This electronic thesis or dissertation has been downloaded from the King's Research Portal at https://kclpure.kcl.ac.uk/portal/

# Studies on human spectrin and its interactions with other cytoskeletal proteins.

Calvert, R A

**The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.**

END USER LICENCE AGREEMENT

**Unless another licence is stated on the immediately following page** this work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International licence. https://creativecommons.org/licenses/by-nc-nd/4.0/

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

**Take down policy**

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 09. Jan. 2025

# STUDIES ON HUMAN SPECTRIN AND ITS INTERACTIONS WITH OTHER CYTOSKELETAL PROTEINS

BY

ROSALEEN CALVERT

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of London at King's College

1980

BEST COPY

AVAILABLE

Poor text in the original thesis.
Some text bound close to the spine.
Some images distorted

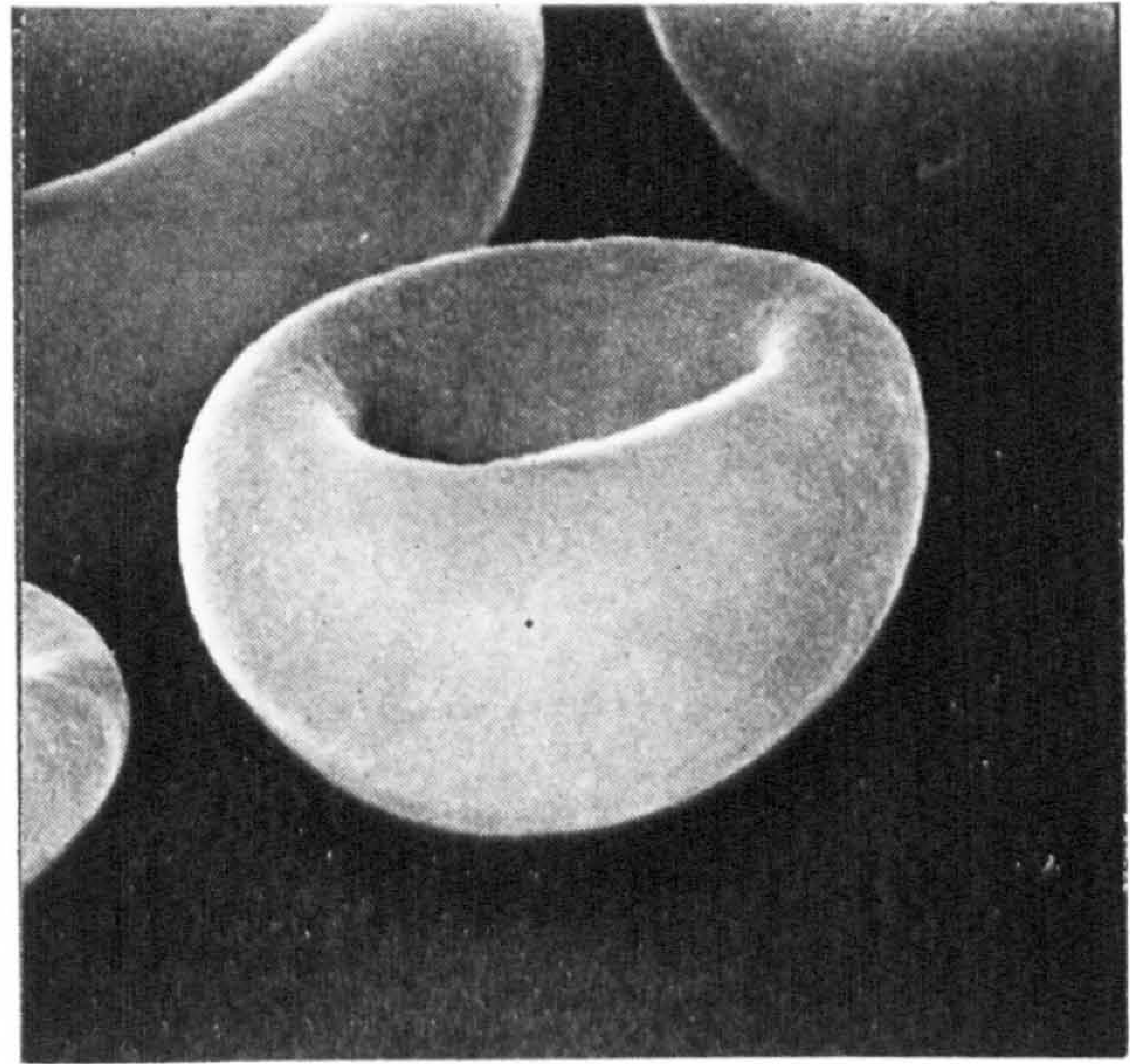
Frontispiece :

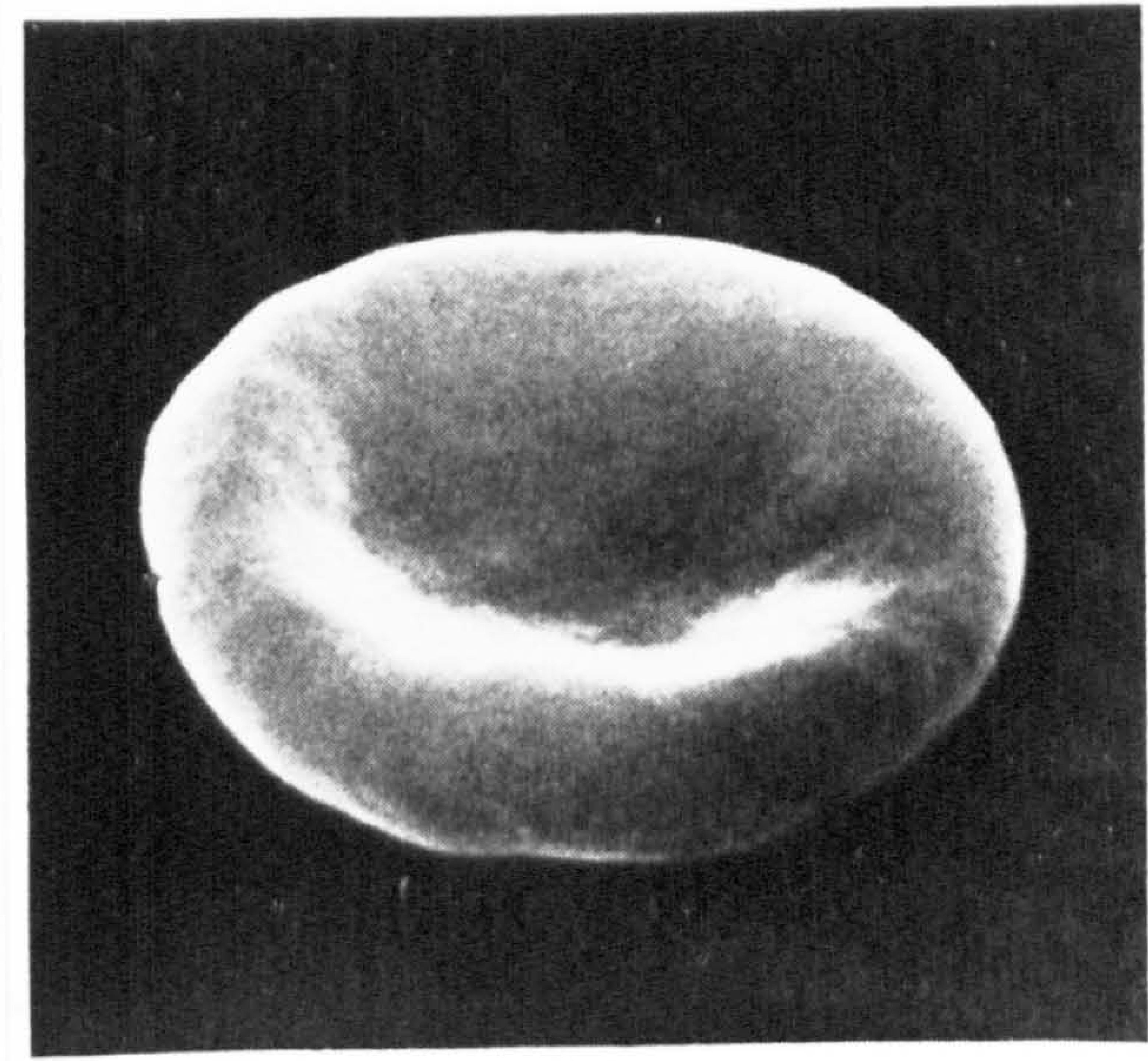
Stomatocyte

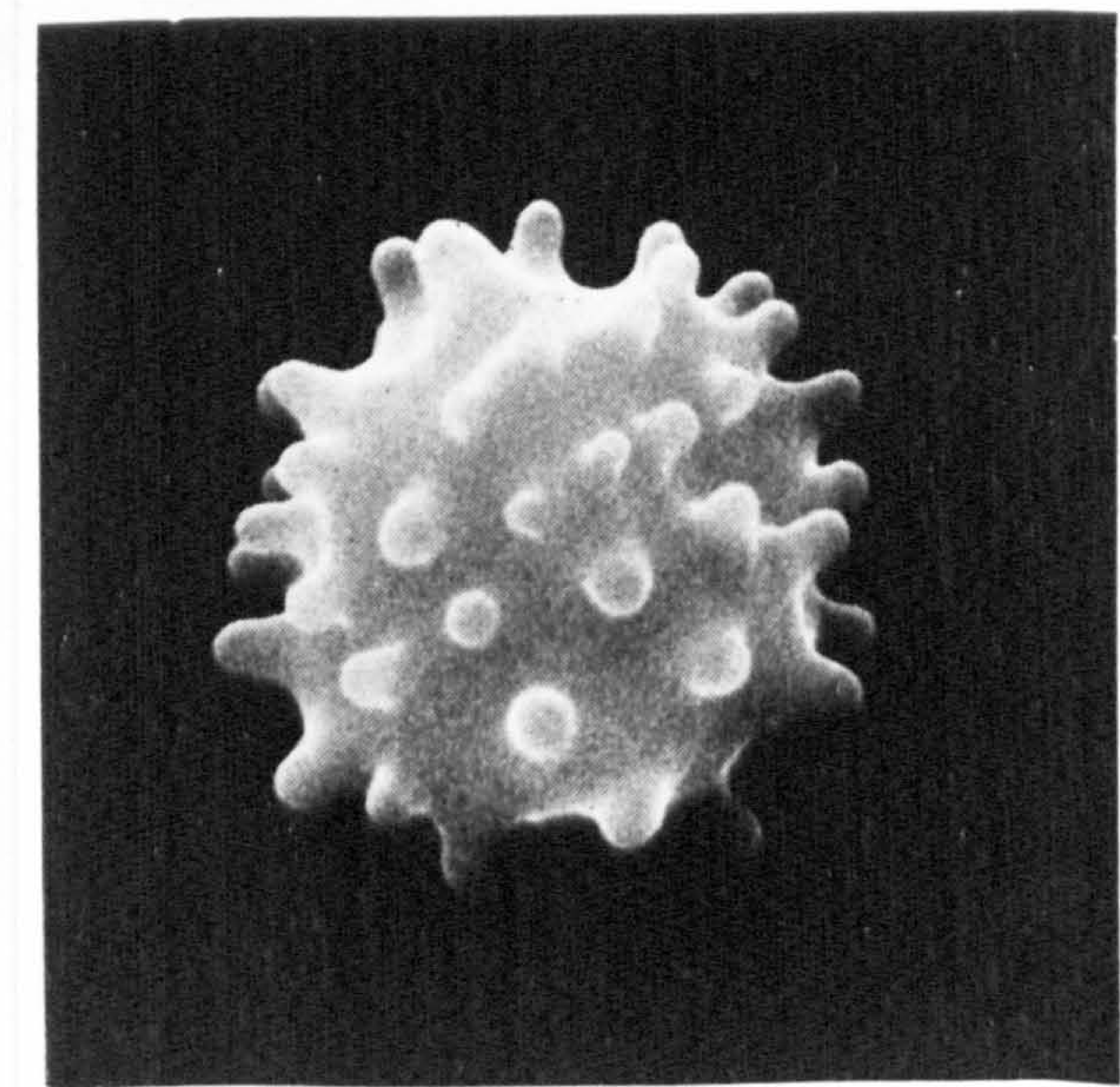
Discocyte

Echinocyte

(From Red Blood Cell Shape, Bessis, M., Weed, R.I. and Leblond, P.F., eds. Springer Verlag 1973)







## ACKNOWLEDGEMENTS

I would like to thank Walter Gratzer for help and encouragement throughout the course of this work. Jenny Fordham, Ernst Ungewickell and Vasken Ohanian have taken part in many useful discussions and given me their help. I would also like to thank Pauline Bennett for electron microscopy, Zoltan Gabor and Steve Boorman for photography and Vivienne Marvell and Vera Calvert for typing.

Professor M.H.F. Wilkins kindly provided me with working facilities and the Science Research Council awarded me a grant.

Finally I would like to thank both my parents for their support during seventeen years of my education and for the interest they have taken since.

The properties of spectrin, the major cytoskeletal protein of the human erythrocyte, and its interactions with the other cytoskeletal elements, have been studied.

A low ionic strength extract from the erythrocyte membrane can be fractionated into pure spectrin and oligomeric complexes containing spectrin. Interactions occur between muscle F-actin and this extract, though not with pure spectrin in physiological conditions. The interaction is shown to be contingent on a minor component in the extract referred to as protein 4.1. Spectrin in its tetrameric (native) state forms a gel with F-actin and 4.1. However, if the dimer (protomer) of spectrin is substituted for tetramer, a complex is formed, but no gelation occurs. These interactions can be interpreted in terms of the formation of a two dimensional cytoskeletal network in the cell between the three proteins.

Spectrin contains equimolar proportions of two polypeptide chains differing slightly in molecular weight. Digestion of each chain with papain in the presence of sodium dodecyl sulphate gives rise to a pattern of fragments which indicate a similarity in sequence between the two chains.

A conformational characterization of spectrin dimer has been carried out. Evidence is given for the presence of both rigid and flexible domains in the molecule. When exposed to increasing concentrations of urea, dissociation of the dimer precedes major unfolding. The two chains can be fractionated

by ion exchange chromatography on a hydroxyapatite column in the presence of urea. They will recombine to give dimer which is indistinguishable from the native protein. Spectrin dimer binds to a proteolytic fragment of protein 2.1 and it is shown that the smaller, but not the larger, subunit is also able to bind to this fragment. The relationship of each subunit to the ternary complex of spectrin, 4.1 and actin has been investigated.

The importance of these results in determining the role played by spectrin in the erythrocyte cytoskeleton is discussed.

# CONTENTS

| | Page |
|---|---|
| Title | 1 |
| Acknowledgements | 2 |
| Abstract | 3 |
| Contents | 4 |
| List of Tables | 10 |
| List of Figures | 10 |
| List of Equations | 11 |
| Abbreviations | 12 |

## Chapter 1 - INTRODUCTION

| | | |
|---|---|---|
| I | Red blood cell membrane function | 14 |
| II | The characterization and arrangement of red blood cell membrane proteins | 20 |
| III | The characterization and arrangement of red blood cell lipids | 24 |
| IV | The molecular properties of actin-binding proteins | 27 |
| V | The properties of actin | 34 |
| VI | The interaction of actin with other proteins | 38 |
| VII | Metabolic and cytoskeletal determinants of red cell shape and deformability | 44 |
| VIII | The cytoskeleton and transmembrane events in red blood cells | 50 |

Page

Chapter 2 - SPECTRIN AND ACTIN IN THE ERYTHROCYTE CYTOSKELETON

I Introduction 66

II Materials and methods

1. The preparation of spectrin dimer, tetramer and oligomer 66
2. The preparation of Triton-extracted ghosts 68
3. The preparation of cAMP independent kinase from red blood cells 68
4. Phosphorylation and dephosphorylation of purified spectrin dimer 69
5. Phosphorylation and dephosphorylation of spectrin in whole red cells 70
6. Preparation of rabbit muscle G-actin 71
7. Estimation of the fraction of denatured actin in a preparation 72
8. The preparation of protein 4.1 73
9. SDS gel electrophoresis 74
10. Electron microscopy 75
11. The binding of crude and pure spectrin to muscle F-actin 75
12. The binding of spectrin to G-actin below the critical concentration 77
13. Gelation of solutions containing mixtures of muscle F-actin and various spectrin-containing preparations 77
14. Cross-linking of spectrin oligomer 78
15. The binding of spectrin dimer to 4.1 and F-actin 79

Page

III Results

1. The binding of spectrin dimer to muscle F-actin — 79
2. The interaction between spectrin and muscle G-actin at physiological ionic strength — 81
3. The interaction between crude spectrin preparations and muscle F-actin — 82
4. The formation of gels between spectrin preparations and muscle F-actin — 83
5. The presence of 4.1 in oligomer and an investigation of its relationship to spectrin and actin in oligomer and Triton-extracted ghosts — 87
6. Interactions between spectrin, 4.1 and actin — 91

IV Discussion

1. Participation of 4.1 in the cyto-skeletal complex — 92
2. Filamentous or monomeric actin in the erythrocyte ? — 96
3. The orientation and accessibility of actin in the erythrocyte membrane — 100
4. The association state of spectrin in the cytoskeleton — 101
5. The regulation of complex formation — 103
   a) Cytochalasin
   b) Phosphorylation
   c) Ionic strength
   d) Temperature
   e) Divalent metal ions

V Summary — 107

Page

Chapter 3 - THE STRUCTURE OF SPECTRIN AND ITS RELATIONSHIP TO THE CYTOSKELETON

I Introduction 133

II Materials and methods

1. The preparation of myosin 133
2. The preparation of filamin 133
3. The preparation of a proteolytic fragment of protein 2.1 135
4. The preparation of hydroxyapatite 136
5. Analytical ultracentrifugation 137
6. Circular dichroism experiments 138
7. Proton magnetic resonance 140
8. Cross-linking experiments 140
9. Digestions of proteins in SDS 141
10. Cyanogen bromide fragments of proteins in SDS 143
11. Determination of the extinction co-efficients of the spectrin subunits 143
12. Determination of tyrosine: tryptophan ratios 144
13. The iso-electric precipitation of the spectrin subunits 145
14. The interconversion of dimer and tetramer 146
15. The binding of spectrin subunits to 4.1 and F-actin 146
16. The binding of the subunits to the proteolytic fragment of ankyrin 146

Page

III Results

1. The conformation of spectrin - a urea denaturation study 148

2. High-resolution proton magnetic resonance of spectrin dimer 157

3. A comparison of the digestion patterns of the two subunits of spectrin, myosin heavy chain and filamin 158

4. The separation of the two subunits of spectrin 162

5. The properties of the two separate subunits 167

6. The interaction between the subunits and other proteins in the erythrocyte membrane 170

IV Discussion 174

V Summary 184

REFERENCES 220

## LIST OF TABLES


| Table | Page |
|---|---|
| 1.1 | 63 |
| 1.2 | 64 |
| 1.3 | 65 |
| 3.1 | 219 |


## LIST OF FIGURES


| Figure | Page |
|---|---|
| 1.1 | 58 |
| 1.2 | 59 |
| 1.3 | 60 |
| 1.4 | 61 |
| 2.1 | 109 |
| 2.2 | 111 |
| 2.3 | 112 |
| 2.4 | 113 |
| 2.5 | 114 |
| 2.6 | 115 |
| 2.7 | 116 |
| 2.8 | 117 |
| 2.9 | 117 |
| 2.10 | 118 |
| 2.11 | 120 |
| 2.12 | 121 |
| 2.13 | 123 |
| 2.14 | 124 |
| 2.15 | 126 |
| 2.16 | 126 |
| 2.17 | 127 |
| 2.18 | 128 |
| 2.19 | 131 |
| 2.20 | 131 |
| 2.21 | 132 |
| 3.1 | 187 |
| 3.2 | 188 |
| 3.3 | 188 |
| 3.4 | 189 |
| 3.5 | 190 |
| 3.6 | 191 |
| 3.7 | 192 |
| 3.8 | 193 |
| 3.9 | 194 |
| 3.10 | 195 |
| 3.11 | 197 |
| 3.12 | 198 |
| 3.13 | 200 |
| 3.14 | 201 |
| 3.15 | 202 |
| 3.16 | 203 |
| 3.17 | 204 |
| 3.18 | 205 |
| 3.19 | 206 |
| 3.20 | 207 |
| 3.21 | 209 |
| 3.22 | 210 |

| | Page |
|---|---|
| 3.23 | 210 |
| 3.24 | 211 |
| 3.25 | 212 |
| 3.26 | 213 |
| 3.27 | 214 |
| 3.28 | 215 |
| 3.29 | 216 |
| 3.30 | 217 |

LIST OF EQUATIONS

| | |
|---|---|
| 3.1 | 137 |
| 3.2 | 139 |
| 3.3 | 139 |
| 3.4 | 144 |
| 3.5 | 147 |
| 3.6 | 147 |
| 3.7 | 147 |
| 3.8 | 152 |
| 3.9 | 156 |
| 3.10 | 156 |
| 3.11 | 160 |
| 3.12 | 160 |
| 3.13 | 168 |
| 3.14 | 169 |
| 3.15 | 169 |

## ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AMP | Adenosine monophosphate |
| AMP-PNP | Adenylyl imidodiphosphate |
| ATP | Adenosine triphosphate |
| Bicine | N,N-bis (2-Hydroxyethyl)glycine |
| Bis | N,N'-methylene bisacrylamide |
| cAMP | Cyclic adenosine monophosphate |
| C D | Circular dichroism |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| E | Extinction coefficient |
| EDTA | Ethylenediamine tetra-acetic acid |
| EGTA | Ethyleneglycol-bis-(β-amino-ethylether) N,N'-tetra acetic acid |
| e.s.r. | electron spin resonance |
| Fab | Fragment (antigen binding) |
| Hepes | N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| HMM | Heavy meromyosin |
| $K_a$ | Association constant |
| n.m.r. | Nuclear magnetic resonance |
| PMSF | Phenylmethylsulphonyl fluoride |
| S | Svedberg unit |
| S1 | Myosin subfragment 1 |
| SDS | Sodium dodecyl sulphate |
| T.C.A. | Trichloroacetic acid |

| | |
|---|---|
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| Tris-HCl | Tris-hydroxymethylaminomethane hydrochloride |
| U.V. | Ultra violet |
| $V_{max}$ | Extrapolated maximum velocity of enzymic reaction at infinite substrate concentration |

# Chapter 1

# INTRODUCTION

## I. Red blood cell membrane function

Red blood cells are containers for the haemoglobin which carries oxygen from the lungs to the tissues. The membrane confers three important attributes on these cells without which function is severely impaired: the non-spherical contour of the membrane (Fig. 1.1) which enables the cell to adopt different shapes without a volume change, the deformable nature of the membrane itself which, together with the cell shape, allows cells to pass through the narrow sinuses of the spleen and through the peripheral circulatory system, and the control of solute migration through the membrane, essential for the regulation of osmotic pressure in the cell. Spectrin, a red blood cell membrane protein which has not yet been found in any other cell type (Hiller and Weber, 1977) and is involved in at least the first two of the membrane functions outlined above, is the subject of this thesis. Advances in an understanding of its structure and interactions with other membrane proteins are described. This is a step towards a full understanding, in molecular terms, of the shape, deformability and communication system of red blood cells.

The most dramatic change observed in red blood cells upon storage is their transformation from discocytes to echinocytes. *In vitro* manipulations can also produce another change: from discocyte to stomatocyte (frontispiece).

Many theories have been put forward to account for the maintenance of red cell shape. Early suggestions of a three-dimensional cage-like structure extending throughout the cell fail to explain that a cell lying on a substratum marked at a point on its surface then subjected to a shearing force can be rolled round whilst maintaining a discoid shape so that the marker migrates with respect to the contour of the cell (Bull, 1973). Murphy (1965) asserted that a differential intercalation of cholesterol into the rims and dimples of the cell led to different interfacial tensions and that this gave rise to the discoid shape. Other workers (e.g. Seeman et al., 1973) have disputed this uneven distribution of cholesterol. A change in the surface area of the inner or outer leaflet of the lipid bilayer with respect to the outer leaflet, either by an electrostatic process or by a change in the bulk of the constituents can explain the effects of most externally added reagents on cell shape. Deuticke (1968) has noted that reagents which induce crenation (the formation of echinocytes) are anionic whereas cationic reagents lead to invagination. Sheetz and Singer (1974) explained these effects by postulating that easily diffusible cations were attracted to the inner leaflet by phosphatidyl serine (the only red blood cell phospholipid with a net negative charge at neutral pH), thus expanding the inner layer, and leading to stomatocytes. Anions, on the other hand, remain in the outer leaflet. The importance of the diffusibility of the reagents used was demonstrated using positively charged tertiary and quaternary amines: the quaternary amine

apparently remained in the outer leaflet causing crenation, whereas the less bulky tertiary amine led to stomatocytosis. The same reagent can produce opposite effects depending on experimental conditions, although equal amounts may have more effect on one monolayer than the other. Mohandas et al. (1978) found that echinocytosis produced by a measured amount of lysophosphatidyl choline in the outer leaflet could be reversed by a much smaller amount in the inner leaflet. The action of phospholipases on the outer leaflet can be used to manipulate cell shape (Fujii and Tamura, 1979). Removal of the phospholipid head groups with phospholipase C or D reduces charge repulsion and allows invagination due to a contraction of the outer layer. On the other hand, phospholipase $A_2$ detaches one fatty acid chain from the rest of the molecule, supposedly increases the bulk of the outer layer and causes crenation. The two effects can be balanced to retain the biconcave disc. Although these reports are reasonable explanations of the effects of added reagents on red blood cells, and of ageing, where there is evidence of phospholipase action (Allan and Michell, 1976), they do not explain the effect of ATP levels on cell shape or the apparent ability of the red cell to regain its shape when subjected to shear stresses _in vivo_ and _in vitro_.

Lux et al. (1976) can remove the majority of lipids from normal and irreversibly sickled cells using Triton X-100 (Yu et al., 1973) leaving proteinaceous cytoskeletons which retain the shape of the cells from which they originated. In 1962, Ohnishi applied the standard prep-

arations of actin and myosin from muscle to red blood cells, obtaining two extracts which showed a viscosity rise when mixed together. Addition of ATP led to a temporary fall in viscosity, just as would be expected for actomyosin. Furthermore, the observation that restoration of the level of ATP in ageing intact red cells led to an echinocyte-discocyte conversion (Nakao et al., 1960) has stimulated an interest in red cell membrane proteins and their response to the metabolic state of the cell, as the key to the mechanism of shape change.

In order to supply sufficient oxygen to the tissues, blood contains about 40% by volume of red blood cells Solid particles at this concentration would have the flow properties of a brick (see Ralston, 1978), so the fluid nature of the cells is essential. As well as lowering the viscosity of blood, the deformability of red blood cells allows their passage through the narrow (0.2 - 0.5μm) sinuses of the spleen (see Weinstein, 1974); the rigid cells of hereditary spherocytotic patients are broken up, causing anaemia. Both red cell shape and the mechanical properties of the membrane contribute to the rheological properties of blood. Spherical cells could not deform without a change in volume whereas this is not true of a biconcave disc. Hochmuth et al. (1979) find that the membrane exhibits an elastic solid-like behaviour, not at all what would be expected from a fluid lipid bilayer. Evidence for the involvement of proteins in these elastic properties comes firstly from Dubbelman et al. (1977) and Fischer et al. (1978) who have found that sulphydryl reagents can decrease

the deformability of red cells, an effect reversible by DTT. Cross-linking shows that spectrin is the main protein involved. Secondly, Evans et al. (1976) have measured the resistance to area expansion of red cells which have been pre-incubated at 50°C and find a similar value to that for a lipid bilayer. Brandts et al. (1977) have shown that spectrin unfolds at about 49°C. Thirdly, spectrin antibody can also decrease deformability although Fab fragments have no effect (Nakashima and Beutler, 1978). The echinocytic shape of senescent cells has already been discussed but there are reasons to believe that this is not the factor leading to their decreased deformability. The effect of calcium on red cells, first described by La Celle et al. (1973) has been explained by Lorand et al. (1978) in terms of a calcium-activated transglutaminase which leads to the irreversible cross-linking of several membrane proteins, into a rigid structure. Despite the well established dependence of deformability on red cell membrane proteins, almost nothing is known about the special properties of these proteins which make this behaviour possible.

Although the red cell membrane must be an important barrier to regulate information and molecular flow between the cell interior and the environment, there is very little evidence, apart from the transport of ions, for *in vivo* transmembrane events which regulate cell function. The sodium/potassium pump maintains a low sodium ion concentration and a high potassium ion concentration inside the cell, working against a constant leakage of each of these cations down a chemical gradient across the membrane.

If the pump is inhibited and passive leakage is allowed, the membrane potential causes a net influx of cations, and therefore water, leading to haemolysis. The cell has also a calcium pump requiring ATP. In depleted cells, an influx of calcium leads to irreversible rigidity due to the action of the transglutaminase (described above) and the proteolysis of many membrane proteins (Tökes and Chambers, 1975; Anderson et al., 1977). There is a system for anion exchange which is important for the release of carbon dioxide from cells in the form of the bicarbonate ion. Prostaglandins are hormones which normally exert a regulatory function by modulating the action of other hormones. At a level of only one or two molecules per red cell, they have been shown by electron spin resonance to alter the microviscosity of the lipid bilayer of red cells (Kury et al., 1974). Although they are known to regulate blood flow, as yet there has been no explanation of their effects on red blood cells. Recently Nelson et al. (1979) have found that μM noradrenaline stimulates the phosphorylation of several red blood cell proteins. Strangely the kinase involved is cAMP independent, implying that this is not the usual adrenergic response. The phenomenon of patching, capping and endocytosis which occurs in lymphocytes and fibroblasts (Taylor et al., 1971) is not observed in red blood cells. Only a limited endocytosis is observed in special conditions ( Schekman and Singer, 1976) suggesting interesting differences between these membranes and those of fibroblasts and lymphocytes (see 1.VIII).

## II. The characterization and arrangement of red blood cell membrane proteins

Since 99% of the protein in a red blood cell is haemoglobin, its removal is necessary before the membrane proteins can be studied. Fortunately, the remarkable phenomenon of hypotonic osmotic lysis, in which haemoglobin is lost from the cell through pores between 10 and 25nm in diameter (Baker, 1967) after which the membrane can be substantially resealed by incubation with divalent cations or in isotonic buffer, allows the preparation of "ghosts", the properties of which are often similar to those of intact cells.

The characterization of membrane proteins is difficult, due to the hydrophobic nature of some of them, but studies have been greatly advanced by the use of detergents. The discovery that electrophoretic mobility of proteins complexed with the anionic detergent, SDS, bore a simple relationship to their molecular weight (Shapiro et al., 1967) has enabled the major erythrocyte membrane proteins to be enumerated (Fairbanks et al., 1971). Fig. 1.2 shows an SDS gel of the membrane proteins using the buffer system of Fairbanks et al. alongside another using the system of Laemmli and Favre (1973). In addition, there are two proteins containing such a high carbohydrate content that they do not stain with Coomassie Brilliant Blue but can be revealed using periodic acid Schiff reagent. These are glycophorins A and B. The part of glycophorin A on the outside of the cell carries most of the red blood cell sialic acid, the MN blood group antigens and receptors for

influenza virus and for lectins such as phytohaemagglutinin, wheat germ agglutinin and fucose binding protein. Glycophorin B carries various blood group antigens and the wheat germ agglutinin receptor. The function of these glycophorins is unknown, since mutants which lack them show perfectly viable red cells. Table 1.1 (from Lux, in press) shows the approximate number of copies of each protein in one cell, as estimated from SDS gel densitometry. Numbers must be regarded with caution as Potter (1972) has shown a discrepancy between the affinity of different proteins for Coomassie Brilliant Blue. The observation that some proteins could be detached from the membrane by variations in pH or ionic strength while the solubilization of others required either non-ionic detergents such as Triton X-100 or powerful denaturants such as guanidine hydrochloride, led to the categorization of membrane proteins as either "integral" or "peripheral" (see Singer, 1974). Spectrin and band 5 are extracted from ghosts in low ionic strength buffers (Marchesi and Steers, 1968) and are therefore regarded as peripheral proteins. The observation of filaments in these preparations and their decoration with myosin subfragment 1 (Tilney and Detmers, 1975) show the presence of actin. The loss of these two proteins results in the disintegration of the ghosts into small vesicles, suggesting that a protein structure is responsible for supporting the membrane under normal circumstances. Band 6 is also a peripheral protein, which can be eluted at a high ionic strength, (above 0.1M) and has been identified as glyceraldehyde-3-phosphate dehydrogenase, an enzyme

involved in glycolysis (Kant and Steck, 1973). In contrast, Triton X-100 solubilizes part of band 3 and the glycophorins which are therefore considered to be partly or wholly intercalated into the lipid bilayer (Yu et al., 1973). The residue after Triton extraction at an ionic strength of 0.04M contains the remainder of the membrane proteins and retains a ghost-like morphology affording evidence for a cytoskeleton.

The disposition of the major membrane proteins has been studied by comparing the effects of various reagents on intact cells and leaky ghosts. Lactoperoxidase-catalyzed iodination (Phillips and Morrison, 1970), covalent labelling with a low molecular weight probe (Bretscher, 1971a,b,c) and enzymatic digestions (Triplett and Carraway, 1972), all indicate that band 3 and glycophorin span the bilayer, protruding at either side, displaying their carbohydrate groups to the extracellular space. Band 3 has been identified as the anion exchange protein and also contains the specific receptor for concanavalin A. The N-terminal, cytoplasmic portion has specific binding sites for band 6, aldolase, 4.2 and 2.1 (Steck, 1978). Spectrin, actin, 6 and 4.1 are located exclusively on the inner face and the dispositions of 4.2 and 7 are uncertain. Further experiments with ferritin conjugated antispectrin antibody confirm the position of spectrin on the inside of the membrane (Nicolson et al., 1971) (Fig. 1.3). In the technique of freeze fracture, a frozen sample of membrane is cut with a razor blade, and a platinum replica is made of the exposed surfaces. It has been shown (Pinto da Silva and Branton,

1970) that the lipid bilayer of the erythrocyte is often cleaved into monolayers by this treatment and that numerous intra-membrane particles, about 8nm in diameter, more densely distributed on the inner than the outer half of the bilayer can be seen on the surface of the replicas. These are homogeneous in size, and randomly distributed.

What constitutes the intra-membrane particles is not clear. According to Sharom et al. (1977), either band 3 or glycophorin will, when reconstituted with lipids, give rise to intra-membrane particles. Lutz et al. (1979), on the other hand, by Triton X extraction of ghosts, obtain glycophorin rich vesicles which have no intra-membrane particles. Edwards et al. (1979) freeze fractured a single layer of red blood cells between a poly-lysine coated cover clip and a copper plate, a system which cleaves the membrane such that enough of the external monolayer remains attached to the coverslip to allow biochemical analysis. This monolayer contains the greater part of glycophorins A and B whereas band 3 is found in the inner leaflet. It is suggested that the strong negative charge of sialic acid causes the glycophorin to remain bound to the positively charged coverslip whereas band 3, linked to the intracellular cytoskeleton (1.VIII), remains in the inner leaflet. Some small fragments observed on SDS gels show that the glycophorin has been covalently cleaved. The different particle densities observed on each fracture face can be explained by this differential distribution of band 3 and glycophorin between the monolayers. The situation is further complicated by the observation of calcium-induced lipidic particles which

closely resemble intra-membrane particles (Verkleij and Ververgaert, 1978).

Bifunctional cross-linking (Wold, 1967) has been favoured as a technique to probe the juxtaposition of proteins in the red cell membrane. Many reagents lead to the cross-linking of bands 1 and 2 and to the dimerization of band 3. In addition, Steck (1972) has observed $(1)_2$ and $(2)_2$ as well as multiples of 2.1. Wang and Richards (1974) obtain dimers and trimers of actin as well as a complex containing spectrin and 4.1. The only work which has demonstrated the juxtaposition of actin and spectrin is that of Liu et al. (1977). Other unusual links such as 1+3, 2+4.9 and 4.9+5 also appear. Some of these phenomena may be due to the low pH (less than 5) at which the reaction was carried out. Such perturbations of the membrane by the reaction conditions and also the inconclusiveness of negative results in cross-linking experiments, limit the usefulness of these results to confirmatory evidence for other experiments. More direct proof of the interactions between some of these polypeptides will be discussed (1.VIII, 2.III.6, 3.III.6) as will their possible dependence on the metabolic state of the cell (1.VII, 2.IV.5).

## III. The characterization and arrangement of red blood cell lipids

The lipid bilayer model of cell membranes, proposed by Gorter and Grendel in 1929, where the polar head groups of phospholipids are exposed to the aqueous medium inside and outside the cell, and the fatty acid chains are sequestered

in the heart of the bilayer, still provides the most satisfactory explanation for most membrane phenomena.

The phospholipid content of the red blood cell membrane consists mainly of four different molecules: 28% is phosphatidyl choline, 26% phosphatidyl ethanolamine, 25% sphingomyelin and 13% phosphatidyl serine, each of which contain two fatty acid chains attached to a glycerol backbone (Turner and Rouser, 1970). About 25% of red blood cell lipid by weight is cholesterol (see Van Deenen and de Gier, 1974).

The phospholipid components are distributed asymmetrically between the two monolayers; about 80% of the sphingomyelin and 75% of the phosphatidyl choline are found in the outer leafet while 80% of the phosphatidylethanolamine and all the phosphatidyl serine are in the cytoplasmic layer (Verkleij et al., 1973). There is some evidence that the outer layer contains slightly more cholesterol (Fisher, 1976). Nmr and esr signals from spin labels in cell membranes reveal different classes of signals, consistent with the presence of an annulus of immobilized lipid surrounding the integral proteins. There is some evidence for more specific lipid-protein interaction: β-hydroxybutyrate dehydrogenase has a higher activity in mixtures of phosphatidyl choline and phosphatidyl ethanolamine than in phosphatidyl choline alone (Lee, 1977). However the binding between single lipid molecules and proteins has not been demonstrated. Thus, lipid asymmetry may exist within monolayers, as well as between them.

Spectroscopic and photobleaching techniques have also shown that lateral motion of lipids takes place with a

diffusion constant of about 2-5 x $10^{-8}$ $cm^2$/sec. (Lee et al., 1973) and experiments demonstrating the redistribution of proteins in the plane of the membrane (1.VI, 1.VIII) have led to the idea of membranes as a two-dimensional solution of integral proteins dispersed in a fluid lipid matrix. In addition to lateral motion, the fatty acid chains themselves are waggling about $10^9$ times/sec. with increasing amplitude towards the end furthest away from the phospholipid head group (Hubbell and McConnell, 1969). In contrast, the movement of phospholipids between the two halves of the bilayer (flip-flop) in both phospholipid bilayers (Rothman and Dawidowicz, 1975) and red blood cells (Rousselet et al., 1976; Renooij et al., 1976) is much slower and has a half-time of several hours. However, some mechanism is still required to maintain this type of phospholipid asymmetry during the 120 day life span of the red blood cells.

Theories as to the functional significance of phospholipid motion and asymmetry are at present speculative. It is possible that the binary mixture of phosphatidyl serine and phosphatidyl ethanolamine in the inner leaflet of the erythrocyte membrane must be screened from the serum because it is particularly efficient in activating intravascular clotting (Zwaal et al., 1977). The possible effect of the negatively charged phosphatidyl serine on cell shape has been discussed (1.I). Although flip-flop is slow in erythrocytes, it is actually about ten times faster than in pure phospholipid bilayers. In order to explain this, and also the otherwise unfavourable apposition of membrane lipids during the fusion of two bilayers, Cullis and De

Kruijff (1979) have suggested that other phospholipid phases, such as inverted micelles, could allow these processes to take place, without the large free energy change needed to transport a phospholipid head group through the hydrophobic part of the membrane.

## IV. The molecular properties of actin binding proteins

The large molecular weights of the two spectrin subunits, reports of an ATPase activity (Weidekamm and Brdiczka, 1975 and Kirkpatrick et al., 1975), and the coexistence of spectrin with actin on the membrane led to speculations on the myosin-like nature of spectrin and the possibility of acto-myosin like shape changes in the cell. Other, recently isolated, high molecular weight actin binding proteins such as filamin from smooth muscle (Wang, 1977; Shizuta et al., 1976) and actin binding protein from macrophages (Hartwig and Stossel, 1975) show some properties in common with spectrin. Table 1.2 summarizes a comparison between myosin, spectrin, filamin and actin binding protein, and Fig.1.4 shows their subunit structures.

Variable molecular weight estimates both from SDS gels and equilibrium sedimentation as well as reports of multiple N-terminal groups of spectrin (Knüfermann et al., 1973; Dunn et al., 1975) led to a theory that spectrin consisted of a number of small polypeptide chains which SDS was unable to disaggregate. However, Hudson and Ralston (1978), using quantitative transamination, observed only one N-terminal group per 240,000 daltons. Furthermore, Pinder et al. (1976) found that the molecular weight of the spectrin subunits

could not be reduced below 2-2.5 x $10^5$ by either guanidine thiocyanate or trifluoroacetic acid. The molecular weights of the subunits of spectrin, myosin heavy chain, filamin and actin binding protein are all similar and are some of the largest known polypeptides.

The solubility of spectrin does not depend on ionic strength. It displays the rather unusual behaviour of a sudden decrease over a pH range of only 0.3 units, culminating in complete precipitation at a pH of about 5.2 (Gratzer and Beaven, 1975). The association state is dependent on the temperature at which spectrin is extracted: 37°C yields a dimer (Gratzer and Beaven, 1975) with polypeptide chains of molecular weight 240,000 and 220,000 as estimated from SDS gels (Clarke, 1971) whereas at 4°C, tetramer is extracted (Ralston et al., 1977). The two can be interconverted by a manipulation of temperature and ionic strength, low temperatures and physiological ionic strengths favouring the tetramer. At 37°C and an ionic strength of less than 0.01M conversion to dimer is complete within 10 minutes. Tetramer formation at 23°C and physiological ionic strength, however, takes several days. The high activation energies for this interconversion are unusual for proteins. Further association can occur in millimolar concentrations of calcium or magnesium but the results are irreproducible (Ungewickell and Gratzer, 1978).

The skeletal muscle myosin molecule consists of six subunits: two heavy chains of molecular weight 190,000 each and four light chains of molecular weight about 20,000. The larger part of the heavy chains forms a rigid rod whose

structure is that of a coiled-coil α-helix (Cohen and Holmes, 1963). Two globular domains, the heads, both at the same end of the rod, and each carrying two light chains, are thought to be involved in the interactions between actin and myosin, whereas the rod takes part in the aggregation of myosin to form filaments. Myosin can be digested with trypsin, chymotrypsin, or papain, producing single globular heads (subfragment-1) or the two heads joined to a short stretch of rod (heavy meromyosin) (Fig. 1.4). Both these fragments have been particularly useful because unlike myosin, they are soluble at physiological ionic strength (see Lowey, 1971).

At physiological ionic strength, myosin assembles into thick filaments closely resembling those found in muscle. In 0.5M potassium chloride and 0.2M phosphate buffer the majority of the protein is in a monomeric or dimeric form although the slow formation of an aggregate has been observed. The equilibrium between monomer and dimer is concentration, pH and salt dependent (Godfrey and Harrington, 1970).

Attempts to make filaments from filamin (Shizuta et al., 1976) have failed. The sedimentation coefficient and Stokes radius yield a value for the molecular weight of about 500,000 whereas only one band of molecular weight 250,000 appears on SDS gels, indicating a dimeric state. Actin binding protein is soluble at high and low ionic strength but not in 0.1M potassium chloride. The subunit molecular weight on SDS gels is 270,000 and the Stokes radius the same as spectrin tetramer but the molecular weight of the

protein is not known (Hartwig and Stossel, 1975; Stossel and Hartwig, 1976). So far as is known, only myosin out of these four proteins possesses light chains.

The sedimentation coefficient for an unhydrated globular protein of molecular weight 500,000 would be 23S. The value for myosin: 6.6S is compatible with a rigid rod structure whereas the rather higher values for filamin and spectrin (10S and 9.7S respectively) indicate more flexibility or a lower asymmetry. Light scattering studies of spectrin, Elgsaeter (1978) have shown that the radius of gyration of spectrin changes appreciably when the ionic strength is reduced from 0.1M to 0.001M making a rigid structure unlikely. The conclusion that spectrin contains flexible elements is strengthened by its appearance in the electron microscope after negative staining (Fig. 3.20, Tilney and Detmers, 1975) and after shadowing (Shotton et al., 1979). Images of shadowed dimer indicate that the subunits lie alongside one another, are usually joined at their ends and show varying numbers of contact points in between. The pictures of tetramer are most easily interpreted as an end-to-end association of the dimer, with the two subunits often separated where they bind to the other dimer. The energy required to bring about this separation could explain the large activation energy for dimer to tetramer conversion.

Although Bjerrum et al. (1974) found an immunological cross-reaction between antibodies to the two subunits of spectrin, Sheetz et al. (1976) and Kirkpatrick et al. (1978) found none. Wallach et al. (1978) found a cross-reaction between filamin and actin binding protein but none between

filamin and spectrin, and Wang et al. (1975) found none between myosin and filamin. A weak cross-reaction was found between band 2 of spectrin and uterine muscle myosin (Sheetz et al., 1976).

Table 1.3 shows the amino-acid compositions of the four proteins. There are no striking differences, which is not surprising for a set of proteins of about the same size, which are all non-globular.

The fuel used directly by the contractile apparatus in muscle is ATP, and it is actin and myosin as molecules which allow the hydrolysis of ATP and the extraction of work from it. Skeletal muscle myosin alone in the unphysiological environment of 0.6M potassium chloride and the absence of magnesium and calcium at 25°C has an ATPase as high as 30 moles phosphate/mole active site/sec., although values for smooth muscle and non-muscle myosins can be three orders of magnitude lower (see review by Korn, 1978). In the more realistic environment of 0.15M potassium chloride, 5mM magnesium chloride and 5mM ATP, pH 7 only 0.02 moles phosphate/mole active site/sec. are hydrolyzed. The presence of actin in muscle allows the coupling of ATP hydrolysis to the production of mechanical work and the ATPase rises to about 17 moles phosphate/mole active site/sec. This value is not attained by mixtures of actin and myosin in solution, probably because of steric factors, since a much higher value is obtained with heavy meromyosin (see Tonomura, 1972).

There have been several reports of an ATPase in the low ionic strength extract from erythrocyte ghosts, activated or inhibited by various combinations of calcium and magnesium

(Rosenthal et al., 1970; Weidekamm and Brdiczka, 1975; Kirkpatrick et al., 1975). However, in no case has the ATPase been convincingly located on spectrin. In fact, White and Ralston (1976) separated the proteins in the low ionic strength extract by native electrophoresis and located an ATPase by incubation of the gel with ATP and lead chloride, followed by sodium sulphide. The gel band showing a lead sulphide precipitate was further analyzed by SDS gel electrophoresis where it was found to have a subunit molecular weight of 100,000. Gel filtration yielded a molecular weight of about 640,000. The level of this ATPase was not measured. It had a requirement for magnesium and was inhibited by calcium, but only at low magnesium concentrations. Thus, there is no evidence for a myosin-like ATPase on spectrin. Neither filamin nor actin binding protein have a potassium/EDTA ATPase similar to that of myosin.

Over a hundred proteins are known to undergo phosphorylation and dephosphorylation in the presence of specific kinases and phosphatases respectively. However, in very few cases is the function of this process at all clear, a notable example being the control of glycogen breakdown by a cascade of enzymes involving two different phosphoproteins, allowing amplification of the original hormonal stimulus and also control of glycogen breakdown both by this stimulus and by muscle contraction (see Fischer et al., 1974). Filamin and actin binding protein can each be phosphorylated by a cAMP dependent enzyme. In myosin, it is one species of light chain (which has no analogue in the other actin-binding proteins) which is phosphorylated, and this can be regulated

by a specific light chain kinase and phosphatase. *Acanthamoeba castellani* myosin II is an exception, where phosphorylation of the heavy chain takes place (Maruta and Korn, 1977).

