ELECTRON SPIN RESONANCE COMPARISON OF WHOLE HUMAN ERYTHROCYTE CELLS AND CELL MEMBRANES WITH ASCITES TUMOR CELLS AND TUMOR CELL MEMBRANES

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in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY
December, 1977

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ACKNOWLEDGMENTS

The author wishes to express her appreciation for stimulating discussions and guidance received from her major adviser, Dr. Kermit L. Carraway. It has been a rare privilege to work under his direction. Sincere appreciation is also expressed to the committee members, Dr. Darrell Berlin, Dr. Ed Grula, Dr. Roger Koeppe, Dr. Ulrich Melcher, and Dr. H. Olin Spivey. Each has contributed knowledge, advice, and the moral support necessary to allow successful completion of this work.

Acknowledgment must also be given to Dr. Larry Halliburton for his timely advice and expertise in instrument modification. A special thanks is given to Dr. William J. Leivo, Dr. Robert Floyd of OMRF, Dr. Richard Powell, Dr. Z. Al-Shieb, Bill Smith, and my dear friends and coworkers, Mark and Trecia Markes. Appreciation is expressed to George Shier and Julienne Adams for their excellent clinical work and contribution of blood samples.

The author extends her warmest affection and appreciation to her three daughters, Mary, Rachael, and Sarah Bertler, for they have suffered much and contributed greatly to this work. But most of all, thanks to my dear little friend, Fang, who made many late-night and weekend expeditions to the lab. He will always be remembered with a tear and a smile, for he gave everything and asked nothing in return.

LIST OF ABBREVIATIONS

ANS	1-Anilinonaphthalene-8-sulfonate
BSA	Bovine serum albumin
°C	Degree centigrade
cm	centimeter
DMSO	Dimethyl sulfoxide
DTBP	3,3'-dithiobispropionimidate
ESR	Electron spin resonance
GEM	5 mM glycine, 1 mM EDTA, 5 mM 2-mercaptoethanol buffer
g•min	gravity times minutes
Hb	Hemoglobin
Hct	Hematocrit
HEPES	Hepes buffered salts
HL	Hyperlipidemia
IP	interperitoneal
KRP	Krebs Ringer Phosphate, 10 mM glucose, pH 7.4
LIS	Lithium diiodosalicylate
μ	micro
MSL	4-maleimido-2,2,6,6,-tetramethylpiperidinooxyl
M	Molar
MATA	13762 mammary adenocarcinoma tumor cells, strain A
MCV	Mean corpuscular volume
mg	milligram

ml	milliliter
mM	millimolar
NMR	Nuclear magnetic resonance
NS	Stearic acid nitroxide
PMSF	Phenylmethyl sulfonyl fluoride
PC	Phosphatidyl choline
PS	Phosphatidyl serine
PL	Phospholipid
RBC	Red blood cell
rpm	revolutions per minute
% (w/w)	percent composition by weight

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CHAPTER I

INTRODUCTION

Biological membranes are sheetlike, noncovalent asymmetric assemblies 60-100 Angstroms thick. They are composed of lipids which are small, amphipathic molecules with a thermodynamic propensity for spontaneous bilayer formation, and proteins of varied structure, which function as pumps, gates, receptors, energy transducers, and structural components. The plasma membrane also plays an integral role in control of cell growth and differentiation (1, 2, 3). In general, the concept of the plasma membrane as a bimolecular lipid leaflet throughout which the protein components form a fluid mosaic has been accepted (4). It is becoming increasingly evident that membranes must also be regarded as dynamic rather than static entities, and that the interactions between the protein and lipid constituents are exceedingly complex. While there are excellent reviews available concerning the nature of membrane constituents (5, 6, 7), and extensive studies on the characteristics of isolated components (8, 9, 10), it is still exceedingly difficult to relate a particular structure to a specific function. But it is obvious that we must first delineate structure more clearly before attempting to assign function. In order to do this, it is necessary to learn more about the protein-lipid interactions.

The simplest systems for the study of protein-lipid interactions are synthetic vesicles. Bilayers formed from synthetic phospholipids

display properties which depend upon the species of fatty acid residues attached to them (11). These may be combined and controlled in synthetic systems to mimic specific situations which are known to arise in real systems. Much attention has been given in vesicle systems to the effect of various isolated protein components on the phase transitions (12, 13, 14), size, density and permeability (15). Chemically induced phase transitions involving cations such as calcium (16, 17, 18, 19), diverse molecules such as cholesterol (20, 21), Mg^{2+}/Ca^{2+}-activated ATPase from the sarcoplasmic reticulum (22), cyclopropane (19), and polypeptides (23, 24) have been studied in an attempt to understand the thermodynamic parameters of lipid bilayers. These transitions and phase separations may also be shifted by pH changes (25), and seem to consist of highly cooperative crystalline to liquid crystalline order-to-disorder transitions in which the bilayer undergoes lateral expansion with concomitant decrease in thickness and density (26, 27, 28). These observed transitions occur with ordered and fluid domains still coexistent (29, 30) although the precise composition of these domains is largely unknown.

Some membrane-associated enzymatic activities require the maintenance of a "fluid" lipid phase. These include sugar transport in bacteria (31, 32) and Na^{+}/K^{+}-dependent ATPase in animal cells (33, 34). At the temperature of transition from a more fluid to a more gel-like membrane state, there is an increase in the apparent Arrhenius activation energy for these reactions. Although lipid phase transitions probably do not generally play a significant role in most biological processes (35), they do provide an avenue for examining lipid-lipid and lipid-protein interactions. Scott (36, 37) has proposed a model for lipid phase transitions in monolayer and bilayer systems based upon

calculations of hydrocarbon chain pressure, interactions among head groups and enthalpy changes. As our understanding of the protein-lipid interactions increases, it should be possible to perform similar calculations on more complex systems.

Erythrocytes and Erythrocyte Membranes

The study of plasma membrane architecture in red blood cells is of paramount importance in elucidating the function of membrane components in the erythrocyte. The erythrocyte provides the simplest mammalian plasma membrane system available for study, since there are no nuclear or reticular membranes associated with the mature human red blood cell. Erythrocytes are discoid cells of fairly constant size, roughly 7.5 microns in diameter and 2 microns in thickness, which arise as erythroblasts in the red marrow of bone and lose their nuclei shortly before entering circulation. This membrane structure is altered upon aging of the cells resulting in an increase in membrane rigidity. This increase in rigidity ultimately prohibits passage of the aged cells through the microcirculatory system and leads to removal of these cells from systemic circulation (38, 39). The maintenance of a high degree of viscoelastic deformability appears to be an energy-dependent phenomenon which is lost with depletion of cellular ATP (40, 41). Under such conditions, cells become spiculated, fragile, leaky, and tend to sequester calcium (40, 42). This metabolic dependence of cell deformability has been shown to be reversible within certain limits (40). It has been postulated that this regulation of deformability is achieved through chelation by ATP of intracellular membrane-bound Ca^{2+} (40, 43), or through activation of a membrane-bound Ca^{2+}-dependent ATPase (44, 45).

It is thought that there is a causal relationship between these factors and membrane microviscosity (46), but the mechanism of action is still unknown.

There are numerous hematological anomalies which may also involve the red cell membrane directly. Among these are hereditary spherocytosis (47), sickle cell anemia (48, 49, 50, 51), hyperlipidosis, polycythemia (52), and muscular dystrophy (53, 54). Evidence has been rapidly accumulating over the last few years that there are certain changes occurring in the membranes of erythrocytes from these patients aside from the other clinical manifestations of the diseases. These changes involve either the lipid or the protein components of the membrane, or both. Very little is yet known about the specific interactions producing alteration of membrane characteristics.

Since the complexity of the whole cell system clouds the issue somewhat, efficient procedures have been developed for isolation of red cell ghosts, the most widely used of which is the method of Dodge *et al*. (55). Isolation of plasma membranes has been reviewed by Wallach and Steck (56), and for red blood cells (RBC's), usually consists of subjecting the cells repeatedly to hypotonic conditions, followed by centrifugation to pellet the membranes. Ghost membranes are composed of a lipid bilayer (30% of which is cholesterol), and seven major proteins. Although the red cell lipids remain with the membrane upon repeated washing, and are therefore tightly associated with the membranes, it has been shown that cholesterol and phospholipids exchange rapidly *in vivo* and *in vitro* with serum cholesterol and serum lipoprotein lipids (57, 58). Indeed, erythrocyte ghosts are able to incorporate fatty acids into phospholipids if ATP, Mg^{2+} and CoA are provided (59, 60). In the

review by Juliano (8) on membrane proteins, he points out some of the problems encountered with isolation of plasma membranes. The similarity of function and permeability of RBC ghost membranes depends heavily upon the method of isolation, and Brown and Harris (61) have shown that even such subtle factors as buffers of the same molarity and pH but different structure can have drastic permeability effects.

Amounts of the various proteins present also vary with the method of preparation. In the hypotonic method of preparation, the membrane bound enzymes of the glycolytic pathway are released to a large extent (8), and almost all the hemoglobin is washed away. If, as Marfey (62) has proposed, hemoglobin is involved in membrane structure, then certainly the structure is altered. There appears to be an oriented "layer" of hemoglobin near the cytoplasmic surface of the membrane.

Erythrocyte Spectrin

Of the seven major membrane proteins, spectrin comprises the greatest proportion. Spectrin consists of a complex of two polypeptides (Bands 1 and 2) with molecular weights of about 240,000 and 220,000 Daltons respectively. These high molecular weight polypeptides make up about 20% of the total membrane protein content of human erythrocytes. The most common method of extraction is that of Marchesi et al. (63). Aggregation states of dimer, tetramer, and polymer seem to be favored. The difference between low and high osmomolarity preparations of red blood cell membranes does not seem to significantly alter the two major spectrin bands. Each of the two major bands of spectrin contains multiple isoelectric, antigenic, and N-terminal components which may or may not be due to natural multiplicity (64, 65, 66). It is possible

that these may be collections of related polypeptides having different ATPase activities. Suffice it to say, no one can agree on what the N-terminal amino acid is in either case. Spectrin has a weak ATPase activity with a Mg^{2+}-ATPase and a Ca^{2+}-ATPase component. These may be separated by partial precipitation with calcium (67). Redissolving the precipitated material gives the fibril-forming peptides. The Mg^{2+}-ATPase may be associated with the torus form of the protein. These may, then, be different polypeptides, or subpopulations of the same polypeptide in differing stages of aggregation. In any case, whether by variable fractionation, EDTA extraction, alteration of pH, or shift in ionic strength, it would be most advantageous to have a homogenous spectrin preparation from which subunits could be easily sorted out. Spectrin is thought to be the major structural protein of the membrane, and as such, should be carefully studied to determine what organizational properties it imparts to the intact membranes.

MATA 13762 Tumor Cells and Isolated Plasma Membranes

There are a large number of alterations of cell surface characteristics thought to arise upon neoplastic transformation. Nicolson and Poste (68) have reviewed these changes recently. A few of these generalized differences are listed below:

1. Decrease in the more complex glycolipids.
2. Deletion of glycolipid terminal saccharide residues.
3. Increase in accessibility of these moieties to antibodies, lectins, and enzymes.
4. Loss of a 210,000-250,000 MW glycoprotein after

transformation. (Names for this protein include LETS, galacto-protein a, CSP, component Z, or SF 210.)

5. Difference in antigens present.
6. Increased susceptibility to patching and capping.
7. Shedding of antigen-antibody complexes.
8. Alteration of cell adhesion, recognition, growth control.
9. Alteration of response of cell to serum factors.
10. Difference in transport properties.
11. Changes in permeability.
12. Altered cell surface enzymes.

The molecular bases, the significance, or interrelationships among these observations are still unknown.

Locomotion of cells is generally thought to be controlled by cytoskeletal organization. When contact inhibition of cell movement is lost, it may arise from an altered cytoskeleton, and this change may be mediated by changes in receptors or receptor patterns. Response to serum-borne hormones may also depend upon altered interaction of these hormone receptors with the internal portion of the membrane. There is also the possibility that specific patterning of receptors is essential for adhesion and cell-cell recognition, and that during transformation, the trans-membrane communication is disrupted.

Transformed cells are more easily agglutinated by plant lectins than are normal cells. They display altered cell surface morphologies, often including increased numbers of surface microvilli and decreased amounts of high molecular weight cell surface glycoprotein (69). The

increased lectin agglutinability may arise from an increase in membrane fluidity within the phospholipid bilayer, or from increased cell-cell interaction due to the greater surface contact area available with large numbers of microvilli. In either case, the cell surface appears to be involved.

Movement of the receptors on the cell surface include three types of mobility:

1. Large lateral movements.
2. Molecular rotation.
3. Small lateral movements.

This mobility would be affected if the postulated increase in membrane fluidity upon transformation exists. Not everyone agrees that it does exist. Fuchs et al. (70), using a fluorescent probe, found decreased lipid fluidity in transformed fibroblasts. Yau et al. (71) in ESR studies of chick fibroblasts transformed by Rous Sarcoma virus, also noted a decrease in membrane fluidity accompanied by a decrease unsaturation index. An increase in membrane fluidity was noted by Shinitzky and Inbar (72) in a malignant lymphoma cell line relative to normal lymphocytes, as shown by fluorescence polarization. These transformed cells also displayed a decreased cholesterol content. Gaffney (73) found no difference in the ESR spectra of normal and transformed fibroblasts. Barnett et al. (74, 75) in ESR and freeze fracture studies of contact inhibited and transformed cells found an increase in the membrane fluidity of transformed fibroblasts. Much of the apparent disparity of these results arises from differences in culturing, isolation, or labeling techniques. Even so, the very inconsistency of the data indicate that changes in the fluidity of the membrane bilayer are

necessarily correlated with malignant transformation. However, that possibility is not conclusively eliminated; the implication is that the methods of detection were perhaps not sensitive enough. Further, with ESR and fluorescence probes it is difficult to determine where probes are located within the cell in its various membrane domains and whether significant structural alteration of these domains is induced by the labeling process.

Eucaryotic cells are far more complex than human erythrocytes; isolation of plasma membranes from these sources is more difficult. Care must be taken to avoid contamination of plasma membranes with other membranes from within the cell, as well as with DNA from the nuclei. The mammary adenocarcinoma 13762 ascitic tumor cell, strain A, (MATA 13762), used in our lab as a cell and membrane source, were obtained from the Mason Research Institute Tumor Bank (Worcester, Mass.). They are eucaryotic transformed cells which grow as single or ascites cells in the intraperitoneal space of female Fischer-Strain 344 rats. The culture is maintained by injection of 0.2 to 0.3 ml ascites fluid into the host, transferring to a new host every 6-10 days. The doubling time is 24 hours; the cells have a heterogeneous size distribution with 50% of the cells in clumps of from 2-10 cells. They are epithelial in appearance, nearly spherical, with large nuclei bearing multiple nucleoli, and granular cytoplasm. This system provides us with an easily maintained source of cells and plasma membranes of a transformed eucaryotic cell type. Membranes are isolated from these cells by Huggins' modification (77) of the method of Warren and Glick (78).

Proteolysis of Plasma Membranes

Early spectroscopic data concerning structure and correlation between structure and function in biological membranes indicated a lack of well developed order yet plasma membranes carry out exceedingly complex, closely regulated biochemical processes. There were obviously many elusive structural features which were not easily identified solely by these methods. However, biochemical techniques may be combined with physical methods to obtain bits of information about membrane organization. Membranes are composed of proteins in a phospholipid matrix, therefore controlled proteolysis may be used to partially characterize membrane proteins.

In 1971, Steck et al. (79) attempted to resolve the disposition of the major proteins in RBC ghosts by what he termed proteolytic dissection using trypsin. He attempted to answer the following questions:

1. Is the distribution of proteins asymmetric?
2. Do any of the proteins penetrate from side to side?
3. If so, are these proteins randomly or symmetrically oriented?

The selective enzymatic digestion of protein from inside out and right side out vesicles from human RBC's indicated definite differences in accessibility, implying asymmetry of protein distribution. Band III was found to penetrate the membrane. The third question was not satisfactorily answered.

One of the assumptions made in the above studies and by Bretcher

(80, 81) was that the distribution and organization of major membrane protein in the vesicles does not vary from that found in the intact erythrocyte. Boxer et al. (82) combined radioiodination of membrane proteins and controlled trypsin digestion method of Phillips et al. (83, 84) in an attempt to justify this assumption. Data obtained in our laboratory tends to refute this assumption, but there is little question that the distribution of membrane protein is asymmetric in the intact cell. Jenkins et al. (85) using trypsin and lactoperoxidase in further investigations of Band III, have shown that this protein probably has an S-shaped structure in intact membranes. Shin and Carraway (86) have shown that trypsin digestion can release glyceraldehyde-3-phosphate dehydrogenase from RBC membranes before there is significant digestion of spectrin. Lepke and Passow (87), examining the effects of trypsin on anion exchange in RBC membranes found that when the enzyme is added externally, anion exchange is not affected. It appears to degrade mostly glycophorin. However, internalization of the enzyme causes fragmentation of the ghosts into small vesicles, apparently without lysis. Under these conditions, spectrin is the first protein digested, followed by Band 3, and then the rest of the proteins. There is an apparent asymmetry in the sensitivity of the anion transport system to trypsin modification, and Band 3 appears to be involved. These results indicate that spectrin either controls the spatial arrangement of Band 3, thereby protecting it from digestion, or Band 3 and spectrin have widely opposing susceptibilities to trypsinization. Since the fragmentation of the membrane takes place while spectrin and Band 2.1 are being digested, the evidence supports the theory that spectrin controls the shape of the membrane. It should also be noted

that there is a naturally occurring proteinase (88) in human erythrocyte membranes with chymotrypsin-like activity which may function in hemolysis.

An increase in proteolytic activity is often found in tumors as compared with their normal counterparts, and it is possible that this activity is involved in transformation and altered growth control. Mild trypsinization can release cells from density-dependent growth inhibition (89) and growth control. Trypsin has a mitogenic effect on B lymphocytes from nude and AKR mice (90). Indeed, mild proteolytic cleavage in whole cultured fibroblasts mimics transformation (91) by altered morphology (92), lectin agglutinability (93), and receptor distribution (94). Huggins et al. (95) noted the loss of two cell surface glycoproteins and a high molecular weight (HMW) protein with controlled proteolysis of sarcoma 180 tumor cells, which also produced rapid and profound morphological alterations. These polypeptides are thought to contribute membrane stabilization and maintenance of cell shape (96). If cell morphology is controlled by an internal cytoskeletal-transmembrane system, some element must communicate information from the cell exterior. While transmembrane connections have been found in many instances, no direct relationship to changes in shape have been determined. Changes produced in the phospholipid bilayer by proteolysis are even more obscure, though the change in receptor mobility implies an alteration. We have used ESR labeling techniques to examine protein-lipid interactions when cell membranes from human RBC's and MATA 13762 cells are completely proteolyzed.

Detergent Solubilization of Membranes

Solubilization of membranes by detergents and organic solvents is useful both for isolation of membrane components and for probing membrane structure. Kirkpatrick et al. (97) have determined that different detergents solubilize membrane constituents by different mechanisms, and that it is possible to differentially solubilize proteins, phospholipids and cholesterol from RBC membranes. Furthmayr et al. (98) have isolated Band 3, the major intrinsic protein in human erythrocyte membranes, by extraction with lithium diiodosalicylate (LIS). Solutions of LIS, which releases glycoproteins from membrane fragments, drastically alter the gross morphology of RBC ghosts (99), causing them to fragment into small vesicles at 0.01 M LIS, and dissolve entirely at 1.0 M. Some workers (100) use a buffer system containing chloral hydrate to dissolve biological membranes for separation of components by electrophoresis. Membranes or membrane components may be solubilized by 2-chloroethanol (101, 102) for subsequent incorporation into liposomes. Steck and Yu (103) selectively solubilized membrane proteins from RBC membranes with NaOH, LIS, acid anhydrides, and organic mercurials. The nonionic detergent Triton X-100 may be used to selectively disrupt hydrophobic interactions (104), releasing glycerolipids and glycoproteins. SDS (sodium dodecylsulfate), which acts upon both protein and lipid components, binds first to positive hydrophilic groups (105), or specific hydrophobic sites, and then with non-specific hydrophobic binding to form a complex with a specific binding ratio and uniform structure in the complexed state (106).

We examined the changes in constraint of mobility of membrane

constituents by comparing the ESR spectra of spin probes in unperturbed membranes and under conditions where the concentration of SDS insured complete destruction of gross membrane structure.

Reversible and Irreversible Crosslinking of Membrane Proteins

The technique of crosslinking membrane constituents to their nearest neighbors, then identifying the products has been used in painstaking attempts to unravel the spatial distribution of these components. Electrostatic and hydrophobic interactions between spectrin and phospholipids in the cytoplasmic leaflet of the bilayer would play a major role in membrane integrity if spectrin is, indeed, the major structural protein. The same would be true in MATA cells for the HMW protein and its lipid associations.

Reagents have been developed for linking aminophospholipids to each other (107) or to adjacent proteins (108) in synthetic and intact biological membranes. Marinetti et al. (109) using difluorodinitrobenzene have determined that there is a 5-8% crosslinking among the aminophospholipids and 20% linking of lipids to membrane proteins in erythrocyte membranes.

Proteins may be crosslinked by use of photoactivated reagents (110), the pattern for which is altered under different pH conditions. In erythrocytes, spectrin is almost exclusively crosslinked at neutral pH, arising from reaction of lipophilic probes such as 1,5-diazidonaphthalene, 4,4'-diazidobiphenyl, and 4,4'-dithiobisphenylazide with the hydrophobic faces of spectrin (110), particularly at points of monomer contact. Various bifunctional imidoesters have been synthesized (111,

112, 113) for formation of protein oligomers or dimers, with particular attention to the reversibility of the crosslinking reaction and the length of the bridge (112). Wang et al. (114) have used 3,3'-dithiobispropionimidate (DTEP) (113) with SDS-PAGE techniques to determine the distribution of human RBC membrane proteins.

Another common crosslinking agent, long used as a biological fixitive is gluteraldehyde, which polymerizes and reacts with primary amino groups (lysine), as well as other functional groups. Surface-charge associated properties are altered by such crosslinking, as evidenced by partition characteristics of the treated cells in dextran/polyethylene glycol aqueous phase systems (115). Gluteraldehyde treatment of human RBC membranes results in different electrophoretic patterns, and marked increases in immobilization of stearic acid ESR probes (116). The only fatty acid analog unaffected is the 16 NS probe which monitors mobility deep within the lipid core. Crosslinking of membrane proteins by gluteraldehyde is essentially irreversible.

Extraction of Water Soluble Membrane Proteins with EDTA

There are two approaches to the study of biological membrane structure. One may completely disassemble the membrane, purify its components, then attempt to reassemble them to achieve functional integrity. Alternatively, one may use chemical modifiers to determine alteration of structure and function resulting from a specific perturbation. These two approaches are combined in EDTA extraction of the water soluble membrane proteins under hypotonic conditions (117), when the effect on the residual membrane components is monitored by

physical methods.

The chelating agent EDTA has a variety of uses in the exploration of membrane characteristics, as well as in the action of divalent cations on membrane-bound enzymes. It is also used to prevent clotting of blood samples drawn for various clinical tests. Pinteric et al. (118) have shown that EDTA at low concentrations induces formation of stromalytic forms of RBC membranes. Addition of Ca^{2+}, Zn^{2+}, or Mg^{2+} reverses perturbation and the effectiveness of the reversal is in the order given. Carraway et al. (119) have shown that behavior of the 5'-nucleotidase in MATA 13762 membranes, as monitored spectrophotometrically, is altered upon membrane disruption with EDTA. This change to cooperative Con A inhibition of 5'-nucleotidase activity from the non-cooperative inhibition pattern observed in unperturbed membranes and whole cells implies that there is a perturbation involving increased mobility of this ectoenzyme at the membrane surface. This change may be due to disruption of protein-protein interaction of the enzyme with structural elements within the membrane, or to a change in the fluidity of the phospholipid bilayer.

Effects of Zn^{2+} on Plasma Membranes

Isolation of plasma membranes from tumor cells by the Zn^{2+} stabilization method (77, 78) is commonly used to obtain large membrane sheets and whole membrane envelopes. In view of the fact that such membranes are stabilized by this divalent cation, and as mentioned in an earlier section, Zn^{2+} reverses spiculation in RBC membranes induced by treatment with EDTA (118), the ion obviously interacts with plasma membranes. However, we have found by examination of the membranes with

atomic absorption spectrophotometry that the Zn^{2+} does not remain with the membranes after isolation (unpublished observations). The mechanisms of its interaction is unknown.

Arcasoy and Cavdar (120) have shown that serum zinc is significantly decreased in patients with thalassemia, a syndrome characterized by an inherited defect in the synthesis hemoglobin peptide chains. The significance of this observation is unknown. Zinc is an essential trace element in plants and animals, and the zinc concentration within human red blood cells is approximately 12 times that found in the serum. Possibly Zn^{2+} is involved with the stabilization of plasma membranes in vivo.

Sato and Gyorkey (121) have shown that glycosaminoglycans bind varying amounts of ^{65}Zn, as shown by gel filtration on Sephadex G-25 at pH 4.0 and 7.0. It is possible that Zn^{2+} binds to membranes at the glycoprotein residues.

Ionophore A23187

The ability of the antibiotic A23187 to act as a selective ionophore toward divalent cations was discovered by Reed and Lardy in 1972 (122). These initial studies conducted on mitochondrial preparations were followed by further efforts to characterize the ionophore (123, 124, 125, 126, 127), as well as to study the effects and mechanisms of divalent cation accumulation by these organelles (128, 129, 130, 131, 132, 133). There are two theories as to how the antibiotic catalyzes movement of divalent cations. Binet and Volfin (134) propose an increase in membrane permeability as a secondary effect of Mg^{2+} depletion, while Pfeiffer et al. (127) have indicated that A23187 catalyzes an electro-

neutral exchange of divalent cation for 2 H^+ by forming $(A23187)_2$-Me^{2+} complexes. This produces a transmembrane Δ pH as well as perturbing the transmembrane distribution of divalent cations. It is possible to adjust the concentration of ionophore so that the Ca^{2+} pump may be overridden, allowing accumulation of Ca^{2+} inside a plasma or mitochondrial membrane.

In our studies, it was hoped that A23187 might be used in lieu of hemolysis to introduce high levels of Ca^{2+} into the interior of the cells studied. In order to insure that the antibiotic itself produced minimal perturbation to the membrane system, the effects of A23187 on the microviscosity of the RBC membrane were examined by ESR.

Cytochalasin B and Its Interaction with the Cell Surface

Cytochalasin B is a fungal alkaloid which inhibits cellular movements and interacts with actomyosin causing a decrease in viscosity of that complex and inhibits acto-heavy meromyosin ATPase activity (135). It appears not to interact with myosin or with the ATPase activity of heavy meromyosin alone. Spudich (135) believes that the inhibition of cellular movements by cytochalasin B may arise from inhibition with actin-like filaments, changing their structure. Cytochalasins cause sustained cell contraction resulting in rounding up of cells and retraction of cell extensions. Weber et al. (136) propose that this is due to the disintegration of the actin-containing microfilaments and myofilaments, i.e., actin and tropomyosin. The cytochalasin-induced rearrangement occurs only in vivo, and does not occur in glycerinated cell contractility models, therefore, it is probably not a direct, specific inter-

action. The interaction is possibly an indirect effect mediated by changes in the plasma membrane which interfere with the anchoring of actin-containing structures on the inner aspect of the membranes. It is also possible that contraction may be due to a cytochalasin-induced increase of the concentration of Ca^{2+} ions in the cytoplasm of the cell. It has been shown that cytoplasmic bundles of microfilaments are not destroyed during the primary phase of alkaloid interaction (137), and that the action of colchicine is very similar to cytochalasin B in its interaction with cells from rat mammary adenocarcinoma and rat erythrocytes. This supports the idea that the primary site of action is the membrane itself. Also, when mouse oocytes are treated with this compound, pseudocleavage is induced in which one lobe of the oocyte contains all the microvilli, indicating that there has been a redistribution of cell surface elements (138).

