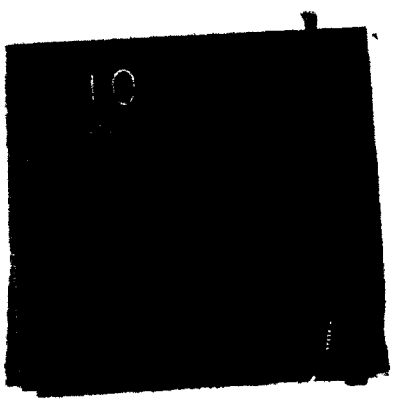
1 2

OF/DE



18578

NATIONAL LIBRARY
OTTAWA

CANADA

BIBLIOTHÈQUE NATIONALE
OTTAWA

NAME OF AUTHOR... Michael Channon .....

TITLE OF THESIS... Trypsin Digestion of Human Erythrocyte Membrane Proteins .....

UNIVERSITY..... Dalhousie University .....

DEGREE FOR WHICH THESIS WAS PRESENTED... Ph.D. .....

YEAR THIS DEGREE GRANTED..1973 .....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed) [redacted] .....

PERMANENT ADDRESS:

841 Ste Marie Rd.
Ste Anne de Bellevue, 820
Quebec

DATED..September, ... .1973

NL-91 (10-68)

TRYPSIN DIGESTION OF HUMAN ERYTHROCYTE MEMBRANE PROTEINS

by

Michael Channon

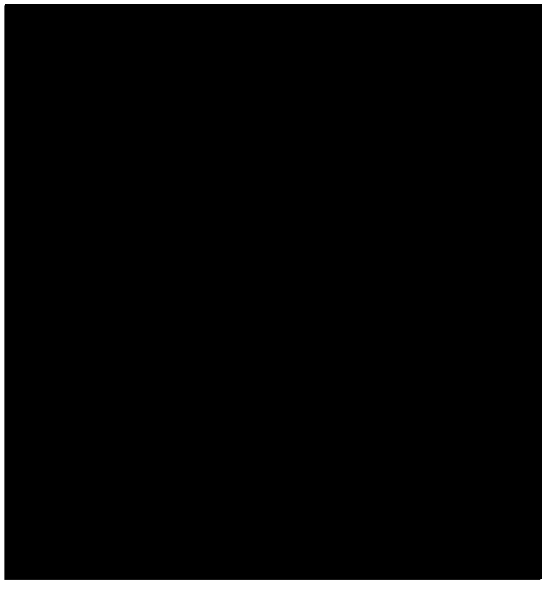
Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
at Dalhousie University

September, 1973.

Dalhousie University

Faculty of Graduate Studies

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled, "Trypsin Digestion of Human Erythrocyte Membrane Proteins", submitted by Michael Channon in partial fulfillment of requirements for the degree of Doctor of Philosophy.



2 Oct. 1973

DALHOUSIE UNIVERSITY

Date September, 1973

Author Michael Channon

Title Trypsin Digestion of Human Erythrocyte Membrane Proteins

Department or School Biochemistry

Degree Ph.D. Convocation Fall Year 1973

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.

[redacted]

Signature of Author

TABLE OF CONTENTS

		Page
SECTION I	GENERAL INTRODUCTION	1
A.	Membrane Structure	1
i.	Introduction	1
ii.	Unit Membrane Concept	1
iii.	Lipoprotein Subunit Models	7
iv.	Bimodal Concept	9
B.	The Erythrocyte Membrane	13
i.	Lipids	13
ii.	Proteins	17
a)	Tektins	19
b)	Glyceraldehyde-3-phosphate dehydrogenase	20
c)	Glycoproteins	21
iii.	Inorganic Ions	23
C.	Disposition of Components Within the Membrane	24
D.	Use of Protease For Studying Membranes	30
E.	Optical Rotatory Dispersion and Circular Dichroism	31
F.	Fluorescent Probes	38
SECTION II	MATERIALS AND METHODS	45
A.	Materials	45
B.	Methods	46
i.	Preparation of Erythrocyte Membranes	46
ii.	Preparation of Membrane Protein Solution	47
iii.	Protein	47
iv.	Carbohydrate	47
v.	Sialic Acid	47
vi.	Amino Acids	47

		Page
vii.	Lipid	48
viii.	Hemoglobin	48
ix.	Phosphorous	48
x.	N-terminal Amino Acids	48
xi.	Anti MN Activity	48
xii.	Polyacrylamide Gel Electrophoresis	49
xiii.	Peptide Mapping	49
xiv.	Turbidity	49
xv.	Viscosity	49
xvi.	Optical Rotatory Dispersion and Circular Dichroism	50
xvii.	Assay of Tryptic Activity	50
xviii.	Sedimentation and Diffusion Studies	50
xix.	Column Chromatography	51
xx.	Trypsin Digestion	51
1	pH stat	51
2	Buffer digestions	51
xxi.	Extent of Digestion	51
1	Alkali consumption	51
2	Determination of α-amino groups	52
3	Determination of C-terminal lysine and arginine	52
xxii.	Cyanogen Bromide Treatment	53
xxiii.	Fluorescence Measurements	53

SECTION III EXPERIMENTAL DATA 54

A. Introduction 54

B. Data 54

I. Characterization of Membrane and of Membrane Protein Solution 54
a) Results 54
b) Discussion 55

II. Extent of Digestion of Human Erythrocyte Membrane Protein by Trypsin 57
a) Results 57
i. Extent of digestion of isolated membrane protein 60
1 by alkali titration 60
2 by determination of amino groups 60
ii. Effects of pretreatments and of digestion conditions on the extent of digestion 60
iii. Extent of digestion of proteins in isolated membranes 61
1 by alkali titration 61
2 by determination of amino groups 61
3 by determination of the increase in C-terminal lysine and arginine 61

		Page
iv.	Extent of digestion of membrane protein in intact erythrocytes	62
1	by determination of amino groups	62
2	by determination of C-terminal lysine and arginine	63
b)	Discussion	63
III.	Effect of Trypsin Digestion on Chemical Composition of Membranes	66
a)	Results	66
b)	Discussion	76
IV.	Effect of Trypsin Digestion on Aqueous Protein Solutions	80
a)	Results	80
i.	Digest of protein solution of normal membranes	80
1	Fraction A	80
2	Fraction B	88
3	Fraction C and D	89
ii.	Digests of protein solutions of membranes of trypsin treated erythrocytes	89
b)	Discussion	90
V.	Effect of Trypsin Digestion on Gel Electrophoretic Patterns	91
a)	Results	91
b)	Discussion	95
VI.	Effect of Trypsin Digestion on the Interaction of Membranes with Salt as Shown by Changes in Optical Activity, Viscosity and Light Scattering	95
a)	Results	95
i.	Turbidity and pH	96
ii.	Viscosity	96
iii.	Optical rotatory dispersion and circular dichroism	99
b)	Discussion	107
VII.	Effect of Trypsin Digestion on the Interaction of Membranes with the Fluorescent Probe 1-anilino-naphthalene-8-sulphonate	110
a)	Results	110
b)	Discussion	125
VIII.	Summary	128
IX.	Bibliography	130

LIST OF TABLES

		Page
I	Lipid Composition of Human Erythrocyte Membrane	13
II	Phospholipid Components of Human Erythrocytes	15
III	Fatty Acid Composition of Major Bhospholipids of Human Erythrocyte Ghosts	16
IV	Buffers for Preparation of Membranes	46
V	Composition of Human Erythrocyte Membrane and Protein Solution	55
VI	Amino Acid Composition of Human Erythrocyte Membrane and of Protein Solution	56
VII	Extent of Digestion of Human Erythrocyte Membrane Proteins by Trypsin	58
VIII	Composition of Membranes: Effect of Trypsin Digestion of Erythrocytes	66
IX	Composition of Aqueous Protein Solutions	68
X	Composition of Membranes: Effect of Trypsin Inhibitor	70
XI	Hemoglobin Contents of Membranes	71
XII	Effect of Trypsin Digestion of Membranes on Compositions	72
XIII	Composition of Released Peptides	74
XIV	Inhibition of Agglutination of Erythrocytes by Anti-M and Anti-N Sera	75
XV	Analyses of Membrane, Membrane Fragments, Membrane Protein Solutions and Digests	76
XVI	Analyses of Fractions From Sephadex G-100 Chromatography	85
XVII	Amino Acid Analyses of Various Membrane Protein Fractions	86

		Page
XVIII	Viscosity of Human Erythrocyte Membrane Suspensions	98
XIX	Effect of Sodium Chloride on Binding of ANS by Human Erythrocyte Membranes	116
XX	Dissociation Constants for ANS and Sodium	124

LIST OF FIGURES

		Page
1	Schematic representation of the organization of the phospholipids and proteins of membranes	3
2	Structure of human glycosphingolipids	18
3	Structure of the major membrane glycoprotein	22
4	Molecular orbital representations of the n- and π-orbitals of the peptide link	33
5	Location of ANS in protein-lipid-water systems	42
6	Release of sialic acid during digestion of human erythrocytes by trypsin	59
7	Scheme of treatement of erythrocytes	67
8a	Elution profile of WSP-1	81
b	Elution profile of WSP-2	82
c	Elution profile of P-1	83
d	Elution profile of P-2	84
9	Schematic diagram of gel electrophoretic separation of membrane proteins	93
10	Densitometer tracings of polyacrylamide gels stained with Coomassie Blue	94
11	Effect of sodium chloride on absorbance at 600 nm and on pH	97
12	Optical rotatory dispersion of a suspension of human erythrocyte membranes (M-1) in distilled water and in sodium chloride	100
13	Optical rotatory dispersion of a suspension of human erythrocyte membranes (M-2) in distilled water and in sodium chloride	101
14	Optical rotatory dispersion of a suspension of human erythrocyte membranes (M-3) in distilled water and in sodium chloride	102
15	Circular dichroism of a suspension of human erythrocyte membranes (M-1) in distilled water and in sodium chloride	104

		Page
16	Circular dichroism of a suspension of human erythrocyte membranes (M-2) in distilled water and in sodium chloride	105
17	Circular dichroism of a suspension of human erythrocyte membranes (M-3) in distilled water and in sodium chloride.	106
18	Excitation and emission spectra of ANS-membrane (M-1) suspension	111
19	Rate of development of fluorescence after addition of ANS to membrane suspensions	113
20	Double reciprocal plot of membrane concentration versus fluorescence	115
21	Effect of sodium chloride on the emission spectrum of membranes M-1	118
22	Effect of sodium chloride on F^o	119
23	Effect of sodium chloride on turbidity, pH and F^o	120
24	Fluorescence of ANS-membrane (M-1) suspension during trypsin digestion	121
25	Double reciprocal plot of fluorescence versus ANS concentration	122
26	Double reciprocal plot of fluorescence versus sodium chloride concentration	123

ABSTRACT

The tryptic digestion of human erythrocyte membrane proteins has been investigated. About 70% of the peptide bonds involving lysine and arginine were hydrolyzed by trypsin. This extent of digestion was observed for both membranes and isolated protein indicating that membrane structure did not limit the availability of bonds to trypsin. The intact erythrocyte was resistant to trypsin, only 1 to 4% of the peptide bonds being hydrolyzed.

Trypsin digestion of aqueous protein solutions led to the formation of a high molecular weight aggregate comprising about 20% of the dry weight of the protein. This aggregate contained over 60 mole% apolar amino acids and might have been derived from hydrophobic portions of intrinsic membrane proteins. Trypsin digestion of membranes resulted in the release of 40% of the protein, 35% of the carbohydrate, 65% of the sialic acid and 14% of the lipid into solution. Membranes isolated from erythrocytes which had been digested with trypsin were found to contain 24 times the usual amount of hemoglobin. This increase could be prevented if the cells were washed with trypsin inhibitor prior to hemolysis, indicating that it was due to digestion of membranes during hemolysis rather than to digestion of the exterior surface of the erythrocyte.

Sequential digestion of erythrocytes and of membranes produced from them indicated that there were substantial changes in the membrane during hemolysis.

Trypsin digestion did not remove the response of membranes

to added salt, as measured by changes in pH, turbidity, viscosity, ORD and CD. Studies with the fluorescent probe 1-anilino-naphthalene-8-sulphonate, which indicated an increase in fluorescence efficiency on addition of salt could be explained by an expulsion of water from the membrane.

Abbreviations

ANS	1-anilinonaphthalene-8-sulphonate
ATP	adenosine triphosphate
$(Na^+ + K^+)$-ATPase	sodium, potassium stimulated adenosine triphosphatase
Å	angstrom
CD	circular dichroism
pCMB	parachloromercuribenzoate
^{o}, ^oC	degree centigrade
EDTA	ethylenediaminetetraacetate
F^o	fluorescence intensity obtained when all dye molecules are bound.
FMMP	formylmethionylsulphone methyl phosphate
M-1	membranes prepared from human erythrocytes
M-2	membranes prepared from trypsin treated erythrocytes
M-3	membranes prepared by trypsin treatment of M-2.
M-4	membranes prepared by trypsin treatment of M-1.
M-2I	membranes prepared from trypsin treated erythrocytes washed with lima bean trypsin inhibitor
M-3I	membranes prepared by trypsin treatment of M-2I
ORD	optical rotatory dispersion
PC	picrylchloride
P-1	peptides released during trypsin treatment of intact erythrocytes
P-2	peptides released during trypsin treatment of M-2
SITS	4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid
TNBS	trinitrobenzene sulphonate
TNP-amino acid	trinitrophenyl-amino acid

WSP-1	water soluble protein from membranes M-1
WSP-2	water soluble protein from membranes M-2
(n=)	number of determinations equals ...
Tris	Tris (hydroxymethyl) aminomethane

Acknowledgements

I would like to express my sincere gratitude to Dr. J.A. Verpoorte, my research director, for his guidance and helpful criticism throughout the course of this study.

I am grateful to Miss L. Saulnier, Mrs. R. Breckon, Mrs. D. Dockerell and Mr. A. Edwards for their technical assistance, especially during preparation of membranes and also to Mrs. R. Breckon for performing many amino acid analyses. Thanks are also extended to Mrs. D. Mullins for typing of the manuscript and to Mr. I Fraser and Mr. E.R. Amiro for proof reading.

Financial support from the Medical Research Council made this work possible.

SECTION I: GENERAL INTRODUCTION

A. MEMBRANE STRUCTURE

1. Introduction:

It was recognized as early as 1855 by Carl Nägeli (1) that the cell surface could be distinguished from the cytoplasm. He discovered that many cells including those of plants, algae, fungi and moss were impermeable to pigments either in their cytoplasm or in the external medium. The surface was noted to be more dense, viscous and otherwise distinguishable from the cytoplasm. Nägeli called this outer layer the plasma membrane. He demonstrated, by observing plasmolysis of plant cells, that the plasma membrane possessed osmotic properties.

Overton (2) studied the permeability properties of membranes and suggested that they were primarily lipid in nature. The same conclusion was reached by Fricke (3-5) from studies of the electrical properties of various cells. Mudd and Mudd (6) postulated a lipid nature for the erythrocyte membrane.

ii. Unit Membrane Concept:

Gorter and Grendel (7) proposed that the lipids extracted from erythrocytes would form a monolayer of twice the area of the intact cell. This observation lead them to suggest the existance of a bimolecular layer of lipid at the cell interface.

Danielli and Harvey (8) showed that the tension at the surface of certain cells was much less than at ordinary oil-water interfaces. This was attributed to the presence of a layer of protein adsorbed onto the membrane lipid. From these observations, and from the bilayer nature of the lipid as suggested by Gorter and Grendel (7), Danielli (9) proposed his classic model for the structure of cellular membranes, composed of a lipid bilayer to which a protein coat is bonded by ionic forces or by hydrogen bonds.

The presence of a lipid bilayer was supported by the observations of Schmidt and his co-workers who studied birefringence of erythrocyte membrane (11-13) and of myelin (14). They showed that within these membranes there were molecules whose axes were arranged perpendicular to the membrane surface. Perturbation of this birefringence by organic solvents, and its presence in phosphatide bilayers suggested that it arose due to the arrangement of hydrocarbon chains as proposed in the Danielli-Davson model.

Small angle X-ray diffraction studies of peripheral nerve myelin (14) demonstrated the presence of concentric layers 180 Å apart. A comparison with X-ray diffraction data for mixed nerve lipids led to the conclusion that the layers in myelin represented two concentric bimolecular lipid leaflets each with its surface covered by a protein coat.

Finean (15) found a period half that previously observed. This was thought to be consistent with a repeating lipid bilayer of 51 Å in width. The Danielli hypothesis has been incorporated into Robertson's (10) unit membrane concept, illustrated in figure 1.

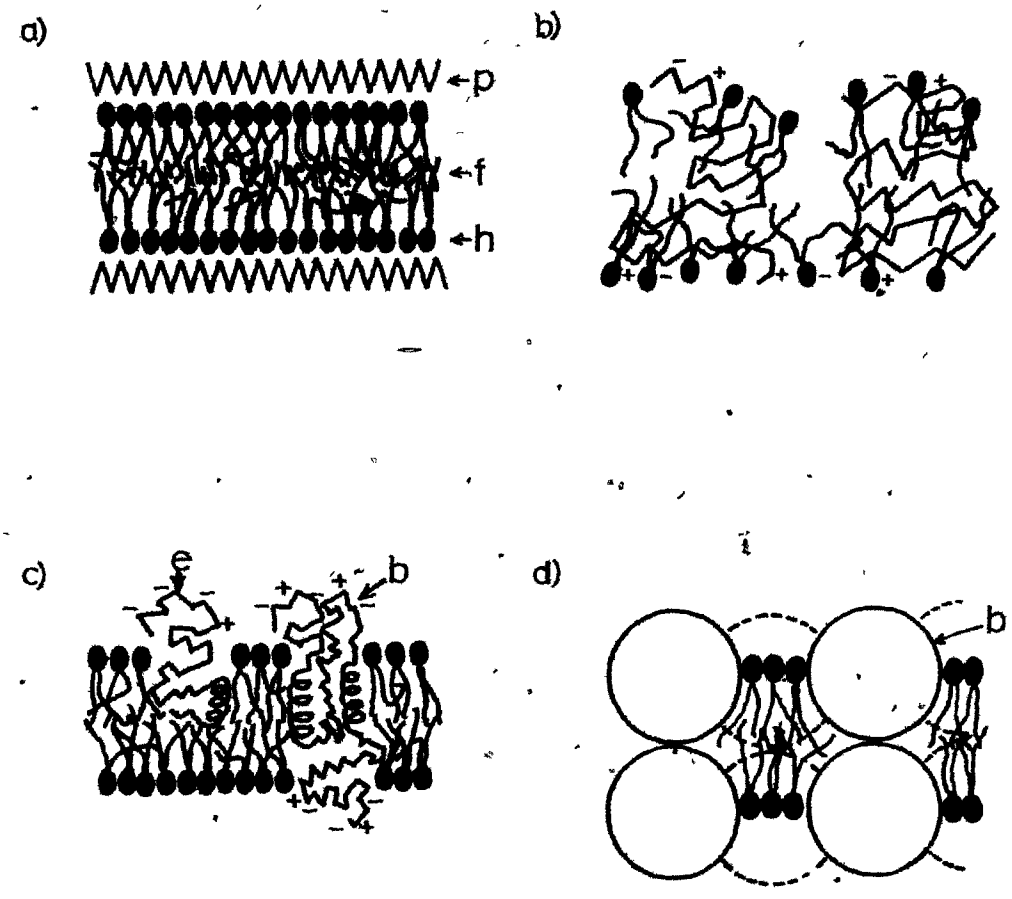


Fig. 1: Schematic representation of models for the organization of the phospholipids and proteins of membranes. (a) The Davson-Danielli-Robertson model, (b) the Benson lipoprotein subunit model, (c) the Singer lipid-globular protein mosaic model, and (d) the Green protein crystal model. Symbols p, h and f denote the polypeptide chains of the protein, the polar and ionic heads of the phospholipids and the fatty acid tails of the phospholipids. Symbols b and e denote bimodal, intrinsic, proteins and extrinsic proteins. The + and - signs represent the ionic residues of the protein. a - c from (54), d from (250).

Much of the evidence on which Robertson based this concept was gathered from observations of membranes with the electron microscope. Cellular membranes, when appropriately stained, appeared as trilaminar structures, consisting of two electron dense regions separated by a less dense zone. Robertson (10) observed that all cell membranes and also artificial phosphatide bilayers had this general characteristic. When allowances were made for changes during fixation and staining of myelin preparations, the electron microscopic observations agreed with the X-ray diffraction patterns (16). Because myelin arises from the plasma membrane of Schwann cells, it was suggested (17) that myelin might serve as a general model for cellular membranes.

The unit membrane concept, however, is not so general as was first thought. It cannot, as was believed, account for the movement of various solutes through cellular membranes. Maddy, Huang and Thompson (18) have made impedance measurements of plasma membranes. The results were not consistent with a continuous lipid bilayer.

Doubt has been cast on the measurements of Gorter and Grendel. It has been suggested (19) that they underestimated the surface area and lipid content of the red cell membrane. Bar et al (20) have shown that the area of a lipid bilayer is dependant on the surface pressure. Thompson (21) demonstrated that phospholipid bilayers themselves may have a low surface tension, thus in part neutralizing the argument of Danielli and Harvey (8).

Measurements of membrane birefringence (11-14), while demonstrating the presence of molecules aligned perpendicular to the membrane surface, did not provide information about the nature of these molecules. Results

based on perturbation by organic solvents (11-13) were equivocal as these agents can denature protein as well as dissolve lipid. In the case of myelin it seemed likely that the birefringence was due to lipid as it accounted for 80% of the membranes mass (22). Many other membranes, however, have protein contents of 60 to 70% (22) and it seemed likely that in these cases the birefringence may have arisen from the membrane protein as well.

X-ray diffraction studies have been restricted mostly to the lamellar systems, especially myelin. The results obtained with myelin, while compatible with the Robertson model, are subject to other interpretations. Low-angle X-ray diffraction gave only an estimate of the electron density distribution in a direction perpendicular to the membrane plane. They did not define the nature of the molecules giving rise to the observed distributions. Recent diffraction analyses at 7 Å resolution by Kirschner and Caspar (23) have gone further toward a correlation of the electron density distribution with the specific arrangement of the molecular constituents within the membrane. Their data have been further substantiated by comparison with neutron diffraction analyses. Kirschner and Caspar (23) provide evidence for the presence of a lipid bilayer.

The unit membrane concept is primarily dependant upon the interpretation of the electron microscopic images of cellular membranes. Korn (19) has questioned what this image actually represents in molecular terms. It has been shown (24) that electron microscopic images of various cellular membranes differ greatly in their width. It is possible that they cannot be assumed to represent a single type of molecular arrangement. Fleischer, Fleischer and Stoeckenius (25) and Napolitano, Lebaron and Scaletti (26) have shown that the trilaminar

arrangement of inner mitochondrial membrane and of myelin remained unchanged after removal of lipid. It was further pointed out that the mechanism of osmium tetroxide and of potassium permanganate fixation have been subject to little scrutiny. Conclusions as to the native molecular arrangements could not be drawn as the changes accompanying fixation were not known. Korn (19) has observed that there was increasing electron microscopic evidence for globular substructures in many membranes.

The above observations raise doubts as to the validity of much of the evidence on which the Danielli-Davson and Robertson hypotheses were founded. As well, there has been an accumulation of data apparently incompatible with the unit membrane concept. Implicit in Robertson's model is ionic bonding between the membrane lipid bilayer and the associated protein. There appears to be little evidence of this type of interaction in biological membranes. Many workers (27-33) have postulated non-polar or hydrophobic interactions between the proteins and lipids of many membrane systems.

The unit membrane concept of Robertson requires that a portion of the membrane protein be in the β-conformation. Infra-red studies of erythrocyte membranes (34), of tumor cell membranes (28) and of myelin (35) revealed no evidence of protein in this conformation. Studies employing optical rotatory dispersion and circular dichroism of tumor cell membranes (28-30), erythrocyte ghosts (35,36), bacterial membranes (35,36), mitochondria (37) and of myelin (35) all indicated the presence of a considerable portion of the membrane protein in an

α-helical conformation. Their optical spectra appeared to exhibit certain characteristics initially attributed to the hydrophobic environment of the protein (28-30,37). Lately these characteristics have been attributed to optical artifacts including Mie scattering (38), and absorbtion flattening (40). It has also been shown that the inorganic cation content of the membrane can influence the optical rotatory dispersion and circular dichroism spectra (41).

Studies of various membranes by nuclear magnetic resonance techniques has added further information concerning the nature of lipid-protein interactions in biological membranes. Chapman et al. (31,32), using erythrocyte membranes, have shown that the choline residues of membrane lecithins are in an aqueous environment. In addition, they suggested a close association between the hydrocarbon chains of membrane lipid and proteins. These observations do not appear to be consistent with the requirements of the unit membrane concept.

iii. Lipoprotein Subunit Models:

A new model was proposed to accommodate experimental evidence which was incompatible with the unit membrane concept of Robertson. The essential features leading up to this model were presented by Singer (42) and by Benson and Singer (43). The proposed model, based on specific lipid-protein interactions, shown in figure 1, was presented by Benson (44). The protein was presumed to be largely globular and to be mostly in the interior of the membrane, to maximize hydrophobic interactions. In contrast to the Danielli-Davson and Robertson models, Benson proposed that the lipid was not in a bilayer, but that their individual fatty acid chains were intercalated into folds of the protein chain. The

polar heads of the lipids were thought to be in contact with water. This type of structure resulted in a more or less uniform lipoprotein complex. These complexes were presumed to exist as morphological subunits and were thought to be held together in the plane of the membrane mainly by hydrophobic interactions.

Similar types of models, incorporating repeating lipoprotein subunits were also proposed by Park and Pon (45) and by Green and Perdue (46). These models were derived from chemical and biochemical studies of the internal membranes of mitochondria and chloroplasts. All these models considered the lipid and protein to be arranged in some manner in subunits. The presence of lipid in a continuous bilayer was excluded.

At this time, however, evidence from four areas resulted in a revival of the lipid bilayer concept. Studies (47) of the response of both artificial phospholipid bilayers and natural membranes to agents including valinomycin and other peptide antibiotics suggested that they possessed identical structures, that is, a bilayer structure.

Studies (48) of the passive permiability properties of inner mitochondrial membrane indicated that it behaved as though its lipid was in a bilayer.

Electron microscopy, in conjunction with the techniques of freeze-cleaving and etching, supported the hypothesis that the lipid of several membranes was in the bilayer form (49). The membranes studied showed cleavage planes similar to those seen with artificial lipid bilayers (49).

The thermotropic change in state of membrane lipids has been measured by differential scanning calorimetry. Steim (50) has shown that a marked phase transition in the lipid of Mycoplasma laidlawii occurred at the same temperature whether the lipids were in membrane or in a protein free water dispersion.

Studies by electron spin resonance of paramagnetic probes such as nitroxide-labelled fatty acids and steroids incorporated into membranes and phospholipid suspensions demonstrated the fluidity of certain lipid components within the membrane (51). These observations were consistent with the presence of lipid as a bilayer. While it is now likely that the lipid of biological membranes exists in a bilayer (52), it appears that the bilayer may not be continuous (53).

iv. Bimodal Concept:

Recent evidence has suggested that proteins associated with membranes may be divided into two general categories, peripheral or extrinsic and integral or intrinsic (54). Proteins of the peripheral class are easily dissociated from the membrane. Much of the cytochrome c of mitochondria may be dissociated by high salt concentrations, and is thus thought to be peripheral. Spectrin (55), which may constitute up to 20% of the hemoglobin-free red blood cell membrane can be isolated by mild treatment involving chelating agents. It too is thought to be peripheral.

Peripheral proteins may also be characterised by a low or negligeable content of lipid when dissociated from the membrane. In normal aqueous buffers they would be expected to be soluble and to be molecularly dispersed. In the membrane they would be expected to be

globular in order to maximize their hydrophobic and hydrophilic interactions (56).

To dissociate integral proteins from membranes, on the other hand, requires much more drastic treatments, such as detergents, bile acids, or protein denaturants. Usually some lipid remains associated with these proteins and when delipidated they become highly insoluble and tend to aggregate in aqueous buffers at neutral pH.

Richardson et al (57) postulated the presence of specific structural proteins in membranes. These proteins were to provide the matrix of the lipoprotein subunits. It was thought that they would have no other function beside their structural role. Recent evidence (58, 59) however is not consistent with the view that a discrete structural protein characterises each membrane, but rather it suggests that membrane proteins are grossly heterogeneous. It appears that the integral membrane proteins may fill the role of structural proteins, though at the same time they may have enzymatic function.

Thus integral proteins are distinguished from peripheral proteins by their intimate association with membrane lipid. This association may be due to some structural feature of the protein (36). The intrinsic proteins would have an amino acid sequence which allows hydrophobic interactions of the protein molecule to be maximized by association with the hydrophobic environment provided by the lipids of the interior of the membrane. The ionic groups of the protein and lipid must be in contact with the bulk aqueous phase in order to maximize hydrophilic interactions. This suggests that the integral proteins of the membrane will have ionic amino acid residues distributed asymmetrically

over the surface of the molecule, concentrated at those portions of the surface exposed to the aqueous phase and absent from those portions in contact with the lipid phase. This results in a protein which is essentially bimodal, having an area predominantly hydrophobic and an area predominantly hydrophilic. On the other hand, the amino acid sequence of extrinsic proteins must be such that it allows hydrophobic interactions to be maximized internally within the individual polypeptide chain or within subunits of protein molecules. At the same time, hydrophilic interactions are satisfied externally by the presence of sufficient numbers of ionic charges more or less evenly distributed over the external surface of the molecule.

The concept of bimodality defines the backbone on which the functional membrane is built, in terms of invariant components, that is, the bimodal proteins and lipids. The proteins and lipids may be bonded together in several ways, permitting different types of arrangements. The unifying concept of bimodality deals with the properties of the components rather than with their structural arrangements.

While bimodality may represent a new unifying concept of general membrane structure, there does not appear to be any general agreement as to the molecular nature and structure of the proteins associated with the lipid bilayer. It is generally agreed that the protein has a high helical content and is largely globular in nature. Urry (62) has suggested the presence of other conformations in proteins of axonal and oxyntic cell membranes.

Different models of membrane structure, based on the concept of bimodality, aportion varying amounts of structural role to the

different components. Singer (63) has proposed a lipid-globular protein mosaic model (see fig. 1). In this model intrinsic membrane proteins are thought to be floating freely in a lipid bilayer. The ionic and polar groups of the proteins are largely located on the membrane surface, in contact with the aqueous medium, while the non-polar residues are oriented away from the water phase, toward the membrane interior. The amphipathic protein units are intercalated into a discontinuous lipid bilayer. Singer proposed (63) that the lipid would form the basic structural element of the membrane and be in a fluid state, rather than in a crystalline one, under normal physiological conditions. The mosaic was expected to be a dynamic one with the components being able to undergo translational diffusion in the plane of the membrane. As a result it was thought that no long range order would be imposed on the protein.

Green (60) on the other hand has presented a protein crystal model (see fig. 1) for membrane structure. He proposed that the intrinsic proteins were linked together by protein-protein interaction into sets. One set might be linked to other sets, resulting in protein domains. These protein domains would form the backbone of the membrane structure. The lipid, as a bilayer, would fill the interstices between sets or domains of protein. Green's model then differs from that of Singer's in proposing a long range order for the protein, which forms the backbone of the membrane. Nevertheless, both models are similar in that they employ the concept of bimodality.

B. THE ERYTHROCYTE MEMBRANE:

The erythrocyte membrane is composed of a combination of lipids, proteins, carbohydrates, metal ions and water. To characterize each of these components and to define their location within the membrane structure is a problem of great magnitude. Present knowledge of some of these aspects as related to the human erythrocyte membrane will be summarized.

i. Lipids

It has been shown that lipids constitute from 35 to 40% of the dry weight of the human erythrocyte membrane (64). The nature of the lipid has been the subject of a number of reviews (65-67). The lipid composition is summarized in Table I.

TABLE I

LIPID COMPOSITION OF HUMAN ERYTHROCYTE MEMBRANE*

	gm x 10^{-13} per cell	μ moles per cell	mole %
Total lipid	4.76	7.41	100
Cholesterol	1.20	3.20	43.2
Phospholipids	3.20	4.01	54.1
Glycolipid	0.18	0.20	2.7

* Modified from table 3 ref. (67).

The study of erythrocyte membranes is complicated by the fact that they are heterogeneous. Erythrocytes have a life span of about 120 days during which time they can undergo structural and dimensional changes that can be associated with a loss of lipid (68). In general, young cells contain more lipid than do older cells (69,70). Most notably, older cells have lost cholesterol and phospholipid (71). There is a general decrease in the amount of exchangable glycerophospholipid with age (72). Not only is lipid lost, it may also undergo changes. For example, phosphatidylethanolamine is converted to lysophosphatidylethanolamine (73). Peroxidation of some lipids has been shown to occur (74). There is also evidence of a rapid exchange of lipid between the membrane of the plasma, especially of cholesterol, fatty acids and phosphatidylcholine (65). The glycosphingolipids on the other hand are relatively inert (65).

