

INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.
2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.
3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again beginning below the first row and continuing on until complete.
4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.
5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

**University
Microfilms
International**

300 N. Zeeb Road
Ann Arbor, MI 48106

8302491

Bartelt, Diana Claire

**THE CYTOSKELETAL SYSTEM OF THE NONMAMMALIAN VERTEBRATE
ERYTHROCYTE**

City University of New York

Ph.D. 1982

**University
Microfilms
International** 300 N. Zeeb Road, Ann Arbor, MI 48106

Copyright 1982

by

Bartelt, Diana Claire

All Rights Reserved

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print ✓
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

University
Microfilms
International

THE CYTOSKELETAL SYSTEM OF THE
NONMAMMALIAN VERTEBRATE ERYTHROCYTE

by

DIANA C. BARTELT

A dissertation submitted to the Graduate Faculty in Biology in
partial fulfillment of the requirements for the degree of Doctor of
Philosophy, The City University of New York

1982

© COPYRIGHT BY

DIANA C. BARTELT

1982

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

JUNE 10, 1982

Date

31 August 1982

Date

William D. Cohen

Chairman of Examining Committee

Dr. William D. Cohen, Hunter College

James A. Organ

Executive Officer

Dr. James A. Organ, City College

Robert Grant

Dr. Robert Grant

Peter Lipke

Dr. Peter Lipke

Raymond Gavin

Dr. Raymond Gavin

Claude Klee

Dr. Claude Klee

George Scheele

Dr. George Scheele

Hunter College

Institution

Hunter College

Institution

Brooklyn College

Institution

National Institutes of Health

Institution

Rockefeller University

Institution

Institution

Institution

The City University of New York

Abstract

THE CYTOSKELETAL SYSTEM
OF THE NONMAMMALIAN VERTEBRATE ERYTHROCYTE

by

Diana C. Bartelt

Advisor: Professor William D. Cohen

Nonmammalian vertebrate erythrocytes are flattened elliptical nucleated cells with relatively few cytoplasmic organelles. Their cytoskeleton consists of a peripheral bundle of microtubules (marginal band;MB),surrounded by a cell surface associated cytoskeleton (SAC) and contains transcellular structures attached to the nucleus which include intermediate filaments. Dogfish erythrocyte cytoskeletons were examined for structural organization by phase contrast light microscopy and transmission electron microscopy. Anucleate dogfish erythrocyte cytoskeletons, consisting of MB and SAC, were prepared. Microtubules of dogfish erythrocyte MBs are cold labile in vivo. Anucleate cytoskeletons prepared from cooled cells devoid of MBs, contained spectrin-like proteins, actin, and possibly vimentin and goblin as their major protein constituents. Marginal bands, isolated from cytoskeletons, contained tubulin as their only major protein constituent.

Dogfish erythrocytes exhibited calcium-dependent changes in

morphology when incubated with ionophore A23187. Calmodulin, a known mediator of the effects of calcium on both soluble and cytoskeletal proteins, was detected in erythrocyte lysates. Anucleate dogfish erythrocyte cytoskeletons bound calmodulin in a calcium-dependent manner, and contained one major calcium-dependent calmodulin-binding protein (CBP). CBP, with an apparent molecular weight of 245,000, comigrated with the upper bands of both dogfish and human spectrin. CBP was localized to the SAC, and was extracted with dogfish spectrin under conditions of low ionic strength. CBP was identified as fodrin, a protein localized in the SAC of non-erythroid cells of both non-mammalian and mammalian origin. Antibody to fodrin reacted strongly with CBP and weakly with human spectrin. Anti-human spectrin antibody bound to the lower band of dogfish spectrin and goblin, but not to CBP. These data suggest that spectrin diverged from fodrin during the evolution of erythrocytes from elliptical nucleated cells containing MBs in non-mammalian vertebrates to anucleate biconcave discoid cells, lacking these structures, in mammalian vertebrates.

Foreword

The writing of this thesis has allowed me to record scientific findings and to hypothesize relatively freely on their meaning, a rare opportunity in a time when pressure to limit space in scientific journals prohibits this type of expression. Given such an opportunity, I cannot resist the temptation to record some personal reflections as well. The period of my graduate education has represented six years, perhaps at the midpoint of my life, devoted to intensive study. It has been a hiatus from the mundane, teaching me that such intensity promotes growth, not only in the field of study, but in other areas as well. As with meditation, the real education has come ancillary to the discipline of the academic course of study. Truths have surfaced and superficial values have been washed away. The study of life has progressed on a multitude of levels, from molecular to global.

The principle to be extracted from the scientific research is that rather than becoming what we do, we, as living beings, do what we are. Tubulin, once coded for and translated, forms microtubules, and not intermediate filaments or microfilaments. Our intrinsic properties predetermine, to a large extent, our behavior and role in life. Each has its role, no one more important than the other. Keeping all of this in balance, is the ultimate interdependence of living things upon each other,

each exerting a life-forming influence on the other. The tremendous diversity built into the system, prevents life from becoming predictable or even subject to definition.

The principle of interdependence extends to the many levels at which we experience life. Everything that I have done during this period has influenced my formal education, and the appreciation of life, gained on a biological level, has enriched my total life experience. The contributions of an extended family of people, especially some adopted siblings, to the total education process, can not be overestimated. The experiences which led to my decision to pursue a graduate education also have had their effect upon the process. Two women, who, against all of my best arguments, urged me to get my degree, finally have their way, even though neither is alive to see it. A year's research in the south of France, working with people who have a special talent for enjoying life and science, supplied the necessary inducement to overcome my reservations. Finally, there are two people who have been there from the beginning and who have had the greatest influence, providing support, love and understanding throughout, my mother and sister Jean, to whom this thesis is dedicated.

Acknowledgements

As is true for any report of scientific research, this thesis represents the end result of work performed with the cooperation, guidance and assistance of a number of colleagues. It is a pleasure to acknowledge and thank them, and to recognize their contributions, not only to this research, but to my education as well.

I owe a tremendous debt of gratitude to Dr. William D. Cohen my thesis advisor, for a teacher/student relationship which I consider ideal. By fostering an open and honest exchange of ideas, and offering an atmosphere conducive to collaborative research, he insured that my graduate education was a positive joy instead of a potential chore. His respect for life, in all of its forms, gave me an insight into and appreciation for biology which, I must admit, I did not have prior to this encounter. His talents as a morphologist and microscopist are evident in this thesis in that he prepared almost all of the electron micrographs presented here. I thank him for introducing me to the world of the Marine Biological Laboratory at Woods Hole, where the body of this research was performed, and thank both he and his wife, Dr. Marion Cohen, for pleasant summer evenings spent together at Woods Hole.

I am indebted to Dr. George A. Scheele of The Rockefeller University for the opportunity to perform research in his laboratory. Techniques acquired there were invaluable in obtaining the results reported in this thesis. I thank him for his valuable

counsel and for his time, patience and generosity during my entire graduate education.

It is with pleasure that I thank Dr. Richard Carlin of The Rockefeller University for a very fruitful collaboration on the work involving the detection and identification of calmodulin-binding proteins. His generosity and enthusiasm greatly facilitated this research and I am indebted to him for continuing the project while I was preparing this thesis.

I thank the faculty of the Department of Biological Sciences of Hunter College, in particular Professor Katherine Lyser, for their contributions to my graduate education and Drs. Dennis Grab, Thomas Carne, Victoria Iwanji, and D. Martin Watterson of The Rockefeller University for their advice and many fruitful discussions. I thank Elena Siphicas for her technical assistance in preparing some of the electron micrographs presented here. The enthusiasm and moral support of fellow graduate students Iris Nemhauser, Gail Zeevalk, Joanne Carroll, Don Burmeister, Jackie Joseph-Silverstein and Jacqui Carter have often provided the incentive to continue. I am especially grateful to Liz Wood and Jackie Joseph-Silverstein for their valuable assistance in preparing this manuscript.

Table of Contents

	PAGE
Copyright	ii
Approval	iii
Abstract	iv
Foreword	vi
Acknowledgements	viii
Table of Contents	x
List of Tables	xvi
List of Figures	xvii
Introduction	1
I. Historical Background	1
II. The Non-Mammalian Erythrocyte as a Model System	2
A. Marginal bands of non-mammalian and mammalian blood cells	4
B. Transcellular Structures	9
C. Cell surface associated cytoskeleton (SAC)	10
III. Objectives and Initial Approach	14
A. Characterization of the dogfish erythrocyte cytoskeleton	15
B. Effects of calcium on the dogfish erythrocyte cytoskeleton	17
C. Calmodulin and cytoskeletal calmodulin receptors in the dogfish erythrocyte	19
IV. Summary of specific experimental objectives	20

	PAGE
Materials	21
Methods	23
Preparation of washed dogfish erythrocytes	23
Treatment of dogfish erythrocytes with calcium ionophore A23187	23
Nucleated dogfish erythrocyte cytoskeletons	24
Cell lysates before and after MB disassembly	25
pH 3 extracts of nucleated dogfish erythrocyte cytoskeletons	25
Calcium extracts of cytoskeletons	26
Preparative isolation of MBs	26
Anucleate dogfish erythrocyte ghosts and cytoskeletons	28
Low-ionic strength extraction of dogfish anucleate ghosts	29
Human erythrocyte ghosts	29
Anucleate chicken erythrocyte ghosts	30
Cell and cytoskeletal morphology	30
Electron microscopy	31
Gel electrophoresis	33
Preparation of bovine brain calmodulin	33
Assay of calmodulin activity in dogfish erythrocyte lysate	39
Iodination of calmodulin and protein A with Na ¹²⁵ I	39
¹²⁵ I calmodulin binding to anucleate dogfish erythrocyte cytoskeletons	40

	PAGE
^{125}I calmodulin binding to proteins separated by polyacrylamide gel electrophoresis	41
Photoaffinity labelling of calmodulin binding proteins in erythrocyte ghosts	41
Experiments and Results	43
I. Structural components and organization of the dogfish erythrocyte cytoskeleton	43
A. Living dogfish erythrocytes and nucleated cytoskeletons	43
B. <u>In vivo</u> temperature cycling of dogfish erythrocyte MBs	46
C. Preparation and structural organization of anucleate dogfish erythrocyte cytoskeletons	48
D. Isolated plasma membrane and SAC preparations	54
E. MB isolation and structure	57
II. Protein components of the dogfish erythrocyte cytoskeletal system	60
A. Solubilized cytoskeletal protein components of nucleated cytoskeletons	60
B. Anucleate ghosts and cytoskeletons	62
C. Localization of protein components to cytoskeletal structures	71
1. MB components	71
2. SAC components	73
III. Correlation between cytoskeletal and cell morphology	75
A. "Spindle cell" formation	75

	PAGE
B. Effect of calcium and ionophore A23187 on erythrocyte morphology	77
IV. <u>In vitro</u> calcium effects on cytoskeletal structures	81
A. Effect of calcium during lysis	81
B. Calcium lability of the dogfish erythrocyte cytoskeleton	83
V. Calmodulin and cytoskeletal calmodulin-binding proteins in dogfish erythrocytes	84
A. Presence of calmodulin	84
B. In vitro binding of calmodulin to dogfish erythrocyte cytoskeletons	86
C. ¹²⁵ I calmodulin binding to proteins in anucleate erythrocyte ghosts and cytoskeletons	86
D. Location of CBP in the anucleate dogfish erythrocyte cytoskeleton	90
E. Presence of CBP in erythrocytes of another non-mammalian species	93
F. Photoaffinity labelling of calmodulin-binding proteins in erythrocyte ghosts	94
VI. Identification of CBP	96
A. Extraction of CBP from anucleate dogfish erythrocyte ghosts	96
B. Immunoreactivity of dogfish erythrocyte CBP with antibody to mammalian CBP-I	98
C. Identification of dogfish CBP (S1) as alpha-fodrin	98
D. Immunoreactivity of anti-fodrin with human and chicken erythrocyte ghost proteins	102

	PAGE
E. Cross-reactivity of Fab' fragments of anti-human spectrin antibody with dogfish spectrin and goblin and canine PSD fodrin	102
Discussion	106
I. The dogfish erythrocyte cytoskeletal system	106
A. Major structural components	106
1. Cell surface associated cytoskeleton (SAC)	106
2. Marginal Band (MB)	107
3. Lack of evidence of transcellular structures	108
B. Function of erythrocyte cytoskeletal components	109
1. The role of MBs in maintenance of morphology	109
2. Contribution of the SAC to cell morphology	111
II. Erythrocyte membrane and cytoskeletal protein comonents	112
A. MB components	112
B. Components of the erythrocyte SAC	114
1. "Spectrin" - Identification of nonmammalian S1 as alpha fodrin	114
2. "Goblin"	119
3. Other cytoskeletal proteins	120
a. "vimentin"	120
b. Actin	121

	PAGE
c. Comparison of dogfish and human erythrocyte proteins	122
III. Effects of calcium on cell morphology and cytoskeletal structures	123
A. Calcium and ionophore A23187 effects on erythrocyte morphology.	123
B. In vitro effects of calcium on dogfish erythrocyte MBs	125
IV. Functions of fodrin and fodrin-like proteins	126
References	130

List of Tables

I. Amino acid composition of bovine brain calmodulin.	Page 37
II. Comparison of major protein components of dogfish anucleate and human erythrocyte ghosts.	65
III. Cross-reactivity of cytoskeletal proteins.	105

List of Figures

	PAGE
1. Purification of calmodulin-procedure.	34
2. DEAE sephadex chromatography of calmodulin.	35
3. Affinity chromatography of calmodulin.	36
4. Dogfish (<u>Mustelus canis</u>).	44
5. Dogfish erythrocytes and erythrocyte cytoskeletons in phase contrast.	45
6. Dogfish erythrocyte cytoskeleton in whole mount and thin section.	45
7. Temperature cycling of dogfish erythrocytes - phase contrast.	47
8. Temperature cycling of dogfish erythrocytes in whole mount and thin section.	49
9. Preparation of anucleate dogfish erythrocyte cytoskeletons - procedure.	51
10. Preparation of anucleate dogfish erythrocyte cytoskeletons - phase contrast.	52
11. Anucleate dogfish erythrocyte cytoskeleton - whole mount.	53
12. Anucleate dogfish erythrocyte ghosts and cytoskeletons - thin section.	55
13. Marginal bands in anucleate ghosts.	56
14. Anucleate dogfish erythrocyte ghosts from cooled cells - phase and thin section.	58
15. Marginal bands isolated from dogfish erythrocyte - phase and whole mount.	59
16. Isolated marginal bands - thin section.	61
17. Protein components of anucleate dogfish ghosts and cytoskeletons.	64

	PAGE
18. Dogfish erythrocyte and rat vimentin migration.	67
19. Immunoreactivity of dogfish spectrin with antibody to human spectrin.	69
20. Immunoreactivity of dogfish vimentin with antibody to BHK vimentin.	69
21. Localization of protein components in the anucleate dogfish erythrocyte ghost.	70
22. Human and anucleate dogfish erythrocyte ghost protein composition.	70
23. Solubilization of protein components of dogfish erythrocyte ghosts.	72
24. Cold and calcium extracts of dogfish erythrocyte cytoskeletons.	74
25. Extraction of anucleate dogfish erythrocyte ghosts.	76
26. Vesicles formed from anucleate dogfish erythrocyte ghosts - phase contrast.	78
27. Dogfish erythrocyte spindle cells - phase contrast.	78
28. Effect of calcium and A23187 on dogfish erythrocyte morphology.	80
29. Effect of calcium during lysis of dogfish erythrocytes in Ringer's solution.	82
30. Effect of calcium during lysis of dogfish erythrocytes in lysis medium A.	82
31. Presence of calmodulin in dogfish erythrocyte lysate.	85
32. ¹²⁵ I calmodulin-binding to dogfish erythrocyte anucleate cytoskeletons.	87
33. Calmodulin-binding proteins of anucleate dogfish erythrocyte ghosts.	88

	PAGE
34. Localization of calmodulin-binding protein in the anucleate dogfish erythrocyte ghost.	91
35. Calmodulin-binding proteins in mammalian cytoskeletal structures.	92
36. Calmodulin-binding proteins of anucleate chicken erythrocyte ghosts.	95
37. Azido- ¹²⁵ I calmodulin-binding to erythrocyte ghosts.	97
38. Immunoreactivity of dogfish and human spectrin with antibody to brain calmodulin binding protein.	99
39. Identification of dogfish erythrocyte CBP as fodrin.	101
40. Immunoreactivity of anti-fodrin antibody with spectrins.	103
41. Immunoreactivity of anti-spectrin antibody with brain proteins.	103

Introduction

I. Historical Background

Nonmammalian erythrocytes were among the many biological specimens observed by van Leeuwenhoek in the late seventeenth century (Gillespie, 1973). He measured the size of erythrocytes and their nuclei in fish and amphibians and observed circulation in the tail of a live eel. The large size of amphibian erythrocytes made them a popular subject of study by early microscopists. In 1773, Hewson described nuclei in fish and amphibian erythrocytes after lysis in water. Boettcher (1866) observed fibers between the nucleus and the cell periphery after tannic acid fixation. Ranvier (1875) reported studies on frog, axolotl and salamander erythrocytes which included the effects of dilute alcohol on the shape of the lysed cell, and the appearance of a marginal layer beneath the membrane which he interpreted as a thickening of the membrane. He also reported observations on the apparent resealing of the ghost after spontaneous enucleation. Adolf Dehler (1895) studied the role of centrioles in cell division during differentiation of chicken erythrocytes. He reported the existence of a marginal ring structure, 0.25-0.5 microns thick, which encircled the interior of the cell, but interpreted this ring as a thickened portion of the ectoplasm. Meeves (1911) correctly identified the marginal band as a filamentous structure which could be isolated from amphibian erythrocytes. Despite this early interest, relatively few studies

of nonmammalian erythrocyte structure were reported after the early part of the twentieth century.

II. The Nonmammalian Erythrocyte Cytoskeleton as a Model System.

Nonmammalian vertebrate erythrocytes are flattened nucleated cells with a characteristic biconvex elliptical morphology. Their biconvexity is due to the protrusion of the nucleus beyond the plane of cell flattening. They vary in size with lengths ranging from 6 microns, (carolina chickadee; Bartsch et al, 1937) to 65 microns in the urodele amphibian *Amphiuma* (Hartman and Lessler, 1964). Their degree of ellipticity varies independently of size; the ratio of major to minor axes ranges from 1.1, or barely elliptical, to greater than 2.0. Immature erythrocytes contain mitochondria, Golgi and endoplasmic reticulum as well as a nucleus, but cells lose their cytoplasmic organelles during maturation leaving a relatively simple nucleated cell (Shinagawa, 1960). Their cytoskeletons consist of a peripheral bundle of microtubules known as a marginal band (MB), and transcellular structures which include intermediate filaments (Woodcock, 1980) attached to the nucleus, both of which are enclosed within a cell-surface associated cytoskeleton (SAC). Although there are species differences in the size and characteristics of these structures, when considered as a cell type, nonmammalian vertebrate erythrocytes offer the following advantages as a model system for the study of cytoskeletal composition and function in nucleated cells: 1. simplicity of intracellular structure, 2. easy accessibility relative to cells of other tissues, 3. availability in pure populations and 4. size sufficiently large to provide a

cytoskeleton that can be visualized by phase contrast microscopy. However, little is known about the organization of their cytoskeletal components, either on a structural or molecular level.

Mammalian erythrocytes have been used extensively as a model system for the study of cytoskeletal organization and molecular composition. Detailed investigations have been facilitated by their availability in quantity and by the ease with which cytoskeletal elements can be prepared from these cells. Although significant progress has been made toward understanding how the principle cytoskeletal proteins are arranged in the mammalian red cell, application of this information to cytoskeletons of nucleated non-erythroid cells is made difficult by the fact that cytoskeletons of nucleated cells contain transcellular structures including microfilaments, microtubules and intermediate filaments while mammalian erythrocyte cytoskeletons are limited to a cell-surface associated meshwork. In addition, non-erythroid cells do not contain sufficient amounts of spectrin (Hiller and Weber, 1977), a protein comprising 75 percent of the erythrocyte cytoskeletal mass (Lux et al, 1976) and essential to the integrity of the erythrocyte (Marchesi and Steers, 1968; Greenquist et al, 1978). Mammalian erythrocytes are therefore not suited to the study of how transcellular structures interact with the cell surface associated cytoskeleton (SAC) or of proteins that constitute the SAC responsible for maintenance of shape in non-erythroid cells.

Nonmammalian erythrocytes represent an intermediate cell type between anucleate mammalian erythrocytes and more complex, less

accessible non-erythroid cells. As such, their cytoskeletons resemble those of other nucleated cells more closely than do the cytoskeletons of the mammalian erythrocyte and therefore provide a more appropriate model cytoskeletal system. Nonmammalian erythrocytes lend themselves to the study of (1). microtubule systems in general and, in particular, marginal bands of flattened blood cells, (2). transcellular cytoskeletal elements associated with the nucleus, and (3). the cell surface associated cytoskeleton of nucleated cells. In addition, since nonmammalian vertebrate erythrocytes undergo distinct changes in morphology during embryonic development from sphere to disc to flattened ellipse (Barret and Dawson, 1974), and erythroid cells of some species have been reported to develop in synchrony in the embryonic circulation (Chan, 1977), these cells provide a suitable model system for the study of cytoskeletal structure formation during development and the role of these structures in determining cell morphology.

A. Marginal bands of nonmammalian and mammalian blood cells.

Marginal bands of microtubules (MBs) occur in some invertebrate erythrocytes and clotting cells (Nemhauser et.al., 1980), and mammalian platelets (Behnke, 1965) and primitive fetal circulating blood cells (van Deurs and Behnke, 1973), as well as nonmammalian vertebrate erythrocytes and thrombocytes (Behnke, 1970). There is a general correlation between the presence of MBs in these cells and their flattened morphology. This correlation has been the subject of a number of investigations with a view toward determining whether

the marginal band is responsible for the generation and/or maintenance of cell shape. Meeves, the first to describe MBs as filamentous structures and isolate them from the erythrocytes of *Amphiuma*, observed that when the MB of a cell collapses the cell becomes spherical, and that isolated MBs retain their elliptical shape (Meeves, 1904). He concluded that the flattened elliptical shape of the cell was due to tension exerted on the membrane by the MB. Using transmission electron microscopy, Fawcett (1959) described the MB of toadfish erythrocytes as being composed of parallel tubular elements about 30 nm. in diameter located immediately beneath the plasma membrane. He concluded that "the hoop-like form of these elements, their equatorial location and their fine-structural resemblance to supporting elements of other cells lend plausibility to Meeves's suggestion that they may play a significant role in maintaining the flattened elliptical form of avian, amphibian and piscian erythrocytes" (Fawcett, 1962). The parallel tubular elements of the MB were later identified as microtubules (Fawcett and Witebsky, 1964). The number of microtubules comprising the MB of nonmammalian erythrocytes increases with increasing cell length (Goniakowska-Witalinska and Witalinski, 1976) which is consistent with the MB providing increased structural support in larger cells. These authors suggested that in amphibia and fish, where erythrocytes are exposed to wide variations in osmolality, the MB might stabilize the cell, protecting it from excessive swelling.

The view that MBs play a role in maintenance of cell morphology has been disputed. Behnke observed that some blood cells could maintain their shape after MB disassembly (1970). In a study of MBs in chick erythrocytes, he induced microtubule disassembly by cooling cells to 0 C. and found that chick erythrocytes remained flattened and elliptical despite the disappearance of microtubules. He concluded that the MB was not responsible for maintenance of shape in all MB-containing cell types.

Barrett and Dawson (1974) arrived at a similar conclusion concerning mature erythrocytes, but demonstrated that MBs are essential to the development of the flat elliptical shape of avian erythrocytes during erythroblast maturation in bone marrow. Studies on camel erythrocytes (Cohen and Terwilliger, 1979) and erythropoiesis in llamas (Goniakowska-Witalinska and Witalinski, 1977) support the view that MBs are involved in the determination of erythrocyte shape. The erythrocytes in species of the camel family are unique among mammals in that they are not round, but elliptical. Cohen and Terwilliger observed that at least three percent of camel erythrocytes contain MBs, a structure not found in other mature mammalian erythrocytes. These MB-containing cells were identified as circulating reticulocytes in camel. They were also observed in immature llama erythrocytes in bone marrow (Goniakowska-Witalinska and Witalinski, 1977). These findings are consistent with the involvement of MBs in the formation of the elliptical shape of these cells during differentiation followed by

loss of MBs as the cells mature. A parallel but partial loss of MB microtubules during erythrocyte maturation has been reported in fish (Sekhon and Beams, 1969) and birds (Barrett and Dawson, 1974), but apparently does not occur in amphibian erythrocytes (Small, 1972). While a role for MBs in maintenance of nonmammalian erythrocyte morphology is not generally accepted, it is clear that MBs are important in the determination of the flattened elliptical shape of the cell.

Marginal bands are essential to the maintenance of correct morphology of mammalian platelets and invertebrate and vertebrate thrombocytes (clotting cells; Nemhauser et al., 1980; Behnke, 1970). The use of the term MB for microtubules in nonmammalian thrombocytes is in question. Behnke (1970) described a marginal bundle of microtubules lying directly beneath the plasma membrane of chick thrombocytes. However fish thrombocytes contain an annular bundle of microtubules encircling the nucleus which Fawcett and Witesbsky (1964) have called the endoplasmic ring. Unlike MBs of chick thrombocytes and invertebrate clotting cells, these bands contain well over one hundred microtubules. Endoplasmic rings always lie in the plane of flattening of the thrombocyte and a correlation exists between the tightness of the microtubule bundle and the ellipticity of the cell. It appears then that endoplasmic rings, like MBs, have a role in the maintenance of cell morphology. Chick thrombocytes and rat and human platelets, lost their normal disk shape concurrent with MB disassembly during incubation in the cold and, for the most part, regained their normal morphology with MB reassembly upon

rewarming (Behnke, 1970). The effect of low temperature on the shape of rat platelets was confirmed by Bennett and Lynch (1980) using scanning electron microscopy. Shape changes caused by cold-induced MB disassembly closely paralleled morphological changes seen during platelet activation. Disorganization of platelet MBs following activation and during spreading has been observed using fluorescent antibodies to tubulin (Debus, et al, 1981).

Fundamental differences exist between MBs of nonmammalian erythrocytes of different species and also between MBs of different cell types within the same species. For example, chicken erythrocyte MBs are cold-labile while those of frog are not. Treatment with N-ethylmaleimide had no effect on chicken erythrocyte MBs but caused disorganization of chick thrombocyte MBs with concurrent changes in cell shape. Mammalian platelet MBs were labile to cold, colcemid, N-ethylmaleimide and vinca alkaloids. All of these agents caused platelets to form spheres or irregular shapes (Behnke, 1970). These results raise the question of whether there are differences in the microtubules of these MB systems or whether these cells contain different mechanisms for the control of microtubule assembly. The role of MBs in the determination of nonmammalian erythrocyte morphology and the maintenance of thrombocyte and platelet morphology demonstrates the importance of these structures to the cells in which they occur. Despite the differences in MB characteristics, MBs provide a relatively simple microtubule structure for the study of microtubule assembly *in vivo* and the effect of microtubular structures on cell morphology.

B. Transcellular structures

Early observations of nonmammalian erythrocytes included conflicting reports on the presence of transcellular structures in these cells. Boettcher (1866) observed fibers between the cell periphery and nucleus of avian and amphibian erythrocytes after treatment with tannic acid, whereas Meeves (1911) in a review of the literature concluded that filaments seen associated with the nucleus of the nonmammalian erythrocyte resulted from staining artifacts. Dawson (1929), using a variety of vital dyes and heavy metal stains, observed that the cytoplasm of salamander erythrocytes was filled with a close-meshed network. This finding was confirmed by Shinagawa (1960) who described silver staining of a three dimensional network within amphibian, fish and avian erythrocytes.

The nature of the transcellular network in avian erythrocytes was first described by Harris and Brown (1971) who reported the presence of 100 Å filaments extending between the outer nuclear membrane and the plasma membrane in thin sectioned chicken and turkey erythrocyte ghosts. Similar observations were made by Virtanen et al. (1979) who observed a network of intermediate filaments that formed a "nucleus-anchoring" cytoskeleton in chicken erythrocytes. Intermediate filaments associated with isolated chicken erythrocyte nuclei were shown to contain vimentin, a protein of intermediate filaments found in muscle and many types of tissue culture cells (Woodcock, 1980). In addition to vimentin, intermediate filaments of avian erythrocytes have recently been

shown to contain synemin, a high molecular weight protein found associated with desmin of intermediate filaments present in smooth and skeletal muscle (Granger *et al.*, 1982). Synemin was shown to co-localize with vimentin in a cytoplasmic network of filaments that extended from the nucleus to the plasma membrane and resisted aggregation by colcemid, an inhibitor of microtubule assembly.

The association of intermediate filaments with erythrocyte nuclei is consistent with their localization in other nucleated cells (Lehto *et al.*, 1978; Small and Celis, 1978). To date, intermediate filaments in erythrocytes have been described only in avian species and it is not known if they occur in the erythrocytes of other nonmammals. Other types of transcellular structures such as microfilaments, non-MB microtubules or microtrabeculae (Wolosewick and Porter, 1979) have not been described in nonmammalian erythrocytes. With the exception of non-MB microtubules (Cohen *et al.* 1982), the possibility that they are present in nonmammalian erythrocytes has not been eliminated. If these other structures are absent, then avian erythrocytes and perhaps erythrocytes of other nonmammals represent an appropriate cell type for the study of intermediate filaments and their association with both the nucleus and plasma membrane or cell surface associated cytoskeleton (SAC).

C. Cell surface associated cytoskeleton (SAC)

The term cell surface associated cytoskeleton (SAC) is defined here as those cytoskeletal elements located directly beneath and

associated with the plasma membrane of a cell that form a structure delimiting the cytoplasmic space. This definition attempts to reconcile the current operational definition of this structure as the residue remaining after detergent extraction of mammalian erythrocytes, and the need to define a comparable structure in other cells in which it is but a part of a three dimensional cytoskeletal system. The operational aspect of this definition defines the SAC of any cell as the structure remaining after detergent extraction of a plasma membrane fraction derived from that cell type.

Until recently, the SAC of nonmammalian erythrocytes has been studied only as a part of the plasma membrane. As is the case for transcellular elements, almost all of the studies of nonmammalian erythrocyte plasma membranes have been performed using avian erythrocytes. In 1971, both Harris and Brown and Zentgraf *et al* described the preparation of chicken erythrocyte plasma membranes. Zentgraf and coworkers determined their protein and lipid content and demonstrated that, like human erythrocyte membranes, those of chicken contain both Mg^{++} -dependent and $Na^{+}-K^{+}$ activated Mg^{++} -dependent ATPases. Electron micrographs showed that the plasma membranes had formed right-side-out vesicles with fibrous material located on their inside surface. This material is very similar in appearance to that seen on the cytoplasmic surface of human erythrocyte ghosts (Tsukita *et al.*, 1980). The vesicles occasionally contained intermediate filaments, material which, the authors noted, could not be removed by high salt extraction. Blanchet (1974) prepared plasma membranes from chicken erythrocytes

and compared their protein content with those of human erythrocytes by polyacrylamide gel electrophoresis in SDS. He reported the comigration of a number of protein bands in chicken erythrocyte membranes with those of human erythrocyte ghosts. Among these were a high molecular weight protein doublet which he identified as bands I and II described by Fairbanks et al , 1971 for human erythrocytes. He concluded that these proteins were the same as spectrin which had been described as the major cytoskeletal protein of human erythrocytes by Marchesi and Steers (1968).

Chicken erythrocyte plasma membranes have been used to study the changes in protein content of membranes during development. Chan (1977) showed that qualitative and quantitative changes in their protein content occur during embryonic erythroid cell maturation. Chicken spectrin and band 3 (using the nomenclature devised for human erythrocyte proteins by Steck in 1974) are present in low levels in early erythroblasts and increase in relative amounts during development. Band 2 of spectrin is present in half the amount of band 1 in the 2.5 day embryo but increases at a faster rate than band 1 such that they both reach the level present in the adult erythrocyte membrane by day 6. In a subsequent study, (Weise and Chan, 1978) it was shown that synthesis of spectrin in primitive erythroblasts predominated over synthesis of band 3 whereas synthesis of both of these proteins was more balanced in definitive cells.

The use of nonmammalian erythrocytes to study the effects of hormones on anion and cation transport has led to the identification

of a major membrane protein constituent not found in mammalian erythrocytes. Experiments using frog (Rudolph and Greengard, 1980) and turkey erythrocytes, (Beam *et al.*, 1979; Alper *et al.*, 1980) have shown that a high molecular weight membrane protein is preferentially phosphorylated when sodium potassium cotransport is stimulated by catecholamines. The protein, named goblin, has an apparent molecular weight of 240,000, and migrates between two high molecular weight protein bands that comigrate with human spectrin during electrophoresis in SDS. On this basis, as well as the fact that like human spectrin, they were extracted from the erythrocyte membrane under conditions of low ionic strength, the latter two bands were identified as turkey spectrin. Spectrin has been reported to be present in the erythrocytes of the marine invertebrate Terebella lapidaria on the basis of cross-reactivity of a single 270,000 molecular weight protein with antibodies directed against human spectrin, and in newt erythrocyte membranes (Sloboda and Dickersin, 1980) by comigration with human spectrin. Other than these reports, there have been few characterizations of the protein composition of nonmammalian erythrocyte plasma membranes.

The first description of the SAC of nonmammalian erythrocytes was reported by Cohen (1978) who, after lysis of goldfish erythrocytes with Triton X-100 under microtubule stabilizing conditions, observed a network of trans marginal band material (TBM) in uranyl acetate-stained whole mounts. The TBM was described as enclosing the nucleus and either attached to or wrapped around the MB. MBs, when released from the TBM in these cytoskeletons tended to

circularize. As a working hypothesis, it was proposed that the TBM applied asymmetric tension across a circular MB resulting in the elliptical shape of the nonmammalian erythrocyte. In a subsequent study, Cohen et al. (1982) described the TBM as a subsurface layer equivalent to the SAC of mammalian erythrocytes. Similiar networks of SAC have been observed in erythrocyte cytoskeletons of toad (Bufo marinus; Sloboda and Dickersin, 1980) and blood clams (Cohen and Nemhauser, 1980).

Excluding the cytoskeletons of platelets (Rosenberg et al., 1981) and mammalian erythrocytes (Gratzer, 1981), the isolated brush border of intestinal epithelial cells which includes the terminal web and associated microvilli, represents the only SAC currently being characterized (Howe et al, 1982; Glenney et al., 1980, 1982). Despite the availability of brush border as an isolated structure, their cytoskeletal system is quite complex. The nonmammalian erythrocyte provides a relatively simple SAC which may be better suited to the study of protein composition and structural organization of its components.