A cAMP independent spectrin kinase can be eluted from red blood cell membranes in 0.4M sodium chloride (Hosey and Tao, 1977) and the incorporation of $^{32}P$ from $\gamma^{32}P$-ATP into band 2 of purified spectrin dimer can be demonstrated (Pinder et al., 1977). Under these conditions about 0.4 moles of phosphate per mole of spectrin dimer are incorporated. Although patterns of phosphorylation often differ greatly between intact cells and extracts from them, the same result is obtained from red cell membranes incubated with $^{32}P\ \gamma$-ATP and in intact cells incubated with radiolabelled inorganic phosphate (Wolfe and Lux, 1978). Phosphatase activity using partially purified spectrin as a substrate has been demonstrated in the haemolysate (Graham et al., 1976). There is a cAMP dependent kinase which affects bands 2.1 and 4.1 as well as two other bands between 4 and 5 but this enzyme has little effect on spectrin (Hosey and Tao, 1976a). Incubation of intact red cells with $^{32}P$ phosphate at 37$^{o}$C leads to the incorporation of almost 4 moles of phosphate per mole of band 2 but the number of phosphorylation sites involved is not known (Harris, 1980; Anderson and Tyler, in press). All the phosphorylation sites occur within 20,000 daltons of the C-terminal end (Harris, 1980)

## V. The properties of actin

The properties of actin have been reviewed (Oosawa and Kasai, 1971). In a low ionic strength medium, rabbit skeletal muscle actin exists as a soluble globular protein of molecular weight 42,000. It has a binding site for ATP with a $K_a$ of $2.3 \times 10^6 M^{-1}$ (at low ionic strength), 5 to 7 low affinity sites for divalent cations and one high affinity site where the ratio of the association constants for calcium and magnesium is about 5:1 (Strzelecka-Golaszewska, 1973). Since intracellular magnesium concentrations are about $10^3$ times those of calcium, this site is occupied *in vivo* by magnesium. Removal of either the nucleotide or tightly bound metal ion from actin leads to its denaturation. Addition of 0.1M salt or a moderately high concentration of magnesium leads to the polymerization of actin monomers into filaments. This change can be monitored by the dramatic rise in viscosity which ensues, by light scattering, and in several other ways. Electron microscopy shows that two helical chains are twisted round each other in the filament. *In vitro* polymerization leads to F-actin of variable length whereas in skeletal muscle the length of F-actin filaments is determined by the structure of the sarcomere. Oosawa and Kasai (see 1971) have predicted the length distribution of *in vitro* formed filaments. The observation of an exponential distribution of lengths in an electron microscope study (Kawamura and Maruyama, 1970) confirms the theory. At magnesium concentrations of about 15mM or more, interactions occur

between filaments resulting in sheet-like paracrystals (Hanson, 1966). Similar structures can be induced by many proteins which bind to F-actin, such as nerve growth factor (Calissano et al., 1978), and two unidentified polypeptides in the acrosomal process of Limulus sperm (Tilney, 1975). Polymerization of actin is accompanied by the release of inorganic phosphate and the nucleotide bound to F-actin is ADP. However, if ATP is replaced by the non-hydrolyzable nucleotide, AMP-PNP, polymerization occurs at the same rate, and the AMP-PNP-F-actin is not known to differ from ADP-F-actin (Cooke and Murdoch, 1973). The polymerization of actin is similar to the condensation of gas to liquid. There is always a critical concentration of G-actin below which polymerization does not take place and in solutions of F-actin, this critical concentration of G-actin is always present. Polymerization is a cooperative phenomenon as is shown by the dependence of the initial rate on the third or fourth power of the actin concentration and the acceleration that can be produced by adding small fragments of F-actin obtained by sonication. Thus, the presence of substances which stabilize actin nuclei can give rise to F-actin in a solution in which it would otherwise be kinetically trapped in the monomeric state in an analogous way to a super-cooled liquid. The polymerized state of actin can also be thermodynamically favoured in the presence of, for example, phalloidin to which it can be bound in equimolar proportions (Dancker et al., 1975). The interaction of F-actin with heavy meromyosin or subfragment 1 gives the appearance of a series of arrow-

heads in the electron microscope after negative staining (Huxley, 1963). If this so called "decorated" actin is incubated with G-actin under polymerizing conditions, filaments with a bare end can be seen with negative staining. The barbs of the arrow heads always point towards the bare end of the filament, showing that it is here that growth takes place (Woodrum, 1975).

At intermediate salt concentrations, if the ratio of G to F-actin is allowed to stabilize at about 1, ATP hydrolysis can still be observed, suggesting that a steady state condition exists, involving constant polymerization and depolymerization of the actin. It is possible that actin monomer is constantly being added to the "barbed end" of the filament and removed from the "pointed end" in a dynamic equilibrium (Wegner, 1976). Attachment of a protein or protein complex to either end of the filament could then disturb the equilibrium in favour of the G or F form. In most cases where F-actin is attached to cell membranes as for instance in intestinal brush borders (Tilney and Mooseker, 1971), the "barbed end" is proximal to the membrane. A number of motile events such as the formation of the acrosomal process of the Thyone sperm (Tilney, 1976a) and the movement of fibroblasts (Goldman et al., 1975) are associated with the formation of bundles of F-actin filaments. It is not clear whether these result from an actual polymerization of G-actin or from the aggregation of previously formed filaments. Only in platelet activation by thrombin has the G to F transformation been demonstrated, using a newly developed biochemical test

for the presence of monomeric actin (Carlsson et al., 1979).

Actin is the second most abundant protein known and has been found in most cell types ranging from the unicellular *Acanthamoebae* to the highly differentiated vertebrate skeletal muscle cell. The non-muscle actins have been reviewed by Korn (1978). They are highly conserved: only 6% of the residues vary between actins from *Acanthamoeba castellani* and rabbit skeletal muscle. The molecular weight is always about 42,000 and the requirement for nucleotide and divalent metal ion for the maintenance of native structure is invariant. All non-muscle actins are able to polymerize though most have a higher critical concentration than that of skeletal muscle. The critical concentration of erythrocyte actin has not been determined. The technique of isoelectric focussing yields three actins: α, β and γ. In addition, two unstable forms δ and ε which are thought to be metabolic intermediates leading to β and γ actin respectively, can be seen. Skeletal muscle actin is exclusively of the α variety whereas most non-muscle cells contain both β and γ actin (Garrels and Gibson, 1976 and see Hunter and Garrels, 1977). Pinder et al. (1978) found only β-actin in the erythrocytes whereas both Garrels and Gibson (1976) and Nakashima and Beutler (1979) claim to see β and γ actin. However, the former authors do not show evidence for their conclusion and the latter show an isoelectric focussing pattern which lacks two, clearly distinguishable bands.

Less than 5% of the protein in liver cells and erythrocyte membranes is actin whereas the highly motile

amoebae and human blood platelets contain as much as 20-30% actin. These values lead to a cellular actin concentration well above the critical value and, since actin can be extracted in a non-polymerized form from these cells at concentrations up to 10 mg/ml, factors which retain it in this form must be present.

## VI. The interaction of actin with other proteins

Myosin and heavy meromyosin each bind to F-actin but dissociation takes place in the presence of ATP. In the electron microscope, filaments lose their decoration if ATP has been added to a mixture of actin and heavy meromyosin. At physiological ionic strength, conditions in which myosin aggregates into filaments, the addition of ATP to a mixture of actin and myosin results in the formation of large clusters composed of both proteins which settle to the bottom of the containing vessels, a phenomenon named super-precipitation. This inhomogeneous system is not well understood; heavy meromyosin has proved more useful in studies of actin-myosin interactions. Actin-activated ATPase can, however, be demonstrated with either heavy meromyosin or myosin (see Tonomura, 1972). In whole skeletal muscle, it is well established that actin and myosin are arranged in sets of interdigitating filaments. The sliding of the actin and myosin filaments over each other, by means of constant attachment and detachment of the globular heads of the myosin molecule on the actin filament concomitant with acto-myosin ATPase activity is the molecular explanation for the contraction of muscle

(see review by Huxley, 1969). Thin (actin) filaments *in vivo* are associated with the two proteins tropomyosin and troponin. Tropomyosin is an elongated molecule consisting of two dissimilar subunits of molecular weight about 34,000 lying in the groove of the actin helix. There is one tropomyosin and also troponin complex for every 7 actin monomers. Troponin consists of 3 subunits, one of which binds calcium, and it has been shown that the interaction between thick and thin filaments is prevented unless this calcium binding site is occupied. In resting muscle, the calcium concentration is about $10^{-8}$M and activation of concentration takes place at about $10^{-6}$M calcium (Ebashi et al., 1969). A variety of other mechanisms which regulate the interaction of actin with myosin have been found in other cell types. Molluscan muscle is thick filament regulated by means of calcium binding to one of the light chains. Gizzard smooth muscle myosin undergoes calcium dependent light chain phosphorylation which controls its contractile function (see Szent-Gyorgyi, 1975).

Both actin binding protein and filamin are found in cells which also contain myosin. Both form bridges between actin filaments giving rise to gels containing actin bundles (Hartwig and Stossel, 1975; Wang and Singer, 1977). Several tenths of a mg/ml of filamin and F-actin are needed to form the gel. Actin binding protein has more potent cross-linking abilities; gelation can occur at a molar ratio of 750 actins to 1 actin binding protein (Brotschi et al., 1978). Cultured cells have been labelled with antibodies to myosin, actin and filamin (Heggeness et al., 1977) and

tropomyosin (Lazarides, 1976). Particularly interesting was the distribution of these proteins in the ruffles, microspikes and areas of cell-cell contact. There was very little myosin or tropomyosin, whereas filamin and actin were present here as elsewhere in the cell. This result is consistent with the inhibition of tropomyosin-actin interaction by filamin which has been found *in vitro* (Maruyama and Ohashi, 1978; Zeece et al., 1979).

Very little is known about the distribution of actin binding protein in macrophages. However, the amount of this protein which is extracted at low ionic strength increases in phagocytozing macrophages over that in resting cells (Stossel and Hartwig, 1976). Presumably, inextractable protein is either aggregated or membrane-bound. Pseudopodia formed by macrophages grown on nylon wool fibres can be separated from the cell bodies and contain the greater part of the cell myosin and actin binding protein and an increased amount of actin (Hartwig et al., 1977). Exactly how these proteins are organized during ingestion is not known.

Whereas filamin inhibits actin-activated myosin ATPase (Davies et al., 1977), as does a proteolytic fragment, heavy merofilamin (Davies et al., 1978), neither actin binding protein (Stossel and Hartwig, 1976) nor spectrin (Puszkin et al., 1978) have any effect. This could mean that, in the presence of filamin, actin may have a cytoskeletal role, in contrast to its contractile role in the presence of myosin (Pollard, 1976). In many motile cells, gelation of crude cell extracts containing actin and other

proteins takes place (see Taylor and Condeelis, 1979) and is inhibited by μM calcium. Recently, two specific calcium-binding proteins responsible for this inhibition have been isolated (Yin and Stossel, 1979; Mimura and Asano, 1979). Thus, if actomyosin based contractility in these systems is calcium activated and gelation does occur *in vivo*, the same conditions which release actin from gels could lead to its participation in contraction. Further, Condeelis and Taylor (1977) have found that gelled extracts from *D. discoideum* are unable to contract until calcium is added, when the gel breaks down. However, contraction can be brought about when the gel is dissolved by lowering the temperature from 25°C to 6°C in the absence of calcium. Thus, the contraction, thought to be due to an actomyosin system, may be controlled through dissolution, which may or may not be calcium dependent *in vivo*. Taylor proposes this mechanism of contraction regulation to explain the apparent absence of tropomyosin and troponin in many non-muscle cells. Other regulatory processes may, however, be involved (see review by Hitchcock, 1977).

In some cells, actin has been found in a depolymerized state, bound to several different proteins which could perform a regulatory function by preventing polymerization and explain the isolation of G-actin from these cells at high concentrations (1.V). DNAse I from calf spleen with a molecular weight of 30,000 (Lindberg, 1967) binds to G-actin with a $K_a$ of $10^8 M^{-1}$ whereas $K_a$ for F-actin is only $10^4 - 10^5$. DNAse depolymerizes F-actin, even in the presence of tropomyosin-troponin, but not if heavy meromyosin is

added (Lazarides and Lindberg, 1975; Hitchcock et al., 1976). The isolation of DNAse was closely followed by that of profilin from spleen (Carlsson et al., 1976) with a molecular weight of about 16,000. This protein accounts for all the actin extracted in unpolymerized form from human platelets (Harris and Weeds, 1978) and has also been isolated from *Acanthamoeba castellani* (Reichstein and Korn, 1979). Recently, F-actin depolymerizing activity has been found in human serum (Norberg et al., 1979).

The involvement of actin in the patching and capping of cell surface receptors has become more firmly established in the last few years. If B lymphocytes, which display immunoglobulins at their cell surface, are labelled with fluorescent anti-immunoglobulin, a cap of fluorescent material can be seen at one end of the cell (Taylor et al., 1971). If labelling is performed at $4^{o}C$, patches of fluorescent label form and after subsequent warming at $37^{o}C$ the patches gather into a cap. Pinocytosis follows, giving rise to fluorescent vesicles inside the cells and after several hours, new cell surface receptors appear presumably as a result of *de novo* protein synthesis. If the cell surface is labelled with monovalent Fab fragments, the receptors are randomly distributed, showing that a cross-linking process is necessary for both patching and capping. *In vivo*, B lymphocytes are not exposed to anti-immunoglobulins, but the antigens for which they are designed as receptors can be multivalent. These antigens also produce capping, showing that it is not an artefact. Other cells such as fibroblasts exhibit the same phenomenon, which can

also be induced by the binding of the multivalent lectin, concanavalin A. After the difficulties of raising antibodies to such a well conserved protein as actin had been overcome, fluorescent antibody labelling has shown that filaments of actin underlie caps produced by both immunoglobulins (Gabbiani et al., 1977) and concanavalin A (Toh and Hard, 1977). Biochemical evidence for the involvement of actin has come from Condeelis (1979) who has shown that detergent extraction of *D. discoideum* to which concanavalin A has been bound, results in a complex containing concanavalin A, actin and myosin. The same amount of the latter two proteins remain in the complex throughout capping, though their release begins as soon as the cap is formed. Myosin can be dissociated by the addition of ATP, whereas actin remains bound even after extraction at low ionic strength, suggesting that F-actin filaments, which can be seen by electron microscopy, are stabilized by the presence of several other proteins which are contained in the complex. These proteins also form part of the gel which can be induced in extracts of *D. discoideum* (Condeelis and Taylor, 1977). Some actin and myosin do not take part in cap formation and so are presumably available for the capping of other surface receptors, or for other motile processes in the cell.

It can be seen from the above discussion that actin is able to bind to an astonishing diversity of other proteins. However, the mechanisms controlling its redistribution during cellular events are largely unknown.

## VII. Metabolic and cytoskeletal determinants of red cell shape and deformability

Nakao et al. (1960) made a study of the shape and ATP content of red cells stored in acid citrate dextrose. After about two weeks, when the ATP content had fallen to 35% of its original value, many of the cells had been transformed from biconcave discs to disc-shaped echinocytes. Five weeks later, with only 5% of the ATP remaining, the cells were all smooth spheres. However, incubation in a mixture of glucose, inosine and adenine, which the cell is able to absorb and use to synthesize ATP, followed by 4mM EDTA, led to the restoration of normal cell shape. The disc to sphere transformation could be brought about within 6 hours using 20mM sodium fluoride to inhibit glycolysis. A 2 hour incubation with glucose, adenine and inosine was sufficient for a reversal of the effect and the experiment could be repeated several times. This was the first demonstration of a relationship between red blood cell shape and ATP levels.

Weed et al. (1969) have observed a similar correlation between cell viscosity and filterability and ATP level. A loss in deformability became apparent after 4 to 6 hours incubation when ATP values were at 70% of their original value. Cells stripped of their surface charge by neuraminidase did not show decreased deformability. Haemoglobin-free ghosts from depleted cells showed a decreased deformability when compared with ghosts from fresh cells. Since the change which occurs on depletion has no effect on cell volume, this shape change alone would actually lead to a

viscosity decrease. If deformability is not changed by a decrease in surface charge, by haemoglobin, or by cell shape, then the mechanical properties of the membrane itself must respond to ATP depletion. The question therefore arises as to how ATP exerts its effects on the shape and deformability of the cell.

Reports of a correlation between membrane deformability and a calcium-ATPase by Quist and Roufogalis (1976) have been criticized by Feo and Mohandas (1977) who observed that cells could be depleted of ATP at 37$^{o}$C by the addition of iodoacetate, and still retain the same deformability and disc-shape. Several hours of further incubation were needed to produce echinocytes whereas the direct effects of ATPase cessation should be detectable immediately. Red blood cells can undergo endocytosis if ATP levels are high enough (Penniston and Green, 1968) or if the drug chlorpromazine is added. The assertion by Feo and Mohandas (1977) that because chlorpromazine can cause endocytosis, the event cannot be energy-dependent, is spurious. Birchmeier et al. (1979) have produced endocytotic vesicles by the addition of 2-10mM Mg-ATP to red cell ghosts. These vesicles can be separated from the membrane within which they are enclosed by extrusion from a syringe and have the same protein composition as ghosts. Phosphorylation of spectrin on their surface occurs at the same rate as in ghosts. However, a magnesium-ATPase is identified, which has a much smaller concentration on the endocytotic vesicles or freeze-thawed vesicles than the remainder of the membrane. Endocytosis and the ATPase are both inhibited by pyrophos-

phate, and chlorpromazine alone is unable to induce endocytosis in depleted cells. Hardy et al. (1979) have found that dephosphorylation of ghosts with alkaline phosphatase inhibits endocytosis but, in contrast to Birchmeier et al. (1979), their vesicles are spectrin-free. In summary, evidence to date is inconsistent with the involvement of an ATPase in either cell shape or deformability, whereas the situation with endocytosis is inconclusive.

When ghosts are prepared by lysis into hypotonic buffers, they appear crenated. Resealing with isotonic buffer followed by incubation at $37^{o}C$ does not produce any morphological change unless magnesium-ATP at a concentration between 0.5mM and 2mM is added at lysis, in which case the echinocytes are partly transformed into discs (Sheetz and Singer, 1977). AMP-PNP was ineffective, suggesting that hydrolysis of ATP took place during the shape change. If $\gamma^{32}P$-ATP was used, label appeared in band 2 of spectrin, establishing that phosphorylation had taken place. The amount of label incorporated reached a constant value well before the shape change. A further demonstration of the involvement of spectrin was provided by the addition of small amounts of anti-spectrin antibody which accelerated the formation of discs. The mechanism of this phenomenon is obscure since at higher concentrations the antibody was inhibitory. Phosphorylation of spectrin has a requirement for magnesium but no calcium, as does the shape change. Birchmeier and Singer (1977a) found that phospholipids, as well as band 2, incorporated $^{32}P$ upon incubation but that *E. coli* alkaline phosphatase incorporated into ghosts at

lysis was able to reverse the shape change and the phosphorylation of band 2, without having any effect on the phospholipids. An ouabain analogue, strophanthidin, which inhibits sodium/potassium ATPase activity, and the calcium chelator, EGTA, had no effect on the shape change, making phosphorylation of spectrin the most likely causative factor. The inhibition of alkaline phosphatase by high ATP concentrations, the loss of $^{32}P$ from intact cells incubated in the absence of ATP (Greenquist and Shohet, 1975) suggesting endogenous phosphatase activity, and the level of incorporation of $^{32}P$ into spectrin (0.5 gm atoms/mole of spectrin at 2mM ATP) in the presence of in vivo ATP levels are consistent with shape maintenance by the dynamic interplay of kinase and phosphatase activity on the membrane. On the other hand, Anderson and Tyler (in press) have recently observed the completion of the discocyte to echinocyte transformation after about 6 hours incubation of red cells in a glucose free medium, at which point the phosphorylation of band 2 retains its original value, apparently ruling out changes in spectrin phosphorylation as a causative factor of shape change.

The inhibition of the shape change by G-actin below its critical concentration (Birchmeier and Singer, 1977b) is evidence that it too participates in red cell morphology.

The difficulty of interpreting results obtained with whole cells or ghosts encouraged the identification and separation of possible cytoskeletal constituents as a prelude to reconstitution experiments. In addition, metabolic effects on protein-protein interactions have been

investigated. The low ionic strength extract from ghosts contains spectrin, actin, 4.1, 4.9 and other trace proteins such as an ATPase (1.IV). Passage through a Sephadex G-200 column separates the main part of the actin from spectrin, which elutes in the void volume along with most of the other proteins. These, however, are almost invisible on SDS gels due to their low concentration. The use of this preparation for a number of experiments has led to a certain amount of confusion about the properties of spectrin which have been resolved by the use of Sepharose 4B in place of Sephadex G-200.

The original extraction of spectrin and actin from ghosts (Marchesi and Steers, 1968) was designed as a search for actin in red cells and the formation of helical filaments on the addition of salt to an extract signified success. Confirmatory evidence by decoration of these filaments with S1 came from Tilney and Detmers (1975), who also prepared G-actin by low ionic strength extraction of an acetone powder of ghosts. Although homopolymers of actin or spectrin are attractive candidates for a cytoskeletal structure, attempts to demonstrate the presence of F-actin in erythrocyte ghosts by sectioning, negative staining (Yu et al., 1973) or freeze-fracturing (Tillack and Marchesi, 1970) have failed. Tilney and Detmers show a fibrillar network which they claim consists of spectrin on the grounds that the only other protein present is actin and the fibrils are not helical. However, self-association of purified spectrin beyond the tetrameric state has not been observed except in the presence of millimolar divalent metal ions when

aggregation takes place (Ungewickell and Gratzer, 1978). Kam et al. (1977) were able to reproduce the appearance of the filaments shown by Tilney and Detmers by applying stain to a grid without first washing the protein with buffer. These fibrils are therefore probably produced by the pH dependent precipitation of spectrin.

Another possible role for spectrin as a structural element of the cytoskeleton was suggested by the increase in viscosity observed when a spectrin extract was added to skeletal muscle F-actin (Tilney and Detmers, 1975). Pinder et al. (1975) found that skeletal muscle G-actin in a buffer containing 10mM sodium phosphate, which remained unpolymerized after prolonged incubation at 30°C, could be induced to polymerize within about 15 minutes by the addition of spectrin, extracted at 37°C and purified on a Sephadex G-200 column. This induced polymerization was also observed by Cohen and Branton (1979) using inside-out vesicles to which an extract of spectrin had been reconstituted. None of these manifestations of an interaction between G-200 spectrin and actin gave rise to a structure which resembled a cytoskeleton or two-dimensional network.

There is some evidence for an effect of phosphorylation on intact cytoskeletons. Triton-extracted ghosts incubated with erythrocyte kinase and ATP at 37°C rapidly form a stiff gel whereas in the absence of kinase, there is no effect. The participation of actin was demonstrated by the introduction of DNAse I in approximately equimolar proportion to the amount of actin in the cytoskeleton preparation, leading to a complete inhibition of gelation. It is not known

whether a change in actin-actin or in some other interaction is involved in this phenomenon (Pinder et al.,1977). In another experiment, cytoskeletons from fresh and depleted cells were cross-linked with the bifunctional dimethylsebacimidate which can span a distance of upto 14Å. Whereas all the cytoskeletal protein from the depleted cells remained soluble, about 60% of the protein from the fresh cells was insoluble in boiling SDS. This suggests that parts of the cytoskeleton move closer to one another in the phosphorylated cell although whether spectrin, actin or both are involved, remains obscure (Pinder et al., 1978).

## VIII. The cytoskeleton and transmembrane events in red blood cells

The observation of Schekman and Singer (1976) that clustering and endocytosis of ferritin-labelled concanavalin A could occur in neonatal but not adult erythrocytes was followed up by Tokuyasu et al. (1979) who used ferritin-labelled anti-spectrin to show that invaginating areas of the membrane and endocytotic vesicles were depleted of spectrin. Further studies on reticulocytes (Zweig and Singer, 1979a) showed a decrease in the amount of endocytosis as the reticulocytes matured. These experiments suggest that spectrin has an effect on the lateral mobility of concanavalin A receptors (band 3). More direct evidence for spectrin involvement was first produced by Nicolson and Painter (1973) who introduced anti-spectrin antibody into red cell ghosts at lysis and observed the clustering of colloidal iron hydroxide binding sites on the outer surface. Since colloidal iron

hydroxide is mostly bound to sialic acid and 60-80% of this is on glycophorin, the inference is that, in these particular conditions, glycoprotein is constrained to cluster by a rearrangement of spectrin in the cytoskeleton. Conversely, Ji and Nicolson (1974) found that the binding of *Ricinus communis I* lectin to ghosts leads to a change in the organization of cytoskeletal proteins, as measured by cross-linking with the reagent dimethyl malonimidate. In particular, there is a considerable decrease in the amount of spectrin, 4.1 and actin appearing on SDS gel of lectin-treated membranes implying the formation of a high molecular weight complex unable to enter these 4% acrylamide gels. It must be emphasized, however, that the distance spanned by the bifunctional cross-linker, dimethyl malonimidate, is only about 4.9 Å so that quite a small change in cytoskeletal organization could produce this effect. If part of the spectrin is removed from the membrane by low ionic strength extraction, and the pH lowered to 5.5, freeze-etching reveals a clustering of the intra-membrane particles. The clusters increase in size depending on the extent of prior removal of spectrin until about 30% of the spectrin has been released (Elgsaeter and Branton, 1974). The process is reversible if the pH is raised. Low pH, millimolar concentrations of divalent metal cations or the addition of polylysine, all of which led to intramembrane particle aggregation, were also the optimum conditions for precipitation of the spectrin extract from erythrocyte membranes (Elgsaeter et al., 1976). Since the composition of intra-membrane particles is disputed, Shotton et al. (1978)

covalently labelled the surface of the erythrocyte with the non-specific hapten p-diazoniumphenyl-β-D-lactoside. After aggregation of the intra-membrane particles by partial extraction of spectrin and adjustment of the pH to 5.5, the introduction of ferritin conjugated antibodies against both the hapten and spectrin showed corresponding clusters on each side of the membrane. Moreover, all the ferritin-labelled hapten sites were in aggregates, making it likely that all membrane proteins with extracellular portions co-aggregate with spectrin under these circumstances. It is pointed out in these papers that the low pH used to bring about aggregation may perturb the membrane and that lateral constraint of all the integral proteins may not represent the *in vivo* situation. Gerritsen et al. (1979) observe the aggregation of intra-membrane particles in spectrin-depleted vesicles, suggesting that spectrin is not required for this aggregation but rather that the integral proteins are sterically confined when all the spectrin is present. This is consistent with the finding of Nigg and Cherry (1979) that removal of spectrin and actin by low ionic strength extraction of red blood cell membranes has no effect on the rotational diffusion of band 3. A population of ghosts labelled with fluorescein isothiocyanate can be mixed with an unlabelled population and fused with Sendai virus, giving rise to a proportion of cells with a fluorescent hemisphere. If these cells are incubated at 37°C, redistribution of the fluorescent label takes place and, with fresh blood, is essentially complete in several hours (Fowler and Branton, 1977). The diffusion

coefficient for the lateral mobility of the fluorescently-labelled proteins is about 4 x $10^{-11}$ $cm^2$/sec. At room temperature, it is 6 x $10^{-12}$ $cm^2$/sec., two orders of magnitude smaller than the value of 1.4 - 2.8 x $10^{-10}$ $cm^2$/sec. for fibroblasts (Edidin et al., 1976) and even less compared with proteins in the muscle fibre membrane (Edidin and Famborough, 1973) or with rhodopsin (Poo and Cone, 1974). Thus although there appear to be constraints on lateral mobility in red blood cell membranes, diffusion nevertheless takes place, making strong binding between the major part of the integral proteins and the cytoskeleton unlikely.

When spectrin is extracted from ghosts, the remaining vesicles have an "inside-out" configuration (Steck et al., 1970). Inside-out vesicles can be distinguished from right-side out vesicles by their appearance in freeze fracture; their extracellular fracture face (Branton et al., 1975) is convex and it is this half of the bilayer which contains most of the intra-membrane particles. Spectrin can bind to inside-out vesicles in a saturable manner with an association constant of about $10^7$. If the vesicles are digested with trypsin or treated with 0.1M acetic acid, however, binding is destroyed, suggesting that a protein is involved (Bennett and Branton, 1977). Ion exchange chromatography of digested polypeptides from the vesicles produces a 72,000 dalton fragment which competitively inhibits the binding of spectrin to inside-out vesicles. Elution of a mixture of spectrin dimer and the fragment from a gel filtration column shows binding between spectrin and the

fragment in a molar ratio of 1 : 0.7 (Bennett, 1978). Although two dimensional peptide mapping of the 72,000 dalton fragment, and all the polypeptide chains which migrate more slowly on SDS gels, has been used to identify which protein is the source of the fragment (Yu and Goodman, 1979; Luna et al., 1979) some of the allegedly similar patterns do not appear to show a high degree of correspondence and far more convincing evidence comes from the work of Bennett and Stenbuck (1979a). Erythrocytes were phosphorylated with $^{32}Pi$ and cleaved with α-chymotrypsin on their outside surface. This produces phosphorylated fragments of Band 3 and glycophorin A with molecular weights of less than 72,000. However, phosphorylated ankyrin fragment was still produced by digestion of the inside-out vesicles from this preparation leaving 2.1 as the only phosphorylated polypeptide of sufficient molecular weight to be the fragment precursor. Furthermore, spectrin binding to inside-out vesicles was not affected by the extraction of 4.1, whereas if both 4.1 and 2.1 were removed, binding was inhibited. 2.1 has been named ankyrin. Yu and Goodman (1979) propose that since there appears to be twice as much spectrin in ghosts as can be bound in the form of dimer to inside-out vesicles, each ankyrin binds to one tetramer in vivo. The amount of the 72,000 dalton fragment which can be produced from the digestion of inside-out vesicles is consistent with this stoichiometry. However, with this arrangement, half the ankyrin binding sites on spectrin would be unused.

Absorption of Triton X-100 solubilized spectrin-depleted vesicles with anti-ankyrin gave complexes which, when precipitated with protein A-bearing staphylococci, were shown to contain both bands 3 and 4.2. Band 3 was present in a 1 : 1 molar ratio with ankyrin, representing only 20% of the band 3 present in ghosts. However, the 80% of free band 3 was similar to the bound; it gave rise to the same set of cyanogen bromide fragments. Spectrin was unable to bind to band 3 in the absence of ankyrin (Bennett and Stenbuck, 1979b).

It is interesting that this protein, which links the cytoskeletal protein spectrin with the transmembrane band 3 has also been found in other tissues by radio-immunoassay (Bennett, 1979). An estimated 120,000 copies in one red blood cell compares with $3 \times 10^3$ per platelet and $3 \times 10^4$ per fibroblast. Brain, liver, kidney and fat cells also gave a positive reaction. Partially purified myosin and filamin did not displace $^{125}$I-labelled fragment from its antibody, showing that ankyrin is distinct from either of these proteins.

If the 72,000 dalton fragment of ankyrin is incubated with fused erythrocytes at 24$^{o}$C, it is able to diffuse though the leaky membrane and compete with ankyrin for its spectrin binding site. Under these conditions, the lateral mobility of the integral membrane proteins is increased two-fold as judged by the rate at which the fluorescent label spreads out from one hemisphere of fused cells when they are incubated at 30$^{o}$C (Fowler and Bennett, 1978).

If intact red cells are incubated in isotonic buffer

in the absence of glucose for more than 12 hours, both phospholipid and protein are lost by exocytosis. An analysis of the vesicles formed during this process shows that they contain no spectrin but, as measured against the amount of phospholipid present, a normal complement of both band 3 and glycophorin (Lutz et al., 1977). The ease with which these two proteins evidently detach from the cell suggests that at least part of their population does not directly interact with the cytoskeleton.

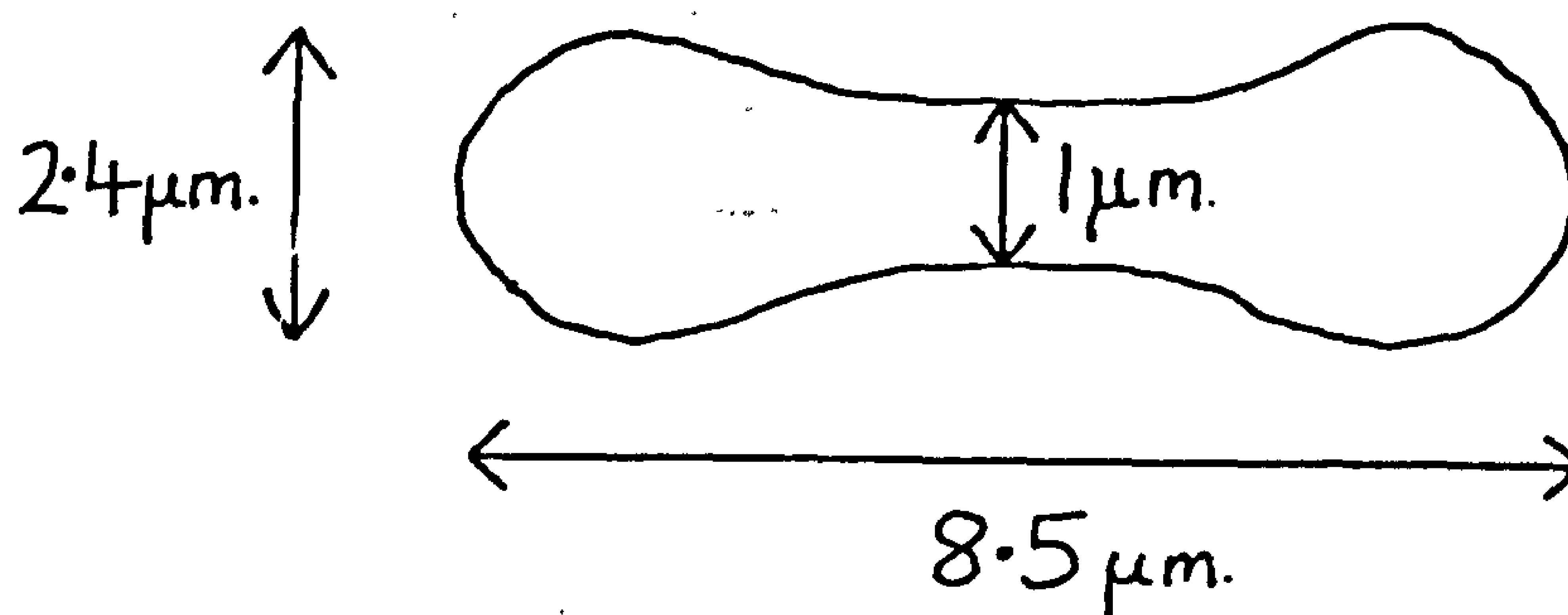
Thus there remains an apparent contradiction between the behaviour of the integral membrane proteins which, on the one hand, are able to diffuse laterally though the membrane or leave the cell completely in vesicle formation and, on the other hand, are unable to cluster as a response to the binding of multivalent ligands to their surface.

<u>Summary</u>

The red blood cell membrane has a unique shape and deformability which, together with the regulation of inside-outside communication, allow the cell to fulfil its oxygen transport function. The peripheral membrane proteins are arranged as a cytoskeleton underneath the membrane, whereas the major integral proteins traverse the lipid bilayer, projecting at either side. Most of these integral proteins undergo restricted lateral diffusion through the fluid-like lipid bilayer. The cytoskeleton is composed mainly of spectrin and actin. Spectrin has not been detected in other cell types but has some features in common with proteins which bind actin. However, whereas most of these proteins

contain two or more large polypeptide chains with the same molecular weight, the two chains in spectrin dimer differ in size. Very little is known about the relationship between these two subunits. Erythrocyte actin has similar molecular properties to skeletal muscle actin but its aggregation state in red blood cells is unknown. Other cells contain actin in the monomeric and filamentous states and also in bundles of filaments. There is often a change in the organization of actin associated with different cellular activities. There is some evidence for an interaction between spectrin and muscle actin, but no complexes which are geometrically capable of forming a cytoskeleton have been found. Spectrin is attached to the red cell membrane by protein 2.1 or ankyrin which, in its turn, is bound to a fraction of the transmembrane protein, band 3. It is not known whether any of these protein-protein interactions are affected by the *in vivo* metabolic state of the cell.

FIGURE 1.1




SURFACE AREA 163 $\mu m^2$

VOLUME 87 $\mu m^2$

Dimensions of the human red blood cell (From Ponder, 1948).

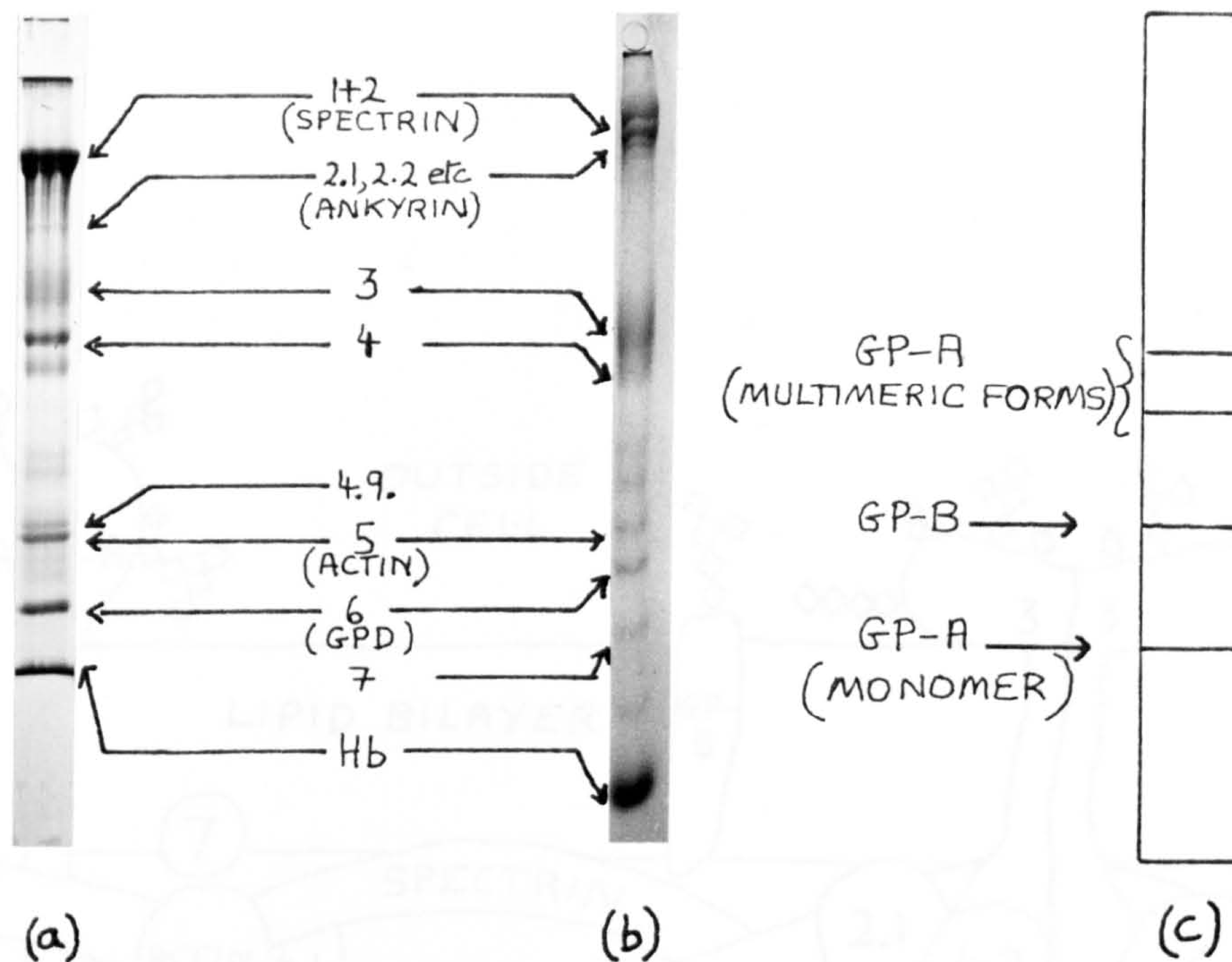



Figure 1. 2.

Polyacrylamide gels, containing SDS, of red blood cell membrane proteins. Electrophoresis was performed using the buffer systems of (a) Laemmli and Favre (1973) and (b) Fairbanks et al. (1971). Both are stained with Coomassie Brilliant Blue G-250. Bands are enumerated according to the system of Fairbanks et al. (1971). (c) Shows the relative positions of the sialoglycoproteins which can be visualized by Periodic Acid Schiff staining.

GP-A Glycophorin A.
GP-B Glycophorin B.
GPD Glyceraldehyde - 3 - phosphate dehydrogenase.

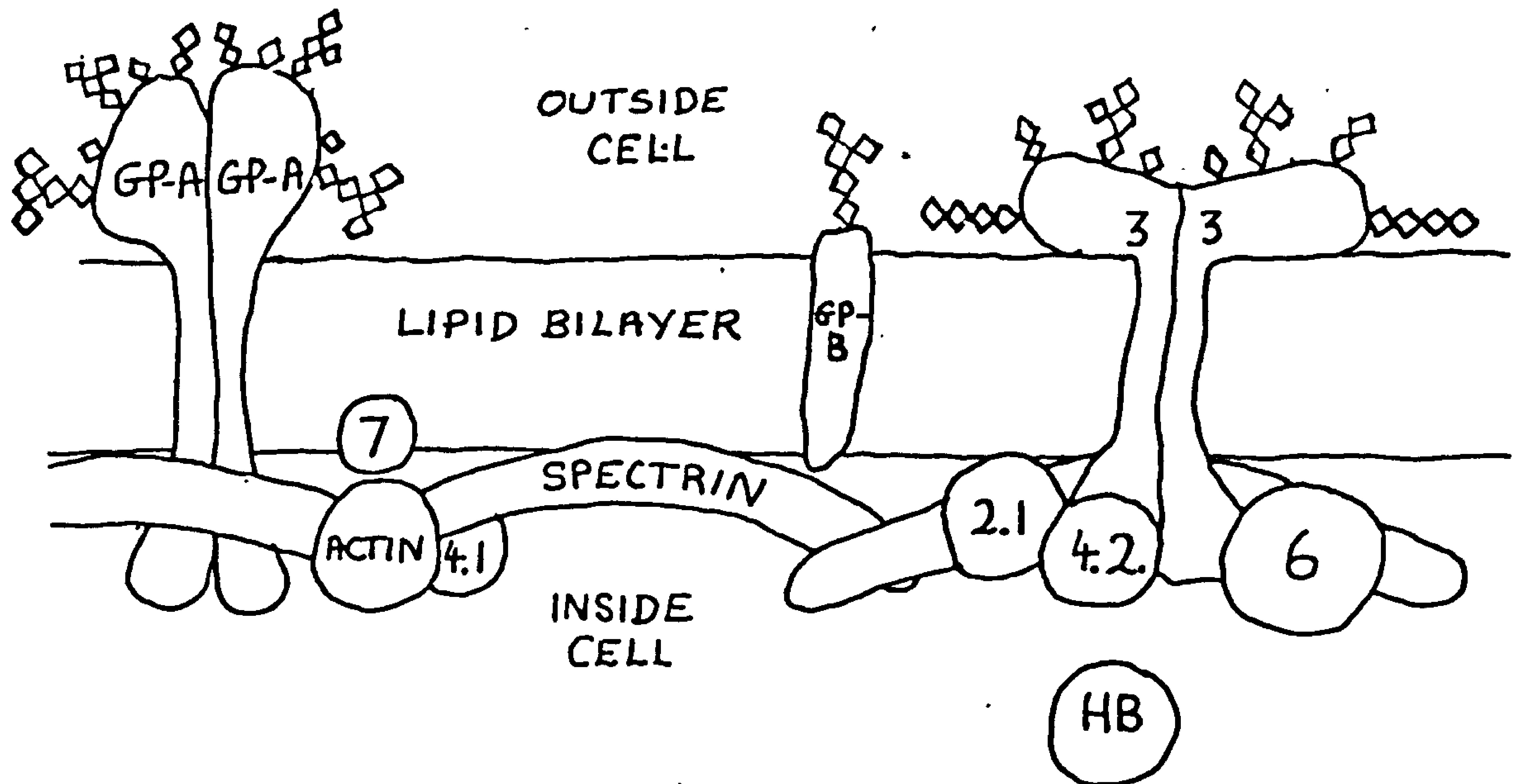



Figure 1. 3.

Schematic illustration of the organization of the major proteins and glycoproteins of the red blood cell. (After Lux, in press). The specific proteins are not drawn to scale. Proteins whose properties are consistent with those of integral proteins are shown partially or completely penetrating the lipid bilayer. Peripheral proteins are depicted as binding to the surface of the lipid bilayer, or to integral proteins.

GP-A Glycophorin-A.
GP-B Glycophorin-B.
HB Haemoglobin.
◇ Carbohydrate.

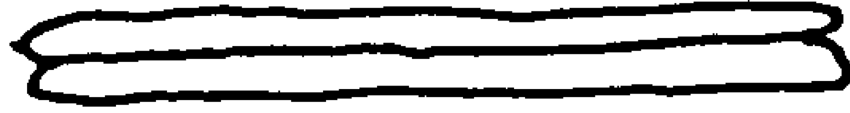
Figure 1. 4.

(a) Diagram of the arrangement of polypeptide chains in spectrin and filamin. The aggregation state of actin-binding protein in solution is not known.

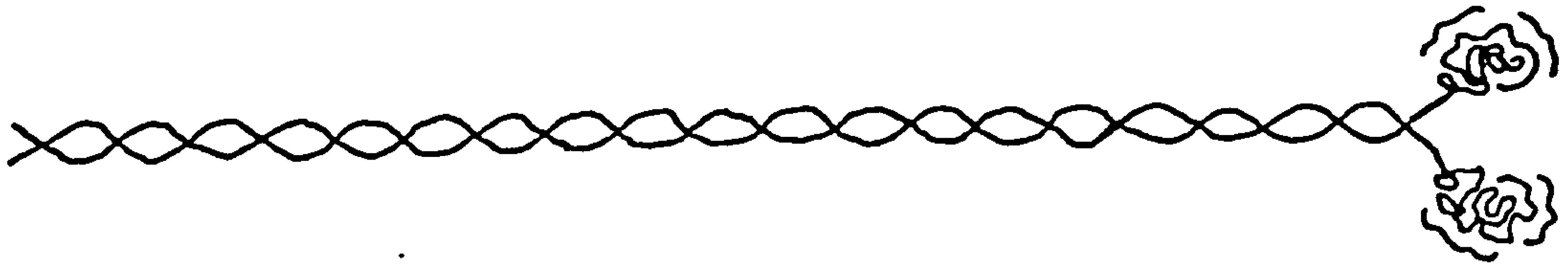
(b) Diagram of the arrangement of polypeptide chains in the myosin molecule. The two heavy chains stretch from one end of the molecule to the other. The four light chains are present only in the heads. (From Offer, 1974).

(c) The myosin molecule and the products of cleavage by proteolytic enzymes. (After Lowey, 1971).