Cholesterol accounts for more than 99% of the neutral lipids of the mature human erythrocyte (75). The remainder of the neutral lipids are made up of cholesterol esters, triglycerides and free fatty acids (73,76,77). Substantial amounts of diglyceride have also been reported (78).

The majority of the membrane cholesterol is exchangeable with that in the plasma, about eight hours being required for complete exchange (79-82).

Cholesterol and phospholipid are present in close to 1:1 molar ratio. Cholesterol and phosphatidylcholine in particular are thought to be closely associated (65). Chapman and Wallach (83)

have speculated that cholesterol may alter the fluidity of the fatty acid chains of the phospholipid molecules.

The phospholipids comprise 50 to 60% of the total lipid of the membrane (84). The major phospholipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin while the lysophosphatides, plasmalogens, phosphatidylinositols, phosphatidic acids and cardiolipins appear as minor constituents. The phospholipid composition of the human erythrocyte membrane is summarized in Table II.

TABLE II

PHOSPHOLIPID COMPONENTS OF HUMAN ERYTHROCYTES*

	% Total Lipid Phosphorous
Phosphatidylethanolamine	26.03
Phosphatidylcholine	28.25
Sphingomyelin	24.57
Phosphatidylserine	13.38
Phosphatidic acid	2.07
Phosphatidylinnositol	1.13
Lysophosphatidylcholine	1.06
Minor components	3.52

* Modified from table 4, ref. (67).

It is of interest that human erythrocyte membranes contain equimolar amounts of choline and non-choline containing phospholipids.

The fatty acids of the phospholipid fraction have been characterized as shown in Table III (74,85).

TABLE III

FATTY ACID COMPOSITION OF MAJOR PHOSPHOLIPIDS OF HUMAN ERYTHROCYTE GHOSTS*

Fatty Acid	Percent Total Fatty Acid			
	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Phosphatidyl-choline	Sphingo-myelin
16:0	15.5	4.4	34.7	41.3
18:0	14.1	39.7	13.8	9.1
18:1	17.2	9.8	21.1	5.2
18:2	5.6	2.6	21.4	3.7
20:4	21.8	23.5	6.7	0.1
22:0				8.0
22:4	7.8			
22:5	4.6	2.9		
22:6	8.9	7.0		
24:0		4.1		15.0
24:1		3.7		15.5

* Modified from table 5, ref. (67).

The fatty acids of the phospholipid fraction turn over rapidly, exchanging with those in the serum (86). Involved especially are linoleate (18:2), oleate (18:0), and arachidonate (20:4).

While the glycolipids comprise less than 5% of the total lipid, they appear to be of great importance because of their blood group activity (87,88). Sweely and Dawson (65) have shown that human membranes contain four glycosphingolipids called GL-1, GL-2, GL-3 and GL-4 of 0.50, 1.43, 1.27 and 7.05 μmoles per 100 ml ghosts respectively. Their structures have been determined and are shown in Figure 2.

In addition to these four glycosphingolipids, other carbohydrate containing lipids include gangliosides and blood group substances. Of the more than sixty known blood group substances at least five are known to be glycosphingolipids (88,89), the most important being the A, B and Lewis activities. The main function of the glycolipids is likely to be their role as tissue antigens and their involvement in the recognition process between cells.

ii. Proteins:

Protein accounts for about 50% of the dry weight of the erythrocyte membrane (90). It is not clear how many proteins are located in the erythrocyte membrane. The best studies are based on dissolution of the membranes, followed by electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulphate. Not only does this technique give an estimate of the numbers of polypeptide chains in the membrane, but also it gives information about their molecular weights. There are, however, certain complications involved with this technique. The presence of either carbohydrate (91) or lipid (92) may result in anomalous mobilities, resulting in error in molecular weight determination. The presence of lipid as well may lead to the appearance of multiple bands (92).

GL-4

N-Acetylgal-(1-3)-gal-(1-4)-gal-(1-4)-glu-ceramide

GL-3

(1-4)-gal-(1-4)-glu-ceramide

GL-2

gal-(1-4)-glu-ceramide

GL-1

glu-ceramide

Fig. 2: Structure of human glycosphingolipids.
The symbols gal and glu represent galactose and glucose,
N-Acetylgal represents N-Acetylgalactosamine.
From ref. (65).

Protein charge has also been shown to affect the electrophoretic mobility of protein in the presence of sodium dodecylsulphate (93). Despite these potential problems, however, electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulphate can give useful information concerning the proteins of the erythrocyte membrane.

Several reports have appeared on the distribution of polypeptide chains in the erythrocyte membrane (94-106). There are from seven to nine major bands corresponding to polypeptide chains ranging from 15,000 to 250,000 daltons. There also appear to be three carbohydrate containing bands.

The following proteins have been identified.

a) Tektins

Gel electrophoresis showed two major bands of about 250,000 daltons. These are amongst the largest known polypeptide chains (107), and they were extracted from the membrane by washing with hypotonic solvents (67,97,108-110). These extracts contained two chains of about 250,000 daltons plus a chain of about 45,000 daltons (67-90). These chains have been resolved and analysed. Mazia and Ruby (111) have called the large polypeptides tektins. Although it has been suggested that the 45,000 dalton component was associated with the tektins (122), this was not supported by data from cross-linking of membrane proteins (123). Clarke (110) has examined the tektins from bovine erythrocytes. Bovine tektin A had two polypeptide chains α and α^1 of 220,000 and 240,000 daltons respectively. The material had a sedimentation coefficient of approximately $s_{20,w} = 8.0$ S. Viscosity measurements indicated that the molecule was highly asymmetric, being rod-like with an axial

ratio of about 45. Cross-linking studies using the bifunctional reagent dimethylsuberimidate suggested that the native molecule consisted of two polypeptide chains, of the form $(\alpha\alpha^1)$, or possibly of four chains of the form $(\alpha_2\alpha_2^1)$. It has been demonstrated that the tektins are located at the inner surface of the erythrocyte membrane (95,96,102,114-119).

Amino acid analyses of the high molecular weight chains from human erythrocytes showed that they had an amino acid composition similar to rabbit muscle myosin and very different from muscle actin (67). The complex extracted from erythrocytes may correspond to spectrin, isolated by Marchesf and Steers (112), which appears fibrous in the electron microscope (55,112-113), The complex may also be related to hollow cylinders of protein described by Harris (120,121).

b) Glyceraldehyde-3-phosphate dehydrogenase

Another protein of the erythrocyte membrane can be extracted by 0.1 M EDTA at pH 7.9 or by 0.15 M sodium chloride (87). This component has a molecular weight of 35,000 daltons and has been identified as glyceraldehyde-3-phosphate dehydrogenase (124). Tanner and Gray (124) have, however, suggested that this enzyme may not in fact be a component of the membrane, but that it is a soluble enzyme which is reversibly bound to the membrane. It may be associated with two other components of the membrane of molecular weights 88,000 and 72,000 daltons (108).

The component of 72,000 daltons might represent a polypeptide chain of a protein responsible for glucose transport (123). The 88,000 dalton component, which has been shown (125) to contain a small amount

of carbohydrate, may be related to membrane acetylcholinesterase (123).

c) Glycoproteins

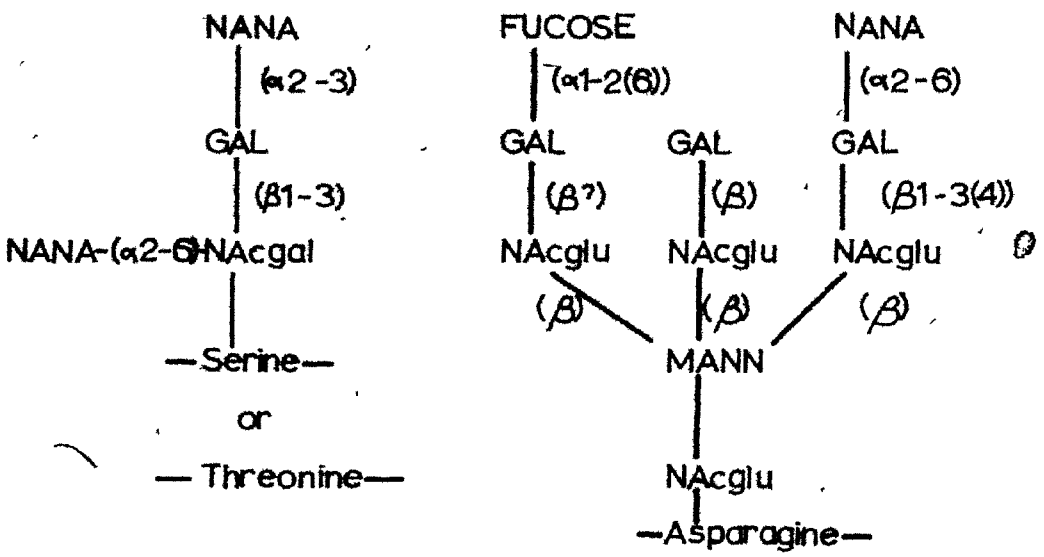
Kobylka et al. (126) have examined erythrocyte membrane proteins by electrophoresis in the presence of sodium dodecylsulphate. The patterns for human, dog, cat, cow, pig and sheep proteins were similar, showing nine major bands which were not affected by the method of hemolysis. Differences were noted in the glycoprotein components. The human membrane appeared to contain 3 or 4 glycoproteins (97,126,127).

The major glycoprotein has been isolated and extensively characterized (91,116,128-135). However, there has been some controversy concerning its molecular weight. Early studies of the glycoprotein by electrophoresis in the presence of sodium dodecylsulphate suggested that it had a molecular weight of about 100,000 daltons (100,116) whereas studies by ultracentrifugation and by column chromatography indicated a molecular weight of 31,000 to 55,000 daltons (129,132, 136,137). Analysis of the chemical composition of the glycoprotein and of glycoprotein fragments suggested a molecular weight of 50-55,000 daltons (128).

This glycoprotein is 60% carbohydrate and contains sialic acid (25%), galactose (10-12%), N-acetylglucosamine (6%) and N-acetylgalactosamine (12%) plus small amounts of mannose and fucose, as well as about 200 amino acid residues (128).

There are two types of carbohydrate chains attached to the polypeptide backbone as shown in Figure 3. These chains are attached to the N-terminal half of the peptide chain and are exposed to the external

a)



b)

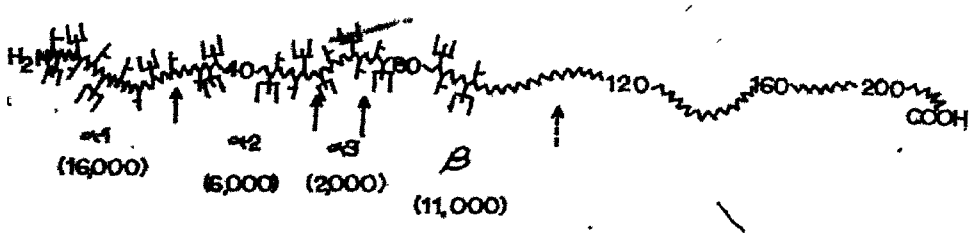


Fig. 3: Structure of the major membrane glycoprotein.
a) Carbohydrate Units: The symbols are: NANA = sialic acid; GAL = galactose; NAcgal = N-Acetylgalactosamine; NAcglu = N-Acetylglucosamine; MANN = mannose. From Ref. (136).

b) Polypeptide backbone: Oligosaccharide units are represented by ⊣ and ⊢ . The arrows indicate sites of cleavage by trypsin, the broken arrow being a site not attached in the intact erythrocyte. From Ref. (128).

environment of the cell. The C-terminal third of the polypeptide chain appears to be located internal to the lipid barrier of the membrane and may in part extend into the cytoplasm of the cell. These two portions are connected by a segment composed predominantly of non-polar and hydrophobic amino acids, about 35 residues which span the membrane (131).

Another glycoprotein of about 100,000 daltons has been shown to contain 10% carbohydrate by weight (90). More than 50% of its amino acid residues are non-polar (90). This component appears to be exposed to the exterior surface of the membrane (90,116,117,139,140) and possibly also to the interior surface (116,117).

iii. Inorganic Ions:

The role of inorganic ions in the erythrocyte membrane is not clear. Rosenberg and Guidotti (94) have observed that the presence of Ca^{++} tends to stabilize the membrane structure, preventing the extraction of some membrane proteins. Reynolds (141) and Reynolds and Trayer (99) have examined the solubility of membrane in aqueous media. They found that up to 90% of the erythrocyte membrane proteins could be solubilized by exposure to either 5×10^{-3} M EDTA, pH 7.5 or 0.1 M tetramethyl-ammonium bromide. They suggested that inorganic cations played a major role in stabilizing protein-lipid interactions in the membrane. Reynolds (141) suggested three possible modes by which such a stabilizing effect could be achieved. First by formation of a ternary complex involving protein, cation and lipid polar head group. The protein could be external to the lipid structure, or it could be partially intercalated. Secondly, a lipid-protein complex could be bound to the lipid bilayer through ternary cationic bridges. Finally, a binding site for protein within the membrane lipid bilayer could be stabilized by a ternary cationic bridge

between lipid head groups.

Forstner and Manery (142,143) found that most Ca^{++} of the membrane was bound to protein carboxyl groups rather than lipid.

Horvath and Sovak (144) have suggested that calcium binding may serve to regulate the activity of membrane bound enzymes. This was thought to occur by coarctation, that is a shrinking and hardening of the membrane due to an increase in the degree of cross-linking when the calcium concentration was increased in the surrounding solution.

C. DISPOSITION OF COMPONENTS WITHIN THE MEMBRANE:

Proteolytic enzymes may be used to study the proteins exposed on the outside of intact erythrocytes. There appears to be more than one class of protease susceptible protein exposed on the exterior surface. Carraway and co-workers (145,146) have shown that trypsin hydrolyses only one protein on the erythrocyte exterior surface. This is the major glycoprotein of the membrane. Pronase on the other hand has been shown to cleave 2 or more proteins (117,118,122,139,146-148). The same has been shown for chymotrypsin (148). At least one of the proteins hydrolysed by pronase is responsible for the acetylcholinesterase activity of the erythrocyte membranes (117,147).

Another method of attacking the question of the topological distribution of proteins within the membrane is by the use of specific protein labeling reagents. Maddy (149) outlined the requirements for such reagents. The reagent must be selected so that it will not pass through the osmotic barrier of the membrane. This may be achieved by making the molecule sufficiently hydrophilic, either by introduction of hydrophilic groups such as sugars, or by introduction of charged groups such as the sulphonate group. Also, the reagent must react with the

membrane under physiological conditions of temperature, pH and tonicity without disrupting the cell. Finally, it must be possible to detect the label in small quantities. In essence, this means the label must be fluorescent or radioactive. Of course the reagent must also be reactive toward some part of the protein.

Maddy (149) proposed the use of 4-acetamido-4^1-isothiocyano-stilbene-2,2^1-disulphonic acid (SITS), and described its reaction with bovine erythrocytes. At that time no attempt was made to identify the reactive proteins.

Another labeling reagent which has been employed is the diazonium salt of sulphanilic acid. Berg (115) using this reagent showed that one band of the polyacrylamide gel electrophoretic pattern of the membrane proteins was reactive in intact human erythrocytes. This band was assigned a molecular weight of 140,000 on the basis of its electrophoretic mobility.

The same reagent was used by Carraway et al. (145) to show that two components were labeled in bovine erythrocytes, one was the major glycoprotein, and the other a polypeptide chain of 108,000 daltons. Carraway (145) did suggest that the 108,000 dalton component was less accesible than the glycoprotein.

Bretscher (116,139,140,150,151) has used formylmethionylsulphone-methyl phosphate (FMMP) for labeling procedures. Using this reagent, in combination with proteolytic digestion, he was able to demonstrate the labeling of two components, one a protein of 105,000 daltons, the other the major glycoprotein. He further showed (140) that both these components appeared to span the thickness of the membrane, being exposed at both surfaces. Based on the reactivity of lipid with FMMP

he also suggested (150,151) that phosphatidylethanolamine and phosphatidylserine were distributed asymmetrically in the membrane since neither was reactive in the intact ghost.

The related reagents trinitrobenzene sulphonate (TNBS) and picrylchloride (PC) have also been used for labeling of membranes (148,152,153). Bonsall and Hunt (152) described the reaction of erythrocyte membranes with TNBS. They suggested that the reagent did not penetrate the membrane. Arrotti and Garvin (153), on the other hand, showed that TNBS penetrated the membrane and could react with hemoglobin within the intact cell. Picrylchloride (153), lacking the charged sulphonate group, penetrated the membrane more rapidly than did TNBS. Both Arrotti and Garvin (153) and Steck (148) found that TNBS labeled protein components in the region of 90-100,000 daltons. Picrylchloride, on the other hand, labeled the high molecular weight bands. It was thought that the difference in reactivity might represent different pathways through the membrane. The picrylchloride, being lipophilic, was thought to diffuse through the lipid bilayer while TNBS was thought to be conveyed through the membrane by hydrophobic channels.

Schmidt-Ullrich et al. (154) have used dansylchloride to label membranes. They have shown that this reagent labels similar classes of proteins as do other reagents. This labeling pattern was maintained even though dansylchloride which is lipophilic, readily penetrated the membrane. They suggested that this observation cast some doubt on the use of small molecular labels, at least in terms of their use as vectorial probes.

Their results, however, are in contrast with those obtained using other lipid soluble reagents, notably picrylchloride and acetic anhydride. As mentioned previously, picrylchloride was found to label only the

two high molecular weight bands (153). Acetic anhydride on the other hand has been found to label all the polypeptide chains of the membrane, either in intact cells or in ghosts (155). The pattern of labeling, as well as depending on the availability of reactive groups will also depend on the amount of reagent, the rate at which it penetrates the membrane and the rate at which it reacts both with membrane components and also with intracellular components, especially hemoglobin (153).

The fact that only a limited number of components are exposed on the outer surface of intact erythrocytes is supported by the work of Phillips and Morrison (119, 156, 151). They have labeled tyrosine residues in proteins with I^{125}, using a reaction catalysed by the enzyme lactoperoxidase. This enzyme is not likely to penetrate the membrane due to its high molecular weight (75,000 daltons).

At least two enzymatic activities have been assigned to proteins exposed on the outer surface of intact erythrocytes. As mentioned previously, digestion with pronase has been shown to destroy acetylcholinesterase activity in intact cells (117,147). Herz and Kaplan (158) have also shown that this enzyme is present at or near the cell surface. Acetylcholinesterase has a molecular weight of 90-108,000 daltons (159-160). Bellhorn et al (160) have labeled acetylcholinesterase in ghosts with tritiated diisopropylfluorophosphate and found a molecular weight of 180,000 which reduced to 90,000 in the presence of sulphydryl reagents. Steck (123) has shown that a band of molecular weight of 88,000 daltons may be cross-linked by several reagents to give a dimer of 165,000 daltons and suggests that this might represent acetylcholinesterase. However,

while Bellhorn et al (160) estimated that there were less than 10^4 acetylcholinesterase sites per ghost, both Bretscher (116) and Fairbanks et al. (97) have suggested that there were 10^6 chains of the 88,000 dalton component per ghost.

Ohta et al (161) have coupled pCMB to a high molecular weight carrier (250,000 daltons). Using this reagent they have shown that there are sulphydryl groups on the exterior of the erythrocyte which are essential for the activity of the $(Na^+ + K^+)$-ATPase. Marchesi et al. (162), on the other hand, have shown that the ATPase is localized at the inner surface of the ghost. This suggests that the ATPase may in fact penetrate the membrane.

The ATPase may also be labeled with γ-^{32}P-ATP (163,164). Arruch and Fairbanks (163) reported that incubation of erythrocyte ghosts with γ-^{32}P-ATP resulted in the labeling of 3 bands on polyacrylamide gel electropherograms of the membrane dissolved with sodium dodecyl-sulphate. Of the three bands labeled only one was believed to be polypeptide in nature. This had a molecular weight of 105,000 daltons. The other bands were ATP and phospholipid.

Williams (164) has reported that a polypeptide of about 100,000 daltons could be labeled with γ-^{32}P-ATP. He also found that a high molecular weight polypeptide (220,000 daltons) was labeled. This chain corresponds to tektin α (110,111). Williams (164) suggested that the fact that one of the two tektins was enzymatically labeled with ^{32}P from γ-^{32}P-ATP may indicate that the protein was possibly operating in some energy related process such as erythrocyte shape maintenance.

Both the acetylcholinesterase and ATPase have mobilities corresponding to molecular weights of about 90,000 daltons. It has been shown that a protein of [redacted] molecular weight penetrates the membrane (117). Evidence suggests that this may be the ATPase. It is not clear if the acetylcholinesterase also penetrates the membrane.

Lipases have also been used in studies of the erythrocyte. Lankish and Vogt (165) observed that phospholipase A would not lyse normal erythrocytes. If the cells were exposed to conditions which caused them to swell they became susceptible to lysis by phospholipase A. This was taken as an indication that there was some fundamental change in the structure of the membrane during lysis in hypotonic solutions. A similar conclusion has been reached by others using phospholipase C and A_2 (166,167).

Information has also been gained with respect to the distribution of components within the plane of the membrane. Murphy (168) has shown that cholesterol is clustered around the periphery of the normal biconcave disc, especially on the convex surface of the cell.

Studies with phospholipase A_2 have shown that there are discrete areas on the cell surface which are susceptible to hydrolysis (169). These areas do not appear to coalesce and they appear to be randomly distributed.

The antigenic sites of the cell membrane have also been studied (170,171). It has been shown that both Rh_o(D) and A sites are randomly distributed over the cell surface.

D. USE OF PROTEASES FOR STUDYING MEMBRANES:

Studies of membranes using proteolytic or lipolytic enzymes may be directed toward two areas. First, enzymatic digestion may be used as a probe to study the effects of lipids on the availability of proteins to protein reagents or to proteolytic enzymes. The reverse may also be examined, that is, the effect of protein on the availability of lipids to lipases. The second area which may be investigated is the topological distribution of individual components within the membrane structure.

The use of proteolytic enzymes in studies of the surface components of the erythrocyte was discussed in section IC. The effect of low concentrations of proteolytic enzymes on ghosts has also been studied. Avruch et al. (172) have examined the effect of low levels of trypsin on the erythrocyte membrane. Digestion of erythrocyte membrane with 1 µg trypsin per ml. of ghosts resulted in the formation of small closed vesicles by a process of exocytosis. The vesicles so formed were less leaky to hexoses than were the parent ghosts. They have a particle density of 1.01, a decrease from 1.06 for normal ghosts. Such a decrease has also been noted by Okhuda et al. (173) who observed the formation of two discrete populations, with bouyant densities 1.106 and 1.125, following trypsin treatment of ghosts of density 1.149.

The vesicles prepared by Avruch et al.(172) were found to be right side out as shown by the accesibility of their sialic acid to neuraminidase. Only two of the major polypeptides, of 89,000 and 77,500 daltons, were attacked. No protein was released from the membrane under these conditions. Avruch et al. (172) also found that the onset of

glucose transport by the vesicles could be correlated with the digestion of these two membrane proteins. Extensive trypsinization (100 µg per ml. of ghosts) did not abolish this glucose transport.

Jung et al. (174) have noted a similar effect of limited proteolysis on ghosts. They found that pronase treatment which resulted in the release of up to 50% of the ghost protein did not cause ghosts to lose their glucose carrier activity. The changes in membrane structure which the limited proteolysis is causing were not clear.

Another area in which proteases may be employed is the study of the effect of lipid on the protein structure. Pasquali et al. (175) have observed that the protein of electron transport particles, isolated from mitochondria, is hydrolyzed by proteolytic enzymes to the same extent and at the same rate either in the presence or in the absence of lipid. This was taken to suggest that there was no strict interdigitation of membrane lipid and protein, as might be expected, for example, for lipo-protein subunit models.

E. OPTICAL ROTATORY DISPERSION AND CIRCULAR DICHROISM:

Membrane proteins have been studied by the techniques of optical rotatory dispersion and circular dichroism in the ultraviolet region of the spectrum. A discussion of the theory of optical rotatory dispersion and circular dichroism has been given by Levine (176).

The absorption bands of the peptide bond (observed between 195 and 250 nm) are used in the study of protein conformation. The

longest wavelength band, corresponding to the lowest energy, is due to the n-π^* transition resulting from the promotion of an electron from the non-bonding atomic orbital of an oxygen to an antibonding molecular orbital involving the oxygen, carbon and nitrogen atoms separately (see Figure 4). The band centered around 220-240 nm is characterized by a very low extinction coefficient. The π-π^* transition of the electron cloud in a π non-bonding orbital into the π^* antibonding orbital (Fig. 4) gives rise to an absorbtion band around 200 nm.

When the polypeptide chain is in an unordered conformation the optical activity is dominated by the π-π^* transition at 198 nm giving rise to an intense, negative CD band. There is a small positive band at 223 nm due to a weak n-π^* transition. When in the α-helical conformation the π-π^* transition gives rise to two strong CD bands, a negative band at 206 nm and a positive one at 192 nm. The n-π^* transition at 224 nm overlapping the negative component of the π-π^* transition gives rise to a negative minimum at 225 nm. In the β-conformation there is a positive band at 195 nm due to the π-π^* transition and a single negative band at 218 nm due to the n-π^* transition.

The ORD spectra have been discussed by Chapman and Wallach (83). In unordered polypeptide chains the π-π^* transition at 198 nm results in a large negative ORD extremum at 205 nm. In the right handed α-helical conformation the ORD spectra are characterized by a large negative band at 233 nm, a point of zero rotation at 223 nm, a shoulder near 210 nm and a positive band at 198 nm. The negative band is due primarily to the n-π^* transition. Polypeptides in the β-conformation

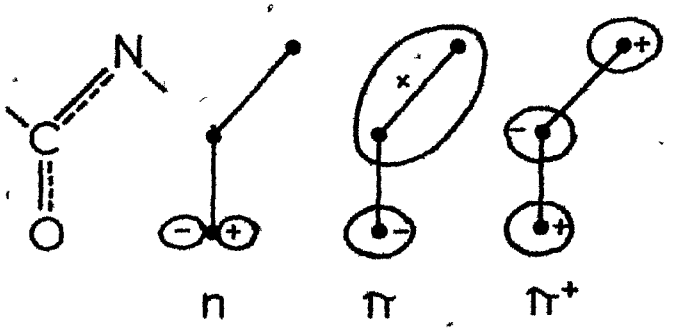


Fig. 4: Molecular orbital representations of the n-and π-orbitals of the peptide link. Only the upper lobes of the π orbitals are shown. Lower lobes are mirror images of the upper lobes in the plane of the paper but opposite in sign. From ref. (176).

show a negative peak at 230 nm, a cross over at 220 nm and a positive maxima at 205 nm.

The CD spectra obtained for suspensions of membranes were found to be similar to those of α-helical polypeptides with two exceptions (38,40,177). Although the shape closely approximated that of a right handed α-helix, the n-π* band was red shifted from 222 nm to 223-225 nm. The value of the ellipticity at the maximum at 194 nm was lower than expected.

There has been much speculation as to the cause of these anomalies in the CD spectra. Gordon et al. (177) have stated that the CD spectra can be explained as arising from proteins containing 50-60% α-helical regions with the rest of the peptide chains assuming a random coil conformation. They did not feel that the light scattering properties of membrane suspensions could explain the anomalies of the observed spectra.

The red shift of the π-π* transition has been attributed by Wallach and Zahler (28) and Urry et al. (37) to the localization of helical segments in a hydrophobic environment. Lenard and Singer (36) attributed the red shift to packing of the α-helices in parallel, a situation, analogous with aggregates of helical polypeptides.

An equal mixture of left and right handed α-helices could account for the low amplitudes and for the shape of the curve. However, according to Chapman and Wallach (83) such a mixture would not produce the red shift observed in the spectra.

Both Chapman and Wallach (83) and Gordon et al. (177) have shown that contributions to the spectrum from chromophores other than the peptide linkages are quite small. The large band width of the n-π* transition has also been attributed to lipid-protein and protein-protein interactions which are sensitive to lipid. Gordon et al (177) have shown that this band narrows after treatment with phospholipase A, lysolecithin and digitonin. They also observed a broadening of the band after treatment with phospholipase C. Such a change, however, was not observed by Glaser et al. (178).

The observed anomalies in the spectra have recently been attributed to optical artifacts. Gordon and Holzworth (38) and Gordon (39) have applied classical Mie scattering theory to the ORD and CD of red blood cell ghosts. The optical constants of the suspension were calculated from the optical constants and geometry of the suspended particles. For red blood cells, the optical constants were derived from measured optical spectra of ghosts dissolved in 0.1% sodium dodecylsulphate. The ghosts were considered to be spherical shells with a thickness of 70 Å and a radius of 3.5 μ. The red shifts and relative amplitudes of the bands at 205-210 and 220 nm were quite well reproduced by these calculations. A similar set of calculations were made for the ORD curves. As with the CD, application of Mie scattering theory resulted in a reproduction of the observed anomalies.

Mie theory was also applied to differential scattering and absorbtion of left and right circularly polarized light for the CD of

red cell ghost suspensions. The absorbtion component of the CD was flattened relative to the CD of the ghost in solutions of sodium dodecylsulphate. This flattening was most pronounced at lower wavelengths. The scattering component resembled the ORD curve. From this analysis it seemed that the red shift of the spectra could be accounted for by scattering. The effects on the relative amplitude of the negative bands was a result of the combined effects of scattering and flattening. Gordon and co-workers concluded that the observed anomalies need not be attributed to unique protein conformation, nor to interactions with neighbouring lipid or protein molecules, but that they may be directly accounted for by scattering.

Urry (40), on the other hand has attributed the anomalies to two absorption dampening effects: absorption flattening of Duysens and absorption obscuring resulting from light scattering, and to an increase in measured absorbance due to scattering of light in directions away from the phototube. These effects were thought to be operating in circular dichroism as well. They could be treated as a simple difference in suspension absorbance for left and right circularly polarized light. Therefore, in CD of optically active membrane suspensions, there would be differential absorption flattening, differential absorption obscuring and differential light scattering.

Urry (40) has described absorption flattening as being due to the shadow cast by a particle occluding particles behind it from the light path. In CD measurements this effect would be augmented by differences in particle absorption for left and right circularly polarized light.

Differential light scattering would arise because the refractive index of the particle is slightly different for left and right circularly polarized light and because light scattering depends on the square of the difference of particle and solvent refractive index.

Urry (40) has described a method for correcting CD data for optical artifacts using a pseudoreference state approach. Utilization of this technique is hampered by the fact that it requires simultaneous determination of absorption and circular dichroism using the same phototube.

Verpoorte and Smith (41) have examined the optical rotation and circular dichroism of membrane preparations. They found that the optical properties were dependant on salt concentration. Changes observed in the optical rotation and circular dichroism as a result of the addition of sodium chloride were accompanied by an increase in turbidity and a decrease in pH and viscosity of the membrane suspensions. Solubilization of membranes by sodium dodecylsulphate resulted in a lower viscosity and loss in turbidity but had little effect on optical activity. Membrane protein solutions prepared by extraction of membrane lipid with n-butanol had optical activities similar to those of salt-free membrane suspensions. Both the addition of sodium dodecylsulphate and extraction with n-butanol eliminated the effect of salt on the rotation and dichroism spectra. They proposed that salt reduced the electrostatic repulsions between phospholipid molecules favouring the formation of a more compact membrane structure with reduced hydration. The effect of

reduced hydration on CD and ORD spectra of films of poly amino acid has been demonstrated by Fasman and co-workers (249). On the other hand, Schneider and Schneider (251) have shown that hydration had no effect on the CD of films of membrane protein between 92% and 0% relative humidities. Therefore there is still some question as to the effect of hydration on the optical properties of membrane proteins.

F. FLUORESCENT PROBES:

The use of fluorescence as a tool in the study of membranes has been the subject of a number of recent reviews (176,179,180). The theory of fluorescence is discussed in these reviews.

One of the most widely used, non-covalently bound fluorescent probes is 1-anilinonaphthalene-8-sulphonate (ANS). In aqueous media ANS has a very low quantum yield of 0.004 (181). On binding to protein or membrane the quantum yield increases up to 200 fold. At the same time the wave length of the emission maximum is shifted from 525 to about 470 nm (180). The main factors involved in changes of this type are solvent viscosity, temperature, proton transfer to the excited state, the rates of intersystem crossing and internal conversion, possible inversion of the energy levels of $n-\pi^*$ and $\pi-\pi^*$ states and the polarity of the medium (148). The significance of these effects with respect to the fluorescence of ANS in membrane suspensions is not yet known with certainty (148).

