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INTERACTION OF POLYPEPTIDES WITH LIPID BILAYERS

by

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in partial fulfillment of the requirements for the degree of
Doctor of Philosophy.

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Abstract

A novel application of competitive labelling was developed for the study of lipid bilayer-protein interactions. The rationale behind this approach is that the reactivities of protein and polypeptide functional groups will be altered upon association with bilayers. From the distribution of functional groups and the differential changes in their reactivities, the regions that are involved in the association can be deduced. The interactions of insulin, glucagon and melittin with large unilammellar vesicles were studied in detail.

In the presence of liposomes, functional group reactivities in monomeric insulin were found to undergo decreases related to their proximity to the dimer-forming surface. It was concluded that the region involved in adsorption encompasses this surface.

The relative reactivities of glucagon's amino-terminal histidine, solitary lysine and two tyrosine residues were concentration dependent. It is concluded that glucagon exists as a trimer in solution at 10^{-6}M and pH 7.5 and dissociates to the free monomer in more dilute solution ($K_{trimerization} = 4.11 \times 10^{13}\ M^{-2}$). In the presence of liposomes the reactivities decreased in a pattern consistent with both trimeric and monomeric forms of glucagon adsorbing to bilayers via the hydrophobic patches of their amphiphilic segments.

The amino groups in the amino and carboxy terminal regions of melittin display similar concentration dependency in their reactivity trends in the presence of liposomes. On the basis of this data and the X-ray crystal structure it is concluded that the dimer is the primary membrane-active species. In the absence of lipid, the relative reactivities of the melittin functional groups showed concentration-dependent changes consistent with a monomer-dimer-tetramer transition ($K_{dimerization} = 7.57 \times 10^{4}\ M^{-1}$; $K_{tetramerization} = 3.25 \times 10^{3}\ M^{-1}$).

DEDICATION

May the 360,000 children who were aborted in Canada during the course of my six years of study be finding themselves in a far better place.

Acknowledgements

I would like to thank my supervisor, Dr. Harvey Kaplan, for his aid and advice, and for putting up with a certain amount of nonsense. The many enjoyable hours of conversation (not only concerning science) will be remembered, as will hopefully some of the attitudes and methods of approaching and solving problems. It was through Dr. Kaplan that the Medical Research Council provided the funds for the research and the researcher; whoever it was up there: my thanks.

Dr. George Oda deserves special mention for always and patiently providing a wealth of experience to the solution of the everyday practical problems that arise in laboratories. I also appreciate the introduction to country music.

Among my peers I must single out Laura Stewart, Ian Park, Mary Hefford and Indira Vishnubhatla for hours of stimulating discussion and speculation regarding experiments, science, the world, and everything else. These people among others (you know who you are) provided at times my only social outlet, and for this I am grateful.

In department administration Brian Stoqua and Helene Amyot have aided and abetted my work. Drs. M. Kates, L. Benoiton, F. Chen and D. Brown have always offered valuable advice and references when asked. In particular I would like to thank Dr. Brown and technician Anne Kroeker for their friendly assistance with the electron microscopy.

At NRC, Drs. A. Storer and D. Rose introduced me to protein computer graphics and Dr. Rose spent hours teaching me, enabling me to learn my way around this system. I would also like to thank Dr. D. Bundle for providing incentive for the rapid completion of this work.

History runs in strange patterns. For several years I have worked in the building which once housed the office and laboratory of my late father-in-law, Dr. George (Jerzy) Glinski, after whom the adjacent street is named and at whose former desk I often pored over data. I never had the honour of meeting this endearing man, save through his family, and I have considered it a privilege to be able to stalk the rooms he once used. In the end it is to his daughter, my wife Margie, mother of our three children and an accomplished professional in her own right, that I must thank the most for her support, encouragement and love.

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ABBREVIATIONS

BLM	bilayer lipid membrane (black lipid membrane)
cAMP	cyclic adenosinemonophosphate
CD	circular dichroism
CRF	corticotropin-releasing factor
DDP	dodecylphosphocholine
dH_2O	distilled water
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG-diether	1,2-ditetradecyl-rac-glycero-3-phosphocholine
DNP	2,4-dinitrophenyl
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
EM	electron microscop(y/ic)
EPC	egg phosphatidylcholine
EPR	electron paramagnetic resonance
ESR	electron spin resonance
FDNB	1-fluoro-2,4-dinitrobenzene
GHRF	growth hormone-releasing factor
HVPE	high voltage paper electrophoresis
HPLC	high pressure liquid chromatography
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
NMR	nuclear magnetic resonance
PA	phosphatidic acid
PC	phospatidycholine
PG	phosphatidylglycerol
PHI	peptide with N-terminal histidine and C-terminal Ile
PI	phosphatidyinositol
PP	pancreatic polypeptide
PS	phosphatidylserine
PTH	parathyroid hormone
QLS	quasi-elastic light scattering
r_H	hydrodynamic radius
R_i	lipid-to-protein molar ration
REV	reverse phase evaporation
SUV	small unilamellar vesicle

T_c	gel-to-fluid phase transition temperature
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
VIP	vasoactive intestinal peptide

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INTRODUCTION: Part 1

1.0 Background and Rationale

1.1 First Principles

Nucleic acids are generally assumed today to hold the informational content that allows living organisms to manifest themselves in the phenomenological world. Although this assumption may or may not be an oversimplification, among the physical structures by which living creatures mediate the interface between their inherent/inherited genetical structure and the rest of the universe, we find that proteins and lipids dominate over other chemical and molecular forms.

In recent years there has been renewed interest in the specific ways in which proteins and lipids interact, especially within the context of biological membranes. Membranes are the basic structural unit in cell biology (constituting at least 90% of dry cell mass), and their biochemical characterization is a fundamental challenge. Integral membrane proteins in particular, with the recognition of their central mediating role in both energy and signal transduction processes, are the subject of intense biochemical and biophysical research.

Techniques for the extraction, isolation and purification of lipids and proteins have been around for decades, and the increasingly sophisticated materials for separation procedures that are now commercially available indicate that separation science is a specialty of its own. The difficulty with membrane-associated proteins is that, in order to maintain the integrity of the system, quite dissimilar substances must be co-purified. Thus, the rate limiting step in

elucidating the structure-function relationships of membrane proteins seems to lie in the difficulties encountered when manipulating mixtures of two chemically distinct classes of compounds which nevertheless form a physically contiguous and functional unit in vivo. The wide range of amphiphiles and detergents introduced in recent years by commercial suppliers has simplified many isolation and reconstitution protocols, but these compounds cannot provide the specificity of molecular interaction that makes lipid-protein systems unique. Similarly, a major difficulty is the inability of researchers to isolate good crystalline arrays of the proteins of interest in their native membrane-bound state for analysis by X-ray crystallography. In fact it was only in 1974 that the first crystal structure of a natural phospholipid (phosphatidyl-ethanolamine) was determined (Hitchcock et al., 1974). Some progress is being made in the cocrystallization of membrane proteins with synthetic amphiphiles (Eisenberg, 1984), but until a general method for the crystallization of these types of proteins is found, we must content ourselves with the data obtained from other sources.

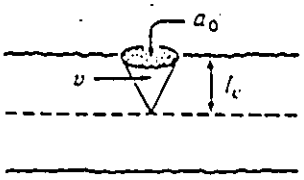
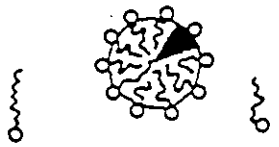
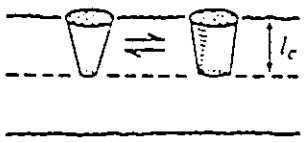
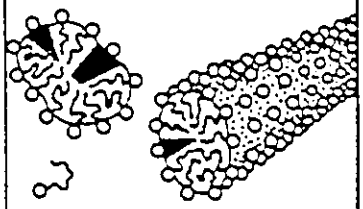
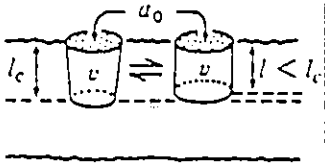
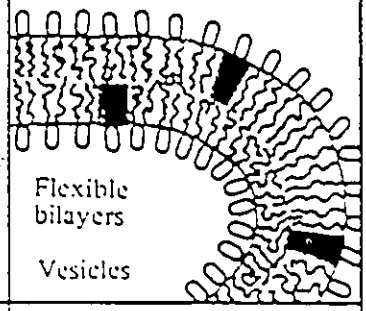
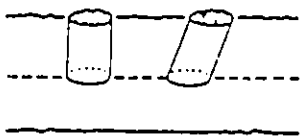
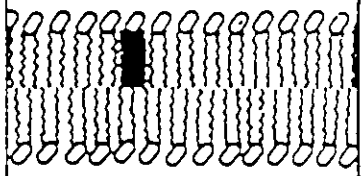
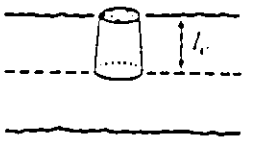
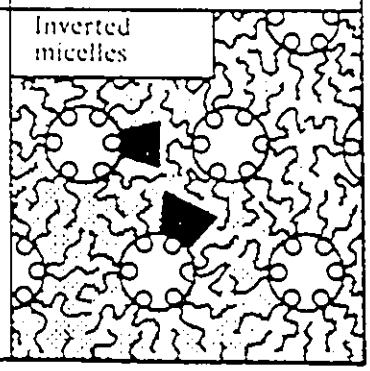
We must be careful to avoid oversimplifying the manner in which proteins and lipids interact, since their modes of association are varied and complex. The basis for this complexity lies in the diversity of structures that can be formed in both the lipid and protein classes. Small changes in substituents amount to large changes that take on macroscopic significance if the compounds form part of a larger ultrastructure. In proteins it is the side chains and their sequence that confer most of this variability, while in lipids, headgroups, backbone and acyl chains can be altered and interconverted.

Biologically occurring lipids are able to form many types of

thermodynamically stable, structurally defined aggregates besides bilayers, from spherical, globular and cylindrical micelles, to variously textured bilayers and inverted micelles (Figure 1). The thermodynamically stable structure of a particular aggregate is largely determined by the spatial and energetic constraints that are laid upon the entropically favoured dissociation of all things. The chemical composition and molecular dimensions of particular lipid species determines the magnitude and directionality of the forces that may stabilize these structures. These forces include van der Waals ionic and dipole interactions, hydration, and lack thereof. (It has in the past been convenient for many authors to speak of 'hydrophobic bonds' to describe the forces that result in the association of non-polar species in polar environments. This is not, properly speaking, a bond at all, and this misleading terminology will not be used here.) The spatial constraints are relatively invariant, and lipids can therefore be classified according to their geometry, specifically their "critical packing shape" (Israelachvili et al., 1977, 1980), or "dynamic molecular shape" (Cullis & DeKruijff, 1979). The geometry of the lipid molecules can oppose the dissociating tendency of entropy by requiring certain minimal packing configurations. Thus, particular lipid species cannot be forced to assemble into smaller (or larger) dimensional aggregates because their shape will not permit them to orient themselves relative to one another in the required manner.

Aggregates with different numbers of constituent monomers will interact with the solvent to varying degrees and a range of variously sized aggregates would have to be represented as having a range of different chemical potentials (Israelachvili et al., 1980). Those

Figure 1: Structures of lipid assemblies arising from the packing shapes of lipids and the resultant steric constraints (from Israelachvili et al., 1980).

Lipid	Critical packing parameter $v/a_0 l_c$	Critical packing shape	Structures formed
Single-chained lipids (detergents) with large head-group areas: NaDS in low salt Some lysophospholipids	$< \frac{1}{3}$	Cone 	Spherical micelles 
Single-chained lipids with small head-group areas: NaDS in high salt Non ionic lipids lysolecithin	$\frac{1}{3} - \frac{1}{2}$	Truncated cone or wedge 	Globular or cylindrical micelles 
Double-chained lipids with large head-group areas, fluid chains: Lecithin, sphingomyelin Phosphatidylserine in water Phosphatidylglycerol Phosphatidylinositol Phosphatidic acid Disugardiglycerides Some single-chained lipids with very small (uncharged) head-groups.	$\frac{1}{2} - 1$	Truncated cone 	
Double-chained lipids with small head-group areas, anionic lipids in high salt, saturated frozen chains: Phosphatidylethanolamine, Phosphatidylserine + Ca^{2+}	~ 1	Cylinder 	Planar bilayers 
Double-chained lipids with small head-group areas, nonionic lipids, poly(cis) unsaturated chains, high T: Unsat phosphatidylethanolamine Cardiolipin + Ca^{2+} Phosphatidic acid + Ca^{2+} Monosugardiglycerides Cholesterol	> 1	Inverted truncated cone 	Inverted micelles 

aggregate structures in which the free energy is minimized will be favoured over other aggregates. At this point other factors such as hydrogen ion, salt and solute concentration can modify the properties of the lipid assembly, especially insofar as these factors affect the surface polarity and interfacial hydration at the lipid-water interface (Cevc, 1987). Additional considerations of chemical potential are then necessary since a new equilibrium state will be obtained.

Hydrophobic and lipophilic solutes can interact with the hydrocarbon core of the lipid structures, while hydrophilic solutes can influence the solvent-exposed polar groups. Whatever the solute type however, both polar and apolar regions of a lipid assembly will be affected, to varying degrees. For example, a lipophilic inclusion such as cholesterol in a phophatidylcholine (PC) bilayer will directly restrict the acyl chain motions above the gel to liquid crystal phase transition temperature (T_c)(Dufourc et al., 1984). Indirectly, the cholesterol will also perturb the relative orientation of the phosphorycholine headgroups, since it separates neighbouring headgroups, thus modifying their interactions (Yeagle, 1978). Conversely, hydrophilic solutes (such as some proteins) that interact electrostatically with the headgroups can also affect the dynamics of the acyl chains (Boggs, 1983). Amphiphiles, as their name implies, directly interact with both polar and apolar regions of the bilayer (Seelig et al., 1987). Proteins can be hydrophilic, hydrophobic and amphiphilic at the same time, depending on their primary structure, and therefore interact with bilayers in all of the above ways. The interactions are complex and interdependent, and experiments have shown that all components of a lipid-protein system, including counter-ions and solvent

must be considered (Davis et al., 1983).

These facts, and the previously mentioned structural diversity of lipids, show that protein-lipid interactions are not confined to the classical globular proteins that float, iceberg-like, in the two-dimensional surface of the Singer-Nicholson bilayer. Some of the more obvious protein-lipid associations that occur in vivo are listed in Table I.

Due to turnover and recycling (which can be considered as quality control and parsimony on the part of the cell), the individual molecules in these complexes have finite lifespans. However, specific examples of each of the above classes of protein-lipid complexes are always present in the organism (allowing for their expression according to developmental timetables), and past a certain developmental point in the life of the organism, they will always be present at the required levels. Effectively, they exist as a functional unit, on a timescale that is of the same order of magnitude as the life of the organism.

There also exist proteins whose associations with lipids are no less intimate, but considerably briefer. These include proteins that are secreted (and so must pass through a bilayer at some point in time), and membrane tropic proteins. Secretory proteins require a large battery of accessory structures in order to translocate a membrane bilayer, including 'leader' or 'signal' sequences, appropriate membrane-bound receptors for the preprotein, and peptidases for the leader peptide. Often, the process of membrane insertion and translocation is coupled to translation and a trans-membrane electrical potential is also frequently required (Wickner & Lodish, 1985).

Membrane-tropic proteins can be defined very broadly to include any

TABLE I: Lipid-Protein Interactions in vivo.

1. Membrane-associated Proteins

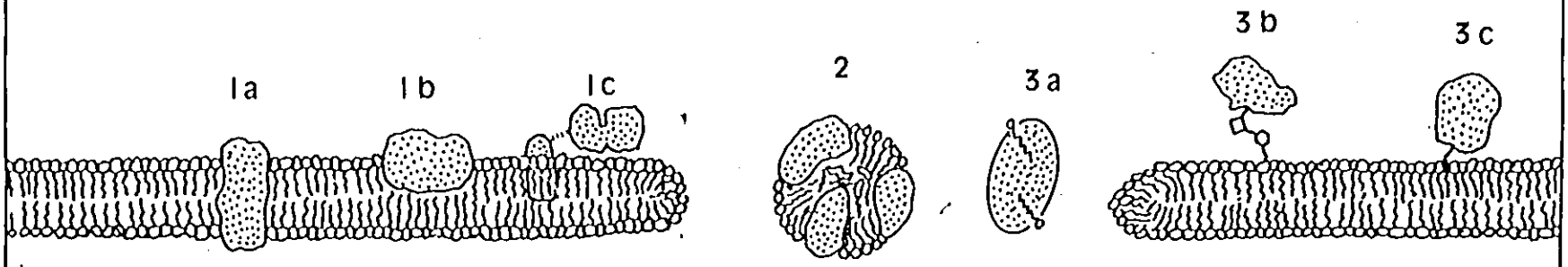
-Integral membrane proteins are embedded in the membrane
  -trans- proteins: ion channels, ATPases, hormone receptors (1a).
  -cis- proteins: various cytochromes and their oxidases (1b).
-Peripheral proteins are bound to membranes non-covalently via other membrane constituents: ankyrin, spectrin (1c).

2. Micelle-associated Proteins

-Apolipoproteins are bound to, or form, the nuclei for micelles and colloidal particles: HDL, LDL (2).

3. Monomer-associated Proteins

-Non-covalently bound lipid-protein complexes found in serum: serum albumin with adhered palmitate.
-Covalently bound lipid-protein complexes found in the cell membrane as glucosyl-phosphatidylinositol membrane anchors: Thy-1 glycoprotein.
-N-terminus acylation to myristate: Rous sarcoma $p60^{v-src}$ protein.



References: (1a,b,c) Bretscher, 1985; Weber & Osborne, 1985; (2) Edelstein et al., 1979; (3a) Kragh-Hansen, 1981; (3b) Low & Saltiel, 1988; (3c) Wilcox et al., 1987.

protein whose ultimate goal is to interact with some component of a membrane. Such an interaction can be constructive, as in the intracellular communication mediated by regulatory peptides, or destructive, as in the transfer of toxins from one species to another. Constructive interactions are mediated by other membrane components such as receptors and second messengers. Destructive interactions can be mediated, or not. The binding of cholera toxin to GM_1 ganglioside is an example of a membrane constituent-mediated process. Alternatively, the phospholipases found in venoms simply breach the membrane directly, delivering their message without mediation.

It has been suggested in recent years that transient associations of proteins and peptides with cell membranes are an important element in the initial stages of certain kinds of signal transduction. In particular, this has been proposed as a preliminary step to receptor binding by polypeptide hormones. There are several lines of reasoning which make this hypothesis attractive, one based on physical theory, and the other derived from observed structure-function relationships.

1.2 "Reduction of Dimensionality in Biological Diffusion Processes"

Once a polypeptide hormone is secreted into the bloodstream, it reaches its target cell by convection and diffusion. Convection carries the hormone with other solutes in the flowpath of the bloodstream. At the same time it diffuses throughout the volume of solvent available to it. The binding sites on receptor proteins are very small; out of the kilometers of blood vessels and the litres of blood, the vacant receptor sites represent a very small fraction of the available surface area available for the hormone to recognize and bind to. Biological responses operate under stringent time limits so the organism can deal effectively

with whatever stimulus triggered the initial release of hormone. Yet, for all this, hormones effectively induce their responses at very low concentrations. No one is quite sure how all the various factors involved --flow velocity, viscosity, shear stress at the cell surface, microviscosity in the 'bound water', receptor affinity, hormone solubility and diffusion coefficients-- contrive together for the smooth functioning of the organism.

Adsorption of a hormone to its target cell's membrane can reduce the time required to bring about receptor binding and activation, providing the distance between the site of release and the site of capture is quite large. If the number of dimensions can be reduced without restricting access to its target molecule (receptor), then the mean time of diffusion should be reduced, thus shortening the time required to effect binding. Further, by increasing the effective concentration of hormone near the receptors, signal transduction will also be facilitated. These ideas have been suggested before by Trurnit in 1945, who suggested that surface diffusion of acetylcholine may increase the speed whereby it reaches acetylcholinesterase, and by Bucher in 1953, who suggested that surface diffusion may also increase the turnover numbers for membrane-bound multienzyme complexes (see Adam & Delbruck (1968) for references). By restricting the volume available to a molecule, the collisional frequency is increased. Similarly, if we restrict the volume to an extreme degree, to the extent of being effectively confined to a two-dimensional space, the collisional frequency is greatly increased.

In such a case it is only necessary that diffusion on the two-dimensional surface be significant with respect to the diffusion in

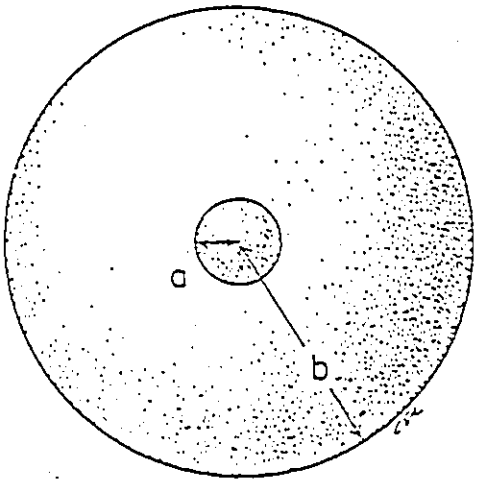
three dimensions (Kaissling, 1983). The properties of such a mechanism were explored theoretically by Adam and Delbruck (1968) for spherical and cylindrical model systems (Figure 2). It was found that if the receptor site was less than one-tenth the size of the diffusion distance ('a' < 0.1'b'), combined two- and three-dimensional diffusion would be faster providing the diffusion coefficients in both phases were equal. If diffusion through the medium was ten times that on the surface, the combined processes would still be faster if 'a' < 0.01'b'. Considering that 'a' could equal 1 nm for a receptor binding site, then 'b' need only be greater than 0.1 μm.

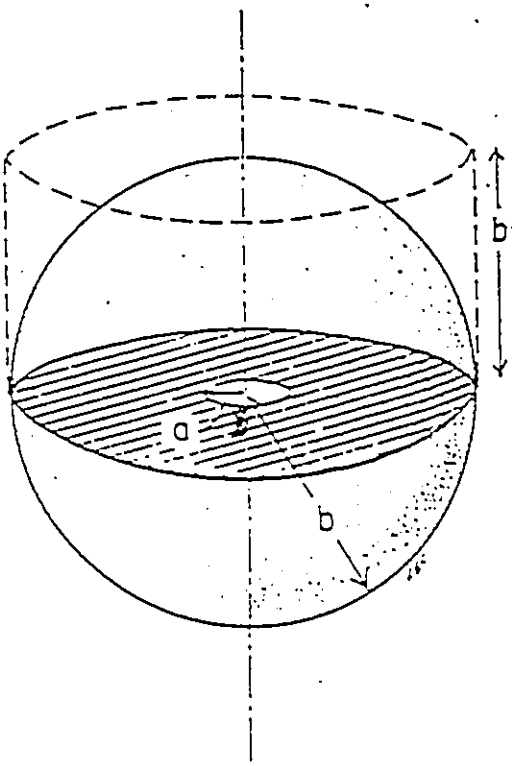
There are two reasonably distinct types of interaction between lipid bilayers and soluble proteins. First, there is an initial adsorption of the peptide to the membrane surface, defined by the extrinsic properties of the system (such as component concentration) and non-specific physical forces (van der Waals and electrostatic) arising between bilayer surfaces and molecules in solution. Once protein and lipid components are brought together, specific inter and intramolecular interactions will be dictated by their unique chemical and physical properties. At this short range, hydrophobic forces and the low dielectric of the immediate environment would be of primary importance in determining the orientation of amino acid side chains relative to each other, followed by van der Waals forces and hydrogen bonding.

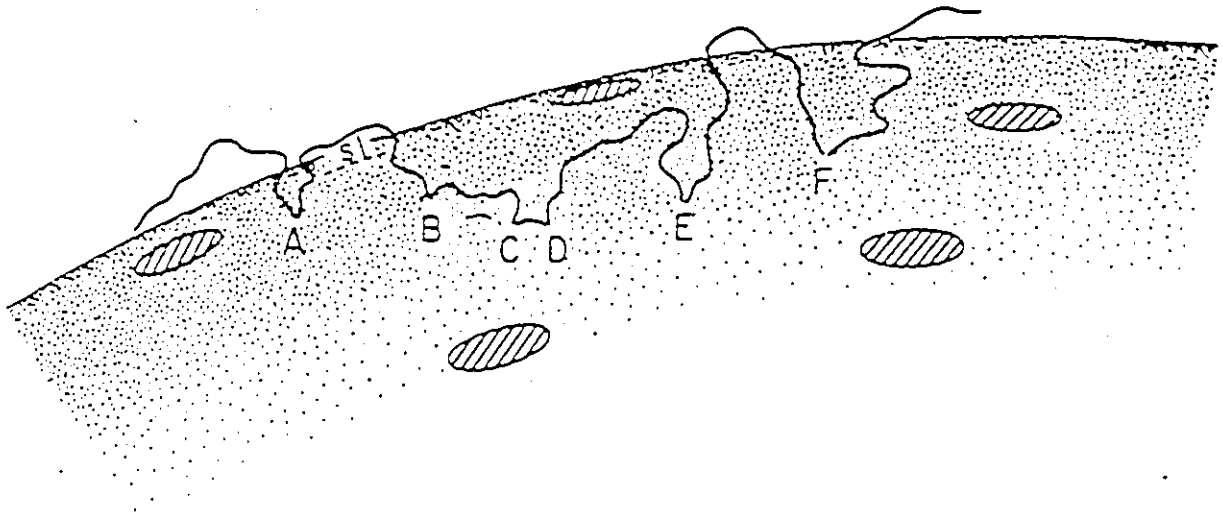
Diffusion of a polypeptide hormone on the membrane surface would depend on the lateral dynamics of the membrane components as well as the affinity of the hormone for the membrane. A corollary to this scenario is an equilibrium adsorption/desorption process (Figure 3). It is also possible that adsorption and surface diffusion could operate in

Figure 2. Models used by Adam and Delbruck (1968) for diffusion in two- and three-dimensional space. (A) Molecules are initially uniformly distributed between concentric spheres of radii 'a' and 'b', and are adsorbed onto the surface of the small sphere (the 'receptor') upon first contact. (B) as in (A), but molecules are initially distributed within the dashed cylinder and adsorb to the equatorial plane of the large sphere, diffusing in two dimensions to reach the receptor.

Figure 3. The path of a diffusing molecule that has some affinity for a cell membrane surface. If the mean distance travelled away from the surface between adsorption events is small, the process approaches two-dimensional diffusion (from Berg & Purcell, 1977).


a
b


b
a
b


SL
A
B
C
D
E
F

different membrane regions; at the polar/apolar interface, the headgroup, or via weak binding to surface antigens. At present there is evidence that many polypeptide hormones may use the mechanism of surface diffusion and that the adsorption is mediated at the polar/apolar interface of the membrane.

1.3 Peptides with Membrane Affinity

It has been known for some time that peptide conformation is influenced by environment. Additionally, it has been observed that many polypeptide hormones are capable of assuming the secondary structural feature known as the amphipathic helix, in which one side of the helical 'cylinder' has predominantly apolar, or hydrophobic, residues, while the opposite side has mostly polar, or hydrophilic, residues. The observation that the association of apolar lipoidal substances (e.g. detergents) with peptides can stabilize alpha-helix formation (Blundell et al., 1982) then suggests that proximity to the membrane bilayer can promote the adoption of an active, receptor binding conformation in the peptide. In this scenario, the membrane bilayer plays a role in inducing an active conformation, broadly analogous to the 'induced fit' mechanisms between enzymes and their substrates.

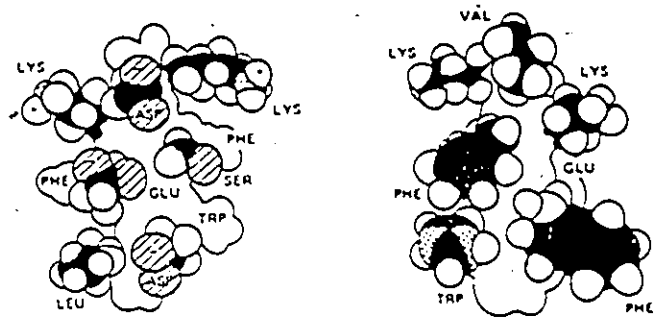
The evidence for surface-mediated diffusion is based on patterns of peptide folding that result in secondary structures possessing amphipathic and amphiphilic character. An amphiphile is something that has affection for two sides, and in chemistry refers to compounds with segregated polar and apolar regions. 'Amphipathic' is a term appropriated by some investigators (Segrest et al., 1974) to denote a stretch of alpha helix that has its charged side chains adjacent to one another, arranged longitudinally and parallel to the helix axis.

Similarly, the hydrocarbon-like side chains run longitudinally down the opposite side of the helix. In a true amphipathic helix (Segrest & Feldman, 1977) the charged side chains are arranged so that positive charges bracket negative charges, with like charges forming rows that interact via ion-pairs (Figure 4). A 'reversed' amphipathic sequence reverses the ordering of the positive and negative charges and consequently has fewer ion-pairs. A 'nonspecific' amphipathic sequence results in a random distribution of charged and polar residues on the polar face of the helix. All of these amphipathic helices have been observed in proteins; the 'nonspecific' type is a general feature of globular proteins containing alpha helices, and has been known for some time (Perutz et al., 1965).

The apolipoproteins A-I, A-II, C-I and C-III are especially rich in amphipathic sequences, and studies using synthetic peptides have shown that both the arrangement of charged residues on the polar face (Segrest et al., 1983) and the relative hydrophobicity of the apolar face (Ponsin et al., 1984) are important parameters for the specific interactions they form with lipids. The relation of amphipathic helical segments to the rest of the protein of which they are a part is also important but not clearly identified, since isolated amphipathic segments of the native proteins do not always mimic the properties of the native proteins as well as some model peptides, which often have scant, if any, homology (Kroon et al., 1978; Yokoyama et al., 1980).

The lipid binding properties of apolipoproteins, coupled with their disproportionate number of amphipathic segments, beg the question of the possible function dipolar secondary structures may have in other proteins. Eschewing the use of the word 'amphipathic' for only the

Figure 4. Association of an amphiphilic helix with phospholipids. CPK representations of residues 58-67 of apolipoprotein C-III in helical conformation, showing the hydrophilic (a) and hydrophobic (b) faces. Rotation of (a) and (b) about their axes by 90° yields (c) and (d) respectively, when associated with a phospholipid. The complementarity of charge and polarity is expected to lead to a stable configuration at the interfacial region of the membrane (e). (from Segrest, 1977).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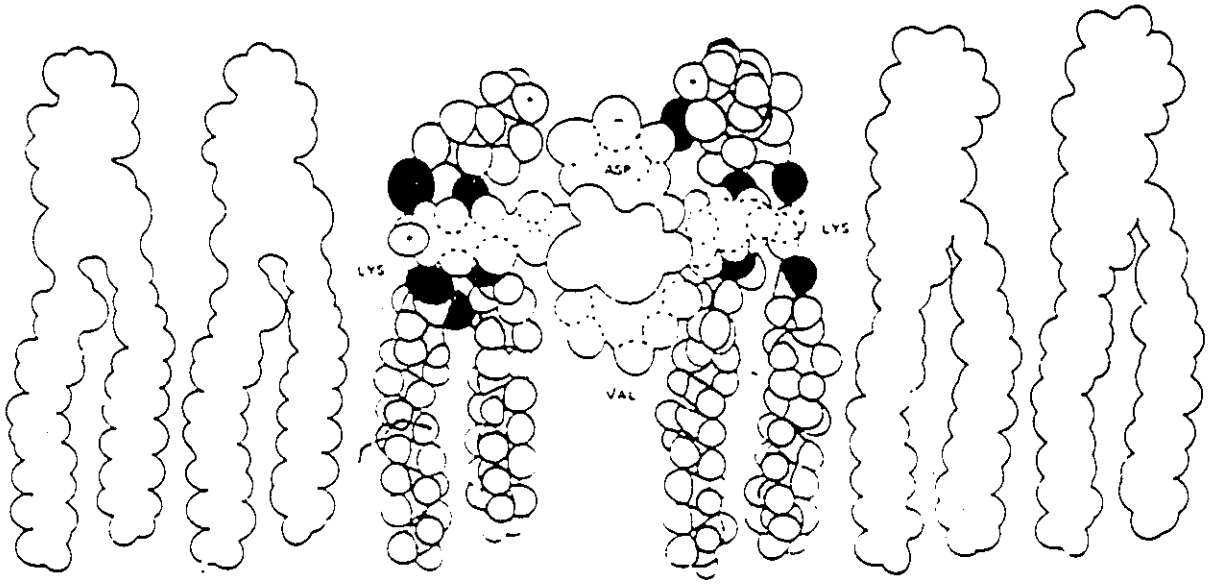
ASP
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particular structure identified by Segrest and Feldmann (1977), the term 'amphiphilic' will be used to describe such structures in general. Neither word is wholly satisfactory, since amphipathic is a word of greater generality than the object to which it has been assigned, and amphiphilic does not sufficiently distinguish as to the relative size or placement of the characteristics it seeks to denote. Note that the termini of an alpha-helical polypeptide can differ in their affinity for aqueous solution, or can have this affinity partitioned laterally, depending on the primary structure. An appropriate terminology might specify between primary and secondary amphiphilicity to differentiate between the two forms when necessary.

Primary amphiphilicity has been identified in several hormones, and is a well known structural motif in integral membrane proteins such as bacteriorhodopsin (Eisenberg, 1984), where hydrophobic helices completely span the membrane bilayer. Secondary amphiphilicity is only present when a peptide assumes a well defined secondary structure, such as one of the helix forms or beta-sheet. In the former case, hydrophobic residues must be present every 3 or 4 residues (3.6 residues to be precise), alternating with polar residues. In an amphiphilic beta-sheet, polar and apolar residues simply alternate one after the other (Kaiser & Kezdy, 1984). Primary amphiphilicity is not dependent on the size of a peptide, occurring in small peptides as well as large membrane-bound enzyme complexes. Secondary amphiphilicity is by its very nature restricted to small peptides (10 to 50 residues) since these alone possess the required flexibility, lacking the tertiary structure that domains and disulfide bridges confer.

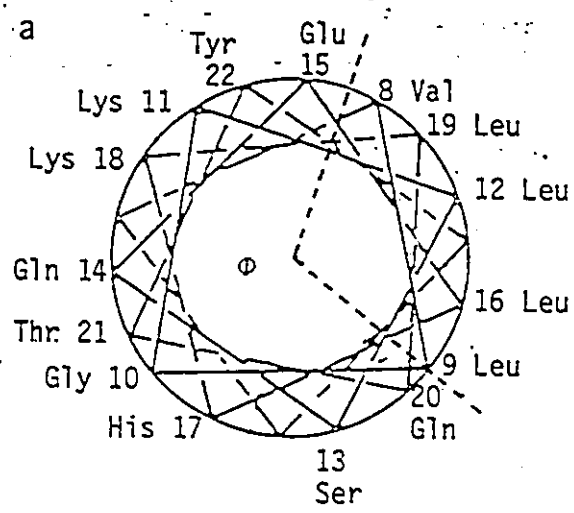
Several polypeptide hormones contain sequences that will, upon

helix formation, be aligned in the manner of amphiphilic secondary structures. These include the enkephalins, calcitonin, corticotropin-releasing factor (CRF), beta-endorphin, glucagon, growth hormone-releasing factor (GHRF), parathyroid hormone (PTH), and pancreatic polypeptide (PP) (Kaiser & Kezdy, 1983, 1984, 1987). Diagrams of some of these structures are presented in Figure 5. It can be seen that there are few similarities between these peptides apart from the commonality of secondary amphiphilicity. It can also be seen that the degree of hydrophobicity varies, as do the number and types of inappropriate residues , i.e. residues which do not conform to the polarity of the region they find themselves in. This may be an artifact of diagrammatic representation, or it may be real, perhaps due to some receptor specificity requirement.

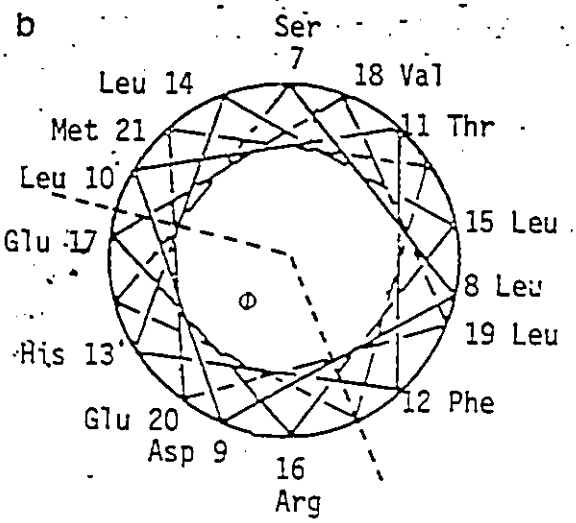
Synthesis of peptide hormone analogues containing minimal sequence homology but preserving the amphiphilicity of the original hormones has confirmed the importance of this secondary structure in promoting receptor binding and pharmacological effects (Kaiser & Kezdy, 1987, and refs. therein), but a full and complete appraisal of the role these structures play in the molecular mechanism of receptor binding and activation remain to be assessed.

By contrast, a much more quantitative assessment of the structure-function relationships in primary amphiphilic hormones has been carried out by Schwyzer's laboratory. The peptides studied by Schwyzer et al. have primary amphiphilicity at either their C- or N-termini, and the resulting hydrophobic helices have been shown by various techniques (attenuated total reflectance IR spectroscopy, capacitance minimization, chemical modification, theoretical calculations) to lie perpendicular to

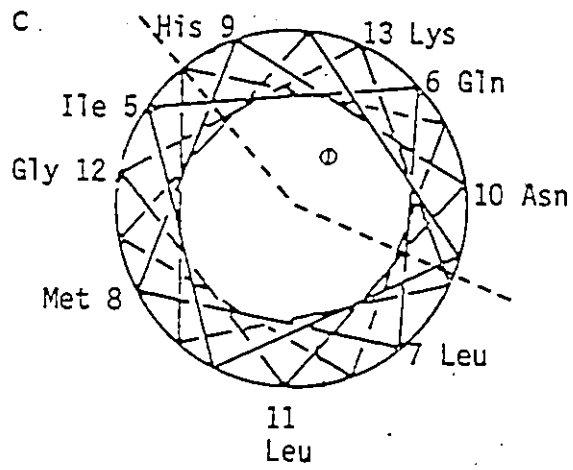
Figure 5. Amphiphilic alpha-helices of some polypeptide hormones, adapted from published sequences. (a) salmon Calcitonin I, resiudes 8-22; (b) Corticotropin Releasing Factor, residues 7-21; human Parathyroid Hormone, residues 7-13; (d) human Pancreatic Polypeptide, residues 15-24. Dashed lines separate hydrophilic regions (Φ) and hydrophobic ones.



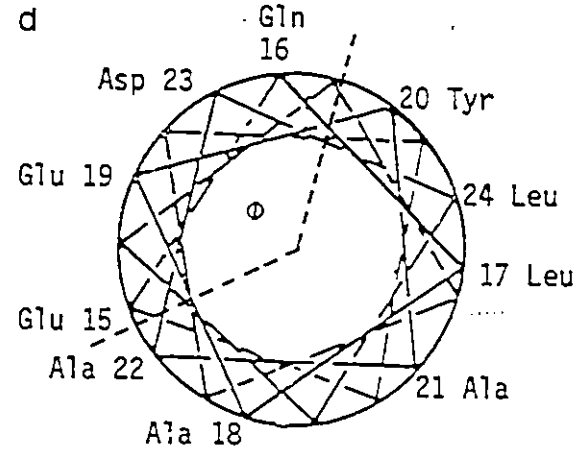
Salmon Calcitonin I



CRF



human PTH



human PP

the plane of the bilayer. The peptides so far studied are $ACTH_{1-24}$ (Gremlich et al., 1984), $dynorphin_{1-13}$ (Erne et al., 1985) and related opioids (Schwyzer, 1986a), substance P (Schwyzer et al., 1986), bombesin and neuromedins B and C (Erne & Schwyzer, 1987). One of the most significant results of these studies is the proposed molecular basis for receptor selectivity for opioid peptides (Schwyzer, 1986b). Since all of the opioid peptides in this study possess the same (enkephalin)message sequence, differences in agonist potency towards the different receptor subclasses could be correlated directly between peptides based on their structural differences. Positive correlations are made between published pharmacological potencies for the different opioid receptor types (delta, mu and kappa) and the structual differences in terms of the net positive charge and amphiphilic moment. The amphiphilic moment of a helix is a vector quantity indicating the relative magnitude of the hydrophobic interactions (based on the Gibbs free energy change for the transfer of a residue in a helical conformation from water to a hydrophobic phase) and the preferred orientation of the amphiphilic helix axis relative to the bilayer. Both the effective charge of an opioid peptide and its amphiphilic moment were found to make contributions to the specificity of the peptide towards a particular receptor subclass. Since all of the opioid peptides studied possessed the same (enkephalin) message sequence, differences in agonist potency towards the different receptor subclasses could be correlated directly, based on the structural differences between the peptides. Quantitative structure-function relationships defined with these parameters have allowed predictions to be made regarding the location of the receptor's binding site with respect to the membrane, thereby reducing the concept

of receptor specificity to a combination of receptor and membrane requirements (Schwyzer, 1986b). The result of such a successive, stepwise interaction involving the membrane and leading to receptor occupancy is an overall increased efficiency due in large part to the large number of weak 'binding sites' that a membrane surface can provide as a preliminary step to productive receptor binding (Schwyzer, 1985). Thus the lipid phase becomes a catalyst for the peptide-receptor interaction (Sargent & Schwyzer, 1986).

If the results obtained by Schwyzer et al. prove to be applicable to other families of amphiphilic hormones, we should not be surprised. Analogous processes are seen throughout the biological world, whenever diffusible substances must traverse large distances. A well known example is found amongst many species of moths, in which the male must correctly follow a stream of pheromones to its source in order to mate. The impressive fan-like antennae of these creatures provides a large surface are to aid them in their capture of the diffusing airborne mating hormone (Kaissling, 1983).