Cytochalasin B is known to decrease the number of available insulin and human growth hormone receptor sites. It appears, then, that cytochalasin-sensitive microfilamentous structures modulate the exposure of cell-surface hormone receptors (139). There is further indication that regulation of the receptor distribution on the cell surface is a transmembrane cytoplasmic control mediated by these microfilaments.

Cytochalasin B reversibly and noncompetitively inhibits glucose transport in human RBC's (140, 141). There appear to be binding sites with at least two affinities, and the degree of inhibition depends upon the concentration of the compound. Lin et al. (142) synthesized tritiated cytochalasin B to studying inhibition of hexose transport in hopes of elucidating the binding characteristics. Binding and inhibition

occurred at concentrations which did not affect cell mobility or cause gross morphological alteration. Czech (143) has used tritiated cytochalasin B to examine cytochalasin binding to the fat cell plasma membrane. He found both high and low affinity binding sites. Inhibition of hexose transport appeared to be secondary to interaction with membrane protein sites distinct from D-glucose binding sites.

Genetically Derived Membrane Anomalies

There are a number of membrane anomalies occurring in vivo in red blood cells from patients with congenital disease states such as myotonic muscular dystrophy, congenital myotonia (144, 145, 146), polycythemia, hyperlipoproteinaemia (147, 148), hereditary spherocytosis (149, 150), and sickle cell anemia (151, 152, 153, 154, 155, 156). These membrane changes are not in most instances the primary lesion, and are poorly understood. However, since they arise from specific perturbations, these membrane alterations are useful in understanding membrane structure.

Butterfield et al. (144, 145, 146, 157, 158, 159) have used spin labeling techniques to study the increased membrane fluidity of erythrocyte membranes in muscular dystrophy and congenital myotonia. Microfilaments are thought to be involved in the altered cellular morphology characteristic of the hereditary spherocytosis syndrome (149). Vinblastine, colchicine, and strychnine, which precipitate microfilament protein, reversibly interact with normal erythrocytes to simulate the syndrome. The presence of Ca^{2+} enhances the effect. These data indicate that microfilaments play a role in maintaining cell shape and deformability. Isoelectric focusing of spectrin components from HS

patients revealed no significant changes in this structural protein component (150).

The most thoroughly investigated of these syndromes is sickle cell anemia, which is due to a genetic mutation that results in the replacement of a Glu with a Val in the beta chains of hemoglobin. This seems to drastically reduce the solubility of deoxy-HbS. Sickle cells are markedly less deformable than normal cells (151, 152, 153, 154), contain higher calcium levels (155), decreased sialic acid content (156), and differences in albumin binding (160). Rumen claims that sickling of cells can be inhibited by asparagine, glutamine, and homoserine (161). Hb A and F can participate in the polymerization of sickle hemoglobin (162). Palek (163) has suggested that there is a primary membrane lesion in these cells which may or may not be induced by Ca^{2+} accumulation. It is possible that during the transformation from normal to sickle shape, the opposing membrane surfaces, which are then in close proximity to one another, undergo irreversible interactions through their spectrin networks. Lux et al. (163) have made Triton X ghosts from irreversibly sickled cells. These cells retain their shape through a membrane defect possibly acquired subsequent to membrane deformation induced by oriented microfilaments of aggregated HbS. The hemoglobin does not remain with the spectrin, actin, Band 4.1 and components of Band 3 found in the Triton X ghosts, nor do these other proteins differ significantly from those found in normal ghosts. These data, however, give direct evidence for the involvement of spectrin in maintaining cell shape.

Electron Spin Resonance; Theory and Applications

ESR and nitroxide-bearing spin labels have been widely employed for the examination of biological systems. ESR studies of whole cells and tissues give a direct approach to determining the existence, role, and importance of free radicals in cells. Spin labeling techniques may be used to study microviscosity of lipids in cell membranes, phase transitions, lateral diffusion of components, and many other physical aspects of plasma membranes. There are several excellent reviews of the area (164, 165, 166, 167, 168, 169, 170, 171, 172, 173). It is a very sensitive, non-destructive, and easily implemented method. The phenomenon of electron spin resonance is based on the fundamental properties of the electron, which are (1) mass, (2) charge, and (3) spin, or intrinsic angular momentum. When an electron is introduced into a uniform magnetic field of strength H, the electron magnetic dipole produced by the spinning of the electron will precess about the axis of the field (Larmour precession). This precession has a finite frequency, omega, given by

$$\omega = \gamma H \tag{1}$$

where γ is the gyromagnetic ratio of the dipole, or more specifically, the ratio of the magnetic moment to the angular momentum. The magnetic energy of this interaction, E, is given by

$$E = \mu_s H \cos \theta \tag{2}$$

where μ_s is the magnetic dipole, H is the external field, and θ is the angle between the axis of the dipole and the field. Since an electron

possesses a spin, S, of ½, there can be only two values of θ. These angles are 35° 15" and 144° 45" (174). If one has two electrons spinning in the same orbit, according to the Pauli exclusion principle, the spins would be opposed, and therefore the magnetic moments will cancel one another out. In the case of a free radical, which is defined in general for spectroscopic examination as a species containing a net spin, there can be two degenerate energy levels of $+\frac{1}{2}$ and $-\frac{1}{2}$. When a magnetic field is applied, these energy levels are split into Zeeman lines with two energy states. The higher energy state is that in which the electron has aligned itself antiparallel to the field and has an energy given by $-g_e\beta H$, where g_e is the g-matrix (discussed later) for a single electron, and has a value of 2.0023, β is the Bohr magneton and H is the applied field strength in Gauss.

The relationship between magnetic field, H, and required frequency, ν, for a given resonance condition is

$$\Delta E = h\nu = g_e\beta H, \tag{3}$$

where ΔE is the difference in energy levels, and ν is the frequency of incident radiation. During an ESR experiment, microwave energy is absorbed by the sample. Many factors influence the character of the absorption giving detail to the spectrum recorded. The absorption is basically due to electrons contained within the sample, and the most simple sample which could be considered is an infinite potential well which encloses an electron. However, a macroscopically detectable absorption could occur only if many electrons were considered. In order to achieve this, the macroscopic sample could be considered to be composed of many such potential wells. Each of the electronic

charges has an internal motion similar to the rotation of a macroscopic body about some axis; this motion is the basis for the energy absorption.

If a magnet is exposed to a magnetic field with magnetic induction $\vec{H}$, its energy can be described through the magnetic moment $\vec{\mu}$ associated with it. If the energy of the interaction is W, then

$$W = -\vec{\mu} \cdot \vec{H} \tag{4}$$

and the energy varies with the orientation of the magnet with respect to the field $\vec{H}$. Indeed, this may be related to anisotropic motion within a molecule (orientation dependent) in that the magnitude of the induced field depends on the orientation of the molecule itself with respect to the applied field.

The magnetic moment of a charged rotating body is proportional to its rotational angular momentum A,

$$\vec{\mu} = \gamma \vec{A}, \tag{5}$$

where γ is again the gyromagnetic ratio by definition from Equation (1). Equation (4) can be combined with Equation (5) using some rules of quantum mechanics for free electrons. In quantum mechanics, angular momentum $\vec{A}$ is expressed in units of Planck's constant h over 2π, designated as $\hbar$, which has a value of 1.05×10^{-34} Joule-sec. The magnetic moment is expressed in units of the Bohr magneton, β, which has a value of 9.27×10^{-28} Joule/Gauss. Also, a free electron is known to assume only two orientations in a magnetic field, as previously indicated; therefore,

$$\vec{A} \cdot \vec{H} = \pm \tfrac{1}{2} \hbar H. \tag{6}$$

The gyromagnetic ratio can be expressed in terms of β and $\hbar$ as

$$\gamma_e = g_e \frac{\beta}{\hbar} \tag{7}$$

where g_e is dimensionless in the case of a free electron, and has a measured value of 2.00232. The energy can now be written as

$$W = \pm \frac{g_e \beta H}{2} . \tag{8}$$

When the energy of the incident microwave photons is equal to the difference in energy between the two orientations, the magnetic field component of the photon can induce a change in the spin orientation. In the process the photon is either absorbed, as in the transition from low to high energy, or it stimulates the emission of a second photon, as in the transition from high to low energy. The energy of the incident photons of frequency υ is hυ, and the separation in electron energy levels is g_e βH. Therefore, free electrons can interact with the microwave radiation field when the equation, hυ = g_e βH, is satisfied.

Real samples differ in many important ways from a collection of electrons in potential wells (175, 176, 177). First, one must consider pairing of electrons. Electrons in atoms and molecules occupy specific spatial regions called orbitals. These orbitals are probably more accurately described as probability distributions describing where the electron is most probably found with respect to a given nucleus. The Pauli exclusion principle, as mentioned before, restricts the number of electrons in an orbital to no more than two with opposing spins. This results in no net magnetic moment for the pair, and they cannot interact with the microwave field. Due to the fact that most, if not all,

electrons in a sample are paired, only a small fraction of them contribute to the energy of the absorption as outlined above.

Another consideration is power saturation and relaxation times (178). Power absorption stops when the two energy levels, separated by the action of the magnetic field become equally populated. In this case, since the populations of the two states are equal, just as many photons will interact with low energy unpaired electrons and be absorbed as interact with high energy electrons and stimulate emission of a new photon of the same energy, and no net photons will be absorbed. In this case, the sample is said to be power saturated.

A natural difference in population exists for samples with unpaired electrons. Such "paramagnetic" samples have the unpaired electrons distributed as follows:

$$\frac{N^+}{N^-} = e^{\left[\frac{w^+ - w^-}{KT}\right]} \tag{9}$$

where N^- is the number of unpaired electrons in the lower energy level and N^+ is the number of unpaired electrons in the higher energy level, $w^{\pm}$ is the energy value, K is the Boltzmann constant, and T is the absolute temperature.

If this distribution is momentarily disturbed, there is a tendency for it to be restored, since the electrons tend to interact with their surroundings. It is through the forces of this interaction that the energy absorbed by the electrons from the microwave field is dissipated, allowing a continuous absorption of energy by the sample. However, if these forces are weak and the incident power intense, then the population difference tends to zero, and the sample is said to be power

saturated. Partial saturation can occur, and in this case, the population difference is smaller than normal at that temperature, and the absorption of power is less intense.

The ESR spectrum of many samples shows a dependence on molecular orientation with respect to the applied magnetic field (179, 180). If the energy level separation of two spin states is designated by means of a "g-factor" as was done for a free electron, it would be found that this effective g-factor would be given by

$$g_{eff} = \frac{h\nu}{\beta H_r} \tag{10}$$

where H_r is the field at which the resonance is observed to occur and is dependent on the orientation of the molecule absorbing the microwave photon, and as a result, no single g-factor could be assigned as characteristic of the molecule. This "g-factor" which is determined by the angle between the axis of symmetry and the magnetic field, represents the position of the center line. The exact dependence of the g-factor as related to line splitting was mathematically developed by Libertini (181).

In order to find something which is characteristic of the molecule, the local field at the absorbing electron must be considered. As before, the energy W can be written

$$W = -\vec{\mu} \cdot \vec{H} \ . \tag{11}$$

However, in a real sample, $\vec{H}$ is often not the applied external field $\vec{H}_a$. The orbital in which the electron travels can contain some orbital angular momentum, and due to its motion about the charged nuclear core,

the electron feels an additional magnetic force. The actual field at the electron still depends on the applied field, but only through a transformation matrix $[T_1]$ as

$$\vec{H} = [T_1]\ \vec{H}_a. \tag{12}$$

Now if the electron spin angular momentum $\vec{A}$ is written as

$$\vec{A} = \vec{S}\ h, \tag{13}$$

the energy levels can be written as

$$w = -\vec{\mu} \cdot \vec{H} = -g_e\ \beta\vec{S}\ [T_1]\ \vec{H}_a, \tag{14}$$

since μ and H are known from Equations (4), (5) and (12). It is customary to drop the "applied" notation on the magnetic field, and combine $-g_e$ and $[T_1]$ to obtain what is called the "g-tensor"; g:

$$[g] = -g_e\ [T_1]. \tag{15}$$

With these modifications, the energy level becomes

$$W = \beta\vec{S} \cdot [g] \cdot \vec{H} \tag{16}$$

and the quantum mechanical "spin-Hamiltonian" can be written

$$\mathcal{H} = \beta\vec{S}_{op} \circ [g] \cdot \vec{H} \tag{17}$$

by making the formal substitution

$$\vec{S} \rightleftarrows \vec{S}_{op}. \tag{18}$$

The g-tensor can be simplified by a suitable choice of coordinates. If an arbitrary set of axes is defined for a molecule, and all the nine

components found by means of an ESR experiment, the g-tensor would be symmetric. This symmetric tensor can be diagonalized by a transformation of axes. The spin Hamiltonian with the g-tensor in diagonal form is

$$H = \beta(S_{xop}\ S_{yop}\ S_{zop}) \begin{bmatrix} g_{xx} & 0 & 0 \\ 0 & g_{yy} & 0 \\ 0 & 0 & g_{zz} \end{bmatrix} \begin{bmatrix} H_x \\ H_y \\ H_z \end{bmatrix}. \tag{19}$$

The g-factor observed at a particular angle of applied H can be found if the three components of the diagonalized g-tensor are known. This is done by writing H in terms of its direction cosines relation to the diagonalization axes:

$$\vec{H} = H \cos\alpha\ \hat{x} + H \cos\beta\ \hat{y} + H \cos\gamma\ \hat{z}. \tag{20}$$

Now the energy levels are written:

$$W = \beta\ \vec{S} \cdot [H^2 \cos^2\alpha\ g_{xx}^2 + H^2 \cos^2\beta\ g_{yy}^2 + H^2 \cos^2\gamma\ g_{zz}^2]^{\frac{1}{2}}\ \hat{H} \tag{21}$$

and since

$$\vec{S} \cdot \vec{H} = \pm \frac{1}{2}\ , \tag{22}$$

the measured energy separation is

$$\Delta E = \beta\ H\ [\cos^2\alpha\ g_{xx}^2 + \cos^2\beta\ g_{yy}^2 + \cos^2\gamma\ g_{zz}^2]^{\frac{1}{2}} \tag{23}$$

and the effective g-factor is defined as

$$g_{eff} = [\cos^2\alpha\ g_{xx}^2 + \cos^2\beta\ g_{yy}^2 + \cos^2\gamma\ g_{zz}^2]^{\frac{1}{2}}\ . \tag{24}$$

The spin Hamiltonian for the system may be related to the g-factor as follows:

$$H = |\beta|\ \hat{S}\ g\ H + \hat{S}\ T\ \hat{I} + [{}^{\text{Electron-Electron}}_{\text{Dipole Term}}] + [{}^{\text{Electron-Electron}}_{\text{Exchange Terms}}] \tag{25}$$

where g is the g-matrix, H is the laboratory magnetic field, $\hat{S}$ is the electron spin operator, T is the hyperfine matrix, and $\hat{I}$ is the nuclear spin operator.

The hyperfine matrix encompasses the effect of the magnetic moment of the nucleus at the electron. The labels employed in this study show the effect of the ^{14}N nucleus. The ^{14}N nucleus has three possible spin states; that is, the nuclear magnetic field can add to, subtract from, or fail to modify the applied magnetic field. Any sample will contain approximately equal numbers of the label molecule in the three different states, and the result will be three lines in the spectrum instead of one. In addition, this effect depends on the orientation of the applied field relative to the label molecule. The separation between the three equally spaced lines will vary depending upon molecular orientation. This orientation-dependence, as in the case of the g-factor can be completely specified by a 3 x 3 matrix, which can be diagonalized by choice of the proper coordinate system for the expression of H, the applied magnetic field. The spin Hamiltonian with hyperfine interaction included takes the form:

$$H = \beta\ \vec{\hat{S}} \cdot \overleftrightarrow{g} \cdot \vec{H} + \vec{\hat{S}} \cdot \overleftrightarrow{T} \cdot \vec{\hat{I}} \tag{26}$$

where $\hat{\vec{I}}$ is the nuclear spin operator. In the case of the nitroxide spin labels, it also happens that the principal coordinate system that diagonalizes g also diagonalizes T. These axes can be determined experimentally because they relate directly to maximum and minimum splittings, as in the case of the g-factor.

The ESR spectra of free radicals depends upon the rate of tumbling, and this is also true for the nitroxide radical (173, 182, 183, 184). There are a number of different types of nitroxide radicals which have been used as labels in various synthetic bilayer preparations (187, 188, 189, 190, 191) as well as in several types of biological membranes (192, 193, 194, 195, 196, 197, 198). The line widths of a given ESR spectrum are a measure of anisotropies of the hyperfine interaction and g-factor, and these are increasingly averaged out as the solvent system decreases in viscosity. This phenomenon has been extensively studied for ^{14}N (165, 182, 191, 200). The nitroxide radical in water shows three distinctly separated lines, appearing equidistant from each other. As the viscosity of the solvent increases, the peak-to-peak separation increases, and there is line broadening with concomitant alteration in the peak intensities. The information which we may glean from introduction of a nitroxide label into a biological membrane is as follows:

1. Since the electron spin resonance spectra are sensitive to the rate at which the label is able to reorient, the degree of mobility permitted by the immediate environment may be evaluated.

2. The g-factor and hyperfine splitting vary with the polarity of the solvent; therefore we may examine the polarity of the label environment.

3. Since the area under the absorption response is proportional

to the total number of unpaired spins in the samples, assuming no saturation is taking place, one can theoretically quantitate the amount of probe present in the samples. So, by following the changes of the ESR spectrum of the free radical in the free and bound states, we can deduce information about the environment close to the binding site.

Nitroxides are stable, relatively inert, and give sharp, well resolved spectra, sensitive to the molecular environment. They have the general form:

in which there is an odd electron, localized almost entirely on the -N-O group and exhibiting magnetic hyperfine interaction, containing both isotropic and anisotropic components, with the nitrogen nucleus. If the nitroxide radical is present in low concentrations in aqueous or nonviscous solvent, the spectrum observed is three equally spaced lines of approximately equal height. This is due to the fact that the nitrogen nucleus can also be aligned parallel, antiparallel, or perpendicular to the laboratory magnetic field. The electron then experiences the sum of the external field and three different local magnetic field values from the nitrogen nucleus, and each of these latter field values give rise to a line in the absorption spectrum. As shown below, the spectra are exhibited as first derivatives of the absorption spectrum instead of Lorentzian lines (179).

The spectrum of a labeled sample includes the effects of label motion and random orientation of these rapidly moving labels. Fig. 1a shows the general outline of an absorption spectrum of a sample with various label orientations. The spectrum is the result of superimposing the lines of the three-line spectra (Fig. 1b) for each of the orientations present. For certain molecular orientations, the splitting is maximum, and only this orientation will contribute to the extreme high and low field portions of the spectrum. This separation often called $T_{\parallel}$, can be obtained by a measure of the overall spectrum width. Most often the spectrum is recorded as the approximate first derivative of the absorption as shown in Fig. 1c, and in this case, we would measure $T_{\parallel}$ as shown in Fig. 2 (Results section).

In a similar fashion, the separation marked as $T_{\perp}$ can be attributed to those molecules oriented so that a minimum splitting results. The rationale for this choice as well as the choice for the $T_{\parallel}$ measurement can be seen by comparison of the first derivative curve, Fig. 1c, with the absorption curve, Fig. 1a.

There are several parameters which may be used to interpret changes in biological membranes as monitored by spin-label techniques. In our experiments we have measured the change in field separation between high and low fields which corresponds to $2T_{\parallel}$ and $T_{\perp}$ (Fig. 2), both of which are components of the motionally averaged hyperfine tensor of nitrogen. As the viscosity of the environment increases and approaches a rigid glass spectrum, the field separation, or $2T'_{\parallel}$, increases. There is a shift in the splitting of the lines and line broadening, as shown by increases in $T'_{\perp}$. These spectral parameters characterize the rigidity with which the label is held. Changes in $T'_{\parallel}$ and $T'_{\perp}$ also indicate a

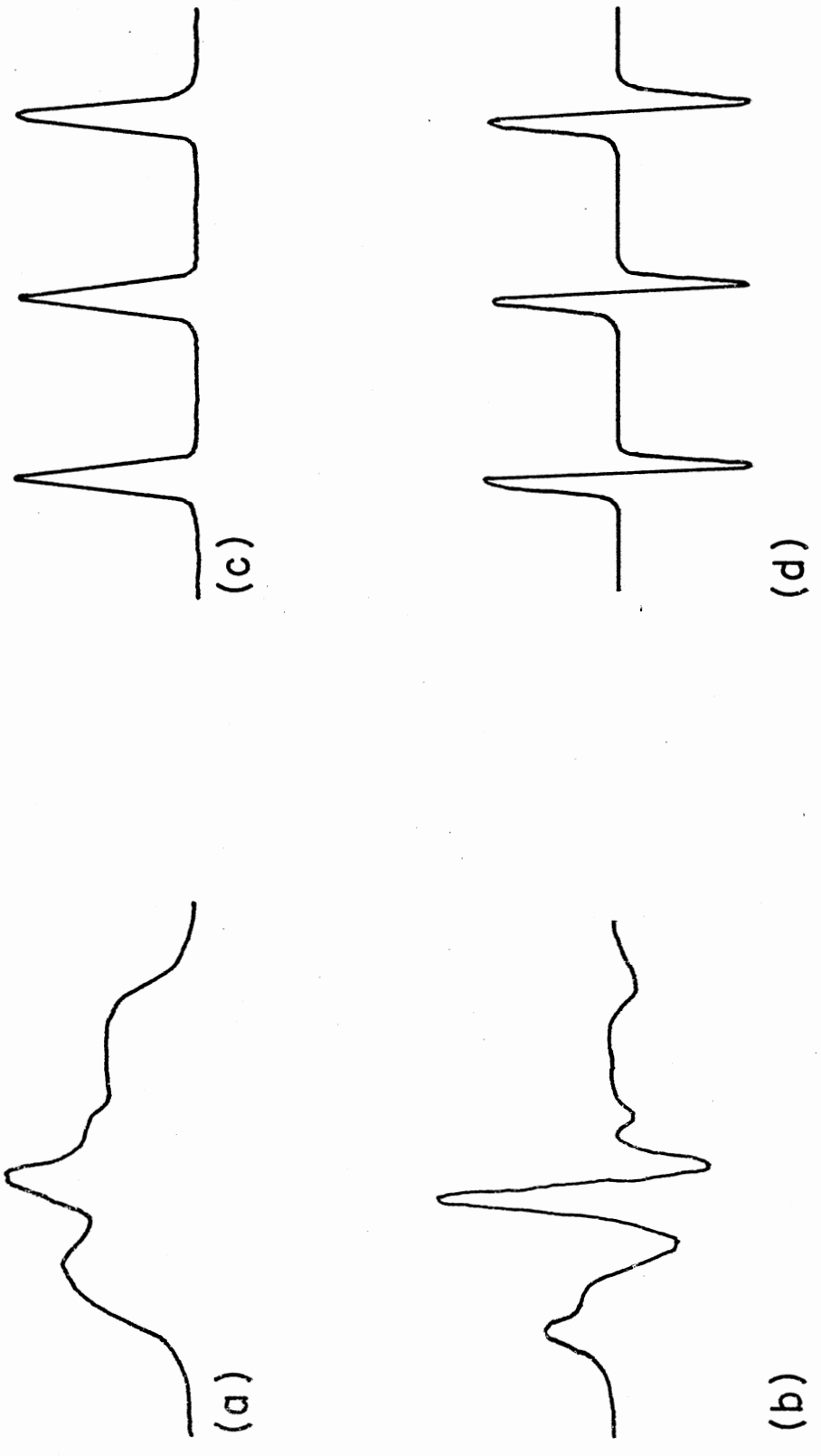
Figure 1. Types of Spectral Representations

a) Idealized absorption spectrum

b) First derivative presentation of the spectrum (a)

c) Absorption spectrum of maleimide label in 310 mOsM phosphate, pH 7.4

d) Maleimide label in 310 mOsM phosphate buffer, pH 7.4, ambient temperature, first derivative curve


(c)
(d)
(a)
(b)

change in the extent of the partial motional averaging in a given sample. McConnell (180) has related changes in $T'_{\parallel}$ to the mean angular deviation of the hydrocarbon chain in the vicinity of the nitroxide label from the axis of symmetry of the rotation of the chain. For lipid hydrocarbon chains, this is simply a measure of the average angular deviation of the molecular axis from the principal axis, perpendicular in the membrane at the nth methylene on the chain. The more rigid the environment, the more restricted the fatty acid chain becomes and, therefore, the smaller the mean angular deviation. This is of particular significance when dealing with stearic acid spin labels in which the nitroxide group is rigidly bound to the stearic acid chain through a spirane linkage. The nitroxide group then accurately reflects the motion of the hydrocarbon chain. The equation expressing the relationship between this angle, θ, and $T_{\parallel}$ is

$$\langle \cos^2\theta \rangle = \left(\frac{T'_{\parallel} - T'_{\perp}}{T_{\parallel} - T_{\perp}} \right) \qquad (27)$$

where $T_{\parallel}$ and $T_{\perp}$ refer to the hyperfine tensor parameters measured for the same nitroxide label fixed in a rigid crystal structure, and $T'_{\parallel}$ is the effective parallel hyperfine component resulting from motional averaging. The values used for $T_{\perp}$ and $T_{\parallel}$ are taken from McConnell *et al.* (179), as applied by Butterfield *et al.* (144, 145). These values are based on measurements on cholestane label in a cholesterol chloride crystal.

Order within the membrane is also often characterized by the order parameter, S, which may be defined in terms of the mean angular deviation as follows:

$$S = \frac{1}{2}\,(3\langle\cos^2\theta\rangle - 1). \tag{28}$$

S can also be expressed directly in terms of $T'_{\perp}$ and $T'_{\parallel}$:

$$S = \left(\frac{T'_{\parallel} - T'_{\perp}}{T_{\parallel} - T_{\perp}}\right)\left(\frac{a_{nxl}}{a'_n}\right) \tag{29}$$

where the factor (a_{nxl}/a'_n) is a solvent polarity correction employed when the parameters are measured under differing polarity conditions. In this case, a'_{nxl} is the coupling constant for the label in a crystal, and a'_n is the coupling constant for the label in the sample. Being directly dependent upon the square of the cosine of the mean angular deviation, the order parameter also changes with alteration of the viscosity of the medium. In short, S is found to decrease as a direct consequences of decreases in $\langle\cos^2\rangle$. By definition, S is bounded between 1, for a completely ordered label (stationary), and 0 for a label undergoing rapid isotropic motion.

Since the extreme high and low field region of the spectrum is enhanced during limited motion and the center region is enhanced during more isotropic motion, relative peak intensities of these two regions can be used to quantitate changes in molecular motion. Verma et al. (200) have used this method in the examination of the effects of melittin on the erythrocyte membrane. The peaks are labelled in Fig. 2. As the ratio of b/c approaches 1, there is a perfect ordering of the long axis of the probe with respect to the membrane. A decrease in the intensity of b, then, indicates a disordering of the long axis and a decrease in the ratio, with an increase in mobility.

The anisotropic ^{14}N hyperfine coupling constant, a'_n, may be

calculated from measurements of $2T'_{\parallel}$ and $2T'_{\perp}$ by the equation

$$a_n = \frac{1}{3} (2T'_{\perp} + T'_{\parallel}) . \qquad (30)$$

This coupling constant shows a small dependence on the polarity of the solvent, ranging from 14.1 Gauss in hexane to 15.1 in water (33). It follows that it will also show a dependence on the polarity of the environment of the nitroxide in the membrane.