McClure and Edelman (182) and Oster and Nishijima (183) have shown that the quantum yield of ANS dyes increased with increasing solvent viscosity at constant dielectric constants. Oster and Nishijima (183)

have also shown that the microscopic rather than the bulk viscosity is of importance. The viscosity effect was smaller than that produced by lowering the dielectric constant. This may be attributed to suppression of vibronic deactivation of the excited state by intramolecular motion between the phehyl and naphthyl rings (184,185).

The major effect on ANS fluorescence appeared to arise from solvation of the excited state and was related to the dielectric constant of the medium (186,187). The long wavelength band of ANS is due to a π-π^* transition. The excited state, π^*, is more polar than the ground state (188). Polar solvents therefore interact more strongly with molecules in the excited state than with those in the ground state. This excited state solvation lowers the energy of the excited state, thus decreasing the energy difference between excited and ground state. A red-shift is therefore expected as solvent polarizability increases. A strong solvent interaction with the excited state assists the vibronic dissipation of the excited state energy and results in a vibrationally excited ground state. Thus in a polar solvent ANS would be expected to have a broad emission band of low quantum yield, as was observed. The fluorescence of ANS may be used to determine the microscopic polarity of complex heterogeneous biological systems (179).

ANS interacts with erythrocyte membranes and membrane protein resulting in enhanced fluorescence (189-194). There is some question as to the location of the ANS within the membrane. The fluorescence spectra of ANS in erythrocyte membranes was found to be similar to that of ANS in lipid (195). Furthermore, the fluorescence of ANS in membranes and in lipids responded to alterations in pH and ionic strength (191, 196). These observations suggested that membrane lipids were important sites for ANS binding.

The effect of solvent quenching of ANS fluorescence has been examined. Radda and co-workers (180,197) have shown, by comparing the fluorescence of ANS in membranes suspended in water or in D_2O, that the ANS is sensitive to solvent.

The removal of phospholipid by treatment of membranes with phospholipase C was shown to result in decreased fluorescence (194,196, 198,199). While these experiments indicated that the presence of lipid significantly contributed to the binding of ANS by membranes, they did not show that lipid provided the only binding domain. In fact Weidekam et al. (194) suggested that the data supported the hypothesis that the ANS was binding to protein. Lesslauer (252) has suggested on the basis of X-ray studies that ANS may also be binding to the membrane protein. In addition, he has suggested that at higher concentrations ANS may perturb the lipid region of the membrane.

The transfer of energy from tryptophan residues of the erythrocyte membrane protein to ANS has been demonstrated (199-201). These data indicated that the average distance between ANS and tryptophan was 20-25 Å. In other words, the ANS binding sites were close to membrane protein (197).

Gulik-Krzywicki et al. (202) have studied the location of ANS in model systems. In simple lipid-water systems of the L α lamellar phase, that is of stacked and equivalent lipid lamellae separated by water layers, the ANS was embeded in the lipid leaflet, likely at or near the lipid-water interface. In lipid-protein-water systems in which the bonding was primarily electrostatic, type e, the ANS seemed to be located at the lipid-water interface, especially where the negative charges of the lipids were neutralized by positive charges of protein.

On the other hand, in lipid-protein-water systems of type h, that is stabilized by hydrophobic interactions, the ANS was located at even less polar sites than in systems of type e, possibly at areas of hydrophobic contact between protein and lipid. These possibilities are summarized in Figure 5.

Gulik-Krzywicki et al. (202) suggested that the above conclusions could only be reached with systems where the affinity of ANS for the interface between the polar and hydrophobic regions was much higher than for protein, and that the situation was likely to be much more complex with membranes or model systems containing more than one type of protein.

It therefore appears that ANS will be located at polar-apolar interfaces, probably at areas of lipid-protein, lipid-water or lipid-protein-water contacts (180).

The fluorescence of ANS is enhanced and the wavelength of the emission is shifted in the presence of membranes (189-192,194,199-201, 203,204). The interaction of ANS with membranes is sensitive both to external conditions and to the state of the membrane.

The interaction between ANS and membranes has been shown to be biphasic (192,199,203,205,206). There is a fast reaction of 1-2 seconds duration and a slow reaction with a half time of 6-8 seconds (193). The slow change has been shown to constitute about 10% of the total enhancement (192,205,206). When the membrane was disrupted by sonication only the fast reaction was observed (192). If, on the other hand, the ghosts had been resealed, as judged by the decreased rate of K^+ efflux from loaded cells, then the slow phase was increased, contributing almost 50% of the total enchancement (205,206). It was also found that in this case the rate of enhancement was slower (205,206).

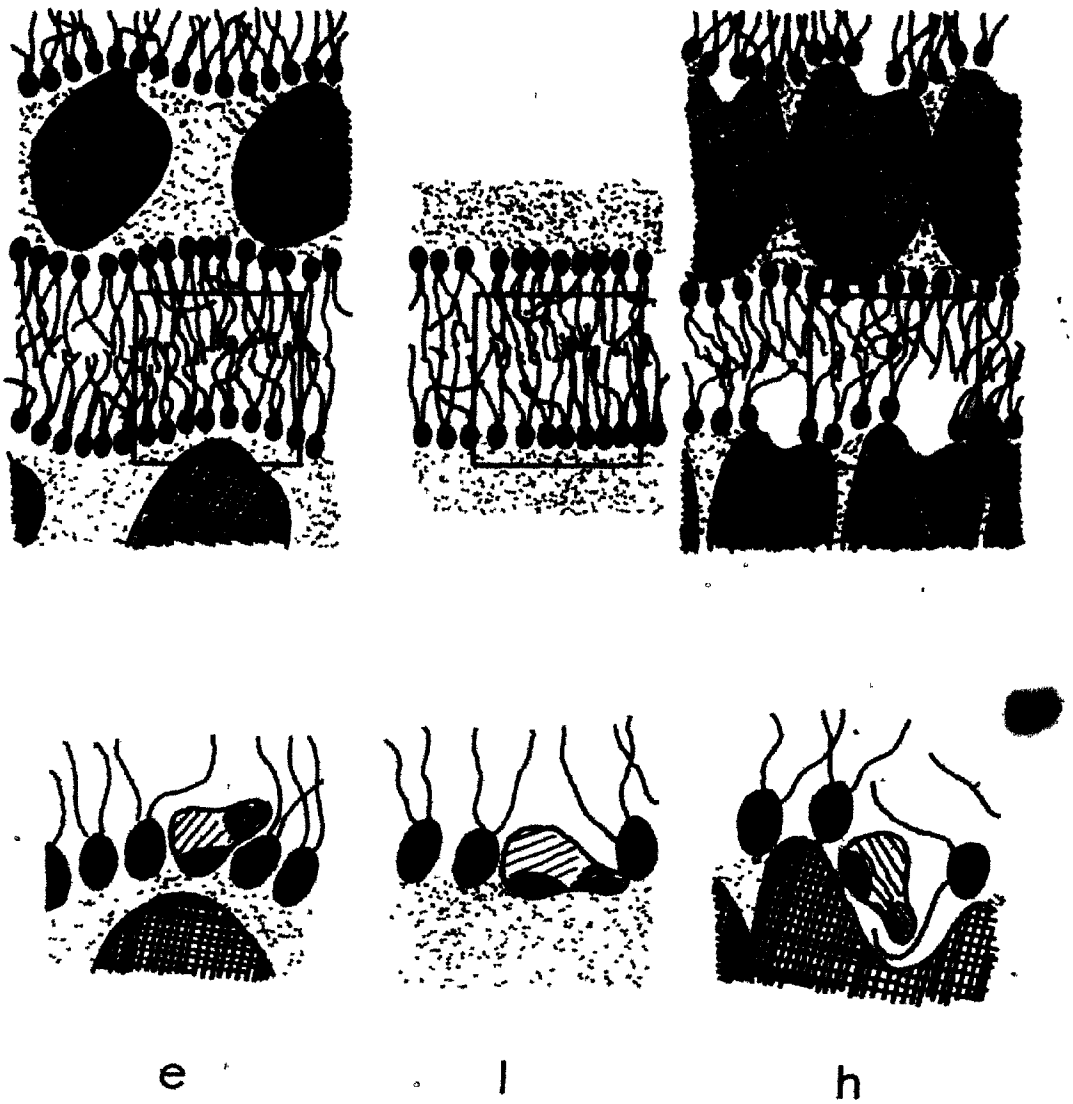


Fig. 5: Location of ANS in protein-lipid-water systems. The cross-hatched areas represent protein, the stippled areas represent water. ANS molecule is shown in the lower panels. The dark area represents the polar portion of the molecule. The phases shown are: e, lipid-protein primarily electrostatic bonding; l-lipid water; and h-lipid protein, primarily hydrophobic bonding. From ref. (202).

Fortes and Hoffman (193) have shown that added organic anions decreased the amount of ANS bound and that this ANS had contributed primarily to the slow component of the enhancement. These studies suggested that ANS was bound at polar-nonpolar regions, within the membrane, that contained charged groups as well as water (180). These sites may also be the anion binding sites of the membrane (180).

The fluorescence intensity of membrane-ANS suspensions is strongly influenced by salt concentration. Addition of salt increased the fluorescence of ANS-membrane suspensions (190). It was found that cations of different valencies increased the fluorescence in the order: tetravalent > trivalent > divalent > monovalent (190). With microsomal membranes from brain it was found that monovalent cations increased the fluorescence in the order: $NH_4^+ > Li^+ > Na^+ > Rb^+ > Cs^+$ (207). This is in the same order as the size of the non-hydrated cation. This suggested that during the process of binding to the membrane there was displacement of water molecules from the hydration shells. The increased fluorescence was attributed to increases in the amount of dye bound.

Both Lantelme and Stock (207) and Vanderkooi and Martonosi (196) have suggested that the effect of cations was not simply related to the ionic strength of the medium. It has been suggested that Debye shielding of charged groups of the membrane may be involved (208).

The pH of the medium influences the fluorescence of ANS-membrane suspensions (190,192), as does the addition of agents such as the local anaesthetic, butacaine (191).

All these observations suggest that the amount of ANS bound depends on membrane charge (180). Membrane charge, while including the number of cationic and anionic groups on the membrane surface, may also

include other areas of the membrane bearing charges such as channels (180) and intrinsic groups associated with specific components of the membrane (193,199,209).

The binding of ANS to the membrane appears to be complex. While some reports (190,191) have determined binding constants and numbers of binding sites by Klotz type plots (212) or by Scatchard plots (213), others (193,196) have shown that these plots are linear over only a narrow range of membrane or ANS concentrations. This would suggest that there may be a heterogeneity of membrane regions with respect to their affinity for ANS. This is also supported by the observation that at low ANS concentations the quantum yield of bound dye is increased by the addition of added ions (193). It was suggested that under these conditions only regions with high affinities for ANS would be involved in the binding and that as a result differences would be observed in the average quantum yields (193).

Even though the binding of ANS to membranes is quite complex, Radda and Vanderkooi (180) felt that fluorescent probes of this type were suited for detecting changes in membrane structure.

SECTION II: MATERIALS AND METHODS

A. MATERIALS:

All chemicals used were of reagent grade unless otherwise specified. Water used in all experiments was distilled and deionized.

n-Butanol (Fischer Spectranalyzed) was redistilled prior to use, the fraction distilling between 118-119^{o} C being collected.

Trypsin used had been pretreated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone to inhibit chymotryptic activity, and was obtained in this form from Worthington Biochemicals. Lima bean trypsin inhibitor was also obtained from Worthington.

Carboxypeptidase B, treated with diisopropylfluorophosphate was also obtained from Worthington.

ANS was obtained as the sodium salt, technical grade, from Eastman Kodak Company. It was recrystallized as described by Weber and Young (232).

Polyamide sheets were obtained from the Chen-Chin Trading Company, No. 75, Sec. 1, Hankow St., Taipei, Taiwan.

TNBS was obtained from Pierce Chemicals.

Anti-M serum was obtained from Dade, anti-N from Hyland.

Dansylchloride was obtained from Sigma Chemicals.

B. METHODS:

i. Preparation of Erythrocyte Membranes:

Normal human O positive erythrocytes were used in all experiments. The cells were washed three times in 0.9% sodium chloride using a clinical centrifuge (International Clinical Centrifuge, Model CL, International Equipment Company) at maximum speed. The supernatant and buffy coat were removed.

For the preparation of membranes, washed cells were made up to a hematocrit of 30% with saline. The cell suspension was treated by the method of Langley and Axell (212) as described by Smith (213). The suspension was mixed with seven volumes of buffer I (Table IV). The membranes were separated from the hemolysate by centrifugation at 10,000 x g for 15 minutes in a Sorval RC2B centrifuge at 4^0. The membrane pellet was then washed once with buffer II and three times with buffer III. The membranes were dialysed overnight against distilled water. Following dialysis the membranes were collected by centrifugation at 20,000 x g for 30 minutes. The membrane pellet thus obtained was suspended in distilled water to the desired concentration.

TABLE IV

BUFFERS FOR PREPARATION OF MEMBRANES

Buffer I	Buffer II	Buffer III
5×10^{-5} M EDTA	9.6×10^{-2} M Tris	4.8×10^{-2} M Tris
3.7×10^{-3} M Na_2HPO_4	2.0×10^{-2} M NaCl	1.114×10^{-2} M NaCl
1.3×10^{-3} M KH_2PO_4	1.0×10^{-3} M disodium EDTA	1.03×10^{-3} M disodium EDTA
1.0×10^{-3} M NaCl		

All buffers to pH 7.2

ii: Preparation of Membrane Protein Solution:

Erythrocyte membrane protein solution was prepared by a modification of the procedure of Maddy (214) as described by Smith (213). Any precipitate which formed at the butanol-water interface was discarded. The aqueous phase was dialysed against distilled water rather than against 1 mM phosphate, pH 7.2, as used by Smith (213).

iii: Protein:

Protein was determined by the procedure of Lowry et al. (215) using bovine serum albumin as standard.

iv. Carbohydrate:

Carbohydrate (neutral sugars) were determined by the phenol-sulphuric acid method as described by Dubois et al. (216) using glucose as standard.

v. Sialic Acid:

Sialic acid was determined by the thiobarbituric acid method described by Warren (217) following hydrolysis of the sample by 0.1 N sulphuric acid, at 80^{0} for one hour. N-acetylneuraminic acid was used as standard. When membrane samples were analysed, absorbance at both 549 and 532 nm were measured to correct for interference by lipid (217).

vi. Amino Acids:

Protein samples were hydrolysed in 6 N hydrochloric acid, under nitrogen, at 110^{0} for 24 hours. Analyses of hydrolysates were performed with a Beckman Model 120 C automatic amino acid analyser with the system of Moore and Stein (218).

Separate samples were oxidized with performic acid according to Hirs (219) and assayed for cysteic acid and methionine sulphone following acid hydrolysis.

vii. Lipid:

The lipid content of membrane samples was determined gravimetrically following the extraction procedure as described by Reed et al (76).

viii. Hemoglobin:

Hemoglobin was determined by the benzidine method as described by Wainwright (220).

ix. Phosphorus:

Phosphorous was determined by the method of Bartlett (221)

x. N-terminal Amino Acids:

N-terminal amino acids were qualitatively determined by chromatography of dansyl derivatives, prepared according to Gross and Labouesse (222), on polyamide sheets as described by Woods and Wang (223).

xi. Anti MN Activity:

The ability of samples to inhibit the agglutination of fresh human O positive, MN erythrocytes by anti-M and anti-N sera was determined following preincubation of 0.1 ml. sample with 0.1 ml. of the antisera at room temperature for 30 minutes. The sample-antisera solutions were then mixed with an equal volume of erythrocytes, 0.2% in phosphate buffered saline pH 7.2. After 5 minutes the samples were observed for signs of agglutination.

xii. Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel electrophoresis was performed as described by Neville (224) using gels containing 11% (w/v) acrylamide and 0.1% (w/v) methylenebisacrylamide. Following electrophoresis, gels were stained for protein with Coomassie Blue and for carbohydrate by a periodic acid Schiff stain, both as described by Fairbanks et al. (97). Gels were scanned using a Joyce-Loebel scanner.

xiii. Peptide Mapping:

Samples were applied to sheets of Whatman 3 MM chromatography paper. Ascending chromatography was carried out with butanol:acetic acid: water (4:1:5). High voltage electrophoresis was carried out using a Savant cold plate apparatus (Savant Instruments Ltd.) using pyridine acetate buffer pH 3.6 at 6000 volts. The peptide maps were stained with cadmium acetate-ninhydrin as described by Heathcoate and Washington (225).

xiv. Turbidity:

Turbidity of membrane suspensions was estimated from the optical density at 600 nm using a Zeiss PM-QII spectrophotometer, as described by Verpoorte and Smith (41).

xv. Viscosity:

The reduced viscosity of samples was determined from flow times measured in a Ubelhode viscosity pipette as described by Verpoorte and Smith (41). Measurements were performed at 26^{0}. No corrections for end effects or kinetic effects were applied.

xvi. Optical Rotatory Dispersion and Circular Dichroism:

The optical rotation and circular dichroism of samples were measured on a Cary model 6001 recording spectropolarimeter with circular dichroism attachment. Measurements were performed at 26°. The rotation and ellipticities were calculated on the basis of dry weight of membrane. The units of specific rotation and ellipticity are in degrees cm^2 per gram. No attempts were made to correct the spectra for optical artifacts since various theories are not in accord (see discussion, reference (38)). The spectropolarimeter was not equipped to read absorbance as is required to perform some corrections (40).

xvii. Assay of Tryptic Activity:

Trypsin activity was assayed by the spectrophotometric method described by Schwert and Takanka (235), based on the hydrolysis of benzoyl-arginine ethyl ester.

xviii. Sedimentation and Diffusion Studies:

Measurements of sedimentation and diffusion coefficients were carried out with a Spinco Model E ultracentrifuge equipped with an electronic speed control. Sedimentation velocity measurements were carried out at 56,000 rpm with a double sector cell with a synthetic boundary centerpiece at 20°. Sedimentation coefficients were converted to the value that would be obtained in pure water at 20° using corrections from International Critical Tables (226).

Diffusion coefficients were estimated according to the method described by Schachman (227) using a double sector cell with a synthetic boundary

centerpiece. The sample was centrifuged at 6,000 rpm. Photographs of the schlieren pattern were taken at 8 minute intervals. Diffusion coefficients were calculated by the height area method and by the second moments method.

xix. Column Chromatography:

Samples were chromatographed on columns of Sephadex G-100 (1.5 x 90 cm) as described by the manufacturers (246). The eluting buffer was 0.1 M ammonium bicarbonate. Fractions were collected using an LKB fraction collector (RadiRac, LKB Produkter, AB Stockholm Bromma-1, Sweden). The optical density of fractions was measured at 280 nm with a Zeiss PMQII spectrophotometer. Aliquots were analysed for carbohydrate by the procedure of Dubois et al. (216).

xx. Trypsin Digestion:

1) pH stat: Trypsin digestion of membrane protein preparations was carried out at the desired pH using a ratio of protein to enzyme of 100:1 (w/w). The pH was maintained by the addition of 0.1 N NaOH, controlled by a Radiometer Titrator II equipped with a syringe burette type SBU 1a, the pH being measured with a Radiometer pH meter, model 26, using a combination electrode.

2) Buffer digestions: Tryptic digestions were also performed in phosphate buffer as described by Winzler et al. (228). If digestions were to be stopped by trypsin inhibitor, a three fold excess was added to the sample suspension.

xxi. Extent of Digestion:

1) Alkali consumption: The extent of digestion of samples by trypsin, using the pH stat, was calculated from the amount of base

consumed. A pK of 7.5 was assumed for the new α-amino groups created by hydrolysis of a peptide bond. As titrations were carried out at pH 7.0, this meant that 0.24 H^+ were released for each bond cleaved. The total number of lysine and arginine residues was determined by amino acid analysis of an aliquot of the sample.

2) Determination of α-amino groups: α-Amino groups were determined by reaction with TNBS as described by Satake et al (229). Reaction was carried out at pH 8.5 in 0.2 M borate buffer. The molar extinction coefficient of the TNP-amino acid was assumed to be 1.0×10^{-4} (229). The number of new amino groups created was determined and the extent of hydrolysis was calculated from the amino acid composition of an aliquot of sample.

3) *Determination of C-terminal lysine and arginine:* C-terminal lysine and arginine were determined following hydrolysis with carboxypeptidase B as described by Ambler (230). Digestion was carried out at pH 7.0-7.4 for at least 5 hours at 37^0. Digestion was terminated by addition of trichloroacetic acid. Excess trichloroacetic acid was removed, after the protein precipitate had been removed, by extraction with ether. Following lyophylization, lysine and arginine were determined with the automatic amino acid analyser. The extent of digestion was estimated from the increase in C-terminal lysine and arginine following trypsin digestion and from the total lysine and arginine content determined on an aliquot of sample.

xxii. Cyanogen Bromide Treatment:

Samples were treated with cyanogen bromide as described by Gros (231). The extent of digestion by cyanogen bromide was determined from the decrease in methionine and from the formation of homoserine. After acid digestion homoserine lactone was converted to homoserine by heating in 0.1 M pyridine-acetic acid at pH 6.5 for one hour at 110°. Homoserine was determined on an amino acid analyser by the method of Schroeder et al. (247).

xxiii. Fluorescence Measurements:

Fluorescence measurements were performed with an Aminco Bowman Spectrofluorimeter. In most experiments excitation was at 370-375 nm and emission at 470 nm. In energy transfer experiments excitation was at about 285 nm, emission at 470 nm. A 1 cm. cell was used in all experiments. All samples were buffered at pH 7.0-7.4 with 0.01 M tris-hydrochloric acid buffer.

Apparent binding constants were determined from double reciprocal plots as described by Gomperts et al. (207).

The binding of ANS to membranes was also determined as described by Collier (204). Membranes were exposed to ANS, at room temperature for at least 30 minutes, after which time they were centrifuged at 20,000 x g for one hour in a Sorval RC2-B centrifuge. The optical density at 350 nm of the supernatant was determined using a Zeiss PMQII spectrophotometer. The amount of bound ANS was determined as the difference between the observed optical density and that of a control sample to which no membranes had been added. The molar extinction coefficient of ANS was assumed to be 4950 (232).

SECTION III: EXPERIMENTAL DATA

A. INTRODUCTION

Proteolytic enzymes have been employed in several studies of cell membranes. Little is known about either the extent of proteolytic digestion of membrane proteins or of its effect on their physical properties (175). With this in mind, the tryptic digestion of human erythrocyte membrane proteins has been examined. The extent of digestion of erythrocyte membrane proteins in aqueous solution, in membranes or in intact erythrocytes was estimated by the following methods: determination of new α-amino groups by alkali titration and by reaction with trinitrobenzene sulphonate and by the increase in C-terminal lysine and arginine released by carboxypeptidase B.

The effect of trypsin digestion on the composition and on some physical properties of the membrane were studied. Properties examined included viscosity, optical rotatory dispersion and circular dichroism. The effect of trypsin digestion on the interaction of membranes with 1-anilinonaphthalene-8-sulphonate was also investigated.

B. DATA

I. Characterization of Membranes and of Membrane Protein Solution

a) Results

Membranes were prepared as indicated in Section II, B.

Examination of the membranes, following dialysis against distilled water, showed that they had retained their discoid shape.

Analyses of membrane suspensions and of membrane protein solutions, for protein, neutral carbohydrate and sialic acid are given in Table V. Amino acid analyses are presented in Table VI.

TABLE V

COMPOSITION OF HUMAN ERYTHROCYTE MEMBRANE AND PROTEIN SOLUTION

	Protein[a]	Carbohydrate[a]	Sialic Acid[a]
Membrane	1.90	0.093	0.116
Protein Solution	1.16	0.040	0.074

a) values are reported as mg. per ml. packed erythrocytes. Values are mean of 6 determinations.

b) Discussion

Membranes prepared from one ml. of packed erythrocytes were found to contain 1.90 mg. protein, 116 µg. sialic acid and 93 µg. neutral carbohydrate. Hoogeveen et al. (95) have isolated a similar membrane preparation which contained 1.84 mg. protein per ml. packed cells, with 160 µg. sialic acid and 101 µg. neutral carbohydrate.

TABLE VI

AMINO ACID COMPOSITION OF HUMAN ERYTHROCYTE MEMBRANE AND OF PROTEIN SOLUTION

	Membrane*	Protein Solution*
Lysine	5.16	5.36
Histidine	2.12	2.77
Arginine	4.94	5.10
Aspartic acid	8.75	7.80
Threonine	5.18	5.40
Serine	7.68	6.43
Glutamic acid	12.34	12.34
Proline	4.88	5.01
Glycine	6.73	6.93
Alanine	8.11	7.99
Valine	6.71	7.17
Methionine	2.44	2.38
Isoleucine	5.06	5.25
Leucine	12.42	12.24
Tyrosine	2.51	2.43
Phenylalanine	4.30	4.70
Cysteic acid	0.66	0.70
Tryptophan	not determined	not determined

*values reported as mole%, are the mean of 3 determinations.

The aqueous protein solution contained 1.16 mg. protein, 40 µg. neutral carbohydrate, and 74 µg. sialic acid per ml. packed cells. Thus only 65% of the membrane protein was recovered in the aqueous phase following the extraction of membrane lipids with n-butanol. Smith (213) has reported that 85% of the protein could be recovered. Part of this difference may be accounted for by the fact that material which accumulated at the butanol-water interface was discarded, rather than being re-extracted. An additional factor contributing to the lower recovery of protein could be the method used to obtain salt free membrane suspensions. Smith (213) washed the membranes by high speed centrifugation. She observed, by phase-contrast microscopy, that following this treatment the membranes were fragmented. Salt free membranes prepared by dialysis, on the other hand still retained the discoid shape. It is possible that the fragmentation of the membranes, observed following high speed centrifugation, accounted for the higher recovery of protein in the aqueous phase following butanol treatment.

The amino acid compositions of the membranes and of the protein solution derived from them were not significantly different, as shown in Table VI. The compositions were similar to those reported by Smith (213).

II. Extent of Digestion of Human Erythrocyte Membrane Protein by Trypsin

a) Results

The data on the extents of digestion of membrane protein are summarized in Table VII.

TABLE VII

EXTENT OF DIGESTION OF HUMAN ERYTHROCYTE MEMBRANE PROTEINS BY TRYPSIN

Protein	Method	Extent of Digestion (%)
Aqueous protein solution	Alkali titration, pH 7.0	69.2
	pH 8.0	71.0
	Amino group determination	97.0
Isolated membranes	Alkali titration, pH 7.0	64.8
	Amino group determination	107.0
	C-terminal determination	74.0
Intact erythrocytes	Amino group determination,	
	no trypsin inhibitor	6.8
	with trypsin inhibitor	4.8
	C-terminal determination,	
	no trypsin inhibitor	10.2
	with trypsin inhibitor	1.6

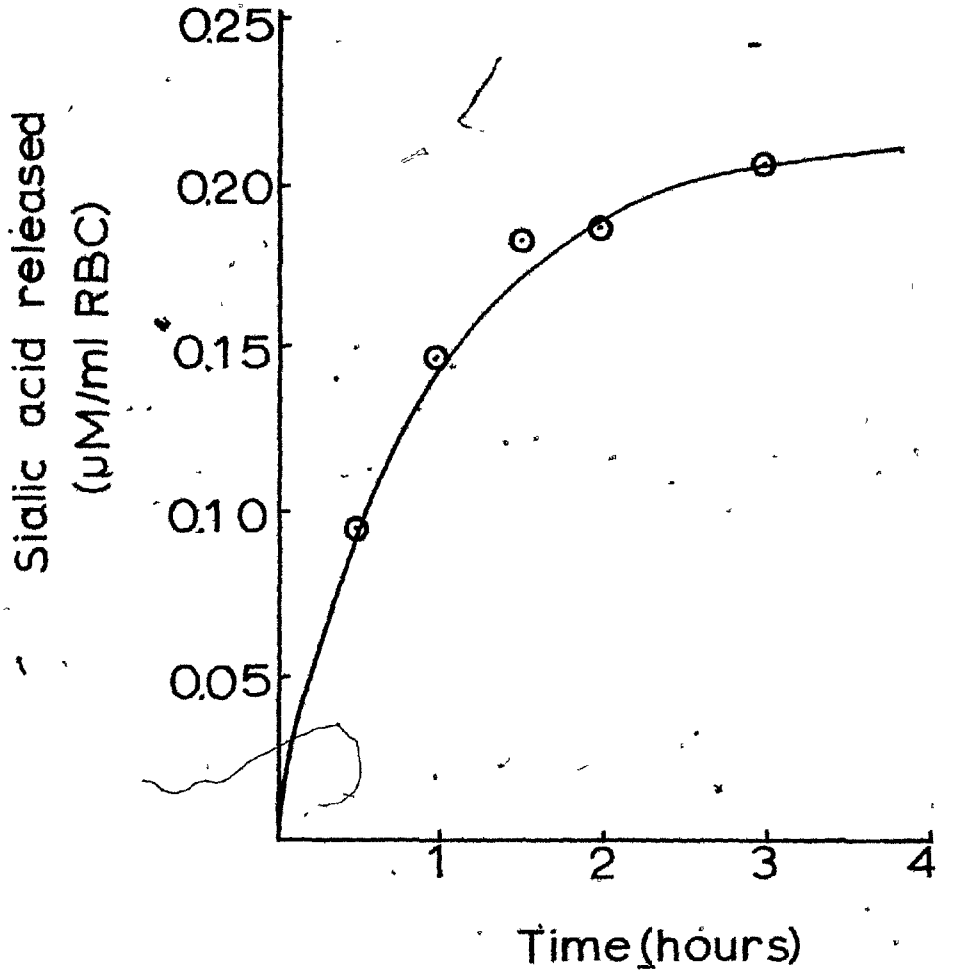


Fig. 6: Release of sialic acid during digestion of human erythrocytes by trypsin (0.25 mg. trypsin per ml. packed cells at 25°).

(i) Extent of digestion of isolated membrane protein

1. by alkali titration

Aqueous solutions of human erythrocyte membrane protein were prepared by butanol extraction of membrane lipid (Section II, Bii). The digestion of the protein solution by trypsin was followed with a pH stat at pH 7.0. The extent of digestion was estimated from the amount of alkali consumed and from the total number of lysine and arginine residues. It was found that 69.2% (mean of 6 determinations) of the bonds were cleaved. This figure was based on the assumption that the pK of a newly created α-amino group was 7.5 (234).

2. by determination of amino groups

The extent of digestion of aqueous membrane protein solution was also determined from the increase in α-amino groups. These were measured colorimetrically following reaction with 2,4,6-trinitrobenzene sulphonate. By this method it was determined that 97% (n = 3) of the total peptide bonds involving lysine and arginine residues were hydrolysed.

(ii) Effects of pretreatments and of digestion conditions on the extent of digestion

Attempts were made to increase the extent of digestion of aqueous solutions of membrane proteins, as measured with the pH stat. Pretreatment of membrane protein with 0.1 N sulphuric acid at 80° for one hour, which removed essentially all the sialic acid residues, did not increase the extent of digestion over that observed for non-treated protein. Pretreatment with cyanogen bromide which resulted in the loss of 70-75% of methionine residues also did not increase the extent of digestion.

Extraction of the protein, prior to digestion, with chloroform:methanol: concentrated hydrochloric acid (200:100:1), which removed most of the remaining phospholipid (5), also had no effect on the extent of digestion.

The digestion proceeded to the same extent as normal in the presence of 2M urea, whereas in 25% propanol only 25% of the bonds were hydrolysed, and in the presence of 0.02-0.2 M Ca^{++} the digestion was completely inhibited. When the digestion was followed with the pH stat at pH 8.0 it was found that 71% of the bonds were cleaved.

(iii) Extent of digestion of proteins in isolated membranes

1. by alkali titration

It was found, using the pH stat, that 64.8% (n = 6) of lysine and arginine bonds were hydrolysed by trypsin at pH 7.0.

2. by determination of amino groups

The extent of digestion of proteins in membranes was determined by reaction of amino groups with trinitrobenzene sulphonate, as for isolated protein. It was found that 107% (n = 3) of the possible bonds were hydrolysed.

3. by determination of the increase in C-terminal lysine and arginine.

The extent of digestion of proteins in isolated membrane by trypsin was estimated by determining C-terminal lysine and arginine residues released by carboxypeptidase B. From the increase in C-terminal lysine and arginine following trypsin treatment, it was estimated that 74% (n = 3) of the bonds were hydrolysed.

(iv) Extent of digestion of membrane proteins in intact erythrocytes

It was difficult to follow the digestion of intact erythrocytes, in physiological saline, with the pH stat. The rate of consumption of alkali in the absence of added trypsin was high and several hours were required before this approached zero. At this time the erythrocytes hemolysed after only brief exposure to trypsin. For this reason, digestion of proteins of intact erythrocytes was not followed with the pH stat but rather was performed in 0.1 M phosphate buffer at pH 7.0. Under these conditions less than 1% of the cells had hemolysed, as estimated from the release of hemoglobin, after 6 hours exposure to trypsin. The progress of digestion was followed by release of sialic acid (Fig. 6) and by release of peptide material. The digestion was essentially complete within 3 hours. The addition of more trypsin at that time did not result in release of more material from the erythrocyte.