The use of a surface to provide a locus for molecular reactions is not in itself a novel idea. Surface catalyzed reactions (heterogeneous catalyses) have been extensively studied and used in physical and engineering chemistry. The difference in the processes underlying, for example, palladium/carbon catalyzed hydrogenation of alkenes and the putative membrane-catalyzed binding of glucagon to its receptor lies in the scale of the reactions. In the former case there is the spatial reallocation and reassignment of bonding electrons to a more stable configuration, in the latter, the spatial reorganization of entire macromolecules to form a stable complex.

1.4 Rationale

The possible importance of membrane-catalyzed hormone-receptor binding in vivo makes detailed studies of the process a reasonable topic for investigation. The experimental difficulties inherent in studying the process in vivo require that suitable model systems be used; preferably a model system that is flexible and amenable to further exploration of all important parameters, should results warrant it. It is proposed that the model membranes afforded by liposomes can provide a model membrane with a suitable balance of structural simplicity and flexibility (section 2.3). The association behaviour of a variety of proteins should be studied, including those for which a physiologically relevant interaction with the membrane prior to, or during, receptor binding, could be postulated. It is proposed that the protein hormone insulin (section 2.4), the peptide hormone glucagon (section 2.5), and the cytolytic peptide melittin (section 2.6), are suitable candidates for study. Insulin and glucagon both regulate intermediary metabolic processes via receptor-mediated processes, but differ markedly at the level of their secondary and tertiary structures. Glucagon and melittin are both peptides of 25-30 amino acids, with secondary amphiphilic helical regions, yet glucagon's membrane-assisted receptor binding is speculative, whereas the membrane tropic activity of melittin is widely known, but not fully understood.

Above all, an appropriate method is required, preferably one that is widely applicable and from which novel structural information can be obtained. The method of competitive labelling, also known as differential chemical modification, is a technique used to measure the chemical reactivities of individual functional groups, thereby providing

site-specific information on side chain properties and microenvironments. Perhaps the most appealing aspect of this approach is that it addresses lipid-protein interactions from the perspective of the protein (section 2.2).

INTRODUCTION: Part 2

2.0 Methodology and Components of the Model System

2.1 Techniques for Studying Protein-Lipid Interactions

The adsorption of lipids to proteins can be measured by such classical biochemical techniques as equilibrium dialysis, gel filtration chromatography and analytical centrifugation (Reynolds, 1982). These techniques can be applied to studying protein adsorption to bilayer liposomes (Kroon et al., 1978; Sparrow & Gotto, 1980), but the information obtained lacks resolution at the molecular level and may compromise liposome stability.

The application of spectroscopic techniques to lipid-protein interactions (fluorescence, fourier-transform infrared spectroscopy, Raman spectroscopy) can be extremely valuable, but in complex mixtures may be hampered by background noise originating from other chromophores in the system. Membrane environments obscure protein properties and make direct measurement difficult by conventional protein analytical techniques like circular dichroism (CD) (Long & Urry, 1981). Consequently, experimental dataare often obtained on the physical properties of lipids in the system and extrapolations subsequently made to deduce what is happening to the protein structure. For instance, intact membranes are compared with lipid extracts or reconstituted systems, and protein effects are then measured as a function of various lipid properties (e.g. Seelig et al., 1982).

Measurements are made of monolayer surface pressures, areas, and permeabilities; techniques using X-ray diffraction, differential

scanning calorimetry (DSC), and spectroscopic probes are used to study perturbations in a given membrane property, such as 'fluidity'. These techniques have provided a great deal of knowledge concerning bilayers, but less so in terms of their intrinsic proteins. This is because lipophilic probes, are confined to the region around the protein and have limited access to only a portion of the protein's structure. Multidimensional NMR of selectively labelled protein nuclei is a promising technique and is now being applied to these systems in various ways (Wuthrich & Wagner, 1984); however, identification of chemical shifts can still be ambiguous in even small (penta) peptides (Deber & Behnam, 1984), and the technique is limited to relatively concentrated protein solutions (1 mM).

Little is known about the microenvironment at the protein-lipid interface, and since the physico-chemical properties of protein surfaces are not well understood in simple aqueous solutions (Ninham, 1982), it is hardly surprising that the interactions between these two interfaces are so poorly resolved.

2.2 Competitive Labelling

The term 'structure-function relationships' recognizes that a complete understanding of how proteins accomplish their physiological roles must be accompanied by detailed knowledge of their underlying structure. Macromolecular structure is present at various levels of atomic and molecular complexity, and the relationships among these levels is frequently elusive. The chemical properties of polypeptides are usually not specifically probed, although these properties are clearly of fundamental importance in biological systems. Atom- and group-specific properties mediate the interactions that arise between

biomolecules and help provide the basis for the specificity of all biochemical recognition processes.

The present work describes studies employing the method of competitive labelling (Kaplan et al., (1971), which allows site-specific measurements to be made of the chemical properties of individual functional groups in proteins (for reviews, see Glazer, 1976; Bosshard, 1977). Chemical reactivity is an intrinsic property that can be influenced by several factors, including medium effects, steric constraints (March, 1968) and microenvironment. For example, if a group is involved in hydrogen bonding, ionic interactions, or is buried in the interior of the protein in a nonpolar environment, its chemical properties will differ from those of the same group interacting freely with solvent.

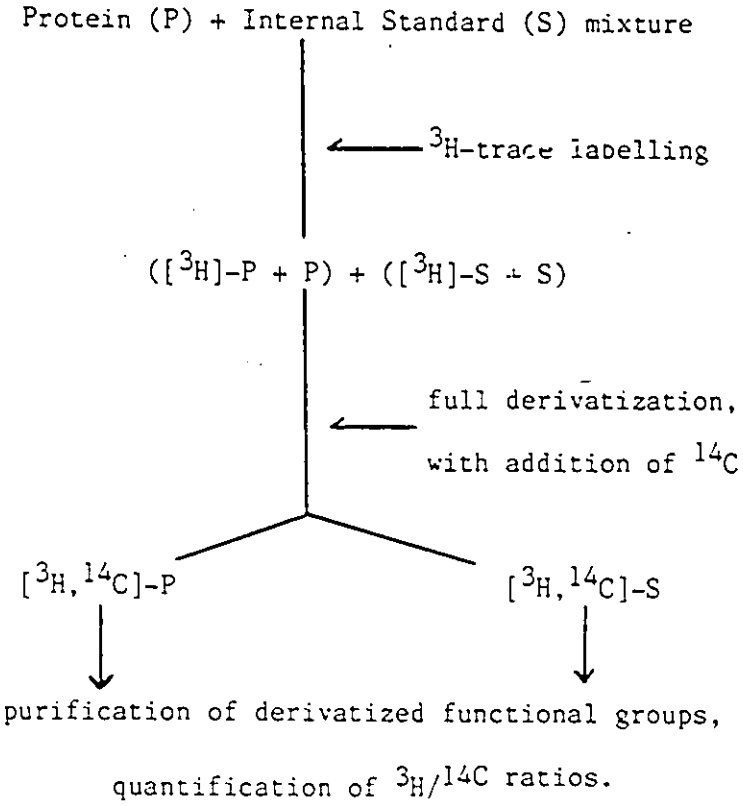
Competitive labelling has been used extensively in recent years to map contact areas in protein complexes (cytochrome c_2:cytochromes bc_1 complex, Bosshard et al., 1987; antibody-bound lysozyme , Burnens et al., 1987), and can be applied to the study of protein folding intermediates (Ghelis, 1980). Competitive labelling has also been used to measure the pK and relative reactivities of a number of protein ionizable groups (Doggleby & Kaplan, 1975; Hefford et al., 1984).

2.2.0 Theory

If a radioactive labelling reagent is added to a large excess of protein, the functional groups will compete for the reagent, and the amount of label incorporated into any specific group will be a function of it's chemical properties, i.e. it's pK and reactivity. As the ratio of protein to label is large, only a fraction of the protein molecules, and an even smaller fraction of each functional group, will react with

the reagent. Unlike many conventional chemical modification procedures in which the perturbation of native structure by the incorporated label cannot be unequivocally ruled out, the low levels of derivatization during the competitive 'trace' labelling ensures that the chemical properties of the protein's native structure are being measured. In order to provide a scale of reference against which the protein's reactivities may be compared, an 'internal standard' nucleophile can be introduced into the reaction mixture.

Upon completion of the trace labelling reaction, the unreacted nucleophiles in the proteins are modified with an excess of non-radioactive labelling reagent. In this way, one obtains a population of protein molecules that is chemically homogeneous, but heterogeneously radiolabelled according to the relative reactivities of the functional groups. Although the amount of incorporated label could now be quantified, the measured radioactivity may not reflect the initial distribution of label, due to incomplete recovery during isolation, and comparison between functional groups and the standard nucleophiles would be impossible. If the isotope used in the trace-labelling was tritium, this problem may be overcome in two ways. The first requires the determination of the specific radioactivity of each isolated peptide or functional group. A more convenient method involves adding an aliquot of 14C-labelled protein and internal standard solution. By counting both isotopes, the reactivities of functional groups relative to the internal standard can be determined, based on $^{3}H/^{14}C$ ratios. If material is lost during recovery, the ratio of ^{3}H to ^{14}C remains intact. The main points of the procedure are summarized in the following diagram:



Measuring reactivities relative to an internal standard nucleophile allows one to (1) measure individual pK values for specific groups, or (2) make comparisons between different labelling experiments.

When pK values are sought, the protein (P) and internal standard (S) nucleophiles are labelled over a range of pH values. Since the reaction is pseudo-first order, the amount of reagent (R) incorporated into each group will be proportional to the product of the fraction of unprotonated nucleophile (α) and the second-order rate constants (k_P,k_S). If PR and SR are the labelled forms of P and S after completion of the procedure, then

$$^3H/^{14}C \text{ of PR} / {}^3H/^{14}C \text{ of SR} = \alpha_P k_P / \alpha_S k_S,$$

where $\alpha = K_{P,S}/(K_{P,S} + [H^+])$, and $K_{P,S}$ is the acid dissociation constant

for the ionizable groups P or S, respectively.

K_S is readily obtained by titration of the internal standard. The value of k_S can be determined independently, but the ratio (r) of the rate constants k_P/k_S can usually suffices as a measure of the protein nucleophile's relative reactivity. Upon rearragement of the above equation,

$$\alpha_{pr} = \alpha_S(^3H/^{14}C \text{ of PR}/\ ^3H/^{14}C \text{ of SR}).$$

A plot of α_{pr} versus pH will yield the pK of functional group A and the value of r (Kaplan et al., 1971).

When determining the steric shielding of a residue upon complex formation, the reactivity of the internal standard can provide an objective point of reference by which to gauge any reactivity changes that may occur. For instance, two groups may have similar reactivities in the free protein, whereas in the complexed protein they may differ from one another. By relating the reactivities in each case to a non-interacting standard that is subject to the same experimental conditions, it is possible to determine the absolute direction and magnitude of the observed reactivity changes. If the protein is labelled when bound to a ligand (L) and when free in solution (F), changes in reactivity are proportional to:

$$(^3H/^{14}C \text{ of } PR_L/\ ^3H/^{14}C \text{ of } PR_F) \times (^3H/^{14}C \text{ of } SR_F/\ ^3H/^{14}C \text{ of } SR_L).$$

The last term is unity for an internal standard that does not interact with any of the components of the system. It can readily be seen that the inclusion of an internal standard in a complex-formation study is not mandatory, and they are frequently not used (Giedroc et al., 1985). Instead, individual groups within the protein may be used as a standard of reference provided they are not affected by complex formation.

Adsorption or binding of a protein to a liposome surface may involve several different groups. However, the site specific nature of competitive labelling offers an advantage in that only a small region of a complex biopolymer is probed at any one time. In this way the complexity of the interaction may be reduced by dissection to the relevant functional groups. The reactivities of these functional groups will depend on their accessibility to reagent, inherent reactivity, and microenvironment. The reactivity data can be combined with a knowledge of secondary structural constraints, allowing changes in group-group interactions, local structural rearrangements, and the surface topography of proteins to be deduced.

2.2.1 Protein Studies in Dilute Solution

Besides the ability to measure reactivities at specific sites, a second important feature of competitive labelling is that it allows very dilute (submicromolar) protein solutions to be examined. Although it is now routine to detect picomolar quantities of amino acids and peptides, the measurement of physicochemical properties is limited to solutions that are much more concentrated. Apart from fluoresence spectroscopy, the sensitivity of most techniques (ORD, CD, or NMR) does not allow for the study of proteins in very dilute solution (less than 10^{-6} M). At concentrations greater that 10^{-6} M however, many peptides oligomerize, and exist as equilibrium mixtures of different multimeric forms. Insulin monomers associate into dimers, tetramers and hexamers as their concentration increases from 10^{-6} M to 10^{-2} M (Blundell et al., 1972). Glucagon likewise associates into trimers, with accompanying changes in secondary structure (Blundell & Wood, 1982). The physiological concentrations of these peptides is very low (10^{-9} M to 10^{-13} M; Ganong,

1981), and most likely are monomeric in the circulation. Due to the sensitivity of the competitive labelling technique, studies can be carried out at concentrations approaching the physiological. Adsorption to liposomes should be accompanied by changes in the environment of some of the functional groups, and shielding may also occur. Stabilization of particular secondary structures would also be expected.

A thorough understanding of protein-lipid interactions will require the evaluation and synthesis of data obtained from a variety of experimental approaches. Competitive labelling has the potential to contribute novel information to this problem. In order to assess the utility of this approach, proteins exhibiting important structural similarities at different levels of biological complexity were studied (section 1.4). Competitive labelling studies should be able to (1) propose plausible structure-function relationships for the specific peptides under study, and (2) point to some general methodological principles that might be useful for future protein-membrane interaction studies.

Ultimately, the long-range goal of this line of research would be to apply the methodology to the study of receptor-bound hormones. If structure-function relationships can be successfully probed with the liposome model system, further studies using solubilized or reconstituted receptors would be feasible.

2.3 Liposomes as Model Membranes

In aqueous systems, vesicular structures can be made from a wide variety of chemical compounds, providing such compounds exhibit some degree of amphiphilicity (Fuhrhop & Mathieu, 1984). Vesicles made from biogenic lipids, particularly phospholipids, are generally known as liposomes ("lipid bodies") and are frequently used to model biological membranes. The rationale implicit in their use is that the lipid bilayer, which is the main structural feature of liposomes, is also the main structural feature of Singer and Nicolson's fluid mosaic model (Singer & Nicolson, 1972). Once it was demonstrated that lipid bilayers formed spontaneously from phospholipids in aqueous solution, the controlled variation of such parameters as phospholipid type, cholesterol content, and degree of fatty acyl chain saturation became inevitable. The incorporation of appropriate proteins further extended the experimental use of liposomes, as they could then be made to mimic many membrane-associated cell processes (Darszon, 1983). The results of these studies have generated the paradigms that now govern our understanding of membrane structure, function and dynamics in vivo.

Although liposomes provide a flexible model for investigating a range of membrane phenomena, they differ from biological membranes in many respects. Geometrical and topological characteristics (shape, surface area, radius of curvature, stability of surface structures) limit their size, so that the largest single-bilayer liposomes which can be conveniently and reproducibly made are well under a micron in diameter, less than a fifth the size of erythrocytes. In solution, the minimization of interfacial tension at the membrane/solvent interface dictates that liposomes are predominantly spheroid, a shape that is

ideal in terms of geometry, but not very similar to most cell types. At the molecular level, liposomes and biomembranes differ in terms of their phospholipid packing, resulting in different intramembrane dynamics and more exposed hydrocarbon acyl chains. Surface charge density and concomitant changes in water structure will also be affected by differences in the packing of the membrane phospholipids. These properties vary between cell types, and also differ between liposomes, depending on their manner of preparation (Abbott & Nelsestuen, 1987).

In addition to size and shape differences, liposomes also lack intrinsic membrane protein, glycoprotein, and glycolipid. Since protein and carbohydrate frequently make up to at least 50% of the dry weight of cell membranes, it may well be that important physico-chemical properties present in biological membranes are not present in liposomes. These large structures on the membrane surface might seem to provide a physical barrier that would prevent soluble molecules, such as peptides, from approaching the bilayer surface. However, non-specific physical shielding by large molecules does not necessarily affect accessibility to the lipids of the membrane. In a study by Peters et al. (1983), dextran and serum albumin were derivatized with fatty acids so as to bring them as close to the lipid matrix of the liposomes in which they were incorporated. No evidence was found for any shielding of glycolipid headgroups from hydrolysis by soluble neuraminidase. Nevertheless, the degree of subtle complexity found in natural membranes, in terms of total lipid composition and distribution in the bilayer, and especially their dynamic associations with other molecular species, may require that extrapolations from results of research based on liposome models be done carefully and conservatively.

2.3.0 Liposome Design

Liposome preparation and design is an active area of research, driven by the applications that liposomes have in both basic and applied science. For example, by the judicious choice of the molecular species to be used as the liposome components, it is possible to make liposomes that are temperature, pH, and light sensitive (Weinstein et al., 1979; Yatvin et al., 1980; Pidgeon & Hunt, 1983). These properties, combined with their innate capacity to contain a finite volume of solution, make them promising drug delivery systems (Knight, 1981). Besides the use to which researchers have been putting liposomes, it is this latter application that no doubt has encouraged most current liposome research.

There are many ways to make liposomes, each method possessing certain advantages and limitations. The type of lipids to be used, and the size and lamellarity of the final product are of primary importance if models of biological membranes are desired. Technically more challenging are limitations that may be imposed if compounds (such as nucleic acids or drugs) are to be encapsulated within the liposome, since these compounds may not be physically stable to the processes used, for instance, during sonication, or if exposure to organic solvents is necessary. The encapsulation efficiency, which is very much dependent of the method of preparation, is also an important parameter in such cases. Traces of detergent or organic solvent may or may not be acceptable contaminants and their possible presence will influence the choice of method.

Liposomes can be classified as multilamellar vesicles (MLVs) or unilamellar vesicles. For practical purposes, such single membrane liposomes have been divided (somewhat arbitrarily) into two groups on

the basis of size: small unilamellar vesicles (SUVs) have a diameter of less than 1000 angstroms, while large unilamellar vesicles (LUVs), are larger (Szoka & Papahadjopoulos, 1980). Other definitions of SUV and LUV are in use (Hope et al., 1986), and many investigators choose their definitions according to their own specific criteria.

Liposomes are characterized by 3 main properties: their physical size, lamellarity, and their trapped volumes. Electron microscopy using negative staining is a simple and convenient method of assessing the approximate diameter of the vesicles; it is often possible to detect the presence of multilamellar structures as well. In recent years quasi-elastic light scattering has been used to measure the sizes of liposome preparations (Schurtenburger, 1984). Freeze fracture electron microscopy is a good technique to obtain information on surface morphology (Verkleij, 1984), and although it can also reveal the multiple layers present in MLV, it does not provide conclusive proof that a preparation is free of multilamellar structures. Thin sections of liposomes can be made for electron microscopy by embedding the vesicles in a gelatin or agar matrix (Hamilton et al., 1980). This technique can size the vesicles and give an indication of their lamellarity. The mean dimensions of a liposome population can be combined with entrapped volume determinations to allow for the calculation of liposome surface area (Pidgeon & Hunt, 1981). Entrapped volume can be determined by encapsulating a detectable marker into the vesicle during formation. Non-entrapped marker can be removed by gel filtration or dialysis and the entrapped marker quantified. The marker should be a nonionic and nonpermeable solute, to avoid artifacts due to non-specific binding.

If a model of the plasma membrane is desired, LUVs are currently

the best approximation simply by approaching the gross dimensions of cell membrane surfaces. The related parameter of surface curvature is thereby minimized and lipid packing can be assumed to be more or less 'wild-type'. The single bilayer separating the encapsulated volume and external bulk solution approximates a cell better than a multilamellar structure, in which interbilayer forces (Rand, 1981) may distort the properties of the outer leaflet of the outermost bilayer.

Much work has been done on membrane systems where the phospholipid fatty acid composition is homogeneous (e.g. dilauroyl, dimyristoyl, or dipalmitoyl PC), a situation not regularly encountered in natural membranes. A more heterogeneous lipid mixture might better reflect the biophysical properties of naturally occurring membranes and thereby mimic their ultrastructure and dynamics. The large percentages of PC and cholesterol found in mammalian cells suggests that these two species alone may have substantial influence on the overall architecture of the plasma membranes, and this fact, combined with the physical stability of PC:cholesterol mixed bilayers, makes them suitable components for a model membrane.

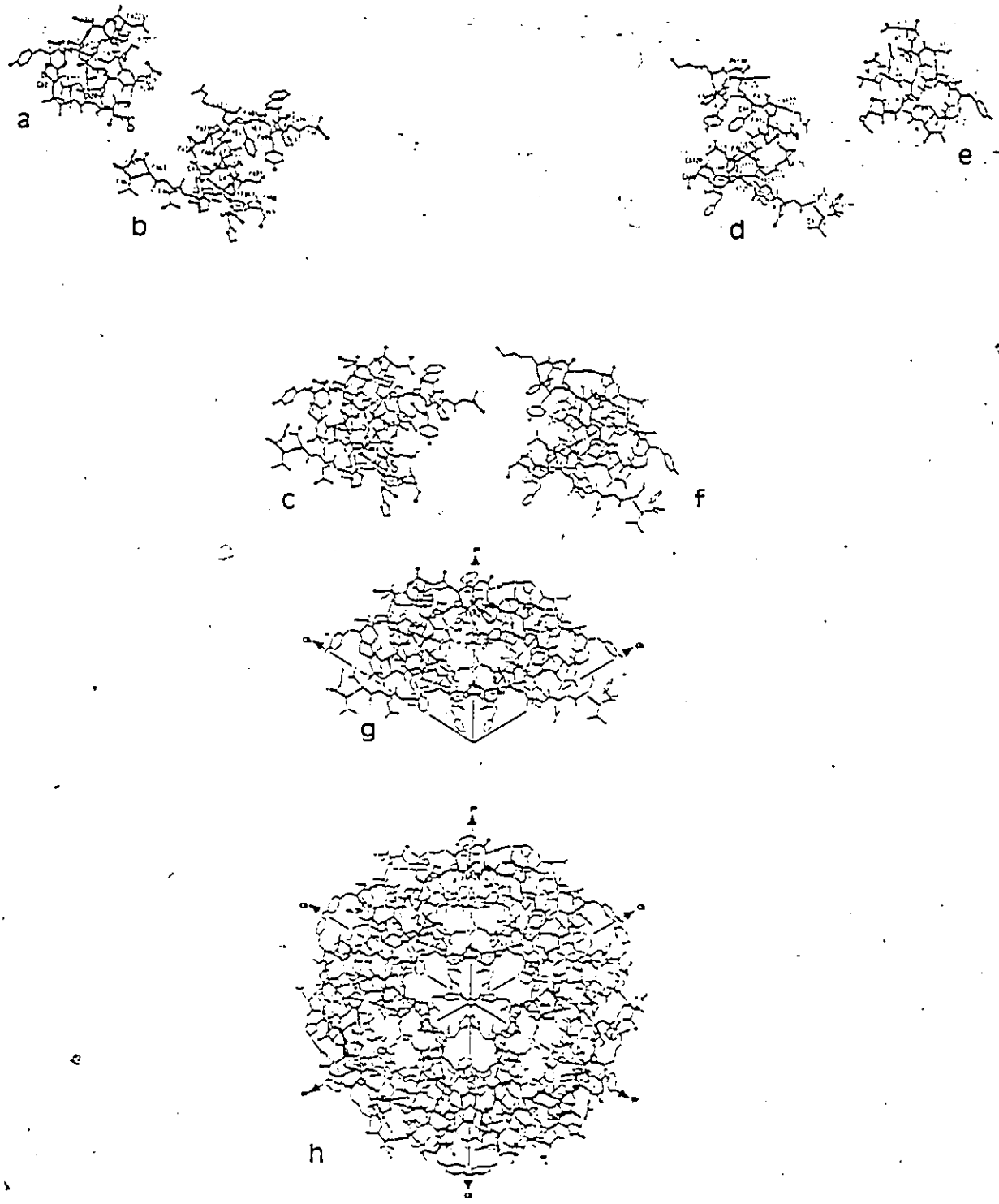
2.4 INSULIN

The anabolic role of insulin in intermediary metabolism is well known and the delivery of appropriate therapeutic regimes to diabetics has been one of the great successes of modern biochemistry. The mechanism of action of insulin at the cellular and molecular level remains to be determined however, and to this end site- and region-directed chemical modifications continue to be used to probe structure-function relationships at the level of the hormone-receptor interaction (Nakagawa & Tager, 1987). This research has been based on the crystal structure of insulin as determined by Dorothy Hodgkin and co-workers in 1969 (for an informative and personal review see the contributions to the dedication volume to Dr. Hodgkins, e.g. Perutz, 1981).

2.4.0 Crystal and Solution Structure

The crystal structure of insulin is built from X-ray diffraction images of the insulin hexamer, which forms rhombohedral crystals in vitro and in vivo (Blundell et al., 1982). When the storage granules in the pancreatic beta-cells are exocytosed, the insulin hexamers dissociate to lower oligomers via the hexamer-tetramer-dimer-monomer scheme outlined in Figure 6 (Blundell et al., 1972). Plasma concentrations of insulin are subnanomolar, normal levels not exceeding 0.5 nM (Ganong, 1981). Published values for the dimerization constant lie between 1.4×10^{-5} (Pekar & Frank, 1972) and 7.5×10^{-5} M (Pocker & Biswas, 1981), indicating that the circulating form of insulin is the monomer. It is the monomeric form that binds to the insulin receptor and presumably elicits the biological effects. Although the insulin receptor possesses a dimeric subunit arrangement and is therefore bivalent

Figure 6. Exploded view of the porcine 2Zn insulin hexamer. One A-chain (a) and one B-chain (b) make up molecule 2; another B-chain (d) and A-chain (e) made up molecule 1 (f). Molecules 1 and 2 combine to form the pseudo-symetrical dimer (g), three of which combine to give the hexamer (h). (from Cutfield et al., 1981).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h

(Czech, 1985) there is no conclusive evidence that both sites need to be filled, or that dimerization of bound insulin is required. In any case, it is clear that a monomer is physiologically favoured to bind to the receptor, whereas a dimer is not.

Indirectly, these ideas have been confirmed by the extensive modification studies involving the dimer-forming surface of the insulin monomer, which appears to be a part of the larger receptor-binding region (Pullen et al., 1976; Blundell et al., 1982; Gammeltoft, 1984). The dimer forming surface is hydrophobic, comprising residues Val-B12, Tyr-B16 & B26, and Phe-B24 & 25, and is quite invariant between all known insulins (Dodson et al., 1983). The receptor-binding region is postulated to involve the dimer-forming surface as well as Gly-A1, Tyr-A19 and Asn-A21, which lie adjacent to it (Pullen et al., 1976). Blundell et al. (1982) have included other residues in the proposed binding region, which lie on the periphery of the region defined above. It should be mentioned that the above named residues, A1, A19 & A21, form part of a second interspecies invariant region along with Glu-A4 and Gln-A5. The involvement of these more polar residues in hydrogen bonding and other polar interactions with the receptor's binding site may help to account for the greater affinity of the insulin monomer for the receptor (ca. 10^{-9} M), rather than for itself (ca. 10^{-6} M) (Gammeltoft, 1984).

As with the other proteins, the structure of monomeric insulin in solution is generally assumed to be similar to that found in the crystal. The only direct evidence supporting this assertion has been obtained by competitive labelling (Kaplan et al., 1984; Hefford et al., 1986), though the situation is complicated by the fact that a number of

different crystal structures are known for a single primary sequence (Cutfield et al., 1981). The first insulin crystal structure was done on the '2Zn' insulin hexamer, made up of three identical dimers coordinated about two zinc atoms. The monomers making up each dimer are not identical but nearly so, displaying only slight shifts in the relative positions of the secondary structural elements, with correspondingly minor differences in the side chains that interface these elements (Chothia et al., 1983). A few side chains show a more significant deviation between monomers, notably Phe-B25, resulting in the pseudo-symmetry at the dimer-forming surface of the 2Zn structure (Blundell et al., 1972). In the dimer form the 4Zn insulin hexamer, one of the monomers is quite similar to one of the 2Zn monomers, and is also very similar to both monomers in the symmetrical hagfish insulin dimer. This common structure is known as molecule 1 according to the nomenclature of the Beijing Insulin Research Groups (1981), and provides a useful reference structure (Figure 7).

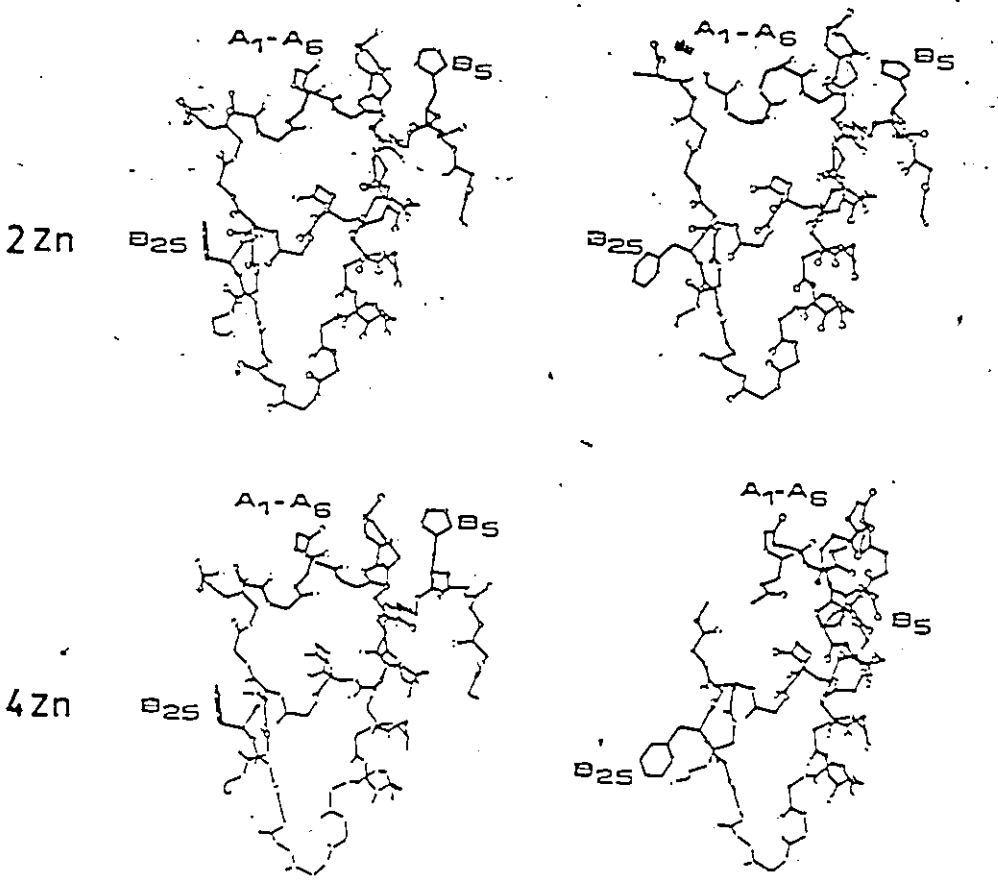
In the 4Zn dimer, molecule 2 is drastically different from the other structural forms (Chothia et al., 1983). The B-chain N-terminal residues B1-8 are folded into an alpha helix that extends the B9-B19 helix common to all structures. The result of this new B-chain conformation is to shift the relative position of the A-chain and alter the dimer forming surface of the molecule, resulting in a substantially different hexameric form (Smith et al., 1984). Incidentally, the 4Zn structure also suggests a molecular mechanism to explain the pharmacodynamics of this species of insulin. A detail of the B-chain conformational change is shown in Figure 8.

The structural flexibility of the insulin monomer, perhaps

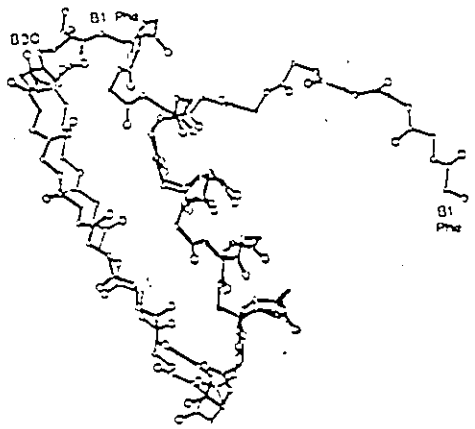
Figure 7. Backbone structures of the four independent porcine insulin molecules, viewed perpendicular to the two-fold axis (from Cutfield et al., 1981).

Figure 8. Detail of the conformational change involving residues B1-B8. The structure from the 4Zn molecule 2 (heavy line) is superimposed on that of the 2Zn molecule 1 (light line) (from Smith et al., 1984).

7)



8)



surprising in view of the three disulfide bridges present in the small protein, has been cited as being important for receptor binding and activation (Dodson et al., 1983). This is a particularly interesting possibility when one considers that the 4Zn form of insulin was first discovered by Schlichtkrull when preparing crystals in sodium chloride concentration of 6% or more (Schlichtkrull, 1958; Bentley et al., 1976). Contrary to its designation, it is not the stoichiometry of zinc to insulin which is the determining factor in formation of the '4Zn' crystal structure, but rather the overall ionic strength, (Chothia et al., 1983). Specific binding has been shown to be increased in 6% NaCl solution (i.e. about 1.0 M salt) (Tatnell & Jones, 1981) and non-specific binding to glass surfaces has been shown to be abolished at this same concetration (Kaplan et al., 1984). The importance of the B-chain N-terminal conformation change with respect to solution conformation of the monomer is not known, but there is some reason to believe it may be present in solution (Blundell et al., 1982).

Molecular dynamics simulations using energy minimization algorithms have been performed on the 2Zn monomers, resulting in a common low energy configuration that closely resembles molecule 1 (Wodak et al., 1984). These findings were qualitatively confirmed by another group (Kruger et al., 1987), though the similarity of 2Zn molecules 1 and 2 to each other and to 4Zn molecule 1 is perhaps sufficient testament to the thermodynamically stable conformation.

It should be noted in passing that one study has suggested that a loss of helical content in the insulin molecule upon dissolution of the dimer to the monomers (Pocker & Biswas, 1980). The helixes postulated as being affected are in the A-chain, involving residues 1-4 or 12-19. The

results of this study, which really pushes the limits of the technique employed (CD) as far as signal-to-noise is concerned, has not been substantiated as yet. Blundell & Wood (1982) have suggested that the technical limitations coupled with possible adsorption of insulin to container surfaces may invalidate this study.

2.4.1 Adsorption Phenomena of Insulin

At the very dilute physiological concentrations where the free monomer is the predominant species, insulin is known to strongly adsorb to many different surfaces. Cuatrecasas & Hollenberg (1975) showed that ^{125}I-labelled insulin could bind to a wide variety of materials in both a non-specific and specific manner. Specific interactions with talc were found to be saturable, specific, reversible, and of high affinity, all of these characteristics being common criteria reserved for hormone-receptor binding phenomena. Binding was also observed with silica, protein-agarose derivatives and glass test tubes. The study of insulin adsorption to glass tubes was extended by this group (Hollenberg & Cuatrecasas, 1976), but the effect did not receive attention again until recently. Studies of the concentration-dependence of functional group reactivities have been done in vessels of various materials (Kaplan et al., 1984). For each material tested (pyrex glass, Tefzel, polystyrene), functional group reactivity was effectively abolished below insulin concentrations of 1.0 μM, although there was some variation as to which functional groups were affected first. These experiments were done in 0.1 M KCl, but when the salt concentration in the glass test tubes was increased to 1.0 M, no reactivities were observed to disappear completely, even at 10^{-7} M. Thus the interaction of insulin with glass surfaces appears to have a major electrostatic component, which can be

eliminated by charge shielding from high salt concentrations. With glass adsorption eliminated, concentration-dependent reactivities could be ascribed to dimer-monomer transitions (Kaplan et al., 1984), and structure-function studies of the insulin monomer were now made possible (Hefford et al., 1986).

The binding characteristics of insulin to synthetic surfaces reveals that the molecule has noteworthy adsorptive properties, and it is natural to ask whether or not these are of any physiological significance. The ion content of blood plasma is dominated by dissolved sodium and its counter-ions, chloride and bicarbonate, with an osmolarity of about 300 mOsm. Near the electrical double layer of the plasma membrane the ionic strength must be greater (McLaughlin, 1977). As noted above, the insulin monomer in crystal form can undergo ionic strength-induced reversible conformational changes, even in the restrictive environment of the crystal lattice (Bentley et al., 1978), and these may be important in the stabilization of a receptor-binding conformation.

Insulin is known to have effects on the membranes of many receptor-containing cell types, affecting translational diffusion of lipids and membrane proteins (Stuschke & Bohar, 1985) as well as lipid structure (Murray & Nelson, 1981; Hyslop et al., 1984). Although there is no evidence that these effects are mediated by anything else but the hormone-induced activation of the insulin receptor, the binding process itself can be altered by the membrane structure (Ginsberg et al., 1982), a finding consistent with possible membrane involvement in the receptor recognition step.

Studies concerned with the feasibility of using liposome-entrapped

insulin as a therapeutic vehicle for diabetes would certainly benefit from a thorough knowledge of insulin-membrane interactions, but there a very few published reports dealing with this question. At concentrations where some dimer would still be present (2.75 uM), insulin was found to increase the permeability of planar PC bilayers to glucose (Kafka, 1974). The increase in permeability was modest (2-3 fold) and could not be increased by having insulin present on both sides of the membrane. Wiessner & Hwang (1982) found that at concentration between 2.7×10^{-4} and 1.7×10^{-5} M, insulin was found to bind to small unilamellar vesicles (SUVs) made of either DMPC or DPPC. The binding was temperature-dependent, becoming negligible at temperatures approaching the T_c of the SUVs. Insulin did not bind to large multi-lamellar vesicles (MLVs), and binding was also adversely affected by increasing amounts of membrane cholesterol. The conclusion was that binding was dependent on the packing of the PC acyl chains, especially insofar as they may be accessible to the solvent in such a highly curved structure. Since binding was only observed below physiological temperature, at greater than physiological concentrations, and with lipid structures of marginal physiological significance, the results of this study cannot rule out an effective insulin-membrane interaction in vivo.

Monolayer studies using a variety of natural and synthetic lipids found that penetration of monomeric insulin (ca. 10^{-7} M) into a monolayer was dependent on the initial surface pressure (Schwinke et al., 1983), as has been found for many proteins (e.g. Kimelberg & Papahadjopoulos, 1971). Decreased penetration of insulin to the air-water interface was observed when saturated phophatidylcholines were employed. In the case of DPPC, this effect could not be reversed by

addition of 33 mol% cholesterol, even though insulin could readily penetrate cholesterol monolayers alone. Phospholipid headgroup types did not affect the surface active behaviour (PC = PS = PI, with unsaturated acyl chains) with the exception of phosphatidic acid (PA), which excluded insulin from the interface. Thus, although hydrophobic interactions are of primary importance in the interaction of insulin with monolayers, it is not the only one, and undefined polar group interactions may also be important.

The finding by Schwinke et al., (1983) with respect to PA is intriguing because PA-containing liposomes can be induced to fuse with the addition of monomeric insulin between pH 3.0 and 5.3 (Farias et al., 1985). Binding of insulin to mixed lipid liposomes with concomitant decreases in membrane 'fluidity' (or lipid 'order'), and fusion were all influenced similarly by temperature and salt concentration (Farias et al., 1986). These effects were enhanced when DMPC was substituted for EPC, but were abolished when pure EPC vesicles were used.

The differential findings when EPC was used as lipid have been further complicated by a demonstration that mixed EPC/cholesterol (4:1 mol/mol) will bind insulin monomers (Hianek et al., 1987). Using bilayer lipid membranes (BLM), insulin was found to increase the negative surface charge (at pH 7.4) on the cis- side of the membrane, concomitant with a cooperative decrease in the modulus of elasticity and the related dynamic viscosity of the acyl chains. These latter effects can be interpreted as resulting from insulin binding to the bilayer and causing a local change in the membrane structure that facilitates further insulin binding. It is difficult to assess the importance of these results: the method is based on electrostriction of the membrane,

induced by an alternating transmembrane voltage, with simultaneous measurement of the first three harmonics of the induced transmembrane current. The original references are published in Russian (Hianek et al., 1987, and references therein) and the precision of this biophysical technique is not known.

In summary, the information on the possible interactions of insulin with bilayer membranes is quite limited. This may be related to the technical difficulties in studying proteins in very dilute solution and to the fact that insulin monomers adsorb strongly to the surfaces of the containing vessels, complicating the interpretation of results.

2.4.2 Experimental Approach

As noted above, the presence of 1.0 M salt prevents adsorption to the reaction vessel surface (Kaplan et al., 1984, Hefford et al., 1986) and makes it possible to study insulin at monomer concentrations. The competitive labelling procedure, using FDNB can be used to probe several sites on the insulin molecule. Of the nine possible labelling sites in the insulin molecule, two (Tyr-B16 & Tyr-B26) are present on the dimer-forming surface (Blundell et al., 1972), and two (Gly-A1 & Tyr-A19) are adjacent to it, part of the proposed receptor binding region (Pullen et al., 1976). Three residues are located on the hexamer-forming surface (Tyr-A14, Phe-B1, His-B10) (Dodson et al., 1983), and two remaining residues are not associated with any known functionally distinguishable surface (His-B5, Lys-B29). The large number of nucleophiles in insulin are distributed widely over the protein surface, so it should be possible to determine whether a specific region of the molecule is involved or whether the process is a non-specific physisorption.

2.5 GLUCAGON

Glucagon is a peptide composed of 29 amino acids, and in terms of its physical chemistry, is the best characterized member of a family of related hormones, including secretin, VIP, gastrointestinal inhibitory peptide (GIP), and others (Bodanszky & Bodanszky, 1986). Despite the impressive sequence homology between these regulatory peptides (Table II) they do not all cross-react at the level of their adenylate cyclase-activating receptors. Rather, cross reactivities are restricted to members of subgroups of this peptide family. Glucagon forms one subgroup, GIP another, and secretin, VIP, PHI and GHRF form the third group (Rosselin, 1986). However, while glucagon stimulated adenylate cyclase activity, mediated by a specific receptor and a GTP-binding protein, has long been a paradigm of hormone second messenger systems, the actual situation is far more complicated. In 1986, Wakelam et al. demonstrated that liver cells possess, in addition to the classic cAMP second messenger system, a second signal transduction mechanism involving phosphodiesterase-catalyzed formation of diacylglycerol and inositol-1,4,5-triphosphate. At physiological concentrations, it is this newly discovered system which is of primary importance in signal transduction (Peterson & Bear, 1986). This finding requires a re-evaluation of all structure-function studies that have been done to date on the glucagon family of hormones.

