenine protons of ATP can be resolved in the spectra of the chromaffin granules and the adrenal medulla, but are substantially broadened compared to the resonances in the simulated mixture; 57Hz and 43Hz compared to 5Hz and 5Hz respectively in the mixture (5Hz is the minimum resonance width that can be measured from these spectra). The H_{1'} ribose proton of ATP is also resolved in the medulla spectrum, and also in the granule spectrum, though it is most clearly seen in that of the medulla. The relative shifts are approximately equivalent in the three relevant spectra. The adrenaline resonances are more easily seen than those of ATP since the concentration of adrenaline present in the tissue is very much larger. The catechol ring protons are well resolved. However, it is interesting that the apparent composition of these resonances is somewhat different in the granule and medulla spectra when compared to the simulated contents. The adrenaline:ATP mixture results in what may be described as a 'singlet' and two 'doublets', with the 'singlet' on the low field side separated by 0.054 and 0.145ppm from the midpoints of the two 'doublet peaks'. The approximate width at half height of the composite resonance is 43Hz. In the chromaffin granule spectrum, the 'singlet' appears to have moved down field and the 'doublet' resonances broadened and partially merged. This results in a peak that appears to be composed of three major 'singlet' resonances. The relative shifts of the low field 'singlet' from the other two are now 0.16 and 0.30ppm respectively. The approximate line width is now 100Hz. The shape of this composite peak in the adrenal medulla spectrum is very similar to that in the granule spectrum, except that the low field 'singlet' is less significant. The

FIGURE 4.4.21.

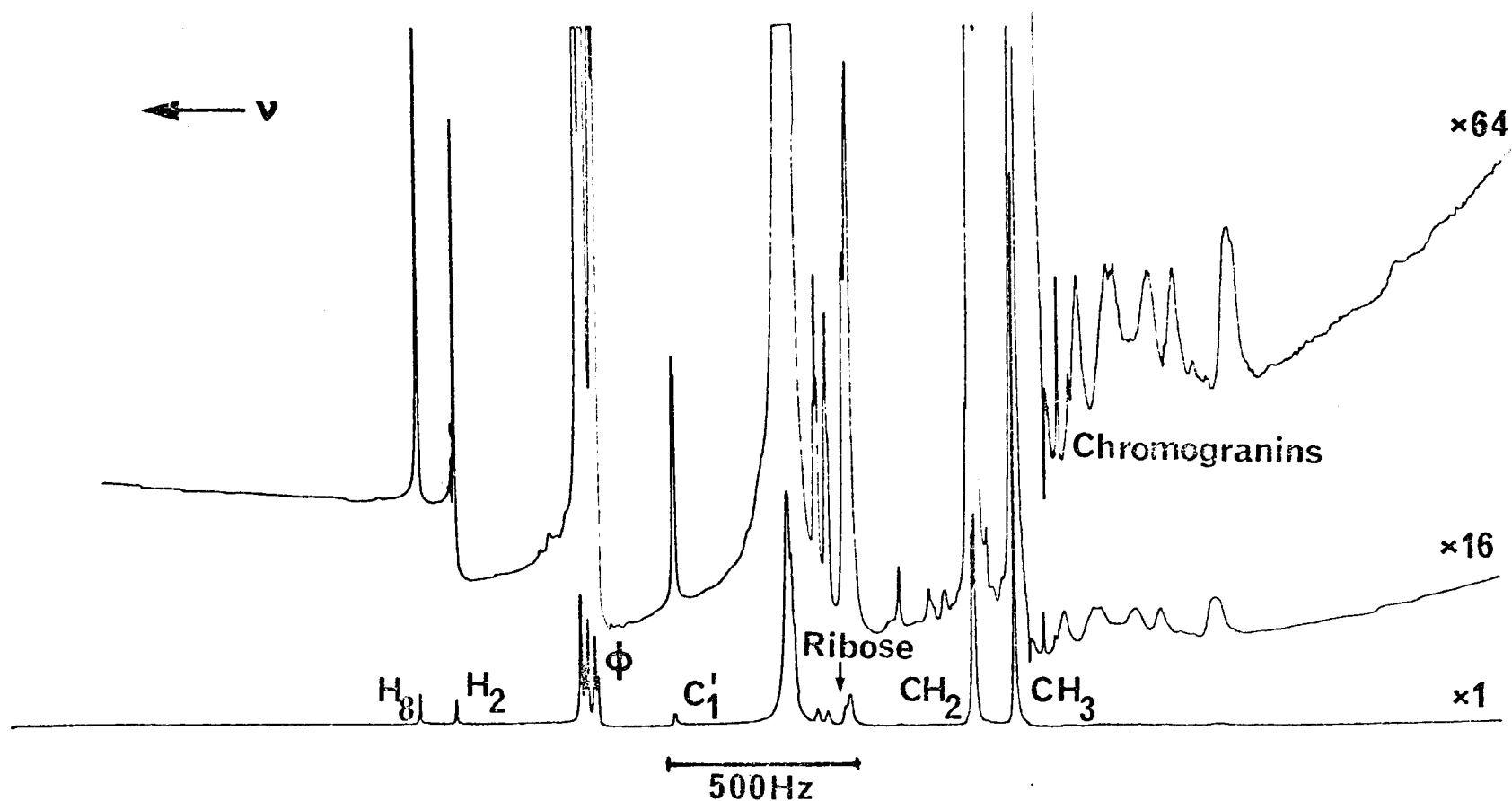
(a)



(b)



(c)



^1H NMR spectra of

- (a) porcine adrenal medulla in Tyrode solution/ D_2O ,
- (b) porcine chromaffin granules in 0.3M sucrose/ D_2O ,
- (c) adrenaline, 550mM, ATP, 122mM and chromogranins at pH 5.5 in D_2O .

Spectra (a) and (b) are accumulations of between 7000 and 8000 scans, whilst (c) is 2000 scans. (pulse interval 0.6s, pulse angle 70°)
Temperature = 16°C $\nu = 270\text{MHz}$.

width at half height is 57Hz since the 'singlet' is not contributing to it.

It is difficult to speculate on the reason for this change. There is no contribution from chromogranins at this frequency. The shoulder on the low field side can, however, be attributed to chromogranins. The methyl and methylene resonances of adrenaline are well resolved in all spectra. The $\Delta\nu_{1/2}$ values of these resonances are also increased in the granule and medulla spectra. The methyl proton resonance broadens from 5Hz to 20Hz in both systems, whilst the methylene broadens from 13 to 36Hz. There is very little difference in the frequencies of these resonances in the different spectra.

The simulated complex does not contain the correct concentration of chromogranins, nor any phospholipids or metal ions, therefore it should not be considered as an accurate indicator of the viscosity. It was not possible to examine the complete, simulated complex looked at by phosphorus NMR in the proton spectrometer. Unfortunately, it is very difficult to prepare sufficient quantities of lipids and proteins to make up an accurate simulation. It is essential that this be done, however, in order that the cause of the broadening of the lines may be determined.

The main conclusion is that there is little difference between the frequencies and line widths of the ATP and adrenaline resonances in the chromaffin granule and adrenal medulla spectra, but these spectra differ significantly from those of ATP and adrenaline, at the correct concentrations and approximately correct physiological pH, but in the presence of a slightly less than physiological concentration of chromogranins. The chromogranin resonances in this spectra, however, agree very well with those in the granule and the medulla spectra.

(a) The Effect of Paramagnetic Ions on the Proton Resonances of ATP.

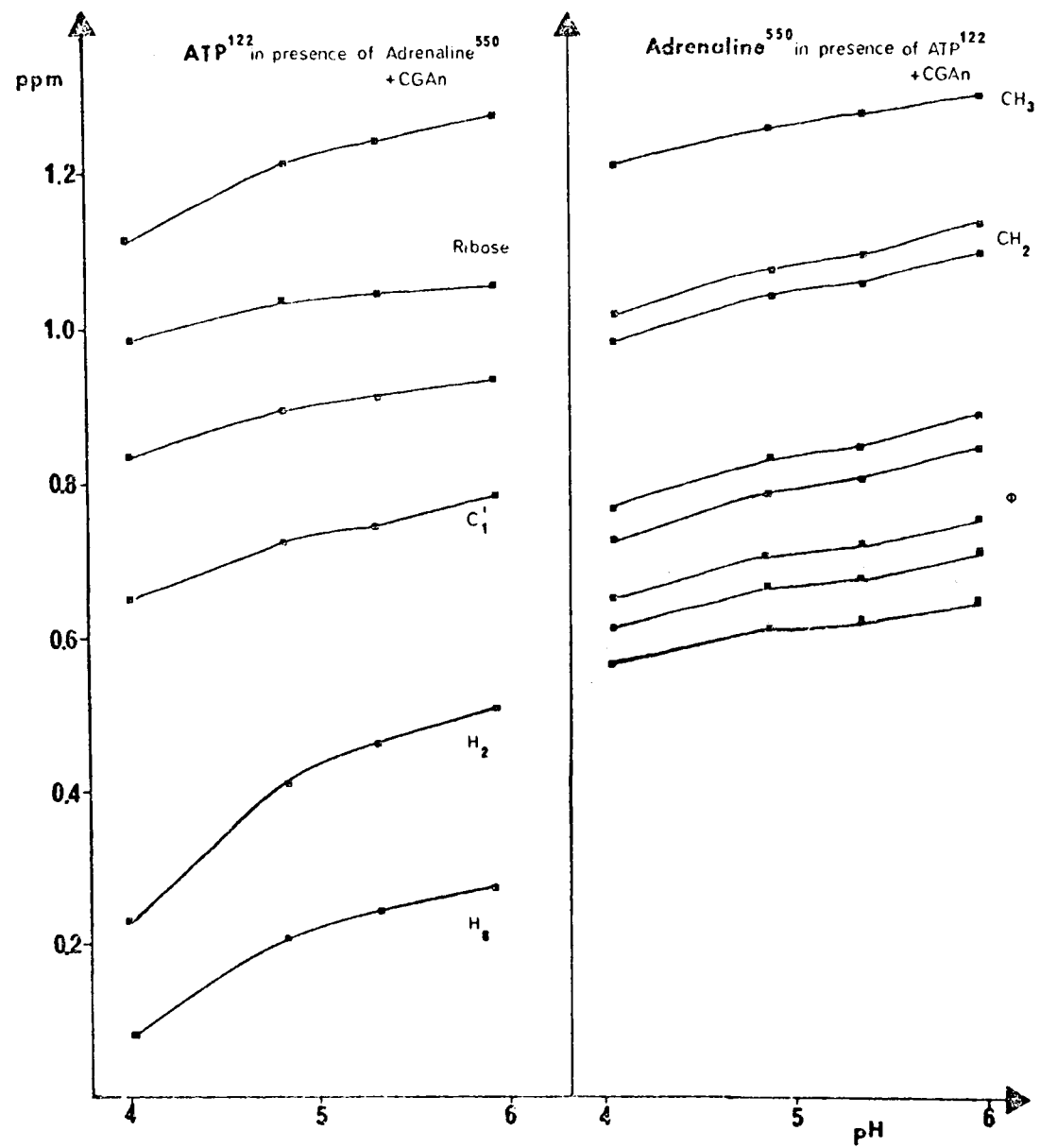
ATP, 122mM, was titrated against copper ions in order to correlate the degrees of broadening observed in the phosphorus and proton

TABLE 4.4.6.

Copper Concentration (μ M)	C ₈	$\Delta\nu_{1/2}$ (Hz)	C ₂	Relative Peak Heights (C ₂ / C ₈)
0	4.40		4.35	0.635
1.17	4.65		4.60	0.635
4.67	4.70		4.68	0.635
18.67	5.00		5.00	0.633
135.40	5.36		5.36	0.782
602.25	28.57		16.96	1.666
835.65	51.79		27.68	1.842
1070.15	101.79		57.14	2.000

The effect of low concentrations of copper ions on the line widths ($\Delta\nu_{1/2}$) and relative peak heights of the protons attached to the C₂ and C₈ atoms of the adenine moiety of ATP.

FIGURE 4.4.22.



The pH titrations of the proton resonances of ATP, 122mM, and Adrenaline, 550mM, in the presence of Chromogranins.

The assignment of the resonances is as in Figure 4.4.21.(c). The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions are as in Figure 4.4.21.

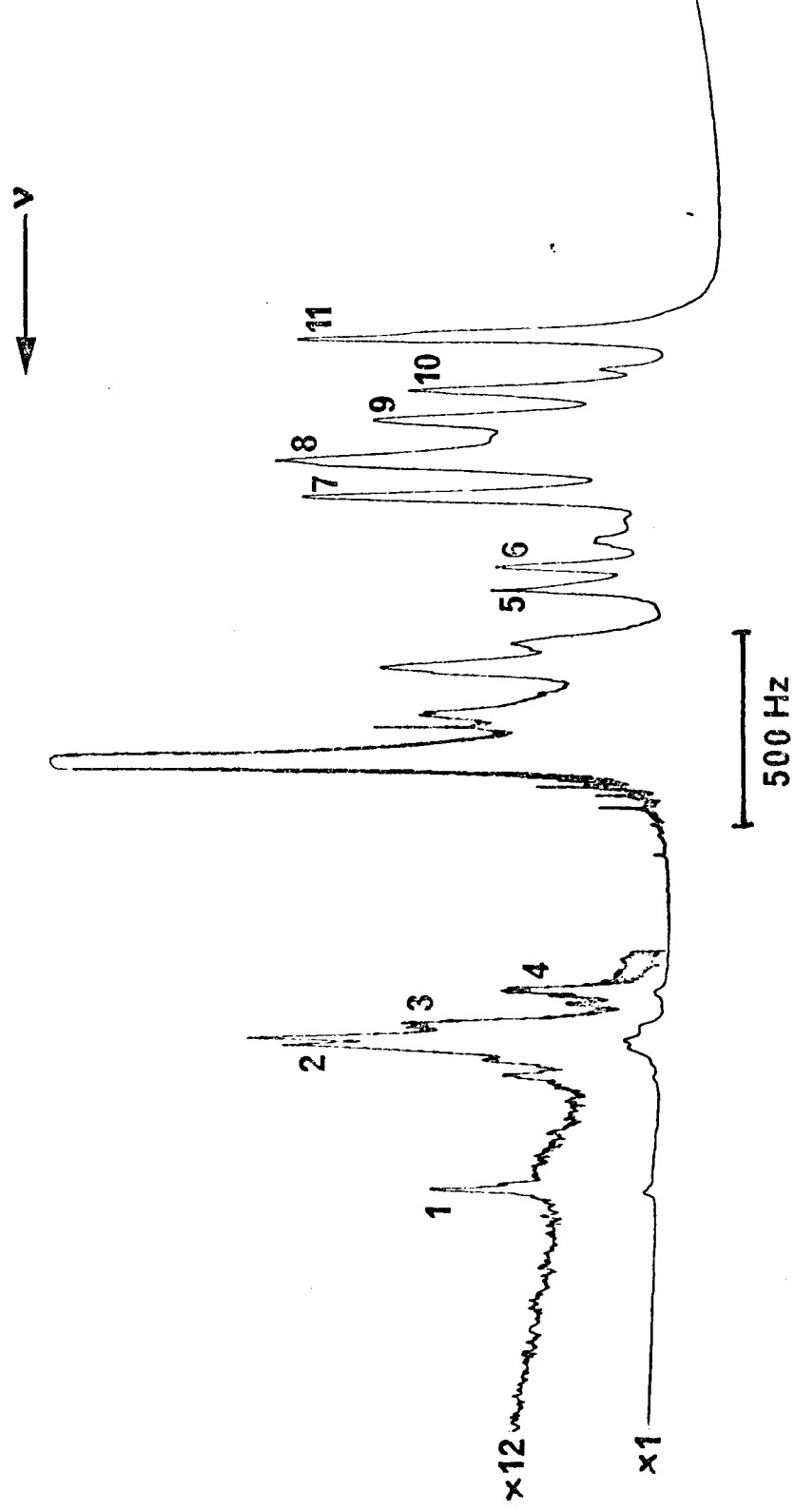
spectra of the intact granules. When the phosphate resonances of ATP were titrated against Cu^{2+} , an effect was observed on the γ -phosphate resonance at a copper concentration as low as $3\mu\text{M}$. However, in the case of the H_8 and H_2 of the adenine ring, significant broadening is not observed until the concentration of copper ions exceeds $100\mu\text{M}$ (Table 4.4.6.). This is obviously greatly in excess of the concentration within the granule, since no phosphate resonances would be visible if this was the true concentration. In addition, in the granule spectrum, the H_2 protons are broadened more than the H_8 which is the opposite of the effect of the paramagnetic ions. Thus the broadening observed in the proton spectra must be due to something other than paramagnetic ions, possibly viscosity.

(b) The Effect of pH on the Chromogranin, ATP and Adrenaline Proton Resonances.

The effect of pH on the resonance frequencies of ATP (122mM) and adrenaline (550mM) in the presence of chromogranins was investigated. The titration curves are shown in Figure 4.4.22.

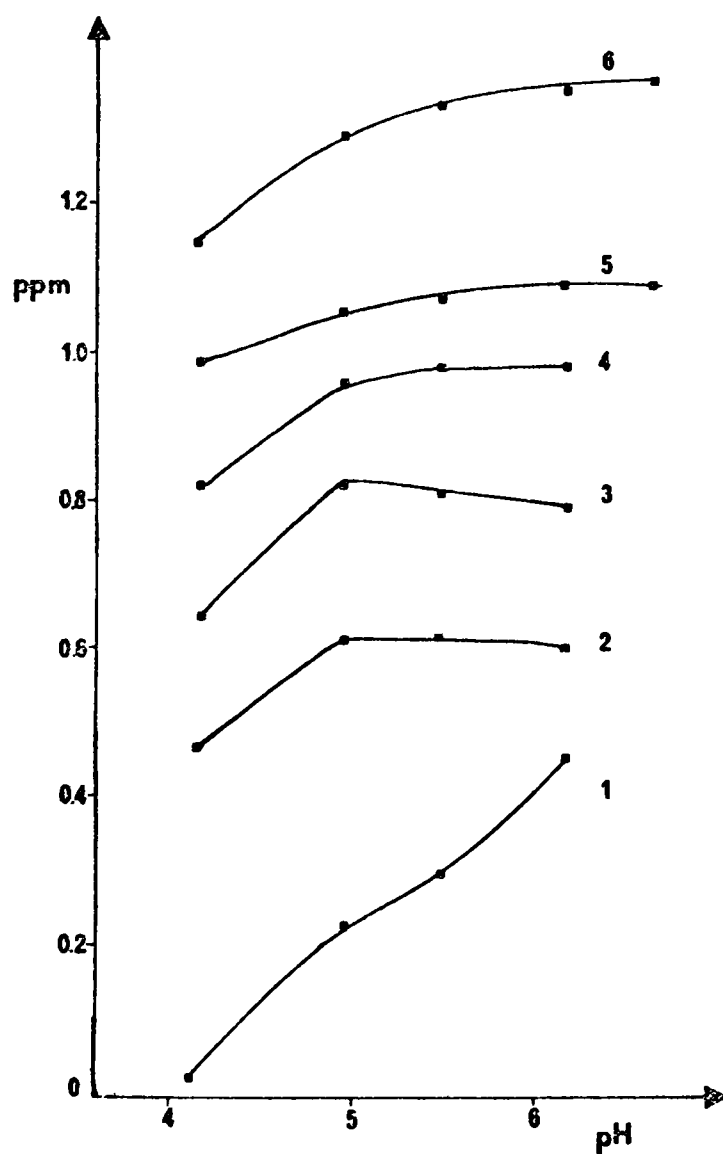
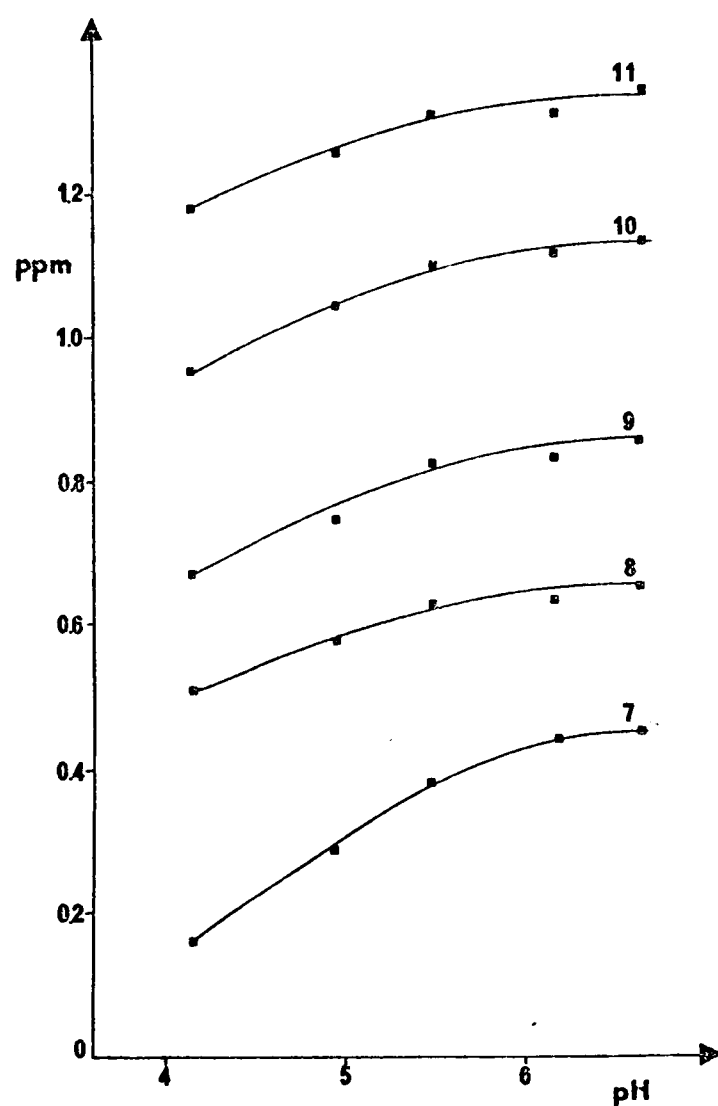
The adrenaline resonances shift in a parallel fashion across the pH range 4 to 6. The ribose resonances of ATP do not shift to the same degree, but the differences are not very significant. However, the adenine resonances of ATP, especially H_8 , shift quite markedly over the lower part of this range (pH 4 to 5). The overall shift of the H_8 resonance is 0.2ppm between pH 4.02 and 5.94. A separate pH titration of a more concentrated solution of chromogranins was also made. These titrations are shown in Figure 4.4.24. The chromogranin spectrum (Figure 4.4.23.) is dominated by five prominent peaks, contributed to by many resonances, which are shifted substantially upfield from the HOD resonance and also from the sucrose resonances in the granule spectrum. These resonances, attributable to aliphatic residues, shifted very little across the pH range examined. The furthest downfield resonance shifted 0.2ppm whilst the furthest upfield shifted 0.012ppm across the pH range 4.14 to 6.64. Since these are

FIGURE 4.4.23.



^1H NMR spectrum of partially purified chromogranins. Conditions as in Figure 4.4.21. 2000scans. The numbers refer to the 'resonances' which shift across the pH range (Figure 4.4.24.)

FIGURE 4.4.24.



The pH titration of the proton resonances of the Chromogranins, assigned as in Figure 4.4.23. These are not frequency shifts of individual protons, but are the shifts of multiple peaks prominent in the spectrum.

The chemical shifts are expressed in p.p.m. units w.r.t. an arbitrary zero. Conditions are as in Figure 4.4.21.