1. by determination of amino groups

The extent of digestion of proteins in intact erythrocytes was estimated using the reaction of amino groups with trinitrobenzenesulphonate. Following trypsin treatment the supernatant and the cells were separated and each was reacted with trinitrobenzene sulphonate. No hemolysis of the cells was observed after reaction with trinitrobenzene sulphonate. Membranes were prepared from these cells. The amounts of α amino groups in each fraction were determined and the extent of digestion was estimated.

It was found that when trypsin inhibitor was added to the cell suspension to stop digestion, 4.8% ($n = 3$) of the susceptible bonds in the membrane were hydrolysed. On the other hand, if no inhibitor was

used and the cells were only washed with saline, then 6.8% of the bonds were hydrolysed.

2. by determination of C-terminal lysine and arginine

Membranes were prepared from trypsin treated and control erythrocytes and each was digested with carboxypeptidase B. The lysine and arginine residues released were measured. The same measurements were performed on the peptides released from the cell during the trypsin treatment. It was found that 1.6% (n = 3) of the total peptide bonds involving lysine and arginine were hydrolysed in membranes of intact cells. This value was only obtained when trypsin inhibitor was added to the digest before preparation of the membranes. In other experiments in which the trypsin treated cells were washed with saline, it was found that 10.2% (n = 3) of the bonds were hydrolysed.

b) Discussion

The isolated proteins of the human erythrocyte membrane have been found to be susceptible to trypsin digestion. When digestion was followed with the pH stat, at pH 7.0, it was found that about 69% of the peptide bonds were hydrolysed. The value of 69% was based on the assumption that the pK of the new α-amino groups was 7.5 (234). On this basis, 0.24 hydrogen ions would be released for each peptide bond hydrolysed. On the other hand, at pH 8.0, 0.72 hydrogen ions would have been titrated for each peptide bond broken. However, at this pH the rate of consumption of alkali in the absence of added trypsin was high, making estimation of the amount of alkali used less reliable than at pH 7.0. Estimates at both pH 7.0 and 8.0 were, however, similar being 69% and 71% respectively.

Estimates of the extent of digestion of membrane protein by trypsin by the colorimetric determination of amino groups following reaction with trinitrobenzene sulphonate were complicated by the fact that this reagent also reacts with other amino groups, especially amino groups of lipids. Bonsall and Hunt (152) have shown that 58% of phospholipid amine groups in ghosts react with trinitrobenzene sulphonate while one-third of the protein amino groups react. Solubilization of the membrane with Triton X-100 resulted in the reaction of all the phospholipid amine groups, while there was essentially no change in the reactivity of the protein amino groups.

It is quite possible that many amino groups are not reactive in the protein prior to tryptic digestion but they become exposed during digestion and then react with trinitrobenzene sulphonate, resulting in an overestimation of the amount of new α-amino groups. This may account for the difference between the estimates based on alkali titration and on reactivity with trinitrobenzene sulphonate.

The determination of C-terminal lysine and arginine by hydrolysis with carboxypeptidase B should give a reliable estimate of the extent of digestion of membrane protein. The estimate may be low if some of the C-terminal lysine and arginine are not susceptible to hydrolysis by carboxypeptidase B. However, the estimates based on the determination of C-terminal amino acids agreed with those estimated from alkali consumption.

The proteins of the human erythrocyte membrane do not appear to be especially resistant to tryptic hydrolysis. About 70% of the peptide bonds in the isolated membrane, involving lysine or arginine, are susceptible to tryptic hydrolysis. This value could not be increased by altering the conditions of digestion or by various pretreatments of the protein.

The proteins appear to be equally susceptible to tryptic hydrolysis either when part of the membrane, or when isolated in aqueous solution. The presence of the lipid of the membrane doesn't appear to hinder the action of trypsin. A similar observation has been made by Pasquali et al. (175) for mitochondrial membrane protein. The rates of digestion and extents of hydrolysis of mitochondrial particles and of lipid depleted particles by both trypsin and pronase were similar (175). These authors also reported that digestion by pronase was not enhanced by the addition of detergent or by denaturation of the protein.

It thus appears that in at least two membrane systems the presence of lipid does not hinder the action of proteolytic enzymes on membrane proteins. The fact that lipid does not protect proteins from digestion by trypsin would seem to rule out the possibility of close association of protein chains with individual phospholipid molecules, as would be expected in lipoproteins. Also, since the removal of lipid by butanol extraction does not alter the extent of digestion of membrane proteins it could be suggested that this extraction had little effect on the protein structure. Such a conclusion has been reached by others (236,237).

Conversly, the membrane proteins in intact erythrocytes are relatively inert to trypsin. This has been observed by others (117,118,146), from the effects of proteolytic digestion as revealed by gel electrophoresis. It is felt (117,118, 146) that only two proteins are cleaved by trypsin in the intact erythrocyte, these being the major glycoprotein and a protein of 100,000 daltons molecular weight. This is brought about

by the hydrolysis of 1 to 4% of the susceptible bonds of the membrane protein.

It has been found that addition of trypsin inhibitor to trypsin treated erythrocytes prior to lysis, resulted in a reduction of the observed digestion from 8-10% to 1-4% of the potentially susceptible bonds in the membrane. This suggests that in the absence of trypsin inhibitor, trypsin is not completely removed by washing the cells and that it could still be active during the preparation of the membranes. This possibility was examined further (Section III, p.69).

III. Effect of Trypsin Digestion on Chemical Composition of Membranes

a) Results

The various membrane preparations and digests analysed are outlined below (see Figure 7).

Analyses of membranes prepared from erythrocytes treated with trypsin are given in Table VIII. No trypsin inhibitor was used in these experiments.

TABLE VIII

COMPOSITION OF MEMBRANES: EFFECT OF TRYPSIN DIGESTION OF ERYTHROCYTES*

	M-1 (control membranes)	M-2 (membranes from trypsin treated cells)
Protein	1.90	2.15
Carbohydrate	0.093	0.120
Sialic Acid	0.116	0.058
Lipid	1.46	1.48
Hemoglobin	0.0057	0.1368

*All values are mean of 6 determinations except lipid and hemoglobin which are of three and are given in mg. per ml. packed cells.

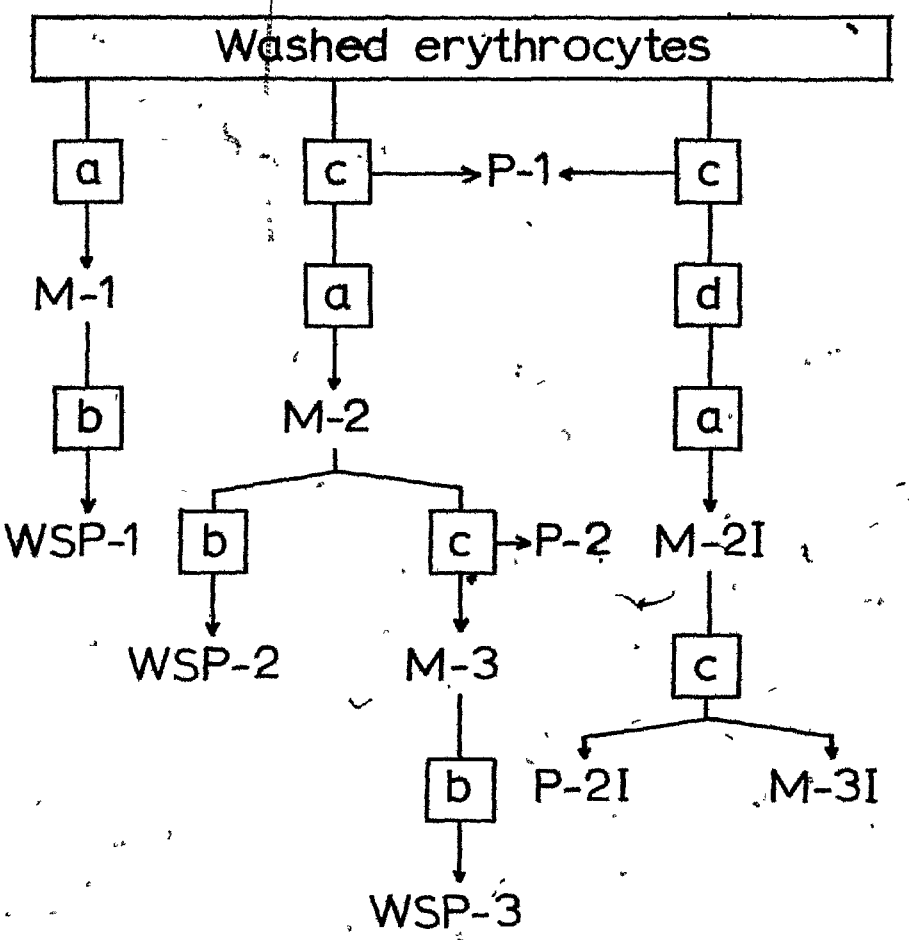


Fig. 7: Scheme of treatment of erythrocytes, where a represents preparation of membranes, b represents butanol extraction, c represents trypsin digestion and d represents treatment with trypsin inhibitor.

Digestion of intact erythrocytes resulted in a loss of 50% of the sialic acid. These membranes contained only 0.058 mg. per ml. packed cells while the normal content was 0.116 mg. per ml. packed cells. At the same time, the amount of protein and carbohydrate in these membranes was increased. They contained 2.15 mg. protein per ml. of packed cells as compared to 1.90 mg. per ml. packed cells for normal membranes. This increase was partly due to an increase in the hemoglobin content. Membranes prepared from trypsin treated erythrocytes contained 136.8 µg. hemoglobin per ml. packed cells, a 24 fold increase over that of normal membranes which contained 5.7 µg. per ml. packed cells.

Digestion of intact erythrocytes did not result in loss of lipid. Membranes prepared from trypsin treated erythrocytes contained 1.46 mg. of lipid per ml. packed cells while normal membranes were found to contain 1.48 mg. per ml. packed cells.

The protein solutions prepared from normal membranes and from membranes of trypsin treated erythrocytes were also compared. These results are shown in Table IX.

TABLE IX

COMPOSITION OF AQUEOUS PROTEIN SOLUTIONS*

	WSP-1 (Protein from normal membranes)	WSP-2 (Prepared from membranes of trypsin treated erythrocytes)
Protein	1.16	0.89
Carbohydrate	0.040	0.027
Sialic Acid	0.074	0.025

*Values are mean of 6 determinations, reported as mg./ml. packed cells.

In contrast to the results obtained with membranes, the protein solutions prepared from membranes from trypsin treated erythrocytes did not contain greater amounts of protein than expected. The extra material which had been isolated with membranes M-2 might either have precipitated at the water-butanol interface, or have remained associated with lipid in the butanol phase during the butanol extraction.

It has previously been shown that trypsin digests about 10.2% of the susceptible bonds in the membrane of intact erythrocytes. However, a value of 1.6% was obtained when the erythrocytes were washed with trypsin inhibitor prior to membrane preparation. The increased number of bonds broken when the cells were not washed with trypsin inhibitor suggested that erythrocytes had bound trypsin which continued to digest protein during preparation of the membranes. Therefore, the tryptic activity of saline washes of erythrocytes, of the hemolysis buffer and of the subsequent buffer washes, was determined. Of the total tryptic activity added to the erythrocyte suspension only 62% was recovered in the saline solutions used to wash the erythrocytes. Thus 40% of the activity, or about 100 µg. trypsin per ml. packed cells remained bound to the erythrocytes. Of this bound trypsin 74% was recovered in the buffer after hemolysis. This left 10% of the total activity still attached to the membrane. A large amount of this activity was recovered in the first buffer used to wash the ghosts. No detectable tryptic activity was found in the subsequent buffers used to wash the membranes.

To inhibit the bound trypsin, lima bean trypsin inhibitor was added to the erythrocyte suspension at the end of the digestion period.

The cells were then washed with saline and membranes were prepared in the usual manner. Membranes prepared in this manner are compared with normal membranes in Table X. The hemoglobin contents of the membrane preparations are given in Table XI.

TABLE X

COMPOSITION OF MEMBRANES: EFFECT OF TRYPSIN INHIBITOR*

	Normal Membranes	Membranes From Trypsin Treated Erythrocytes	
	M-1	M-2 (no inhibitor)	M-2I (with inhibitor)
Protein	1.90	2.15	1.65
Carbohydrate	0.093	0.120	0.078
Sialic Acid	0.116	0.058	0.081

*Values are mg. per ml. packed cells and are the mean of 6 determinations except for M-2I which are the mean of 3 determinations.

Membranes prepared from cells washed with trypsin inhibitor after the trypsin digestion was completed contained about twice the normal amounts of hemoglobin, 0.12% compared to 0.06%. Similar preparations in which trypsin inhibitor was not used contained 1.4% hemoglobin, about 24 times the normal amount. The further digestion of any of these membranes by trypsin resulted in the release of hemoglobin.

TABLE XI

HEMOGLOBIN CONTENTS OF MEMBRANES*

	% dry weight	µg. per ml. packed cells
M-1 (normal membranes)	0.06	5.67
M-2 (membranes from cells treated with trypsin)	1.40	136.80
M-2I (membranes from cells treated with trypsin then with trypsin inhibitor)	0.12	10.08
M-4 (M-1, treated with trypsin)	0.08	4.59
M-3 (M-2, treated with trypsin)	0.14	6.03
M-3I (M-2I treated with trypsin)	0.06	2.97

*Values are the mean of 3 determinations

The effect of trypsin inhibitor on the composition of membranes from trypsin treated cells was noticeable (Table X). Instead of an increase in protein from 1.90 to 2.15 mg. per ml. packed cells, this value decreased to 1.65 mg. per ml. packed cells. This represents a reduction of about 13.2% in protein. The amounts of carbohydrate and sialic acid also decreased, the former from 0.093 to 0.078 mg. per ml. packed cells, the latter from 0.116 to 0.081 mg. per ml. packed cells. The increase previously seen in the carbohydrate content of membranes prepared from trypsin treated erythrocytes where no trypsin inhibitor

was used, was not evident when inhibitor was added. Also, the decrease in sialic acid content was less when trypsin inhibitor was added, than in its absence.

As well as resulting in a loss of hemoglobin as shown in Table XI, the digestion of any of the membranes by trypsin resulted in loss of more protein, carbohydrate and sialic acid as shown in Table XII.

TABLE XII

EFFECT OF TRYPSIN DIGESTION OF MEMBRANES ON COMPOSITIONS[a]

	Protein	Carbohydrate	Sialic Acid	Lipid[c]
M-1 (normal membranes)[b]	1.90	0.093	0.116	1.46
M-4 (M-1, treated with trypsin)[b]	1.21	0.059	0.040	1.25
M-3 (membranes from trypsin treated erythrocytes, treated with trypsin)[b]	1.25	0.097	0.038	1.48
M-3I (membranes, from trypsin treated erythrocytes washed with trypsin inhibitor, treated with trypsin)[c]	1.13	0.064	0.035	

a) all values are mg. per ml. packed cells.

b) mean 6 determinations.

c) mean 3 determinations.

In all cases the sialic acid content of membranes was reduced by trypsin treatment to about 30% of the amount usually found in membranes. The amount of carbohydrate was also reduced by trypsin treatment to about 65% of the normal amount, except when the membranes were prepared from erythrocytes which had been treated with trypsin but not with trypsin inhibitor. In this case the amount of carbohydrate was slightly greater than normal, 0.097 mg. per ml. packed cells as compared to 0.093 mg. per ml. packed cells. This increased amount of carbohydrate was a reflection of the already increased carbohydrate content of the membranes, prepared from trypsin treated erythrocytes without the use of inhibitor, which had 0.120 mg. per ml. packed cells as was shown in Table XI.

Digestion of intact erythrocytes had little effect on the lipid content of the membranes. Normal membranes contained 1.46 mg. lipid per ml. packed cells while membranes from trypsin treated cells contained 1.48 mg. per ml. packed cells. On the other hand, digestion of membranes resulted in a substantial loss of lipid, these membranes containing only 1.25 mg. lipid per ml. of packed cells. This represented a loss of 14.4% of the total lipids.

The peptides, P-1, which were released during the digestion of intact erythrocytes, and P-2, which were released during the digestion of membranes prepared from the trypsin treated erythrocytes were analysed (Table XIII).

TABLE XIII

COMPOSITION OF RELEASED PEPTIDES*

	P - 1	P - 2
Protein	0.18	0.47
Carbohydrate	0.014	0.013
Sialic Acid	0.055	0.017

*Average of 6 determinations, reported as mg. per ml. packed cells.

Both sialic acid and carbohydrate were released in P-2, even though these peptides were obtained from membranes prepared from erythrocytes which had been treated with trypsin to completion. That the digestion of the erythrocytes had gone to completion was shown by the failure of added trypsin to effect the release of additional material.

Both P-1 and P-2 were tested for their ability to inhibit the agglutination of erythrocytes by anti-M and anti-N sera. Results are presented in Table XIV.

P-1 at concentrations of 1.2 to 2.5 mg. protein per ml. was able to inhibit the agglutination of erythrocytes by anti-M serum. All dilutions of P-1 tested (0.6 to 9.8 mg. per ml.) were able to inhibit the agglutination of erythrocytes by anti-N serum. P-2 was not as effective as P-1. The agglutination of erythrocytes by anti-M serum

TABLE XIV

INHIBITION OF AGGLUTINATION OF ERYTHROCYTES BY ANTI-M AND ANTI-N SERA

		Dilution Tested				
		Stock	1:1	1:4	1:8	1:16
P-1	M	-	-	+	++	+++
	N	-	-	-	-	-
P-2	M	++	+++	+++	+++	+++
	N	-	++	+++	+++	+++

- indicates no agglutination; +, ++, +++ indicate increasing agglutination.

P-1: 9.8 mg. protein/ml.; 0.14 mg. carbohydrate/ml. of stock.

P-2: 1.9 mg. protein/ml.; 0.01 mg. carbohydrate/ml. of stock.

was only partially inhibited by the most concentrated solution of P-2, 1.9 mg. per ml. Furthermore, only the most concentrated solutions of P-2, 0.95 and 1.9 mg. per ml., were able to inhibit the agglutination of erythrocytes by anti-N serum.

A summary of the analyses of the various membrane preparations is given in Table XV.

TABLE XV

ANALYSES OF MEMBRANE, MEMBRANE FRAGMENTS, MEMBRANE PROTEIN SOLUTIONS AND DIGESTS*

	Protein	Carbohydrate	Sialic Acid	Lipid
M-1	1.90	0.093	0.116	1.46
M-2	2.15	0.120	0.050	1.48
M-3	1.25	0.097	0.038	
M-2I	1.65	0.078	0.081	
M-3I	1.13	0.064	0.035	
M-4	1.21	0.059	0.040	1.25
WSP-1	1.16	0.040	0.074	
WSP-2	0.89	0.027	0.025	
WSP-3	0.38	0.017	0.008	
P-1	0.18	0.014	0.055	
P-2	0.47	0.013	0.017	

*All values are mg. per ml. packed cells and are the mean of 6 determinations except for M-2I and M-3I which are the mean of 3 determinations.

b) Discussion

Membranes prepared from erythrocytes which had been digested to completion with trypsin were found to contain elevated levels of protein and carbohydrate while sialic acid levels were reduced by 50% as compared to membranes from non-treated erythrocytes. A large part of the increased protein content could be attributed to increased hemoglobin content. If, on the other hand, trypsin inhibitor was added to the

erythrocyte suspension at the end of the digestion period, the membranes from these cells did not contain elevated levels of protein or carbohydrate. The loss of sialic acid was also less than observed when no trypsin inhibitor was used.

That trypsin digestion of intact erythrocytes leads to the retention of hemoglobin has been noted previously (238), though no explanation was offered.

Weed et al. (239) have observed that digestion of erythrocytes with neuraminidase resulted in the isolation of membranes with increased hemoglobin contents. The removal of sialic acid, however, cannot be the major factor in the increase in hemoglobin observed here, because when trypsin inhibitor is added, much less hemoglobin is retained in the membranes even though they have lost 30% of their sialic acid.

The observation that addition of trypsin inhibitor prevented the increase in hemoglobin content of the membranes suggested that the enzyme was able to act on the membrane during or following hemolysis. It was found that after the first hemolysis step, the membranes still contained 700 µg. of the 250 µg. of trypsin added per ml. packed cells. Digestion of ghosts by lower concentrations of trypsin than this have been shown (172,174) to result in the production of vesicles with enhanced capacity for glucose. It is possible that the digestion of the membranes by remaining trypsin is resulting in the formation of similar vesicles which retain hemoglobin. These vesicles would have to be more stable to hypotonic buffers than are normal erythrocytes as the subsequent buffer washes failed to remove the hemoglobin. However, as opposed to intact erythrocytes, the proteins of these vesicles must still be susceptible to hydrolysis by trypsin, as digestion of M-2 released protein, carbohydrate, sialic acid and hemoglobin.

The reduction in sialic acid content of membranes by trypsin digestion of intact erythrocytes was not as great when trypsin inhibitor was added to stop the digestion as compared to digestions where none was added. In the former case the sialic acid content was reduced by 30% while in the latter it was reduced by 50%. The loss in sialic acid observed when no trypsin inhibitor was used was similar to that reported by Carraway et al. (145) who reported that a total of 60% of the sialic acid was lost. They observed that of the total amount lost, half was recovered from the supernatant of the digestion while half appeared to be lost during the preparation of the membrane. They suggested that the sialoglycopeptides produced by trypsin cleavage were differentially released from the membrane, 30% during the digestion of the intact cell, 30% during membrane preparation and 40% not released. In view of the results presented here, it seems likely that the 30% of the total sialic acid lost during the preparation of the membranes was actually lost due to the action of bound trypsin.

Further digestion of membranes prepared from trypsin treated erythrocytes still resulted in the loss of additional sialic acid and carbohydrate in fraction P-2. The peptides in this fraction had a similar, though less potent, ability to inhibit the agglutination of human erythrocytes by anti-M and anti-N sera, indicating their similar nature. This finding, plus the observation of Eylar et al. (240) that all sialic acid is on the exterior of the erythrocytes, suggested that at least some of the material of P-2 was derived from the outer surface of the membrane. This occurred even though the digestion of the cells from

which these membranes were isolated had proceeded to completion as indicated by the failure of added trypsin to release more sialic acid. This may be explained in either of two ways. First, digestion of the isolated membrane from the inner surface could produce structural changes exposing additional peptide bonds on the outer surface, or secondly, the process of hemolysis could produce changes in the membrane structure resulting in the exposure of more external peptide bonds to trypsin. This latter possibility is supported by the observations of other workers (165-167) which indicate profound changes in the structure of the membrane during hemolysis.

Marchesi et al. (128) have described the structure of glycophorin, the major glycoprotein of the human erythrocyte membrane. Glycophorin is known to have at least one peptide bond that is resistant to trypsin in the intact erythrocyte. It is quite possible that this is the bond being hydrolysed in M-2, resulting in the release of peptide material into fraction P-2. If this is the case the released glycopeptide is the β-peptide of Marchesi (see Fig. 3). It has recently been shown (241) that this peptide has similar antigenic activity as the other peptides designated α_1.

Tryptic digestion of the isolated erythrocyte membrane resulted in the release of 14% of the membrane lipid. Okhuda et al. (172) have reported that no phospholipid or cholesterol was released during the tryptic digestion of ghosts prepared by the Dodge method. (242) The differences in results may be due to the differing methods of membrane preparation. The lipid released here may be lipid which is closely associated

with protein, rather than being in a lipid bilayer. The existance of such a lipid fraction has been suggested by Jost et al.(243) for membranous cytochrome oxidase.

IV. Effect of trypsin digestion on aqueous protein solutions

a) Results

Aqueous protein solutions were prepared by n-butanol extraction of normal membranes, M-1, membranes, M-2, prepared from erythrocytes treated with trypsin without the use of trypsin inhibitor, and of membranes, M-3, prepared by digestion of M-2 with trypsin. These protein solutions were subsequently digested with trypsin, at pH 7.0 with the pH stat. The digests were examined by gel filtration on a Sephadex G-100 column (1.5 x 90 cm.). Typical elution profiles are shown in Fig. 8. The digest could be divided into four fractions, A, B, C, D.

i) Digest of protein solution of normal membranes.

The elution profile of the tryptic digest of the aqueous protein solution, WSP-1 prepared from normal erythrocyte membranes is shown in Figure 8a. The results of chemical analyses are given in Tables XVI and XVII.

1) Fraction A:

Fraction A eluted at the void volume of the Sephadex G-100 column. It was found that a fraction of similar chromatographic behaviour could be isolated by centrifugation of the total digest of WSP-1 at 12,000 g. for 15 minutes. This removed essentially all material which eluted at this position from the digest. About 20% of the dry weight of the digest was sedimented in this manner.

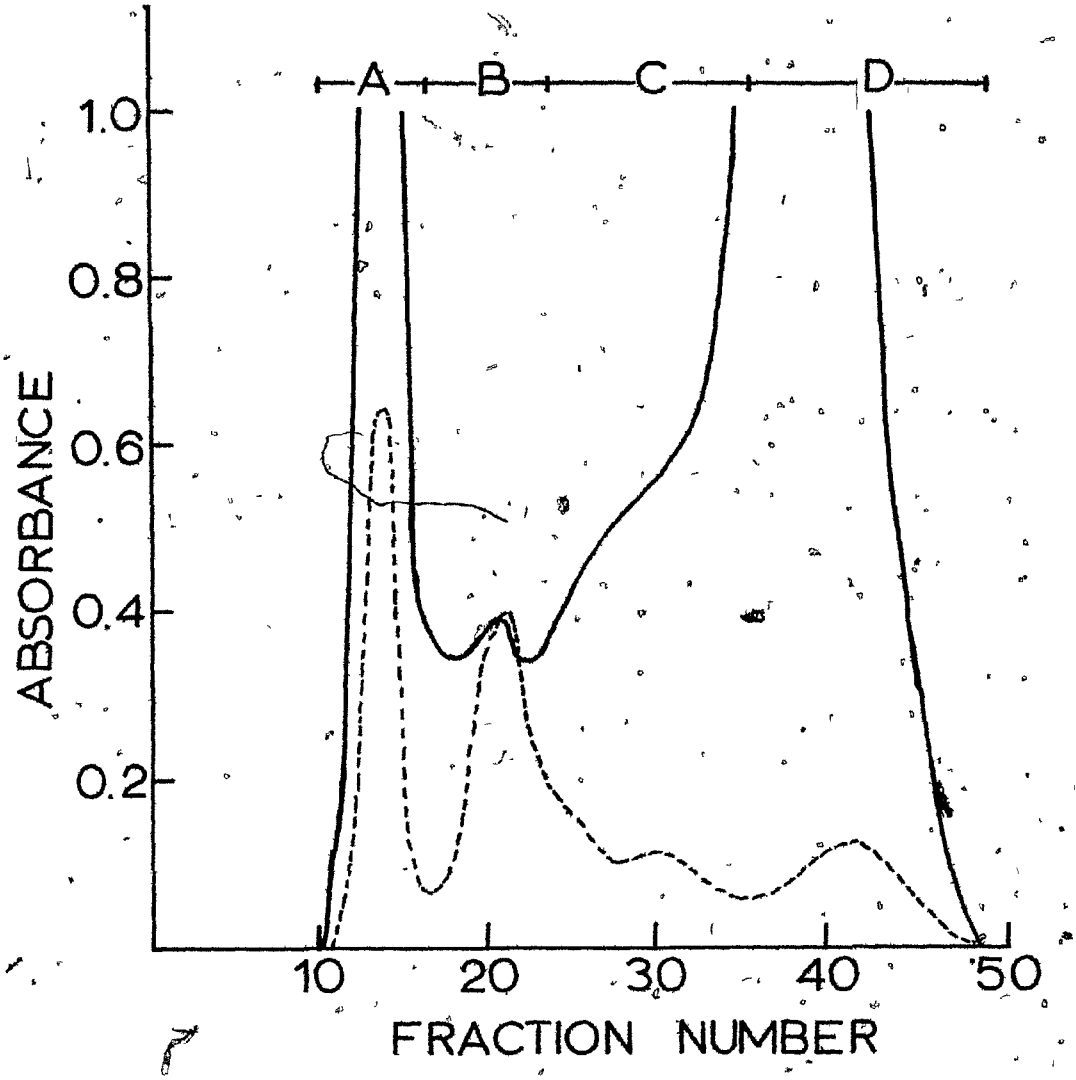
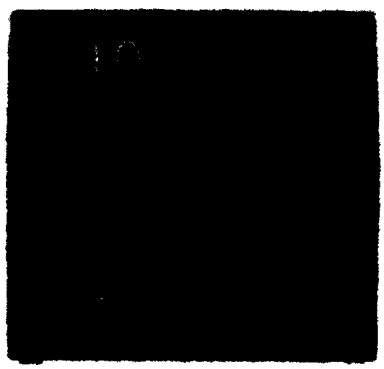


Fig. 8a: Elution profile of WSP-1 following chromatography on a Sephadex G-100 column (1.5 x 90 cm.) developed with 0.1 M ammonium bicarbonate. The solid line represents absorbance at 280 nm, the broken line absorbance developed with the Dubois reaction for neutral sugars.

2 2

OF/DE



AEC-4374

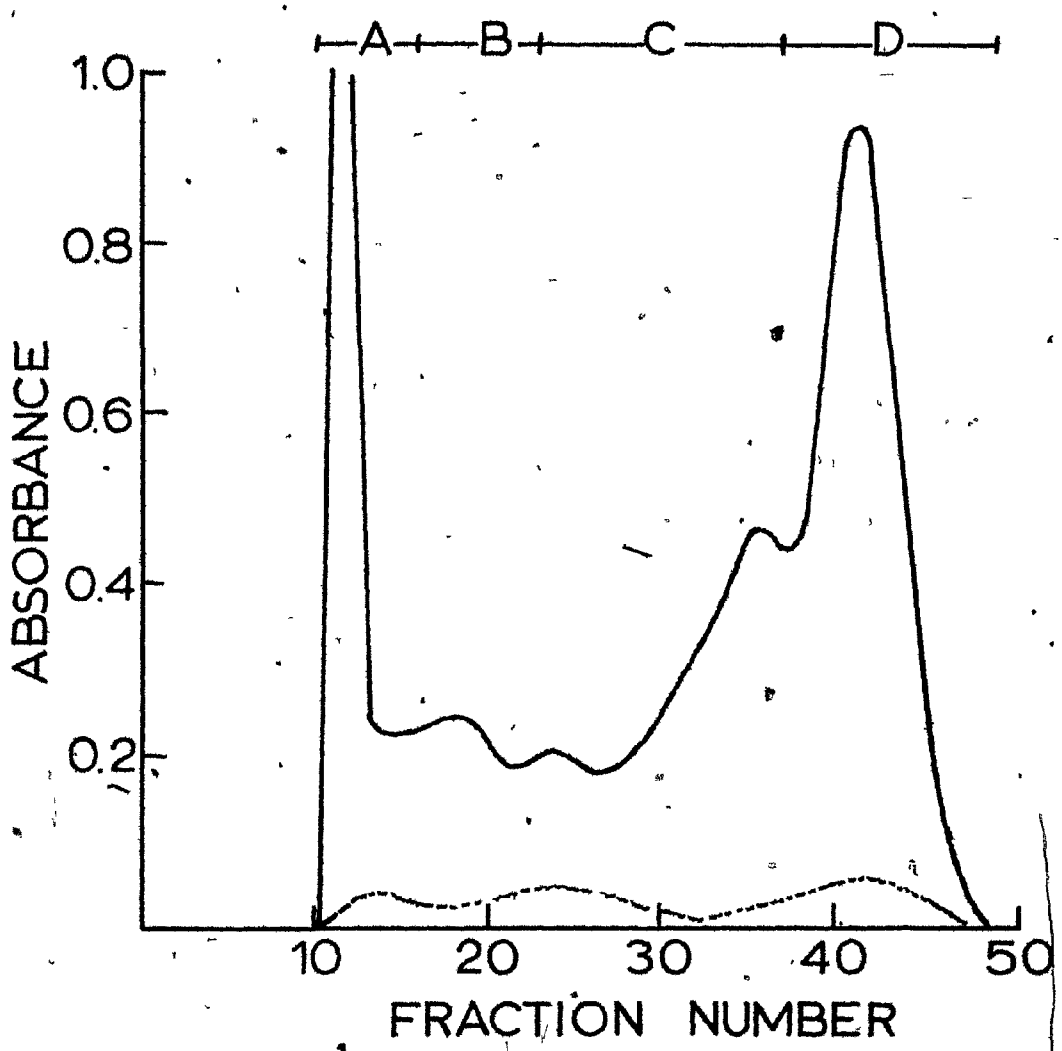


Fig. 8b: Elution profile of WSP-2 following chromatography on a Sephadex G-100 column (1.5 x 90 cm.) developed with 0.1 M ammonium bicarbonate. The solid line represents absorbance at 280 nm, the broken line absorbance developed with the Dubois reaction for neutral sugars.