III. Objectives and Initial Approach

The three main objectives of this research were (1) to characterize the cytoskeletal system of erythrocytes of the smooth dogfish (*Mustelus canis*) on a structural and molecular level; (2) to determine whether calcium could be involved in the regulation of cytoskeletal structure formation and function by studying the effects of calcium on the system; and (3). to determine whether

calmodulin could be involved in the mediation of these calcium effects on cytoskeletal structures. The dogfish erythrocyte was chosen for this study for a number of reasons. Erythrocytes of this species are relatively large (approximately 20 μ in length), yielding a cytoskeleton which is readily visible by phase contrast microscopy. Dogfish and hence dogfish erythrocytes are physiologically accustomed to laboratory temperatures which is convenient for the study of erythrocytes close to in vivo temperatures, eliminating temperature effects on cell and cytoskeletal morphology. The blood volume of a moderately-sized dogfish (3 to 4 feet in length) is sufficient to withdraw adequate volumes to prepare erythrocytes for cytoskeletal fractionation and biochemistry, without sacrificing the animal.

A. Characterization of the dogfish erythrocyte cytoskeleton

At the outset of this project, dogfish erythrocyte cytoskeletons had been examined by phase contrast microscopy and shown to retain the characteristic flattened elliptical morphology of the cell and to contain MBs and nuclei interconnected by material that was nearly transparent in phase contrast (Cohen et al., 1977). A subject of primary interest in the cytoskeleton was the MB, with a view toward using it as a model system for the study of microtubule assembly/disassembly and the role of microtubules in overall cell morphology. A number of questions concerning this structure in the dogfish erythrocyte existed at that time or arose during this study. These included the number and polarity of the constituent microtubules, whether the MB contained structures other than

microtubules; what held the MB microtubules together in a bundle; and whether there were centrioles associated with the MB as was the case for MBs of erythrocytes of the blood clams (Cohen and Nemhauser, 1980). Important to the study of MB assembly/disassembly was the question of whether dogfish erythrocyte MBs were cold labile as in avian erythrocytes or cold stable as the MBs of amphibian erythrocytes (Behnke, 1970). The protein composition of dogfish MBs was of interest. Although tubulin was expected to be the major protein component, the presence of other proteins such as microtubule-associated proteins (MAPs) was in question. Sloboda and Dickersin (1980) reported the presence of MAP 2 in MBs of newt erythrocytes. Since this protein had been implicated in the regulation of microtubule polymerization (Sloboda et al., 1976) and stability to calcium (Sandoval and Weber, 1979, Schliwa et al., 1981) it seemed important to determine whether MAP 2 or any other MAP was a component of dogfish MBs.

With the apparent effect of the SAC on MB morphology in other species (Cohen, 1978), it soon became evident that the SAC played an equally important role in the determination of cell shape. Of particular interest was the comparison of the SAC of dogfish erythrocytes with that of human erythrocytes with respect to overall structural organization, biochemical characteristics and protein content. As noted above, the human erythrocyte SAC has been characterized in great detail. It would be impossible to review the vast body of literature on this subject here, but pertinent reviews have been made recently by Gratzer (1981), Branton et al. (1981) and Lux, (1979).

No regular pattern exists in the structure of the human erythrocyte SAC (Tsukita *et al.*) which would make it difficult to compare the dogfish SAC with that of the human solely on a structural basis. Anesthetics (Sheetz and Singer, 1974), sulfhydryl reagents (Steck and Yu, 1973) and metabolic conditions such as ATP depletion (Nakao *et al.*, 1960) cause dramatic changes in mammalian erythrocyte morphology. The effects of these agents on dogfish erythrocytes could provide insight into how the characteristics of the SAC of these cells compare with that of mammalian erythrocytes. The protein composition of the human erythrocyte SAC is well characterized. As noted previously, protein patterns of various nonmammalian erythrocytes after separation by electrophoresis in the presence of detergent have been compared with that obtained for human erythrocytes. It has generally been concluded that the protein compositions of all of these membranes are similar, to the extent that the system of nomenclature for the human proteins (Steck, 1974) has been adopted for the erythrocyte membranes of all of the species examined thus far. Examination of the protein composition of erythrocyte membranes and SAC of dogfish, one of the more primitive vertebrates would provide evidence either for or against this conclusion and test the degree of conservation of erythrocyte membrane-associated proteins through evolution.

B. Effects of calcium on the dogfish erythrocyte cytoskeleton

Generation and maintenance of correct cytoskeletal organization is a subject of primary interest in cells such as erythrocytes in

which specific mechanical properties and asymmetric morphology are vital to cell function. Evidence obtained in recent years implicates calcium ion in the control of such organization. Assembly of both microtubules (Schliwa *et al.*, 1981; Weisenberg, 1972) and microfilaments (Yerna *et al.*, 1979; Glenney *et al.*, 1980) has been shown to be calcium-mediated in vitro, and localized intracellular calcium levels are believed to regulate formation of microtubules of the mitotic spindle in vivo (Hepler, 1977; Salmon and Segall, 1980). Barrett and Dawson (1974) have shown that the sphere to disc transformation in avian erythroblasts occurs concomitant with the formation of the MB. The question arises as to what controls the formation of cytoskeletal structures such as the MB. In view of its effects upon cytoskeletal structures, calcium ion is a prime candidate as a controlling factor in the formation of these structures. One of the objects of this research was to explore the possibility that calcium might control the organization of the nonmammalian erythrocyte cytoskeleton. A minimal requirement for such an hypothesis would be some effect of calcium ion upon the cytoskeletal structures of the nonmammalian erythrocyte. For this reason the in vivo and in vitro effects of calcium on the dogfish erythrocyte were examined.

There are potentially many mechanisms by which calcium ion can exert an effect upon cytoskeletal structures. Calcium-activated ATPases such as in erythrocyte membranes (Lynch and Cheung, 1979), and sarcoplasmic reticulum (Le Peuch *et al.*, 1979) control intracellular levels of free calcium which ultimately could regulate

the formation of calcium labile structures. Calcium-activated proteases are involved in myofibrillar protein turnover in muscle (Dayton *et al.*, 1976) and degradation of cytoskeletal proteins in platelets (Phillips and Jakabova, 1977). Calcium-activated transglutaminases cross-link cytoskeletal proteins such as spectrin (Siefring *et al.*, 1978), causing the erythrocyte membrane to lose its elastic properties. Kinases responsible for the phosphorylation of myosin light chain are stimulated by calcium and represent the mechanism by which calcium controls smooth muscle contraction (Dabrowska *et al.*, 1978) and non-muscle actomyosin interactions such as occur in platelets (Hathaway and Adelstein, 1979). With the exception of transglutaminases, the control of at least some form of each of the types of enzymes listed above by calcium is mediated by calmodulin.

C. Calmodulin and cytoskeletal calmodulin-receptors in the dogfish erythrocyte

Since its description as an activator of calcium-dependent phosphodiesterase by Cheung in 1971, calmodulin has been found in every eukaryotic cell type thus far examined and has been shown to mediate the effects of calcium on both cytoplasmic and cytoskeletal proteins. The literature on calmodulin is far too extensive to discuss in any detail. Recent reviews of the topic have been made by Klee (1981) and Cheung (1979). Reports made by Marcum *et al.*, (1978) implicated calmodulin in the control of microtubule assembly *in vitro*. Subsequently calmodulin was localized in association with the half spindles during mitosis and was postulated to be involved

in mediating the effects of calcium on the microtubules of the mitotic apparatus (Welsh et al., 1978). With this in mind, the question arose as to whether effects of calcium on the dogfish erythrocyte cytoskeleton, and MB in particular, could be mediated by calmodulin. A minimal requirement for such mediation would be to demonstrate the presence of calmodulin in the dogfish erythrocyte and calmodulin-receptor proteins associated with the MB in particular and the cytoskeleton in general.

IV. Summary of specific experimental objectives

The major experimental objectives were as follows: (1) Examination of dogfish erythrocyte cytoskeletons for structural organization by phase contrast light microscopy and transmission electron microscopy; (2) Determination of the protein composition of the dogfish erythrocyte cytoskeleton; (3) Development of fractionation techniques to isolate component structures of the cytoskeleton; (4) Localization of proteins to component structures by determining the protein composition of isolated MBs and the SAC; (5) Investigation of in vivo and in vitro effects of calcium on cytoskeletal structures; (6) Documentation of the presence of calmodulin in the dogfish erythrocyte; (7) detection and possible identification of cytoskeletal calmodulin-binding proteins using several independent techniques.

MATERIALS

Bovine serum albumin (BSA), diisopropyl fluorophosphate (DFP), ethylene glycol-bis-aminoethyl ether N,N'-tetraacetic acid (EGTA), N-2-hydroxyethylpiperazine-N' 2 ethane sulfonic acid, (HEPES), p-hydroxymercibenzoate (pHMB), piperazine- N-N'-bis 2-ethane sulfonic acid (PIPES), p-tosyl arginine methyl ester (TAME), Triton X-100 polylysine (Mr>400,000), rabbit muscle actin, porcine pancreatic elastase and lactoperoxidase were obtained from Sigma Chemical Co. Acrylamide, bisacrylamide and sodium dodecyl sulfate (SDS) were from BDH Chemicals Ltd., Poole England. Standard molecular weight markers and Protein Assay Kit were obtained from Bio-Rad Laboratories, Richmond CA. Leupeptin and rat vimentin were products of Transformation Research Laboratories, Framingham, Mass. Ionophore A23187 was from Calbiochem. Na¹²⁵I, 2 Ci/mmol, was obtained either from Amersham Corp. or New England Nuclear, Boston, MA. Du Pont Cronex x-tra Lite intensifying screens used in autoradiography were the product of E.I. Du Pont de Nemours and Co., Willimington DE. Fuji-RX X-ray film was a product of Fuji Photo Film Co. Ltd., Japan. Protein A, diethylaminoethyl (DEAE) Sephadex and CNBr activated Sepharose were from Pharmacia Fine Chemicals, Piscataway, NJ. Nitrocellulose was obtained from Schleicher and Scheull, Keene, NH. Goat antirabbit IgA, IgG, and IgM was purchased from Cappel Laboratories, Cochranville, PA. Chicken erythrocytes, free of leukocyte contamination, were obtained from Pel-Freeze, Inc.,

Rogers, AR.

It is with pleasure that I acknowledge and thank the following investigators for their cooperation in supplying materials needed to perform this research. Trifluoperazine (TFP) was the generous gift of Dr. Carl Kaiser of Smith Kline and French Laboratories. 2CI,10 (3 aminopropyl) phenothiazine (CAPP) was generously donated by Dr. Albert Mannian of the National Institutes of Health, Bethesda, MD. Bovine and dogfish brain tubulin, temperature cycled twice according to the method of Shelanski (1973), were the gift of Dr. George Langford of the University of North Carolina. Bovine brain MAP 2, prepared by the method of Fellous and coworkers (1977), was the gift of Dr. Robert Zackroff of Northwestern University. Canine cortical postsynaptic density preparations (Carlin et al, 1980b) and azido ^{125}I calmodulin (Andreasen et al, 1981) were the generous gift of Dr. Richard Carlin of the Rockefeller University, New York, N.Y. Rabbit antiserum to guinea pig fodrin and a sample of partially purified fodrin (Levine and Willard, 1981) were generously provided by Dr. Mark Willard, Washington University, St. Louis, MO. Rabbit antiserum to bovine brain CBP I (Davies and Klee, 1981) was kindly donated by Dr. Claude Klee, National Institutes of Health, Bethesda, MD. ^{125}I Fab' fragments (0.95 mg/ml of rabbit IgG directed against human spectrin were the generous gift of Dr. Julie Olson, Rockefeller University, New York, NY. Rabbit antiserum directed against BHK vimentin was kindly donated by Dr. Eugenia Wang, Rockefeller University, New York, N.Y.

Methods

Preparation of washed dogfish erythrocytes

Blood was freshly drawn from smooth dogfish (Mustelus canis) for each experiment and either heparinized (20-50 U/ml) or immediately diluted at least 1:10 into Elasmobranch Ringer's solution (Cavanaugh, 1975). Cells were washed by four or more centrifugation (750g, 1 min)/resuspension cycles in Ringer's, carefully removing the leukocyte (upper) layer each time, after which there was less than 1% leukocyte contamination. Alternatively, essentially complete leukocyte removal was achieved by layering 10 ml aliquots of diluted heparinized blood onto 25 ml of 25% Ringer's solution containing 0.5M sucrose. Red cells were sedimented by centrifugation at 750 g for one min while the majority of the leukocytes remained in suspension and were removed by aspiration. The pelleted erythrocytes were washed with 35 volumes of Ringer's solution and sedimented. The final erythrocyte pellets, obtained by either method, were resuspended in 35 volumes of Ringer's solution. Dogfish of moderate size (3 ft.) readily survived withdrawal of 40 ml blood, containing approximately 10 ml of packed erythrocytes.

Treatment of dogfish erythrocytes with calcium ionophore A23187

Washed erythrocytes were prepared by centrifugation through sucrose as described above and suspended in modified Elasmobranch

Ringer's solution containing no urea, no calcium and 0.4M NaCl. Aliquots containing 50 μ l of packed cells were sedimented by centrifugation at 2,000 g for 1 min and suspended in 1.0 ml aliquots of modified Ringer's solution containing either 1mM EGTA or 0.1, 1.0 or 10mM CaCl_2 . These suspensions were made 10 μ M in ionophore A23187 and 1% in ethanol with immediate rapid mixing. A control cell suspension containing 1% ethanol was prepared for each Ringer's solution. After 1, 2, 5 and 30 min at room temperature (20 °C), two aliquots were removed from each tube. The first set of aliquots were lysed in 100 mM PIPES, 1 mM MgCl_2 , 5 mM EGTA, 10 mM TAME, 0.1mM DFP, 0.4% (v/v) Triton X-100, pH 6.8, pH adjusted with KOH (lysis medium A; Weisenberh, 1972, Rebhun *et al*, 1974). The second set of aliquots were fixed in the appropriate Ringer's solution containing 0.1% glutaraldehyde. All samples were examined by phase contrast microscopy.

Nucleated dogfish erythrocyte cytoskeletons

Erythrocyte pellets or suspensions were diluted at least 1:10 with lysis medium A. Alternately, media were buffered with 10 or 100 mM HEPES at either pH 6.8 or 7.3. As in other species, media immediately cleared the cells of hemoglobin, revealing MB, nucleus, and SAC (Cohen, 1978). In experiments studying the effect of calcium on cytoskeletal morphology, cells were lysed using 0.4% Triton in either elasmobranch Ringer's solution or in lysis medium A containing 5 mM CaCl_2 in place of 5mM EGTA. Leupeptin (10 μ g/ml) and sodium tetrathionate (1 mM) were often included in lysis medium A as additional protease inhibitors. The

presence of these protease inhibitors did not discernably alter either cytoskeletal structure or protein composition.

Cell lysates before and after MB disassembly

Control cells (room temperature) and cells incubated 60 min at 0 ° C. to disassemble MBs (cooled cells) were lysed in lysis medium A. As a further precaution against proteolysis, lysis medium A contained 1mM benzamidine, 64uM pepstatin, 100 µg/ml soybean trypsin inhibitor, and 25 K.I.U. trasylol/ml. Equal numbers of cells (0.09 ml packed volume) were lysed in 0.45 ml PIPES medium + inhibitors at 0 ° C. Preparations were centrifuged 2 min at approximately 750 g and the supernates were analyzed by 5-12% gradient polyacrylamide gel electrophoresis in SDS.

pH 3 extracts of nucleated dogfish erythrocyte cytoskeletons

Erythrocyte pellets (= 1 volume) were suspended in approximately 10 volumes of the same medium used for MB isolation and centrifuged 2 min at approximately 350g at room temperature. The nucleated cytoskeleton pellets were suspended uniformly in 10 volumes McIlvaine 0.08M citrate-0.04M phosphate buffer at pH 3.0 (Cavanaugh, 1975), after which only intact nuclei were present (see Figure 23a). The nuclei were removed (10 min, approximately 750g) and the supernate adjusted to pH 7.3 with NaOH. Ice-cold TCA was added to 5% final concentration, and the preparation stored on ice for 1 hour. The precipitate was collected (10 min, approximately 750 g), washed twice with ice-cold acetone, and air-dried prior to storage at -20 ° C.

Low temperature extracts of cytoskeletons

Washed erythrocytes (1.25 ml) were suspended in ten volumes of lysis medium A at pH 6.8, containing 150mM KCl. Cytoskeletons were sedimented through 25 volumes of wash medium (lysis medium without detergent) containing 10% glycerol by centrifugation for 2 min at 750 g. The supernate was removed, the cytoskeletons were resuspended in 10 volumes of wash medium and collected by centrifugation. The 0.5 ml pellet was resuspended in 1 ml of wash medium and incubated at 0° C. for two hours or until the marginal bands were no longer visible by phase contrast microscopy. The extract was clarified by centrifugation for 10 min at 2,000 g and stored at -20°C.

Calcium extracts of cytoskeletons

Erythrocytes were lysed in 5 volumes of 10mM HEPES pH 7.3, 5mM EGTA, 10mM TAME, 1mM MgCl, 150mM KCl, 0.4% Triton X-100. The resulting cyoskeletons were washed five times in 10 volumes of lysing medium containing no Triton (wash medium). The washed cytokeletons were suspended in wash medium containing 5mM CaCl instead of 5mM EGTA and incubated at room temperature for 150 min or until the marginal bands were no longer visible by phase contrast microscopy. The extract was clarified by centrifugation at 2,000 g for 5 min, dialyzed vs. 10mM HEPES pH 7.3, and lyophilized.

Preparative isolation of MBs

Unless otherwise noted, all operations were performed at 20-22° C. In Method I, erythrocytes from 1 volume blood were

suspended in 10 volumes of 100mM PIPES, 1mM $MgCl_2$, 5mM EGTA, and 2.5% Triton X-100, pH 6.8 ("isolation medium"), and the nucleated cytoskeletons sedimented in 1 min at approximately 750 g. After resuspension in the same medium, the preparation was gently poured between beakers, and the resulting release of both MBs and nuclei was monitored in phase contrast microscopy. Additional fluxing or vortex mixing was used if necessary. The suspension was centrifuged 5 min as before to sediment free nuclei and any intact cytoskeletons. The supernate, containing free MBs, was slowly transferred to another tube via a 1mm wide Pasteur pipet (to minimize MB breakage), and centrifuged as before to remove additional nuclei. The supernate was transferred to 50 ml polycarbonate tubes and centrifuged 10 min at approximately 11,000 x g. Pellets consisted largely of intact and broken MBs, with some contamination (see Figure 15a). MB sedimentation and subsequent washes in isolation medium lacking Triton were tested both at 0-4 °C and at 15-20 °C; no major MB dissolution occurred in either case.

This procedure (Method I) appeared to be facilitated by increased leukocyte contamination, presumably a source of protease activity. Initial removal of essentially all leukocytes prevented MB release, yielding nucleated cytoskeletons which were stable. In an alternative procedure (Method II) these stable cytoskeletons were incubated in isolation medium containing 0.5 U/ml elastase (Sigma Chemical Co.), which released MBs and nuclei in about 10 min at room temperature. The nuclei were removed and the MBs

sedimented and washed as in Method I. The MBs in these preparations were remarkably intact and free of contamination (see Figure 15e), and remained intact in the presence of elastase for at least 1 hour. MBs were prepared from anucleate dogfish erythrocyte ghosts using Method II.

Anucleate dogfish erythrocyte ghosts and cytoskeletons

Erythrocytes were lysed osmotically in 50 mM PIPES, 0.5 mM MgCl_2 , 5 mM TAME, 2.5 mM EGTA, 50 μM DFP, 1 mM sodium tetrathionate, 5 $\mu\text{g/ml}$ leupeptin pH 6.8 (lysis medium B). The preparation was drawn twice through a 22-gauge syringe needle, producing a suspension of anucleate ghosts, particles, free nuclei, nucleated ghosts and some unlysed cells (figure 10b). After centrifugation for 3 min at 750g, the supernate contained only anucleate ghosts and particles (figure 9, 10e). These were either sedimented together in 10 min at 11,000g at 15-20^o C., or were separated by discontinuous sucrose density gradient centrifugation for 15 min at 750g. as shown in figure 9. Anucleate ghosts, suspended in the 0.5M sucrose layer were then collected by centrifugation at 11,000g for 10 min., and washed twice in lysis medium A without Triton. Samples of anucleate ghosts prepared without sucrose gradient centrifugation and containing minor particle contaminants were further purified by resuspending the upper part of the pellet, containing the anucleate ghosts, in lysis medium A without Triton. The lower part of the pellet containing a mixture of particles and anucleate

ghosts was discarded.

Anucleate dogfish erythrocyte cytoskeletons were prepared from anucleate ghosts by washing in lysis medium A. These cytoskeletons were collected by centrifugation at 10,000 g for 10 min and washed twice with medium without Triton. Anucleate ghosts devoid of marginal bands were prepared by cooling whole cells at 0°C. for two hours prior to anucleate ghost preparation. For these experiments, all manipulations were performed at 4°C.

Low ionic-strength extraction of dogfish anucleate ghosts

Anucleate ghosts were washed with five volumes of 1mM Tris•HCl pH 7.8 and sedimented (Fairbanks et al, 1971). Aliquots of ghosts corresponding to 1 ml of packed cells and containing 2.7 mg protein were extracted for 30 min in 5 ml 1mM Tris•HCl containing either 0.2mM EDTA pH 7.8 at 37°C. (Marchesi and Steers, 1968) or 5mM pHMB pH 9.0 at 23°C. (Ralston, 1975, Steck and Yu, 1973). pHMB was insoluble below pH 8.8 at this concentration. Samples were examined by phase contrast microscopy. Extracted ghosts were sedimented by centrifugation at 100,000g for 15 min (figure 26). Soluble and particulate fractions were collected, assayed for protein content (Bradford, 1976) and for protein composition by polyacrylamide gel electrophoresis in SDS. Soluble fractions containing the same protein components were combined prior to assay for the presence of calmodulin-binding proteins.

Human erythrocyte ghosts

Human erythrocytes were obtained by diluting a fresh sample of

heparinized blood with one volume 20mM sodium phosphate, 150mM sodium chloride pH 7.3 (PBS), and sedimenting the red cells through 25% PBS containing 0.5M sucrose at 750 g for 5 min. The supernatant containing suspended leukocytes was removed by aspiration and the erythrocytes were washed in 15 volumes of PBS. Human erythrocyte ghosts were prepared by hypotonic lysis of washed cells in 10 volumes of lysis medium B. The lysate was centrifuged at 11,000 g for 15 min and the sedimented ghosts were washed with ten pellet volumes of lysis medium A without Triton. The ghosts were collected by centrifugation, resuspended in one volume of this medium, and stored at -20°C .

Anucleate chicken erythrocyte ghosts

Chicken erythrocytes, maintained at 0°C during shipping, were lysed in Hanks HBSS medium (Hanks, 1948) diluted 1:10 and containing 1mM MgCl and 50uM DFP. Anucleate ghosts were prepared and washed as described for dogfish erythrocyte ghosts, except that leupeptin and sodium tetrathionate were omitted from the wash medium.

Cell and cytoskeletal morphology

Cell morphology was assessed by phase contrast examination of both living and fixed cells. For the latter, approximately 25 μl of cell suspension was diluted into 0.5 ml fixation medium consisting of 1 volume 10% glutaraldehyde, 9 volumes Ringer's, which faithfully preserved cell morphology. Fixed samples were a convenience for observation, and, more importantly, avoided "glass

effects" which can grossly distort living erythrocyte shape. For standardized surveys of cytoskeletal structure, approximately 25 μ l of cell suspension was diluted into 0.5 ml lysis med.A. A Zeiss phase contrast microscope equipped for photomicrography was used throughout. For some critical phase contrast observations, samples were viewed through small air bubbles intentionally produced under the coverslip, enhancing contrast considerably (see Figure 15f). Specimens under bubbles are effectively in miniature moist chambers, and do not dry out.

Electron microscopy

Anucleate ghosts prepared from normal dogfish erythrocytes and cells that had been incubated at 0°C for 2 hours (cooled cells) were fixed in lysis medium A containing leupeptin and sodium tetrathionate and 1% glutaraldehyde with and without 0.4% Triton for 2 hrs. at room temperature. The ghosts and cytoskeletons were sedimented by centrifugation and washed with lysis medium A without Triton, followed by 0.1 M KH_2PO_4 , pH 6.8. The material was post-fixed in 1% OsO_4 in phosphate buffer for 1 hour, washed twice in buffer, and dehydrated in ethanol. Samples were then treated with 0.1% tannic acid prior to embedment in Epon. Isolated MBs were fixed in isolation medium containing 1% glutaraldehyde (18 hours, room temperature), washed twice in isolation medium followed by 10mM NaH_2PO_4 at pH 6.0 (= "buffer"), post-fixed in cold buffer containing 0.5% OsO_4 (30 min, 3 °C), washed twice in cold buffer, dehydrated in ethanol and embedded in Epon.

For whole mounts, isolated MBs on Formvar-coated grids were rinsed with isolation medium, fixed in the same medium containing 1% glutaraldehyde (5 min), rinsed with water, and stained with 2% aqueous uranyl acetate. Nucleated cytoskeletons on grids were rinsed twice with lysis medium A lacking Triton and TAME, fixed in the same medium containing 2.5% glutaraldehyde (15 min), rinsed twice as before and once with water, and stained with 2% uranyl acetate. For anucleate cytoskeletons, anucleate ghosts were extracted on grids with lysis medium A (2 min) and processed as above for nucleated cytoskeletons except for fixation (5 min). To improve retention of material in some cases, Formvar-coated grids were treated for 5 min with 1% polylysine solution ($M_r > 400,000$), and washed and dried prior to use.

Simultaneous lysis and fixation (Cohen and Nemhauser, 1980) was used to examine cytoskeletal structure in thin section. In temperature-cycling experiments, samples included room temperature controls, cells incubated 1.5 hours at 0 °C, and similarly cooled cells rewarmed 1.5 hours at room temperature. Cells were sedimented at their respective temperatures and the pellets resuspended in lysis medium A containing 1% glutaraldehyde at room temperature (1:20). A loose hemoglobin gel formed in which the cytoskeletons were suspended, eliminating the need for centrifugation during subsequent steps. After 48 hours at room temperature, small pieces of the gel were washed twice in 0.1M NaH_2PO_4 , pH 6.0, post-fixed 1 hour in the same medium containing 1% OsO_4 , washed again in the 0.1M buffer, and dehydrated and embedded

as above.

Thin sections were cut on a Sorvall MT-2 ultramicrotome using diamond knives, and stained with uranyl acetate and lead citrate. Specimens were examined in the Hitachi HS-8 transmission electron microscope operating at 50kV or the JEOL 100B at 60kV.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Maizel (1969) using 20x30 cm slabs of either 1 or 2 mm. thickness. Gels contained either 5% or 5-12% or 5-15% linear gradients of acrylamide. They were stained with Coomassie blue and destained by diffusion. The presence of calmodulin was detected by electrophoresis of samples in 12.5% polyacrylamide gels in the absence of SDS using a discontinuous buffer system as described by Watterson et al, (1976).

Preparation of bovine brain calmodulin

Initial steps in the preparation of calmodulin from bovine brain were as described by Watterson et al (1976) and are summarized in figure 1. Frozen tissue was thawed and homogenized in 3 volumes 0.1 M sodium acetate, 1mM EDTA, 1 mM B mercaptoethanol, pH 7.2. The homogenate was centrifuged at 10,000 g for 45 min. The soluble fraction was brought to 30% saturation with respect to ammonium sulfate. The resulting precipitate was collected by centrifugation as described above and discarded. The soluble fraction was brought to 60% saturation and centrifuged. The precipitated protein was collected by centrifugation and saved for the preparation of calmodulin-dependent phosphodiesterase.

Figure 1. Isolation of calmodulin from bovine brain.

Figure 1

Procedure for Isolation of Calmodulin
from Bovine Brain

1. Tissue homogenization (3 volumes 0.1M sodium acetate, 1mM EDTA, 1mM beta-mercaptoethanol, pH 7.2).
2. Post-mitochondrial fraction.
3. Ammonium sulfate fractionation at 60% saturation.
4. Isoelectric precipitation at pH 4.0 of proteins soluble in 60% saturated ammonium sulfate.
5. Dialysis against 10mM Tris HCl, 0.3M NaCl, 1mM EDTA, 1mM beta-mercaptoethanol.
6. DEAE Sephadex chromatography at pH 8.1; 0.35-0.6M NaCl gradient; calmodulin eluted at 0.4M KCl.
7. Affinity chromatography with 2-chloro-10-(3-aminopropyl) phenothiazine (CAPP) sepharose. Calmodulin bound in 1mM CaCl_2 , eluted in 2mM EGTA.

Figure 2. Purification of bovine brain calmodulin by gradient elution ion-exchange chromatography on DEAE-sephadex A-50 at 4°C. The column, 4.4X35 cm, was equilibrated with 10mM Tris, 300mM NaCl, 1mM EDTA, 1mM beta-mercaptoethanol, pH 8.1 (conductivity = 8.1 mmho/cm). The sample of pH 4.0 precipitated bovine brain proteins containing 16.4 mg calmodulin in 39 ml column buffer ($A_{280} = 16.5/\text{ml}$) was applied at 40 ml/hr, after which the column was eluted with column buffer. Ten milliliter fractions were collected. At fraction 64 the eluant was changed to column buffer 0.35M in NaCl (14.4 mmho/cm). At fraction 124, a linear gradient (G) of 900 ml each of this buffer and buffer 0.6M in NaCl (28.5 mmho/cm) was applied to the column. Calmodulin was detected and quantitated by scanning of polyacrylamide gels on which aliquots of fractions and a calmodulin standard had been electrophoresed. The fractions indicated by the bar were combined for purification of calmodulin by affinity chromatography.

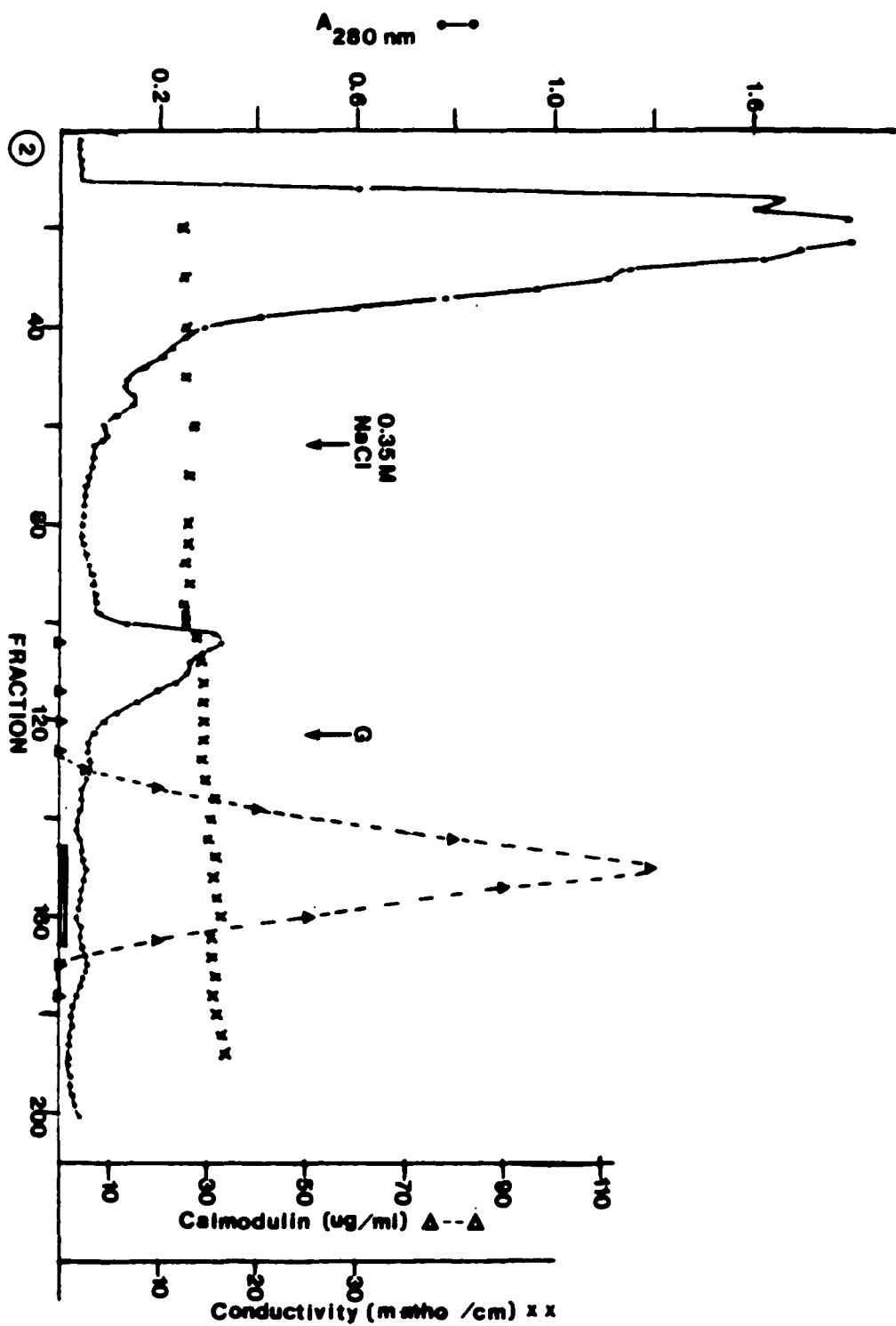


Figure 3a. Affinity chromatography of bovine brain calmodulin on 2-chloro-10-(3-aminopropyl)phenothiazine (CAPP) sepharose at 23°C. Calmodulin, eluted from DEAE-sephadex in 206 ml 10mM Tris, 350 mM NaCl, 1mM EDTA, 1mM beta-mercaptoethanol, pH 8.1, was made 1mM in $MgCl_2$ and 2mM in $CaCl_2$ and applied to a 2.3X6.5 cm column of CAPP-sepharose equilibrated with 20mM Tris, 350 mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 1mM beta-mercaptoethanol (buffer F). The sample was applied at 40 ml/hr and 6.4 ml fractions were collected. At fraction 32, the column was eluted with buffer F until no further material was eluted from the column. At fraction 56 the eluant was changed to 20mM Tris, 350mM NaCl, 1mM $MgCl_2$, 1mM beta-mercaptoethanol, 2mM EGTA (buffer E). At fraction 63 the eluant was changed to buffer E containing 8M urea. Calmodulin was detected and quantitated by scanning of polyacrylamide gels in which aliquots of fractions and a calmodulin standard had been electrophoresed. The fractions indicated by the bar were combined.

Figure 3b. Electrophoresis of combined fractions (2a) in a 5-15% gradient polyacrylamide gel in SDS.