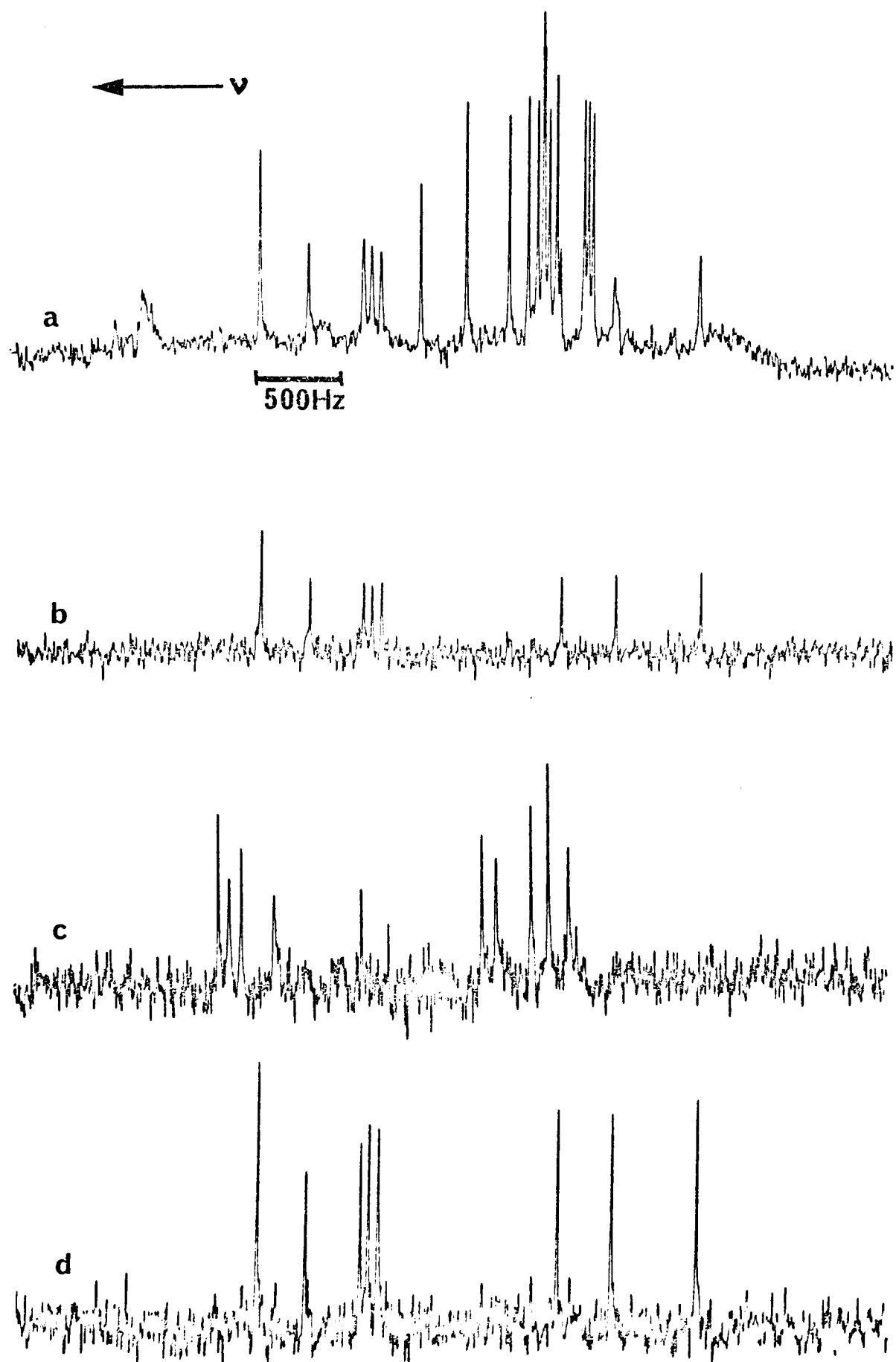
multiple resonances and are, as a comparison of the spectra in Figure 4.4.21., shows, relatively unaffected by the presence of other molecules, they act as a crude but useful internal standard. At much higher magnification, resonances on the high frequency side of the HOD peak can be seen. These peaks are dwarfed in the chromaffin granule spectrum by the ATP and adrenaline resonances. Even without deconvolution, however, several resonances attributable to single protons can be seen. The furthest downfield peak, which is composed of several resonances, moves markedly upfield as the pH increases and also 'broadens' due to the contributory resonances shifting to different degrees. The 'resonance' is 18Hz wide at pH 4.15 and 100Hz wide at pH 6.64, over which pH range, its midpoint has shifted by 0.31 ppm.

Longer accumulations followed by deconvolution and other techniques, in the presence and absence of small concentrations of ATP and adrenaline, may yield information about the role of the chromogranin protein in the storage complex, since there are obviously resolvable resonances present in the spectra.

(iii) Carbon NMR Studies.

Whilst the chemical shift range of carbon nuclei is approximately thirty times that of protons, this advantage is counterbalanced by the much lower, approximately four fold, frequency at which ^{13}C studies are usually made. Thus, though ring current shifts might be expected if there is a complexing of ATP and adrenaline, these, in practice, may be difficult to determine. The carbon spectra of adrenaline, ATP, chromaffin granules and an adrenaline:ATP mixture are shown in Figure 4.4.25. The chemical shifts, at natural abundance, detected for the resonances in these and other related spectra, are compared in Table 4.4.7. The resonance from the adrenaline methyl group, the furthest upfield, is not affected by increasing concentration of adrenaline or the presence of ATP. It has been used as the internal standard. The other shifts are related to it.

FIGURE 4.4.25.



^{13}C NMR spectra of

- (a) bovine chromaffin granules in 0.3M sucrose,
- (b) adrenaline, 550mM,
- (c) ATP, 550 μM ,
- (d) adrenaline, 550mM, and ATP, 122mM.

Spectra are accumulations of 20,000, 1600, 4800, and 5000 scans resp.
(pulse interval 1.5s, pulse angle 60°)

Temperature = 16°C ; $\nu = 22.63\text{MHz}$.

TABLE 4.4.7.

	Adrenaline							ATP (Unassigned)									
	C7&C8	C4	C5	OR C6	OR C9	C3	C2	C1									
Adrenaline, 100mM (x3)	110.68	98.40	84.85	82.73	80.21	35.08	21.40	0									
Adrenaline, 100mM	110.69	98.47	84.96	82.85	80.36	35.06	21.40	0									
Adrenaline, 700mM (x3)	110.58	98.32	84.85	82.70	80.17	35.05	21.40	0									
Adrenaline, 700mM	110.58	98.37	84.93	82.79	80.27	35.06	21.40	0									
Adrenaline, 550mM	111.01	98.82	85.23	83.18	80.59	35.17	21.47	0									
Adrenaline, 550mM, + ATP, 122mM	111.01	98.72	85.12	82.95	80.59	35.17	21.57	0	121.58	114.79	84.36	54.05	41.54	32.04			
Chromaffin Granules	111.01	98.82	85.12	82.95	80.59	35.17	21.47	0									
ATP, 550mM									120.83	114.79	106.35	84.58	53.83	50.27	41.32	37.00	31.93
ATP, 1M									120.94	114.36	105.83	84.26	53.94	49.84	41.32	36.90	32.15

The frequencies of the ¹³C resonances of Adrenaline and ATP at various concentrations and in combination. The frequencies of the Adrenaline resonances are compared to those in Chromaffin Granules. The frequencies are expressed as chemical shifts, in p.p.m. units, from the C1 resonance of Adrenaline, the absolute frequency of which, in each set of experiments, stayed constant. The error in each value is ±0.11p.p.m. The conditions are as in Figure 4.4.25. The Adrenaline spectra labelled (x3) were obtained with a threefold less spectral width, and hence greater resolution.

The non-aromatic adrenaline resonances do not shift in any of the spectra. There are small but significant shifts in the aromatic resonances, however. When the concentration of adrenaline is increased from 100 to 700mM, there are shifts of approximately 0.10, 0.08, 0.01, 0.03 and 0.04 ppm in the frequencies of the aromatic resonances from highest to lowest frequency respectively. These changes may be due to aromatic ring proximity. A comparison of the shifts observed in the chromaffin granule, ATP:adrenaline and adrenaline, 550mM, spectra indicates that there are no significant shifts observed except possibly in resonance 4. The resonance furthest shifted when the concentration of adrenaline was increased was unaffected by the presence of ATP.

It is difficult to detect the ATP resonances in the adrenaline: ATP and chromaffin granule spectra, since the resonances are not much greater than the noise level. The chemical shift values indicated in Table 4.4.7., whilst useful values for comparison, are not as accurate as the other values shown. The concentration dependence of the resonances of ATP when its concentration is increased from 550mM to 1M indicates that, as with the adrenaline resonances, the non-aromatic are unaffected but changes are detectable in the frequencies of some of the aromatic resonances.

(iv) Relaxation Measurements.

A postulated model of the storage complex suggests 'exchangeable' and 'non-exchangeable' pools of catecholamines (61). Daniels et al vaguely infer the presence of substantial aggregation within the granule from the large width of the resonances in the proton and phosphorus NMR spectra of intact chromaffin granules (60). Since accurate measurements of the relaxation times for NMR signals can provide detailed information about molecular dynamics, preliminary studies of the storage complex have been made using these methods. This section is not intended to do more than show the direction of future research.

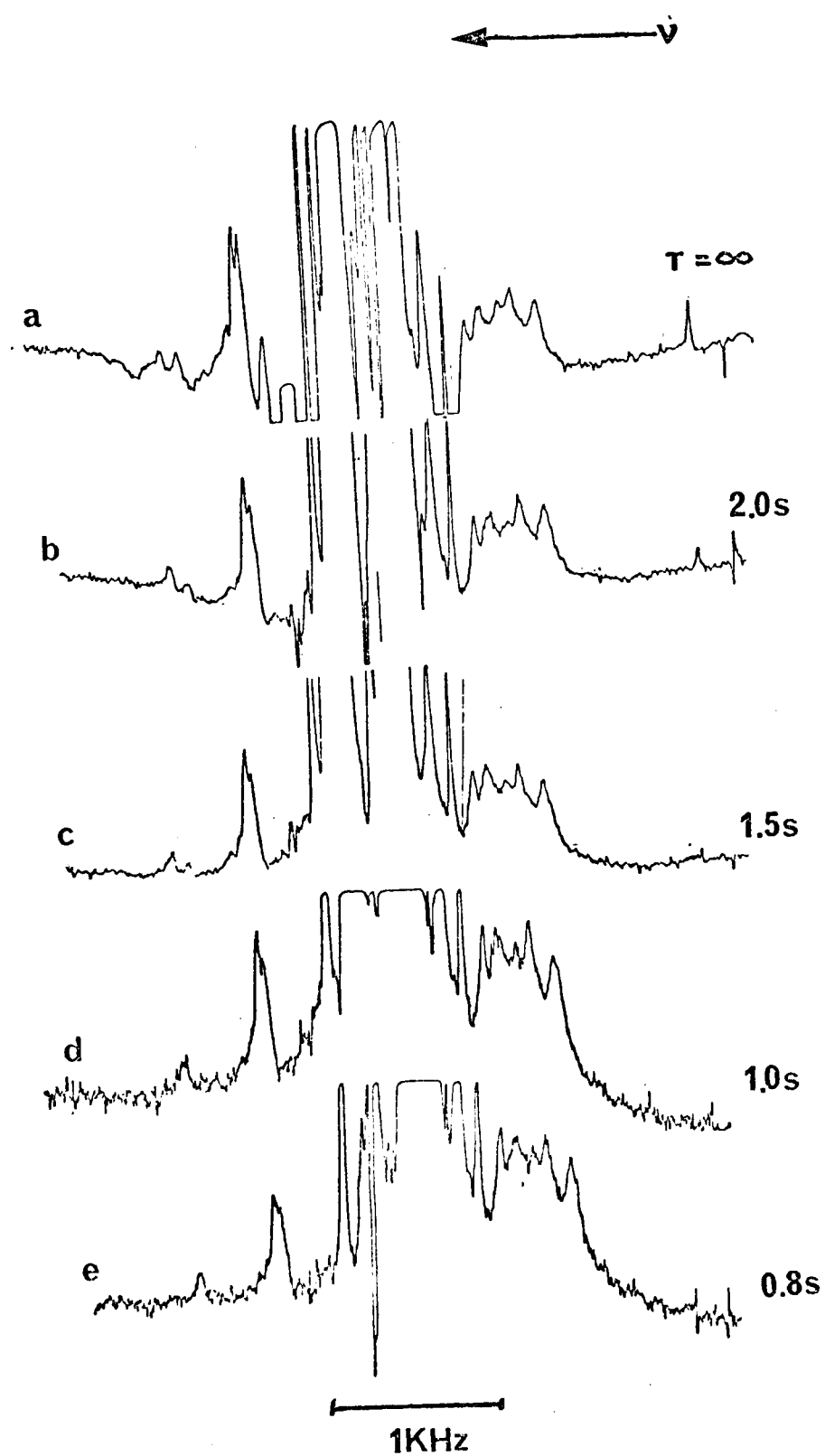
The line widths of the phosphorus NMR signals of bovine and ovine granular ATP are estimated as 42, 54, and 48 Hz compared to those of porcine granules which are 37.5, 48 and 37.5 Hz. This, on a crude basis might be taken to indicate different T_2 's in the different systems. The line widths in the simulated complex are 35.5, 46 and 33Hz.

The resonances of the bovine granule could not be saturated by 50° pulses at 0.2s intervals, therefore T_1 measurements were made of the ATP phosphates of both porcine glands and granules which could be saturated using the inversion recovery technique described in the appendix to this chapter. A value of 1.0 ± 0.3 secs was determined for all three phosphate resonances. This relatively long T_1 suggests that the nucleotide molecules are fairly mobile. Why then are the line widths so large? The significantly greater transverse relaxation rate ie ($T_2^{-1} \propto \Delta\nu_{1/2}$) of the β -phosphate cf. the α - and γ -phosphates tends to rule out a broadening mechanism based on inhomogeneity of the local magnetic field. Three possible explanations remain:

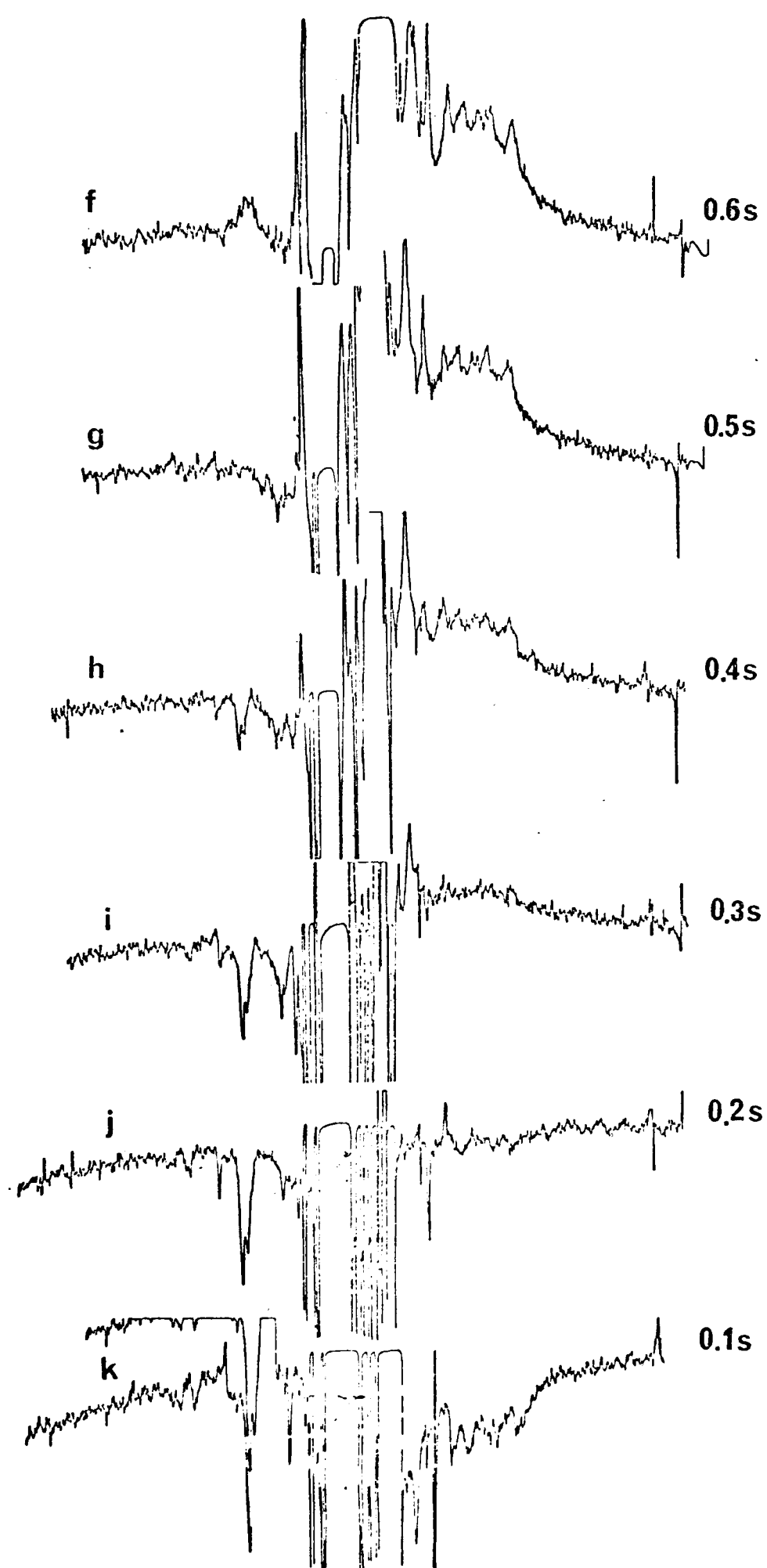
- i) Paramagnetic broadening,
- ii) Slow intermediate exchange of divalent cations,
- iii) Heterogeneity of granule composition.

Although it has been demonstrated that bovine glands may contain more paramagnetic ions than porcine ones, paramagnetic relaxation of the ATP phosphates in the porcine gland cannot be ruled out (Figure 4.4.15. shows that the β - and γ -phosphates are more strongly relaxed than the α -). Diamagnetic divalent metal ions eg. Mg^{2+} , Ca^{2+} have been shown to cause exchange broadening of the β - and γ - phosphate resonances of ATP under certain conditions (62). Since the total magnesium and calcium concentration within the bovine chromaffin granule is probably only 25% of that of ATP, this mechanism is a possible, though perhaps an improbable, mode of relaxation for the ATP. Results earlier in this chapter indicate that it is the frequencies of the β - and γ -phosphate resonances which are most

FIGURE 4.4.26.



A ^1H NMR inversion-recovery T_1 sequence of porcine chromaffin granules in 0.3M sucrose/ D_2O . The values of τ , for each spectrum, are indicated on the right-hand side. Other conditions are as in Figure 4.4.21.



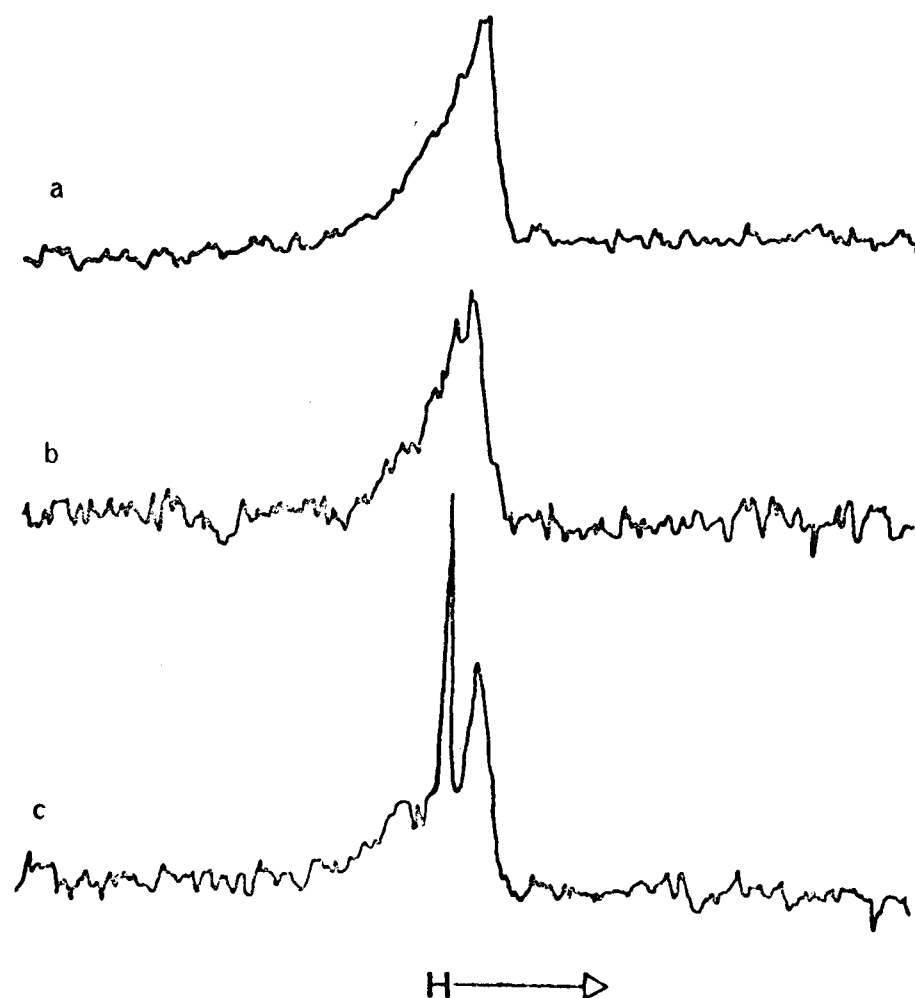
sensitive to the cationic composition of the environment. Heterogeneity is another possible cause. The existence of noradrenaline and adrenaline containing granules is well documented (63). The existence of further heterogeneity with respect to the ratio of protein:catecholamines:ATP has also been demonstrated (64). It is probable that, in addition, there is a heterogeneity in the metal ion composition.

An inversion-recovery T_1 sequence on the porcine chromaffin granule proton resonances is shown in Figure 4.4.26. The aromatic protons will be relaxed, to a very good approximation, by the dipole-dipole interactions modulated by tumbling in the complex. The measured T_1 values of approximately seconds, indicate a rotational correlation time, τ , of seconds and a T_2 of seconds. T_2 values calculated from the line widths are in good agreement with this value. These rates of tumbling are compatible with the initial proposal from the simulation studies that the storage complex is a viscous solution rather than an ordered crystalline or gel structure.

More detailed relaxation measurements are required for confirmation of the 'viscous solution model'. Multiple pulse measurements of the inherent T_2 's for the phosphate signals should produce a more accurate estimate of their rates of motion and clarify the possible compositional heterogeneity. Accurate measurements of the T_2 's of the proton signals and of the T_1 values at an additional frequency eg 90MHz should similarly clarify the situation with regard to the aromatic rings of the granule constituents.

A further complication should be mentioned. Since the resonances from solids are very broad when examined in a conventional NMR spectrometer, it is possible that a proportion of the storage complex of the granule exists in a solid or semi-solid matrix. Present results, based on calculations of the effective concentration of ATP and nucleotides in the suspensions indicate that at least 90% of the granule contents are

FIGURE 4.4.27.



^{31}P NMR spectra of

- (a) chromaffin granule membranes suspended in 10mM HEPES, pH 7.4, 5mM EDTA,
- (b) sarcoplasmic reticulum suspended in 10mM histidine, 100mM KCl, 0.5mM EDTA, pH 7.2,
- (c) bovine spleen nerve suspended in 0.25M sucrose, 10mM Tris, pH 7.4.

The total spectral width in all cases is 50KHz.
 Temperature = 20°C ; $\nu = 129\text{MHz}$.

'fluid' and hence detected in the signal. This is a very difficult calculation to make accurately as the included volume has to be determined if more than an approximation is to be made.

F NMR Studies on the Chromaffin Granule Membrane.

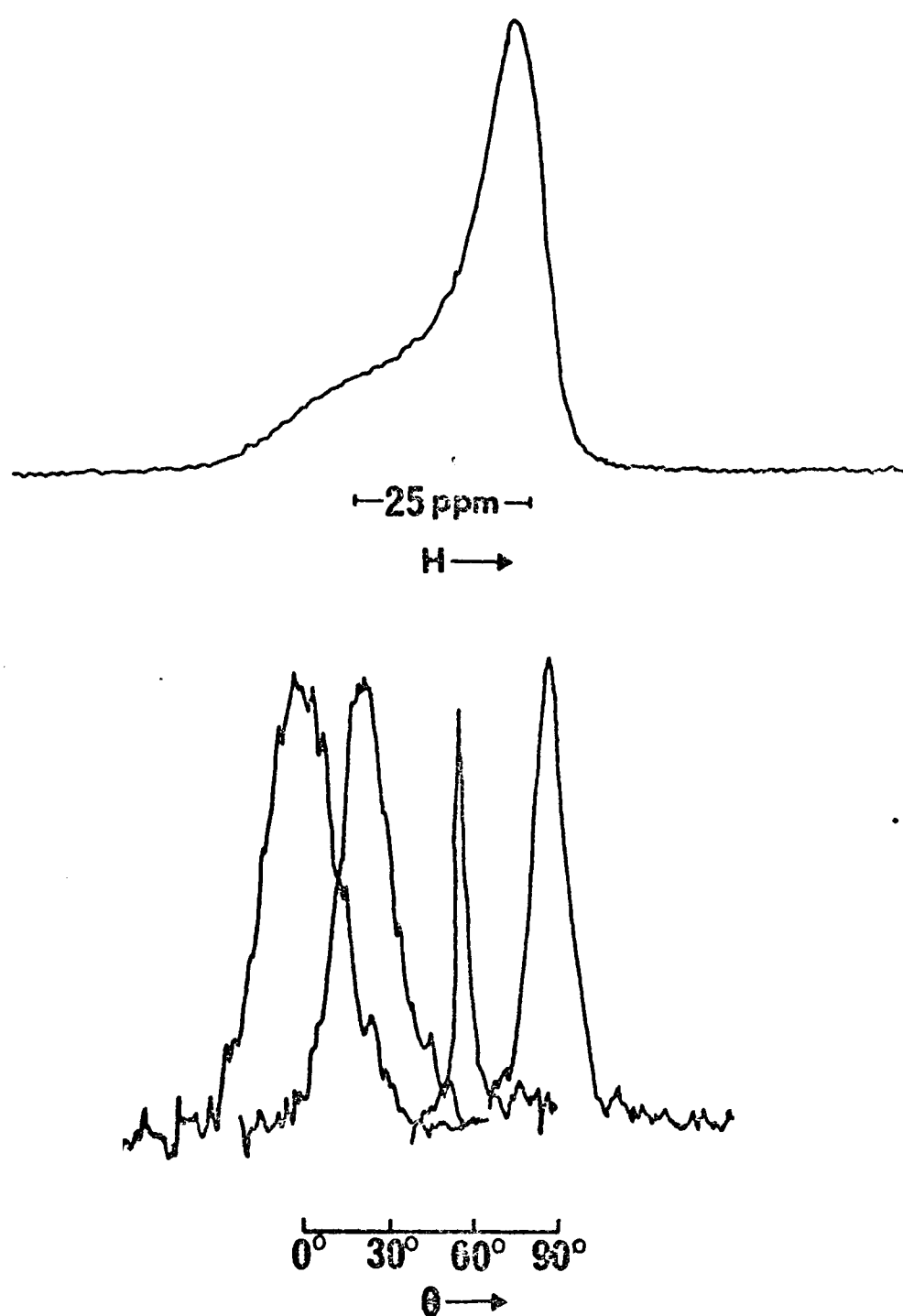
The chromaffin granule membrane has been studied primarily with phosphorus NMR, though proton NMR studies have also been made. Carbon NMR was not used as a probe of the membrane structure, although a membrane sample was examined and the spectrum confirmed that it was unlikely that high resolution detail could be observed and hence information obtained.