The dual hepatic receptor system is also significant for the observation that fragment glucagon$_{19-29}$ has been observed to specifically inhibit the liver Ca^{++} pump without effecting adenylate cyclase activation (Mallet et al., 1987). Proteolytic cleavage on the C-

TABLE II: Sequence comparisons in the glucagon hormone family[a].

Residue #:	5	10	15	20	25
Glucagon:	H S Q G T - F T S D Y - S K Y L D - S R R A Q - D F V Q W - L M D T				
Secretin:	H S D G T - F T S E L - S R D R D - S A R L Q - R L L Q G - L Vamide				
VIP:	H S D A V - F T D N Y - Y R L R K - Q M A V K - K Y L N S - I L N				
PHI:	H A D G V - F T S D F - S R R L L - G Q L S A - K K Y L G - S L Iamide				
GIP:	Y A E G T - F I S D Y - S I A M D - K I R Q Q - D F V N W - L L A...				

[a]Amino acids are designated by their standard IUPAC one-letter symbols. Charged side chains are indicated by (*) above, hydrophobic side chains by (^) below.

terminal side of basic residues is a common occurrence in the processing of polypeptide hormones (Docherty & Steiner, 1982) and cleavage of glucagon C-terminal to Arg-17 & 18 requires a new appreciation of the numerous regulatory activities that glucagon may give rise to. It is useful to note here that glucagon may also be a neurotransmitter, since glucagon immunoreactivity has been found in various brain regions, including hypothalamic synaptosomal fractions (Sasaki et al., 1985). These revolutionary findings occur hard on the heels of the discovery that fragment glucagon$_{1-6}$ is not active in the in vitro liver adenylate cyclase assay (Pelton et al., 1983), as had been previously thought, and upon which much speculation had been built regarding glucagon's structure-function relationships (Blundell et al., 1982; Rodbell, 1983).

2.5.0 Crystal and Solution Structures

The 48% and 32% sequence homology of secretin and VIP to glucagon may be significant in the hormonal regulation of the diacylglycerol-inositol-1,4,5-triphophate effector system. Further physico-chemical studies on glucagon should therefore be carried out, as they must form the basis for any comparative biochemical analysis of secretin and VIP. The importance of such studies in providing a foundation of knowledge lies chiefly in the fact that the information obtained will be relevant regardless of the relative completeness of understanding of hormonal signal transduction at the molecular, cellular, or clinical levels.

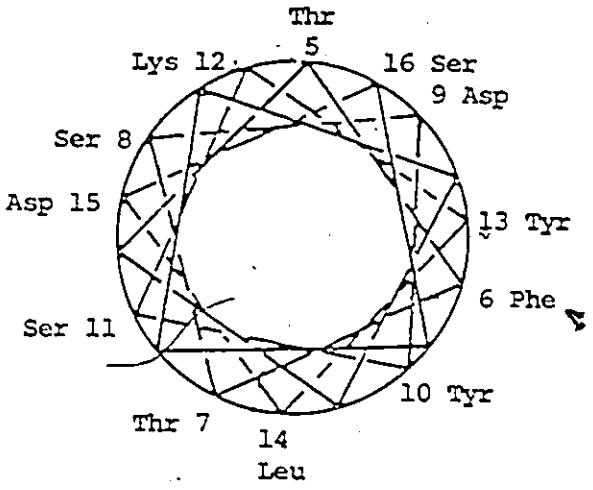
Like insulin, glucagon is a pancreatic hormone, though synthesis and storage, in the form of amorphous zymogen granules, takes place in the alpha cells, rather than the beta cells (Blundell et al., 1982). The non-crystalline form of the granules is a likely result of the extreme mobility of the monomeric unit, particularly the side chains, and has

been the source of many problems associated with its crystallization for the purpose of X-ray diffraction (Sasaki et al., 1975).

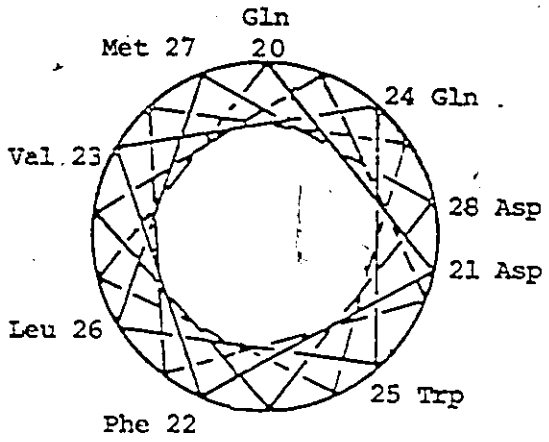
Two crystalline forms of glucagon are known and have been resolved to 3.0 Å resolution (Sasaki et al., 1975). In each case the unit cell is built up from four trimers, in which the glucagon monomer is in a helical configuration. The primary sequence of glucagon confers secondary amphiphilicity in two regions of the molecule (Figure 9). In trimer 1, both hydrophobic patches are involved in stabilization of the complex, with heterologous contacts being made between the region involving Phe-6, Asp-9, Tyr-10 & -13, and the region involving residues Arg-18 (the methylene groups), Phe-22, Trp-25, and Leu-26 (Blundell, 1983). This order of contacts results in a three-sided closed loop structure, with hydrophilic side chains extending into the centre (Figure 10).

The intramolecular forces stabilizing trimer 2 are homologous hydrophobic contacts between residues Ala-19, Phe-22, Val-23, Trp-25, Leu-26 and Met-27. These contacts hold the carboxy-termini of the three monomers in close proximity, forming the apex of a tripod-like structure of which the amino termini form the base (Figure 11). Progressing from the apex of the trimer towards the amino termini, there is an increase in main chain flexibility starting at Arg-17 & -18 which continues to the point where residues 1-5 are highly mobile, even in the crystalline state (Sasaki 1975). This disorder may explain the amorphous nature of the precipitated glucagon in its zymogen storage form. It should be pointed out that between residues 1 and 18 an alpha-helical structure is not required for the maintenance of the trimer 2 configuration (Blundell, 1983), although prediction methods do indicate a high

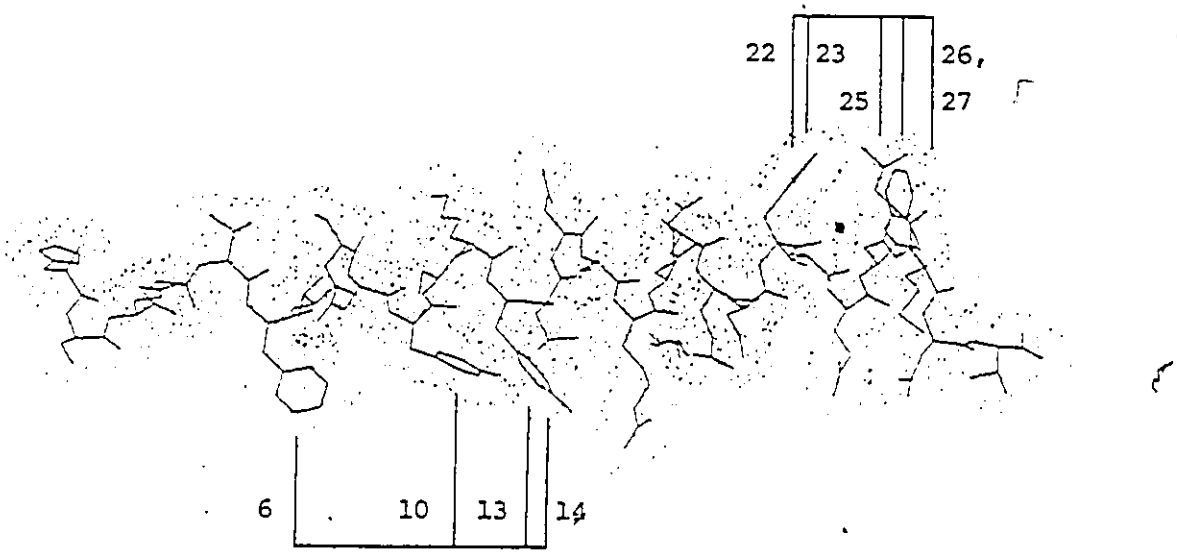
Figure 9. Amphiphilicity in glucagon. Helical wheel diagrams of the N- and C-terminal segments of glucagon, (A) and (B), respectively. (C) The position of the hydrophobic side chains (numbered according to residue) in the helical monomer of glucagon. Coordinates were obtained from the Brookhaven Protein Data Bank and displayed on an Evans and Sutherland PS 300 colour graphics workstation. Van der Waal's radii generated by FRODO.


Thr
5
Lys 12
16 Ser
9 Asp
Ser 8
Asp 15
13 Tyr
6 Phe
Ser 11
10 Tyr
Thr 7
14
Leu

A


Gln
Met 27
20
24 Gln
Val 23
28 Asp
21 Asp
Leu 26
25 Trp
Phe 22

B


22
23
26,
25
27
6
10
13
14

C

Figure 10. The crystallographic structure of the glucagon type 1 trimer, in which the protomers are joined via heterologous contacts. The trimer is viewed down the three-fold axis of rotation (from Blundell, 1983).

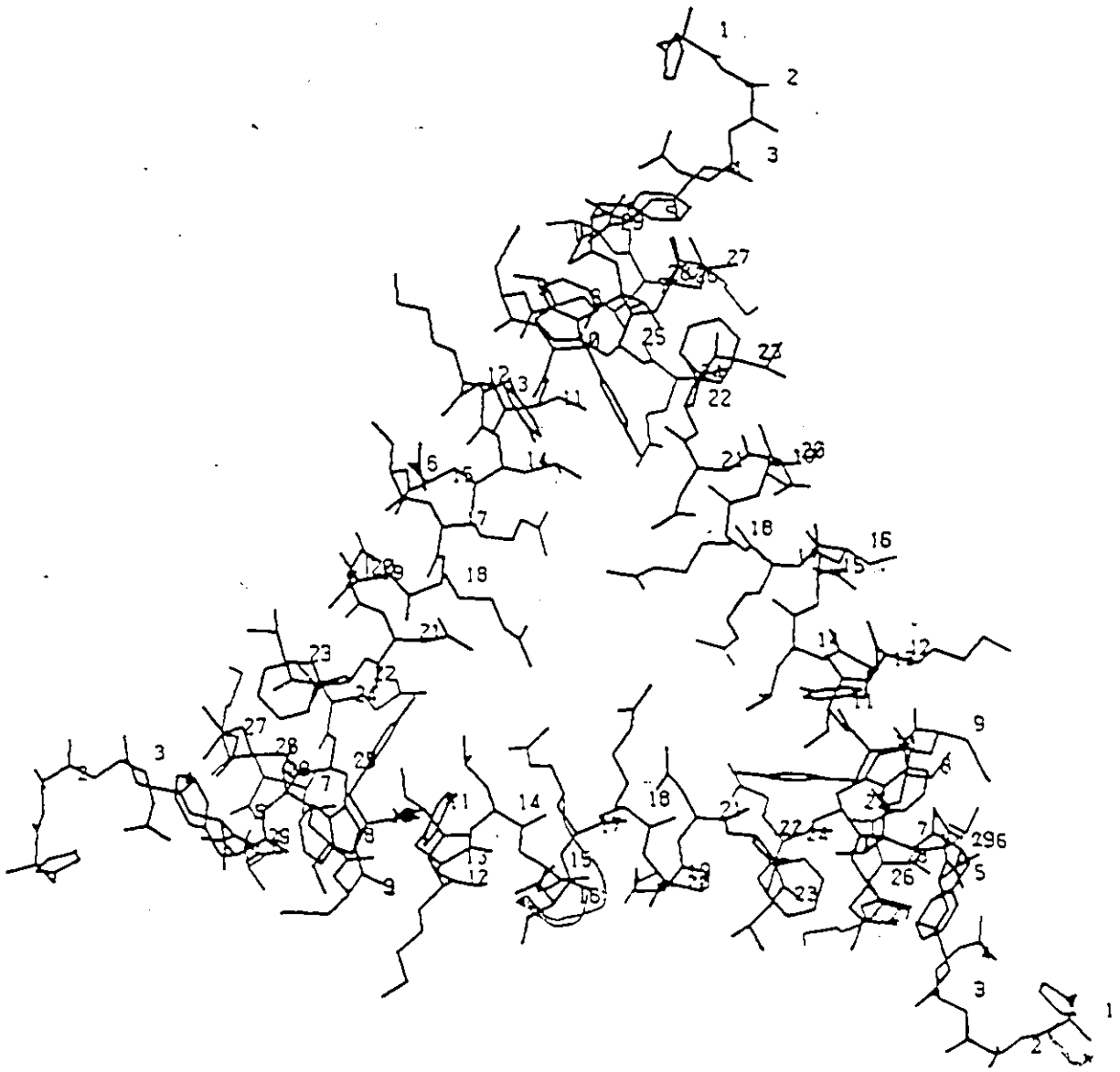
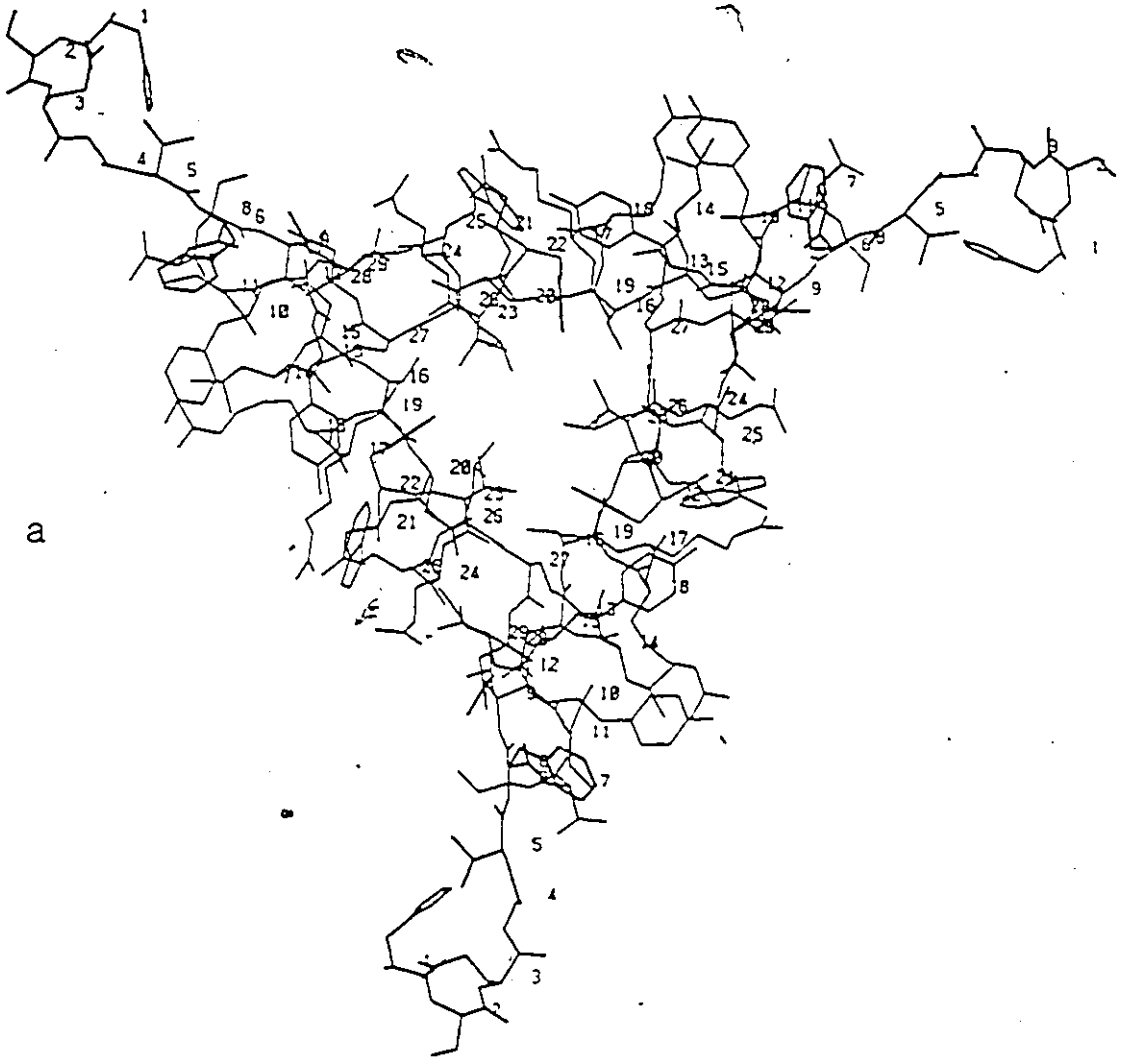
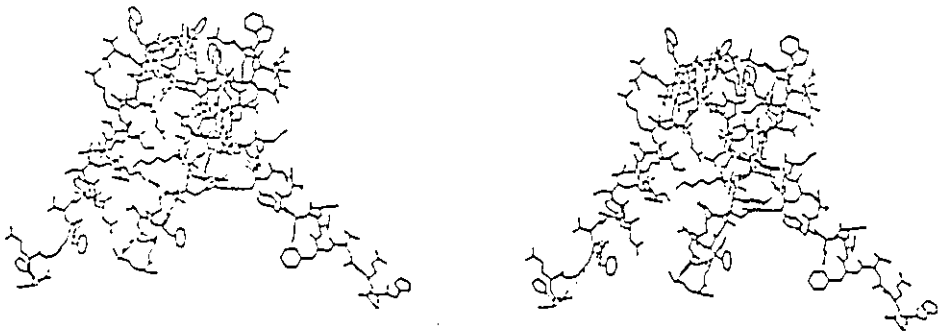


Figure 11. The glucagon type 2 trimer, viewed down the 3-fold axis of rotation (a), and perpendicular to this axis (b) in stereo projection. (b) was generated by FRODO as described in figure 9.

a



b



probability of ordered structure in residues 5-10, either beta-sheet (Chou & Fasman, 1975) or alpha-helix (Schiffer & Edmundson, 1970; Lim, 1974).

The value of the trimerization constant of glucagon at physiological pH and temperature values has not been reported. At pH values between 3.5 and 8.5, the solubility of glucagon in less than 50 ug/mL (1.43×10^{-5} M), while at extremes of pH (less than 3.0 and greater than 9.0) solubility is greatly increased to about 2.87×10^{-3} M (Bromer, 1972). The assumption that the monomeric form predominates at concentrations below 3 mg/mL (i.e. 8.6×10^{-4} M) (Panijpan & Gratzer, 1974) is probably based on structural changes accompanying aggregation of glucagon trimers. Recent studies have all been performed at pH 10.6 and 25°C and give a range of values. Formisano et al. (1977) have employed CD data to arrive at a trimerization constant of 1.06×10^{6} M^{-2}, though the lowest concentration of glucagon used was about 1.0 mM. A later report from this group (Formisano et al., 1978) used sedimentation equilibrium studies in conjunction with lower concentration CD studies to obtain a constant of 2.2×10^{7} M^{-2}. Johnson et al. (1979) measured the heats of dilution of millimolar solutions of glucagon to determine a trimerization constant of 2.4×10^{5} M^{-2}. Wagman et al. (1980) found a constant of 1.3×10^{6} M^{-2} on the basis of concentration dependent changes in chemical shift. The relevance of these constants to the trimerization of glucagon at physiological pH has not been assessed, though the low solubility of glucagon at intermediate pH values of 6-9 (Sasaki et al., 1975) indicates that glucagon trimerization occurs much more readily at pH 7.5 than at pH 10.6.

Whatever the precise value of the trimerization constant, at

physiological concentrations (below 10^{-10} M) glucagon will be a monomeric 'random coil', probably a mixture of different conformers, the relative proportions of which depend on the solvent, ionic strength of the solution and the internal energy of the system. At higher concentrations (above 10^{-5} M to 10^{-6} M), the trimeric form will predominate (Blundell, 1979). Of the two known trimers, most experimental data support the existence of the type 2 (autologous contacts) trimer in solution. Early CD and ORD studies indicated that an intitial 10-15% helical content could be increased to 35% in more concentrated solutions, though addition of 2-chloroethanol was found to increase the helicity even further, especially with more highly concentrated solutions (Srere & Brooks, 1969). It was postulated that 2-chloroethanol stabilizes a largely helical configuration which probably self-associated. Optically detected NMR studies localized a 15-20% helical content to the C-terminal end of the molecule (Ross et al., 1977).

Proton NMR studies have found that the sequence corresponding to residues 22-25 (Phe-Val-Gln-Trp) possess a non-helical, but quite specific conformation in both a model pentapeptide with a C-terminal leucine and glucagon (Boesch et al., 1978). As this sequence is also found in PTH, the possibility exists that this is an important structural feature with a functional role, and may provide a locus for the initiation of other conformational transitions. Line width broadening of proton NMR resonances are significant for C-terminal residues but not near the N-terminal, indicating low mobility (Wagman et al., 1980). Monomeric glucagon would seem to be 'primed' for specific interactions involving the C-terminal hydrophobic residues while the

absence of similar evidence for structure in the region encompassing the N-terminal hydrophobic patch must be a reflection of disorder in this region (vide supra). It should be noted however that competitive labelling suggests that whatever the structure of the monomer in solution, it is sufficient to perturb the chemical reactivity of the Lys-12 N^{ϵ} amino group, resulting in a lower than average pK (Hefford et al., 1985). This may be the result of hydrophobic interactions of the side chain methylene groups (as per the Korn & Ottensmeyer (1983) model, vide infra) but there are a sufficient number of polar groups in the vicinity to allow for some type of polar interaction as well. This study did not find the reactivity of the N-terminal histidine nucleophiles (the N^{α}-amino and the N^{π}-imidazole group) to differ from that in the model compound histidylglycine, thus supporting the notion of an unordered N-terminal. In general, entropic effects are thought to favour the type 2 trimer in solution rather than the more structured type 1 trimer (Blundell, 1983).

A recent attempt to compile the bulk of experimental findings on glucagon has led to a detailed model for a solution conformation which takes into account secondary structure prediction methods and data from fluorescence quenching, CD, NMR and high resolution dark field EM (Korn & Ottensmeyer, 1983). The molecule is posited to be almost globular, with residues 1-16 being wrapped around a helix involving residues 19-29. A large hydrophobic 'groove' exists between residues Tyr-10 and Phe-22, and a fixed orientation of the amino terminal histidine is proposed. Good steric complementarity between the hydrophobic groove and the aromatic ring portion of guanosine-5'-triphosphate (GTP) suggests that this groove may be a transient binding site for GTP, possibly a prior

and necessary condition for subsequent adenylate cyclase activation. Since high concentrations of GTP tend to inhibit glucagon receptor binding (Rosselin, 1986), this is a reasonable and interesting proposition.

2.5.1 Interactions with Lipids

In addition to concentration and solvent dependency, CD studies on glucagon show that helical content is also sensitive to the presence of amphiphiles, being stabilized by dodecylsulphate (Robinson et al., 1982), lyso-PC (Blundell et al., 1982), and other single chain surfactants (Wu & Yang, 1980). Lipid induced helix formation has also been found in secretin and VIP (Robinson et al., 1982).

Glucagon penetrates surface monolayers composed of the dimyristoyl and dipalmitoyl derivatives of PC and PG in a surface pressure-dependent manner (Hendrickson et al., 1983). Penetration was predictably greater at lower surface pressures, but penetration (measured as the change in surface area) was maximal during the phase transition, indicating that the transient packing defects thought to occur in the membrane structure at this point could be exploited by glucagon.

Monomeric glucagon undergoes structural changes upon interaction with micellar dodecylphosphocholine, forming stoichiometrically well defined complexes with a glucagon:lipid ratios of about 1:40 (Bosch et al., 1980). NMR and ESR data has been presented as showing that the glucagon helix runs approximately parallel to the micelle surface, with the C-terminal half being embedded to the level of the phosphates of the phosphocholine headgroup (Brown et al., 1981). A more complete and detailed conformation of micelle-bound glucagon was subsequently worked out using spatial constraints generated by two-dimensional nuclear

Overhauser enhancement spectroscopy, with bond angle optimization of the resultant structure (Braun et al., 1983). A major conclusion of this study was that glucagon conformation at a lipid-water interface was determined by the topology of the lipid supporting structure to which it was bound. For some reason however, it was also asserted that this membrane-bound form is similar to the conformation adopted when bound to a lipid bilayer (Braun et al., 1983). This is an unreasonable assertion given the relative dimensions of glucagon versus the DDP micelle. These micelles would have an approximate radius of 25 Å (the length of the DDP monomer, Bosch et al., 1980; Brown et al., 1981), whereas the glucagon helix itself measures about 36 Å. Given the small size of the detergent micelles, and the presence of possible binding sites for them on the hydrophobic patches of glucagon, it is not clear which species should be considered the ligand. At the root of this largely semantic argument is the assumption that is made regarding the structure of the micelle, taken to be a sphere with surface polar groups interiorizing the hydrophobic hydrocarbon chains. This model is quite old and was never meant to be taken as anything else but as a physically inaccurate simplification (Hartley, 1936). A more reasonable micelle model would have a poorly defined outer boundary due to dynamic motions of the lipid monomers, and a rough surface which would have water pockets and solvent-exposed hydrocarbon chain segments (Menger, 1984). It is doubtful whether either structure is topologically similar to membrane bilayers in any way.

The rationale for studying the interaction of monomeric glucagon with lipids and other amphipathic molecules was initially the suggestion by Rodbell et al.(1971) that hydrophobic interactions are an important

component of glucagon binding to its membrane bound adenylate cyclase-activating receptor. In earlier studies, no differences in the CD or fluorescence spectra of glucagon were observed when small multilamellar PC liposomes were added to micromolar solutions of glucagon (Schneider & Edelhoch, 1972), yet a number of reports have shown that glucagon does interact with phospholipid and phospholipid vesicles (Epand, 1980, Epand et al., 1981, Ernandes et al., 1983). Structures that are quite similar to serum lipoprotein particles can be formed from glucagon and DMPC when the two are co-solubilized (Epand et al., 1977; Jones et al., 1978). The structure of glucagon in these complexes is only slightly affected by temperature, with decreasing aromatic side chain mobility occurring at lower temperatures. In another study, DMPC-glucagon complexes were found to transform into oligolamellar vesicles when the temperature was raised to the phase transition temperature (Epand et al., 1981). Unfortunately, neither the physical structure of these glucagon lipoproteins, nor the association state of glucagon were determined in these studies. Although glucagon clearly interacts with DMPC, it is premature to make generalizations as to the 'condensing' effect (Epand et al., 1981) of this peptide on membrane bilayers.

In an attempt to identify some of the specific structures important for the interaction of glucagon with lipids, two glucagon analogues were synthesized (Musso et al., 1983). The analogues were made with minimal sequence homology over residues 19-29 but the amphiphilic character of this sequence was maintained. One of the peptides did not displace ^{125}I-glucagon at all, while the other, which retained Phe-22 and Trp-25, displayed reduced but specific binding behaviour. Further studies on the membrane-associated configuration of these peptides would be indicated,

but have not been reported. In general, the specific structural features responsible for the interactions between phospholipid bilayers and monomeric glucagon can be tentatively identified as involving portions of the hydrophobic patches, but uncertainty as to their relative contributions and conformation at the bilayer-solvent interface persists.

2.5.2 Experimental Considerations

The extremely low solubility of glucagon at neutral pH makes the detection and measurement of its chemical and physical properties difficult at physiological pH values. The sensitivity of the physical methods employed to date have required that relatively high concentrations of glucagon (> 10.0 μM) be used (Bornet & Edelhoch, 1971, Schneider & Edelhoch, 1972, Wu & Yang, 1980, Ernandes et al., 1983). In these studies the association state of glucagon was not clearly defined and therefore the results may not always be applicable to the physiologically active monomeric form of the hormone. Application of the competitive labelling procedure to the study of dilute glucagon solutions are possible (vide supra). Extending published studies (Hefford et al., 1985) to lower concentrations in the presence and absence of lipid bilayer surfaces may provide insight into a putative interaction with membrane surfaces. There are five FDNB-sensitive nucleophiles on glucagon that may be probed, and two of these are found in one of the hydrophobic patches (Tyr-10 & -13), with a another on the opposite face of this amphiphilic helix segment (Lys-12). The N-terminal histidine provides two other reporter groups in its N^{α}-amino and N^{τ}-imidazole functions.

2.6 MELITTIN

Melittin is a 26 amino acid peptide that makes up 50% of the dry weight of the venom of the honeybee, Apis Millifera (King et al., 1976). The primary sequences of melittin in all four Apis sp. are homologous for the most part, and substitutions are conservative and complementary (Kreil, 1975). The distribution of amino acids in the primary sequence immediately suggests an amphiphilic character for the molecule, and the high surface activity, comparable to lysolecithin and digitonin (Habermann, 1972), confirms this. Strong interfacial activity is also seen at the membrane surface, conferring upon melittin marked lytic activity. Melittin induces hemolysis and disrupts leukocytes, lysosomes and mitochondria, as well as liposomes made of purified lipids and reconstituted membranes (Sessa et al., 1969; Mollay et al., 1976). The similarity of melittin to the 26-mer delta-hemolysin peptide of Staphylococcus aureus is also remarkable in terms of the distribution of apolar, polar and charged residues (Figure 12). The two peptides have similar lytic behaviour in many ways (Bhakoo et al., 1985), indicating that the overall design is uniquely suitable for membrane-lytic activity. A peptide incorporating the general structural features of these peptides has been synthesized and exhibits similar cytolytic and membrane-lytic activity (DeGrado et al., 1981).

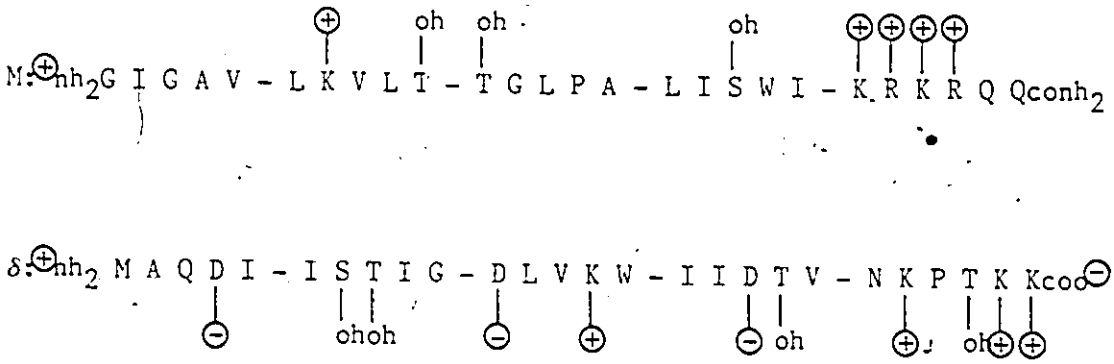
2.6.0 Crystal and Solution Structure

Despite its high content of apolar amino acids, melittin is quite soluble in aqueous solution, oligomerizing at high concentrations in order to shield its hydrophobic side chains.

The tetramerization of melittin is not well characterized, and most

Figure 12. (A) Sequences of melittin [M] and the δ-hemolysin [δ] from Staphylococcus aureus sp., indicating distribution of charged (+, −) and hydroxyl (oh) residues. (B) Drawing of the melittin monomer in the helical conformation as found in the type I and II crystals (from Terwilliger & Eisenberg, 1982a).

A)


M⊕nh2G I G A V - L K V L T - T G L P A - L I S W I - K R K R Q Qconh2
δ⊕nh2 M A Q D I - I S T I G - D L V K W - I I D T V - N K P T K Kcoo⊖
oh oh
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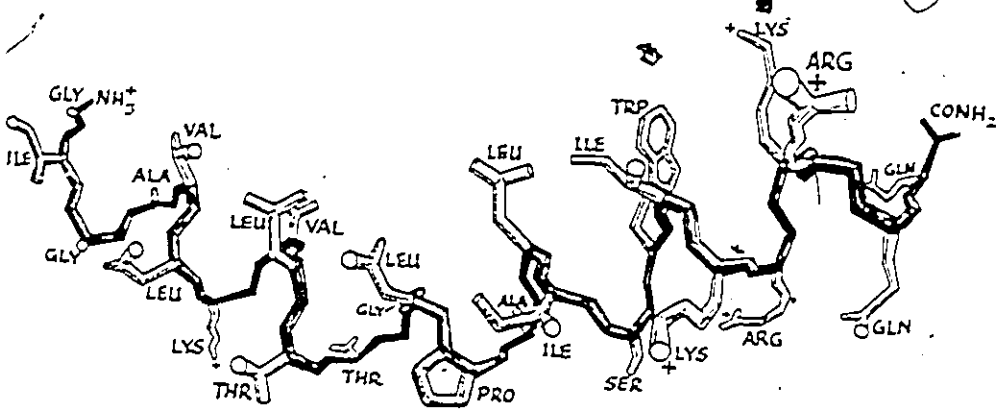
B)


GLY
NH3+
ILE
VAL
ALA
GLY
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LEU
VAL
LYS
THR
THR
LEU
GLY
PRO
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ALA
ILE
TRP
ILE
SER
LYS
+LYS
ARG
ARG
CONH2
GLN
GLN

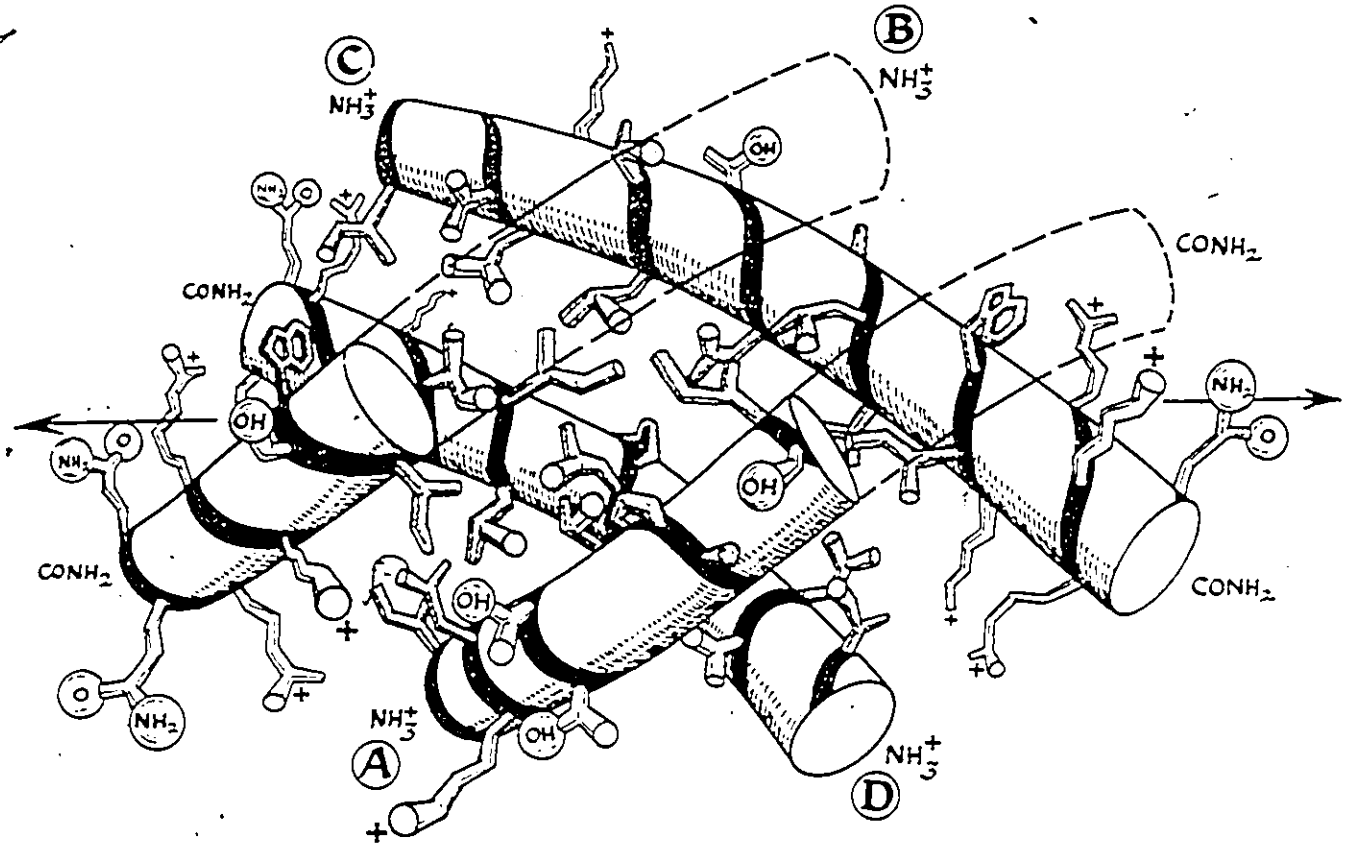
investigators consider the self-association process as a monomer-tetramer transition (Talbot et al., 1979; Quay & Condie, 1983). However, the tetramerization must take place via aggregates of intermediate size, either built up stepwise (eq. 1), or by subunit assembly (eq. 2):

$$4M \rightleftharpoons M_2 + 2M \rightleftharpoons M_3 + M \rightleftharpoons M_4 \qquad (1)$$

$$4M \rightleftharpoons 2M_2 \rightleftharpoons M_4 \qquad (2).$$

In (1) and (2), nM represents a stoichiometric number of monomers, and M_2, M_3, and M_4 represent dimeric, trimeric and tetrameric aggregates of monomer, respectively. In either case the presence of dimers is mandatory (3-body collisions being quite rare). Equation (1) does not seem likely for at least two reasons. The first reason is based on the known crystal structures of the tetramer, which are composed of two equivalent groups of dimers, and not four equivalent monomers (Terwilliger & Eisenberg, 1982a). In both the form I and II crystals, a crystallographic two-fold axis of symmetry relate the AB dimer to the CD dimer (Terwilliger et al., 1982), whereas chains A and B are related by a non-crystallographic two-fold symmetry, the same being true for the C and D chains) (Terwilliger & Eisenberg, 1982a; Figure 13). This implies that the association of A with B is not primarily a consequence of the crystallization process; as a result, the tetramer has pseudo-222 symmetry. The arrangement of the unit cells themselves in the form I and II crystals brings equivalent dimers into close spatial arrangement, their positions being described by simple translation in the direction of their helix axes by a distance of one monomer length (approximately

Figure 13. Drawing of the melittin tetramer showing the relative orientations of the four protomers A, B, C, and D, and the shielding of the hydrophobic side chains (from Terwilliger & Eisenberg, 1982a).



Hydrophobic sidechain

Hydrophilic sidechains

the length of the unit cell in that direction). Thus, the repeating units of equivalent dimers can be viewed as 'strings' or 'ribbons' of dimers, the distance between the ribbons being approximately equal to the monomer width. These ribbons have a hydrophilic and hydrophobic side, and, since (form I) crystals have been observed to grow directly from the surface of melittin-containing droplets (Terwilliger et al., 1982), the latter side has been postulated to be exposed to the atmosphere at the gas-water interface, by analogy to the surfactant behaviour of fatty acids. In terms of structure then, there is no structural evidence for a metastable trimeric melittin aggregate.

A second reason for favouring equation (2) is based on thermodynamic considerations. If dimer formation is at all favourable (as the crystal structure indicates), dimerization would compete with trimerization and tetramerization for monomers according to equation (1). In the absence of any mechanism for the transmission of conformational change (apart from alteration in the chemical potentials of solvent and solute), the only factor that might bear on the cooperative oligomerization of melittin would be the availability of a binding site for successive monomers. The driving force in protein-protein binding is almost exclusively the result of the stabilizing effect of decreasing the free energy of apolar residues on the protein surface. The decrease in free energy, and hence stability of the complex, is proportional to the surface area at the interface between the bound proteins (Chothia & Janin, 1975). Since the cohesive forces in the melittin tetramer are wholly hydrophobic in nature (Terwilliger & Eisenberg, 1982a), the contributions to binding energy are derived from the unfavourable entropy caused by the exposed hydrophobic residues. The

combined decrease in free energy for the apposition of two dimers would exceed the free energy decrease for addition of a monomer to a dimer, so it would be energetically more favourable to appose two dimers than add monomers stepwise onto a dimer to obtain an increasingly smaller gain in free energy. Such a process would mark an uncooperative transition, in which case tetramer formation could only be achieved with difficulty (Klotz et al., 1975), contrary to the observations of experimental workers.

The conformation of melittin in solution is extremely sensitive to the type and concentration of co-solutes. In pure water, free melittin adopts a largely random coil configuration at pH 3.3 (Lauterwein et al., 1980), as judged by the similarity of melittin's ^{1}H-NMR resonances to resonances of a mixture of appropriate model tripeptides. Resonances for residues at positions 1-3, 10-12, 25, and 26 were found to be consistent with a random coil or extended conformation. Differences in chemical shift and coupling constants were found for Val-5 & 8, Pro-14, and the segment Ile-Ser-Trp-Ile (17-20), but were not indicative of helical structure. The observation that deuterium exchange from the backbone amides is complete in a matter of minutes is taken to mean that there is no significant intrachain hydrogen bonding or chain folding. Dawson et al. (1978) have analyzed the alpha-helix and beta-sheet propensities for melittin and have found no general correspondence to the distinctions made between residues according to NMR. This would indicate that some non-random structure is adopted by particular residues, but that it is neither alpha-helix nor beta-sheet. Some residues, for which the NMR resonances are not clearly assignable, are likely in partial alpha helical form. Talbot et al. (1979) detected 12% helix in the monomer,

corresponding to about 3 residues. These residues probably provide the nucleation sites for further helix formation and would be situated somewhere over positions 4-9 and/or 13-17.

Although melittin is monomeric in pure water even at the relatively high concentration of 3mM, even slight changes in the solvent can induce aggregation to tetramers. Increasing the concentration of either melittin, salt, or hydrogen ions will result in a decrease in the Trp-19 fluorescence maximum, an increase in CD ellipticity, and the formation of a higher molecular weight species as measured by gel filtration (Talbot et al., 1979; Bello et al., 1982; Tatham et al., 1983). These results taken together indicate that the peptide undergoes a coil-to-helix transition, and then associates into tetramers. In Tris-HCl buffer (pH 7.5) the maximum helical content for the tetramer was reported at 65% (Talbot et al., 1979), while in phosphate buffer a helix content of 80% was found (Bello et al., 1982). These results, while being qualitatively similar, reflect a real difference in the manner in which counterions can influence the conformation of melittin.