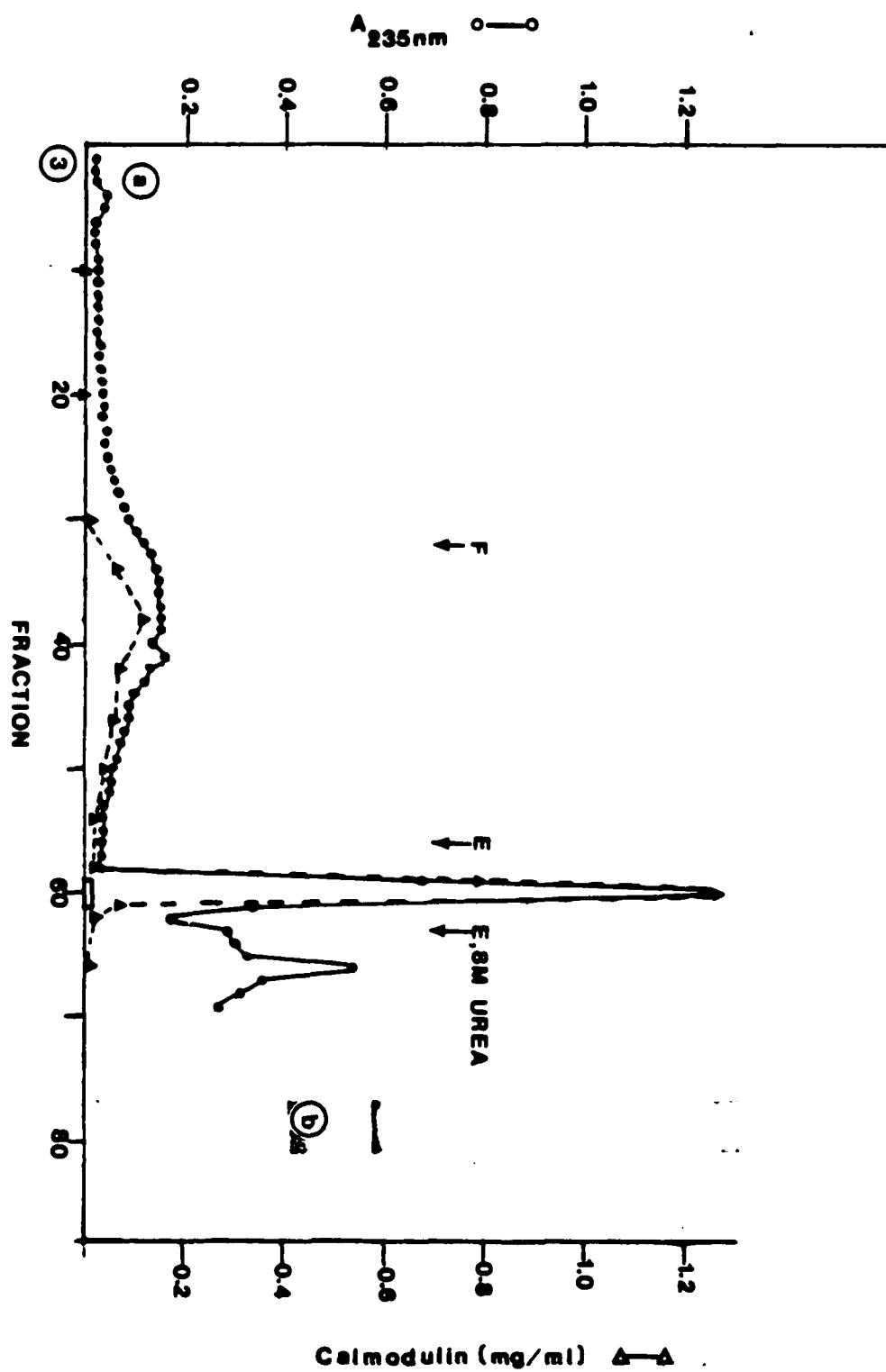


TABLE I
Amino Acid Composition of Bovine Brain
Calmodulin

Amino Acid	Moles/Mole Protein ¹ .	Composition ² .
Aspartic Acid	24.86	24
Threonine	12.62	12
Serine	4.42	5
Glutamic Acid	28.45	29
Proline	1.81	2
Glycine	11.85	12
Alanine	11.63	12
Half-Cystine	-	-
Valine	6.84	8
Methionine	6.50	10
Isoleucine	7.90	8
Leucine	9.74	10
Tyrosine	1.60	2
Phenylalanine	8.22	8
Trimethyl lysine	1.02	1
Histidine	1.00	1
Lysine	6.44	8
Arginine	6.17	7

1. Average of duplicate 22-hour hydrolysates. Calculation based on moles/mole reported for glutamic acid 29, Glycine 12, Alanine 12, phenylalanine 8, by Watterson et al.
2. Watterson, D.M. et al. 1976.

The resulting supernate was acidified to pH 4.0 using 1 N sulfuric acid which precipitated a fraction containing calmodulin. This fraction was solubilized and dialyzed against 10 mM Tris·HCl, 0.3

M NaCl, 1 mM EDTA, 1 mM β mercaptoethanol, pH 8.1 and chromatographed on DEAE Sephadex as described in the legend to figure 2. The fractions shown to contain calmodulin but not S 100 protein (a mixture of low molecular weight calcium-binding proteins that bind phenothiazines in a calcium-dependent manner), by polyacrylamide gel electrophoresis and indicated by the open bar in figure 2, were combined, and applied to a column of 2,Cl,10-(3 aminopropyl)phenothiazine (CAPP) Sepharose prepared by the method of Cuatrecasas and Anfinsen (1971). Affinity purification of calmodulin was performed as described in the legend to figure 3. Fractions eluted with EGTA and indicated by the open bar in figure 3 contained only calmodulin and were combined. This preparation was shown to be homogeneous by SDS polyacrylamide gel electrophoresis (figure 3b) and, as shown in Table I, had an amino acid composition comparable to that reported for bovine brain calmodulin by Watterson et al. (1976).

Preparation of crude calmodulin-dependent bovine brain phosphodiesterase

Using the 30-60% ammonium sulfate fraction of bovine brain described in the preceding paragraph as starting material, phosphodiesterase free of contaminating calmodulin was prepared by chromatography on DEAE Sephadex in a modification of the procedure

described by Klee and Krinks, (1978). The column was equilibrated with and the sample applied in 50 mM Tris HCl, 1mM EDTA, 1 mM B mercaptoethanol, pH 7.5. Calmodulin-dependent phosphodiesterase was eluted with buffer containing 0.2M NaCl. The enzyme was stored in this buffer in aliquots at -20° C.

Assay of calmodulin activity in dogfish erythrocyte lysate .

A lysate of dogfish erythrocytes was prepared in 10 volumes of medium consisting of lysis medium A. The calcium-dependent activation of partially purified calmodulin-sensitive bovine brain phosphodiesterase (PDE) was used to assay this lysate for the presence of calmodulin. PDE was depleted of calmodulin as described above. PDE activity was measured as described by Grab and coworkers (Grab et al , 1979). Levels of endogenous phosphate and PDE activity were subtracted from the total phosphate released to determine the extent of activation of the bovine brain PDE by calmodulin. Incubations were carried out in either 1mM EGTA, 1mM CaCl₂, or 1mM CaCl₂ plus 40 uM trifluoperazine. Calmodulin content was estimated by comparing the stimulation of PDE to one-half maximal velocity by the dogfish erythrocyte lysate with that obtained using known amounts of purified bovine brain calmodulin. The molarity of the calmodulin standard was determined by amino acid analysis. Iodination of calmodulin and protein A with Na¹²⁵I

Proteins were iodinated as described by Carlin et al.

(1980a). One mg. of either calmodulin or protein A was dissolved in 1 ml. 50 mM sodium phosphate pH 7.5 containing 1 mM CaCl_2 , 10 ug. lactoperoxidase and 1 mCi. Na^{125}I . The reaction was initiated by the addition of 5 ul. 0.06 % H_2O_2 at 23 C. After 7 min., an additional 5 ul. of hydrogen peroxide was added. After a total of 15 min., the reaction mixture containing protein A was gel filtered on a 0.8x20 cm. column of Sephadex G 25 in phosphate buffer to remove free Na^{125}I . ^{125}I calmodulin was purified by affinity chromatography using 1 ml of CAPP Sepharose. Iodinated proteins routinely had specific activities of approximately 4×10^5 cpm./mg. ^{125}I calmodulin was able to stimulate the activity of bovine brain phosphodiesterase in a calcium-dependent manner to the same extent as the unlabeled protein.

^{125}I calmodulin binding to anucleate dogfish erythrocyte cytoskeletons

Cytoskeletons derived from anucleate ghosts containing 400 ug protein were incubated with 30-2550 ng (1.8-150 nM) biologically active ^{125}I calmodulin (128 cpm/ng) in 1.0 ml of a solution containing 20 mM PIPES.KOH, 100 mM KCl, 1mM MgCl_2 , 1mM DTT, 0.1 mM DFP, 8 mg/ml bovine serum albumin, 0.4% Triton X-100, pH 6.8, and either 0.1 mM CaCl_2 or 1mM EGTA for 30 min at 25° C with shaking. Cytoskeletons were collected by centrifugation at 12,500 g for 10 min. The supernates were removed by aspiration and the pellets were washed twice with 1 ml of incubation medium. The supernates, washes and washed

pellets were counted in a Packard Gamma scintillation counter.

^{125}I calmodulin binding to proteins separated by polyacrylamide gel electrophoresis in SDS

Binding of ^{125}I calmodulin to proteins separated by polyacrylamide gel electrophoresis in SDS was performed as described by Carlin and coworkers (1981) with slight modification. Gels were washed in 25% isopropanol:10% (v/v) acetic acid to fix proteins in the gel and remove SDS. Proteins were allowed to renature by incubating gels at 23°C. for 24 hrs. in 50 mM Tris pH 7.5, 200 mM NaCl, 1 mg./ml bovine serum albumin and either 1 mM CaCl_2 or 1 mM EGTA. Gels were incubated in buffer containing 50 nM ^{125}I calmodulin in either calcium or EGTA for 24 hrs. with vigorous shaking. Following incubation, gels were washed in four changes of buffer over a 24 hr. period, stained with coomassie blue, destained, dried and subjected to autoradiography. Degredation of the high molecular weight calmodulin-binding protein, CBP, was estimated by scanning autoradiograms of dried gels.

Photoaffinity labeling of calmodulin binding proteins in erythrocyte ghosts

In experiments performed by Dr. Richard Carlin, azido- ^{125}I - calmodulin was prepared by the method of Andreasen et al (1981). Dogfish, chicken and human erythrocyte ghosts (100 ug protein) were incubated for 15 min at 23 °C with 480 nM

azido-125I calmodulin in 20mM Tris.HCl, 100 mM NaCl, pH 7.5 containing either 0.6 mM CaCl₂ or 0.2 mM EGTA in the presence or absence of 0.15% Triton X-100. Cross-linking of the photoaffinity label to calmodulin-binding proteins was achieved by UV irradiation (5 min. each at 253 and 375 nm) at 23°C. Proteins in the samples were then separated by gradient polyacrylamide gel electrophoresis in SDS. To detect calmodulin-protein complexes, dried gels were autoradiographed at -80°C using an intensifying screen.

Experiments and Results

I. Structural components and organization of the dogfish erythrocyte cytoskeleton

A. Living dogfish erythrocytes and nucleated cytoskeletons

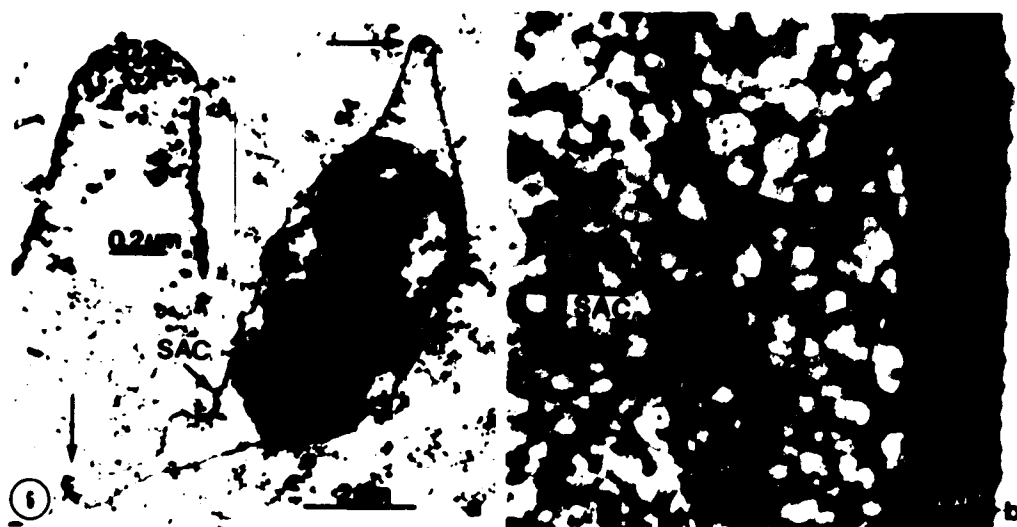
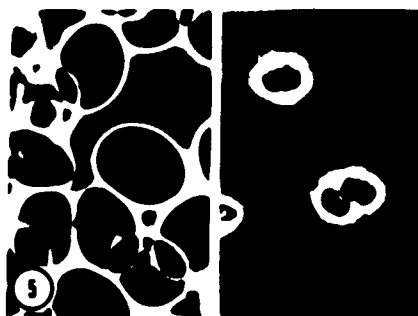
The erythrocytes of M. canis (figure 4) are flattened, elliptical, and nucleated (figure 5a), with biconvexity due to the nuclear bulge, typifying erythrocyte morphology in all non-mammalian vertebrates (Andrew, 1965). They are relatively large, with major and minor axes in the range of 17-22 μm and 13-15 μm respectively. Cell morphology is stable during washes and several hours of storage in Elasmobranch Ringer's solution at room temperature (20-22 C). When the cells are lysed in lysis medium A, the marginal band (MB) and nucleus immediately become visible in phase contrast (figure 5b). The cell surface associated cytoskeleton (SAC) is essentially phase-transparent, but is readily visualized by electron microscopy of whole mounts and thin sections. In thin section (figure 6a), the SAC is seen as a surface delimiting component which completely encloses nucleus and MB. In tangential section the SAC appeared to be a continuous layer. Whenever MB cross sections were found, as in Figure 6a, they characteristically occupied the tapered extremities of the SAC indicating that the MB lies in the plane of cell flattening. In whole mounts, figure 6b, the SAC appeared as

Figure 4. A smooth dogfish (Mustelus canis). Blood was routinely drawn from the region of the haemal arch.



Figure 5. Dogfish erythrocytes and their cytoskeletons. (a) Living cells in fresh blood sample, with flattened elliptical morphology typical of all non-mammalian vertebrate erythrocytes. (b) Nucleated cytoskeletons prepared by Triton lysis under microtubule-stabilizing conditions (lysis medium A, see Methods). Nuclei and MBs (arrow) become visible immediately as hemoglobin is lost. Phase contrast, X 825.

Figure 6. The cytoskeletal system of the dogfish erythrocyte in thin section and whole mount. (a) Thin section of simultaneously lysed and fixed erythrocyte. The cell surface associated cytoskeleton (SAC) is seen as a surface delimiting layer completely enclosing nucleus and MB, with the MB characteristically lying in its tapered extremities (unlabelled arrows). Inset - higher magnification view of upper MB cross-section, showing the microtubule bundle in close proximity to the SAC. Fuzzy background material is dispersed hemoglobin. TEM X 9,500; inset, X 50,000. (b) Part of a nucleated cytoskeleton whole mount. The SAC appears as a rough, irregular network occupying all of the space between MB and nucleus. Individual microtubules (MT) are evident in some places within the densely-stained MB, and are occasionally splayed from it. Uranyl acetate staining, TEM X 50,000.



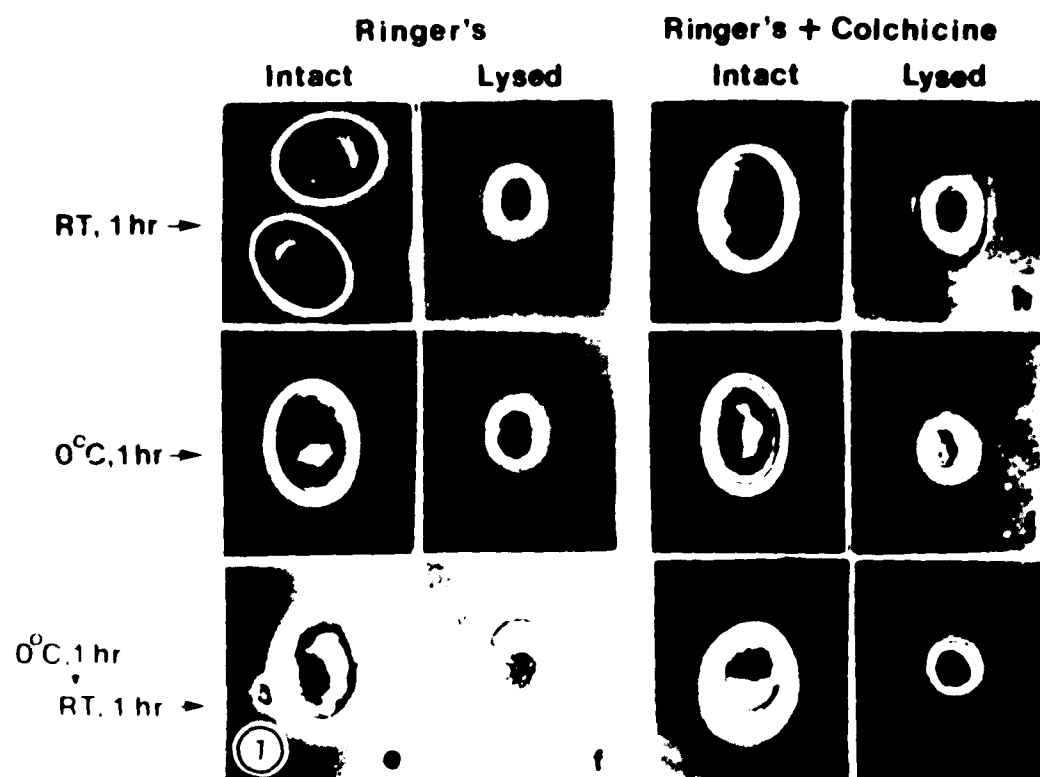
a rough irregular network completely spanning the space between the MB and nucleus. The MB was electron dense, but occasionally individual microtubules were visible (figure 6b).

B. In vivo temperature cycling of dogfish erythrocyte MBs

To determine whether dogfish erythrocyte MBs were cold-labile structures, cells were incubated in Ringer's at 0°C for 30 min or more. Cell morphology remained normal but examination following lysis showed that the MBs were no longer present (Figure 7, a,b vs. c,d). Upon rewarming for 1 hour at room temperature, MBs reassembled with much the same appearance as non-cooled controls (figure 7, e,f vs. a,b). The MB of this species is thus low-temperature labile in vivo. Multiple disassembly/reassembly cycles were also obtained, and even after 5 cycles, many MBs were of normal appearance. Erythrocytes could be stored in Ringer's for 21 hours at 0°C (longer periods were not tested) with essentially normal MB reassembly occurring in most cells after rewarming.

Colchicine (10^{-3}M) and colcemid (10^{-3}M , 10^{-4}M) did not produce MB disassembly after 1-2 hours at room temperature, but inhibited MB re-assembly after 0°C -induced disassembly, as illustrated in figure 7, g-l. After 1 hour at room temperature, both cell and cytoskeletal appearance were normal (figure 7g,h). After 1 hour at 0°C , the MBs disappeared as in controls (i,j vs. c,d). However, in the presence of colchicine (k,l) MBs failed to reappear after rewarming, in contrast to controls (e,f).

Figure 7. Effect of temperature cycling (0°C -room temperature) and colchicine (1mM) on cell morphology and the cytoskeleton. Erythrocytes were incubated in Ringer's (a-f) or in Ringer's containing colchicine (g-l). Paired photographs show representative intact cells and cells lysed in PIPES medium. Samples: (a-b, q-h) Incubation at room temperature, 1 hour; MBs are not disassembled by colchicine. (c-d, i-j) 0°C , 1 hour; MBs disassemble with or without colchicine. Faint cytoskeletal remnants are sometimes detectable near nuclei (j, arrow; see also Fig. 8c). (e-f, k-l) 0°C , 1 hour, transferred to room temperature, 1 hour. MBs reassemble in the absence (f) but not presence (l) of colchicine. Note cytoskeletal remnant adjacent to nucleus (l, arrow). Flattened, elliptical cell morphology is retained in all cases. Except for (a), which shows living cells, intact cells were fixed in Ringer's containing 1% glutaraldehyde. Phase contrast, X 800.



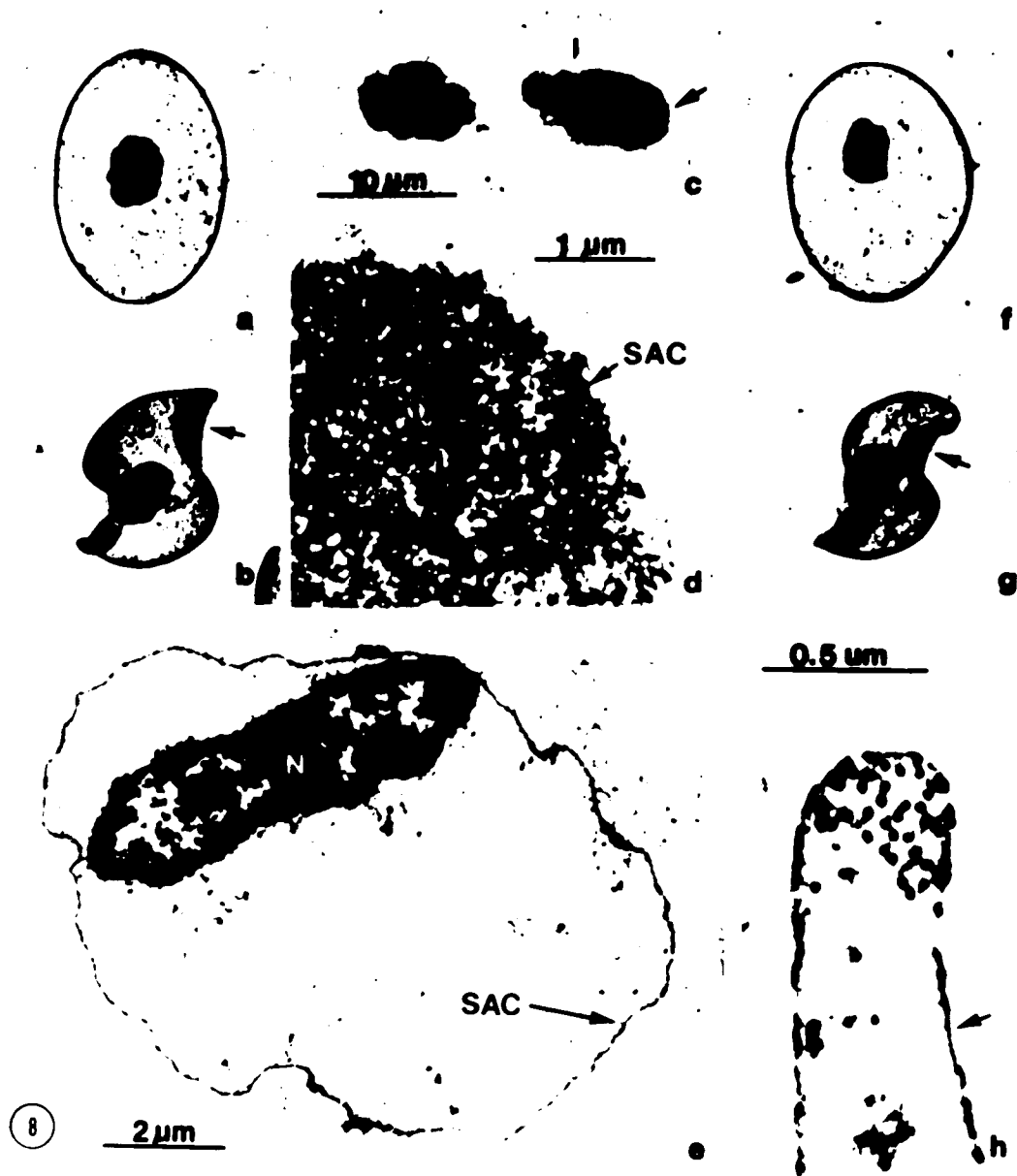
Inhibitor-treated cells retained flattened elliptical morphology throughout, though wrinkling of the cell surface was sometimes observed.

Cytoskeletal structure during temperature cycling was further examined by electron microscopy (Figure 8). In whole mounts, typical structure was evident in non-cooled controls (figure 8a,b), with the SAC most apparent where it overlapped itself due to MB twisting. After incubation of cells at 0°C the MB was no longer present, leaving the SAC collapsed inward into a denser mass (figure 8c,d). Upon rewarming (figure 8f, g), cytoskeletal structure again resembled that of controls. Thin sections of simultaneously lysed and fixed material confirmed the presence of the SAC after incubation of living cells at 0°C. (figure 8e). Neither MBs nor the tapered configuration of the SAC typical of controls (Figure 6a) were found in any sections of cooled cells, and no microtubules were observed anywhere in these lysed cells examined at higher magnification. Rewarmed cells regained essentially normal cytoskeletal structure (figure 8h; compare with Figure 6a) indicating a correlation between normal cytoskeletal morphology and the presence of MBs.

C. Preparation and structural organization of anucleate dogfish erythrocyte cytoskeletons

Examination and manipulation of dogfish erythrocyte cytoskeletons was hindered by the fragility of nuclei after Triton extraction. Nuclei often ruptured releasing a chromatin gel which caused aggregation of cytoskeletons and prevented observation of

Figure 8. Effect of temperature cycling as observed in whole mounts and thin sections, TEM. Whole mount controls: (a) cells lysed at onset of experiment, or (b) after incubation 5 hours at room temperature. When MBs are twisted, SAC visibility is enhanced where it overlaps itself (b, arrow). Twisting is common in all whole mount preparations, not peculiar to long-term incubations. Comparable thin sectioned material is shown in Figure 6a. Bars as in (c); X 2,100. (c) Incubation 3 hours at 0°C; MBs have disappeared, but the SAC remains collapsed into a relatively dense mass around nuclei. Area near arrow is shown at higher magnification in (d). X 2,100 and 18,000 respectively. (e) Thin section of cell simultaneously lysed and fixed after incubation at 0°C for 1.5 hours. The SAC is present encompassing the nucleus (N), but the MB is absent. Examination at higher magnification shows that no mts are present, and the SAC no longer displays tapered extremities in any view (compare with Figure 2a). Fuzzy background material is dispersed hemoglobin. X 9,500. (f,q) Whole mounts of cells incubated 3 hours at 0°C followed by rewarming 2 hours at room temperature; cytoskeletons are comparable to controls (a,b), with the SAC again more apparent at overlap (g, arrow). Background contamination in these and other whole mounts (a-d) is due to polylysine coating on Formvar. Bars as in (c); X 2,100. (h) Part of thin section of cell incubated 1.5 hours at 0°C followed by rewarming 1.5 hour at 20°C; the MB has reassembled, and tapered morphology of the SAC (arrow) is restored (compare with Figure 2a, inset); X 50,000.

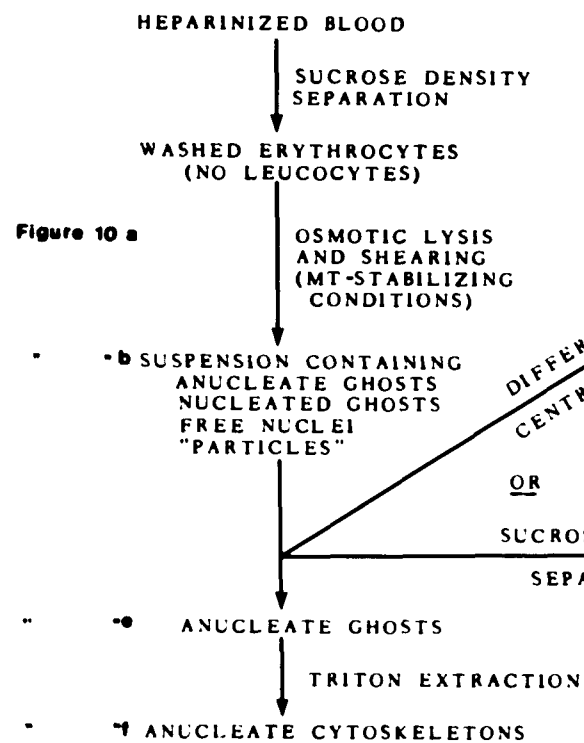


cytoskeletal morphology. The procedure for the preparation of anucleate erythrocyte cytoskeletons in quantity, described in "Methods", was therefore developed (figure 9). Nuclei of ghosts prepared by hypotonic lysis were far more stable than those of cytoskeletons prepared by Triton lysis and withstood shearing which resulted in enucleation of the ghosts. The separation of cellular components by differential gradient centrifugation is documented in figure 10. Although the initial fraction of anucleate ghosts obtained by sucrose density centrifugation was purer than that obtained by differential centrifugation, contaminating "particles" (possibly mitochondria) could be separated from anucleate ghosts by collecting only the upper part of the anucleate ghost pellet. After three suspension/centrifugation cycles to remove contaminating hemoglobin as well as particles, anucleate ghost preparations were of comparable purity to those obtained by sucrose density fractionation.

Anucleate ghosts were then extracted in lysis medium A to yield anucleate cytoskeletons (figure 10 f). Like nucleated cytoskeletons (figure 5b), anucleate cytoskeletons retained the flattened elliptical morphology characteristic of nonmammalian erythrocytes. As seen in whole mounts, (TEM; figure 11), anucleate cytoskeletons consist of an MB and SAC, with a few microtubule segments resulting from MB breakage during shearing. No intermediate filaments were observed associated with either nucleated or anucleate dogfish erythrocyte cytoskeletons. Despite

Figure 9. Preparation of dogfish erythrocyte ghosts and cytoskeletons. Flow diagram and separation of fractions by either differential centrifugation or sucrose density separation. Numbers and letters refer to phase contrast micrographs of fractions shown in Figure 10. Final anucleate ghost preparations obtained by either method were of comparable purity.

PREPARATION OF ANUCLEATE GHOSTS
AND CYTOSKELETONS



DIFFERENTIAL
CENTRIFUGATION

OR

SUCROSE DENSITY
SEPARATION

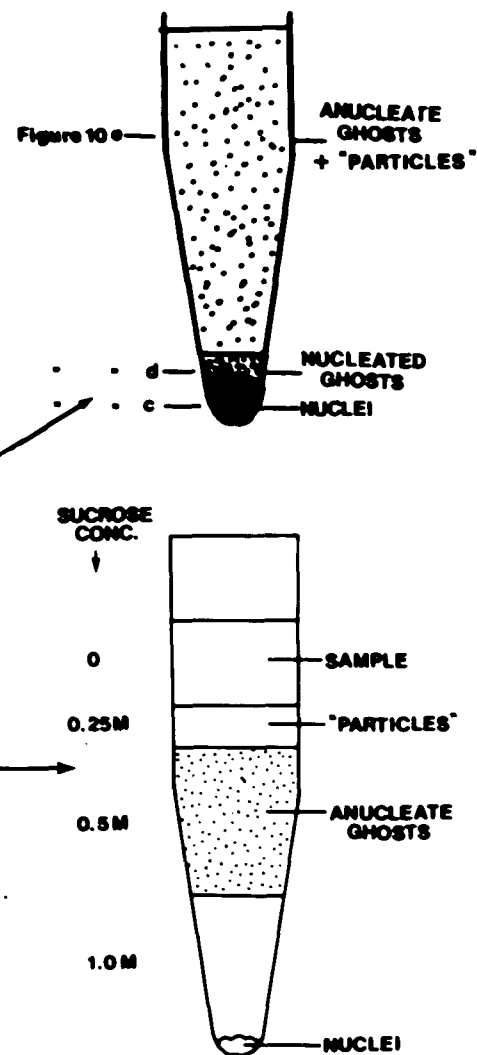


Figure 10. Phase contrast microscopy of fractions obtained during preparation of dogfish erythrocyte anucleate cytoskeletons. (a) ghosts and erythrocytes in lysing medium (arrows indicate unlysed cells); (b) Preparation after shearing containing anucleate ghosts, free nuclei, and nucleated ghosts; (c) lower layer of pellet resulting from centrifugation of 750 g for 3 min, containing nuclei; (d) upper layer of pellet containing nucleated ghosts; (e) anucleate ghosts and particles in supernate; (f) anucleate cytoskeletons prepared from ghosts by Triton extraction, consisting of SAC and MB. X 1,120.

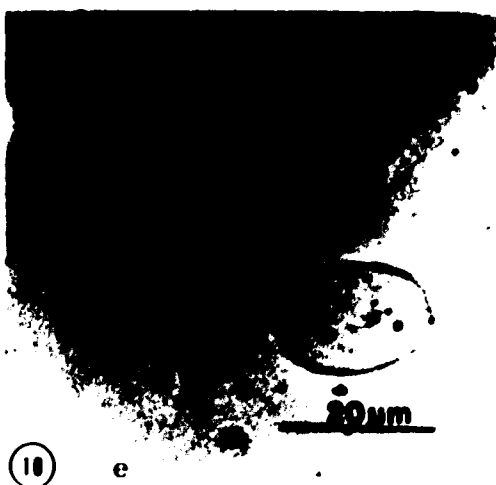
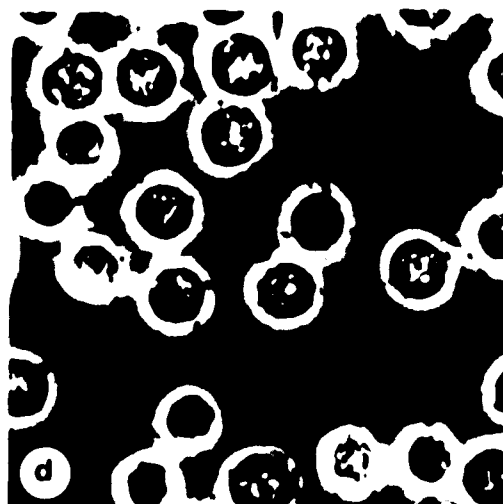
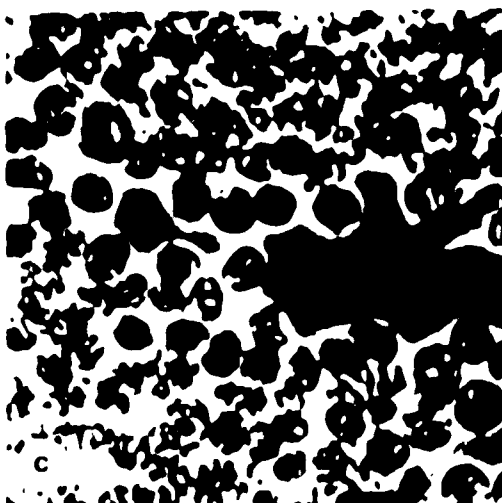
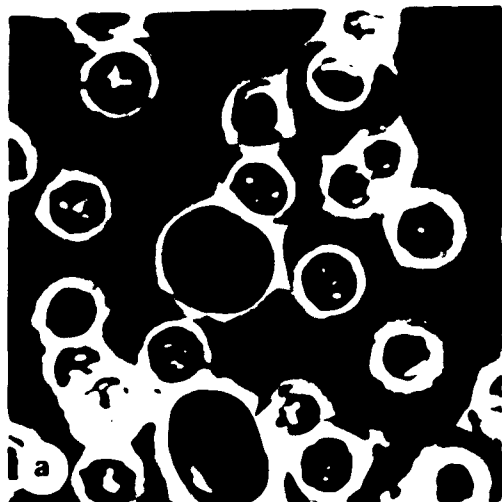


Figure 11. Anucleate cytoskeleton in whole mount, TEM. The anucleate cytoskeleton consists only of marginal band (MB) and surface associated cytoskeleton (SAC) with broken microtubule segments sometimes present (MT, verified at higher magnification). Note netlike appearance of SAC over holes in Formvar (unlabelled arrows). Uranyl acetate staining, TEM X 11,300. Inset SAC at higher magnification showing netlike structure, X 53,500.



the fact that the SAC had been ruptured during enucleation of the cell, it appeared as a continuous structure in whole mount, suggesting that the ghost had resealed. The appearance of the SAC as an irregular meshwork was further complicated by the fact that it was viewed as a double layer. The "netlike" structure can best be seen over the holes in the Formvar coating of the grid.