SPECTRIN



FILAMIN



MYOSIN

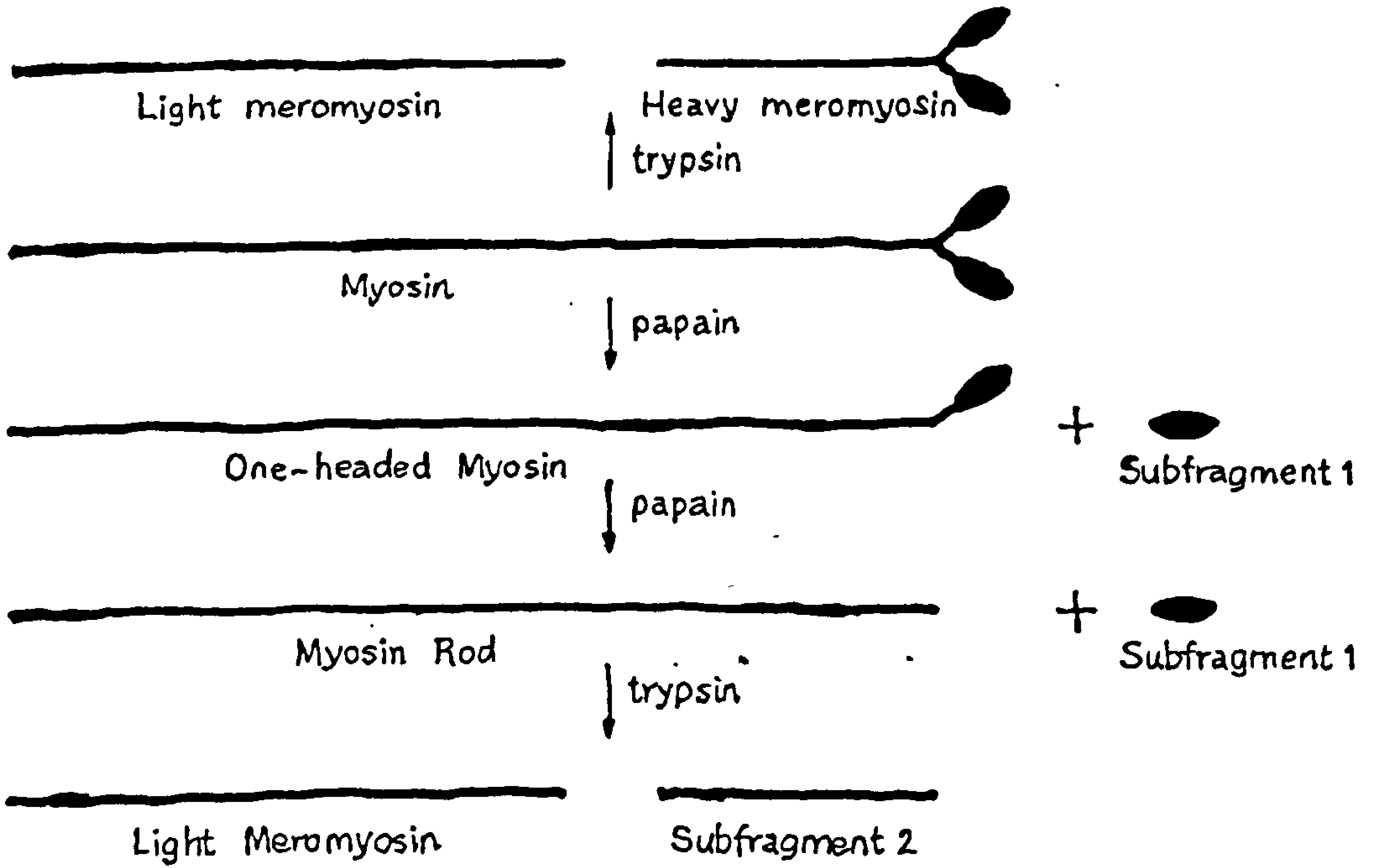


Table 1.1

Major erythrocyte membrane proteins

| Band | Appropriate Molecular Weight | Identification | Integral of Peripheral | Approximate Proportion | Approximate Copies/Ghost |
|---|---|---|---|---|---|
| 1<br>2 | 240,000<br>220,000 | Spectrin (dimer) | P | 25% | 200,000 |
| 2.1<br>2.2<br>2.3 | 200,000<br>190,000<br>180,000 | Ankyrin | P | 5% | 100,000 |
| 3 | 93,000 | Anion exchange protein* | I | 25% | 1,000,000 |
| 4.1 | 78,000 | ? | P | 4-5% | 200,000 |
| 4.2 | 72,000 | ? | P | 4-5% | 200,000 |
| 5 | 43,000 | Actin | P | 4-5% | 400,000 |
| 6 | 35,000 | Glyceraldehyde-3-$PO_4$ dehydrogenase | P | 4-5% | 500,000 |
| 7 | 29,000 | ? | P | 4-5% | 500,000 |
| 8 | 23,000 | ? | P | 1% | 150,000 |
| GP-A | 31,000 | Glycophorin A | I | 1-2% | 400,000 |
| GP-B | ~24,000 | Glycophorin B | I | 0.5% | 100,000 |

* Minor components include acetylcholinesterase (0.25% of membrane protein) and $Na^+$, $K^+$-ATPase (0.005%).

Table 1.2

A comparison of the properties of spectrin, myosin, filamin and actin-binding protein

| Property | Protein | | | |
|---|---|---|---|---|
| | spectrin | skeletal muscle myosin | filamin | actin-binding protein |
| molecular weight of dimer | 480,000 | 500,000 | 500,000 | 540,000 |
| appearance in the electron microscope | worm-like | rod | worm-like* | rings |
| sedimentation coefficient | 9.7 | 6.6 | 10.0 | ? |
| subunits | 250,000<br>230,000 | 2 x 210,000<br>4 x 20,000 | 2 x 250,000 | 2 x 270,000 |
| aggregation state at physiological ionic strength | tetramer + dimer | filaments | dimer | ? |
| α-helix content | > 65% | > 65% | ? | ? |
| phosphorylation | 230,000<br>4 sites | regulatory light chain<br>1 site | 500,000<br>1 known site | ?<br>? sites |
| $K^+$/EDTA ATPase μM/mg/min. | not significant | 22.5 | <0.002<br>probably not significant | not significant |

| Amino-acid | Human erythrocyte spectrin | Skeletal muscle myosin | Chicken gizzard filamin | Rabbit macrophage actin binding protein |
|---|---|---|---|---|
| | mol % | mol % | mol % | mol % |
| Lys | 6.6 | 10.6 | 9.9 | 6.0 |
| His | 3.0 | 1.9 | 1.6 | 2.2 |
| Arg | 5.8 | 5.0 | 5.4 | 4.1 |
| Asp | 9.4 | 9.8 | 10.0 | 8.7 |
| Thr | 4.6 | 5.1 | 5.0 | 6.2 |
| Ser | 5.0 | 4.5 | 5.3 | 6.8 |
| Glu | 15.8 | 18.2 | 18.7 | 11.4 |
| Pro | 3.3 | 2.5 | 2.8 | 7.1 |
| Gly | 5.8 | 4.6 | 5.4 | 11.8 |
| Ala | 8.0 | 9.0 | 8.6 | 7.4 |
| ½-Cystine | 0.54 | | 1.1 | 0.46 |
| Val | 5.9 | 5.0 | 4.6 | 8.5 |
| Met | 1.8 | 2.7 | 2.3 | 1.3 |
| Ile | 3.4 | 4.9 | 4.1 | 4.4 |
| Leu | 13.9 | 9.4 | 9.8 | 6.2 |
| Tyr | 2.4 | 2.3 | 1.8 | 3.1 |
| Phe | 4.5 | 3.4 | 3.0 | 3.2 |
| Trp | | | 0.74 | 1.2 |

Table 1.3

Amino-acid compositions of spectrin (taken from Fuller et al., 1974), skeletal muscle myosin (calculated from Lowey, 1971), chicken gizzard filamin (taken from Wang, 1977) and macrophage actin-binding protein (calculated from Stossel and Hartwig, 1976).

# Chapter 2

# SPECTRIN AND ACTIN IN THE ERYTHROCYTE CYTOSKELETON

## I. Introduction

Considering the strange polymorphism of red blood cells, and the demonstration of the presence of actin in trypsinized ghosts by the appearance of actin filaments which can be decorated by heavy meromyosin (Marchesi and Palade, 1967; Tilney and Detmers, 1975), it is not surprising that the idea of an actomyosin system in red blood cells (Ohnishi, 1962) has been strongly promoted. High molecular weight proteins such as filamin (Wang et al., 1975; Shizuta et al., 1976) and actin binding protein (Hartwig and Stossel, 1975) which have been isolated recently, have molecular properties closer to those of spectrin than do those of myosin. The conditions under which all these proteins are able to interact with actin is at present under investigation (e.g. Wang and Singer, 1977; Stossel and Hartwig, 1975b) and suggest ways in which spectrin might be associated with actin. The evidence of Pinder, Bray and Gratzer (1975) of a direct effect of spectrin on muscle G-actin was used as a starting point for this study of the binding of spectrin to muscle actin.

## II. Materials and Methods

### 1. The preparation of spectrin dimer, tetramer and oligomer

Cells from fresh or out-dated blood bank blood were

washed four times with an equal volume of 0.15M sodium chloride, 5mM sodium phosphate pH 8, and the buffy coat layer discarded each time. The cells were lysed twice in 15 volumes of 5mM sodium phosphate, 0.1M EDTA pH 8 and centrifuged for 25 minutes at 15,000 x g (Dodge et al., 1963). EDTA inhibits a calcium-activated protease which otherwise degrades protein 2.1 (Anderson et al., 1977) and could also affect spectrin and 4.1. Ghosts were washed three times with an equal volume of 0.3mM sodium phosphate, 0.1mM EDTA pH 7.6 and centrifuged for 15 minutes at 30,000 x g. Spectrin was extracted by addition of $^{1}/3$ volume of the same buffer containing 0.5 mg/ml of PMSF, followed by a 15 minute incubation at 37°C in the preparation of dimer, or an overnight dialysis against one litre of extraction buffer at 4°C in the case of tetramer (Ralston et al., 1977; Ungewickell and Gratzer, 1978). The mixture was centrifuged for 1 hour at 80,000 x g to give vesicles which could be used to prepare protein 4.1 or the proteolytic fragment of ankyrin, and a supernatant of crude spectrin. The spectrin was purified by passage through a 90 x 2.5 cm Sepharose 4B column, equilibrated with 0.1M sodium chloride, 10mM sodium phosphate, 30mM sodium azide pH 7.6. The elution profiles from this column for dimer and tetramer preparations from fresh cells are shown in Fig. 2.1. Depeleted cells give very low yields of tetramer. Dimer and tetramer were concentrated when necessary by addition of an equal volume of ice cold, saturated ammonium sulphate solution, centrifugation at 10,000 x g for 15 minutes, and rapid dissolution of the pellet in a buffer containing 0.1mM DTT followed by

overnight dialysis against the same buffer. Spectrin prepared by this method can be stored at $0^{o}C$ for several weeks without detectable proteolysis. Spectrin concentrations were measured spectrophotometrically assuming that $E^{1\% \ 1cm}_{280 \ nm} = 10.7$ (Kam et al., 1977; Schechter et al., 1976).

2. <u>The preparation of Triton-extracted ghosts</u>

Pelleted ghosts were suspended in 7 volumes of 25mM sodium chloride, 5mM sodium phosphate, 1% Triton X-100 pH 8. After incubation for 15 minutes on ice and centrifugation for 15 minutes at 3,000 x g (Yu, Fischman and Steck, 1973), the ghosts were resuspended in the same buffer, omitting the Triton X, gently homogenized and recentrifuged. The pellet was resuspended in the same buffer by homogenization. This preparation results in the loss of at least half the phospholipid and part of band 3 from the ghosts (Fig. 2.2).

3. <u>The preparation of cAMP independent kinase from red blood cells</u>

Ghosts from 50ml of red blood cells, which had been lysed in 5mM sodium phosphate, pH 8, were added to an equal volume of 0.5M sodium chloride. The mixture was stirred on ice for 10 minutes. After centrifugation for 30 minutes at 65,000 x g, the pellet was re-extracted and centrifuged. The pooled supernatants were precipitated with an equal volume of ice-cold saturated ammonium sulphate solution and the pellet resuspended in 7ml of 20mM Tris-HCl, 1mM DTT pH 7.5. After an overnight dialysis against this buffer, the impure kinase was frozen in liquid nitrogen and stored

at -20$^{\circ}$C (Hosey and Tao, 1977).

4. Phosphorylation and dephophorylation of purified spectrin dimer

In a typical experiment, 20mg of spectrin dimer in 50mM Tris-HCl, 5mM magnesium chloride, 0.2mM DTT, pH 8, were incubated with 0.5ml of red blood cell cAMP independent kinase in the presence of 0.2mM ATP for 30 minutes at 37$^{\circ}$C. In order to check the potency of the kinase, a pilot experiment using $\gamma$ $^{32}$P-ATP was performed.

The same weight ratio of spectrin to kinase was used but the molar ratio of spectrin dimer to ATP was 1 : 1 to avoid dilution of labelled with unlabelled ATP. Labelled ATP was added to the spectrin and two 50 µl aliquots of the mixture removed, spotted onto Whatman glass fibre filters (GFC) which were air-dried, placed in scintillation vials and used to determine counting efficiency. A further two 50 µl aliquots were added to 50 µl of bovine serum albumin (1 mg/ml) and 10 µl of 55% TCA, vortex-mixed and incubated on ice to precipitate the protein. Kinase was then added to the spectrin and 50 µl aliquots removed every two minutes during the incubation at 37$^{\circ}$C, and treated as above. A pasteur pipette was used to deposit each protein precipitate onto a Whatman glass fibre filter which was then washed twice with 5% TCA, 3% sodium pyrophosphate, once with ethanol, and dried using a suction pump. The filters were placed in scintillation vials for counting. Fig. 2.3 shows the time course of $^{32}$P incorporation into spectrin. The final level of incorporation is approximately 0.4gm atoms of phosphorus per mole of spectrin dimer. Spectrin was

separated from the kinase by passage through a Sepharose 4B column. The conditions used for the phosphorylation result in the formation of some tetramer (Ungewickell and Gratzer, 1978) which can be reconverted to dimer by incubation in a low ionic strength buffer at 37$^{o}$C.

Dephosphorylation of spectrin dimer was carried out under conditions where it has been shown by radio-labelling experiments that exchangeable phosphate is lost from the protein (J.C. Pinder, personal communication). Spectrin was dialyzed overnight into 50mM Tris-HCl, 15mM sodium chloride, 10mM magnesium chloride, pH 8. A 5 mg/ml suspension of Worthington *E. coli* alkaline phosphatase in ammonium sulphate was used. A small volume was centrifuged to pellet the protein, which was dissolved in dephosphorylation buffer (see above), heated for 5 minutes at 80$^{o}$C, to inactivate possible proteolytic enzymes remaining in the preparations, cooled on ice for 5 minutes, and centrifuged for 5 minutes at 7,000 x g to remove aggregates. One volume of alkaline phosphatase was added to 100 volumes of spectrin and the mixture incubated at 20$^{o}$C for 30 minutes. Spectrin was repurified by passage through a Sepharose 4B column, and converted to dimer when necessary.

5. Phosphorylation and dephosphorylation of spectrin in whole red cells

Incubation of washed cells in 130mM sodium chloride, 25mM sodium bicarbonate, 3.7mM potassium chloride, 2.4mM magnesium chloride, 1.2mM calcium chloride, 1mM adenosine, 12mM glucose, 400 units/ml of benzylpenicillin and 0.62mM radio labelled sodium phosphate pH 7.5, leads to

incorporation of radioactive phosphorus into the spectrin (Bennett and Branton, 1977). In order to phosphorylate spectrin from outdated blood bank blood, cells were incubated for 15 hours at 37°C in this buffer, and the change in cell shape from echinocyte to discocyte observed using a phase contrast microscope (Fig. 2.4).

If washed, whole cells are incubated for 18 hours at 37°C in Tris or phosphate buffered saline, in the presence of 1mM DTT to prevent oxidative damage, transformation to the echinocytic form takes place (Nakao et al., 1960). This change is paralleled by a loss of radioactive $^{32}P$ from the spectrin, if the intact cells have been prelabelled (Greenquist and Shohet, 1975). To prepare spectrin with a low exchangeable phosphate content, cells were incubated for 18 hours as described above, the shape observed in a phase contrast microscope, and the spectrin extracted as described in 2.II.1.

6. Preparation of rabbit muscle G-actin

Actin was extracted from 10g of a Straub acetone powder (Katz and Hall, 1963) into 350ml of 0.2mM ATP, 0.2mM $CaCl_2$, 2mM Tris-HCl, 0.5mM DTT pH 8 for 1 hour at 0°C with constant stirring (Drabikowski and Gergely, 1964). The mixture was filtered through a PG2 sinter and then clarified by passage through a 3μ pore-size Millipore filter at 4°C. Sodium chloride was added to 10mM, and $MgCl_2$ to 0.5mM and the solution incubated at 30°C for 30 minutes to polymerize the actin. In these conditions, tropomyosin does not bind to F-actin. The solution was centrifuged for 3 hours at

108,000 x g to pellet most of the F-actin. The pellets were resuspended in extraction buffer by homogenization and dialyzed overnight against 2 litres of extraction buffer. Observation of the actin between crossed polaroids showed it to be depolymerized. It was clarified by centrifugation at 170,000 x g for 1½ hours at $4^{o}C$. The concentration of G-actin can be measured from its absorbance at 290nm using $E^{1\% \; 1cm}_{290 \; nm} = 6.3$ (Houk and Ue, 1974), taking care to use dialysis buffer in the reference cell to balance the contribution of ATP in the solvent. For storage, actin was added to an equal volume of spectroscopic grade glycerol, stirred at $4^{o}C$ until complete mixing had taken place, and kept at $-20^{o}C$.

7. Estimation of the fraction of denatured actin in a preparation

The addition of EDTA to G-actin causes denaturation and a concomitant change in the fluorescence spectrum of the protein. From the magnitude of this change, the proportion of the actin which was denatured before EDTA addition can be estimated (Lehrer and Kerwar, 1972).
Actin at a concentration of 2 - 5 mg/ml in 2mM Tris-HCl, 0.2mM ATP, 0.2mM $CaCl_2$, 0.2mM DTT pH 8 was diluted with 2mM Tris-HCl pH 8.2 to a final actin concentration of 0.1 mg/ml. The fluorescence emission spectrum from 300-400nm was recorded using a Perkin Elmer MPF-3 spectrofluorimeter with excitation and emission slit widths of 10nm and an excitation wavelength of 290nm. The temperature was maintained at $20.0^{o}C$. The actin denatured in about 4 minutes after the addition of 1mM EDTA, the fluorescence

spectrum was recorded again and the ratio of the peak maxima of the original to the denatured actin noted. If the original actin is 100% native, this ratio should be 1.30 ± 0.05; for denatured action, the ratio is 1.00. Fig. 2.5 shows the change in fluorescence, starting with native G-actin. The ratio is 1.22 for this particular preparation which is therefore 63-88% native.

8. <u>The preparation of protein 4.1</u>

5ml of packed vesicles remaining after the extraction of spectrin at low ionic strength, were further extracted with 0.8M potassium chloride, 10mM sodium phosphate, 0.1M DTT and 3mM PMSF pH 8 in a total volume of 20ml at $0^{o}C$ with stirring for 5 hours. The vesicles were then removed by centrifugation at 80,000 x g for 1 hour. The supernatant was dialyzed against 7.5mM sodium phosphate and 0.1mM DTT pH 8.0 and applied to a DE52 ion-exchange column (10 x 0.9 cm) equilibrated with the same buffer. The column was eluted first with 40ml of the initial buffer, followed by two steps with added potassium chloride. Fig. 2.6 shows a typical elution profile. 0.16M potassium chloride at the second step gave optimal resolution. The haemoglobin and most of band 6 were eluted before the addition of salt. Protein 4.1 and lower molecular weight contaminants were eluted by 0.1M potassium chloride, then protein 4.1 with minor contaminants at 0.16M potassium chloride. Only spectrin was eluted at higher ionic strength. The 0.16M salt eluate was concentrated by vacuum dialysis against 7.5mM sodium phosphate 0.1mM DTT pH 7.5, frozen in liquid nitrogen and stored at $-80^{o}C$.

9. SDS gel electrophoresis

Three different electrophoretic systems were used. Red blood cell membrane proteins were numerically classified by Fairbanks et al. (1971). When whole membrane preparations were analyzed or when unknown polypeptides were to be identified, this system was used. The electrophoresis buffer was 40mM Tris, 20mM sodium acetate, 2mM EDTA, 1% SDS pH 7.4. The acrylamide:bis ratio was 40 : 1.5 and gels were polymerized by the addition of 0.5ml ammonium persulphate at 15 mg/ml and 12 µl of TEMED for every 10 ml of gel. Although the TEMED concentration was increased five-fold over that of Fairbanks et al. (1971) resolution of polypeptide chains was not affected.

The discontinuous buffer system of Laemmli and Favre (1973), usually gives rise to the largest number of bands from a given preparation. The lower SDS concentration (0.1% SDS), as well as other differences in buffer composition, lead to striking changes in the red blood cell membrane protein profile : band 3 is hardly visible whereas a doublet can just be distinguished in place of 4.1, and an additional band, 4.9, is visible just above actin. This band appears in spectrin oligomer. Fig. 1.2 shows acrylamide gels of red cell proteins using each of these systems, for comparison. The reasons for the dissimilarities are not clear although glycoproteins such as band 3 are known to behave anomalously on SDS gels.

The large size and therefore presumably low mobility of all the ions in the system used by Kendrick-Jones et al. (1976) means that a large potential gradient can be applied

without generating high currents leading to excessive ohmic heating. This allows rapid protein separations. For this reason, a 0.1M Tris, 0.1M bicine, 0.1% SDS buffer was used for all routine work.

The ratio of acrylamide to NN' methylene bis acrylamide was 37.5. Protein samples were made 1% in SDS, 1% in β-mercaptoethanol and 10% in glycerol containing a trace of bromophenol blue. They were immersed in a boiling water bath for 5 minutes before loading onto the gel. Gels were stained for 1 hour in Coomassie Brilliant Blue G-250 (Serva) and destained in 5% methanol, 7.5% glacial acetic acid. Densitometry was performed on a Joyce Loebl instrument. The area under the densitometer trace is proportional to the amount of spectrin in the gel band if less than 12 μg of spectrin is present (J.C. Pinder, personal communication).

10. Electron microscopy

Complexes of F-actin with spectrin tetramer and 4.1 were prepared at a total protein concentration of about 1 mg/ml. After 10-fold dilution they were applied to carbon-coated grids, negatively stained with 1% uranyl acetate and examined in a Philips EM 200 instrument at 80 kV.

11. The binding of crude and pure spectrin to muscle F-actin

Spectrin dimer was centrifuged at 95,000 x g for 45 minutes to remove any aggregated material. The concentration of the supernatant was measured and it was mixed with F-actin to give final concentrations of 0.6 - 1.0 mg/ml and 0.3 - 0.6 mg/ml respectively. 1 ml of the mixture was centrifuged for

45 minutes at 95,000 x g and the absorbance of the supernatant at 290nm was noted. Any binding of spectrin to actin would lead to a decrease in absorbance when compared with the sum of the absorbances of the supernatants in the control tubes containing spectrin and actin alone. Binding was assayed at $0^{o}C$ in the absence of ATP (0.1M potassium chloride, 5mM sodium phosphate, 10mM imidazole, 0.1mM magnesium chloride pH 7), in the presence of 0.2mM ATP and the same buffer at $0^{o}C$, and after an incubation in the presence of ATP at $37^{o}C$ for 30 minutes prior to centrifugation. Both phosphorylated and dephosphorylated spectrin prepared by the incubation of dimer with kinase and phosphatase respectively, were used.

In experiments with crude spectrin, 0.1M potassium chloride, 10mM sodium phosphate, 0.2mM ATP, 0.2mM DTT, 0.2mM calcium chloride pH 8.2 was used as a buffer. Crude spectrin and F-actin were dialyzed overnight into this solvent. Spectrin was centrifuged for 45 minutes at 96,000 x g and the supernatant used for binding studies. Mixtures of spectrin at 0.2 mg/ml and actin at 0.3 mg/ml were left to stand on ice for 30 minutes before centrifugation at 95,000 x g for 45 minutes. Binding was estimated from the absorbances of the supernatants. Due to the presence of a substantial amount of haemoglobin in the crude spectrin, necessitating the measurement of relatively small differences between large absorbances, aliquots of the pellets and supernatants after centrifugation were run on SDS gels and analyzed by densitometry.

12. The binding of spectrin to G-actin below the critical concentration

Spectrin dimer was dialyzed and G-actin diluted into 0.1M sodium chloride, 10mM sodium phosphate, 0.2mM ATP, 0.2mM DTT, 0.2mM calcium chloride pH 8 and the two mixed to give final concentrations of 0.34 mg/ml and 0.03 mg/ml respectively. The latter value is below the critical concentration for actin polymerization. 0.4ml aliquots were incubated at 37°C for 15 minutes and loaded onto 4.4 ml, 10-20% sucrose gradients with 0.5ml 50% sucrose cushions. They were centrifuged for 3 hours at 240,000 x g in a Beckmann SW65 rotor. 10 fractions were collected from each gradient by inserting the thin end of a pasteur pipette carefully into the bottom of the tube and then withdrawing liquid using a peristaltic pump. The fractions were analyzed on SDS gels containing 7.5% acrylamide.

13. Gelation of solutions containing mixtures of muscle F-actin and various spectrin-containing preparations

0.5ml samples, in a "gelation" buffer containing 30mM sodium chloride, 10mM imidazole, 1mM ATP, 1mM DTT, 1mM EGTA, 1mM magnesium chloride, pH 7 were incubated at 30°C for 10 minutes and inspected for gelation. In equivocal cases, the samples were centrifuged at 20,000 x g for 15 minutes, followed by analysis of the pellets and supernatants on SDS gels containing 7.5% acrylamide. Where gelation was carried out under different conditions, these are mentioned in the text.

To check whether either spectrin or 4.1 bound to F-actin in the conditions of the experiments, spectrin

tetramer at a concentration of about 0.2 mg/ml or 4.1 at a concentration of about 0.025 mg/ml in the presence, and absence of 0.45 mg/ml of F-actin were centrifuged for 45 minutes at 96,000 x g to pellet the F-actin. Pellets and supernatants were examined on SDS gels. Fig. 2.7 shows that actin has no effect on the sedimentation of 4.1 but that as much as 40% of the tetramer is found in the pellet with F-actin. Binding constants can be estimated under an arbitrary assumption of a ratio such as 1 : 1 or 1 : 10 of spectrin tetramer to actin in a putative complex, leading to values for $K_a$ of $6.3 \times 10^4$ and $6.7 \times 10^4$ respectively. These findings are in agreement with those of Brenner and Korn (1979) who have found a weak interaction between spectrin and actin at magnesium concentrations of 2mM and intermediate ionic strengths. On the other hand, at physiological ionic strength, in the presence of 0.1mM magnesium, there is negligible interaction (Ungewickell et al., 1979).

14. Cross-linking of spectrin oligomer

Cross-linking was performed according to the method of Davies and Stark (1970). Oligomer with an absorbance of 0.25 at 280 nm in one of a variety of buffers containing detergents and denaturants was added to an equal volume of 0.2M triethanolamine hydrochloride pH 8.5, (containing 7M urea when required). 1/8 volume of dimethyl suberimidate at a concentration of 20 mg/ml was added and the reaction allowed to proceed for 45 minutes at 22°C. Samples of cross-linked and uncross-linked material were dissolved in

SDS and applied to SDS gels containing either 5% or 7.5% acrylamide.

15. The binding of spectrin dimer to 4.1 and F-actin

Centrifugation through a sucrose cushion leads to good separation of free spectrin dimer or tetramer from F-actin or a complex of spectrin with 4.1 and F-actin. 0.35ml of spectrin dimer in 0.1M sodium chloride, 10mM sodium phosphate, 0.5mM DTT pH 7.5 was added to 0.1ml of 4.1 in 7.5mM sodium phosphate pH 7.5, and 0.05 ml of F-actin in 0.1M sodium chloride 2mM Tris-HCl, 0.2mM magnesium chloride, 0.2mM DTT, 0.2mM ATP pH 7.4. The mixture was incubated for 1 hour at room temperature, applied to a 0.3ml 10% sucrose cushion and centrifuged for 1¼ hours at 100,000 x g. Pellets and supernatants were analyzed on 7.5% acrylamide gels.

## III. Results

### 1. The binding of spectrin dimer to muscle F-actin

It is impossible to explore all combinations of variables of solvent, temperature and concentration in which protein-protein binding might take place. The experiments described use solvent conditions close to physiological. Myosin-actin and filamin-actin interactions as well as the conditions under which spectrin and actin interact (Pinder, Bray and Gratzer, 1975) were used as models to study actin binding to spectrin.

Myosin will bind strongly to F-actin only in the absence of ATP (Tonomura, 1972). Binding was therefore assayed in the presence and absence of ATP.

Pinder, Bray and Gratzer (1977) have found that interactions between red cell cytoskeletal proteins are markedly affected by the phosphorylation state of the spectrin, and effects of filamin phosphorylation on actin-activated heavy meromyosin ATPase (Davies et al., 1978) have been reported. Both the phosphorylated and dephosphorylated forms of spectrin were used for binding.

Although most protein-protein interactions have low activation energies and occur within minutes of mixing on ice, the self association of spectrin has evidently an activation energy of some 300 kJ per mole so that, starting from the dimer, equilibrium between dimer and tetramer takes several hours at 26°C (Ungewickell and Gratzer, 1978). In order to take into account possible kinetic effects on an interaction between spectrin and F-actin, in one experiment the two proteins were incubated at 37°C before centrifugation. Experiments were performed in the presence of 0.1mM magnesium, but no calcium since Gratzer and Beaven (personal communication) have found that there is no strong binding site for calcium on pure spectrin, making an effect of this cation on the aggregation state of spectrin unlikely.

No matter what conditions were used, no binding was detected between spectrin dimer and F-actin: less than 2% discrepancy was found between the absorbance of the supernatant in the tube containing spectrin and actin and the sum of the absorbances of the supernatants in tubes containing actin alone and spectrin alone.

## 2. The interaction between spectrin and muscle G-actin at physiological ionic strength

G-actin denatures more quickly than F-actin in the absence of added nucleotide (Martonosi et al., 1960): when G-actin was exchanged into a buffer without ATP, by passage through a 170 ml G-100 Sephadex column, it was 100% denatured by the criterion of Lehrer and Kerwar (1972). All experiments involving spectrin and G-actin were therefore performed in the presence of ATP. Phosphorylated spectrin was used, as it was the species implicated in interactions with actin (Pinder et al., 1977).

It has been reported that a single tyrosine residue can be modified on G-actin, giving rise to a species which is unpolymerizable (Bender et al., 1976) yet binds ATP and also myosin subfragment 1 (Bender et al., unpublished results). With the assumption that spectrin and actin binding sites on the actin monomer do not overlap, this modified G-actin could be used to study interactions between the two proteins at physiological ionic strength. However, treatment of actin with 5-diazonium-(1H)tetrazole in our hands, produced a large increase in the number of modified tyrosine residues for a small change in reagent concentration and the reaction was thus difficult to control. Moreover, even when a sample containing an average of only 1.2 modified residues per molecule was prepared, it eluted in the void volume of a G-100 column, indicating that aggregation or polymerization had taken place.

Equilibrium constants for the reaction between two interacting molecules can be estimated from perturbations of

their migration during electrophoresis (Eisinger and Blumberg, 1973). In particular, if there is an interaction between spectrin and G-actin, a retardation of the G-actin zone relative to its position in the absence of spectrin might be expected. 180 µl aliquots of G-actin in a buffer of physiological ionic strength at concentrations ranging from $5 \times 10^{-4}$ mg/ml to 0.3 mg/ml were applied to acrylamide gels in the absence of SDS, electrophoresed, stained and destained. At the lowest actin concentrations giving rise to a visible gel band ($5 \times 10^{-3}$ mg/ml), aggregated material was observed at the top of the gel. One can only assume that concentration of the actin occurs on entry into the gel, leading to polymerization, thus rendering the method unsuitable for this experiment.

Using sucrose gradients, containing a solvent of physiological ionic strength, followed by SDS gel electrophoresis, it was found that the sedimentation profile of G-actin was not altered by the presence of spectrin dimer. Assuming that 20% binding of the actin could be detected as a depletion of the existing zone, the maximum association constant for a complex in the absence of a visible redistribution of actin would be $1 \times 10^{5}$. Strong binding between G-actin and spectrin did not, therefore, occur.

3. <u>The interaction between crude spectrin and muscle F-actin</u>

Analysis of pellets and supernatants from this binding experiment by SDS gel electrophoresis and densitometry showed that, for instance, with initial concentrations of 0.6 mg/ml actin and 0.3 mg/ml spectrin, 16% of the spectrin

pelleted in the presence of actin with only 4% in the control. Inspection of the elution profile of spectrin from a Sepharose 4B column (Fig. 2.1) shows that there are only two peaks containing spectrin, making it likely that oligomer was involved in the binding experiment described above. When samples from each peak in the elution profile were incubated with G-actin at intermediate ionic strength, only the oligomer fraction was active in polymerizing the actin (Pinder et al., 1979). Earlier experiments (Pinder, Bray and Gratzer, 1975, 1977) used spectrin from the void volume of the Sephadex G-200 column, which contained oligomer.

4. The formation of gels between spectrin preparations and muscle F-actin

When 0.9 mg/ml of F-actin is mixed with 0.4 mg/ml of crude spectrin (extracted at 4°C overnight) in a buffer containing 30mM sodium chloride, 10mM imidazole, 1mM ATP, 1mM DTT, 1mM EGTA, 1mM magnesium chloride pH 7.0 on ice, a gel is formed. After low speed centrifugation (2.II.13) the gel forms a tight pellet (Fig. 2.8) Fig. 2.9 shows an SDS gel of the pellet and supernatant. Gelation also occurs in the presence of 1mM EDTA and no magnesium chloride. Incubation at 30°C for ten minutes does not affect the interaction. If the spectrin is extracted at 37°C or if purified dimer or tetramer is used, no gel is obtained. The minimum concentration of actin required is between 0.33 and 0.48 mg/ml and that of spectrin extract 0.14 to 0.41 mg/ml. If a 37°C extract is incubated for 10 minutes at 30°C in the presence of F-actin, and then left on ice for

several hours, gelation will occur. These results are in substantial agreement with those of Fowler and Taylor (unpublished work). They measured viscosity increases and concomitant gelation of mixtures of G-actin and spectrin at intermediate ionic strength. They used 0.15 - 0.25 mg/ml of spectrin extract and found that a minimum of 0.4 mg/ml of G-actin was required for gelation. In their hands, optimal gelation of 37$^{o}$C extracts and of oligomer prepared from them took place after incubation in the presence of actin for four hours at 32$^{o}$C. They emphasized that under these conditions small amounts of spectrin tetramer would be formed.

Triton-extracted ghosts prepared either as in Section II.2 or in isotonic solutions by a modification of the method of Sheetz and Sawyer (1978) will also form gels with F-actin.

G - actin in a buffer of ionic strength 10mM is not active in gelation, but the addition of 30mM sodium or potassium chloride followed by a 15 minute incubation at 30$^{o}$C and overnight incubation on ice, gives rise to the same amount of rapidly sedimenting protein as with F-actin (Fig. 2.10).

The ionic strength of the above "gelation buffer" is about 40mM. No investigation was made into the effects of lowering the salt on gelation with F-actin, since possible depolymerization of the actin would have had to be taken into account. However, additions of sodium chloride to the reaction mixture, resulting in ionic strengths of up to 0.14M gave rise to an increasing proportion of rapidly sedimenting protein. In this experi-

ment, mixtures were incubated at 30°C for 10 minutes before centrifugation. 1% SDS was added to the pellets to give a total volume of 1ml and the absorbances of the resulting solutions were monitored at 290 nm. The percentage of sedimented protein is plotted against ionic strength in Fig. 2.11. This result shows that gelation at lower ionic strengths is not an artefact due to non-specific interactions between the proteins.

Whilst spectrin dimer has no strong binding site for calcium, many gelation phenomena involving crude cell extracts (e.g. Condeelis and Taylor, 1977) or indeed interactions between purified proteins,(Yin and Stossel, 1979), are calcium dependent. The effect of calcium on the gelation between a crude spectrin extract and F-actin was investigated using a buffer containing 30mM sodium chloride, 2.5mM imidazole, 0.06mM DTT, 1mM magnesium chloride, 1mM ATP, 0.1mM EGTA and either 0, 0.1 or 1mM calcium. The presence of the four interacting species $Mg^{++}$, $Ca^{++}$, EGTA and ATP makes calculation of the free calcium concentration a difficult task. However, using the association constants between calcium and magnesium and EGTA given by Portzehl et al. (1963), and those with ATP given by Smith and Alberty (1956), calculations show that the free calcium concentration with 0.1mM total calcium is greater than $10^{-6}$M. Whereas concentrations of calcium as low as $10^{-6}$M inhibit gelation in, for example, extracts from D. discoideum (Taylor et al., 1977), sea urchin eggs (Kane, 1976) and Xenopus oocyte extracts (Clark and Merriam, 1978) and in a mixture of actin, actin binding protein and gelsolin from

macrophages (Yin and Stossel, 1979), it was found that 0.1mM calcium did not effect and 1mM calcium slightly increased the amount of sedimenting protein in mixtures of F-actin and crude spectrin at $0^{o}C$ (Fig. 2.10). There is a trans-glutaminase in intact erythrocytes (Lorand et al., 1978) which is calcium dependent, and if it is present in spectrin extracts, could lead to some cross-linking between the proteins present in this experiment, giving rise to the observed effect. In contrast, Fowler and Taylor (unpublished work) have found an inhibition of gelation in a mixture containing G-actin and crude spectrin, by micromolar levels of calcium and persisting at $10^{-4}$M calcium. The reason for this difference is not clear but may involve the polymerization of the G-actin, or differences in the constitution of their buffer: the absence of both magnesium and ATP might be responsible.

Calcium-dependent regulatory factors for gelation could exist in intact cells, but remain attached to the membrane when spectrin is extracted. Triton-extracted ghosts prepared in isotonic buffers contain all the red blood cell membrane proteins which are normally visible on SDS gels, except band 6 (Sheetz and Sawyer, 1978), and thus represent the preparation capable of gelation which is most likely to retain any calcium binding regulatory proteins. These Triton-extracted ghosts gelled in the presence of F-actin. Low speed centrifugation pellets all the ghosts and any F-actin which is bound to them. No difference in sedimentable actin could be detected between samples in the presence and absence of 0.1mM calcium chloride, but there

was always evidence of proteolysis in the preparations (Sheetz, 1979). Thus, it is possible that interactions in the cytoskeleton are regulated by calcium in the presence of a protein or proteins which are absent in spectrin extracts.

Crude spectrin from fresh cells was dephosphorylated as described in 2.II.4. When this and a control sample were incubated with F-actin, there was no difference in the amount of sedimentable protein between the two samples, as measured by inspection of SDS gels of the pellets and supernatants after centrifugation (Fig. 2.10).

Micromolar concentrations of the drug, cytochalasin B can inhibit the gelation of cell extracts (Kane, 1975: Pollard, 1976a; Hartwig and Stossel, 1976; Weihing, 1976). F-actin was added to a mixture of $2 \times 10^{-4}$M cytochalasin in dimethylsuphoxide and spectrin oligomer in "gelation buffer" giving a final concentration of cytochalasin of $2 \times 10^{-6}$M. A ten minute incubation at 30$^{o}$C, followed by centrifugation and analysis on SDS gels revealed that cytochalasin had not affected the protein-protein interactions (Fig. 2.10).

5. <u>The presence of 4.1 in oligomer and an investigation of its relationship to spectrin and actin in oligomer and Triton-extracted ghosts</u>

An SDS gel of oligomer eluted from a Sepharose 4B column (Fig.2.1)shows that there is a substantial amount of a protein which migrates in a position approximately half-way between spectrin and actin. A comparison of oligomer with the proteins from intact red blood cell

membranes using the electrophoretic system of Fairbanks, Steck and Wallach (1971) shows that this protein comigrates with band 4.1 in their indexing system (E. Ungewickell, personal communication). On these gels, 4.9 is also visible, but it comigrates with actin in the Tris-bicine system. The interaction between crude, but not pure, spectrin and actin and the presence of 4.1 in oligomer opens up the possibility that 4.1 is the missing factor which could link spectrin and actin into a two-dimensional network. The conditions which regulate the interactions between these proteins in oligomer and Triton-extracted ghosts were explored. If an extract of spectrin is analyzed by means of sucrose gradient sedimentation in extraction buffer, there is a rapidly sedimenting peak containing spectrin, actin and 4.1, which represents from 20 to 30% of the total weight of protein in the extract (S. Lux, personal communication). If the ionic strength of the extract is increased to physiological values, spectrin, actin and 4.1 become incorporated into additional oligomer. Furthermore, the removal of "low salt oligomer" from the extract, followed by concentration of the remainder and elevation of the ionic strength to 0.15M results in the formation of high molecular weight material (E. Ungewickell, personal communication).

0.3ml aliquots of crude spectrin extracted from fresh and depleted cells respectively at 37$^{o}$C, were loaded onto 4.4ml 10-20% sucrose gradients with 0.5ml 45% sucrose cushions in spectrin extraction buffer. The gradients were centrifuged for 14 hours at 180,000 x g and 0.5ml fractions were collected.

Fig. 12a shows the absorbance profiles of the two sucrose gradients and Fig. 12b shows SDS gels of each of the fractions. The extract from fresh cells contained approximately 23% oligomeric material and from depleted cells 31%. Thus the metabolic state of the cells from which spectrin has been extracted does not greatly affect the amount of this complex which has been formed. On the other hand, when samples from the two crude extracts were applied to Sepharose 4B columns equilibrated with 0.1M sodium chloride, 50mM sodium phosphate pH 7.5 and eluted with the same buffer, different profiles were obtained in each case (Fig. 2.13): 38% of the protein was found in the oligomeric peak from the extract from depleted cells compared with only 24% from fresh cells. The total protein concentrations were comparable: viz. 1.40 mg/ml and 1.62 mg/ml respectively.

Oligomer dialyzed overnight against 20mM sodium chloride, 2mM sodium phosphate, 0.5mM DTT, 10mM sodium deoxycholate pH 7.5 was cross-linked with dimethyl suberimidate as described in 2.II.14. Fig.2.12 shows that a substantial amount of the spectrin is not cross-linked to the complex either in the presence or absence of deoxycholate. This could be due to an inefficient cross-linking reaction or to a partial breakdown of the oligomer due to lowering the ionic strength to about 40mM. Spectrin subunits are seen in the presence of deoxycholate, in agreement with the results of Schechter et al. (1976) (the efficiency of cross-linking between the two subunits in dimer is almost 100%). Most of the 4.1 and about half of

the actin remains in the oligomer or as an aggregate in the presence of deoxycholate.

Spectrin dimer in 7M urea, 50mM Tris-HCl, 0.5mM DTT, pH 8, is almost completely dissociated into subunits (see Chapter 3) but whether oligomer was treated with 7M urea, 6M guanidine hydrochloride followed by 7M urea, 10mM sodium deoxycholate and 7M urea or 0.2% Triton X-100 and 7M urea in the same buffer, subsequent cross-linking showed that band 1 was the only polypeptide chain released in substantial quantities from the complex, accompanied by some band 2 and a barely discernible band of actin (Fig. 2.14).

If crude spectrin is incubated at 37$^{o}$C for 15 minutes in 1M sodium chloride, very little oligomer remains in the pellet after centrifugation at 100,000 x g for 1 hour (Fig. 2.15). The diminution of the pellet with respect to that formed under physiological salt conditions may be slightly exaggerated due to the higher viscosity and density of the buffer as well as possible partial unfolding of the proteins. (The corrected sedimentation coefficients of both dimer and tetramer are 0.5S smaller in 1M than in 0.1M sodium chloride). However, these effects could scarcely diminish the pellet by more than a factor of two. The identity of the breakdown products of the oligomer is unknown but if separate proteins result, a preparation of 4.1 from the oligomer could be achieved.

Triton-extracted ghosts (2.II.2) from 4ml of ghosts were washed three times in 0.2mM imidazole, 0.1mM DTT, 0.1mM ATP pH 7.7, and extracted for 15 minutes at 37$^{o}$C in

3ml of the same buffer. After centrifugation for 15 minutes at 63,000 x g, the pellet was dissolved for analysis by SDS gel electrophoresis and the supernatant sedimented through a sucrose gradient, fractions from which were also analysed on SDS gels (Fig. 2.16). The pellet represents only about one tenth of the total protein. The gradient fractions in which 4.1 is visible all contain spectrin and some actin, though the actin is present in less than equimolar proportion to the 4.1. Whether the 4.1 and spectrin merely comigrate on the gradient or whether they are bound to one another has not been determined, though the latter would not be surprising in view of the observations of Tyler et al. (1979) that spectrin binds to 4.1 in 20mM potassium chloride. Since spectrin and 4.1 do not interact detectably at physiological ionic strength (Ungewickell et al., 1979), this treatment of Triton-extracted ghosts followed by further sucrose gradients or column chromatography may provide another means for the preparation of 4.1. None of these conditions described, however, leads to a complete dissolution of high molecular weight material.

6. Interactions between spectrin, 4.1 and actin

When Bennett (1978) treated spectrin-depleted vesicles with 1M potassium chloride bands 6, 2.1 and 4.1 were liberated. Ungewickell et al. (1979) used this as the basis for a successful preparation of 4.1 which was described in 2.II.8. When 0.9 mg/ml of F-actin is mixed with 0.4 mg/ml of spectrin tetramer and approximately 0.005

mg/ml of protein 4.1 in "gelation buffer", on ice, a gel is formed. No gelation occurs in any binary combination of the proteins, nor when the spectrin is dimeric. When two such mixtures containing respectively spectrin dimer and tetramer were centrifuged at low speed, 15% and 60% of the total protein pelleted.

Centrifugation of mixtures containing spectrin dimer through a sucrose cushion (see 2.II.15) show that although gelation does not occur, a ternary complex is nevertheless formed (Fig. 2.17).

Fig. 2.18d shows an electron micrograph of a complex formed between spectrin tetramer, 4.1 and F-actin. Cross-links, identified as tetramers, can be seen in places, bridging F-actin filaments, thus causing them to run in parallel for short stretches. Unbound tetramer was separated from the complex by passage through a 1 x 10 cm Sepharose 4B column. Figs. 2.18a and b show electron micrographs of spectrin tetramers and 4.1 for comparison. If dimer instead of tetramer is used to form the complex, a random arrangement of actin filaments is seen, but the spectrin is still visible as short curved protruberances from the filaments (Fig. 2.18c).

## IV. Discussion

### 1. Participation of 4.1 in the cytoskeletal complex

Much of the evidence presented in this chapter indicates that pure spectrin and F-actin do not bind to one another at physiological ionic strength and interact only weakly in a total magnesium concentration of 1mM at

intermediate ionic strength. Presence of protein 4.1, however, leads to the appearance of high molecular weight complexes which are capable of forming an extended two-dimensional network such as the cytoskeleton. The polymerization of actin by spectrin observed by Pinder et al. (1975) used spectrin purified on a G-200 Sephadex column where oligomer and dimer or tetramer elute together in the void volume. Gels of this fraction reveal a certain amount of actin, but the spectrin:4.1 ratio is so high that 4.1 is barely, if at all, visible. Subsequent experiments using Sepharose 4B purified material showed that only oligomer was active. Brenner and Korn (1979) achieve gelation of mixtures containing only tetramer and F-actin, when the F-actin concentration is above 0.7 - 1.0 mg/ml. In the gelation described in 2.III.4, actin concentrations were 0.9 mg/ml or less, and the total magnesium concentration only 1mM, which could explain why no gelation was observed in mixtures of tetramer and F-actin. Brenner and Korn (1979) also obtain viscosity rises in mixtures of tetramer and F-actin where 0.1M potassium chloride is substituted for 2mM magnesium chloride. The extent of interaction represented by this viscosity rise is not clear, so that this result cannot be directly compared with the gelation reported above. In all preparations where spectrin is associated with actin, i.e. erythrocyte ghosts, Triton cytoskeletons and oligomers, there is always at least 0.5 moles of 4.1 for every mole of spectrin dimer (J.C. Pinder, personal communication). This observation, coupled with the low binding constant for the spectrin-actin inter-

action, suggests that 4.1 mediates interactions between the other two proteins in the erythrocyte membrane

No gelation of solutions containing G-actin and oligomer occurs, in a buffer of ionic strength 10mM containing 1mM magnesium-ATP. This result and the absence of a high molecular weight complex when G-actin, spectrin and 4.1 are mixed (Ungewickell et al., 1979) does not exclude an interaction between G-actin, 4.1 and spectrin or oligomer, but indicates that extensive networks cannot be built up in this way. Brenner and Korn (1979), measured spectrin binding to G-actin by assuming that any bound actin would not be able to polymerize, thus increasing the apparent critical concentration of the actin. However, since they claim that spectrin binds to F-actin, bound G-actin might be expected to polymerize quite normally.