The existence in many, if not all, membrane systems of a bilayer (201, 202, 203) has been demonstrated by comparison of spectra from biological membrane systems with those from synthetic systems. The fluidities of these bilayer regions differ significantly, with the erythrocyte membrane being less rigid than viral membranes, and more rigid than nerve cell membranes (30, 202, 205, 209). In general, lipid labels are more useful in examining polarity within the bilayer, and are more easily incorporated. Interpretive problems may arise due to the fact that spin-labeled hydrocarbons may be sterically unable to participate in normal lipid-protein interactions and might be sterically excluded from highly ordered regions of the membrane. Evidence against this is the biosynthetic incorporation of these labels into mitochondria of Neurospora crassa (210), mycoplasma (207), and transformed cells (211).

Phospholipids and cholesterol are essential to structure and function of biological membranes. The variation from cell to cell in proportions of these and other membrane components, as well as variation in fatty acid chains on phospholipids may dictate functional properties such as permeability of a given membrane. Cholesterol and water affect the rigidity and order of phosphatidyl choline bilayers in opposite

directions. Cholesterol increases orientation and decreases the fluidity of the membrane up to a certain point, while water will disorient an ESR spin label and seems to decrease the thickness of the bilayer (180, 212, 213). Insertion of protein into a synthetic phospholipid bilayer causes an increase in the order parameter of phospholipids (206, 207, 214), or a decrease in the fluidity.

It has also been demonstrated that on a phosphotidylcholine (PC) and phosphatidylserine (PS) membrane, calcium ion causes aggregation of the PS molecules and a remarkable increase in membrane rigidity (215, 216, 217). There is actually a calcium-induced phase separation, observed with Ba^{2+} and Sr^{2+} also, which is evident from the exchange-broadening in ESR spectra of PS-PC membranes. There is a rapid reversible aggregation of PS bridged by Ca^{2+} chelation and a fluid phase of PC (219) which can be reversed or attenuated by the presence of Mg^{2+} or tetracaine, a local anesthetic (218).

Localization of amphiphilic probes such as fatty acid labels depends upon the interaction of the hydrocarbon chain with lipid molecules of the bilayer, and electrostatic interaction of the carboxylate end group with the polar head groups at the water interface. The existence of a net electrical charge at the polar head groups depends upon the nature of the phospholipids and the pH of the surrounding medium. There is a constant interaction of the probes with polar groups such as peptides, proteins, and other charged entities. Maleimide derivatives bind covalently to membrane protein through sulfhydryl groups. The characteristics of spin probes may be manipulated in order to examine such diverse interactions as that of the cholesterol side-chain with egg lecithin (220), interactions of proteins and cholesterol

with lipids in synthetic bilayers (221), effects of pesticides on membrane-bound acetylcholinesterase (222), the presence of lipid oxidation in membranes (223), or alteration of agglutinability due to cell surface changes in mouse ascites cells (196).

We have used stearic acid probes to compare the microviscosities of the phospholipid bilayers of whole human RBC's, RBC membranes, whole MATA 13762 tumor cells, and tumor cell membranes in the presence and absence of chemical perturbants in an effort to understand structural interactions among the protein and lipid moieties contained therein. Similarly, we have examined the effects of these perturbants on protein conformation through use of the covalently bound MSL label. We have also made preliminary studies on membrane alterations in congenital hematological anomalies in red blood cells through spin labeling techniques.

Fluorescence Measurements of Membrane Viscosity and Tryptophan Quenching in Membranes

Fluorescence depolarization is often used as an alternative or confirmatory method for measurement of small changes in molecular motion. This is also a very sensitive, non-destructive spectroscopic method. Fluorescence probes may be used to study a variety of membrane systems, including normal lymphocytes and lymphoma cells (224, 225), cholesterol levels in leukemia cells (226), transformation of fibroblasts (227), viral transformation of BHK cells (228), and erythrocyte membranes (229). Spectral shifts of fluorescent emission from extrinsic fluorophores may be used to indicate polarity of probe environment (230, 231). Energy transfer is useful for detecting very minute

interactions among molecules through changes in absorption and quantum yield of the donor molecule (232). Changes in fluorescence lifetimes of protein-bound probes may be used to detect conformational changes in these molecules upon polymerization (233). Bieri et al. (234) have used paramagnetic fluorescence quenching of intrinsic fluorescence in conjunction with crosslinking agents to determine spatial relationships of binding sites on the erythrocyte membrane. Wallach et al. (235) have also used similar fluorescence quenching to study the effects of other membrane perturbing agents. Indeed, the compatibility of electron spin resonance and fluorescence methods is such that Stryer and Griffith (252) have synthesized probes bearing both a nitroxide and a fluorophore. This insures that both spectroscopic methods are measuring the same region within the membrane.

We have taken fluorescence depolarization measurements, using the probe 1-anilinonaphthalene-8-sulfonic acid (ANS), to confirm results obtained with ESR upon extraction of plasma membranes from RBC's and MATA 13762 tumor cells with EDTA under hypotonic conditions, and upon solubilization with 1% SDS. We have examined spectral shifts attendant upon these membrane perturbations. We have also used paramagnetic quenching of tryptophan fluorescence in an effort to determine the location of the spin probe in protein extracts from these two membrane systems.

CHAPTER II

METHODS AND MATERIALS

Spin Labeling of Cells and Isolated Cell Membranes

All spin labels were obtained from Syva Associates, Palo Alto, California. There were four labels used in this series of experiments: 2-(14-carboxytetradecyl)-2-ethyl-4,4'-dimethyl-3-oxizolidinyloxyl (16 NS), 2-(10-carboxydecyl)-2-hexyl-4,4'-dimethyl-3-oxizolidinyloxyl (12 NS), 2-(3-carboxypropyl)-2-tridecyl-4,4'-dimethyl-3-oxizolidinyl-oxyl (5 NS), and 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MSL). The structures of the labels are given in Figure 2 (Results section).

The stearic acid labels were prepared for incorporation by a variation of the method of Landsberger *et al.* (236), which consists of dissolving each label in CCl_4 at a concentration of 1 mg/ml. This stock solution is used with the samples by removing an aliquot of this pale yellow solution, placing it in a glass container, and evaporating the solvent with a gentle stream of nitrogen, which leaves a thin film coating the inside surface of the container. The aqueous suspension of the sample which was to be labeled was then added to the flask. The flask was either covered and allowed to stir overnight at 4°C, or incubated at room temperature for 3-4 hours, depending upon the sample in question. Sample spectra were always run on the labeling suspension

as well as upon the washed sample to insure that adsorption had occurred and that the label had been present in excess.

The maleimide label was suspended in ethanol (95% or absolute) in a stock solution of 1 mg/ml. If the volume of the suspension to be labeled was large, and the concentration of cells was low (e.g. 5 ml of RBC's at 10% Hct), then the stock was added to the suspension directly. When spectrin was labeled, the aqueous suspension of spectrin had to be added to a tube which had been coated with the label, as described for the stearic acid labels above. Spectrin will form a rubbery, completely insoluble mass at low concentrations of ethanol, although some workers have indicated that it is soluble in 80% ethanol. Also, if ethanol is added directly to suspensions of erythrocytes at 50% Hct, spiculation occurs immediately, along with lysis of many cells. There are deformations of the MATA 13762 whole cells in the presence of ethanol. For all these cases, the label was coated onto the container, and the aqueous suspension of cells or protein was added as for the stearic acid probes.

Erythrocyte Preparation

The erythrocytes and erythrocyte ghosts were prepared from fresh blood obtained from either the Dallas Blood Bank or locally from healthy volunteers. Whole red cells were washed three times in 10 mM Tris buffer, 0.154 M NaCl, pH 7.4. The buffy coat and debris were removed by aspiration. Both erythrocytes and ghosts were labeled in this isotonic Tris buffer. For stearic acid labels, the ghost membranes were prepared in Tris buffer as described below. Each sample consisted of 0.5 ml packed membranes (3-5 mg protein/ml as determined by Lowry assay) (237) per 0.5 ml stock solution of spin label. Following

incubation at 4°C for 10-12 hours with occasional stirring, the membranes were carefully washed until the supernatant was free of signal. These membranes were then diluted and used for perturbation experiments. Erythrocytes were labeled in a similar manner, except that the incubation time was much shorter (maximum of 6 hours), and labeling was performed at room temperature. Care had to be taken to keep the concentration of the spin label at 10^{-4}M or less, or severe blebing occurred at the surface of the red cell membranes. This is discussed further in the results section.

Erythrocytes from hematologically abnormal patients were kind gifts of Mrs. J. L. Adams of Pruitt Labs, Lufkin, Texas, and Mr. George Shier, Stillwater, Oklahoma. The fresh blood was drawn from donors in EDTA-containing vacuutainers, and the clinical analyses were run in these two medical laboratories prior to spectrophotometric examination in our lab. Samples from Lufkin were either transported in an ice chest in spin-probe coated glass tubes following washing as previously described, or shipped in a cold-pack container by Special Delivery. Samples obtained locally were transported in an ice bucket directly to our lab. Membranes and whole cells were prepared as described previously, and used as soon as possible. We noted that when labeled membranes were frozen prior to examination by ESR that the signal intensities were diminished for both controls and sample, therefore this was not a common practice.

Isolation of Spectrin From Human Erythrocytes

Spectrin was isolated and purified by a modification of the method of Marchesi *et al.* (63). Washed, fresh whole human RBC's were lysed by

suspending in 10 volumes of 10 mM Tris or 20 mM phosphate buffer, pH 7.5 and allowing them to incubate in the cold for 10-20 minutes. The lysate was centrifuged at 12,000 rpm (SS-34 rotor) for 15 minutes, and the supernatant was aspirated off. This process was repeated until the desired level of hemoglobin was removed. The membranes were usually pink in color. Repeated washing until all red color was removed apparently stripped out much of the spectrin. The washed membranes were placed in a flask containing at least 10 volumes of GEM extraction buffer (5 mM glycine, 5 mM mercaptoethanol, and 1 mM EDTA, pH 9.5). Extraction was carried out at either 37°C for 15 minutes or at 4°C overnight (10-12 hours). The former procedure resulted in lower yields, but the overall preparation was more pure; the latter method resulted in more complete extraction of spectrin, but purification was more difficult. The residual membranes were sedimented by centrifugation in an SS-34 rotor for 90 minutes at 15,000 rpm. This pellet remains light and flocculant, therefore the supernatant was removed carefully. The supernatant was concentrated by either $(NH_4)_2SO_4$ precipitation at 50% saturation (35 g/100 ml cold supernatant, added slowly with gentle stirring), or by diaflow filtration using XM-50 filters. When ammonium sulfate was used, the resultant precipitate was centrifuged at 8,000 rpm for 10 minutes, and the supernatant discarded. The pellet was resuspended in 10 ml or less of extraction buffer. The final concentrate was placed in a dialysis bag and dialyzed against 10 mM Tris, 0.1 mM EDTA (714) or 20 mM phosphate, 0.1 mM EDTA, pH 7.4 for 12 hours. The sample was removed from the dialysis bag and centrifuged for 15-20 minutes at 17,500 rpm (SS-34 rotor) to remove any residual solid matter. The soluble protein was removed and made 10% with sucrose. Either

bromphenol blue tracking dye or the residual hemoglobin were used to monitor the passage of the sample through a Sepharose 4B column. After equilibration of the column with GEM buffer, the sample was added. A small aliquot was retained for electrophoresis. Column fractions (3.0 ml each) were analyzed, plotting A_{280} vs fraction number. Fractions under each peak were pooled and concentrated by diaflow filtration. Each peak was analyzed by SDS electrophoresis. Often, when separation was less complete than desired, electrophoresis was run on column fractions before pooling them. Samples were stored with 0.2% NaN_3 to retard bacterial growth, or filter sterilized and stored cold.

Isolation of Plasma Membranes From MATA 13762 Tumor Cells

Plasma membranes were isolated from MATA 13762 tumor cells by the membrane stabilization method of Warren and Glick (79), as modified by Huggins et al. (77). Cells were removed from the peritoneal cavity of one or two rats at 7-8 days after implantation, centrifuged at 750 rpm for 3 minutes at 4°C, and the fluid decanted. The cells were washed with HEPES buffer at pH 7.4, the washed cells were suspended in 10 volumes of 40 mM Tris, pH 7.4, at 4°C and allowed to swell for 4 minutes (1928 g•min). This swelling step was repeated to obtain swollen tumor cells in the pellet and hemolyzed erythrocyte ghosts in the supernatant. The swollen cells were suspended in approximately 11 volumes of cold 1 mM $ZnCl_2$ for 2-3 minutes, then homogenized by 20 strokes of a Dounce homogenizer, or until microscopic examination by phase contrast revealed that about 85% of the cells were broken. This homogenate consisting of large sheets of plasma membrane was diluted with an equal volume of cold

40 mM Tris, pH 7.4, and centrifuged at 4.0×10^4 g•min in a Sorvall HB-4 rotor, as monitored by a Power Instruments (Skokie, Ill) Optical Tachometer, model B891. At this speed, most of the plasma membranes remain in the supernatant. Note that when cells are bloody, this speed must be carefully monitored by microscopy and altered accordingly. This supernatant was then recentrifuged at 2.44×10^5 g•min in a HB-4 rotor in order to pellet the membranes. The pellet was resuspended in 5 ml each of 45% and 40% sucrose in 20 mM Tris pH 7.4. Centrifugation was completed at 4°C in a Beckman SW-27 rotor in a model L-5 Beckman ultracentrifuge at 15,000 rpm for 60 minutes. All bands were removed with a bent needle, washed twice by suspending in 40 mM Tris, and centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 10 minutes. Protein concentrations were determined by the method of Lowry et al. (237).

MATA 13762 Whole Cell Stability in Various Buffers

When whole MATA 13762 cells were used, they were washed with HEPES buffer, pH 7.4 following removal from the peritoneal cavity of the host rats, and maintained cold. Since preliminary studies indicated some difficulty in getting sufficient label incorporated into whole cells within short time intervals, protein release in isotonic Tris and Kreb's Ringer phosphate with 10 mM glucose was monitored for up to 5 hours by Lowry protein assay of supernatants from the cells followed by SDS-polyacrylamide electrophoresis. Cell viability was monitored by trypan blue exclusion. For lengthy incubations of cells with stearic acid spin probes, the suspending medium was cold Kreb's Ringer phosphate, 10

mM in glucose, pH 7.4. The viability of cells in this buffer was 85% at the end of 10 hours, although some morphological changes were evident at the end of this time.

Sodium Dodecylsulfate Solubilization of Plasma Membranes

Membranes from either system were solubilized in 1.3% SDS in 0.1 M phosphate (pH 7.8) and 1% in mercaptoethanol at room temperature as described by Carraway et al. (61), and subjected to electrophoresis on 5% polyacrylamide gels in 0.1% SDS (238). All gels were stained with Coomassie blue by the procedure of Fairbanks et al. (239). In all cases, the labeled membranes displayed electrophoretic patterns identical with unlabeled membranes; membranes which had been frozen and thawed also showed no change in electrophoretic pattern. Electrophoresis on spectrin preparations were conducted in a similar manner, and displayed bands 1a, 1b, and 1c, with an occasional slight contamination with band 5.

Trypsin Treatment of Plasma Membranes

For proteolysis studies on RBC ghosts, EDTA-extracted RBC membrane fragments, and MATA 13762 plasma membranes, trypsin (Sigma, 2 X recrystallized from bovine pancreas, salt free crystals) was carefully weighed to give a stock solution of 5 mg/ml in the appropriate buffer. The activity of the enzyme was 11000 BAEE units/mg protein, based on $E^{1\%}_{280} = 14.4$. Trypsin was added to the spin labeled membrane samples at a ratio of 5 µg enzyme/mg protein and the samples gently shaken to insure thorough mixing. An aliquot was placed in the instrument cavity and the

spectrophotometer was adjusted so that the central peak emerged at 15 minutes incubation time for the first sample, 30 minutes for the second, and one hour for the third. All incubations were performed at room temperature. At the end of each interval, one drop of each of these samples was placed on a glass slide and crosslinked with 25% glutaraldehyde. The sample was then sealed under a coverslip with petrolatum for later microscopic examination. The samples were quenched at the appropriate time intervals with hot 1% SDS, and placed in a boiling water bath, then reserved and frozen for subsequent electrophoresis.

Reversible Crosslinking Studies

Crosslinking with reversible crosslinking agent dimethyl-3,3-dithiobisproprionimidate (Pierce Chemical Co., MW 281.2) was accomplished in both membrane systems by adding the reagent to the membrane suspension to give a final concentration of 2.2×10^{-6} M. This corresponded to approximately 0.5 mg/mg protein. The samples, which had been previously labeled with either MSL or 12 NS, were allowed to incubate at room temperature for 15 minutes, then maintained at 4°C until spectra could be taken. This was accomplished by beginning the scans immediately upon completion of the incubation period. At no time was the time elapsed by the end of the scan more than 30 minutes. When slides were prepared as above for microscopic examination, there was no observable difference between controls and crosslinked samples. Aliquots were removed for electrophoresis. These samples were solubilized by adding 0.2 ml membranes, 0.05 ml 20% SDS in electrophoresis buffer, 0.025 ml of a stock solution of 40 mg/ml n-ethyl maleimide,

incubated at 37°C for 6 hours, and run on 3.5% gels at 5 ma/gel for 5.5 hours.

Extraction of Water Soluble Membrane Proteins by EDTA Under Hypotonic Conditions

The water soluble proteins from 12 NS labeled MATA 13762 and erythrocyte membranes were EDTA-extracted by adding cold glycine-EDTA buffer (5 mM glycine, 1 mM EDTA, 0.1 mM mercaptoethanol, pH 9.5) to each sample at 1 ml extraction buffer/0.5 mg membrane protein. The samples were allowed to extract at 4°C overnight. The extracted membranes were centrifuged at 16,000 rpm for 90 min in a Sorvall SS-34 rotor to pellet the fragments. Spectra were taken of the supernatants, the pellets, and of washed whole membranes retained previously for this purpose. The latter had been washed free of excess label. Care was taken to monitor the distribution of protein in the extract and in the pellet. In the red blood cell ghosts, 30.6% of the total protein was removed under these conditions, and 30.9% was extracted from MATA membranes. The pellets from the MATA samples were quite small. The extract was concentrated by Amicon filtration using XM50 filters and spectra were run on the concentrate and the filtrate.

Fluorescence Measurements

Preliminary fluorescence quenching experiments were run on samples of RBC whole membranes, EDTA extracted membrane fragments, and the water soluble extract which were prepared as outlined above. Samples of whole membranes with and without 12 NS and 12 NS fragments were adjusted to 0.5 mg/ml protein in 10 mM Tris, 0.154 M in NaCl, pH 7.4. Fluorescence

measurements of tryptophan fluorescence were made at ambient temperature by exciting at 280 nm with a xenon lamp and monitoring emission at 340 nm. The concentration of the extract was not commensurate with obtaining useful spectra.

SDS Solubilization of Whole Cells

Detergent solubilization of whole erythrocytes, RBC ghosts, MATA 13762 whole cells and isolated plasma membranes were performed at room temperature in either 10 mM Tris, 0.154 M NaCl, or in Kreb's Ringer phosphate, 10 mM in glucose, pH 7.4. Aliquots of 4% SDS stock solution in the appropriate buffer were added to membrane or whole cell suspensions previously labeled with 12 NS, which contained 2-3 mg total membrane protein. These samples were brought to a 0.5 ml volume with buffer. The final SDS concentrations ranged between 0-1%, and incubation time was 15 minutes at room temperature.

ESR Study of Zn^{2+} Stabilization of Plasma Membranes

Stabilization of membranes by Zn^{2+} was examined by ESR for MATA 13762 cells by first labeling the washed whole cells with 12 NS or MSL as previously described, and then following the hypotonic swelling and Zn^{2+} stabilization procedure normally used persuant to membrane isolation. ESR spectra were taken of sedimented unswollen cells, after the first and second swelling steps, and following Zn^{2+} stabilization.

Introduction of Ionophore A23187 Into Whole RBC's

Washed, fresh whole red blood cells labeled with 12 NS or MSL were suspended in a minimal volume of cold isotonic Tris, pH 7.4 and titrated with a stock solution of ionophore A23187 (2.25 mg/ml in absolute ethanol which was 25% in DMSO) from 0-10 μM. The ESR spectra of these cells were run on a Varian E-9 spectrophotometer through the generosity of Dr. Robert Floyd of OMRF. Cells which had been treated with 1.15×10^{-8} M ionophore were titrated with 0.05 M $CaCl_2$ in 10 mM Tris, 0.154 M NaCl, pH 7.4 from 0-5 mM. Additions of $CaCl_2$ were made at 15 minute intervals and spectra were taken at each time point. MSL labeled whole cells were then incubated at 25°C with 15 μg A23187/ml packed cells, the threshold value found to show no spectral alterations, and spectra were taken at 0, 30, and 40 minutes. Samples were reserved for electrophoresis.

Cytochalasin B Treatment of MATA 13762 Cells

Treatment of 12 NS labeled whole, washed MATA 13762 cells with the microfilament disruptor cytochalasin B (Aldrich Chemical Co.) was performed using a stock solution of cytochalasin B (MW 479) prepared by dissolving 5 mg/ml in DMSO (Grade I, Sigma Chemical Co.). Samples of cells were prepared to contain 8.7×10^7 total cells in a 2 ml volume. Aliquots from the stock solution were added to give 0, 1×10^{-5}, 2×10^{-5}, and 2.5×10^{-5} M final concentration of cytochalasin B. Incubations proceeded at 4°C for one hour in isotonic Tris at pH 7.4, and ESR spectra were taken. An aliquot from each sample was reserved for

electrophoresis. Incubation of 12 NS labeled whole erythrocytes at 50% Hct with cytochalasin B were performed in a similar manner.

DMSO Treatment of MATA 13762 Cells

DMSO titrations of 12 NS labeled MATA 13762 cells were performed by adding aliquots of DMSO to a cell suspension containing 1.64×10^7 cells/ml. DMSO concentrations ranged from 0-0.17%; spectra were taken at each point, and samples were reserved for electrophoresis.

Gel Scans

Scans were made of gels on an autoscanner (Helena Laboratories, Dallas, Texas) at 525 nm. Peaks were automatically integrated from these scans by the Quick Quant II computer attachment and reported as either % total protein added to the gel or on a percentage of total area under all peaks present.

ESR Measurements

The electron spin resonance spectra were obtained on an electron spin resonance spectrophotometer constructed in the Physics Department of Oklahoma State University. This instrument is composed of the following units:

Varian 100 KHz field modulation and control unit V4560

Varian V4531 rectangular cavity

Varian V153/6315 reflex klystron tube (90 Mwatts)

Varian 4007-1 electromagnet

Varian V2200 regulated power supply

Varian E-248-1 aqueous solution sample cell

The instrument is an X-band spectrophotometer utilizing 100 KC modulation and phase-sensitive detection, probably roughly equivalent to a Varian E-3, but much more amenable to modification. The modulation and control unit is applied to signal filtering and phase-sensitive detection for a rectangular microwave resonant cavity. The loss of microwave energy due to the polarity of water molecules is a major problem with aqueous samples. When it was necessary to minimize this effect, a Varian E-248 aqueous solution sample cell was used. This enables accurate positioning of the sample in the planar region of minimun electric field intensity. When signal level permitted, samples were placed in a sealed Pasteur pipette instead of the flat cell. This permitted easier retrieval of the sample, and less sample was required to fill the capillary portion of the pipette than the flat portion of the aqueous sample cell.

Fluorescence Measurements

Fluorescence lifetimes were taken using the single photon counting method on an instrument constructed in the Physics Department of Oklahoma State University from the following purchased elements:

Single Photon Counting Setup

Ortec 920-00 Sample Chamber

Ortec 920-01 Phototube Base

Ortec 715 Dual Counter/Timer

" 9290 Power Supply (Spark Gap)

" 456 Power Supply (Phototube)

" 457 Biased Time-to-Pulse Height Converter

" 454 Timing Filter Amplifier

Ortec 463 Constant Fraction Discriminator

" 436 100 MHz Discriminator

" 425 Delay

" 451 Spectroscopy Amplifier

" 420A Timing Single Channel Analyzer

" 113 Preamplifier

Multichannel Analyzer

Nuclear Data, Inc. Series 1100 Analyzer System

Phototube

RCA 8850 Photomultiplier Tube

Filters

Corning Glass CS3-60 (excitation); CS 3-73 (emission)

The samples were excited with a free-running spark gap oscillator in air at atmospheric pressure. Fluorescent photons were detected with a fast response RCA 8850 phototube. Corning band pass filter CS 7-60 was used for excitation and CS3-73 cut-off was used for emission. The timing sequence is initiated with a pick-off signal from the spark gap. The signal is processed by an Ortec 436 discriminator and 425 delay circuit, and sent to the 457 time-to-height pulse height converter to begin the timing sequence. Fluorescent photons detected by the phototube are processed by an Ortec 454 timing filter amplifier and a 463 constant fraction discriminator, and sent to the time-to-pulse height converter to halt the timing sequence. To eliminate distortion of the decay curve due to detection of multiphoton events a signal is picked off the last dynode, and after processing by a 113 scintillation preamplifier and a 451 spectroscopy amplifier, is sent to a 420A timing single channel analyzer. Here, single photon signals are separated from

low level noise and multiphoton events. A start-stop sequence is processed only if the time-to-pulse height converter receives a signal from the 420A within its reset time. The output from the 457 is analyzed with a Nuclear Data series 1100 multichannel analyzer system. A statistical signal is built up and printed with a series 2000 Omnigraphic X-Y recorder from Houston Instruments.

Fluorescence spectra and depolarization data were taken on a second unit composed of a xenon lamp (Illumination Industries, Inc., 150 watt xenon short arc lamp with power supply Model CA-150), monochrometers (Spex Industries, Inc., Spex Minimate 1670, 22 f/4.0, 1200 grooves/mm, 300 mm blaze, 5 mm slits to give a 20 nm bandpass, and a Jarrell-Ash, ¼ m f/3.6, 1180 grooves/mm, 300 mm blaze, 5 mm slits to give a 17 nm bandpass), filters (Corning Glass, excitation, CS3-60, and excitation, CS3-73, CS3-74, CS0-51), and a phototube (RCA 1P28 photomultiplier). A model 125 100 Hz mechanical light chopper (Princeton Applied Research Corp.) was used to chop the beam, and power was supplied by a Model 415B power supply (John Fluke Mfg. Co., Inc.). A Model 128 lock-in amplifier was used (Princeton Applied Research Corp.), and spectra were recorded with a Model EU-205-11 strip chart recorder (Heath Co.). The polarizing filters were simple film filters mounted in movable holders.

CHAPTER III

RESULTS

Introduction

The most readily observable characteristics of any membrane system are the gross morphological perturbations brought about by aging, genetic anomalies involving membrane alteration, transformation, or those induced by the application of perturbing agents. For example, human red blood cells, the simplest of all mammalian cells, are known to undergo spiculation, crenation, sphering, sickling, or lysis in response to ATP depletion, Ca^{2+} accumulation, or to certain disease states such as hereditary spherocytosis, sickle cell anemia, or polycythemia. MATA 13762 cells, one of many transformed cell types, also respond to certain perturbants by exhibiting alterations of gross morphology and cell surface properties. It is possible that any or all of these cell surface changes, which are often observable with phase contrast or electron microscopy, are due to changes in the physical state of certain proteins in the cell membrane. There is also the question of whether these proteins are primarily structural in nature, or perhaps interact with structural proteins, or only with adjacent phospholipids. In short, these morphological changes may be related to either an alteration in the interaction of the surface components with structural protein, disruption of structural components within the membrane, or from a marked shift in the microviscosity of the membrane bilayer.