The effect of phosphate anions is remarkably different from simple salts. The potency of phosphate anions to facilitate tetramerization is more than an order of magnitude greater than for chloride ions (Tatham et al., 1983). An explanation for this effect lies in the guanidinium functions of Arg-22 & 24, which likely form doubly hydrogen bonded complexes to the phosphate oxyanion in solution. There is considerable support for such interactions from crystallographic studies of guanidinium-phosphate complexes and from observations of increased helical content in both poly-(L)-Arg and proteins (Cotton et al., 1973, and references therein). The difference between the previously cited

helical contents of 65% and 80% can almost certainly be ascribed to the effect that hydrogen-bonded phosphates would have in reducing the electrostatic repulsions between the neighbouring side chains of the Lys-Arg-Lys-Arg sequence in the C-terminal (residues 21-24). The reduction in effective net charge on the peptide would also facilitate aggregation of the monomers and therefore, tetramerization.

2.6.1 Interactions with Lipids

The manner in which melittin interacts with membranes is not clearly defined, but the known stimulatory effects that melittin has on phospholipase A_2 (Mollay et al., 1976) and guanylate cyclase (Lad & Shier, 1979), suggest that membrane components are rearranged on the scale of binding and active site dimensions (Angstroms), as well as in terms of the previously cited gross morphological perturbations that are implicit in cell lysis.

A number of models for melittin association and insertion into lipid bilayers have been proposed (Dawson et al., 1978; Kempf et al., 1982; Terwilliger et al., 1982; Posch et al., 1983; Vogel et al., 1983; Vogel & Jahnig, 1986; Dufourc et al., 1986a). Not surprisingly, experimental support for all of these models has been obtained at some time or another. It is safe to say however, that the complexity of the melittin-phospholipid association problem has only recently become apparent. This is largely the result of the application of more sophisticated techniques, notably quasi-elastic light scattering (QLS) (Prendergast et al., 1982) and ^{2}H- and ^{31}P-NMR (Dufourc et al., 1986b). These techniques have indicated that melittin-phospholipid vesicle systems are dynamic and heterogeneous, with reversible associations and dissociations of various combinations of the component molecules,

varying with temperature and concentration.

Prendergast et al. (1983) first showed that DMPC vesicles of 750 Å diameter could be broken down by melittin to non-vesicular structures with diameters of 200-300 Å. The effect was dependent on the ratio of lipid to peptide (R_i) and showed variation with lipid type. Negatively stained preparations showed the presence of multiple rouleaux that could be formed by heating the mixtures past the T_c (about 41°C). Raman spectroscopy of the C-C and C-H streching modes of DMPC and DPPC in the presence of melittin at R_i=5 indicated that discoidal particles with high conformational order, decreased acyl chain packing, and lower cooperativity during phase transition (Lafleur et al., 1987). The observations were dependent on the thermal history of the samples, as discoidal DPPC/melittin complexes formed larger structures upon heating. There were noticeable differences in the affinity of melittin for DMPC and DPPC, which were ascribed to the greater stability of DPPC intramolecular chain packing relative to DMPC.

Early ^{2}H-NMR studies of lipid-protein interactions had generally found that lipid conformation is not appreciably altered by the presence of even large amounts of membrane proteins (Seelig et al., 1982). However, melittin was found to induce large R_i-dependent changes in bilayer parameters by ^{2}H- and ^{31}P-NMR (Dufourc et al., 1986b). At $R_i >= 20$, and above T_c, the melittin-lipid system was composed of large (thousands of Angstroms) structures, while below T_c these structures were in reversible equilibrium with much smaller (hundreds of Angstroms) assemblies. With greater amounts of melittin (R_i=4), only the smaller assemblies were observed.

Freeze-fracture EM studies, in conjunction with QLS were used to

characterize the small melittin-DPPC complexes as a function of temperature and R_i (Dufourc et al., 1986a). For R_i between 10 and 30, the diameters of the interconverting discs and vesicles were 100-400 Å and 3000-6000 Å, respectively. With high melittin concentrations (R_i=5.2), the transition was abolished, particle sizes ranging from 100-250 Å. These results imply that the lipids undergo reversible 'disc-to-vesicle' transitions.

DPPC and EPC systems were characterized with QLS, and indicated that lipid type affected the transition (Dufourc et al., 1986). Increasing the melittin concentration ($R_i > 100$), induces the formation of particles with MLV dispersions of DPPC, their maximal radius being proportionately dependent of their initial size. Presumably DPPC is forced, at certain melittin concentrations ($50 < R_i < 100$) to fuse into larger bilayer sheets, reflecting some gel-phase property of the lipid, such as its critical packing shape. Melittin may in this instance be simply a fusogenic agent as it has been observed to do by others at similar concentrations (Morgan et al., 1983). At lower R_i, small discs are again present, visible by EM freeze fracture, their size correlating with the QLS hydrodynamic radius.

Direct information on the protein (i.e. melittin) portion of these systems has come from fluorescence studies of the intrinsic chromophore Trp-19 (Georghiou et al., 1982) as well as fluorophore-conjugated melittins (Hermetter & Lakowicz, 1986; Vogel & Jahnig, 1986). Polarized infrared spectroscopy (Vogel et al., 1983) and chemical crosslinking studies (Hider et al., 1983) have also been employed. As can be judged from the characteristic types of information obtainable by these methods, the properties of the peptide are not resolved at a level much

finer than the size of the entire molecule.

The high resolution ^{1}H-NMR work from the laboratory of K. Wuthrich (Brown et al., 1982) is not included with the above techniques. Their studies on melittin, similar to their studies on glucagon (vide supra), involve the use of non-physiological amphiphiles (e.g. DDP) to form well-defined melittin-amphiphile complexes. Such studies clearly generate useful and detailed information (perhaps most importantly in developing methodologies to study lipid-protein interactions) at the level of those individual side chain spin systems for which assignments can be made. However, because of the flexible nature of both the protein and lipid species involved, it is impossible to pass beyond generalities about the relative perturbation of particular residues in the lipid complex.

2.6.2 Experimental Considerations

Unlike insulin and glucagon, the interaction of melittin with lipids has generated an extensive literature. No consensus on the mechanisms of melittin-induced bilayer perturbation exists, despite a number of available models (Bernheimer & Rudy, 1986). In one model, the Gly-1 amino terminus is believed to extend into the hydrocarbon phase of the bilayer (Terwilliger et al., 1982), while in another the Lys-7 N^{ϵ}-amino groups is placed in this region (Tostéson & Tosteson, 1981). Competitive labelling studies of melittin at different concentrations, and in the presence and absence of liposomes should be able to differentiate between these two models, as well as provide information on the tetramerization of the peptide. In addition to probing the Gly-1 and Lys-12 amino groups, the two lysines at positions 21 & 23 can be used as reporter groups for the C-terminal segment of the peptide.

3.0 EXPERIMENTAL PROCEDURES

3.1 Materials

The following compounds were obtained from Sigma Chemical Co. (St. Louis, MO): glucagon, porcine pancreatic insulin, melittin, pepsin, egg phosphatidylcholine (type V-E), 1-fluoro-2,4-dinitrobenzene, all amino acids and peptides and their derivatives unless otherwise stated. The purity of the porcine pancreatic insulin was verified by reverse phase HPLC. Purity of the melittin was assessed by amino acid analysis. Purity of the EPC was checked by thin layer chromatography (TLC) using chloroform/methanol/28% ammonia (65:25:5), and found to be free of lysoPC. All of the above named Sigma products were used without further purification. Ultrapure urea (Schwarz/Mann, Cambridge, MA) was used. Water used in HPLC was distilled, deionized and filtered through a 0.45 um membranes from Millipore. The acetonitrile and methanol used as organic modifiers for HPLC were HPLC grade and obtained from either BDH, J.T. Baker, or Caledon. All other chemicals and solvents used were reagent grade or better. [^{14}C]-FDNB and [^{3}H]-FDNB were obtained from Amersham Corp.(Oakville, Ontario, Canada) and NEN Canada (Lachine, P.Q., Canada) supplied the Aquasol-2 for scintillation counting.

A standardized buffer was adopted for the aqueous phases in both liposome preparation and incubation of the protein-liposome systems. It consisted of 5 mM Na_2HPO_4 at pH 7.5, with KCl present at either 1.0 M (insulin experiment) or 0.1 M (glucagon and melittin experiments).

3.2 Equipment

High voltage paper electrophoresis (HVPE) was carried out on equipment based on the original apparatus of Michl.

Samples were spotted on Whatman 3MM paper, and run in a pH 2.1 buffer composed of formic acid/glacial acetic acid/ dH_2O (1:4:45, v/v).

Thin layer chromatography of DNP-amino acids was carried out in Gelman chromatography chambers, using silica-coated plastic sheets (20 cm x 20 cm) from Eastman Kodak.

Two high-pressure liquid chromatography (HPLC) systems were employed during the course of this work. HPLC system #1 consisted of Beckman HPLC pumps, models 110A and 112 and model 421 pump Controller. The injection port was made by Altex, and a Hitachi 100-40 variable wavelength spectrophotometer was used in conjunction with a Spectra-Physics SP4270 Integrator.

HPLC system #2 consisted of Waters/Millipore solvent delivery modules, injection port and dual wavelength spectrophotometer interfaced with a Digital Corporation controller/data collection station.

Fractions were collected in polypropylene wells with a Gilson FC80 MicroFractionator.

3.3 Sample Preparation

Liposomes and protein solutions were prepared separately. Large unilamellar liposomes were made by two methods: the reverse phase evaporation (REV) method of Szoka & Papahadjopoulos (1978) was used for the preliminary, insulin, and glucagon experiments (sections 4.1, 4.2 and 4.3, respectively), while a method based on vesicle extrusion (Hope et al., 1985) was devised for the melittin experiment (section 4.4).

3.3.0 Reverse-Phase Evaporation Liposomes Egg PC (120 µl and cholesterol (60 µl) were dissolved in $CHCl_3$/MeOH, combined, and dried under nitrogen. After redissolving in 4.5 ml of peroxide-free ether, 1.5 mL of the appropriate standard buffer was added, and the two-phase

system sonicated with a Branson sonicator using a needle tip probe set at maximum allowable power in spaced bursts of 1 minute duration. The temperature of the preparation during sonication was maintained at room temperature with a water bath. The ether was then removed on a Buchi rotary evaporator at 37-40°C under aspirator-reduced pressure. The process was continued for 5 minutes past the point at which ether could no longer be detected by odor. The preparation was diluted with 1.5 mL buffer, further evaporated (30 min) and set aside as a stock liposome solution.

Liposomes as prepared by this method are predominantly unilamellar (Szoka & Papahadjopoulos, 1978). Examination of negatively stained samples (uranyl acetate, 2%) by electron microscopy (micrographs not shown) indicated most liposomes (> 85%) had a diameter of 250 Å, the remainder being larger, up to 1500 Å or more.

Although the REV method produced acceptable liposomes in terms of their characterization by negative staining, it was felt to be somewhat cumbersome, requiring a degree of coordination between reagent preparation and equipment availability that was difficult to manage in our laboratory. Additionally, although the procedure outlined above significantly reduced the possibility of ether contamination of the finished product (Allen, 1984), a preparation method that avoided the use of organic solvents altogether was felt to be desirable.

3.3.1 Extrusion Liposomes The second procedure used for LUV formation arose from attempts to duplicate the rapid extrusion technique developed in the laboratory of P. Cullis (Hope et al., 1985). This technique involves forcing an aqueous dispersion of large multilamellar vesicles through thin polycarbonate filters of defined pore size, using

nitrogen pressures of 100-500 lb/in^2 as the driving force. Liposomes for the melittin experiment were made using an HPLC solvent pump, injector, and pre-column inline solvent filter, with sample collection after the filter and before the column (Figure 14a). The inline filter (Upchurch Scientific, Inc.) consisted of a passivated stainless steel frit (pore size 0.5 μm, thickness 1.6 mm) contained in a stainless steel fitting (Figure 14b).

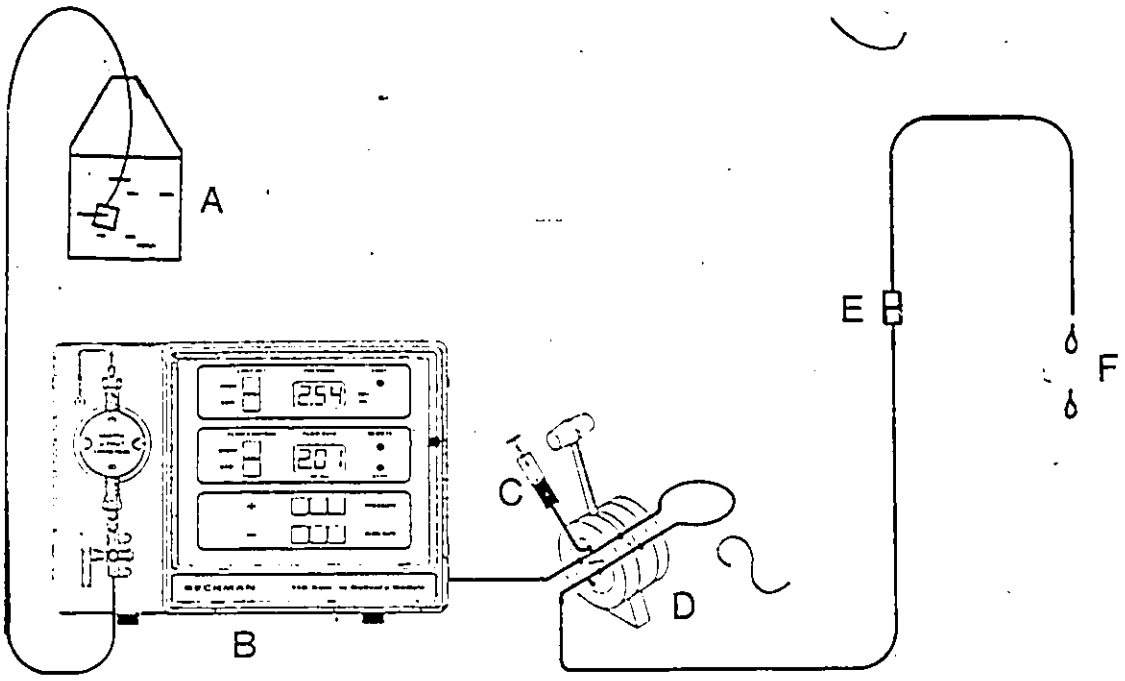
For a typical liposome preparation, egg PC and cholesterol (2:1, mol/mol) were 'shelled' on bottom of a glass test tube (16 x 125 mm) using nitrogen gas. Care was taken to confine the dried lipid to only that part of the tube that would be in contact with aqueous solution when 1.0 mL of standard (see above) buffer would be added to the tube. The buffer was added and the dispersion was vortexed for one minute. A measured volume of this mixture was then taken up into a calibrated syringe and loaded into the sample loop of an HPLC injector. With an ongoing flow rate of 1.0 mL/min, a polydisperse MLV suspension was injected into the flow path and forced through the frit at operating pressures, breaking down the large multilamellar and other hydrated lipid structures, and producing large unilamellar vesicles generally no larger than the pore size of the frit (0.5 μm) (Figure 15). Depending on the sample loop size, volumes of 0.25 to 2.0 mL could be injected in this manner.

Fractions of LUV suspensions prepared in this way were collected as a function of volume and analyzed. Measurements of absorption at 500 nm (i.e. turbidimetry) indicated that dilution of the lipid during the extrusion process was minimal, with the injected lipid being recovered in a volume 1.5-2.0 times the initial sample volume. Recovery of PC was

Figure 14a. Apparatus used to make extrusion LUVs. (A) buffer reservoir, (B) HPLC pump, (C) syringe for loading MLV dispersion into sample loop of injector, (D) injector, (E) inline solvent filter containing porous steel frit, (F) eluate containing LUVs.

Figure 14b. Specifications (in inches) of the inline solvent filter (A) and replaceable frit (B). The outer part of the frit is made of Kel-F plastic, and the central filter is made of passivated stainless steel, with pore sizes of 0.5 micron.

a)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b)

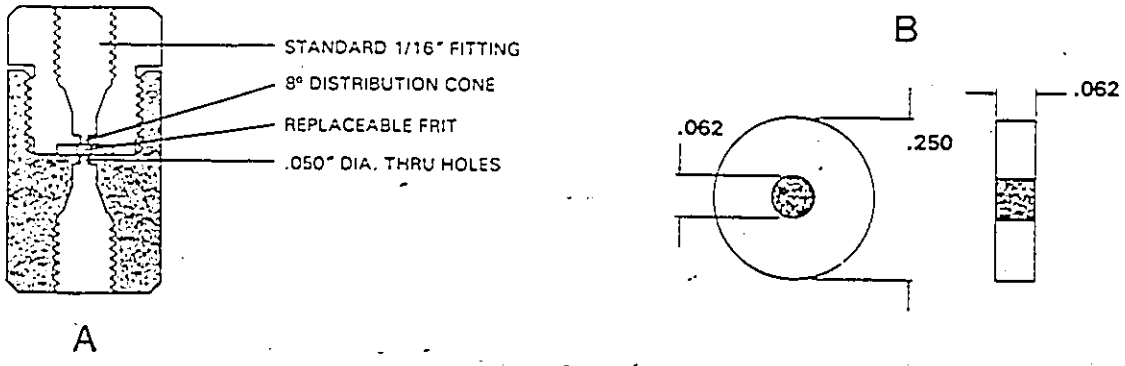
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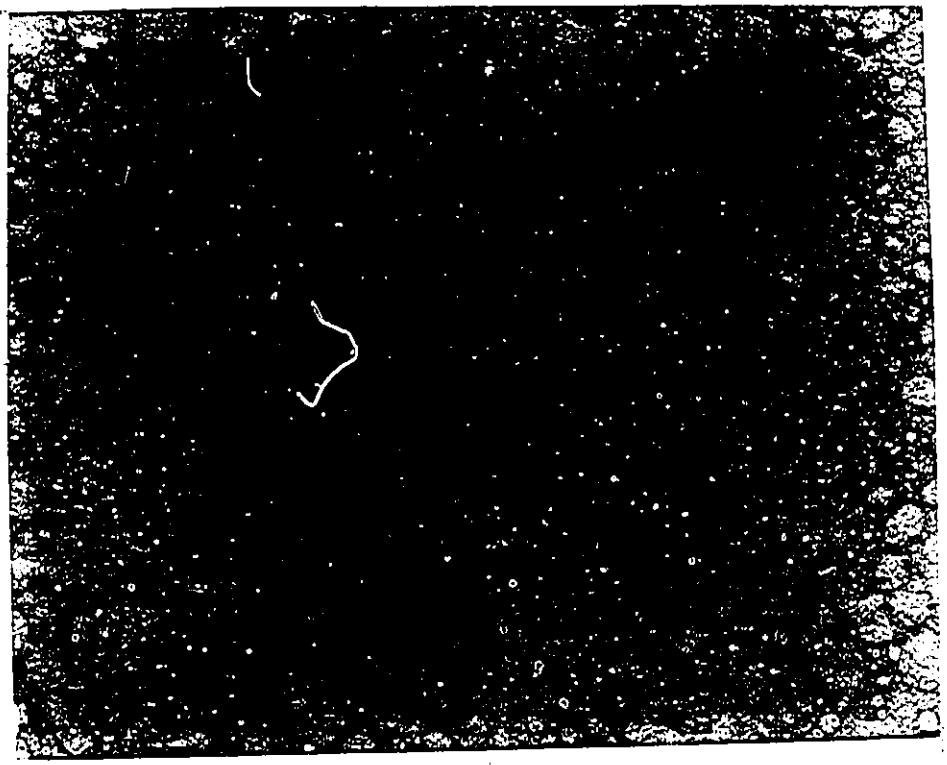
Figure 15. Electron micrographs of negatively stained liposome preparations made of EPC:cholesterol (2:1, mol/mol). (A) Multilammellar vesicles obtained upon hydration of the dried lipid and vortexing. Total magnification: 13125 X. (B) Same preparation as in (A) after passage through the porous steel frit shown in figure 14. Total magnification: 39375 X.

A

B



quantitive by phosphate analysis and choline determination (Kates, 1986). Cholesterol recovery was also found to be quantitative according to a modified Zak procedure (Zak, 1957). Trapped volume measurements were made by including ^{14}C-glucose into the buffer prior to extrusion and dialyzing out unencapsulated glucose afterwards. With an initial lipid concentration of 5 mg/mL, the average captured volume was 3.5 +/- 2.3 µL/umol lipid (n = 11). Starting with an initial lipid concentration of 100 mg/mL, the captured volume was 4.8 +/- 0.2 µL/umol lipid (n = 4). These results compare very well with those obtained by Hope et al. (1985).

3.3.2 Protein Solutions

In general, stock solutions with equimolar concentrations of the protein under study and the corresponding internal standard(s) were made up in the appropriate buffer (section 3.1). When necessary, working solutions were made from the stock solution and aliquots were then transferred to screw cap Pyrex test tubes to give the desired final concentrations. Except in the case of the melittin experiment, the remaining stock solutions were lyophilized in preparation for ^{14}C labelling. For the melittin experiment a fresh solution of equimolar peptide internal standard was made up.

For liposome containing samples, aliquots of stock liposome preparation (vide supra) were added to aliquots of the respective (protein + internal standard) solutions and made up to a final volume of 2.0 mL with buffer. The pH of the samples was fine-adjusted if necessary with 1.0 N NaOH or 1.0 N HCl to pH 7.5. For the control samples, buffer was substituted for the liposome preparation. Before ^{3}H-trace labelling, all samples except those for the melittin experiment

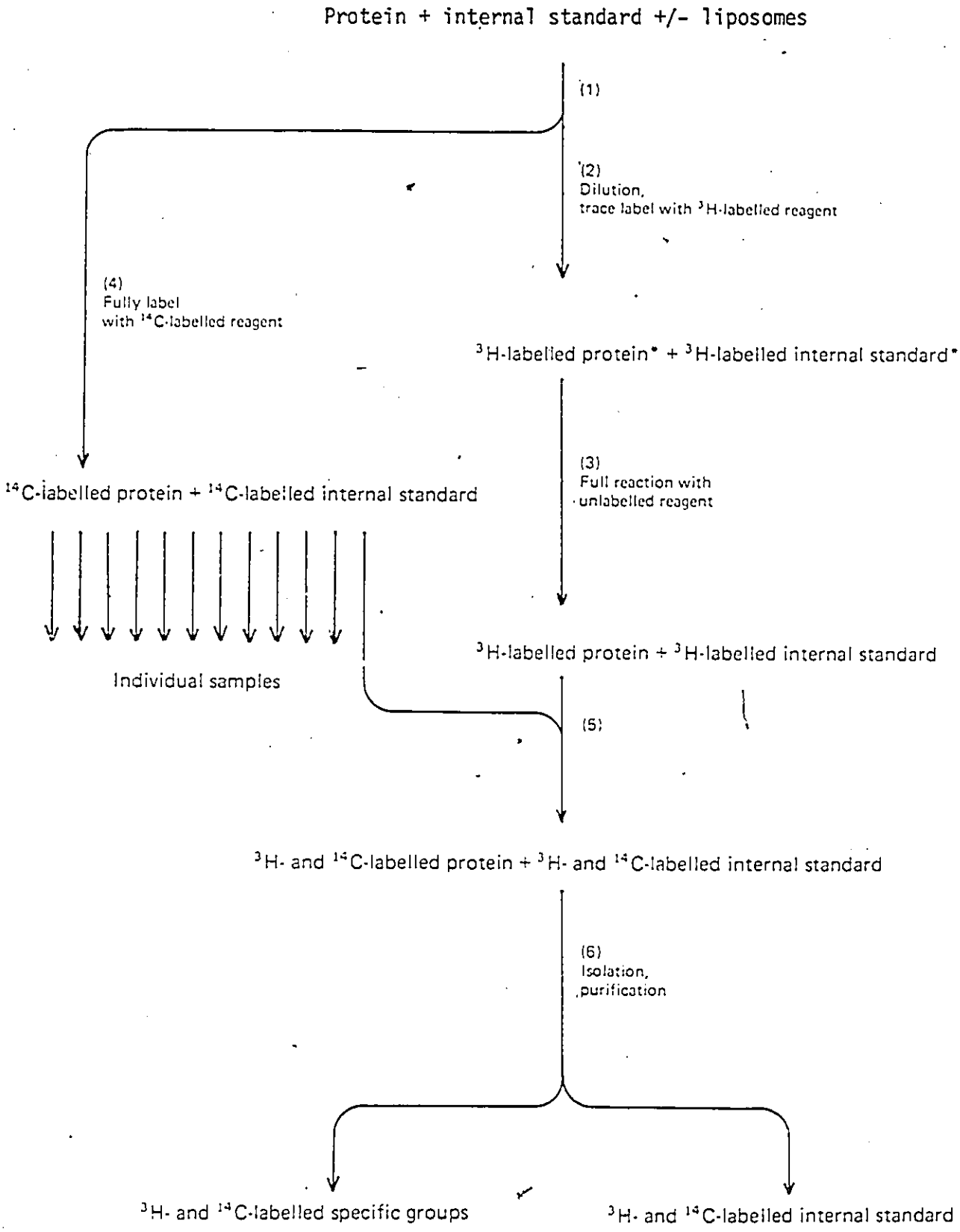
were incubated for 3 h in a 37°C temperature-controlled water bath with gentle agitation to ensure equilibrium for any protein-lipid interaction. Melittin samples were incubated for 15 minutes at room temperature. This incubation period was chosen because it corresponds to the endpoint of the initial 'fast phase' of hemoglobin release from erythrocytes under melittin induced lysis (DeGrado et al., 1982). After the incubation period, trace-labelling was commenced.

3.4 Competitive Labelling.

A schematic diagram of the competitive labelling procedure as employed in these experiments is shown in Figure 16.

3.4.0 [^{3}H]-Trace-Labelling In the preliminary experiment, as well as the insulin and glucagon experiments, an aliquot (10 µL) of acetonitrile containing [^{3}H]-FDNB (1.09 nmol, specific radioactivity 16.6 Ci/mmol) was added to each sample with vigorous stirring and the reaction left to proceed in the dark for 18 h with shaking. In the case of the melittin experiment, the 10 uL aliquot had a calculated specific activity of 14.8 Ci/mmol, and probably contained somewhat less than 1.09 nmol due to radiolysis. After the ^{3}H-trace labelling, the reactions were left shaking in the dark overnight at either 37°C or room temperature. Complete derivatization of all groups was accomplished by making each sample approximately 8 M in urea (through addition of 2.0 g urea), adding 0.25 g $NaHCO_3$ and 50 µL of 50% FDNB in acetonitrile (v/v) and reacting for 18 h at room temperature in the dark with shaking. Single labellings were done for each data point in the preliminary experiment, in anticipation of trends in the data. Labellings were done in triplicate for the insulin and glucagon experiments, and in duplicate for the melittin experiment.

Figure 16. Flow chart of the competitive labelling procedure (from Hefford et al., 1986).


Protein + internal standard +/- liposomes
(1)
(2)
Dilution,
trace label with ^{3}H-labelled reagent
(4)
Fully label
with ^{14}C-labelled reagent
^{3}H-labelled protein* + ^{3}H-labelled internal standard*
(3)
Full reaction with
unlabelled reagent
^{14}C-labelled protein + ^{14}C-labelled internal standard
^{3}H-labelled protein + ^{3}H-labelled internal standard
Individual samples
(5)
^{3}H- and ^{14}C-labelled protein + ^{3}H- and ^{14}C-labelled internal standard
(6)
Isolation,
purification
^{3}H- and ^{14}C-labelled specific groups
^{3}H- and ^{14}C-labelled internal standard

3.4.1 Preparation and Addition of [^{14}C]-DNP-Protein and [^{14}C]-DNP-Internal Standards Lyophilized stock solutions were dissolved in a minimal volume of $NaHCO_3$ saturated 8 M urea. To this was added 200 µL of a 25% [^{14}C]-FDNB solution in acetonitrile (v/v) (2.0 mmol, 125 µCi/mmol), and the reactions were allowed to proceed overnight as above. The ^{14}C-labelled protein and internal standard mixtures were separated into equal aliquots, one being added to each ^{3}H-trace labelled sample. For the melittin experiment a fresh solution of equimolar melittin and Ala_2 was prepared, which was dinitrophenylated with ^{14}C-FDNB as above. Aliquots of this solution, containing approximately 0.93 mg ^{14}C-labelled melittin and 0.05 mg of ^{14}C-labelled Ala_2, were added to each sample.

3.4.2 Internal Standard Isolation and Purification With the exception of the melittin experiment, carrier was added to each of the samples in the form of non-radioactive DNP-bovine serum albumin (5 mg), and 0.5 mg each of the appropriate DNP-internal standards. The samples were extracted with three 5 mL aliquots of $CHCl_3$ to remove the bulk of the lipid, and then brought to pH 2 with concentrated HCl. Further extractions with ether (4 x 5 mL) were carried out, and the extracts, containing the DNP- internal standard, were combined and dried down. The internal standards were then dissolved in 95% ethanol, spotted on TLC silica plates (Kodak) and separated with a $CHCl_3$/benzyl alcohol/glacial acetic acid (70:30:3, v/v) solvent system, according to Brenner et al. (1961). Alternatively, purification of the internal standards was accomplished on HPLC system #1 using a 5 um reverse-phase C_{18} column (Beckman Ultrasphere-ODS, 0.46 x 25 cm), with a 10 µm C_{18} precolumn (3.2 x 40 mm), at room temperature. The solvent system employed various gradients of HPLC grade acetonitrile against an ammonium formate buffer

(35 mM formate, pH 3.0; Cockle et al., 1982) at a flow rate of 1.0 ml/min. Eluate was monitored at 320 nm.

3.4.3 Functional Group Isolation and Purification After ether extraction of the acidic aqueous phase, excess ether was evaporated with a stream of nitrogen in order to precipitate DNP-protein at the air/ether-water interface. The mixture was then centrifuged on a bench top centrifuge, and the pellet washed with successive 5 mL aliquots of dH_2O, 50% acetone, and (v/v in dH_2O, twice), and 100% acetone. At this point the insulin and glucagon samples were prepared for acid hydrolysis; melittin samples were dissolved in a small amount of concentrated formic acid which was then diluted to 10% with dH_2O and digested with pepsin as described in section 3.3.5). Hydrolysis of the insulin and glucagon samples was accomplished by adding 1.0 mL of 6 M HCl to the dried pellet, sealing the tubes under vacuum and subjecting them to 110°C in a heating block. Insulin hydrolyses were stopped after 4 hours, extracted with ether (4 x 2 mL) to remove DNP-glycine and DNP-phenylalanine, dried down again and further subjected to hydrolysis for another 14 h. The [$^3H/^{14}C$]-DNP-glycine and [$^3H/^{14}C$]-DNP-phenylalanine N-termini were separated on silica plates by two dimensional TLC using toluene/pyridine/2-chloroethanol/0.8 N ammonia (100:30:60:60,v/v) for the first dimension and benzene-pyridine-glacial acetic acid (80:20:2,v/v) for the second dimension (Brenner et al., 1961). Alternatively, the N-termini were separated by HPLC as above.

After complete hydrolysis, the hydrolysate was extracted with four 2.0 mL aliquots of diethyl ether to remove $^3H/^{14}C$-dinitrophenol. Isolation of the insulin functional groups was done by high-voltage paper electrophoresis (pH 2.1, 3500 V, 50 min). The N^{τ}-DNP-histidine, N^{ϵ}

-DNP-lysine and O-DNP-tyrosine bands were cut out, stitched onto another sheet of paper and electrophoresed a second time (pH 2.1, 3500 V, 75 min), to completely remove cross contamination. The bands were eluted into counting vials with 10% ammonia and dried down in preparation for liquid scintillation counting.

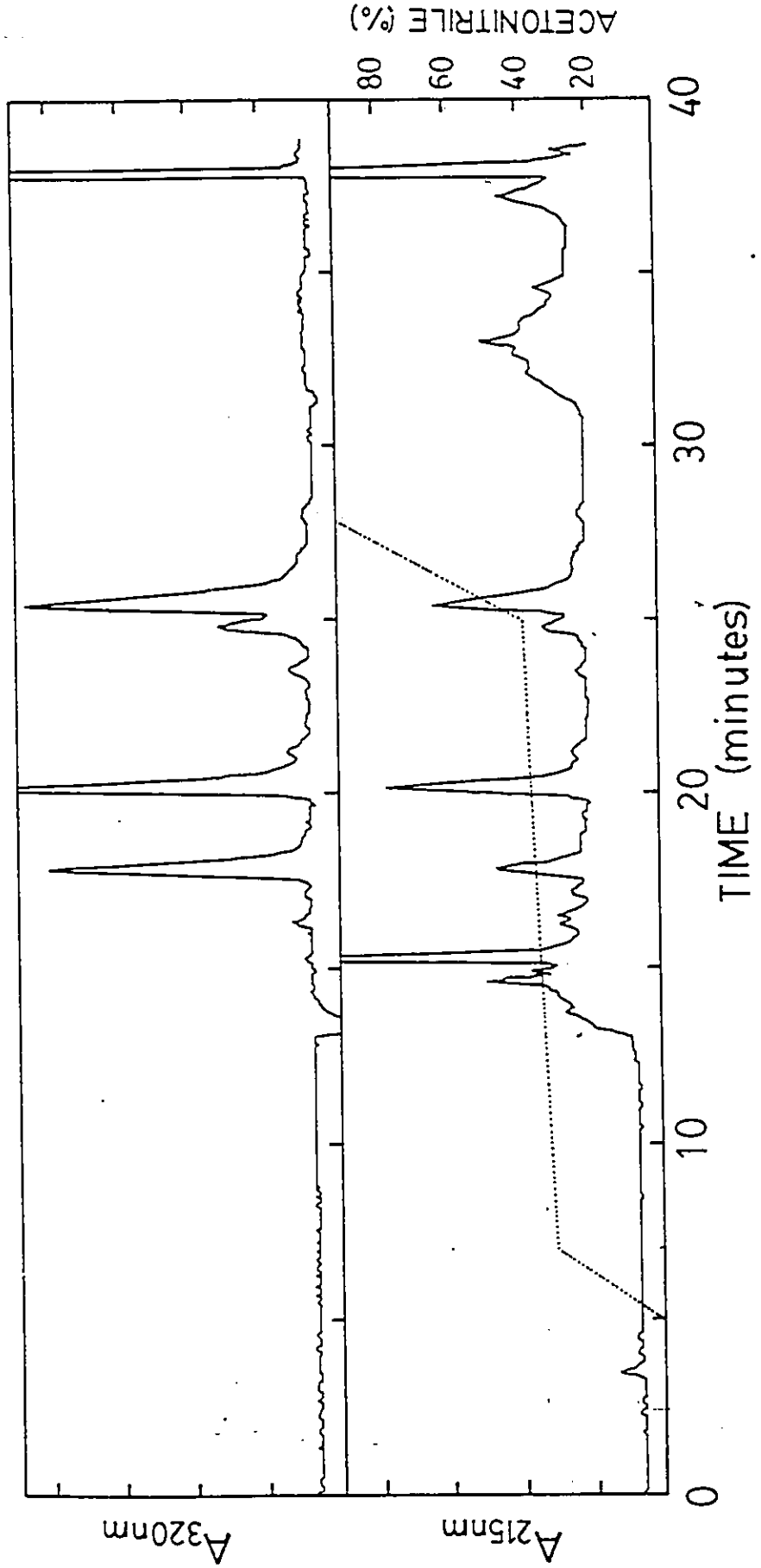
Isolation of the glucagon functional groups was carried out after complete hydrolysis as above; the N^{α},N^{τ}-di-DNP-histidine was well resolved upon paper electrophoresis, while the O-DNP-tyrosine and N^{ϵ}-DNP-lysine bands were cut out and purified by HPLC as above.

3.4.4 Functional Group/Peptide Mapping of Melittin Melittin (5mg) was dissolved in 2.0 mL of 8M urea, to which was added ca. 1.0 g $NaHCO_3$, followed by 10 µL of 50% FDNB in acetonitrile (v/v). The mixture was shaken in the dark at room temperature overnight, and then brought to pH 2 with concentrated HCl. DNP-OH was removed by ether extractions, and the DNP-melittin was precipitated upon evaporation of residual ether by a nitrogen stream. The precipitated protein was pelleted on a bench top centrifuge, washed with dH_2O, and lyophilized.

DNP-melittin (2.3 mg) was first dissolved in 1.0 mL of 99% formic acid, and the solution then diluted to 10% formic acid with dH_2O. Pepsin (approx. 0.25 mg) was added and the solution incubated at 37°C overnight with gentle swirling. Two lyophilizations yielded a fluffy yellow powder.

Separation of the DNP-peptides was carried out on HPLC systems #1 and #2 using an Ultrasphere ODS reverse-phase C_{18} column (0.46 x 25 cm) using 0.01 N HCl with a gradient of AcCN (Figure 17). Eluate was monitored at 320 and 215 nm for detection of DNP-derivatives and peptides, respectively. Fractions (0.5 mL), corresponding to peaks A, B,

Figure 17. HPLC separation of DNP-peptides from a peptic digest of fully dinitrophenylated melittin, using an aqueous phase of 0.01 N HCl with acetonitrile as organic modifier (dotted line). O.D. units at 215 and 320 nm are arbitrary. See text for details.


A320nm
A215nm
0
10
20
30
40
TIME (minutes)
80
60
40
20
ACETONITRILE (%)

and C were pooled from several runs, recycled with the same solvent system, lyophilized and hydrolyzed (1.0 mL 6N HCl, 110°C, 18 h, in vacuo). The hydrolysates were split into two portions, one of which was analyzed for the presence of DNP-Gly and N -DNP-Lys by HPLC system #1 using 0.01N HCl with a gradient of MeOH, while the second portion of the hydrolysate was used for amino acid analysis. The results of these analyses are listed in Table III.

Based on these compositions, it was possible to assign peptides A, B and C to the primary sequence of melittin (Figure 18). The peptic cleavage sites correspond to those in the literature for melittin (Habermann, 1972), and are designated by arrows. There is some ambiguity as to whether Leu-6 should be included in peptide B (see Table III); dinitrophenylation of Lys-7 may have altered the pattern of peptic digestion.

The methodology for the purification of the melittin peptides was devised on HPLC system #1, but the purification of the actual $^{3}H/^{14}C$-DNP-peptides was performed using HPLC system #2, though the same column was used each time. During the course of peptide purification a peak with absorbance at 320nm was observed to elute closely with peptide B (data not shown). The identity of this anomalous peak was not determined, and its $^{3}H/^{14}C$ ratios did not have any identifiable pattern. To ensure that the anomalous peak had not contaminated the desired peptides, a few samples were isolated in duplicate, the second being hydrolyzed. Purification of the resultant DNP-lysines by HPLC (as above) and subsequent counting showed that the DNP-peptides were sufficiently pure without recourse to a further purification step (Table IV).

TABLE III. Composition of DNP-peptides from peptic digest of DNP-melittin.

Peptide	DNP-amino acid (from HPLC)	Amino acid composition (molar ratio: obtained/expected)
A	DNP-Gly	Gly (1.6/1.0), Ile (1.0/1.0)
B	DNP-Lys	Glx (0.3/0.0), Gly (1.6/1.0), Leu (2.6/3.0), Ser (0.6/0.0), Thr (2.0/2.0), Val (1.0/1.0)
C	DNP-Lys	Arg (2.3/2.0), Glx (1.9/2.0), Ile (1.0/1.0)

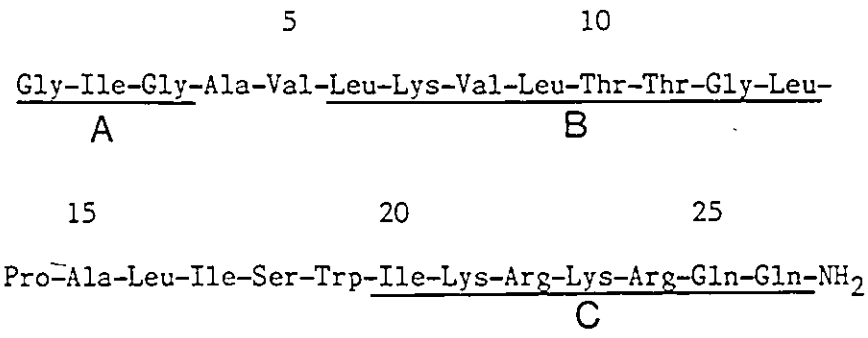


Figure 18. Assignment of peptic peptides A,B and C to the primary sequence of melittin.

Table IV: Radioactive purity of selected DNP-peptides.

Sample	$^{3}H/^{14}C$-DNP-peptide	$^{3}H/^{14}C$-DNP-amino acid
A1 K-7	18.3	16.7
K-21 & 23	33.4	33.9
A2 K-7	18.2	17.9
K-21 & 23	35.1	34.8
AL2 K-21 & 23	15.5	15.6

3.5 Liquid Scintillation Counting

All samples were dried in borosilicate counting vials, dissolved in 100 µl of 0.01 M HCl, and made up to 5 or 10 mL with Aquasol-2. Scintillation counting was carried out on a programmable Beckman 1800 scintillation counter equipped with automatic quench correction and dpm converter.

4.0 RESULTS AND DISCUSSION

4.1 Preliminary Experimentation

In order to test the feasibility of the proposed methodology a preliminary experiment was carried out in which the reactivities of three test proteins (lysozyme, insulin, and glucagon) were measured as a function of total lipid concentration (as REV liposomes).

4.1.0 Internal Standards The internal standards used in this experiment were the dipeptide arginyl-aspartate (Arg-asp) and imidazolyl-lactic acid (Imlac). Neither Arg-asp nor Imlac yielded consistent $^{3}H/^{14}C$ ratios (data not shown). The extreme photolability of Imlac and its DNP derivative, and the low recovery yields of the latter, are thought to be responsible for the results obtained. Arg-asp was originally chosen for its high charge density, which, it was thought, would minimize interactions with the liposome amphiphiles. Since the present work was initiated however, a body of evidence has been compiled indicating that the pairing of an acidic side chain with a nearby arginyl side chain can provide proteins with potent recognition and binding sites (Kazim & Atassi, 1980; Eggleston & Hodgson, 1985), and are responsible for the adhesive properties of such proteins as fibronectin (Hynes, 1986). The inconsistent results obtained for Arg-asp may therefore be due to polar and hydrogen bonding interactions peculiar to this dipeptide with the EPC headgroups and proteins.