The absence of 4.1 binding to actin was noted in 2.II.13. Ungewickell et al. (1979) have also found that in 0.1M sodium chloride, 30mM Tris-HCl, 0.1mM magnesium chloride, 0.5mM DTT pH 8, there is no binding between spectrin and 4.1 as measured by their migration in sucrose gradients. However Tyler et al. (1979) report the appearance of a new zone on sucrose gradients when spectrin is present in addition to 4.1. The apparent discrepancy in results is probably due to the low ionic strength buffer used by Tyler et al., viz. 20mM potassium chloride, 1mM sodium phosphate, 0.5mM EDTA, 0.5mM DTT pH 7.6. Tyler et al. further suggest from rotary shadowing results that 4.1 binds near one end of spectrin dimer. If this can be firmly established and if the 4.1 is bound to the end of

dimer which does not participate in tetramer formation, then a ternary complex such as is indicated in Fig. 2.19 would be geometrically feasible.

The stoichiometry of the spectrin-4.1-actin complex is at present unclear. Although Ungewickell et al. have found 1 : 1 molar ratios of spectrin tetramer : 4.1 and similar ratios exist in oligomer, triton shells and erythrocyte ghosts, as already mentioned, it does seem that very small quantities of 4.1 can lead to the presence of substantial amounts of, for instance, spectrin dimer in a complex, as shown in Fig.2.17. Perhaps the formation of the ternary complex induces a change in the F-actin filament which increases its binding constant for pure spectrin. Skeletal muscle tropomyosin binds to F-actin with a stoichiometry of one molecule of tropomyosin for every six actin monomers. The resulting filament gives rise to a lower activation of heavy meromyosin ATPase than does F-actin alone (Eaton et al., 1975). Thus one tropomyosin molecule can influence six actin monomers, and in this respect may be analogous to 4.1. It is interesting to note that the presence of tropomyosin inhibits ternary complex formation (E. Ungewickell, personal communication) and that tropomyosin inhibits the gelation of filamin with F-actin (Maruyama and Ohashi, 1978).

A search for 4.1 in other cell or tissue types, has not yet been made. Other actin binding proteins, such as that found in macrophages (Hartwig and Stossel, 1975) and filamin from smooth muscle (Wang et al., 1975; Shizuta et al., 1976) can bind directly to actin so it would be

interesting to know whether 4.1 or spectrin contain any sequence homologies with the other two proteins. A part of this question is considered in the next chapter.

2. Filamentous or monomeric actin in the erythrocyte?

Sedimentation in sucrose gradients of mixtures of spectrin tetramer and 4.1 with G-actin below the critical concentration, did not reveal the formation of high molecular weight complexes, so that although binding may take place, extended networks are not formed. These experiments were also performed with erythrocyte actin and there is no reason to believe that it behaves differently from muscle actin (Ungewickell et al., 1979). No gelation was observed in mixtures of oligomer with G-actin although gelation might have been affected by the low ionic strength of the buffer.

Despite this indirect evidence for the existence of F-actin in the cytoskeleton, the original *in vitro* demonstrations of spectrin-actin interactions involved G-actin. Examination of the high viscosity material from this experiment in the electron microscope (Pinder et al., 1979; Sato et al., 1979) revealed that it was not a network of spectrin and G-actin but filaments of F-actin, often with protein complexes (which are probably oligomers) at their ends. It is easily shown that a complex binding only to the ends of F-actin filaments has an extremely small effect on the thermodynamic stability of the filaments : the effective equilibrium constant $k = \sigma s^N$ (where N is the number of monomers in a long filament, $\sigma$ the binding

constant for initiation of an aggregate and s that of addition of monomers at the end) can only be minimally affected by a change in $\sigma$ . Furthermore, Lin et al. (1980) have achieved the same polymerization effects using either oligomers or actin "nuclei" consisting of 2 - 4 actin monomers cross-linked by p-NN'-phenylene bismaleimide, showing that the actin in these experiments is above its critical concentration.

Since it has been demonstrated that this viscosity effect is due to oligomer, and not dimer or tetramer, and oligomer contains erythrocyte actin, it seems most likely that actin "nuclei" in the oligomer initiate polymerization of actin which would otherwise be kinetically trapped in the G state. Assuming this mechanism, no interaction between muscle G-actin and spectrin or 4.1 is necessary. Evidence for the involvement of erythrocyte actin, rather than spectrin or 4.1 with added muscle G-actin will be discussed in 2.IV.4.

Puszkin et al. (1978) have succeeded in coating Lytron particles with both spectrin and erythrocyte G-actin. Although G-actin coated particles did not bind spectrin, the spectrin bound G-actin in a 1 : 8 molar ratio and some binding was also obtained using F-actin. However, it is not clear whether the spectrin in these experiments contained oligomer, in which case binding of G-actin could be explained as the phenomenon described by Pinder et al. (1975) and an interaction with F-actin could be similar to the gelation described above.

Although the existence of G-actin in the erythrocyte

is not required to explain the results of Pinder, Bray and Gratzer (1975), Dunbar and Ralston (1978) have analyzed the nucleotide bound to actin in erythrocyte ghosts. Its identification as ATP and not ADP suggests G and not F-actin. However, the analysis is not altogether convincing since AMP as well as ATP appears on the thin layer chromatograms.

The inability of Marchesi and Palade (1967) and Tilney and Detmers (1975) to observe F-actin filaments in ghosts using the electron microscope (except in cases where the ghosts had been treated with trypsin or heavy meromyosin) suggests an aggregation state of actin containing less than about 14 monomers.

An SDS gel analysis of proteins from red blood cell ghosts gives a molar ratio of dimer : actin of only 1 : 2 or 1 : 3 (J.C. Pinder, personal communication). The association of spectrin with actin by way of 4.1, the probable existence of only short strands of actin, and the inability of spectrin to polymerize beyond its tetrameric form (Ungewickell and Gratzer, 1978) suggest a network in which from 3 to 6 tetramers radiate from a short filament of F-actin (Fig. 2.20).

Short strands of actin have not yet been found in other cell or tissue types but a clue to the mechanism whereby they could be retained, comes from a recent publication by Grazi and Magri (1979) who have demonstrated that phosphorylated G-actin is unable to polymerize and that F-actin, upon phosphorylation, exhibits a small drop in viscosity. It is possible that phosphorylated species

could be "capping" short strands of actin in the red blood cell.

Investigation into the form of actin in other cells has only just begun. Thin sections of Physarum in isotonic conditions show fibrils of F-actin parallel to the long axis of the plasmodial strand whilst in the contracting or elongating phase, whereas in the contracted state, only feltlike networks can be seen in this ectoplasmic region (Nagai et al., 1975).

The sol to gel transformations observed in cell extracts can lead to optically isotropic structures (Condeelis and Taylor, 1977) in which filaments of actin are not visible but which, on addition of $10^{-6}$M calcium, rearrange to form F-actin and simultaneously display contractility. Kane (1975), on the other hand, obtains birefringent gels containing structures resembling paracrystals. In the sols, the presence of G-actin may not be physiologically meaningful as the constituents have been extracted at low ionic strength.

When certain echinoderm sperm come into contact with egg jelly, an acrosomal process is produced, which consists of bundles of F-actin (Tilney et al., 1973). Before contact with the egg jelly, the actin can be traced to the 'periacrosomal material", thin sections of which resemble G rather than F-actin (Tilney, 1976). Moreover, if a Triton extract of sperm is centrifuged at 80,000 x g for 3 hours, all the actin appears in the supernatant (Tilney et al., 1973). Polypeptide chains of molecular weight 250,000 and 230,000 are found with the actin in the periacrosomal material and Tilney (1976b) has suggested that these proteins

prevent the actin from polymerizing.

Lazarides and Lindberg (1975) identified the inhibitor of DNAse I in calf spleen as a non-muscle actin. This inhibitor can be crystallized and analysis shows that it is a complex containing equimolar amounts of actin and a protein of molecular weight 16,000 named profilin. Spleen actin not complexed with profilin polymerizes to form F-actin.

From these experiments, it is clear that actin exists *in vivo* in a number of different forms, often changing from one to another within a particular cell. However, the proteins with which actin is associated and the stimuli for changes in its supramolecular structure are often obscure.

### 3. The orientation and accessibility of actin in the erythrocyte membrane

F-actin filaments have a polarity which can be established by decoration with myosin subfragment 1 or heavy meromyosin which, when they bind to F-actin, give rise to a structure with the appearance of arrow heads in the electron microscope. Cohen and Branton (1979) have found that G-actin can polymerize onto inside-out vesicles (containing spectrin oligomer) and that the arrowheads formed by heavy meromyosin decoration point towards the membrane, in contrast to skeletal muscle where arrowheads point away from the Z-disc (Huxley, 1963). Elongation of F-actin takes place only at one end of the filament (see Pollard, 1975), in this case, the end distal to the membrane.

Reports of an increase in deformability of erythrocytes subjected to DNAse (Nakashima and Beutler, 1979), the finding that DNAse can induce an echinocyte to discocyte shape change in ghosts (E. Ungewickell, personal communication) and the inhibition of gelation between phosphorylated Triton-extracted ghosts by DNAse (Pinder, Bray and Gratzer, 1977), suggest that erythrocyte actin is accessible to this protein. However, attempts to decompose the oligomer present in the low salt extract from erythrocyte ghosts with DNAse I met with no success (E. Ungewickell, personal communication). Sheetz (1979) observed the dissolution of Triton-extracted ghosts, prepared with an isotonic buffer, by DNAse, but never without concomitant proteolysis. Evidently DNAse binds to the cytoskeleton, but in a way which does not destroy its integrity.

4. The association state of spectrin in the cytoskeleton

*In vitro* reconstitutions of the three purified proteins: spectrin, 4.1 and actin, suggest that a two-dimensional network cannot be formed unless spectrin is tetrameric. The ability of the 4°C low ionic strength extract, but not the 37°C extract, to gel in mixtures with F-actin, in agreement with the results of Fowler and Taylor (unpublished work), is consistent with the existence of two different types of oligomer with only the "4°C oligomer" containing tetrameric spectrin.

The extraction of spectrin as tetramer at 4°C suggests that this is it's *in vivo* association state, although

in vitro at concentrations of 1 - 4 mg/ml, 0.15M ionic strength and at 37°C, dimer is slightly favoured. Presumably, the immobilization of tetramer on the membrane results in a higher effective concentration which shifts the equilibrium.

Triton-extracted ghosts can be prepared from ghosts which have been incubated at intermediate ionic strength at 37°C in order to effect tetramer to dimer conversion. These Triton shells are more fragile than those prepared from "tetramer ghosts", but they do not disintegrate altogether, intimating that other proteins can give rise to a stable network (Liu and Palek, unpublished work). The known association of spectrin with ankyrin and band 3 (Bennett, 1978; Bennett and Stenbuck, 1979) and the ease with which band 3 can dimerize (Wang and Richards, 1974; Yu and Steck, 1975; Kiehm and Ji, 1977) could lead to a network independent of tetramer (Fig. 2.21).

It is unlikely that the 10-30% of spectrin extracted at 37°C which remains oligomeric at low ionic strength contains tetramers unless they are stabilized by another protein, such as 4.9. However, with a sedimentation coefficient of about 27 S (Lin and Lin, 1979), "37°C oligomer" could have a low enough molecular weight to consist of a short filament of actin with dimers bound to it by means of 4.1. There is no evidence that it forms a lattice.

It is unlikely that there is any tetramer in the pellet which remains after the extraction of hypotonic Triton-extracted ghosts in low ionic strength solution at 37°C, but in this case, 2.1 and part of band 3 remain, so that the pellet

probably contains an extended structure depending on these two proteins (e.g. Fig.2.21). In the absence of other proteins, it seems that tetrameric spectrin is essential for a network between spectrin 4.1 and actin to exist.

5. <u>The regulation of complex formation</u>

a. <u>Cytochalasin</u>

Hartwig and Stossel (1976) have reported the disruption of gels of F-actin and actin binding protein by cytochalasin B. They proposed (Hartwig and Stossel, 1979) that the drug caused breakages of actin filaments between the cross-links formed by actin binding protein. However, Lin and Lin (1979) found inhibition of the rate of polymerization, but not the final viscosity of actin added to oligomer, with concomitant binding of cytochalasin to oligomer. Subsequent work (Lin et al., 1980) has shown that short filaments of F-actin or trimers cross-linked with p-NN'-phenylene bismaleimide also bind cytochalasin with the same effect, but that there is no binding with G-actin. Thus, cytochalasin B probably binds to actin nuclei in oligomer, preventing or slowing down the addition of further monomers. The binding of G-actin to inside-out vesicles presumably takes place by the same mechanism since it, too, is cytochalasin dependent (Cohen et al., 1978). If the gelation of oligomer with F-actin involved any interaction between muscle and erythrocyte actin, cytochalasin would at least be expected to have some effect on the rate of gelation. The similarity between gels in the presence and absence of the drug suggests that the

interaction is similar to that between the separate proteins: tetramer, 4.1 and actin. The gelation of Triton-extracted ghosts with F-actin probably takes place by the same mechanism, implying that actin binding sites in these ghosts are not saturated by endogenous actin. It is thus likely that conditions affecting gelation with the oligomer, with Triton-extracted ghosts, or with the three separate proteins will be similar.

b. Phosphorylation

Despite the correlation between the phosphorylation of band 2 of spectrin and the echinocytic to discocytic shape change in ghosts (Birchmeier and Singer, 1977a) the effect of phosphorylation on oligomer formation (2.III.5) and the gelation of hypotonic Triton-extracted ghosts upon incubation with erythrocyte cAMP-independent kinase (Pinder, Bray and Gratzer, 1977), neither gelation with oligomer, nor complex formation between tetramer, 4.1 and actin (Ungewickell et al., 1979) were affected by the state of phosphorylation of spectrin. Birchmeier and Singer (1977a) reported that no other species in ghosts was phosphorylated in parallel with spectrin. 4.1 only responds to a cAMP dependent kinase (Hosey and Tao, 1976a). J.C. Pinder (personal communication) has recently observed the incorporation of $^{32}P$ from labelled ATP into muscle actin incubated with a spectrin extract and erythrocyte kinase, in conditions where the spectrin extract induces the polymerization of the actin. A histidine residue may be involved since this protein phosphate bond is alkali-

labile. This explains why autoradiography of SDS gels (Hosey and Tao, 1976b; Birchmeier and Singer, 1977a) where samples have previously been heated in alkaline solution fail to indicate the presence of labelled actin. It is possible that processes affected by phosphorylation all involve actin-actin interactions whereas gelation with oligomer or spectrin and 4.1, involve interactions between actin and other proteins. Further experimental work is required to determine whether actin phosphorylation occurs in the red cell and whether it is involved in regulating the properties of the cytoskeleton.

c. Ionic strength

Complex formation at physiological ionic strength requires the presence of all three proteins: spectrin tetramer, 4.1 and actin. In contrast, Tyler at al. (1979) find a binary complex between spectrin and 4.1 in 20mM potassium chloride. The weak interaction between spectrin tetramer and actin which took place in 70mM potassium chloride and 1mM Mg-ATP was described in 2.II.13, and agrees with the results of Brenner and Korn (1979). Under these conditions no interaction between 4.1 and actin was detected. The effect of low ionic strength solutions (favouring the depolymerization of actin) on the complex, have not been investigated.

It is under these conditions of low ionic strength that spectrin is extracted from the membrane, and persistence of oligomer in these extracts after overnight incubation at low ionic strength which would lead to the

depolymerization of F-actin suggests that short strands of F-actin are stabilized in oligomer, though structures containing only G-actin cannot yet be ruled out and extraction conditions in which divalent metal cations are absent encourages caution in treating the actin-containing oligomer as a native structure. A closer comparison between the reconstituted complex and oligomer persisting at low salt may throw some light on the nature of the cytoskeleton.

The reason why part of the cytoskeletal complex apparently remains intact during extraction at low ionic strength, while another part disintegrates, leaving a considerable amount of 4.1 in the membrane, is obscure. Partial denaturation of the actin is a possibility as is also the existence of two native populations of actin, spectrin or 4.1.

d. Temperature

The effects of temperature on tetramer and oligomer and consequent interactions with other proteins have been discussed (2.III.4, 2.III.6). In a complicated environment such as the red cell membrane, the low yields of pure spectrin obtained from depleted membranes extracted at 4°C, but not at 37°C, are difficult to explain. However, the effect of the dephosphorylation of dimer and tetramer on their binding to 2.1 has never been investigated.

e. Divalent metal cations

Calcium has little effect on the gelation of oligomer with F-actin. In this respect, this system resembles

extracts from baby hamster kidney cells (Schloss and Goldman, 1979). Many gelation phenomena using purified proteins exhibit the same behaviour but recently Yin and Stossel (1979) and Mimura and Asano (1979) have isolated proteins which confer calcium sensitivity on gelation. However, those cells in which gelation is inhibited by micromolar calcium concentrations, are all cells in which myosin is found. Furthermore, Condeelis and Taylor (1977) have shown that the conditions which inhibit gelation are just those which favour contraction of the extract after actomyosin formation: calcium binding may release actin from actin binding proteins for use in contractile events (Pollard, 1976a). Although we have little knowledge of the molecular events accompanying shape changes in erythrocytes, gross supramolecular structures do not turn over as they do in, for instance, cytoplasmic streaming (Nagai et al., 1975). The absence of calcium regulation is not, therefore, surprising. On the other hand, there is evidence of molecular rearrangement on phosphorylation (Pinder et al., 1977, 1978) and the possibility of calcium regulated events has not yet been excluded.

Gelation is unaffected by magnesium.

## Summary

At physiological ionic strength, no interaction between spectrin dimer and either muscle F-actin or G-actin below its critical concentration was detected. At intermediate ionic strength and in the presence of 1mM Mg-ATP weak binding of spectrin tetramer to F-actin occurred.

Impure spectrin, extracted from ghosts at 37°C, on the other hand, binds to F-actin as shown by their cosedimentation. With a 4°C extract, above certain limiting protein concentrations, gelation occurs. The active factor in this process is spectrin oligomer formed in a 4°C extract which is evidently different from "37°C oligomer". Hypotonic and isotonic Triton-extracted ghosts also form gels with F-actin.

Spectrin oligomer consists of spectrin, erythrocyte actin, 4.1 and 4.9, though other trace components may be present, which are not detectable by SDS gel electrophoresis. Treatment of oligomer with low or high (1M) salt, whether the spectrin is phosphorylated or dephosphorylated, does not lead to its complete dissociation, nor do sodium deoxycholate, 7M urea, or mixtures of 7M urea and detergents. Triton-extracted ghosts are also not completely dispersed by either low or high salt. However, all these changes in condition lead to an alteration of the proportion of protein in the complexed form.

Tetramer, 4.1 and F-actin mixed together form a gel. In the absence of any one of the proteins, or with spectrin in the dimeric state, no gel is formed. However sedimentation studies show that dimer can still bind to 4.1 and F-actin.

Electron microscopy of the complex shows F-actin filaments which are often bridged by spectrin tetramers. 4.1 is not visible. With the dimer in place of tetramer, spectrin can still be seen attached to the actin filaments but no cross-links are visible. This complex has the potential to form a two-dimensional network resembling the cytoskeleton of the erythrocyte.

Figure 2. 1.

Elution profile of spectrin extracts from a Sepharose 4B column. 4°C extract ( —— ). 37°C extract ( - - - - ). Peak (a) contains oligomer, (b) spectrin tetramer, (c) spectrin dimer, (d) mostly actin, (e) haemoglobin. 7.5% SDS gels of oligomer and dimer are shown.

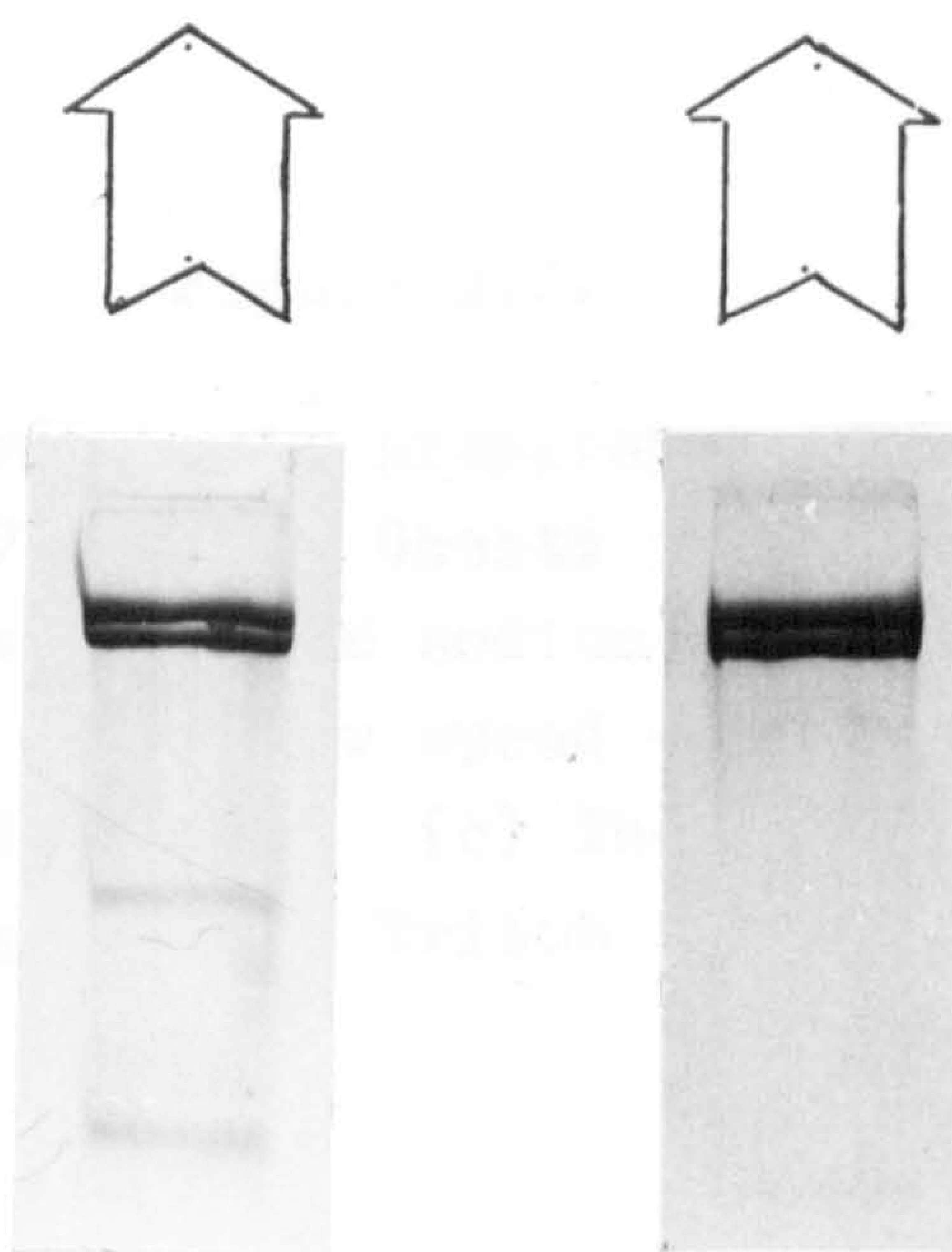
a
b
c
d
e

ABSORBANCE (280 nm)

0.20
0.15
0.10
0.05
0

100
200
300
400

ELUTION VOLUME (ml)



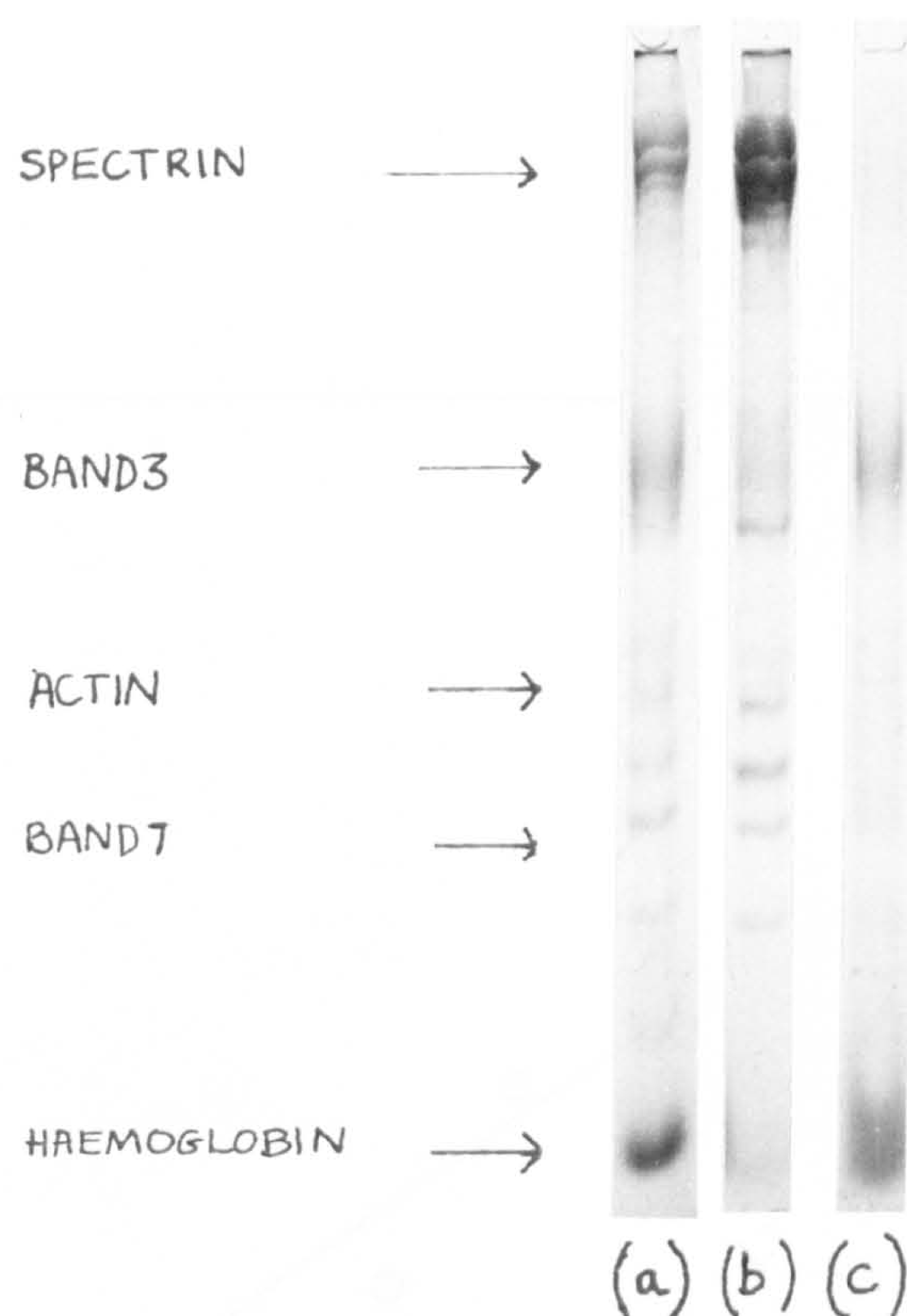



Figure 2.2.

Triton-extracted ghosts prepared by the method of Yu et al. (1973). (a) Ghosts prepared by the lysis of red blood cells with 5mM sodium phosphate, pH7.5. (b) The pellet after low speed centrifugation of Triton X-100 extracted ghosts. (c) The supernatant after low speed centrifugation of Triton X-100 extracted ghosts.

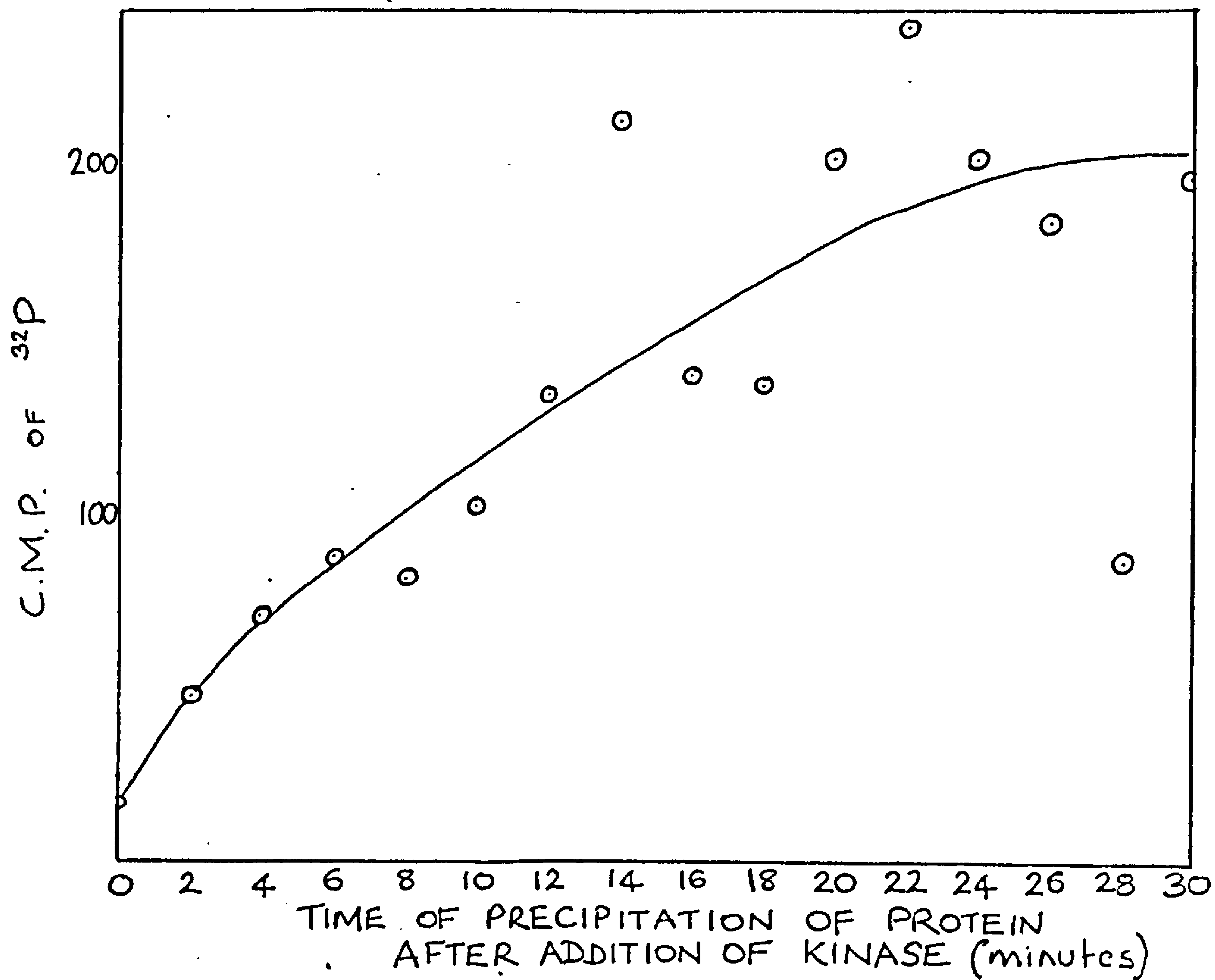
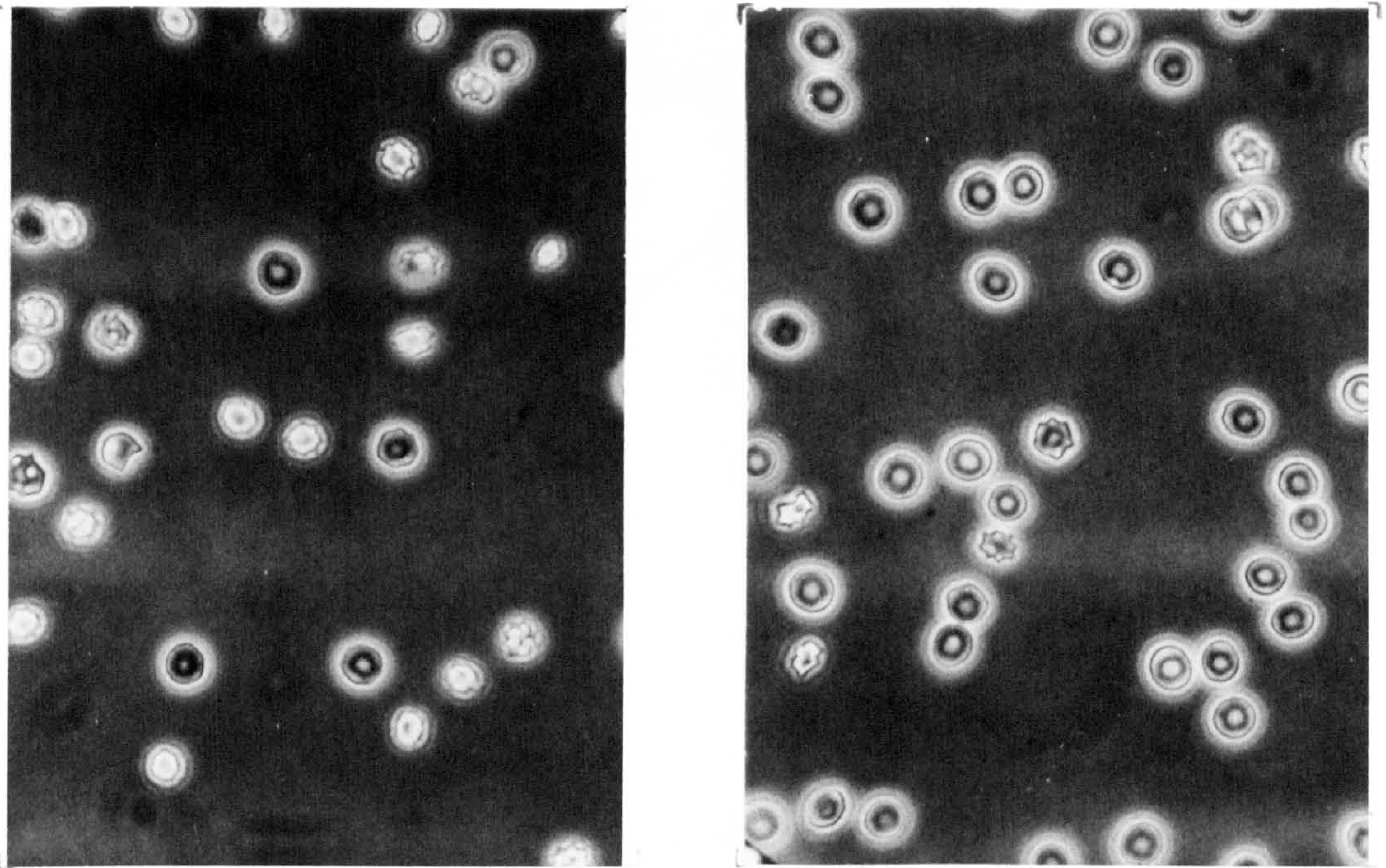



Figure 2. 3.

The incorporation of $^{32}P$ into purified spectrin dimer upon incubation with $^{32}P$ - ATP and erythrocyte membrane kinase.



(a) (b)

Figure 2. 4.

The conversion of echinocytes from outdated blood bank blood to discocytes by incubation of the cells in a medium containing adenosine and glucose. (a) outdated blood bank blood, (b) the same after incubation. Magnification X 400.

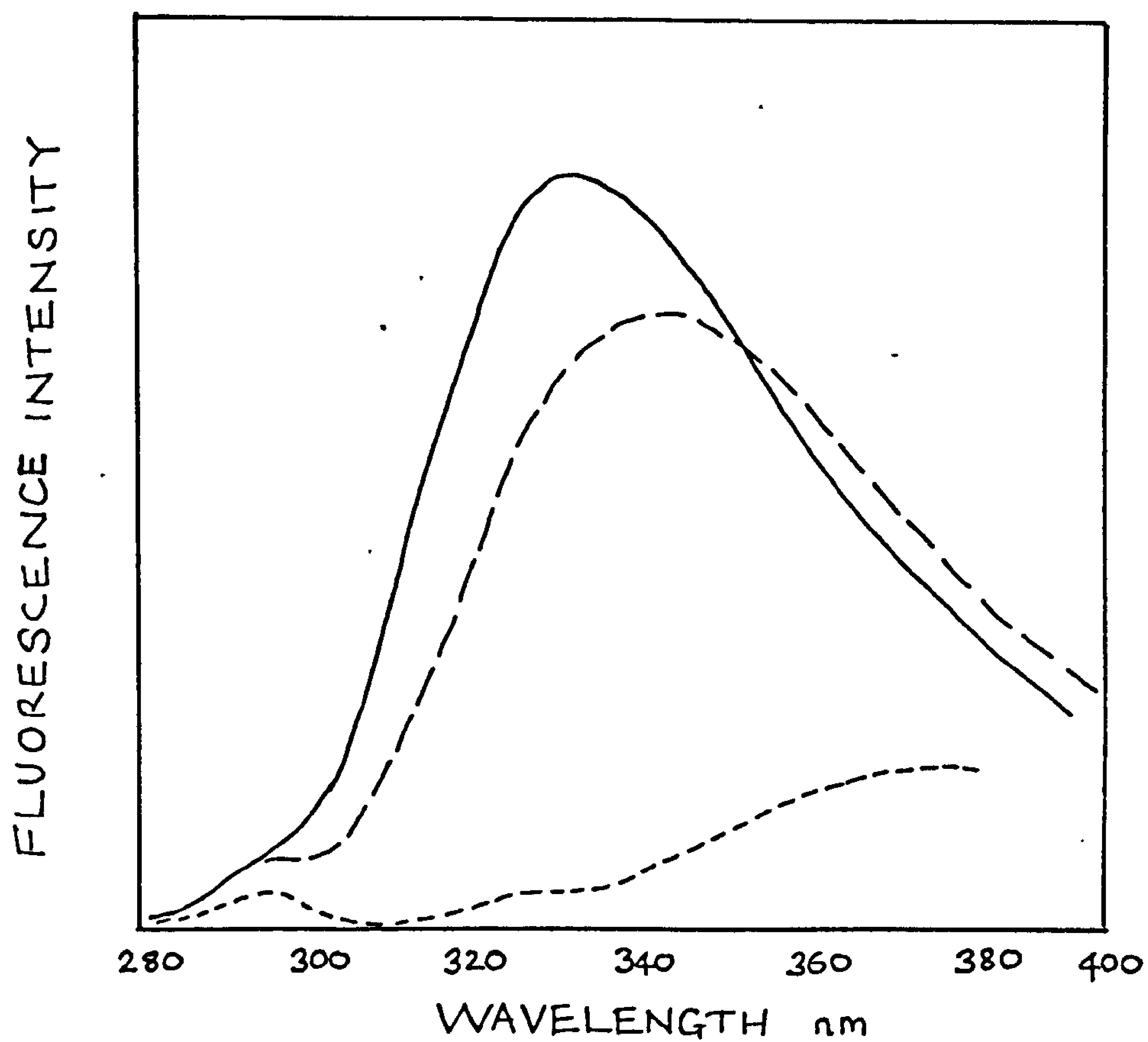



Figure 2.5

Fluorescence spectrum of actin before (———) and after (– – – –) the addition of EDTA. Buffer blank (-----).

0.1M KCl 0.16M KCl 0.3M KCl 0.4M KCl

0.3
0.2
0.1
0
absorbance

2 4 6 8 10 22 24 26 28 51 53 70 72 74

Fraction number

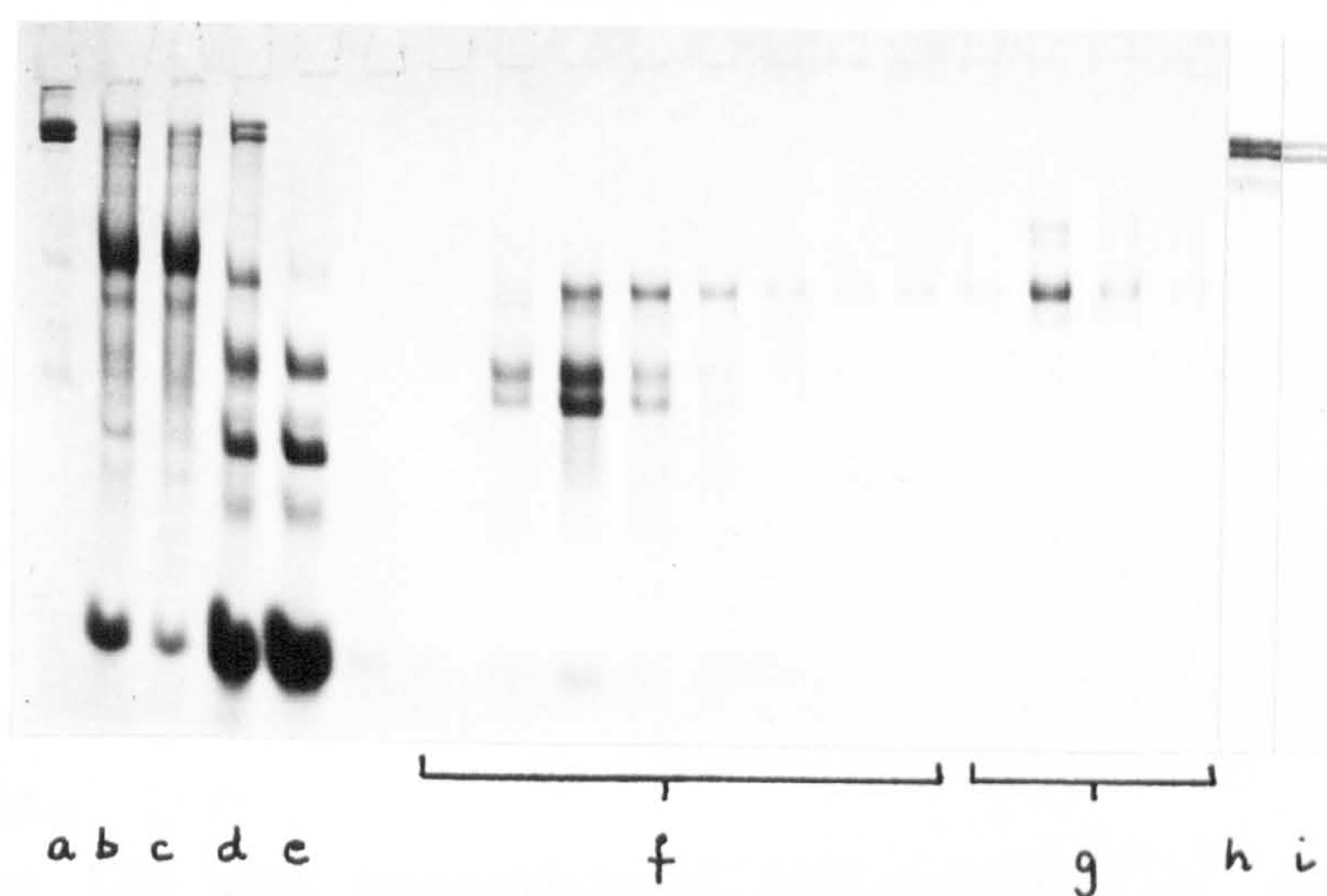



Figure 2.6

Elution profile of crude 4.1 from DE52 and SDS gels of (a) oligomer (b) spectrin-depleted vesicles (c) 4.1 - extracted vesicles (d) crude 4.1 (e) 7.5 mM phosphate effluent from DE52 (f) 0.1M potassium chloride step (g) 0.16M potassium chloride step (h) 0.3M potassium chloride step (i) 0.4M potassium chloride step.

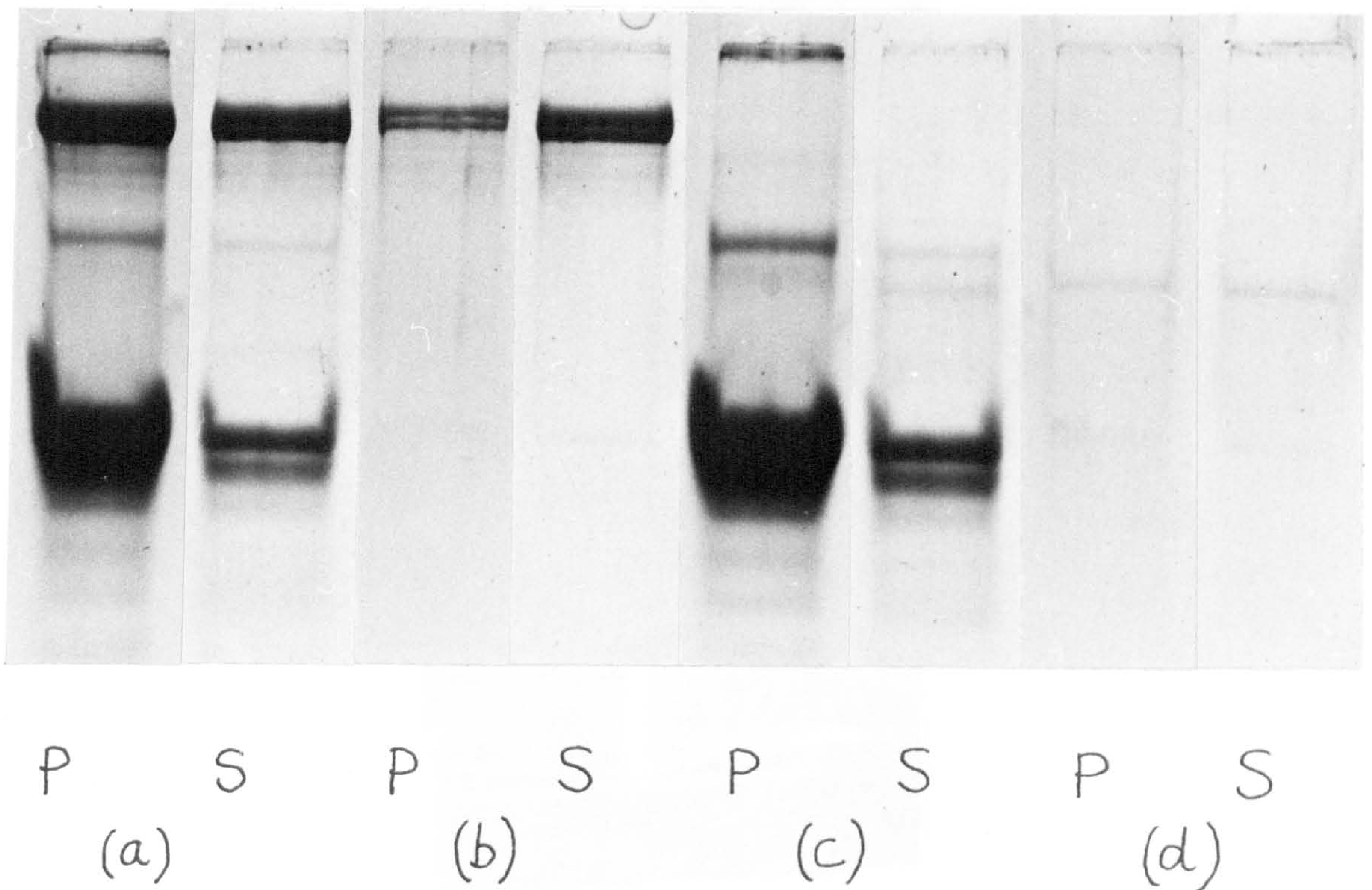



Figure 2.7

An assay for binary interactions between spectin and F-actin, and 4.1 and F-actin in the presence of 1mM magnesium. SDS gels of the pellets and supernatants after low speed centrifugation of the binary mixtures. Gel samples represent 20% of the pellet and 4% of the supernatant.