Since electron spin resonance spectroscopic methods are particularly sensitive to the rate of reorientation of a spin label in a given system (molecular motions on the order of 10^{-11} seconds) the method is particularly well-suited to detecting changes in mobility as well as in lipid order and protein conformation. We have used spin probes in the form of stearic acids bearing nitroxide groups at varying positions along the hydrocarbon chain (Figure 2), which allowed observation of the phospholipid bilayer at different levels. Shown in Figure 3 is the nitroxide-bearing maleimide derivative, used to reflect changes in protein conformation. Examples of the spectra obtained from spin-labeling biological systems are given in Figure 2.

Spin Labeling of Intact Cells

Whole RBC's and MATA 13762 ascites tumor cells were labeled with the fatty acid spin probe 12 NS. There is a reasonable consistency among the order parameter values obtained for these cells. As noted in the Introduction section, even slight variation in isolation procedures or buffering systems employed gives rise to marked changes in the condition of the resultant cell population. Very early in these studies, it became evident that the Tris buffering system characteristically used in the RBC studies was unsuitable for the MATA cells. Care had to be taken to maintain these cells in a favorable environment. We found that the optimal buffer system for labeling was Krebs Ringer Phosphate, pH 7.4, which was 10 mM in glucose. Cells incubated in this buffer in the presence of fatty acid spin label for up to ten hours maintained a 90% viability, as shown by trypan blue exclusion. Regardless of the difference between the isolated plasma membrane and the whole cell systems,

Figure 2. Spectra of Stearic Acid Spin Labels Incorporated into Normal RBC Ghosts

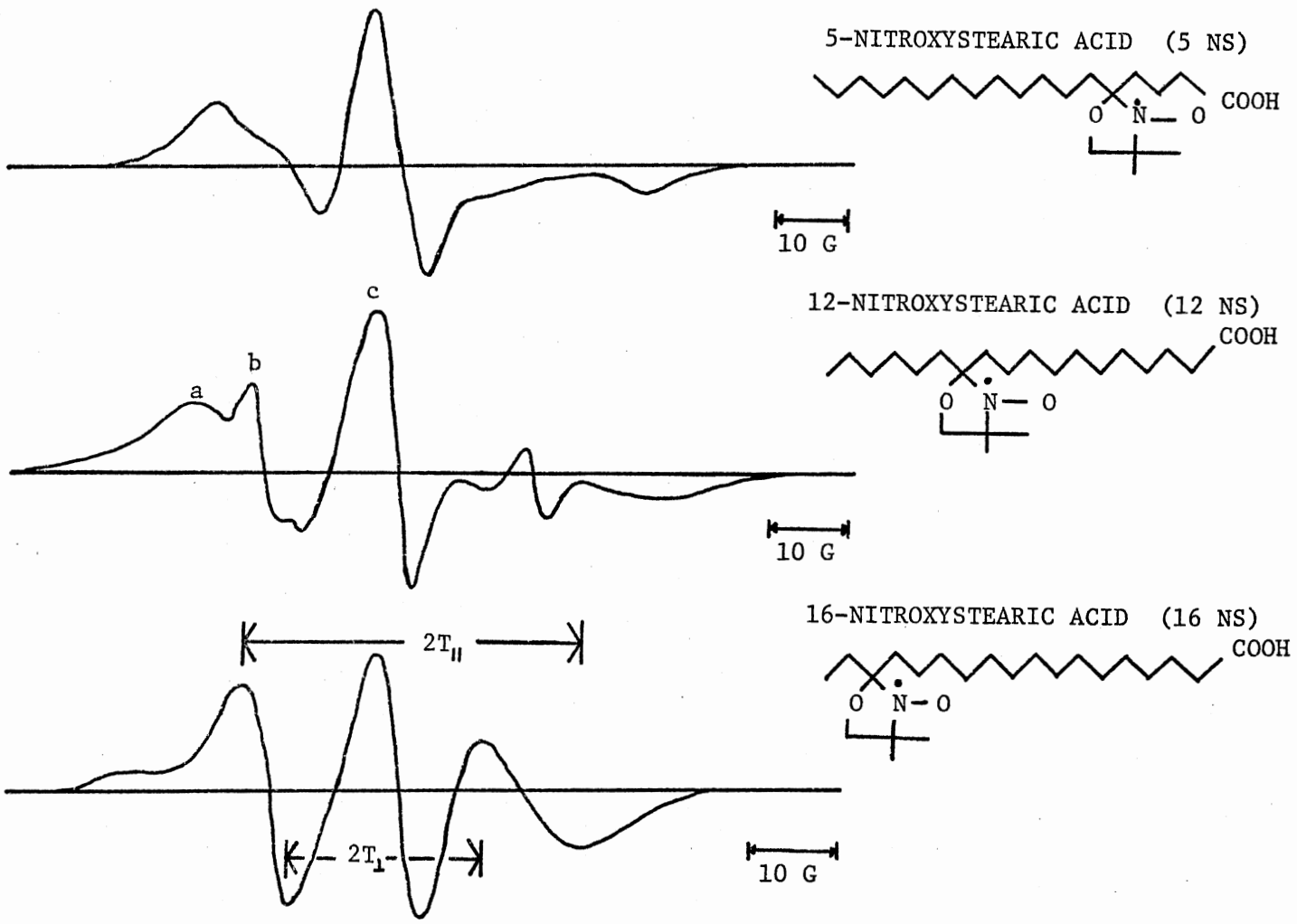
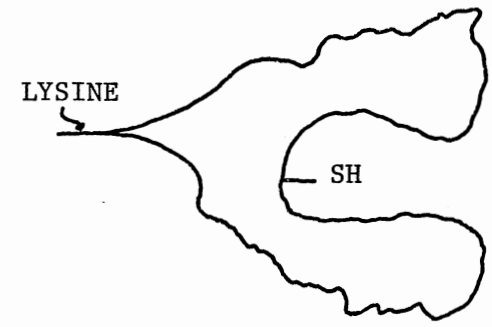
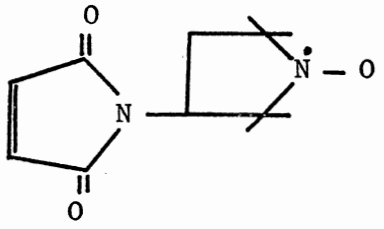
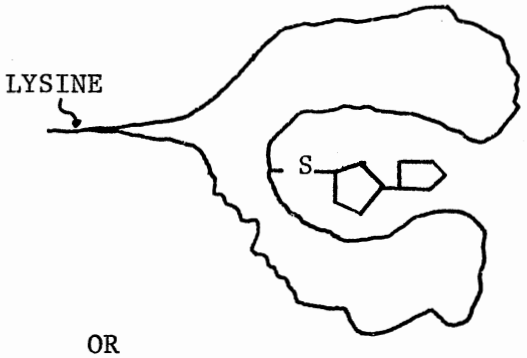

5-NITROXYSTEARIC ACID (5 NS)
COOH
O N O
10 G
12-NITROXYSTEARIC ACID (12 NS)
COOH
O N O
a
b
c
10 G
16-NITROXYSTEARIC ACID (16 NS)
COOH
O N O
$2T_{\parallel}$
$2T_{\perp}$
10 G

Figure 3. Schematic Representation of Incorporation of the Spin Label MSL Into Protein

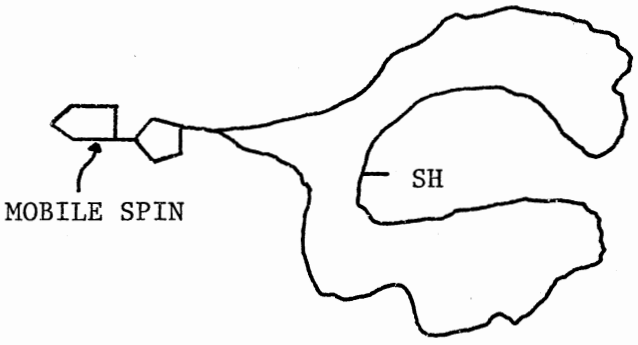




N-(1-OXYL-2,2,5,5-TETRA-METHYLPYRROLIDINYL)-MALEIMIDE (MSL)



OR



definite trends may be established with the several perturbing agents, as shown in later sections, which may ultimately give some insight into the structure-function relationships in the intact cell as compared with the isolated cell membrane.

There is an additional problem with maintaining cells in buffering medium for extended periods of time. (The optimum labeling time for our tumor cells was 6 hours in the cold.) Even in an optimized buffering system, there is a steady release of protein from the cells, which levels off at the end of four hours. The amount of protein assayed in the supernatant from the cells at the end of five hours was 19% of the original total protein, as determined by Lowry assay. It is possible that some of the protein released was actually serum protein adsorbed to the surfaces of the cells throughout the isolation procedure, but this does not account for the large quantity of protein released, nor the period of time over which the protein was released. Another source for protein might be the cytoplasm and ruptured membranes of lysed cells. However, there were not a significant number of membrane fragments visible by phase contrast microscopy in the suspensions. We did note that the cells themselves exhibited some aneurisms on the plasma membrane of the type designated as "blebs" in RBC membranes.

The concentration of the label, itself, is also important in maintaining the membranes of whole cells in a reasonably native state. During early experiments to determine the optimum concentration of fatty acid probe for adequate labeling of RBC's, we noticed that rather severe membrane blebing occurred when more than 10^{-3} M probe was present in the labeling suspension. This is shown by electron microscopy with negative staining in Figures 4a and 4b. Later, this finding was

Figure 4a. Membrane Blebbing Induced by Spin Probe In Erythrocytes as Shown by Electron Microscopy (15,000 X)

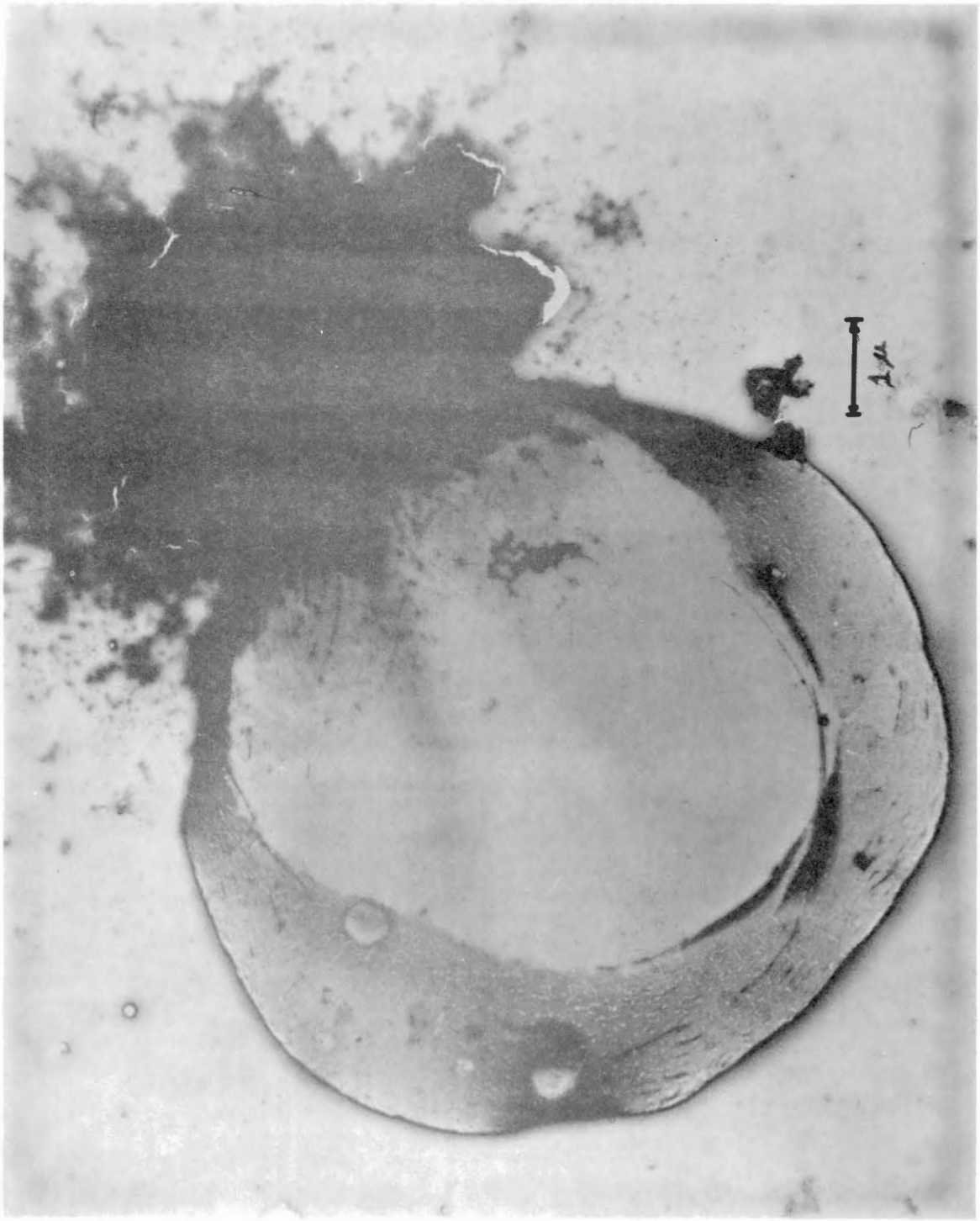
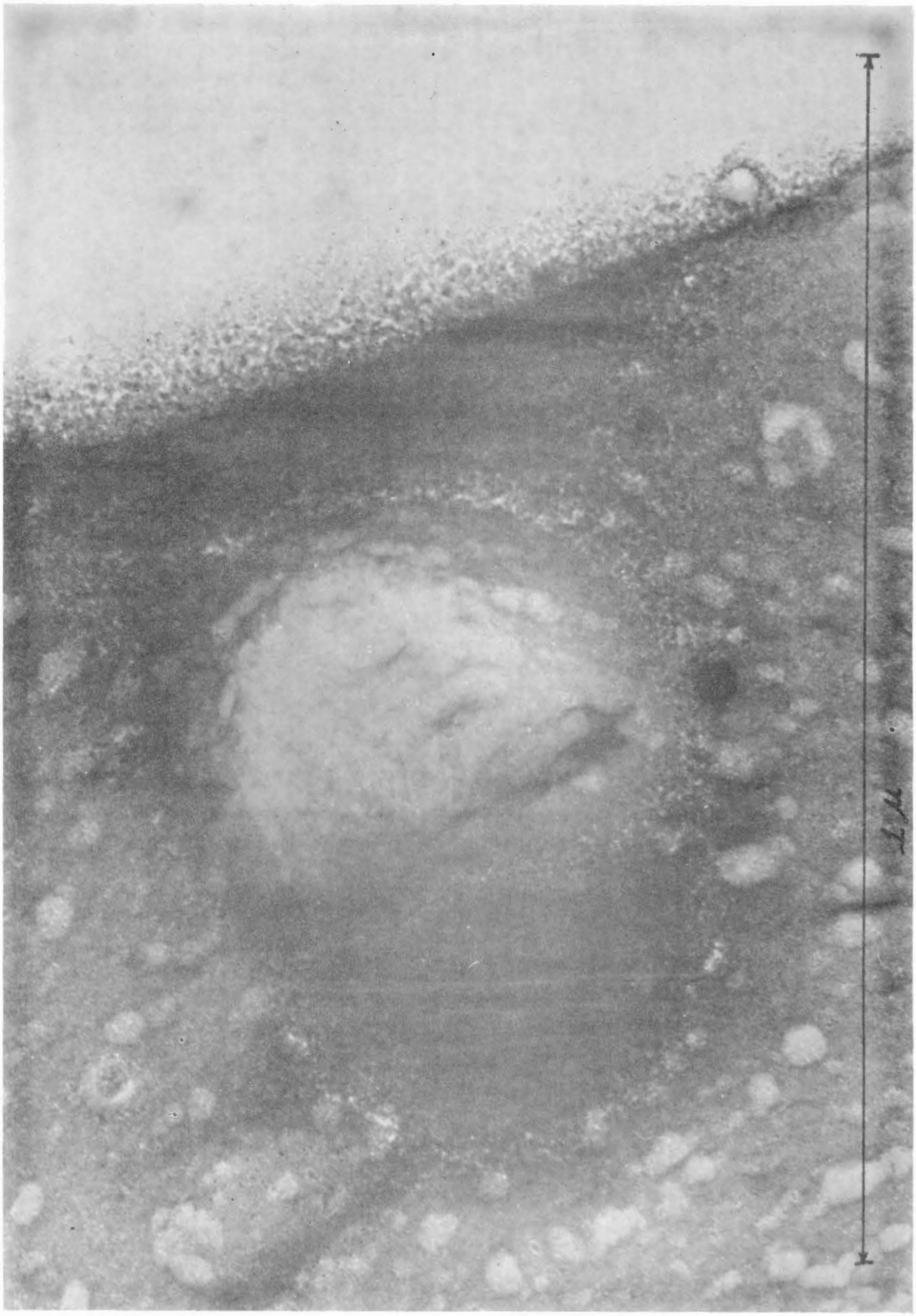

1 μ

Figure 4b. Membrane Blebbing Induced by Spin Probe In Erythrocytes as Shown by Electron Microscopy (200,000 X)



confirmed by Wallach and Lin (240), who had performed similar experiments. Their result differed from ours in that they demonstrated blebbing at fatty acid probe concentrations of 10^{-4} M. Further studies indicated that it was possible to label the cells with 10^{-3} M probe, which allowed a great excess of label, by washing out the excess after labeling was complete and incubating the cells at 37°C in isotonic medium for 30 minutes to restore the normal morphology. There was an increase in hemolysis, indicating that such treatment rendered the cells more fragile. This blebbing phenomenon seemed to vary in severity with the age of the blood sample. Fresh blood samples were less susceptible to damage under the specified conditions.

Labeling of Membranes and Comparisons with Intact Cells

Plasma membranes from either cell system are easily labeled with fatty acid probes by the method of Landsberger et al. (236), following membrane isolation. Isolated membranes provide a less complex system for the study of protein-lipid interactions than intact cells, and are useful for comparisons with the intact cells.

Of the three stearic acid probes used, the 12 NS derivative most consistently gave measurable differences among the systems examined. Both 5 NS and 16 NS gave only negligible responses when most perturbants were applied to the membranes. However, in labeling membranes isolated either from whole human RBC's or from MATA 13762 ascites cells, there seemed to be some variability among samples aside from the applied perturbations, regardless of the label used. Indeed, this same variability was observed on studies performed on whole cells.

In addition, there is a discrepancy in order parameters between RBC membranes and whole cells of 0.34 units (Table I). This is negligible considering the standard deviations shown in Table I for the two different systems over a period of more than two years. However, there is a variation of 0.200 units between the values for MATA 13762 cells and isolated membranes which cannot be discounted by standard deviation. It is due to labeling of different components in the two systems, such as the organelle membranes within the whole cells in addition to the external membrane. These tumor cells also characteristically contain fat droplets in the cytoplasm which are also labeled when the probe penetrates the cell. The spectrum (Figure 5) of whole MATA cells is clearly more mobile, differing from a simple three line spectrum of 12 NS in solution primarily in line width. Examination of the remaining spectra in Figure 5 reveals the qualitative differences among the systems with respect to spin populations. In each case, there are at least two superimposed spectra, reflecting vastly different mobilities. These populations are shifted due to differences in microviscosities among these systems.

Proteolysis of Plasma Membranes

Shin and Carraway (96) have described a set of membrane polypeptides which are thought to contribute to membrane stabilization and maintenance of cell shape. As pointed out in Chapter I, mild proteolytic cleavage in cultured fibroblasts produces alterations which mimic those seen in transformation (91). In whole cells, such proteolysis brings about altered morphology (92), lectin agglutinability (93), and receptor distribution (94). Furthermore, it has been shown that trypsin

TABLE I

ORDER PARAMETERS FOR 12 NS LABEL IN RBC AND MATA 13762 MEMBRANES AND WHOLE CELLS

Sample	Mean Value of S	Standard Deviation	n	Variance
MATA 13762 membranes	.518	± .04	16	.002
MATA 13762 whole cells	.321	± .04	6	.001
Human RBC membranes	.532	± .03	9	.001
Human RBC whole cells	.566	± .05	7	.002

Incubation of cells and membranes with 12 NS probe followed essentially the method of Landsberger. The mean, standard deviation and variance were calculated as follows:

$$\text{Mean} = \bar{X} = \frac{\sum_{i=1}^{N} X_i}{N}$$

$$\text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{N} (X_i - \bar{X})^2}{N-1}}$$

$$\text{Variance} = \sum_{i=1}^{N} \frac{(X_i - \bar{X})^2}{N}$$

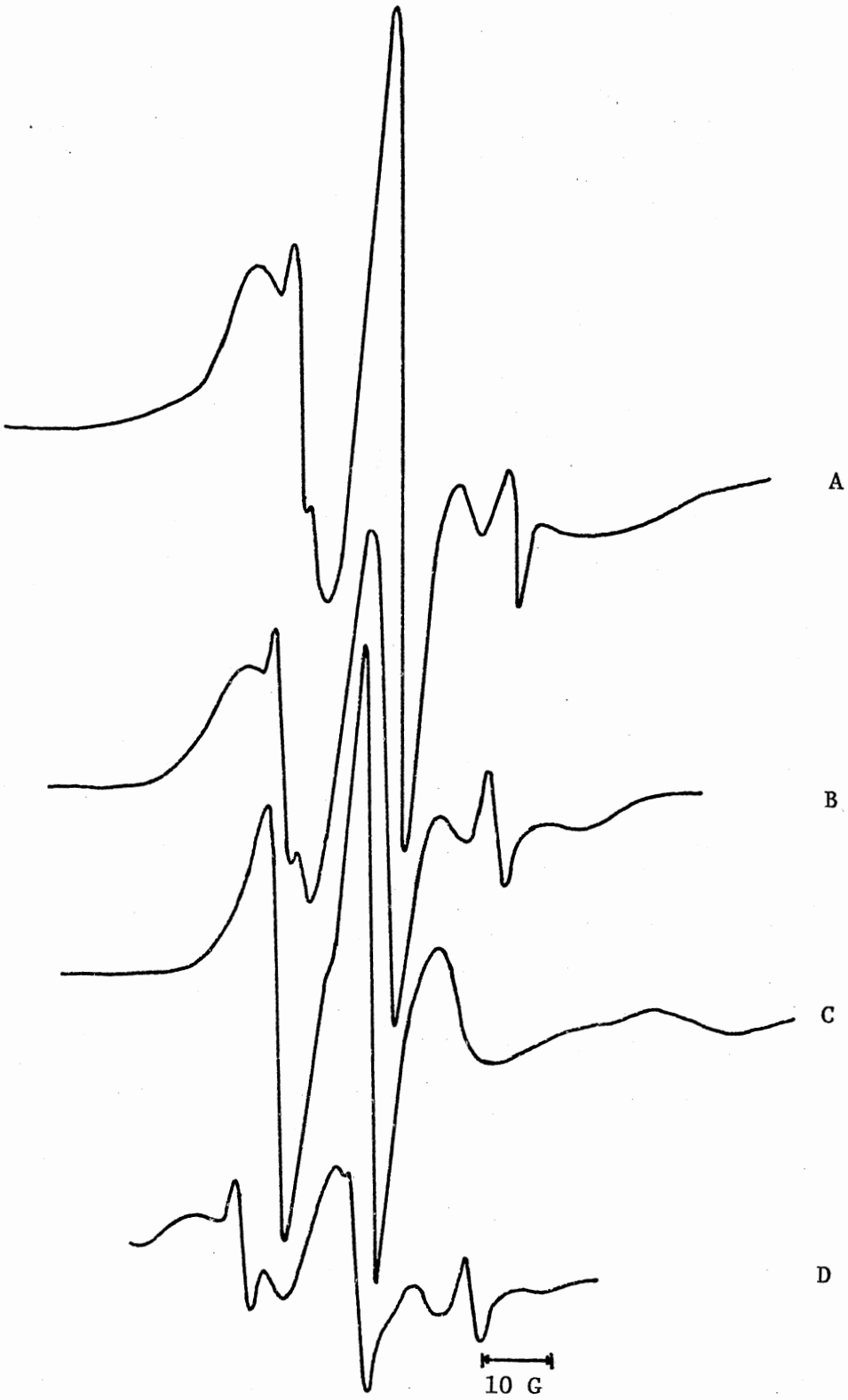
Figure 5. Comparison of Spectra From Whole Cells and Isolated Plasma Membranes Labeled with 12 NS

A) Whole Red Blood Cells

B) Red Blood Cell Membranes

C) Whole MATA 13762 Cells

D) MATA 13762 Membranes


A
B
C
D
10 G

treatment of RBC's inhibits the membrane contraction which may be induced by Ca^{2+} in the presence of ATP (241). This is probably due to spectrin modification. Proteolysis of isolated erythrocyte membranes leads to vesiculation and sealing of the membranes. Similarly, proteolysis of isolated ascites cell envelopes leads to fragmentation with preferential cleavage of high molecular weight cytoskeletal polypeptides attached to the membranes. Thus, the proteolysis may aid in assessing effects of cytoskeletal elements on the membrane.

Proteolytic cleavage of the protein moieties accessible to the peptidase trypsin when applied to erythrocyte ghosts resealed by incubation in isotonic medium following lysis resulted in a decrease in the order parameter. These ghosts, which had been previously labeled with 12 NS, were incubated with trypsin for 30 minutes. The spectra indicated a rather small, but distinct difference in order parameter, from 0.571 in the control to 0.535 in the trypsinized sample (Table II). There was an enhancement of the high field liquid line with a general narrowing of the liquid components, further indicating disordering of the membrane. There was no further increase in membrane fluidity after the 30 minute interval. PAGE-SDS electrophoresis of the samples indicated that there was a significant amount of cleavage of the high molecular weight (HMW) membrane proteins.

Membrane envelopes isolated from MATA 13762 cells and labeled with 12 NS were subjected to similar treatment with similar results, with the exception that the change in order parameters was greater (Table II). The order parameter ranged from 0.563 in the control to 0.507 in the trypsin-treated samples within 15 minutes, and then to 0.497 at the end of 30 minutes. Again there was no further change at the end of this

TABLE II

PROTEOLYSIS OF 12 NS LABELED RBC AND MATA 13762 TUMOR CELL MEMBRANES BY TRYPSIN

Sample	$2T_{\parallel}$	$2T_{\perp}$	a_n'	S
Control, RBC Membranes	54.48	21.57	16.27	.571
RBC Trypsinate	52.81	22.12	16.17	.535
Control MATA Membranes	52.35	21.75	15.98	.541
MATA Trypsinate	51.75	22.68	16.19	.497

Incubation of plasma membranes with 12 NS, followed by trypsinization for 30 minutes at room temperature. The a_n' values were calculated as follows:

$$a_n' = 1/3\ (2T_{\perp}' + T_{\parallel}')$$

S values were calculated from the following relationship:

$$S = \left(\frac{T_{\parallel}' - T_{\perp}'}{T_{\parallel XL} - T_{\perp XL}}\right)\left(\frac{a_{n_{XL}}}{a_n'}\right)$$

time interval. Electrophoresis showed degradation of high molecular weight components, although the cleavage seemed to be more generalized. Since there were a multitude of peptide fragments, it was difficult to ascertain from the gels which components were cleaved. Since these membranes were not resealed, it is probable that more protein was easily accessible to the enzyme; since its specificity is only for arginine and lysine residues, but not for a particular protein, the cleavage products were a result of this increased accessibility. This factor probably accounts to some extent for the increase in fluidity over that observed in the erythrocyte. However, it still implies a requirement for protein-lipid interaction for maintenance of membrane structure and ordering of the phospholipids. Indeed, microscopic examination of these trypsinates revealed extensive fragmentation of the membranes with no intact sheets of MATA membrane or whole RBC ghosts visible.