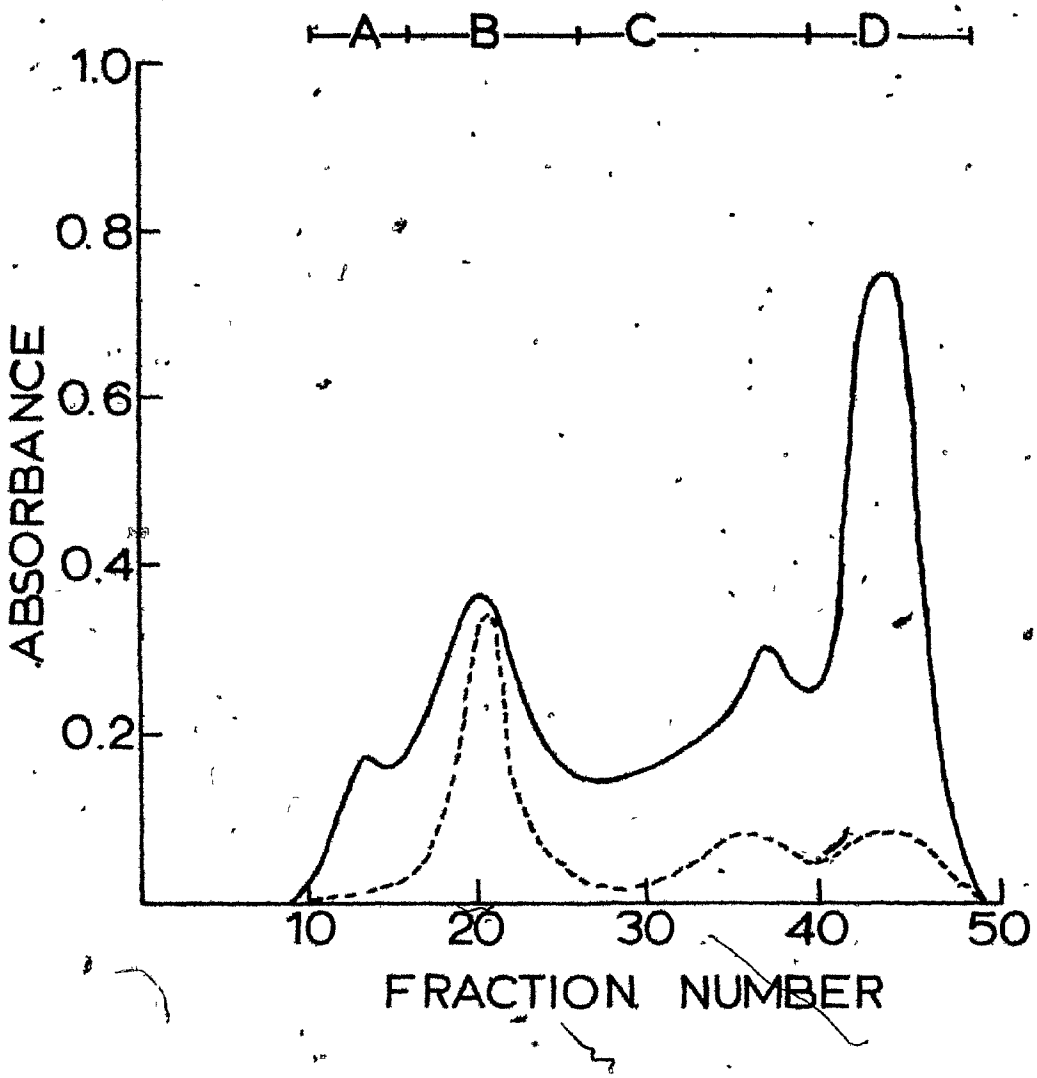


Fig. 8c: Elution profile of P-1 following chromatography on a Sephadex G-100 column (1.5 x 90 cm.) developed with 0.1 M ammonium bicarbonate. The solid line represents absorbance at 280 nm, the broken line absorbance developed with the Dubois reaction for neutral sugars.

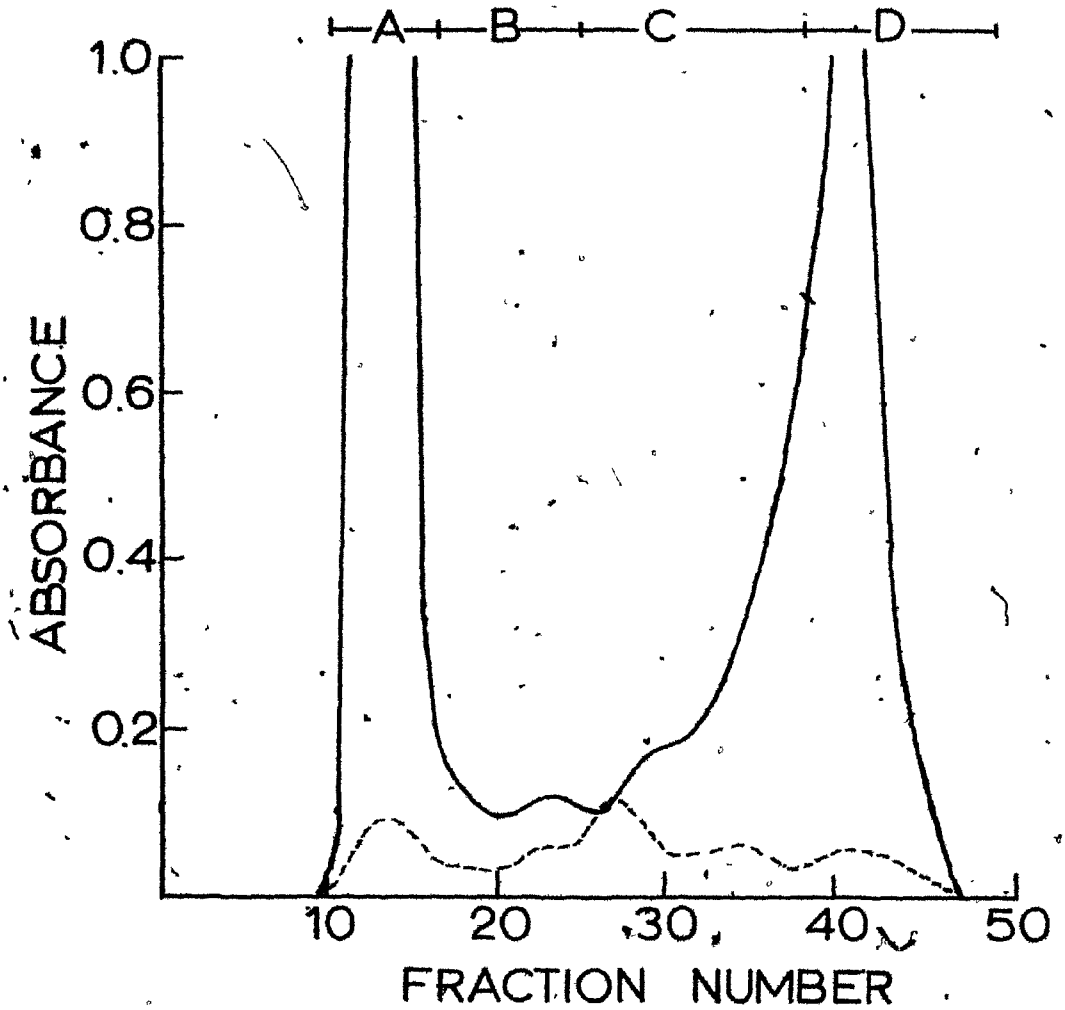


Fig. 8d: Elution profile of P-2 following chromatography on a Sephadex G-100 column (1.5 x 90 cm.) developed with 0.1 M ammonium bicarbonate. The solid line represents absorbance at 280 nm, the broken line absorbance developed with the Dubois reaction for neutral sugars.

TABLE XVI

ANALYSES OF FRACTIONS FROM SEPHADEX G-100 CHROMATOGRAPHY

		Protein		Carbohydrate		Sialic Acid	
		1	2	1	2	1	2
WSP-1	A	.385	20.3	.0126	13.5	.0115	9.9
	B	.096	5.1	.0104	11.2	.0608	52.4
	C	.406	21.4	.0154	16.6	.0176	15.2
	D	.357	18.8	.0064	6.9	.0026	2.2
P-1	A	.012	0.6	.0009	1.0	.0047	4.1
	B	.044	2.3	.0097	10.4	.0462	39.8
	C	.084	4.4	.0030	3.2	.0041	3.5
	D	.030	1.6	.0019	2.0	.0016	1.4
WSP-2	A	.296	15.6	.0098	10.5	.0070	6.0
	B	.063	3.3	.0050	5.4	.0098	8.4
	C	.237	12.5	.0038	4.1	.0069	5.9
	D	.220	11.6	.0018	1.9	.0011	0.9
P-2	A	.073	3.8	.0032	3.4	.0035	3.0
	B	.081	4.3	.0024	2.6	.0045	3.9
	C	.223	11.7	.0057	6.1	.0070	6.0
	D	.123	6.5	.0024	2.6	.0014	1.2

1) mg./ml. packed red blood cells.

2) % total in membrane M-1.

TABLE XVII

AMINO ACID ANALYSIS OF VARIOUS MEMBRANE PROTEIN FRACTIONS

	Amino Acid Composition (mole %)						
	WSP-1	WSP-2	WSP-3	A	B	C	D
Lys.	5.36	5.50	4.18	3.87	4.47	7.82	6.54
His.	2.77	2.42	2.02	1.89	4.58	2.38	1.35
Arg.	5.10	4.70	3.50	4.03	4.11	4.22	7.67
Asp.	7.80	8.54	8.06	7.07	10.22	10.59	9.36
Thr.	5.40	5.04	5.03	4.98	16.92	5.32	4.70
Ser.	6.43	5.90	6.44	5.22	18.21	5.56	6.47
Glu.	12.34	12.74	11.66	9.49	9.28	17.54	14.92
Pro.	5.01	4.99	4.95	4.88	4.23	4.82	2.89
Gly.	6.93	6.94	7.63	7.79	4.47	6.45	7.22
Ala.	7.99	8.20	8.22	7.63	6.11	8.80	8.57
Val.	7.17	7.35	7.70	8.82	6.35	6.65	5.68
Met.	2.38	2.31	2.59	1.35	0.12	1.43	0.83
Ile.	5.25	5.31	5.99	7.90	3.17	3.95	4.59
Leu.	12.24	12.18	13.07	14.74	3.76	10.76	12.41
Tyr.	2.43	2.56	2.66	2.17	3.06	1.81	2.71
Phe.	7.41	4.93	5.70	6.50	0.59	2.59	4.10
Cya.	0.70	0.40	0.59	1.67	0.35	0.36	-

A-D: Fractions from Sephadex G-100 chromatography of WSP-1.

Once fraction A had been lyophylized it could not be redissolved in aqueous solution. A suspension of Fraction A was not solubilized by exposure to trypsin, pepsin or pronase, nor did it seem to be digested by pronase in the presence of 0.1% sodium dodecylsulphate as shown by the lack of alkali consumption with the pH stat. On the other hand, fraction A was susceptible to attack by cyanogen bromide, 70% of its methionine residues being destroyed.

The material of fraction A, when studied with the ultracentrifuge prior to freeze-drying, was very heterogeneous. The aqueous solution, at low salt concentration, showed a peak with a sedimentation coefficient of 60S which increased to 76S in the presence of 1M sodium chloride. Addition of formic acid to aqueous solutions of fraction A reduced the sedimentation coefficient to 30S in 30% formic acid and to 10S in 70% formic acid. In 4M guanidine hydrochloride the sedimentation coefficient was 20S. Following freeze-drying, fraction A was poorly soluble in guanidine hydrochloride, even at concentrations of 6M.

Fraction A was analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. A single band was observed which moved very near the buffer front. This corresponded to a molecular weight of 15,000 or less.

Examination of fraction A by high voltage electrophoresis and paper chromatography revealed 7 to 10 components although most of these showed extensive tailing.

The amino acid composition of fraction A (Table XVII) showed it to contain an exceptionally high percentage of apolar amino acids, (244)

which accounted for over 60 mole%. N-terminal amino acids were found to include isoleucine, leucine, phenylalanine, valine, glycine, arginine, histidine, glutamic acid and threonine.

Analysis of the total digest of WSP-1, showed it to contain 0.12% of its dry weight as phosphorus. Of this, 67% was recovered in fraction A. Up to 90% of the phosphorus of fraction A could be extracted with chloroform:methanol:concentrated hydrochloric acid (200:100:1).

2) Fraction B:

This fraction contained at least 50% of the total sialic acid of the membrane. Nevertheless, 57% of the dry weight of this fraction was protein, 6% neutral carbohydrate and 36% sialic acid.

Pooled material of fraction B was chromatographed a second time on a Sephadex G-100 column (1.5 x 90 cm.) and eluted as a single peak. When examined in the ultracentrifuge it appeared to be a homogenous material with a sedimentation coefficient of 1.70S and a diffusion coefficient of 8.60×10^{-7} cm^2 sec^{-1}. Assuming a partial specific volume of 0.63, this corresponds to a molecular weight of about 13,000 daltons.

Despite the apparent homogeneity in the ultracentrifuge, this material gave three bands after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate.

Analyses of the amino acid composition (Table XVII) showed that 35% of the total amino acids were accounted for by threonine and serine, while there was a low amount of sulphur containing amino acids. About 32% of the residues were apolar. No N-terminal amino acids could be detected.

This material was found to inhibit the agglutination of erythrocytes by both anti-M and anti-N sera.

3) Fractions C and D:

Peaks C and D, which were distinguished by their carbohydrate contents, could not be separated by measuring the optical density at 280 nm. The distribution of carbohydrate in C and D varied more between different membrane preparations than that of A and B. In most experiments sialic acid was not detected in peak D, although this material did contain neutral carbohydrate.

Many N-terminal amino acids were detected in peak C-D, including isoleucine, leucine, phenylalanine, valine, glycine, glutamic acid, serine, threonine, arginine and histidine.

ii) Digests of protein solutions of membranes of trypsin treated erythrocytes.

Aqueous protein solutions from trypsin treated erythrocytes (WSP-2) and from similar membranes digested with trypsin (WSP-3) were examined in the same way as aqueous protein solutions of normal membranes (WSP-1). The WSP-3 became insoluble after freeze-drying as did WSP-1 and WSP-2. However, WSP-3 did not redissolve on exposure to trypsin as did the others. No uptake of alkali was observed with the pH stat, at pH 7.0, indicating that WSP-3 was not digested by trypsin. WSP-3 was not examined chromatographically as were WSP-2 and WSP-1.

A typical elution profile of a digest of WSP-2 is shown in Figure 8b. The chemical analyses of this fraction are given in Tables XVI and XVII. The chromatographic pattern of the peptides released during the digestion of intact cells, P-1, and of membranes prepared from these cells, P-2, are also presented in Figure 8c,d.

b) Discussion

Fraction A appeared to be of high molecular weight as shown by its behaviour on the Sephadex G-100 column, and by its behaviour in the ultracentrifuge. It was, however, obviously an aggregate of a large number of small peptides, because it contained many N-terminal amino acids, showed several spots on peptide maps and yet gave only one fast moving band following electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulphate. That the material was aggregated was also shown by the effect of various solvents on its sedimentation coefficient. Aggregation might be expected in aqueous solution, due to the large percentage of apolar amino acids present.

Palmer and Verpoorte (24) have reported that the human erythrocyte membrane contains about 0.03% phosphoprotein phosphorus. The amount of non-extractable phosphorus in fraction A is sufficient to account for 50% of this phosphoprotein phosphorus.

At least 90% of the peptides of fraction A remained associated with the membrane lipid. Very little of this material was found in the peptide fraction P-1, obtained by digestion of intact erythrocytes. Some material of the peptide fraction P-2 chromatographed at the position of fraction A. However, this material represented only about 10% of the total fraction A obtained from WSP-1.

It has recently been suggested (54) that intrinsic membrane proteins are bimodal in nature (see Figure 1). These proteins have a predominantly hydrophobic domain which is oriented toward and interacts

with the lipid bilayer. This portion, in effect, is assumed to be dissolved in the lipid bilayer. It has also been suggested (244) that intrinsic membrane proteins are characterized by a higher percentage than usual of apolar amino acid residues. Because of the high percentage of apolar amino acids found in fraction A and also because of its association with the membrane lipids, it seems likely that these peptides may be derived from the hydrophobic domain of intrinsic membrane proteins.

The material of peak B is mostly released into fraction P-1. This suggests that these peptides are located on the exterior of the membrane as might be expected from their sialic acid content.

The majority of the components in C and D are released in P-2 which suggests that they are mainly associated with the inner surface of the membrane, with the possible exception of those containing carbohydrate. These peptides have a lower content of apolar amino acids than usual. It is possible then that these peptides are derived from the hydrophilic portion of bimodal, intrinsic membrane proteins, or that they are derived from extrinsic membrane proteins.

V. Effect of trypsin digestion on gel electrophoretic patterns

a) Results

The effect of trypsin digestion of intact erythrocytes was examined by polyacrylamide gel electrophoresis of membrane proteins in the presence of sodium dodecylsulphate. Normal membranes (M-1), membranes prepared from trypsin treated erythrocytes either with (M-2I) or without (M-2) the use of trypsin inhibitor, and the corresponding membranes digested with trypsin (M-4, M-3I and M-3) were examined. A

schematic diagram of the separation obtained is shown in Figure 9, while densitometer tracings are shown in Figure 10. The major bands stained by Coomassie Blue were numbered consecutively starting from the origin. The same numbering was used for the other patterns as well. Any additional bands were labeled alphabetically. Bands staining for carbohydrate were labeled separately with roman numerals.

The pattern for M-1 and for M-2I were quite similar except that M-2I did not show bands 1, 11, 13 and 16 but instead had bands 13a, 16a and 16b. The pattern for M-2 was very different from either M-1 or M-2I. Bands 2 and 3 were reduced in intensity while 5, 6, 9, 10, 13 and 15 were missing. New bands 1a and 1c were found between 1 and 2, 3a and 3b between 3 and 4, 9a between 8 and 11, and 13a between 12 and 14.

Membranes M-3, M-3I and M-4, all of which had been digested to completion with trypsin, showed a great reduction in the number of bands. Except for band 2, none of the remaining bands corresponded to those of M-1.

Gels were also stained for carbohydrate. In membranes M-1, only one major band was observed, band IV, which corresponded to band 14 of the protein pattern. A less intensly stained band, III, was observed which corresponded to band 11 of the protein pattern. Two very faint bands, I and II, were observed which corresponded to bands 2 and 3 of the protein pattern. All of these bands disappeared following exposure to trypsin. They were replaced by a single band which moved faster than the hemoglobin band. All other membrane samples only showed the faint bands corresponding to 1 and 2.

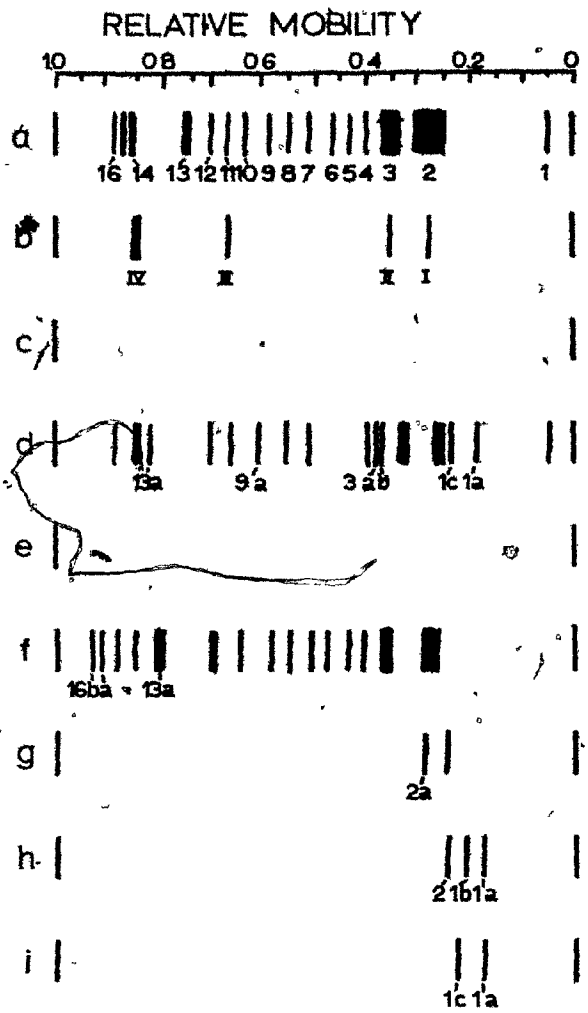


Fig. 9: Schematic diagram of gel electrophoretic separation of membrane proteins. Samples are a, b; M-1; c, d, M-2; e, f-M-2I; g,M-3; h,M-3I; i,M-4. Gels a, d, f, g, h, i are stained by Coomassie Blue, gels b, c, e periodic acid-Schiff stain. Samples run from right to left, and mobilities are relative to that of hemoglobin. The origin is at 0, hemoglobin at 1.0.

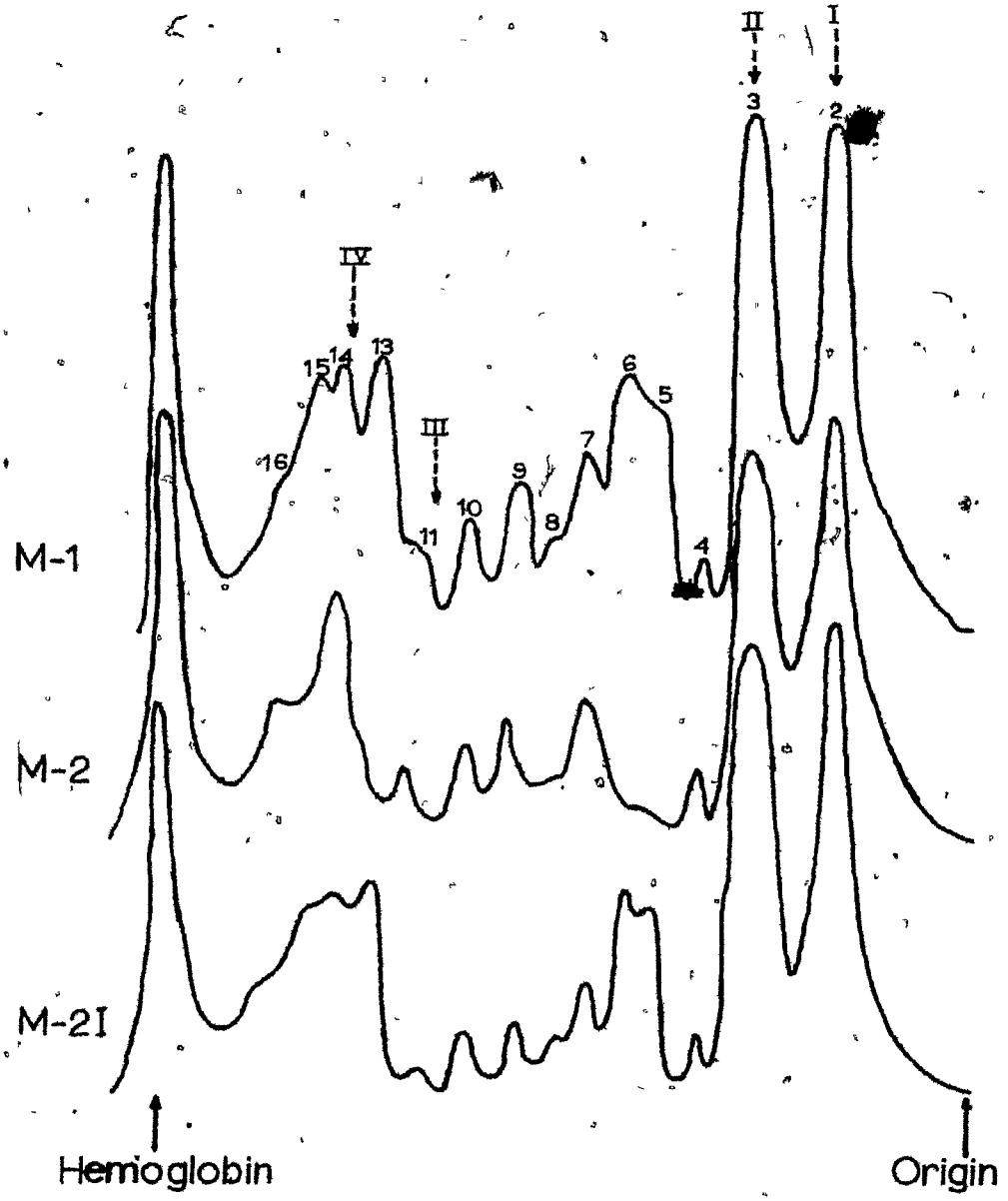


Fig. 10: Densitometer tracings of polyacrylamide gels stained with Coomassie Blue. Migration is from right to left. The broken arrows indicate the position of bands stained by periodic acid-Schiff stain.

b) Discussion

The gel electrophoretic pattern observed for membranes M-1 following staining with Coomassie Blue was similar to that reported by Kobylka et al. (126). Several workers (122,145-147) have observed that tryptic digestion of intact erythrocytes produces little alteration in the protein pattern. It was found that to keep the alterations to a minimum it was necessary to inhibit trypsin by adding trypsin inhibitor. On the other hand, once the membranes had been prepared from erythrocytes, none of the bands seemed to be resistant to proteolysis, confirming the observation of Carraway et al. (145) and of Steck et al. (122).

It was observed that when trypsin inhibitor was not used, there was a selective proteolysis of only a few bands, notably 2, 3, 5, 6, 9 and 10. The digestion of some or all of these polypeptide chains may have been responsible for the changes observed with membranes M-2, such as the increased binding of Hemoglobin.

VI. Effect of trypsin digestion on the interaction of membranes with salt as shown by changes in optical activity, viscosity and light scattering.

a) Results

Verpoorte and Smith (41) have shown that the optical properties of erythrocyte membranes prepared by osmotic hemolysis in the presence of EDTA are dependant on the salt concentration of the medium. Changes in optical rotation and circular dichroism as a result of addition of sodium chloride were accompanied by an increase in turbidity and a decrease in both pH and viscosity. The effects of trypsin digestion of membrane proteins on these changes has been examined.

i) Turbidity and pH.

The optical density at 600 nm, at constant pH, and the pH of suspensions of normal erythrocyte membranes (M-1), of membranes from erythrocytes treated with trypsin (M-2) and of these membranes digested with trypsin (M-3) were measured. The results are shown in Figure 11. Changes in both optical density and in pH had reached their maxima at 0.1 M sodium chloride, with the largest change occurring before 0.05 M. The digestion of membranes did not significantly influence the effect of salt on these properties.

ii) Viscosity

The viscosities of normal membranes (M-1), of membranes prepared from trypsin treated erythrocytes, (M-2 → M-2I), and of these membranes digested with trypsin (M-4, M-3, M-3I) were determined in aqueous suspension and in 0.05 M sodium chloride. Results are presented in Table XVIII.

The reduced viscosity of normal membranes was found to be about 60 ml. per g. at a concentration of about 10 mg. membrane per ml. suspension. Addition of sodium chloride to 0.05 M decreased this to 44 ml. per g. The membranes prepared from erythrocytes digested with trypsin (M-2 and M-2I) exhibited a higher reduced viscosity. In aqueous suspensions this was 140-160 ml. per g. while in sodium chloride it was 40 to 60 ml. per gm. The membranes which had been digested with trypsin all exhibited increased values of reduced viscosities. The digestion of M-1 increased the reduced viscosity from 60 ml. per g. to 340 ml. per gm. (see M-4), digestion of M-2 increased the value from 160 to 420 ml. per gm. (see M-3) while digestion of M-2I increased it from 140 to 354 ml. per gm.

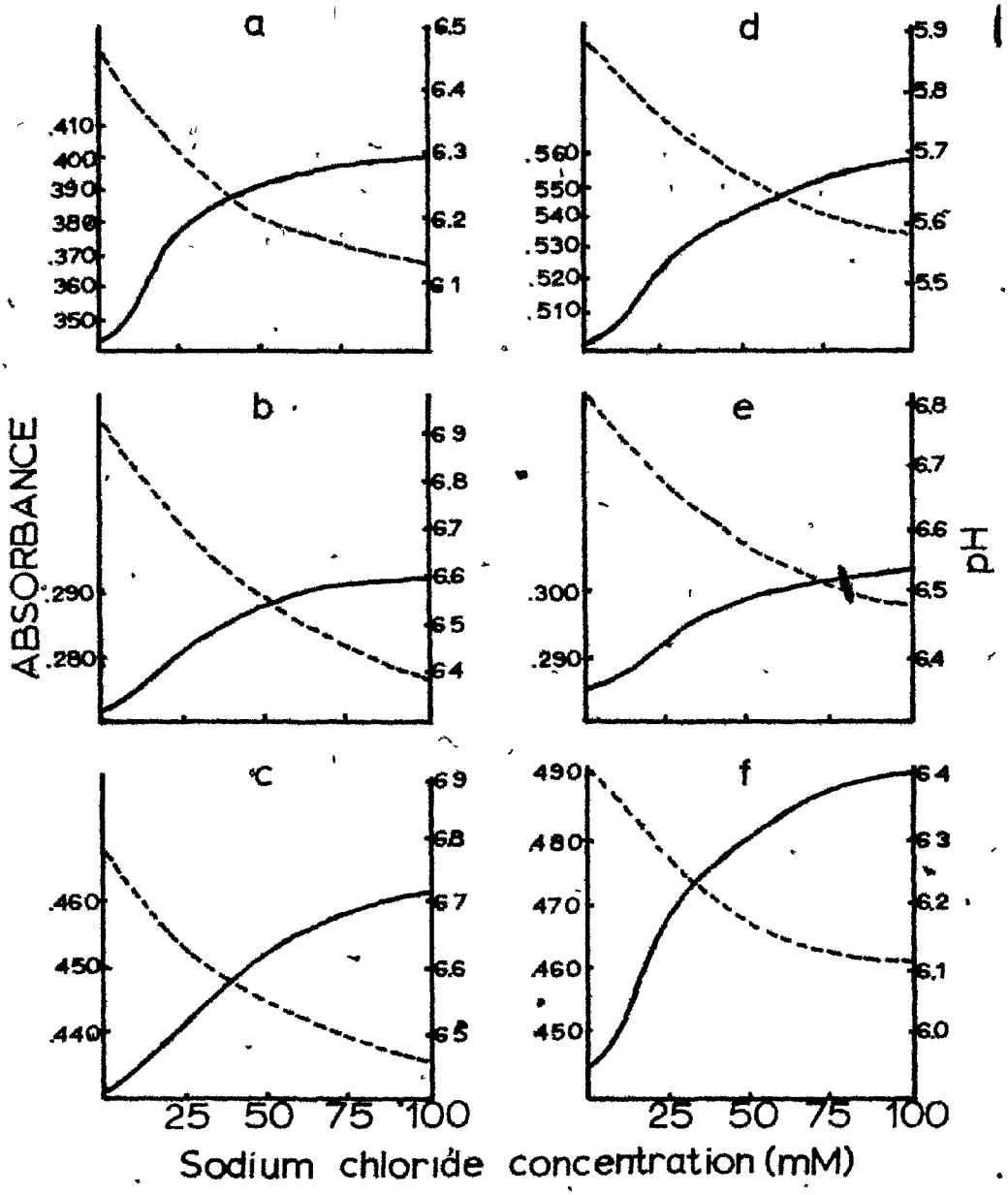


Fig. 11: Effect of sodium chloride on absorbance at 600 nm (solid line) and on pH (broken line).. Sample are: a, M-1; b, M-2 c, M-3; d, M-4; e, M-2I; f, M-3I.

(see M-3I). In the presence of 0.05 M sodium chloride all these values were reduced, M-3 to 54 $ml.g^{-1}$, M-3I to 109 $ml.g^{-1}$ and M-4 to 107 $ml.g^{-1}$

TABLE XVIII

VISCOSITY OF HUMAN ERYTHROCYTE MEMBRANE SUSPENSIONS*

	Reduced Viscosity ($ml.g^{-1}$)	
	in distilled water	in 0.05 M sodium chloride
M-1; normal membranes	61.5	44.1
M-2; membranes from trypsin treated erythrocytes	161.2	39.3
M-3; M-2 digested by trypsin	424.1	54.5
M-2I; membranes from trypsin treated erythrocytes, trypsin inhibitor added before membrane preparation	139.1	60.8
M-3I; M-2I digested by trypsin	354.1	109.8
M-4; M-1 digested by trypsin	343.3	107.0

*All membranes at a concentration equivalent to about 10 mg./ml. Values mean 6 determinations on each of 3 different preparations.

iii) Optical rotatory dispersion and circular dichroism.