4.1.1 Proteins The $^{3}H/^{14}C$ ratios of the total DNP-proteins were plotted as a function of total lipid concentration (Figure 19). The total amount of label incorporated into each protein during the ^{3}H-trace

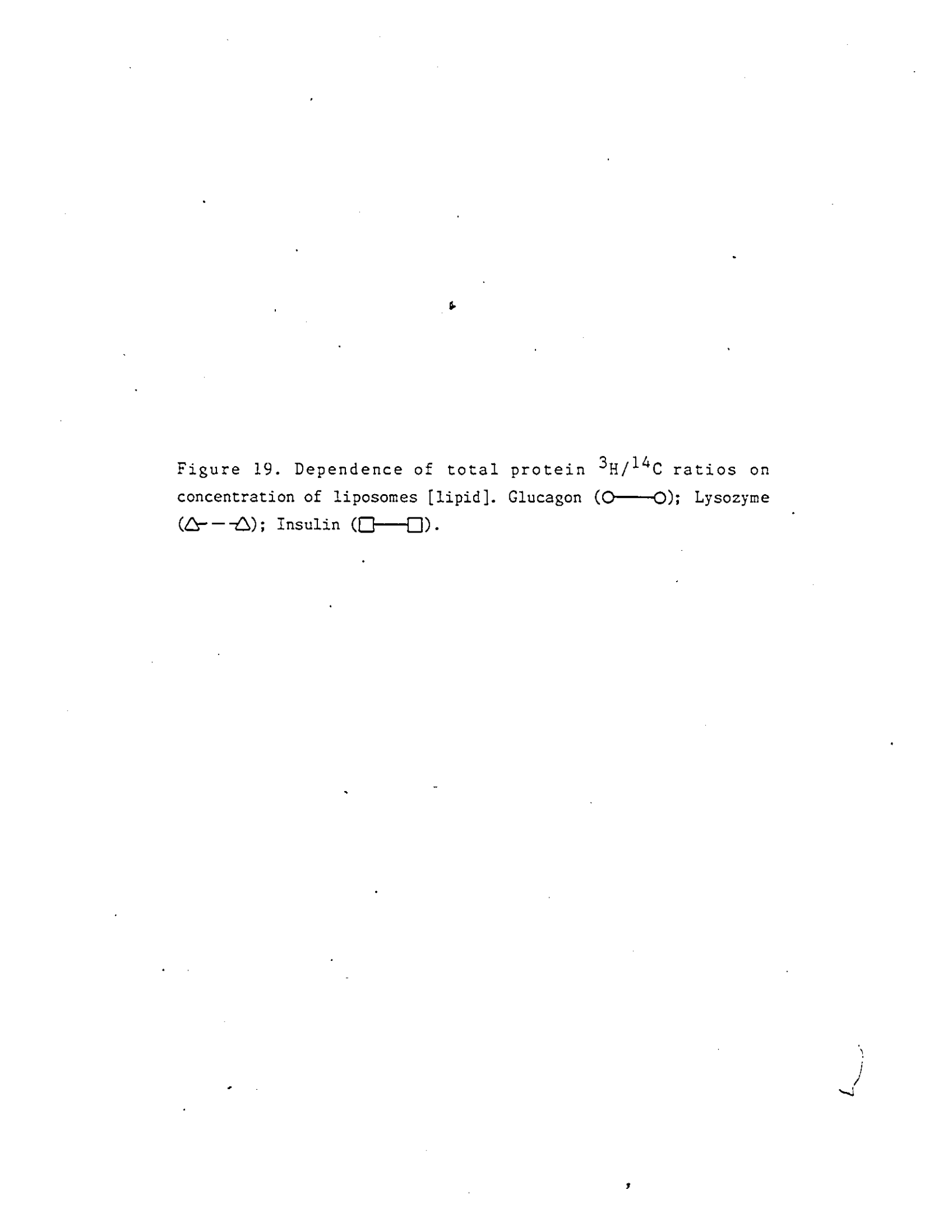
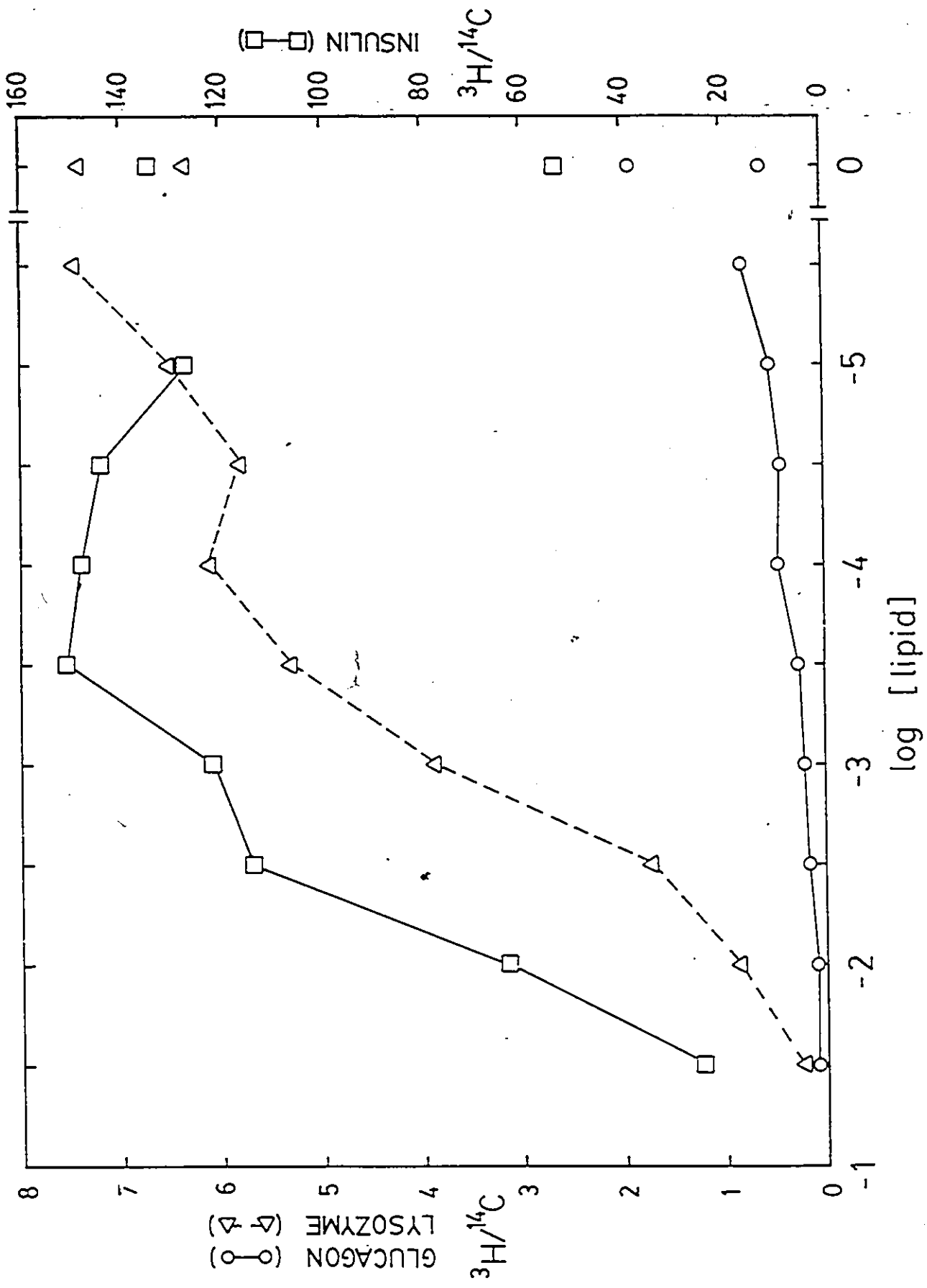


Figure 19. Dependence of total protein $^{3}H/^{14}C$ ratios on concentration of liposomes [lipid]. Glucagon (○——○); Lysozyme (△– –△); Insulin (□——□).


INSULIN (□—□)
$^3H/^{14}C$
160
140
120
100
80
60
40
20
0
GLUCAGON (○—○)
LYSOZYME (△—△)
$^3H/^{14}C$
8
7
6
5
4
3
2
1
0
-1
-5
-4
-3
-2
log [lipid]

labelling decreased as the concentration of liposomal lipid increased. One explanation for this behaviour is that the labelling reagent was partitioning into the liposomes, resulting in an apparent decrease in overall protein reactivity. If this were true however, all the proteins would exhibit similar reactivity behaviours towards the labelling reagent. Lysozyme and insulin, the latter being present as the dimer at this concentration, had similar patterns of label incorporation relative to one another, but glucagon's behaviour differed from both. The greatest decrease in the rate of label incorporation as a function of liposome concentration for insulin and lysozyme occurs at the highest concentrations of liposome. Conversely, the greatest decrease for glucagon was observed to occur at very low concentrations of liposome (about 10^{-5} M). These results can be explained on the basis of the structures of the protein molecules. Glucagon has regions of exposed hydrophobic residues, so its association with liposomes will be entropically driven, as it seeks to diminish the free energy of its interaction with the aqueous environment. Lysozyme and dimeric insulin, which do not have significant degrees of their surface occupied by apolar residues, will probably interact with the liposomes elecrostatically, a process that will be concentration-dependent.

4.2 Insulin

4.2.0 Internal Standards

The ideal internal standard is one which does not interact with the other components of the system, viz. liposomes and insulin. The $^{3}H/^{14}C$ ratios will give some indication as to whether the standards are interacting with the liposomes. Table V shows that the reactivity of Ala_2 is not changed at higher concentrations, and only slightly at lower concentrations when liposomes are present. Therefore, the reactivity of Ala_2 does not seem to be greatly affected by the introduction of liposomes into the buffer, except at the lowest concentration, and then only slightly. The dipeptide Ala_2 therefore, does not appear to interact with liposomes and is therefore suitable for use as an internal standard under these reaction conditions.

Glycine that is free in solution has an approximate 5-fold lower reactivity than Ala_2 due to the higher pK value of its alpha-amino group (Table V). In the presence of liposomes a 16-19% decrease in free glycine reactivity is observed at higher concentrations, with a 50% decrease at 3×10^{-8} M. Since the decrease is concentration-dependent, adsorption is most likely taking place at the low concentrations. This is evidence that free glycine adsorbs to the liposomes, with increasing adsorption at lower concentrations. Free glycine in solution can not be used as an internal standard in this case.

4.2.1 Functional Groups of Insulin

Previous concentration-dependence studies have been carried out under similar conditions, though with different internal standards and specific radioactivity of isotopes (Kaplan et al., 1984). Examination of

TABLE V: Internal Standard $^{3}H/^{14}C$ Ratios (Insulin Experiment)[a]

internal standard	concentration (M)	control	liposome	L/C[b]
Ala_2	1×10^{-6}	4.20 ± 0.04	4.23 ± 0.21	1.01 ± 0.05
	3×10^{-7}	3.91 ± 0.10	4.18 ± 0.91	1.07 ± 0.23
	1×10^{-7}	6.48 ± 1.78	4.52 ± 0.42	0.70 ± 0.20
	3×10^{-8}	4.38 ± 0.68	3.75 ± 1.43	0.86 ± 0.35
glycine	1×10^{-6}	0.84 ± 0.02	0.70 ± 0.01	0.83 ± 0.02
	3×10^{-7}	0.62 ± 0.07	0.54 ± 0.07	0.87 ± 0.15
	1×10^{-7}	0.68 ± 0.24	0.40 ± 0.14	0.59 ± 0.29
	3×10^{-8}	0.53 ± 0.09	0.26 ± 0.12	0.48 ± 0.23

[a]Average of three experiments with standard deviation.
[b]See text for definition.

Table VI indicates that the ranking of functional groups $^{3}H/^{14}C$ ratios in the control samples at 10^{-6} M is qualitatively similar to the order found previously (Kaplan et al., 1984). With the exception of Lys-B29 all functional groups experience a diminution in reactivity at lower concentrations; again, this mirrors the findings of the previous study, and indicates that dimer dissociation is not complete at 10^{-6} M.

The effect of liposomes on the chemical properties of insulin at each concentration can be quantified by normalizing all reactivities with respect to the reactivity of the internal standards. This must be done at each concentration for both the control (C) sample and the liposome-containing (L) samples. The L/C ratio is a useful expression that describes the alteration of functional group reactivity in the presence of liposomes, with reference to a standard (section 2.2.0). If L/C is greater than unity (1.0), the presence of liposomes in the solution has resulted in an increase in the chemical reactivity; if L/C is less than unity the reactivity has been diminished.

Figures 20(a) and (b) show the concentration-dependence of the L/C ratios for all of the insulin functional groups tested, relative to the internal standards Ala and glycine. The tyrosine and histidine ratios represent the average reactivities of the four tyrosyl and two histidyl residues of insulin. In each case the reactivities of all functional groups except the Phe-B1 amino terminus decreases as the concentration of insulin becomes more dilute and the free monomer becomes the major and perhaps only, species. The Gly-A1 amino-terminus shows the largest reduction in reactivity over the concentration range studied, decreasing to approximately 20% of the control values. It should be noted that for most of the data points, there is an apparent relative increase in

TABLE VI: Insulin Functional Group $^{3}H/^{14}C$ Ratios[a]

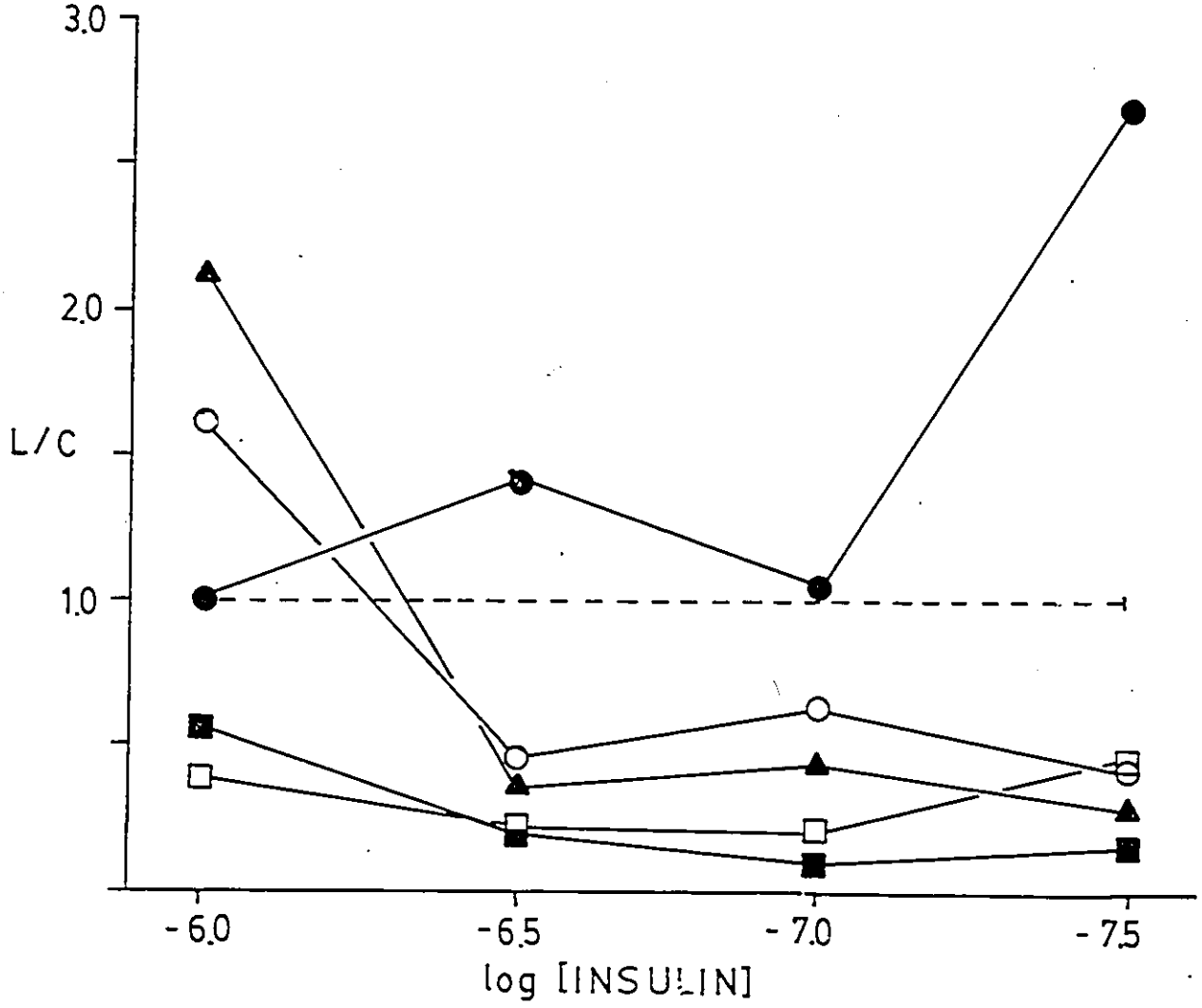
group	concentration (M)	control	liposome	L/C[b]
Gly-A1	1×10^{-6}	1.999 ± 0.052	0.667 ± 0.059	0.56 ± 0.05
	3×10^{-7}	0.681 ± 0.022	0.133 ± 0.004	0.19 ± 0.01
	1×10^{-7}	0.510 ± 0.080	0.054 ± 0.002	0.11 ± 0.01
	3×10^{-8}	0.153 ± 0.001	0.023 ± 0.003	0.15 ± 0.02
Phe-B1	1×10^{-6}	0.613 ± 0.038	0.618 ± 0.005	1.01 ± 0.06
	3×10^{-7}	0.117 ± 0.010	0.164 ± 0.009	1.40 ± 0.14
	1×10^{-7}	0.066 ± 0.012	0.069 ± 0.005	1.05 ± 0.21
	3×10^{-8}	0.042 ± 0.001	0.112 ± 0.003	2.68 ± 0.09
Lys-B29	1×10^{-6}	0.227 ± 0.016	0.480 ± 0.089	2.11 ± 0.41
	3×10^{-7}	0.218 ± 0.004	0.078 ± 0.014	0.36 ± 0.06
	1×10^{-7}	0.128 ± 0.013	0.055 ± 0.013	0.43 ± 0.11
	3×10^{-8}	0.228 ± 0.013	0.063 ± 0.003	0.28 ± 0.02
His-B5, -B10 (average of 2)	1×10^{-6}	0.378 ± 0.012	0.148 ± 0.001	0.39 ± 0.01
	3×10^{-7}	0.168 ± 0.001	0.037 ± 0.002	0.22 ± 0.01
	1×10^{-7}	0.076 ± 0.011	0.016 ± 0.001	0.21 ± 0.03
	3×10^{-8}	0.047 ± 0.007	0.021 ± 0.001	0.45 ± 0.01
Tyr-A14, -A16, -B19, -B26 (average of 4)	1×10^{-6}	0.244 ± 0.008	0.392 ± 0.009	1.61 ± 0.01
	3×10^{-7}	0.161 ± 0.005	0.074 ± 0.004	0.46 ± 0.03
	1×10^{-7}	0.088 ± 0.002	0.055 ± 0.002	0.63 ± 0.03
	3×10^{-8}	0.075 ± 0.001	0.032 ± 0.004	0.42 ± 0.05

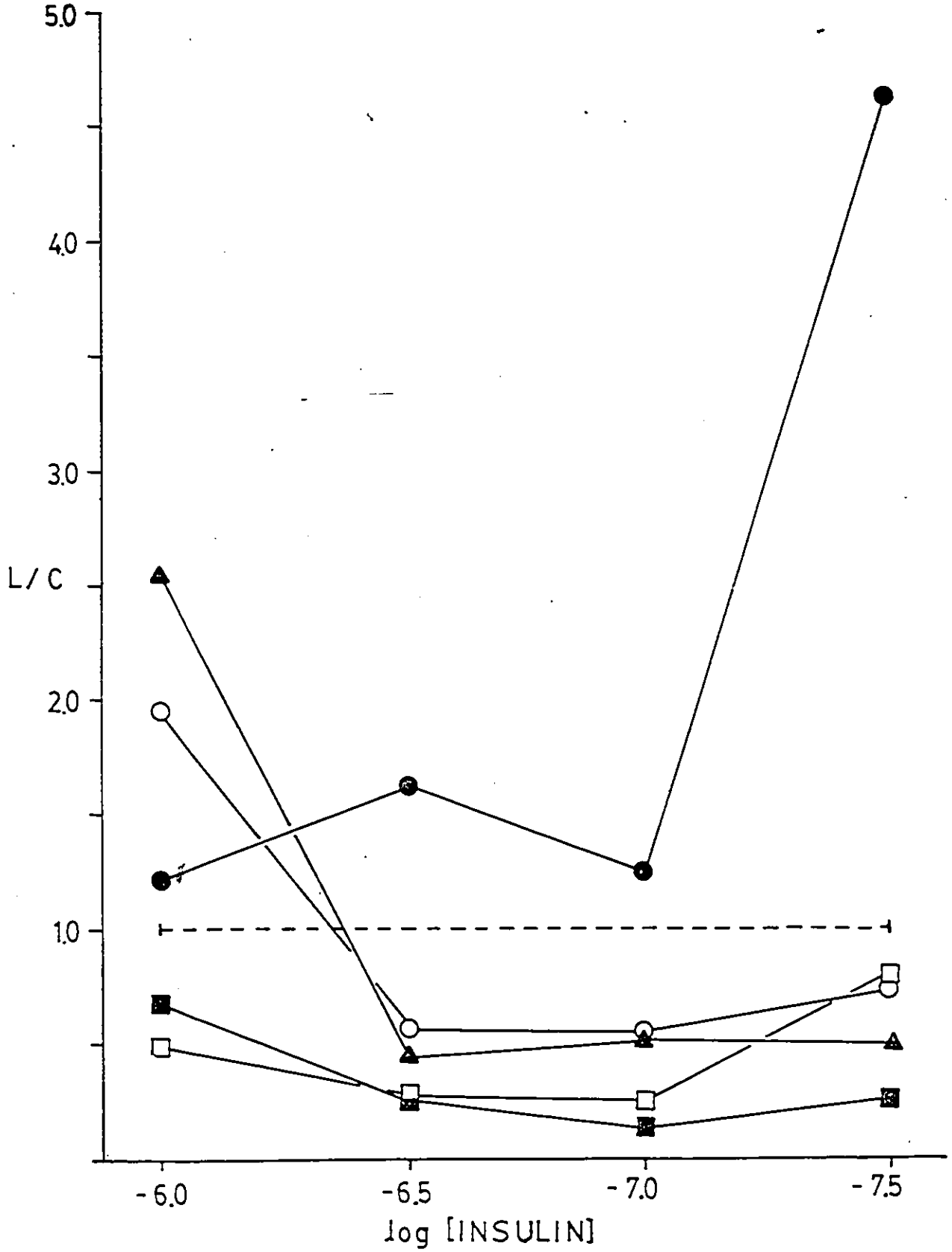
[a]Experiments were done in triplicate and normalized with respect to Ala_2.
[b]See text for definition.

Figure 20a (facing page). Reactivity L/C-concentration profiles of insulin functional groups with respect to internal standard Ala_2;

Figure 20b (following page). Reactivity L/C-concentration profiles of insulin functional groups with respect to internal standard glycine:

(⊘) phenylalanine-B1; (○) average of four tyrosines; (□) average of two histidines; (■) glycine-A1; (▲) lysine-B29.


3.0
2.0
1.0
L/C
-6.0
-6.5
-7.0
-7.5
log [INSULIN]


5.0
4.0
3.0
L/C
2.0
1.0
-6.0
-6.5
-7.0
-7.5
log [INSULIN]

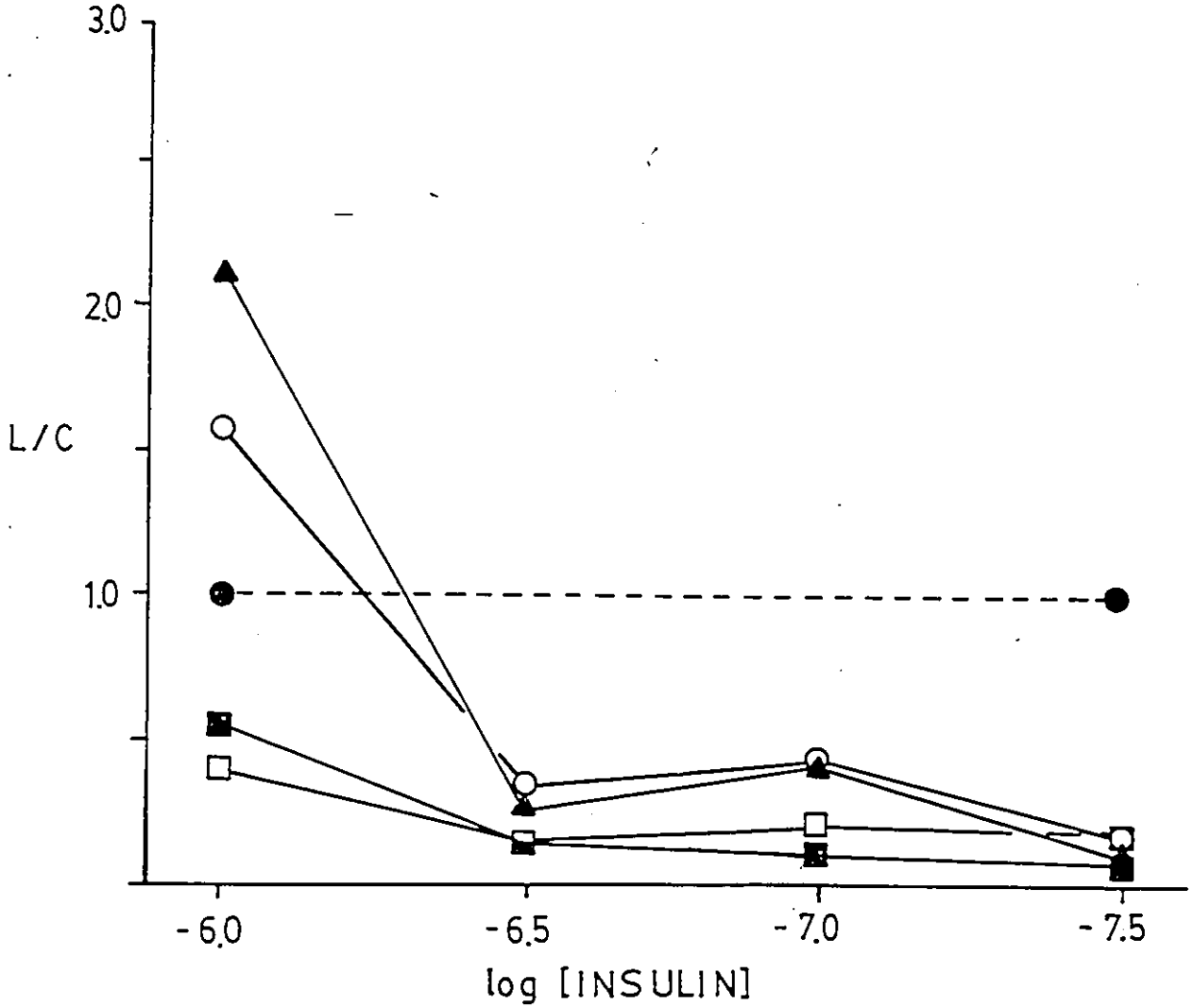
functional group reactivity observed at the lowest concentration when ratios are calculated with respect to the glycine internal standard. This is due to the latter's adsorption to the liposomes (vide supra).

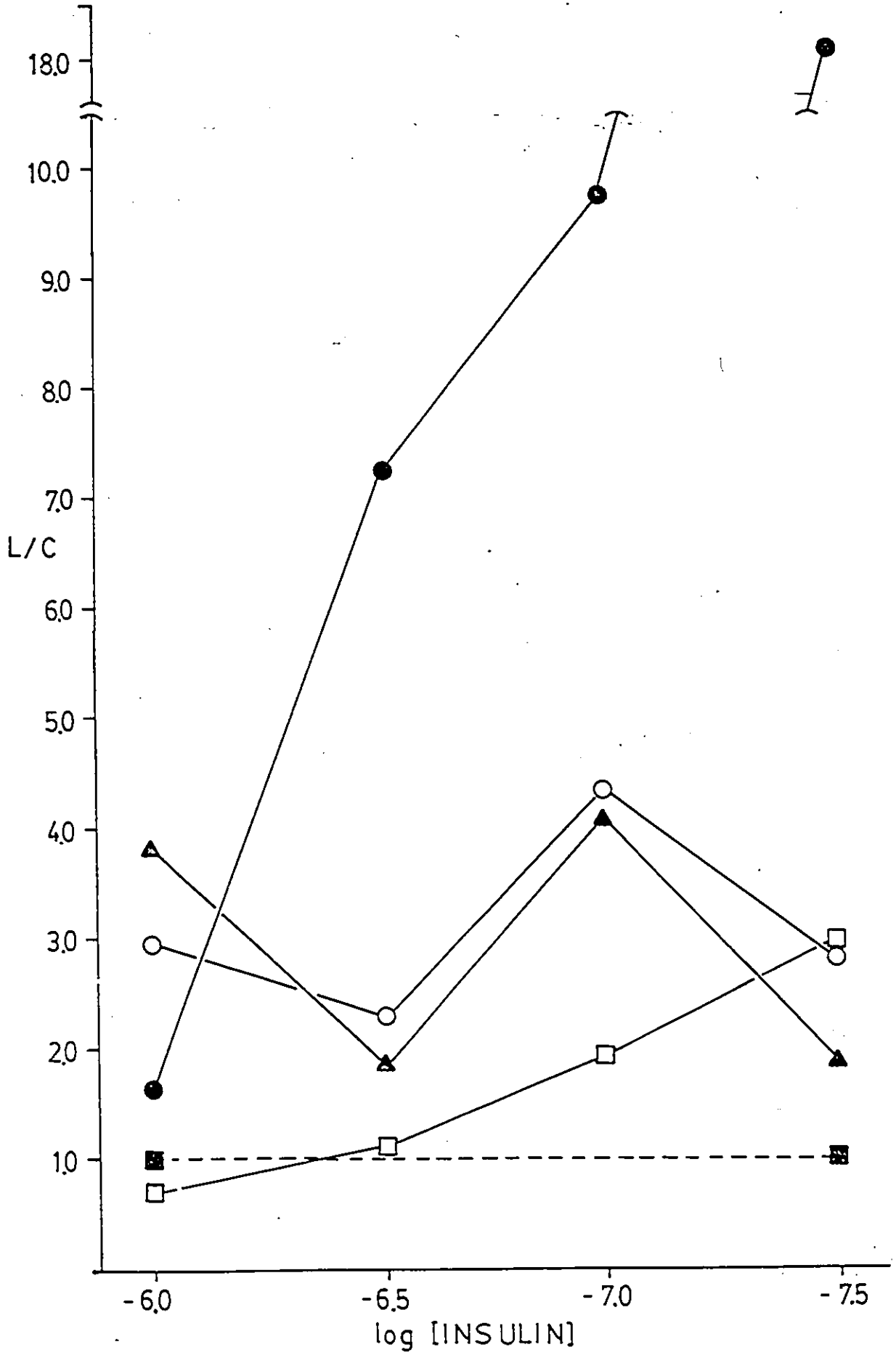
The internal standards are useful in establishing that adsorption of insulin takes place, but do so from the perspective of an outside observer. Alternatively, a method of determining whether there are changes in the chemical properties of specific groups relative to other groups is to treat each of the functional groups in the molecule as an internal standard. If this is done in turn using each functional group, then, depending on the location and number of groups, it should be possible to obtain a semi-quantitative assessment of the chemical topology of the bound molecule.

Figures 21(a) and (b) show the L/C profile of the functional groups after normalization with respect to Phe-B1 and Gly-A1. The most striking feature of the Phe-B1 L/C profile is the resemblance to the profiles obtained using the internal standards as the reference nucleophiles. Quantitatively, it appears that access of the labeling reagent to the B chain N-terminal, in both free and bound states, is as unrestricted as the access to the internal standard nucleophiles. The relative decrease in reactivity of the other functional groups immediately suggests that the Phe-B1 has a similar environment in the free and bound states of insulin.

Examination of the 2Zn insulin crystal structure shows that Phe-B1 is located at the opposite end of the molecule from the dimer-forming surface in both molecules 1 and 2 of 2Zn insulin and molecule 1 of 4Zn insulin (Beijing Insulin Structure Research Group nomenclature). An earlier suggestion that upon dissociation of the dimer, residues B1-3

Figure 21a (facing page). Reactivity L/C-concentration profiles of insulin functional groups with respect to Phe-B1.
Figure 21b (following page). Reactivity L/C-concentration profiles of insulin functional groups with respect to Gly-A1. (●) phenylalanine-B1; (○) average of four tyrosines; (□) average of two histidines; (■) glycine-A1; (▲) lysine-B29.


3.0
2.0
1.0
L/C
-6.0
-6.5
-7.0
-7.5
log [INSULIN]


18.0
10.0
9.0
8.0
7.0
L/C
6.0
5.0
4.0
3.0
2.0
1.0
-6.0
-6.5
-7.0
-7.5
log [INSULIN]

may pack more closely to the surface of the molecule (Blundell et al., 1972), is based on the 2Zn crystal structure, in which Phe-B1 is in a surface pocket bordering the dimer-dimer contact region of the insulin hexamer. A large body of evidence, including atomic temperature factor analysis (Tainer et al., 1985), antigenicity studies of exposed surface areas, (Schroer et al., 1983), and earlier labelling and modification studies (Blundell et al., 1972), supports the idea that in aqueous solution, the Phe-B1 N^{α}-amino group extends into solution, and is probably not tightly packed against the insulin molecule; the 4Zn molecule 2 structure would probably not satisfy these requirements (Figures 7, 8). Therefore, in the 2Zn conformation, the amino group of Phe-B1 is an excellent vantage point from which to gauge the change in chemical reactivities that the other functional groups of the insulin molecule undergo in binding of insulin.

If the Gly-A1 amino terminus is taken as the reference nucleophile, the L/C profile in Figure 21(b) is obtained. Relative to Gly-A1, the B chain N-terminus has a greatly augmented reactivity at all concentrations, increasing sharply at the lowest concentration. This apparent increase may reflect a continuing decrease in Gly-A1 reactivity, or, conversely, may reflect a real increase attributable in part to some membrane-associated conformational change that increases Phe-B1 reactivity. All the other functional groups also show an enhanced reactivity over that of glycine.

Like Phe-B1, Gly-A1 is also accessible to the solvent in the monomer, though situated in a "compact, surface pocket" (Blundell et al., 1972). Although the position of Gly-A1 is altered by the conformational changes represented in the 2Zn and 4Zn crystal structures

(Chothia et al., 1983), it is somewhat constrained by its function as a shielding group for the hydrophobic core of the insulin molecule (Blundell et al., 1982). The Gly-A1 amino group is part of a postulated receptor binding region (Pullen et al., 1976; Gammeltoft, 1984) and is adjacent to the hydrophobic dimer-forming surface of the molecule. It is not unreasonable to propose that if the insulin monomer binds to the liposome by apposition of the largely hydrophobic dimer-forming surface, the reactivity of Gly-A1 will be diminished. However, the flexibility and conformation of the molecule at positions B29-30 has to be critically assessed since these residues represent a topological projection separating the dimer-forming face from the vicinity of Gly-A1 in the 2Zn dimer crystal model.

It can be seen from Figure 21 that Lys-B29 undergoes the greatest relative decrease in reactivity. Previous studies have shown that the pK of Lys-B29 in monomeric insulin is 9.80 (Hefford et al., 1986), close to what might be expected for this kind of group in solution. The X-ray crystallographic structure shows that Lys-B29 is close to the dimer-forming surface, though it is one of the most flexible parts of the molecule (Tainer et al., 1985; and references therein). The implication is that the lysyl side chain of monomeric insulin goes from a fairly solvent-accessible environment to one which is sterically shielded from reagent in the presence of liposomes. Its decrease in reactivity is therefore consistent with the dimer-forming surface being part of the site at which insulin binds to liposomes.

Conceptually, it may be useful to consider Gly-A1 and Phe-B1 as lying on opposite ends of a solvent and electrophile accessibility spectrum when the protein is adsorbed onto the liposome surface, and

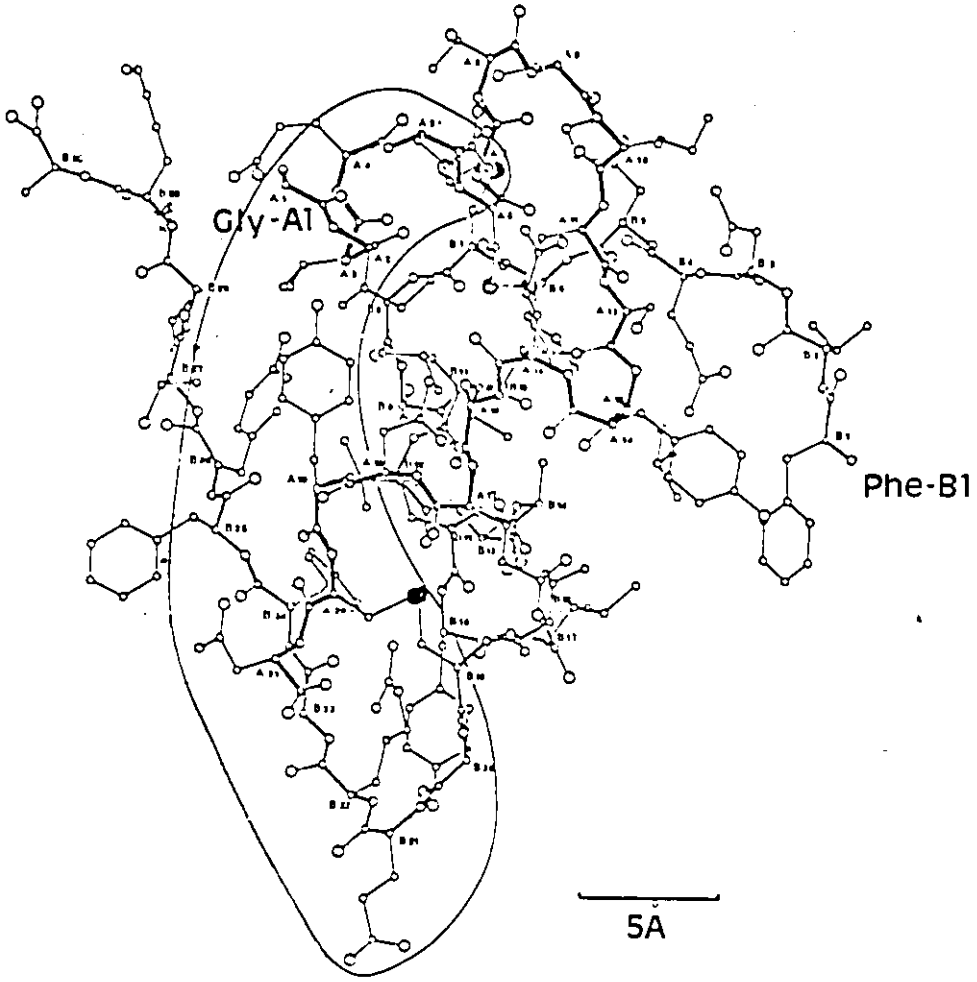
that as Phe-B1 may be a sensitive indicator of conditions external to the bulk of the molecule, (in the monomeric form), so Gly-A1 may be a suitable reporter group for the defined region of the molecular surface that is adjacent to the hydrophobic dimer-forming surface and which could reasonably be involved in adsorption or binding to the liposome surface (Figure 22).

Changes in the other functional groups are intermediate between those of the A- and B-chain amino-termini, though it is not possible to draw any specific conclusions since only the average reactivities were measured. The sharp decrease in tyrosine reactivity is consistent with a specific shielding effect on the B-chain tyrosines (B16 and B26) that are directly involved in dimerisation (Pullen et al., 1976). Tyr-A19 lies on the periphery of this region and should also experience decreased reactivity if the dimer-forming surface becomes adsorbed to a surface. Of the four insulin tyrosyl residues, only Tyr-A14 should maintain a substantial reactivity, as it is situated on the opposite side of the molecule, close to Phe-B1.

The average reactivity of the two histidine residues (B5 and B10) decreases upon liposome association, but not to the same degree as found with the tyrosine, glycine, or lysine residues. The decrease is almost certainly due solely to a less accessible His-B10 side chain, as it lies closer to the putative adsorption surface than His-B5.

At 10^{-6} M the four tyrosines and lone lysine residue show enhanced reactivity in the presence of liposomes. Although it seems possible that the high tyrosine reactivity could be due to additivity of experimental errors, a more satisfying explanation may be that the presence of a small population of insulin dimers modifies the

Figure 22. View of the insulin monomer showing the putative receptor-binding site of insulin (smooth thin line), and the relative positions of the two N-terminal residues. The dimer forming surface comprises approximately the middle half of the putative receptor-binding region (from DeMeyts et al., 1978).


Gly-A1
Phe-B1
5A

interaction of insulin with the liposome surface. A similar explanation may account for the apparently enhanced reactivity of the Lys-B29 N^{ϵ}-amino group, which would be sensitive to the changes in local dielectric that are found at membrane/solvent interfaces.

The explanation for the enhanced reactivity of the Phe-B1 N-terminus at 3×10^{-8} M can only be speculative. It may be that the unusual reactivities of Lys-B29 and Phe-B1 are linked via the coil-helix transition involving residues B1-B8. Proximity of the Phe-B1 aromatic ring to the lysyl N^{ϵ}-amino group may promote deionization and a higher reactivity in the 4Zn molecule 2 structure. Such an effect has been noticed before for the reactivity of the Phe-B1 N -amino group itself (Friesen, 1980). At low concentrations the B1-B8 helix may undergo a helix-coil transition, thereby exposing the Phe-B1 amino group to solvent and labelling reagent.