(i) Phosphorus Studies.

The phosphorus spectrum of the chromaffin granule membrane is that of a typical membrane spectrum. It is compared in Figure 4.4.27. to the spectra of sarcoplasmic reticulum membrane and bovine splenic nerve. The spectrum shows a broad assymmetric resonance consisting of a narrow component with a broad shoulder on the low frequency side. The overall shape of this partially immobilised phospholipid spectrum is a direct result of chemical shift anisotropy (13). The angle dependence of the frequency and width of the phosphate resonance from oriented phospholipid bilayers is shown in Figure 4.4.28. At the magic angle, approximately 55° , the resonance goes through a minimum width. Unfortunately, as will be described in Chapter Five, it has not been possible to orient the granule membrane to the degree necessary to observe this angle dependence, and hence obtain information about the orientation and mobility of the phospholipid head group in the membrane.

The temperature dependence of the membrane spectra of both bovine and porcine chromaffin granule membranes, prepared from granules isolated through 1.6M sucrose, were investigated. The spectra of the bovine granule membrane, over the temperature range 18° to 45°C , are shown in Figure 4.4.29. Since it is very difficult to prepare a sufficiently concentrated membrane sample, it is not practicable to obtain spectra

FIGURE 4.4.28.

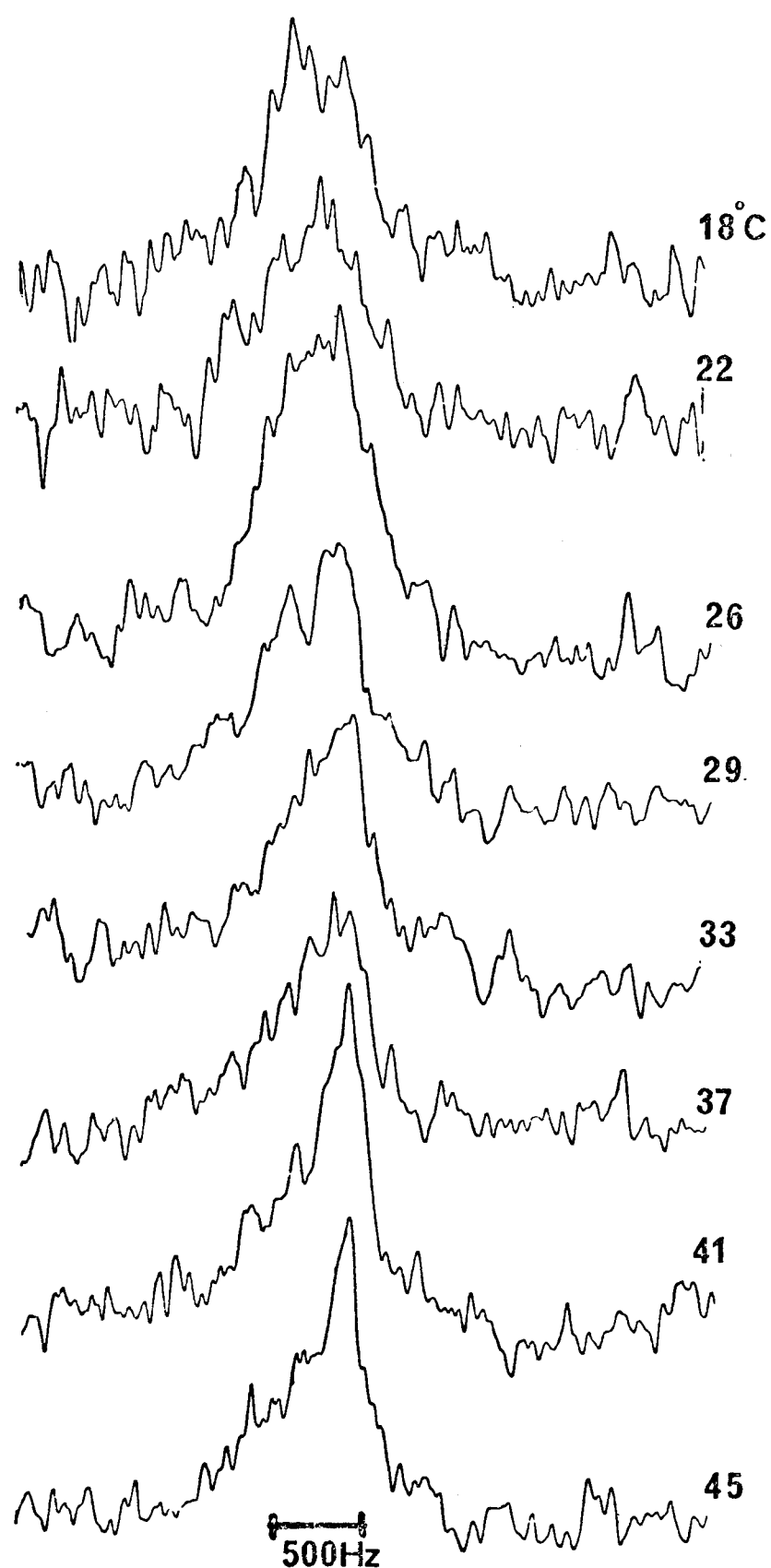


^{31}P NMR spectra of

- (a) unoriented dipalmitoyl lecithin/cholesterol (1:1) bilayer membranes,
- (b) planar oriented dipalmitoyl lecithin/cholesterol (1:1) multilayers at various orientations w.r.t the applied magnetic field. θ is the angle between the magnetic field and the normal to the plane of the membranes. The membranes were hydrated at 75% relative humidity. (13).

Temperature = 20°C : $\nu = 129\text{MHz}$.

FIGURE 4.4.29.



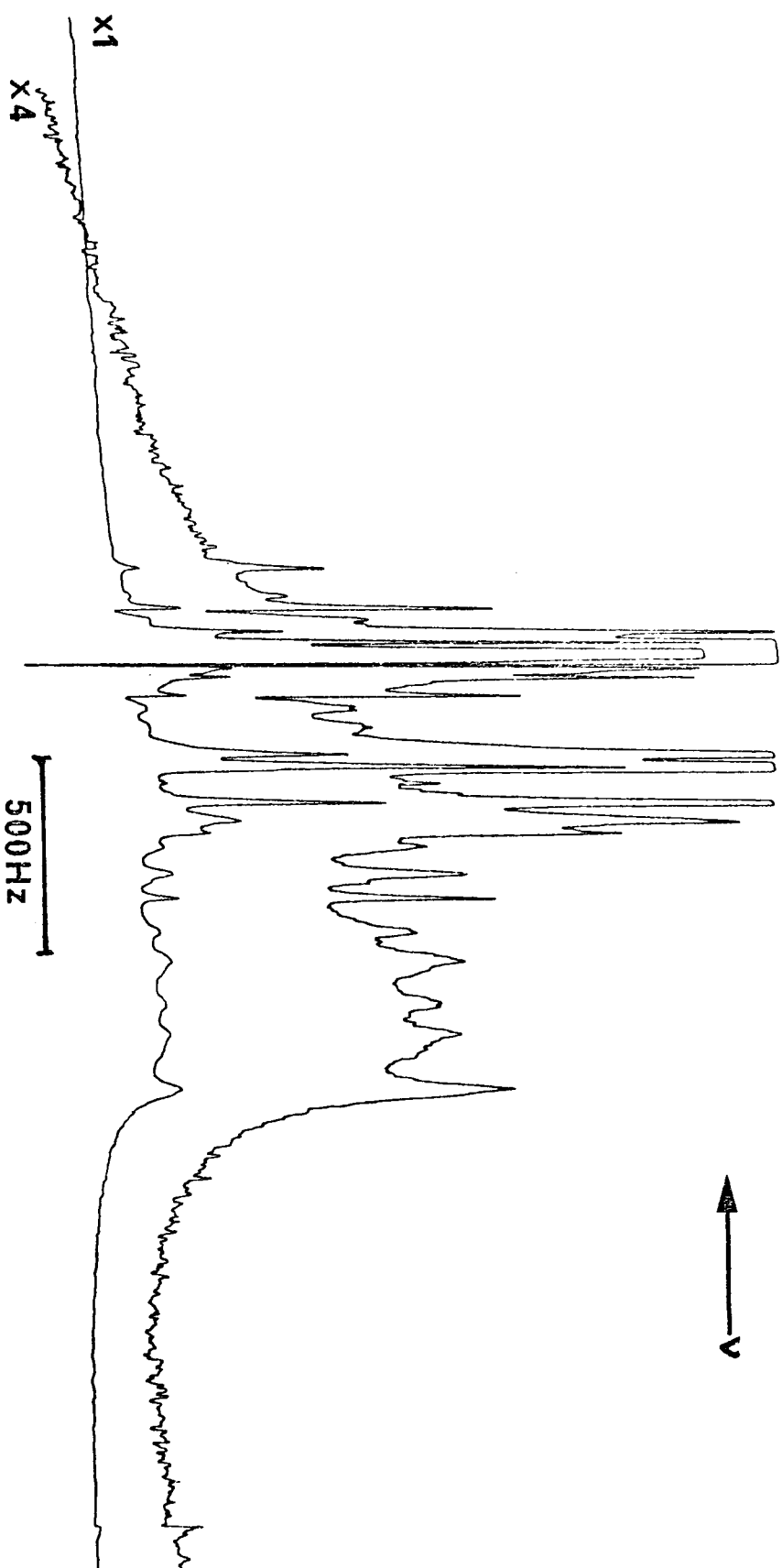
The temperature dependence of the ^{31}P NMR spectrum of bovine chromaffin granules, suspended in 10mM HEPES, pH 7.0, 5mM EDTA. The spectra are all 1200 scans, with the same phase correction applied on each. $\nu = 36.43 \text{ MHz}$.

with a very high signal:noise ratio when a broad temperature range is being investigated. The high noise level results in phasing difficulties; therefore, all spectra shown have had the same phase correction applied to permit a comparison to be made. It is not feasible to analyse these spectra in terms of line width since the shape of the resonance also alters as the temperature changes. However, certain trends in the spectra are apparent. In the crudest analysis, the eventual narrow component is shifted upfield between 13° and 29°C. In the spectrum at 29°C, a second component may arise and this appears to also shift upfield until 45°C when it merges with the initial component. However, the phasing will radically alter the shape of the resonance. This result should be treated with caution, but the sharpening up of the resonance does seem to occur between 33° and 37°C. The ESR and fluorescence studies (Chapter Three) indicate that an alteration in the membrane occurs between 32° and 37°C. It is possible that the development and shift of the second component with the resultant sharpening of the resonance, is a reflection of this change. This is purely speculation and until the experiment has been repeated with a greatly improved noise level in the spectra no conclusions can be made.

(ii) Proton Studies.

The proton NMR spectrum of bovine chromaffin granule membranes, prepared by successive hypo- and hypertonic shocks, is shown in Figure 4.4.30. Two observations can be made immediately. Firstly, apart from the EDTA resonances, the spectra is dominated by the five low frequency resonances from the chromogranins (compare to Figure 4.4.23.). Secondly, no broad lipid resonances are apparent. This latter result would be expected from the ESR studies (Chapter Three) which indicated that the membrane was not unusually fluid. The proteins must have considerable mobility since their resonances are no broader than the comparable resonances in the spectra of the soluble chromogranins. There are no aromatic resonances present in the spectrum of the membrane. This is an interesting observation

FIGURE 4.4.30.



The proton NMR spectrum of bovine chromaffin granule membranes
suspended in 5mM EDTA/ D_2O .
Temperature = 20°C : ν = 270MHz : 2320 scans.

since it may mean that the aromatic residues are buried in the hydrophobic region of the membrane and are, therefore, partially immobilised.

It has not been possible to pursue this preliminary, but interesting, result as yet, but further studies are to be made to determine how tightly bound this protein is to the membrane. If it can be shown that the protein resonances are definitely from a membrane-bound protein, not just protein enclosed in the membrane vesicles, it will be interesting to see if the aromatic resonances reappear on solubilisation of the membrane. This is important in view of Helle's proposal that the chromogranins in vivo are associated with phospholipids (45).

4.5 Discussion.

These studies have demonstrated the multiple applications of phosphorus NMR as a probe of biological systems, with particular reference to the chromaffin granule system. The simplicity of the spectra and the considerable sensitivity of the phosphate resonance to its environment have been clearly shown.

The initial comparison of the spectrum of an intact adrenal gland those of its dissected cortex and medulla, and subsequently with that of chromaffin granules obtained from medullary tissue (Figures 4.4.1 and 2,) permitted the tracking, localisation and assignment of the resonances from the intact tissue to be made. This is extremely important since a knowledge of the state of the system in situ is invaluable to the more usual in vitro studies. In this case, it was possible to compare the nature of the ATP resonances in the intact glands and in the isolated granules of different animals. The similarity of the frequencies and the line widths of these resonances in the two systems is a very critical finding, since it justifies the detailed research that has been made on the isolated subcellular system. The spectra from the glands (or medullae) and the granules from pigs, sheep and cows (Figures 4.4.4 and 5.) show that small differences exist in the nature of the storage complexes in the granules of these animals. The bovine and ovine spectra are very similar, but the resonances in the spectra of the porcine granules and glands were narrower and the resolution in the hyperfine splitting was greater. These differences are, in all probability, not due to major differences in the structure of the storage complexes in these animals, though it is likely that the chromogranin proteins from the three species, though similar, are not identical. The more probable explanation is that the metal ion contents of the granules from the different sources are not the same. Small variations in the concentration and the nature of the metal ions liganding to the phosphate moieties of ATP may cause large differences in the line

widths and the frequencies of the resonances (62).

The use of phosphorus NMR in this type of study obviously has a limited application. It can only be used to investigate tissues in which moderately high (greater than 1mM) concentrations of phosphates are present. It does, however, allow impure preparations to be examined, if the contaminants do not contain the same phosphate-containing compounds. This is very useful when examining preparations contaminated with mitochondria, as the initial chromaffin granule pellet is, since mitochondria contain very low levels of phosphates, including ATP. The other major limitation is that the system under study must be small enough to fit into an NMR tube. Hence, it was not possible in this study to examine the intact bovine adrenal gland. Despite these restrictions, the applications of the technique in comparative biochemistry are diverse.

The extreme sensitivity of the phosphorus nucleus in a phosphate group to its charge environment means that the frequency of the resonance is highly dependent on the pH of the surrounding medium. This pH sensitivity is present whether other molecules are interacting with the phosphate group or not. In the presence of other interactions, the pH curve shifts in frequency. The various titration curves for disodium ATP show that across the pK range, the extents of the shifts of the resonances of the phosphate groups are in the order: $\gamma \rightarrow \beta \rightarrow \alpha$ -. Unfortunately, at the very high concentrations used in this study, it was not possible to titrate the resonances across the full chemical shift range, since the mixture of 122mM ATP and 550mM adrenaline is not soluble at pHs greater than 6. This means that it is difficult to determine whether the shifts of the phosphate resonances in the presence of catecholamines and other metabolites are pK_a shifts or otherwise.

With the knowledge of the resonance frequencies of the phosphates in the granule complex, in addition to the composition of the complex, it was possible to compare these frequencies to those of the

titration curves of the various simulated mixtures. Whilst not providing a precise value for the internal pH of the bovine chromaffin granule, these studies indicated a range in which the pH might lie. The problems encountered with this method of pH determination have been discussed thoroughly in the text. It is important to appreciate that although the value of 5.45 ± 0.25 has been deduced on the basis of several antagonistic effects, it is probable that the error range quoted is generous since an estimation was made using the titrations of both the β - and γ -phosphate resonances. The β - and γ -resonances act as two separate probes since their susceptibilities to alterations in the phosphorus charge environment are different. The assessment of pH using phosphorus NMR permits its measurement in intact tissue; no other technique can do this. It is most accurate when several different phosphate-containing molecules with a range of pK_a values are present in the tissue. The technique has been applied to the study of muscle (8). Its application could be extended to many other systems.

The value of 5.45 ± 0.25 determined using this method is in excellent agreement with the value of around 5.5 proposed on the basis of completely independent biochemical studies of the ATPase activity and the ANS response (Chapter Two). The determination of pH inside mitochondria and chloroplasts is usually made using the methylamine distribution method (65). Preliminary attempts were made to use this method but the inulin accessible included volume has to be determined very accurately if the technique is to be applied successfully. This study is continuing. It will be interesting to see if the value for the internal pH obtained eventually using the methylamine distribution method is in agreement with the values from the other methods.

'Are the contents of the chromaffin granule present in a paracrystalline form, a gel state or as a viscous solution of freely moving molecules? It has not been possible to provide an absolute answer to this very crucial question. Previous workers have suggested highly

conflicting models for the interaction of ATP with adrenaline in the storage complex; Weiner and Jardetzky (57) propose that the phosphate resonances of ATP form ionic and hydrogen bonds with the side chain of adrenaline, whilst Daniels et al prefer a structure in which there is stacking of the aromatic rings by hydrophobic forces (60). The results described in this chapter illustrate the dangers of immediately interpreting chemical shifts of resonances in terms of specific interactions. Only Weiner and Jardetzky (57) acknowledge the existence of non-specific interactions which are solely the result of the presence of high concentrations of aromatic rings. Clearly, it is not accurate to compare spectra where the ratios of the components are similar, if the concentrations of the molecules are not also similar when working with such abnormally high levels of metabolites. These studies indicate that there is a general ring current shifting of the resonances due to this very large concentration of aromatic rings present in the complex, and hence to the very close average proximity of all atoms to an aromatic ring.

The proton NMR studies suggest that an additional interaction may exist since the chemical shifts of the adrenaline protons are further shifted in the mixture of 122mM ATP and 550mM adrenaline compared to 672mM adrenaline. The ATP resonances from this mixture are not further shifted compared to 672mM ATP (in the absence of divalent metal ions). Another interpretation of this result is that the adenine ring of ATP is larger than the catechol ring of adrenaline, and hence has a greater ring current shifting capacity. This means that a direct comparison of the shifts in the solutions of the individual metabolites and in mixtures of them, whilst a useful approximation, is not entirely accurate.

The phosphorus NMR studies showed that the β - and γ -, and to a lesser extent the α -, resonances of ATP were shifted to higher frequencies in the presence of adrenaline. This is in the same direction as the shifts caused by divalent metal ion liganding. It is possible that an ionic

interaction exists between the negatively charged phosphate groups and the positively charged nitrogen of the adrenaline. This localised bonding would not have a large effect on the other resonances of the molecules since it only involves the terminal groups of each molecule. This would be in agreement with the proton NMR results and also with the findings of Weiner and Jardetzky (57).

Preliminary studies of the porcine chromaffin granules and the porcine adrenal medulla indicate that the 'complex' is a dynamic system and that specific interactions, if they do occur, are short lived. The relaxation studies once complete should clarify this point conclusively.

The problem of osmotic stability still exists. In order to be isotonic with the cytoplasm of the cell, approximately 2,5 adrenaline molecules must interact with one ATP molecule. Only the model proposed by Weiner and Jardetzky (57) is capable of fulfilling this criterion. In their model, up to three molecules of adrenaline might interact with each ATP molecule, though it is most likely that the β - and γ -phosphates would bind most readily. Multiple, non-specific interactions might also account for the osmotic stability.

The studies on the chromaffin granule membrane were very preliminary. The phosphorus NMR spectrum has no unusual features. It is very similar to the spectra obtained from unoriented lipids and other membrane systems. It is unfortunate that it was not possible to obtain a better signal:noise ratio in the study of the temperature dependence of the phosphorus resonances. It is always difficult to decide whether to use several different membrane samples and obtain the spectra at each temperature on separate occasions but with reduced spectral noise due to long accumulations, or else to use one preparation for the whole temperature range, hence having an internal standard through repetition at several temperatures. In this case, the latter approach was used. Changes obviously occur but it is both difficult and unwise to quantify them since

the phasing is uncertain and the signal:noise ratio poor. It is essential , therefore, to use the former method in addition. This should permit the changes to be characterised in a detailed manner and their reproducibility ascertained. It is encouraging that the initial study indicated that the major part of the 'sharpening of the resonance' occurs between 30° and 40°C, in the same range as the changes detected by ESR and fluorescence studies.

The proton NMR study of the membrane, though also preliminary, suggests that further investigation of the protein resonances might be very worthwhile. It should be possible to determine how tightly bound the protein is to the membrane and whether the resonances are affected by the presence of different reagents including calcium ions. It might also be profitable to examine the temperature dependence of these protein resonances to determine whether there is a transition in the amplitudes of motional freedom of the amino acid residues of the proteins. It might additionally be possible to label the membrane with aromatic molecules analogous to the covalently binding spin label molecules and investigate the behaviour of these resonances under a variety of conditions.

Thus the NMR studies of the chromaffin granule and the chromaffin granule membrane, whilst answering certain problems have posed many new ones and revealed several areas to which future research might profitably be directed.

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Appendix to Chapter Four

A.1 Chemical Shifts.

In liquids, dipole-dipole interactions are usually averaged out, due to the rapid molecular tumbling, and hence sharp lines are observed. The precise frequency of the absorption line of a particular nucleus is dependent on the nature of the molecule containing the nucleus. The frequency differences are termed chemical shifts. These arise because of the differences between the applied field, H_0 , and the magnetic field experienced by the nucleus because of the weak diamagnetic moments due to the surrounding electrons in the molecule, This is expressed as :

$$H = H_0(1-\sigma) \quad A1.1$$

where σ is the magnetic shielding constant.

Chemical shifts are usually expressed as shifts from some standard resonance. Tetramethylsilane (TMS) is often used as the standard in proton spectra. The shifts are generally expressed in dimensionless p.p.m. units ie.

$$\delta = 10^6 \left(\frac{\nu_{\text{sample}} - \nu_{\text{standard}}}{\nu_{\text{standard}}} \right) \quad A1.2$$

at constant H_0 .

The chemical shift range of ^{13}C nuclei is 250ppm, of protons is 10-20ppm and of ^{31}P nuclei is around 200ppm.

A.2 The Spin-Lattice Relaxation Time, T_1 .

It is necessary to maintain a population difference between the two energy states. The transitional probability, P , is the same for transitions from $|\alpha\rangle$ to $|\beta\rangle$ and vice versa. The rate of change of population of the $|\beta\rangle$ state is, in the presence of H_1 radiation, given by :

$$\begin{aligned} \frac{dN}{dt} |\beta\rangle &= P(N_{|\beta\rangle} - N_{|\alpha\rangle}) \\ &= -Pn \end{aligned} \quad A2.1$$

where $n = N_{|\beta\rangle} - N_{|\alpha\rangle}$.

In macroscopic samples with N spins,

$$N_{|\beta\rangle} = \frac{1}{2}(N + n) \quad ; \quad N_{|\alpha\rangle} = \frac{1}{2}(N - n) \quad A2.2$$

Hence,

$$\frac{dN}{dt}|\alpha\rangle = \frac{1}{2} \frac{dn}{dt} = -Pn. \quad A2.3$$

Therefore,

$$\frac{dn}{dt} = 2Pn. \quad A2.4$$

The solution to this differential equation gives the population difference at time t , i.e.

$$n = n_0 e^{-2Pt} \quad A2.5$$

where n_0 is the population difference at zero time.

The rate of energy absorption from H_1 depends on the difference between 'upwards', endergonic transitions and 'downwards', exergonic transitions, i.e.

$$\frac{dE}{dt} = N|\beta\rangle P(E|\alpha\rangle - E|\beta\rangle) + N|\alpha\rangle P(E|\beta\rangle - E|\alpha\rangle) \quad A2.6$$

$$= nP\Delta E \quad A2.7$$

Equation A2.5 shows that on the application of H_1 , n decays exponentially and is eventually zero, a phenomenon known as saturation. Equations A2.6&7 show that energy is absorbed only when n is finite. Together, these equations show that the absorption line will eventually disappear. In order to maintain n , it is necessary that a means of non-radiative transition between the $|\alpha\rangle$ and $|\beta\rangle$ states exists in which excess magnetic energy is converted to other forms of energy. This process is called Spin Lattice Relaxation. It is due to the interaction of the nuclear spins with oscillatory magnetic fields produced by molecular motion in the sample. This provides a mechanism whereby the magnetic energy of the nuclear spins can be converted into the thermal energy of agitation of the molecule. Thermal fluctuations in the system cause the various magnetic moments of the system to undergo a variety of motions, which produce oscillatory magnetic fields. If this range overlaps with the Larmor Precession Frequency of the nucleus, it can cause a transition from the upper energy state to the lower.