In thin section (figure 12), the plasma membrane and SAC of anucleate ghosts and cytoskeletons can be seen to completely enclose the MB which, when viewed in cross section, is always found in the extremity of either of these structures. In glancing section of an anucleate ghost (figure 13), the SAC can be seen underlying the lipid bilayer of the plasma membrane. Without the nucleus present, the SAC collapses upon itself except where the MB is present. Cross-bridges between component microtubules of the MB are also visible (figure 13).

D. Isolated plasma membrane and SAC preparations

Taking advantage of the cold-lability of dogfish erythrocyte MBs, anucleate dogfish erythrocyte ghosts and cytoskeletons were prepared from cooled cells devoid of marginal bands. When seen in phase contrast (figure 14a), these ghosts were not unlike MB-containing anucleate ghosts (figure 10e) in that they retained their elliptical shape. However, when treated with Triton, the resulting cytoskeletons became flaccid, either folding or rolling upon themselves (figure 14b) unlike their MB-containing counterparts (figure 10f) which remained planar and elliptical.

Figure 12. a. Dogfish erythrocyte anucleate ghost in thin section (TEM). Arrows show marginal band (MB) microtubules in close proximity to the plasma membrane (PM; double headed arrow) at the end of the long axis of the ghost; double arrows, MB microtubules in longitudinal section X 22,000.

b. Dogfish erythrocyte anucleate cytoskeletons in thin section (TEM). MB microtubules (arrows); surface associated cytoskeleton (SAC, double arrows) that underlies the plasma membrane. X 17,000.

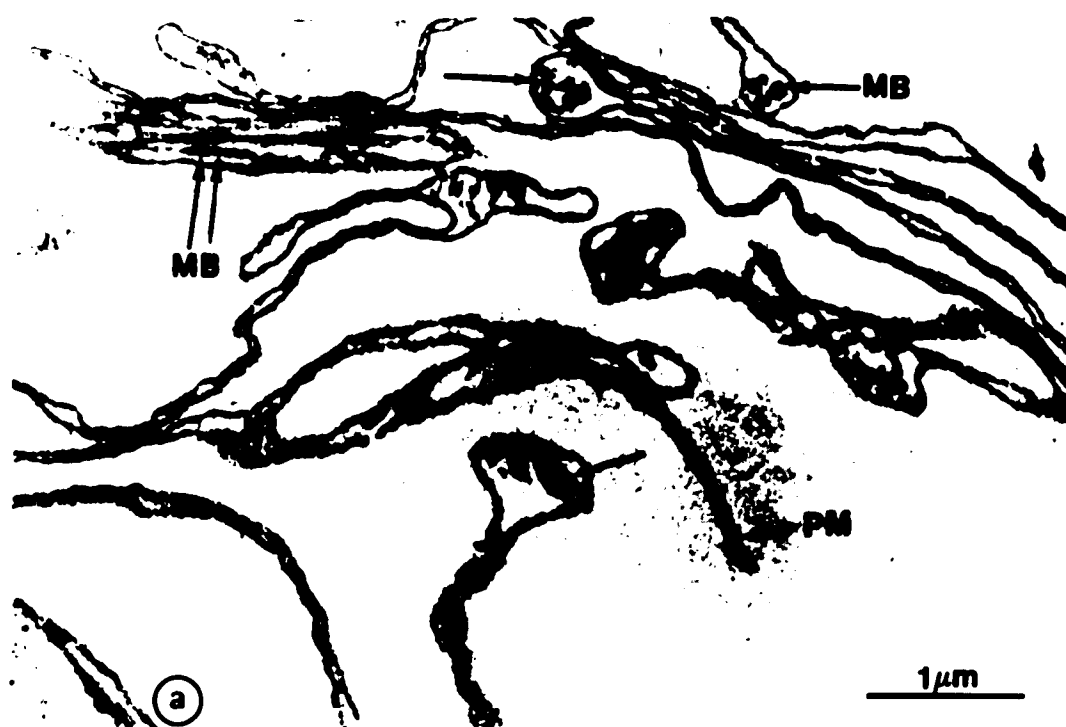
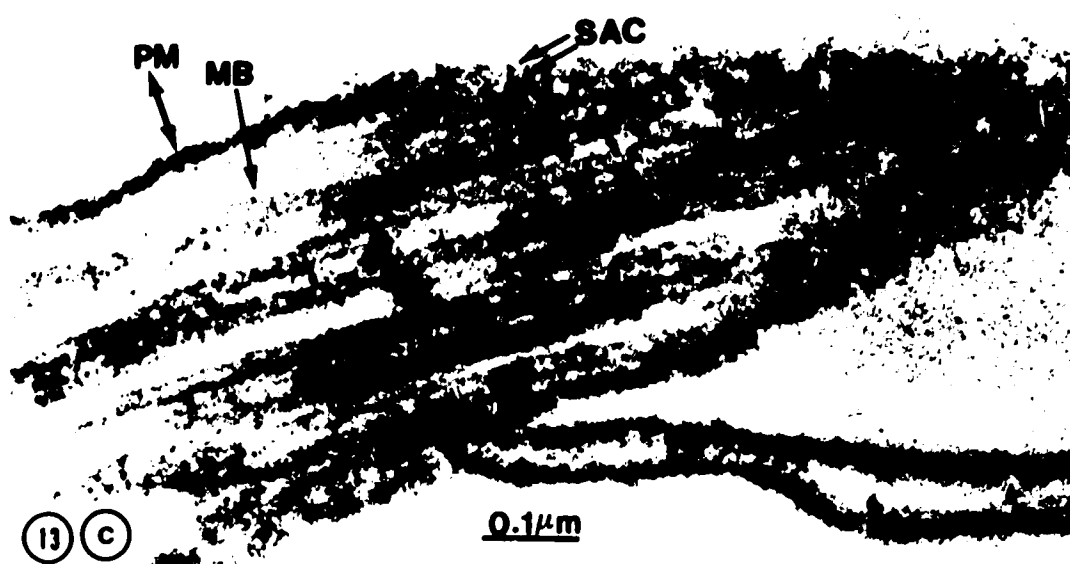
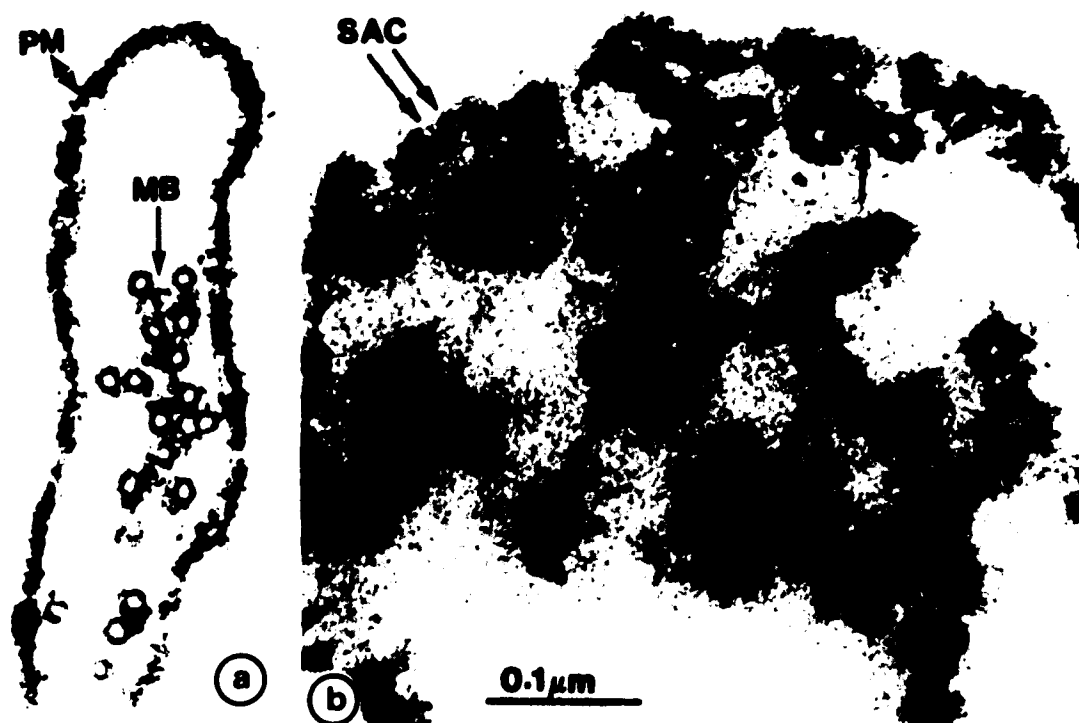


Figure 13. Marginal bands (MB) of dogfish erythrocyte anucleate ghosts and cytoskeletons in thin section (TEM). (a) End of ghost showing the closely applied microtubules of the MB within the plasma membrane (PM). (b) MB microtubules in cytoskeleton are occasionally linked by cross bridges (arrows) and lie in close proximity to the surface associated cytoskeleton. (SAC; double arrows). (c) MB microtubules in ghosts surrounded by plasma membrane (PM). SAC is revealed underlying PM in glancing section. a,c, X 135,000; b, X 210,000.



Examination of anucleate ghosts and cytoskeletons prepared from cooled cells in thin section reveals the complete absence of microtubules. These preparations contain only plasma membrane and SAC respectively (figure 14 c and d).

E. MB isolation and structure

The first procedure for the mass isolation of MBs (Method I, Methods) was developed empirically based upon the spontaneous appearance of free MBs in some cytoskeleton preparations. The mechanism of MB release from cytoskeletons was investigated by testing variations on the successful procedure. Free MBs were not obtained if the isolation medium was used at 0°C (though MBs remained stable), or if it contained TAME as a protease inhibitor in very high concentration (0.1M). This suggested that proteolysis was involved in the release of MBs from cytoskeletons, with contaminating leukocytes as a possible source of activity. When essentially all leukocytes were removed by sucrose density centrifugation prior to cell lysis, the erythrocyte cytoskeletons were highly stable and MBs could not be released even with excessive mechanical agitation. However, incubation with elastase produced intact free MBs within a few min, at room temperature, and these MBs were stable for at least an hour in the presence of elastase (MB isolation Method II).

MBs isolated by elastase treatment of nucleated cytoskeletons (figure 15 e,f) were nearly all intact, and the preparations were relatively free of visible contaminants.

Figure 14. Anucleate ghosts and cytoskeletons prepared from cooled dogfish erythrocytes devoid of marginal bands. (a) ghosts and (b) cytoskeletons in phase contrast X 1,120. Ghosts in (a) are comparable to those containing MBs (Figure 10e) while cytoskeletons in (b) are folded or rolled (double arrows) in contrast to cytoskeletons containing MBs (Figure 10f) which maintain their shape. (c) ghosts and (d) cytoskeletons in thin section (TEM). Unlabelled arrows denote position MB microtubules would normally appear. X 22,800.

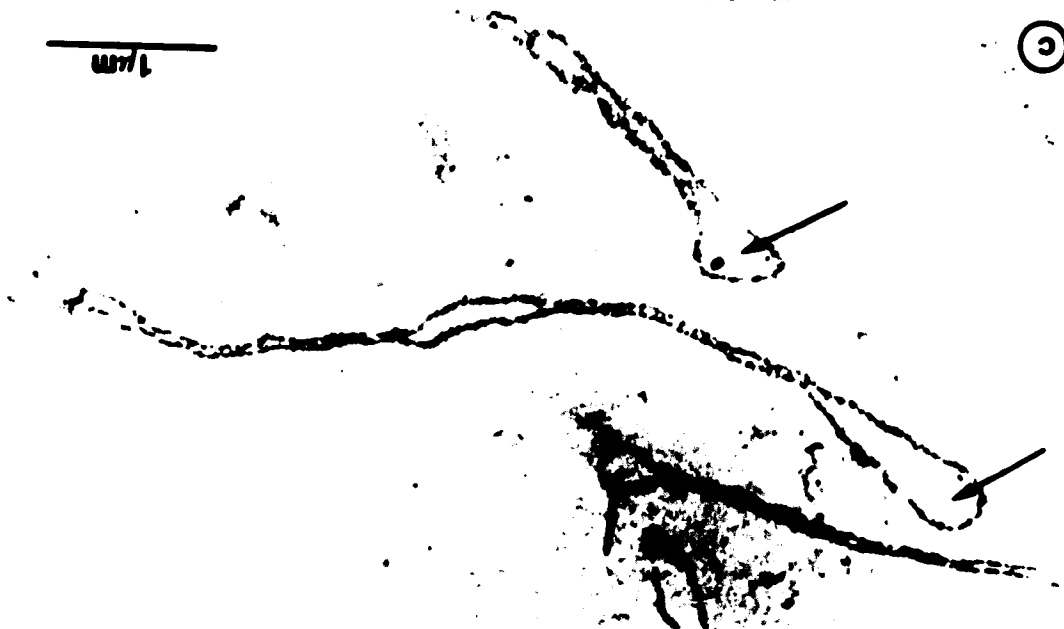
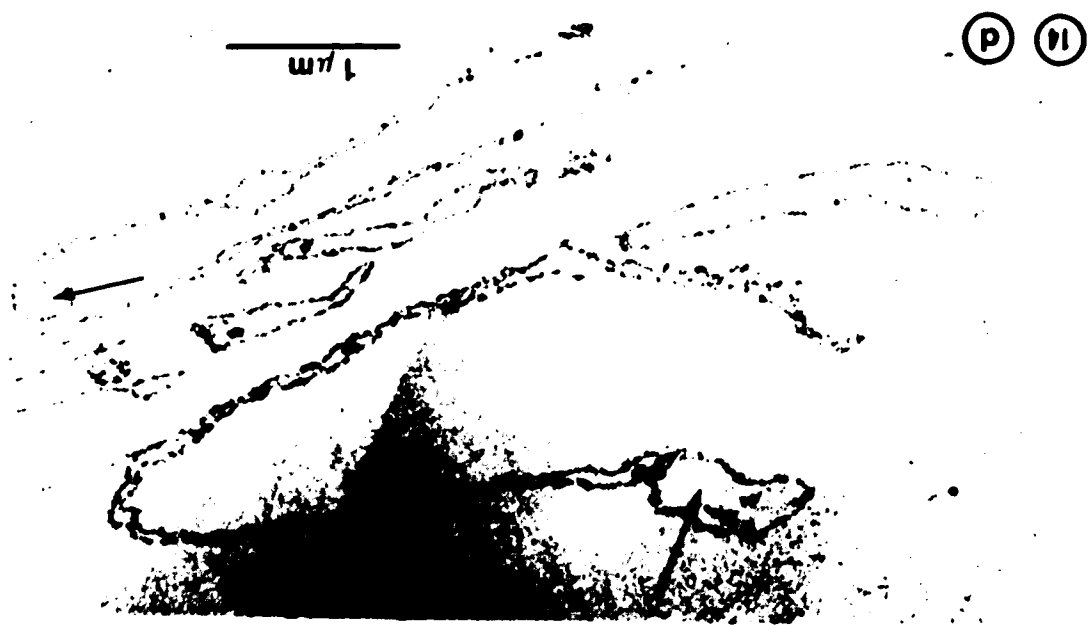
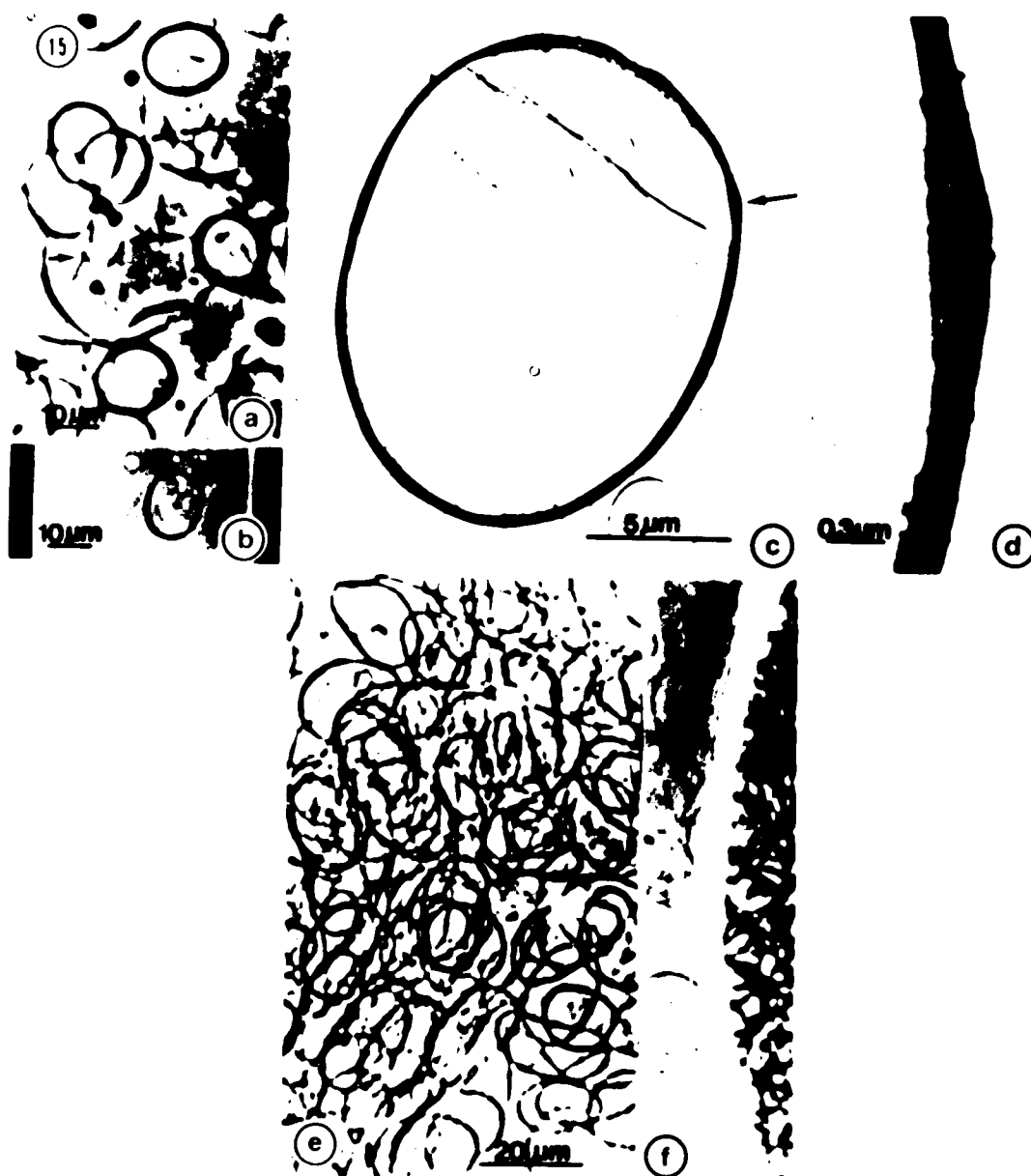


Figure 15. (a-d) Isolated dogfish erythrocyte MBs (Method I).

(a) Sample from MB pellet, viewed through air bubble, showing whole MBs plus smaller and thinner MB segments (arrows); phase contrast, X 880. (b) Whole mount of one isolated MB on Formvar-coated grid after uranyl acetate staining; phase contrast, X 760. (c,d) Same MB as shown in (b), viewed in the electron microscope. The MB is a continuous bundle of mts appearing ribbon-like in construction. A "fold" or buckled point (c, arrow) is shown at higher magnification in (d). TEM X 4,800 and X 32,000 respectively. (e,f) MBs mass-isolated by elastase treatment of nucleated cytoskeletons (Method II). (e) Sample from MB pellet, viewed in air bubble in phase contrast. Most MBs are intact, with no apparent contamination by TBM or nuclei. (f) Illustration of contrast enhancement in bubble: liquid medium at left, air at right. Phase contrast, X 850.



Isolated MBs of this species are large enough to permit pre-selection of whole mounts on grids under phase contrast (Figure 15a,b). In TEM they appeared ribbon-like, with closely applied microtubules (Figure 15c,d). Thin sections of MB pellets (Method I) contained bundles of microtubules in all orientations, with some contaminating fibrillar and granular material (Figure 16a). Typically, 40-50 mts were counted in well separated cross-sections, although lower numbers were frequently encountered. At higher magnifications cross-bridges were evident between many of the microtubules (Figure 16b). No filaments of other kinds or internal "dots" (Behnke, 1967) were observed in regular association with the microtubules.

II. Protein components of the dogfish erythrocyte cytoskeletal system.

A. Solubilized Cytoskeletal protein componets of nucleated dogfish erythrocyte cytoskeletons

Total protein composition of nucleated cytoskeletons could not be determined using polyacrylamide gel electrophoresis in SDS due to the rapid swelling of nuclei producing chromatin gels during the preparation of samples for electrophoresis. A method was therefore sought for selectively solubilizing MB and SAC while keeping nuclei intact. Various salt solutions also produced chromatin gels, but very low pH proved effective. Using lysis medium A in which 0.1M citrate was substituted for PIPES, MB and SAC were present at pH 6, pH 5, and pH 4. Increased MB

Figure 16. Isolated MB pellet (Method 1) in thin section. (a) Microtubule bundles are present in various orientations, with some contaminating granular (g) and fibrillar (f) material. (b) Separate cross-sectioned bundles typically contain 40-50 mts, though lower numbers are encountered. Cross-bridges are frequently observed between adjacent microtubules of the isolated MBs (b, arrows). TEM X 44,500 and X 160,000 respectively.



twisting occurred with pH reduction, and at pH 4 the cytoskeleton appeared to be collapsed tightly around the refractile nuclei. However, lysis at pH 3, or transfer of nucleated cytoskeletons from normal PIPES medium (pH 6.8) to pH 3 medium, immediately dissolved MB and SAC leaving morphologically intact nuclei (Figure 23a). The latter procedure eliminated hemoglobin and other soluble components in the initial lysate, simplifying the "pH 3 extract".

Electrophoretic analysis of pH 3 extracts in gradient polyacrylamide gels containing SDS (figure 23 b, lane 7) revealed proteins comigrating with human erythrocyte spectrin and actin (lane 8) and with 3 bands in the C_{15} fraction of temperature cycled dogfish brain microtubule proteins (Langford, 1980) containing dogfish brain tubulin (lane 6). Another protein band in the tubulin region was present with several other major unidentified components. High molecular weight bands corresponding to MAPs 1 and 2 (lane 1) were not observed.

B. Anucleate Ghosts and Cytoskeletons

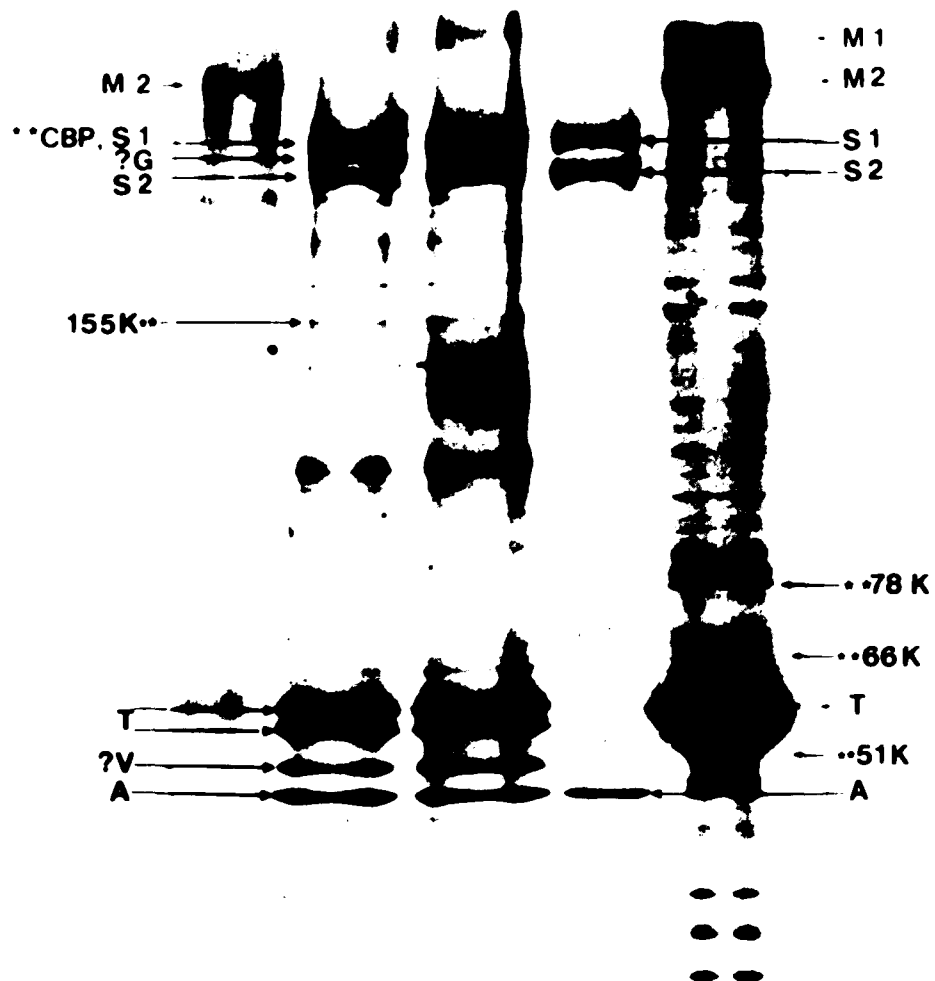
Although pH 3 extraction of nucleated cytoskeletons appeared to solubilize MB and SAC selectively, the nuclei could not be dismissed as a possible source of extract components. The method for mass preparation of anucleate cytoskeletons consisting only of MB and SAC (Figures 9, 10 and 11) was therefore developed as an independent approach to cytoskeletal composition. Anucleate dogfish erythrocyte ghosts and cytoskeletons were directly soluble in SDS electrophoresis buffer and were electrophoresed with known

cytoskeletal proteins in gradient polyacrylamide gels in SDS (figures 17 and 18). Where applicable, the nomenclature devised for human erythrocytes by Steck (1974) and based on the mobility of proteins during polyacrylamide gel electrophoresis was used here to describe the proteins of the dogfish erythrocyte membrane and cytoskeleton. For comparison, dogfish anucleate and human erythrocyte ghost samples were electrophoresed in a 5-15% polyacrylamide gradient gel in SDS (figure 22). A quantitative comparison of the coomassie blue staining profile obtained for anucleate dogfish erythrocyte ghosts with that reported for human erythrocyte ghosts by Steck (1974) is presented in Table II.

The only discernible difference between the electrophoretic patterns for ghosts and cytoskeletons (figure 17, 18, and 25) was the presence of a broad band with an apparent molecular weight of 110-130,000 in the ghosts which was greatly decreased after Triton-extraction. Due to its solubility in detergent, its heterogeneity during electrophoresis, the fact that it represents the largest percentage of total ghost protein as judged by coomassie blue staining (Table II) and that it remains in membrane vesicles formed during low ionic strength extraction of anucleate ghosts (figure 25), this band has been designated as dogfish erythrocyte band 3. Other than a difference in molecular weight, it shares all of the above properties with human erythrocyte band 3.

Identification of the two spectrin bands and the actin band is made on the basis of their comigration with purified human

Figure 17. Identification of the major constituents of dogfish anucleate cytoskeletons by electrophoretic comigration with known cytoskeletal proteins. Coomassie blue staining pattern of a 5-15% polyacrylamide gradient gel in SDS. Lane (a) bovine brain MAP 2, (b) anucleate dogfish erythrocyte cytoskeletons, (c) anucleate dogfish erythrocyte ghosts, (d) human erythrocyte spectrin and actin, and (e) bovine brain microtubule protein. M1, MAP 1; M2, MAP 2; S1 and S2, spectrin; G, goblin; V, vimentin; CBP, calmodulin-binding protein; A, actin; T, tubulin; Hb, hemoglobin. 17.5K, 51K, 66K, 78K and 155K indicate positions of other calmodulin binding proteins with corresponding apparent molecular weights (cf. figure 33).



17.5K••
 Hb

17

a

b

c

d

e

TABLE II

COMPARISON OF MAJOR PROTEIN COMPONENTS OF DOGFISH ANUCLEATE AND HUMAN ERYTHROCYTE GHOSTS

<u>PROTEIN</u>	<u>M.W.app.</u>	<u>DOGFISH</u>		<u>PROTEIN</u>	<u>M.W.</u>	<u>% STAINED PROTEIN</u>
		<u>+MB</u>	<u>-MB</u>			
		<u>% STAINED PROTEIN</u>	<u>% Stained PROTEIN</u>			
Spectrin ₁	245,000	11.4	14.6	1	240,000	15.1
Goblin	235,000	7.0	9.4	-	-	-
Spectrin ₂	220,000	10.3	19.0	2	215,000	14.7
3	103-128,000	37.0	39.3	3	88-105,000	24.1
	96,000	7.4	6.4	-	-	-
4.1	78,000	2.	-	4.1	78,000	4.2
-	-	-	-	4.2	72,000	5.0
Tubulin ₁	58,000	7.3	0.8	-	-	-
Tubulin ₂	52,000	9.2	2.1 ³	-	-	-
Vimentin	51,000	5.7	3.6	-	-	-
Actin	45,000	4.5	4.8	5	43,000	4.5
-	-	-	-	6	35,000	5.5
7	30,500	2.	-	7	29,000	3.4

1. From Steck (1974) J. Cell Biol 62, 4.

2. Minor bands representing less than one percent of the total coomassie blue stain.

3. Overestimate due to incomplete separation from vimentin.

erythrocyte spectrin and actin (figures 17, 18 and 22). As another criterion for identity, dogfish spectrin was tested for immunoreactivity with antibody directed against human spectrin (figure 19). The lower band (S2) but not the upper band (S1) of dogfish spectrin bound ^{125}I Fab' fragments of IgG raised against

human spectrin. Control preimmune IgG Fab' fragments gave no reaction with either dogfish or human spectrin.

Substantial amounts of spectrin and actin are released from mammalian erythrocyte ghosts by incubation at alkaline pH in low ionic strength media (Marchesi and Steers, 1968) and with p-hydroxymercuribenzoate (pHMB; Ralston, 1975) leading to the formation of sealed inside-out membrane vesicles (Steck and Kant, 1974). In order to determine whether dogfish erythrocyte spectrin had similar solubility characteristics, anucleate ghosts were incubated in 0.2 mM EDTA, pH 7.8 and with 5 mM pHMB at pH 9.0. Both of these treatments caused the partial release of spectrin and actin as well as other cytoskeletal proteins (figure 25, lane d and f). Exposure to pHMB caused the immediate disruption of anucleate ghosts and the formation of membrane vesicles shown in figure 26.

Identification of the tubulin bands was made on the basis of comigration with the major band of a sample of temperature-cycled bovine brain microtubule protein (figures 17 and 18) and the presence of microtubules in the anucleate ghosts and cytoskeletons (figure 13). No protein bands comigrating with high molecular weight MAPs were observed.

Figure 18. Gradient polyacrylamide gel electrophoresis in SDS of dogfish erythrocyte ghosts and cytoskeletons and cytoskeletal protein standards. Coomassie blue staining pattern of 5-15% gel. Lane (a) bovine brain microtubule proteins; (b) human erythrocyte spectrin and actin; (c) rat vimentin; (d) dogfish erythrocyte anucleate cytoskeletons; (e) dogfish erythrocyte anucleate ghosts. M2, MAP2; S1, S2, spectrin; T, tubulin; V, vimentin; A, actin; G, goblin; 3, dogfish erythrocyte band 3. Migration of putative dogfish erythrocyte vimentin is similar but not identical to rat vimentin. Lower molecular weight bands in the vimentin standard are fragments of vimentin.

18

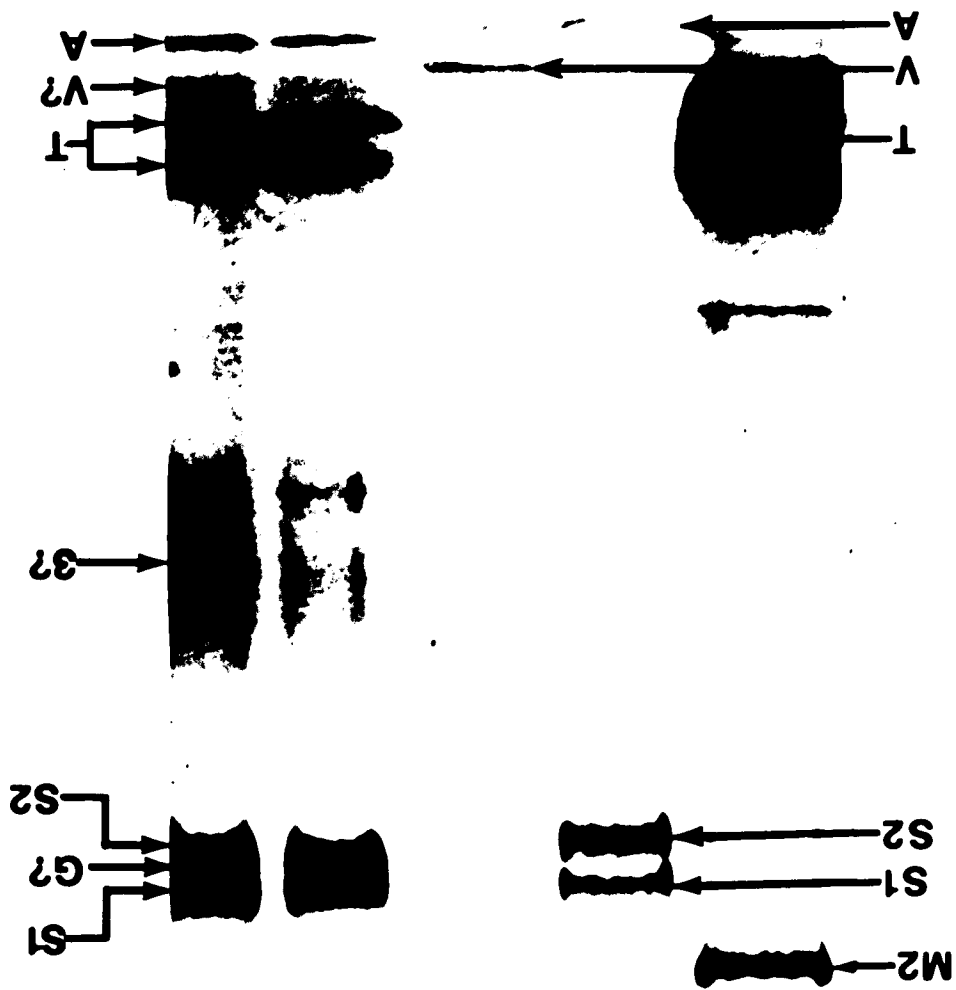
a

b

c

d

e



Dogfish goblin is tentatively identified by comparison with the electrophoretic patterns published for turkey and frog erythrocyte plasma membranes (Beam et al., 1979; Rudolph et al., 1980) where it is shown to migrate between the two bands of spectrin of these species. Dogfish erythrocyte goblin bound anti-human spectrin antibody ^{125}I Fab' fragments with an intensity equivalent to that obtained for dogfish S2 (figure 19) and was extracted with spectrin from anucleate ghosts by incubation in both EDTA and pHMB (figure 25).