4.3 Glucagon

4.3.0 Internal Standards In the control samples, the $^{3}H/^{14}C$ ratios of the Ala_2 internal standard do not vary greatly over the concentration range studied (Table VII), similar to the results obtained with the insulin experiment (section 4.2.0). However, although there is no concentration-dependent interaction between Ala_2 and the glass surface of the reaction vessel, there is a moderate decrease in reactivity below concentrations of 1×10^{-7} M, when liposomes are present.

The previous experiment with insulin has shown that in the presence of 1.0 M KCl, there is only slight adsorption of Ala_2 to the liposomes at concentrations below 1×10^{-7} M. Thus the interaction between this dipeptide and the lipid bilayers is mainly electrostatic in nature, and the small degree of hydrophobicity characteristic of the methyl side chain is not a major factor in adsorption. Recent NMR studies on the partitioning of model tripeptides of the form Ala-X-Ala-O-tert-butyl into liposome bilayers (Jacobs & White, 1986), have also shown that, by comparison with phenyl or tryptophanyl side chains, a sole methyl substituent will not cause appreciable partitioning into bilayers.

The reactivity behaviour of glycine was more complicated. Decreases in the $^{3}H/^{14}C$ ratios of free glycine were observed at low concentrations, while in the presence of liposomes, substantial and almost exponential increases in reactivity were observed (Table VII). These results are contrary to those obtained in the insulin experiment (section 4.2.0) and must be due to the different KCl concentrations used in each experiment. A concentration of 1.0 M salt, would generate a substantial electrical double-layer on the membrane surface (McLaughlin,

TABLE VII: Internal Standard $^{3}H/^{14}C$ Ratios (Glucagon Experiment)[a]

internal standard	concentration (M)	control	liposome	L/C[b]
Ala_2	1×10^{-6}	2.43 ± 0.26	2.75 ± 0.44	1.13 ± 0.22
	3×10^{-7}	2.19 ± 0.63	2.12 ± 0.44	0.97 ± 0.34
	1×10^{-7}	2.43 ± 0.37	1.63 ± 0.40	0.67 ± 0.19
	3×10^{-8}	2.34 ± 0.57	1.49 ± 0.39	0.64 ± 0.22
glycine	1×10^{-6}	0.58 ± 0.06	0.54 ± 0.02[b]	0.93 ± 0.10
	3×10^{-7}	0.21 ± 0.01[b]	0.38 ± 0.13[b]	1.78 ± 0.61
	1×10^{-7}	0.32 ± 0.09	0.97 ± 0.08	3.03 ± 0.88
	3×10^{-8}	0.17 ± 0.05	1.49 ± 0.40	8.75 ± 3.48

[a]Average of three experiments with standard deviation.
[b]Mean of two experiments.

1977), shielding the headgroups and reducing the range of their electrostatic force fields. In the absence of this high salt concentration (i.e. at 0.1 M KCl), interactions with charged solutes would be facilitated. Being quite small, glycine may be able to interact with the dipoles of the EPC headgroups without disturbing their packing arrangements. In this position, the pK of the glycine amino group would be lowered, stabilized by the low dielectric of the immediate environment.

4.3.1 Functional Groups of Free Glucagon in Solution

The chemical reactivity of the amino terminal histidine was measured as the sum of the N^{α}-amino and N^{τ}-imidazole (Bell & Jones, 1974) reactivities. Similarly, tyrosine reactivity is expressed as the average of the phenolic hydroxl reactivities of Tyr-10 and Tyr-13 (Table VIII). Figure 23 summarizes the $^{3}H/^{14}C$ ratios obtained for the N-terminal histidine, tyrosines 10 and 13, and the lysine-12 N^{ϵ}-amino group both in the presence and absence of liposomes. In the control samples, there is little change in reactivity for the histidine and tyrosine groups over the concentration range studied (100 to 3×10^{-8} M), though an increase in Lys-12 reactivity occurs in the middle of this range.

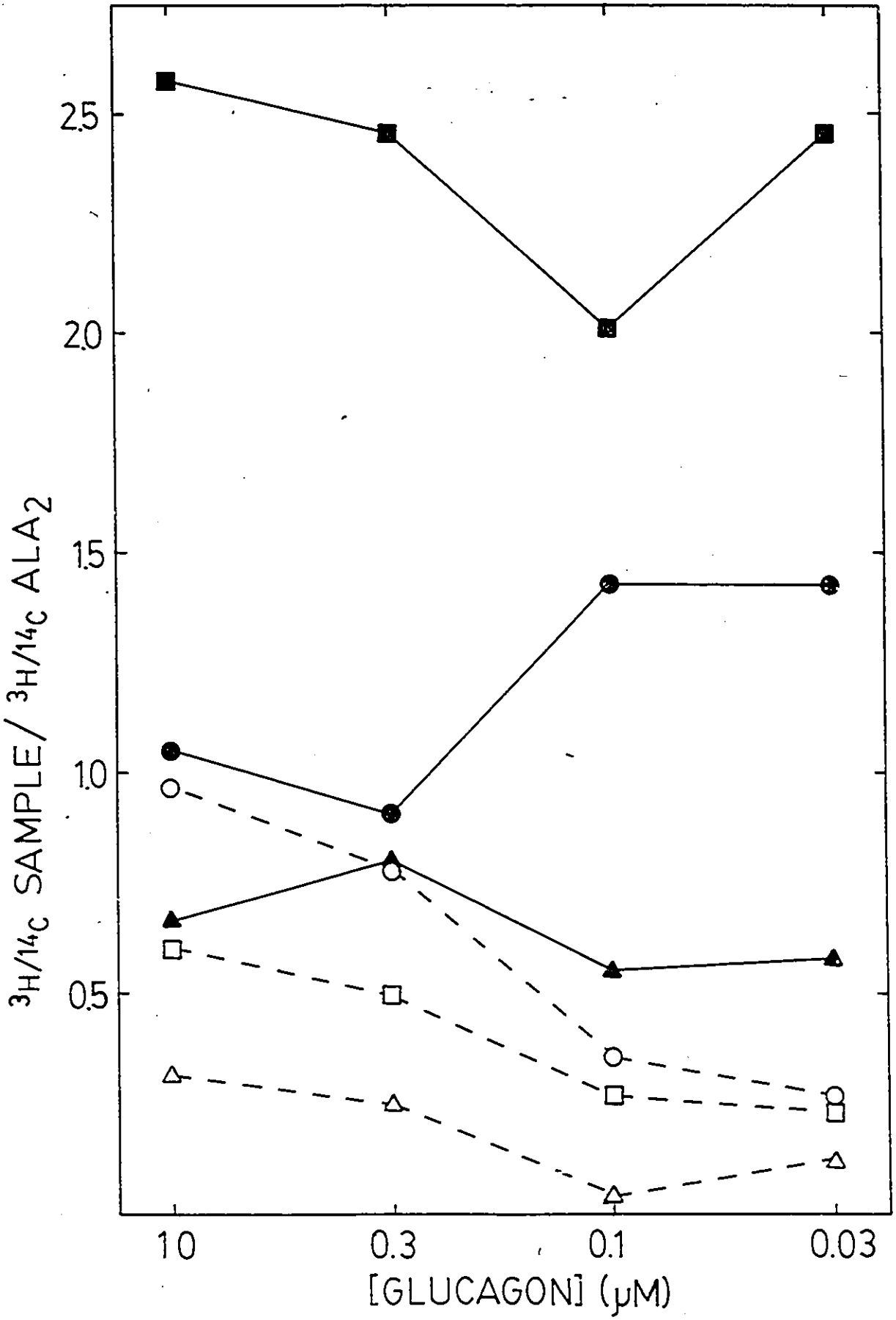
The pattern of reactivities observed is consistent with the dissociation of type 2 trimers to give the free glucagon monomer (section 2.5.0). In trimer 1 the tyrosyl side chains are involved in the trimer-stabilizing hydrophobic contacts, thus, dissociation of trimer 1 should result in greatly increased accessibility to both tyrosines and a consequent increase in reactivity. Side chain accessibility to aqueous solvent has been calculated by the methods of Lee & Richards (1971) and

TABLE VIII: Glucagon Functional Group $^{3}H/^{14}C$ Ratios[a]

group	concentration (M)	control	liposome	L/C[b]
His 1	1×10^{-6}	2.56 ± 0.26	0.59 ± 0.08	0.23 ± 0.01
	3×10^{-7}	2.46 ± 0.23[b]	0.49 ± 0.17	0.20 ± 0.07
	1×10^{-7}	2.01 ± 0.32	0.27 ± 0.18	0.13 ± 0.09
	3×10^{-8}	2.46 ± 0.85	0.23 ± 0.01	0.09 ± 0.03
Lys-12	1×10^{-6}	1.05 ± 0.12	0.97 ± 0.32	0.92 ± 0.03
	3×10^{-7}	0.91 ± 0.04[b]	0.78 ± 0.11	0.86 ± 0.12
	1×10^{-7}	1.43 ± 0.27	0.36 ± 0.17	0.25 ± 0.13
	3×10^{-8}	1.43 ± 0.23	0.27 ± 0.13	0.19 ± 0.09
Tyr-10, -13 (average of 2)	1×10^{-6}	0.67 ± 0.22	0.31 ± 0.05	0.46 ± 0.16
	3×10^{-7}	0.80 ± 0.00	0.25 ± 0.02	0.31 ± 0.02
	1×10^{-7}	0.55 ± 0.20	0.04 ± 0.02	0.07 ± 0.04
	3×10^{-8}	0.58 ± 0.08	0.12 ± 0.12	0.20 ± 0.20

[a]Experiments were done in triplicate and normalized with respect to Ala_2.
[b]Results obtained from duplicates only.

Figure 23. Concentration dependence of $^{3}H/^{14}C$ ratios of glucagon functional groups in the absence (full symbols) and presence (open symbols) of liposomes. The ratios were normalized relative to internal standard Ala_2. (■,□) His-1; (●,○) Lys-12; (▲,△) Tyr-10 & -13.


2.5
2.0
1.5
1.0
0.5
$^{3}H/^{14}C$ SAMPLE / $^{3}H/^{14}C$ ALA$_2$
10
0.3
0.1
0.03
[GLUCAGON] (μM)

Finney (1978) for both trimer configurations as well as the helical monomer from the X-ray crystal analysis (Blundell, 1983). For the residues whose reactivity we have characterized (His-1, Tyr-10, Lys-12, Tyr-13), there is no calculated increase in solvent accessibility for the dissociation of trimer 2 to the monomer. However, dissociation of trimer 1 should result in greatly increased accessibility for the two tyrosines, but not for His-1 or Lys-12 (Blundell 1983).

On the basis of our reactivity data, there is no increase in tyrosine accessibility over the concentration range studied. If trimer dissociation occurs between 3.0 and 1.0 x 10^{-7} M, our results would indicate that trimer 2 would be the species involved, supporting the conclusion of Blundell (1983) that at neutral pH, trimer 1 is likely to be present in solution in only very small amounts, if at all. The slight increase in Lys-12 reactivity may be explained by trimer 2 dissociation, since, although this trimer does not reduce accessibility of the side chain to the solvent, the motions of the adjacent protomers sweep out a large volume, restricting access to labelling reagent in the bulk solution. Removal of these bulky neighbouring groups would provide a number of new angles of attack for approaching FDNB molecules. The lack of comparable increase for the two tyrosine groups (Figure 23) may be additional evidence for a local non-random structure involving these groups, as has suggested by NMR experiments (Ph.D. thesis of M.E. Wagman, 1981, Harvard University, Cambridge, Massachusetts, as cited by Blundell, 1983). The Lys-12 N^{ϵ}-amino groups is known to have an unusually low pK of approximately 8.5, as well as a lower reactivity than might normally be expected (Hefford et al., 1985). These data also support the existence of some sort of localized structure.

It should be kept in mind that the reactivities obtained are the average of two residues and changes in one may be masked by changes in the other. On the basis of these results, it seems likely that the conformation of monomeric glucagon in solution is similar to its conformation in the trimer, that is, a combination of locally ordered segments that can be stable (residues 22-26; Braun et al., 1981), or transitory (residues 10-13; vide supra), with a considerable amount of multi-conformational random coil.

4.3.2 Glucagon Trimerization Constant

The concentrations used in this investigation of glucagon-model membrane interactions were initially chosen with the expectation that glucagon would be present as the free monomer below concentrations of 1 x 10^{-6} M, in accordance with literature values for the association constant (section 2.5.1). The results we have obtained by assessing the chemical reactivities of glucagon are inconsistent with a single molecular species of glucagon being present below this concentration, indicating that trimerization may occur at lower concentrations under conditions approaching the physiological. Although trimerization will proceed via a dimeric intermediate, the lifetime of this species will likely be very short, since the addition of the third and last monomer will be even more energetically favourable than the initial dimerization. In dimerization, only half of each protomer's hydrophobic patch becomes apposed, leaving what is essentially a complete hydrophobic patch still exposed to solvent. The addition of the third protomer's hydrophobic patch seals off a total of two hydrophobic patches from unfavourable solvent interactions at one time; this provides a decrease in free energy approximately twice that of dimer

formation. Due to the highly cooperative nature of the trimerization process, it seems quite practical to deal with it in terms of a simple monomer-trimer transition, neglecting the fleeting existence of the dimer in solution.

Assuming that the pattern of chemical reactivity changes seen in the absence of liposomes (Figure 23) is consistent with a monomer-trimer transition, the midpoint concentration for this process may be estimated as being about 1.8×10^{-7} M. Thus, if the transition is represented as

$$3M \rightleftharpoons T,$$

where M and T are the concentrations of monomer and trimer, respectively, then a trimerization constant $K_{1,3}$, may be defined:

$$K_{1,3} = T/M^3 \qquad (I).$$

The total concentration of protomers at any time P_o, may be expressed as

$$P_o = M + 3T.$$

At the midpoint of trimerization, M = 3T, so that

$$P_o = 2M = 6T \qquad (II).$$

Substituting equation (II) into (I):

$$K_{1,3} = (P_o/6) \; / \; (P_o/2)^3,$$

$$= (4/3) \; P_o^{-2}.$$

Using the estimated midpoint concentration for the trimerization,

$$K_{1,3} = 4.11 \times 10^{13} \; M^{-2}.$$

This value is approximately six orders of magnitude greater than the highest previously reported value (Formisano et al., 1978), which was measured at pH 10.6 using glucagon concentrations of 6.3×10^{-5} M and greater. The known relative insolubility of glucagon in neutral solution as compared to extremes of pH (Bromer, 1972) makes such a high value for trimerization constant seem reasonable. In all previous

estimates of the trimerization constant, the values obtained seem to approach the sensitivity of the experimental methodology employed; this is not the case in the present work. A trimerization constant of 4.11 x 10^{13} M^{-2} corresponds to a free energy change of about -6.4 kcal/mol protomer, entirely consistent with free energy changes observed for the self-association of other proteins (Schulz & Schirmer, 1979).

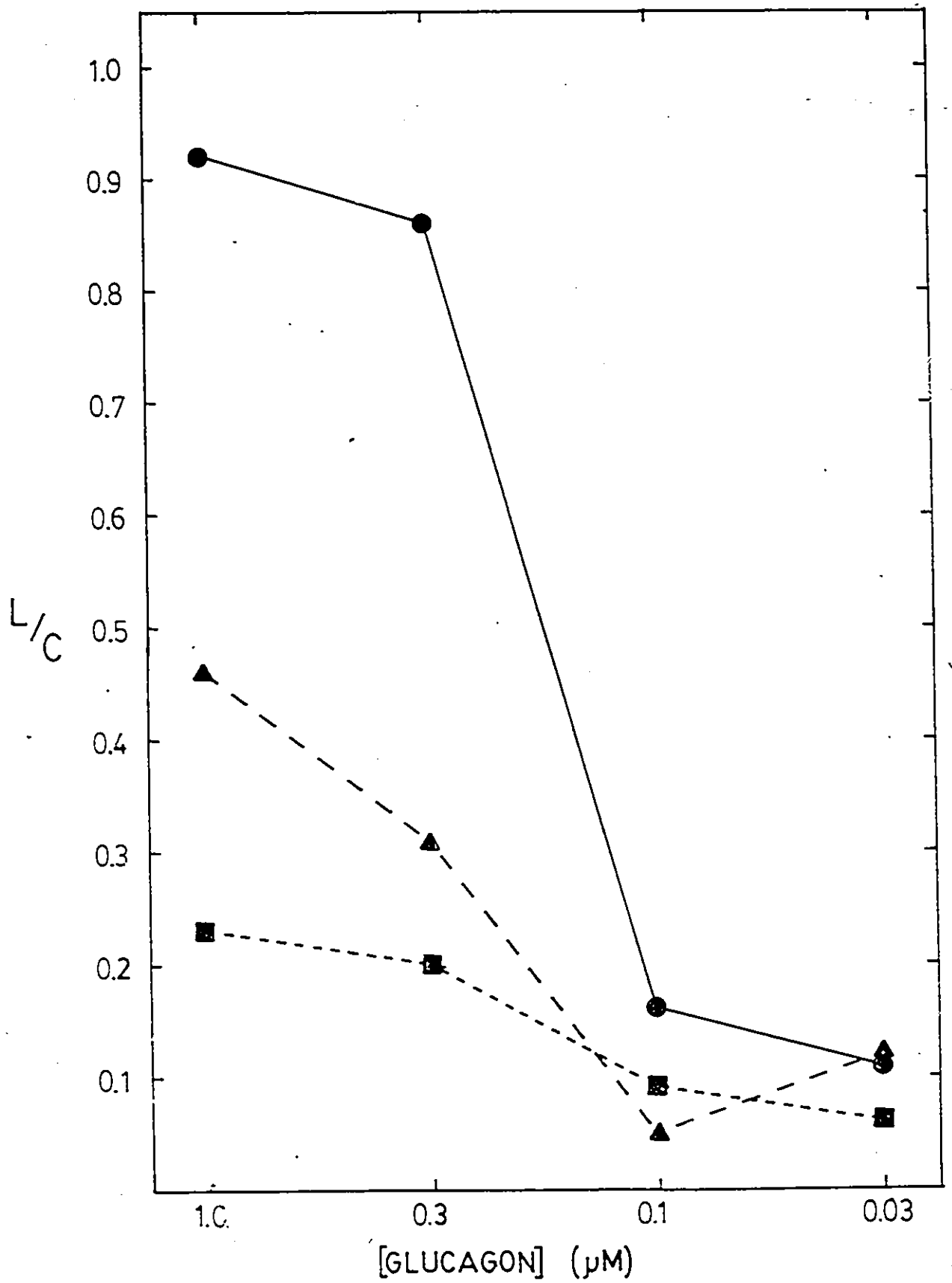
4.3.3 Functional Groups of Liposome-Associated Glucagon

The functional group reactivities underwent a drastic reduction in the presence of liposomes (Figure 23). As the glucagon concentration is lowered, there is a trend towards a further reduction in chemical reactivity. The amino terminal histidine and the two tyrosine residues are affected to a much greater degree than the Lys-12 residue, which, over the concentration range of 1.0 x 10^{-6} to 0.3 x 10^{-8} M, does not have an appreciably different reactivity than in the control samples. Below this concentration however, Lys-12 reactivity drops markedly.

Correction for the adsorption of Ala_2 to the liposomes at the lowest concentrations, yields numbers that can be used to calculate a corrected L/C ratio. The corrected L/C ratios are shown in Figure 24, which shows the pattern of reactivity changes that take place in glucagon upon association with liposomes.

Although there is a significant and parallel decrease in reactivity for the His-1, Tyr-10 and Tyr-13 groups at all concentrations studied, the reactivity of Lys-12 shows no change at the higher concentrations, but only falls markedly at lower concentrations. These results are inconsistent with only one form of glucagon existing over the concentration range studied. The fact that the reactivities do not decrease in parallel (Figure 24) demonstrates that, between 1.0 x 10^{-6}

Figure 24. Concentration dependence of corrected L/C ratios of the functional groups of glucagon. (■) His-1; (●) Lys-12; (▲) Lys-21 & -23.

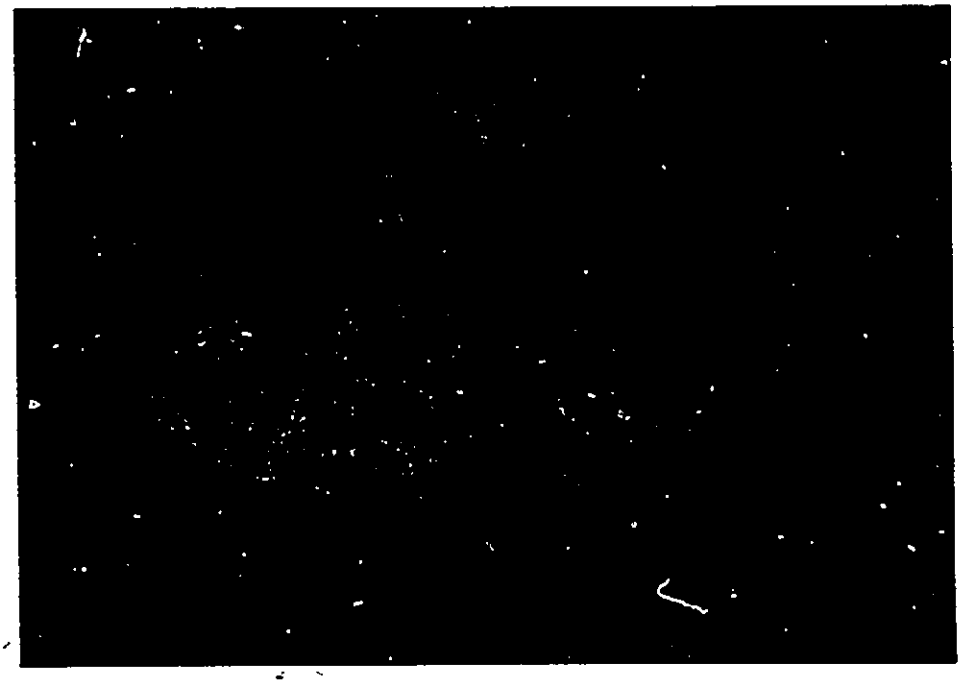

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
L/C
1.0
0.3
0.1
0.03
[GLUCAGON] (μM)

and 3.0×10^{-7} M, glucagon binds in a specific orientation, and does not simply adsorb to the liposomes in a random manner. The change in Lys-12 reactivity with concentration could be a consequence of two (or more) conformational states, one at 1×10^{-6} M, and another at 3×10^{-8} M in which the lysine side chain has different environments. A model consistent with these phenomena requires a conformation in which the N-terminal histidine and the relatively hydrophobic tyrosine residues may interact preferentially with the bilayer.

The observed results are consistent with those expected on the basis of the structure of trimer 2. Since the sequence positions of residues Phe-6, Tyr-10, Tyr-13 and Leu-14 satisfy the requirements for an amphiphilic helix (Figure 9), it is plausible to suggest a helical conformation for this region in the presence of an asymmetric hydrophobic force field. Association with the liposome surface would sequester the tyrosyl side chains from the bulk solution, resulting in their decreased reactivity. The conformation of the amino terminal tetrapeptide (residues 1-4) is highly variable, so it is not reasonable to postulate a specific conformation for this segment. The data indicate however, that His-1 interacts strongly with the membrane and is effectively removed from interaction with the aqueous phase.

The simplest explanation for the decreased reactivities is that binding of glucagon type 2 trimers-(Figure 11) to the membrane surface is mediated by the N-terminal region of the protomers, specifically involving residues Phe-6, Tyr-10 & 13, and Leu-14 of the hydrophobic patch (Figure 25). Inspection of the N-terminal residues of the type 2 trimer reveals that when residues 5 to 15 are in the helical conformer of the crystal structure, they may easily provide points of contact

Figure 25. The hydrophobic patch of glucagon's N-terminal amphiphilic segment. The van der Waals surface of hydrophobic amino acids Phe-6, Tyr-10 and -13, and Leu-14 are shown, colour-coded according to atom (carbon: yellow ; oxygen: red ; nitrogen: blue). The position of the Lys-12 N^{ϵ}-amino group (at the top center of the page, labelled as 'NZ 12') is clearly to be seen as lying on the opposite side of the hydrophobic patch.



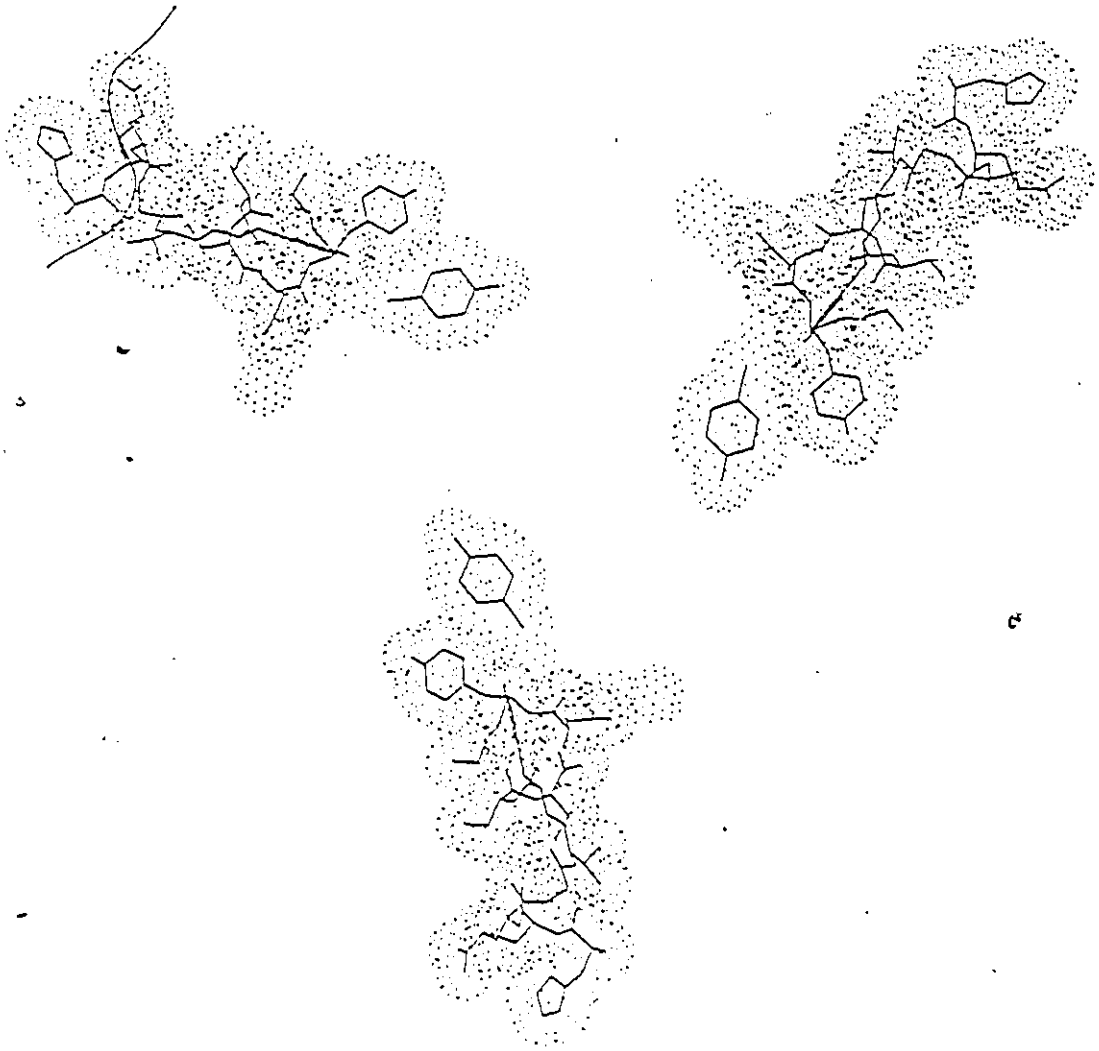
between the trimer and the liposome surface. Figure 26 is a computer generated view of the N-terminal region as it might present itself to the bilayer. Once one of the hydrophobic 'feet' of the trimerbecomes strongly adsorbed, presumably via hydrophobic stabilization through contact with the bilayer hydrocarbon phase, the other two 'feet' of the trimer would tend to adsorb in a cooperative manner.

The relatively unchanged reactivity of Lys-12 from 1.0×10^{-6} to 0.3×10^{-6} M suggests that the environment of this residue remains essentially the same, whether the trimer is free in solution, or associated with the liposome surface. In a helical conformation, Lys-12 is on the opposite side of the hydrophobic region comprising Phe-6, Tyr-10 & 13, and Leu-14 (Sasaki et al., 1975), and would therefore be fully accessible to the labelling reagent.

At lower concentrations (1.0×10^{-7} M and below), Lys-12 reactivity decreased dramatically (Figure 24), indicating a profound change in environment, structure, or both. By analogy to the changes observed in the control samples, a possible interpretation is that dissociation of glucagon trimers, whether in solution or membrane bound, results in the release of monomers which associate with the membrane in such a way as to hinder Lys-12 reactivity. Proximity to a membrane surface could reduce the N^{ϵ}-amino group reactivity in a number of ways: the protonated amine could form a salt bridge to the phosphate group of a neighbouring PC molecule, or the high dielectric environment of the headgroup region (Raudino & Mauzerall, 1986) could stabilize the ionized, unreactive, amine.

It might be argued on the basis of helix stereochemistry that Lys-12 should extend into the solvent and maintain its reactivity if the

Figure 26. Residues 1 to 13 of each protomer are shown in this cross-sectional view of the glucagon type 2 trimer as seen down the central 3-fold axis of rotation. The remainder of the trimer, which would extend away from the viewer and into the page, is omitted for clarity. If the trimer were to adsorb to the liposome surface after the manner of a tripod, the threee N-terminal hydrophobic patches shown here would be presented to the bilayer surface first.



glucagon monomer is membrane-bound. This argument assumes a strictly alpha-helical conformation, but there is evidence suggesting the possibility of 3_{10} helix over residues 5-11 (Blundell, 1983). In the most detailed physical study to date, Braun et al (1983) have shown that the helices of glucagon bound to DDP micelles are not ideally alpha-helical, but may be extended. As well, interaction of the C-terminal hydrophobic patch with the lipid bilayer may cause a reorientation of previously bound segments relative to the membrane surface.

Although there is no evidence for His-1 interaction with lipids in micelle-bound glucagon (Braun et al., 1983) the topology of the relatively planar LUV surface is vastly different from that of the quasi-spherical micelles containing about 40 lipid molecules, and this may explain why such an interaction has not been previously observed. The structural flexibility of glucagon is such that a number of conformations are possible, particularly in the amino terminal half of the molecule. Prediction methods support the possibility of beta-turns over residues 2-5, 10-13, and 15-18 (Chou & Fasman, 1975; Korn & Ottensmeyer, 1983). Whether or not these conformations are likely to occur at a membrane/solvent interface is not known, but these segments may provide 'hinges' with threshold activation energies that are sensitive to their local environment. These hinges could be locked into or out of beta-turn conformations. The presence of two arginine residues at positions 17 and 18 in one of the predicted 'turn' segments may have important structural consequences for membrane-bound glucagon, since guanidinium groups are known to form doubly hydrogen-bonded structures with phosphates (section 2.6.1).

4.4 Melittin

4.4.0 Free Ala_2 in Solution

A pertinent question to the interpretation of the $^{3}H/^{14}C$ data in this experiment is whether or not the high concentration of nucleophiles in solution exceeded the capacity of the tritiated trace labelling reaction to label the nucleophiles at the relatively high concentrations employed in this experiment (above 1×10^{-4} M). Here 'capacity' is defined as the upper limit for label incorporation using a small and finite amount of reagent with an increasing amount of substrate (nucleophile). With constant amounts of ^{14}C-derivative as background, the effect of such a dilution of tritium amongst an increasing number of nucleophiles would be a net reduction in the $^{3}H/^{14}C$ ratios for all the nucleophiles. This is in fact observed: the N-terminals of both Ala_2 and melittin, as well as the latter's three lysyl amino functions all have lower $^{3}H/^{14}C$ ratios at concentrations above 1×10^{-4} M (Table IX). Since the dilution of ^{3}H-trace label would be distributed evenly over these groups, the use of Ala_2 as an internal standard is not affected, but is in fact made necessary.

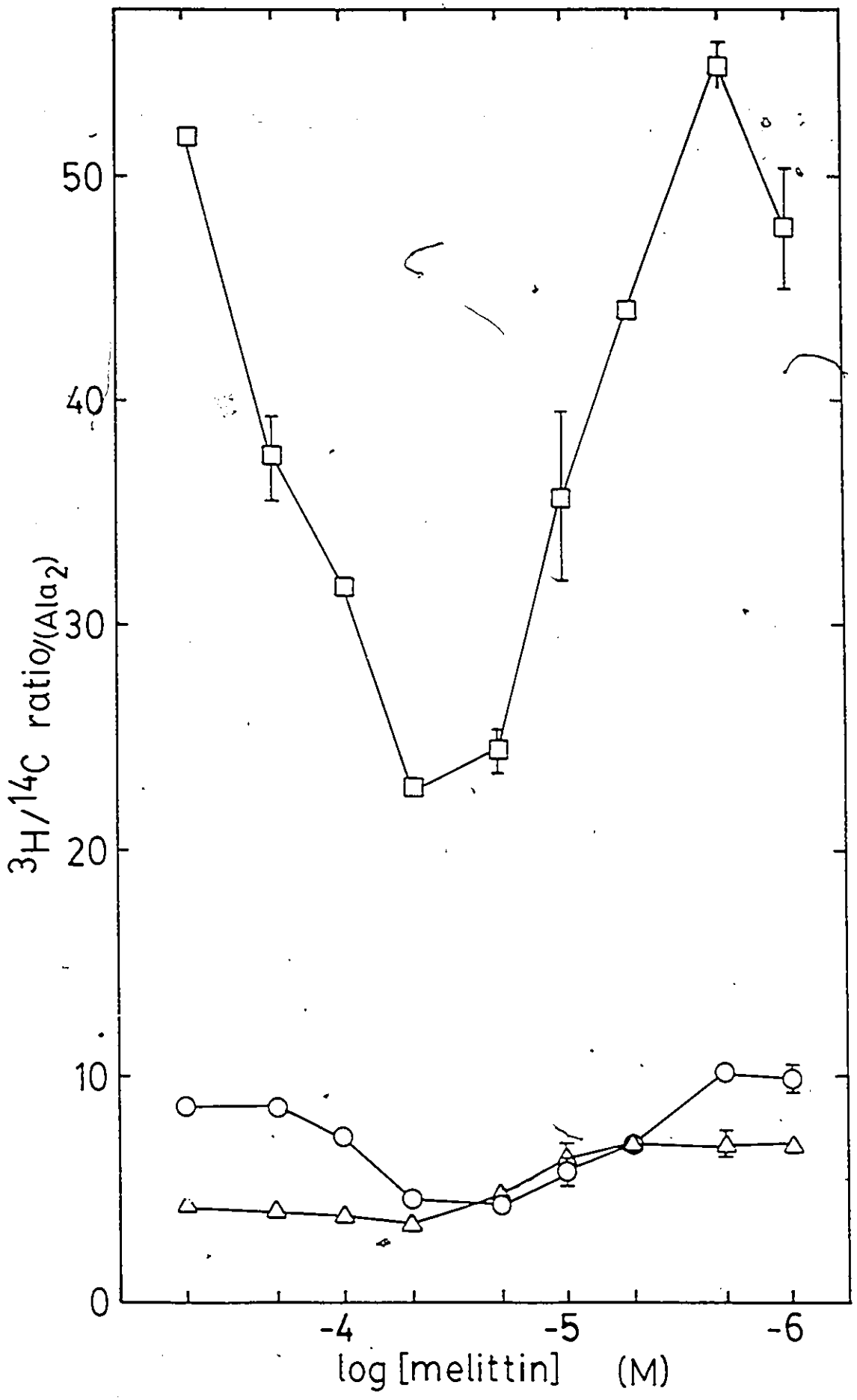
4.4.1 Functional Groups of Free Melittin

The concentration-dependent reactivity profiles for the melittin functional groups relative to the internal standard are shown in Figure 27. The reactivity/concentration profiles for Gly-1 and Lys-21 & -23 are bimodal, with 50% reductions in reactivity over the first half of the concentration range; the reactivity is then recovered in the latter half. This behaviour implies the existence of at least three molecular species over the concentration range studied. The reactivity behaviour

TABLE IX: $^{3}H/^{14}C$ Ratios of Ala_2 and Melittin Functional Groups in Solution.

CONCENTRATION (M)	ALA_2	GLY-1	LYS-7	LYS-21 & 23
7×10^{-4}	3.99	205.69	16.75	33.86
	4.15	214.69	17.88	34.83
3×10^{-4}	5.22	204.70	22.18	44.28
	5.77	204.48	23.21	47.51
1×10^{-4}	7.73	243.79	28.38	49.71
	7.13	232.30	29.23	47.94
7×10^{-5}	7.34	167.17	25.58	32.12
	6.91	155.37	24.66	30.51
3×10^{-5}	4.14	97.34	18.69	16.43
	4.08	103.35	20.15	18.35
1×10^{-5}	2.26	89.20	15.98	14.33
	2.39	76.19	13.59	12.78
7×10^{-6}	1.34	59.38	9.03	9.43
	1.30	57.11	8.80	8.79
3×10^{-6}	0.66	35.42	4.20	6.61
	0.56	31.54	4.19	5.73
1×10^{-6}	0.36	18.34	2.83	3.83
	0.37	16.79	2.44	3.44

Figure 27. Concentration-dependence of functional group $^{3}H/^{14}C$ ratios obtained from free melittin in solution. The ratios are normalized relative to the internal standard Ala_2. (□) Gly-1; (△) Lys-7; (○) Lys-21 & -23.


50
40
30
20
10
0
$^3H/^{14}C$ ratio/(Ala_2)
-4
-5
-6
log [melittin] (M)

of the Lys-7 residue is somewhat different, with a less well defined decrease at intermediate concentrations. A reasonable explanation is that the bimodal reactivity pattern is due to the presence of three concentration-dependent and interconvertible forms of melittin, the tetramer, dimer, and monomer. The tetrameric form of melittin, present at high concentrations of the peptide, must be in equilibrium with the dimeric form, which in turn must equilibrate with the monomer at lower concentrations.

Glycine-1. The N-terminal glycine is the most reactive functional group in melittin towards FDNB (Figure 27). As an aside, this group is reportedly not reactive towards trinitrobenzenesulfonate (TNBS) (Quay & Tronson, 1983), which also reacts via an aromatic nucleophilic substitution reaction. NMR studies indicate that the pK of Gly-1 is 7.7 in monomeric melittin (Lauterwein et al., 1980); though pK values of 7.2 and 6.8, for melittin concentrations of 8×10^{-5} and 6.3×10^{-4} M, respectively, have also been reported (Bello et al., 1982). Despite these lower pK values, the N-terminal amino group is largely deprotonated in the tetramer according to NMR chemical shift titration experiments (Brown et al., 1980). The absence of a charge at the amino terminus could promote monomer aggregation by reducing charge repulsions between the N-terminal and the polycationic C-terminal segments of adjacent protomers.

At the extremes of aggregation, that is, either as a tetramer or as a monomer, Gly-1 reactivity is the same. This fact must reflect a balance of contributing factors: although Gly-1 in the tetramer is unprotonated and thus highly reactive, it also experiences long-range steric hindrance due to the bulk of the tetramer. By contrast, in the

monomeric form of melittin the Gly-1 reactivity is of similar magnitude, though the relative importance of the restrictions is reversed: without the suppression of charge formation in the tetramer, the amino terminal would be free to ionize, giving rise to the less reactive protonated amine. Concurrently, the degree of steric shielding is greatly reduced. At the midpoint between these extremes, one might expect to find a point where the contributions from ionization state and steric factors both figure prominently. In the dimeric form it would seem that the greater accessibility of the Gly-1 N atom to FDNB is offset by the degree of ionization, resulting in a net decrease in reactivity. The number of degrees of freedom lost in dimer formation from a randomly coiled monomer is much greater than the number of degrees of freedom lost when going from dimer to tetramer. This aspect likely contributes to the decreased steric hindrance to reactivity. In the monomer, both protons and FDNB have relatively unrestricted access to the N-terminal.

Lysine-7. The Lys-7 side chain extends directly out into solution in the tetramer (Terwilliger & Eisenberg, 1982a), and this would also be the preferred configuration in the dimer and monomer. The micro-environment of Lys-7 is not expected to change upon dissociation; any effect on reactivity due to dissociation must be due to long-range shielding effects. The mass of aggregated protein in the tetramer sweeps out a large volume in the bulk solution relative to the Lys-7 N amino groups, restricting the exposure to bulk solution and the number of angles of approach for FDNB. This long-range steric hindrance is removed at low concentrations of melittin, a slight increase in reactivity is observed (Figure 27). The effect is similar to the one observed for glucagon's Lys-12 side chain, which had increased reactivity after

trimer dissociation (section 4.3.1).

Lysines 21 & 23. The interpretation of the reactivity data for Lys-21 and -23 is not straightforward, as the data represents the average of two groups which, despite their similarity in structure and location in the melittin molecule, may not be equivalent. The atomic coordinates of the four positively charged residues near the C-terminus are not clearly resolved in the crystal structure (Terwilliger & Eisenberg, 1982b). In the crystal tetramer, the orientation of the four helices relative to one another separates the positively charged chain segments from one another, contributing to the high solubility of the melittin tetramer. Although the side chains of Lys-21 and Arg-22 and -24 extend out into the solvent, the Lys-23 side chain appears to be inaccessible to the solvent. (Solvent accessibility is here defined according to the method of Richards (1974), where water molecules have a van der Waals radius of 1.4 Å.) This may be partly an artifact of crystallization, since these side chains will repel one another resulting in a random coil in the absence of phosphate. Conversely, the presence of phosphate will favour helix formation (section 2.6.1). C-terminal segment conformations that are intermediate between random coil and helix are most likely at the low phosphate concentrations employed in the present work.

In solution, the tetramer structure obtained by crystallography would be less constrained, and the number of allowable side chain configurations may be expected to increase. Nevertheless, the assignment of qualitatively different environments for the Lys-21 and -23 residues is a distinct possibility. The data indicate that Lys-21 and -23 are more reactive in the monomer than in the tetramer, but that one or both of these side chains becomes less reactive in the dimer.