Application of a strong H_1 can cause n_0 to alter to some value n . If H_1 is switched off, n will decay back to the equilibrium value n_0 because of spin lattice relaxation. It will approach the equilibrium value exponentially, according to the equation

$$\frac{dn}{dt} = - \frac{(n-n_0)}{T_1} \quad \text{A2.8}$$

T_1 is the spin lattice relaxation time. Large values of T_1 , minutes or longer indicate a very slow relaxation. The relationship in Eqn. A2.8 can be rewritten in terms of the net magnetisation along H_0 :

$$\frac{dM_z}{dt} = - \frac{(M_z - M_0)}{T_1} \quad \text{A2.9}$$

where M_0 is the equilibrium value of M_z corresponding to n_0 .

An important property of the line-width of the resonance follows from the Heisenberg Uncertainty Principle,

$$\Delta E \cdot \Delta t \approx \hbar \quad \text{A2.10}$$

Any process which decreases the lifetime, Δt of the excited state increases the uncertainty of E , the energy of the line, and hence broadens the resonance line.

A.3 Line Broadening.

There are several processes which affect the line-width of the absorption signal. The first, spin lattice relaxation was discussed above. The others are also of importance.

(i) Non-secular or lifetime broadening. Due to the processes of spin-lattice relaxation which govern the decay of M_z , the nuclear spin states have a finite lifetime. For a line whose half-height width is $\Delta\nu_{\frac{1}{2}}$, since a nucleus remains in a given energy level no longer than T_1 , on average, then $(\hbar\Delta\nu_{\frac{1}{2}})T_1 \geq \hbar$ or $\Delta\nu_{\frac{1}{2}} \geq \frac{1}{2}\pi T_1$.

(ii) Secular broadening. Secular broadening is the process of spin exchange between two nuclear dipoles. There is no loss of total magnetic energy in the system, therefore, M_z remains unchanged.

(iii) Dipolar broadening. In solids, absorption lines are often much

broader than would be predicted from secular and non-secular broadening.

This is due to the dipole-dipole interactions of proximal nuclei.

(iv) Inhomogeneity broadening. It is difficult to achieve a perfectly homogeneous applied magnetic field, H_0 , across the sample, and generally there will be some range of ΔH_0 . Thus nuclei in different parts of the sample will experience slightly different applied magnetic fields, and hence will precess at different frequencies. Also, if the sample itself is non-uniform, then local fields, H_{local} , will vary from one part of the sample to another, resulting also in a range of precession frequencies.

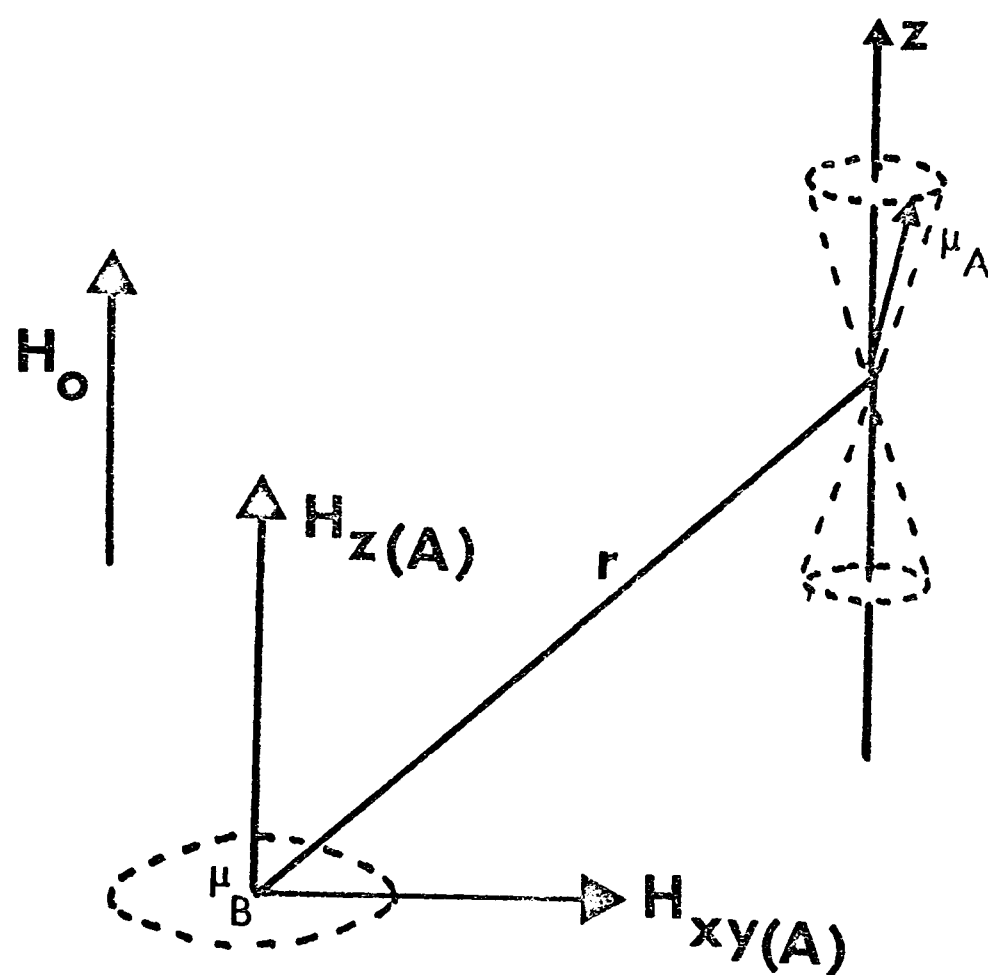
(v) Chemical shift effects. Nuclei at different positions in a molecule may resonate at slightly different magnetic field values. The observed absorption line corresponds to overlapping lines due to several chemically shifted nuclei. This introduces an apparent line broadening.

A.4 The Spin-Spin Relaxation Time, T_2 .

The magnetic fields produced at any one nucleus due to neighbouring magnetic nuclei have important effects. At nucleus B, there may be a component of the magnetic field due to nucleus A, which oscillates in the plane perpendicular to H_0 . If the nucleus B has a component at A in the same direction, at the same frequency, energy may be passed from one spin another and the effective lifetimes of the spin states decreased. By the Heisenberg Uncertainty principle, this results in a line broadening of the absorption signal. In this spin exchange, there is no net energy change in the spin state, merely a redistribution within it. This process is most effective for identical spins. For non-identical spins, the Larmor precession frequencies will be different and the spin exchange correspondingly less efficient.

Since in the process of spin exchange, there is no net energy difference, spin exchange cannot contribute to the T_1 . Spin exchange does affect the equilibrium within the spin system, and such processes are defined by a relaxation time, the spin-spin relaxation time.

FIGURE A.5.1.



The interaction between a pair of spin $I = \frac{1}{2}$ nuclei, A and B, in a magnetic field, H_0 .

T_2 is a much less well defined concept than T_1 . Whilst T_1 corresponds to a single process, T_2 corresponds to several. Experimentally, it is useful to define T_2 in terms of the width of the absorption line at half height, $\Delta\nu_{1/2}$, the exact relationship depending on the nature of the shape of the absorption line. In general, any of the factors listed in A3 because they affect line-broadening, can contribute to the line-width. Spin-spin relaxation times defined in this way are often defined as T_2^* .

In liquids, absorption signals usually have a Lorentzian shape, therefore,

$$T_2^* = \frac{1}{\pi\Delta\nu_{1/2}} \quad \text{A4.1}$$

In solids, absorption signals are often Gaussian, and T_2^* is defined as:

$$T_2^* = \frac{(\ln 2\pi^{-1})^{1/2}}{\Delta\nu_{1/2}} \quad \text{A4.2}$$

T_2 is also called the transverse relaxation time since it defines the relaxation of the components of magnetisation in the plane perpendicular to H_0 . T_1 is correspondingly called the longitudinal relaxation time.

A.5 Dipole-Dipole Interactions.

The magnetic field at a distance, r , from a dipole with a magnetic moment, μ , is approximately μ/r^3 . Thus when an external field is applied to a real system, the actual field experienced by a particular spin is the resultant of H_0 and all the other spins in the sample ie.

$$H_{\text{total}} = H_0 + \sum H_{\text{local}}. \quad \text{A5.1}$$

It is necessary to know $\sum H_{\text{local}}$, since spin transitions occur when:

$$\nu_0 = \left(\frac{\gamma}{2\pi}\right) H_{\text{total}} \quad \text{A5.2}$$

Consider a pair of nuclei A and B at an internuclear distance, r . Spin A will produce at B a magnetic field which can be resolved into two components, a steady field, $H_z(A)$, with a constant component parallel to H_0 , and a rotating component, $H_{xy}(A)$, perpendicular to H_0 (Figure A.5.1.).

At a fixed frequency, ν , the resonance of nucleus B occurs at a value of the magnetic field, H^* , given by:

$$\nu = \frac{\gamma H^*}{2\pi} \quad \text{A5.3}$$

The effect of nucleus A is to produce a pair of lines at field values given by:

$$H = H^* \pm \frac{3}{2}\mu(3\cos^2\theta - 1)/r^3 \quad \text{A5.4}$$

One line corresponds to each of the two possible orientations of the nucleus A, ie. $M_I = \pm\frac{1}{2}$ or $|\alpha\rangle$ and $|\beta\rangle$.

For a single crystal containing pairs of nuclei, A and B, with the internuclear vectors unidirectional, the splitting of the two lines observed will be dependent on the orientation of the crystal in the magnetic field, since this will determine the value of θ . If a powdered sample is used, the absorption line obtained is described by the sum of the values of $3\mu(\cos^2\theta - 1)/r^3$ averaged over all values of θ .

If the nuclei are together in a more complex arrangement than the pairs considered above, then the absorption line will be a broad hump due to the dipole-dipole interactions between all the nuclei in the sample.

A.6 Measurement of Spin-Lattice Relaxation Time, T_1 .

T_1 measurements are usually made using the $(180^\circ, \tau, 90^\circ)$ pulse sequence. This is illustrated in Figure A.6.1.

(i) Molecules containing one type of chemically shifted nucleus.

A 180° pulse is applied along the x'axis, inverting the magnetisation along the z'axis, and the system left for a time, τ , in which longitudinal relaxation occurs. M_z decays from the value $-M_0$ to reach its equilibrium value, $+M_0$. After time τ , a 90° pulse is applied along the x'axis and the magnetisation remaining at that time is rotated into the y'axis and a free-induction decay observed. The initial height of this signal is proportional to the value of M_z at time τ . The system is allowed to return to equilibrium by waiting at least $5T_1$, and the pulse sequence repeated for a

FIGURE A.6.1.

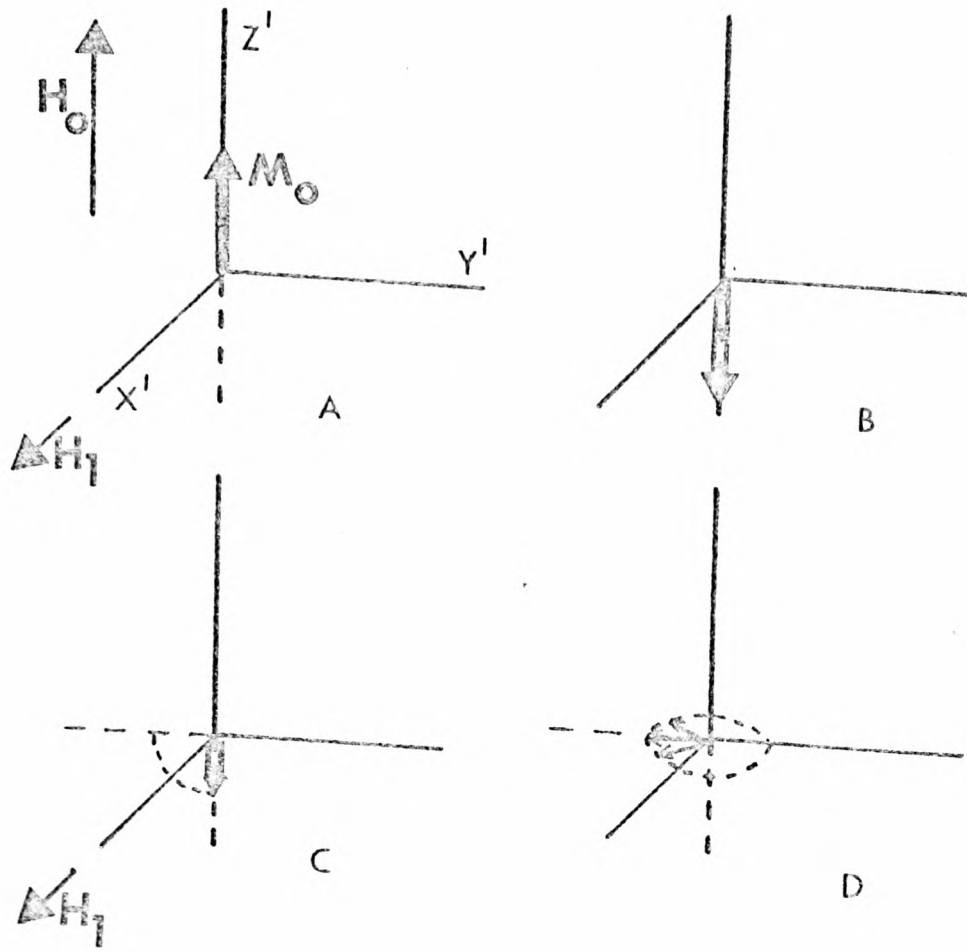
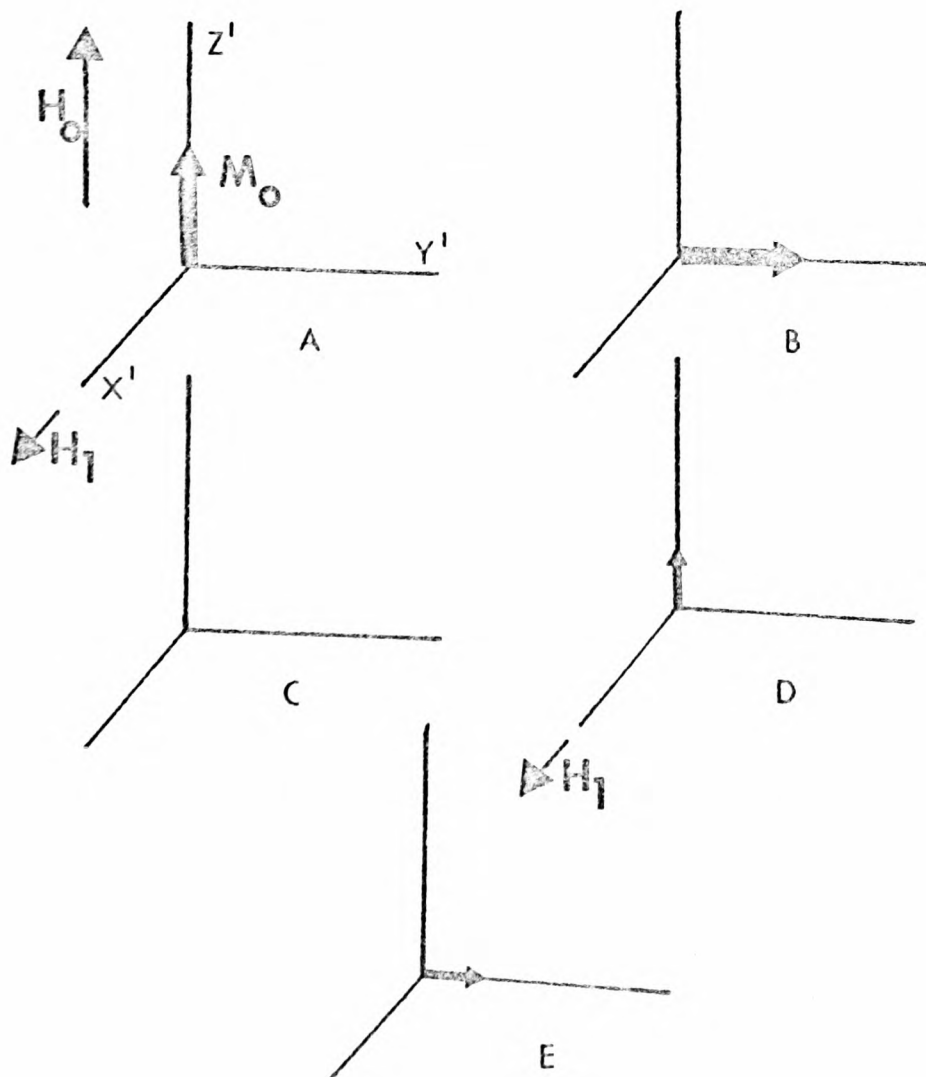
The $(180^\circ, \tau, 90^\circ)$ pulse sequence.

FIGURE A.6.2.

The $(90^\circ, \tau, 90^\circ)$ pulse sequence with a homogeneity spoiling pulse.

different value of τ . The relationship between the initial amplitude of the free-induction decay and τ is:

$$A_{\tau} = A_{\infty} [1 - 2\exp(-\frac{\tau}{T_1})] \quad \text{A6.1}$$

where A_{∞} is the equilibrium value of the amplitude.

The value of T_1 is obtained by plotting $\ln(A_{\infty} - A_{\tau})$ against τ .

(ii) Molecules containing several types of chemically shifted nucleus.

When the spectrum consists of several absorption lines, then Fourier transformation of the free-induction decays obtained using the above sequences gives a spectrum containing a number of partially relaxed nuclei. The rate of change of the intensity of each resonance with τ gives T_1 for the chemically shifted nuclei contributing to each particular resonance. In studies where repeated accumulation must be made to improve the signal: noise ratio, the $(180^{\circ}, \tau, 90^{\circ})$ sequence is repeated the requisite number of times with a delay of at least $5T_1$ between each pulse sequence.

Using the pulse sequence $(180^{\circ}, \tau, 90^{\circ}, t, 90^{\circ}, t)_n$, where t is greater than $5T_1$, the transient signal, A_{τ} , may be measured alternately with the measurement of A_{∞} . The deviation from equilibrium, $A_{\infty} - A_{\tau}$, is then displayed directly. Plots of $\ln(A_{\infty} - A_{\tau})$ against τ are straight lines of slope $-1/T_1$ since:

$$A_{\infty} - A_{\tau} = 2A_{\infty} \exp(-\tau/T_1) \quad \text{A6.2}$$

Spectra obtained in this manner for various values of τ are equivalent to time-lapse photographs; the shorter the value of the T_1 of a nucleus, the faster the intensity of the resonance decreases as τ increases.

T_1 can also be measured using a $(90^{\circ}, \tau, 90^{\circ})$ pulse sequence. This is shown in Figure A.6.2. After the initial 90° pulse has shifted magnetisation into the $x'y'$ -plane, a pulsed field is applied along the z' axis spoiling the field homogeneity and causing a rapid dephasing of the spins in the $x'y'$ -plane. This results in zero magnetisation. After an interval, τ , in which the spin system is allowed to relax, the resulting

magnetisation is sampled with another 90° pulse. The free-induction decay is then collected, followed by a further application of the pulsed field gradient to remove any remaining magnetisation in the $x'y'$ -plane. The pulse sequence can be repeated immediately. The variation of signal intensity is given by:

$$A_\tau = A_\infty [1 - \exp(-\tau/T_1)] \quad \text{A6.3}$$

This system is particularly useful when studying nuclei with long relaxation times as it is not necessary to have a delay of $5T_1$ between each pulse sequence.

CHAPTER FIVE

X-ray Diffraction Studies of the Chromaffin Granule Membrane

5.1 Introduction

X-ray diffraction studies of biological membranes provide a potentially non-destructive, analytical approach which, in principle, can produce structural information about membranes in atomic detail. The subject has been comprehensively reviewed by Levine(1) and Worthington(2). While it is possible using this technique to obtain information about any membrane system which can be ordered by orientation, the detail and the value of the information derived from different systems is diverse. A survey of the various membrane systems that have been studied so far shows that the greatest detail has been obtained from the simplest membrane systems.

A. Membrane Systems Studied by X-ray Diffraction

Photoreceptor membranes are amongst the simplest membranes and have been much studied by X-ray diffraction. The outer segments of retinal rod cells contain stacks of flattened membrane vesicles(3). The protein:lipid ratio of these membranes is approximately 1:1, with rhodopsin the major protein present(4). X-ray diffraction studies were first applied by Finean et al(5) to osmium fixed outer rod segments. Blasie et al(6) on diffraction of oriented membrane discs, detected three orders of a lamellar array with a period of $300\text{\AA}$ and an equatorial diffraction. Improvement in the techniques of X-ray diffraction permitted higher orders of the lamellar array to be detected, and accurate intensities and phases to be obtained (7,8,9). In order to determine the localisation of the rhodopsin molecule in the disc membrane, Blasie oriented frog disc membranes in media of different electron density. It was concluded that the rhodopsin molecule is $42\text{\AA}$ in diameter and that $28\text{\AA}$ protruded, leaving $14\text{\AA}$ in the lipid

region. Upon bleaching, the rhodopsin sank into the lipid phase by approximately $7\text{\AA}$ ⁰(10). In a subsequent study, Blasie investigated the mechanism of interaction of rhodopsin molecules in the disc membrane. The average nearest neighbour separation for the protein molecules was computed from Fourier analysis of the diffraction data obtained under various conditions (11).

Similarly detailed studies have been carried out on the purple membrane from Halobacterium Halobium, first identified by Stoekenius and Rowan(12). The only protein present in the membrane, and representing 75% of the membrane mass, is an opsin-like protein, M.Wt. 26,000 daltons, which binds a pigment identical to retinol(13). The initial studies by Blaurock and Stoekenius(14) on both purple membrane dispersions and dried oriented specimens resulted, in the former case, in diffuse scatter and a series of sharp rings, extending from $54\text{\AA}$ ⁰ to $7\text{\AA}$ ⁰, accounted for by a hexagonal array with a centre to centre distance of $62\text{\AA}$ ⁰, and in the latter case, by a series of sharp equatorial reflections proving that the hexagonal array extends in the plane of the membrane. These X-ray findings were substantiated by electron microscopy and freeze-etch data. More recently, Henderson has extended these studies and has obtained an electron density map of the protein lattice in the plane of the membrane from X-ray diffraction and optical diffraction studies(15,16). This study and that of Blaurock(17) show the presence of α -helices in this membrane protein. Studies on gap junction membranes(18) and acetylcholine receptor membranes(19) also show clear evidence of protein lattices in agreement with earlier electron microscopy studies(20,21). These are all highly atypical membranes which may be likened to two-dimensional protein crystals in a lipid matrix. However, it is on such systems that high resolution studies on membrane proteins must be made.

Less detailed results have been obtained from studies on the sarcoplasmic reticulum membrane. The sarcoplasmic reticulum(SR) contains

several identified proteins but the ATPase, M.Wt. 100,000 daltons, comprises 50% of the membrane protein(22). Ultrastructural studies of SR using negative stain methods indicated the presence of $40\text{\AA}$ knobs on short stalks on the outside of the membrane(23). Early studies on air-dried SR showed four orders of a $265\text{\AA}$ repeat(24,25,26), and that it contained a phospholipid bilayer(27). Two more recent studies have been made on oriented wet samples by Worthington and Lui(28) and by Dupont et al(29). The former authors interpret their results in terms of a 62-65 $\text{\AA}$ thick membrane with bilayer structure, with greater electron density on the inside of the membrane but with a further electron density maximum, width 40 $\text{\AA}$, on the outside of the membrane. The equatorial spacing was ascribed to proteins on the membrane surface, by analogy to rhodopsin. The centre to centre spacing of the proteins was estimated to be 73 $\text{\AA}$. However, the map of Dupont et al was very different. Qualitatively they placed the bulk of the electron density towards the outside, not the inside of the membrane. The ultimate differences may probably be ascribed more to the different choice of phases, than to a different evaluation of the intensities. In a subsequent paper Lui and Worthington(30) constructed an absolute electron density map and conclude that their original electron density map and interpretations were correct. There has been no defense by Dupont et al. Thus even when a well defined X-ray diffraction pattern is present, there is considerable difficulty in establishing correct, unambiguous phasing.

The membranes discussed above are all of relatively simple constitution and all contain one major protein. It is clear that as the quantity of the major protein in the membrane is reduced or the number of proteins present increased, the X-ray diffraction detail and the ultimate structural information derived are in turn reduced.

Studies of more complex systems of biological interest have been made. The erythrocyte ghost membrane, which is 50% protein by weight, (31), has been studied by several workers(32,33,34,35). There is much

conflict between the most recent of these results (35) which concludes that the membrane profile is a symmetric one, and biochemical studies which indicate that there is considerably more protein on the cytoplasmic side of the membrane (36,37,38,39,40). Much of this discrepancy is thought to arise from the processes of ghost preparation and membrane orientation (41). Until detailed studies are carried out on the effect of these and other essential preparative procedures on the integrity and properties of the membranes, it will be increasingly more difficult to reconcile results obtained using different techniques.