Dogfish vimentin was tentatively identified by its mobility relative to tubulin and actin (Brown et al., 1976), and by analogy with chicken erythrocytes where vimentin is present in ten nanometer filaments associated with the nucleus (Woodcock, 1980; Granger et al.). Electrophoresis of a commercially available sample of rat vimentin (figure 18, lane c) showed the presence of three bands (figures 18, lane c and 20a, lane 2), the upper band of which migrated with an apparent molecular weight of 51,500, close to the value of 52,000 observed by Brown et al. (1976) for chicken vimentin. The migration of putative dogfish erythrocyte vimentin was similar but not identical to this protein band. All three bands present in the rat vimentin preparation bound antibody raised against BHK vimentin (figure 20b, lane 2), however no antibody binding was detected for putative dogfish vimentin.

Figure 19. Binding of anti-human spectrin ^{125}I Fab' fragments to human (lane 1) and dogfish (lane 2) spectrin. (a) Coomassie blue-stained gel; (b) autoradiogram of nitrocellulose transfer after incubation with anti-spectrin ^{125}I Fab' fragments. S1, S2, spectrin; G, goblin. Dogfish erythrocyte S2 and goblin but not dogfish S1 cross-react with antibody to human spectrin.

Figure 20. Incubation of anti-BHK vimentin antibody with dogfish erythrocyte ghost proteins (lane 1) and vimentin (lane 2). (a) Coomassie blue-stained gel; (b) autoradiogram of nitrocellulose transfer after incubation with anti-vimentin and ^{125}I protein A. T, tubulin; V, vimentin; A, actin. All three protein bands in the vimentin standard react with the antibody while putative dogfish erythrocyte vimentin showed no reaction.

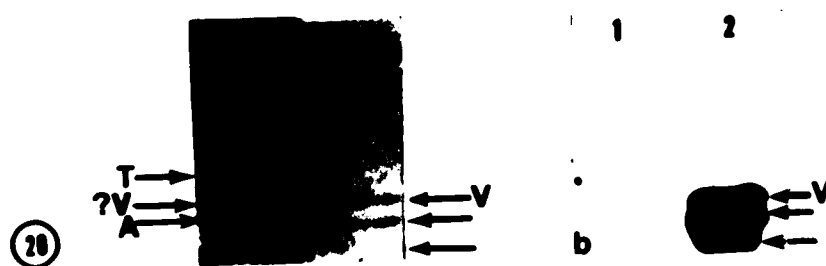
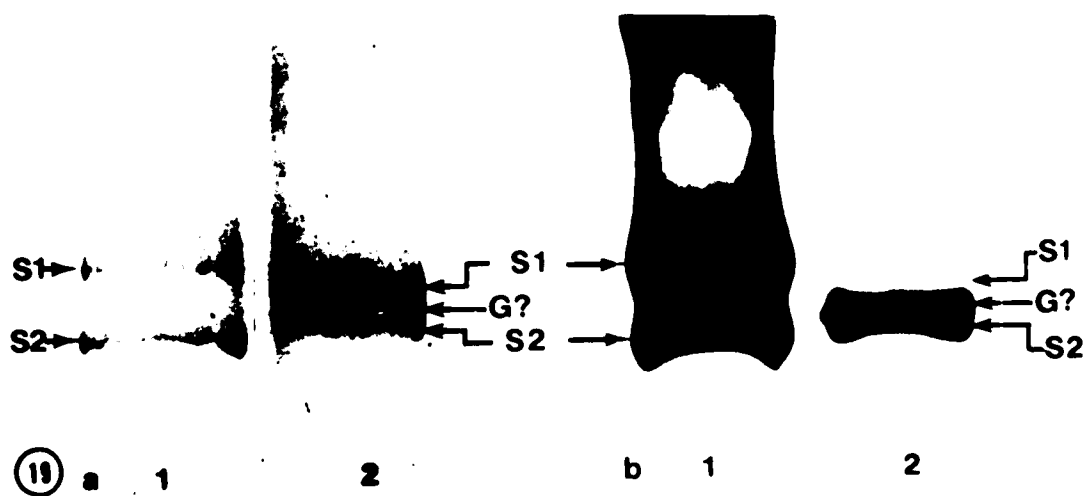
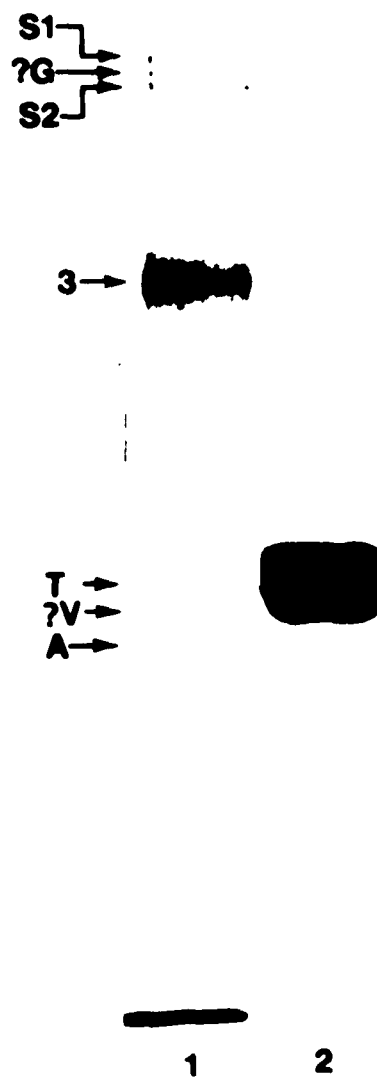
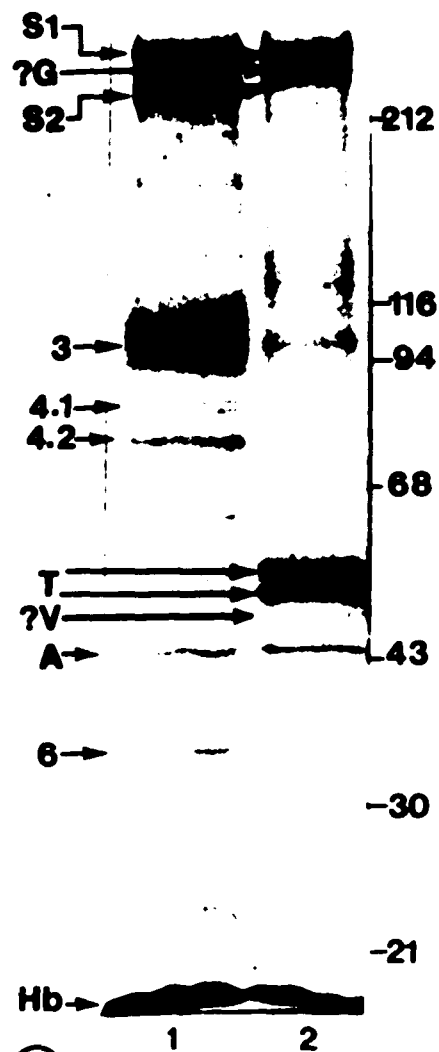


Figure 21. Protein components of the dogfish erythrocyte plasma membrane and marginal band (MB). Coomassie blue staining pattern of proteins separated by electrophoresis in a 5-15% gradient polyacrylamide in SDS. Lane 1, anucleate ghosts devoid of marginal bands and tubulin (see Figure 14); lane 2, isolated marginal bands as shown in Figure 15e. S1, S2, spectrin; G, goblin; 3, dogfish erythrocyte band 3; T, tubulin; V, vimentin; A, actin. Tubulin is localized in the MB while spectrin, goblin, vimentin and actin are located in the SAC associated with the plasma membrane.

Figure 22. Comparison of human erythrocyte ghost and dogfish erythrocyte cytoskeletal proteins. Coomassie blue staining pattern of proteins separated by electrophoresis in a 5-15% gradient polyacrylamide gel in SDS. Lane 1, human erythrocyte ghosts; lane 2, anucleate dogfish erythrocyte cytoskeletons. S1 and S2, spectrin; G, dogfish goblin; T, tubulin; V, vimentin; A, actin; 3, 4.1, 4.2, and 6, human erythrocyte ghost proteins according to the nomenclature of Steck (1974); numbers to the right of the figure denote the positions of protein molecular weight standards with molecular weights given $\times 10^{-3}$.



(21)



(22)

C. Localization of protein components to cytoskeletal structures

I. MB components

Isolated MBs (Method I) exhibited the same four tubulin region bands found in the pH 3 extract (Figure 23b, lane 4; bands a,b,c,d), three of which comigrated with major bands in the C 5 fraction containing dogfish brain tubulin (lane 6). Isolated MB pellets (Method I) did not contain spectrin, but various other bands were present. Most of these, including some actin, were judged to be contaminants since they were also present in a pellet obtained by further centrifugation of the supernate after MBs had been sedimented (figure 23a, lane 4 vs. 5). High molecular weight MAPs were not observed in these isolated MB preparations, even on overloaded gels.

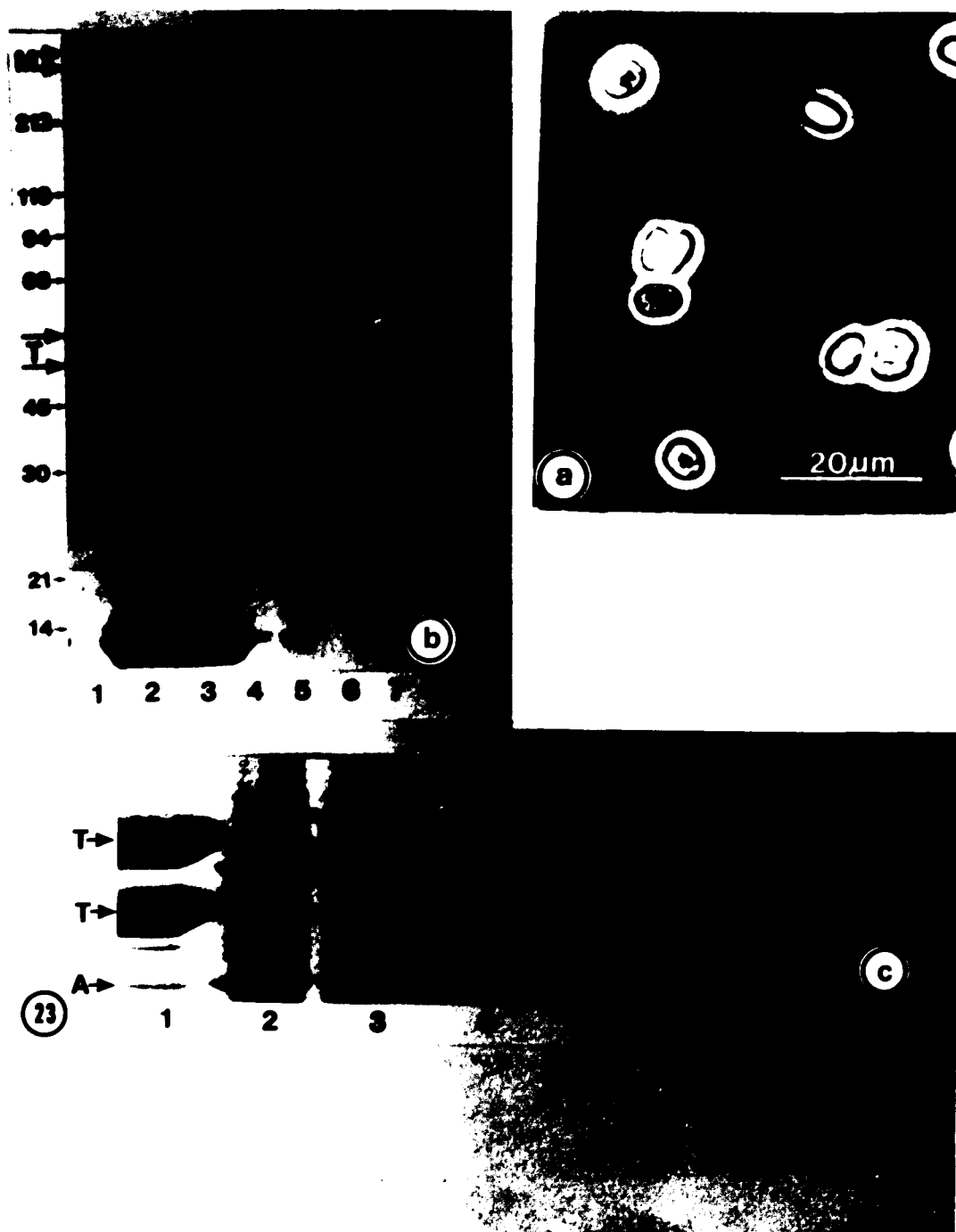
MBs isolated by elastase treatment of nucleated cytoskeletons (Method II; Figure 15e) were nearly all intact, and the preparations were relatively free of visible contaminants. Their electrophoretic pattern was correspondingly simple, even on overloaded gels (Figure 21, lane 2), consisting almost entirely of protein in the tubulin region. A minor protein band with an apparent molecular weight of 35,000 was the only other component detected in the preparation. There were no protein bands in the region above the spectrin bands where high molecular weight MAPs would migrate.

An independent in vivo approach to identification of major MB components was also utilized. Control cells at room temperature

Figure 23. Solubilization of cytoskeletal protein components.

(a) Effect of pH 3 media on nucleated cytoskeletons; MB and SAC are solubilized, leaving free nuclei. Phase contrast, X 1,000.

(b,c) SDS-PAGE separation of dogfish erythrocyte cytoskeletal proteins. (b) 5-12% gradient gel. Lanes: 1) C₁S (crude) fraction of dogfish brain tubulin (T) overloaded to show high molecular weight MAPs (M), 2) control cell (room temperature) lysate supernate, 3) lysate supernate from cells incubated 2 hours at 0°C to disassemble MBs, 4) isolated MB pellet (Method I), containing four "tubulin region" (T) bands, 5) pellet obtained by further centrifugation (approximately 11,000 g, 60 min) after MB removal (11,000 g, 10 min). This is the probable contaminant fraction for lane 4. 6) C₁S fraction of dogfish brain tubulin, underload, 7) pH 3 extract containing solubilized components of nucleated cytoskeletons; S, dogfish erythrocyte spectrin, A, erythrocyte actin. 8) Human erythrocyte ghost standards; S, spectrin, A, erythrocyte actin. Molecular weight standards (MW X 10⁻³) are given at left. (c) Higher magnification of the "tubulin region" (T) of lanes 1-7. Four bands (a,b,c,d) were identified by enhancement in the lysate supernates from 0°C-incubated cells (lane 3) versus controls (lane 2). Enhancement of bands a and b is most evident here, with c and d more apparent in other experiments. The four bands identified by in vivo MB dissolution (lane 3) comigrate with the four tubulin region bands of isolated MBs (lane 4), and are not present in the isolated MB contaminant pellet (lane 5). Bands a, b, and d are also evident in the C₁S fraction of dogfish brain tubulin, with a trace of band c (lane 6). All four bands (a-d) are prominent in the pH 3 extract (lane 7) containing both MB and SAC components.



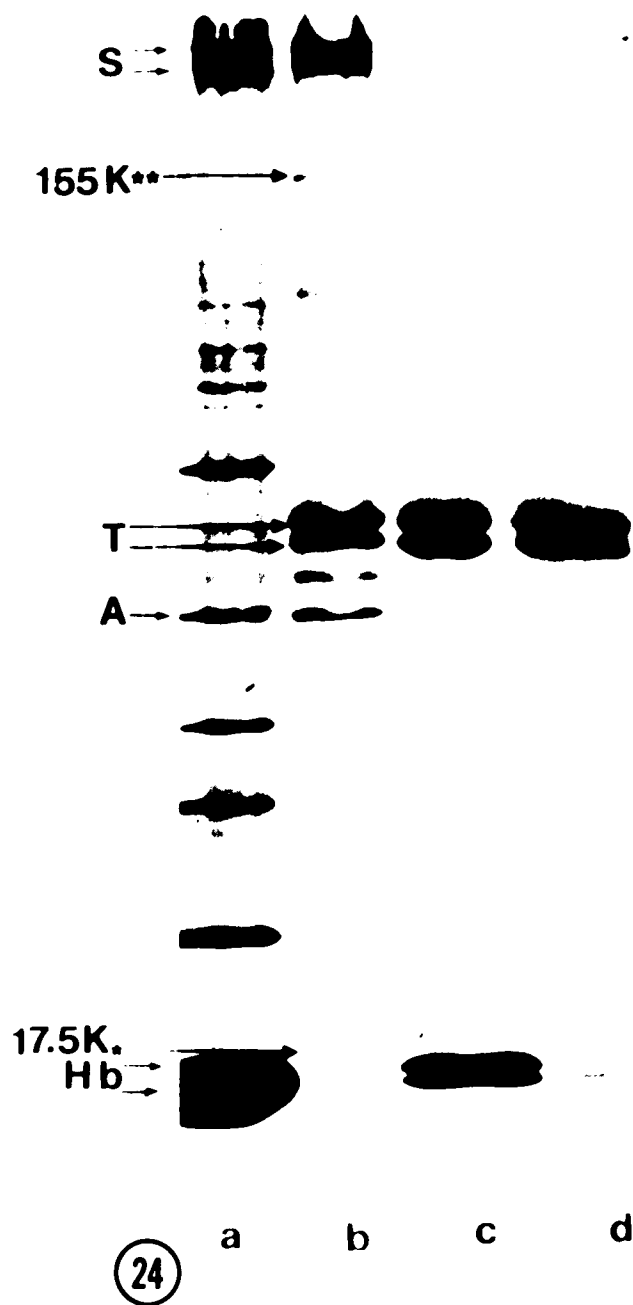
were lysed in lysis medium A and the solubilized proteins compared with those of an equal number of cells lysed after MB disappearance at 0°C (60 min). Of the numerous bands present, only four were enhanced in supernates from cells with 0°C-dissociated MBs (figure 23b, lane 3 vs. 2; bands a,b,c,d). These correlated closely with the four tubulin region bands of isolated MBs (lane 4). Enhancement of bands a and b is apparent in the gel shown (figure 23b, lane 3 vs. 2), while bands c and d were more evident in other experiments. The actin band was not noticeably affected, and spectrin did not appear in either supernate (lanes 2 and 3).

As noted above, isolated MBs were stable at 0°C. in lysis medium A. MBs of nucleated cytoskeletons were similarly cold stable in lysis medium A. However, incubation of nucleated cytoskeletons at 0°C in the presence of 150 mM KCl caused MB disassembly. Electrophoresis of the resulting extract confirmed the presence of tubulin as the only major protein component other than the hemoglobin contaminant, and the absence of proteins migrating above spectrin (a minor contaminant in the extract) in the region of high molecular weight MAPs (figure 24, lane c). Actin is also present as a minor contaminant.

2. SAC components

Anucleate ghosts prepared from cells incubated at 0°C so that they were devoid of marginal bands (figure 14) were greatly depleted of tubulin (Figure 21, lane 1). There was no other major

Figure 24. Electrophoresis of human and dogfish erythrocyte ghosts and cytoskeletal extracts. Coomassie blue staining pattern of 5-15% polyacrylamide gradient gel in SDS. a) human erythrocyte ghosts, b) anucleate dogfish erythrocyte ghosts, c) low temperature extract of nucleated dogfish erythrocyte cytoskeletons and d) calcium extract of nucleated dogfish erythrocyte cytoskeletons. S spectrin, T tubulin, A actin, Hb hemoglobin. Tubulin is the major component of both the low temperature and calcium extracts.



difference in protein composition between these ghosts and MB-containing anucleate ghosts (figure 18, lane e; Table II), confirming that the only major cytoskeletal protein localized in the MB is tubulin. All other cytoskeletal proteins are associated with the SAC.

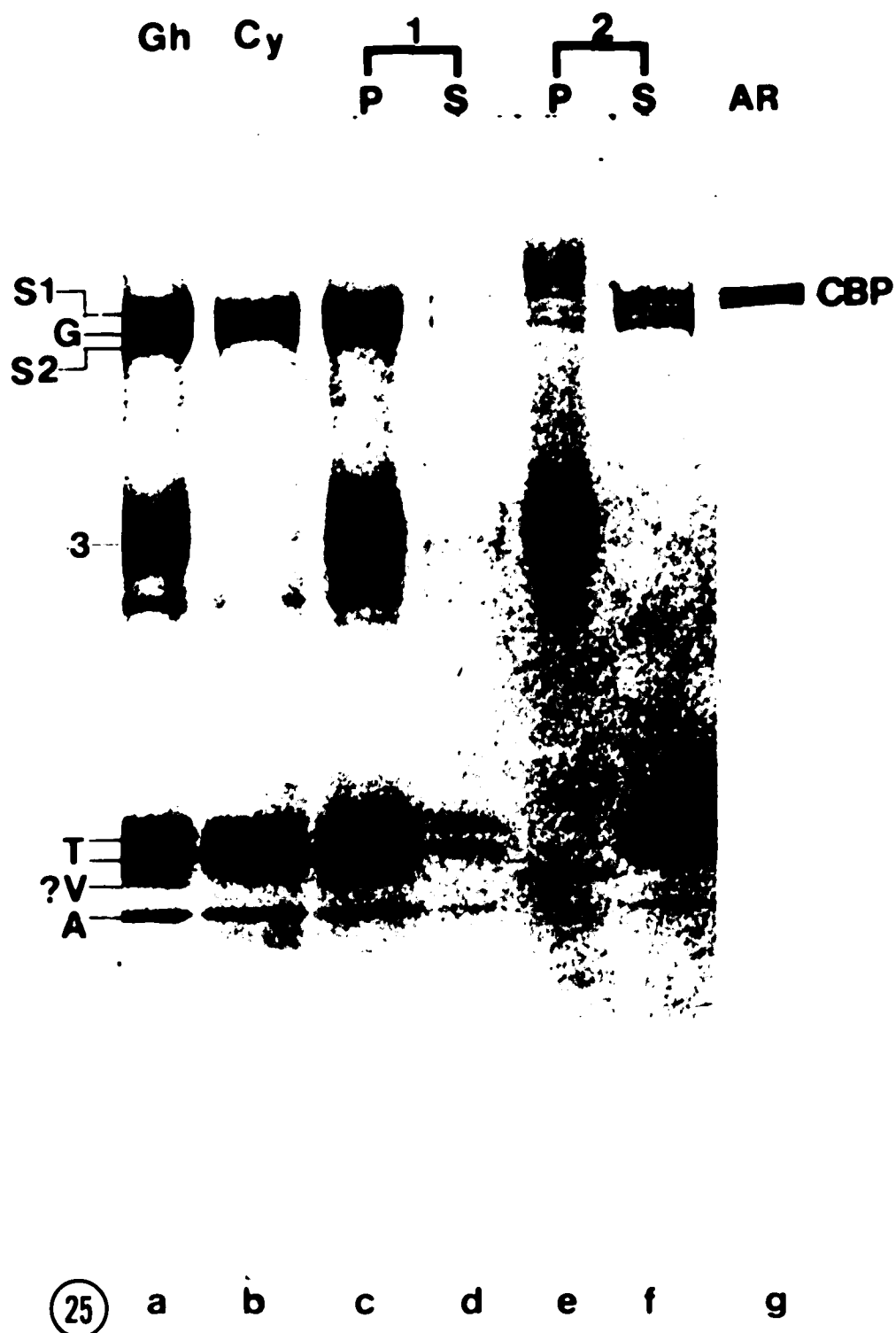
Protein bands in the region of band 3, the major protein component of anucleate ghosts both containing and devoid of MBs, were still present in anucleate cytoskeletal preparations, though in amounts greatly diminished by Triton extraction. (figure 17, lane b; figure 18, lane d). The protein bands remaining in the cytoskeletal preparations may be cytoskeletal proteins comigrating with band 3 and masked by its presence in ghost preparations. Using the operational definition of the cytoskeleton as the Triton insoluble components of the ghost, by definition, the Triton soluble material in band 3 is not part of the SAC. The assignment of band 3 as an integral membrane protein and not a component of the SAC is supported by its presence in membrane vesicles formed by pHMB extraction of anucleate ghosts (figure 25, lane e; figure 26).

III. Correlation between cytoskeletal and cell morphology.

A. "Spindle cell" formation

When dogfish erythrocytes were stored in Ringer's at room temperature for long periods (4 or more hours) with or without prior temperature cycling, increasing numbers of singly and doubly-pointed cells appeared with time. A few singly-pointed

Figure 25. Low ionic strength and pHMB extraction of anucleate dogfish erythrocyte ghosts. Electrophoresis of anucleate dogfish erythrocyte ghosts, cytoskeletons and extracts. Coomassie blue staining patterns of proteins separated in a 5-15% polyacrylamide gradient gel containing SDS. (a) anucleate ghosts (Gh); (b) anucleate cytoskeletal preparation (Cy); (c) pellet (p) and (d) soluble fraction (s) of ghosts treated with 1) Tris HCl pH 7.8, 0.2mM EDTA at 37⁰; (e) pellet and (f) soluble fraction of ghosts treated with 2) 1mM Tris pH 9 containing 5mM pHMB at 23⁰; (g) autoradiogram (AR) resulting from ¹²⁵I calmodulin binding to proteins present in combined extracts shown in lanes d and f. S1, S2, spectrin; G, globin; 3, dogfish erythrocyte band 3; T, tubulin; V, vimentin; A, actin; CBP, dogfish calmodulin-binding protein.



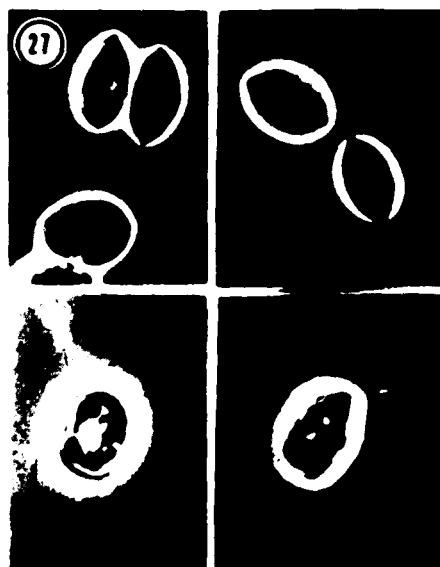
cells can usually be found in fresh erythrocyte suspensions, but the doubly-pointed "spindle cells" are rare. In some stored preparations however, spindle cells represented over 20% of the population (figure 27a,b). Lysed samples contained a similar percentage of anomalous doubly-pointed MBs (figure 27c,d). These MBs were not continuous, but consisted of separated fibers (microtubule bundles) of varying thickness converging at cell "poles". These fibers lay essentially in the plane of cell flattening and the pointed cells retained their flatness. Occasionally the fibers could be traced slightly beyond the polar convergence point (figure 27c,d), presumably accounting for polar projections observed in some of the intact spindle cells (figure 27b). Singly-pointed teardrop-shaped MBs were also found, corresponding to singly-pointed cell morphology. Would normal cell shape be regained if these anomalous MBs were disassembled at 0 °C? Cell suspensions were incubated at room temperature until significant numbers of spindle cells were present, and samples placed at 0 °C. Ellipticity was restored as nearly all pointed cells disappeared within 1-2 hours, while being retained in room temperature control cells.

B. Effect of calcium and ionophore A23187 on erythrocyte morphology

Dogfish erythrocytes treated with calcium ionophore A23187 underwent calcium-dependent changes in morphology. Generally the cells changed from a normal flattened ellipse (figure 28a),

Figure 26. Vesicles formed upon extraction of anucleate dogfish erythrocytes with pHMB at pH 9.0. Vesicles were spherical when formed but appear concave after centrifugation at 100,000 g. Phase contrast $\times 1,120$. (cf figure 25, lane e).

Figure 27. "Spindle cells" and their cytoskeletons. Doubly-pointed erythrocytes are rare in fresh blood samples, but appear in increasing numbers with long-term storage in Ringer's at room temperature (4 or more hours). (a) Two spindle cells. Like normal erythrocytes (cell at lower left) the spindle cells are flattened. Bar as in (b). (b) Two spindle cells, one with two antenna-like projections at one end (arrow). Note that projection angles follow cell surface contours. (c) A spindle cell cytoskeleton with 3 major fibers (mt bundles) meeting at the "poles" and crossing at upper pole (arrow; crossed fibers very thin and faint). Bar as in (d). (d) A spindle cell cytoskeleton with 2 major fibers crossing at "poles", and a third very thin fiber (f) visible. Erythrocyte shape appears to conform to the shape of the marginal band. Phase contrast, $\times 750$ (a,b) and $\times 1,700$ (c,d).

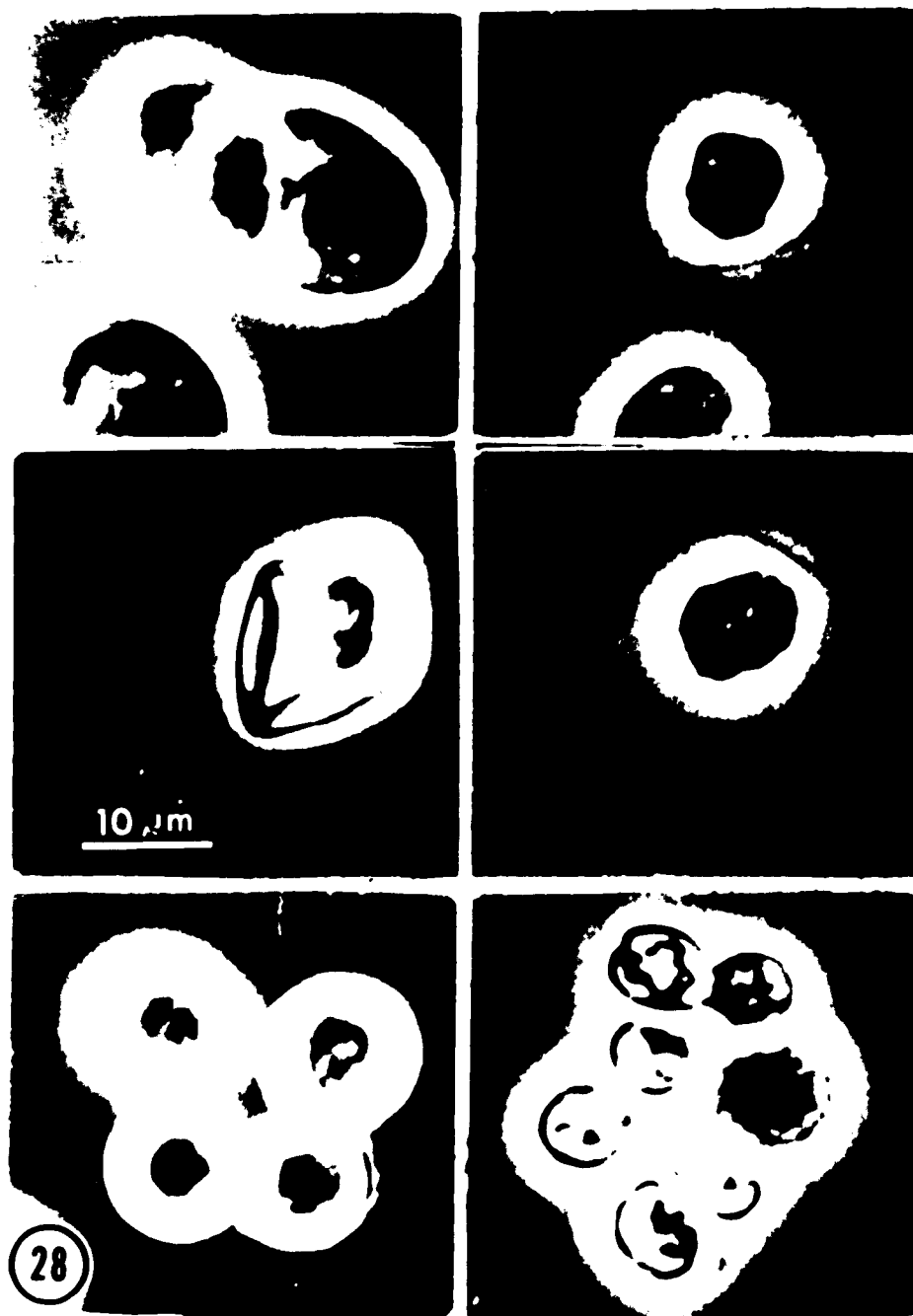


through angular shapes which were no longer planar (figure 28b), to spheres (28c). These changes were both time and calcium-concentration dependent. The formation of spheres was more rapid in 10mM calcium than in 1 or 0.1 mM calcium and was essentially complete after 30 min. Changes in cell morphology were observed as early as 1 min after ionophore addition to cell suspensions in 10 mM calcium. In 1mM calcium there were no morphologically normal cells after 30 min and some cells were spherical. Most cells in 0.1mM calcium had abnormal shapes after 30 min, but no spheres were evident. When EGTA was substituted for calcium in the presence of ionophore, cells retained their normal flattened elliptical morphology although incubation in this medium for periods longer than 30 min caused hemolysis, due, presumably, to a lack of calcium required for membrane stability.