Optical rotatory dispersion and circular dichroism spectra are presented in Figures 12-17. The optical rotation of salt free erythrocyte membranes suspended in distilled water was characterized by a maximum negative rotation at 233-234 nm of -260 deg cm^2 g^{-1}, and a maximum positive rotation at 202 nm of 1020 deg cm^2 g^{-1}. Zero rotation was found at 225-226 nm. The positive peak exhibited a shoulder in the region 210-220 nm. In the presence of 0.05 M sodium chloride the spectra were shifted to longer wavelengths and the values of the observed rotations were decreased. The maximum negative rotation which now occured at 237 nm had decreased to -120 deg cm^2 g^{-1}. The point of zero rotation was shifted to 229 nm. The positive peak was shifted to 204 nm and had decreased to 480 deg cm^2 g^{-1}.

Membranes M-2, prepared from trypsin treated erythrocytes showed an optical rotatory dispersion similar to that for M-1. The same red shifts in the spectra were seen when in 0.05 M sodium chloride though the reductions in rotation were less. The spectra had a maximum negative rotation of -270 deg cm^2 g^{-1} at 234 nm and a positive maximum of 990 deg cm^2 g^{-1} at 202 nm. In the presence of 0.05 M sodium chloride these reduced to -201 deg cm^2 g^{-1} at 237 nm and to 780 deg cm^2 g^{-1} at 204 nm respectively. The point of zero rotation shifted from 226 nm to 228 nm.

The membranes M-3, resulting from the tryptic digestion of membranes M-2 had lower rotations than either M-1 or M-2 and their spectra had shifted to the red. Though addition of sodium chloride

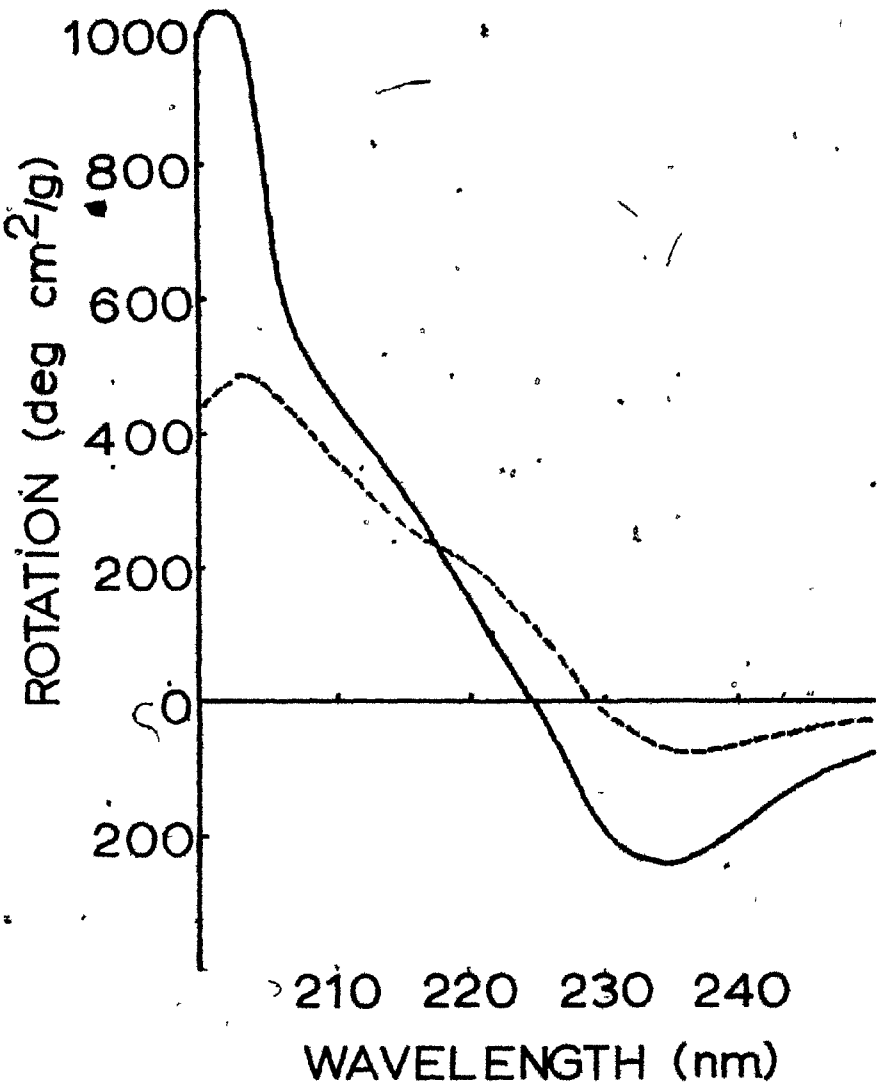


Fig. 12: Optical rotatory dispersion of a suspension of human erythrocyte membranes (M-1) in distilled water (solid line) and 0.05 M sodium chloride (broken line).

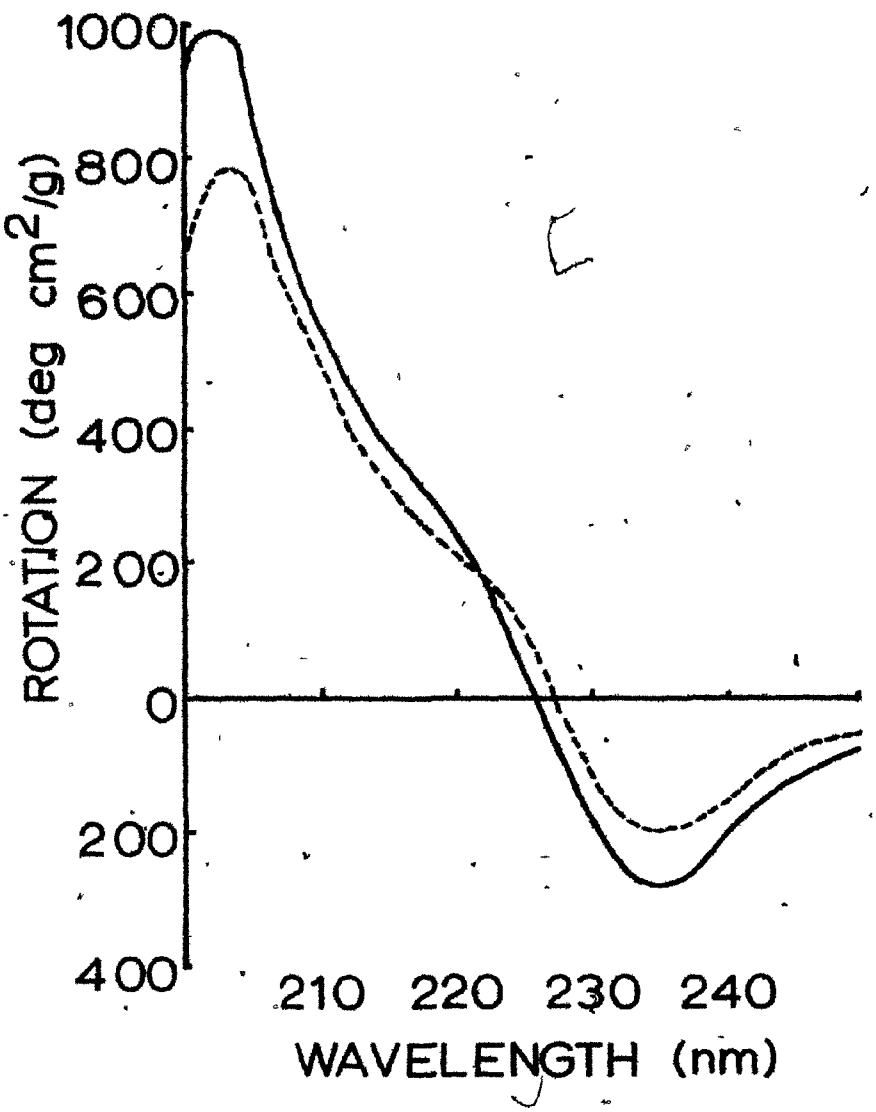


Fig. 13: Optical rotatory dispersion of a suspension of human erythrocyte membranes (M-2) prepared from trypsin treated erythrocytes, in distilled water (solid line) and in 0.05 M sodium chloride (broken line).

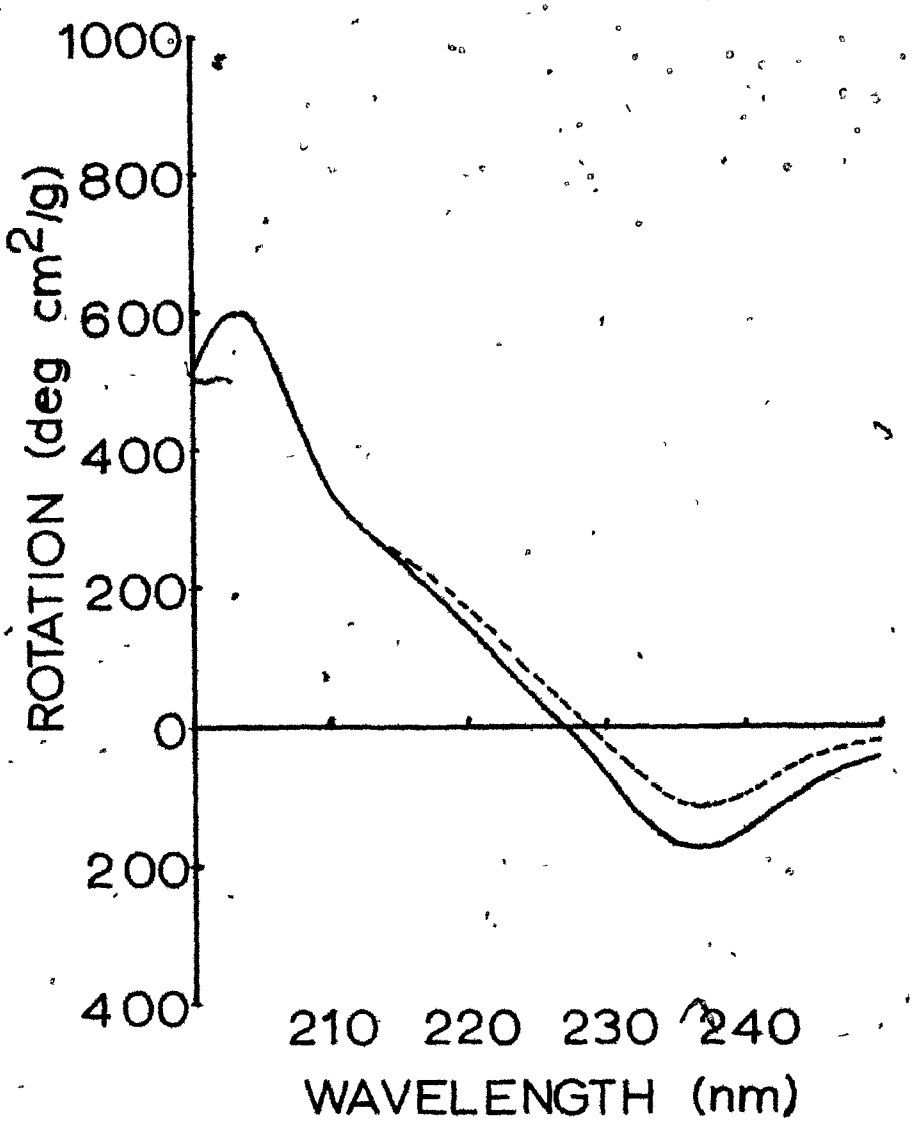


Fig. 14: Optical rotatory dispersion of a suspension of human erythrocyte membranes (M-3), prepared by digestion of M-2, in distilled water (solid line) and in 0.05 M sodium chloride (broken line).

reduced the magnitude of the negative maxima from -175 deg cm^2 g^{-1} to -99 deg cm^2 g^{-1}, the wavelength of the trough remained at 237-238 nm. The point of zero rotation shifted from 226 nm to 228 nm. There was no change in either the position or the magnitude of the positive maxima. It remained 580 deg cm^2 g^{-1} at 204 nm.

The circular dichroism of aqueous suspensions of membranes (M-1) was characterized by two negative bands centered at 222 nm with an ellipticity of -495 deg cm^2 g^{-1} and at 208 nm with an ellipticity of -425 deg cm^2 g^{-1}. Addition of sodium chloride to 0.05 M resulted in a reduction of the ellipticites, that at 222 nm decreasing to -380 deg cm^2 g^{-1} while that at 208 decreased to -250 deg cm^2 g^{-1}. The band at 208 also seemed to be shifted to 209-210 nm.

Membranes M-2, prepared from trypsin treated erythrocytes exhibited similar circular dichroism. The two negative bands occured at 222 nm and 209 nm and had ellipticities of -550 deg cm^2 g^{-1} and -480 deg cm^2 g^{-1} respectively. In the presence of 0.05 M sodium chloride these decreased to -470 deg cm^2 g^{-1} at 222 nm and to -405 deg cm^2 g^{-1} at 210 nm.

Membranes M-3, prepared by trypsin treatment of M-2, exhibited lower ellipticities. In aqueous suspension these membranes had negative bands of -360 deg cm^2 g^{-1} at 222-223 nm and of -299 deg cm^2 g^{-1} at 212 nm. Although these reduced to -330 deg cm^2 g^{-1} and -220 deg cm^2 g^{-1} respectively when sodium chloride was added, no wavelength shift was observed.

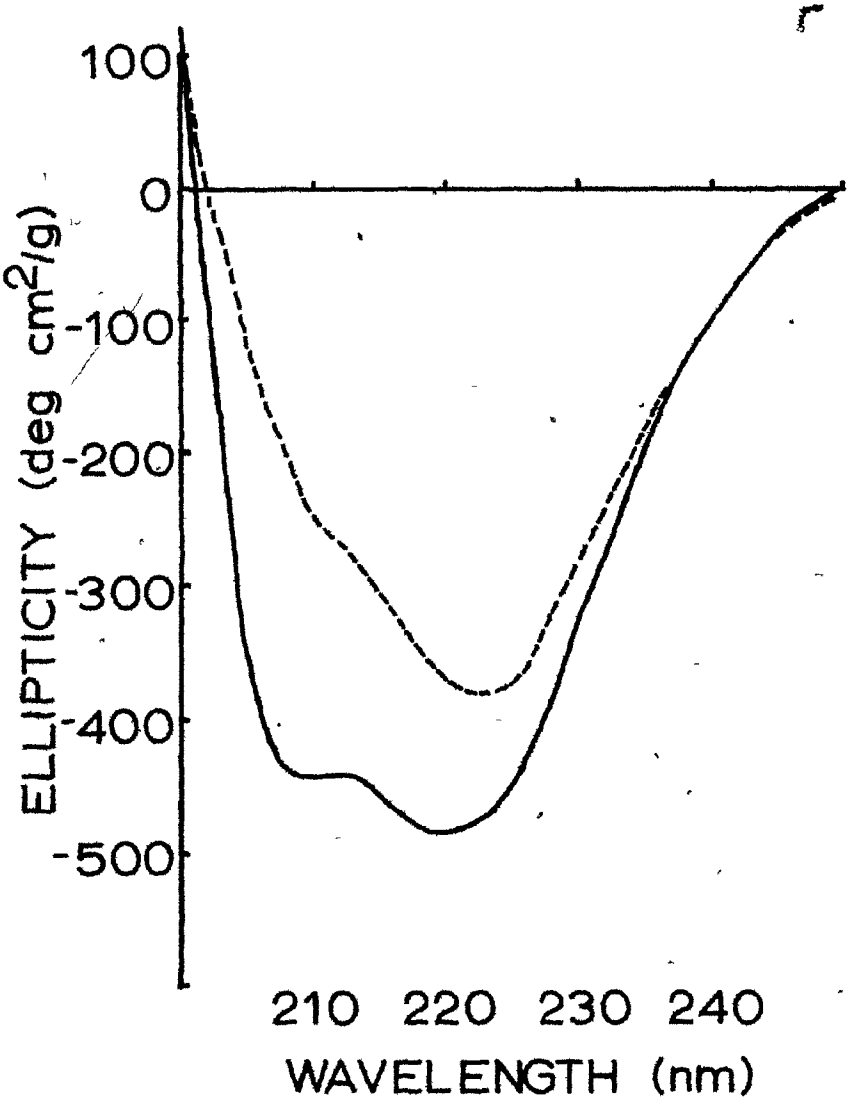


Fig. 15: Circular dichroism of a suspension of human erythrocyte membranes (M-1) in distilled water (——) and in 0.05 M sodium chloride (----).

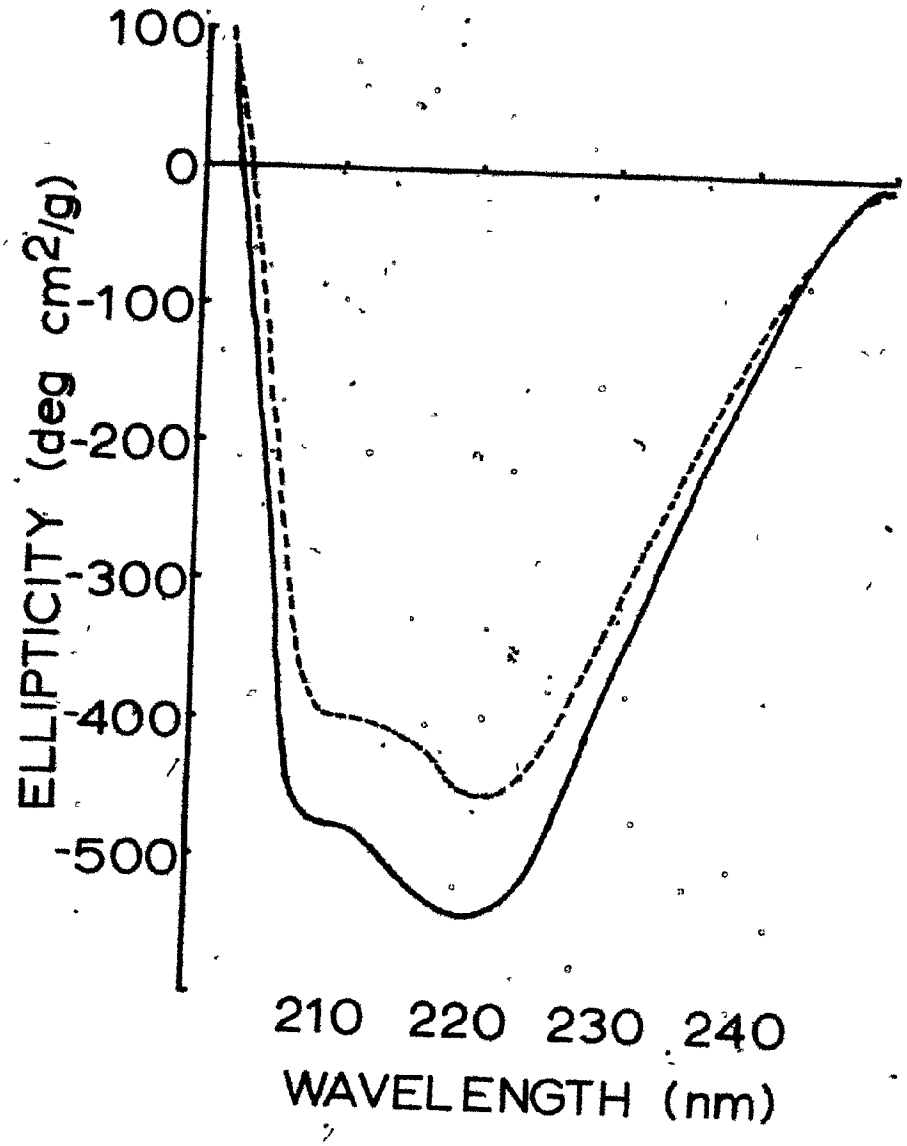


Fig. 16: Circular dichroism of a suspension of membranes (M-2), prepared from erythrocytes treated with trypsin, suspended in distilled water (——) and in 0.05 M sodium chloride (----).

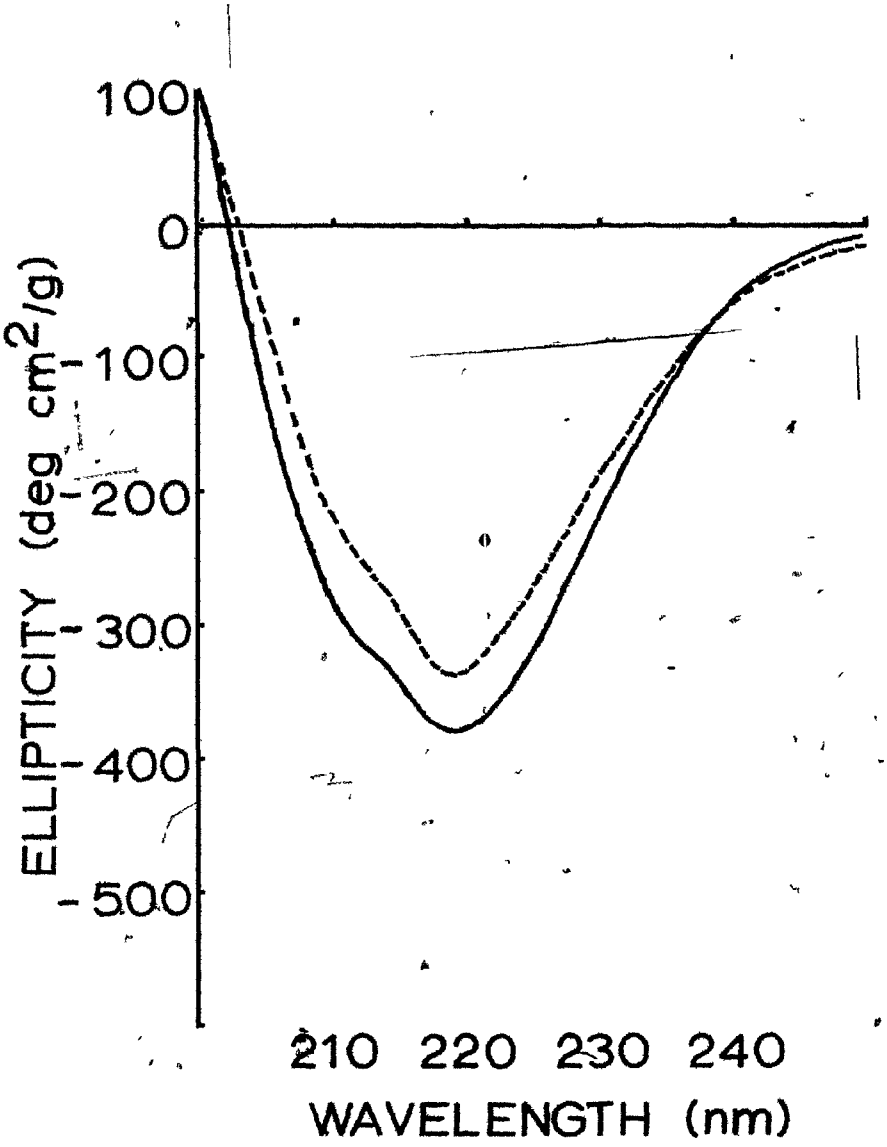


Fig. 17: Circular dichroism of a suspension of membranes (M-3), prepared by treating M-2 with trypsin, suspended in distilled water (——) and in 0.05 M sodium chloride (---).

b) Discussion

It appeared that tryptic digestion of erythrocyte membranes while resulting in the release of about 60% of the sialic acid, 40% of the protein and 15% of the lipid did not greatly influence the observed changes in turbidity and pH when sodium chloride was added. The pattern of the changes in turbidity and pH with increasing salt concentration were similar for the membranes examined, M-1, M-2 and M-3. The portions of the membrane removed by trypsin therefore do not participate significantly in the observed action of salt. This lack of effect of tryptic digestion on these properties provides indirect support for the hypothesis of Verpoorte and Smith (41) who have proposed that electrostatic repulsion of the negative charges of the phospholipid groups may hold the membrane in an expanded form. Addition of salt is thought to decrease the electrostatic repulsion between charged groups and thus to cause a shrinking effect. This could result in a decrease in pH and an increase in turbidity as was observed.

The reduced viscosity of erythrocyte membranes suspended at a concentration of 10 mg. per ml. in distilled water was about 60 ml. g^{-1}. This decreased by 28% to 44 ml. g^{-1} in 0.05 M sodium chloride. These viscosities are lower than those reported by Verpoorte and Smith (41) who observed a value of 90 ml. g^{-1} for membrane suspensions of 2-4 mg. per ml. in distilled water. Their membrane preparations were likely more fragmented, (see section IIIB Ib). This and the differences in concentrations may have resulted in lower reduced viscosities. They observed a decrease of 33%, from 90 to 60 ml. g^{-1} in 0.05 M sodium chloride, similar in magnitude to that reported here.

Digestion of intact erythrocytes resulted in membranes with substantially increased viscosities, about 140 ml. g^{-1} for M-2I and 160 ml. g^{-1} for M-2. These viscosities decreased by 56% and 76% respectively when measured in 0.05 M sodium chloride. This decrease was much larger than that observed for normal membranes.

Capaldi (248) has shown that the glycoprotein of beef erythrocyte membranes is not cross-linked by glutaraldehyde. He suggested that the high negative charge of these molecules would prevent their association with other proteins due to electrostatic repulsion. The removal of about 60% of the sialic acid by tryptic digestion might decrease this repulsion, allowing aggregation of the residual peptide. Gingell (245) has postulated that aggregation of mobile membrane proteins, such as the glycoprotein, may lead to changes in some properties of the membrane including local increases in membrane permeability resulting in exocytosis, pinocytosis and functional coupling. This could be a reason for the increased values of the reduced viscosities of membranes from trypsin treated erythrocytes.

Membranes M-3, M-3I and M-4 produced by trypsin digestion of M-2, M-2I and M-1 all showed greatly increased values of reduced viscosity. The digestions which produced these membranes could have increased still further the interaction between the membrane fragments to result in the higher values of reduced viscosity observed. All showed a reduction in values of reduced viscosities of 70 to 90% in sodium chloride solutions.

The effect of trypsin inhibitor, added following the digestion of intact erythrocytes, is also of interest. The membranes M-2I prepared from these cells had a lower reduced viscosity than did membranes M-2, prepared without inhibitor, 139 ml. g^{-1} and 161 ml. g^{-1} respectively.

Similarly M-3I had a lower reduced viscosity, 354 ml. g^{-1}, than did M-3 which had a reduced viscosity of 424 ml. g^{-1}. Thus the effect produced by trypsin inhibitor persisted even after the membranes were again exposed to trypsin.

All membranes, either digested with trypsin or not, still exhibited a decrease in viscosity when exposed to 0.05 M sodium chloride although the magnitude of the effect did vary. Since removal of proteins by tryptic digestion does not eliminate the effect of salt on viscosity, lipids are believed to be involved.

The optical rotatory dispersion and circular dichroism of membrane suspensions were not greatly affected by trypsin digestion of the erythrocytes from which they were prepared, even though the viscosity was affected. Likely, the material removed from the erythrocytes was located on the exterior surface of the membrane and did not contribute significantly to the optical activity.

It was noticed that with M-2 the decrease in magnitude of the optical rotation at 235 and 204 nm and of the ellipticities at 222 and 208 nm on addition of sodium chloride was not as great as seen with M-1. It has previously been shown that these membranes contain significant amounts of hemoglobin (see Table XI). As this hemoglobin is not an intrinsic part of the membrane its optical activity would not likely be affected by expulsion of water from the lipid bilayers.

While the observed rotations of membrane suspensions were decreased by trypsin digestion, there was little change in the rotation when expressed on the basis of protein rather than dry weight of the membrane. With membranes M-3 there was less reduction in rotation and little shift in wavelengths of the bands when sodium chloride was added. These results were confirmed by circular dichroism experiments which also

showed little change in ellipticities and no change in the position of the optically active bands.

The effects of trypsin digestion on the response of membrane suspensions to added salt, as seen by the optical rotatory dispersion and circular dichroism could possibly be explained by postulating a close association of the remaining peptides with lipids. Due to this close association the remaining optically active residues would be in a hydrophobic environment. The addition of sodium chloride, which is thought (41) to cause a shrinkage of the membrane with concomitant expulsion of water therefore might not result in a shift in the parameters of the optical effects. Such a close association of lipid and protein is in fact postulated by recent theories of membrane structure which emphasize hydrophobic interactions between bimodal proteins and lipids (see section I).

The fashion in which trypsin digestion alters the response of membranes to sodium chloride, as revealed by changes in pH, turbidity, viscosity, optical rotatory dispersion and circular dichroism is consistent with the hypothesis of Verpoorte and Smith (41).

VII. Effect of trypsin digestion on the interaction of membranes with the fluorescent probe 1-anilinonaphthalene-8-sulphonate

a) Results

The effect of trypsin digestion on the interaction of erythrocyte membranes with the fluorescent probe ANS has been studied. The excitation and emission spectra of a membrane-ANS suspension are shown in Fig. 18.

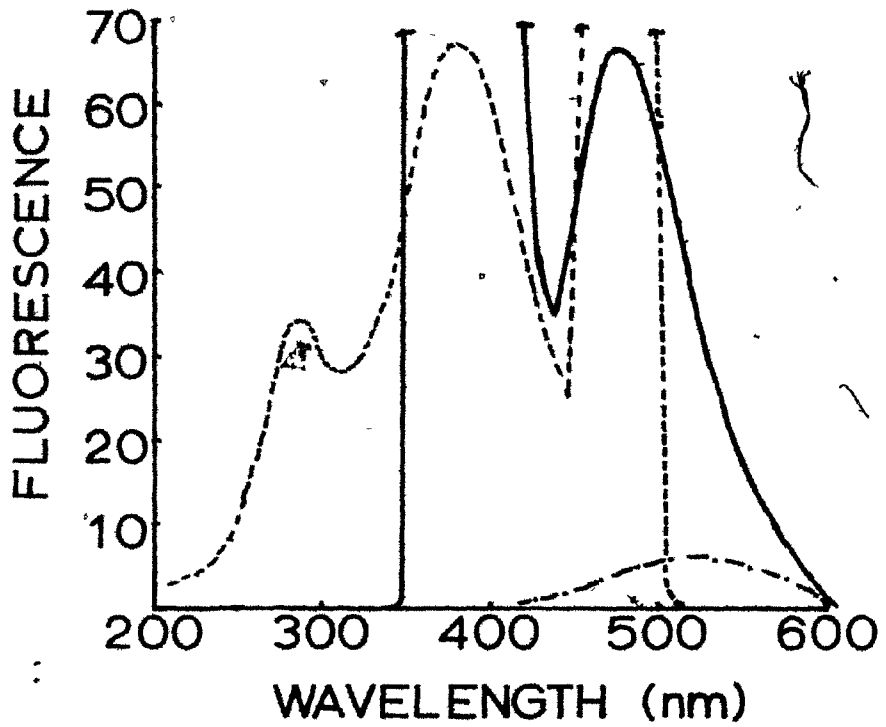


Fig. 18: Excitation (---) and emission (——) spectra of ANS-membrane (M-1) suspension in 0.01 M Tris-HCl buffer pH 7.2. Excitation spectrum was measured with emission at 470 nm, emission spectrum was measured with excitation at 375 nm. Fluorescence is in arbitrary units. The emission spectrum of ANS is represented by (-·--).

The addition of 0.5 mg. of membrane to 8-80 μM ANS resulted in 5 to 10 fold increases in fluorescence. The emission maximum of ANS fluorescence was shifted from 525 nm to 470-475 nm. Similar characteristics were observed with trypsin treated membranes. Although these observations were similar to those reported by others (192) the degree of enhancement of fluorescence was less.

The change in fluorescence with time, following the addition of ANS to membrane suspensions is shown in Figure 19. Normal and trypsin treated membranes in the presence and absence of sodium chloride were used. The reaction with normal membranes, M-1, was essentially complete within 20 seconds. Addition of sodium chloride to the membrane suspension, while resulting in enhanced fluorescence, did not alter the rate of interaction of ANS with membrane. Membrane M-2 showed both a fast and a slow reaction. The slow reaction was most evident from 10 to 60 seconds after addition of ANS and still persisted after 120 seconds. Membranes M-3 also showed both a fast and a slow reaction. As with M-1, however, the addition of sodium chloride to suspensions of either M-2 or M-3 did not alter the rate of interaction.

Membranes M-2I and M-3I, prepared from trypsin treated erythrocytes to which trypsin inhibitor was added, did not give the extensive slow reaction seen with M-2 and M-3.