Reversible Crosslinking of Membrane Proteins with DTBP

The number and diversity of protein components interacting directly with cytoskeletal elements in plasma membranes from either the erythrocyte or tumor cells is not yet defined. The suggestion has been made by Hasketh *et al.* (242) that certain of the erythrocyte proteins are surrounded with a shell of closely associated lipid which is perturbed with protein modifications. The structure of these shells depends upon the protein architecture. Use of crosslinking agents (111) to form oligimers or dimers gives an idea as to what proteins are adjacent to each other. Dimethyl-3,3'-dithiobisproprionimidate (DTBP) provides a disulfide bridge bifunctional imidoester crosslinking agent which may

be used to reversibly crosslink proteins. Wang et al. (114) have shown that spectrin 220,000 and 240,000 molecular weight units exist as dimers, and further, that they are on the cytosolic side of the membrane, since spectrin also crosslinks to hemoglobin (Hb). Their work shows that Band 5 crosslinks to itself, but not to Hb or spectrin, though it is thought to be located on the internal aspect of the membrane, and to interact with spectrin.

The effect of DTBP on the microviscosity of the phospholipid bilayer was examined with 12 NS labeled membranes from both sources at a ratio of 5 mg DTBP per mg protein. In the erythrocyte membranes, there was a slight decrease in the immobilization of the spin probe (Table III) ranging from an order parameter value of 0.571 in the control to 0.530 and 0.521 in treated membranes. This rather small change in erythrocytes is in the opposite direction to that observed in the tumor cell membranes, which gave order parameter values of 0.439 in the control and 0.560 in treated membranes at t = 30 minutes. Furthermore, the change in MATA membrane was virtually immediate, beginning at S = 0.460 at t = 0. It takes roughly 7.8 minutes for the central peak of the spectrum to emerge once a scan is begun, and the first sweep was initiated as soon as the reagent was added to the sample. These differences are related to the tendency of RBC ghosts to reseal under isotonic conditions as compared with MATA membrane envelopes which remain ruptured. A greater proportion of the protein-associated label is readily available to the crosslinking agent, hence the increase in probe immobilization in the MATA membranes. The cytoskeletal elements of the RBC ghosts are less accessible and are probably perturbed more indirectly by crosslinking of transmembrane elements at the cell surface.

TABLE III

DTBP CROSSLINK OF 12 NS LABELED RBC AND MATA 13762 TUMOR CELL MEMBRANES

Sample	$2T_{\parallel}$	$2T_{\perp}$	a_n'	S
Control RBC Membranes	54.48	21.57	16.27	.571
DTBP X-link	54.66	23.43	16.92	.521
DTBP X-link	54.10	22.87	16.64	.530
Control MATA Membranes	46.27	22.59	15.24	.439
DTBP X-link (t = 0)	47.17	22.30	15.29	.460
DTBP X-link (t = 30 min)	48.76	19.35	14.58	.570

Incubation of 12 NS labeled isolated plasma membranes from human erythrocytes and MATA 13762 ascitic tumor cells with dimethyl-3,3'-dithiobisproprionimidate at room temperature.

A comparison of these spectra is given in Figure 6.

In both RBC and MATA membranes which had been crosslinked with DTBP, electrophoresis indicated definite aggregate formation. No attempt was made to quantitate the extent of aggregation, since the DTBP crosslinks are extremely labile, and one can assume that some of the bonds were disrupted during sample preparation.

The response of RBC membranes to the crosslinking agent is similar to that observed in previous work (52) involving Ca^{2+}-mediated aggregation of the membrane protein spectrin. The interaction of the Ca^{2+} ion and DTBP appears to be at the polar ends of the phospholipid molecules, such that the head groups are drawn together more tightly, inducing a randomization of the associated hydrophobic chains lying within the interior of the membrane. The difference in response of the MATA membrane to DTBP reflects differences in the type of crosslinking present, and organizational differences, as well as the increased protein accessibility mentioned earlier.

Labeling of these membranes with MSL indicated restriction of molecular motion of the spin probe, which was covalently attached to available membrane proteins through reaction of the free sulfhydryls or ε-amine groups (Figure 3). Since there are potential binding sites on any protein accessible to the label, it is not possible to identify the protein which is labeled by spectrophotometric observation. However, there is always a contribution from weakly immobilized radicals which are bound at the surface, and a contribution from strongly immobilized nitroxides which have penetrated further into the membrane protein. This is true both of the controls and the treated samples, so any change in the ratio of the strongly (a) to the weakly (b) immobilized

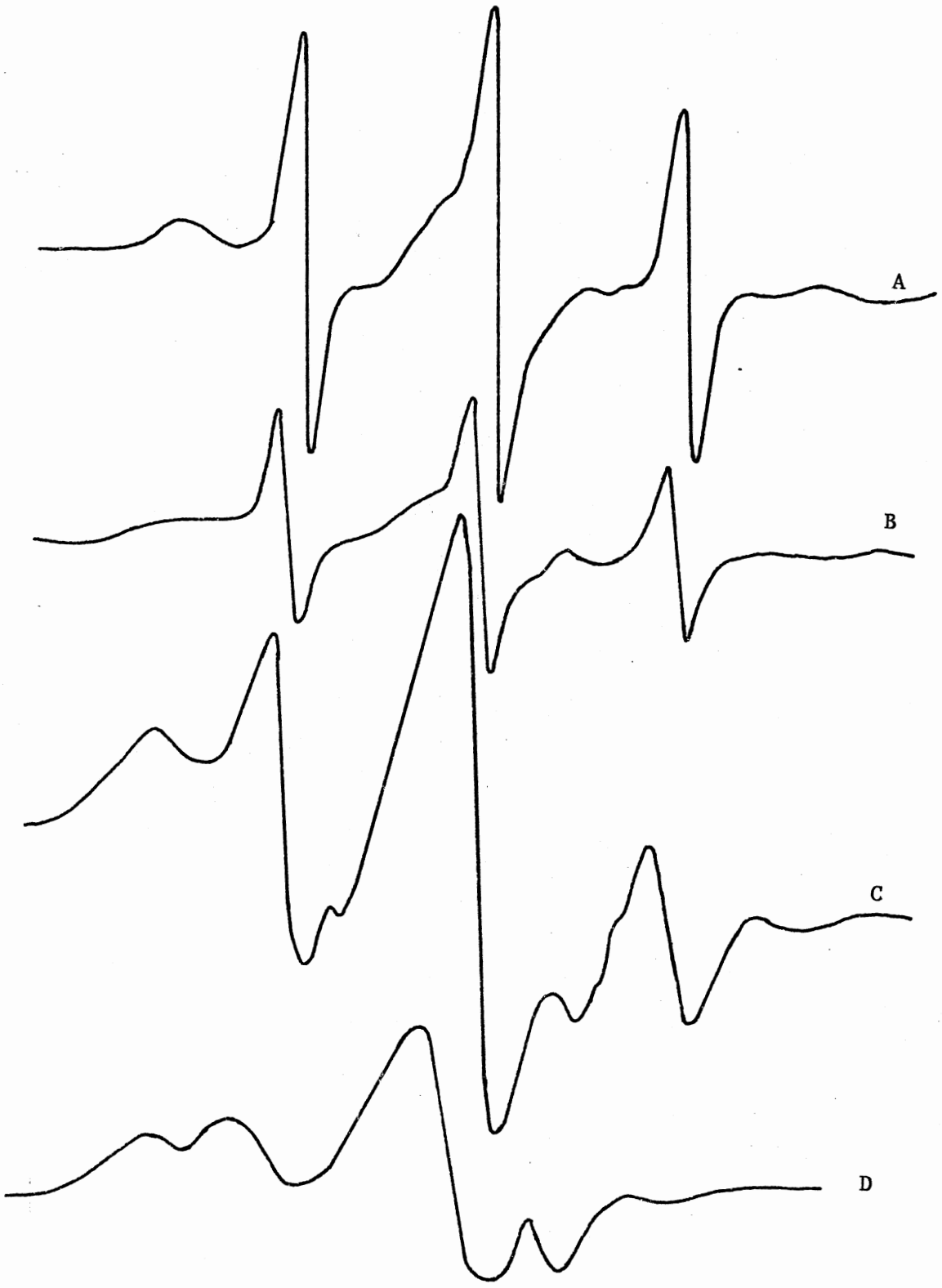
Figure 6. Comparison of Spectra from 12 NS Labeled Membranes Crosslinked with DTBP

A) Red Blood Cell Membranes (Signal Level 5000)

B) DTBP crosslinked RBC Membranes (Signal Level 5000)

C) MATA 13762 Membranes (Signal Level 8000)

D) DTBP Crosslinked MATA Membranes (Signal Level 8000)


A
B
C
D

contribution to the spectrum is indicative of a change in the state of aggregation of the protein. The a/b ratios in both instances varied in the same direction (Table IV), and the difference between control and crosslinked protein is 0.19 in RBC's and 0.280 in MATA membranes. A comparison of the spectra is given in Figure 7. These data are indicative of an altered protein in the membranes upon crosslinking. There is nothing remarkable in this observation, although this particular result does not resemble the magnitude of the change observed in similar studies conducted with this labeling system in Ca^{2+} treated RBC membranes. The Δ value for increased immobilization in the Ca^{2+} treated membranes was 0.260, implying a larger conformational change.

A remarkable result of these MSL studies was the observation that the spectra of the RBC and MATA crosslinked samples showed a vast reduction in liquid signal and signal intensity in general. The reduction of the central peak height is drastic, approaching 58%, but as noted in Figure 7, in the case of the MATA crosslinked sample, the peaks from the immobilized component remain at approximately the same height. The high field liquid line height is depressed. When samples were spun down to determine whether or not some of this label had been dislodged, only a small amount of signal was detectable in the crosslinked sample, and none at all was found in the supernatant from the control. It is possible that some of the weakly immobilized, and therefore more labile MSL molecules were displaced by the DTBP, released into solution, and subsequently reduced. If such reduction occurred, then it seemed to require that MSL be free in solution. Attempts to show reduction of MSL by DTBP in aqueous solution were inconclusive.

TABLE IV

REVERSIBLE CROSSLINKING OF MEMBRANE COMPONENTS IN MATA AND RBC PLASMA MEMBRANES WITH DTBP*

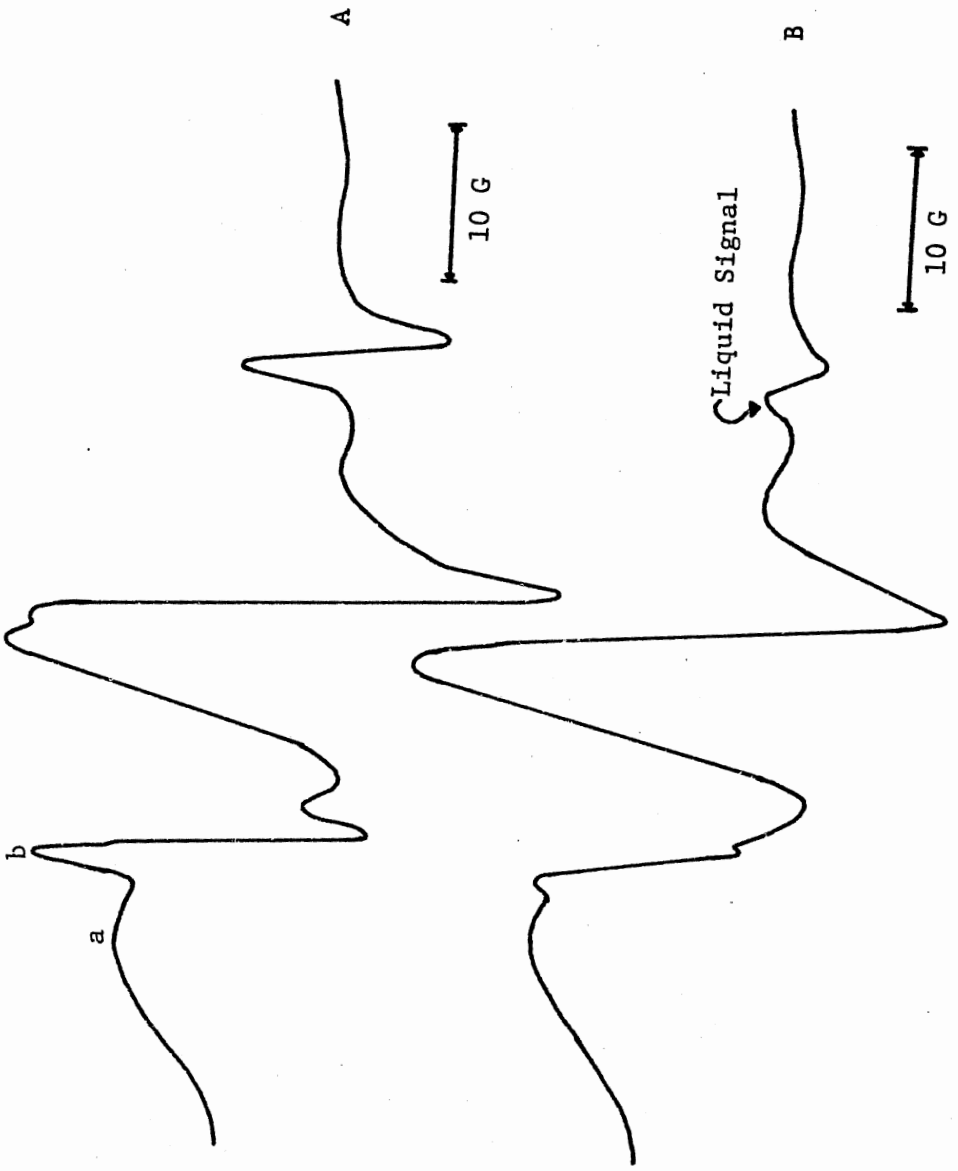
Sample	a/b Ratio	Difference (Δ)
MATA membranes	0.51	0.29
MATA crosslink	0.81	
RBC membranes	0.13	0.19
RBC crosslink	0.32	

*Membranes labeled with MSL prior to crosslinking.

Figure 7. DTBP Crosslinking of MSL-Labeled MATA 13762 Membranes

A) Control

B) DTBP Crosslinked Membranes (Signal Intensity x 2)


A
B
10 G
10 G
Liquid Signal
a
b

Irreversible Crosslinking of Membrane Protein with Glutaraldehyde

Glutaraldehyde is another widely used crosslinking agent, which is also utilized as a fixative for biological samples. It is thought to crosslink proteins more extensively than DTBP, and the bonds formed are not readily labile. Indeed, treatment of 12 NS labeled RBC membranes with glutaraldehyde gave an increase in membrane viscosity, as shown by S = 0.499 in the control and S = 0.549 in the treated sample. This represents no greater perturbation than the shift produced by DTBP, but it is in the opposite direction from that observed with DTBP, and it is reproducible. SDS gels run on these samples showed a definite aggregate formation. That the two different reagents produce opposing effects may reflect their differing modes of action, or alternatively, the lack of sensitivity of the method to the changes produced. It is quite possible that any significant alteration of membrane microviscosity simply does not occur in the region of the bilayer accurately monitored by the 12 NS probe. However, upon examination of the shapes of the spectra in Figure 8, it is readily apparent that a massive change has occurred. The spectrum of the glutaraldehyde crosslinked sample resembles a powder spectrum, i.e., almost totally immobilized.

Similar results are obtained with the MATA membranes, with an average control value of S = 0.550 and S = 0.602 in the presence of 0.5% glutaraldehyde (Figure 9). The differences in order parameter here between RBC and tumor cell membranes are almost identical, and are in the same direction. The spectra (Figures 8 and 9) in both cases reflect greater changes than one might have deduced from the order parameters alone.

Figure 8. Spectral Comparisons Among Perturbations of 12 NS Labeled RBC Membranes

A) RBC Membranes, Untreated

B) RBC Membranes Solubilized with 1% SDS

C) RBC membranes Crosslinked with Glutaraldehyde (2X)

D) RBC Membranes Proteolyzed with Trypsin (2X)

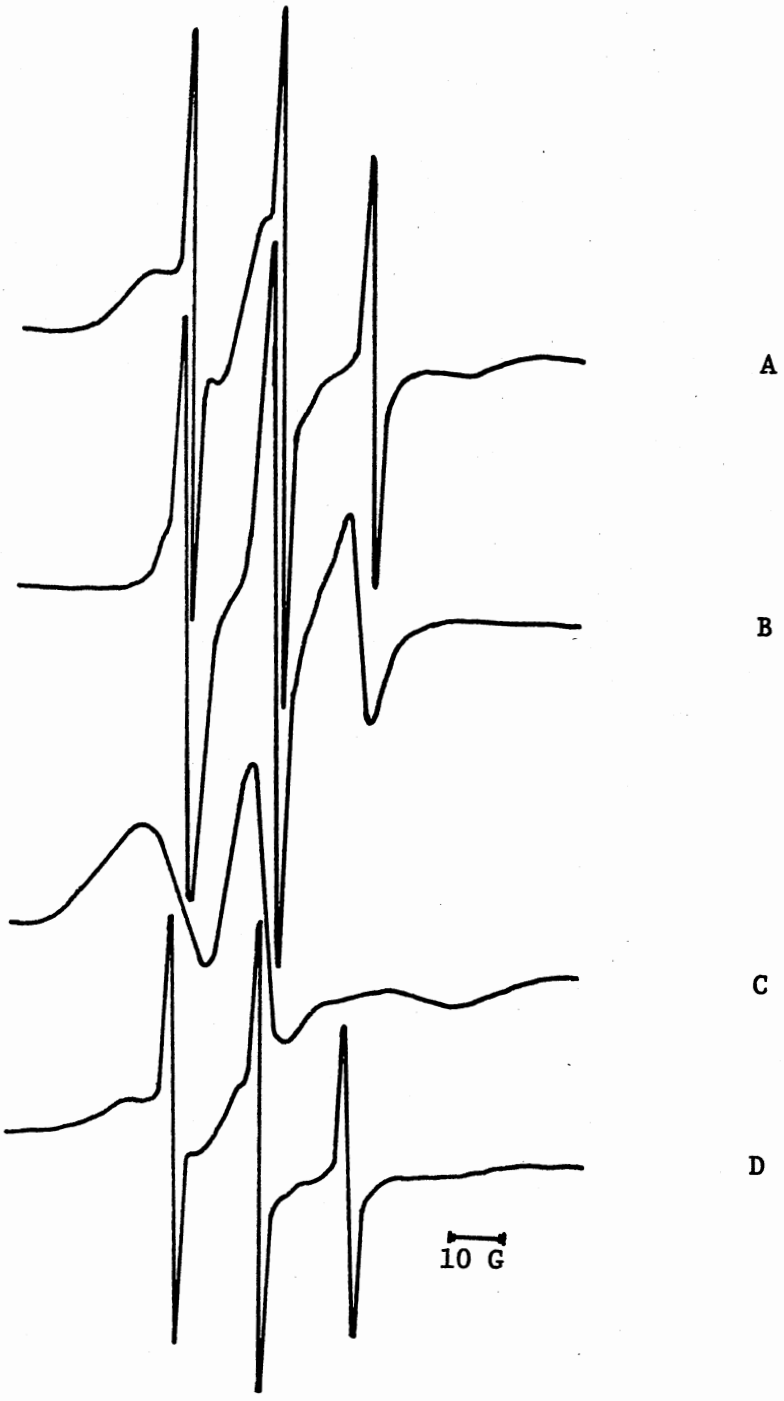

A
B
C
D
10 G

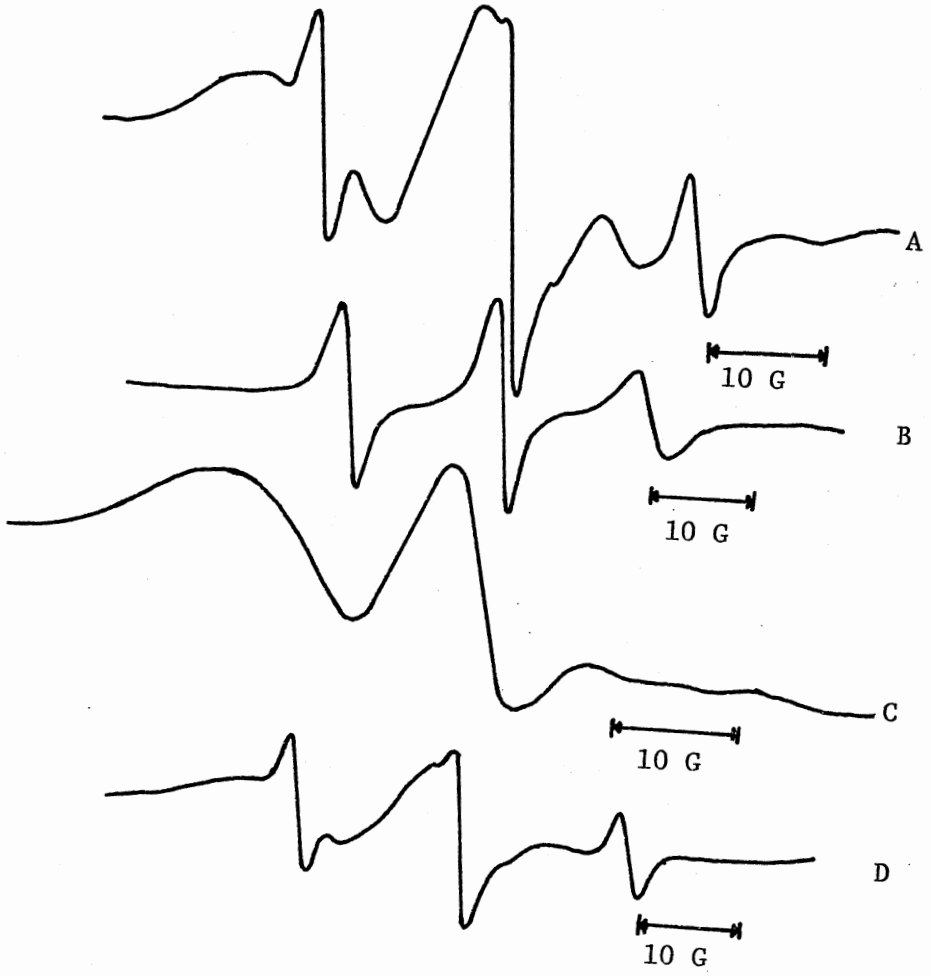
Figure 9. Spectral Comparisons Among Perturbations of 12 NS Labeled MATA 13762 Membranes

A) MATA Membranes, Untreated

B) MATA Membranes Solubilized with 1% SDS

C) MATA Membranes Crosslinked with Glutaraldehyde

D) MATA Membranes Proteolyzed with Trypsin


A
10 G
B
10 G
C
10 G
D
10 G

MSL studies of glutaraldehyde crosslinking indicated a possible immobilization of the membrane protein, but the most significant observation was the fact that the signal level again decreased, as with DTBP. Studies of the maleimide derivative in the presence of glutaraldehyde showed a slightly diminished signal intensity over a 30 minute interval, but it was still not great enough to account for the degree of quenching observed in membrane systems.

EDTA Extraction of Soluble Proteins

Water-soluble proteins may be extracted from plasma membranes from either RBCs or MATA 13762 tumor cells by incubation of these membranes with EDTA-containing low ionic strength buffers. In the red blood cell membranes treated with GEM buffer, the spectrin doublet and a lower molecular weight species of 45,000 MW coextract. As mentioned in the Introduction, spectrin has been more completely characterized than have the HMW proteins similarly extracted from other sources, but even for spectrin, such studies are far from complete. Structural roles for both spectrin from RBCs and HMW protein in MATA cells have been proposed. EDTA extractions of the two 12 NS labeled membrane systems were carried out in an effort to determine what effect the removal of these presumably structural elements might have on the phospholipid bilayer.

In our earliest attempts to examine these effects, we encountered instrumental difficulty in that the signal to noise ratio was very poor, and the high field lines were ill-defined. The order parameters obtained from the vesicles remaining after EDTA extraction of RBC membranes gave an average value of $S = 0.520$ as compared with $S = 0.510$ in unextracted control membranes. This was unremarkable, and might have been dismissed

as random error in the measurements had these not been averages of 3 separate samples. The spectra also exhibited very distinctive characteristics. The early work with MATA 13762 extractions was fraught with sensitivity problems to the point that it was not possible to calculate order parameters at all, since measurement of 2 $T_{||}$ could not be made with any confidence. Again, there were spectral changes which indicated that there were, indeed, changes taking place.

Examples of spectra obtained from EDTA extraction of RBC membranes are presented in Figure 10. There are several distinctive spectral characteristics which should be noted. First, the signal intensity, as indicated by the reduction of the central peak height, was diminished in the extracted fragments as compared with the whole RBC membranes. Second, unlike the crosslinked samples discussed previously, there is spin label present in the supernatants from the extracted membranes. The spectrum of this extracted label was a typical three-line spectrum similar to that observed when 12 NS is free in solution. Third, the spectrum of the extracted membranes showed an increase in the immobilized component, with the central peak clearly splitting into a doublet, as well as a more complete resolution of high and low field liquid lines. In short, there were two completely different spectra superimposed upon one another, and these were much more completely resolved from each other than similar components observed in the control sample.