Examination of lysed cell preparations showed corresponding changes in cytoskeletal morphology. The cytoskeletons of cells incubated with ionophore in the presence of EGTA appeared to be normal, as were the untreated controls, consisting of the elliptical MB and SAC enclosing the nucleus within (figure 28d). In most cells incubated for 30 min with ionophore in the presence of 0.1 and 1.0 mM calcium, the MBs were angular in shape (figure 28e). This angularity appeared to correspond to that observed for the intact cells, demonstrating a correlation between the shape of the erythrocyte and the shape of the MB. After treatment with ionophore for 30 min, the cytoskeletons of all the cells in 10mM

Figure 28. Effect of calcium and ionophore A23187 on erythrocyte morphology. Dogfish erythrocytes treated with calcium ionophore A23187 underwent calcium-dependent changes in morphology. Generally the cells changed from a normal flattened ellipse (Figure 28a), through angular shapes which were no longer planar (Figure 28b), to spheres (28c). These changes were both time and calcium-concentration dependent. The formation of spheres was more rapid in 10mM Ca^{2+} than at 1 or 0.1 mM Ca^{2+} and was essentially complete after 30 min. In 1mM Ca^{2+} there were no morphologically normal cells after 30 min and some cells were spherical. Most cells in 0.1mM Ca^{2+} had abnormal shapes after 30 min, but no spheres were evident. When EGTA was substituted for calcium in the presence of ionophore, cells retained their normal flattened elliptical morphology although incubation in this medium for periods longer than 30 min caused hemolysis.

Examination of lysed cell preparations showed corresponding changes in cytoskeletal morphology. The cytoskeletons of cells incubated with ionophore in the presence of EGTA appeared to be normal as were the untreated controls, consisting of the elliptical marginal band, trans-marginal band material (SAC) and nucleus within (Figure 1d). In most cells incubated for 30 min with ionophore in the presence of 0.1 and 1.0 mM Ca^{2+} , the marginal bands were angular in shape (Figure 1e). This angularity appeared to correspond to that observed for the intact cells, demonstrating a correlation between the shape of the erythrocyte and the shape of the marginal band. After treatment with ionophore for 30 min, the cytoskeletons of all the cells in 10mM Ca^{2+} and some of those in 1mM Ca^{2+} consisted only of nuclei with remnants of SAC attached (Figure 1f). The disappearance of the marginal band and SAC as observed by phase microscopy paralleled the formation of spheres in the living cells.



calcium and some of those in 1mM calcium consisted only of nuclei with remnants of SAC attached (figure 28f). The disappearance of the MB and SAC as observed by phase microscopy paralleled the formation of spheres in the living cells.

IV. In vitro calcium effects on cytoskeletal structures

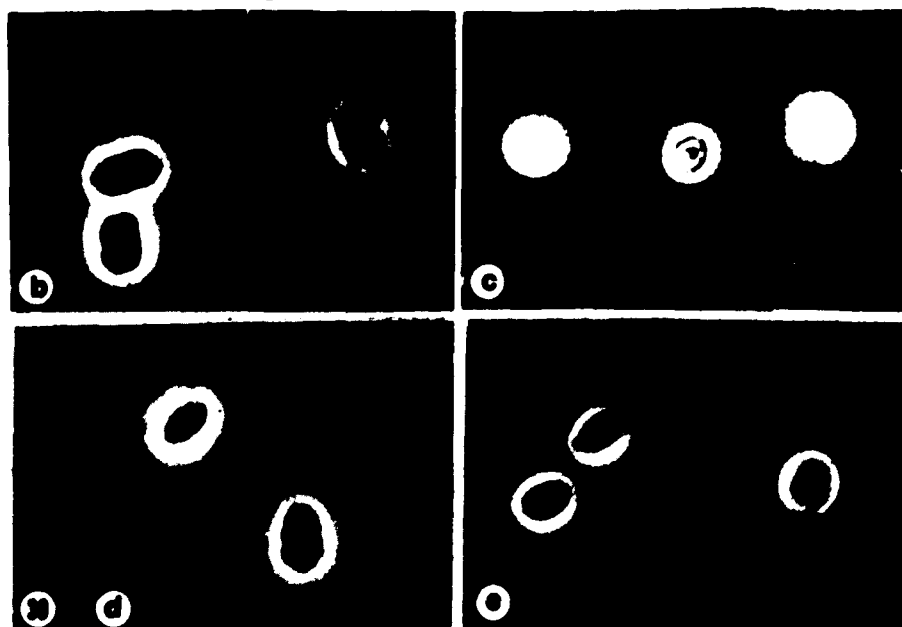
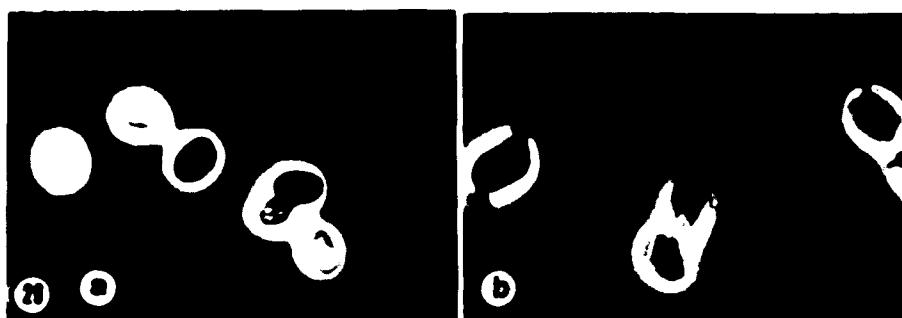
A. Effect of calcium during lysis

Since calcium is known to cause microtubule depolymerization in vitro (Weisenberg, 1972), lysis medium A (Rebhun et al., 1974) or variations on PIPES medium containing 5 mM EGTA as a chelator of calcium were used throughout this study for the preparation of dogfish erythrocyte cytoskeletons and cytoskeletal structures. To test the in vitro effect of calcium on cytoskeletal structures, dogfish erythrocytes were lysed with 0.4% Triton in Elasmobranch Ringer's solution which contains 10 mM calcium, and cytoskeletons were observed immediately after lysis. A suspension of naked nuclei with an occasional remnant of SAC was observed (figure 29a) indicating that the MB and possibly SAC are labile in this medium. Inclusion of 15 mM EGTA in the Ringer's solution containing Triton, did not prevent MB disassembly indicating that the effect of lysis in this medium on the MB was not due solely to calcium. Lysis in EGTA-Ringer's yielded nuclei with remnants of SAC attached, suggesting that some of the effects on the SAC were calcium-dependent (figure 29b).

To eliminate differences in ionic composition between Ringer's and lysis medium A as a cause of cytoskeletal

Figure 29. Effect of calcium on the dogfish erythrocyte cytoskeleton during lysis with 0.4% Triton X-100 in Elasmobranch Ringer's. Cells were lysed either in Elasmobranch Ringer's which was 10mM in CaCl_2 (a) or in Ringer's containing 15mM EGTA (b). Only nuclei remain after lysis in Ringer's while remnants of the surface associated cytoskeleton (arrow) are present with nuclei in samples lysed in EGTA Ringer's. No marginal bands are visible in either sample.

Figure 30. Effect of KCl and protease inhibitors on cytoskeletons of dogfish erythrocytes lysed with 0.4% Triton in lysis medium A containing calcium. (a) cytoskeletons prepared in medium containing 5mM EGTA and 150mM KCl; (b,c) samples lysed in medium containing 5mM CaCl_2 without (b) and with (c) 150mM KCl; (d,e) as in b and c except that media contained TAME, DFP, leupeptin and tetrathionate as protease inhibitors. Cells lysed in calcium in the absence of KCl contain marginal bands while those lysed in the presence of KCl do not. Protease inhibitors do not inhibit this effect.



disassembly, cells were lysed in lysis medium A containing 5 mM calcium in place of EGTA. Lysis in this medium did not cause MB disassembly (figure 30b). Consistent with this result, when dogfish erythrocyte cytoskeletons were prepared and washed in PIPES medium, both the SAC and MB were stable in 5mM calcium over long time periods (data not shown).

The effect of calcium and ionophore A23187 on dogfish erythrocytes indicated that the MB and to some extent the SAC were calcium-labile in vivo. One major difference between the in vitro conditions and the in vivo state was the higher ionic strength of the in vivo milieu. Therefore, cells were lysed in PIPES medium containing 150 mM KCl and either EGTA or calcium. MBs and SAC were present in cytoskeletons prepared in EGTA (figure 30a), but absent from samples lysed in the presence of calcium (figure 30c).

In vitro effects of calcium on microtubules (Sandoval and Weber, 1979) and other cytoskeletal structures (Phillips and Jakabova, 1977) have been reported to involve calcium-activated proteases. To determine if MB disassembly in the presence of calcium and KCl could be affected by protease inhibitors, cells were lysed in lysis medium A containing calcium and either with or without TAME, DFP, sodium tetrathionate and leupeptin. The presence of these protease inhibitors had no effect. Cytoskeletons prepared in the absence of KCl contained MBs (figure 30 d), while those prepared with KCl did not (figure 30e).

B. Calcium lability of the dogfish erythrocyte cytoskeleton

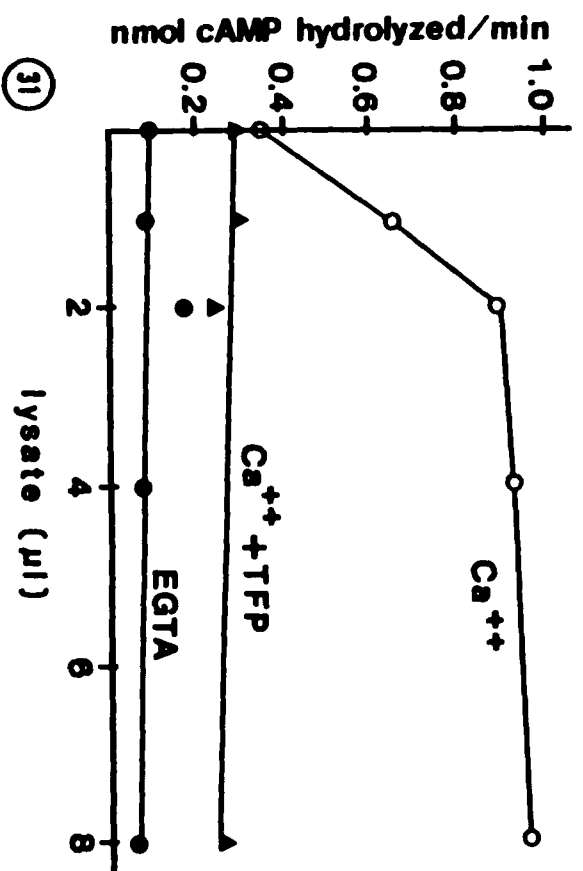
As noted above, cytoskeletons prepared in lysis medium A were stable in 5 mM calcium. However inclusion of 150mM KCl in lysis and incubation media and was found to greatly enhance the calcium lability of the cytoskeleton. When cytoskeletons, prepared either in the presence or absence of KCl, were incubated in the presence of KCl and calcium for 30 min, the MB was no longer visible under phase contrast. Proteins extracted from isolated cytoskeletons with calcium were separated by polyacrylamide gel electrophoresis in SDS. As shown in figure 24, lane d, the major component in this extract is tubulin, except for hemoglobin which contaminates the cytoskeleton preparations. Minor bands that comigrated with human erythrocyte spectrin and actin were also observed.

V. Calmodulin and cytoskeletal calmodulin-binding proteins in dogfish erythrocytes

A. Presence of calmodulin

A lysate of dogfish erythrocytes was prepared in ten volumes of lysis medium A and assayed for calmodulin activity using a preparation of bovine brain phosphodiesterase (PDE) which had been depleted of calmodulin. As shown in Figure 31, microliter quantities of the lysate were sufficient to activate the PDE in a calcium-dependent manner. This activation was blocked by 40uM trifluoperazine, a known inhibitor of calmodulin, in the presence of 1mM calcium. The level of activity present in the lysate was equivalent to 34ug calmodulin/ml of packed erythrocytes. Since only free calmodulin would be detected by this assay, the minimal

Figure 31. Presence of calmodulin in dogfish erythrocyte lysate. Activation of bovine brain PDE by dogfish erythrocyte lysate (58 mg protein/ml) in the presence of 1mM CaCl_2 (○-○), 1mM EGTA (●-●), 1mM CaCl_2 + 40uM trifluoperazine (TFP; ▲-▲). Activation is calcium-dependent and inhibited by TFP.



concentration of calmodulin in the dogfish erythrocyte is approximately 2 μ M.

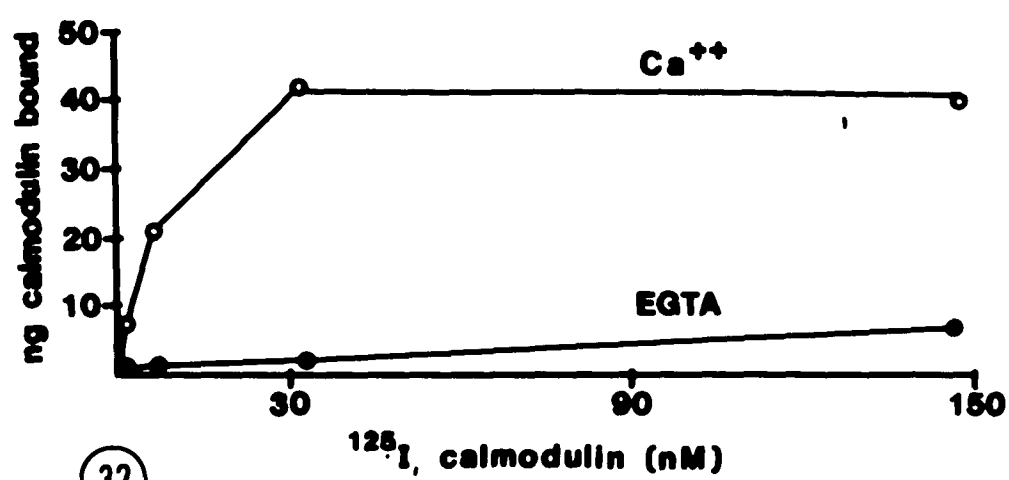
B. In vitro binding of calmodulin to dogfish erythrocyte cytoskeletons

In order to determine whether the dogfish erythrocyte cytoskeleton contained calmodulin receptors, anucleate dogfish erythrocyte cytoskeletons were incubated with increasing amounts of ¹²⁵I calmodulin in the presence of either calcium or EGTA. Anucleate dogfish erythrocyte cytoskeletons bound labelled calmodulin in a calcium-dependent manner (Figure 32). The binding was calmodulin concentration-dependent, with saturation achieved above 30 nM.

C. ¹²⁵I calmodulin binding to proteins in anucleate erythrocyte ghosts and cytoskeletons.

Calmodulin-binding proteins present in anucleate dogfish erythrocyte ghosts and cytoskeletons were detected using a recently developed procedure in which proteins separated by polyacrylamide gel electrophoresis in SDS were incubated with ¹²⁵I calmodulin after removal of the detergent and renaturation in the gel (Carlin et al., 1980a). To determine whether the binding was calcium-dependent, duplicate gels were incubated in 1mM CaCl₂ and 1mM EGTA. The autoradiograms in figures 33b and c and 35b and c correspond to the gels in figures 33a and 35a respectively which had been incubated with ¹²⁵I calmodulin prior to coomassie blue

Figure 32. Binding of ^{125}I -calmodulin in vitro to whole anucleate dogfish erythrocyte cytoskeletons. Cytoskeletons derived from anucleate ghosts containing 400ug protein were incubated for 30 min at 25°C . with biologically-active ^{125}I calmodulin as described in Materials and Methods. Incubations were performed either in the presence of 1mM CaCl_2 (o-o) or 1mM EGTA (●-●).



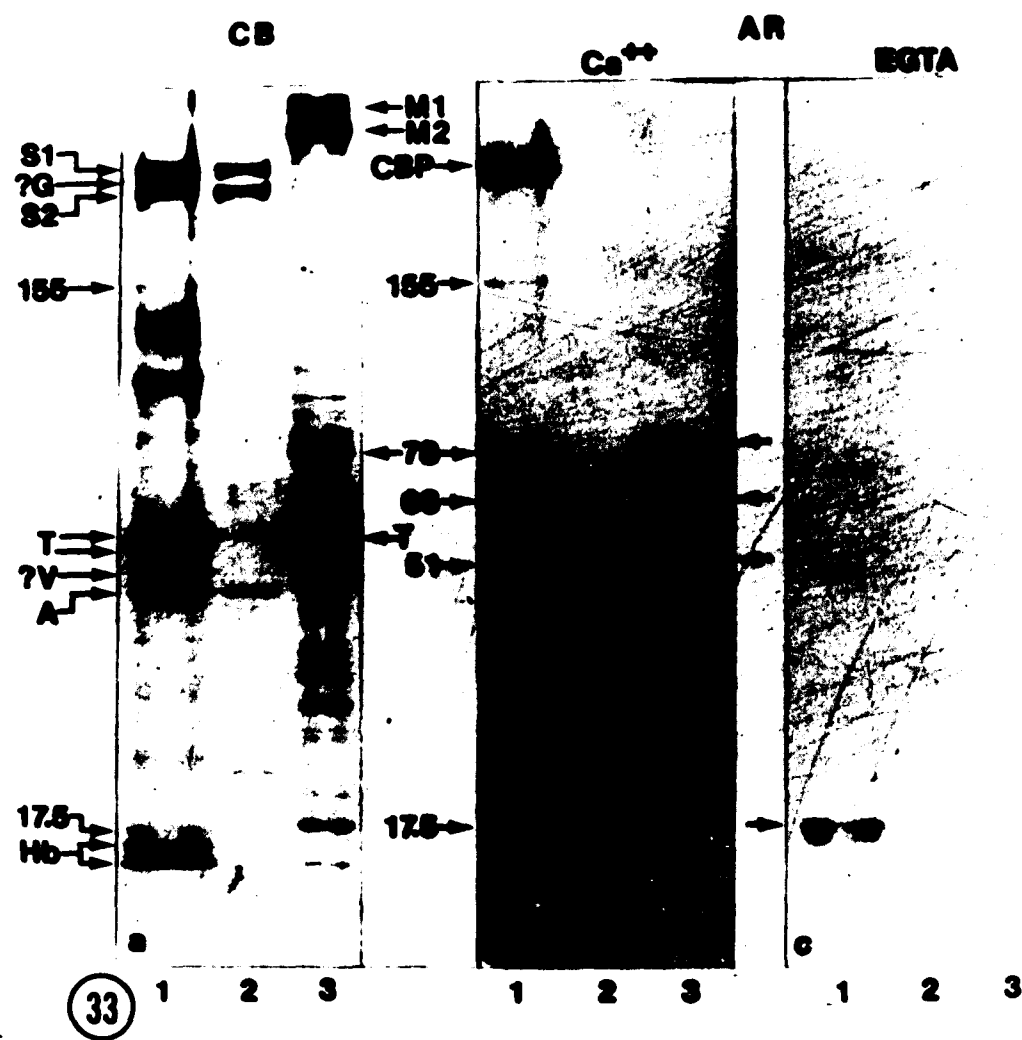
(32)

staining. Human erythrocyte ghost proteins (figure 35, lane 1) served as standards for protein identification, and canine cortical PSD proteins (figure 35, lane 3) were used as an internal standard for calmodulin binding (Carlin et al , 1980b).

Anucleate ghosts (figure 33, lane 1) and cytoskeletons (figure 35, lane 2) of dogfish erythrocytes contain one major calcium-dependent calmodulin-binding protein (CBP). Binding of ¹²⁵I calmodulin to CBP was inhibited in the presence of an excess of unlabelled calmodulin (data not shown). CBP comigrated with the upper band (S1) of human and dogfish spectrin and had an apparent molecular weight of 245,000. Neither band of human spectrin bound calmodulin (figure 33b, lane 2; figure 35b, lane 1). CBP also comigrated with a calmodulin-binding protein present in canine cerebral cortical postsynaptic densities (Figure 35b, lane 3). The calmodulin-binding pattern for the PSD sample was consistent with results previously obtained using this technique (Carlin et al ., 1981).

Since MAP 2 had been reported to bind calmodulin (Burgess et al ., 1980) and to be present in marginal bands of amphibian erythrocytes (Sloboda and Dickerson, 1980), calmodulin binding by dogfish anucleate ghost proteins was compared with that of bovine brain microtubule proteins containing MAPS 1 and 2 (Figure 33, lanes 1 vs. 3). Unlike CBP, neither MAP 1 nor MAP 2 bound calmodulin using this method of detection. In addition, purified bovine brain MAP 2 did not bind calmodulin using this assay (data not shown). Calmodulin did bind in a calcium-dependent manner to

Figure 33. Calmodulin binding proteins associated with anucleate dogfish erythrocyte ghosts and microtubule proteins. (a) Coomassie blue staining pattern (CB) of proteins separated by electrophoresis in a 5-15% polyacrylamide gel in SDS. (b) and (c) Autoradiograms (AR) resulting from incubation of gels in ^{125}I calmodulin in the presence of 1mM calcium (b) or 1mM EGTA (c). Lane 1, anucleate dogfish erythrocyte ghosts; lane 2, human erythrocyte spectrin and actin standards; lane 3, bovine brain microtubule proteins. M1, MAP1; M2, MAP2; S1, S2, spectrin; G, goblin; T, tubulin; V, vimentin; A, actin; Hb, hemoglobin. Numbers indicate molecular weights $\times 10^{-3}$ of calmodulin-binding proteins and arrows show their location on the gel. Dogfish erythrocyte ghosts contain one major calcium-dependent calmodulin-binding protein (CBP) which comigrates with dogfish S1. In addition to CBP, two minor calmodulin binding proteins were detected. Calmodulin binding is not observed for MAPs 1 and 2 or for human spectrin.



three minor proteins present in the microtubule preparation (Figure 33b, lane 3). These proteins had apparent molecular weights of 78,000, 66,000, and 51,000.

Two minor calmodulin-binding proteins were also detected when the loading of dogfish erythrocyte ghost or cytoskeletal protein was heavier than that shown in Figure 35. As can be seen for ghosts in figure 33, one of these had an apparent molecular weight of 155,000, and bound calmodulin in a calcium-dependent manner. This protein is thought to be a proteolytic fragment of CBP since it increased in amount relative to CBP, as judged by scanning of autoradiograms, in successive experiments with repetitive freezing and thawing (data not shown). Binding of calmodulin to the 155 K protein was minor compared to that of CBP due to the low amounts of the 155,000 molecular weight protein, and could only be observed after relatively long exposures of autoradiograms. The 155,000 molecular weight band also comigrated with a calmodulin-binding protein present in canine PSDs. This band was observed in longer exposures of the autoradiogram in figure 35b,(data not shown). The second minor calmodulin-binding protein (figure 33, lane 1) had a molecular weight of 17,500 and bound calmodulin both in the presence and absence of calcium. This protein was not present in all preparations.

D. Location of CBP in the anucleate dogfish erythrocyte cytoskeleton

In order to determine whether CBP was associated with

Figure 34. Localization of CBP in the dogfish erythrocyte ghosts. (a) Coomassie blue staining pattern (CB) of proteins separated in a 5-15% gradient gel by SDS-PAGE. (b) Autoradiogram (AR) resulting from incubation of gels in ^{125}I calmodulin in the presence of 1mM calcium. Lane 1: anucleate ghosts devoid of marginal bands and tubulin; lane 2: marginal bands isolated from anucleate ghosts. S1, S2, spectrin; G, goblin; T, tubulin; A, actin. CBP is present in anucleate ghosts lacking marginal bands but is absent from isolated marginal bands.

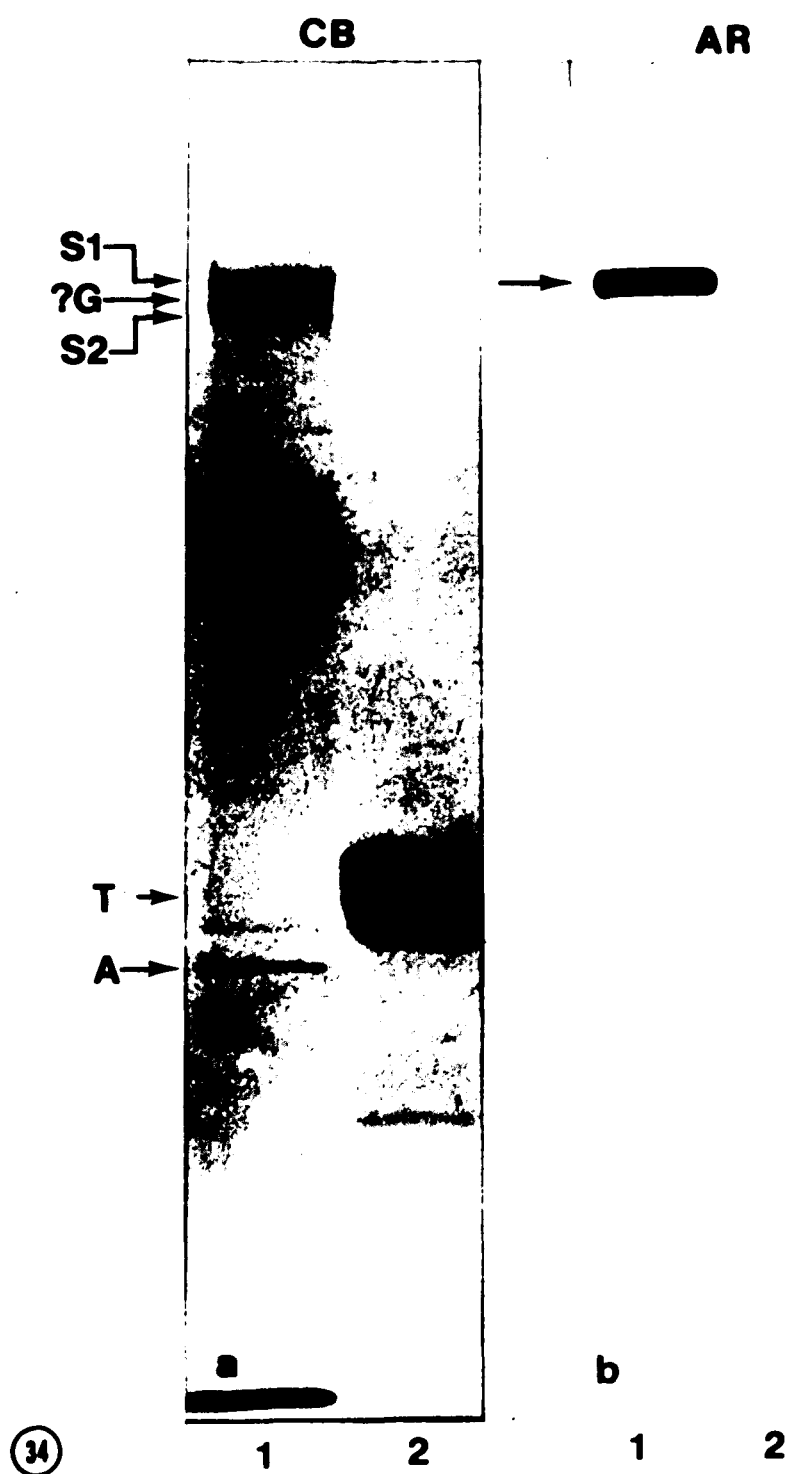


Figure 35. Calmodulin-binding proteins in mammalian cytoskeletal structures - comparison with dogfish erythrocyte CBP. (a) coomassie blue staining pattern (CB) of cytoskeletal proteins separated in a 5-15% polyacrylamide gel in SDS. (b) and (c) autoradiograms (AR) resulting from incubation of gels with ^{125}I calmodulin in the presence of 1 mM calcium (b) or 1 mM EGTA (c). Lane 1, human erythrocyte ghosts; lane 2, anucleate dogfish erythrocyte cytoskeletons; lane 3, canine cortical postsynaptic densities. S1 and S2, spectrin; G, goblin; T, tubulin; A, actin; Hb, hemoglobin. Numbers indicate molecular weight $\times 10^{-3}$ of protein standards. Dogfish CBP bound calmodulin in a calcium dependent manner and comigrated with dogfish and human S1 and with a calmodulin binding protein present in postsynaptic densities. Human spectrin did not bind calmodulin.



the marginal band or was a component of the SAC, anucleate ghosts, prepared from cells incubated at 0 °C and devoid of marginal bands (figure 14) and greatly depleted of tubulin (figure 24a, lane 1), were tested for the presence of CBP (figure 34b, lane 1). The amount of CBP present in these ghosts was comparable to that in anucleate ghosts or cytoskeletons containing marginal bands (figure 33b, lane 1; figure 35, lane 2), indicating that CBP was a component of the SAC. Localization of CBP to the SAC was further supported by the absence of calmodulin-binding to proteins in marginal band preparations isolated by treatment of anucleate ghosts with elastase (Figure 24b, lane 2). Tubulin was the primary component of such preparations. Calcium extracts of nucleated cytoskeletons, having tubulin as their major protein components, contained trace amounts of CBP; however the ratio of tubulin to CBP in these extracts compared to that of anucleate ghost preparations, indicated that CBP was not an integral MB component.

E. Presence of CBP in erythrocytes of another nonmammalian species

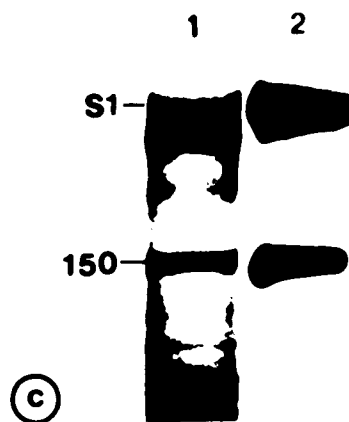
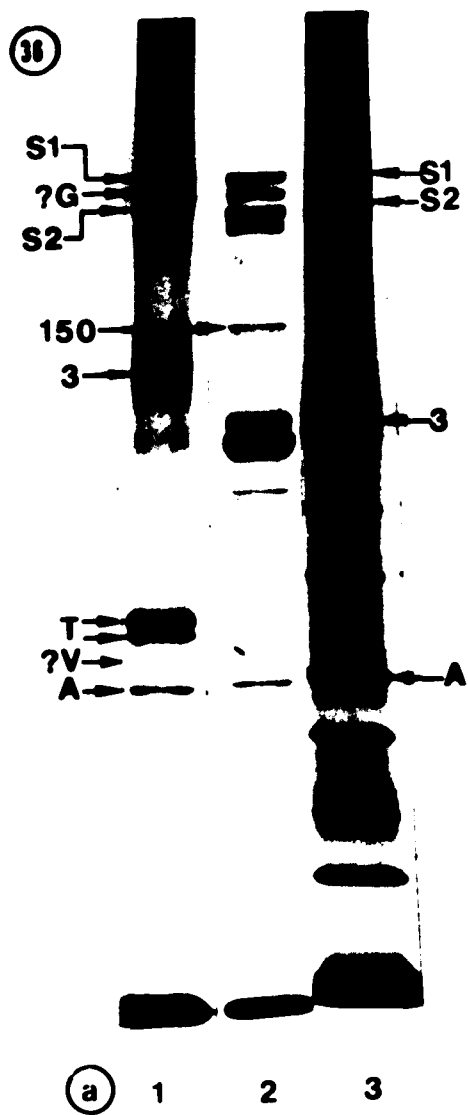
Anucleate ghosts were prepared from chicken erythrocytes as described in "Methods". Ghosts were flat and essentially

elliptical when observed in phase contrast (figure 36a). They contained no MBs, probably as a result of storage overnight at 0° C., and resembled ghosts prepared from dogfish erythrocytes after incubation at 0° C. (figure 14a). As expected, these chicken erythrocyte ghosts contained essentially no tubulin when analyzed by polyacrylamide gel electrophoresis in SDS, (figure 36 lane 2). The protein pattern in the spectrin region was somewhat more complex than either anucleate dogfish (figure 36, lane 1) or human (figure 36, lane 3) erythrocyte ghosts. However the upper bands of spectrin (S1) of all three species comigrated. Chicken erythrocyte ghost proteins were assayed for their ability to bind ¹²⁵I calmodulin using the gel binding technique. Two protein bands bound calmodulin (figure 36c); one which comigrated with S1 in the position of CBP, and the other with an apparent molecular weight of 150,000. Binding of calmodulin to both of these proteins was calcium-dependent (data not shown).

F. Photoaffinity labeling of calmodulin-binding proteins in erythrocyte ghosts

As an independent test of the validity of the above results for calmodulin-binding to ghost (Figure 33, lane 1) and cytoskeletal proteins, (Figure 35, lane 2), and as a means of determining whether CBP would bind calmodulin in situ, anucleate dogfish, chicken and human erythrocyte ghosts were reacted with azido ¹²⁵I calmodulin (Andreasen et al., 1981). The proteins to which calmodulin was covalently-linked were detected after

Figure 36. Calmodulin-binding proteins of anucleate chicken erythrocyte ghosts. (a) Coomassie blue staining pattern of proteins separated by electrophoresis in a 5-15% gradient polyacrylamide gel in SDS. Lane 1, anucleate dogfish erythrocyte ghosts; lane 2, anucleate chicken erythrocyte ghosts; lane 3, human erythrocyte ghosts. S1, S2, spectrin; G, goblin; 150, 150,000 Mr calmodulin-binding protein; 3, dogfish and human erythrocyte band 3; T, tubulin; V, vimentin; A, actin. Low amount of tubulin in chicken ghosts may be due to storage of cells at 0°C. (b) Anucleate chicken erythrocyte ghosts in phase contrast x 2400. (c) ^{125}I calmodulin-binding to chicken erythrocyte ghost proteins separated by electrophoresis in 5-12% gradient polyacrylamide gel in SDS. Lane 1, Coomassie blue staining pattern; lane 2, autoradiogram resulting from incubation of the gel with ^{125}I calmodulin. Both chicken S1 and 150,000 molecular weight protein bind calmodulin.



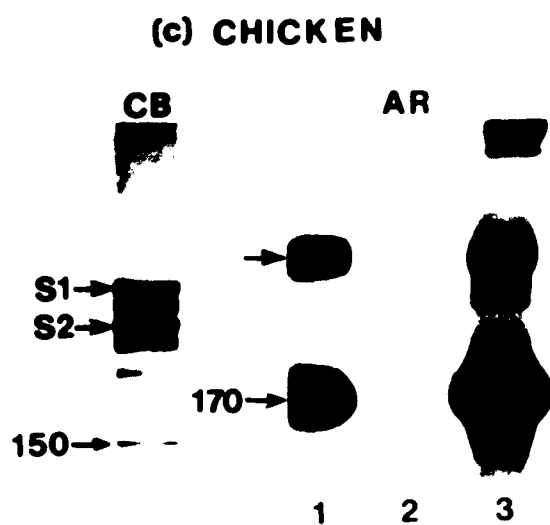
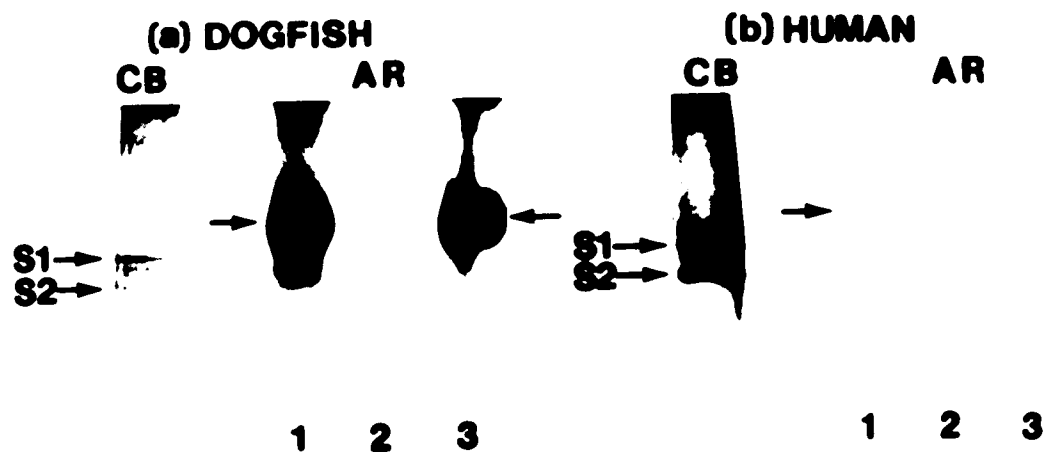
separation by electrophoresis in polyacrylamide gels by autoradiography of dried gels (figure 37). Calcium-dependent binding of calmodulin to CBP in both ghosts and cytoskeletons of dogfish (figure 37a) and chicken (figure 37c) erythrocytes was indicated by the appearance of a band of reactivity of molecular weight higher than S1, corresponding to a CBP-calmodulin complex (Figure 37a and c, lanes 1 and 3). The binding of calmodulin to CBP in the absence of Triton is presumably due to permeabilization of the ghosts by freezing and thawing prior to their use. No binding was observed for spectrin in human ghosts (Figure 37b), consistent with results obtained by ^{125}I calmodulin binding to proteins separated by SDS-PAGE (Figure 33, lane 2, and Figure 35, lane 1).