(a) Pellet (P) and supernatant (S), actin + spectrin tetramer.

(b) Spectrin tetramer alone (the total spectrin concentration is only $\frac{2}{3}$ of that in (a) ).

(c) 4.1 + actin.

(d) 4.1 alone.

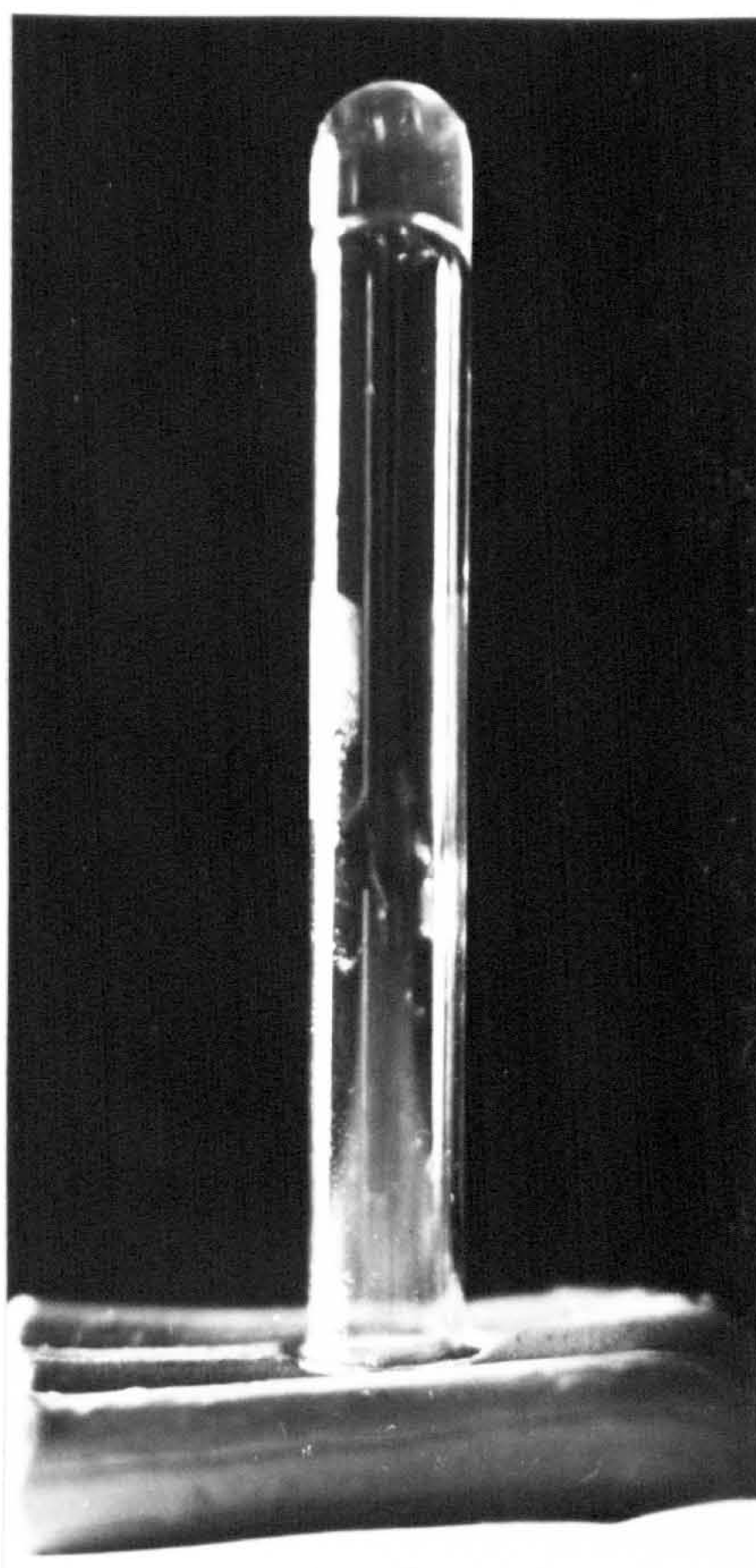


Figure 2. 8.

Gel formed between F-actin (0.9 mg/ml) and crude spectrin (0.4 mg/ml).

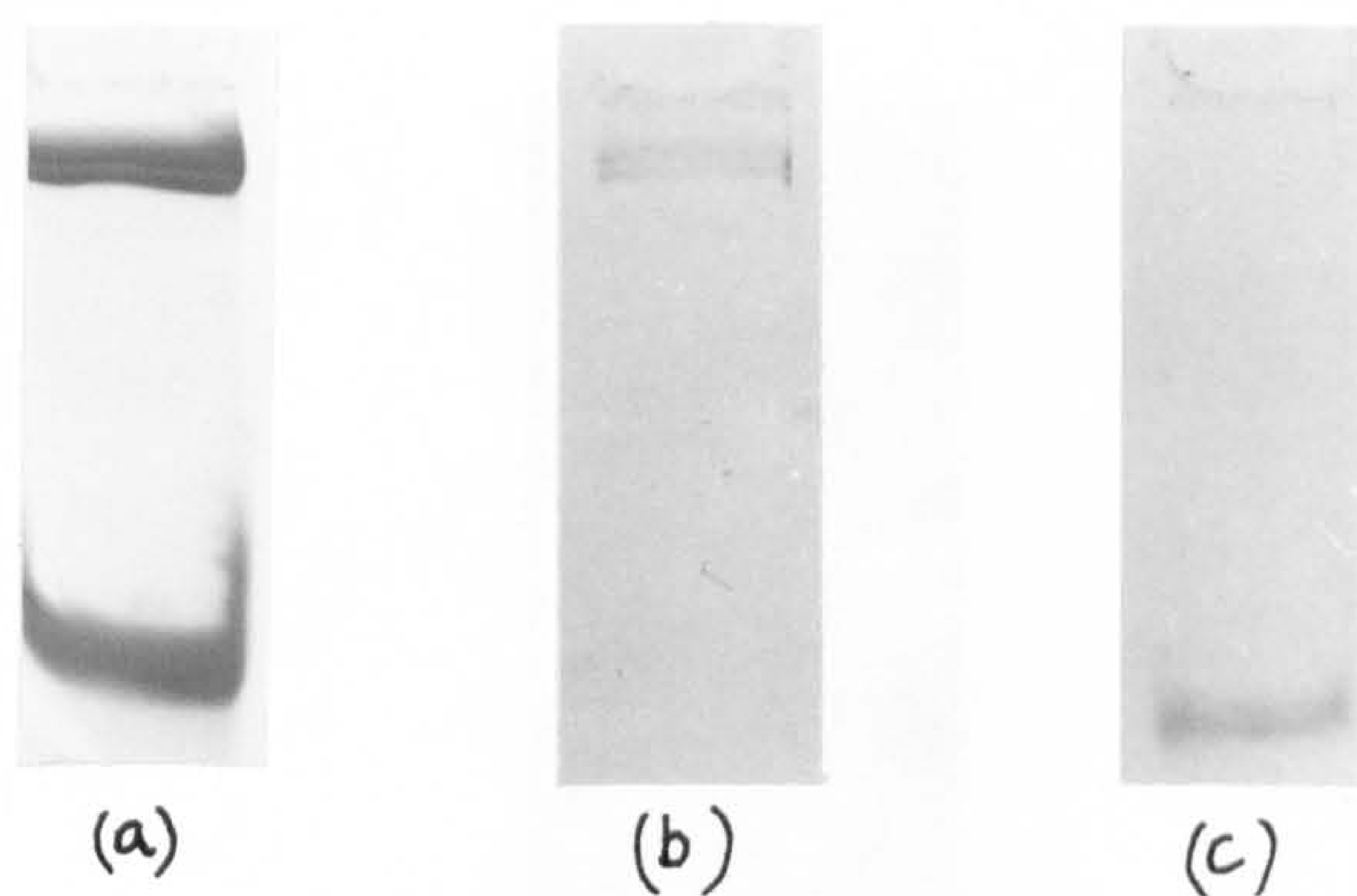



Figure 2. 9.

SDS gels of pellets from the low speed centrifugation of gels formed between crude spectrin and actin. Gel samples represent 10% of the pellet.

(a) Crude spectrin + F-actin.
(b) Crude spectrin alone.
(c) F-actin alone.

Figure 2.10

SDS gels of pellets and supernatants from the low speed centrifugation of mixtures of crude spectrin and actin in a variety of conditions.

(a) (i) Control. F-actin + spectrin.

(ii) G-actin + spectrin, followed by 30mM potassium chloride.

(b) (i) F-actin + spectrin; no calcium.

(ii) F-actin + spectrin; 0.1mM total calcium.

(iii) F-actin + spectrin; 1mM total calcium.

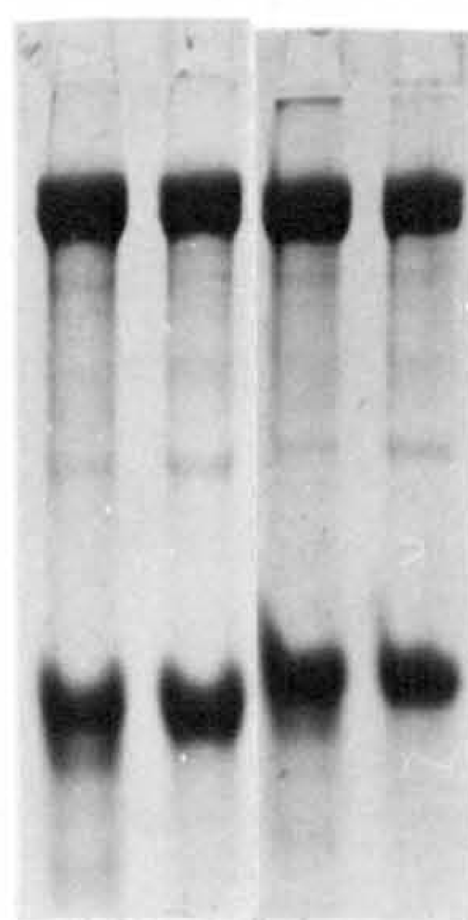
(c) (i) F-actin + spectrin.

(ii) F-actin + dephosphorylated spectrin.

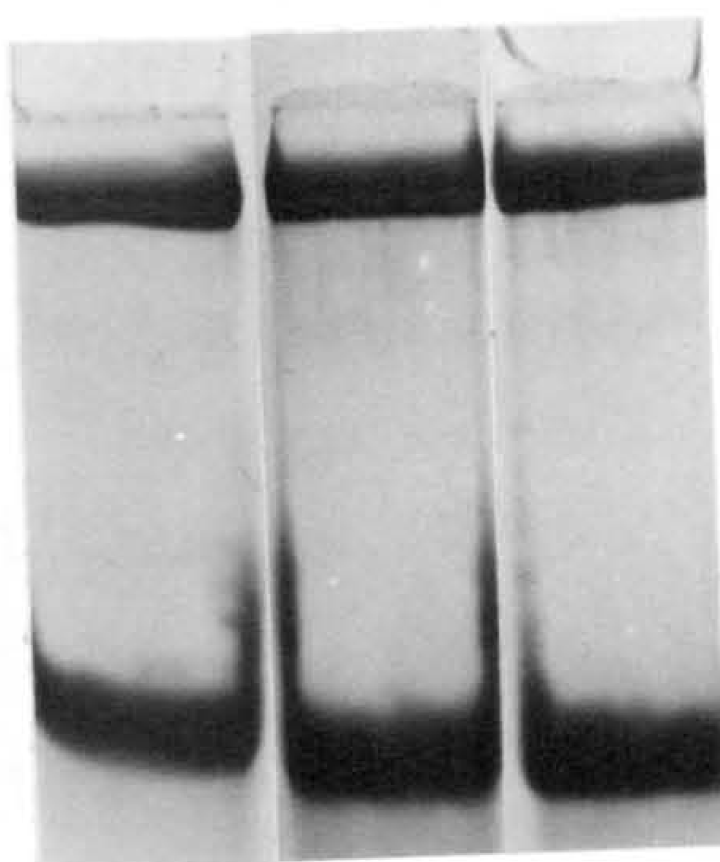
(d) (i) F-actin + spectrin.

(ii) F-actin + spectrin in the presence of cytochalasin B.

(a)

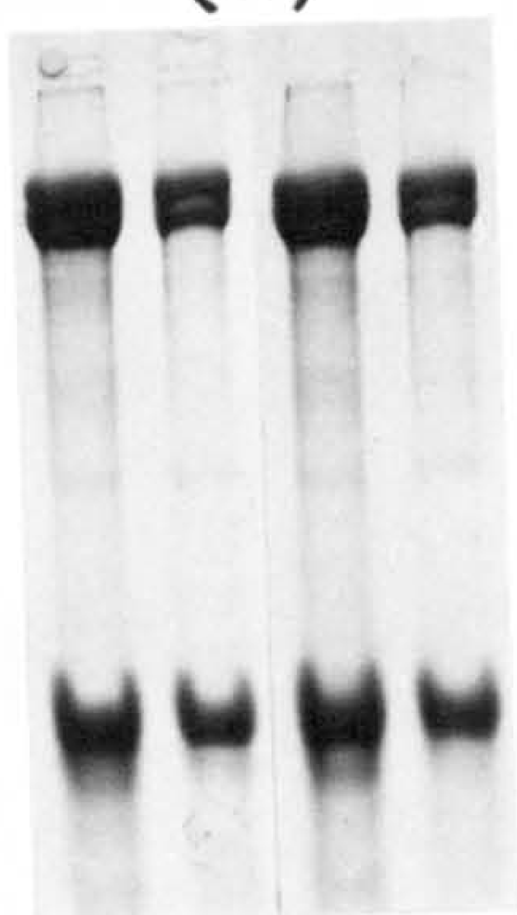


(b)



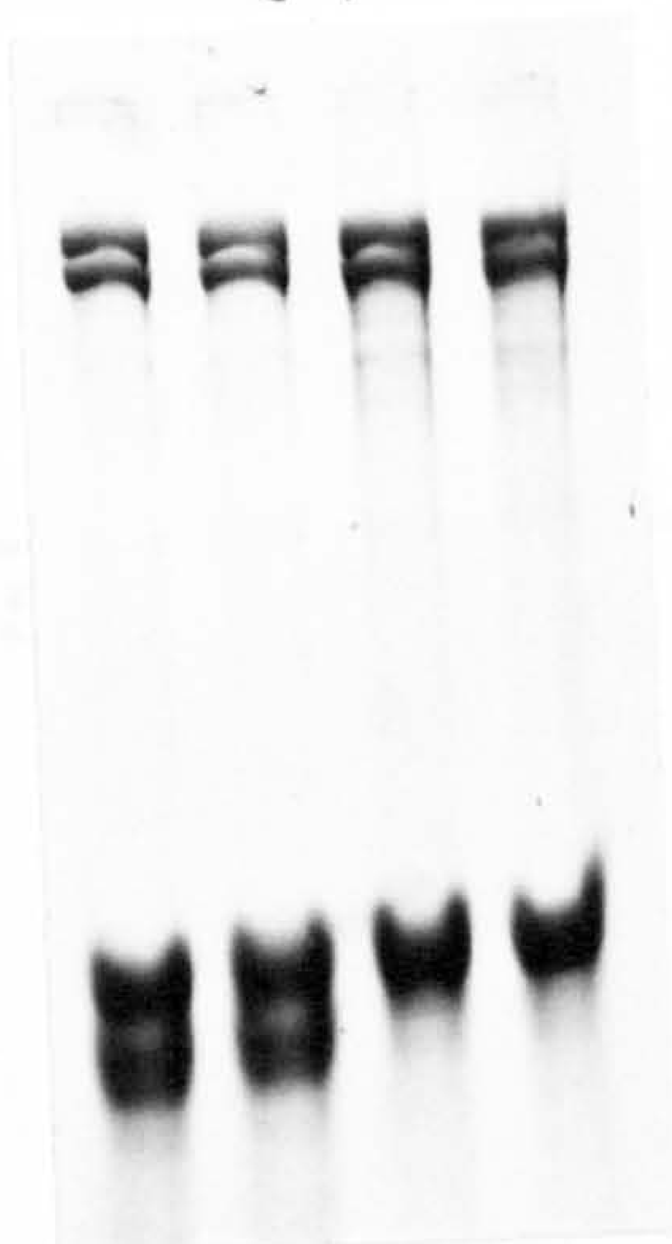
P P P
(i) (ii) (iii)

(c)



S P S P
(i) (ii)

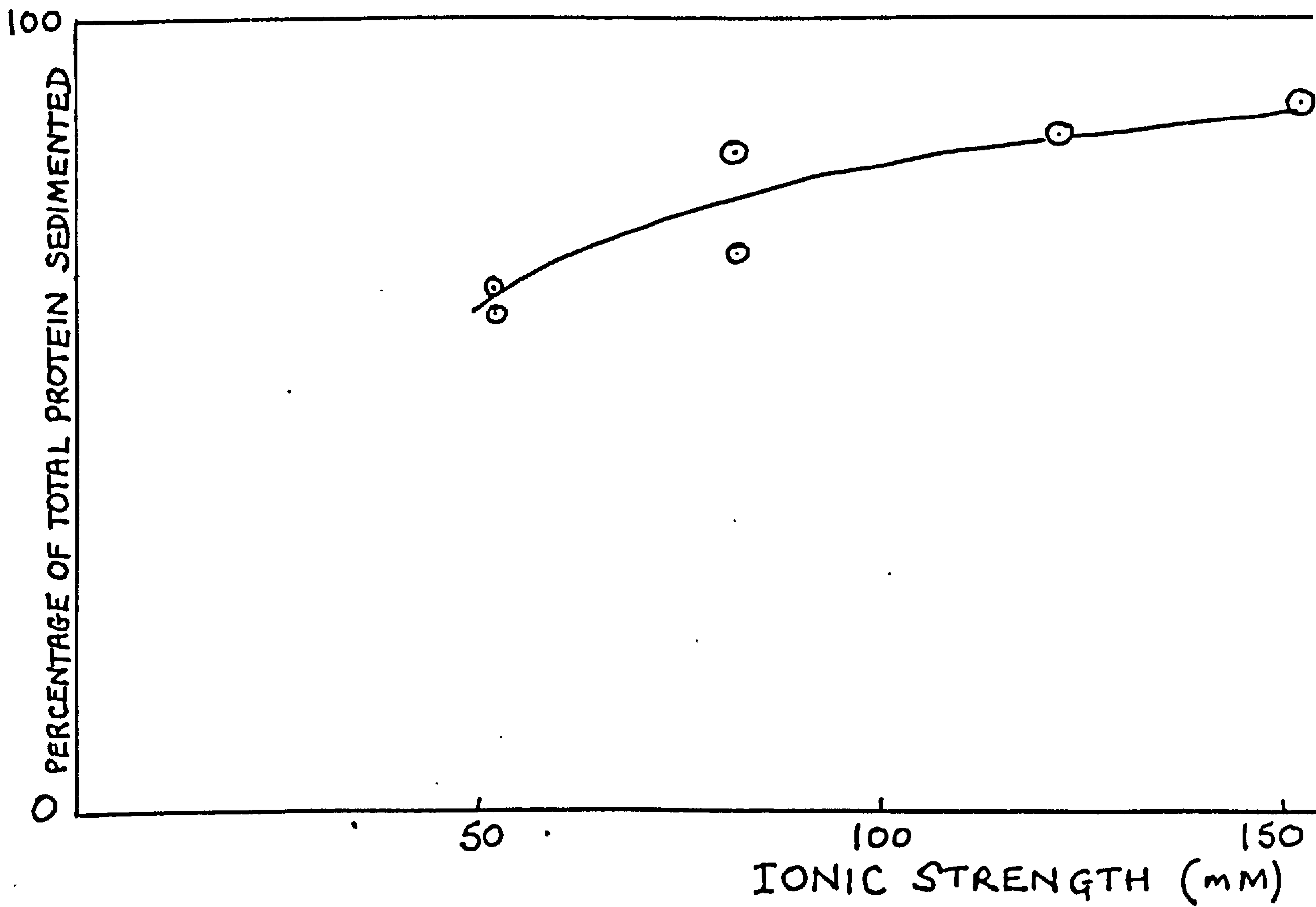
(d)



P P S S
(i) (ii) (i) (ii)

Figure 2.11

The effect of ionic strength on the percentage of total protein sedimented at low speed from a mixture of crude spectrin and F-actin.



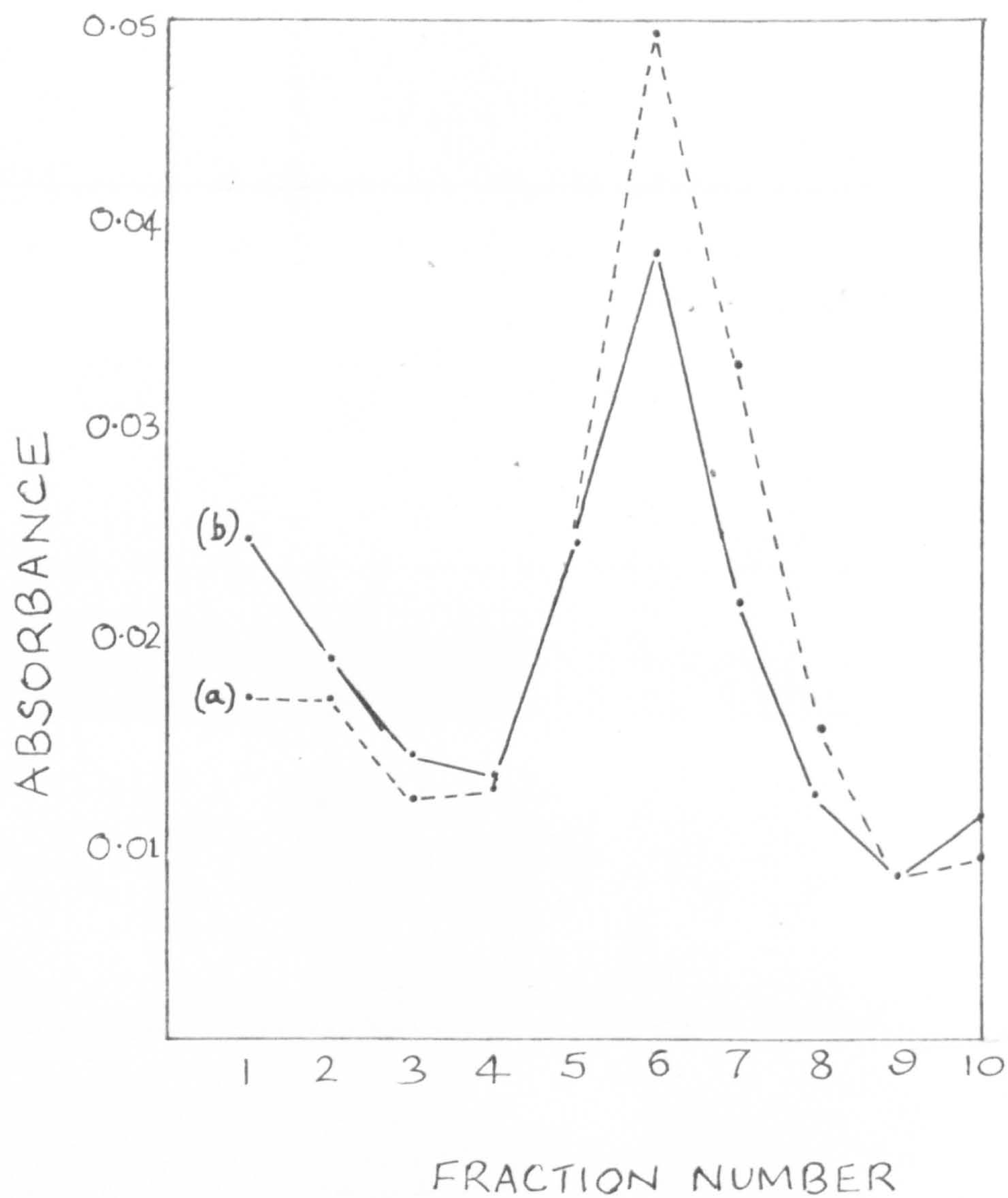
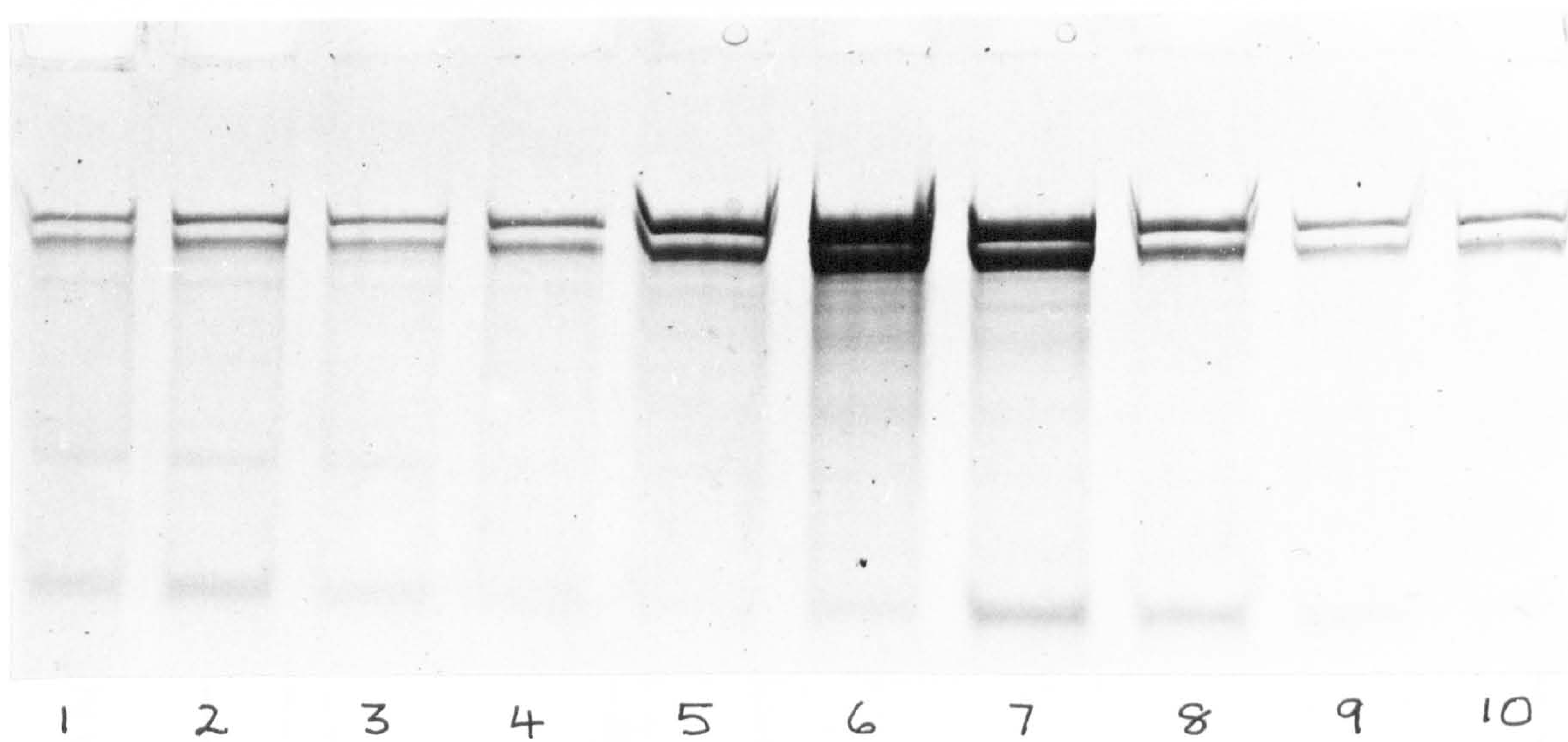
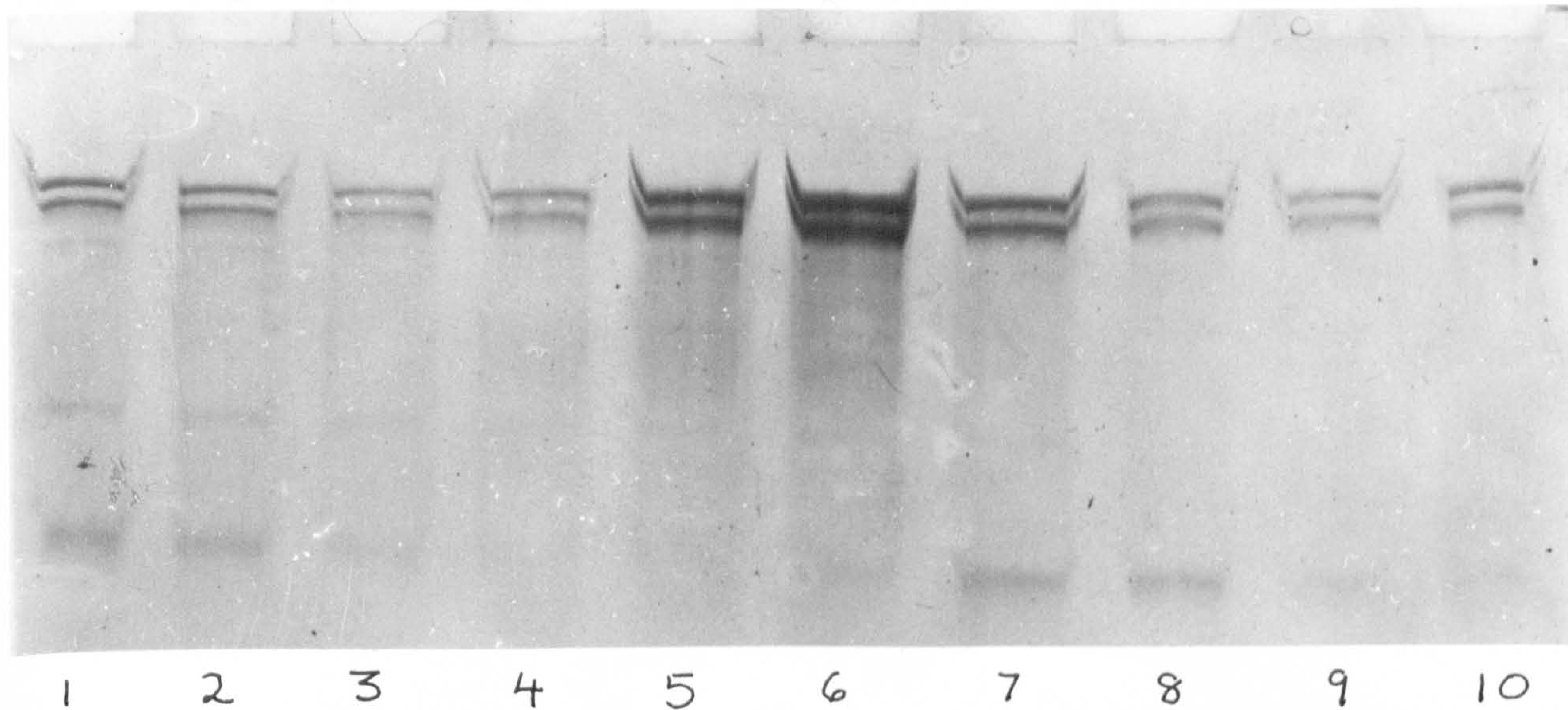



Figure 2. 12.

Sedimentation of crude spectrin in sucrose gradients at low ionic strength. Absorbances and SDS gels of each fraction using extracts from (a) fresh and (b) depleted red blood cells.




(a)




(b)

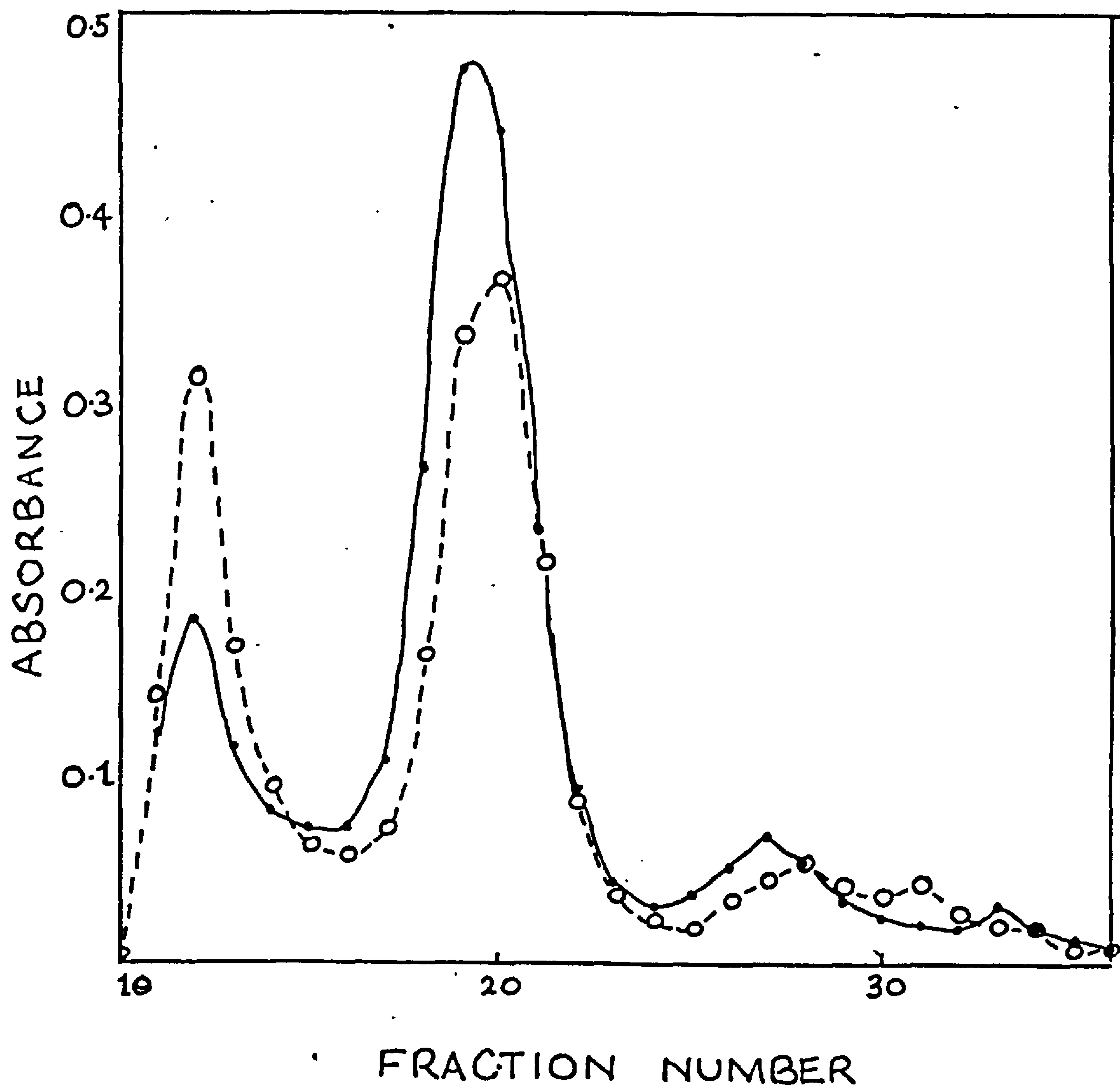



Figure 2.13

Elution profiles of crude spectrin from Sepharose 4B. A preparation (a) from fresh cells, (b) from depleted cells.

Figure 2. 14.

SDS gel of oligomer, cross-linked by dimethyl-suberimidate under a variety of conditions.

(a) Oligomer in aqueous solution with (X) and without (O) cross-linker. (5% gel).

(b) Dimer in aqueous solution. (5% gel).

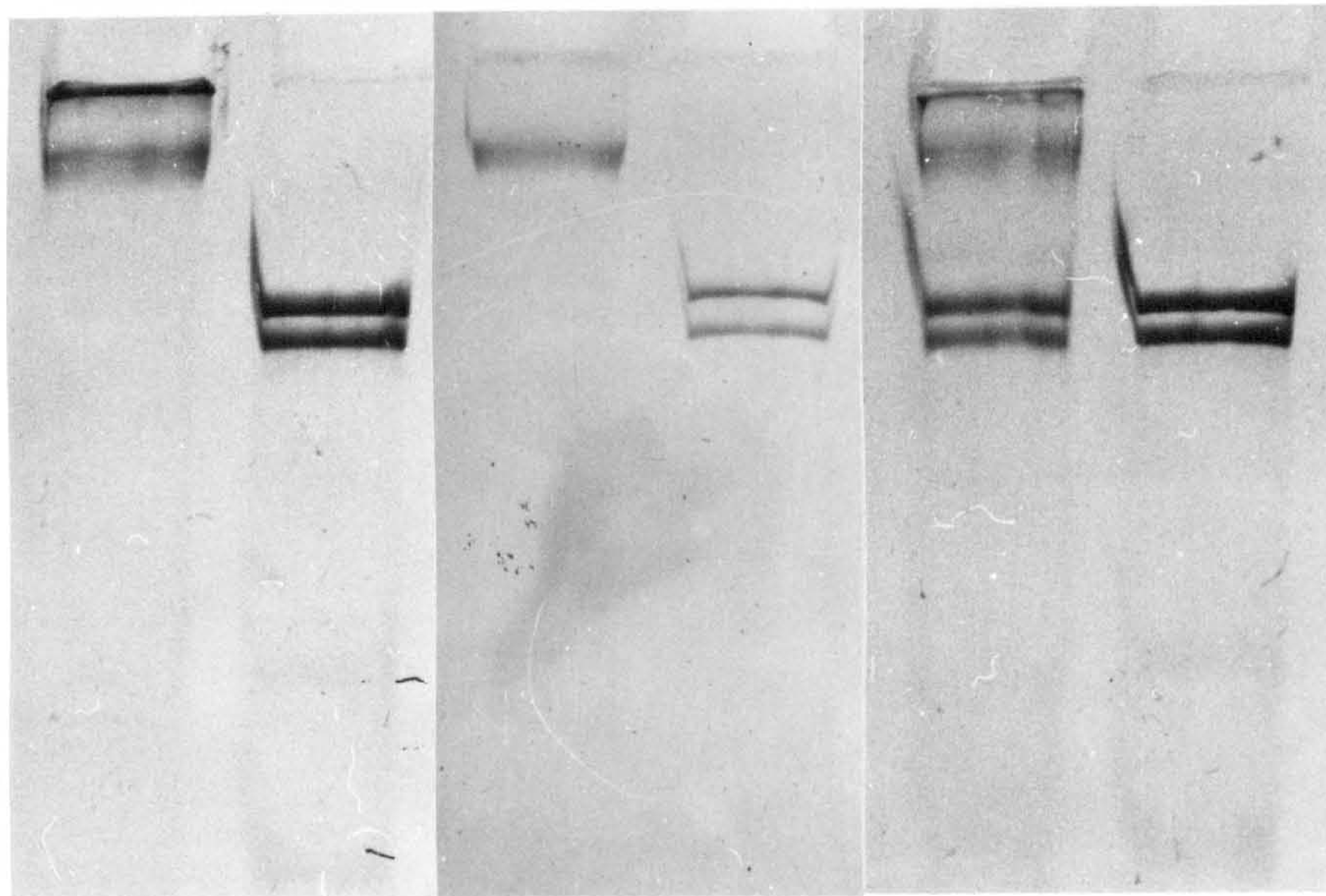
(c) Oligomer in sodium deoxycholate. (5% gel).

(d) Oligomer dialyzed into 6M guanidine hydrochloride and then 7M urea. (7.5% gel).

(e) Oligomer in 7M urea. (7.5% gel).

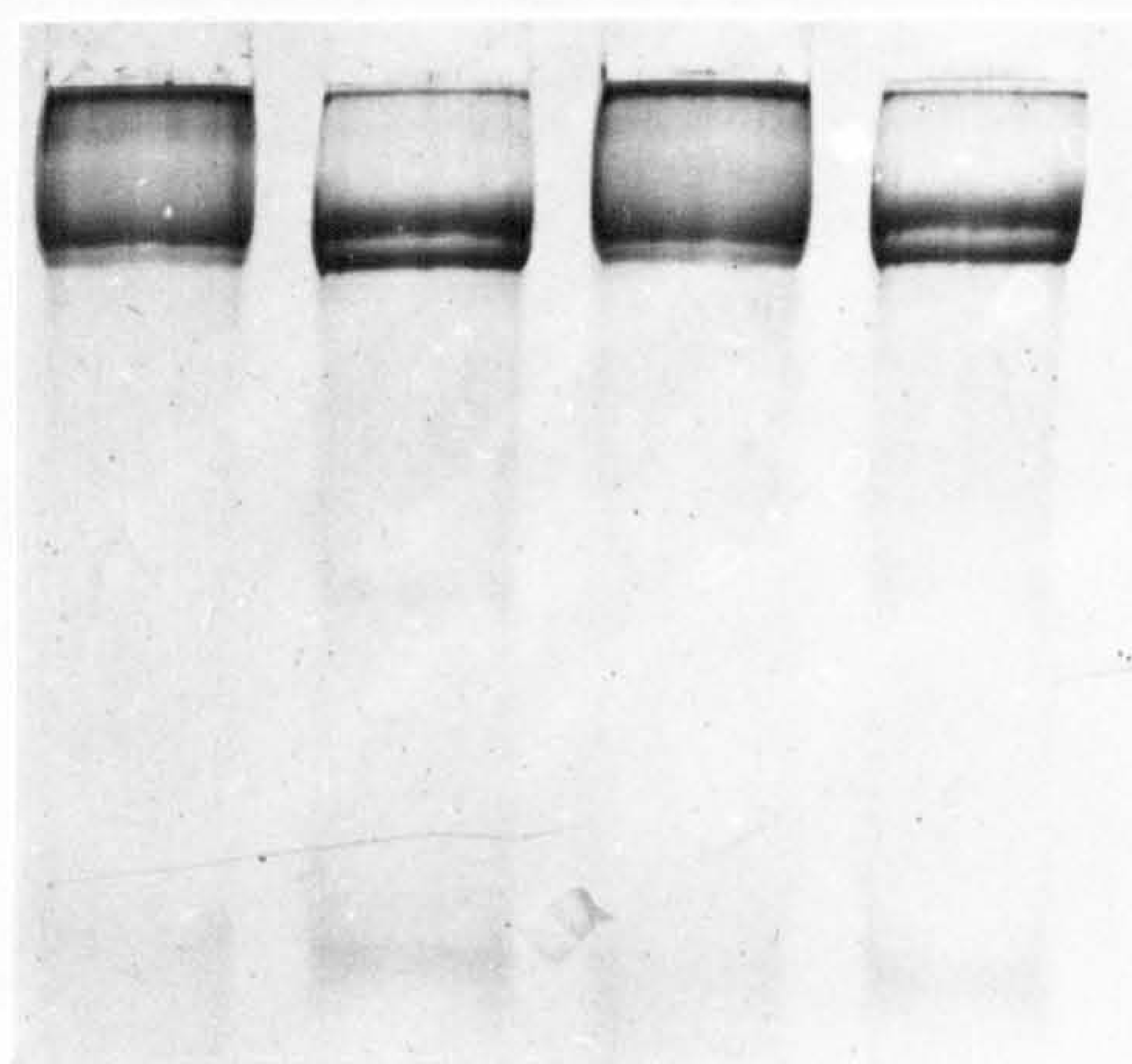
(f) Oligomer in deoxycholate and 7M urea. (7.5% gel).

(g) Oligomer in Triton X-100 and 7M urea. (7.5% gel).



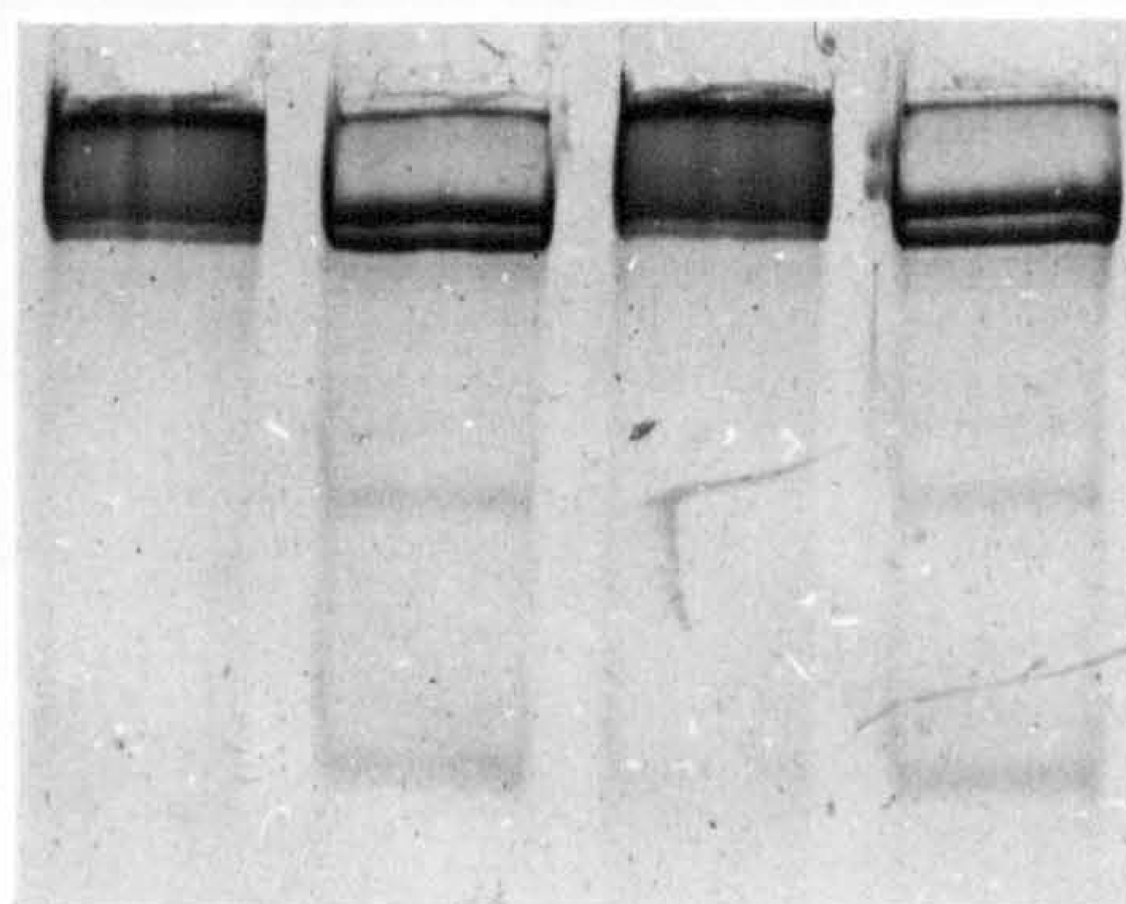
X O X O X O

(a) (b) (c)



X O X O

(d) (e)



X O X O

(f) (g)

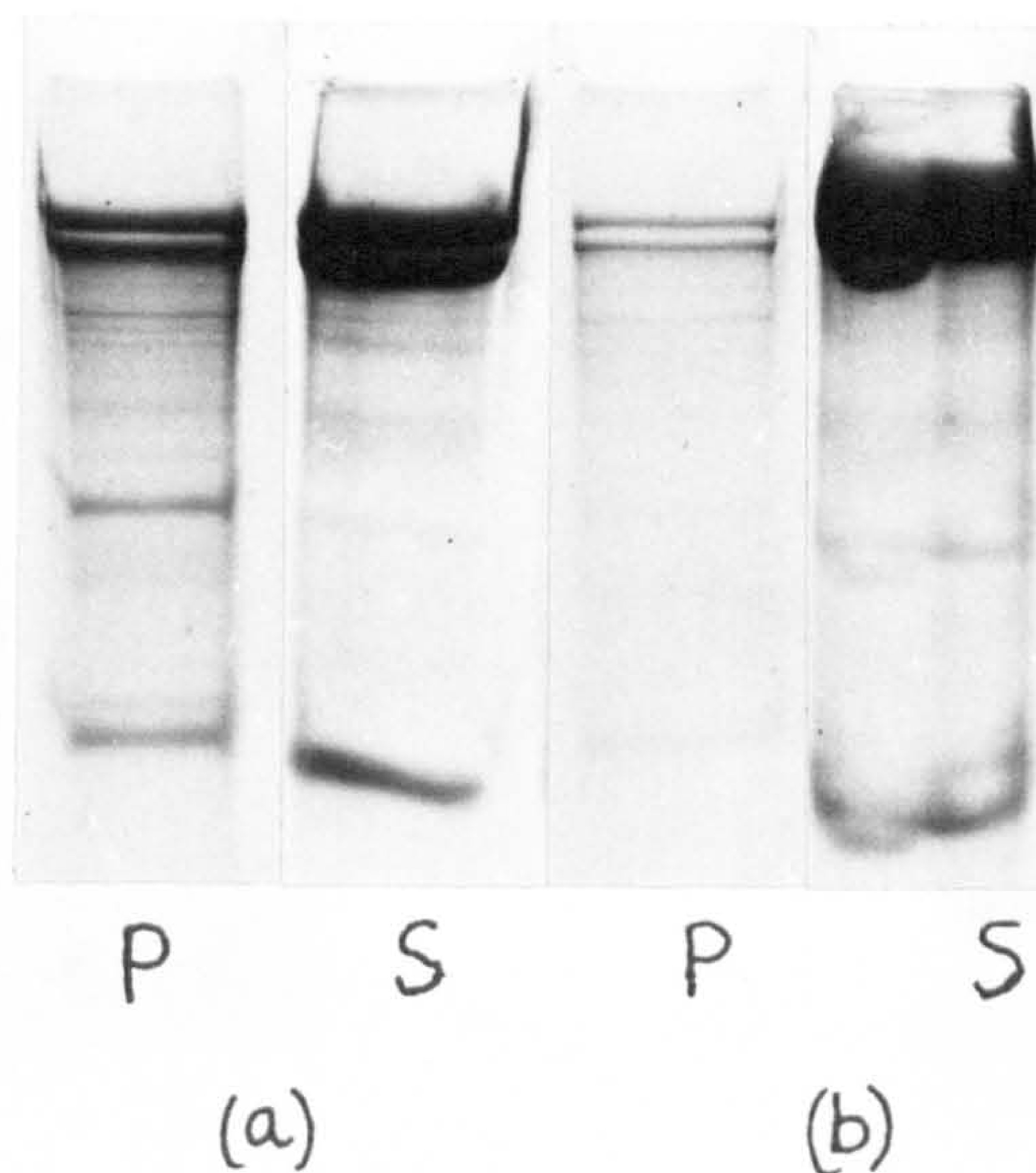



Figure 2.15

SDS gels of the pellets and supernatants after centrifugation of crude spectrin incubated (a) at physiological ionic strength, (b) in 1M sodium chloride.