Spectra taken of the MATA membranes, extracted membranes and supernatants are shown in Figure 11. There was again a reduction in signal intensity attendant with extraction, and a similar separation of the low field immobilized component from the weakly immobilized peak. The central peak is also distinctly split into a doublet. These findings

Figure 10. Spectra of EDTA Extracted 12 NS Labeled RBC Membranes and Protein Extract

A) Whole RBC Membranes

B) Membrane Fragments from EDTA Treatment

C) Protein Extract

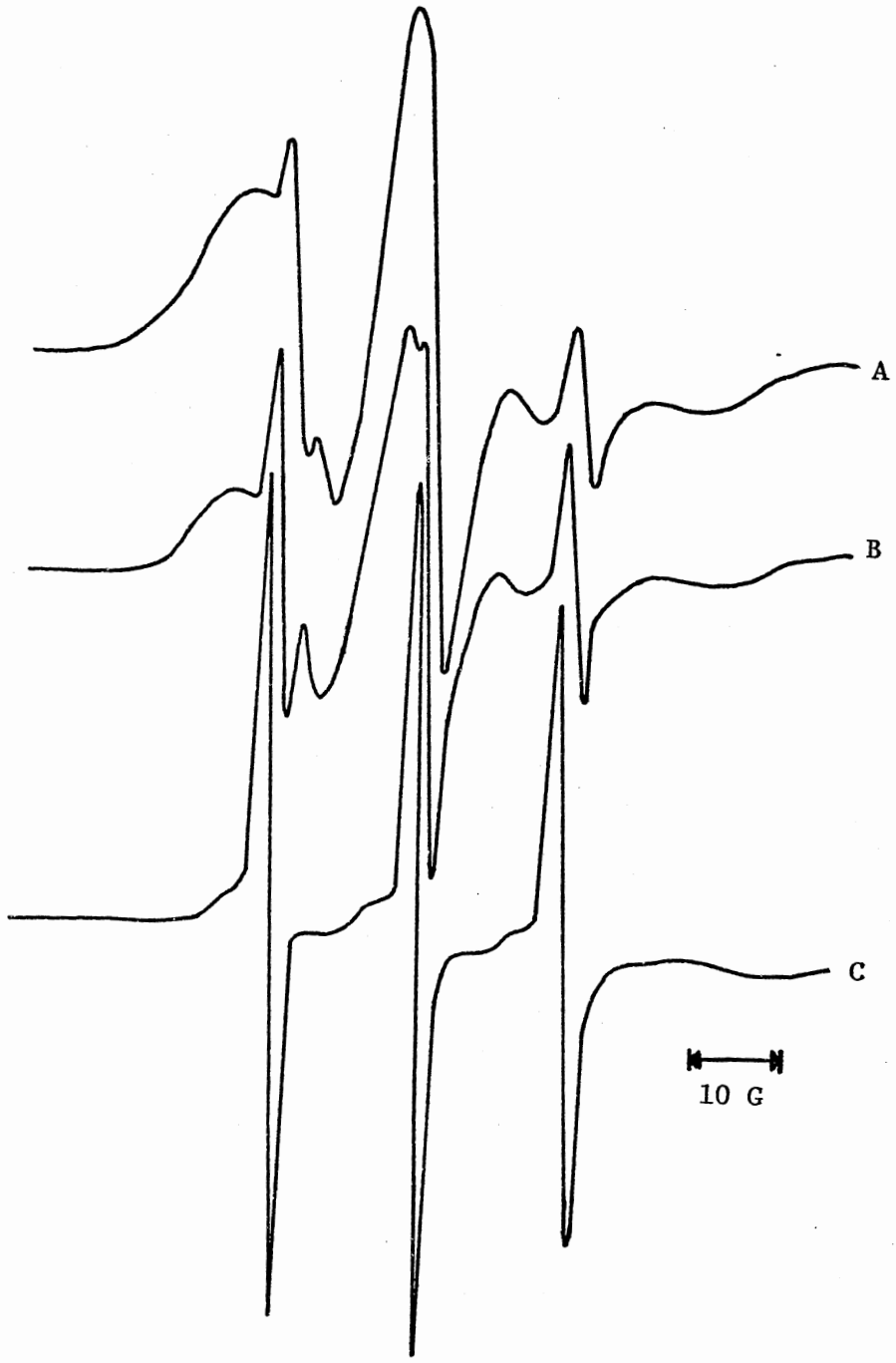
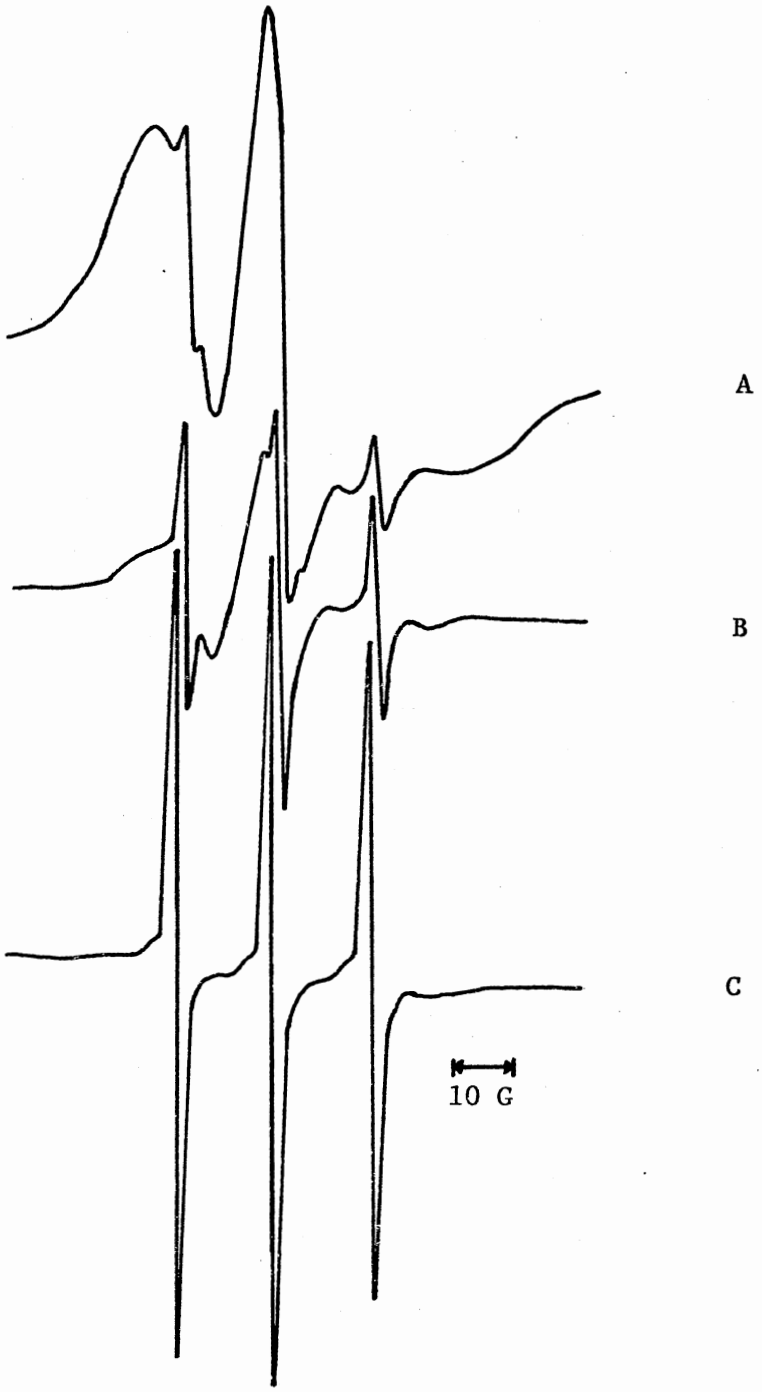

A
B
C
10 G

Figure 11. Spectra of EDTA Extracted 12 NS Labeled MATA Membranes and Protein Extract

A) Whole MATA Membranes

B) Membrane Fragments

C) Protein Extract


A
B
C
10 G

indicated that some of the spin probe was being removed during the extraction process, and that the weakly immobilized spin population remaining with the membrane fragments was becoming more distinct from the strongly immobilized fraction.

Following a modification of the instrument by Dr. Larry Halliburton which significantly increased the sensitivity, these extraction studies were repeated. Parameterization of the values obtained for RBC membranes is given in Table V. Note that these values for S indicate an ordering of the phospholipid bilayer in the EDTA extracted "vesicles" as compared with the whole membranes. Again, there was quite a significant amount of label released upon extraction with EDTA, and this extract gave a fairly typical liquid spectrum. The shape of this spectrum would have led us to believe that a significant fraction of the probe was simply being released into the suspending medium as free label. However, there was a tiny shoulder on the low field side of the spectrum of the supernatant (Figure 10) which had been dismissed as an artifact in earlier studies, and it was shown to remain when that portion of the spectrum was enlarged. This spectral characteristic was observed in all EDTA extractions of 12 NS labeled RBCs, though it is barely visible above the noise.

In order to determine whether or not this label was bound in some fashion to the extracted protein, contained in some solubilized lipid system, or free in solution, as we had previously supposed, the extract was concentrated by Amicon filtration. The filter used has a pore size which will retain any molecule above a MW of 50,000. Spectra of the concentrated extract showed a very intense liquid signal which still contained the small shoulder on the low field side, and a very

TABLE V

EDTA EXTRACTIONS OF MATA 13762 AND HUMAN RED BLOOD CELL MEMBRANES

Sample	$2T_{\parallel}$	$2T_{\perp}$	a_n'	S
MATA Membranes #1	45.67	21.65	14.83	0.457
MATA Vesicles #1	43.51	18.65	13.47	0.522
MATA Membranes #2	49.77	20.02	14.97	0.562
MATA Vesicles #2	52.666	18.47	14.93	0.647
RBC Membranes #1	49.29	19.61	14.75	0.569
RBC Vesicles #1	51.28	18.65	14.76	0.624
RBC Membranes #2	48.31	20.06	14.74	0.542
RBC Vesicles #2	51.28	18.13	14.59	0.641

ill-defined component on the high field region, indicative of immobilization of a small portion of the spin label present. The signal remained in the soluble portion of the concentrate, even when centrifuged at 100,000 x g for 90 minutes. Electrophoresis of these samples showed that the concentrate contained predominately spectrin, while the filtrate, which had only a barely detectable amount of spin probe, contained smaller protein fragments, including most of the 45,000 MW component. The very weak signal from this filtrate appeared to be entirely liquid. The ratio of central peak heights from the Amicon concentrate/filtrate was 9.25, indicating further that the vast majority of the extracted spin label remained with spectrin.

Data from parallel EDTA extractions of MATA 13762 membranes (Table V) showed a greater variability among the samples than did the data from the RBC system, but the difference or "Δ" values from the sample sets indicate a definite ordering of the region probed by the 12 NS label as compared with the whole membrane. ($S_{vesc} - S_{memb} = 0.065$ for set 1; $S_{vesc} - S_{memb} = 0.085$ for set 2.) This apparent increase in viscosity of the internal portion of the phospholipid bilayer of the EDTA extracted membrane fragments could have several origins. One possibility is that such severe membrane perturbation renders a greater portion of the hydrophobic region of the membrane subject to labeling with the probe. That is, the probe becomes more completely dissolved in the membrane. This is contraindicated by the more complex resolution and increased separation of the liquid lines present in the extracted membranes. Finally, since there was a significant amount of the protein removed from the remaining vesicles, lipids which were previously associated with these moieties may have been either removed or remained

behind to assume a more linear packing array of the fatty acid chains in the absence of stearic disruption induced by the presence of the protein.

As in the RBC samples, a portion of the 12 NS extracted with the HMW proteins, and imparted a liquid signal to the suspension. Again, a significant portion of the signal remained with the HMW protein upon concentration by Amicon filtration. The proportion remaining with the concentrate as shown by the concentrate/filtrate peak height ratio of 2.0 is much less than that observed in the former case, and there is no apparent immobilized component in either of the spectra from these samples. It was unclear at the time whether this spin label, which is undoubtedly bound in some fashion to spectrin from red blood cells, and to the HMW protein in MATA 13762 cell membranes, associates with "annular lipids," as described by other workers (242), for other systems, which copurify with these proteins, or with small lipid vesicles which may have been present in the extract, or is adsorbed to the protein through direct hydrophobic interaction. We still have insufficient knowledge about the location of the probe in the intact membrane, which is, of course, compounded when these membranes are perturbed. All that one can really determine from simple spectral analysis is that there are at least two spin populations present: one which is weakly immobilized, and one which is strongly immobilized. There is incomplete mixing of the spectral lines, making quantitation of these separate species extremely difficult.

Samples of RBC membranes were again labeled with 12 NS. The fluorescence intensity of the tryptophan residues was measured in these and in unlabeled membranes by exciting at 280 nm with a Xenon lamp and

monitoring fluorescence emission at 340 nm. Since free radicals lying within 4-6 Angstroms of a fluorescent chromophore are known to quench fluorescence (234), measurements of the extent of quenching of tryptophan fluorescence would indicate the proximity of some of the spin label to protein moieties. This same quenching assay was run on 12 NS labeled EDTA extracted vesicles. The results shown in Figure 12 indicate that significant quenching occurs in both the whole membranes and in the extracted membranes, and that this quenching is greater per milligram protein present in the vesicles than it is in the intact membranes.

Trypsinization of RBC vesicles isolated from EDTA extracted 12 NS labeled membrane fragments shed little light on the interaction of remaining protein moieties with the phospholipid bilayer. The S values for the vesicles themselves gave an average value of 0.598 ± 0.03 and an average a_n' value of 13.62. The four trypsinized samples gave varying values, as shown in Table VI, with $\bar{S}$ = 0.588 ± 0.03 and $\bar{a}_n'$ = 14.22. The spectral characteristics are shown in Figure 13; note the poor definition of the high field line. There does not appear to be a significant variation in the order of the phospholipid bilayer upon trypsinization of these vesicles. Trypsin treatments were carried out at precisely the same protein/enzyme concentrations used for unextracted membranes. The total protein concentration of the samples, however, was maintained at 2 mg/ml to enhance signal intensity, since this was shown to diminish considerably upon EDTA extraction as discussed above. The a_n' values, which are indices of the polarity of the environment experienced by the spin label, show a shift from a relatively nonpolar environment in the vesicles to a more hydrophobic one in the trypsinates. This does imply a change in the protein-lipid interaction within the

Figure 12. Comparison of Tryptophan Fluorescence Intensity at 340 nm of the EDTA Extraction Series. (Protein concentrations of all samples = 0.5 mg/ml.)

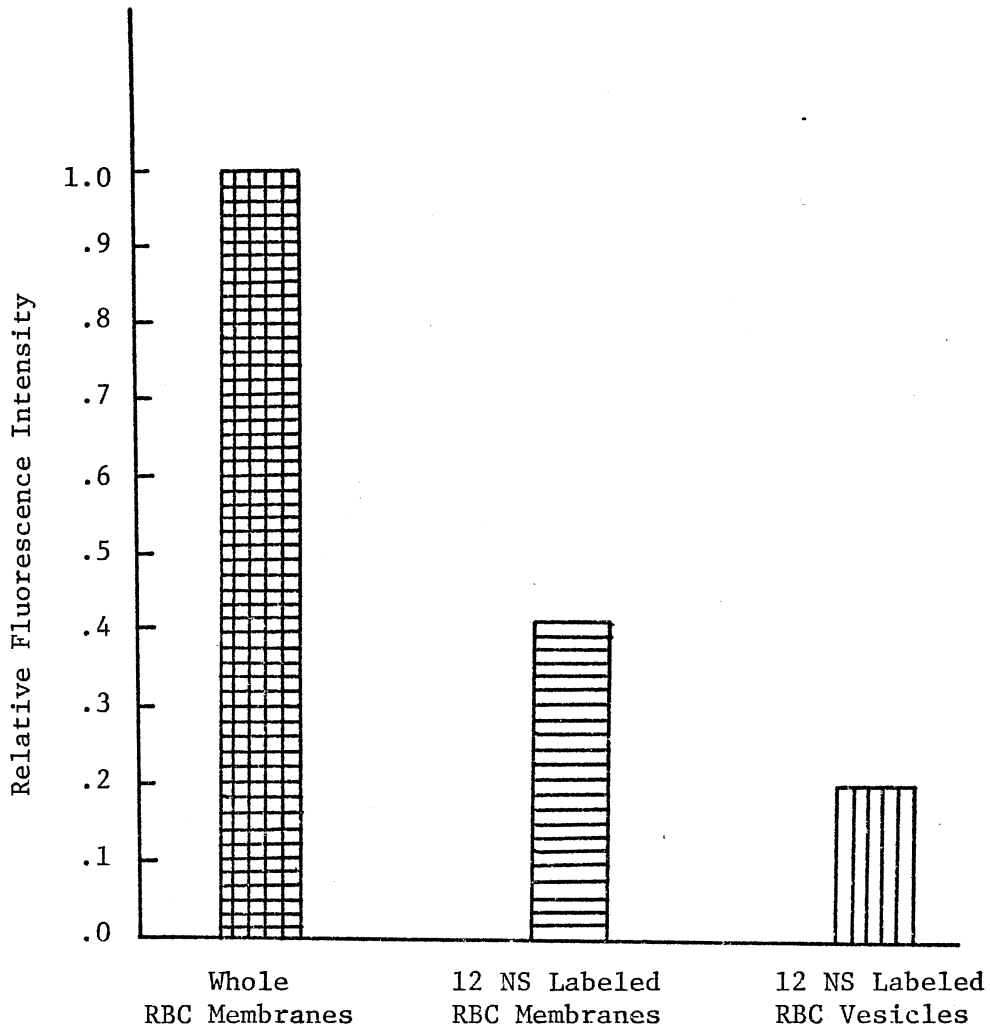

Relative Fluorescence Intensity
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.0
Whole
RBC Membranes
12 NS Labeled
RBC Membranes
12 NS Labeled
RBC Vesicles

TABLE VI

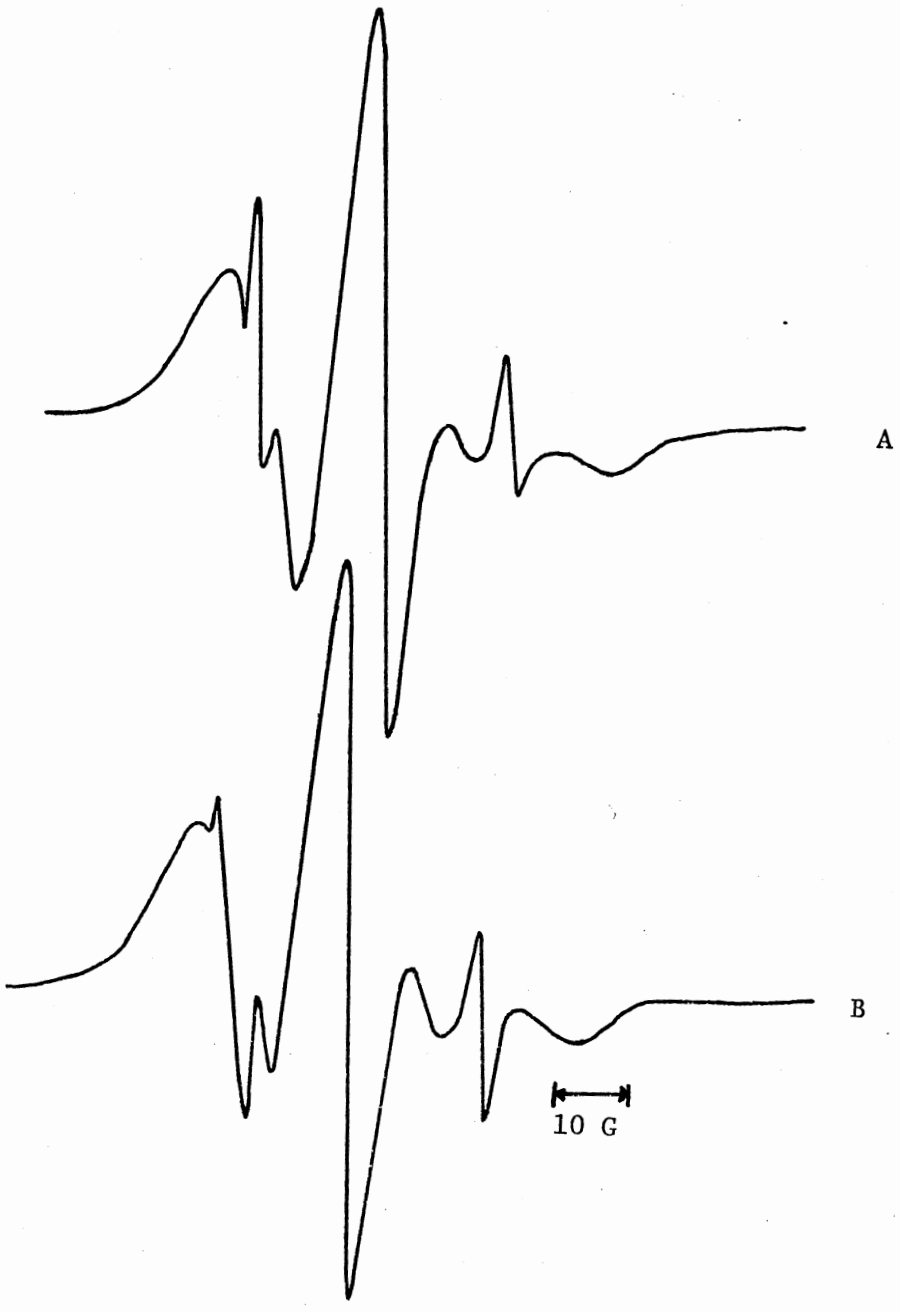
TRYPSINIZATION OF 12 NS LABELED VESICLES FROM EDTA-EXTRACTED RBC MEMBRANES

Sample	$2T_{\parallel}$	$2T_{\perp}$	a_n'	S
RBC Vesicles	46.05	17.82	13.62	0.598
Trypsinate #1	45.94	18.84	13.94	0.547
Trypsinate #2	46.53	17.67	13.64	0.595
Trypsinate #3	50.34	18.85	14.68	0.606
Trypsinate #4	50.07	18.85	14.62	0.603

Figure 13. Trypsinization of 12 NS Labeled RBC Membrane Fragments

A) RBC Membrane Fragments

B) Trypsinized RBC Membrane Fragments


A
B
10 G

vesicles in that as the proteins are hydrolyzed, the fatty acid probe seems better able to dissolve more completely in the hydrophobic domains of the bilayer. Microscopic examination of the vesicles in the control samples revealed a high degree of variability in vesicle size and integrity. Similar examination of the trypsinates showed fragmentation of the membranes and a general reduction in vesicle size. This size distribution may be partly responsible for the variation in the spectral parameters among the samples.

Fluorescence Depolarization Measurements of ANS-Labeled Whole Cells and Membranes

EDTA Extraction Series

Confirmation of the apparent rigidification of the phospholipid bilayer upon EDTA extraction was sought by an alternative method. Fluorescence depolarization measurements were made on samples containing 0.5 mg/ml protein as determined by Lowry assay. When isolated RBC ghosts are incubated for one hour with 10^{-4} M ANS and fluorescence measurements made immediately, a depolarization value of 0.223 is obtained. The emission peak obtained using a Corning filter CS3-73 and exciting at 370 was 477 nm. Measurements taken on these samples after storage at 4°C for 48 hours gave the same spectral peak, but the depolarization value had decreased to 0.198. Spectral shifts and depolarization data for the EDTA extraction system are given in Table VII. Note the small increase in fluorescence polarization of ANS in the EDTA membrane fragments as compared with unperturbed RBC ghosts. The extracted protein suspension gave a fluorescence depolarization of 0.128, indicative of lack of constraint of molecular motion. The

TABLE VII

FLUORESCENCE EMISSION PEAKS AND DEPOLARIZATION VALUES FOR ANS FLUORESCENCE IN RBC GHOSTS AND MEMBRANE FRAGMENTS

Samples	Emission Peak in Nanometers	Depolarization (P)
Red Blood Cell Ghosts	477	0.223
Red Blood Cell Ghosts at 48 Hr. Incubation	485	0.198
RBC-EDTA Membrane Extraction Mixture	481	0.207
RBC Membrane Fragments from EDTA Extraction	482	0.229
EDTA-Extracted Protein from RBC Membranes	490	0.128
RBC Ghosts Solubilized in 1% SDS	485	0.085
ANS in 10 mM Tris Buffer	518	0.076

Emission spectra were obtained using Corning filter CS3-73, exciting at 370 nm. Depolarizations were obtained using Corning filter CS3-73, film polarizing filters, and exciting at 370 nm. Ghosts were labeled with ANS prior to perturbation.

depolarization value for the EDTA-RBC extraction mixture appears to be intermediate (0.207) between that for the membrane fragments and the extracted protein. Comparison of the spectral peaks for these three samples shows that the ANS extracted with the HMW protein is in a markedly more polar environment, as evidenced by the peak at 490 nm, as compared with approximately 482 nm for the other two samples. The emission peak for ANS in Tris buffer is 518 nm, and the depolarization of the probe in this aqueous suspension is 0.076.

MATA 13762 whole cells were incubated with 10^{-4} M ANS at a concentration of 0.5 mg protein/ml in HEPES buffer for one hour at room temperature. These cells remained viable during the incubation period. Fluorescence spectra were taken of each sample using Corning filter CS3-74 and exciting at 360 nm. Polarizations were taken by exciting at 360 nm. A set of non-viable cells were similarly labeled and the appropriate measurements made (Table VIII). There was no shift in the emission peak, and the depolarization values for the two systems were remarkably alike. The fluorescence lifetimes were identical. Spectra taken of ANS labeled MATA membrane envelopes gave an emission peak at 478 nm; the depolarization value was 0.217, indicating a more restrictive environment than that experienced by the probe in whole cells. The fluorescence lifetimes were similar to those obtained previously, within the limits of experimental error. Measurements on the MATA membrane fragments following EDTA extraction of the membrane envelopes gave a spectral shift to 470 nm, indicative of a decrease in the polarity of the ANS environment, as well as a depolarization value of 0.244, indicating definite increase in the membrane microviscosity. The spectral peak for the supernatant containing the HMW protein extracted

TABLE VIII

LIFETIMES, DEPOLARIZATIONS, AND EMISSION PEAKS OF ANS FLUORESCENCE IN MATA 13762 TUMOR CELLS AND MEMBRANES

Sample	Emission Peak in nm	Depolarization (P)	Lifetime (τ)
MATA Whole Cells Labeled When Viable	482	0.175	11.0 ns
MATA Whole Cells Labeled When Non-Viable	482	0.154	-
MATA Membrane Envelopes	478	0.217	10.8
MATA-EDTA Membrane Extraction Mixture	478	0.213	12.3
MATA Membrane Fragments from EDTA Extraction	470	0.244	10.1
Supernatant from EDTA Extraction of MATA Membranes	483	0.190	11.7
MATA Membrane Envelopes Solubilized in 1% SDS	485	0.087	-
ANS in Tris Buffer	518	0.076	-
ANS in Tris Buffer and 1% SDS	491	0.085	-

The emission spectrum was obtained in each case using Corning filter CS3-73, exciting at 360. Fluorescence depolarizations were measured using Corning filter CS3-74, and film polarizing filters by exciting at 360 nm and passing the entire fluorescence band. Cells and membranes were labeled with ANS prior to perturbation.

by EDTA treatment emerged at 483 nm, which is the same as that for the whole cells. The depolarization value of 0.190 indicates great mobility of the probe, approaching that observed in aqueous solution. Again, as in the RBC system, the values obtained for the EDTA-MATA extraction mixture were intermediate between those obtained for the membrane fragments and extracted HMW protein.

ANS, like the stearic acid spin probes, has been used primarily as a probe for the hydrophobic region of the membrane bilayer. However, as observed with the stearic acid analogues, a significant portion of the label coextracts with the water soluble proteins under hypotonic conditions in the presence of EDTA. Lowry assay in both RBC and MATA membrane systems showed that approximately 50% of the total protein was extracted. The fluorescence signal intensity in the pellet and the extract were roughly equal, but the total signal intensity was far below that of the ANS labeled unextracted membranes. A further experiment was performed to determine the distribution of the probe. MATA membranes were first labeled with ANS, extracted, and the components separated as before. The relative fluorescence intensities are given in Table IX. A second set of membranes were first extracted with EDTA, the components separated, and then labeled with ANS. The fluorescence intensities indicate that the quantum efficiency of ANS in the lipid-containing pellet is approximately four times that observed in the supernatant. Titration of the pellet and extract with ANS showed that the ANS binding sites were not saturated, therefore there is roughly four times the amount of ANS extracted with the water-soluble protein than remains with the membrane fragments when labeled membranes are extracted with EDTA. This indicates that most of the ANS label is associated with

TABLE IX

RELATIVE FLUORESCENCE INTENSITIES OF MATA-EDTA EXTRACTION SERIES

Sample	Emission Peak (Excite 375 nm)	Relative Fluorescence Intensity
ANS-Labeled MATA 13762 Membrane Envelopes + EDTA	480	1.0
Extracted Membrane Fragments	477	0.20
Protein from EDTA Extraction	483	0.24
MATA 13762 Membrane Envelopes	-	-
Extracted Membrane Fragments + ANS	478	0.61
Protein from EDTA Extraction + ANS	485	0.15

Spectral peaks were obtained by exciting at 375 nm and using a Corning CS3-73 filter. Membrane sample concentrations were 0.5 mg protein/ml, as determined by Lowry assay. Whole membrane envelopes were labeled in 6×10^{-4} M ANS. Membrane fragments and extracted protein were labeled in 3×10^{-4} M ANS.

protein moieties in the unperturbed membrane. Further, these are probably surface and not integral proteins.

These data confirm the observations made with ESR measurements on EDTA extracted membrane systems in that the residual membrane fragments display a more viscous membrane bilayer than do unextracted plasma membranes.