V. Identification of CBP

A. Extraction of CBP from anucleate dogfish erythrocyte ghosts

To test whether CBP was extracted from anucleate ghosts by incubation in 0.2 mM EDTA or 5 mM pHMB, the extracts shown in figure 25, lanes d and f) were combined and component proteins were separated by polyacrylamide gel electrophoresis in SDS. The gel containing the separated proteins was incubated with ^{125}I calmodulin in the presence of calcium. Autoradiography revealed a single calmodulin-binding protein which comigrated with dogfish S1 in the position of CBP, demonstrating that CBP was extracted with spectrin from anucleate ghosts (figure 25, lane g).

Figure 37. Cross-linking of azido-¹²⁵I calmodulin to proteins in dogfish (a), human (b) and chicken (c) erythrocyte ghosts. Freeze-thawed membrane preparations were photolyzed in the presence of 0.6mM CaCl₂ (lane 1); 0.1mM EGTA (lane 2) and 0.6mM CaCl₂ + 0.15% Triton (lane 3). CB, Coomassie blue staining pattern; AR, autoradiogram; S1, S2, spectrin; 150, 150,000 Mr calmodulin-binding protein. Arrows indicate the position of CBP-calmodulin complex. 170 indicates position of calmodulin 150,000 Mr protein complex. CBP binds calmodulin in whole anucleate dogfish and chicken erythrocyte ghosts.



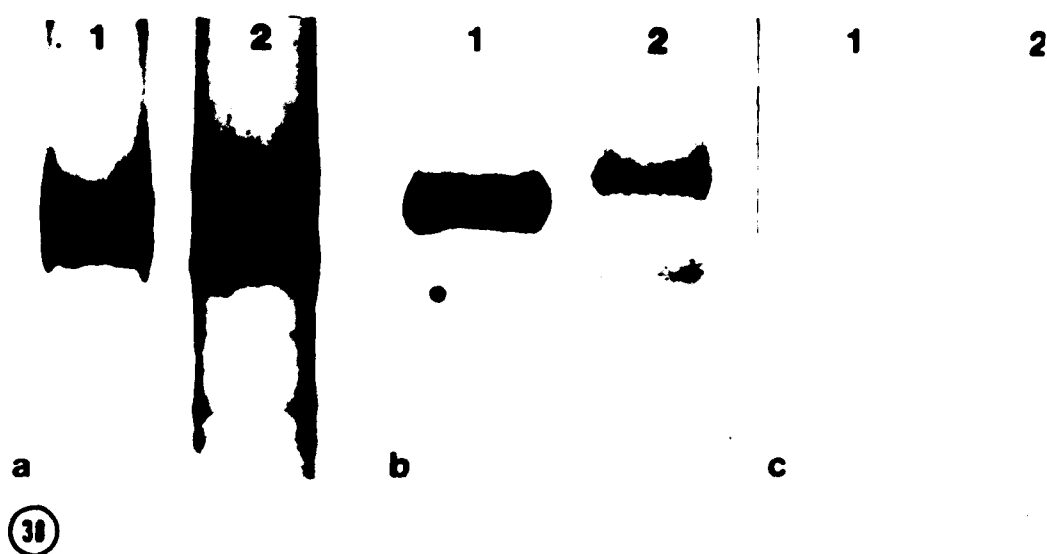
B. Immunoreactivity of dogfish erythrocyte CBP with antibody to mammalian CBP-I

Dogfish erythrocyte CBP has been shown to comigrate with CBP-I present in canine cortical PSDs (figure 35; Carlin et al., 1981), which in turn comigrates with the upper band of bovine brain CBP-I (Davies and Klee, 1981). The latter protein binds actin as well as calmodulin. To test the relationship between the dogfish CBP and bovine brain CBP-I, human spectrin and dogfish spectrin and goblin were separated by polyacrylamide gel electrophoresis in SDS, transferred to nitrocellulose paper, and incubated with rabbit antiserum raised against bovine brain CBP-I and with control pre-immune serum. Antibody binding was then detected using ^{125}I protein A followed by autoradiography as described in "Methods". As shown in figure 38, dogfish erythrocyte CBP bound anti-bovine brain CBP-I (figure 38b, lane 1). In addition, both bands of human spectrin (figure 38b, lane 2) reacted weakly with anti-CBP-I. Neither goblin nor the lower band of dogfish spectrin, S2, bound the antibody. No bands were detected for proteins incubated with pre-immune serum (figure 38c).

C. Identification of dogfish CBP (S1) as alpha-fodrin

CBP-I from both bovine and canine brain is the higher molecular weight band ($M = 245,000$) of a protein doublet (Carlin et al., 1981; Davies and Klee, 1981). A similar doublet has recently been characterized in guinea pig brain by Levine and Willard

Figure 38. Binding of anti-bovine brain CBP-I to dogfish (lane 1) and human (lane 2) spectrin. (a) Coomassie blue-stained gel; (b) autoradiogram resulting from ^{125}I protein A binding to nitrocellulose transfer after incubation with anti-CBP-I serum; and (c) as in b except that incubation was with preimmune serum. Dogfish CBP (S1) reacts strongly with anti-CBP-I while both bands of human spectrin bind the antibody moderately well.

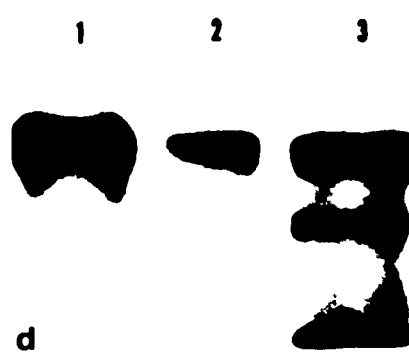
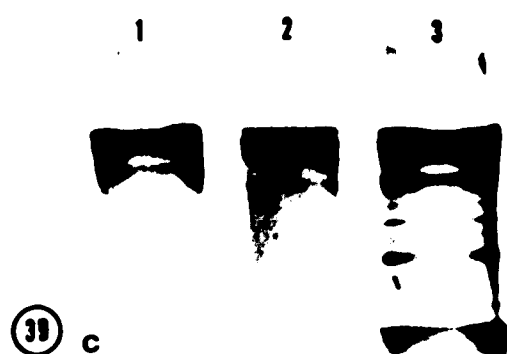
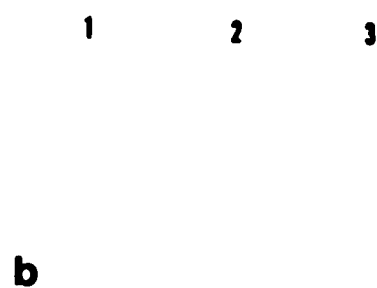
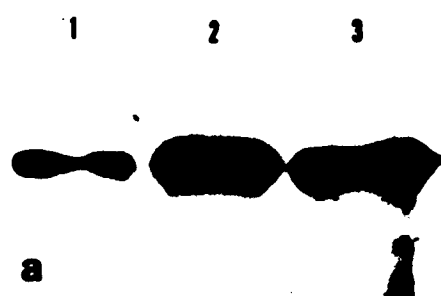


(1981), who named it fodrin. To determine whether dogfish CBP and CBP-I of canine cortical PSDs are related to fodrin, erythrocyte proteins extracted from anucleate ghosts at low ionic strength and PSD proteins were compared with partially purified guinea pig brain fodrin by testing for electrophoretic comigration, calmodulin-binding, and reaction with anti-fodrin. The protein patterns of the samples in the high molecular weight region are quite similar (figure 39c). The autoradiogram (figure 39d) of the same gel after ^{125}I calmodulin binding demonstrates that fodrin binds calmodulin and that the calmodulin-binding proteins present in the two samples comigrated. Calmodulin binding to dogfish erythrocyte CBP was comparable to that obtained for guinea pig fodrin and the PSD protein, CBP-I. (Carlin, et al

1982). Although it was not possible to determine from this experiment whether one or both bands of fodrin bound calmodulin, subsequent higher resolution gels demonstrated that only the upper band of fodrin, fodrin, binds calmodulin (Carlin et al, 1982).

The results obtained when proteins in these three samples were incubated with anti-fodrin antibody are shown in figure 39a. Incubation with ^{125}I protein A and autoradiography revealed that dogfish CBP (S1) reacts strongly with anti-fodrin antibody. Neither dogfish goblin nor S2 showed any reactivity. Of the proteins present in the sample of guinea pig fodrin, only fodrin bound antibody despite the fact that the anti-fodrin antibody was raised against both bands of fodrin. No reactivity was detected for preimmune serum (figure 39b). These results indicate that

Figure 39. Comparison of dogfish erythrocyte proteins (lane 1) with authentic guinea pig brain fodrin (lane 2) and canine cortical postsynaptic density proteins (lane - 3). (a) Autoradiogram of ^{125}I protein A binding to nitrocellulose transfer after incubation with anti-fodrin; (b) same as in a except that incubation was with preimmune serum; (c) Coomassie blue-stained gel (5-15% polyacrylamide gel in SDS); (d) autoradiogram resulting from ^{125}I calmodulin binding to proteins in the gel. Dogfish CBP and a calmodulin binding protein present in postsynaptic densities comigrating with CBP reacts strongly with anti-fodrin antibody. Fodrin binds calmodulin.



dogfish CBP (S1) and fodrin are immunologically closely related.

D. Immunoreactivity of anti-fodrin with human and chicken erythrocyte ghost proteins

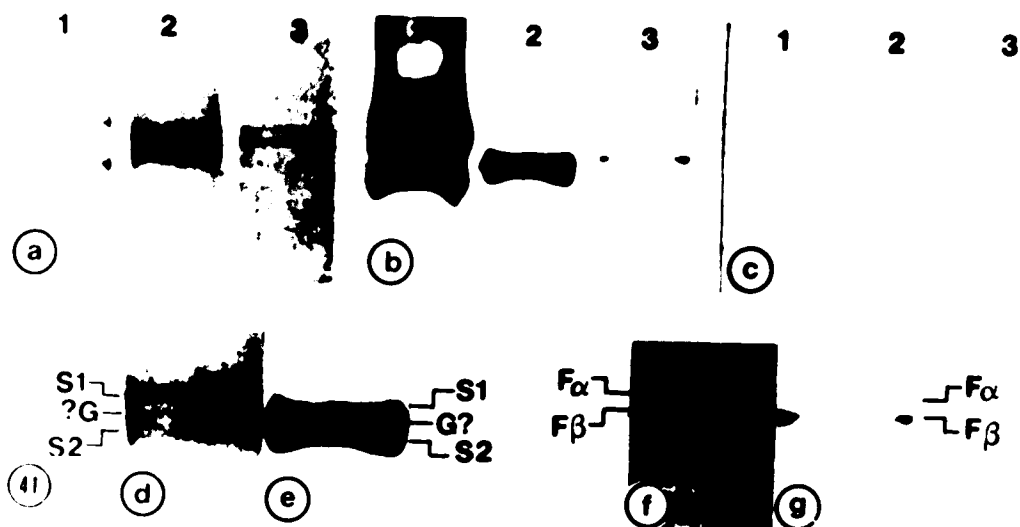
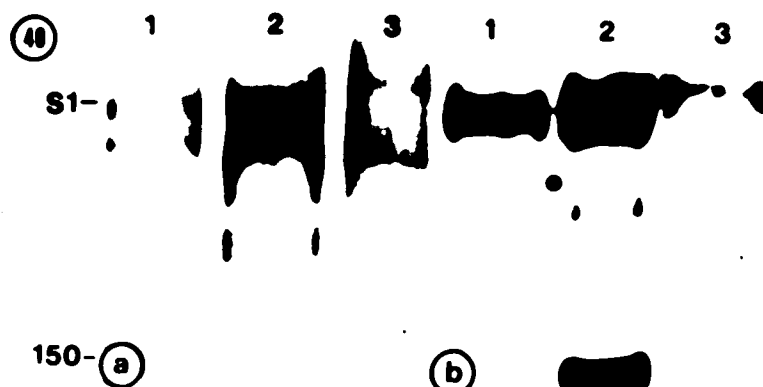
Fodrin has been localized to the SAC of non-erythroid cells in mammalian, amphibian and avian origin (Levine and Willard, 1981). To determine the extent to which fodrin is also present in erythrocytes of other species, high molecular weight human and chicken erythrocyte ghost proteins were assayed for binding of anti-fodrin (figure 40). Chicken erythrocyte ghosts contained two heavy bands of reactivity (figure 40b), one corresponding to chicken S1 and the other having an apparent molecular weight of 150,000. As noted above, a 150,000 molecular weight protein present in anucleate chicken erythrocyte ghosts bound calmodulin (figure 36c). Antibody directed against fodrin reacted weakly with human S1 but not with human S2.

E. Cross-reactivity of Fab' fragments of anti-human spectrin antibody with dogfish erythrocyte spectrin and goblin, and canine PSD fodrin

To test for structural homology between dogfish and human spectrins and CBP-I of canine cortical PSDs, ^{125}I Fab' fragments of anti-human spectrin antibody and preimmune serum were incubated with dogfish spectrin and goblin, human spectrin, and high molecular weight canine brain PSD proteins which had been transferred from a polyacrylamide gel to nitrocellulose paper. The coomassie blue staining pattern and resulting autoradiograms are shown in figure 41. Dogfish S1 did not bind anti-spectrin

Figure 40. Antifodrin antibody binding to dogfish (lane 1), chicken (lane 2) and human (lane 3) erythrocyte cytoskeletal proteins. a) coomassie blue staining pattern of proteins separated by electrophoresis in a 5% polyacrylamide gel in SDS; b) autoradiogram of nitrocellulose transfer from the gel after incubation with anti-fodrin serum and ^{125}I protein A. S1, upper band of spectrin. Anti-fodrin antibody cross-reacts with dogfish and chicken S1 (CBP) and with the 150,000 molecular weight calmodulin-binding protein (150) present in chicken erythrocyte ghosts.

Figure 41. Anti-human spectrin F ab' fragment binding to human (lane 1), and dogfish (lane 2) spectrin, and canine cortical postsynaptic density fodrin (track 3). a) coomassie blue-stained gel; b) autoradiogram of nitrocellulose transfer after incubation with anti-spectrin ^{125}I Fab' fragments; c) as in b except incubation was with ^{125}I Fab' fragments prepared from preimmune serum; d) and e) enlargements of lanes a2 and b2 respectively showing reactivity of dogfish G and S2; f) and g) enlargements of a3 and b3 showing reactivity of PSD beta fodrin. F_{α} , upper band of fodrin; F_{β} , lower band of fodrin; S1 and S2, spectrin; G, goblin.



Fab' fragments, but dogfish S2 and goblin bound them moderately well (figure 41b, lane 2, 2e) in comparison with the strong binding to both bands of human spectrin (figure 41b, lane 1). The lower band of the high molecular weight doublet of canine cortical PSDs of which CBP-I is the upper band gave a faint but definite reaction with anti-spectrin Fab' fragments (figure 41b, lane 3 and figure 41g). No binding was detected for proteins incubated with preimmune ^{125}I Fab' fragments (figure 41c).

The reactivity of dogfish, chicken and human spectrins and canine brain PSD fodrin toward anti-CBP-1, anti-fodrin, and anti-human spectrin is summarized in Table III. The relative reactivities of the antibodies toward these proteins is indicated qualitatively by one plus (+) for a weak reaction, two plus (++) for a moderate reaction, and three plus (+++) for a strong reaction.

TABLE III

Cross - Reactivity of Cytoskeletal Proteins

Antigen		Antibody			
Species	Protein	CBP	Anti-Fodrin	Anti-CBP-I	Anti-Spectrin
Dogfish	S1	+++	+++	+++	-
	G	-	-	-	+
	S2	-	-	-	+
Chicken	S1	+++	+++	N.D.	N.D.
	S2	-	-	N.D.	N.D.
	150	+++	-	N.D.	N.D.
Human	S1	-	+	++	+++
	S2	-	-	++	+++
Canine PSD	F1	+++	+++	+++	-
	F2	-	-	-	+

N.D. = not determined

DISCUSSION

1. The dogfish erythrocyte cytoskeletal system

A. Major structural components

1. Cell surface associated cytoskeleton (SAC)

The major components of the dogfish erythrocyte cytoskeletal system are the marginal band (MB) and the cell surface associated cytoskeleton (SAC). The latter was initially described in Triton-lysed amphibian and goldfish erythrocytes (Cohen, 1978). It is nearly transparent in phase contrast, but in edge view appears to correspond to the cell surface. In thin sections of whole cells the SAC is invisible due to the electron density of hemoglobin, but in lysed cells it is seen as a thin surface-delimiting layer which is external to the MB and nucleus. MB cross-sections are always found in tapered regions of the SAC, giving the impression that the MB maintains pressure against the SAC at these points of contact, flattening the cytoskeleton in the plane of the MB. In lysed cell and anucleate cytoskeletal whole mounts observed by electron microscopy, this surface layer collapses onto itself from opposite sides and appears as a rough planar network traversing the space between MB and nucleus. In appearance, it is not unlike the cytoskeleton of mammalian erythrocytes as seen by scanning electron microscopy (Hainfield and Steck, 1977) or in thin section by transmission electron

microscopy (TEM; Tsukita et al., 1980). Therefore, in terms of cellular location and morphology, the SAC is quite likely the nonmammalian counterpart of the "membrane cytoskeleton" which underlies the mammalian erythrocyte membrane bilayer.

2. Marginal Band (MB) - Isolation and properties

The problem of MB isolation is defined by the morphology of the cytoskeletal system. Since the MB is enclosed within the SAC, the latter must be removed selectively. As a first approach to mass isolation, SAC proteolysis has been satisfactory. Whole MBs are released, and many survive as such during centrifugation. The MB microtubules are presumably stabilized into ribbon-like arrays by the cross-bridges. Ribbon-like construction has also been observed in amphibian MBs (Bertolini and Monaco, 1976; Cohen, 1978). Cross bridges have been noted previously in platelet and amoebocyte MBs (Behnke, 1967; Nemhauser et al, 1980), but nothing is known of their three-dimensional distribution or composition. MBs tend to circularize following digestion of the SAC. It is not clear whether this is due to a release of tension on an elastic MB by the SAC as suggested by Cohen (1978), or due to proteolysis of an MB component responsible for maintenance of ellipticity.

In living cells, the dogfish erythrocyte MB undergoes temperature-induced disassembly/reassembly, with colchicine inhibiting reassembly after cooling but not inducing disassembly prior to cooling. The former property is similar to mitotic spindles (Inoue and Sato, 1967), while the latter resembles

flagella (Rosenbaum *et al.*, 1969). Low temperature presumably alters the state of tubulin molecules throughout MB microtubules, whereas colchicine-induced disassembly would be a function of the number of microtubule free ends, of which there may be few in the MB.

MBs of avian erythrocytes and mammalian platelets have been reported previously to be cold-labile (Barrett and Dawson, 1974; Behnke, 1967, 1970), while those of amphibian erythrocyte are cold-stable (Behnke, 1970). The low-temperature lability of the dogfish erythrocyte MB refutes a possible correlation of MB lability with homeothermy and stability with poikilothermy. Though cold-labile *in vivo*, the dogfish erythrocyte MBs were relatively stable to washes and storage at low temperature after isolation. *In vitro* cold stability could be reversed by inclusion of physiological levels of salt in the incubation medium. The factors involved in MB microtubule assembly/disassembly are unknown and merit further study.

3. Lack of evidence of transcellular structures

No intermediate filaments or other transcellular structures were observed in either nucleated or anucleate dogfish erythrocyte cytoskeletons. Virtanen *et al.* (1979) observed intermediate filaments associated with nuclei of chicken erythrocytes and proposed that these filaments formed a "nucleus-anchoring" network. Granger *et al.* (1982) observed intermediate filaments associated with chicken erythrocyte plasma membranes with similar fixation and staining procedures used here for dogfish anucleate

ghosts. Of interest is the fact that it was not possible to enucleate chicken erythrocyte ghosts under conditions used to prepare anucleate dogfish erythrocyte ghosts, suggesting a correlation between the observation of intermediate filaments and the stability of the nucleus within the cytoskeleton. Although the possibility exists that there are intermediate filaments present in dogfish erythrocytes, their presence may not be necessary to maintain the nucleus in its central position in the cell. Cohen (1978) postulated that pressure of the SAC against the nucleus could be sufficient to account for the positional stability of the nucleus. The appearance of a nuclear bulge seen in cells viewed on edge is consistent with the SAC being in contact with and following the contour of the nucleus.

B. Function of erythrocyte cytoskeletal components

1. The role of MBs in maintenance of morphology

Nearly all studies of erythrocyte MBs support a morphogenetic role but discount a contribution of the MB to the maintenance of cell morphology. Barrett and Dawson (1974) have shown that chicken erythroblasts, upon cold-induced MB disassembly form spheres while mature erythrocytes maintain their elliptical flattened morphology. On the basis of similar experiments, Behnke (1970) proposed that something other than the MB was responsible for cell shape maintenance in at least some species. Like chicken erythrocytes, dogfish erythrocytes retain approximately normal morphology at low temperature in the absence of MBs.

However, these data do not preclude a contribution of MBs to maintenance of erythrocyte morphology in vivo. Closer examination of the basis upon which these conclusions were made reveals that cell morphology in the absence of MBs was only observed under static conditions. Erythrocytes are designed for conditions of blood flow, with appropriately adapted mechanical and rheological properties. When MBs are experimentally disassembled in dogfish or chicken erythrocytes, retention of normal cell morphology is meaningful only for the static conditions of microscopic examination. It says nothing about morphological or mechanical properties during flow or osmotic stress in vivo.

MBs are present in most (possibly all) of the circulating erythrocytes of nonmammalian vertebrates and data presented here indicate that under certain experimental conditions, MBs do contribute to cytoskeletal stability. Results obtained for "spindle cells" and calcium-ionophore treated erythrocytes suggest that nucleated erythrocyte shape corresponds to that of the SAC at all times and that the SAC assumes the shape of the MB. "Spindle cells" similar to those observed here also occur in avian blood (Lucas and Jamroz, 1961), and may be the erythrocyte equivalent of spindle-shaped platelets produced by cooling and rewarming (Behnke, 1967; Zucker and Borrelli, 1954).

The stabilizing influence of the MB on cytoskeletal morphology was readily apparent upon observation of MB-containing and MB-deficient nucleated and anucleate cytoskeletons. With the

exception of simultaneously lysed fixed nucleated cytoskeletons, the SAC of MB-deficient cytoskeletons either partially or completely collapsed upon itself. Cytoskeletons containing MBs retained their flattened elliptical morphology. One interpretation of this data is that the MB acts as a frame or support upon which SAC shape is generated during cellular morphogenesis and by which SAC shape is maintained when stabilizing membrane components are experimentally removed by detergent extraction.

2. Contribution of the SAC to cell morphology

Extraction of SAC proteins from dogfish erythrocyte anucleate ghosts caused the plasma membrane to vesiculate, indicating the importance of the SAC to plasma membrane integrity and therefore erythrocyte morphology. Studies of mammalian erythrocytes similarly demonstrate that the presence of spectrin, the major protein component of the SAC is essential to the integrity of the erythrocyte membrane (Marchesi and Steers, 1968; Ralston, 1975). Some forms of severe spherocytosis in man have been shown to be caused by spectrin deficiency (Agre, 1982), and the presence of spectrin variants have been implicated in elliptocytosis (Tomaselli, 1981), indicating a dependence of cell morphology upon the molecular components of the SAC.

The occurrence of "spindle cells" in dogfish erythrocytes and the changes in erythrocyte shape during treatment with calcium and ionophore indicate that the SAC is deformable. This is consistent with the flexibility of toadfish erythrocytes deformed by

constriction in the capillary lumen. The plasma membrane forms invaginations as the surface to volume ratio of the erythrocyte is decreased during deformation (Fawcett and Witebsky, 1964). As noted above, the SAC is not sufficiently stable to retain its shape following detergent extraction. However cells and anucleate ghosts devoid of MBs retain their flattened elliptical morphology. A frozen lipid bilayer would not account for shape maintenance at 0°C., since shape is also maintained in living

cells which lack MBs after rewarming in colchicine. This suggests that a non-SAC membrane component contributes to the stability of cell morphology. An hypothesis for the maintenance of erythrocyte morphology in the absence of the MB is that neither the lipid bilayer nor the SAC is sufficient to maintain stable cell surface morphology. Both exist in an equilibrium configuration, the SAC lending structural support to the bilayer and the bilayer and integral membrane proteins acting to stabilize the SAC. An extension of this hypothesis would include the MB in shape maintenance in vivo, suggesting that none of the three components mentioned is sufficient for maintenance of morphology. The maintenance of functional erythrocyte morphology would depend upon SAC, MB and integral plasma membrane components.

II. Membrane and cytoskeletal protein components

A. MB components

The four tubulin-region proteins found in isolated MBs and

other cytoskeletal preparations were detectable with underloading of 5-12% gradient gels (figure 23). Although the identification of all four as tubulins must be regarded as tentative, their number is not surprising, as multiple alpha- and beta-tubulins have been found in other systems. Their assignment as MB components is supported by their enhancement in lysates of cooled cells. With increasing amounts of protein applied, resolution was lost and dogfish MB tubulin, like brain tubulin migrated as one broad band. Proteins migrating with or near MAP-1 and MAP-2 standards were not observed even on gels overloaded with samples from apparently intact isolated MBs suggesting that the cross-bridges seen in these MBs are not composed of high molecular weight MAPs. Tubulin preparations from whole mammalian platelets contain several high molecular weight proteins as minor components (Castle and Crawford, 1977), but it is not known whether these are actually present in platelet MBs. A "MAP-2" has been reported in amphibian erythrocyte MBs on the basis of antibody binding (Sloboda and Dickersin, 1980). However Langford (1980) has shown that dogfish brain tubulin preparations contain little or no accessory proteins after purification through two cycles of temperature-dependent assembly/disassembly, indicating that, unlike mammalian microtubule systems, MAPs, if present, do not copurify with microtubules using this procedure. However proteolysis cannot be eliminated as an explanation for the absence of MAPs. The possible presence of potentially protease-sensitive minor components of isolated MBs, cannot be

confirmed or eliminated until other conditions are discovered for selective dissolution of the SAC.

Three calmodulin binding proteins, with molecular weights between 51,000 and 78,000, were detected in a sample of twice cycled bovine brain microtubule proteins. Tau proteins, with molecular weights ranging from 55,000 to 62,000, have been reported to bind calmodulin in a calcium dependent manner (Sobue et al, 1981). However no proteins in this molecular weight range other than tubulin and no calmodulin-binding proteins were detected in samples of dogfish MBs.

The identity of the material forming the cross-bridges seen in dogfish MBs remains unknown. Other than a minor component, all of the protein present in intact isolated MBs was in the molecular weight range of tubulin. The possibility exists that the cross-bridge material has a molecular weight similar to tubulin and therefore is not observed even when large amounts of protein are electrophoresed. Intermediate filaments containing proteins of molecular weight similar to tubulin have been observed in close association with flagellar microtubules (Linck et al. 1981) and brain microtubules (Runge et al, 1979).

B. Components of the erythrocyte SAC

1. "Spectrin" - Identification of nonmammalian S1 as fodrin,

The pH 3 extracts of dogfish erythrocyte nucleated cytoskeletons and whole anucleate cytoskeletons contained proteins comigrating with human erythrocyte spectrin. When less

protein was electrophoresed, the two bands originally observed were resolved into a triplet of bands of equal intensity. The presence of "spectrin" in erythrocytes of other nonmammalian vertebrate species, chicken (Chan, 1977), turkey (Alper, 1980), and newt (Sloboda, 1980) has been reported. The identification of dogfish erythrocyte "spectrin" was made on the basis of its electrophoretic comigration with human spectrin, its location in the SAC, the fact that it represented a major protein component of the SAC, and that it could be extracted from anucleate ghosts under conditions known to extract mammalian spectrin.

Further attempts to confirm this identification using antibody raised against human spectrin yielded an interesting result. The lower band of dogfish spectrin, S2, reacted with the antibody while the upper band, S1, did not, suggesting that differences existed between dogfish S1 and S2 that did not exist between dogfish S2 and human S1 and S2.

CBP, a calcium-dependent calmodulin-binding protein was detected in anucleate dogfish erythrocytes and localized to the SAC. CBP comigrated with dogfish S1. Like mammalian spectrin (Faribanks et al., 1971), the migration of dogfish spectrin varied considerably with the amount of protein electrophoresed. However, the binding of ^{125}I calmodulin to CBP was in every case coincident with the Coomassie blue stained band representing dogfish S1, irrespective of its migration relative to human S1. In addition, incubation of dogfish erythrocyte ghosts under conditions known to extract spectrin from mammalian ghosts caused the extraction of

both dogfish S1 and CBP. From these data it was concluded that dogfish S1 and CBP are the same protein.

The binding of calmodulin to dogfish S1 represented yet another difference between dogfish and human spectrin in that neither band of human spectrin bound calmodulin using either the gel overlay technique or in situ cross-linking of azido-¹²⁵I calmodulin to erythrocyte ghosts. Binding of calmodulin to human spectrin in the presence of 6M urea had been reported by Sobue and coworkers (1980).

Dogfish S1 comigrated with a high molecular weight calmodulin-binding protein present in canine cortical postsynaptic densities (PSDs) as the upper band of a protein doublet. This protein had been shown to comigrate with a similar calmodulin-binding protein, CBP-I, isolated from a particulate fraction of bovine brain as a doublet by affinity chromatography on calmodulin-Sepharose (Davies and Klee, 1981). The relationship between dogfish S1 and mammalian CBPs was investigated using antibody raised against the bovine brain protein doublet. This antibody bound strongly to dogfish S1 but not to dogfish S2 or goblin, suggesting close similarity between dogfish S1 and the upper band of CBP-1. Carlin et al. (1982) have shown that the upper band of the PSD doublet which binds calmodulin also reacts with anti-CBP-I. Like mammalian spectrin, bovine brain CBP-I binds actin (Davies and Klee, 1980).

Fodrin is a high molecular weight protein doublet which binds actin and is located in the SAC of a variety of non-erythroid cell

types of both mammals and nonmammals (Levine and Willard, 1981). Dogfish S1 has been identified here as the upper band of fodrin (α fodrin, F_{α}) by the following criteria: (a) electrophoretic comigration of dogfish S1 with authentic guinea pig brain F_{α} , (b) calmodulin-binding to dogfish S1 and guinea pig fodrin, and (c) strong reactivity of dogfish S1 with anti-guinea pig fodrin. The anti-fodrin antibody, raised against both bands of fodrin, reacted only with F_{α} . Therefore it was not possible to determine whether dogfish S2 or goblin are also immunologically related to fodrin. The presence of F_{α} in both dogfish and chicken erythrocyte ghosts suggests that fodrin is widely distributed among non-mammalian vertebrate erythrocytes. The slight reaction of anti-fodrin with human S1, like the reaction of anti-CBP-I with human S1 and S2, indicates the presence of at least some structure common to spectrin and fodrin. The absence of reactivity of dogfish S1 with Fab' fragments of anti-human spectrin antibody is in agreement with results reported previously for both CBP-I (Davies and Klee, 1981) and fodrin (Levine and Willard, 1981) in that neither of these proteins reacted with antisera raised against mammalian spectrins. These data suggest that mammalian spectrin may have diverged from fodrin during the evolution of erythrocytes from nucleated elliptical cells containing MBs in nonmammalian vertebrates to anucleate biconcave discoid cells, lacking MBs, in mammals. The mammalian erythrocyte is perceived as a gross modification of the nonmammalian one, in which MB and nucleus are eliminated but a sub-surface cytoskeletal layer containing

different, but related proteins, is retained.

The reaction of dogfish S2 with Fab' fragments of anti-human spectrin antibody indicate these proteins share antigenic determinants. The identification of the upper band of dogfish spectrin as alpha-fodrin brings into question the use of the term "spectrin" to identify the high molecular weight proteins of the non-mammalian erythrocyte SAC. This designation can be maintained if spectrin is defined as the major high molecular weight protein extracted from the erythrocyte SAC under low ionic strength conditions.

Spectrin is readily detectable in erythrocytes of various mammals (Hiller and Weber, 1977; Marchesi and Steers, 1968; Tillack et al, 1970) but was not observed in non-erythroid mammalian cells using anti-spectrin antibodies (Hiller and Weber, 1977) until recently when Goodman et al. (1981) detected spectrin-like proteins in a variety of cells by anti-human spectrin immunoprecipitation of radioactively-labelled proteins. Among these proteins was a doublet more closely resembling fodrin in electrophoretic migration than spectrin. Given the reactivity of the lower band of PSD fodrin with Fab' fragments of anti-spectrin antibodies reported here, and the widespread distribution of fodrin (Levine and Willard, 1981), the spectrin-like protein of these cells may well be fodrin.

The cross-reactivity of spectrin with anti-fodrin and fodrin with anti-spectrin, as shown here, is indicative of some structural homology between these proteins. The reaction of

anti-CBP-I with both bands of human spectrin compared with the reaction of anti-fodrin with only the upper band of human spectrin (Table III), illustrates the variability obtained when measuring immunoreactivity. Caution must be observed in interpreting these results. A positive reaction indicates only that an antigenic site recognized by the polyclonal antibody is present in the protein being tested. A negative result cannot be interpreted as an absence of homology, but only of the antigenic sites recognized by the antibody. These results cannot be taken as a measure of the degree of homology between proteins; such an assessment can only be made by structure determination of the proteins in question.