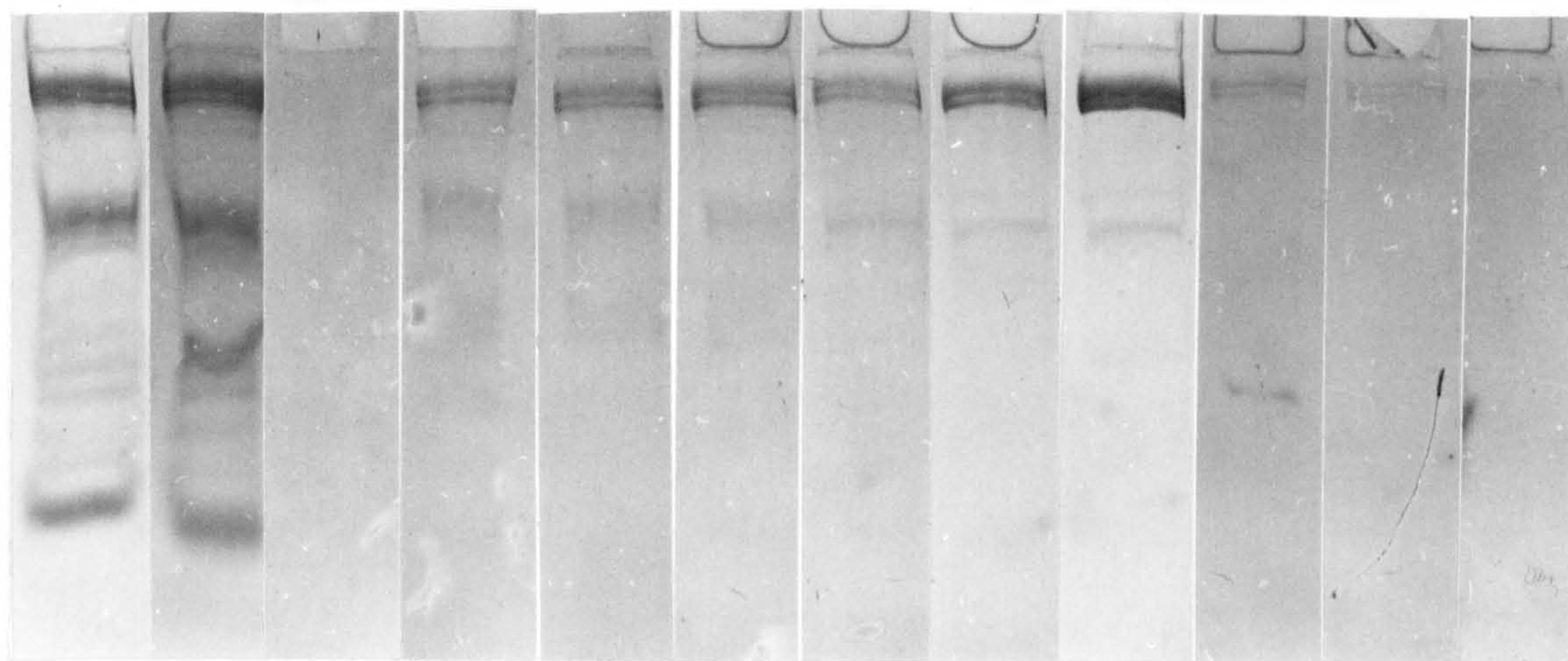



Figure 2. 16.

The partial solubilization of hypotonic Triton-extracted ghosts in a low ionic strength buffer. Pellet (P) after low speed centrifugation and fractions from a sucrose gradient of the supernatant.

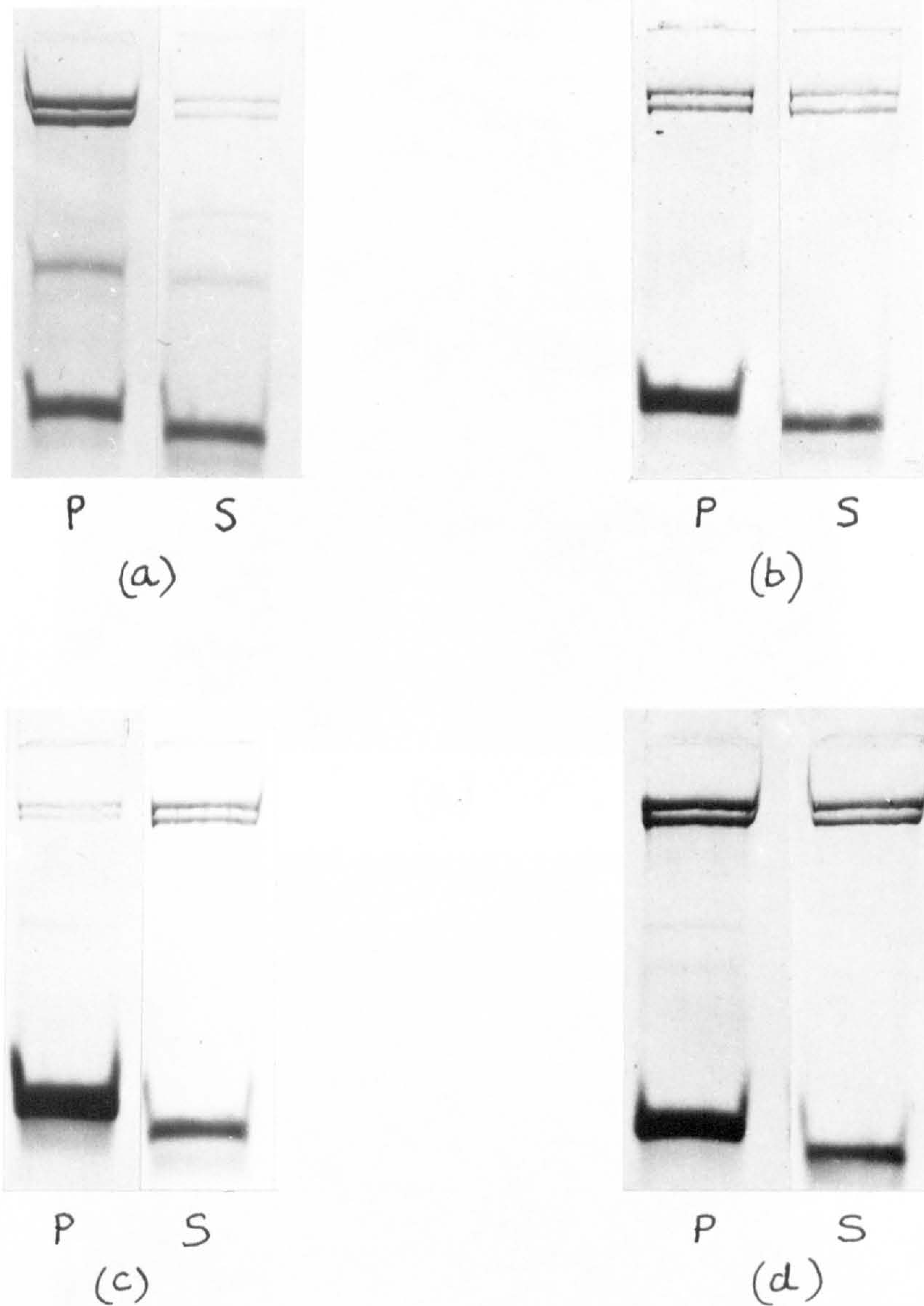



Figure 2.17.

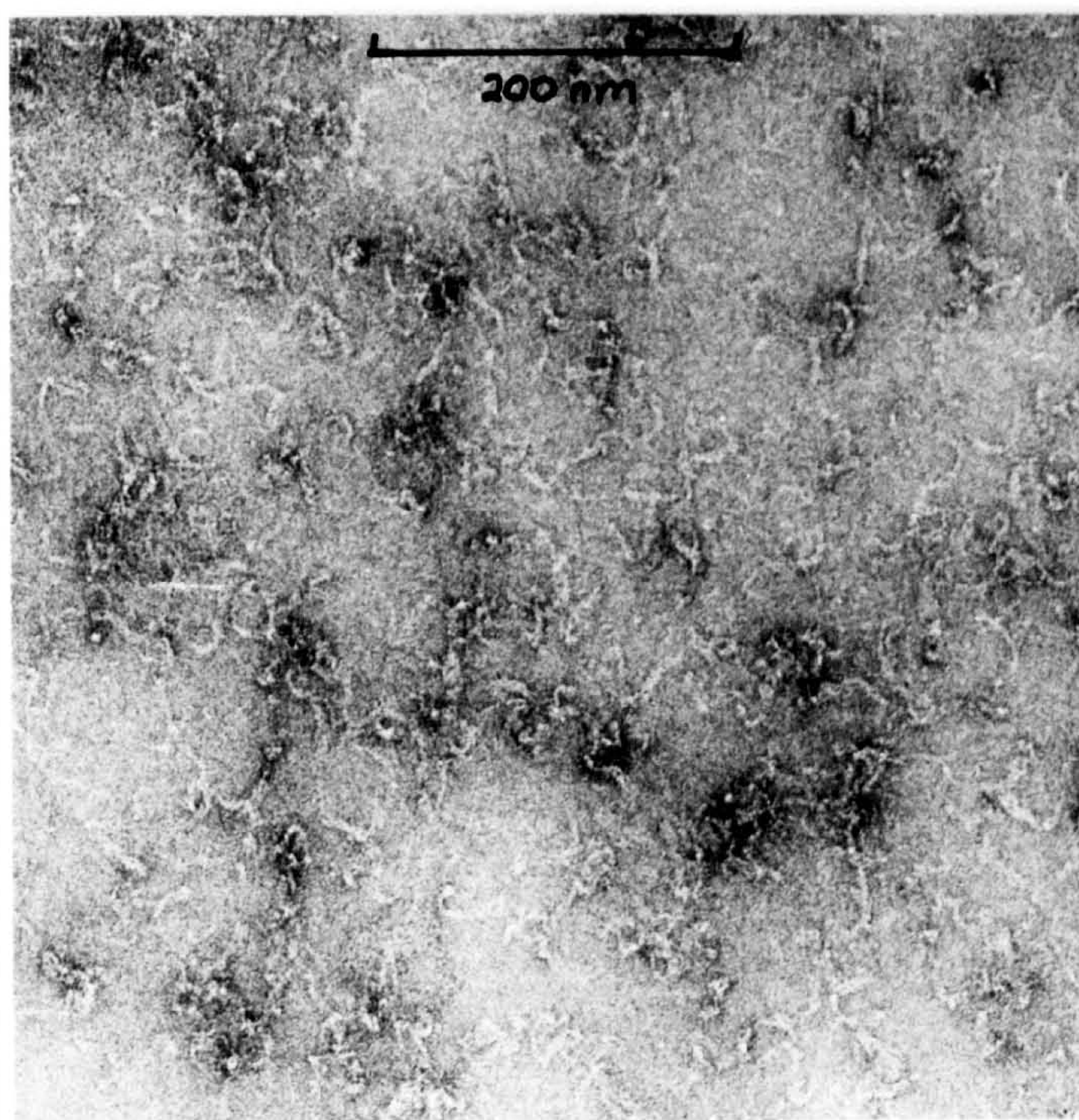
The binding of the spectrin dimer to 4.1 and F-actin. SDS gels of pellets (P) and supernatants (S) after centrifugation of mixtures of the three proteins. Gel samples represent 20% of the pellet and 5% of the supernatant. The total concentration of spectrin dimer was 0.04 mg/ml and of F-actin 0.14 mg/ml.

(a) About 0.02 mg/ml 4.1.

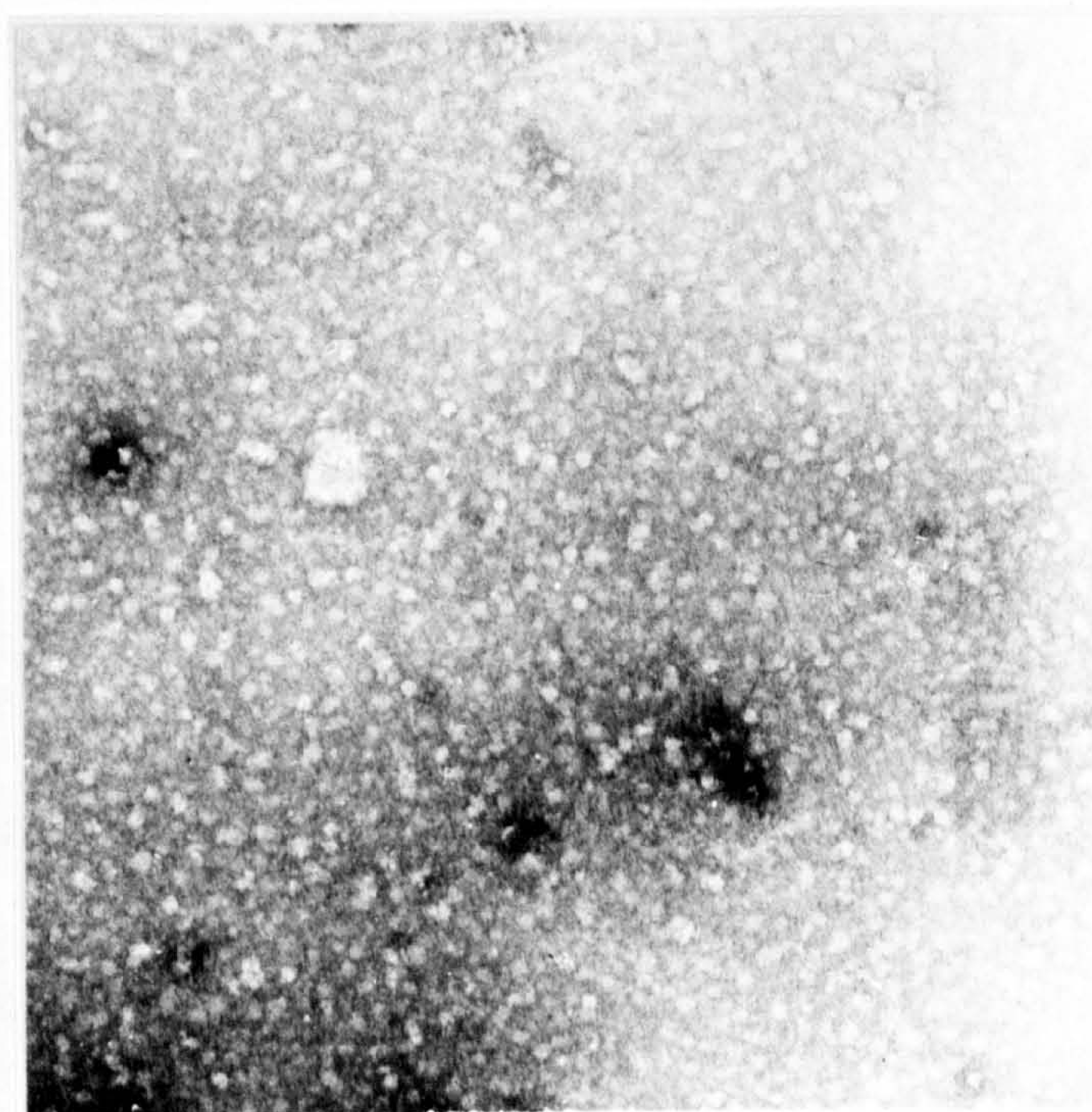
(b) About 0.002 mg/ml 4.1.

(c) No 4.1

(d) About 0.008 mg/ml of another preparation of 4.1. From the staining of the gel, it appears that the molar ratio of dimer: 4.1 in the complex is much larger than 1:1.




(a)



(b)

Figure 2.18

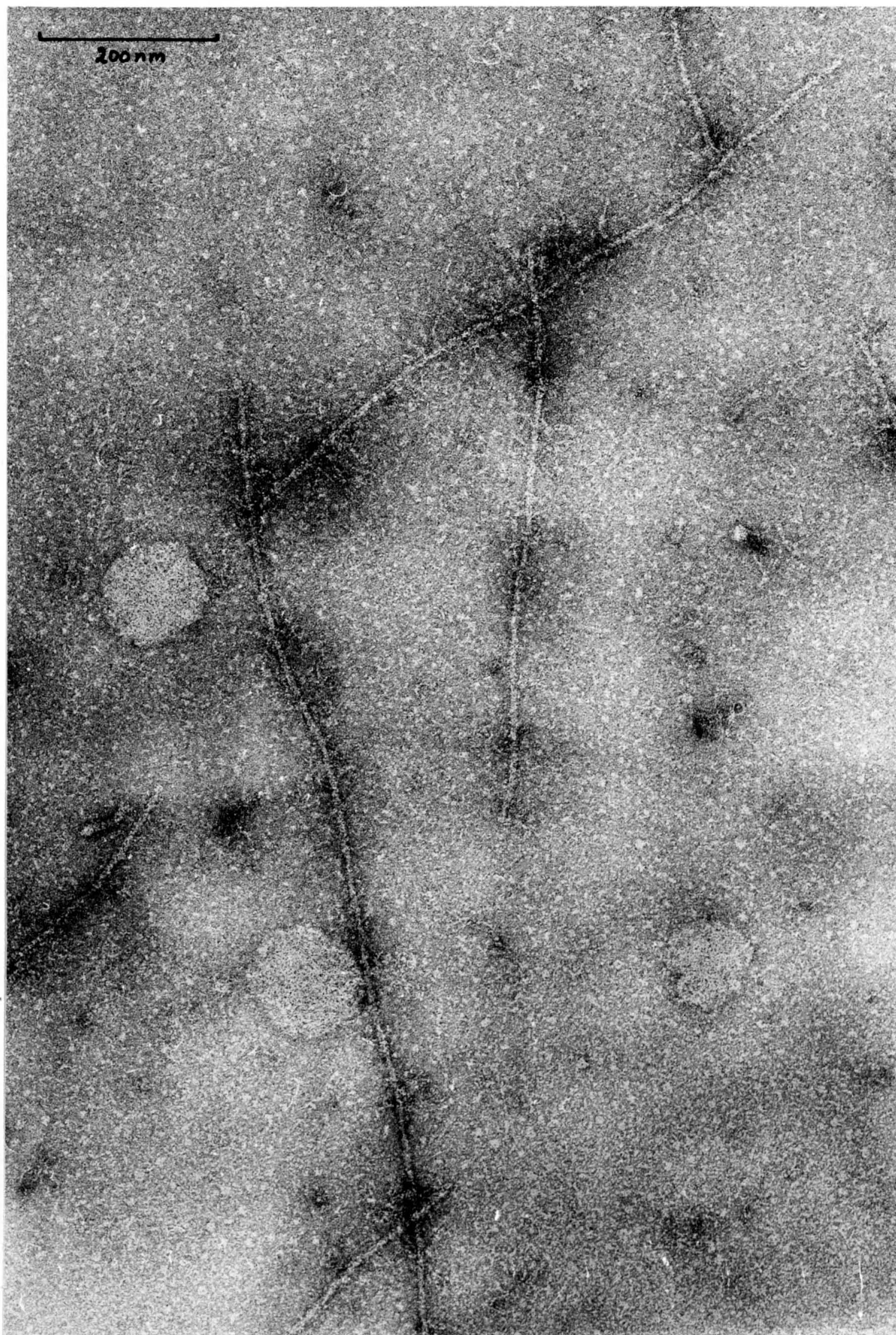
Negatively stained electron micrographs of

(a) Spectrin tetramer.

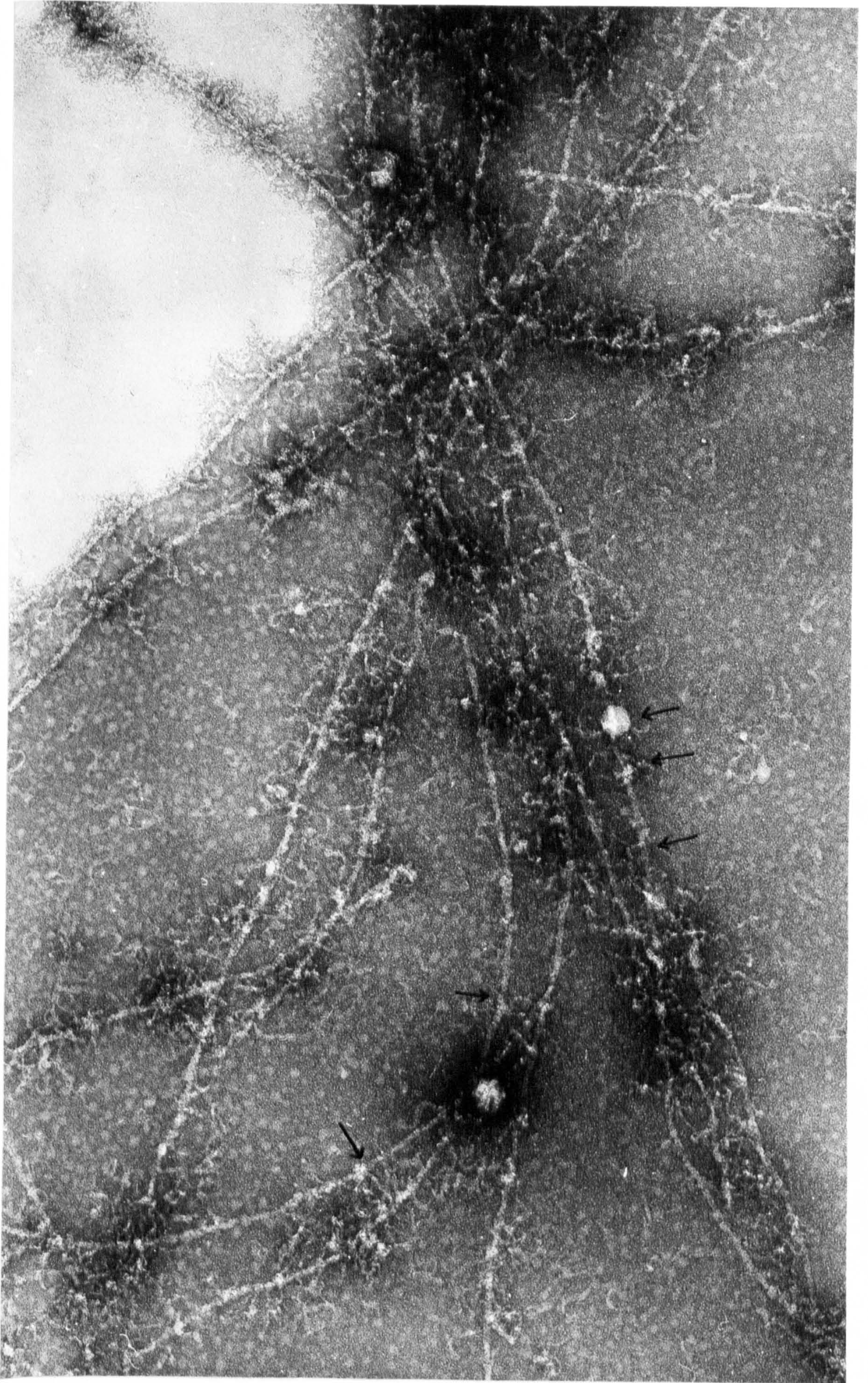
(b) 4.1

(c) Dimer bound to 4.1 and F-actin.

(d) The 2-dimensional complex formed between tetramer, 4.1 and F-actin. Magnification x175,000. Arrows point to spectrin tetramers which can be seen bridging F-actin filaments.




(c)



(d)

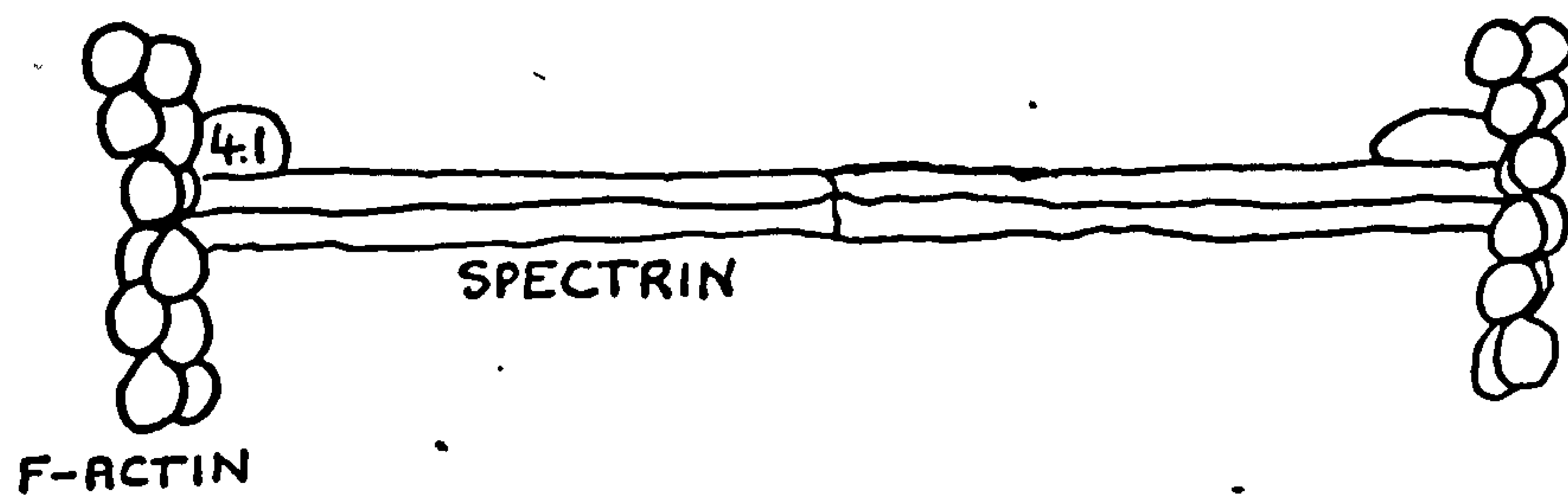



Figure 2. 19.

A speculative arrangement for the three proteins in the ternary complex between spectrin tetramer, 4.1 and F-actin.

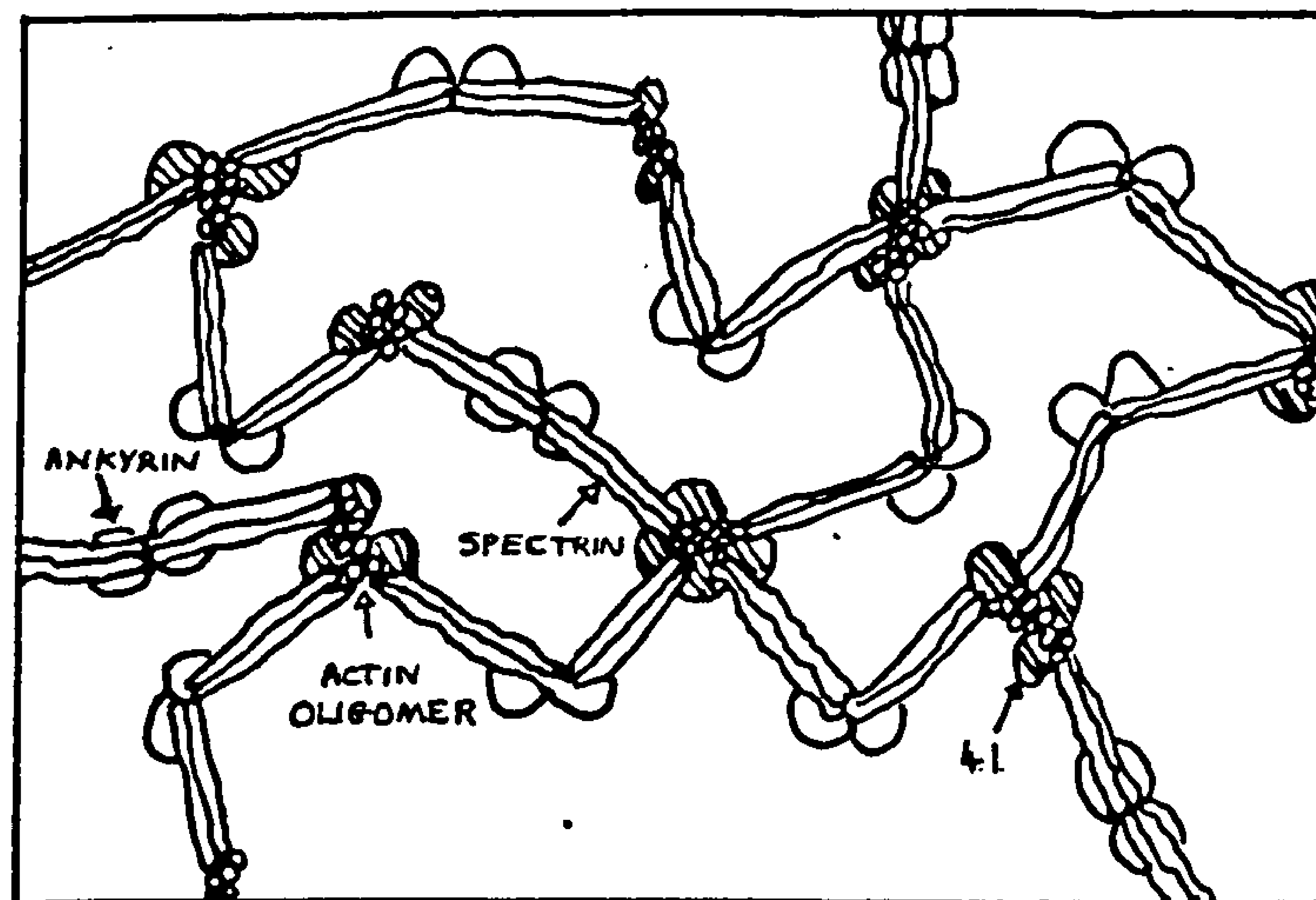



Figure 2. 20.

A speculative view of the cytoskeleton from inside a red blood cell. Small numbers of spectrin tetramers radiate out from each actin oligomer. Ankyrin can just be seen on the membrane side of the cytoskeleton, where spectrin dimers meet. (After Lux, in press).

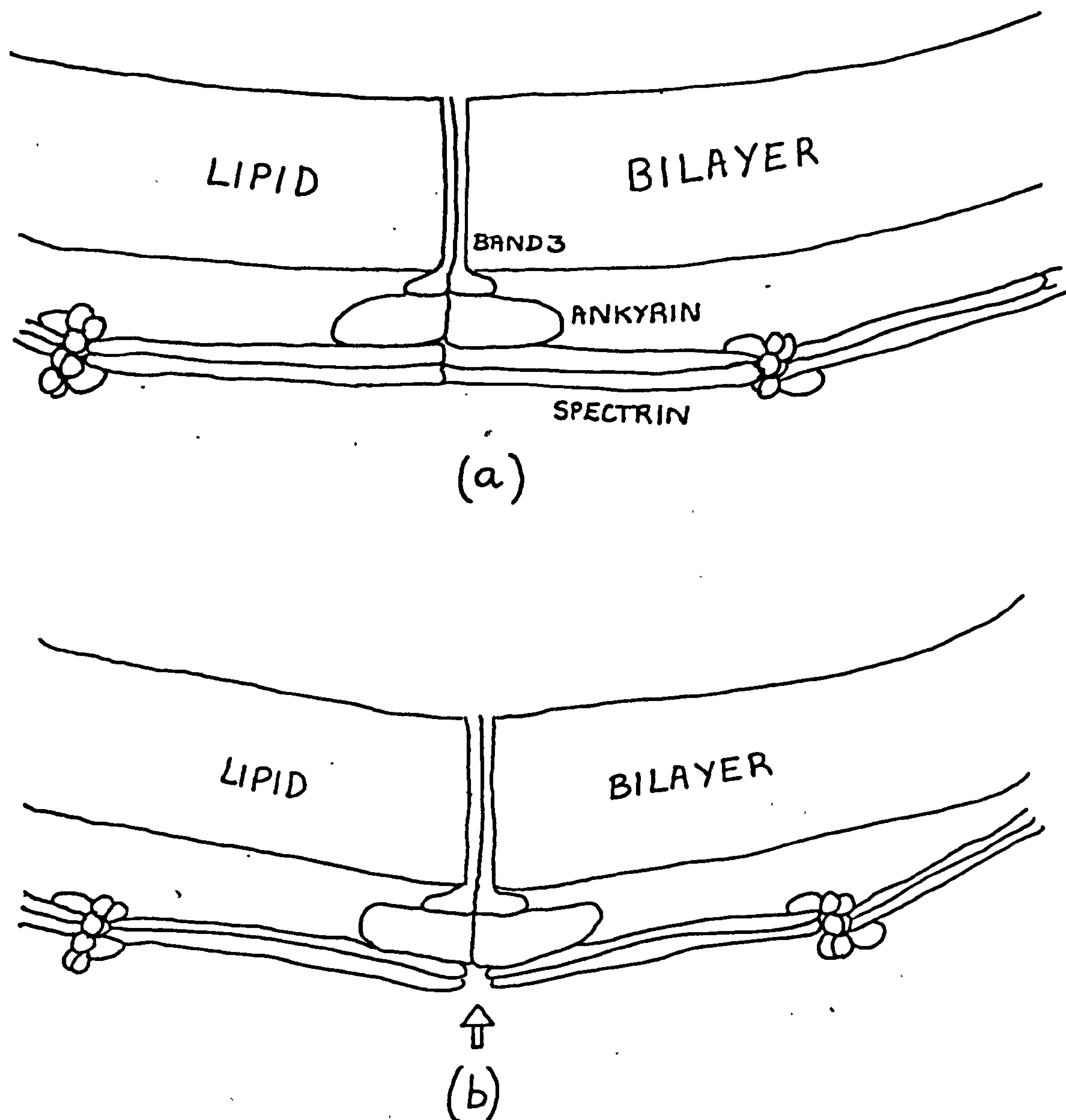



Figure 2. 21.

A section through the cytoskeleton. (a) A possible arrangement for band 3, ankyrin and spectrin.
(b) If band 3 or ankyrin remains as a dimer, the conversion of spectrin tetramer to dimer does not necessarily lead to a breakdown of the cytoskeleton.

# Chapter 3

# THE STRUCTURE OF SPECTRIN AND ITS RELATIONSHIP TO THE CYTOSKELETON

## I. Introduction

The work described in Chapter 2, establishing that the association of 4.1 with spectrin tetramer and F-actin is sufficient to form a cytoskeletal network, and the finding of Bennett , that the cytoskeleton is linked to a transmembrane protein through the medium of ankyrin, leaves unanswered the question of how the quaternary structure and conformation of spectrin equip it to form these protein-protein interactions and perhaps influence the mechanical properties of the cell. The close juxtaposition of spectrin to actin in the red cell and the high molecular weight of its two subunits invite a comparison with actin-binding proteins such as myosin and filamin.

## II. Materials and Methods

### 1. Myosin

Myosin was a gift from Mrs Retnam Rao and was prepared by the method of Perry (1955).

### 2. Preparation of filamin

Filamin was prepared according to the method of Wang (1977). Two chicken gizzards were trimmed of fat and connective tissue using a scalpel to give about 60 grams

of wet muscle. After passing through a meat grinder and washing once in 0.3M sodium chloride, 2mM sodium ATP, 0.5mM $MgCl_2$, 0.5mM DTT, 50mM imidazole, 0.1mM PMSF pH 6.9, the mince was homogenized in a Waring blender with three 15 second bursts at 1 minute intervals in 200ml of the same buffer. The homogenate was centrifuged for 40 minutes at 24,000 x g in an MSE 100ml x 6 rotor. The supernatant was filtered through a Whatman No. 1 filter paper. 32 grams of finely ground ammonium sulphate were added to 200ml of supernatant with stirring. After standing for 30 minutes the suspension was centrifuged for 35 minutes at 17,000 x g. The pellet was dissolved in 6ml of 0.6M sodium chloride, 2mM magnesium chloride, 1mM EDTA, 0.1mM DTT, 10mM Tris-HCl pH 7.4 and left to stand for 30 minutes. It was stirred gently with a glass rod from time to time. After centrifugation at 120,000 x g for 45 minutes in a Beckmann 50Ti rotor, the supernatant was applied to a 2.5 x 90cm Sepharose 4B column equilibrated with the same buffer and 8.7ml fractions collected. All operations were performed at $4^{o}C$. Fig. 3.1 shows a 4% SDS gel (using the buffer system of Fairbanks et al., 1971) of every second fraction from the column between fractions 8 and 44. Firstly, it can be seen that a considerable quantity of myosin is present. It is possible that the use of solid ammonium sulphate, even though finely ground, led to the precipitation of some myosin in 29% saturated ammonium sulphate. However, since the filamin was to be further purified on an SDS gel, it was not necessary to remove the myosin. Secondly, a band corresponding to a molecular weight of

240,000 can be seen, as mentioned by Wang (1977), but as this band represented only 5% or less of the Coomassie Blue Stain, it was not of importance for the proposed digestion studies (3.II.9). Impure filamin was either used within 48 hours or added to an equal volume of spectroscopic grade glycerol, mixed thoroughly and stored at -20°C. The approximate yield of filamin as estimated from the absorbances of fractions containing filamin, and using an $E^{1\%}_{280nm}$ of 6.8 (Wang, 1977) was 18mg.

3. Preparation of a proteolytic fragment of protein 2.1

The 72,000 dalton fragment of ankyrin was prepared according to the method of Bennett (1978). A 10ml volume of the vesicles remaining after extraction of spectrin from ghosts was dispersed in 150ml of 0.3mM sodium phosphate pH 7.5, and incubated a second time at 37°C for 30 minutes to extract residual spectrin. The vesicles were again pelleted by centrifugation at 80,000 x g for 45 minutes, mixed with an equal volume of 7.5mM sodium phosphate, pH 7.5, and 0.5ml aliquots were digested on ice for 30 minutes with chymotrypsin, at a series of enzyme concentrations of up to 60 μg/ml. The reaction was terminated by addition of 5 μl of a solution of 50 mg/ml PMSF in ethanol. The samples were centrifuged at 100,000 g for 45 minutes, the supernatants made 0.1% in SDS, heated in a boiling water bath for 5 minutes and aliquots applied to a 7.5% poly-acrylamide gel for electrophoresis. The stained gel gave the enzyme concentration leading to a maximal yield of the ankyrin fragment (Fig. 3.2). A wide range of enzyme

concentrations gave rise to approximately the same amount of fragment. A chymotrypsin concentration of 50 μg/ml was used to treat the remainder of the vesicles. The mixture was centrifuged at 87,000 g for 1 hour and the supernatant was stirred for half an hour in the cold with DE52 equilibrated with 7.5mM sodium phosphate pH 7.5. The slurry was poured into a 1 x 30cm column and the packed bed was washed with phosphate buffer, followed by 100ml of the same buffer containing 0.1M potassium chloride. The fragment was eluted with 0.2M potassium chloride in the same buffer. Fig. 3.3 shows an SDS gel of the fragment eluted from the DE52 column which was vacuum dialyzed against 20mM potassium chloride, 2mM sodium phosphate, 0.5mM magnesium chloride, 0.4mM DTT, pH 7.5, frozen in liquid nitrogen, and stored at -80$^{o}$C.

4. <u>Preparation of hydroxyapatite</u>

The procedure of Spencer (1978) was used. 2 litres of 0.5M calcium chloride were added at 50 ml/min to 2.4 litres of 0.5M sodium phosphate buffer pH 6.7 in a 9 litre bucket with a bath recirculator (B. Braun, Melsungen G.F.R) pumping at a rate of 4 litres/min. After mixing, the resulting brushite crystals were allowed to settle, washed with 5 litres of glass distilled water and allowed to settle again. The 600ml of brushite produced was transferred to a well-lagged 5 litre beaker for conversion to hydroxyapatite. The brushite was stirred with a 50 x 100mm paddle stirrer at 200 rpm whilst adding 2.5 litres of boiling glass distilled water. When the sediment had

settled, the supernatant was aspirated immediately. The stirrer was restarted and 5ml of 0.5N hydrochloric acid were added, followed by 2.5 litres of boiling glass distilled water. 72ml of 20% sodium hydroxide solution was added using a variable speed peristaltic pump so that the pH of the mixture rose from 6.0 to 7.0, remained at 7.0 ± 0.2, and rose to 7.2 after addition of alkali was stopped. The supernatant was removed, the crystals resuspended twice in 2.5 litres of hot glass distilled water and left to settle. The beaker was covered with polystyrene and the suspension left to cool overnight in an insulated cabinet. This preparation results in hydroxyapatite with faster and more reproducible flow rates than that from previous methods.

5. <u>Analytical ultracentrifugation</u>

A model E Spinco instrument, with schlieren optics, was used. The rotor speed was 59,780 or 60,000 rpm and the bar angle $50^o$.

The dependence of the sedimentation coefficient, s, on molecular parameters is given by:

$$s = \frac{M}{N\ f} (1 - \bar{\upsilon}\rho) \quad \text{(Eqn. 3.1)}$$

where M is the molecular weight of the protein, N is Avogadro's number, f is the frictional coefficient of the protein, $\bar{\upsilon}$ is the partial specific volume of the protein and $\rho$ is the density of the solvent. Sedimentation coefficients were converted to the value ($s_{20}$,w) that would have been obtained in a solvent having the viscosity and density of water at $20^oC$, using a value of 0.733 for the partial specific volume $\bar{\upsilon}$ (Kam et al., 1977) and

viscosities ($\eta$) and densities ($\rho$) of urea solutions from Kawahara and Tanford (1966). The variation in partial specific volume with solvent will be discussed (3.III.1).

6. Circular dichroism experiments

For measurements in the near U.V. (250-320nm) a Jasco JV 40C instrument was used. For the far U.V., either this or a Cary 61 Spectropolarimeter was used. The cell housings were thermostatted at 15 $\pm$ 0.3$^{o}$C and the temperature of the samples measured by inserting a thermistor probe into the neck of the cell. Strain-free cells were selected. In experiments involving the comparison of the ellipticities of various solutions, the cell was not removed from its housing between readings. In comparisons of the ellipticities of tetramer and dimer, and dimer from fresh and depleted cells, protein absorption spectra were run from 260-400nm. If proteins were centrifuged at 3,000 g for 5 minutes to remove aggregates, absorbance due to scattering could be reduced to 0.001 in the region from 320-400nm so that an accuracy of about 1% could be achieved for protein concentrations of 0.1 mg/ml. Samples with absorbances between 0.5 and 1.5 were used. Phosphate and borate buffers do not absorb light in the near or far U.V. and were accordingly used for CD experiments. It was found that the reversibility of spectrin denaturation was dependent on the presence of DTT and this was included in all buffers at a concentration of 0.1mM resulting in an absorbance of only 0.049 at 222nm in a 1cm path length cell. The use of Aristar urea gave rise to an absorbance

of 1.1 at 222nm with an 8M solution in a 1cm path length cell. In denaturation experiments, urea was added as a solid, and the resulting urea concentrations and concomitant dilution of protein were calculated according to the equations of Kawahara and Tanford (1966). If the weight fraction of the urea is w then the density of the solution is given by

$$\rho = 1 + 0.2658\ w + 0.0330\ w^2 \qquad \text{(Equ. 3.2)}$$

hence the molarity of urea and the new concentration of protein can be found.

After addition of urea, solutions were mixed using a pasteur pipette and measurements taken when the ellipticity became steady; less than 15 minutes after addition of urea. No measurements of the kinetics of denaturation were made since, during this time period, the temperature of the samples was equilibrating.

For measurements of 222nm, molar residue ellipticities in degrees.$cm^2$.$decimole^{-1}$ were calculated using the formula

$$[\theta] = \frac{\theta . M}{10.lc} \qquad \text{(Eqn. 3.3)}$$

where $\theta$ is the measured ellipicity, M is the mean residue molecular weight of the protein, taken in the case of spectrin to be 113 as calculated from the amino acid composition from Fuller et al. (1974), l is the path length of the cell in cm. and c is the protein concentration in grams/ml. In the near U.V. the data were expressed in terms of molar ellipticities using a molecular weight of

480,000 for spectrin (Kam et al., 1977)

Spectrin, which had been in 7M urea at 15°C for 1 hour or more was renatured by overnight dialysis out of urea at 4°C.

7. Proton magnetic resonance

Spectra were measured with a 270 $MH_3$ Bruker pulsed Fourier transform spectrometer. The protein was examined in 5mM sodium phosphate buffer with an apparent pH of 7.4 and with added concentrations of potassium chloride and 1mM EDTA in deuterated water, distilled prior to use. Transfer of the protein to deuterated water solution was achieved by precipitation with ammonium sulphate, followed by rapid dissolution in the deuterated water-containing buffer, and dialysis with two changes against the same buffer. Protein concentrations were generally about 5 mg/ml. Analytical sedimentation showed no significant aggregation at this concentration. Generally, $10^4 - 10^5$ free-induction decays were accumulated with a pulse length of 12 μs.