Detergent Solubilization of Plasma Membranes by SDS

While the foregoing EDTA extraction selectively removes water soluble proteins from the membranes in question, it is necessary to examine the obverse procedure. Detergent solubilization of proteins and phospholipids from plasma membranes may be used to define certain aspects of molecular organization by the way in which these detergents disrupt membrane associations. Sodium dodecyl sulfate (SDS) solubilization at 1% concentration should disrupt the phospholipid bilayers virtually completely, liberating protein constituents and lipids. Any fatty acid probe present in these membranes should likewise be released, and hence exhibit a very liquid spectrum. As shown in Table X, this is indeed the case for both RBC and MATA 13762 membranes. Solubilization in the former case seems to be essentially complete at 0.5% SDS, and there is a tremendous decrease in the order parameter, S, which ranges from S = 0.500 in the control to 0.132 in the solubilized system. This approaches the average value obtained for the label free in solution of S = 0.079. The change observed in the tumor cell membranes goes in the same direction with $\bar{S}$ = 0.465 in the control, and $\bar{S}$ = 0.102 at 1% SDS. The magnitude of the decrease in membrane microviscosity is very

TABLE X

DETERGENT SOLUBILIZATION OF 12 NS LABELED RBC AND MATA 13762 TUMOR CELL MEMBRANES

Sample	Cell Membranes				
	$2T_{\parallel}$	$2T_{\perp}$	a_n'	S	$\bar{S}$
MATA 13762 Membranes					
Control #1	46.84	21.49	14.97	0.479	.465
Control #2	46.28	22.04	15.06	0.451	
0.5% SDS #1	33.89	28.38	15.11	0.103	.105
0.5% SDS #2	34.16	28.38	15.15	0.107	
1.0% SDS #1	34.16	28.65	15.24	0.102	.102
1.0% SDS #2	34.16	28.65	15.24	0.102	
RBC Membranes					
Control #1	49.77	22.67	15.85	0.483	.499
Control #2	51.15	22.12	15.89	0.516	
0.5% SDS #1	34.97	25.93	14.47	0.176	.171
0.5% SDS #2	34.84	26.26	14.56	0.166	
1.0% SDS #1	34.28	27.37	14.81	0.132	

similar for the two systems. If these samples are allowed to remain standing for an hour, there is a tendency for an increase in the order parameter to occur. This is undoubtedly indicative of micelle formation of the detergent. These data indicate that most of the fatty acid probe is released into solution, and that it is almost completely stripped away from the protein components.

Fluorescence depolarization data confirm these results. In RBC ghosts, the depolarization value of the ANS labeled membranes is 0.223. It is reduced to 0.085 in the solubilized membranes, and is roughly comparable with the depolarization of 0.076 in 10 mM Tris buffer. In MATA membranes, the depolarization value for ANS is 0.217 as compared with 0.087 for SDS solubilized membranes. The depolarization for ANS in Tris buffer with 1% SDS is 0.085, and for ANS in Tris buffer, 0.076. These depolarizations and the spectral shifts are shown in Tables VII and VIII. The spectral shifts indicate that there is an increase in the polarity of the probe environment upon solubilization of the membranes with SDS.

Effect of Zn^{2+} on Plasma Membranes

The effects of Zn^{2+} on membranes were of considerable interest, since this ion is known to stabilize the MATA 13762 tumor cell membranes during isolation according to the method of Huggins (77). When the ascites cells are removed from the peritoneal cavity of the rat, they are first washed with HEPES buffer, and briefly swollen in two consecutive steps with hypotonic (40 mM) Tris buffer at 4°C. The swelling procedures are undertaken to facilitate removal of contaminating red blood cells by lysing them selectively and removing them

with the supernatant following centrifugation. Since it was not known what effect this membrane stabilization had on the phospholipid bilayer, or if this phenomenon was observable in plasma membranes other than from ascites tumor cells, the effects of Zn^{2+} on 12 NS labeled RBC membranes and 12 NS labeled MATA 13762 plasma membranes were examined by ESR. No change in the S values or a_n' values was observed for RBC membranes over a Zn^{2+} concentration range of 0-2.5 mM when the ghosts were titrated with this ion.

When whole 12 NS labeled MATA 13762 were swollen in hypotonic buffer, the fluidity of the membrane increased, as shown in Table XI, from S = 0.407 in the control to S = 0.266 at the first swelling step, and S = 0.242 in the second swelling step. The cells appear larger upon swelling, as monitored by phase contrast microscopy, seem to be fragile, and exhibit some small protuberances on the cell surface. Treatment with Zn^{2+} at 1 mM concentrations under hypotonic conditions reduces the cell size so that they appear to be slightly crenated. The membrane fluidity as monitored by spin label appears to approximate the value prior to perturbation by hypotonic conditions, as shown by order parameter values of S = 0.411 for the 12 NS probe in the Zn^{2+}-treated membrane system. Note that all these cells were labeled with the spin probe prior to subjecting them to hypotonic conditions and Zn^{2+} stabilization.

Examination of the effects of Zn^{2+} concentrations using MSL reveal an increase in the a/b ratio between 0-1.0 mM with no further increase observed even at concentrations as great as 4.0 mM Zn^{2+} (Table XII). This implies a protein change occuring at 1.0 mM Zn^{2+} and that the probe is experiencing a slight increase in immobilization. When the

TABLE XI

EFFECTS OF Zn^{2+} ON MATA 13762 TUMOR CELLS

Sample	$2T_{\parallel}$	$2T_{\perp}$	a_n'	S
Whole MATA Cells	42.56	31.60	14.38	.407
First Hypotonic Swelling	33.64	21.57	12.80	.266
Second Hypotonic Swelling	33.23	22.15	12.92	.242
Zn^{2+} Stabilization	44.21	22.54	14.88	.411
Zn^{2+} Stabilization (duplicate sample)	44.21	22.54	14.88	.411

Labeled prior to perturbation with 12 NS.

TABLE XII

Zn^{2+} EFFECTS ON MSL LABELED PLASMA MEMBRANES

Zn^{2+} in mM	a/b
0	0.125
.5	0.135
1.0	0.157
4.0	0.160

Labeled as whole cells, then titrated with $ZnCl_2$.

rotational correlation time, τ, was determined for this system in an additional experiment, it was found to be 4.32 ns for both the unswollen MATA cells and the zinc-treated cells. The τ value for both of the swelling steps was 4.16 ns. Atomic absorption analysis of the membranes after isolation showed that the zinc does not remain with the isolated membrane envelopes.

Though we cannot specify a mode of action of the zinc ion when it interacts with the tumor cell membrane, these data definitely indicate that this ion functions as a stabilizing agent by shifting the microviscosity of the phospholipid bilayer back toward that which is present in the unperturbed whole cell. This interaction is of a polar nature, but the information is too scant to allow any further insight. Whatever this interaction may be, it serves to prevent fragmentation of the membrane sheets during the remainder of the isolation procedure, so that they may be collected separately from the nuclei and cellular debris. Maintenance of the plasma membrane integrity is probably due to the fact that the fluidity of the membrane is maintained in the zinc treated cells at a value which approximates that of the unperturbed system.

Effects of the Ca^{2+} Ionophore A23187 on the RBC Membrane

Since the calcium perturbations carried out in previous studies (52) indicated definite structural changes in the membrane, as one would expect due to the massive aggregation of protein material induced by this ion during the hypotonic lysis of red blood cells, we sought ways to monitor the same phenomenon in whole red cells. Other workers

(243) had indicated by deformability assays that the calcium ionophore A23187 might be useful in this regard.

When human erythrocytes which had been previously incubated with 12 NS were titrated with an ethanolic solution of A23187, there seemed to be an increase in membrane fluidity, but the shift was very slight and a singular lack of spectral resolution in the high field portion of the spectrum prevented parameterization. It did appear that a concentration of 5 mM Ca^{2+} was required to reverse these effects when the ionophore was present in concentrations of 1.15×10^{-8} M, which had been described by Kirkpatrick et al. (243) as an acceptable level according to deformability assays. These cells were very fragile and showed a great deal of hemolysis. Microscopic examination revealed spiculation and crenation of an estimated 85-90% of the cells.

When whole human RBC's were labeled at 4°C with MSL and incubated subsequently at 25°C with 15 µg A23187/ml packed cells (which we had determined to be the threshold level for the ionophore), spectra were taken at 0, 30 and 40 minutes to determine the response of the treated cells. There was an obvious increase in lysis of the cells as monitored by phase contrast microscopy. A comparison of the a/b ratios obtained (Table XIII) indicates that after 30 minutes, even at this minimal level, the ionophore in the absence of Ca^{2+} alters the membrane such that the MSL spin probe tumbles more freely. It would appear that we were monitoring MSL in the membrane, since we compared the ionophore treated cells directly with the untreated cells. If we were simply releasing label into the aqueous suspending medium, then an increase in signal in the supernatant should have been evident. It was not, and since the label is known to bind covalently, it is unlikely

TABLE XIII

EFFECTS OF IONOPHORE A23187 AT 15 µg/ml ON WHOLE HUMAN RED BLOOD CELLS

Time in Minutes	A23187	a/b
0	0	.323
0	15 µg/ml	.331
30	15 µg/ml	.273
40	15 µg/ml	.285

Cells labeled with MSL prior to perturbation.

that this occurred. When whole red blood cells are incubated with A23187 in the presence of 5 mM Ca^{2+} this tendency toward increased membrane fluidity over the same 30 minute interval (Table XIV) is not observed. It was shown that 5 mM Ca^{2+} was the minimal concentration which provided this stabilization. This is well above the 1 mM concentration required to produce spectrin aggregation in resealed ghosts. Electrophoresis data from these treated cells revealed that there was only minimal aggregate formation if Ca^{2+} was removed before a 30 minute interval had elapsed.

There are two points of interest here. First, in resealed ghosts, 0-0.5 mM Ca^{2+} alone will produce an increase in membrane fluidity (52); in whole RBCs in the presence of ionophore, which also induces an increase in membrane fluidity, the Ca^{2+} appears to protect the cell against the perturbation induced by the ionophore. This implies that the effect of the ion is antagonistic to the action of the ionophore, which may mean that at these very low levels of A23187, Ca^{2+} is not efficiently transported into the cell, so that it could act on the cytosolic side of the membrane. The Ca^{2+} may be restricted to an electrostatic interaction with the red cell surface. The 30 minute time interval involved may be sufficient to deplete cellular glucose and ATP, whereupon the cell Ca^{2+} pump would fail, allowing Ca^{2+} to leak into the cell at the end of this time. In short, it is quite possible that at these low levels, the ionophore is non-functional. Edmundson et al. have determined that there is no discernible concentration of calcium in erythrocytes at A23187 concentrations of less than 1 μM. Also, we have not ruled out the possibility that the effect we see is on hemoglobin.

TABLE XIV

EFFECTS OF A23187 AND 5 mM Ca^{2+} ON WHOLE HUMAN RBCs

Time in Minutes	a/b
0	.291
5	.288
10	.288
30	.286

Cells labeled with MSL prior to perturbation.

ESR Comparisons of RBCs and RBC Membranes from Hematologically Normal Donors and Donors with Genetic Blood Anomalies

As pointed out previously, the study of plasma membrane architecture in red blood cells and isolated RBC membranes is of paramount importance in elucidating the function of membrane components in the living erythrocyte. Since this is the simplest of all mammalian cellular systems, it provides a logical starting point for attempting to understand mammalian plasma membranes. It is an established fact that the architecture of the RBC is altered, and that these alterations are observable in red blood cells upon aging (38, 39). These changes result in an increase in membrane rigidity, which prohibits passage of these cells through the microcirculatory system and leads ultimately to the removal of the aged cells from systemic circulation. Evidence has been rapidly accumulating over the last few years that there are certain changes occurring in the membrane from erythrocytes from patients with myotonic muscular dystrophy, congenital myotonia (53, 54), and sickle cell anemia (48, 49, 50, 51) aside from other clinical manifestations of these diseases. Some of these changes are subject to observation by electron spin resonance through spin labeling, as we have done with the chemical perturbations. These naturally occurring anomalies may also be used to reveal certain aspects of membrane structure-function relationships. Butterfield et al. (147, 158, 159, 160) have used this method for looking at changes in the membranes of red blood cells from patients with muscular dystrophy. We have conducted preliminary studies on blood samples taken from patients with polycythemia,

hyperlipidosis, and sickle cell anemia, using both whole cells and isolated red cell membranes. The labels used were 5 NS, 12 NS, and 16 NS, along with MSL. The stearic acid probes were used as molecular dipsticks, and the MSL was used again as a covalently bound protein label which mirrors conformational changes.

Before discussing the spin label data on these patients, electrophoretic data and clinical evaluations should be reviewed to briefly acquaint the reader with the types of samples examined. First, it should be noted that the sampling population was quite small, since we were not associated with a hospital complex. There were only two local polycythemic donors and two through Pruitt Labs in Texas. The blood panel and electrophoretic data on the serum proteins for one of these patients is presented in Figure 14 along with that for the normal control. The elution pattern for the polycythemic (PC) serum includes a monoclonic peak in addition to the normally observed albumin, α_1, α_2, α_3, β, and γ fractions. This peak, classified as a gammopathy in medical jargon, signifies a pathological change in bone marrow. The syndrome would logically appear more frequently in polycythemic patients than in the normal population, although incidence in normal individuals increases with age. The third scan from a patient with multiple myeloma presented purely for comparison, provides an example of advanced gammopathy. Cells from PC patients were macrocytic, anisocytic, hyperchromic, and all samples contained some spherocytes. The average MCV was 104 μ^3 as compared with 84 μ^3 in the controls. Elution patterns from electrophoretic analysis of hemoglobin from two sickle cell (HbS) patients who were among the dozen donors located through Pruitt Labs are given in Figure 15. It is of interest to note

Figure 14. Elution Patterns from Electrophoretic Analysis of Serum Proteins.

A) Normal Serum Protein

B) Polycythemic Serum Protein

C) Multiple Myeloma Serum Protein

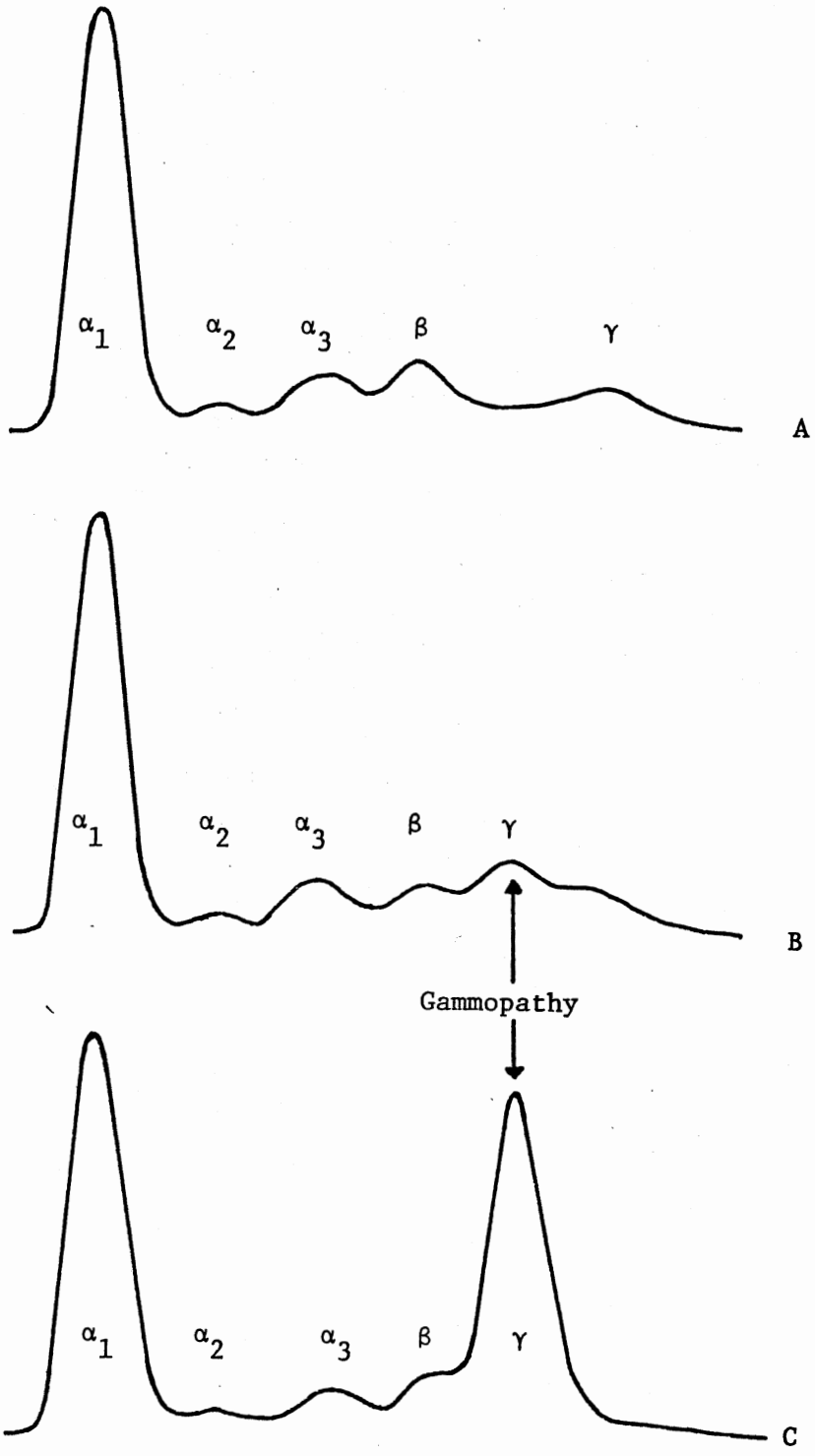
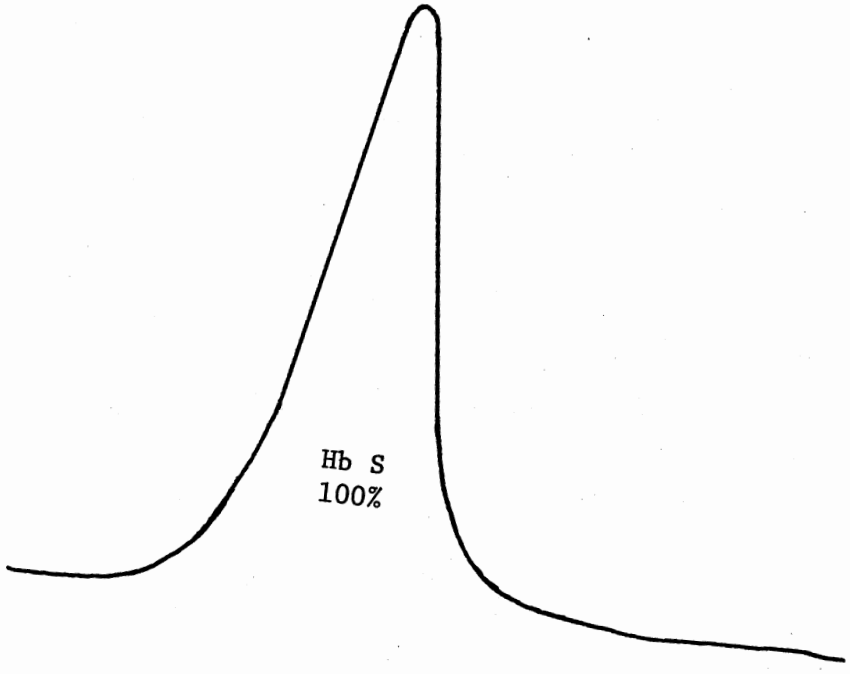
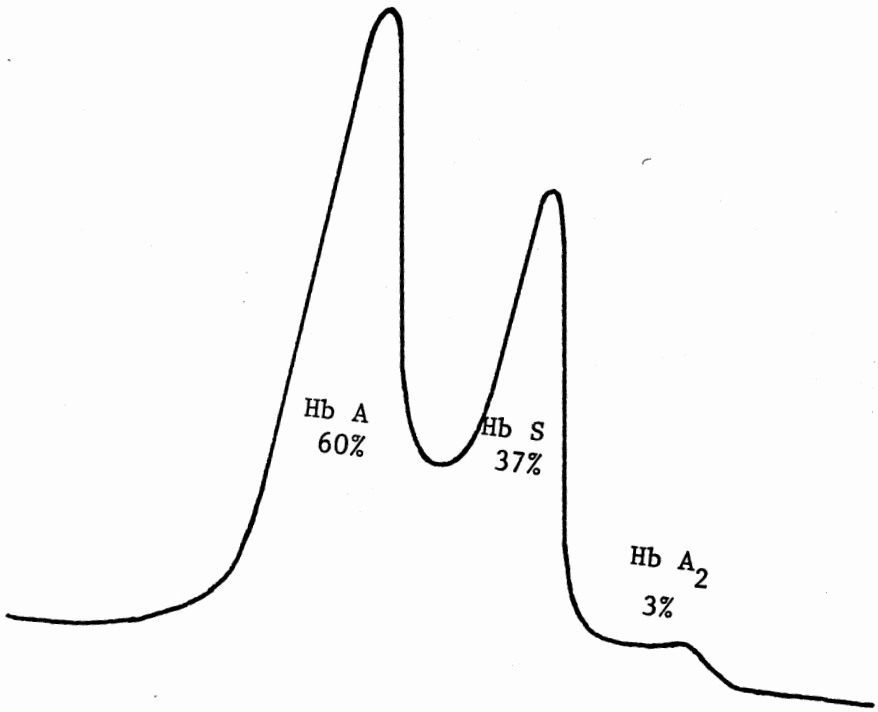

α1
α2
α3
β
γ
A
α1
α2
α3
β
γ
B
Gammopathy
α1
α2
α3
β
γ
C

Figure 15. Elution Patterns from Electrophoretic Analysis of Hemoglobin.

A) Homozygous Sickle Cell Anemia

B) Heterozygous, Sickle Cell Trait


Hb S
100%


Hb A
60%
Hb S
37%
Hb A2
3%

that when normal cells from these sickle cell patients are placed in serum from otherwise hematologically compatible normal donors, the cells will undergo sickling.

As mentioned previously, MSL is a non-specific protein label which covalently binds to free sulfhydryl or ε-amino groups. Again we measured the change in a/b ratio, i.e., the ratio of the strongly immobilized to weakly immobilized component of the low-field component of the three-line spectrum. When whole cells are labeled with MSL, the variations in Table XV may be noted. These data imply that there is a change in a protein component in the cells such that the spin label in the sample from the PC patient and those from the hyperlipidemic (HL) patients show less immobilization than the nitroxides in the controls. Also, there is no difference among the samples when supernatants, which obviously contained hemoglobin were compared. This implies that the shift in protein constraint is not due to differences in Hb. The patients designated as "mildly" hyperlipidemic have a serum triglyceride of about 175; those designated as "severe" have a triglyceride of 1,000 or greater. The latter patients have a deficiency in lipoprotein lipase, hence the high serum triglyceride.

Table XVI gives representative data on membranes isolated from whole cells which have been labeled with MSL. Since MSL labels all protein with available sulfhydryl groups, the samples from the HbS and HbAS (the homo- and heterozygotes for sickle trait), which we know have altered Hb structures, are perhaps better studied as isolated membranes, if it is the membrane alterations which are of interest. Whole cells were, however, labeled and the control value was a/b = 0.280. In the HbS homozygote, it was 0.321. When membranes were isolated from the

TABLE XV

a/b RATIOS OF SPECTRA FROM WHOLE ERYTHROCYTES COVALENTLY LABELED WITH MSL

Sample	a/b
Control	0.454
Polycythemic	0.238
Hyperlipidemic (mild)	0.300
Hyperlipidemic (severe)	0.261
Control	0.280
HbS	0.321
Normal Membranes	0.304

Spectra were run on all the hemoglobin-containing supernatants from these samples, and there were no significant differences among them.

TABLE XVI

a/b RATIOS OF SPECTRA FROM MEMBRANES FROM ERYTHROCYTES LABELED WITH MALEIMIDE SPIN LABEL

Sample	a/b	Frozen Membrane
Control	.630	-
Polycythemic	.167	-
Control	.454	.330
HbS	.351	.240

Membranes were isolated, resuspended in 10 mM Tris, 0.154 M NaCl, pH 7.4 and incubated with MSL in ethanol at 0.1 mg label/mg membrane protein. Membranes were then washed free of excess label, and measurements made at room temperature.

control cells, the a/b ratio was .304. Normal cells lysed more readily than did the HbS under labeling conditions. From the data presented in Table XVI, it appears that the trend is toward an increase in mobility of the spin probe relative to that observed in the control. This occurs for all the PC and the HbS samples examined. Note also that when a set of the membranes was isolated, labeled, and frozen prior to taking spectral data, some fluidization of the membrane occurs, although the difference between a/b ratios for the control and HbS samples remain relatively constant. Though these changes may indeed reflect alteration of the membrane due to disease process, one must bear in mind that there may also be changes brought about by the isolation procedure. In studies on whole cells, the relative spin concentrations contributed by membrane-bound protein and intracellular protein are unknown so no conclusions can be made concerning the shift in the a/b ratio.

Stearic acid probes monitor changes at various levels within the hydrophobic portion of the phospholipid bilayer. For these studies, order parameters were calculated for samples of whole cells which were labeled with 5 NS and 12 NS. The membranes from 5 NS labeled cells were then isolated and the order parameters calculated for the membranes. These order parameters reflect the membrane fluidity in the immediate vicinity of the probe. When HbS membranes are isolated and labeled with stearic acid probes, the order parameters in Table XVII are obtained. Spectra obtained on hyperlipidemic cells with 5 NS, 12 NS, and 16 NS are shown in Figure 16.

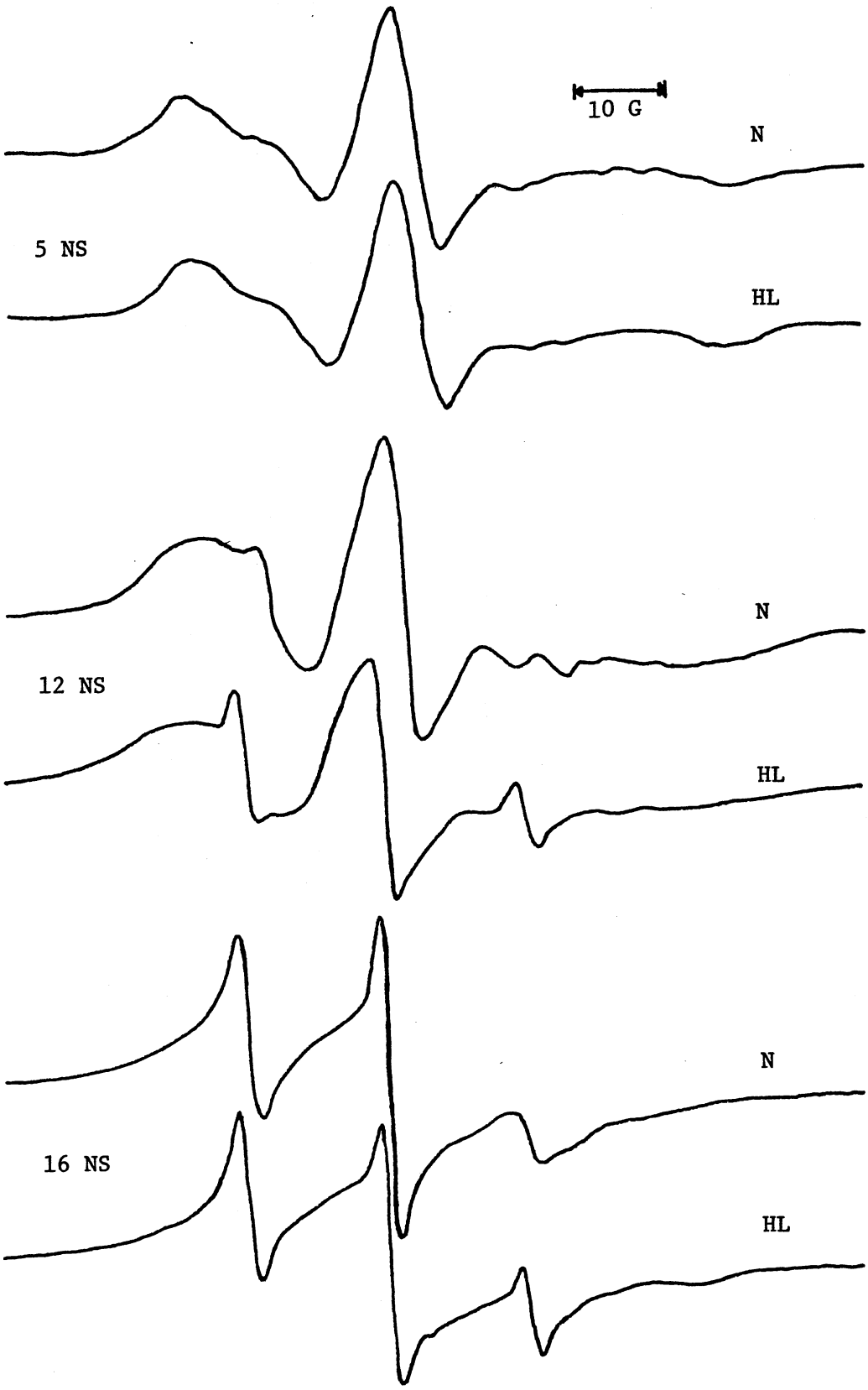
In studies made with membranes from PC blood samples, there is an indication of increases in the membrane fluidity at the 12 NS level. In

TABLE XVII

STEARIC ACID LABELING OF RBCs AND RBC MEMBRANES

Sample	Whole Cells		Membranes
	S, 5 NS	S, 12 NS	S, 5 NS
Control	.735	.592	.704
Polycythemic	.701	.619	.712
HbS	.717	.593	.727
Control	.741	.639	
HbAS	.747	.452	
HbAS	.739	.505	

Figure 16. Stearic Acid Labeled Whole Erythrocytes from Normal (N) and Hyperlipidemic (HL) Donors


10 G
N
5 NS
HL
N
12 NS
HL
N
16 NS
HL

whole cells, as one can see from Table XVII, this does not seem to be the case. The 5 NS probe in PC blood indicates a slight increase in fluidity relative to the control, and the 12 NS probe shows a decrease. This could be rationalized on the basis that a randomization of the region at the polar head group could cause tighter packing in the region of the long hydrocarbon chains at the 12 NS level. Another possibility is that since these cells are rapidly synthesized by an over-stimulated erythropoietic system, there are small differences in the relative amounts of protein, lipid, and lipoproteins such that the fatty acid labels can dissolve into regions of the membrane not usually available to them in normal cells. During preparation of the membranes, a change in the relative fluidity of the 5 NS probe in the control is observable, but there does not appear to be as great a change in the PC membranes. Indeed, if there is a trend, it is small and in the opposite direction.