Not all membranes have been studied purely to locate the proteins and hence obtain an electron density map of the membrane. The effect of variation in the lipid composition of membranes has been investigated through study of Mycoplasma and Escherichia coli membranes. Bacteria are an ideal system in which to study the lipid components since the fatty acid composition and cholesterol content of the membranes can be varied by supplementing the growth medium of the culture. Engelman looked at dispersions of isolated Mycoplasma membranes as a function of lipid composition and temperature (42). He observed the appearance of the 4.2 Å diffraction maximum attributable to the hexagonal array of fatty acid chains previously reported in long chain paraffins (43). The transition temperature was dependent on the degree of saturation of the phospholipid present in the medium. Subsequent studies by Wilkins et al (27) and by Engelman (44) further characterised the lipid bilayer and showed that no equatorial reflections attributable to proteins could be detected. Studies similar to those made by Engelman on Mycoplasma membranes were carried out on the E.coli K12 mutant membrane by Esfahani et al (45) and comparable transitions detected. These did not coincide in temperature range with those present in the Arrhenius plots of the sugar and amino acid transport functions. Analogous studies by Schechter et al (46) on the E.coli ML membranes, using fluorescence techniques in conjunction with X-ray

diffraction reached somewhat different results and conclusions, correlating that the 'melting' temperature of the paraffin chains of the phospholipids was the starting temperature for the active transport process. These studies show the problems of generalising observations made with the one system to properties of other related systems.

The major 'low protein' membrane studied is the myelin nerve sheath. Myelin, which contains about 20% protein, is a highly stable, relatively easily oriented membrane formed from a spirally wrapped cell membrane (47,48,49,50,51). Initial swelling experiments determined the phases of the first five orders of the lamellar diffraction (52,53), but more recent workers have been able to detect meridional spacings down to $10\text{\AA}$ (15th order) (54), and $14\text{\AA}$ (12th order) (55). Small, but significant, differences have been found in myelins from peripheral nerves and central nervous system (56,54). These more recent studies suggest that the membrane profile is slightly asymmetric, perhaps due to cholesterol distribution. Protein is thought to be located in the outer aspect of the membrane. In spite of the high resolution maps available for myelin, relatively little functional information has been derived. This is probably due to the fact that myelin is only about 20% protein, with no evidence of regularly spaced sub-membrane particles (57), and is in addition biochemically quiescent.

In summary, if ordering of the proteins within the plane of the membrane exists, as in the case of the purple membrane, then it is possible to obtain a highly detailed electron density map of the membrane. If, however, the distribution of the proteins is more random, as in the case of myelin, then the best that can be achieved is a one-dimensional electron density profile of the membrane cross-section which may or may not provide useful biological information.

B What can X-ray Diffraction reveal about the Chromaffin Granule Membrane?

The above moderately detailed account of the results and information obtained from studies of other membrane systems is essential to a consideration of the feasibility of applying the technique profitably to other partially characterised membranes. From this account, a description of the 'ideal membrane' for study by diffraction can be assimilated.

The ideal membrane should be

- a) easily and reproducibly prepared in large quantities to permit control experiments to be carried out simultaneously.
- b) capable of being oriented by centrifugation and slight dehydration.
- c) stable, i.e. it should not be disrupted by preparative processes including the alteration of humidity necessary for calculation of the reflections.
- d) simple, i.e. have a large protein to lipid ratio but contain a small number of different proteins with one protein in overwhelming majority.
- e) easily modified.

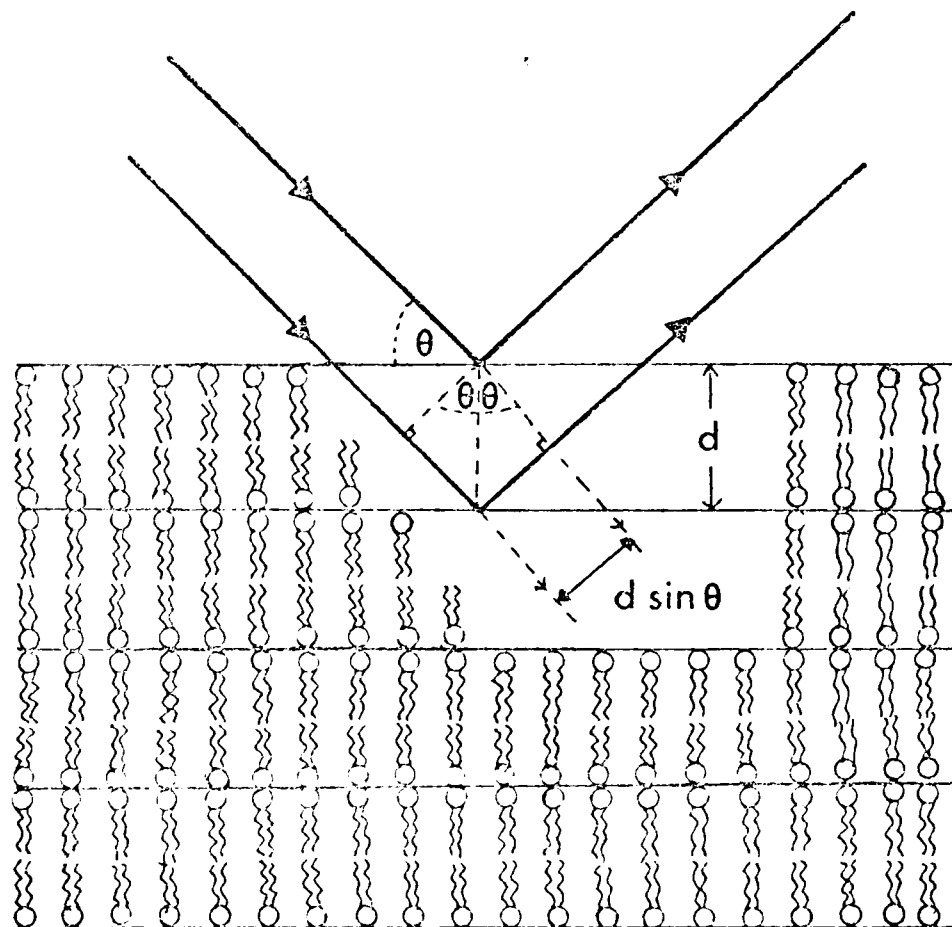
How does the chromaffin granule membrane fit the most superficial of these conditions? The chromaffin granule membrane is relatively easily prepared in sufficient quantity but unfortunately in order to obtain very pure granules a high density sucrose gradient is necessary. This has been shown to affect some of the membrane properties. The membrane is not particularly stable. Helle (58) has reported the breakdown of the membrane into lipid-protein containing multilamellar liposomal structures after freezing and thawing of isolated granule membranes. These structures were observed independently by Mrs C. van Horn and myself. The stability of the membrane under other conditions of stress has not been reported. The membrane has a low protein:lipid ratio of 0.45:1 (59) but contains several different membrane proteins including dopamine- β -hydroxylase, a Mg^{2+} activated ATPase, flavoproteins and cytochrome b 561. Dopamine- β -hydroxylase is reported to be the major protein present in the membrane (60) but the membrane proteins have not been very well characterized. Lipid modification

using phospholipase A has been reported (61,62). The enhanced activity of the ATPase observed was interpreted to be the result of lipid modification but can more readily be interpreted as an uncoupling of the ATPase due to the disruption of the storage granule membrane. Apart from limited freeze-fracture evidence presented by Pollard et al (63), there has been no reported evidence of the presence of a lattice of submembrane particles in the chromaffin granule membrane.

What diffraction detail might reasonably be expected from a study of the chromaffin granule membrane if it is assumed that the vesicle membrane can be oriented satisfactorily in the conventional manner?

The membrane should diffract in a manner similar to erythrocyte ghost membranes and nerve sheath myelin. The protein content of the membrane is close to that of myelin but the vesicles would be expected to pack in a comparable fashion to erythrocyte ghosts. It is unlikely that equatorial reflections will be present since there is no evidence for ordering of the protein molecules in the plane of the membrane. If sufficient resolution can be obtained and phases assigned to the meridional lamellar reflections, it should be possible to derive information about the average electron density profile of the membrane, however meaningful that may be.

The work on the chromaffin granule membrane was initiated in the Laboratory of Molecular Biophysics by H.B. Pollard whilst on a post-doctoral fellowship in the laboratory of Dr L.N. Johnson. His findings (63) will be compared to those observed in my study in the discussion of this chapter.

FIGURE 5.2.1.

The diffraction of an X-ray beam by a lamellar array of membranes of width, d . The Bragg reflections are formed by constructive interference of the reflected beams, whose path lengths in the membrane differ by an integral number of wavelengths. θ , the Bragg angle of diffraction, is the angle between the incident beam and the plane of the membranes.

5.2 Theory of X-ray Diffraction of Membranes and Lipid Bilayers

Oriented arrays of membranes, stacked with their planes parallel and regularly spaced, scatter radiation in easily recorded Bragg reflections which reveal the relative orientations of different regions of the structure. The membranes, therefore, do not pack in the form of a regular, three-dimensional structure like a crystal but as a one dimensional structure in a direction perpendicular to the membrane planes. The disorder in the packing of the membranes greatly restricts the information available from diffraction experiments. Any lateral structure within each membrane diffracts independently and, unless the structures are repetitive and equally spaced, the diffraction will generally be weak and difficult to observe.

The diffraction patterns observed from lamellar arrays of membranes can be interpreted by direct analogy with the optical diffraction grating, each plane of membranes in the array corresponding to a single slit in the grating. The Bragg reflections are the result of constructive interference between the X-rays scattered by the planes, and correspond to the principle diffraction grating maxima. The planes of the array act as a set of parallel mirrors and reflect the X-rays (Fig. 5.2.1.), thereby introducing phase differences because of their spacial separation. If the difference in phase, introduced by the parallel planes, is an integral number of wavelengths then the scattered radiation will interfere constructively, giving rise to a Bragg reflection. The order of a reflection is defined by the integral number of wavelengths required for constructive interference. This condition, Bragg's Law, is stated

$$2d.\sin\theta = h\lambda \quad 5.1$$

where h is an integer,

θ is the angle between the planes of the array and the incident beam,

d is the perpendicular spacing between the planes,

λ is the wavelength of the radiation.

For a given membrane thickness, a discrete set of Bragg orders

will be observed, provided the orientation of the array relative to the incident beam, as determined by the angle, satisfies the Bragg condition for every order. The condition also defines a reciprocal relationship between the angle of diffraction, θ , and the membrane width, d . Bragg reflections are, therefore, measured in the reciprocal space of the diffraction pattern. Diffraction patterns from a lamellar array of membranes are usually observed in the low angle region, where d is greater than $20\text{\AA}$. The spacings of the diffraction orders may be obtained from measurements of the distance (diam) between the equivalent reflections on either side of the direct beam. If f is the specimen to film distance, then

$$\tan\theta = \frac{\text{diam}}{2f} \quad 5.2$$

Hence d , the lamellar spacing, may be calculated from

$$d = \frac{h\lambda}{2\sin\theta} \quad 5.3$$

where h is the order of the reflection,

λ is the wavelength of radiation used (for $\text{CuK}\alpha$, $\lambda = 1.5418\text{\AA}$).

If the diffraction pattern only extends to 9° , then $\tan\theta = \sin\theta$, to within 1%. Hence d is given approximately by

$$d = \frac{h\lambda \cdot 2f}{\text{diam}} \quad 5.4$$

The electron density profile of the membrane may be calculated from these diffraction data by means of classical Fourier Transform theory. The electron density in one-dimension ($p(x)$) is given by

$$p(x) = \frac{1}{\ell} \sum_{h=-\infty}^{\infty} F(h) \exp^{-2\pi i h x} \quad 5.5$$

where ℓ is the unit cell length,

h is the order of diffraction,

$F(h)$ are the structure factors.

The structure factors are related to the observed intensities (suitably corrected for the camera geometry) by $|F(h)|^2 = I(h)$.

However, $F(h)$ is a complex quantity and information can only be obtained for its magnitude, not for its phase, from experimental observations. The determination of the phases of the diffracted X-rays is the major problem in any crystallographic project. If the unit cell contains a centre of symmetry (ie. for every scattering object at position x , there is an equivalent object at $-x$), then the phase is either 0 or π . That is

$$F(h) = \pm |F(h)| \quad 5.6$$

The phase problem is then simplified to the determination of the sign of $F(h)$.

Membranes have an important property in that they swell or shrink as a result of changes in the immersion medium. On occasions, the separation distance between individual membranes can be varied without altering the internal structure of the membrane. The X-ray data from two such membrane assemblies provides measurements on the same Fourier transform, but at different values of X , where $X = \frac{h}{d}$. One set of X-ray data gives values of $|F(X)|$ at $X = \frac{h}{d}$; whereas the other set gives values of $|F(X)|$ at $X = \frac{h}{g}$, where g is the new repeat distance. Both sets of data provide values of the same Fourier transform, but at different sample points. The data is graphed as the square root of the intensity against the distance in reciprocal space ie. $|F(h)|$ vs. $\text{\AA}^{-1}$. The value of the phase changes whenever the transform goes to zero. The frequency of phase changes is limited by the Minimum Wavelength Principle. This states that the maxima and the minima in a diffraction pattern must succeed each other with a certain minimal distance of separation or minimum wavelength. This depends on the overall dimensions of the diffraction grating in the corresponding direction (64). Thus, the phase cannot change more frequently than the minimum wavelength.

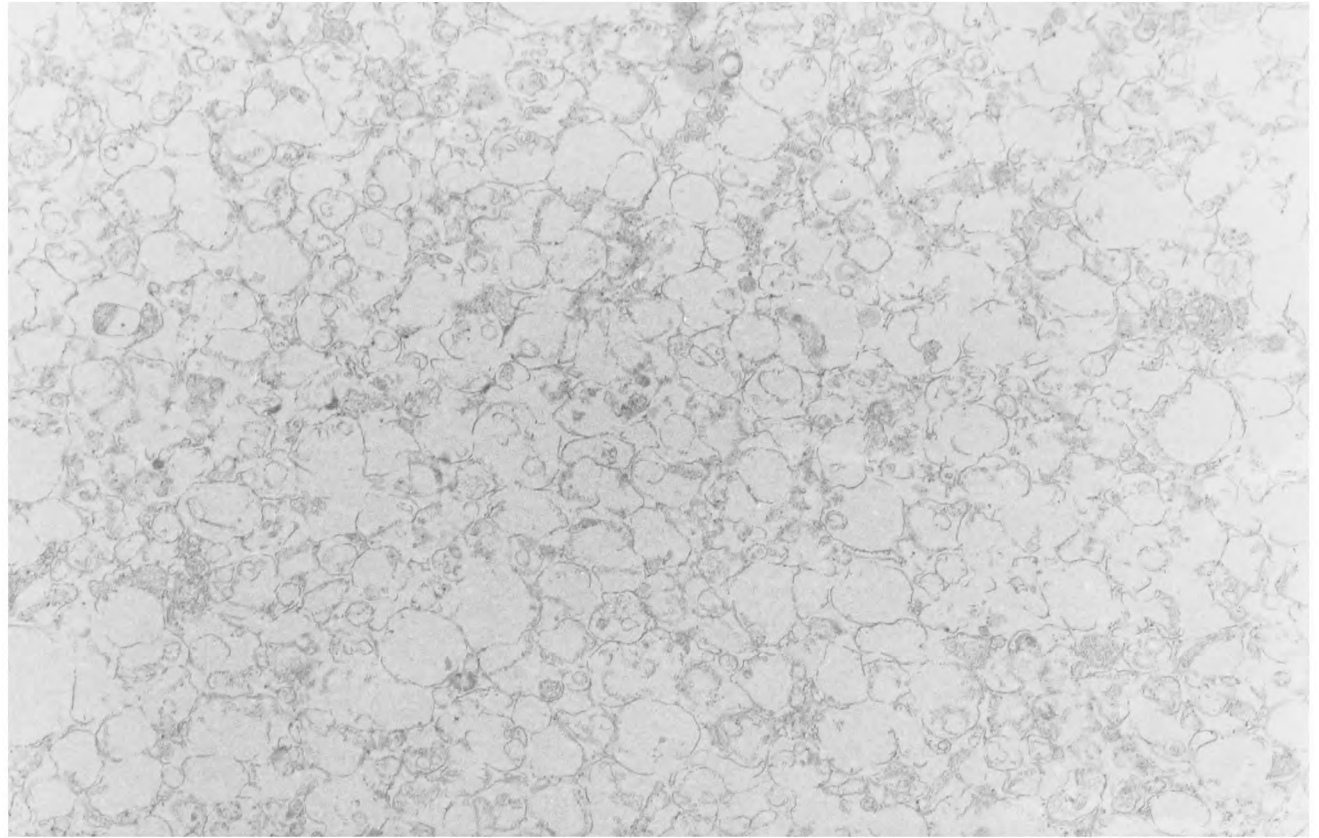
This method, termed the Sampling Theorem (2), is most successful if several sets of data, at different repeat distances are available and if higher orders of diffraction can be recorded, which have sufficient intensity to influence the Fourier transform values at higher values of $\text{\AA}^{-1}$.

5.3 Materials and Methods

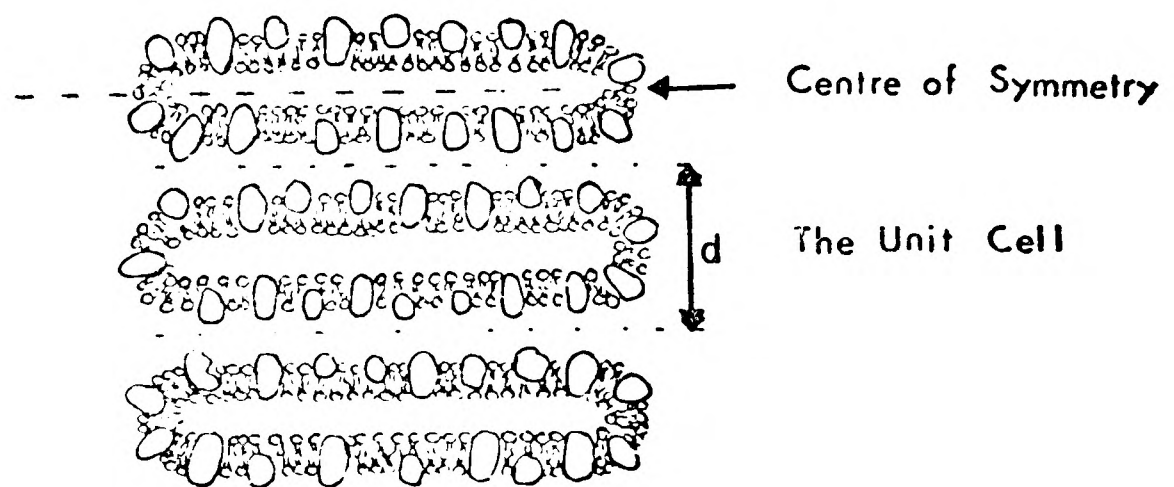
Chromaffin granule membranes (CGMs) and their extracted lipids were prepared as described previously in this thesis. Membranes were spun down onto small pieces of plastic which were fitted into the bottom of a double sector cell of a MSE Analytical Ultracentrifuge Mk II, (50,000rpm for 12 to 24 hours, at 10°C). One sample was immediately placed in fixative for examination by electron microscopy, whilst the other was mounted in the humidity cell for study by X-ray diffraction. Humidity was controlled in two different ways. In the first, nitrogen gas was bubbled through a solution of the required humidity and then passed into the humidity cell which also contained a small amount of the humidity solution. The other method of controlling humidity was by means of a sealed humidity cell, with a reservoir at the bottom of the cell to contain the humidity solution.

The lipids, dissolved in chloroform:methanol (2:1), were spotted onto pieces of perspex. After the solvent had evaporated, the perspex was placed in the latter humidity cell and the humidity of the lipids equilibrated over the appropriate concentration of sulphuric acid, (2.16N H₂SO₄ is equivalent to 95% humidity (69)).

The X-rays, from a rotating anode source, were focussed using a mirror- monochromator camera, based on that of Huxley and Brown (70). The mirror was gold plated and the monochromator was quartz. The specimen to film distance used in virtually all experiments was 11.5cms.

FIGURE 5.4.1.

An electron micrograph of ultracentrifuged chromaffin granule membranes. The membranes were fixed in glutaraldehyde and stained with osmium tetroxide. The total magnification is $\times 20,000$.

FIGURE 5.4.2.

Oriented Membrane Vesicles.

Diagrammatic representation of pelleted, oriented chromaffin granule membrane vesicles.

5.4 Results

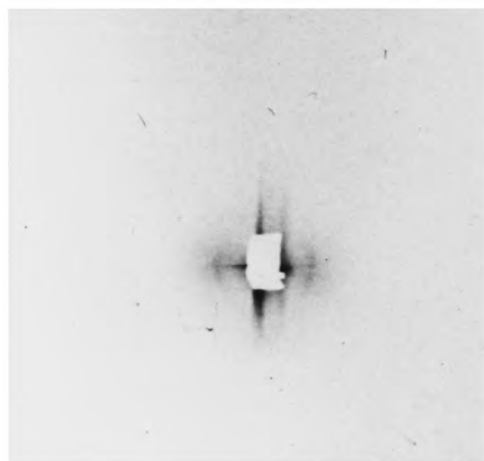
A Studies on the Chromaffin Granule Membrane

Preliminary studies of the chromaffin granule membrane (CGM), using X-ray diffraction showed clearly that it was very difficult to obtain reproducible results from one preparation to another. The work by H.B. Pollard (63) was carried out under conditions described as 'wet' and 'dry'; no precise control of the humidity of the membrane specimen was used. It is not essential for the humidity to be known precisely, but it must be carefully controlled, under known conditions, so that it remains constant throughout an experiment.

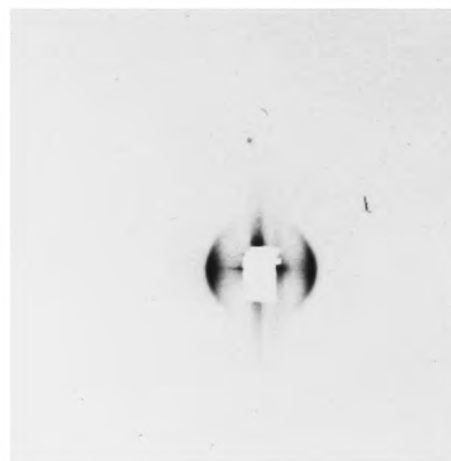
In my experiments it was observed that when the membrane pellet had dehydrated slightly, a lamellar diffraction pattern, with an approximate spacing of $55\text{\AA}$ (which extended to the sixth order, approx. $9\text{\AA}$), appeared in addition to two or three orders of a spherically symmetrical diffraction, with a spacing equivalent to $45\text{\AA}$. No intermediate orders of diffraction, which might indicate that the spacing was larger than $54\text{\AA}$, were observed in the lamellar diffractions. $54\text{\AA}$ is a very small unit cell for a diffracting membrane. Electron micrographs of ultracentrifuged CGMs show that the membranes are pelleted as vesicles, (Fig. 5.4.1.). Unless the membrane is highly symmetrical and very closely packed, the expected repeat is that from two membrane bilayers, (Fig. 5.4.2.). The bilayer repeats for myelin and erythrocyte ghost membranes are about $170\text{\AA}$ and $120\text{\AA}$, respectively (54,32). In view of the smaller lamellar spacing and the presence of non-lamellar diffraction, a series of strictly controlled experiments were carried out correlating X-ray diffraction and electron microscopy findings.

Freshly prepared CGMs were spun down, as described, in a double sector cell; one pellet was immediately fixed and the other pellet placed in the former humidity cell with N_2 equilibrated to 81% humidity bubbled through. It was only necessary to detect the first order of the

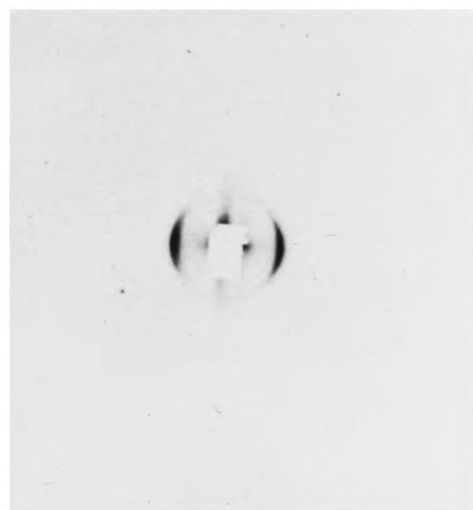
FIGURE 5.4.3.