8. Cross-linking experiments

These were performed as described in 2.II.14. Solutions containing various molarities of urea were prepared by diluting a stock solution containing 10M urea. To check the efficiency of the reagent, dimethylsuberimidate, in 7M urea, cross-linking reactions were carried out on the disulphide-linked protein γ-globulin. The products of the reaction were reduced, heated in SDS and run on an 8% acrylamide gel using 0.1M borate, 0.1M sodium acetate, 0.1%

SDS pH 8.5 as the running buffer, as described by Davies and Stark (1970). Fig. 3.4 shows the uncross-linked protein, followed by cross-linked products in 1 and 7M urea respectively. It was concluded that dimethylsuberimidate cross-links as efficiently in 7M urea as in urea-free solutions. Reactions with spectrin were performed both in borate buffer, to afford comparison with CD results, and in triethanolamine. With borate buffer, it was necessary to add sodium hydroxide to the DMS before mixing with the protein, in order to maintain the pH above 8. Densitometry of gel tracks containing cross-linked and uncross-linked spectrin dimer revealed that the Coomassie Blue stain bound to cross-linked material was only 60% of that bound to the normal gel bands. The possible effects of this unequal staining are discussed separately for each result.

9. Digestions of proteins in SDS

Cleveland et al. (1977) have found that various proteolytic enzymes are able to function in the presence of SDS. This provides a convenient way of comparing the digestion patterns of polypeptide chains without the need to purify them by the usual biochemical methods. Polypeptide chains for digestion were separated on a 5% gel using the discontinuous buffer system of Laemmli and Favre (1973). This system is designed to give a high resolution of SDS-protein complexes by "stacking" the proteins into very thin discs before they are separated by the normal electrophoretic method. Fig. 3.5 shows a diagram of the particular system used. In the low pH stacking gel, glycine has a lower

electrophoretic mobility, and the chloride ion a higher electrophoretic mobility than SDS-protein complexes. The complexes become sandwiched between these two ions at the boundary between the Tris glycine electrode buffer and the Tris-HCl stacking buffer. When the boundary moves into the separating gel, buffered at pH 8.8, the mobility of glycine increases above that of the protein complexes and they migrate in a uniform potential gradient as they do in electrophoresis using a continuous buffer system. The concentration of each ion and the pH in the two gels and the electrode compartments must be carefully chosen to maintain a stable boundary during electrophoresis. The necessary theory is described in Ornstein (1964). All the buffers used by Laemmli and Favre (1973) contain 0.1% SDS. The polypeptide chains for digestion could just be visualized after a one hour stain and 30 minute destain. They were cut out from the gel and soaked in 0.125M Tris-HCl 0.1% SDS, 1mM EDTA pH 6.8. Samples were digested straight away or stored at -20°C. Digestion took place on a second dimension gel. The separating gel was formed from a gradient of acrylamide from 5-20% affording maximal resolution of polypeptide chains of disparate size. The large pore gel was abnormally long (7 cm) allowing a large distance for the stacking of protein-SDS complexes which are eluted slowly from their 5% gel slices on top of the stacking gel. Papain was dissolved in the sample buffer containing additionally 20% glycerol, 2% β-mercaptoethanol, 0.1mM EDTA and a trace of bromophenol blue. The optimal papain concentration for a protein sample of about 70 μg

was about 0.1 μg/ml. 5 μl of this solution was deposited on a piece of parafilm with a micropipette and transferred to a gel slot using a drawn out pasteur pipette. The gel slice containing protein was then placed on top of the papain and electrophoresis was begun. When the bromophenol blue tracking dye had migrated 2-3 cm through the stacking gel, the current was switched off for 30 mintues to allow proteolysis to take place. Electrophoresis was then performed overnight at 6V/cm. To avoid further proteolysis, gels were fixed in 10% trichloroacetic acid, then stained overnight in Coomassie Blue G-250. Band patterns from different polypeptide chains were compared both by eye and by comparison of densitometric scans.

10. Cyanogen Bromide fragments of proteins in SDS

Gel slices containing polypeptides to be digested were soaked in 0.1N hydrochloric acid for 30 minutes and then in cyanogen bromide at 100 mg/ml in 0.1N hydrochloric acid for 24 hours. The slices were then covered in sample buffer used in the Laemmli system and the buffer changed until the bromophenol blue remained purple. A 7 cm stacking gel was used for the second dimension gel as described above and the separating gel contained 15% acrylamide. This provided better resolution than a gradient gel, for the particular size range of fragments produced.

11. Determination of the extinction coefficients of the spectrin subunits

Approximate extinction coefficients for proteins or polypeptide chains at 280nm can be calculated from the

ratio of their absorbances at 210nm and 280nm. Since the absorbance at 210nm is primarily due to a peptide chromophore transition with a peak at 190nm, the extinction coefficients of, for instance, the serum proteins at this wavelength are approximately the same: 205 ± 8.9 standard deviation (Tombs et al., 1959). For example, 85% of the absorbance of ovalbumin at 210nm is due to the peptide bond (Beaven and Holiday, 1952). However, Rosenheck and Doty (1961) found about 20% difference between the absorbances of both poly-L-lysine and poly-L-glutamic acid at 210nm in the α-helical and random conformations respectively. Moreover, tryptophan has a molar extinction coefficient of about 26,000 at this wavelength (Wetlaufer, 1962) so that proteins or peptides with abnormal tryptophan contents can give anomalous results: gramicidin has a molar extinction coefficient of about 340 at 210nm (Tombs et al., 1959). Measurements on the subunits were made in a Perkin Elmer Coleman 575 spectrophotometer.

12. Determination of tyrosine:tryptophan ratios

In the spectral region from 280-310nm the only amino acids which absorb are tyrosine and tryptophan, thus providing a means for the estimation of the number of residues of each in a protein by the method of mixtures (Beaven and Holiday, 1952). Even if the concentration of the protein for analysis is unknown, the ratio of the number of tyrosine to tryptophan residues can be found using the formula :

$$\frac{M_{Tyr}}{M_{Try}} = \frac{0.592A_{294.4} - 0.263A_{280}}{0.263A_{280} - 0.170A_{294.4}} \quad \text{(Eqn. 3.4)}$$

where $M_{Tyr}$ is the number of moles of tyrosine per gram of protein, $M_{Try}$ the number of moles of tryptophan, and A the absorbance of the protein solution in 0.1N sodium hydroxide at the wavelength indicated.

Since the usual procedure for amino acid analysis results in the destruction of tryptophan by acid, this spectroscopic technique provides the most convenient estimation of tryptophan in proteins. The constants used in the above formula contain molar extinction coefficients for the free amino acids tyrosine and tryptophan. In so far as the absorption spectra of these two amino acids are shifted towards a longer wavelength in their protein environment a systematic error is introduced into the Tyr/Try ratio whose size depends on the value of the ratio. The effect is an overestimation of tyrosine and an underestimation of tryptophan. Other errors arise from the limits in the accuracy of the absorbance measurement and in inadequate estimations of scattering. Despite these errors, good agreement between spectroscopic and chemical analyses has been obtained.

13. <u>The iso-electric precipitation of the spectrin subunits</u>

The variation in solubility of each spectrin subunit with pH was measured using the same technique as Gratzer and Beaven (1975). 0.1ml aliquots of sodium acetate buffers with a series of different pH values were added to a series of 1ml samples of spectrin subunit in 0.1M sodium chloride, 5mM sodium phosphate 0.1mM DTT pH 7.5. The samples were centrifuged for 5 minutes at 8,000 x g and the absorbance of the supernatants measured at 280nm.

14. The interconversion of dimer and tetramer

Spectrin dimer and tetramer can be interconverted as described by Ungewickell and Gratzer (1978). At physiological ionic strength, and a protein concentration of 1.5 - 2.5 mg/ml, the ratio of dimer to tetramer at 37°C is approximately 1 : 1 and equilibrium is reached in 15 minutes. Incubation of dimer in these conditions, followed by analysis in the Spinco Model E ultracentrifuge provides a rapid, if rather insensitive, method to assess the competence of this dimer to form tetramer. Higher sensitivity is achieved in circumstances favouring a tetramer: dimer weight ratio of about 9 : 1: incubation for 5 hours at 26°C. Fig. 3.6 shows schlieren traces of preparations containing predominantly tetramer and dimer respectively.

15. The binding of spectrin subunits to 4.1 and F-actin

The method described in 2.II.15 was used.

16. The binding of the subunits to the proteolytic fragment of ankyrin

The ankyrin fragment at 0.15 mg/ml was mixed with spectrin at 0.8 mg/ml or either subunit at 0.4 mg/ml and left to stand for 30 minutes on ice before sedimentation of 0.4ml samples through a sucrose gradient. Fractions were collected as described in 2.II.12 and analyzed on 7.5% acrylamide gels in Tris-bicine buffer.

The sedimentation behaviour for particles in a linear sucrose gradient can be described by combining the equation defining the sedimentation coefficient :

$$s_{T,M} = \frac{dr/dt}{\omega^2 r} \qquad \text{(Eqn. 3.5)}$$

with the relation between the sedimentation coefficient and solvent density and viscosity :

$$s_{20,w} = s_{T,M} \frac{\eta_{T,M}}{\eta_{20,\omega}} \cdot \frac{(1-\bar{\upsilon}\rho)_{20,\omega}}{(1-\bar{\upsilon}\rho)_{T,M}} \qquad \text{(Eqn. 3.6)}$$

where $s_{T,M}$ is the sedimentation coefficient at temperature T and in buffer M, $\omega$ is the angular velocity of the rotor in radians per second, r is the distance of the schlieren boundary from the axis of the rotor and t is the time in seconds (using an arbitrary zero). $\eta$ is the solvent viscosity and $\rho$ its density. $\bar{\upsilon}$ is the partial specific volume of the particle.

Then $$\omega^2 s_{20,\omega} dt = A \frac{\eta(x)_{T,M}}{[1-\bar{\upsilon}\rho(x)_{T,M}]} \cdot \frac{1}{x} \cdot dx \qquad \text{(Eqn. 3.7)}$$

where A is a constant, assuming that the partial specific volume is not dependent on temperature or sucrose concentration. The right hand side of the equation is approximated by a summation by considering the sucrose gradient as a series of narrow discs. The variation in partial specific volume from one protein to another is small so that an average value of 0.725 leads to a maximum error of about 10% in the calculation of the distance moved by a protein with known sedimentation coefficient (Martin and Ames, 1961). For spectrin dimer S = 9.7 (Gratzer and Beaven, 1975); for the ankyrin fragment, Bennett (1978) has found a stokes radius of 39 Å leading to a sedimentation coefficient of 4.4S and for the two subunits s ≈5.5S (3.III.5). For a 4.4ml 5-20% linear sucrose gradient in a buffer at

physiological ionic strength, optimal separation between dimer and the ankyrin fragment should occur after centrifugation for 15 hours at 300,000 x g and between each subunit and the ankyrin fragment after 15 hours at 200,000 x g. In practice, in the case of band 2, it was necessary to separate contaminating dimer and aggregated material from the monomeric band 2 so that centrifugation conditions were the same as for dimer.

The buffer used in these experiments was 20mM potassium chloride, 2mM sodium phosphate, 0.5mM magnesium chloride, 0.4mM DTT pH 7.5 which was used by Bennett (1978) to assay the binding of spectrin to inside-out vesicles. At this rather low ionic strength the primary charge effect was evidently small, since optimum separation was still obtained using the calculated centrifugation conditions.

## III. Results

### 1. The conformation of spectrin - a urea denaturation study

The interaction of proteins with urea is not always reversible and a judicious choice of the conditions is necessary to avoid irreversible changes. It has been known for a long time that urea in solution is in equilibrium with the cyanate ion, $CNO^-$, which reacts with amino, sulphydryl, carboxylic and phenolic groups in proteins as well as with the imidazole ring of histidine, at an optimum pH of 7 (Stark, 1967). Although the equilibrium strongly favours urea, the molarities of urea used for denaturation are so high and the protein concentrations relatively low, that a considerable excess of cyanate is present at equilibrium.

However, starting from urea, it is several weeks before equilibrium is reached at 4°C, and 30 minutes even at 100°C (Stark et al., 1960). Accordingly, Aristar grade urea was added as a solid and circular dichroism measurements made on the same day, to avoid the formation of cyanate. Sedimentation of spectrin in urea at pH 5.8 showed evidence of aggregation so that although sulphydryl groups in denatured proteins are apt to undergo irreversible disulphide interchange at neutral and alkaline pH (Cecil, 1963), experiments were carried out at pH 7.5 in the presence of DTT. Since urea is a neutral molecule, electrostatic effects are still operative in its presence. Buffers of physiological ionic strength were used in these experiments, unless otherwise indicated, in order to minimize interactions between the charges on the spectrin molecules.

Fig. 3.7 shows the CD spectrum of spectrin from 250 - 220nm. The peak with a negative CD at 222nm is due to an $n - \pi^*$ transition corresponding to the promotion of an n electron on the oxygen of the carbonyl group in the peptide bond to the $\pi$ system. This transition gives only a weak absorption but often results in large Cotton effects, for example in proteins with a high α-helical content. The CD of other secondary structures: β-sheet and random coil, contribute so little at this wavelength that several methods for determining the α-helix content of proteins from their CD at 222nm have been developed. Greenfield and Fasman have used the CD spectra of poly-L-lysine in α-helix, β-sheet and random coil conformations as models for

comparison with the spectra of proteins. A combination of the three poly-L-lysine spectra are computed to give a best fit with the protein spectrum. However, lengths of α-helix in proteins are much shorter than in poly amino-acids and the unordered portions of proteins are often rigid and compact, in contrast to the flexibility of a true random coil. Accordingly, Chen and Yang (1971) have used several proteins whose secondary structure is known from X-ray data as standards from which the CD spectra of proteins with different structures can be predicted. Further refinements (Chen et al., 1974) taking helix length into consideration show that for a helix of infinite length, $\theta_{222}$ is -40,000 deg $cm^2$ $decimole^{-1}$ whereas a five residue helix has $\theta_{222}$ = -19,000 deg $cm^2$ $decimole^{-1}$. Spectrin has a molar residue ellipticity between -26 and 127,000 deg $cm^2$ $decimole^{-1}$ at 222nm, based on an extinction coefficient of 10.7 (Kam et al., 1977). The method of Greenfield and Fasman (1969) leads to an α-helix content for spectrin between 65 and 82%. The method of Chen and Yang (1971) gives 75-88%. The model proteins used, however, are all globular and if the method is applied to light meromyosin (Wu and Yang, 1976), the spectrum implies more than 100% α-helical character. It is possible that in fibrous proteins with high α-helix contents, the number of residues per helix is higher, leading to an overestimation of the helix content. At any rate, it would seem that the α-helix content of spectrin is higher than 65%, and is about the same as that of myosin, which has a molar residue ellipticity of -26,000 deg $cm^2$ $decimole^{-1}$ at 222nm (Wu and Yang, 1976).

The near U.V. CD spectrum of spectrin (Fig. 3.8) shows bands due to $\pi$-$\pi^*$ transitions in phenylalanine between 250 and 270nm and in tryptophan between 280 and 300nm. The largest peak in the CD spectrum of acetyl tyrosinamide occurs at 275nm and the spectrum of spectrin in this region has a low intensity.

If the side chains of these aromatic amino acids are freely rotating, asymmetric influences are small. Signals can be enhanced at low temperatures (e.g. $77^{o}$K) or in proteins when the aromatic groups are often buried, and thus immobilized, in a hydrophobic core. However, in large proteins, such as spectrin, intensities can be very small as aromatic groups in a variety of different environments, can give rise to signals of opposite sign from one another, which cancel out. The most intense band has a molar ellipticity of about $1.6 \times 10^{5}$ deg $cm^{2}$ $decimole^{-1}$ containing contributions from about 89 tryptophan groups (3.IV.5). The fine structure between 250 and 270 nm is difficult to interpret but the peak at 292nm is probably a component of the $^{1}L_{b}$ transition of tryptophan. This nomenclature due to Klevens and Platt (1949) is equivalent to the group theory designation $^{1}B_{2u}$ denoting a singlet electronic state which is antisymmetric with respect to its centre of symmetry (ungerade), antisymmetric with respect to rotation through $180^{o}$ about the 6-fold axis of the benzene ring (B), and distinguishable from $^{1}B_{1u}$. The position and intensity of this band is consistent with a transition from the lowest vibrational state of the ground state to the same in the excited state (0 - 0). Another band can be seen at 285nm

which is probably from a transition to the next vibrational state (0 - 850 $cm^{-1}$) in the same $^1L_b$ progression. There is no evidence of the broader $^1L_a$ band. Neither are the contributions from tyrosine possible to analyze (Strickland, 1974).

Fig. 3.8 also shows the near U.V. CD spectrum after the addition of solid urea to a concentration of 7M. The intensity of the 292nm peak has dropped to about one third of its original value consistent with the presence of a random coil structure. Similar fractional decreases have been observed in other proteins: bacterial α-amylase has a molar ellipticity of about 1.3 x $10^2$ deg $cm^2$/decimole in the native state and about 0.3 x $10^2$ after denaturation in 8M urea (Yutani et al., 1969). The ellipticity per decimole of tryptophan residue in denatured spectrin is only slightly greater than the ellipticity per decimole of the model compound acetyl tryptophan amide either in water or in high concentrations of guanidine hydrochloride.

The molar residue ellipticity at 222nm in the presence of 7M urea is about -2,000 deg $cm^2$ $decimole^{-1}$, a value which is also consistent with a random coil structure. Tanford et al. (1967) have found that there is a simple relationship between the sedimentation coefficient of random coil polypeptides in 6M guanidine hydrochloride at 25°C and their molecular weights:

$$\frac{s^o}{(1-\phi' \rho)} = 0.286\, n^{0.474} \quad \text{(Eqn. 3.8)}$$

where $s^o$ is the sedimentation coefficient, corrected for protein concentration only, $\phi'$ is the partial specific

volume of the protein in denaturant, $\rho$ is the density of the solvent and n is the number of amino-acid residues in the coil. The partial specific volume of bovine serum albumin decreases by 1% in 6M guanidine hydrochloride relative to water, and that of rabbit muscle aldolase by 0.5% (Reisler and Eisenberg, 1969) leading to an error in $(1-\phi'\rho)$ of up to 4% if the partial specific volume in aqueous solution is used instead of $\phi'$. The sedimentation coefficient of spectrin in 7M urea at 20°C is 1.8S giving a value for $s^o$ (in 6M guanidine hydrochloride at 25°C) of 1.65S. Hence n is 1858. The mean residue weight for spectrin can be calculated from its amino-acid composition (Fuller et al., 1974) and is 113, leading to a value for the molecular weight of the species in 7M urea of 210,000 which corresponds most nearly to the monomeric species, with molecular weights estimated from SDS gels of 240,000 and 220,000 respectively (Clarke, 1971). Thus, spectrin dimer is dissociated into its two constituent polypeptide chains in 7M urea.

After renaturation, about 90% of the ellipticity at 222nm and at 292nm was regained. Sedimentation of renatured material in the ultracentrifuge showed a peak with an S value of 9.5 (Fig. 3.9). Gratzer and Beaven (1975) found a value of 9.7S for dimer. A small boundary can be seen in the position where tetramer normally sediments. At the concentration of spectrin used in this experiment (0.6 mg/ml) and at 25°C, the mixture would be 66% tetramer at equilibrium. Lower temperatures favour a larger proportion of tetramer (Ungewickell and Gratzer,

1978). When spectrin is prepared using a 37°C extraction, some of the dimer is often damaged and unable to form tetramer (Ungewickell, personal communication) which could partially explain the lack of tetramer in the renatured sample.

The transition from the native to the denatured state was studied using a number of techniques to look further into the conformation of spectrin and the relationship of the subunits to it. Fig. 3.10 shows the fractional change in sedimentation coefficient of spectrin dimer with urea concentration. Dimer and monomer were never observed simultaneously, indicating that a fast equilibrium takes place between the two species, at urea conentrations where they co-exist. Numerical values assigned to the sedimentation coefficients at intermediate urea concentrations are of qualitative significance only. As explained above, errors of up to 4% and possibly more, are introduced by the uncertainty in $\bar{v}$. In addition, the hydrostatic pressure in an analytical ultracentrifuge cell running at maximum speed is 250 atmospheres and this could affect the position of the equilibrium between monomer and dimer.

For these reasons, the monomer-dimer equilibrium was also studied using chemical cross-linking. The fraction of spectrin remaining as dimer as a function of urea concentrations is shown in Fig. 3.10. This curve follows closely the fractional change in sedimentation coefficient. The dissociation has substantially taken place at 4M urea and the mid-point is at about 2.2M urea. It is possible that the cross-linking reagent could shift the equilibrium

towards dimer by preventing dissociation. The magnitude of this effect is not possible to estimate. On the other hand, as described in 3.II.8, cross-linked dimer binds less Coomassie Brilliant Blue than does the uncross-linked species. The denaturation curve has been redrawn assuming that the dimer absorbs only 60% of the stain of the monomer species and the fraction of dimer remaining shows a maximum rise of only 0.09 at 2.4M urea. This difference would not alter the conclusions which have been drawn from this curve.

Fig. 3.10 shows the molar residue ellipticity of spectrin dimer at 222nm as a function of urea concentration. At 2.2M urea, only about 10% of the helical structure has been lost when half of the dimer has already dissociated into subunits. This would seem to indicate that the two chains can separate from one another easily, rather than being twisted round one another as for instance in the coiled coil structure of myosin rod (Cohen and Holmes, 1963).

The molar residue ellipticity begins to fall at the smallest urea concentration at which it was measured (about 0.1M urea), in contrast to the constant values displayed by small globular proteins, sometimes up to quite high denaturant concentration; for example, the mean residue rotation of lysozyme remains constant in up to 3M guanidine hydrochloride (Tanford, 1968). The lability of the structure of spectrin indicates that parts of the molecule are not incorporated into compact globular domains, a finding which is borne out by other experimental data

(3.IV.2).

If the denaturation of a protein can be represented as a two-state process involving only the native and denatured states then the equilibrium constant for unfolding is :

$$K = \frac{[\theta]_o - [\theta]}{[\theta] - [\theta]_\infty} \qquad \text{(Eqn. 3.9)}$$

where $[\theta]$ is the observed ellipticity at 222nm $[\theta]_o$ that in the absence of urea, and $[\theta]_\infty$ that of the unfolded state at high urea concentration. Moreover, a plot of log.K vs. log.denaturant concentration (C) gives a straight line since K and C are related by the equation. $K = AC^\nu$ (Eqn. 3.10) where A and $\nu$ are constants (Tanford, 1968). Fig. 3.11 shows this plot for the denaturation of spectrin. The steep curve indicates that more than one unfolding process is involved in the denaturation, a result which is not surprising in view of the large size of spectrin. The loss in ellipticity with increasing urea at 292nm (Fig. 3.10) bears out this conclusion. The main feature of this curve is a cooperative transition centred on 4.5M urea so that this profile does not coincide with the profile at 222nm. An increase in ellipticity was always observed in the region of 4M urea. This is not an unprecedented phenomenon, particularly in large proteins where CD bands of opposite sign in the native conformation can cancel each other out, whereas their environment becomes increasingly uniform as denaturation proceeds: the phenylalanine fine structure of Horseradish peroxidase-C increases in intensity in guanidine

hydrochloride (Strickland, 1974).

Within the limits of accuracy for determination of protein concentrations, spectrin prepared from fresh and depleted cells showed no difference in molar residue ellipticity at 222nm. The same was true of spectrin in the tetrameric and dimeric states. Gratzer (personal communication) has shown that variation of the ionic strength between 1mM and 100mM also does not affect the ellipticity of dimer. These results are not surprising since protein-protein interactions and the phosphorylation of proteins are not usually associated with significant changes in the α-helix content of proteins.

2. <u>High-resolution proton magnetic resonance of spectrin dimer</u>.

To investigate further the structure of the labile part of spectrin dimer, high-resolution magnetic resonance studies were undertaken. For a protein of this size experience shows that the correlation time, if it behaves as a single kinetic unit, is so large as to preclude the observation of resolved side chain resonances, if these are devoid of independent freedom. Nevertheless, spectrin in aqueous buffer solution gives rise to a sharp-line proton magnetic resonance spectrum at 270 MHz (Fig. 3.12). The area under the envelope is difficult to determine precisely, but can be estimated to correspond to 20 ± 5% of the total population of unexchangeable protons, referred to the spectrum of the unfolded state in acid solution (Fig. 3.12). This is probably an upper limit, because of the presumed presence of a broader signal superimposed on the sharp

resonance. Thus, only a minor part of the chains exhibits segmental flexibility, the remainder being presumably involved in globular structure. The spectrin is not appreciably different in low ionic strength solution (5mM phosphate), so that the change in molecular dimensions which occurs in these conditions (Ralston and Dunbar, 1978) is not associated with loss or gain of globular character, (a conclusion also suggested by the invariance of the circular dichroism in the peptide region). Examination of the proton magnetic resonance spectrum of native spectrin reveals further that the mobile portions of the chain do not have the same amino acid composition as the whole protein. In particular, they are rich in alanine and other hydrophobic residues (peak a containing the contributions of the methyl protons of valine, leucine and isoleucine), and also reveal the presence of threonine, methionine and, in the downfield region, a very little tyrosine and/or tryptophan. Signals due to ionic side chains, especially lysine and arginine, are conspicuously absent.

### 3. A comparison of the digestion patterns of the two subunits of spectrin, myosin heavy chain and filamin

Fig. 3.13 shows the digestion patterns obtained using papain and Fig. 3.14 those from cyanogen bromide cleavage. Fuller et al. (1974) and Anderson (1979) do not agree on the number of methionine residues in each spectrin subunit, so that it is not possible to say whether or not complete CNBr cleavage has taken place. About 20 bands were observed in each pattern. It is clear from both these figures that none of these polypeptide chains is identical to any of the

others. However, the patterns generated by the two spectrin chains appear similar. Accordingly, statistical methods were used to quantify the degree of similarity (Pinder and Gratzer, 1972).

The number of bands in each pattern is counted, and the number of coincident bands between two patterns is estimated by eye or using a densitometer trace and assuming a resolution of 0.3 or 0.5mm. Then the probability of observing this number of coincident bands from two quite unrelated patterns can be calculated by a consideration of the distribution of one set of bands on the other. The gel is divided into a number of cells (N), depending on the chosen value for the resolution. For a gel 7cm long and a resolution of 0.5mm, there are 140 cells. If the number of bands in the first pattern is m, the number in the second is n and the number of coincident bands is x, then, if the bands in each pattern have a random distribution, the number of ways that x bands can be arranged on m bands to achieve the coincidences is

$$\frac{m!}{x!\ (m-x)!}$$

and the number of ways (n-x) bands can be arranged in the (N-m) unoccupied cells of the first track is

$$\frac{(N-m)!}{(n-x)!\ (N-m-n+x)!}$$

Thus, the total number of ways of achieving x coincidences is

$$\frac{(N-m)!\ m!}{(n-x)!\ (M-m-n+x)!\ x!\ (m-x)!}$$

But the total number of ways of arranging n bands on the gel track is

$$\frac{N!}{n!(N-n)!}$$

so that the probability of achieving x coincidences as a random event is :

$$P(x) = \frac{m!\ n!\ (N-m)!\ (N-n)!}{N!\ x!\ (m-x)!\ (n-x)!\ (N-m-n+x)!} \quad \text{Eqn. 3.11}$$

$P(x) < 0.01$ is used as a common criterion of significance. If the probability of the observed number of coincidences is less than 0.01, a relationship between the two patterns is indicated but if it is more than 0.01, no conclusions can be drawn. If information concerning amino-acid sequence homology is to be elicited from pattern similarities, the possibility that one band consists of dissimilar fragments of approximately the same molecular weight, must be considered. Using the parameters from the digestion patterns of the spectrin subunits, the possibility of as many as three overlapping fragments in a pattern is only about 0.002. The effect of having 3 extra bands in each pattern and no extra coincidences between the two patterns has been computed, and does not affect the results obtained for the two spectrin subunits. P (x) was calculated using Stirling's formula as an approximation for B! where B is an integer :

$$B! = \sqrt{2\pi B}\left(\frac{B}{e}\right)^{B} \quad \text{(Eqn. 3.12)}$$

The value of x leading to $P(x) \approx 0.01$ is about 6, and for $B = 6$, the error due to the use of Stirling's formula is less than 3%. A computer program was used so that the effects of different resolutions could be quickly calculated. When the patterns were analyzed by eye, using a photograph

of the gel, with each track lined up side-by-side if necessary and then by using densitometry traces measuring from the origin of the gel, different results were obtained. Measurement of a photograph revealed that the distance from the gel origin to the same fragment in different gel tracks varied by as much as 1mm. If the solvent front does not remain exactly parallel with the gel origin, migration distances will vary in this way. Moreover, due to the mechanical properties of polyacrylamide, gradient gels are trapezoidal rather than rectangular, so that a direction perpendicular to the gel origin, along which to perform densitometry, is difficult to define. Finally, some bands are curved making the track edges rather than centres more reliable for measurements of coincidence. Since the eye can detect the direction of the solvent front by observing the whole gel and also take band curvature into account, more reliable assessments of the number of coincident bands can be made this way. However, in this case, the resolution is difficult to assess. It was assumed to lie between 0.3 and 0.5mm and the results were calculated for each of these values (Table 3.1).

Whichever resolution is used, papain gives rise to a set of fragments from the two spectrin subunits which show similarity well above the level of chance. A comparison between all other sets of fragments shows dissimilarity even by the criterion of $P > 0.05$. Analysis of cyanogen bromide fragments leads to the same conclusion for the spectrin subunits. A relationship is seen between band 2 and filamin and between myosin and filamin, depending on

the method of analysis.

It is concluded that homologies in amino-acid sequence occur between the separate polypeptide chains of spectrin which far exceed those between any other combination of the polypeptides studied.

4. <u>The separation of the two subunits of spectrin</u>

The two subunits have molecular weights of 240,000 and 220,000 respectively, precluding separation on the basis of size.

The anion-exchanger DE52 (Whatman) can be used to effect separations between polypeptide chains with slightly different charge properties. Both continuous and batch processes were used in attempts to separate the spectrin subunits. Crude or G-200 purified spectrin (which contains dimer and oligomer) in 20mM Tris-HCl, 7M urea, 0.1mM DTT pH 8 was applied to a DE52 column equilibrated with the same buffer. Fresh solutions of Analar urea were used to avoid the formation of cyanate ions. A gradient of 0 - 0.5M sodium chloride was then passed through the column and band 1 of spectrin, with a yield of 20% of the total protein present, was eluted at 0.15M sodium chloride. Attempts to recover the protein remaining on the column with strong denaturants such as 0.1N sodium hydroxide or 0.1% SDS were unsuccessful.

For batch separation, crude or G-200 purified spectrin in 7M urea, 7.5mM sodium phosphate, 0.1mM DTT, pH 6.8 was stirred with DE52 equilibrated with the same buffer. No protein remained in the supernatant. Subsequent elution with increasing concentrations of sodium phosphate in steps

of 0.005M led to the appearance of both subunits in the supernatant at the same phosphate concentration (0.055M). All the protein was eluted before the phosphate concentration reached 0.1M.

Further attempts were made to separate the subunits by differentially extracting them from red blood cell ghosts and Triton-extracted ghosts. Triton-extracted ghosts were prepared as described (2.II.2) but were resuspended in 25mM sodium chloride, 5mM sodium phosphate, 1% Triton X-100, pH 8 containing urea at concentrations increasing in steps of 0.25M up to 1.75M. After incubation on ice for 20 minutes, samples were centrifuged for 5 minutes at 8,000 g and the pellets and supernatants analyzed on 5.6% polyacrylamide gels using the method of Fairbanks et al. (1971). Both subunits started to elute together at 1.75M urea.

Ghosts were prepared as described in 2.II.1. They were freeze-thawed 3 times or sonicated on ice for 10 seconds at amplitude 3, medium power using an MSE sonicator to ensure exposure of the inside surface of the membranes. Various amounts of buffer containing 8M urea were added to give urea concentrations increasing in 0.5M steps up to 2M. The samples were incubated at 22$^{o}$C for 35 minutes and centrifuged at 80,000 g for 1 hour. Pellets and supernatants were analyzed on 5.6% SDS gels (Fairbanks et al., 1971). Fig. 3.15 shows that both subunits elute together. Using either freeze-thawed or sonicated ghosts, both subunits started to elute in 1M urea. A similar experiment was performed using freeze-thawed ghosts which were pre-incubated in 0.15M sodium chloride, 5mM sodium phosphate pH 8, but the

two subunits still eluted simultaneously. Hargreaves et al. (1979) have recently reported the selective elution of bands 1 and 5 from erythrocyte ghosts in urea at moderate ionic strength and below physiological pH. In the light of the tendency of band 2 to aggregate, the reasons for which it does not appear in the soluble fraction after extraction should be examined with caution: this finding cannot necessarily be used as evidence that, of the two subunits, band 2 exclusively has a binding site on the membrane.

2 mg of spectrin dimer in 7M urea, 10mM sodium phosphate, 0.1mM DTT pH 6.4 were added to 1.5ml of hydroxyapatite equilibrated with the same buffer and the mixture centrifuged for 3 minutes at 3,000 g. A sample of the supernatant was removed for analysis on an SDS gel and an equivalent volume of buffer containing 1M sodium phosphate pH 6.4 added to give a final phosphate concentration of 60mM. After mixing and centrifugation the process was repeated, with the phosphate concentration increasing by 0.05M at each step. An SDS gel of the samples removed at each step showed that separation of the subunits was taking place.

Fig. 3.16 shows an elution profile of 5mg of spectrin in urea from a 4ml hydroxyapatite column using a gradient of sodium phosphate up to 0.33M. Although there is only one peak in absorbance, SDS gel analysis of every fourth fraction (Fig. 3.16) reveals one peak for each subunit.

The optimum conditions for separation involved two step-wise increases in phosphate concentration : from 10mM to 90mM and hence to 200mM. The yield after this first

separation was up to 87% of band 1 and up to 67% of band 2. To obtain band 1 completely free from band 2, it was always necessary to repeat the purification but the final yield was often 80%. Repurification of band 2, on the other hand, led to substantial losses, caused presumably by the aggregation which led to its affinity for DE52. Fig. 3.17 shows an SDS gel of the two separated subunits.

Renaturation conditions for the separate subunits were optimized using their subsequent ability to form dimer, and also their aggregation state, both measured by cross-linking, as criteria. Dialysis against a urea-containing buffer of higher pH, followed by overnight dialysis against 0.1M sodium chloride, 10mM sodium phosphate, 0.1mM DTT pH 7.5 gave rise to subunits which, when recombined, formed no dimer but only an aggregate containing bands 1 and 2, of a size too large to enter a 5% SDS gel. The temperature at which the two subunits were incubated together ($0^{o}$ - $37^{o}C$) had no effect on the amount of dimer formed. Neither did a momentary exposure of the mixture to a pH of 11.3, attempting to disaggregate band 2 by charge repulsion. However, an intermediate dialysis step using 6M guanidine hydrochloride, 5mM Hepes, 0.1mM EDTA, 0.1mM DTT pH 8 led to the formation of a substantial amount of dimer. Recombination was also favoured by buffers of low ionic strength. Fig. 3.17 shows an SDS gel of a mixture of the subunits in the presence and absence of dimethyl suberimidate, alongside a control using spectrin dimer.

Similar gels were densitometered to assess the percentage of renatured dimer. It can be seen from the gels that the

cross-linking between the subunits in dimer is almost 100% efficient, yet the area under the densitometry trace of the cross-linked species is only 60% of that under the trace of the uncross-linked species. It must therefore be assumed that the binding of Coomassie Brilliant Blue to the two species is different. Taking this into account, the percentage of dimer in the mixture of renatured subunits varied from 50 to 70%.

A mixture of the two subunits in 5mM Hepes, 0.1mM EDTA, 0.5mM DTT, 0.2% sodium azide, pH 8 was left at 23$^{o}$C for 75 minutes, then vacuum dialyzed against 0.1M sodium chloride, 10mM sodium phosphate, 0.1mM DTT, pH 7.5 at 4$^{o}$C until the protein concentration was about 1 mg/ml. Analysis in the analytical ultracentrifuge showed a boundary accounting for most of the material with a sedimentation coefficient of 9.6S. Dimer has an S value of 9.7 (Gratzer and Beaven, 1975). This constitutes strong evidence that the renatured species is indeed native dimer. The small peak with a lower S value is presumably either excess or unreacted band 1 (Fig. 3.18).

When the subunits are cross-linked separately, band 2 forms an aggregate which does not enter the 5% acrylamide gel. Band 1, on the other hand, remains primarily as a monomeric species although a faint band between the position of band 1 and dimer is visible (Fig. 3.17). Griffith(1972) has found that proteins containing disulphide bonds migrate anomalously quickly in SDS gels. Fibrin, which contains separate polypeptide chains linked by γ-glutamyl-lysyl groups, also shows a high mobility (McDonagh et al., 1972).

If band 1 were subject to intramolecular cross-linking, one would therefore expect the appearance of bands with a higher mobility than the uncross-linked species. The band above band 1 remains unexplained.

Various detergents were used to try to disaggregate band 2. 10mM sodium deoxycholate was the most effective but this reagent interferes with dimer formation (Fig. 3.19) in agreement with the results of Schechter et al. (1976) who found that spectrin dimer and monomer coexisted in the presence of deoxycholate by the criterion of sedimentation equilibrium. Both Nonidet P-40 and Triton X-100 had a significant effect on the amount of disaggregated band 2, but only if they were added before renaturation (Fig. 3.19). Since each separate subunit fails to give rise to a cross-linked species with the mobility of dimer, its formation from a mixture of the two constitutes the first direct evidence that spectrin is a heterodimer. Previous evidence has rested on the simultaneous disappearance of bands 1 and 2 to form species of higher molecular weight in cross-linking experiments (Clarke, 1971) and their subsequent reappearance together after ammonolysis of cross-links (Hulla and Gratzer, 1972).

5. The properties of the two separate subunits

In Fig. 3.20 negatively stained electron micrographs of bands 1 and 2 and dimer are shown. The dimer is elongated and curved, often appearing as a C or O shape. Unfortunately, the length of the dimer cannot be compared with that of either of the subunits as the latter are present at rather a high concentration, causing the ends of

each polypeptide chain to merge with part of another chain. Slightly narrower curved shapes are visible in the electron micrographs of band 1 and also of band 2, though band 2 is more heterogeneous: there are negatively stained regions which resemble aggregates and other regions where strands narrower than those of dimer or band 1 are visible. The appearance of the separate chains indicates that they are substantially renatured independently, which is consistent with the dissociation of dimer into subunits in urea before unfolding takes place (3.III.1) and also with the model of spectrin proposed by Shotton et al. (1979) where the two subunits lie alongside one another in the dimer.

Fig. 3.21 shows precipitation curves for each of the subunits with the curve for dimer taken from Gratzer and Beaven (1975) for comparison. It can be seen that the sharp change in solubility beginning at a pH between 5 and 6 is a characteristic of all three species. Most proteins do show a decrease in solubility as their iso-electric point is approached but the change is usually gradual. Whatever structural feature causes dimer to behave this way must also be a property of each subunit.

Band 1 was vacuum dialyzed against 0.1M sodium chloride, 10mM sodium phosphate, 0.5mM DTT pH 7.5 to a concentration of 2.0 mg/ml. A value of 5.5S was obtained for the sedimentation coefficient at this concentration (Fig. 3.22)

Since $$s = \frac{M}{Nf}(1 - \bar{v}\rho) \quad \text{(Eqn. 3.1)}$$

and $$f_o = 6\pi\eta\left(\frac{3M}{4\pi N}\right)^{1/3} \quad \text{(Eqn. 3.13)}$$

where $f_o$ is the frictional coefficient of a spherical unhydrated molecule of molecular weight M, $\eta$ is the solvent viscosity and $\bar{\upsilon}$ the partial specific volume of the protein, then s for a globular, unhydrated protein of molecular weight 240,000 daltons is 13.8S.

The frictional coefficient contains contributions from shape and hydration :

$$F_{observed} = F_{shape} \cdot F_{hydration} \quad \text{(Eqn. 3.14)}$$

and $$F_{hydration} = 1 + \frac{\omega}{\rho \bar{\upsilon}} \quad \text{(Eqn. 3.15)}$$

where $\omega$ is the weight of water immobilized per gram of protein and $\rho$ is the density of the solvent. $\omega$ is generally taken as 0.5 g/g giving a revised S value of 8.2S. Clearly band 1 is not globular.

Calculations (Bloomfield et al., 1967) assuming rod-like behaviour for both dimer and band 1 indicate that the frictional coefficient of the former would be about 10% greater than that of the latter, which agrees with the experimental values. This result is expected if band 1 maintains the same shape in the absence of band 2.

Due to aggregation, it was not possible to obtain band 2 at a high enough concentration for the analytical ultra-centrifuge. Sedimentation in sucrose gradients (3.IV.6), when a comparison can be made between the position of band 2 and dimer, indicate that the S value is less than 7S.

The molar residue ellipticities at 222nm of bands 1 and 2 were $2.37 \times 10^3$ and $1.68 \times 10^3$ deg $cm^2$ $decimole^{-1}$ respectively representing 90% and 64% of the value of native dimer. The relatively low value for band 2 is probably due to some

irreversible denaturation, rather than a part of the structure depending on the other subunit, since mixing of the two did not lead to an increase in the mean residue ellipticity.

The far and near ultraviolet spectra of band 1 are shown in Fig. 3.23. Those for band 2 are similar. Values for $E^{1\%}_{280nm}$ of 11.0 for band 1 and 11.6 for band 2 were calculated.

Fig. 3.24 shows a spectrum of spectrin dimer in 0.1N sodium hydroxide. Sodium hydroxide was added to both sample and reference cells and the spectrum recorded after the signal had equilibrated. The tyrosine/tryptophan ratio for spectrin calculated from this spectrum is 1.22. The number of tyrosines per dimer given by the amino acid analysis of Hsu et al. (1979) is 89 leading to a value of 73 for the number of tryptophans. Addition of alkali to band 2 led to a large increase in absorbance at 330nm indicating considerable aggregation. The tyrosine/tryptophan ratio for band 1 was 1.38 giving 36 residues of tryptophan per band 1. By subtraction band 2 should contain 37 tryptophans per molecule. Neither the spectrum of dimer nor band 1 showed any contribution from scattering.

## 6. The interaction between the subunits and other proteins in the erythrocyte membrane

If one assumes that spectrin dimer consists of one of each subunit lying alongside each other and that two dimers join end-to-end to form tetramer, as might be expected from the shadowed electron micrographs of Shotton et al. (1979), the question arises as to whether both subunits are involved

in the binding site between spectrin dimers and, if so, whether the links are between similar or different subunits or by some other arrangement.

The possibility that band 1 can associate with itself under the conditions optimum for tetramer formation (3.II.14), was investigated. Spectrin dimer and band 1 in 0.1M sodium chloride, 10mM sodium phosphate, 30mM sodium azide, 0.1mM DTT pH 7.5 at a concentration of 1.5 mg/ml were each incubated for 42 hours at 23$^{o}$C, conditions optimum for the formation of tetramer from dimer. Sedimentation in the analytical ultracentrifuge (Fig. 3.25) showed that about 60% of the dimer was converted to tetramer, and that band 1 gave rise to about 10% of a more rapidly sedimenting species. Subsequent overnight dialysis of the two samples against 5mM Hepes, 0.1mM EDTA, 0.5mM DTT, 30mM sodium azide pH 8 followed by incubation at 37$^{o}$C for 15 minutes (the optimum conditions for the formation of dimer from tetramer), cooling, addition of sodium chloride to 0.1M, and sedimentation, showed that the amount of tetramer present had been reduced to about 10% and that there had also been a reduction in the more rapidly sedimenting species in the profile of band 1. This is evidence for the association of band 1 with itself, but considering that the molar concentration of band 1 was twice that of dimer in this experiment and that the schlieren boundary which presumably arose from the self-association of band 1 was so small, it is unlikely that the interaction between two band 1 subunits is the only one involved in tetramer formation. Conditions for the self association of band 2 have not been studied in

detail.

The arrangement of the subunits in the complex containing spectrin, 4.1 and actin was investigated using the method described in 3.II.16. In Fig. 3.26, showing the pellets and supernatants resulting from sedimentation of a mixture of the three proteins, it is clear that where either spectrin dimer or a reconstitute of bands 1 and 2 shows binding to 4.1 and actin, band 1 alone does not. Densitometry reveals that almost 100% of the dimer is binding, about 35% of the reconstitute, and about 11% of band 2.

Since the complex involves three species and the stoichiometry is unknown, it is difficult to use these percentages to calculate possible equilibrium constants for the interactions. In a subsequent experiment, a single band 2 preparation showed no binding to F-actin in the presence of one preparation of 4.1 (Fig. 3.27) but with another preparation, binding was observed. In this case, however, only 10% of the band 2 was pelleted whereas 40% of the dimer pelleted with only $^{1}/40$ of the amount of 4.1. In the pellet, no impurity from the 4.1 preparation was present in stoichiometric amount with band 2. However, in view of the difficulty of obtaining a high degree of purity in 4.1 preparations, the possibility that band 1 or a fragment of band 1 was contaminating the "active" 4.1 preparation was investigated using anti-spectrin antibody (which was a gift from J.C. Pinder). The 4.1 preparation, was mixed with N-ethyl maleimide in excess quantity over the DTT contained in the buffer, to prevent reduction of the

antibody by DTT. 10 µl of affinity column purified anti-spectrin antibody with an absorbance of 0.24 at 280nm was added to 50 µl of 4.1 (absorbance 0.44 at 280nm). The sample was incubated at room temperature for 15 minutes and centrifuged for 5 minutes at 8,000 g. The supernatant and pellet were analyzed on a 7.5% acrylamide gel. When compared with a control containing no antibody, supernatants and pellets were identical for each sample. A negative result in an experiment of this nature cannot be taken as conclusive evidence that 4.1 is not contaminated with fragments of band 1, since the amount of contaminant is unknown, so that antibody titrations would be required to ensure achievement of equivalence point for any antigens present. The F-actin used for these experiments contained α-actinin as an impurity. This actin, in the absence of 4.1 does not bind to band 2. The possible presence of other impurities will be discussed (3.IV.).

Bennett (1978) has shown that spectrin dimer binds to a fragment of ankyrin. Cross-linking was explored as a way of demonstrating this interaction. Spectrin dimer or subunits and the ankyrin fragment (3.II.3) were dialyzed into 20mM potassium chloride, 2mM sodium phosphate, 0.5mM magnesium chloride, 0.4mM DTT pH 7.5. Mixtures of the two were incubated on ice for 30 minutes before the addition of an equal volume of 0.2M triethanolamine pH 8.5 and cross-linker, usually at a final concentration of 2.5 mg/ml. Analysis of the cross-linked material on SDS gels showed that, in the case of dimer, cross-linked by dimethyl suberimidate, a new band appeared above the normal position

of cross-linked dimer, which could be attributed to dimer with ankyrin fragment bound. However, this band represented less than 5% of the protein present, at concentrations where 50% binding of the spectrin would be expected. No new bands were detected in the case of band 1, and no depletion of uncross-linked ankyrin fragment occurred with band 2. Cross-linkers with longer distances between their two functional groups were used. Dimethylsebacimidate gave rise to material which did not enter an SDS gel, as did dithiobispropionimidate. However, using the latter, a new band was observed which, again, accounted for only a small precentage of the total protein. No new bands were observed with dithiobissuccinimidylpropionate. It was concluded that lysine groups on the two proteins are not in suitable dispositions to undergo cross-linking.

The binding of dimer to the ankyrin fragment can be demonstrated using sucrose gradients (3.II.17). Fig. 3.28 shows three SDS gels of the fractions from sucrose gradients containing the ankyrin fragment alone, the ankyrin fragment with dimer and the ankyrin fragment with band 2. Each track was densitometered and a graph of the amounts of protein in each fraction is shown in Fig. 3.29. It is clear that band 2 binds to the ankyrin fragment. A similar graph for band 1 (Fig. 3.30) indicates no interaction between the two polypeptides.