The HbS cells also show a slight tendency toward an increase in membrane fluidity in the whole cells which is reversed upon membrane isolation. Much speculation has been offered about the sickling phenomenon, and some of this has centered about crosslinking of the hemoglobin to the membrane protein on the internal aspect of the RBC plasma membrane. From these data on the 5 NS probe, the membrane fluidity which would certainly be influenced by such crosslinking is remarkably similar to that of the control. This is also true for the heterozygote samples. It is possible that in the whole cells a larger proportion of the stearic acid probe dissolves into the outside half of the bilayer leaflet so that the changes reflected are primarily those at the external surface. When the membranes are first isolated and then labeled, the

5 NS and 12 NS probes clearly show a decrease in membrane fluidity of HbS membranes relative to normal RBC ghosts (Table XVIII). The 16 NS probe shows a slight tendency toward greater mobility, which would be reasonable if the polar head groups of the hydrocarbon chains are restricted by crosslinking. These membranes are probably more uniformly labeled than are those which were labeled prior to isolation. The studies of whole cells from these sickle cell patients indicates that there is an increase in the resolution of the liquid spectral components with the stearic acid probes, which further supports the information gleaned from calculation of order parameters.

The fact that the foregoing are preliminary studies must be stressed. However, suffice it to say that using either the MSL or fatty acid probes, a difference between control and abnormal cells may be noted in either whole cells, membranes, or both, for each of the anomalies. This technique may or may not be useful in the future as a clinical tool. The most striking piece of information from these studies is that the membranes isolated from these cells differ in fluidity from membranes in whole cells, and that caution must be exercised in extrapolating results obtained on red blood cell ghosts to intact cells. Also, membrane fluidity at one level in the membrane may not reflect fluidity at a different level. Last, but not least, we are constantly plagued with the ambiguity of the location of our spin probes.

TABLE XVIII

ESR ORDER PARAMETERS OF MEMBRANES ISOLATED AND THEN LABELED WITH FA PROBES

Sample	5 NS S	12 NS S	16 NS S
Control	.710	.538	.365
Hemoglobin S	.773	.629	.337

CHAPTER IV

DISCUSSION

It is useful at this point to discuss briefly some assumptions which have been made in reduction of the data. In the original development of parameter calculations, a series of approximations were made by Hubbel and McConnell (37). These were outlined as follows:

1. The effective spin Hamiltonian, $\mathcal{H}'$, has axial symmetry.

2. The line shape for each hyperfine signal is Lorentzian (though this is not always strictly true).

3. The line width for each hyperfine signal is isotropic, but m-dependent where m is the component of the nitrogen nuclear spin in the local field direction at the nucleus.

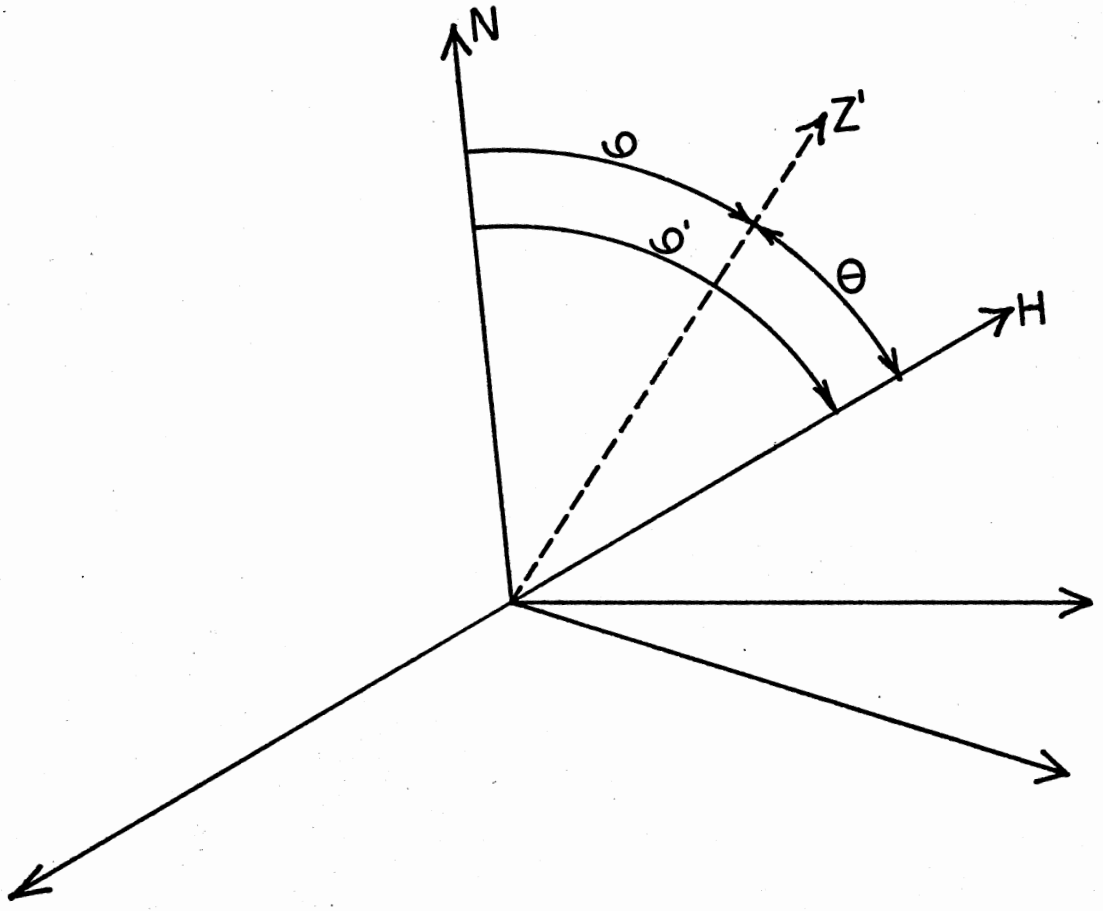
4. The spatial distribution of the average hyperfine axis in oriented multilayers is given by:

$$\rho(\vartheta) = \sin \vartheta^{[-(\vartheta - \bar{\vartheta})^2/2\ \vartheta_o^2]} \tag{31}$$

This is a distribution function for the principle axis, Z', where ϑ is the angle between Z' and $\underline{N}$ which is normal to the planes of the phospholipid bilayers (Figure 17).

There are a series of further approximations also pertinent to the dependence of the spectra on Eigenvalues of the time-dependent Hamiltonian, $\mathcal{H}'$, and perturbations thereof. These obtain under certain

Figure 17. The Axis System Defining the Orientation of the Molecular Z' Axis with Respect to the Magnetic Field Direction H


N
Z'
φ
φ'
θ
H

conditions and include considerations that the individual widths of resonance lines are angular dependent, and that the effective or time-dependent Hamiltonian, H', does not have axial symmetry in either the g' or T' tensor. The calculated spectra employing the above approximations are very similar to the experimentally observed spectra; therefore, we may assume that the approximations made are valid. The method is, as pointed out in the Introduction, a valuable and sensitive method for examination of biological membranes as well as for synthetic systems.

Assumptions must be made about the labeling of the membrane itself. The spin label may possibly be bound nonspecifically at two or more conformationally different sites on the membrane, and could give rise to both a weakly immobilized and a strongly immobilized contribution to the spectrum. Indeed, Wallach and coworkers (27) have observed that 5 NS may be complexing with arginine residues as well as interacting with lipids in the membrane. This would render the analysis of these spectra with respect to specific lipid-protein interactions very difficult indeed.

Labeling of intact red blood cells and MATA 13762 ascites tumor cells with the fatty acid spin probe 12 NS was accomplished by adjusting the probe concentration to prevent membrane blebbing (Figure 3) in red cells, and by incubation of ascites cells in KRP to minimize protein loss and to optimize viability. Aged intact erythrocytes, in contrast to fresh cells, do not label as efficiently with stearate probes, and display an increase in the fluidity of the membrane. This implies some difference in the membranes in the two systems, which is in agreement with the studies of Zwaal et al. (244). In MATA 13762 tumor cells, as

the viability of the cells decreases, the amount of label bound also decreases and the length of time required for label uptake increases.

Isolated plasma membranes, which provide a less complex system for the study of protein-lipid interaction than intact cells, also showed less variability in order parameter than did the intact cells when labeled with 12 NS (Table I). The order parameters, which are representative of time averages over molecular motions, are such that the longer the time involved in averaging, the lower the parameter. The averaging time, in turn, is dependent upon the magnitudes of the anisotropic contributions to the spin Hamiltonian, which involves hyperfine and g-factor interactions. These have been quantitated for the 12 NS label and are shown to vary from 0.566 ± 0.05 in whole human RBCs to 0.532 ± 0.03 in RBC ghosts, and from 0.321 ± 0.04 in intact MATA 13762 tumor cells to 0.518 ± 0.04 in isolated membrane envelopes. Whole red cells are less complex than are intact MATA 13762 cells, hence the difference in order parameter between whole cells and isolated plasma membranes is much less. Also, red cell ghosts will spontaneously reseal when incubated under isotonic conditions, as these were, therefore the accessibility of the internal aspect of the membrane bilayer was somewhat more similar to that in the intact cell than is the accessibility in the unsealed membrane envelopes of the MATA cells as compared with the intact cells. There are more cytoplasmic elements present in the tumor cells, including fat droplets, and additional membrane assemblies, which are labeled along with plasma membranes. It should be noted that isolated plasma membranes do not have the same properties as they do in intact cells, and that some of these properties depend heavily upon the isolation procedure.

The two methods for studying complex biological systems are: 1) degradation of the system to its component parts and reconstruction of the system from isolated components based on theoretical considerations and experimental observation, and 2) perturbation of the system under controlled conditions, and analyzing the changes observed during perturbation. These methods are complementary and ancillary for a thorough understanding of membrane structure. In this work, we have concentrated primarily on the latter, while others in our group have been examining the former.

Proteolytic cleavage by trypsin of isolated RBC ghosts leads to vesiculation and sealing of membranes in RBC ghosts, and fragmentation and preferential cleavage of HMW cytoskeletal polypeptides in MATA membrane envelopes. In both systems, there is an increase in membrane fluidity as shown by decreases in the order parameter, S (Table II). The magnitude of the change is greater in the MATA membranes than in the RBC ghosts, again reflecting possibly the differences in accessibility of the cytoplasmic face of the membranes between the two systems. The observed increase in the central peak intensity implies an increase in accessibility of the lipid layer to the stearate label upon trypsinization. These data indicate that protein-lipid interaction is required for maintenance of membrane structure and ordering of the phospholipids.

Reversible crosslinking of membrane protein with DTBP in RBC ghosts gave a slight increase in the fluidity of the phospholipid, as monitored by 12 NS. The effect in the MATA membrane envelopes was in the opposite direction, and was more immediate. This is again related to the tendency of RBC ghosts to reseal. A greater proportion of the protein-associated label is readily available to the crosslinking agent,

hence the increase in probe immobilization in the MATA membranes, and the decrease in the time required to produce an effect. It is also quite possible that the domains probed are different in the two systems, e.g., that the label in the MATA membrane envelopes is attached to the protein, or lies at the protein-lipid interface. Data obtained with the protein label MSL indicated a great constraint of nitroxide mobility with crosslinking. The difference in magnitude of the change induced in the two systems shows more extensive crosslinking in MATA cells, which reflects either greater numbers of protein moieties in close proximity to one another, a higher degree of organization and interaction of the protein moieties with the cytoskeletal elements, or, again, the increased accessibility of these proteins to the crosslinking agent. During the process of crosslinking a significant portion of the nitroxide label is reduced.

Irreversible crosslinking of membrane protein with glutaraldehyde 12 NS labeled RBC ghosts reduces the mobility of the probe to such an extent that a powder spectrum is simulated. This reflects the difference in the mode of action of the reagent on RBC membranes from DTBP, since the change is in the opposite direction. Results obtained for MATA membranes with glutaraldehyde are almost identical, exhibiting extreme immobilization of the probe. Bieri et al. (234) have shown in similar crosslinking studies with glutaraldehyde that there is a diffusion-limited, temperature dependent paramagnetic quenching of protein fluorescence. Some of the fatty acid apparently binds to the protein, or is associated within 4-6 Angstroms (235) of these tryptophan residues, or else transfer of excitation energy from the lowest singlet state of the fluorophore to the paramagnetic nitroxide could not occur.

Reduction of signal intensity of the ESR probe may be a result of interaction with some product of lipid oxidation, as observed by Brown et al. (223) in studies of lipid oxidation catalyzed by heme proteins. As the hydrophobic nature of the spin label increases, the rate of loss of ESR signal also increases, therefore, one would conclude that the reaction must be more prevalent in the interior of the membrane. Label was not released into solution.

EDTA extraction of 12 NS labeled MATA and RBC membranes results in a decrease in signal intensity in the extracted membrane fragments and a distinct immobilization of the nitroxide probe, suggesting that the lipid bilayer becomes more ordered. There is a greater resolution of spectral components in these membrane fragments, therefore the label is in at least two distinct domains of vastly different viscosities. It is probable that a greater proportion of the hydrophobic region of the membrane becomes accessible to the stearate label upon extraction with EDTA. These data were completely supported by fluorescence polarization measurements made in similarly extracted membrane systems labeled with ANS. The lipid bilayer region becomes more ordered, and the spectral shift of ANS emission in membrane fragments toward the blue definitely indicates a decrease in the polarity of the probe environment.

The diminution of the ESR signal intensity of the membrane fragments in this case is not due to a reduction of the nitroxide. The label is present in the protein extract in bound form, and the shape of the spectrum clearly shows little constraint of probe mobility. The protein extract from the RBC ghosts contains primarily spectrin, and from the MATA membrane envelopes, contains mostly the HMW structural protein. Paramagnetic quenching of tryptophan fluorescence in the

spectrin-containing protein extract from RBCs shows that the nitroxide is indeed bound to the protein. Whether or not this association is with "annular lipids" (242, 235) which copurify with these proteins is not clear.

ANS, like the stearic acid spin probes, has been used primarily as a probe for the hydrophobic region of the membrane bilayer. However, a significant portion of this label also coextracts with the water-soluble proteins under hypotonic conditions in the presence of EDTA from both RBC and MATA membranes. The polarization values show little constraint of probe mobility in the extract, and the red-shifted emission peak in both cases shows that the ANS is in a polar environment. One may conclude that both the ANS and the fatty acid probe are present at the lipid-protein interface as well as in the remainder of the bilayer. Indeed, these data indicate that quite a significant portion of both of these probes lies within close proximity to membrane structural proteins, and coextracts under hypotonic conditions. Whether or not each of these probes monitors the same region of the membrane is irrelevant. The fact remains that neither is distributed uniformly in the membrane bilayer; neither, then, is strictly reflective of general membrane properties, therefore care must be exercised in data interpretation.

Trypsinization of the remaining membrane fragments from EDTA-extracted RBC ghosts as monitored by 12 NS showed little change in the membrane viscosity, although there was a slight change in the clarity of the probe environment as indicated by the increase in a_n' values. This implies a small change in the protein-lipid interaction within the fragments. EDTA, therefore, probably disrupts protein-protein interactions and changes the fluidity of the phospholipid bilayer of the

intact membrane through direct perturbation of the structural protein, rather than by induction of a phase separation.

SDS solubilization of 12 NS labeled RBC and MATA membranes produces a tremendous decrease in membrane microviscosity, which was very similar in both systems. This indicates release of the fatty acid probe upon complete disruption of the phospholipid bilayer. Fluorescence polarization data obtained from ANS-labeled membranes confirmed these results, and the red-shift of the emission peaks reflects a marked increase in polarity of the probe environment, or that the dye molecules were in the process of dissociation, as observed by Easter et al. (230) in fluorescence studies of synthetic vesicles.

ESR studies of the effects of zinc in plasma membranes shows that this ion reverses the disordering of the phospholipid bilayer induced by hypotonic swelling. The order parameters for control and zinc-treated membranes are very similar, as are the rotational correlation times. Atomic absorption spectrophotometry indicates that the ion does not remain with the membrane in either red blood cells or with MATA membrane envelopes. One can speculate that the interaction is electrostatic, but the results are inconclusive with respect to the mode of action of this stabilizing agent.

The calcium-ionophore A23187 produces increases in membrane fluidity in intact red cells, as monitored by 12 NS, and increases the cell fragility. Data obtained with MSL showed a protein conformational change occurring. The presence of Ca^{2+} protects the cell against perturbation by the ionophore. In looking carefully at the two theories presented on the mode of action of the antibiotic, Binet and Volfin (134) postulated an increase in permeability due to Mg^{2+} depletion.

Pfeiffer (127) has proposed a mechanism in which A23187 exchanged divalent cations by formation of (A23187)-Me^{2+} complexes which both perturbs the distribution of divalent cations and produces a transmembrane Δ pH. At low levels of ionophore, Mg^{2+} is thought to be released immediately, and the influx of the Ca^{2+} produced by the Ca^{2+} pump at these low levels reaches a steady state with the efflux. If this is the case, then the time-dependence observed in our work is probably reflective of the gradual depletion of cellular glucose and ATP and subsequent failure of the Ca^{2+} pump, and is supportive of the latter hypothesis. Post and Nicolson (247) have shown that the ionophore in the presence of Ca^{2+} produces a marked decrease in agglutination by lectins of lymphocyte surface immunoglobulins without inhibiting con A binding. This inhibition of agglutinability was similar to the effect produced by colchicine or vinblastine, and was thought to be an indirect action by the ionophores on the polymerization of the microtubule system. Steer et al. (248) have shown that A23187 inhibits up to 70% of the cAMP accumulation in resealed ghosts. These authors suggest that the ionophore induces a shift in the distribution of Ca^{2+} already present in the membrane, increasing the concentration available to adenyl cyclase. In any case, the evidence all points to membrane perturbation by the ionophore, itself.

Examination of RBCs from human donors known to have genetically derived disorders provides another avenue for exploring structural relationships between lipids and proteins. The very striking morphological deviations of cells from polycythemic, hyperlipidemic, and sickle cell patients imply membrane organizational differences, and differences in the spatial arrangements of membrane proteins. Labeling

of the whole red blood cells with MSL, which non-specifically binds to protein -SH groups, reveals in every case a deviation between blood samples from hematologically abnormal patients and control donors. There were no differences in ESR spectra of the hemoglobin from blood samples obtained from each of the above mentioned disorders, therefore, within the limits of our detection system, the difference in protein conformation shown by MSL in whole cells does not arise from free hemoglobin. This does not rule out differences in the amounts or the conformation of bound hemoglobin. However, SDS-PAGE electrophoresis of the several samples from each anomaly indicated no significant differences in hemoglobin retention in isolated membranes from normal samples. The amounts of hemoglobin retained by each set of samples seemed to depend more upon the age of the cells, state of oxygenation, and the method of isolation than upon the disease state. It is also possible that these differences in a/b ratios arose from differences in the amounts of bound serum albumin (160) which is said to stabilize the RBCs against sheer stress.

Stearic acid probes used to examine whole cells from these donors revealed a general fluidization of the membrane bilayer in hyperlipidemic cells with all three labels (Figure 15), but these changes are small. They could arise from an altered exchange of phospholipids between the membrane and the serum so that when the serum is removed and the cells are washed, as they are in these studies, the equilibrium of the exchange is shifted, allowing dissociation of a small portion of the spin probe, or by generally destabilizing the membrane. These cells do, indeed, lyse readily. The cells from polycythemic patients labeled with 5 NS show a decrease in order parameter, and a slight increase in order

parameter with 12 NS. It is probable that these rapidly-produced cells, which characteristically have an increased MCV, also have small differences in the relative amounts of protein, lipid and lipoprotein, allowing the probe to dissolve in regions normally not available to them, or differences in packing densities from those found in normal membranes. Additional caution in interpretation of these particular results must be exercised due to the paucity of available samples. HbS and HbAS whole cells do not reflect significant differences from normal cells, as shown by 5 NS and 12 NS. Since these probes do not appear to bind to Hb, itself, they should reflect only membrane lesions which engender alterations in the fluidity of the PL bilayer. Crosslinking of Hb to the surface of the membrane might logically change this membrane fluidity; it did not appear to do so. Nor was there a significant retention of Hb in the SDS-PAGE electrophoretic analyses of membranes isolated from these cells as compared with normal samples.

Red cell membranes from polycythemic donors, when labeled with MSL, which typically labels the HMW proteins spectrin and band 3, showed a marked decrease in constraint of nitroxide mobility. The same phenomenon, though to a lesser degree, was observed in MSL-labeled HbS membranes as compared with control samples (Table XVI). One must conclude, then, that there are differences in either the protein-lipid organization of these samples, or alternatively, the states of aggregation of the protein moieties present are different.

When membranes from HbS cells are first isolated and then labeled with 5 and 12 NS, the order parameters reflect a restriction in motion of the nitroxide probe. The 16 NS probe shows a slight increase in mobility. This is distinctly different from spectral characteristics

of these probes in the whole cells, and further indicates that the membranes are perturbed upon isolation. The manifestation of this perturbation is different in HbS cells as compared with normal cells. This gives further circumstantial evidence of a membrane lesion involving an increased predisposition of HbS membrane proteins or lipids toward crosslinking.

HbS is a congenital hemolytic anemia which gives rise to rigid, misshapen RBCs. These data show that in the intact cell, this decrease in cellular deformability (151, 154) does not arise from an increase in the viscosity of membrane phospholipids. There are, however, differences in the mobility of MSL probes in both whole cells and membranes which do not seem to be related to free Hb or Hb retention. The matter of Hb retention is in conflict with data obtained by other workers (154, 156), but Fischer et al. (153) have stated that Hb retention is highly dependent upon electrostatic interactions, therefore any shift in pH or ionic strength in our isolation procedure would affect the Hb retention as compared with that observed by others. Some of these samples were frozen, as were the attendant controls, prior to electrophoresis, but as Skabut et al. (244) have shown, there was no change in electrophoretic membrane protein elution as a result of freezing. It is known that HbS cells contain increased amounts of intercellular Ca^{2+} (154, 155), which suggests that there is a primary membrane lesion which may or may not be induced by Ca^{2+}-promoted alteration of the state of aggregation of spectrin. Isoelectric focusing of spectrin from HbS patients (150) shows the presence of 12-15 peptides with differing isoelectric points, but there were no significant reproducible differences in spectrin from HbS as compared with normal samples. The Triton extractions performed

by Lux et al. (163) on ISC cells revealed a sickle-shaped residual protein subshell composed of spectrin, actin, band 4.1a, and band 3. HbS was not present in the Triton ghosts. These sickle cells retain their shape through a membrane defect possibly acquired subsequent to membrane deformation, but definitely involving spectrin. The sickling phenomenon can be simulated by treatment of normal cells with the microfilament disruptors vinblastine, colchicine, and strychnine (150), and Ca^{2+} enhances this effect. As mentioned previously, when normal-appearing cells from HbS patients are placed in serum from otherwise hematologically compatible normal donors, the cells will undergo sickling. This could be a result of differences in ionic strength of the autologous serum from that of the donor. This is indirectly supported by the work of Ralston et al. (245, 246), who have shown that ionic strength has definite effects on the aggregation state of isolated spectrin in that the tetramer (960,000 MW) is stabilized by high salt. It is possible that the primary effect is upon the microfilament system, with subsequent aggregation of spectrin components induced by the rapid influx of Ca^{2+}.

In conclusion, it is helpful to note that of all of these studies, it is the membrane perturbations which most clearly show organizational differences and differences in the spatial distribution of protein in the MATA membranes as compared with red cell membranes. The EDTA extraction series in this work is probably the most significant of the perturbations performed. This reagent is known to affect the mobility of ectoenzymes on the surface of MATA tumor cells (119). This study shows that this effect is from alteration of the structural protein, and not through induction of a phase separation in the phospholipid

portion of the membrane bilayer. It is also clear that both the hydrophobic ESR stearate probes and the ANS fluorophore remains bound to and coextract with water soluble membrane proteins. Therefore, there is some support given to the postulation of an annulus of lipids which surround membrane proteins such that protein interaction in membranes is lipid-mediated (242, 235, 249).

There is present at the cell surface a quantity of hydrophilic carbohydrates closely bound to membranes, which may contribute to the long-term stability which is characteristic of animal cell plasma membranes by extensive solvation of the membrane surface (250). We have not examined this aspect of membrane structure. The binding characteristics of ESR probes and fluorescent dyes should be examined in terms of environmental conditions which alter the negative surface potential of the membrane. Further work should be done to determine what portion of the ESR signal arises from the cell surface by bathing the cells first with ascorbate to reduce the signal, and then with $K_3Fe(CN)_6$, since cells are essentially impermeable to these reagents (251). Since there is an uncertainty in the location of both fluorescent and ESR probes, a double-label similar to the one synthesized by Stryer et al. (252) might be synthesized in order to make these types of measurements more comparable. Different labels may be used to probe other regions of the membrane. Quantitation of the observed fluorescent quenching which would help delineate the spatial distribution of proteins and probe groups might be accomplished by using a standard such as quinine sulfate. In this manner, one might determine the ratio of label bound directly to or near the protein as compared with that present in the remainder of the phospholipid bilayer. One

might also examine the ease of spectrin or structural protein extraction from cells derived from patients with HbS, although in this case, the most important thing would be to have sufficient donors to obtain more data in the areas already examined.

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VITA

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