(a)



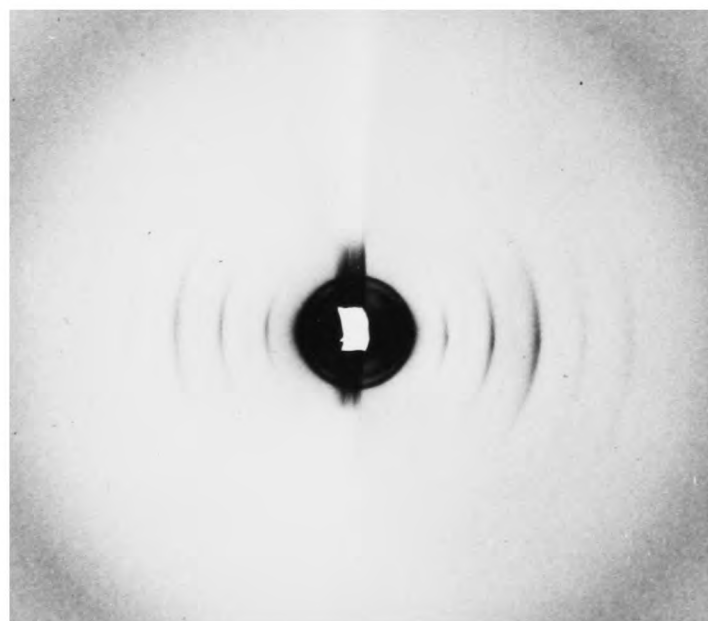
(b)



(c)



(d)



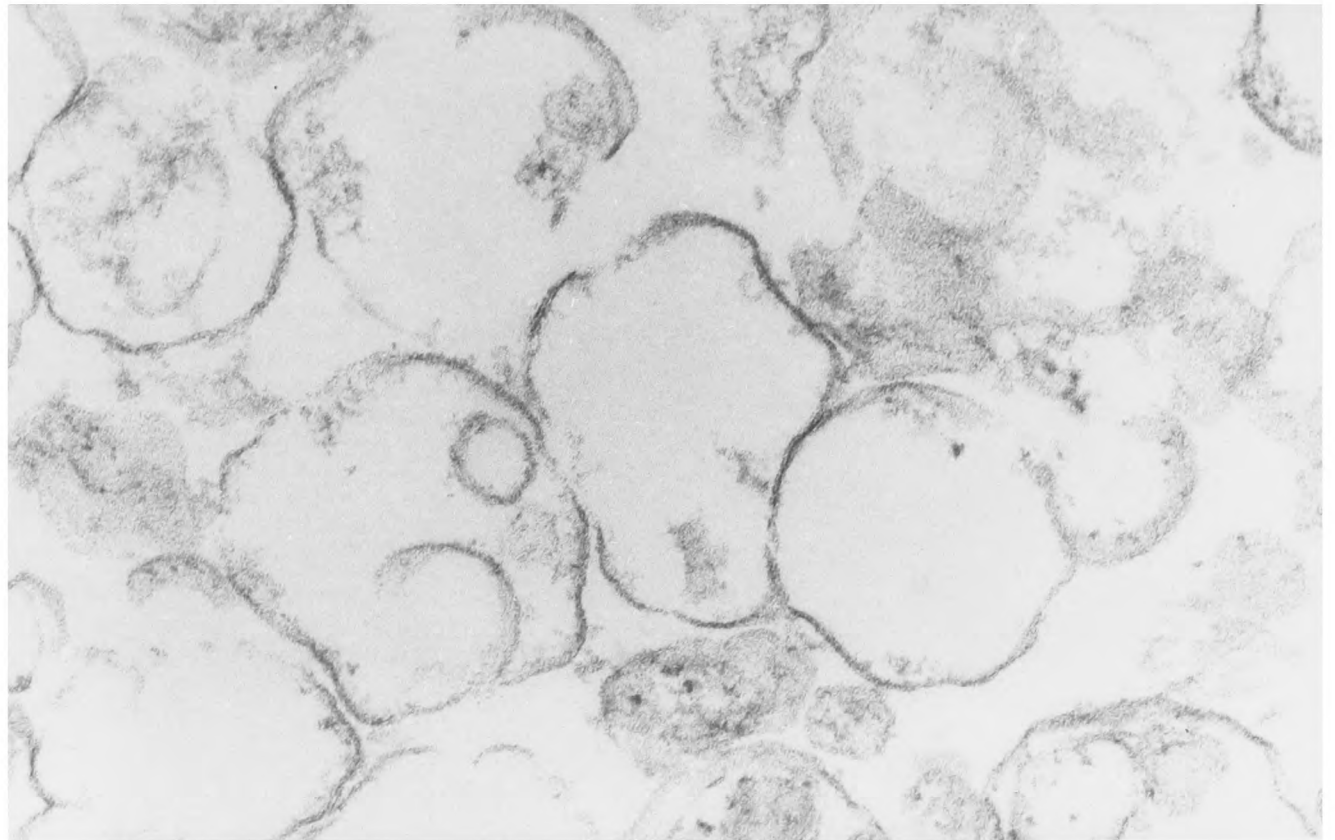
(e)

The X-ray diffraction from chromaffin granule membranes, pelleted and slowly dehydrated to 81% relative humidity.

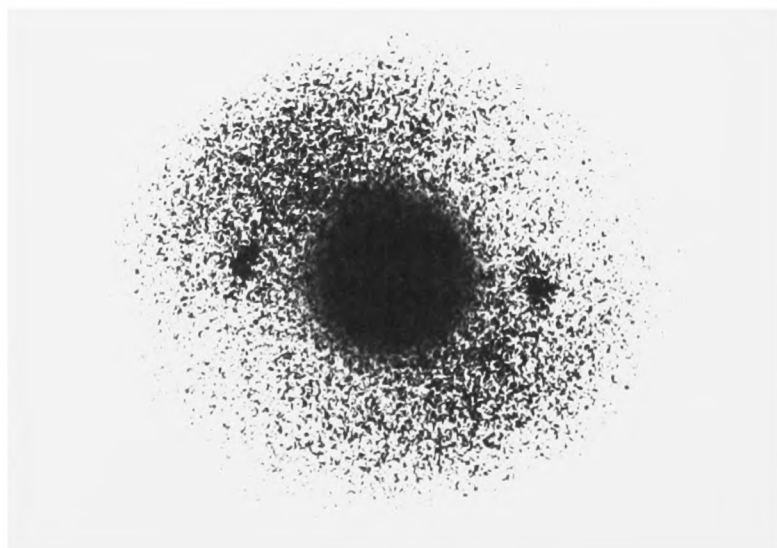
(a)-(d) The first order of the diffraction pattern from onset of diffraction at different times, and hence at different humidities, decreasing from (a) to (d). All are one hour exposures.

(e) The diffraction pattern obtained when the spacing had stabilised. The first order is equivalent to $55\text{\AA}$. The exposure was for 12 hours.

Other conditions as described in the text.

FIGURE 5.4.4.

A comparison of pelleted chromaffin granule membranes, before and after dehydration. The membrane vesicles shown in (a) were fixed in glutaraldehyde immediately after ultracentrifugation, whilst the multilamellar structure, shown in (b), is present in the membrane pellet after dehydration to 81% relative humidity. Pellet (b) is that which produced the X-ray diffraction pattern shown in Figure 5.4.3. and was fixed immediately after diffraction (c) was made. The membranes were stained using uranium and lead salts. Magnification: (a) 116,000, (b) 174,000.

FIGURE 5.4.5.

The highly ordered optical diffraction pattern obtained from the electron micrograph shown in Figure 5.4.4.(b). The spacing is equivalent to $46\text{\AA}$.

FIGURE 5.4.4.

(b)

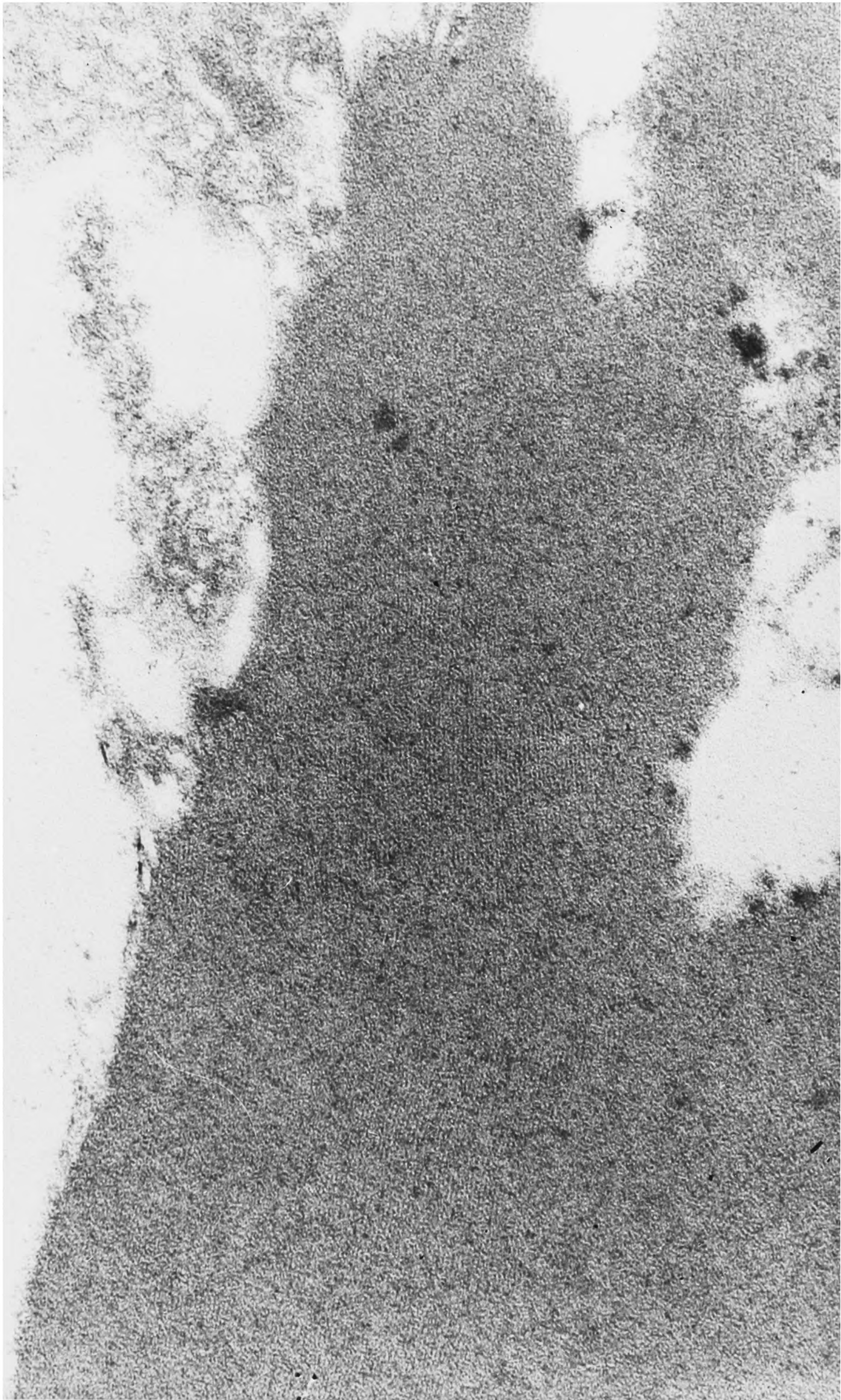
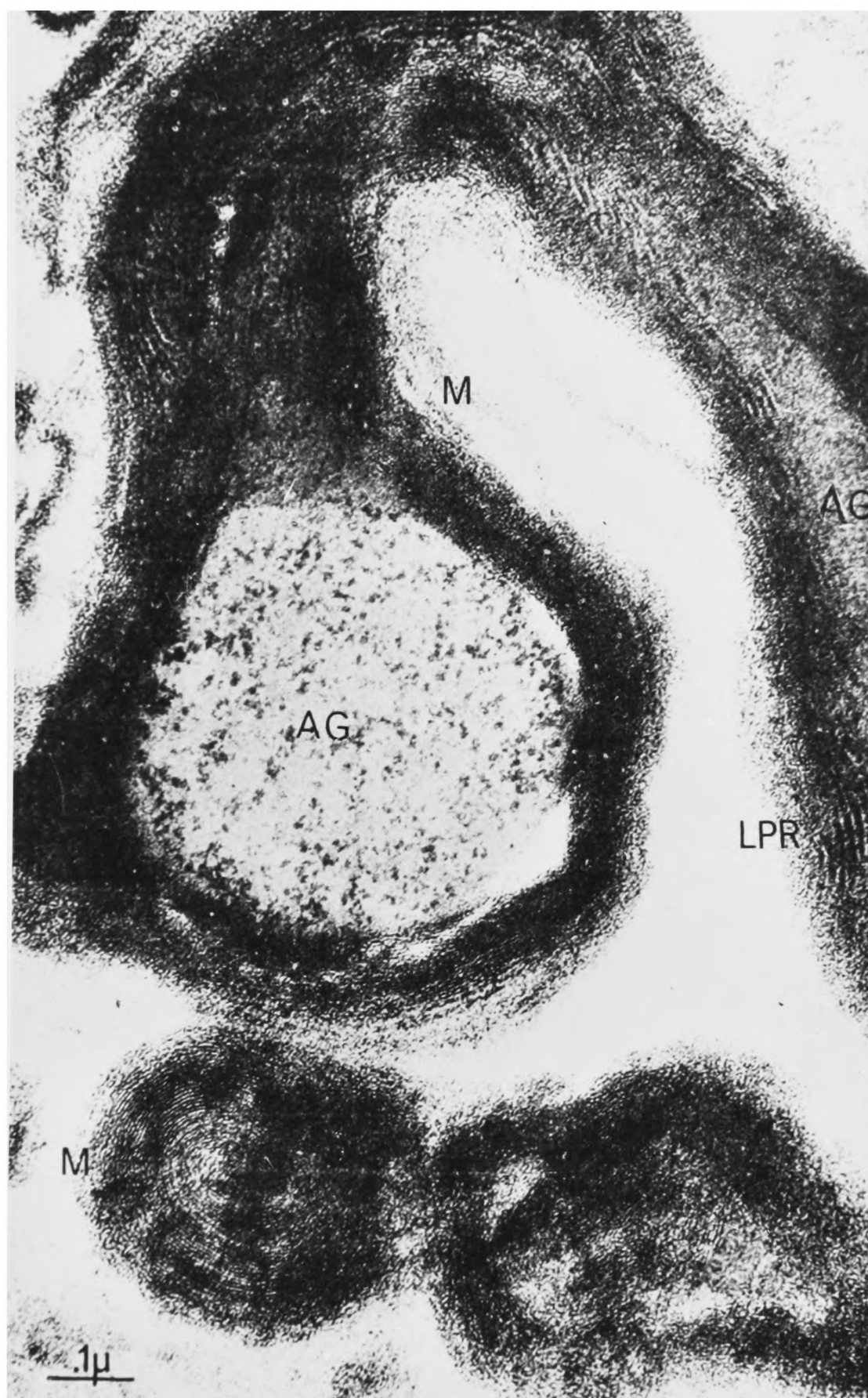


FIGURE 5.4.6.



The Multilamellar structures observed by Helle (58) after freeze-thawing chromaffin granule membrane. Magnification :100,000.

diffraction to index the series. Therefore, short exposure times were used. A series of exposures were made and the appearance and stabilisation of a first order diffraction monitored. When the first order was reduced to a spacing of $55\text{\AA}$ or thereabouts, a longer exposure was made, (Fig. 5.4.3.). The membrane pellet, which still appeared to be moist and fairly pink, was then fixed and embedded. A comparison of the control and partially dehydrated membrane pellets is shown in Fig. 5.4.4. In the control electron micrograph (Fig. 5.4.4.a.), membrane vesicles with the typical tramline appearance, approx. $60\text{\AA}$ across, are the only membrane form present in the areas examined. However, in Fig. 5.4.4.b., these vesicles are entirely absent. Instead, very large liposomal structures and a large amount of granular material are observed. When the optical diffraction pattern of the electron micrograph is examined, a sharp diffraction pattern with a spacing of $46\text{\AA}$ is detected, (Fig. 5.4.5.). This correlates moderately with the X-ray diffraction spacing. There is no other observable diffracting species in any of the electron micrographs made.

It is necessary to speculate on the nature of these multilamellar structures. Are they purely lipid or do they contain both lipid and protein? In view of the findings of Helle (58) and ourselves that the stress imposed on the membrane by freezing it to liquid nitrogen temperature and subsequently thawing it disrupts its integrity, resulting in the formation of multilamellar structures, it is possible that dehydration affects the membrane in a similar manner. The multilamellar structures observed by freezing and thawing are shown in Fig. 5.4.6. to permit comparison. Helle has isolated and analysed these multilamellar structures, and finds that they contain both lipid and protein, 12% of the total membrane protein and 53% of the total membrane lipid phosphate (58). The multilamellar structures produced by freezing and thawing have a repeat unit of $45\text{--}50\text{\AA}$. The similarity between the two multilamellar structures is striking.

Another fact indicates that these arrays are not purely lipid.

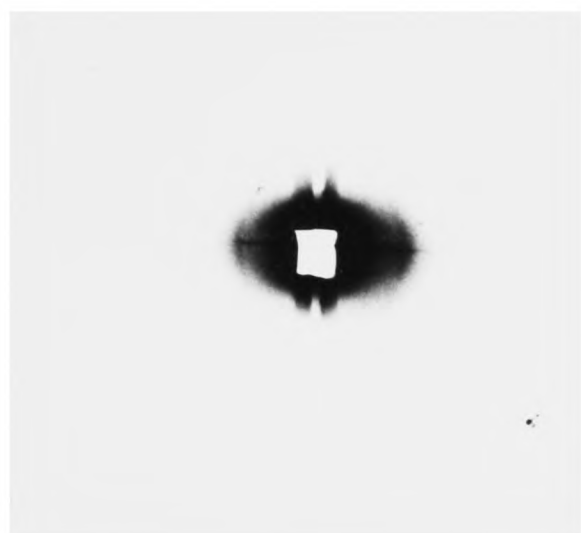
When the X-ray diffraction pattern derived from them (Fig 5.4.3.) is compared to those obtained from the diffraction of the oriented extracted lipids (Fig. 5.4.8.), there is a similarity in the lamellar spacing, but the relative intensities of the diffractions are very different. This indicates that the electron density profiles of the unit cells are very different. The most probable explanation would be the presence of additional molecules, presumably proteins. It is most likely, therefore, that the lamellar structures causing the diffraction are of lipid-protein composition.

In order to observe a diffraction pattern from intact membranes, they must be sufficiently oriented in a regular manner. This does not normally occur at 100% humidity, however, even after a lengthy centrifugation. Most recent evidence(65) suggests that the most successful orientation of biological membranes is achieved by fairly short centrifugation followed by slow controlled dehydration. Thus if X-ray diffraction patterns are to be observed from CGMs, the membranes must orient at a humidity higher than that at which they disintegrate.

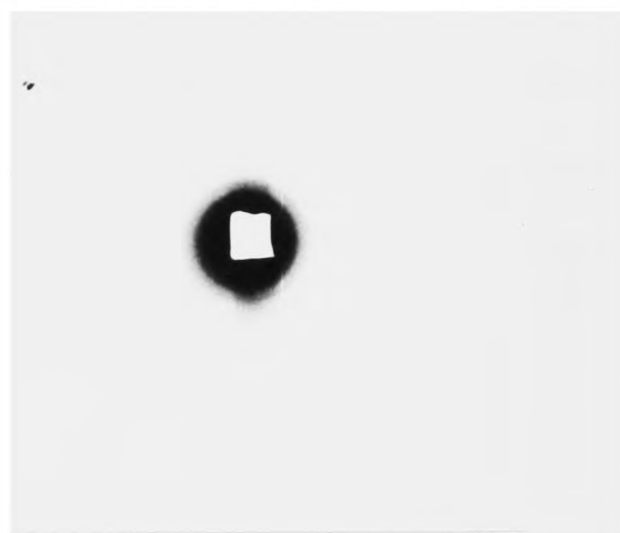
Several studies were made of pelleted CGMs to see if this was possible. Initially, the membranes were fixed in gluteraldehyde before being placed in the X-ray diffraction camera in an attempt to stabilise the membrane structure. In these specimens, a lamellar spacing ($>70\text{\AA}$) was observed. However, at constant humidity, it could not be accurately reproduced. These studies were interrupted by a major breakdown in the rotating anode X-ray generator, therefore work could not be completed before the specimens were too old.

In my most recent studies, the diffraction from pelleted CGMs, equilibrated to 97.5% humidity (ie. $1.08\text{N H}_2\text{SO}_4$), was examined. Three lamellar diffractions are present, the first, second and fourth order of an $82.5\text{\AA}$ spacing. This diffraction pattern is shown in Fig. 5.4.7. along with the corresponding 'face-on' diffraction pattern, which shows little diffraction detail apart from faint rings at approximately $59\text{\AA}$ and $71\text{\AA}$.

FIGURE 5.4.7.



(a)



(b)

The X-ray diffraction obtained when a chromaffin granule membrane pellet was equilibrated to 97.5% relative humidity , and examined with (a) its membrane planes parallel to the beam, and (b) with its membrane planes perpendicular to the beam. The photographs are 1.5 $\times$ actual size. The exposures were for 16 hours.

(cf. Fig. 5.4.12.). It is possible that these are diffraction patterns from intact CGMs. Unfortunately, it was not possible to examine this membrane pellet by electron microscopy in order to confirm this.

It is my conclusion that if meaningful studies are to be made on the chromaffin granule membrane, and indeed on many other membrane systems, very careful correlation of X-ray diffraction patterns and electron micrographs of the diffracting material must be made. So far this has not been reported by workers on any system, even the erythrocyte ghost membrane where the most recent studies are controversial since the state of the diffracting material was unknown (35).

B Studies of the Chromaffin Granule Membrane Lipids

The chromaffin granule membrane lipids were oriented in the humidity cell. The humidity range studied was 81% to 100%. The sequence of humidities used was 95%, 88%, 81%, 95%, 97.5%, 98.75% and 100%. The diffraction patterns obtained at the humidities, 95%, 81%, 95%, 97.5 and 100%, are shown in Fig. 5.4.8. The diffraction pattern altered radically when the humidity was changed. The diffraction is not solely of lamellar origin and, at every humidity, several series of diffractions exist. There is always a dominant lamellar diffraction which at some humidities extends to the eighth or ninth order. The changes in the spacing and in the intensities of the different orders of the dominant lamellar diffraction series were investigated. The lamellar spacings and the number of orders visible at the different humidities are shown in Table 5.4.1.

The films, several exposures for each humidity, were examined using the Optronics system available, and the electron densities printed out as an imaged line-printer display, using a computer programme written by Dr K.R.Leonard. The reflections required were located, and by means of a programme written by Mr S.W.White, which measures the total optical density of arcing reflections, the integrated intensities of the different orders

FIGURE 5.4.8.

The X-ray diffraction patterns from oriented, extracted chromaffin granule membrane lipids at a range of relative humidities. The lipids were prepared by the method of Bertsch et al which is described in Chapter Four. All exposures were were between 12 and 18 hours after the lipids had been allowed to equilibrate. The unevenness of the exposure is a result of the perspex support on which the lipid was held in the beam. All photographs are $\times 1.5$ the actual size. Other conditions as in the text.

Key: (a) 95% , (b) 81%, (c) 95%, (repeat), (d) 97.5%, (e) 100%.

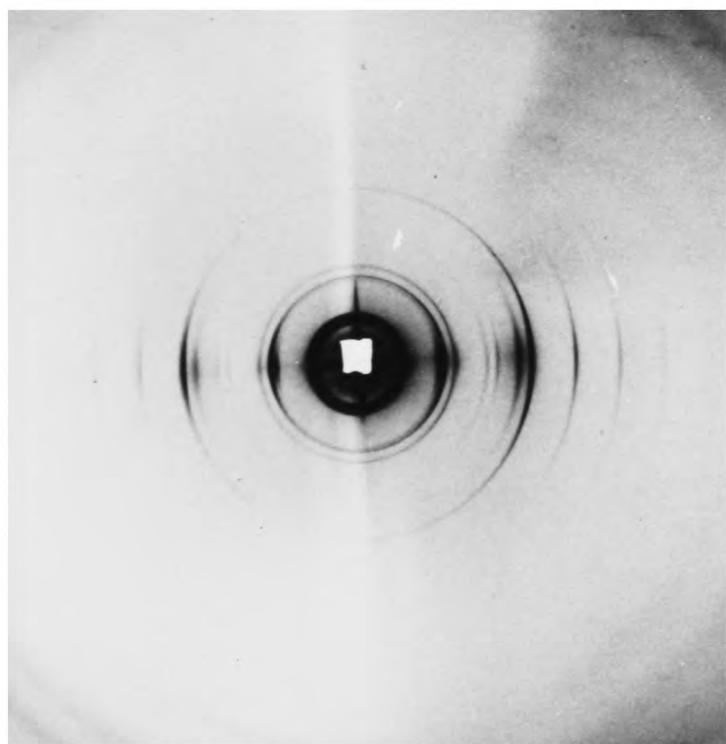
(a)



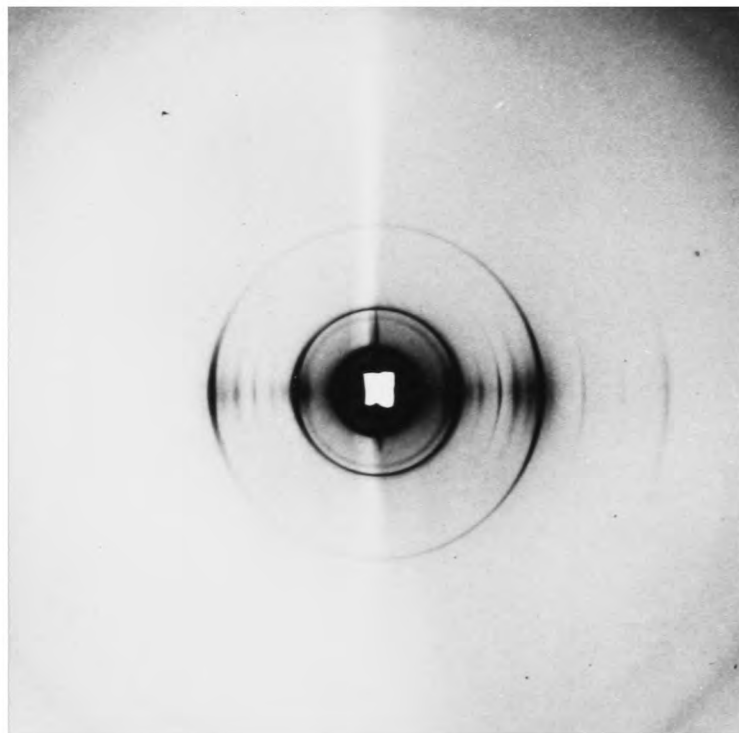
(b)



(c)



(d)



(e)

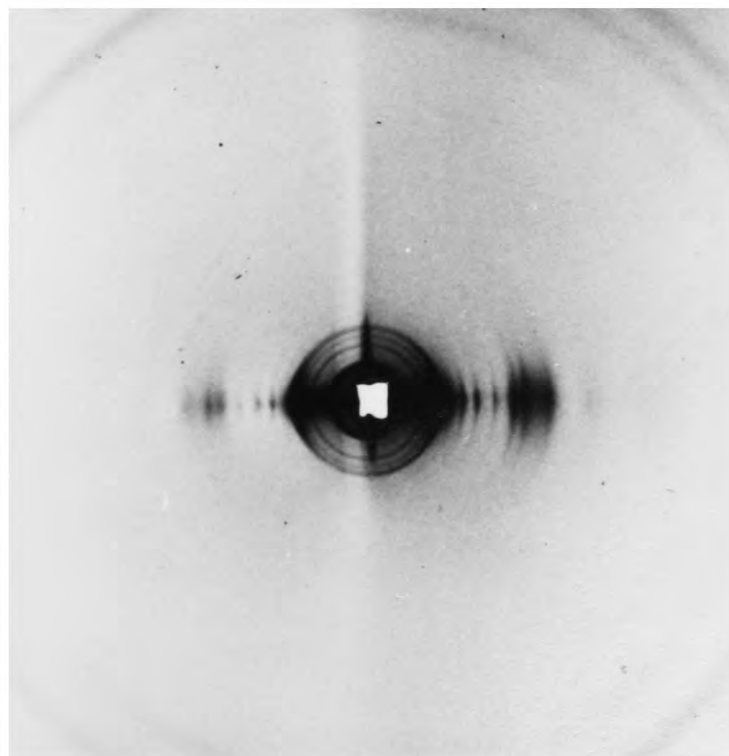


TABLE 5.4.1.