As shown in Figure 19, addition of sodium chloride to ANS-membrane suspensions, at constant pH, resulted in increased fluorescence. This increase could be due to either an increase in the quantum yield of bound dye or to an increase in the total amount of dye bound. Double reciprocal

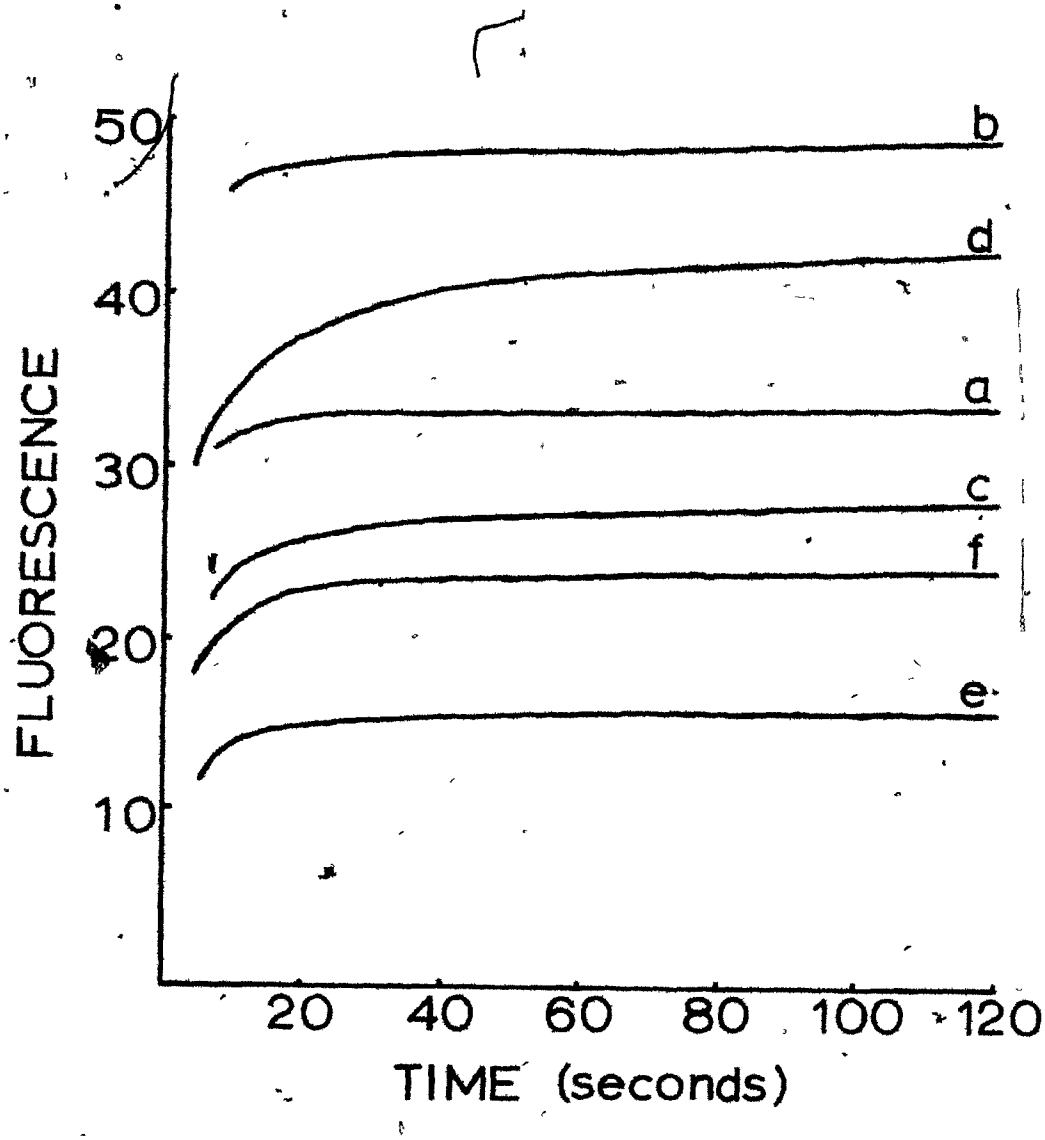


Fig. 19: Rate of development of fluorescence measured by adding ANS to suspensions of membrane in 0.01 M Tris-HCl buffer pH 7.2. Fluorescence is in arbitrary units. Excitation was at 375 nm, emission at 470 nm. Membrane suspensions are: a, M-1 in distilled water; b, M-1 in 0.1 M NaCl; c, M-2 in distilled water; d, M-2 in 0.1 M NaCl; e, M-3 in djstilled water, f, M-3 in 0.1 M NaCl.

plots of fluorescence intensity versus protein concentration at constant concentrations of ANS were used to distinguish between these possibilities. The fluorescence (F^0) at the intercept on the reciprocal fluorescence axis represents the maximum obtainable when all the dye is bound. A change in the value of this intercept on addition of sodium chloride would mean that the fluorescence efficiency of bound dye had changed. A change in slope would indicate that the number of dye molecules bound had changed. An example of such a plot is shown in Figure 20. It was evident from these data that addition of sodium chloride to 0.1 M resulted in a change in both intercept and slope. For all membranes tested, the value of F^0 in 0.1 M sodium chloride was about twice that in distilled water. Digestion of erythrocytes or membranes with trypsin did not affect this increase.

The results suggest that both quantum yield and the number of molecules of dye bound are changing. The effect of sodium chloride on ANS binding was determined by centrifuging the membranes and measuring free ANS remaining in the supernatant. Results are presented in Table XIX. It is evident that the addition of sodium chloride resulted in an increase in the amount of dye bound.

Further insight into the increase in fluorescence was gained by examining the effect of sodium chloride on the emission spectra obtained when the suspension was excited at 286 nm. It has been shown that energy transfer may occur between excited tryptophan and ANS (199-201). The efficiency of this transfer depends on the distance separating the amino acid residue and the ANS. Due to the energy transfer, the emission

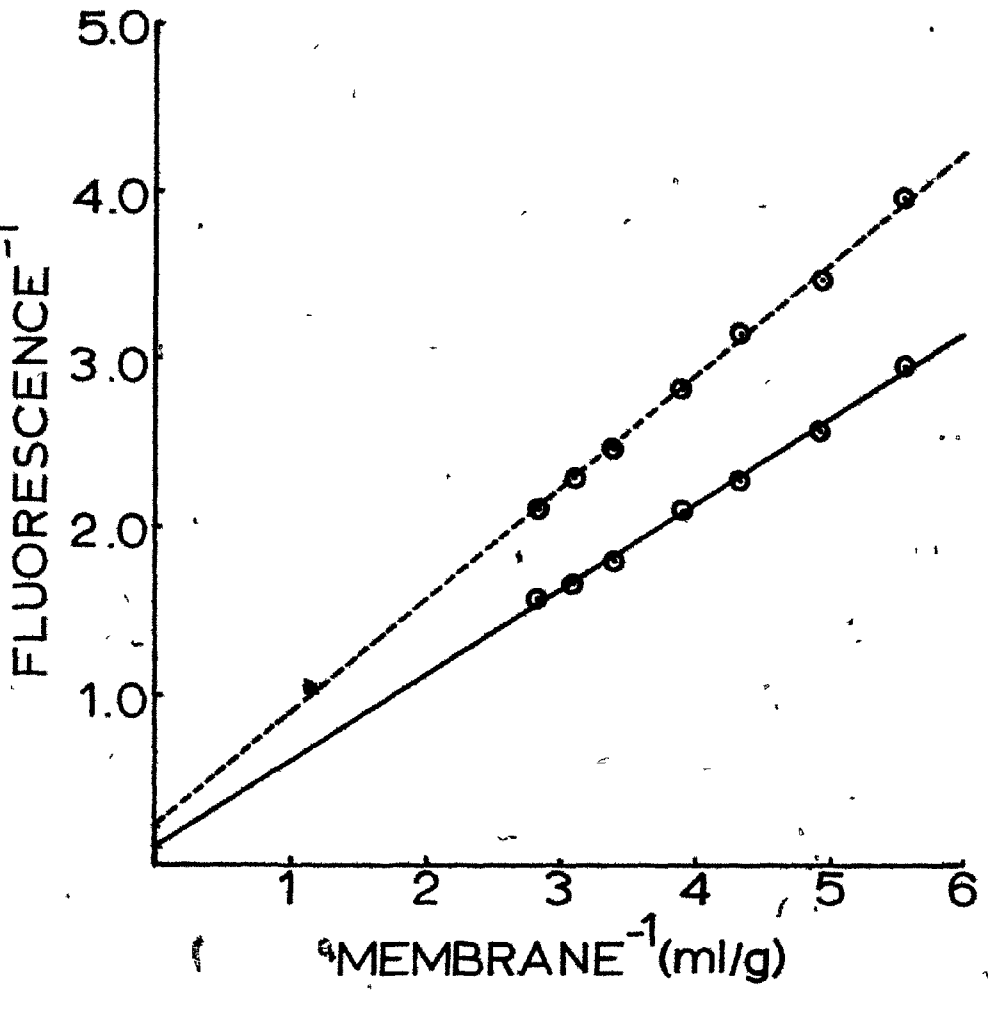


Fig. 20: Double reciprocal plot of membrane concentration on a dry weight basis versus fluorescence (meter reading), in presence (——) and absence (---) of 0.1 M sodium chloride. Normal membranes (M-1) were suspended in 76 μM ANS in 0.01 M Tris-HCl buffer, pH 7.2. Excitation was at 375 nm, emission at 470 nm.

TABLE XIX

EFFECT OF SODIUM CHLORIDE ON BINDING OF ANS BY HUMAN ERYTHROCYTE MEMBRANES*[1]

Sodium Chloride concentration (M)	Fluorescence	ANS bound (μM)
0	0.936	1.42
0.02	1.716	7.88
0.04	1.986	10.11
0.06	2.151	10.71
0.08	2.256	11.92
0.10	2.331	11.52

*Membrane suspension, 5.6 mg., was added to a solution of ANS in buffer at pH 7.2. The final concentration of ANS was 53.5 μM.

[1] These data show a 10 fold increase in bound ANS and only a 3 fold increase in fluorescence. It therefore seems that the ANS molecules which bound after addition of NaCl have a lower quantum yield than the strongly bound ANS molecules which bound already in the absence of salt. Fig. 20 suggests that the quantum yield was doubled in 0.1 M NaCl, however, these values are obtained at infinite protein concentration and thus apply to ANS molecules at high affinity sites in the membrane. Data here are at lower protein concentrations and therefore also apply to ANS molecules at lower affinity sites which could have lower quantum yields. Fig. 21 and the non-linearity of Scatchard and Klotz plots indicate heterogeneity of ANS binding sites.

of tryptophan at 350 nm will be quenched while emission by ANS at 470 nm will increase. Membrane-ANS suspensions would therefore show a decreased fluorescence at 350 nm and an increased fluorescence at 470 nm if more ANS binds at sites close enough to tryptophan for energy transfer to occur.

When sodium chloride was added to membrane-ANS suspensions (Fig. 21) there was no decrease in emission at 350 nm while there was an increase in emission at 470 nm.

Figure 22 shows the change in fluorescence efficiency, as measured by the value of F^0, with increasing sodium chloride concentration. All membranes, whether normal or treated with trypsin, gave similar results. The changes in F^0 occurred at the same concentrations of sodium chloride as did the changes in turbidity and pH (Fig. 23).

The effect of trypsin digestion of membrane protein on the interaction with ANS was examined by digestion of a membrane-ANS suspension. Fluorescence was determined during the course of the digestion (Fig. 24). There was an immediate increase in fluorescence on the addition of trypsin. As digestion by trypsin progressed the fluorescence of the suspension decreased to a value lower than that of the original membrane-ANS suspension.

The apparent dissociation constants of ANS and of Na^+ were determined from double reciprocal plots (Fig. 25,26). Values of the dissociation constants are given in Table XX. In all cases the dissociation constant was essentially independant of membrane concentration.

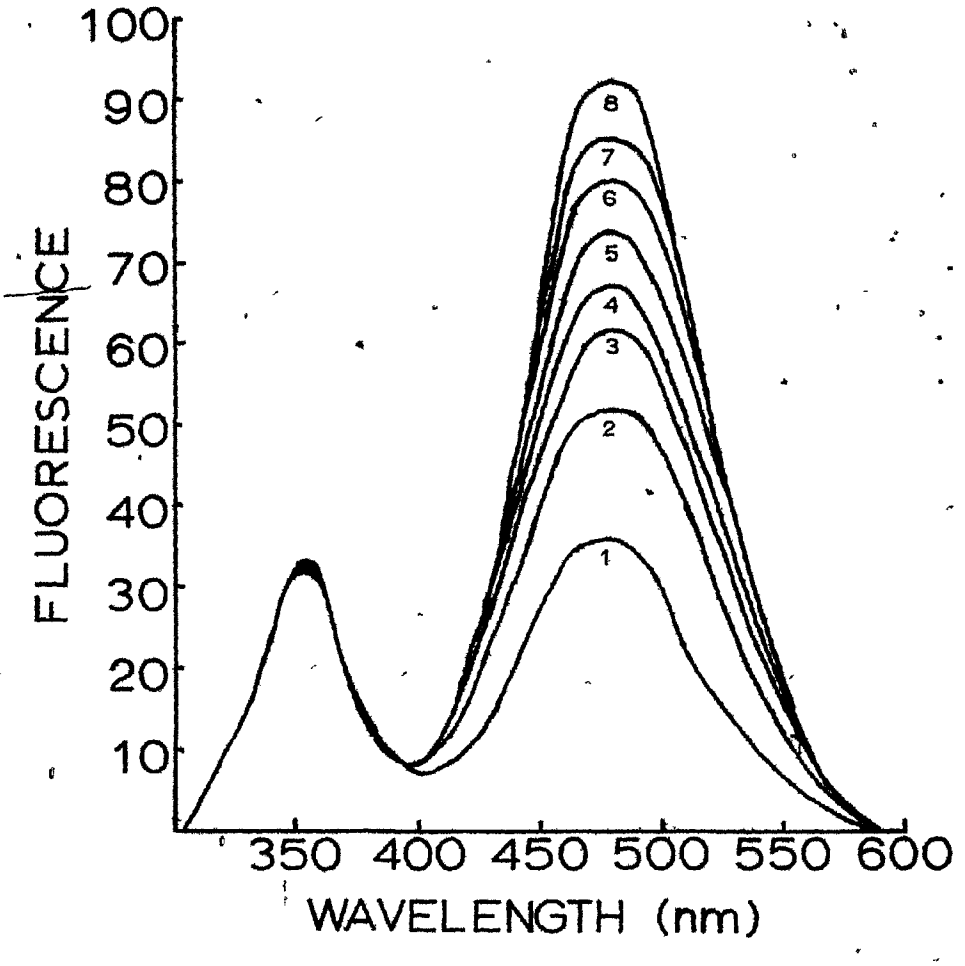


Fig. 21: Effect of sodium chloride on the emission spectrum of membranes M-1 (0.5 mg./ml.) suspended in 60 μM ANS in 0.01 M Tris-HCl buffer, pH 7.2. Sodium chloride concentrations were: 1, none; 2, .04 M; 3, .05 M; 4, .06 M; 5, .07 M; 6, .08 M; 7, .09 M; 8, .10 M. Excitation was at 286 nm.

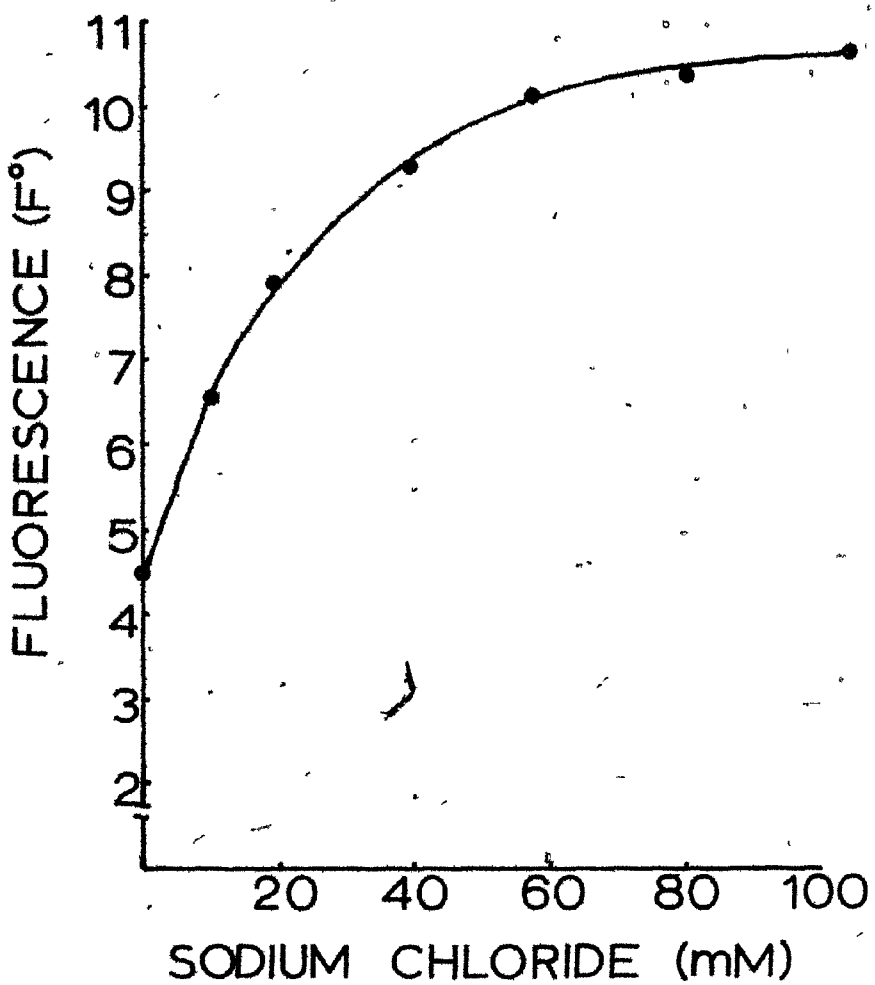


Fig. 22: Effect of sodium chloride on the maximal fluorescent intensity (F^0) of ANS-membrane (M-1) suspensions in 0.01 M Tris-HCl buffer, pH 7.2. Concentration of ANS was 76 μM. Values of the maximal fluorescent intensity were determined as in Figure 20.

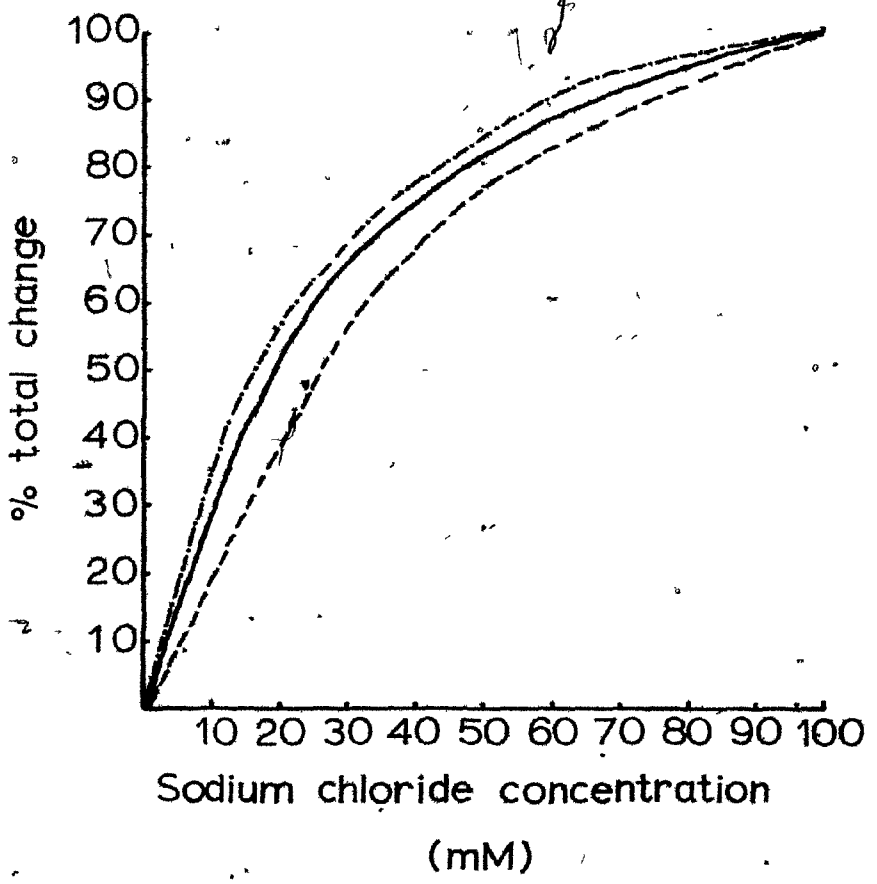


Fig. 23: Effect of sodium chloride on turbidity (——), pH (---) and maximal fluorescent intensity (-.-.) of membranes M-1.

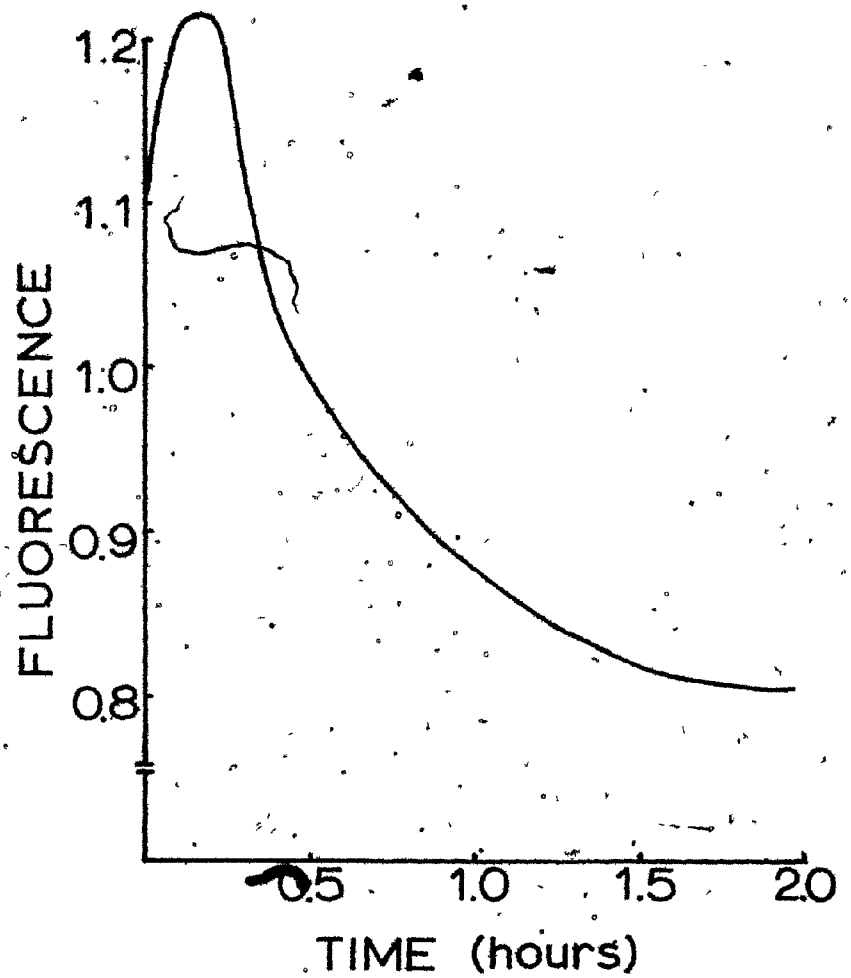


Fig. 24: Fluorescence of ANS-membrane (M-1) suspension in .1 M phosphate buffer pH 7.0, during trypsin digestion. Trypsin was added at 0 hours. Fluorescence was measured at 470 nm, excited at 375 nm.

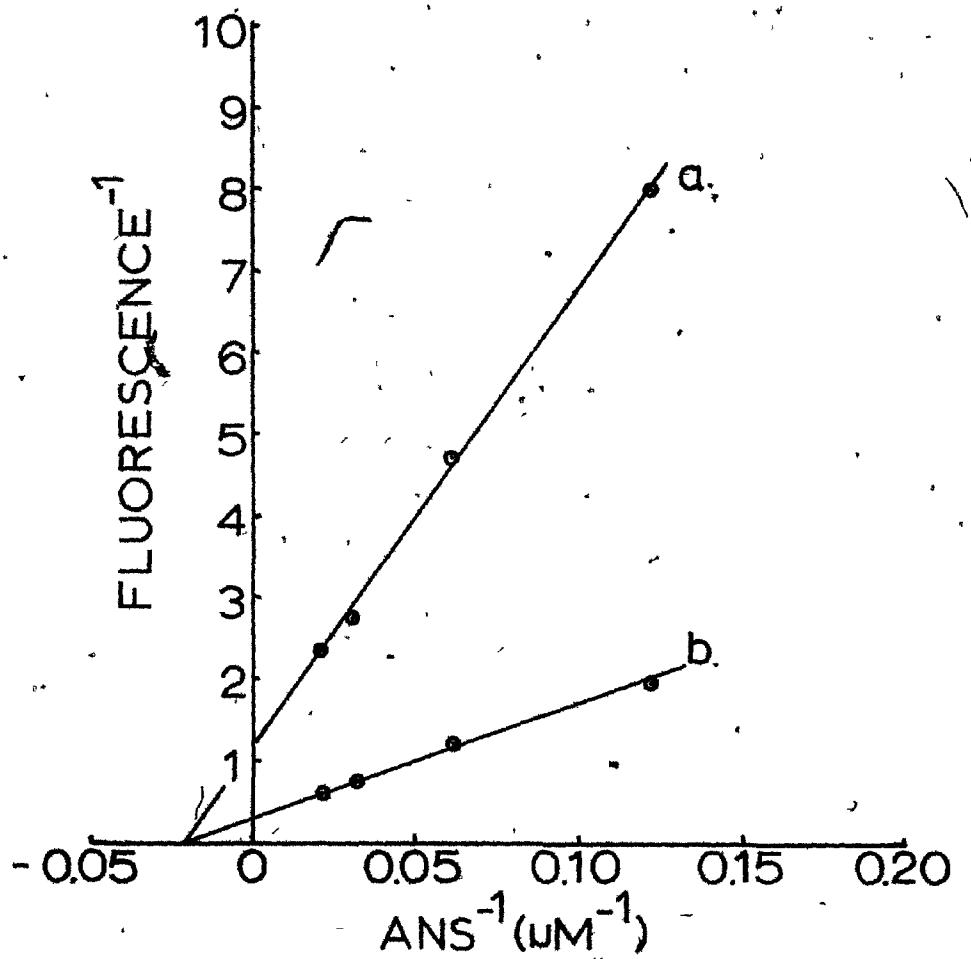


Fig. 25: Double reciprocal plot of fluorescence versus ANS, concentration for membranes M-1 (6.5 mg./ml.) suspended in 0.01 M Tris-HCl buffer, pH 7.2. Fluorescence was measured at 470 nm with excitation at 370 nm. Fluorescence was measured in the absence, a, and presence, b, of sodium chloride (0.1M).

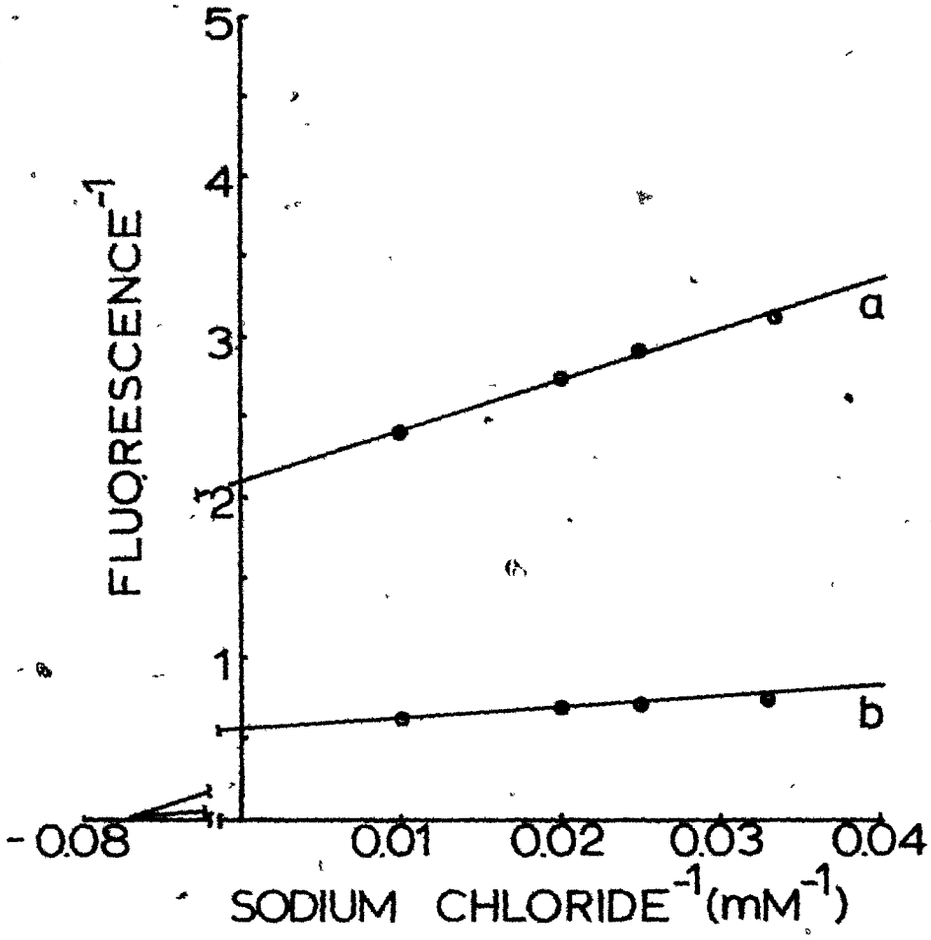


Fig. 26: Double reciprocal plot of fluorescence versus sodium chloride concentration for membranes M-2 (3.2 mg./ml.) suspended in 0.01 M Tris-HCl buffer, pH 7.2. Fluorescence was measured at ANS concentrations of a, 11.5 μM and b, 121.1 μM. Fluorescence was measured at 470 nm with excitation at 370 nm.

TABLE XX

DISSOCIATION CONSTANTS FOR ANS AND SODIUM

	K_{ANS} (μM)	K_{Na} (mM)
M-1, normal membranes	43	9.2
M-2, membranes from trypsin treated erythrocytes	45	13.0
M-2I, membranes from trypsin treated erythrocytes, washed with trypsin inhibitor	48	13.1
M-4, membranes M-1 treated with trypsin	17	9.0
M-3, membranes M-2 treated with trypsin	23	13.1
M-3I, membranes M-2I treated with trypsin	12	12.8

The dissociation constant for ANS was about 43 μM for normal membranes, M-1, or for membranes prepared from erythrocytes treated with trypsin, M-2 and M-2I. Digestion of M-1, M-2 and M-2I resulted in decreased dissociation constants.

Normal membranes had a dissociation constant of 9 mM for sodium whereas membranes M-2 or M-2I, from erythrocytes pretreated with trypsin had dissociation constants of about 13 mM. Further digestion of any membrane did not alter its dissociation constant for sodium. Thus M-4 had a dissociation constant of 9 mM, identical with M-1 from which it was derived. Similarly, M-3 and M-3I had constants of 13 mM identical with those of M-2 and M-2I.

b) Discussion

Freedman and Radda (192) have reported that the interaction of ANS with erythrocyte membranes prepared by the Dodge method (242) occured in two stages. They described a fast reaction of less than 10 seconds duration and a slow reaction of about 200 seconds duration. When membranes were disrupted by sonication only the fast reaction was observed. They suggested that the fast reaction might represent the interaction of the dye at the surface of the membrane while the slow reaction might represent diffusion of the dye into the membrane.

As no slow reaction was observed with membranes M-1 it is possible that membranes prepared with tris-EDTA buffers according to Smith (213) are more fragmented than those prepared by the Dodge method (242).

Membranes M-2 and M-3 prepared from erythrocytes which had been treated with trypsin showed both a fast and slow reaction. In this respect they resembled membranes prepared by the Dodge method (242), suggesting that trypsin digestion of erythrocytes resulted in a significant alteration of membrane structure, possibly resulting in creation of a diffusion barrier to ANS. This could result from production of vesicles during trypsin digestion as was suggested by the increased retention of hemoglobin. As with the retention of hemoglobin, the washing of digested erythrocytes with trypsin inhibitor before the preparation of membranes prevented the effects on the rate of interaction of membranes with ANS. Thus neither M-2I nor M-3I showed the slow interaction with ANS.

Addition of sodium chloride to membrane suspension in ANS resulted in both increased dye binding as well as increased fluorescence efficiency. The increase in fluorescence efficiency seemed to parallel the changes seen in turbidity and pH. An increase in fluorescence efficiency of ANS may be attributed to a decrease in the polarity of the environment of the dye. Such a change could result from expulsion of water from the microenvironment of the dye(192). This is consistent with the observation of Verpoorte and Smith (41) that addition of sodium chloride to membranes results in a shrinkage of the membrane and expulsion of water.

Besides an increase in the fluorescence efficiency of ANS there was also an increase in the amount of bound dye on addition of sodium chloride. None of this extra dye seemed to show energy transfer

with tryptophan. This suggests that these molecules are located at sites in the membrane removed from the membrane protein.