The simple model of tetramer dissociation to yield the crystallographic dimers, and thence to monomers, does not provide any obvious means, steric or otherwise, of reducing the reactivity of these groups in the dimer relative to the tetramer. A reasonable possibility is that the secondary structure of this segment changes upon both tetramer dissociation and dimer dissociation. A second possibility is that the ionization state is shifted in the tetramer in a manner similar to the Gly-1 residue, favouring a lower pK and enhanced reactivity. Perhaps melittin dimers are able to associate in more than one orientation. Alternatively, reactivities may be decreased due to interactions, direct or indirect, with a neighbouring side chain. The aromatic ring system of Trp-19 is a good candidate for this since it is adjacent to Lys-23 in a helix conformation, and could form an energetically favourable weakly polar interaction with the lysyl N amino function via its aromatic pi electrons (Burley & Petsko, 1986).

It should be noted that Quay & Tronson (1983) have measured the apparent pKs of Lys-21 & 23 in both the monomer and tetramer, using TNBS. Their failure to obtain modification at residues Gly-1 and Lys-7 is in direct contradiction to the unequivocal findings reported here. The reasons for the non-incorporation of label in Quay and Tronson's study may be due to the extreme sensitivity of the reagent to steric effects (Quay & Tronson, 1983) since other factors, such as net molecular charge and hydrophobic regions, would tend to facilitate this reaction (Lundblad & Noyes, 1984).

4.3.2 Do Melittin Dimers Occur in Solution?

There are few published reports of melittin dimers. Most studies report only the existence of tetramers or monomers. Wuthrich's group did

not explicitly seek dimers in their NMR studies of melittin self-aggregation (Brown et al. 1980; Lauterwein et al., 1980). Circular dichroism studies (Tatham et al., 1983) only detect changes in secondary structure, in this case, coil-helix transitions, and probably could not differentiate between the dimers and tetramers, both of which are predominantly alpha-helical (ca. 70-90%). It is also doubtful whether the other major physical technique used to study self-aggregation, fluorescence (Talbot et al., 1979; Quay & Condie, 1983; Thompson & Lakowicz, 1984), would be able to detect dimers. The melittin fluorophore, Trp-19, is closely surrounded by both apolar and polar groups in the tetramer, although the $N^{\epsilon 1}$ atom of the indole ring, as well as one or two other ring atoms, are accessible to the solvent. This information is derived from the crystal structure, for which the C-terminal side chains are not well defined (Terwilliger & Eisenberg, 1982a). In solution, the Trp-19 indole ring is likely to be even more solvent accessible due to side chain mobility. Such a microenvironment supports the moderate blue shifting of the Trp-19 λ_{max} observed by Quay & Condie (1983) upon tetramerization corresponds to a 'surface' or 'class II' tryptophan according to the classification scheme of Burstein et al. (1973). In an AB or CD dimer however, the Trp-19 side chain extends away from the monomer-monomer interface and would not experience any major change in microenvironment (Figure 13).

It is difficult to see how the absence of a dimeric intermediate can be assumed in studies of melittin, irrespective of whether or not changes in fluorescence parameters fit models that assume only a monomer-tetramer equilibrium (Quay & Condie, 1983; Vogel & Jahnig, 1986). Furthermore, Bello et al. (1982) have shown that at high pH

(>9.0) the degree of helicity derived from CD measurements is not mirrored by parallel changes in the degree of fluorescence polarization. Hence, fluorescence polarization is not a direct indicator of the coil-helix transition which must necessarily accompany dimerization.

Gel filtration through Sephadex G-50 has been used by various groups studying melittin, and some evidence for the presence of dimers can be adduced from them. Talbot et al. (1979) found that in the presence of 1.5 M NaCl, melittin eluted near cytochrome c with an apparent molecular weight of about 12,000 daltons ($MW_{tetramer}$ = 11,360 daltons), whereas in the absence of salt, the monomeric weight was judged to be about 3500 ($MW_{monomer}$= 2840 daltons). Although a weight of 3500-3600 is about 25% greater than the formula weight of a monomer, this may reflect some interaction with the gel matrix. For example, melittin's behaviour on Sephadex LH-20 is certainly known to be anomalous (see Fig. 6A,B of Dawson et al., 1978). This result should be compared with the results obtained by Bello et al. (1982), who found a species eluting at a concentration of 9×10^{-2}M, with an apparent molecular weight of 7600 daltons. This is likely the melittin dimer, as was interpreted by Bello et al. (1982).

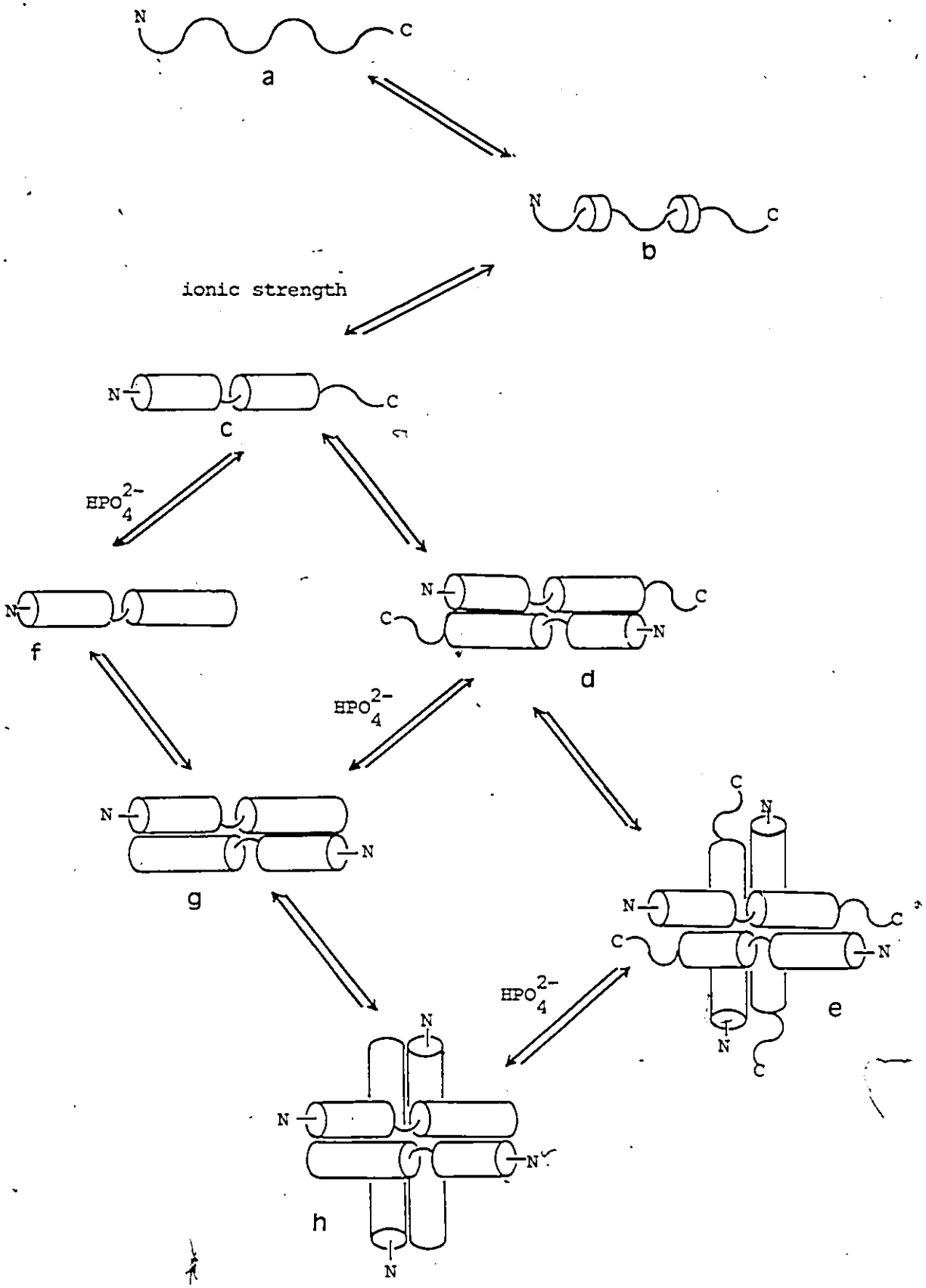
Thus it is reasonable to assume that dimers exist in solution, despite the assertions of Schubert et al. (1985) to the contrary. These workers fit their analytical ultracentrifugation data to a monomer-tetramer model of association and concluded that the concentration of dimer cannot exceed 0.5% of the total protein concentration. Although Schubert et al. successfully applied their method to two other self-associating proteins, the "relative uncertainty... is quite large" (Schubert et al., 1985). Since this technique depends upon "precise data

at very low protein concentrations, the region where experimental difficulties are most severe" (Klotz et al., 1975), the conclusions of Schubert et al. (1985) need not be considered the definitive statement on the association equilibrium of melittin.

In a recent review of amphipathic peptides (Kaiser & Kezdy, 1987), unpublished work by G. Yates and H.P. Tao is described in which the rate of melittin-induced hemolysis depends solely on the concentration of dimer in solution, supporting an earlier study in which the slow phase of erythrocyte lysis was found to be proportional to the square of the melittin concentration (Degrado et al., 1982). Given the source and function of melittin, we can infer that the dimer is at least a major functional form of this 'attack peptide', and should be present in solution. The data presented in Figure 27, and the foregoing discussion, supports this concept. A model of melittin tetramerization is proposed in Figure 28, which summarizes the reported conformational transitions in the literature in the context of the monomer-dimer-tetramer association suggested by the present work.

Approximate association constants for the monomer-dimer and dimer-tetramer associations under the conditions of this experiment can be calculated by the method used to obtain the glucagon trimerization constant (section 4.3.2). If the equilibrium midpoints are halfway between the reactivity maxima and minimum, the midpoint for dimerization from monomers is estimated from Figure 27 to be 1.65×10^{-5} M, yielding a dimerization constant $K_{1,2}$, of 7.57×10^{4} M^{-1}. Similarly, estimating a midpoint for tetramer formation to be 3.85×10^{-4} M, $K_{2,4}$ is 3.25×10^{3} M^{-1}. These values are quite similar to those reported in the literature for the monomer-tetramer transition ($K_{diss} = 7.96 \times 10^{-14}$ M^3; Quay &

Figure 28. Proposed model of melittin self-association. The concentration of melittin increases from the top of the page to the bottom. In dilute solution, melittin exists as a random coil (a), in dynamic equilibrium with a conformer containing small elements of non-random structure, probably helical (cylinders, b). Upon increasing the concentration or ionic strength, a conformer is induced which is approximately 65-70% alpha-helix (c). This form will dimerize (d) and tetramerize (e) with increasing concentrations of melittin. Each of these form can assume conformations which are almost completely alpha-helical in the presence of phosphate (f, g, h).


N
C
a
b
ionic strength
c
HPO_4^{2-}
f
d
HPO_4^{2-}
g
e
HPO_4^{2-}
h

Condie, 1983), but it should be noted that the self-association process is very sensitive to even slight changes in experimental conditions.

4.4.4 Ala_2 in the Presence of Liposomes Results from the glucagon experiment (section 4.3) indicated that in very dilute solution (below 10^{-7} M), and at 0.1 M salt, adsorption of Ala_2 to bilayers can be significant, although this was not found to be the case at micromolar concentrations. In the presence of liposomes, Ala_2 $^3H/^{14}C$ ratios were similar to the control samples at micromolar concentrations (Table X). However, the Ala_2 L/C ratio shows that liposome-associated Ala_2 reactivity decreases in a manner similar to a binding curve (Figure 29).

Gel filtration studies were undertaken to determine whether or not the internal standard was interacting with the melittin. Ala_2 did not significantly alter the pattern of melittin elution (Figure 30), so it was assumed that Ala_2 does not interact with melittin to any appreciable extent, and that the low $^3H/^{14}C$ counts for Ala_2 is the result of other perturbations within the system. These results can be explained on the basis of melittin's lytic effects on liposomes and subsequent reorganization of the lipids involved (vide infra). It seems likely, on the basis of the results presented further on, that lipid-melittin aggregates are formed which bind Ala_2.

Since the labelling of free melittin and Ala_2 was satisfactory (section 4.4.0), these results were taken as the standard by which dilution of the functional groups could be corrected. Functional group $^3H/^{14}C$ ratios were therefore normalized with respect to the Ala_2 $^3H/^{14}C$ ratios from the control samples at the appropriate concentrations. The average $^3H/^{14}C$ ratios were employed for this purpose, and the results give a first approximation of the relative reactivities of melittin's

TABLE X: $^{3}H/^{14}C$ Ratios of Ala_2 and Melittin Functional Groups in the Presence of Liposomes.

CONCENTRATION (M)	ALA_2	GLY-1	LYS-7	LYS-21 & 23
7×10^{-4}	0.85	220.92	6.35	18.52
	0.71	183.05	5.04	15.63
3×10^{-4}	0.55	266.39	8.37	28.99
	0.56	253.11	8.98	25.85
1×10^{-4}	0.49	274.48	8.57	20.92
	0.49	285.85	7.81	20.46
7×10^{-5}	1.01	252.03	5.44	11.38
	0.92	250.98	9.24	14.59
3×10^{-5}	1.17	225.06	6.97	13.41
	1.19	195.73	6.57	11.60
1×10^{-5}	0.97	169.25	4.56	8.49
	0.86	177.51	5.44	8.87
7×10^{-6}	0.77	159.02	4.23	10.42
	0.73	148.38	4.19	9.48
3×10^{-6}	0.55	58.56	2.67	6.20
	0.52	56.33	2.51	5.53
1×10^{-6}	0.34	25.75	1.56	3.19
	0.34	25.40	1.49	2.97

Figure 29. Concentration dependence of Ala_2 $^3H/^{14}C$ ratios in absence (filled circles) and presence (open circles) of liposomes (1 mM total lipid). Resultant L/C profile is sigmoidal (inverted triangles).

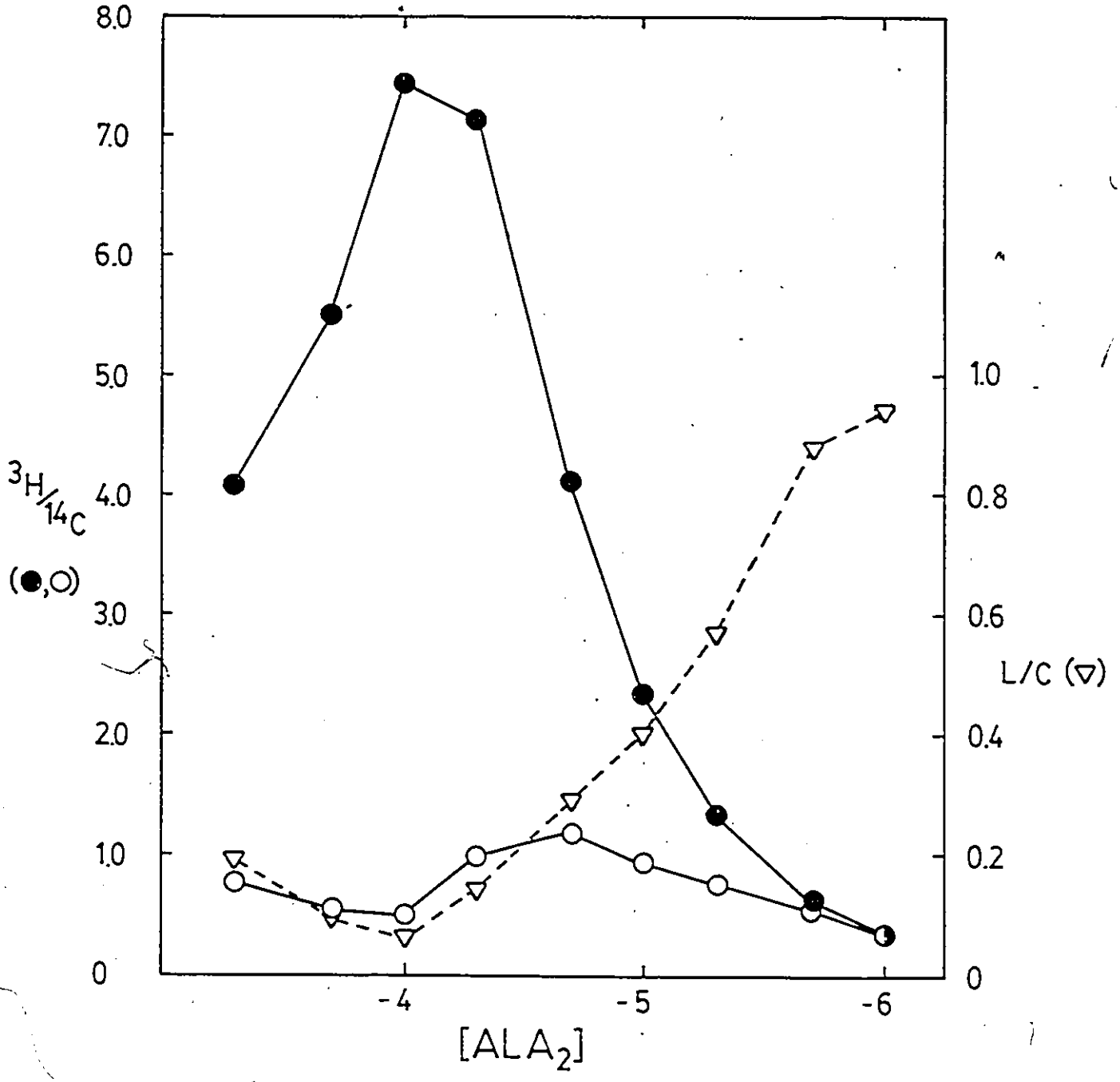
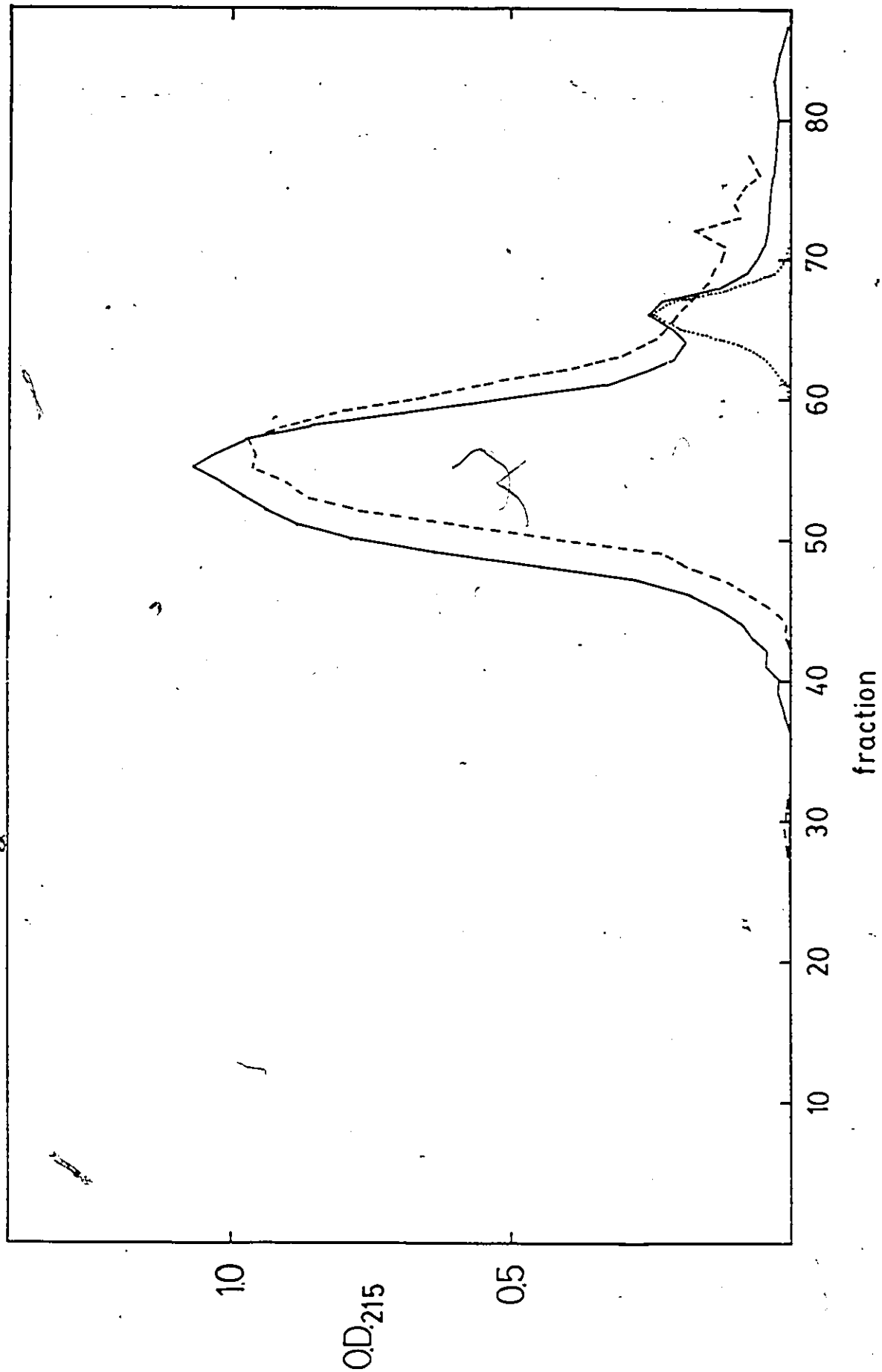

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
$^3H/^{14}C$
(●,○)
1.0
0.8
0.6
0.4
0.2
0
L/C (▽)
-4
-5
-6
[ALA$_2$]

Figure 30. Sepahdex G-50 gel filtration of solutions of melittin (- - - -); Ala_2 (·····); and both melittin and Ala_2 together (———). Concentrations in each case are 2 mM.


1.0
0.5
O.D.215
10
20
30
40
50
60
70
80
fraction

functional groups over the concentrations studied.

4.4.5 Melittin-Induced Perturbation of Liposome Structure In light of the known disruptive effect of melittin on phospholipid bilayers, it would be inappropriate to interpret the data in the same manner as with the insulin and glucagon experiments. In the case of insulin we have a protein with well defined tertiary structure; the interaction with a bilayer surface would appear to be one of simple apposition or adsorption, with possible immersion of some non-polar side chains in the hydrophobic region of the bilayer. In the case of glucagon, the situation may be similar due to the peptide having some amphiphilic structure, though, being small and flexible, glucagon may be able to undergo mutual adjustments with the phospholipids. Melittin differs from the other proteins by virtue of its unique structure: it is designed to associate with and physically disrupt membranes. As such it is a good model for proteins and peptides that have definite interactions with membranes in vivo.

The functional group reactivities share similar trends in their concentration-dependence (Figure 31), as they did in the control samples. They are higher at low concentrations, pass through a minimum at 7×10^{-5} M, and then show slight differences in the degree to which their reactivities increase at higher concentrations. These results would indicate that the whole molecule experiences a markedly different environment at the concentrations near the shared reactivity minimum, and that there are more site-specific perturbations in the microenvironments of the functional groups at high melittin concentrations. It is instructive at this point to consider in more detail the findings of Dufourcq et al., (1986) with liposomes.

Figure 31. Concentration-dependence of functional group $^{3}H/^{14}C$ ratios obtained from melittin in the presence of liposomes. $^{3}H/^{14}C$ ratios are normalized relative to the internal standard Ala_2 ratios obtained from the control experiment (see text). (■) Gly-1; (▲) Lys-7; (●) Lys-21 & -23. Mean values from the duplicate experiments are shown; ranges are 9% or less of the values plotted.

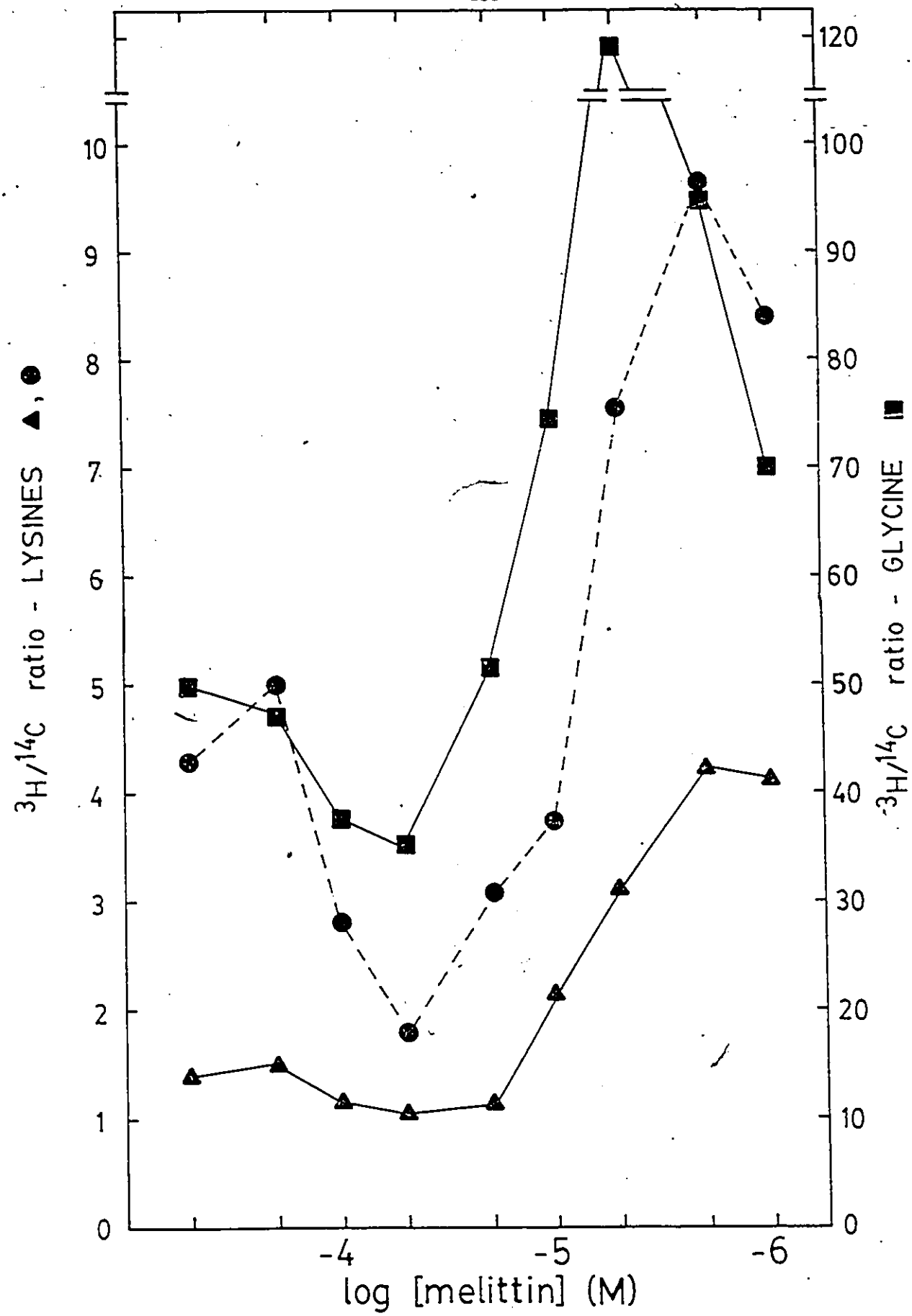
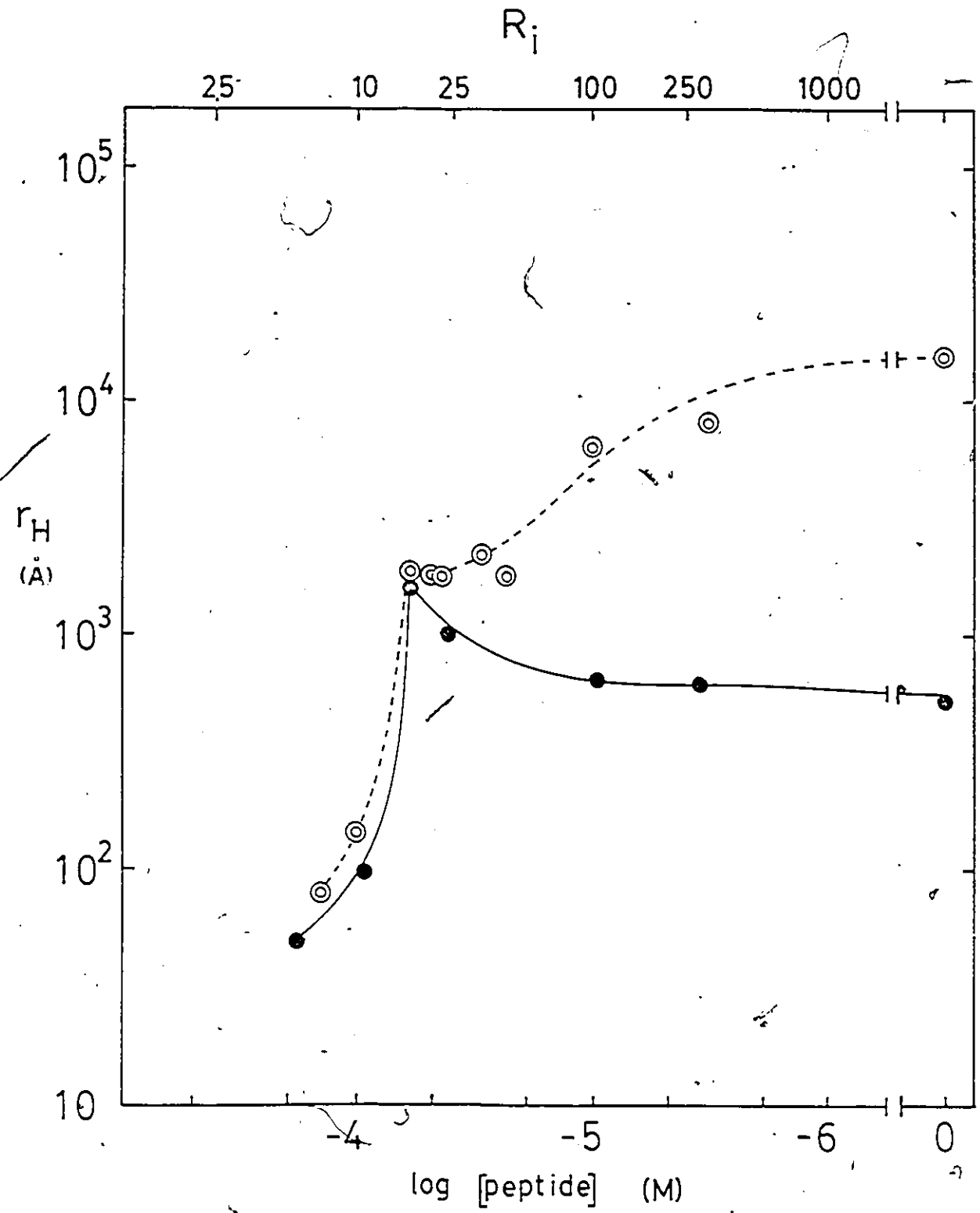

$^3H/^{14}C$ ratio - LYSINES ▲, ●
$^3H/^{14}C$ ratio - GLYCINE ■
log [melittin] (M)
-4
-5
-6
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
70
80
90
100
120

Figure 32. Data from Dufourcq et al. (1986) adapted to the scale used in the present work for comparison. The graph illustrates the effect of increasing the total concentration of melittin ([peptide]) on the hydrodynamic radius (r_H) of EPC liposomes. Size changes are observed for both large multilammellar vesicles (◎) and small unilammellar vesicles (●). The lipid-to-protein ratio (R_i) is indicated along the top of the figure.

In order to assess the data properly, it is necessary to establish as far as possible what the physical state of the system is. The recent work of Dufourcq et al. (1986), provides information relevant to the interpretation of the results obtained in the present work.

Dufourcq et al. (1986) prepared EPC liposomes two ways; MLV via simple dispersion (hydration followed by vortexing), and SUV (the MLV were subsequently sonicated). The QLS-measured hydrodynamic radii (r_H) of their MLV and SUV were 16,000 and 540 angstroms, respectively. Upon increasing the amounts of melittin the r_H values for these two morphologically distinct forms converged until a plateau was reached at R_i between 13 and 25. At this point, the melittin-EPC structures had a r_H of 1700 Å. At lower lipid-to-protein rations, there is a sudden transition to smaller-sized structures of about 150 Å. Further increases in the melittin concentration cause the r_H to diminish even further, until a limiting r_H of about 50 Å is attained at $R_i < 6$. Data from figure 2 of Dufourcq et al. (1986) have been adapted to the coordinates used in the present work and are shown in Figure 32.

Valid comparisons between the system of Dufourcq et al. (1986) and the present one can be made since differences between the two are of a relatively minor nature. Dufourcq et al. used a 50 mM phosphate/ 1 mM EDTA buffer, whereas the present work was done with a 5 mM phosphate/100 mM KCl buffer. In both cases the pH and temperature was the same (pH 7.5 at room temperature), and lipid concentrations were similar: 2.6 mM (Dufourcq et al.) versus 1 mM (present work). Major differences may be the inclusion of 33 mol% cholesterol and the presence of the Ala_2 internal standard. The overall effect of cholesterol would be to confer more order and rigidity on an EPC liposome, but not so much as to mimic


R_i
25
10
25
100
250
1000
10^5
10^4
10^3
10^2
10
r_H
(Å)
-4
-5
-6
0
log [peptide] (M)

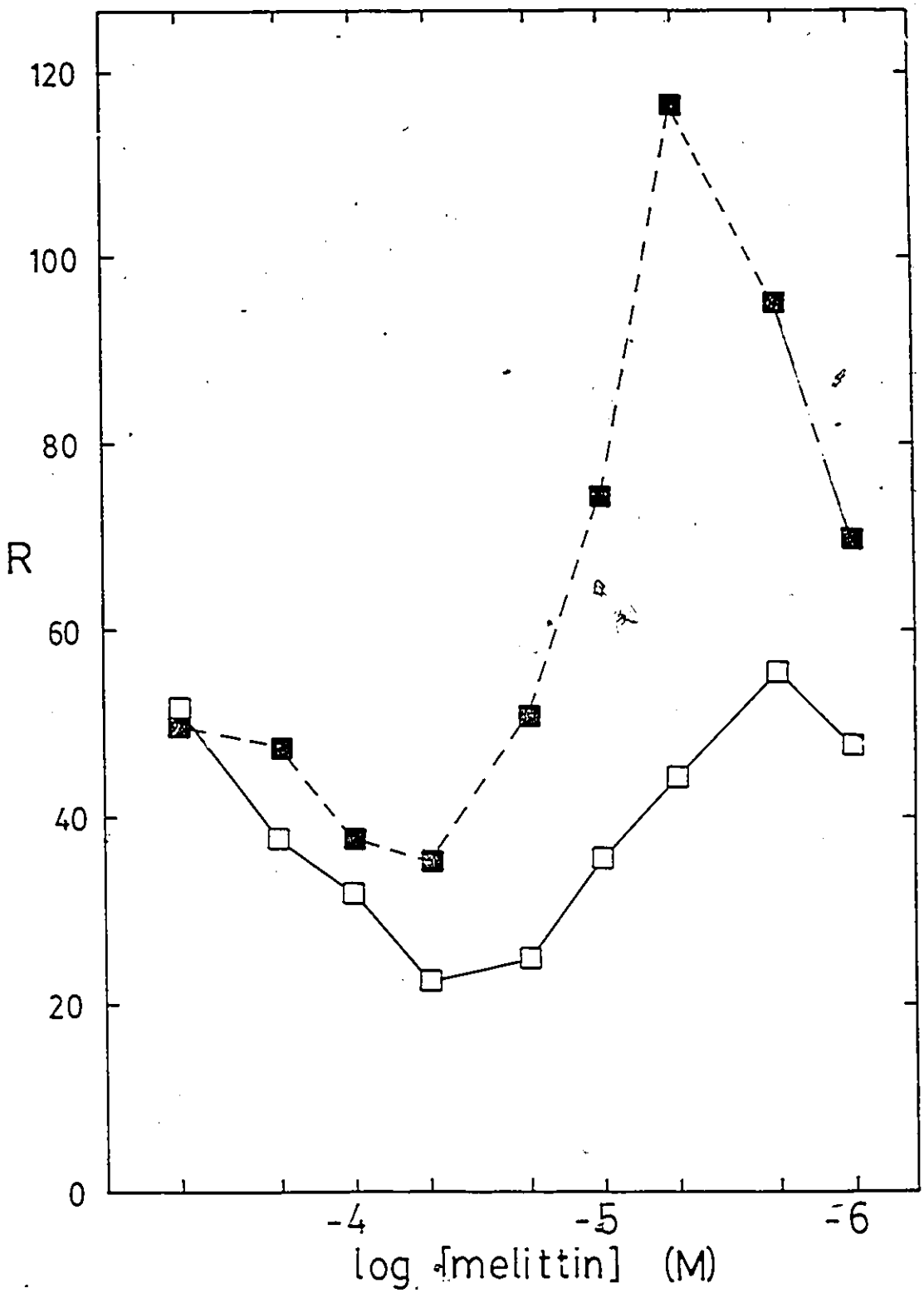
a gel phase. Qualitatively, a metaphase midway between gel and liquid crystalline is reasonable. Assuming this to be the case, one might expect the effects of melittin on EPC:cholesterol as being intermediate to Dufourcq et al.'s EPC- and DPPC-melittin preparations.

Applying the findings of Dufourcq et al. (1986) to the interpretation of the L/C ratios of Figure 31, the reactivity minimum, corresponding to a R_i of about 14.2, could represent the metastable melittin-lipid complex observed at the plateau value for $13 < R_i < 25$ (Dufourcq et al., 1986). The formation of these complexes, which all other evidence suggests is a reversible process (Dufourc et al., 1986a; 1986b), would begin at much lower melittin concentrations, probably at the point where melittin dimers begin to make their appearance.

4.4.6 Functional Groups of Liposome -Associated Melittin

Glycine-1 As with the control samples, ^3H incorporation into Gly-1 is an order of magnitude greater than the level of incorporation into the lysines. This is not surprising considering the pKs are different by over 1.0 pK unit. What is surprising is that the reactivity of the amino terminal is greater in the presence of liposomes than in their absence, at all concentrations except the highest (Figure 33). The dielectric constant of lipid bilayers changes from a value of 78 to 2 over a distance of about 15 Å. Consequently, the deprotonated form of an amine would not need to extend deeply into the bilayer in order to experience a significant change in the effective dipole of the surrounding medium. Such an environment would stabilize this (reactive) form of the nucleophile. The physical properties of bilayers are insufficiently well known to allow a quantitative estimate of the stabilization afforded by the different regions of the bilayer, however, certain structural

Figure 33. Concentration dependence of Glycine-1 reactivity relative to internal standard Ala_2, in the presence (filled squares) and absence (open squares) of liposomes.

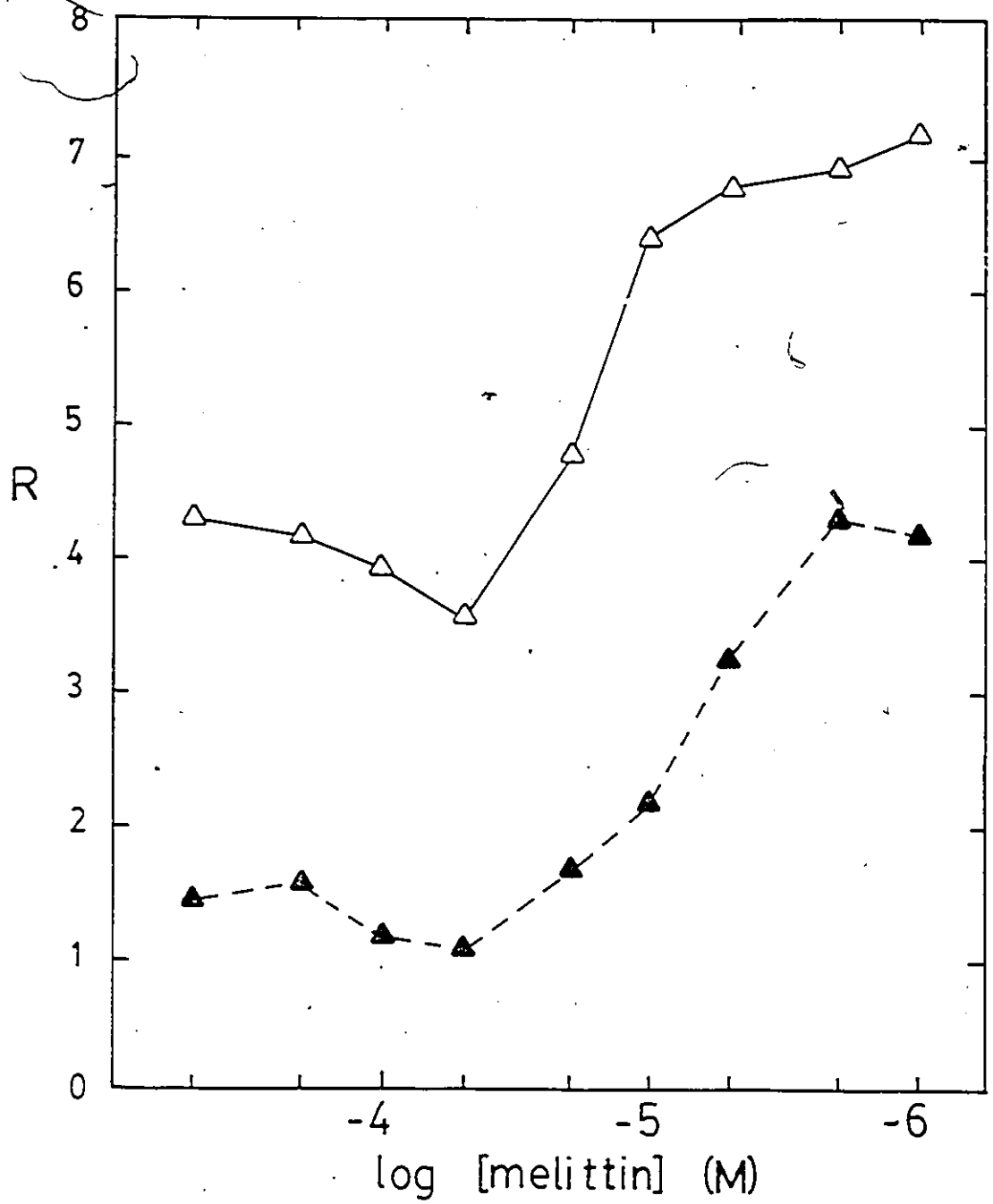

120
100
80
R
60
40
20
0
-4
-5
-6
log [melittin] (M)

features of the lipids may also promote an increased reactivity. Hydrogen bonding of the unprotonated amine to a cholesterol β-hydroxyl group or to one of the glycerol backbone ester carbonyls could increase the nucleophilicity of the nitrogen amine via intermolecular field effects. Enhanced reactivity was also observed for the Lys-B29 group of insulin (section 4.2.0), and the glycine internal standard of the glucagon experiment (section 4.3.0). It is possible that such an effect is characteristic of amines at the lipid-water interface.