Order of the diffraction maximum	81%	95%	97.5%	100%
1	29,756	27,288	21,228	12,521
2	2,550	5,821	12,854	18,720
3	2,550	196	454	1,577
4	3,401	5,821	5,142	4,092
5	850	728	212	2,465
6	893	146	110	625
7	—	120	400	400
8	70	60	—	—
Lamellar spacing($\text{\AA}$)	53.7	57.2	60.1	68.2

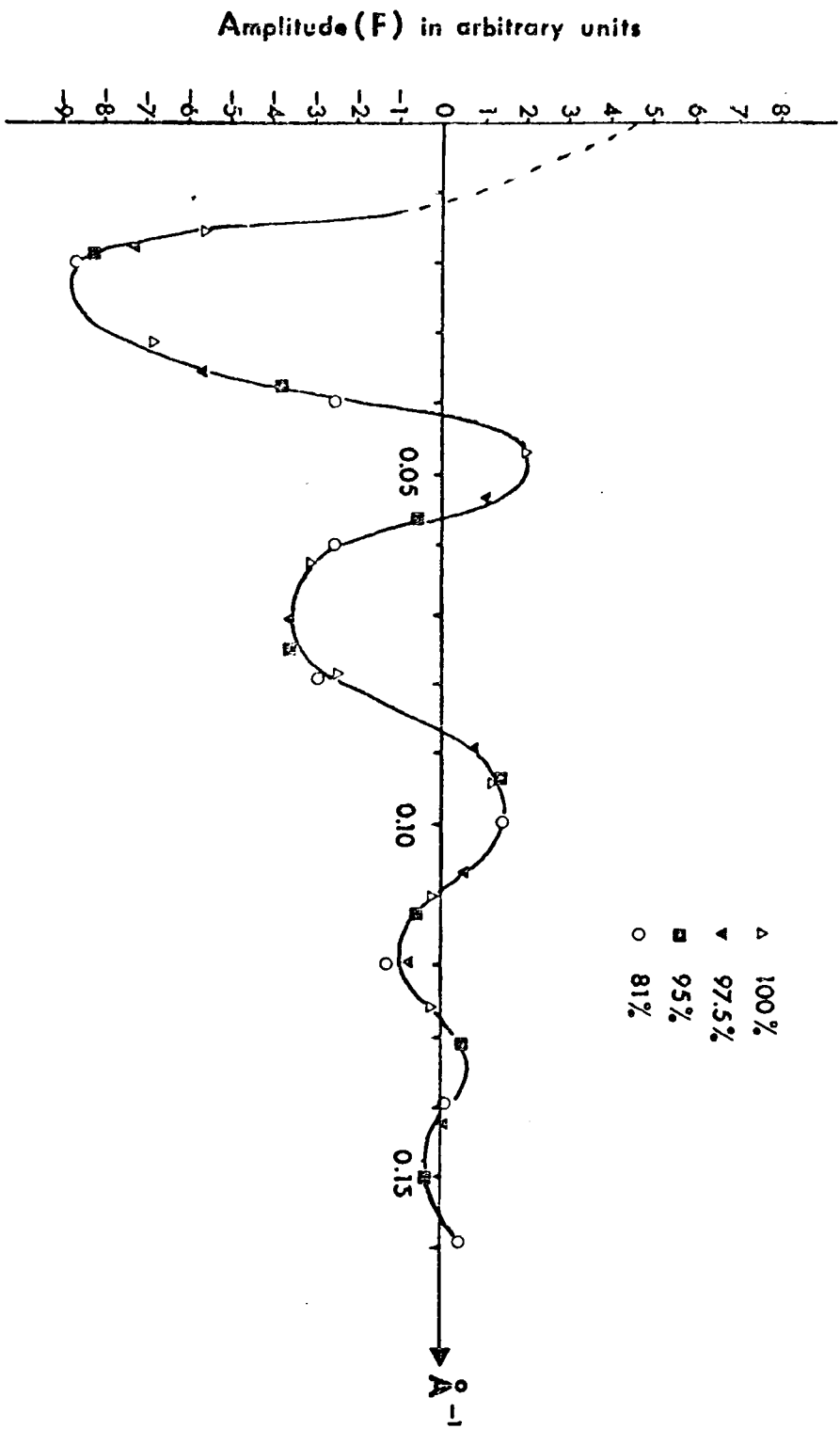
A comparison of the Intensities of the different orders of the diffraction maxima of the Lamellar series produced by the extracted Chromaffin Granule Membrane Lipids, equilibrated at several humidities. The intensities are scaled as described in the text. The total intensity of the first six reflections is assumed in each case to be 40,000 arbitrary units. The size of the Lamellar spacings is also shown.

of the lamellar diffraction at each humidity were obtained. Even using the least heavily exposed film of each set, the first-order reflections were still overexposed. The intensities of the first-orders were, therefore, measured relative to the second and subsequent orders using a Joyce-Loeble microdensitometer. The Lorentz factor applied to the intensities was $(\sin 2\theta)^{-1}$, as for equatorial reflections of a rotation photograph. For small angles, $\sin^2 \theta \approx 2 \sin \theta \propto h$, where h is the order of the reflection. This is the correction applied by Casper and Kirschner (54). No polarisation factor, ie. $p = \frac{1 + \cos^2 2\theta}{2}$, was applied since in the low angle region measured, it is approximately unity.

In order to scale together the films recorded at different humidities, it was assumed that the total intensity of each diffraction series was equivalent. This method is valid since the lipid bilayer itself is unaltered by changes in humidity, as evidenced by the equivalence of the relative intensities of the diffraction series at 95% humidity. This means that the membrane will scatter the same amount of the X-ray beam at each humidity. By using this approximate normalisation procedure and proportioning accordingly, it was possible to compare the intensities of the different orders of the diffraction patterns at each humidity. Errors are likely to be involved, but alternative methods also introduce errors of the same magnitude. The errors introduced by this procedure will be reduced in subsequent calculations, since it is the square root of the intensities that is used in these calculations. This is the method used to compare X-ray crystallography data obtained from different crystals of the same protein.

The related intensities are shown in Table 5.4.1. and the square root of the intensities of the different reflections plotted against θ^{-1} in Fig. 5.4.9.. It was assumed that the lipid bilayer from which these diffraction patterns originated was centrosymmetric. Hence the phases are either 0 or π . The minimum wavelength principle was also used to help make

FIGURE 5.4.9.



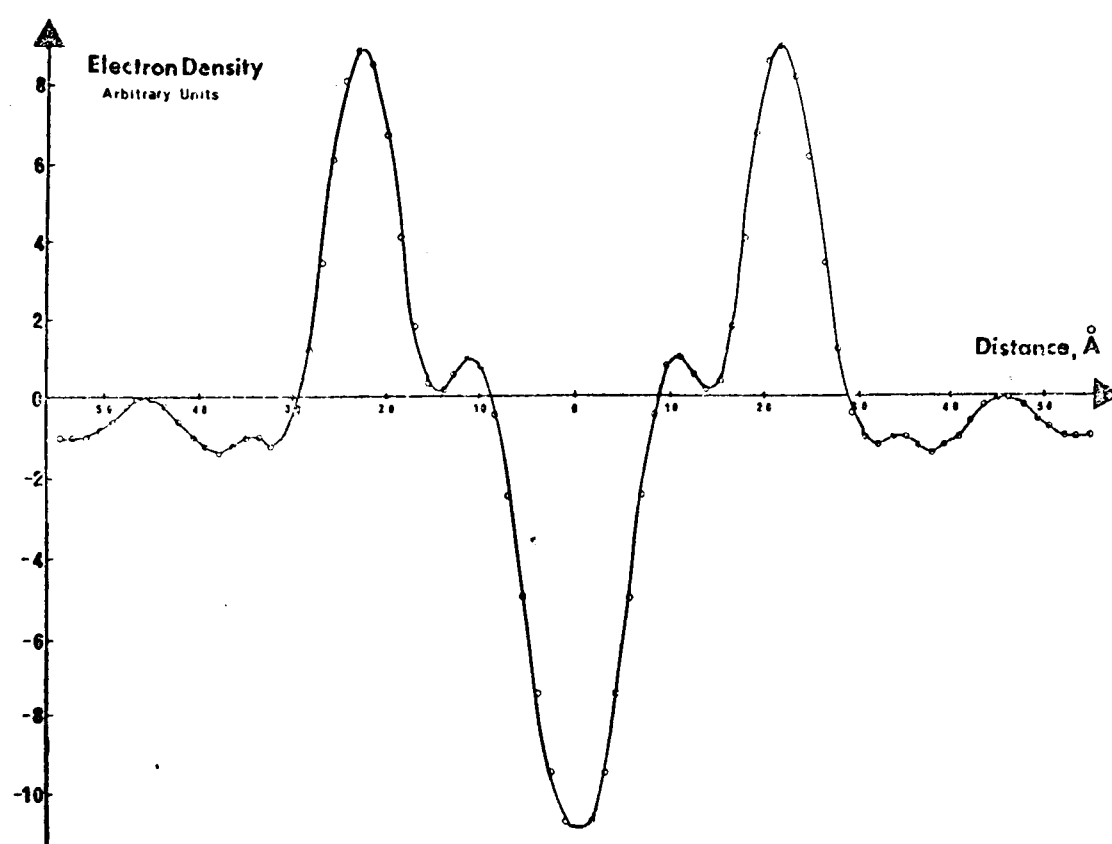
The Fourier transform constructed from the relative intensities and the spacings of the reflections in the series of diffraction patterns from the extracted chromaffin granule membrane lipids at different humidities. Methodology as described in the text.

the phase assignments (64). Since the actual size of the repeating unit is not known, the minimum wavelength was assumed to be the minimum lamellar spacing observed, $54\text{\AA}$.

A one-dimensional 'Fast Fourier Transform' computer programme written by Dr K.R. Leonard, was used to generate an electron density profile of the lipid bilayer. This profile is shown in Fig. 5.4.10.. It is a typical bilayer profile. It can be compared to that of egg lecithin:cholesterol (1:1) bilayers shown in Fig. 5.4.11.. The peak to peak distance across the hydrocarbon region of the CGM lipid bilayer is $45\text{\AA}$. This is the same bilayer thickness as that found for the lamellar phase of total erythrocyte lipids, which have a comparable cholesterol content (65). The bilayer thickness of CGM lipids and total erythrocyte lipids is greater than that of mitochondrial lipids, $40\text{\AA}$, (66). This difference has been attributed to the fact that although the fatty acid composition of mitochondrial and erythrocyte membranes are similar, the mitochondrial lipids do not contain cholesterol (66). These thicknesses may be compared to those of pure lipid systems. Whilst the egg lecithin:cholesterol (1:1) bilayer thickness of $42\text{\AA}$ does not alter appreciably as the humidity is altered, that of egg lecithin bilayers is humidity dependent, and is reduced to $36.8\text{\AA}$ at 21% water content (67). It is $35\text{\AA}$ for sonicated egg lecithin (27).

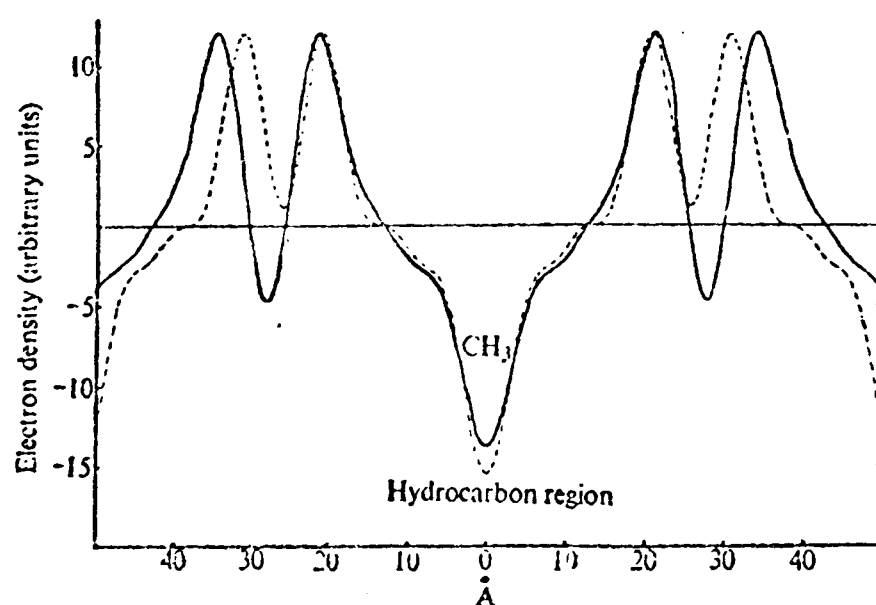
The trough in the centre of the profile corresponds to low electron density in the middle of the hydrocarbon region due to a marked localisation of the terminal methyl groups. This trough is present in the Fourier synthesis of both egg lecithin:cholesterol bilayers and egg lecithin bilayers, but is much more prominent in the presence of cholesterol (67). The shoulders bordering the troughs have not been attributed to any structural feature of the bilayer, although, on first inspection, one might assign them to the presence of cholesterol. However the shoulders, which are prominent in electron density profiles of egg lecithin bilayers at 57% relative humidity, are thought to be artifacts introduced by the

FIGURE 5.4.10.



The electron density profile of the extracted chromaffin granule membrane lipid bilayers in a direction perpendicular to their planes. This profile was calculated from the data in Figure 5.4.8. by fourier transformation.

FIGURE 5.4.11.



The electron density profile of egg lecithin/cholesterol bilayers in a direction perpendicular to their planes. This profile is taken from a paper by Levine and Wilkins (67).

use of only a finite number of diffraction orders in the reconstruction of the profile (1).

It is important to appreciate that this method of obtaining an electron density profile is only applicable to systems whose structure does not alter as the humidity alters. The evidence that this is true for the CGM lipid bilayer is twofold. Firstly, the order of humidity variations used tested the reversibility of the system, which is an important criterion. If the relative intensities and the spacings were not reversible, then it could be assumed that structural changes had occurred. Secondly, a continuous transform was obtained, within experimental error. This is a situation of the result justifying the method but is, in fact, an excellent test of the validity of the assumption. If changes in the structure had occurred, then a continuous transform would not have been observed.

No attempt was made to record accurately the inter-hydrocarbon chain spacing between molecules in the bilayer, since the camera length used was rather too long. Also, the diffraction from the mylar windows and the plastic support occurred in the 4 to 6 Å region, where the interchain spacing occurs at around 4.6 Å. The origin of the other diffraction series were not investigated. However it is worthwhile to consider the diffractions present. The spacings of the observable diffractions, other than those of the major lamellar series, are shown in Table 5.4.2.. The origin of the spherically symmetrical diffractions, with a first order spacing of 50 Å at 95% humidity, is not known. The first order is a very strong diffraction and can be observed in the diffraction pattern from egg lecithin: cholesterol multilayers (67). At 81% humidity, two very sharp meridional reflections appear. These are the first and second order of a 33.7 Å spacing and are due to cholesterol crystallising out of the bilayers. They disappear when the humidity is raised back to 95%. Several of the reflections can be attributed to more minor lamellar structures. There

TABLE 5.4.2.

81%	95%	97.5%	100%
	a	b	
scm 56.3	sch 50.1	sch 51.6	sch 58.2
sch 44.8	scm 25.0	scm 25.6	scm 28.9
scw 26.1		scw 14.5	
scm 22.5			

vsoh 33.7			
vsoh 16.7			

dom 39.4	dom 68.2	dom 61.2	soh 70.9
dom 28.8	dow 33.8	dow 30.9	dom 35.1
dom 23.5	dow 22.5	dow 15.1	dow 23.6
doh 19.3	dom 17.0		dom 17.9
doh 14.5			dow 14.0
dow 11.6			dom 15.5
dow 9.7			

The spacings and descriptions of the reflections from the extracted Chromaffin Granule Membrane Lipids, equilibrated at several humidities, which do not form part of the major lamellar diffraction series.

Key to the descriptions of the diffractions:

o - oriented

w - weak

c - circular

m - medium

s - sharp

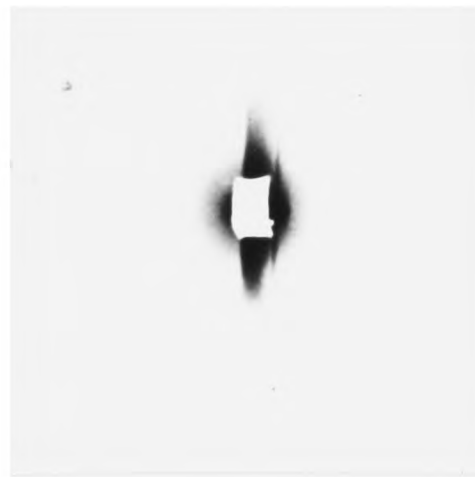
h - heavy

d - diffuse

v - very

FIGURE 5.4.12.

The X-ray diffraction patterns from chromaffin granule membrane lipids irradiated with the beam perpendicular to the plane of the membranes. These reflections were obtained from the same specimens as Figure 5.4.8. (d) & (e). Conditions as Figure 5.4.8. Photographs are 1.5× actual size.

FIGURE 5.4.13.

The X-ray diffraction from pure chromaffin granules mounted in a thin-walled quartz capillary. Exposure was for 8 hours at room temperature of around 14°C. Other conditions as Figure 5.4.8. Photograph is 1.5× actual size.

are at least two possible explanations for the presence of these reflections. Firstly, the lipid sample was fairly thick, therefore, it is possible that equilibration did not occur completely uniformly throughout the sample. Secondly, there may have been slight partitioning of the different lipids into different bilayer systems. Unfortunately, it was not possible to examine these specimens by electron microscopy.

In addition to studying the extracted CGM lipids oriented parallel to the beam, the diffraction produced when the lipids were turned through 90° and diffracted perpendicular to the beam was also examined. The diffraction pattern produced by the lipids at 97.5% and 100% humidity are shown in Fig. 5.4.12.. The spacing of these diffractions are compared to the parallel, 'edge-on' spacings in Table 5.4.3.. Whilst at 97.5% humidity, the perpendicular reflections are closely similar in spacing to the comparable parallel ones, there is a slight discrepancy between those of the lipids at 100% humidity. This is most probably due to the difficulty in reproducing the humidity precisely.

It is evident that when lipids are not completely oriented, as in sheets of parallel bilayers, but are present, for instance, in liposomal forms, then spherically symmetrical diffractions in the form of rings are observed when the lipids are irradiated 'face-on'.

C. Studies on the Intact Chromaffin Granule

Intact chromaffin granules, prepared through 1.6M sucrose, were mounted in a thin walled quartz capillary and examined in the X-ray beam. The diffraction obtained is shown in Fig. 5.4.13.. There is no crystalline diffraction present. This would be present if the contents of the chromaffin granule were present in an ordered, possibly crystalline array.

TABLE 5.4.3.

97.5%		100%	
parallel	perpendicular	parallel	perpendicular
70.9		78.8	
60.1	58.1	68.2	74.2
		45.4	
35.1	34.1	39.0	43.4
30.7	30.0	34.4	36.7
		28.5	
23.6		26.6	
20.3		22.8	

A comparison of the spacings, in Å, of the diffraction maxima detected when the Chromaffin Granule Membrane Lipids were irradiated with the lipids oriented parallel and perpendicular to the X-ray beam at 97.5% and 100% humidity.

5.5 Discussion

The studies of Pollard et al (63) described both X-ray diffraction and freeze-etch results. The X-ray diffraction results were obtained in Oxford. Their 'hydrated' membrane array, irradiated with the beam parallel to the plane of the membranes, produced, by their interpretation, fifteen orders of a $143\text{\AA}$ lamellar repeat. When examined with the membrane planes 'face-on' ie. perpendicular to the beam, six spherically symmetrical diffractions were detected. Five of these were interpreted as representing orders of an hexagonal lattice, with a centre to centre spacing of $131\text{\AA}$. They reported that this organisation in the plane of the membrane was abolished by treatment with trypsin, but not by treatment with phospholipase C or by lipid extraction. However, no details of experimental procedure are described in the paper which was published as a conference report not as a refereed paper.

It is difficult to assess the validity of the interpretation of these results in absence of the X-ray films on which they are based. However, some observations indicate conclusions reached by Pollard et al may not be as unambiguous as claimed. Pollard reported that the plastic used as a membrane support, did not produce diffraction in the low angle region, ie. between $10\text{\AA}$ and $200\text{\AA}$. On subsequently testing this plastic, I found that there were at least four rings, of approximately $48\text{\AA}$, $19\text{\AA}$, $14\text{\AA}$ and $11\text{\AA}$ spacing, of plastic origin. The absolute spacings of these were dependent on the humidity. They are present whether the plastic is parallel or perpendicular to the beam. Pollard et al report that all odd reflections of the $143\text{\AA}$ repeat were 'very weak' except for the seventh order at $20.6\text{\AA}$. Since this spacing is close to one of the spacings produced by the plastic support, the evidence for a $143\text{\AA}$ lattice appears to be tenuous. A more obvious choice would be a $71.5\text{\AA}$ lattice, which is close to that observed for the CGM lipid bilayers at 100% humidity. In the 'face-on' view of the membrane, the diffraction pattern of Pollard et al shows the presence of a $48\text{\AA}$ ring, which they do not assign as a lattice diffraction. This could be

due to the plastic, but it was a relatively weak diffraction compared to the other plastic rings. It is more probable that it is of lipid origin. When observed in diffraction patterns of membranes, this ring is usually taken as an indication that there is some disruption of the membrane. Further, the interpretation of the spherical diffractions observed when the membrane plane is perpendicular to the beam as originating from a regularly spaced, protein lattice in the plane of the membranes is also open to doubt. There are two main reasons for this. Firstly, it is unlikely that the proteins should form a regular lattice when they represent only 30% of the membrane mass, since the distance between the macromolecules will be sufficiently large to permit unimpeded lateral diffusion of the protein molecules to occur. Secondly and more importantly, if a regular protein lattice does exist, it ought to produce equatorial reflections when the membrane planes are parallel to the beam. There was no indication of any low angle equatorial diffraction in any of my studies and Pollard et al did not report their presence in their paper. This is strong evidence that such a protein lattice does not exist.

The 'face on' reflections reported by Pollard et al could, in view of my observation of 'face on' reflections from the partially oriented lipid preparation (and their almost complete absence from my membrane preparation diffracted at 97.5% humidity) be attributed to liposomal structures and also to reflections from the plastic support. If the lipids were not completely removed by chloroform, ethanol or any other lipid extracting method, then once the solvent had evaporated, the liposomes could reform, therefore little difference in the diffraction pattern would be detected. It is surprising that they reported an effect on the diffraction pattern after treatment with trypsin, since Dr P.A.M. Eagles, of this laboratory, has since found that trypsin has virtually no proteolytic effect on the chromaffin granule membrane (71).

Freeze-fracture replicas of chromaffin granules were also examined by Pollard et al. In certain localised areas of a very few granules there appeared to be an indication of an hexagonal array of submembrane particles. However, the conclusion that these particles are highly ordered appears to be an over interpretation. In my opinion, the ordering of the particles shown in their micrographs is not regular, especially in view of the freeze-fracture studies carried out in this laboratory (68), to correlate with my X-ray diffraction results. Many preparations of chromaffin granules and chromaffin granule membranes were examined using the freeze-fracture technique. Figure 5.5.1 shows several of the freeze-fracture micrographs made in this laboratory. Despite careful examination of many photographs, at no time was a regular array of submembrane particles observed. Their distribution in our micrographs was found to be completely random.