2. "Goblin"

Goblin has been found in turkey (Beam *et al.*, 1979), chicken (Granger *et al.*, 1982) and frog (Rudolph *et al.*, 1980) erythrocyte membranes. It was named by Beam *et al.* (1979), who described its electrophoretic mobility as "haunting spectrin". Goblin migrates between the two protein bands identified as spectrin in turkey erythrocytes (Alper, 1980) and has been shown in the reports cited above to be phosphorylated when sodium and potassium co-transport across the erythrocyte membrane is stimulated by catecholamines. Dogfish goblin was tentatively identified on the basis of its electrophoretic mobility compared with the published pattern for turkey erythrocytes. Alper *et al.* (1980) have reported that unlike spectrin, goblin is not extracted from turkey erythrocyte

membranes by low-ionic strength extraction. The protein band identified as dogfish goblin was extracted with spectrin by low-ionic strength and pHMB. Further characterization, including phosphorylation with cyclic AMP-dependent protein kinase in vitro, would be necessary to confirm this identification.

Dogfish goblin bound antibody directed against human spectrin and therefore, like dogfish S2, appears to be related to the mammalian erythrocyte protein. Goblin comigrated with the lower band of PSD fodrin, which also reacted with the antibody to human spectrin. Since the antibody to fodrin, raised against both bands of fodrin only bound to the upper band of guinea pig fodrin (F_{42}) it was not possible to determine whether goblin is the lower band of fodrin (F_{48}). The lack of antibody binding to both of these proteins, though a negative result, is consistent with this possibility.

3. Other cytoskeletal proteins

a. "Vimentin"

Vimentin was tentatively identified in anucleate dogfish erythrocyte ghost preparations on the basis of its mobility relative to tubulin and actin (Brown *et al.*, 1976), and by analogy with chicken erythrocytes which contain vimentin in intermediate filaments (Granger *et al.*, 1982). The amount of vimentin present in chicken erythrocyte plasma membranes was comparable to the amount of putative dogfish vimentin in anucleate ghost preparations. In addition, comparison of chicken and dogfish erythrocyte anucleate ghost preparations in figure 36 show faint bands in both

preparations comigrating above the actin bands. However, Granger and coworkers reported that vimentin was extracted from chicken erythrocyte membranes by incubation at low-ionic strength or in water. Dogfish "vimentin" was not extracted from anucleate ghosts at low-ionic strength, but remained associated with the membrane vesicles. Dogfish "vimentin" did not react with antibody raised against BHK vimentin. However, intermediate filament proteins exhibit species specificity and do not always react with antibodies raised against intermediate filament proteins of an unrelated species (R. Goldman, personal communication). No intermediate filaments were observed either in nucleated or anucleate dogfish erythrocyte cytoskeletal preparations. These latter data bring into question the identification of this protein band as vimentin. Further examination of this question will require using procedures established for chicken erythrocytes for the detection of vimentin and intermediate filaments with dogfish erythrocyte preparations. Alternately, vimentin could be sought in preparations of isolated nuclei.

b. Actin

Dogfish erythrocyte actin comigrated with human erythrocyte actin and was present in the same percentage of the total membrane protein in both species (Table II). In addition, actin was localized to the dogfish erythrocyte SAC.

c. 150,000 molecular weight calmodulin-binding protein

A calmodulin-binding protein of 150,000 molecular weight was detected in dogfish and chicken erythrocyte ghosts. This protein

was thought to be a proteolytic fragment of the higher molecular weight CBPs. Reaction of the 150,000 M_r calmodulin-binding protein present in chicken erythrocyte ghosts with anti-fodrin (Figure 40) demonstrates a structural relationship to F_{α} in support of the above hypothesis. It is not known whether this protein is related to caldesmon, a 150,000 molecular weight actin-binding, calmodulin-binding protein isolated from chicken gizzard by Sobue and co-workers (Sobue et al., 1981).

C. Comparison of dogfish and human erythrocyte ghost proteins

The data in Table II compare the protein compositions for dogfish and human erythrocyte ghosts. In order to achieve resolution of the high molecular weight triplet present in dogfish preparations, only moderate amounts of protein were electrophoresed. As a result it was not possible to quantitate or, in some cases, to detect the minor components present in the preparation. Other than proteins comigrating with human erythrocyte spectrin and actin and band 4.1, the protein pattern obtained for dogfish erythrocyte membranes differed considerably from that of human erythrocytes. The protein band designated dogfish band 3 had an apparent molecular weight of 103,000 to 128,000, while human erythrocyte band 3, the transmembrane anion channel protein, had a molecular weight of 88,000 to 105,000. Confirmation of the designation of this protein as dogfish band

3 would require its identification as the anion transport protein of dogfish erythrocytes.

Band 3 represents almost 40% of the coomassie blue staining in anucleate ghosts devoid of MBs and was almost completely extracted by Triton treatment. It may be the protein component that acts to stabilize the SAC. In the mammalian erythrocyte, spectrin, the major protein component of the SAC is associated with the membrane by binding of S2 to ankyrin (band 2.1; Morrow et al., 1980). Ankyrin in turn is associated with the cytoplasmic domain of band 3 which is a major membrane spanning polypeptide (Bennett and Stenbuck, 1979). If the nonmammalian system is homologous to the mammalian erythrocyte membrane, then the nonmammalian system may contain the equivalents of ankyrin. Band 3 could then bind to nonmammalian ankyrin which would bind to S2, linking the SAC with the cytoplasmic side of the lipid bilayer, and giving stability to the entire structure.

III. Effects of calcium on cell morphology and cytoskeletal structures

A. Calcium and ionophore A23187 effects on erythrocyte morphology

In the study of calcium transport in mammalian erythrocytes, the use of calcium ionophore A23187 is necessitated by the low cation permeability of red cell

membrane. Changes in mammalian erythrocyte morphology from biconcave disc to sphere have been observed concomitant with an increase in intracellular calcium concentration (Sarkadi et al, 1976; White, 1974). The effect of ionophore and calcium on mammalian erythrocyte morphology has been shown to be calcium-dependent and reversible upon removal of ionophore (Sarkadi et al., 1976). The results reported here for dogfish erythrocytes indicate that ionophore-induced, calcium-dependent changes in erythrocyte morphology are not limited to mammalian red cells, but that similar dramatic changes in morphology, from flattened ellipse to sphere, occur in nucleated non-mammalian erythrocytes. As a minimum surface-to-volume and minimum energy configuration, the sphere is indicative of loss of a functional cytoskeleton. The disappearance of the MB and SAC, as observed by phase contrast microscopy in detergent-lysed dogfish erythrocytes, paralleled the formation of spheres, suggesting that one or both are involved in maintenance of cell shape and are calcium-labile in vivo. The generation of angular shapes in whole cells preceded sphere formation. Examination of these cells after detergent lysis showed angular marginal bands with the SAC still present, consistent with the interpretation that nucleated erythrocyte shape corresponds to that of a surface-associated network subject to elastic deformation by the marginal band within (Cohen, 1978).

Barrett and Dawson (1974) have reported that the development of avian erythrocytes involves a spheroid to discoid

transformation in shape and that the discoid shape of the young erythroid cell is dependent upon the presence of the MB in the early stages of postmitotic development. It is tempting to postulate that the flattened ellipse to sphere conversion seen with ionophore A23187 and calcium treatment may represent a reversal of this normal developmental transition and that intracellular calcium concentration controls both of these transitions by controlling the assembly/disassembly of cytoskeletal structures.

B. In vitro effects of calcium on dogfish erythrocyte MB and SAC

The marginal band of the dogfish erythrocyte has been shown here to be labile both in vivo and in vitro to millimolar concentrations of calcium. However this lability was dependent upon the ionic composition of the media in which the experiments were conducted. MBs were stable to 5 mM calcium in lysis medium A, but could be disassembled in a calcium-dependent manner if 150 mM KCl was present in the medium. Lysis in Ringer's solution caused MB disassembly even when EGTA was present in 5 mM excess. These results demonstrate the difficulty in studying calcium effects on microtubules in vitro. Since in vivo conditions are difficult to determine and simulate, the results of in vitro experiments are difficult to interpret in relation microtubule behavior in vivo. Perhaps the most relevant experiment concerning MB calcium-lability would be an attempt to reverse the effects of calcium and ionophore in vivo.

Microtubules present in detergent-extracted BSC-1 monkey cells (Schliwa, 1981) and isolated sea urchin mitotic spindles have recently been shown to be labile to micromolar concentrations of calcium (Salmon and Segall, 1980). Further experiments are required to determine whether the MB and SAC are labile to physiological levels of calcium, and whether their formation could be controlled by the level of free calcium present in the developing nucleated erythrocyte.

IV. Functions of fodrin and fodrin-like proteins

The localization of fodrin in non-erythroid cells (Levine and Willard, 1981) and non-mammalian erythrocytes implicates fodrin as the ubiquitous protein of the nucleated cell SAC analogous to spectrin in the cytoskeleton of mammalian erythrocytes. Fodrin has been shown to localize under caps when lymphocytes are exposed to multivalent ligands that cross-link surface moieties (Levine et al, 1981). This suggests that (1) fodrin can "communicate" with cell surface moieties, (2) it can move withing the SAC, and (3) it may have some role in the mechanism of moving the cross-linked membrane proteins to one location on the cell surface. Calmodulin has similarly been shown to localize under lymphocyte caps (Salisbury et al., 1981), suggesting that perhaps calmodulin is bound to fodrin during lymphocyte capping.

Fodrin belongs to a family of high molecular weight proteins

that bind both actin and calmodulin. Howe et al., (1982) and Glenney et al. (1982) have described similar high molecular weight calmodulin-binding proteins in chick intestinal epithelial brush border. Glenney and coworkers have reported that this protein exists as the lower band of a protein doublet (molecular weight 240,000), that comigrates with the upper band of fodrin and spectrin. Like fodrin, the protein doublet is capable of binding actin. Micrographs of low angle rotary shadowed preparations of this protein show it to exist as a loosely intertwined, flexible double stranded molecule similar to but distinct from spectrin. Localization of this protein by immunofluorescence microscopy shows it to be in the terminal web but not in the microvilli.

Examination of the recent literature on actin binding proteins reveals a number of reports of proteins that may be members of the fodrin family. Tilney (1976) described an actin storage protein in phase dense cups of the periacrosomal region of echinoderm sperm and named the protein actin complex "profil. actin". This protein migrated as a high molecular weight doublet, the upper band of which comigrated with human S1. A high molecular weight actin binding protein doublet from bovine brain, the upper band of which comigrates with human S1, has recently been shown to stimulate the Mg^{2+} -ATPase of actomyosin (Shimo-Oka and Watanabe, 1981). This protein was coprecipitable with actomyosin and stimulated ATPase activity four-fold.

As noted previously, Sobue et al have reported the presence of caldesmon, a 150,000 molecular weight protein doublet of chicken gizzard that binds both calmodulin and actin. In view of the findings reported here for chicken erythrocyte ghost calmodulin-binding proteins, and the results of Glenney et al (1982) concerning the high molecular weight calmodulin-binding actin-binding protein of chicken gizzard, the possibility exists that caldesmon is related to or derived from the fodrin-like protein. In all of these systems, the proteins are high molecular weight doublets with molecular weights that vary slightly. However, one property of these protein doublets is consistent. The 240,000-245,000 molecular weight component, whether the upper or lower band, always comigrates with the upper band of human spectrin (S1) and binds calmodulin. It is interesting to note that both anti-CBP-I and anti-fodrin reacted with the upper band of human spectrin (S1). Perhaps this subunit is the antecedent of human S1, which during evolution, has lost its capability to bind calmodulin, unless a latent calmodulin-binding site is exposed by urea denaturation (Sobue,1980).

Both actin and calmodulin have been detected in every species and tissue examined thus far. The existence of a cytoskeletal protein or family of proteins that interact with both actin and calmodulin and appear to be widely distributed in nature (Levine and Willard, 1981) provides a mutual site of action for both of these proteins. The localization of these

proteins to the SAC of their respective cells implies that fodrin and fodrin-like proteins could, through calmodulin, mediate the effects of calcium on the cytoskeleton as calcium levels are increased from extracellular sources. As the results obtained for lymphocyte capping imply, fodrin may be involved in the coordination of movement of both cell surface proteins and intracellular cytoskeletal structures.

References

- Agre, P., E.P. Orringer and V. Bennett. 1982. Deficient red-cell spectrin in severe recessively inherited spherocytosis. New Eng. J. Med. 306: 1151-1161.
- Alper, S.L., H.C. Palfrey, S.A. DeRiemer and P. Greengard 1980. Hormonal control of protein phosphorylation in turkey erythrocytes. Phosphorylation by cAMP-dependent protein kinases of distinct sites in goblin, a high molecular weight protein of the plasma membrane. J. Biol. Chem. 255: 11029-11039.
- Andreasen, T.J., C.H. Keller, D.C. LaPorte, A.M. Edelman and D.R. Storm. 1981. Preparation of azidocalmodulin: A photoaffinity label for calmodulin-binding proteins. Proc. Nat. Acad. Sci. U.S.A. 78: 2782-2785.
- Andrew, W. 1965. Comparative Hematology. Grune and Stratton, Inc., New York/London.
- Barrett, L.A. and R.P. Dawson. 1974. Avian erythrocyte development: microtubules and the formation of disc shape. Devel. Biol. 36: 72-81.
- Bartsch, P.W., W.H. Ball, W. Rosenzweig, and S. Salman. 1937. Size of red blood corpuscles and their nuclei in fifty North American birds. Auk. 54: 516-519.
- Beam, K.G., S.L. Alper, G.E. Palade and P. Greengard. 1979. Hormonally regulated phosphoprotein of turkey erythrocytes. Localization to plasma membrane. J. Cell Biol. 83: 1-15.
- Behnke, O. 1965. Further studies on microtubules. A marginal bundle in human and rat thrombocytes. J. Ultrastr. Res. 13: 469-477.
- Behnke, O. 1967. Incomplete microtubules observed in mammalian blood platelets during microtubule polymerization. J. Cell Biol. 34: 697-701.
- Behnke, O. 1970. A comparative study of microtubules of disc-shaped blood cells. J. Ultrastr. Res. 31: 61-75.
- Bennett, W.F. and G. Lynch. 1980. Low-temperature induction of calcium-dependent protein phosphorylation in blood platelets. J. Cell Biol. 86: 280-285.
- Bennett, V. and P. Stenbuck. 1979. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. Nature (London) 280: 468-473.

- Bertolini, B. and G. Monaco. 1976. The microtubule marginal band of the newt erythrocyte. Observations on the isolated band. J. Ultrastruct. Res. 54: 57-67.
- Boettcher, A. 1866. Untersuchungen über die rothen Blutkörperchen der W. belthiere. Virchow's Arch. für Path. Anat. und Phys. und für Klin. Med. 36: 340-424.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
- Branton, P., C.M. Cohen and J. Tyler. 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. Cell 24: 24-32.
- Brown, S., W. Levinson and J.A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramolec. Struct. 5: 119-130.
- Burgess, W.H., L.I. Rebhun and R.H. Kretsinger. 1980. Calmodulin interaction with cycle-purified brain tubulin components. J. Cell Biol. 87: 248a.
- Carlin, R.K., D.J. Grab and P. Siekevitz. 1980a. The binding of radio-iodinated calmodulin to proteins on denaturing gels. Ann. N.Y. Acad. Sci. 356: 73-74.
- Carlin, R.K., D.J. Grab, R.S. Cohen and P. Siekevitz. 1980b. Isolation and characterization of postsynaptic densities from various brain regions: Enrichment of different types of postsynaptic density. J. Cell Biol. 86: 831-843.
- Carlin, R.K., D.J. Grab, and P. Siekevitz. 1981. Function of calmodulin in postsynaptic densities III: Calmodulin-binding proteins of the postsynaptic density. J. Cell Biol. 89: 449-455.
- Carlin, R.K., D.C. Bartelt and P. Siekevitz. 1982. Identification of the high molecular weight calmodulin binding protein of postsynaptic densities as fodrin. J. Cell Biol. submitted.
- Castle, A.G. and N. Crawford. 1977. The isolation and characterization of platelet microtubule proteins. Biochim. Biophys. Acta. 494: 76-91.
- Cavanaugh, G.M., ed. 1975. Formulae and Methods VI of the Marine Biological Laboratory, Woods Hole, Mass.
- Chan, L.N.C. 1977. Changes in the composition of plasma membrane proteins during differentiation of embryonic chick erythroid cell. Proc. Nat. Acad. Sci. U.S.A. 74: 1062-1066.

- Cheung, W.Y. 1979. Calmodulin plays a pivotal role in cellular regulation. Science. 207: 19-27.
- Cohen, R.S., F. Blomberg, K. Berzins, and P. Siekevitz. 1977. Structure of postsynaptic densities isolated from dog cerebral cortex. I. Overall morphology and protein composition. J. Cell Biol. 74: 181-203.
- Cohen, W.D. 1978. Observations on the marginal band system of nucleated erythrocytes. J. Cell Biol. 78: 260-273.
- Cohen, W.D., D. Bartelt, R. Jaeger, G. Langford, and I. Nemhauser. 1982. The cytoskeletal system of nucleated erythrocytes I. Composition and function of major elements. J. Cell Biol. 93: 783-794.
- Cohen, W.D. and I. Nemhauser. 1980. Association of centrioles with the marginal band of a molluscan erythrocyte. J. Cell Biol. 86: 286-291.
- Cohen, W.D., I. Nemhauser and R. Jaeger. 1977. Rapid visualization of the marginal band system in blood cells of marine species. Biol. Bull. 153: 420.
- Cohen, W.D. and N.B. Terwilliger. 1979. Marginal bands in camel erythrocytes. J. Cell Sci. 36: 97-107.
- Cuatrecasas, P. and C.B. Anfinsen. 1971. Methods in Enzymology 22: 345.
- Dabrowska, R., J.M.F. Sherry, D.K. Aromatorio, and D.J. Hartshorne. 1978. Modulator protein as a component of the myosin light chain kinase from chicken gizzard. Biochem. 17: 253-258.
- Davies, P.J.A. and C.B. Klee. 1981. Calmodulin-binding proteins: Identification of a high molecular weight calmodulin-binding protein from bovine brain. Biochem. Internat. 3: 203-212.
- Dawson, A.B. 1929. A further study of the reaction of the amphibian erythrocyte to vital dyes, osmic acid, and silver salts, with special reference to basophilia and reticulation. Anat. Rec. 42: 281-300.
- Dayton, W.R., W.J. Reville, D.E. Goll and M.H. Stromer. 1976. A Ca²⁺-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. Biochem. 15: 2159-2166.
- Debus, E., K. Weber and M. Osborn. 1981. The cytoskeleton of blood platelets viewed by immunofluorescence microscopy. Eur. J. Cell Biol. 24: 45-52.

- Dehler, A. 1895. Beitrag zur Kenntnis de feineren Baues der roten blutkörperchen beim hühnerembryo. Arch. Mikroskop. Anat. Entwicklungsmech. 46: 414-430.
- Fairbanks, G., T.L. Steck and D.F.H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochem. 10: 2606-2624.
- Fawcett, D.W. 1959. Electron microscopic observations on the marginal band of nucleated erythrocytes. Anat. Rec. 133: 379.
- Fawcett, D.W. 1962. Physiologically significant specializations in the cell surface. Circulation 36: 1105-1122.
- Fawcett, D.W. and F. Witebsky. 1964. Observations on the ultrastructure of nucleated erythrocytes and thrombocytes with particular reference to the structural basis of their discoidal shape. Z. Zellforsch. 62: 785-806.
- Fellous, A., J. Francon, A.M. Lenon and J. Nunez. 1977. Microtubule assembly in vitro. Purification of assembly-promoting factors. Eur. J. Biochem. 78: 167-174.
- Franke, W.W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc. Nat. Acad. Sci. U.S.A. 75: 5034-5038.
- Gillespie, G.C., ed. 1973. Dictionary of Scientific Biography. Scribner's, New York. pp.126-129.
- Glenney, J.R., Jr., and K. Weber. 1980. Calmodulin-binding proteins of the microfilaments present in isolated brush borders and microvilli of intestinal epithelial cells. J. Biol. Chem. 255: 10551-10554.
- Glenney, J.R., Jr., P. Glenney, M. Osborn and K. Weber. 1982. An F-actin- and calmodulin-binding protein from isolated intestinal brush borders has a morphology related to spectrin. Cell 28: 843-854.
- Goniakowska-Witalinska, L. and W. Witalinski. 1976. Evidence for a correlation between the number of marginal band microtubules and the size of vertebrate erythrocytes. J. Cell Sci. 22:397-401.
- Goniakowska-Witalinska, L. and W. Witalinski. 1977. Occurrence of microtubules during erythropoiesis in llama, Lama glama. J. Zool. (London) 181: 309-313.
- Grab, D.J., K. Berzins, R.S. Cohen and P. Siekevitz. 1979. Presence of calmodulin in postsynaptic densities isolated from canine

- cerebral cortex. J. Biol. Chem. 254: 8690-8696.
- Graf, E., A. Filoteo and J.T. Penniston. 1980. Preparation of 125I calmodulin with retention of full biological activity: Its binding to human erythrocyte ghosts. Arch. Biochem. Biophys. 203: 719-726.
- Granger, B.L., E.A. Repasky and E. Lazarides. 1982. Synemin and vimentin are components of intermediate filaments in avian erythrocytes. J. Cell Biol. 92: 299-312.
- Gratzer, W.B. 1981. The red cell membrane and its cytoskeleton. Biochem. J. 198: 1-8.
- Greenquist, A.C., S.B. Shohet, and S.E. Bernstein. 1978. Marked reduction of spectrin in hereditary spherocytosis in the common house mouse. Blood 51: 1149-1155.
- Hainfield, J.F., and T.L. Steck .1977. The sub-membrane reticulum of the human erythrocyte: A scanning electron microscope study. J. Supramolec. Struct. 6: 301-311.
- Hanks, J.H. 1948. The longevity of chick tissue cultures without removal of medium. J. Cell Comp. Physiol. 31: 235-259.
- Harris, J.R. and J.N. Brown. 1971. Fractionation of the avian erythrocyte: an ultrastructural study. J. Ultrastr. Res. 36: 8-23.
- Hartman, F.A. and M.A. Cessler. 1964. Erythrocyte measurements in fishes, amphibias and reptiles. Biol. Bull. 126: 83-88.
- Hathaway, D.R. and R.S. Adelstein. 1979. Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity. Proc. Nat. Acad. Sci. 76: 1653-1657.
- Hepler, P.K. 1977. Membranes in the spindle apparatus: Their possible role in the control of microtubule assembly. In: Mechanisms and Control of Cell Division. T.L. Rost and E.M. Giffords, eds. Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pennsylvania. pp. 212-232.
- Hewson, J. 1773. Philosoph. Transact. 63: 310. As cited in Boettcher, A. 1866. Untersuchungen über die rothen Blutkörperchen der Wirbelthiere. Virchow's Arch. für Path. Anat. und Phys. und für Klin. Med. 36: 342-424.
- Hiller, G. and Weber, K. 1977. Spectrin is absent in various tissue culture cells. Nature 266: 181-183.
- Hinds, T.R. and T.J. Andreasen. 1981. Photochemical cross-linking of azidocalmodulin to the (Ca²⁺ + Mg²⁺)-ATPase of the

- erythrocyte membrane. J. Biol. Chem. 256: 7877-7882.
- Howe, C.L., T.C.S. Keller III, M.S. Mooseker and R.H. Wasserman. 1982. Analysis of cytoskeletal proteins and calcium-dependent regulation of structure in intestinal brush border from rachitic chicks. Proc. Nat. Acad. Sci. U.S.A. 79: 1134-1138.
- Inoue, S. and H. Sato. 1967. Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. J. Gen. Physiol. 50: 259-292.
- Jackson, R.C. 1975. The exterior surface of the chicken erythrocyte. J. Biol. Chem. 250: 617-622.
- Kakiuchi, S., K. Sobue and M. Fujita. 1981. Purification of a 240,000 Mr calmodulin-binding protein from a microsomal fraction of brain. FEBS Lett. 132: 144-148.
- Klee, C.B. and M.H. Krinks. 1978. Purification of cyclic 3'5' nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to Sepharose. Biochemistry 17: 120-126.
- Klee, C.B., T.H. Crouch and P.G. Richman. 1980. Calmodulin. Ann. Rev. Biochem. 49: 489-515.
- Langford, G.M. 1980. Arrangement of subunits in microtubules with 14 protofilaments. J. Cell Biol. 87: 521-526.
- Lehto, V.-P., I. Virtanen, and P. Kurks. 1978. Intermediate filaments anchor the nuclei in nuclear monolayers of cultured human fibroblasts. Nature 272: 175-177.
- LePeuch, C.J., J. Haiech, and J.G. Demaille. 1979. Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate-dependent and calcium-calmodulin-dependent phosphorylations. Biochem. 18: 5150-5157.
- Levine, J. and M. Willard. 1981. Fodrin: Axonally transported polypeptides associated with the internal periphery of many cells. J. Cell Biol. 90: 631-643.
- Lucas, A.M. and C. Jamroz. 1961. Atlas of Avian Hematology. U.S. Dept. of Agriculture Monograph 25, U.S. Govt. Printing Office, Washington. D.C.
- Lux, S.E., K.M. John and M.J. Karnovsky. 1976. Irreversible deformation of the spectrin-actin lattice in irreversibly sickled cells. J. Clin. Invest. 58: 955-963.
- Lux, S.E. 1979. Dissecting the red cell membrane. Nature 281: 426-429.

- Lynch, T.J. and W.Y. Cheung. 1979. Human erythrocyte Ca^{++} - Mg^{++} -ATPase: mechanism of stimulation by calcium. Arch. Biochem. Biophys. 194: 165-170.
- Maizel, J.V. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids. In: Fundamental Techniques in Virology. K. Habel and N.P. Salzman, eds. Academic Press, Inc., New York, 334-362.
- Marchesi, V.T. and E. Steers, Jr. 1968. Selective solubilization of a protein component of the red cell membrane. Science 159: 203-204.
- Marcam, J.M., J.R. Dedman, B.R. Brinkley, and A.R. Means. 1978. Control of microtubule assembly-disassembly by calcium-dependent regulator protein. Proc. Nat. Acad. Sci. U.S.A. 75: 3771-3775.
- Meeves, F. 1904. Die Hünfeld-Heasenschen Bilder bei roten Blutkörperchen der Amphibien. Anat. Anz. 24: 465-476.
- Meeves, F. 1911. Gesammelte Studien an den roten Blutkörperchen der Amphibien. Arch. mikroskop. Anat. U. Entwicklungsmech. 77: 465-540.
- Nakoo, M., T. Nakoo and S. Yamazoe. 1960. Adenosine triphosphate and maintenance of shape of the human red cells. Nature 187: 945-947.
- Nemhauser, I., W.D. Cohen, A. Milsted and R.D. Goldman. 1979. Marginal band systems in blood cells of marine species: visualization by indirect immunofluorescence. Biol. Bull. 157: 384-385.
- Nemhauser, I., R. Ornberg and W.D. Cohen. 1980. Marginal bands in blood cells of invertebrates. J. Ultrastr. Res. 70: 308-317.
- Phillips, D.R. and M. Jakabova. 1977. Ca^{++} -dependent protease in human platelets. J. Biol. Chem. 252: 5602-5605.
- Ralston, G.B. 1975. Proteins of the camel erythrocyte membrane. Biochim. Biophys. Acta 401: 83-94.
- Ranvier, L. 1975. Recherches sur les elements du sang. Arch. de Physiol. 7: 1-15.
- Rebhun, L.I., J. Rosenbaum, P. Lefebvre and G. Smith. 1974. Reversible restoration of the birefringence of cold-treated isolated mitotic apparatus of surf clam eggs with chick brain tubulin. Nature (London) 249: 113-115.
- Rosenberg, S., A. Stracher and R.C. Lucas. 1981. Isolation and characterization of actin and actin-binding protein from human

- platelets. J. Cell. Biol. 91: 201-211.
- Rudolph, S.A. and P. Greengard. 1980. Effects of catecholamines and prostaglandin E1 on cAMP, cation fluxes and protein phosphorylation in the frog erythrocyte. J. Biol. Chem. 255: 8534-8540.
- Salmon, E.D. and R.R. Segall. 1980. Calcium-labile mitotic spindles isolated from sea urchin eggs (Lytechinus variegatus). J. Cell Biol. 86: 355-365.
- Sandoval, I.V. and K. Weber. 1979. Calcium-induced inactivation of microtubule formation in brain extracts. Presence of a calcium-dependent protease acting on polymerization-stimulating microtubule-associated proteins. Eur. J. Biochem. 92: 463-470.
- Schliwa, M., U. Euteneuer, J.C. Bulinski and J.G. Izant. 1981. Calcium lability in cytoplasmic microtubules and its modulation by microtubule-associated proteins. Proc. Nat. Acad. Sci. U.S.A. 78: 1037-1041.
- Sekhon, S.S. and H.W. Beams. 1969. Fine structure of developing trout erythrocytes and thrombocytes with special reference to the marginal band and cytoplasmic organelles. Am. J. Anat. 125: 353-374.
- Sheetz, M.P. and Singer, S.J. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc. Nat. Acad. Sci. U.S.A. 71: 4457-4461.
- Sheetz, M.P. and D. Sawyer. 1978. Triton sheels of intact erythrocytes. J. Supramolec. Struct. 8: 399-412.
- Shelanski, M.L., F. Gaskin and C.R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. Proc. Nat. Acad. Sci. U.S.A. 70: 765-768.
- Shinagawa, C. 1960. Morphological and cytochemical studies on erythrocytes of vertebrates. Journal of Science of the Hiroshima University, Ser. B, Div. 1, Vol. 19: 1-60.
- Siefring, G.E., A.B. Apostol, T. Velosco and L. Lorand. 1978. Enzymatic basis for the Ca^{++} -induced cross-linking of membrane proteins in intact human erythrocytes. Biochem. 17: 2598-2604.
- Sloboda, R.D., W.L. Dentler and J.L. Rosenbaum. 1976. Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*. Biochem. 15: 4497-4505.
- Sloboda, R.D. and K. Dickersin. 1980. Structure and composition of

- the cytoskeleton of nucleated erythrocytes. The presence of microtubule-associated protein 2 in the marginal band. J. Cell Biol. 87: 170-179.
- Small, J.V. 1972. The significance of marginal band microtubules. J. Ultrastr. Res. 38: 207-208.
- Small, J.V. and J.E. Celis. 1978. Direct visualization of the 10-nm (100-A)-filament network in whole and enucleated cultured cells. J. Cell. Sci 31: 393-409.
- Sobue, K., M. Fujita, Y. Muramoto and S. Kakiuchi. 1980. Spectrin as a major modulator-binding protein of erythrocyte cytoskeleton. Biochem. Internat. 1: 561-566
- Sobue, K., Y. Muramoto, M. Fujita and S. Kakiuchi. 1981. Calmodulin-binding protein of erythrocyte cytoskeleton. Biochem. Biophys. Res. Comm. 100: 1063-1070.
- Starger, J.M., W.E. Brown, A.E. Goldman and R.D. Goldman. 1978. Biochemical and immunological analysis of rapidly purified 10-nm filaments from baby hamster kidney (BHK-21) cells. J. Cell. Biol. 78: 93-109.
- Steck, T.L. and J. Yu. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. J. Supramolec. Struct. 1: 220-232.
- Steck, T.L. 1974a. The organization of proteins in the human red blood cell membrane. J. Cell Biol. 62: 1-19.
- Steck, T.L. and J.A. Kant. 1974b. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Meth. in Enzymol. 31: 172-180.
- Tillack, T.W., S.L. Marchesi, V.T. Marchesi and E. Steers Jr. 1970. A comparative study of spectrin: A protein isolated from red blood cell membranes. Biochim. Biophys. Acta. 200: 125-131.
- Tilney, L.G. and P. Detmers. 1975. Actin in erythrocyte ghosts and its association with spectrin. J. Cell Biol. 66: 508-520.
- Tomaselli, M.B., K.M. John and S.E. Lux. 1981. Elliptical erythrocyte membrane skeletons and heat-sensitive spectrin in hereditary elliptocytosis. Proc. Nat. Acad. Sci. U.S.A. 78: 1911-1915.
- Tsukita, S., S. Tsukita, H. Ishakawa, S. Sato and M. Nukao. 1981. Electron microscopic study of reassociation of spectrin and actin with the human erythrocyte membrane. J. Cell Biol. 90: 70-77.

- Tyler, J.M., W.R. Hargreaves and D. Branton. 1979. Purification of two spectrin-binding proteins: Biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. Proc. Nat. Acad. Sci. U.S.A. 76: 5192-5196.
- van Deurs, B. and O. Behnke. 1973. The microtubule marginal band of mammalian red blood cells. Z. Anat. Entwickl. Gesch. 143: 43-47.
- Virtanen, I., M. Kurkinen and V.-P. Lehto. 1979. Nucleus-anchoring cytoskeleton in chicken red blood cells. Cell Biol. Int. Reports 3: 157-162.
- Watterson, D.M., W.G. Harrelson, P.M. Keller, F. Sharief and T.C. Vanaman. 1976. Structural similarities between the Ca²⁺-dependent regulatory proteins of 3'-5'-cyclic nucleotide phosphodiesterase and actomyosin ATPase. J. Biol. Chem. 251: 4501-4513.
- Watterson, D.M. and T.C. Vanaman. 1976. Affinity chromatography purification of a cyclic nucleotide phosphodiesterase using immobilized modulator protein, a troponin C-like protein from brain. Biochem. Biophys. Res. Comm. 73: 40-46.
- Weise, M.J. and L.N.L. Chan. 1978. Membrane protein synthesis in embryonic chick erythroid cells. J. Biol. Chem. 253: 1892-1897.
- Weisenberg, R.C. 1972. Microtubule formation in vitro in solutions containing low calcium concentrations. Science 177: 1104-1105.
- Welsh, M.J., J.R. Dedman, B.R. Brinkley and A.R. Means. 1978. Calcium-dependent regulator protein: localization in mitotic apparatus of eukaryotic cells. Proc. Nat. Acad. Sci. U.S.A. 75: 1867-1871.
- Wolosewick, J.J. and K.R. Porter. 1979. Microtrabecular lattice of the cytoplasmic ground substance: artifact or reality? J. Cell Biol. 87: 114-139.
- Woodcock, C.L.F. 1980. Nucleus-associated intermediate filaments from chicken erythrocytes. J. Cell Biol. 85: 881-889.
- Yerna, M.J., R. Dabroska, D.J. Hartshorne and R.D. Goldman. 1979. Calcium-sensitive regulation of actin-myosin interactions in baby hamster kidney (BHK-21) cells. Proc. Nat. Acad. Sci. U.S.A. 76: 184-188.
- Zentgraf, H., B. Deumling, E.D. Jarasch, and W.W. Franke. 1971. Nuclear membrane and plasma membranes from hen erythrocytes.

I. Isolation, characterization and comparison. J. Biol. Chem.
246: 2986-2995.

Zucker, M.B. and J. Borrelli. 1954. Reversible alterations in
platelet morphology produced by anticoagulants and by cold.
Blood. 9:602-608.