As the lipid to protein ratio decreases, there is a convergence of reactivity values between the control and liposome-containing samples, until at the highest concentration (7×10^{-4} M), there is no measurable difference between the two. This trend indicates that tetramers predominate in the incubation mixture, or that lipid-melittin complexes with a microenvironment around the Gly-1 N^{α}amino group similar to the microenvironment found in the tetramer, are present at this protein-to-lipid ratio.

Lysine-7 The concentration dependence of the Lys-7 reactivity (Figure 34) indicates that there is a net decrease in reactivity at all concentrations when liposomes are present. The proportional decrease is maintained at all concentrations, being approximately one-third that of the control. This directly proportionality suggests a single unchanging factor is responsible, and that its influence is exerted in the context of the same conformational and chemical changes that account for the reactivity trends of Lys-7 in the controls. Although for the most part the trends in reactivity diminution are parallel and proportionate, the liposome-associated reactivity trends are shifted to a lightly lower concentration. These data are consistent with melittin's monomer-dimer-

Figure 34. Concentration dependence of Lysine-7 reactivity relative to internal standard Ala_2, in the presence (filled triangles) and absence (open triangles) of liposomes.

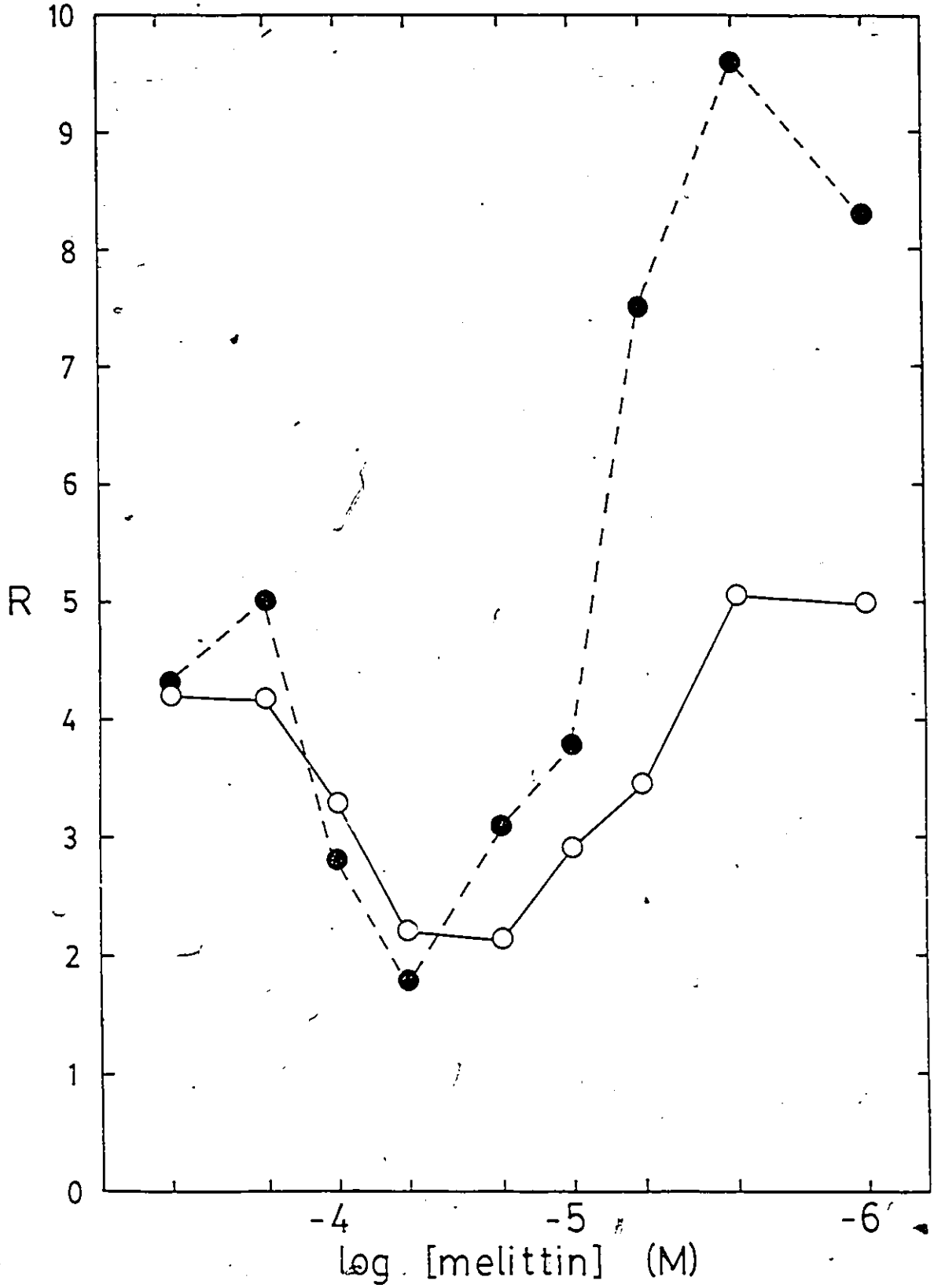

8
7
6
5
4
3
2
1
0
R
-4
-5
-6
log [melittin] (M)

tetramer transition being modified in the presence of lipid.

Lys-7 reactivity is modified by the lipid in two ways. First, the binding of melittin to the liposome surface causes a decrease in Lys-7 reactivity due to long-range steric exclusion. Considering the amphiphilicity of this portion of the molecule, the spatial relationship between the amino group and the lipid phase probably does not change very much, once established. Bound melittin is not likely to undergo major conformational changes, so the relative decrease in Lys-7 reactivity would remain fairly constant. If this were not the case, one might expect to see divergence or convergence of the control and liposome-containing samples. The arrangement of residues about the amphiphilic helix in this region of the molecule supports this idea, as do the energy minimization calculations of melittin in an apolar environment (Pincus et al., 1982).

Lysines 21 & 23 The reactivities of these two groups are qualitatively the same as that of the Gly-1 amino group (compare Figures 32 and 35). At low concentrations of peptide, where the monomer predominates, liposomes markedly increase the reactivity of lysines 21 and 23 relative to the internal standard, possibly for reasons similar to those given for the Gly-1 reactivity behaviour (vide supra). As the concentration of melittin is increased, the reactivities first decrease, and then increase, converging to the values found for the free melittin when in the tetrameric form. These data indicate that the C-terminal segment of melittin is bound to the membrane at low concentrations while still being accessible to labelling reagent. As the concentration of melittin increases, the structural changes accompanying the dimerization process appears to be similar whether lipids are present or not. At the

Figure 35. Concentration dependence of the mean reactivities of Lysines-21 and -23, relative to internal standard Ala_2, in the presence (filled circles) and absence (open circles) of liposomes.


10
9
8
7
6
R 5
4
3
2
1
0
-4
-5
-6
log. [melittin] (M)

highest concentration, the reactivities of the two C-terminal lysyl side chains is indicative of either a large amount of tetramer being present in solution, a melittin-lipid complex that closely mimics the microenvironment of the tetramer, or both.

4.4.7 Model of Melittin-Induced Membrane Lysis

With the above information in hand, it is now possible to formulate a comprehensive model of membrane lysis by melittin. Experiments by other investigators suggest that the mode of melittin interaction with lipid bilayers is qualitatively different below peptide concentrations of 10^{-6} M, since membrane lysis is not observed at these concentrations. At concentrations below 1 μM, melittin adsorbs to bilayers (Schoch & Sargent, 1980), and forms anion-conducting channels (Tosteson & Tosteson, 1982; Tosteson et al., 1987). The incorporation of monomeric melittin into lipid bilayers could follow the scheme propsed by Terwilliger et al. (1982), in which the slightly bent helical rod lies on top of the membrane with the N-terminal segment penetrating into the hydrocarbon layer at a shallow angle while the charged groups remain solvent-exposed. It seems likely that the reactivity data reflects such an interaction, since the relative reactivities appear to peak at the lowest concentrations where the monomeric form of melittin would be present (Figure 31).

Phospholipid bilayer membranes are stable structures with isotropic physical properties that are the result of balanced forces acting on them. Both the interfacial free energy density (surface tension) and the two-dimensional compressibility of the bilayer determine the equilibrium state of lipid packing (Evans, 1974). These forces can be altered by the adsorption of other molecules, resulting in

a new equilibrium state, possibly to the point of membrane disruption.

Insertion of a large molecule such as melittin into the plane of the bilayer will cause changes in the surface chemical potential of the outer leaflet, causing an imbalance between the surface tensions of the outer and inner monolayers (leaflets), resulting in a bending moment. As the outer leaflet attempts to accomodate the peptide on its finite surface area, it will bulge outwards, causing the inner leaflet to thin out, as this latter is forced to cover a larger surface area with the same number of lipid molecules. This destabilizing influence will be compounded by electrostriction of the bilayer immediately under the peptide due to the cluster of positive charges on the C-terminal segment (Parsegian, 1969). Electrostrictive pressure would be even greater on physiological membranes, which have a net negative surface charge.

Data for the Lys-7 side chain (Figure 34) indicates that the liposome-associated change in reactivity is proportional at all concentrations studied, supporting the notion that this (charged) residue does not enter the membrane. The changes in membrane structure following destabilization of the once planar leaflets of the bilayer may leave exposed edges that can be stabilized by melittin's amphiphilic helix. 'Edge-active' detergents have been observed to mediate disc-to-vesicle transitions in dispersions of EPC (Fromherz et al., 1986). Sufficiently destabilized, the membrane would thin out and the full complement of melittin's hydrophobic side chains would seek to minimize their free energy by association with the lipid hydrocarbon phase.

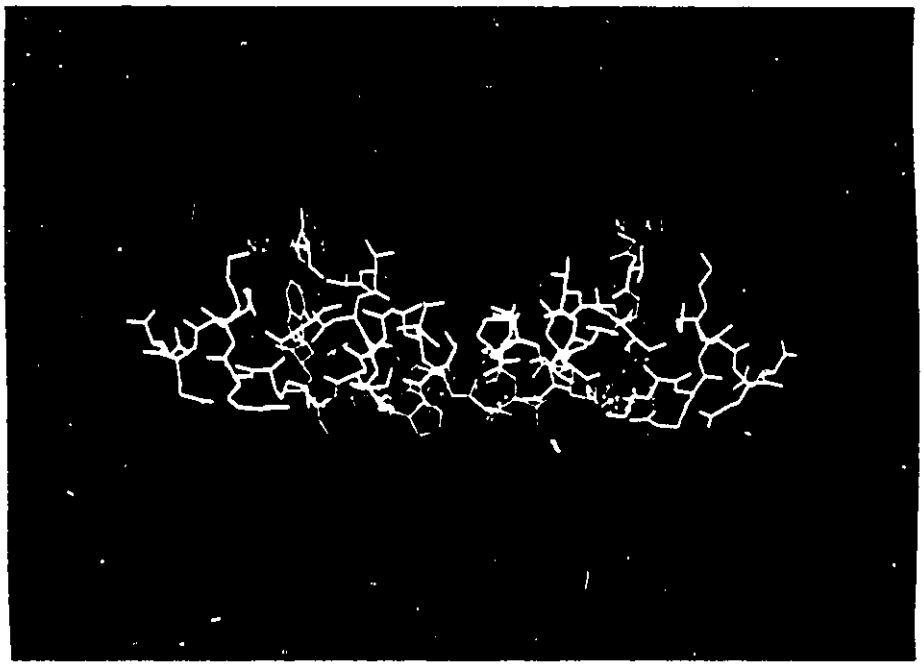
The crystal structure of the melittin dimer immediately suggests the most likely mechanism of melittin-lipid association. When viewed down the crystallographic two-fold axis of symmetry, the apolar residues

of both protomers form a contiguous hydrophobic patch. When the dimer is viewed edgewise (parallel to the non-crystallographic two-fold axis) the segregation of charged and hydrophobic residues is especially notable (Figure 36). The pincer-like shape of the dimer, a consequence of the kink introduced by Pro-14, spans a distance of approximately 30 Å between the N-terminal Gly-1 residues, corresponding to the thickness of the hydrocarbon region of the lipid bilayer. Edge stabilization would occur when a number of membrane-bound monomers would be able to form a small pore. Pore size would be variable, depending on the number of edge-wise associated melittin dimers, but a minimum size may be related to the previously reported tetrameric ion channel observed a low concentrations of melittin (Tosteson et al., 1987). Since the protomers are paired anti-parallel, the only limit as to how many can be associated edge-to-edge would be the radius of curvature of the dimer, a parameter that is probably quite variable, and very large pores could be made in a membrane, stabilized by a ring of amphiphilic peptide. Initiation of pore formation would be sensitive to dimer concentration, explaining the dependency of the fast phase of hemolysis on the square of the monomer concentration (Degrado et al., 1982).

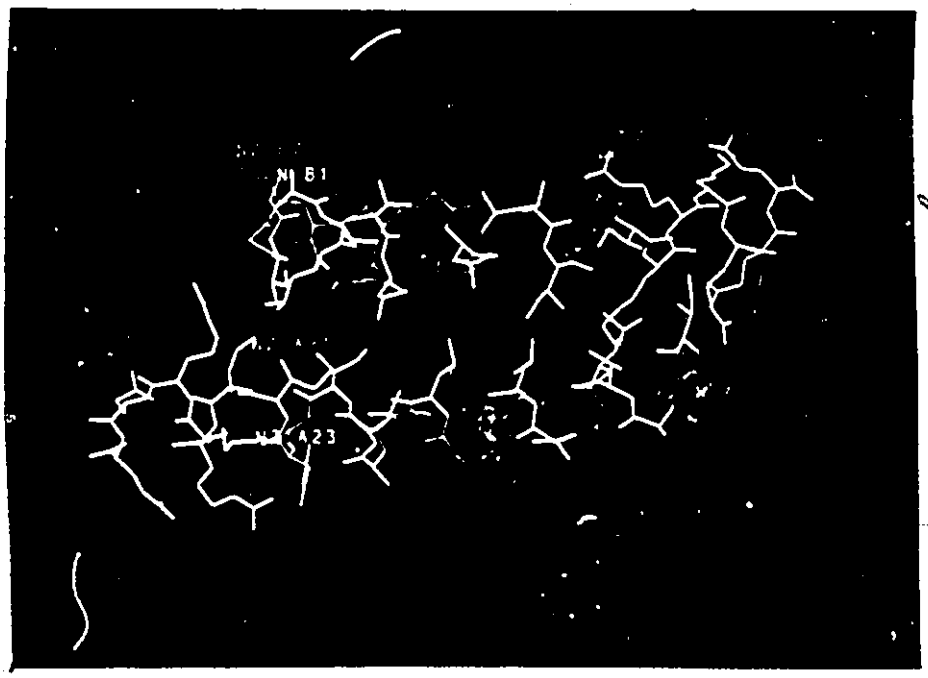
At higher concentrations of melittin, the rings of melittin stabilizing the pore edges would come into contact with one another, and the liposome (or cell) would be broken down to smaller vesicular structures containing melittin pores that are continually changing size. A minimum size for a melittin-containing vesicle would provide the plateau region observed by Dufourcq et al. (1986), corresponding in general to the region of reactivity minima measured in the present work (Figure 31).

Figure 36. The melittin dimer. (A) Viewed down the non-crystallographic axis of the tetramer, only one dimer is shown. All amino groups are labelled 'N' according to protomer (A or B) and residue number. Hydrophobic residues (excluding Trp-19) are light grey, and the six polar C-terminal residues are in green. The shape of the dimer is suggestive of pincers from this angle. (B) View of the dimer after rotating (A) 90° about a horizontal line in the plane of the paper, towards the viewer. The segregation of charged and apolar residues is clearly to be seen, so that if the dimer is taken as a whole, it can be regarded as a bipolar amphiphile. These are photographs of a terminal display generated by FRODO as described in figure 9.

A

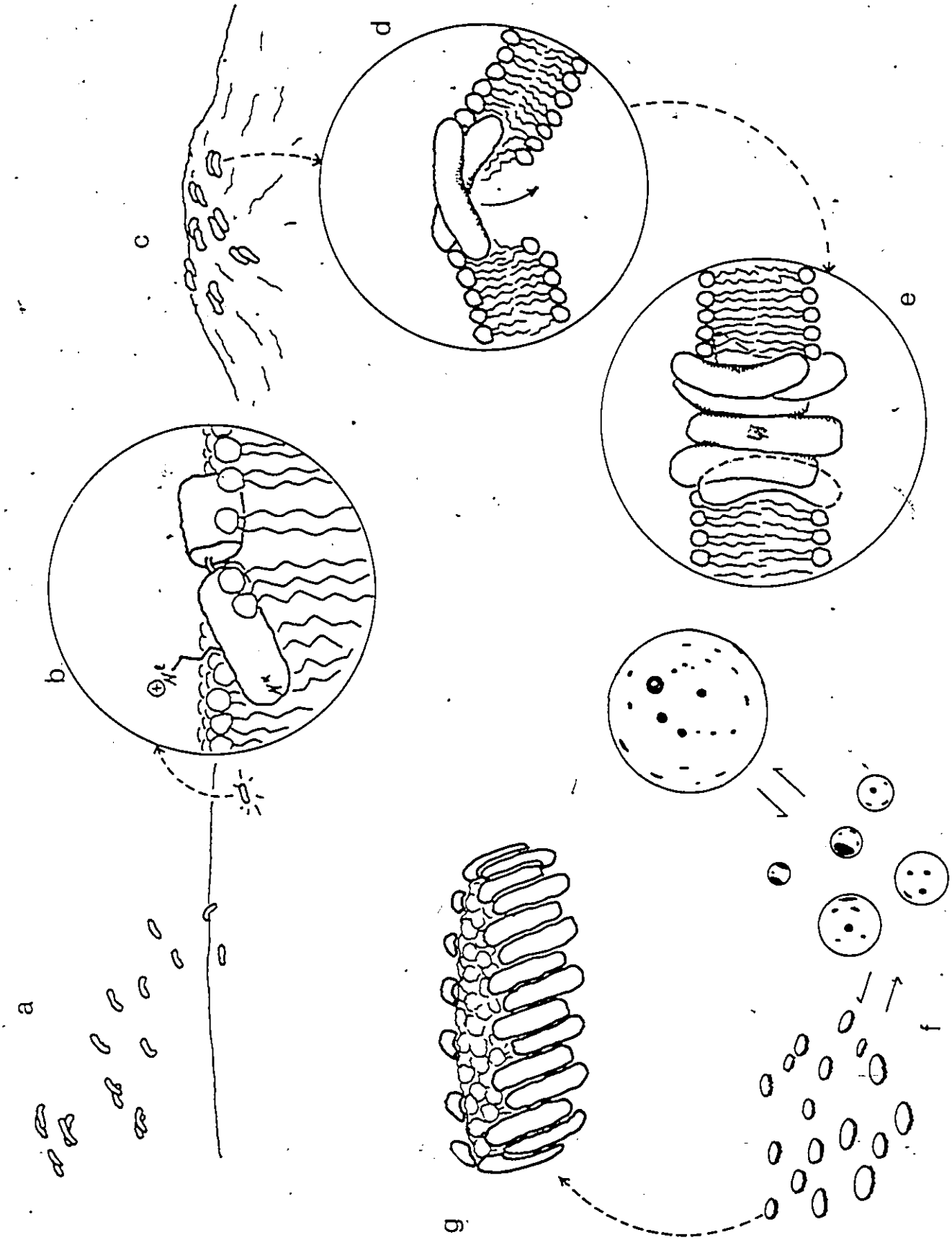


B



Upon further addition of melittin to the system, a point would finally be reached where the formation of vesicular structures could no longer compete with the sequestration of the lipid by melittin. The rapid decrease in r_H below a lipid-to-protein ratio of 13 (Figure 31), coupled with the observed increases in reactivity (Figure 30) suggests that an equilibrium mixture of peptide-delimited bilayer sheets of various sizes might now appear. A model for melittin-encircled bilayer discs has already appeared in the literature (Dufourcq et al., 1986), and have been observed with glucagon:DMPC complexes (Epand & Sturtevant, 1982) and apolipoproteins (Segrest, 1977). Addition of more melittin, to a R_i of 1.4 would give result in a mixture of discoidal particles, mixed micelles and melittin tetramers. Figure 37 is a schematic diagram outlining the main events in membrane lysis by melittin.

Figure 37. Proposed mechanism of vesicle rupture by melittin. (a) melittin diffuses towards the membrane surface and adsorbs; (b) monomeric melittin is partially inserted into the outer leaflet. Clustering of monomers and dimer formation increases the outer leaflet surface area (c), disrupting the membrane structure and leading to dimer insertion (d), and stabilization of the resultant pore edges (e). Pore enlargement upon incorporation of more dimer leads to the breakdown of large vesicles into smaller ones (f), culiminating in the formation of edge-stabilized bilayer discs (g).


a
b
c
d
e
f
g

4.5 Summary of Experimental Findings

The results presented in the preceding sections are representative of a variety of lipid-protein interactions due to the structural diversity of the proteins that were studied. In order to determine the physiological relevance of the observed interactions, the information that has been obtained should be considered from the perspective of current models and expectations.

4.5.0 Internal Standards Although internal standards are not necessary to the success of the competitive labelling method employed in the present work, they were invariably included as an adjunct to interpretation of the experimental data. As a result, a number of interesting observations were made.

The unsuitability of Arg-asp and imidazolelactate as internal standards has been discussed (section 4.1.0). In another early trial, glucosamine was tried as an internal standard, since it was thought that the combination of its high water solubility with a nucleophilic amine would provide an ideal standard. DNP-glucosamine was difficult to purify however, owing to its rapid decomposition in solution (Okotore, 1976), and the large number of by-products formed upon reaction with FDNB (data not shown; Talieri & Thompsom, 1980).

The applicability of the competitive labelling method to the study of very dilute solutions of nucleophiles was readily apparent from the results obtained for the internal standards glycine and Ala_2 in the insulin- and glucagon experiments (Tables V and VII). At high salt concentrations (1.0 M KCl), liposome-induced decreases in the $^3H/^{14}C$ ratios were minor when compared to the perturbations observed at low

salt concentrations (0.1 M KCl). The results suggest that electrostatic interactions are a preliminary step to the adsorption of these molecules to the bilayer surface, as has been suggested for systems involving larger molecules by others (Kimelberg & Papahadjoulos, 1971; Boggs, 1983). The effective electrostatic field of zwitterionic membrane lipids is sensitive to the ionic strength of the solution (Lis et al., 1981; Rand & Parsegian, 1984), as is the degree of polar headgroup hydration (Cevc, 1987). Interrelationships between charged solutes and phospholipid headgroups can result in complex and cooperative changes affecting the ionization state of charged lipids, their intermolecular hydrogen bonding, and their phase transition behaviour (Boggs, 1986). Similarly, effects involving ionization states and changes in pK would also be expected for molecules adsorbed to the surface, or intercalated amongst, phospholipid headgroups.

$^{3}H/^{14}C$ ratios obtained for submicromolar concentrations of glycine indicate that the amino group of this molecule becomes less accessible or reactive towards FDNB in 1.0 M salt, but increasingly reactive when a lower salt concentration is used. These results imply that the glycine amino group can experience one of two distinct microenvironments, depending on the salt concentration. At high salt concentrations, weak electrostatic interactions between the glycine molecule and the surface polar headgroups probably results in a non-specific adsorption. At lower salt concentrations, there is a greater degree of headgroup hydration and less competition for salt interactions with the phosphorylcholine moiety. Under these conditions, glycine is probably able to penetrate into the headgroup layer where hydrogen bonding interactions and the low local dielectric contribute to an increased reactivity.

The changes in the $^{3}H/^{14}C$ ratio for Ala_2 in the melittin-lipid system resembled a binding curve after correction for dilution effects (Figure 30). Mixed micelle formation following the melittin disruption of LUVs could provide an explanation for this pattern, providing that Ala_2 could be bound in some way to the micellar structures that were generated. Discoidal lipid particles are formed by the action of amphiphilic proteins on lipids (Segrest, 1977), and have been visualized by electron microscopy in the case of melittin (Prendergast et al., 1983). These particles are frequently observed to stack upon one another, forming rouleaux, and weakly adsorbed internal standard could be trapped between particles.

4.5.1 Insulin In the case of insulin, the reactivity data supports a model of insulin adsorption to liposome bilayers that involves the dimer-forming surface of the insulin monomer. In retrospect, such a finding should not be surprising, since the dimer-forming surface contains a high proportion of hydrophobic residues that could decrease their entropically unfavourable interactions with the aqueous solvent by immersion into another solvent of lower polarity, in this case, the hydrocarbon region of the lipid bilayer.

Such a model is qualitatively identical to one previously proposed to describe an observed correlation between protein-induced permeability of liposomes to ions and the ability of several proteins to increase monolayer surface pressures (Kimelberg & Papahadjopoulos, 1971). In this study, human serum albumin, cytochrome c and lysozyme were postulated to interact with the membrane electrostatically, whereupon a deformation of tertiary structure would occur in order to maximize the number of

hydrophobic contacts made between the bilayer hydrocarbon phase and the proteins's apolar residues. The hydrophobic residues, which are normally in the protein core, would penetrate into the membrane, thus accounting for the increase in surface pressure. It is well known that proteins undergo structural changes when they are adsorbed to 'hard' hydrophobic surfaces like siloxane carbonate (Iordanski et al., 1983), but these changes are irreversible and are likely the result of denaturation. Whether or not such changes could be induced, even partially, on the more resilient and deformable phopholipid bilayer surface is doubtful.

Indeed, the flexible and dynamic properties of the bilayer's constituent molecules have been cited in what is essentially an extension of the Kimelberg-Papahadjopoulos (1971) model, in an effort to explain the insertion and translocation of apocytochrome c into mitochondrial membranes (Rietveld & DeKruijff, 1986). These authors propose that the headgroups of negatively charged phospholipids (PS, PI) can neutralize positively charged protein residues, moving with them as the protein penetrates and translocates across the bilayer. Unfortunately, theoretical calculations on the transfer of charges into low dielectric membranes indicate that charge 'neutralization' through the formation of ionic bonds has little or no effect on counterbalancing the unfavourable energetics of such a process (Parsegian, 1969), and this matter was not addressed by the authors.

The basic features of the Kimelberg-Papahadjopoulos model can, however, be used to explain the adsorption of the insulin monomer. The insulin monomer has surface hydrophilic groups. Measurement of the dielectric properties and the viscosity of despentapeptide insulin solutions implies that it has a lower water of hydration than most

proteins, the hydration shell being less than one water molecule thick (Laogun et al., 1984). A thinner hydration shell may contribute to the adsorption of insulin to a bilayer, since fewer intervening water molecules would separate the protein from the bilayer surface and require removal. The fact that monomeric insulin does perturb phopholipid monolayers (Schwinke et al., 1983), and can induce vesicle fusion (Farias et al., 1985; 1986) is qualitative proof for an interaction of insulin with at least the outer leaflet of a bilayer. If the electrostriction technique of Hianek et al. (1987) is as precise as the authors suppose, the area of an EPC:cholesterol bilayer that can be perturbed by a single insulin molecule is slightly larger than the cross-sectional area of the protein. Assuming an insulin molecular diameter of 3.2 nm, the data of Hianek et al. (1987) can be used to calculate the diameter of affected membrane surface upon insulin adsorption yielding an affected area of 3.6 nm (calculations not shown).

The results of the present work (section 4.2.1) are consistent with this degree of membrane perturbation, providing evidence that insulin adsorbs to bilayers, most likely via the hydrophobic residues of the dimer-forming surface and those polar groups which immediately surround this region. It is postulated that the hydrophobic groups would extend into the bilayer at least past the hydrogen-belt layer, just below the glycerol backbone region. Local clustering of cholesterol molecules underneath the adsorbed protein, with stabilizing polar interactions on the periphery, would minimize the impact of the protein on the structure of the membrane while holding the protein in a specific orientation. According to this model, no charged groups need enter the bilayer. Drastic conformational changes, in which the protein might unfold to

insert into the bilayer, are extremely unlikely due to the large number of disulfide bridges for a protein of its low molecular weight (Richardson, 1981). However the present study does not rule out the possibility of a conformational change involving residues B1-B8. The reactivity data indicate that this conformational change would likely see the B1-B8 helical extension of the 4Zn molecule 2 structure uncoil outwards and away from the liposome-adsorbed face of the protein, resulting in a structure similar to molecule 1. The high salt concentration employed in this study (1.0 M KCl) favours the 4Zn molecule 2 conformation initially (Bentley et al., 1976; Chothia et al., 1983), while the increased Phe-B1 reactivity at low insulin concentrations suggest the kind of increased accessibility to solvent that such a conformational change would impart (section 4.1). A similar explanation has been proposed to explain reactivity increases observed in a competitive labelling study of calmodulin-endorphin association (Giedroc et al., 1985).

The model of adsorption to lipid bilayers proposed above renders unlikely any analogous process that may occur as a prelude to receptor binding. Since the residues that have been implicated in receptor binding are closely associated with, and in some cases overlap the residues involved in liposome adsorption, such a process in vivo would be counterproductive. The affinity of insulin for the liposome surface has not been measured, but it is bound to be less than it's affinity for the receptor, for which a K_D of about 10^{-9} M has been measured (Hollenberg & Cuatrecasas, 1976). This value is likely due to a high degree of surface complementarity between the insulin receptor binding site and the insulin molecule. However, since the same general surface

of the insulin molecule is implicated in both liposome adsorption and receptor binding (Figure 22), the possibility is opened up that receptor binding may lead to the B1-B8 conformational change, as mentioned above. Although this concept has been mentioned before in a general way (Dodson et al., 1983) there has never been any evidence for it apart from the structural heterogeneity seen in the insulin crystal structures. The present study has shown, via a model system, that this may be a real possibility. More importantly, the results presented here indicate that a direct probing of receptor-bound insulin in situ, using the method of competitive labelling, could provide information on a receptor-induced conformational change. Such a study could employ insulin receptor reconstitued into liposomes. A more detailed analysis of the labelling pattern could be made by isolating each individual tyrosine and histidine residue. Preliminary results (not shown) have shown that performic acid oxidization of fully dinitrophenylated insulin, followed by elastase digestion and separation of the peptide digest on HPLC would yield the desired peptides.

4.5.2 Glucagon The present work confirms previous speculations (Kaiser & Kezdy, 1984) that, by virtue of its amphiphilicity, glucagon may interact with membranes in a stereospecific manner. A major finding was that glucagon associates with membranes both in its trimeric and monomeric form (section 4.3). The results are consistent with the adsorption of the type 2 trimer being mediated by the N-terminal hydrophobic patch (residues 6, 10, 13 & 14) of each protomer. Due to the helical arrangement of the side chains in residues 6-15, and the 'quaternary' structure of the trimer (Blundell, 1979), it is possible to define the orientation of the N-terminal amphiphilic segment of the

trimeric form as being approximately parallel to the membrane normal. The concentration-dependence of Lys-12 reactivity suggests that the orientation of this segment relative to the membrane is not preserved when the monomeric form of glucagon is membrane bound. Possible explanations have been proposed (section 4.2.2), but the primary cause for this reorientation remains speculative. Since glucagon circulates in vivo at nanomolar concentrations, only monomers will be presented to cell surfaces and their receptors.

The C-terminal amphiphilic segment of the glucagon monomer must interact with the lipid bilayer to a greater extent than the N-terminal segment since (i) there are no longer any restrictions imposed by quaternary structure, (ii) the molecule has considerable flexibility, and (iii) there would be a 33% greater decrease in free energy for this segment, as estimated from the mean surface area that could be inserted into the membrane phase (1168.2 A^2 for the C-terminal segment, 889.5 A^2 for the N-terminal segment, as calculated from the amino acid side chain surface area values of Rose et al., 1985). Thus, satisfying the energetic requirements of the C-terminal segment would be expected to have priority over those of the N-terminal segment, and this latter might be induced to roll or be rotated slightly as the C-terminal segment adjusts to a stable configuration on the membrane surface.

What are the implications of these findings for receptor binding? The cAMP second messenger system is the best characterized of the known glucagon signal transduction mechanisms, and consists of three distinct membrane proteins. These are the receptor protein proper, adenylate cyclase, and a GTP-binding protein that regulates the interaction between these two (Rodbell, 1983). A number of studies on glucagon

fragments have shown that productive binding to the receptor requires essentially the entire glucagon molecule. The C-terminal portion appears necesary for binding to the receptor proper, while the N-terminal region is responsible for the signal transduction process (McKee et al., 1986). Specific residues in glucagon play crucial roles as well. Substitution experiments suggest that aromaticity at position 1 (His-1), and a positive charge at position 12 (Lys-12) are essential for signal transduction, but not binding (Súieras-Diaz et al., 1984). Residues 10-13 of glucagon determine the correct positioning of the N-terminal segment of the molecule relative to the receptor, leading to efficient signal transduction (Krstenansky et al., 1986). The hydroxyl groups of Tyr-10 and -13, as well as Ser-11, are also functionally important structural features in this segment (Krstenansky et al., 1985).

There clearly exists a large body of evidence for an alpha helical glucagon molecule in hydrophobic or amphiphilic environments, and this conformer is thought to be similar to the receptor-binding conformation (section 2.4.2). Data from the present work suggests that both hydrophobic patches interact with lipid bilayers. Although the affinity of glucagon for the lipid phase will be lower than for its receptor, the very large surface area of a membrane in vivo, compared to the size of the available binding sites on the receptors, will adsorb some fraction of the total monomeric glucagon in solution. The relative amounts of membrane-bound hormone, as compared to the receptor-bound hormone will depend on the ratio of the association constants for each bound form, multiplied by a factor that relates the proportion of membrane bilayer surface area to the area of the binding site. Calculations by D.F. Sargent addressing this consideration have shown that if the membrane

lipid phase can provide a 'low-affinity high-capacity' site, full receptor occupancy will occur at lower concentrations than if only the receptor's 'high-affinity low-capacity' site were involved (Schwyzer, 1985). If the postulates of Schwyzer (1985) are correct (and they seem reasonable), the binding site on the glucagon receptor must lie adjacent to the lipid-water interface.

A second known glucagon receptor-signal transduction mechanism results in the hydrolysis of PI to yield the second messengers diacylglycerol and inositolphosphate (Peterson & Bear, 1986). The assignment of specific glucagon residues to functional roles in the activation of this receptor have not yet been fully made. Given the full agonism of N^{α}-trinitrophenyl-12-homoarginine glucagon, it is at least certain that the N-terminal segment of the molecule is involved in receptor binding in a manner similar to the cAMP-mediated receptor system.

A second possible role for the membrane adsorption of glucagon may be related to a requirement for further processing of the hormone at the target tissue. The glucagon fragment $glucagon_{19-29}$ is the physiological ligand responsible for inhibition of the liver Ca^{++} pump (Mallat et al., 1987). This fragment could be generated by a membrane-associated trypsin-like protease that has been found in liver plasma membranes (Tanaka et al., 1986). A membrane-stabilized conformation of glucagon may help to bring the hormone into contact with such a protease, from whence the processed fragments may proceed to their respective receptors. Processing of growth hormone and arginine vasopressin by target tissues has already been demonstrated and is known to generate molecules with new biological activities (Mallet et al., 1987).

That the interaction of glucagon with the cell membrane is a prior step to receptor binding for this hormone cannot at present be proven. Considerable insight is first required into the location of the receptor binding site relative to the membrane surface and the conformational requirements that are made on the glucagon monomer.

4.5.3 Melittin Competitive labelling over a range of concentrations has provided evidence for the existence of multiple forms of melittin in solution possessing distinct chemical properties (section 4.4.2). Functional group reactivities in the presence of liposomes indicates that the degree of involvement of the N- and C-terminal primary amines in the mechanism of membrane lysis is similar. The similar behaviour obtained for these groups (Gly-1, Lys-21 and -23) indicates similar changes in microenvironments, and thus, roles, upon association with bilayer-forming lipids. These data therefore provide support for the notion that the dimeric form of melittin is the main active form in the disruption of bilayers and consequent stabilization of the resultant smaller lipid particles.

The structure of the melittin dimer as derived from the crystallographic structure explains the similar behaviour seen for the C- and N-terminal nucleophiles. The melittin dimer can be regarded as a bipolar amphiphile: two antipodal polar regions connected by an apolar one (Figure 36). The precise dimensions of these regions relative to one another provide the basis for melittin's ability to rearrange phospholipids and stabilize them in a different aggregate form. Analogous surface and membrane-lytic activity has been observed in the Staphylococcal δ-hemolysin (Morgan et al., 1986) and the newly discovered 33-residue ichthyotoxic peptides from the sole Pardachirus

pavoninus, the pardaxins (Thompson et al., 1986). The general features of these toxic peptides were incorporated into a synthetic peptide that displays many of their properties , including tetramerization and cytolysis (Kaiser & Kezdy, 1987). Common features of these peptides are (i) a length of about 30 ± 5 amino acids, (ii) a minimal number of hydrophilic residues separating stretches of 2-4 hydrophobic residues, and (iii), C-termini with a high density of charged/polar side chains. Hydrophilic residues in these peptides would interact with water via hydrogen bonding to seryl and threonyl hydroxyl groups, and hydration of the charges on lysyl, arginyl, aspartyl and glutamyl residues, as well as the N- and C-terminal amino and carboxylic functions. The net result of this specific distribution of side chains is to generate a helical peptide that can associate in a limited number of well defined ways.

The concept of 'edge-activity' as applied to these peptides, and possibly the apolipoproteins as well (Segrest, 1977), would introduce a new category for biological peptides, albeit a small one. Surface free energy gives rise to the phenomenon of surface tension concomitant with the minimization of surface area. Similarly, a disc of phospholipid bilayer with exposed acyl chains would have a high 'edge free energy'. The easiest route to minimization of this energy would be via the formation of a concave/convex structure, followed by contraction of the 'rim' to form a closed vesicle. Melittin is able to stabilize the edge of discoid phospholipid bilayers because the peptide provides an interface that satisfies the free energy requirements of the lipid hydrocarbon region, the polar headgroup region, and the surrounding aqueous medium.

5.0 Conclusion

The application of competitive labelling to the study of protein-lipid interactions holds some promise as a general approach for determining the roles of specific protein residues in lipid bilayer-protein complexes as well as during the adsorption or binding process itself. Decreases in relative reactivities due to steric shielding appear to be good indicators of adsorption for proteins in general. Increases in relative reactivities indicate the possibility of finding microenvironments within phospholipid bilayers that may confer specific chemical properties on ionizable groups such as amines. The implications of such interactions for the processes of recognition and catalysis in vivo make this possibility worth exploring further. The observation of relative increases in the functional group reactivities of liposome-bound proteins further suggests that a similar approach could be used to study receptor-bound proteins and perhaps follow any conformational changes that may occur there.

Plausible structure-function relationships have been proposed on the basis of changes in functional group reactivities in various polypeptides and proteins when they were incubated in the presence of large unilammellar vesicles. The results are consistent with the literature, especially the known X-ray crystallographic strucures. The monomeric forms of glucagon and insulin, which only exist at very dilute protein concentrations, could be competitively labelled while involved in interactions with phospholipid bilayer membranes. Thus, the sensitivity of the method has permitted a study of structure-function relationships to be undertaken for proteins and polypeptides at very dilute concentrations; these conditions are currently inaccessible to

analysis by other experimental approaches.

By applying the competitive labelling procedure over a range of concentrations, insight has been gained into some of the structural factors contributing to the roles of specific residues during adsorption. Thus, in trimeric glucagon, the decrease in Tyr-10 and -13 reactivities, concomitant with an unchanged Lys-12 reactivity, indicates the importance of amphiphilicity as a secondary structural element in the context of bilayer-polypeptide interactions. Similar conclusions could be drawn from the reactivity behaviour of Lys-7 in melittin when this polypeptide associated with liposomes. As well, reactivity changes in melittin's N^{α}-amino and Lys-21 and -23 N^{ϵ}-amino groups were shown to be qualitatively identical, indicative of similar roles and conformational constraints during membrane lysis.

In conclusion, the methodology developed in this work can provide detailed quantitative information on protein and polypeptide structure-function relationships in the context of their associations with lipid bilayers. As such, the approach constitutes a useful tool for the further characterization of these interactions, and complements the battery of existing experimental techniques.

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7.0 Claims to Original Research

1) The development of competitive labelling as a general approach to studying lipid-protein interactions, with particular application to the interactions of soluble proteins and peptides with phospholipid bilayers.
2) Specific structure-function relationships in the context of membrane-protein interactions were proposed:
   a) the adsorption of the insulin monomer to phospholipid bilayers via a region encompassing the dimer-forming surface.
   b) evidence consistent with speculations in the literature that monomeric glucagon adsorbs to bilayers in a specific manner, via its hydrophobic patches.
   c) evidence consistent with the adsorption of glucagon type 2 trimers to bilayers.
   d) evidence for a mechanism of membrane lysis by melittin that is based on the dimer as being the primary membrane-lytic species, and that invokes the concept of edge-activity.
   e) a proposed model of melittin-induced membrane lysis that is consistent with the extant literature.
3) Specific structure-function relationships in the context of solution conformation and behaviour were proposed:
   a) evidence consistent with the existence of glucagon type 2 trimers being present in aqueous solution.
   b) the trimerization constant of glucagon at physiological pH and temperature was determined for the first time and found to be $4.11 \times 10^{13}\ M^{-2}$.
   c) the first chemical evidence consistent with the existence of melittin dimers in solution.
   d) the determination of the respective association constants for melittin dimerization ($7.57 \times 10^{4}\ M^{-1}$) and tetramerization ($3.85 \times 10^{3}\ M^{-1}$) under the experimental conditions employed.
   e) a model of melittin self-association is proposed, consistent with the results obtained and the extant literature.
4) A new procedure for the manufacture of large unilammellar vesicles from phospholipid dispersions, utilizing pressure-driven extrusion through porous steel frits of defined pore size.