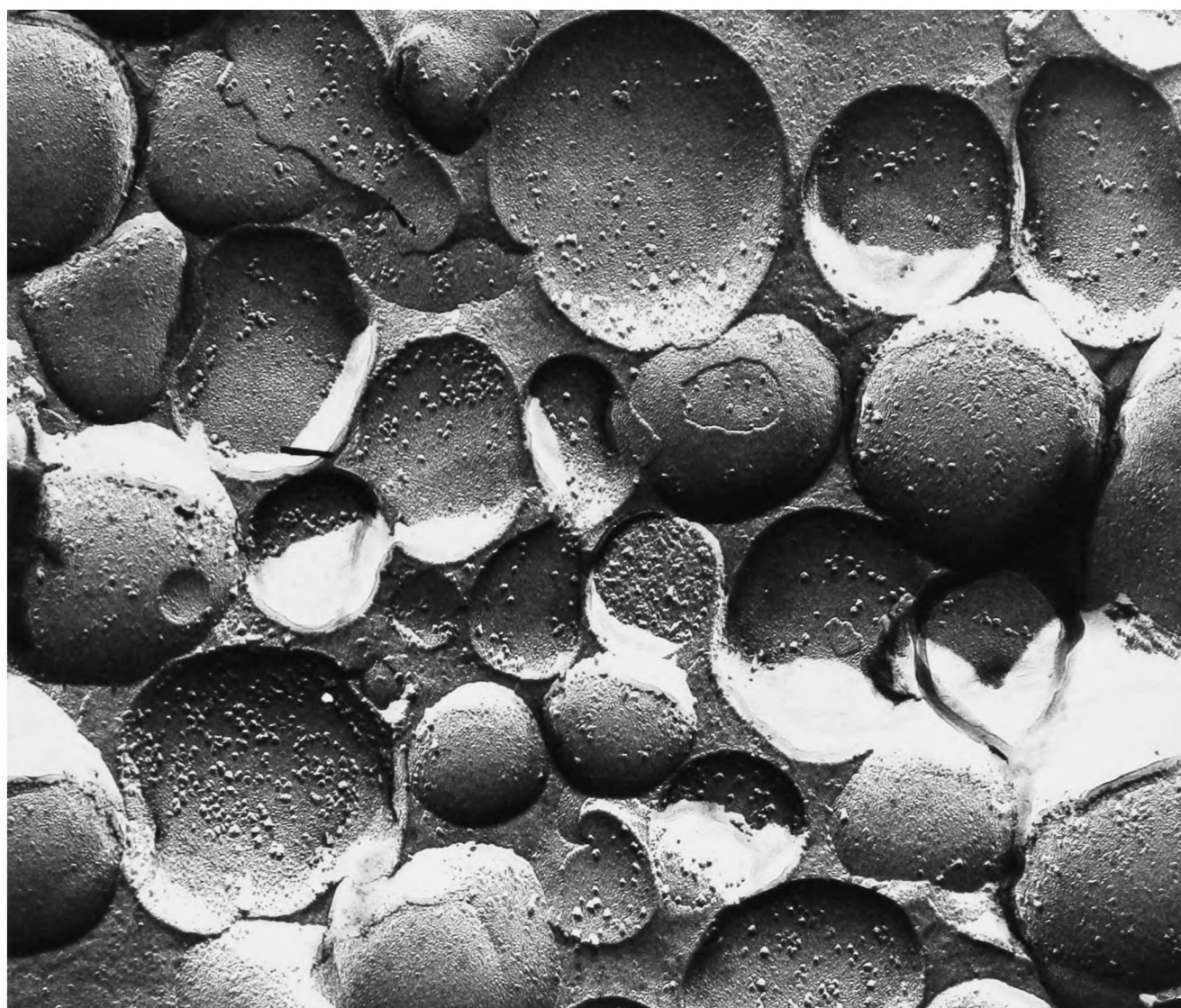
It is always possible to observe any symmetrical arrangement of particles if a sufficiently large area is scanned, but in order to postulate the presence of a lattice which will diffract, the arrangement has to be very regular indeed. It also has to be present in the majority of membrane vesicles. Our results indicate that this degree of order is not present.

In conclusion, my X-ray diffraction and electron microscopy results indicate that the chromaffin membrane does not maintain its integrity when subjected to even minor dehydration. It readily forms multilamellar liposomal structures which diffract strongly to at least $9\text{\AA}$. Diffuse lamellar diffraction from membranes equilibrated at humidities of 95% or greater have been observed. Only three orders of an approximately $83\text{\AA}$ spacing were detected. Whether these are from the intact membrane or from lipids has yet to be ascertained by electron microscopy.

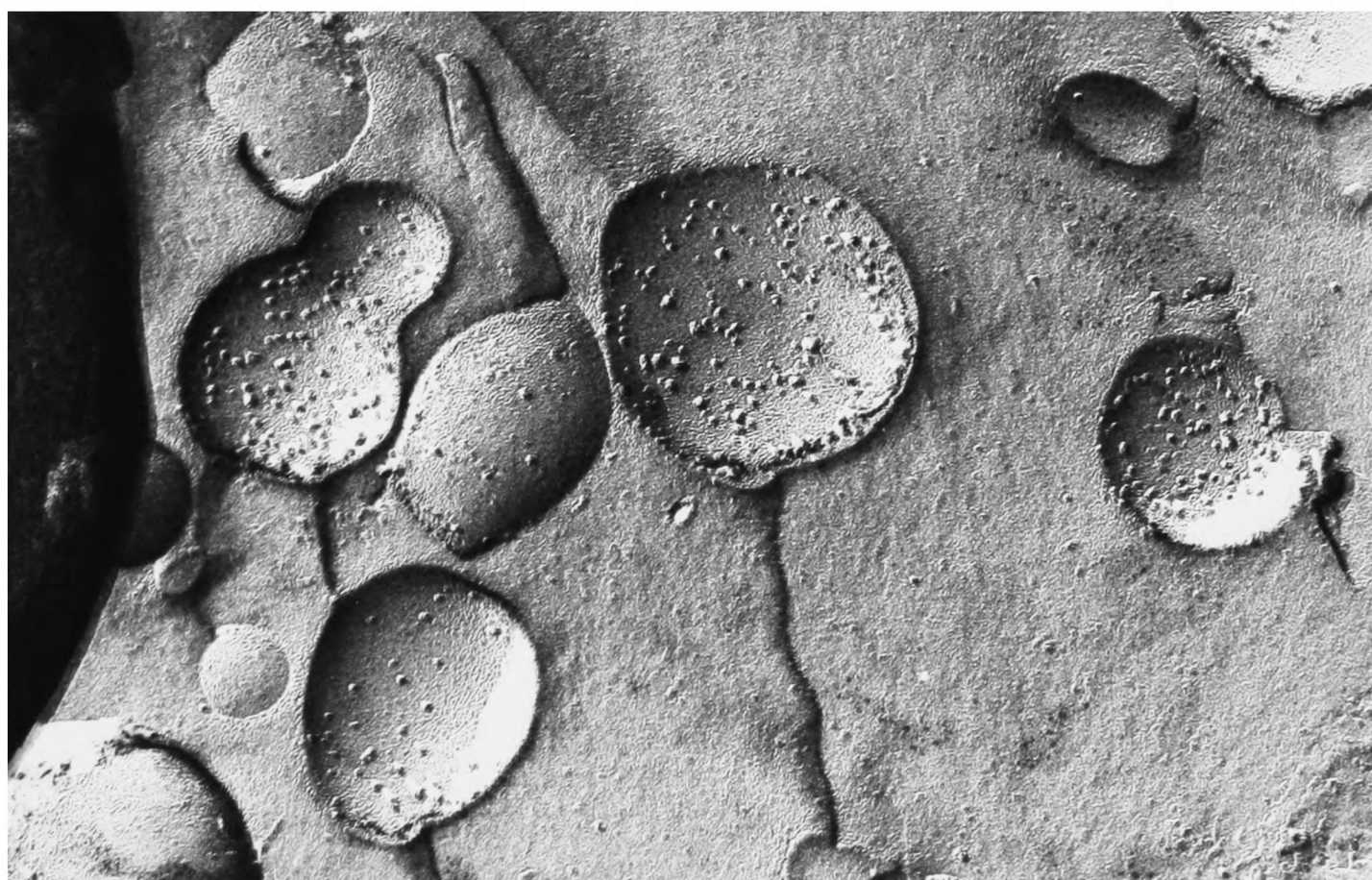
There is no evidence from either my X-ray diffraction or the freeze-fracture studies that a regular protein lattice exists in the membrane and would attribute the 'face on' diffraction observed by them

FIGURE 5.5.1.

(a)



(b)



Freeze fracture replica of chromaffin granules made by the method of Bullivant (72). The shadowing direction is shown by the arrow. The micrographs are printed with the shadows white. Magnification: (a) 70,000. (b) 110,000.

to being of lipid origin.

Finally, it is my conclusion that the Chromaffin Granule Membrane is a very difficult membrane to work with using conventional techniques. It does not orient well by centrifugation and there appears to be a critical humidity at which it disintegrates. If, therefore, meaningful diffraction results are to be obtained from this membrane, the humidity must be carefully controlled and sufficient orientation by dehydration must be achieved at humidities greater than that at which disintegration commences. If, as my results suggest, the proteins are not arranged in a highly ordered form, then even if several orders of a lamellar diffraction are obtained the amount of information derived from a study of the membrane by X-ray diffraction may be very limited. It may be difficult to produce an unambiguous electron density profile for the membrane, unless diffraction to high resolution is obtained at several lamellar spacings. However, as this electron density profile will be an averaged one, it may not be particularly meaningful or helpful in furthering our knowledge of the structure and function of this membrane, which, after all, is the ultimate purpose of such a study.

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CHAPTER SIX

Conclusions and Conjectures

The conclusion, from the studies described in Chapter Two, that the ATPase of the chromaffin granule membrane is capable of generating a potential gradient across the membrane, has important implications in the mechanisms of catecholamine uptake and storage. This potential appears to take the form of a proton or cation gradient. The movement of protons across the mitochondrial and chloroplast membranes, with the resultant development of a proton gradient, is a fundamental tenet of the Chemiosmotic Hypothesis of energy coupling. Mitchell proposes that ATP synthesis in these systems is driven by a bulk proton gradient (1). The practicality of such a system has been questioned by several workers (2,3) who suggest that localised, not bulk effects, may be important since, due to the small volume of these organelles, bulk potentials are virtually meaningless since they involve so few protons. It is conceivable, therefore, that the protons generated by the ATPase, or by the electron transport system, do not leave the membrane but possibly alter the surface charge of it. Such mechanisms are merely ideas and no concrete proof has been offered, as yet, to support them.

The inhibition of the chromaffin granule ATPase, and also of catecholamine uptake, by DCCD lends support to the existence of a proton gradient, rather than a cation gradient, since it has been proposed that DCCD may function by blocking proton conducting channels across the mitochondrial membrane (1,4).

The biochemical and NMR studies, Chapter Two and Chapter Four respectively, indicate that the 'internal pH' of the bovine chromaffin granule is around pH 5.5. In view of the simulation studies described in Chapter Four, this is not surprising since such large concentrations of catecholamines and ATP are insoluble at pH values greater than 6. The

catecholamines are most stable, with less tendency to oxidise, at low pH (5) as indeed is ATP (6). It would, therefore, seem practical to store these molecules at a pH of less than 6.

It is not possible, as yet, to provide a detailed explanation to account for the mechanism whereby the proton gradient drives the catecholamine uptake since several aspects of the system are unknown. One of the major complications is that it is believed that the chromogranins and ATP, both negatively charged species at neutral pH, are synthesised and stored in the granules prior to the synthesis and uptake of the catecholamines (7,8). The chromogranin proteins are thought to be present in the granules at the time of their 'budding off' from the Golgi region of the cell but it is not known how the ATP molecules are accumulated in the granules. Membranes are generally impermeable to ATP since it is such a highly charged molecule: a diffusion based uptake is not, therefore, a practical explanation. The uptake must presumably be an active process, possibly involving a transport system to carry the ATP molecule across the membrane. It is conceivable, though no evidence has been forthcoming from these studies, that the chromaffin granule ATPase, analogous to the mitochondrial ATPase, F_1 , can act as an ATP synthetase under favourable conditions and accumulate ATP in the inside of the granule. More important to the problem of catecholamine uptake is the lack of knowledge about the nature of the ATP and chromogranin storage prior to the uptake of the catecholamine molecules. This means that the state of the system into which catecholamine molecules are transported remains virtually undefined, though possible conditions can be considered.

Presumably the negative charges of the ATP and chromogranin proteins are balanced by positive charges, which may possibly be due to the presence of protons or metal ions. On catecholamine uptake, the charged catecholamine molecules effectively exchange with these other charged species. If the positively charged moiety is a proton, then the pH inside

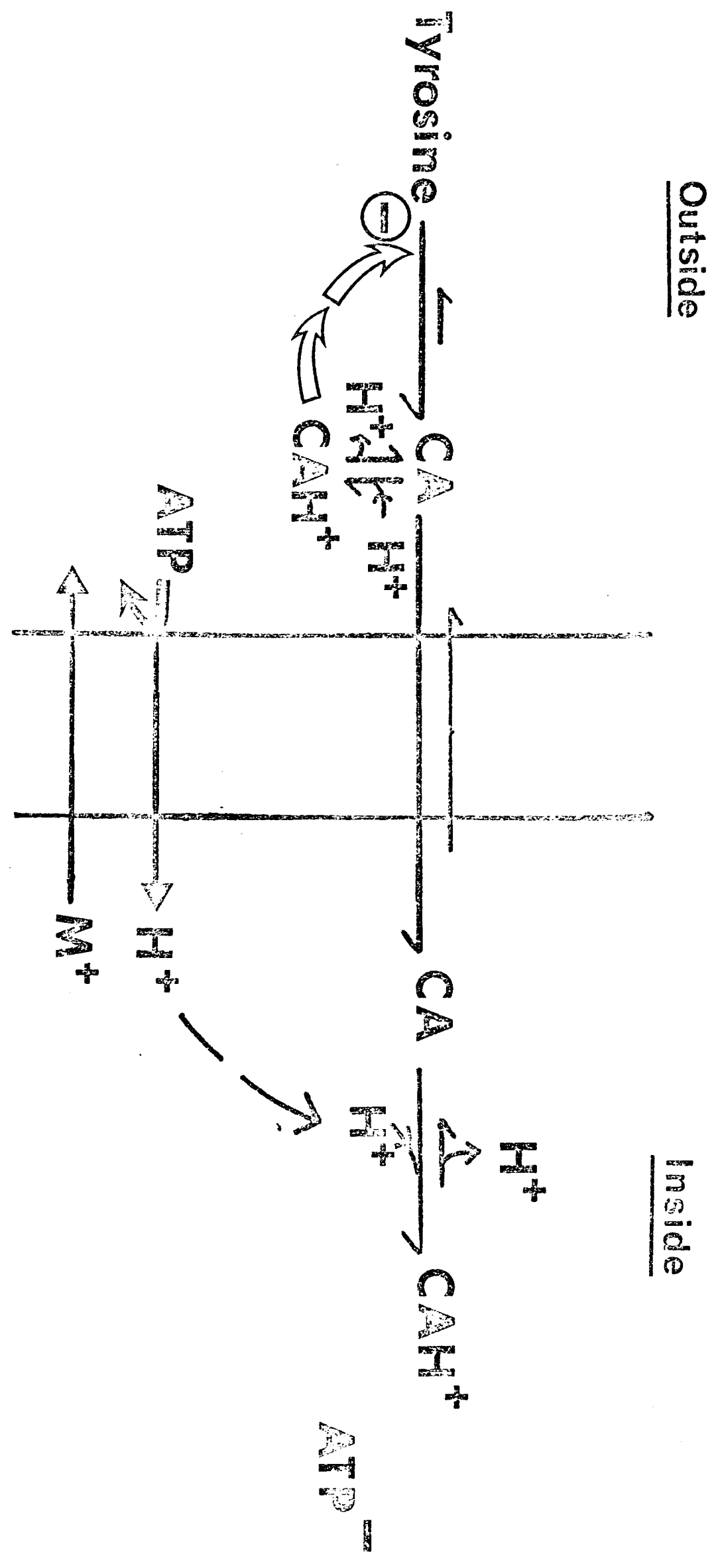
these primary vesicles must be low, since the pK_a of the ATP phosphates is low (pH 6 or less). Obviously the stability of the protein and of the ATP must set a limit to this pH, in practice. In this situation, a very large concentration of catecholamines could enter the granule by a virtually passive mechanism, though it could be proposed that the low pH itself is generated and maintained by an ATP- Mg^{2+} -dependent proton gradient. At neutral pH, the catecholamines are in equilibrium between their protonated and deprotonated form. If a proton gradient exists across an essentially proton impermeable membrane, then the catecholamines can distribute across this membrane: the degree of distribution being determined by the size of the gradient. This is analogous to the methylamine distribution method for determining the pH inside organelles (9). The deprotonated species passively equilibrates across the membrane and, if the pH inside the vesicle is lower, picks up a proton but, as the charged species, is unable to return. An equilibrium is soon set up. Thus, if a large proton gradient exists, a large amount of catecholamines could be accumulated. By this mechanism, as the catecholamines become protonated, the pH within the granules must rise, since the binding of the catecholamines for the protons at this low pH is very strong. If this is the mechanism, which is unlikely, then it may be that an equilibrium is set up at pH 5.5, or thereabouts, and no further catecholamines can be taken up. The major problem with this type of mechanism is an osmotic one. This is a difficulty which is encountered with all the possible mechanisms, but is one which would appear to have been solved by the cell.

If the positively charged species is a metal ion, monovalent or divalent, then a rather different mechanism must exist. In order to accumulate catecholamines by a proton gradient in this case, the proton moved into the vesicle must ultimately exchange with a monovalent cation, if the equality of charge inside the granules is to be maintained. By this mechanism, the internal pH, if it is sufficiently low, should not alter

very much on uptake of catecholamines, since the catecholamines could bind the majority of the protons generated. At a pH of 5 or thereabouts, this would most certainly be the case, since the pK_a of the amine group of the catecholamine is around 9. This mechanism may be capable of explaining the low concentration of metal ions present in the granules prepared through density gradients. (10).

In the chromaffin cell, essentially, a displaced equilibrium must exist. The synthesis of catecholamines is controlled at the level of tyrosine hydroxylation (in the cytoplasm) by end-product inhibition by the 'free' catechol derivatives (11). This means that as catecholamines are taken up by the granules, further synthesis occurs, but should the granules be unable to accumulate any more catecholamines, the resultant rise in their cytoplasmic concentration will turn off their synthesis. The active uptake situation is not, therefore, an equilibrium since when it occurs, the total concentration of catecholamines in the system increases. If the uptake is assumed to involve the deprotonated species, then the situation may be represented by Figure 6.1. This model, based on the diffusion of catecholamines through the membrane, driven by a proton gradient, is obviously highly simplified since it does not explain the inhibition of the $ATP-Mg^{2+}$ enhanced uptake, in vitro, by reserpine (12). Reserpine does not inhibit the uptake by uncoupling, since the ATPase activity of the granules and of the membranes is unaffected by it (Chapter Two). It is thought the reserpine exerts its action by specifically binding to the proposed catecholamine transport protein. This must be incorporated into the model. In order to simplify the model, it can be assumed that the transport protein is specific for the deprotonated species. It is possible to also include the concept of localised charge effects into this model. The nature of the proposed localised charge is undefined, though it may be an alteration of the surface charge in particular portions of the membrane but since there is

FIGURE 6.1.



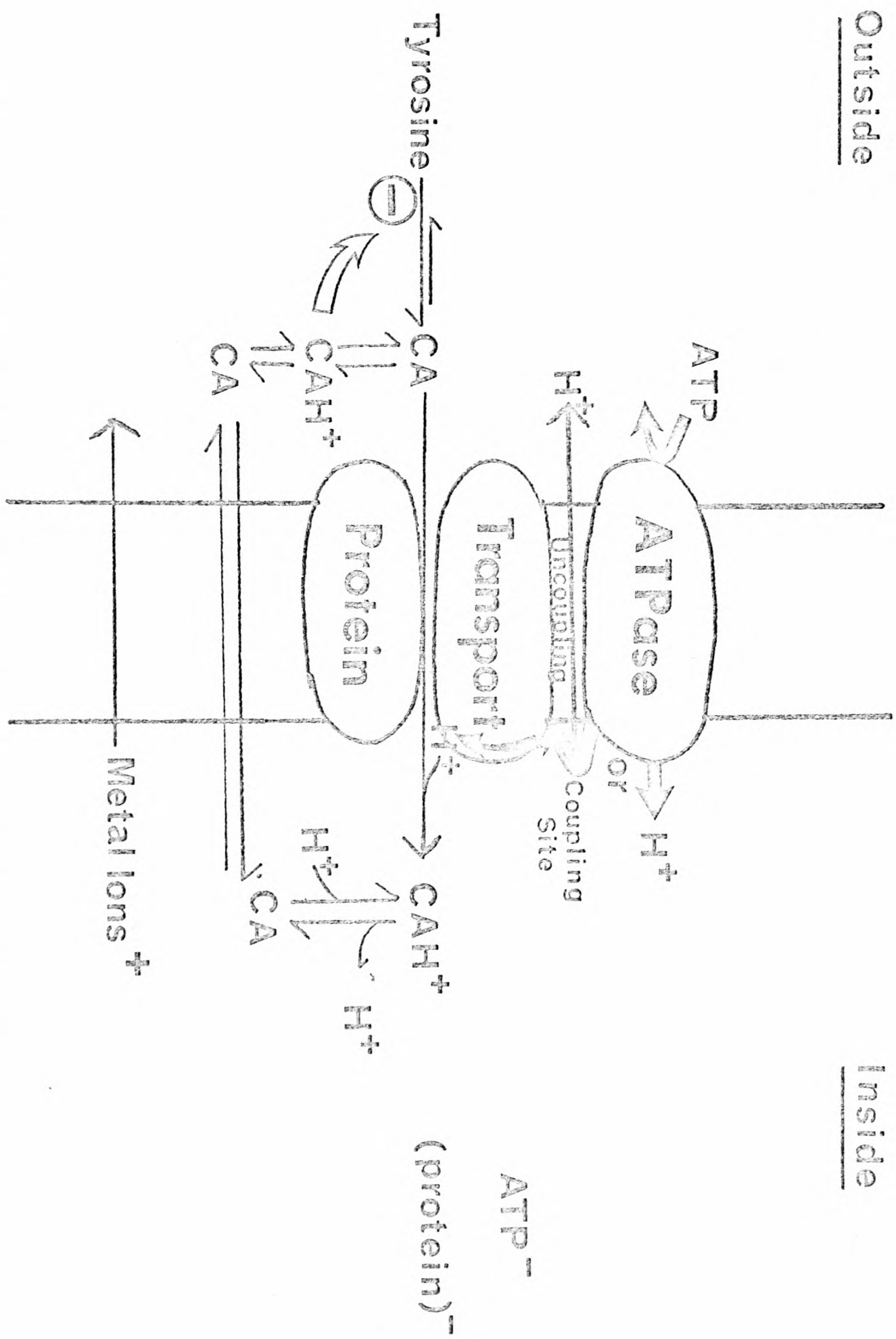
Model for catecholamine uptake involving proton/metal ion exchange.

no evidence to the contrary, it may be, in certain cases, localised within a protein. This could conceivably occur via a charge-transfer mechanism where the ultimate effect is that of proton generation in a different site from the initial reaction. A model incorporating these features is shown in Figure 6.2. In this model, the transport protein is effectively a proton transferase. The proton gradient generated by the ATPase can be dissipated by the uncouplers by acting at a coupling site between the ATPase and the transport protein.

These models of net bulk uptake, which can equally well be applied to the maintenance of the high catecholamine concentration, once accumulated, within the granule, are very much conjecture and are, therefore, open to much criticism and modification. There has been little consideration in these models of thermodynamic details since, because the models themselves are very simplified and the in vivo system so poorly defined, such an analysis would not be practical. Unfortunately, until more is known about the biogenesis of the chromaffin granule, in particular about the method of stabilisation of the charges of ATP and the chromogranin proteins throughout the process, it is unlikely that a solution can be reached. The nature of the final storage complex has been better defined by the NMR studies (Chapter Four) and hopefully, in the near future, a complete description may be achieved from the continuation of this work. It may also be possible to investigate the more immature granules using NMR, but this will be considerably more difficult to do.

The conclusion from the ESR and fluorescence studies (Chapter Three) that a transition occurs, very close to the physiological temperature range, in the structure of the chromaffin granule membrane, which is also reflected in the activity of certain membrane proteins, is very interesting. A possible explanation for this transition, discussed in detail in the text, is the redistribution of cholesterol. This segregation, and the effect of calcium on it, may play a role in the processes of membrane fusion and

FIGURE 6.2.



Model for catecholamine uptake involving a transport protein and proton/metal ion exchange.

catecholamine release. It is also noteworthy that this transition occurs so close to the physiological temperature range, since the release of adrenaline is stimulated in hypothermia, ie. when the body temperature is lowered. This release is obviously controlled by higher centres in the brain, but it is possible that this observed transition is related to this phenomenon.

In conclusion, many of the results and proposals made in this thesis have greatly altered the concepts within which the chromaffin granule, structurally and functionally, must be considered and, hopefully, much meaningful and useful information about the system will be forthcoming as a result.

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