When membranes were digested with trypsin the fluorescence of ANS decreased. This was observed when membranes were digested in the presence of ANS and also with membranes M-2 and M-3 prepared from normal membranes M-1. That the fluorescence decreases suggests that proteolysis is destroying some of the ANS binding sites.

Digestion of intact erythrocytes did not alter the binding constant for ANS. The dissociation constant for normal membranes, M-1, was 43 μM while membranes M-2 and M-2I prepared from trypsin treated erythrocytes had constants of 45 and 48 μM respectively. On the other hand, digestion of isolated membranes resulted in a reduction of the dissociation constant to about 12-23 μM.

On the other hand, digestion of intact erythrocytes altered the binding of sodium. Normal membranes had a dissociation constant of about 9 mM while membranes from erythrocytes treated with trypsin had dissociation constants of about 13 mM. Unlike the binding of ANS, digestion of isolated membranes did not alter the dissociation constant for sodium. Thus M-4, derived from M-1, had a constant of 9 mM while M-3 and M-3I derived from M-2 and M-2I had constants of about 13 mM.

Digestion of the exterior surface of erythrocytes appears to result in some change in the membrane affecting its affinity for sodium ion. This change may have resulted prior to or during hemolysis as digestion of isolated membranes did not alter the dissociation constants, even though in the digestion of M-1 to give M-4 the same bonds would have been hydrolysed as in the digestion of intact erythrocytes

to give M-2 or M-2I.

The alterations in binding of ANS and of sodium do not appear to be related. Digestion of intact erythrocytes gave membranes with decreased affinity for sodium while not affecting ANS binding. On the other hand, digestion of isolated membranes increased the affinity for ANS but did not alter that for sodium. The use of trypsin inhibitor at the end of the digestion of intact erythrocytes did not alter the changes in dissociation constants, therefore they must be unrelated to the changes resulting in increased retention of hemoglobin.

VIII. Summary

It has been shown that the proteins of the human erythrocyte are not especially resistant to proteolysis by trypsin. Up to 70% of the peptide bonds involving lysine and arginine were hydrolysed whether the protein was present in membranes or in aqueous solutions. This suggested that digestion of protein was not affected by the presence of lipid as might be expected if it were associated with lipid as in a lipoprotein complex. These data also support the suggestion by others (236,237) that extraction of lipid with n-butanol does not affect the protein structure.

It has been demonstrated that proteolysis of intact erythrocytes results in binding of hemoglobin by membranes only if removal of trypsin before hemolysis is not complete. The increased retention of hemoglobin could be prevented by addition of trypsin inhibitor. Gel electrophoresis has shown that only a limited number of polypeptide chains need be

hydrolysed to cause the increased retention of hemoglobin. The presence of trypsin during the hemolysis of cells may also explain the observation of Triplett and Carraway (146) that up to 30% of the total sialic acid of erythrocytes exposed to the enzyme may be lost at this stage.

The proteins of the intact erythrocyte were much less susceptible to trypsin than were those of the membrane. A second treatment with trypsin released additional carbohydrate and sialic acid containing peptides from membranes prepared from erythrocytes which had been digested to completion. Both these observations suggested that there were major changes in structure during hemolysis and washing of the membrane. This supports the observation made by others (166,167) that at best the ghost must be regarded as a derivative of the membrane as it exists in the intact, functional cell. Great care must be taken when ghosts are used as models for the behaviour of the membrane of cells.

Trypsin digestion of isolated membrane protein led to the formation of an aggregate of small peptides containing a large percentage of apolar amino acids. It was suggested that these may be derived from hydrophobic portions of bimodal membrane proteins.

Changes in turbidity and pH and in fluorescence of ANS on addition of sodium chloride seemed to be related. The increased fluorescence efficiency suggested an expulsion of water from the membrane. This supported the suggestion by Verpoorte and Smith (41) that changes in viscosity, ORD and CD as well as turbidity and pH could be attributed to changes in water content of membranes as a result of membrane shrinkage. The effect of proteolytic digestion on the ORD and CD of membranes as they responded to changes in salt content were also consistent with the hypothesis of Verpoorte and Smith (41).

BIBLIOGRAPHY

1. Nageli, C. and Cramer, C.: Pflanzenphysiologische Untersuchungen, 1, (1855).
2. Overton, E.: Vischr. Natunforsch Ges. 40, 159 (1895).
3. Fricke, H.: J. Gen. Physiol. 9, 137 (1925).
4. Curtis, H.J. and Fricke, H.: Nature 134, 102 (1934).
5. Curtis, H.J. and Fricke, H. Nature 135, 430 (1935).
6. Mudd, S. and Mudd, E.B.H.: J. Gen. Physiol. 14, 733 (1931).
7. Gorter, E. and Grendel, F.: J. Exp. Med. 41, 439 (1925).
8. Danielli, J.F. and Harvey, E.N.: J. Cell. Comp. Physiol. 5, 483 (1935).
9. Danielli, J.F. and Dawson, H.: J. Cell. Comp. Physiol. 5, 495 (1935).
10. Robertson, J.D. in Cellular Membranes in Development (M. Locke, ed.) p. 1, Academic Press, New York (1964).
11. Schmidt, W.J.: Z. Zellforsch. 23, 261 (1936).
12. Schmidt, W.J.: Z. Zellforsch 23, 657 (1936).
13. Schmidt, F.O., Bear, R.S. and Ponder, E.: J. Cell. Comp. Physiol. 9, 89 (1936).
14. Schmidt, F.O.: Biology 4, 7 (1936).
15. Finean, J.B.: Exp. Cell. Res. 5, 202 (1953).
16. Fernandez-Moran, H. and Finean, J.B.: J. Biophys. Biochem. Cytol. 3, 557 (1957).
17. Geren, B.B.: Exp. Cell. Res. 7, 558 (1954).
18. Maddy, A.H., Huang, C., and Thompson, T.E.: Fed. Proc. 25, 933 (1966).
19. Korn, E.D.: Science 153, 1491 (1966).
20. Bar, R.S., Deamer, D.W., and Cornwell, D.G.: Science 153, 1010 (1966).

21. Thompson, T.E.: In Cellular Membranes in Development (M. Locke, ed.) p. 83, Academic Press, New York (1964).

22. Korn, E.D.: In Theoretical and Experimental Biophysics (A. Cole, ed.) p. 1, Marcel Debber, New York (1969).

23. Kirschner, D.A. and Caspar, D.L.D.: Ann. N.Y. Acad. Sci. 195, 309 (1972).

24. Sjostrand, F.S.: J. Ultrastruct. Res. 9, 561 (1963).

25. Fleischer, S., Fleischer, B. and Stockenius, W.: J. Cell. Biol. 32, 193 (1967).

26. Napolitano, L., Lebaron, F., and Scaletti, J.: J. Cell. Biol. 34, 817 (1967).

27. Green, D.E., Allmann, D.W., Bachmann, E., Baum, H., Kopaczyk, K., Korman, E.F., Lipton, S., Maclennan, D.H., McConnell, D.G., Perdue, J.F., Rieske, J.C., and Tzagoloff, A.: Arch. Biochem. Biophys. 119, 312 (1967).

28. Wallach, D.F.H., and Zahler, H.P.: Proc. Nat. Acad. Sci. U.S. 56, 1552 (1966).

29. Wallach, D.F.H. and Gordon, A. In 15th International Symposium on the Protides of Biological Fluids (H. Peters, ed.) p. 47, Elsevier, Amsterdam (1968).

30. Wallach, D.F.H. and Gordon, A.: In Regulatory Functions of Biological Membranes (J. Jarnefelt, ed.) p. 87, Elsevier, Amsterdam (1968).

31. Chapman, D., Kamat, V.B., deGier, J. and Penkett, S.A.: Nature 213, 74 (1967).

32. Chapman, D., Kamat, V.B., deGier, J. and Penkett, S.A.: J. Mol. Biol. 31, 101 (1968).

33. Chapman, D., Kamat, V.B. and Levene, R.J.: Science 160, 314 (1968).

34. Maddy, A.H. and Malcolm, B.R.: Science 150, 1616 (1965).

35. Steim, J.: 153rd Meeting of the Amer. Chem. Soc., Miami Beach (1967).

36. Lenard, J. and Singer, S.J.: Proc. Nat. Acad. Sci. U.S. 56, 1828 (1966).

37. Urry, D.W., Mednieks, M. and Bejnarowiecz, E.: Proc. Nat. Acad. Sci. U.S. 57, 1043 (1967).

38. Gordon, D.J. and Holzworth, G.: Proc. Nat. Acad. Sci. U.S. 68, 2365 (1971).

39. Gordon, D.J.: Biochemistry 11, 413 (1972).

40. Urry, D.W.: Biochim. Biophys. Acta 265, 115 (1972).

41. Verpoorte, J.A. and Smith F.M.: Can. J. Biochem. 50, 177 (1972).

42. Singer, S.J.: Advan. Prot. Chem. 17, 1 (1962).

43. Benson, A.A. and Singer, S.J.: Abstr. 150th Nat. Meet. Amer. Chem. Soc. p. 8 (1965).

44. Benson, A.A.: J. Amer. Chem. Soc. 43, 265 (1966).

45. Park, R.B. and Pon, N.G.: J. Mol. Biol. 3, 1 (1961).

46. Green, D.E. and Perdue, J.F.: Proc. Nat. Acad. Sci. U.S. 55, 1295 (1966).

47. Henn, F.A. and Thompson, T.E.: Ann. Rev. Biochem. 38, 241 (1969).

48. Pressman, B.C., Harris, E.J., Jagger, W.S. and Johnson, J.H.: Proc. Nat. Acad. Sci. U.S. 58, 1949 (1967).

49. Park, R.B.: Annal. New York Acad. Sci. 195, 262 (1972).

50. Steim, J.M., Tourtelotte, M.E., Reinert, J.C., McChaney, R.N., and Radar, R.L.: Proc. Nat. Acad. Sci. U.S. 63, 104 (1969).

51. McConnell, H.M. and McFarland, B.G.: Annal. New York Acad. Sci. 195, 207 (1972).

52. Stoeckenius, W.: Annal. New York Acad. Sci. 195, 35 (1972).

53. Hechter, O.: Annal. New York Acad. Sci. 195, 506 (1972).

54. Singer, S.J.: In Structure and Function of Biological Membranes. (Rothfield, L.I., ed.) p. 145. Academic Press, New York (1971).

55. Marchesi, V.T. and Steers, E., Jr.: Science 159: 203 (1968).

56. Gulick-Krzywicki, T., Schechter, E., Luzzati, V. and Faure, M.: Nature 223, 1116 (1969).

57. Richardson, S.H., Hultin, H.O. and Green, D.E.: Proc. Nat. Acad. Sci. U.S. 50, 821 (1963).

58. Haldar, D., Freeman, K. and Work, T.S.: Nature 211, 9 (1966).

59. Lenaz, G., Haard, N.F., Silman, H.I. and Green, D.E.: Arch. Biochem. Biophys. 128, 293 (1968).

60. Green, D.E.: Annal. New York Acad. Sci. 195, 173 (1972).

61. Colacicco, G.: Annal. New York Acad. Sci. 195, 224 (1972).

62. Urry, D.W.: Annal. New York Acad. Sci. 195, 108 (1972).

63. Singer, S.J. and Nicholson, G.L.: Science 175, 720 (1972).

64. Parpart, A.K. and Dzieman, A.J.: Cold Spr. Hbr. Symp. Quant. Biol. 8, 17 (1940).

65. Sweeley, C.C. and Dawson, G.: In Red Cell Membrane: Structure and Function (G.A. Jamieson and T.J. Greenwalt eds.) p. 172. Lippincott, Toronto (1969).

66. Cooper, R.A.: Seminars in Hematology 3, 296 (1970).

67. Guidotti, G.: Arch. Int. Med. 129, 194 (1972).

68. Marks, P.A., Johnson, A.B., Hirschberg, E. and Banks, J.: Annal. New York Acad. Sci. 75, 95 (1958).

69. Prankerd, T.A.J.: J. Physiol. 143, 325 (1958).

70. Prankerd, T.A.J.: Folia Haemat. 78, 114 (1962).

71. Westerman, M.P., Pierce, L.E. and Jensen, W.N.: J. Lab. Clin. Med. 62, 394 (1962).

72. Ways, P.O.: J. Lipid Res. 8, 518 (1967).

73. deGier, J. and van Deenen, L.L.M.: Biochim. Biophys. Acta 49, 286 (1961).

74. Dodge, J.T. and Phillips, G.B.: J. Lipid Res. 7, 387 (1966).

75. Nelson, G.J.: J. Lipid Res. 8, 374 (1967).

76. Reed, C.F., Swisher, S.N., Marinetti, G.V. and Eden, E.G.: J. Lab. Clin. Med. 56, 281 (1960).

77. Hanahan, D.J., Watts, R.M. and Pappajohn, D.: J. Lipid Res. 1, 412 (1960).

78. Vacca, J.B., Waring, P.P. and Nims, R.M.: Proc. Soc. Exp. Biol. Med. 105, 100 (1960).

79. Eckles, N.E., Taylor, C.B., Campbell, D.J. and Gould, R.G.: J. Lab. Clin. Med. 46, 359 (1955).

80. Gould, R.G., LeRoy, G.V., Okita, G.T., Kabara, J.J., Keegan, P. and Bergenstal, D.M.: J. Lab. Clin. Med. 46, 372 (1955).

81. London, I.M. and Schwarz, H.: J. Clin. Invest. 32, 1248 (1953).

82. Hagerman, J.S. and Gould, R.G.: Proc. Soc. Exp. Biol. Med. 78, 329 (1951).

83. Chapman, D. and Wallach, D.F.H.: In Biological Membranes (D. Chapman, ed.) p. 125. Academic Press, New York (1968).

84. vanDeenen, L.L.M. and deGier, J.: In the Red Blood Cell (C. Bishop and D.M. Surgenor, eds.) p. 243. Academic Press, New York (1964).

85. Ways, P. and Hanahan, D.J.: J. Lipid Res. 5, 318 (1964).

86. Farquhar, J.W. and Ahrens, E.H., Jr.: J. Clin. Invest. 42, 675 (1963).

87. Lloyd, K.O., Kabat, E.A. and Licerio, E.: Biochemistry 7, 2976 (1968).

88. Watkins, W.M.: Science 152, 172 (1966).

89. Watkins, W.M.: In Specificity of Cell Surfaces (Davis, D.B. and Warren, L., eds.) p. 257. Prentice-Hall, New Jersey (1967).

90. Guidotti, G.: Ann. Rev. Biochem. 41, 731 (1972).

91. Segrest, J.P., Jackson, R.L., Andrews, E.P. and Marchesi, V.T.: Biochem. Biophys. Res. Commun. 44, 390 (1971).

92. Fessendem-Raden, J.M.: Biochem. Biophys. Res. Commun. 46, 1347 (1972).

93. Tung, J. and Knight, C.A.: Biochem. Biophys. Res. Commun. 42, 1117 (1971).

94. Rosenberg, S.A. and Guidotti, G.: J. Biol. Chem. 244, 5118 (1969).

95. Hoogeveen, J. Th., Juliano, R., Coleman, J. and Rothstein, A.: J. Memb. Biol. 3, 156 (1970).

96. Furthmayr, H. and Timpl, R.: Eur. J. Biochem. 15, 301 (1970).

97. Fairbanks, C., Steck, T.L. and Wallach, D.F.H.: Biochemistry 10, 2606 (1971).

98. Trayer, H.R., Nozaki, Y., Reynolds, J.A. and Tanford, C.: J. Biol. Chem. 246, 4485 (1971).

99. Reynolds, J.A. and Trayer, H.: J. Biol. Chem. 246, 7337 (1971).

100. Lenard, J.: Biochemistry 9, 1129 (1970).

101. Lenard, J.: Biochemistry 9, 5037 (1970).

102. Phillips, D.R. and Morrison, M.: Biochemistry 10, 1766 (1971).

103. Carraway, K.L. and Kobylka, D.: Biochim. Biophys. Acta 219, 238 (1970).

104. Blumenfeld, O.O., Gallop, P.M., Howe, C. and Lee, L.T.: Biochim. Biophys. Acta 211, 109 (1970).

105. Neville, D.M. and Glossmann, H.: J. Biol. Chem. 246, 6335 (1971).

106. Hamaguchi, H. and Cleve, H.: Biochim. Biophys. Acta 233, 320 (1971).

107. Gwynne, J.T. and Tanford, C.: J. Biol. Chem. 245, 3269 (1970).

108. Yu, J., Fischmann, D.A. and Steck, T.L.: J. Supramol. Struct. 1, 233 (1973).

109. Marchesi, S.L., Steers, E. Jr., Marchesi, V.T. and Tillack, T.W.: Biochemistry 9, 50 (1970).

110. Clarke, M.: Biochem. Biophys. Res. Commun. 45, 1063 (1971).

111. Mazia, D. and Ruby, A.: Proc. Nat. Acad. Sci. U.S. 61, 1005 (1968).

112. Haggis, G.H. Biochim. Biophys. Acta 193, 237 (1969).

113. Rosenthal, A.S., Kregenow, F.M. and Moses, H.L.: Biochim. Biophys. Acta 196, 254 (1970).

114. Nicholson, G.L., Marchesi, V.T. and Singer, S.J.: J. Cell. Biol. 51, 265 (1971).

115. Berg, H.C.: Biochim. Biophys. Acta 183, 65 (1969).

116. Bretscher, M.S.: J. Mol. Biol. 58, 775 (1971).

117. Bender, W.W., Garan, H. and Berg, H.C.: J. Mol. Biol. 58, 783 (1971).

118. Phillips, D.R. and Morrison, M.: Biochem. Biophys. Res. Commun. 45, 1103 (1971).

119. Phillips, D.R. and Morrison, M.: FEBS Lett. 18, 95 (1971).

120. Harris, J.R.: J. Mol. Biol. 46, 329 (1969).

121. Harris, J.R.: J. Ultrastruct. Res. 36, 587 (1971).

122. Steck, T.L., Fairbanks, G. and Wallach, D.F.H.: Biochemistry 10, 2617 (1971).

123. Steck, T.L.: J. Mol. Biol. 66, 295 (1972).

124. Tanner, M.J. and Gray, W.R.: Biochem. J. 125, 1109 (1971).

125. Guidotti, G.: Annals New York Acad. Sci. 195, 139 (1973).

126. Kobylka, D., Khettry, A., Shin, B.C. and Carraway, K.L.: Arch. Biochem. Biophys. 148, 475 (1972).

127. Glossmann, H. and Neville, D.M., Jr.: J. Biol. Chem. 246, 6339 (1971).

128. Marchesi, V.T., Tillack, T.W., Jackson, R.L., Segrest, J.P. and Scott, R.E.: Proc. Nat. Acad. Sci. U.S. 69, 1445 (1972).

129. Morawiecki, A.: Biochim. Biophys. Acta 83, 339 (1964).

130. Segrest, J.P., Kahane, I., Jackson, R.L. and Marchesi, V.T.: Arch. Biochem. Biophys. 155, 167 (1973).

131. Marchesi, V.T., Segrest, J.P. and Kahane, I.: In Membrane Research (C.F. Fox, ed.) p. 41. Academic Press, New York (1972).

132. Winzler, R.J.: In Red Cell Membrane: Structure and Function (G.A. Jamieson and T.J. Greenwalt, eds.) p. 157. Lippincott, Toronto (1969).

133. Springer, G.F., Nagai, Y. and Tegtmeyer, H.: Biochemistry 5, 3254 (1966).

134. Blumenfeld, O.O.: Biochem. Biophys. Res. Commun. 30, 200 (1968).

135. Zvilichovsky, B., Gallop, P.M. and Blumenfeld, O.O.: Biochem. Biophys. Res. Commun. 44, 1234 (1971).

136. Kathan, R.H., Winzler, R.J. and Johnson, C.A.: J. Exp. Med. 113, 37 (1961).

137. Kathan, R.H. and Winzler, R.J.: J. Biol. Chem. 238, 21 (1963).

138. Hughes, R.C.: Prog. Biophys. Mol. Biol. 26, 189 (1973).

139. Bretscher, M.S.: Nature New Biol. 231, 229 (1971).

140. Bretscher, M.S.: J. Mol. Biol. 59, 351 (1971).

141. Reynolds, J.A.: Annal. New York Acad. Sci. 195, 73 (1972).

142. Forstner, J. and Manery, J.F.: Biochem. J. 124, 563 (1971).

143. Forstner, J. and Manery, J.F.: Biochem. J. 125, 343 (1971).

144. Horvath, C. and Sovak, M.: Biochim. Biophys. Acta 298, 850 (1973).

145. Carraway, K.L., Kobylka, D. and Triplett, R.B.: Biochim. Biophys. Acta 241, 934 (1971).

146. Triplett, R.B. and Carraway, K.L.: Biochemistry 11, 2897 (1972).

147. Martin, K.: Biochim. Biophys. Acta 203, 182 (1970).

148. Steck, T.L.: In Membrane Research (C.F. Fox, ed.) p. 71, Academic Press, New York (1972).

149. Maddy, A.H.: Biochim. Biophys. Acta 88, 390 (1964).

150. Bretscher, M.S.: J. Mol. Biol. 71, 523 (1972).

151. Bretscher, M.S.: Biochem. J. 122, 40p (1971).

152. Bonsall, R.W. and Hunt, S.: Biochim. Biophys. Acta 249, 281 (1972).

153. Arrotti, J.J. and Garvin, J.E.: Biochim, Biophys. Res. Commun. 49, 205 (1972).

154. Schmidt-Ullrich, R., Knufermann, H. and Wallach, D.F.H.: Biochim. Biophys. Acta 307, 353 (1973).

155. Carraway, K.L., Kobylka, D., Summers, J. and Carraway, C.A.: Chem. Phys. Lipids 8, 65 (1972).

156. Phillips, D.R. and Morrison, M.: Biochem. Biophys. Res. Commun. 40, 284 (1970).

157. Phillips, D.R., and Morrison, M.: Fed. Proc. 30, 1065 Abs. (1971).

158. Herz, F. and Kaplan, E.: Proc. Soc, Exptl. Biol. Med. 140, 720 (1972).

159. Simpkins, H., Tay, S. and Panko, E.: Biochemistry 10, 3579 (1970).

160. Bellhorn, M.B., Blumenfeld, O.O. and Gallop, P.M.: Biochem. Biophys. Res. Commun. 39, 267 (1970).

161. Ohta, H., Matsumoto, J., Nagano, K., Fujita, M. and Nakao, M.: Biochem. Biophys. Res. Commun. 42, 1127 (1971).

162. Marchesi, V.T. and Palade, G.E.: J. Cell. Biol. 35, 385 (1967).

163. Avruch, J. and Fairbanks, G.: Proc. Nat. Acad. Sci. U.S. 69, 1216 (1972).

164. Williams, R.O.: Biochem. Biophys. Res. Commun. 47, 671 (1972).

165. Lankish, P.G. and Vogt, W.: Biochim. Biophys. Acta 270, 241 (1972).

166. Woodward, C.B. and Zwaal, R.F.A.: Biochim. Biophys. Acta 274, 272 (1972).

167. Roelofsen, B., Zwaal, R.F.A., Comfurius, P., Woodward, C.B., and van Deenen, L.L.M.: Biochim. Biophys. Acta 241, 925 (1971).

168. Murphy, J.R.: J. Lab. Clin. Med. 65, 756 (1965).

169. Ottolenghi, A.C. and Bowman, M.H.: J. Memb. Biol. 2, 180 (1970).

170. Nicholson, G.L., Masouredis, S.P. and Singer, S.J.: Proc. Nat. Acad. Sci. U.S. 68, 1416 (1971).

171. Pinto da Silva, P., Douglas, S.D. and Branton, D.: Nature 232, 194 (1971).

172. Avruch, J., Price, H.D., Martin, D.B. and Carter, J.R.: Biochim, Biophys. Acta 291, 494 (1973).

173. Okuda, T., Ito, A. and Sato, R.: J. Biochem (Tokyo) 71, 343 (1972).

174. Jung, C.Y., Carlson, L.M. and Balzer, C.J.: Biochim. Biophys. Acta 298, 108 (1973).

175. Pasquali, P., Landi, L., Masotti, L. and Lenaz, G.: J. Supramol. Struct. 1, 194 (1973).

176. Levine, Y.K.: Prog. Biophys. Mol. Biol. 24, 1 (1972).

177. Gordon, A., Wallach, D.F.H. and Strauss, J.H.: Biochim. Biophys. Acta 193, 225 (1969).

178. Glaser, M., Simpkins, H., Singer, S.J., Sheetz, M. and Chan, S.I.: Proc. Nat. Acad. Sci. U.S. 65, 721 (1970).

179. Chapman, D. and Dodd, G.H.: In Structure and Function of Biological Membranes (Rothfield, L.I. ed.) p. 13. Academic Press, New York (1971).

180. Radda, G.K. and Vanderkooi, J.: Biochim. Biophys. Acta 265, 509 (1972).

181. Stryer, L.: J. Mol. Biol. 13, 482 (1965).

182. McClure, W.O. and Edelman, G.M.: Biochemistry 5, 1908 (1966).

183. Oster, G. and Nishijima, Y.: Fortscher Hoch. Polym.-Forsch. 3, 313 (1964).

184. Weber, G. and Laurence, D.: Biochem. J. 51, XXXI (1954).

185. Oster, G. and Nishijima, Y.: J. Amer. Chem. Soc. 78, 158 (1956).

186. Lippert, E.: Z. Electrochem. 61, 962 (1967).

187. Wehry, E.L.: In Fluorescence (G. Guilbault, ed.) p. 37. Arnold, London (1967).

188. Weber, G.: In Light and Life (W. McElroy and B. Glass, eds.) p. 94. John Hopkins Press, Baltimore (1961).

189. Gitler, C., Rubalcava, B. and Caswell, A.: Biochim. Biophys. Acta 193, 479 (1969).

190. Rubalcava, B., Martinez de Munoz, D. and Gitler, C.: Biochemistry 8, 2742 (1969).

191. Feinstein, M.B., Spiro, L. and Felsenfield, H.: FEBS Lett. 6, 245 (1970).

192. Freedman, R.B. and Radda, G.K.: FEBS Lett. 3, 150 (1960).

193. Fortes, P.A.G. and Hoffman, J.F.: J. Memb. Biol. 5, 154 (1971).

194. Weidekamm, E., Wallach, D.F.H. and Fischer, H.: Biochim. Biophys. Acta 241, 770 (1971).

195. Radda, G.K. and Sanadi, D.R.: Current Topics in Bioenergetics 4, 81 (1971).

196. Vanderkooi, J. and Martonosi, A.: Arch. Biochem. Biophys. 144, 87 (1971).

197. Radda, G.K.: Biochem. J. 122, 385 (1971).

198. Vanderkooi, J. and Martonosi, A.: Arch. Biochem. Biophys. 133, 153 (1969).

199. Brocklehurst, J.R., Freedman, R.B., Hancock, D.J. and Radda, G.K.: Biochem. J. 116, 721 (1970).

200. Wallach, D.F.H., Ferber, E., Selin, D., Weidekamm, E. and Fischer, H.: Biochim. Biophys. Acta 203, 67 (1970).

201. Freedman, R.B., Hancock, D.J. and Radda, G.K.: In Probes of Structure and Function of Macromolecules and Membranes (B. Chance, C.P. Lee and J.K. Blaise, eds.) p. 325. Academic Press, New York (1971).

202. Gulik-Krzwicki, T., Shechter, E., Iwatsubo, M., Ranck, J.L. and Luzzati, V.: Biochim. Biophys. Act. 219, 1 (1970).

203. Daniel, E. and Weber, G.: Biochemistry 5, 1893 (1966).

204. Collier, H.B.: Experientia 26, 184 (1970).

205. Smith, D.S.: D. Phil. Thesis, Oxford University (1971), cited in (180).

206. Radda, G.K.: In the Passive Permeability of Cell Membranes (F. Kreuzer, ed.) p. 247. Plenum Press, New York (1972).

207. Gomperts, B., Lantelme, F. and Stock, R.: J. Memb. Biol. 3, 241 (1970).

208. McLaughlin, S.G.A., Szabo, G. and Eisenman, G.: J. Gen. Physiol. 58, 667 (1971).

209. Radda, G.K.: In Current Topics in Bioenergetics (D.R. Sanadi, ed.) Vol. 4, p. 81. Academic Press, New York (1971).

210. Klotz, I.: Chem. Revs. 41, 373 (1947).

211. Scatchard, G.: Annal. N.Y. Acad. Sci. 57, 660 (1949).

212. Langley, G.R. and Axell, M.: Brit. J. Haemat. 14, 593 (1968).

213. Smith, F.: Ph.D. Thesis, Dalhousie University (1971).

214. Maddy, A.H.: Biochim. Biophys. Acta 117, 193 (1966).

215. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: J. Biol. Chem. 193, 265 (1951).

216. Dubois, M., Gilles, K.A., Hamilton, J.K., Robers, P.A., and Smith, F.: Anal. Chem. 28, 350 (1956).

217. Warren, L.: J. Biol. Chem. 234, 1971 (1959).

218. Moore, S. and Stein, W.H.: Methods in Enzymology 6, 819 (1963).

219. Hirs, C.H.W.: Methods in Enzymology 11, 197 (1967).

220. Wainwright, S.D.: Can. J. Biochem. 48, 393 (1970).

221. Bartlett, G.R.: J. Biol. Chem. 234, 466 (1959).

222. Gros, C. and Labouesse, B.: Eur. J. Biochem. 7, 463 (1969).

223. Woods, K.R. and Wang, K.T.: Biochim. Biophys. Acta 133, 369 (1967).

224. Neville, D.M., Jr.: J. Biol. Chem. 246, 6328 (1971).

225. Heathcote, J.G. and Washington, R.J.: Analyst 92, 627 (1967).

226. International Critical Tables. McGraw-Hill, New York (1926).

227. Schachman, H.K.: Methods in Enzymology 4, 32 (1957).

228. Winzler, R.J., Harris, E.D., Pekas, D.J., Johnson, C.A. and Weber, P.: Biochemistry 6, 2195 (1967).

229. Satake, K., Okuyama, T., Ohashi, M. and Shinoda, T.: J. Biochem. (Tokyo) 47, 654 (1960).

230. Ambler, R.P.: Methods in Enzymology 25B, 143 (1972).

231. Gross, E.: Methods in Enzymology 11, 238 (1967).

232. Weber, C. and Young, L.B.: J. Biol. Chem. 239, 1415 (1964).

233. Schwert, G.W. and Takenaka, Y.: Biochim. Biophys. Acta 16, 570 (1955).

234. Margolis, S. and Langdon, R.G.: J. Biol. Chem. 241, 485 (1966).

235. Palmer, F.B. St. C. and Verpoorte, J.A.: Can. J. Biochem. 49, 337 (1971).

236. Rega, A., Weed, R.I., Reed, C.F., Berg, G.G. and Rothstein, A.: Biochim. Biophys. Acta 147, 297 (1967).

237. Schneider, H. and Smith, I.C.P.: Biochim. Biophys. Acta 219, 73 (1970).

238. Quidotti, G.: In Red Blood Cell Membrane: Structure and Function. (G.A. Jamieson and T.J. Greenwalt, eds.) p. 115, Lippincott, Toronto (1969).

239. Weed, R.I.: In Red Blood Cell Membrane: Structure and Function (G.A. Jamieson and T.J. Greenwalt, eds.) p. 115, Lippincott, Toronto (1969).

240. Eylar, E.H., Madoff, M.A., Brady, O.V. and Oncley, J.L.: J. Biol. Chem. 237, 1992 (1962).

241. Jackson, R.L., Segrest, J.P., Kahane, I. and Marchesi, V.T.: Biochemistry 12, 3131 (1973).

242. Dodge, J.T., Mitchell, C. and Hanahan, D.J.: Arch. Biochem. Biophys. 100, 119 (1963).

243. Vanderkooi, J., Jost, P., Griffith, O.H., Capaldi, R.A. and Vanderbooi, G.: Biochim. Biophys. Acta 311, 141 (1973).

244. Vanderkooi, G. and Capaldi, R.A.: Annal. New York Acad. Sci. 195, 135 (1972).

245. Gingell, D.: J. Theor. Biol. 38, 677 (1973).

246. Gel Filtration in theory and practice. Pharamacia Fine Chemicals, Uppsala, Sweden.

247. Schroeder, W.A., Shelton, J.R. and Robberson, B.: Biochim. Biophys. Acta 147, 590 (1967).

248. Capaldi, R.A.: Biochem. Biophys. Res. Commun. 50, 656 (1973).

249. Fasman, G.D., Hoving, H. and Timasheff, S.N.: Biochemistry 9, 3316 (1970).

250. Vanderkooi, G. and Green, D.E.: Proc. Nat. Acad. Sci. U.S. 66, 615 (1970).

251. Schneider, M.J.T. and Schneider, A.S.: J. Memb. Biol. 9, 127 (1972).

252. Lesslauer, W.: Proc. First Eur. Biophys. Symp. IV, 425 (1971).

END

22 • 11 • 74

FIN