## IV. Discussion

The high α-helix content of spectrin calculated from its CD spectrum agrees well with that found by Ralston and

Dunbar (1979). By the method of Chen and Yang (1971), the value for spectrin is 81% which can be compared with 80% for myosin and 76% for myoglobin. However, other features of spectrin suggest that its structure is unlike that of either of the other two proteins. Myoglobin is a compact globular molecule (Rossi-Fanelli et al., 1964) which spectrin dimer, as judged by its sedimentation coefficient of 9.7S and its appearance in the electron microscope after shadowing, giving a length of 97nm (Shotton, Burke and Branton, 1979), is not. The coiled coil α-helical structure found in myosin rod and paramyosin would, if adopted by spectrin subunits of molecular weight 220,000, give rise to a dimer of length 250nm. Paramyosin monomer, of molecular weight 110,000 has a length of 127.5nm (Cohen et al., 1971). The slow but significant decrease in the α-helical content of spectrin with increasing urea at low urea concentrations suggests that part of the α-helical structure is not contained in globular domains, which would be expected to break down cooperatively in denaturant. More knowledge about the distribution of α-helical structure between the globular and non-globular parts of spectrin could be obtained by digestion studies.

Various studies indicate that there is an expansion of the spectrin molecule at low ionic strength. Bennett and Branton (1977) found that the sedimentation coefficient of dimer (as measured in sucrose gradients calibrated with proteins whose S values did not change under these conditions) diminished by about one unit as the ionic strength was increased from 1 to 20mM. Sedimentation velocity

experiments (Ralston and Dunbar, 1979) have confirmed this: the S value changed from 7.5S at ionic strength 10mM to 9.3S at 50mM ionic strength. The radius of gyration of dimer changes from 76 to 140nm as the ionic strength is reduced from 100mM to 1mM (Elsgaeter, 1978). The stability of the α-helix content of dimer with variable ionic strength suggests that the globular elements of spectrin are not disturbed by the expansion of the molecule which takes place at low ionic strength. Together with the evidence for random coil structure provided by the sharp signals of the proton magnetic resonance spectrum of native dimer and the several separate unfolding processes in the presence of denaturant, this expansion suggests a molecule consisting of a number of globular domains, repelled by an electrostatic effect at low ionic strength and linked by flexible regions . The globular domains of serum albumin move apart in this way as the pH is lowered from 6 to 3 (Pederson and Foster, 1969).

Hydrophobic residues are usually sequestered in the core of globular proteins leading to a lower free energy than does their exposure to aqueous solvents. The presence of amino-acids such as alanine, valine, leucine and iso-leucine in an exposed part of the molecule, as shown by proton magnetic resonance, suggests that this flexible part may be inserted into the lipid bilayer when spectrin is membrane bound. Several workers have reported an inter-action between spectrin and lipids: negatively charged liposomes will bind spectrin as measured by its depletion from the supernatant after centrifugation (Sweet and Zull,

1970). The diffusion of glucose from these liposomes is also altered by the presence of spectrin. Insensitivity of spectrin binding to ionic strength was taken as an indication of hydrophobic interaction. Mombers et al. (1977) have observed a change in the enthalpy of phase transition of a mixture of lipids in the presence of spectrin when bovine serum albumin had no effect. In particular, there is reported to be preferential interaction with the inner leaflet component, phosphatidyl serine (Mombers et al., 1979).

Digestion patterns of the subunits, when compared with those of myosin and filamin indicate that the two subunits are probably derived from a common ancestral gene. Exactly what the function of the dissimilar and similar parts of the polypeptide chains could be, will be discussed below. The comparisons involving myosin and filamin led to more variable results, ruling out large areas of sequence homology, but leaving the possibility of short range similarities.

Other workers have reached varying conclusions in similar digestion studies. Anderson (1979) in a comparison of the two subunits, obtained a manageable number of fragments by observing only those cysteine-containing [$^{14}$C]-carboxyamidomethylated tryptic peptides from a digestion performed in SDS. A number of spots were coincident for the two subunits. He concluded that too many $^{125}$I-peptides were produced to allow of their use in assessing sequence homology and since this was the method chosen by Zweig and Singer (1979) in their comparison of the two subunits, it

is not surprising that the latter considered the two subunits to be as dissimilar as they were to myosin and filamin. Dunn et al. (1978), using both methods, claim a similarity on the basis of both [$^{14}C$]carboxyamidomethylated and $^{125}I$-peptides. Hsu et al. (1979) who cleaved the subunits in 6M guanidine hydrochloride at their cystine residues using 2-nitro-5-thiocyanobenzoic acid came to the conclusion that the subunits were different. However, no maps of the spot patterns are given and the actual gels do not allow an easy comparison to be made. Partial sequence homology is not, therefore, ruled out by their work.

The resemblence between the subunits in their sharp precipitation profiles, their appearance in the electron microscope and their sedimentation coefficients is in accord with a partial sequence homology between them. However, they show disparate behaviour in their interactions with other proteins.

The similarities between spectrin, myosin and filamin which might give rise to homologous regions of amino-acid sequence will now be considered.

Only uterine muscle myosin shows an immunological cross-reaction with spectrin (Sheetz et al., 1976).

The three proteins all undergo self-association. The coiled-coil α-helical rod of myosin is involved in extensive aggregation leading to the bipolar filaments observed in muscle (Lowey, 1971). Spectrin, on the other hand, does not associate beyond the tetrameric state (Ungewickell and Gratzer, 1978). Wang (1977) has shown by chemical cross-linking in 0.5M potassium chloride, 0.1M triethanolamine,

1% β-mercaptoethanol pH 8.5 that the association state of filamin in this buffer is dimeric. The sedimentation coefficient of 10S is consistent with a side-by side association of monomers. Shizuta et al. (1976) have observed a small proportion of aggregated material in their preparations, which increases on a time scale of days, with the concomitant release of degraded material. It is not clear whether this process is reversible. Attempts to produce filaments using ionic strengths between 40mM and 600mM, failed. Spectrin dimer and tetramer exist in equilibrium (Ungewickell and Gratzer, 1978) and there has been a report of a monomer-dimer equilibrium in filamin (Lewis et al., 1978) at 0.6M potassium chloride. Thus the self-association properties of myosin and spectrin are quite different from one another, and those of filamin are not well established.

Spectrin, filamin and myosin all interact with F-actin. Each of the two globular heads of a myosin molecule has a binding site for F-actin (Lowey, 1971). Separation of two isoenzymes of S-1 containing different light chain populations, leads to two different values of $V_{max}$ for actin activated myosin ATPase (Weeds and Taylor, 1975). Thus, these light chains, which have no analogues in either filamin or spectrin, appear to be involved in actin binding. Although spectrin requires 4.1 to bind strongly to actin, a weak interaction is observed without 4.1 in the presence of 2mM magnesium, implying that at least one of the spectrin subunits has a binding site for F-actin. It appears from the bridges made by spectrin tetramer between actin filaments

in the presence of 4.1 (Fig. 2.18) that only one end of spectrin dimer is binding to the F-actin. In contrast, the tightly packed bundles of F-actin which can be seen in a mixture of filamin and F-actin (Wang and Singer, 1977) suggest that filamin lies along the F-actin strand. Bundle formation can be prevented by the digestion of a fragment of only 9,500 daltons from the end of each monomer whilst the remainder of the molecule is still bound to F-actin (Davies et al., 1978). Thus both filamin and spectrin have a binding site for actin near one end of the molecule. The rest of the filamin molecule probably lies parallel to the actin filament whereas, in spectrin, it does not. A homologous amino-acid sequence of only about 9,500 daltons might give rise to only one coincident fragment in a comparison between the digestion patterns from two proteins. However, several coincidences over and above the number observed between two random patterns are necessary to obtain a less than 1% probability of the pattern arising by chance. Hence, such limited homologies could not be detected by this method.

Filamin in stress fibres (Heggeness et al., 1977) probably does not interact with the membrane. The flexible hydrophobic region of spectrin might, therefore, be expected to have no parallel in filamin and a similar argument applies to the binding site on spectrin for ankyrin.

The independence of the secondary and tertiary structure of each subunit of the presence of the other, as shown by their ability to dissociate before unfolding, leads to an expectation that the properties each contributes to

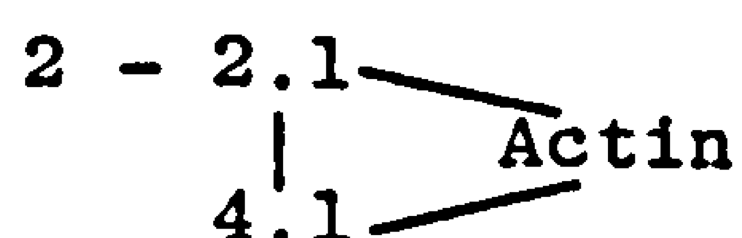
dimer will be retained in the separate subunits. To a large extent, the similarity in the physical characteristics of the two chains and their similarity to dimer bears this out. The appearance of negatively stained electron micrographs and the sedimentation velocity profiles of bands 1 and 2, are compatible with the presence of two thin, flexible strands, narrower than spectrin dimer. Denaturation of band 1 in urea shows just the same profile as dimer and the α-helix content of both the subunits is high. None of this information on single polypeptide chains is available for filamin or for the similar actin binding protein from macrophages. Despite evidence for the integrity of the separate chains, the ease with which band 2 aggregates shows that it is markedly different from band 1 and dimer. There are examples of poor resolution of band 2 in gels, even in the presence of SDS; the digestion patterns of band 2 in the paper by Hsu et al. (1979) show streaking which is not evident for band 1. Although it has been shown (3.III.4) that Triton X-100 and Nonidet P-40 partially prevent this aggregation, it is interesting that sodium deoxycholate, which is so much more effective, prevents dimer formation.

Although some evidence has been presented for a self-association of band 1, such a small amount of the dimeric species is produced, that the arrangement of subunits in the tetramer _in vivo_ cannot be deduced from this result. In this connection, the studies of Schechter et al. (1976) on the shape and aggregation state of spectrin in sodium deoxycholate are of interest. Spectrin was prepared at $4^{o}C$; conditions which lead to the extraction of tetramer.

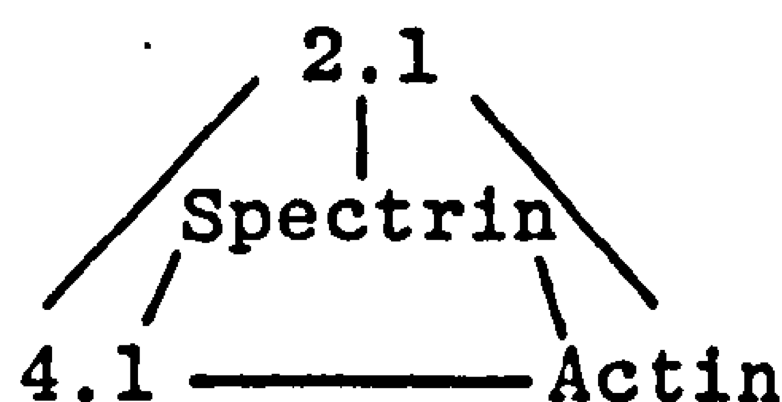
Extracted spectrin was concentrated by various methods, whose effects on the aggregation state of spectrin are not known, and the deoxycholate added to give a final concentration of 5 to 10mM. Sedimentation equilibrium results were consistent with the coexistence of two species of molecular weight 440,000 and 220,000 with the concentration of the larger species varying from 25% to 79% of the total, from one preparation to another. In the cross-linking experiments described in 3.III.4, the cross-linking of a mixture of bands 1 and 2 in sodium deoxycholate did not lead to any dimer formation. Variation of the rotor speed, or protein concentration, in the sedimentation equilibrium experiments did not change the proportions of 440,000 and 220,000 dalton species, suggesting that the two were not interconvertible within the time scale of the experiment. Sedimentation velocity revealed only one peak, leading to a rather variable S value between 5.5S and 6.0S which is about the same as the value for band 1 in aqueous solution (3.III.5). Kam et al. (1979) found a value of 6.9S. This is incompatible with the presence of spectrin dimer prepared in aqueous solution, with an S value of 9.7 (Gratzer and Beaven, 1975). However, an end-to-end association of monomers, with a much higher asymmetry could give rise to such a low sedimentation coefficient. It would be of interest to know if sodium deoxycholate, by virtue of its disruption of the binding between subunits in dimer, can lead to a similar breakdown of tetramer to yield two new "dimers" consisting of monomers associated end-to-end. Cross-linking might then solve the problem of how

bands 1 and 2 are arranged in spectrin tetramer.

The manner of participation of the subunits in the complex involving spectrin, 4.1 and F-actin has been partly established. It is clear that band 1, in the absence of band 2, does not interact with the other proteins. The variable ability of band 2 to form complexes, and in particular the observation that a single preparation of band 2 can interact differently, according to the 4.1 preparation used, suggests that impurities in the 4.1 may be involved. Although the presence of proteolytic fragments of band 1 cannot be ruled out, a protein which undergoes proteolysis with great ease in red blood cell membranes is ankyrin or 2.1 (Bennett and Stenbuck, 1979; Luna, Kidd and Branton, 1979). Moreover, it has been shown that an ankyrin fragment which binds spectrin dimer can interact with band 2 but not band 1 (3,III.6). If any of these fragments were present in a 4.1 preparation and could also bind to 4.1, then band 2 could co-sediment with F-actin by virtue of a complex such as




and a structure such as




could exist in the membrane. Since both subunits are evidently required for ternary complex formation, parts of both the spectrin chains may be associated with a binding region, or the sites for spectrin binding to F-actin and 4.1

may be on different subunits. In this respect, it is interesting that mild oxidation of red cell membranes results in the cross-linking of actin to band 1, but not to band 2 (Liu ét al., 1977). The recent findings of Tyler et al. (1979) that spectrin and 4.1 form a binary complex in 20mM potassium chloride, could be used to investigate the relationship between 4.1 and the subunits. The weak binding of spectrin to actin in 2mM magnesium may also provide an answer as to which spectrin chain is attached to actin.

Experiments with the ankyrin fragment reveal that it is band 2 that uniquely bears the binding site by which the cytoskeleton is secured to the membrane. This site survives the fractionation and renaturation cycle. The failure of cross-linking experiments to reflect the formation of the complex is presumably a consequence of an insufficiency of reactive amino groups in the vicinity of the binding sites, and shows the inadequacy of a negative result in such experiments as a criterion for interaction.

The results in this chapter go some way towards answering the intriguing question of the parts played by the spectrin subunits in the erythrocyte cytoskeleton and suggest further experiments to resolve remaining problems.

V. Summary

A study of the structure of human erythrocyte spectrin has been undertaken. The molar residue ellipticity of spectrin dimer at 222nm leads to an estimation of the α-helix content of the molecule of more than 65%. This value

is not changed by the state of phosphorylation of spectrin, nor by conversion to the tetrameric state. The denaturation of spectrin dimer in urea is largely reversible by the criteria of CD and sedimentation velocity. Denaturation profiles at 222nm and 292nm indicate the presence of more than one unfolding domain and a labile structure, even at 0.1M urea. A comparison of the denaturation curves with the degree of dissociation of dimer into monomers, as measured by chemical cross-linking, shows that dissociation largely precedes unfolding.

The proton magnetic resonance spectrum of spectrin dimer shows that up to 20% of the residues are involved in an unstructured region which is rich in hydrophobic amino-acids. The spectrum is not changed by a reduction in the ionic strength to 20mM. This, and the invariance of $\alpha$-helicity with ionic strength, point to a movement of the globular regions of spectrin with respect to one another by means of flexible links, as the ionic strength is changed.

Digestion patterns of the separate spectrin subunits as well as myosin heavy chain and filamin, in SDS, show that there are sequence homologies between the two subunits but no or little relationship between any other combinations of the polypeptides assayed.

The two spectrin chains can be separated in 7M urea using a hydroxyapatite column. They have a similar appearance in the electron microscope, which compares with that of the dimer. Their sedimentation coefficients are compatible with those of a species of approximately the same shape as dimer, of half the molecular weight. Approximate extinction

coefficients do not differ greatly from that of dimer. Both subunits precipitate sharply as a function of pH, as does dimer. Band 2 is prone to aggregation which probably explains its lower molar residue ellipticity (16.8 deg $cm^2$ $decimole^{-1}$) as compared with band 1 (23.7 deg $cm^2 decimole^{-1}$).

The subunits recombine to form dimer as evidenced by its sedimentation coefficient and position of migration as a cross-linked species on an SDS gel.

Some self-association of band 1 is observed under the conditions of dimer to tetramer conversion. Further evidence is needed to establish whether this observation has physiological significance. The separate subunits are unable to form cytoskeletal complexes with 4.1 and actin, although impurities in the 4.1 give rise to complex formation with band 2. It is band 2 which, through the medium of ankyrin, forms the site of attachment of spectrin to the red blood cell membrane.

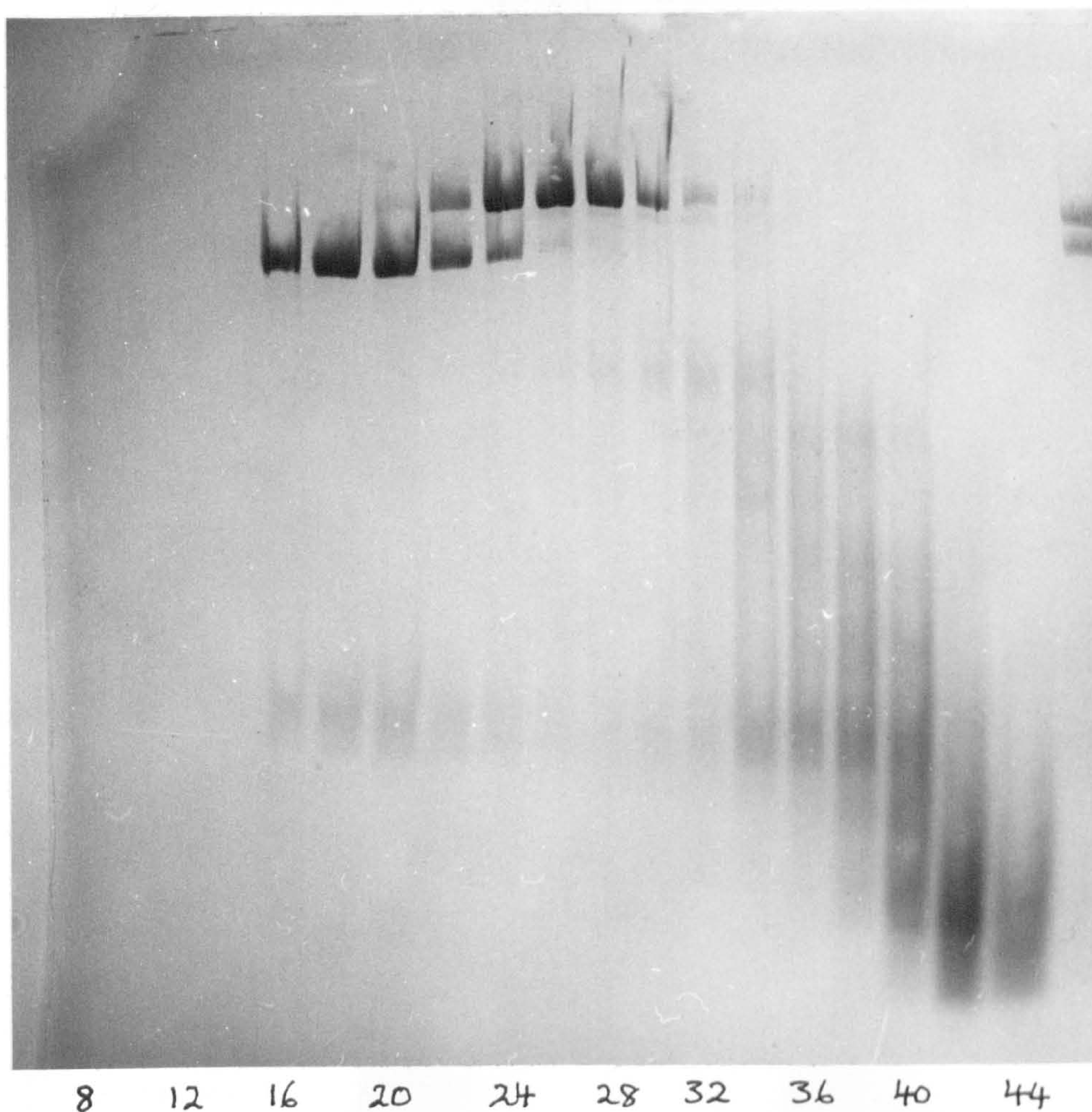



Figure 3. 1.

A 4%, SDS gel of every second fraction from the elution profile of a filamin preparation from a Sepharose 4B column. Fractions 18-30 contain filamin, 26-36 myosin, and 18-24 a small amount of material with a molecular weight of 240,000. The last track shows the two subunits of spectrin for comparison: molecular weights 240,000 and 220,000.

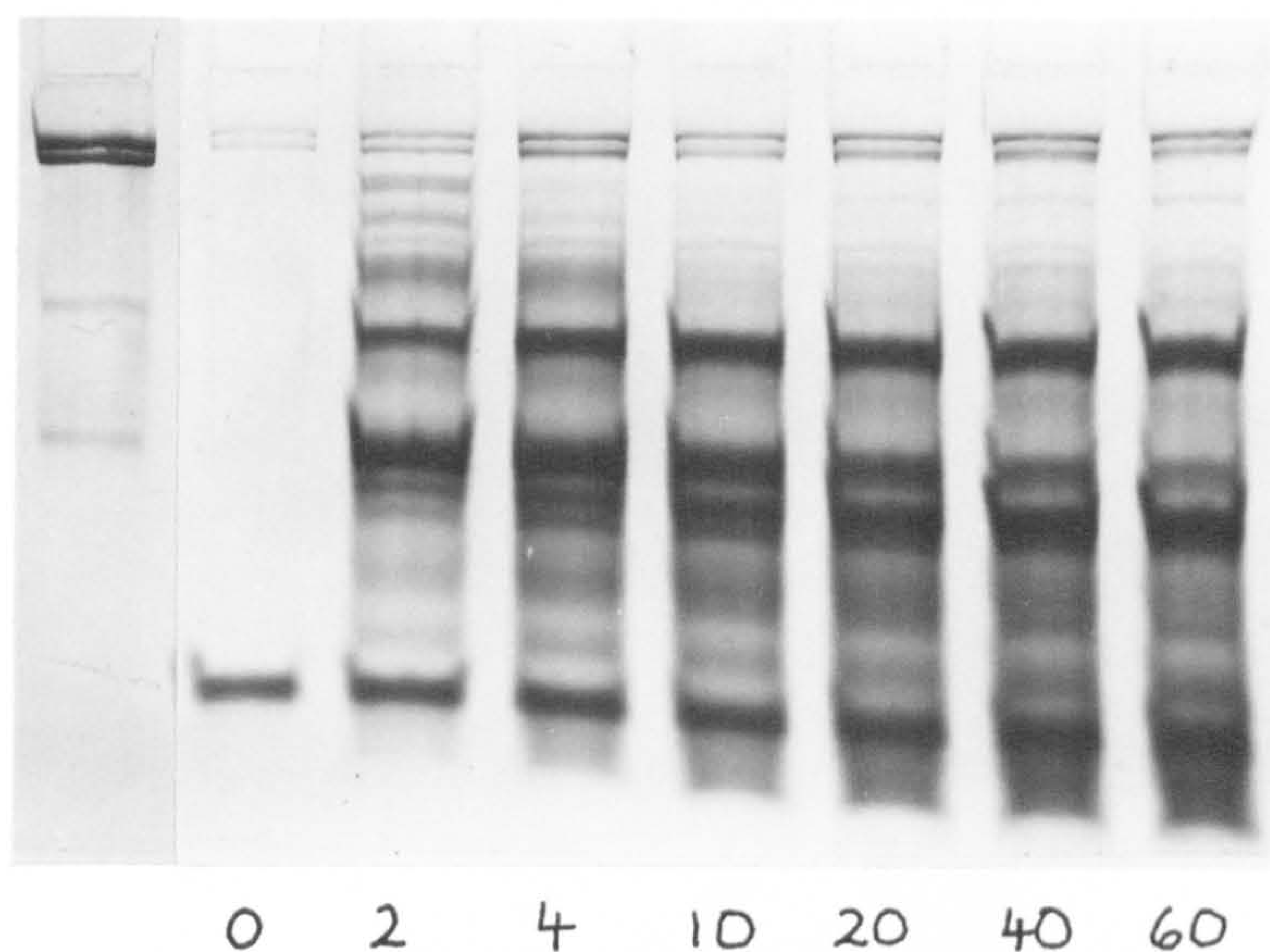



Figure 3.2.

A 7.5%, SDS gel of spectrin-depleted vesicles, digested with increasing amounts of $\alpha$-chymotrypsin. The concentration of enzyme in ($\mu$g/ml) is written under each gel track. The first track contains spectrin oligomer as a molecular weight marker.

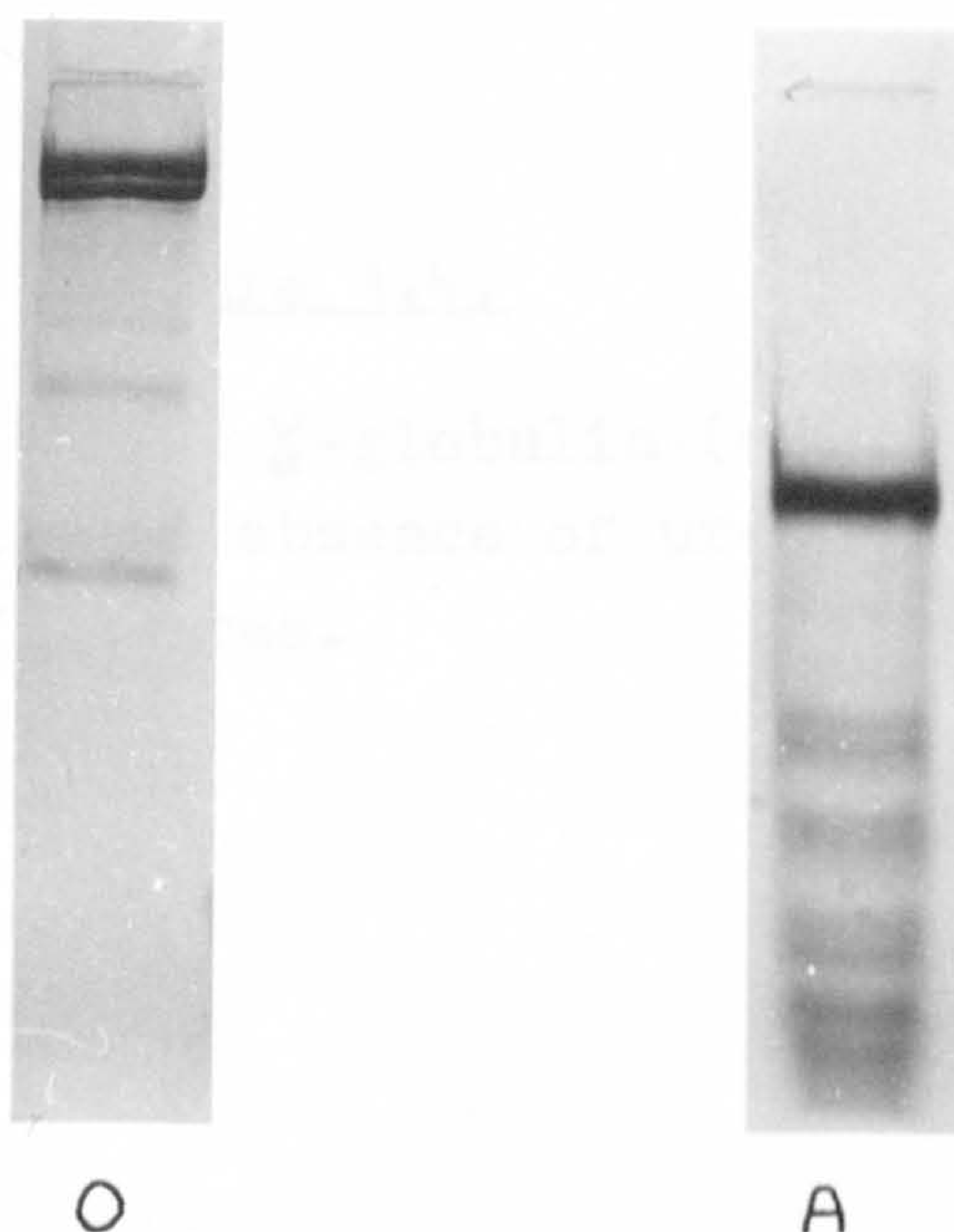



Figure 3.3.

A 7.5%, SDS gel of partially purified ankyrin fragment, eluted from a DE52 column with buffer containing 0.2M potassium chloride. O - oligomer, A - ankyrin fragment.

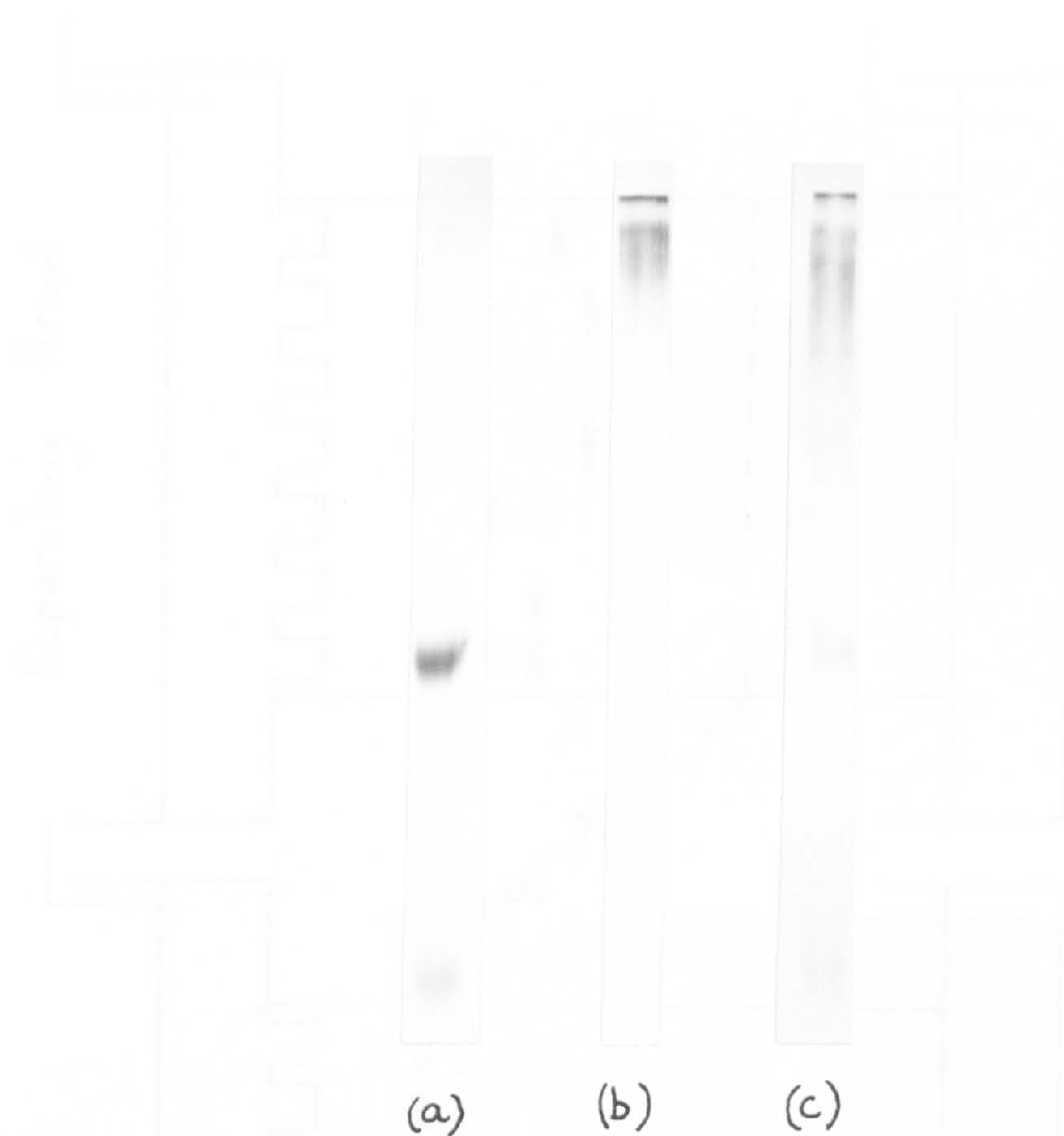



Figure 3.4.

An 8%, SDS gel of bovine $\gamma$-globulin (a) uncross-linked, (b) cross-linked in the absence of urea, (c) cross-linked in the presence of 7M urea.

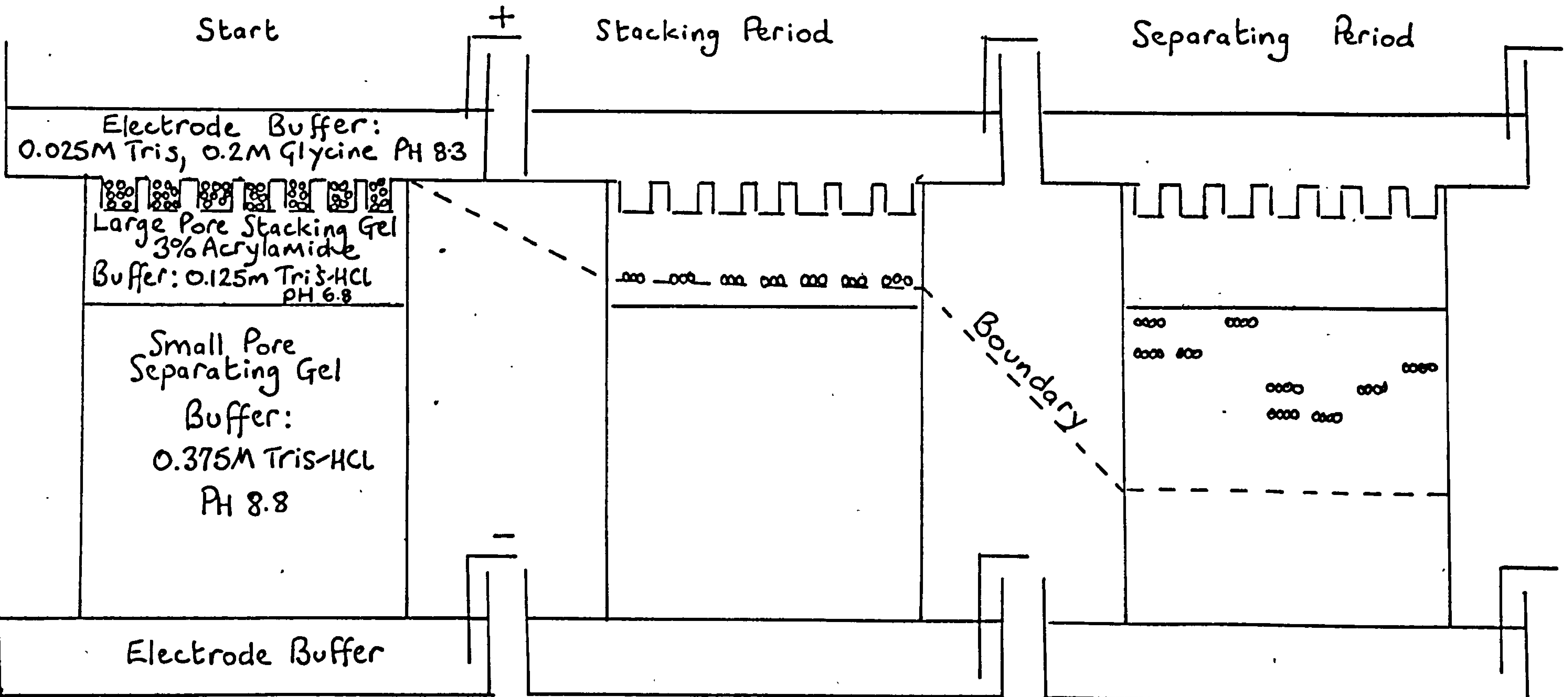
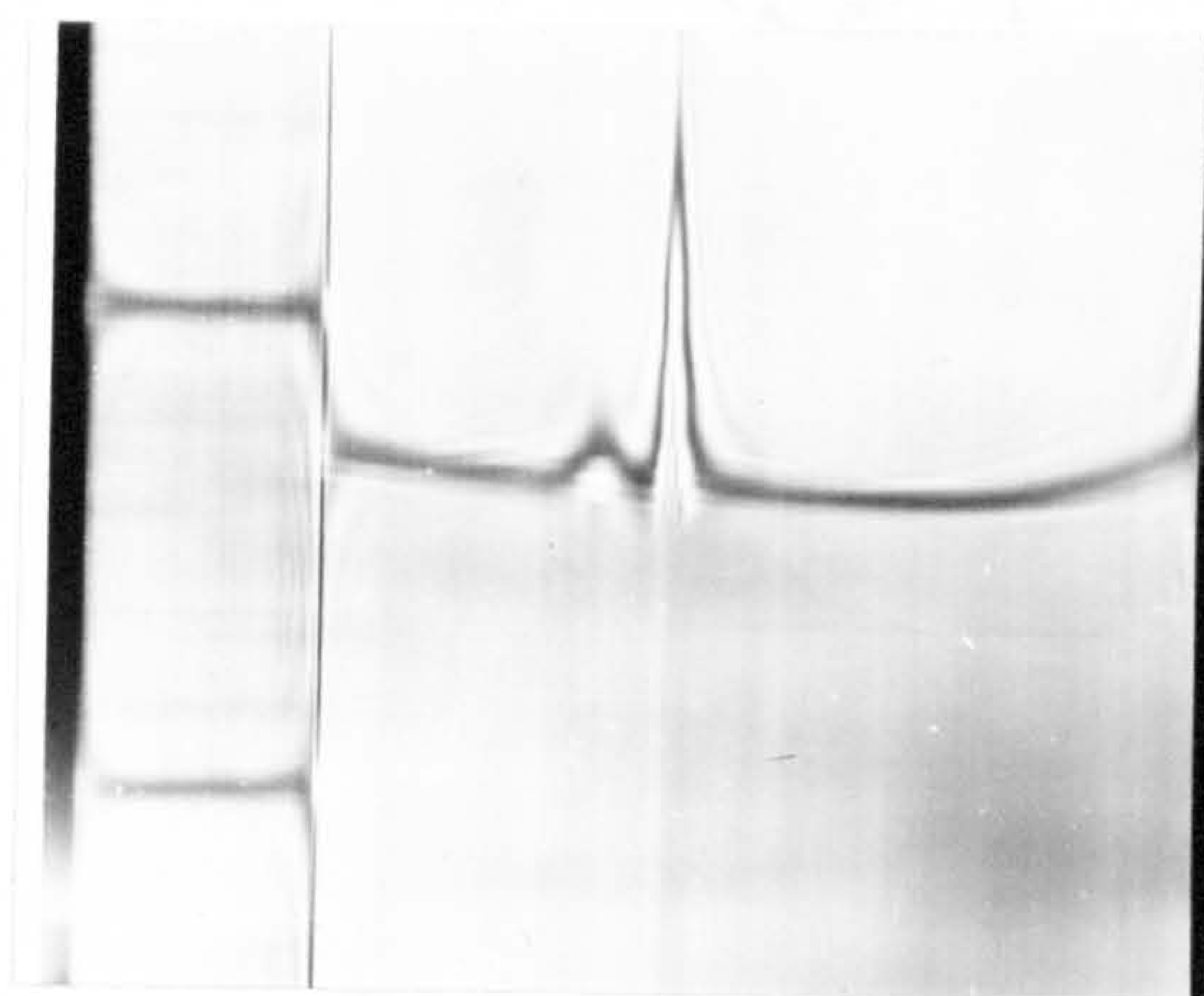
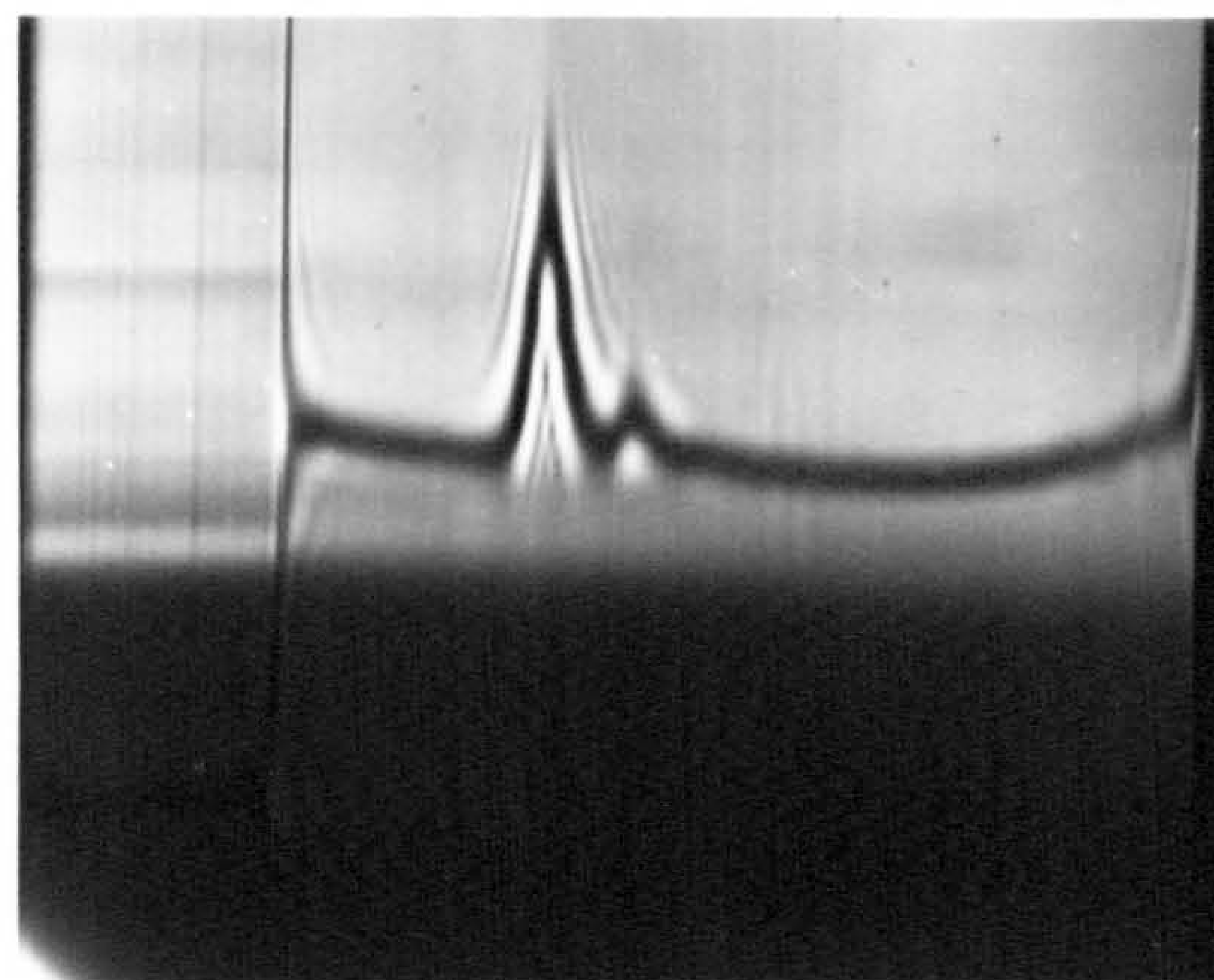



Figure 3. 5.

A diagram to show the behaviour of protein in the discontinuous electrophoretic buffer system of Laemmli and Favre (1973) for SDS gels.



(a)



(b)

Figure 3. 6.

Sedimentation velocity profiles showing spectrin preparations containing predominantly (a) tetramer, (b) dimer.

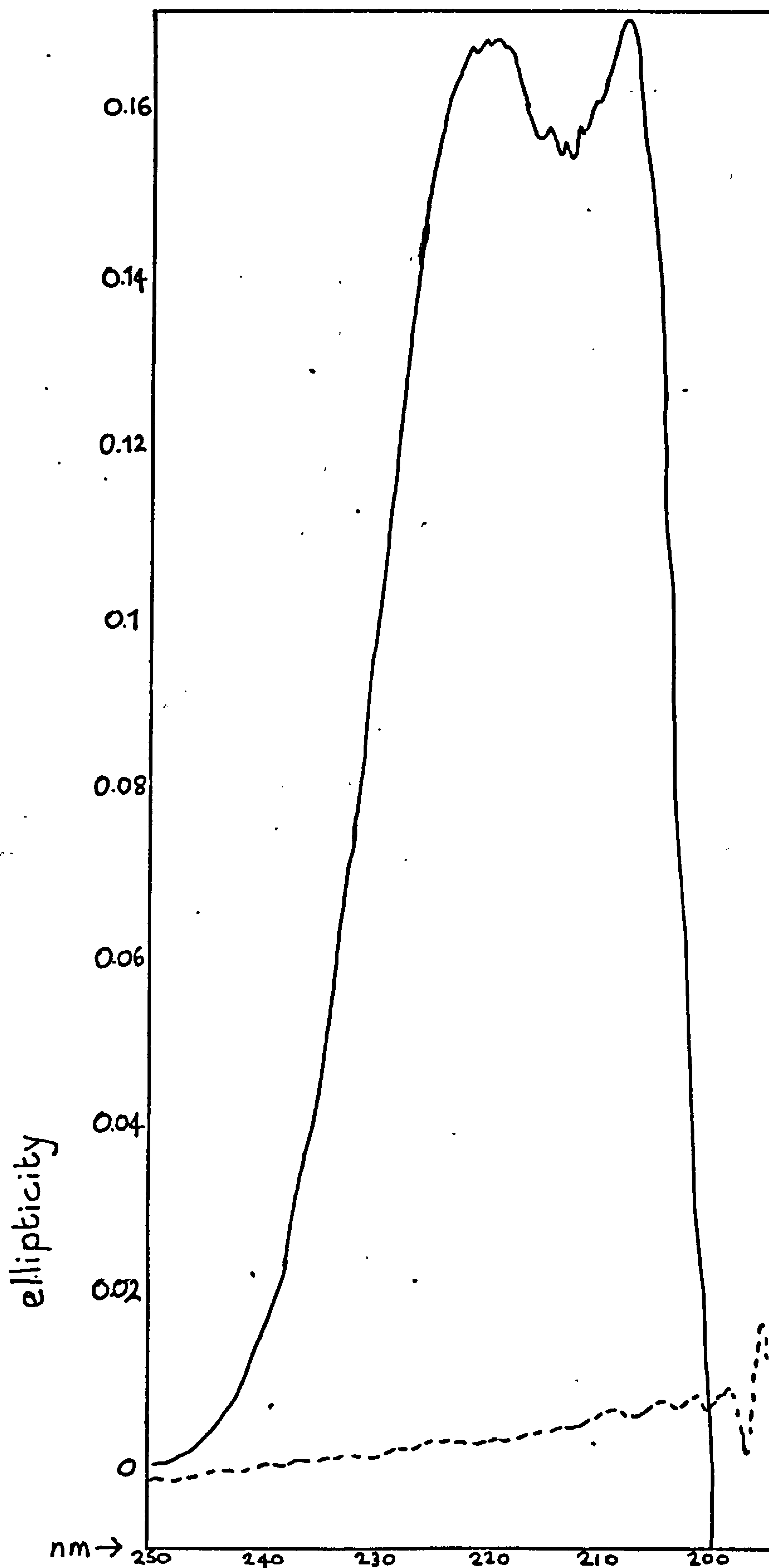



Figure 3. 7.

The far ultra-violet circular dichroism spectrum of native spectrin dimer (———) spectrin; (----) buffer blank.

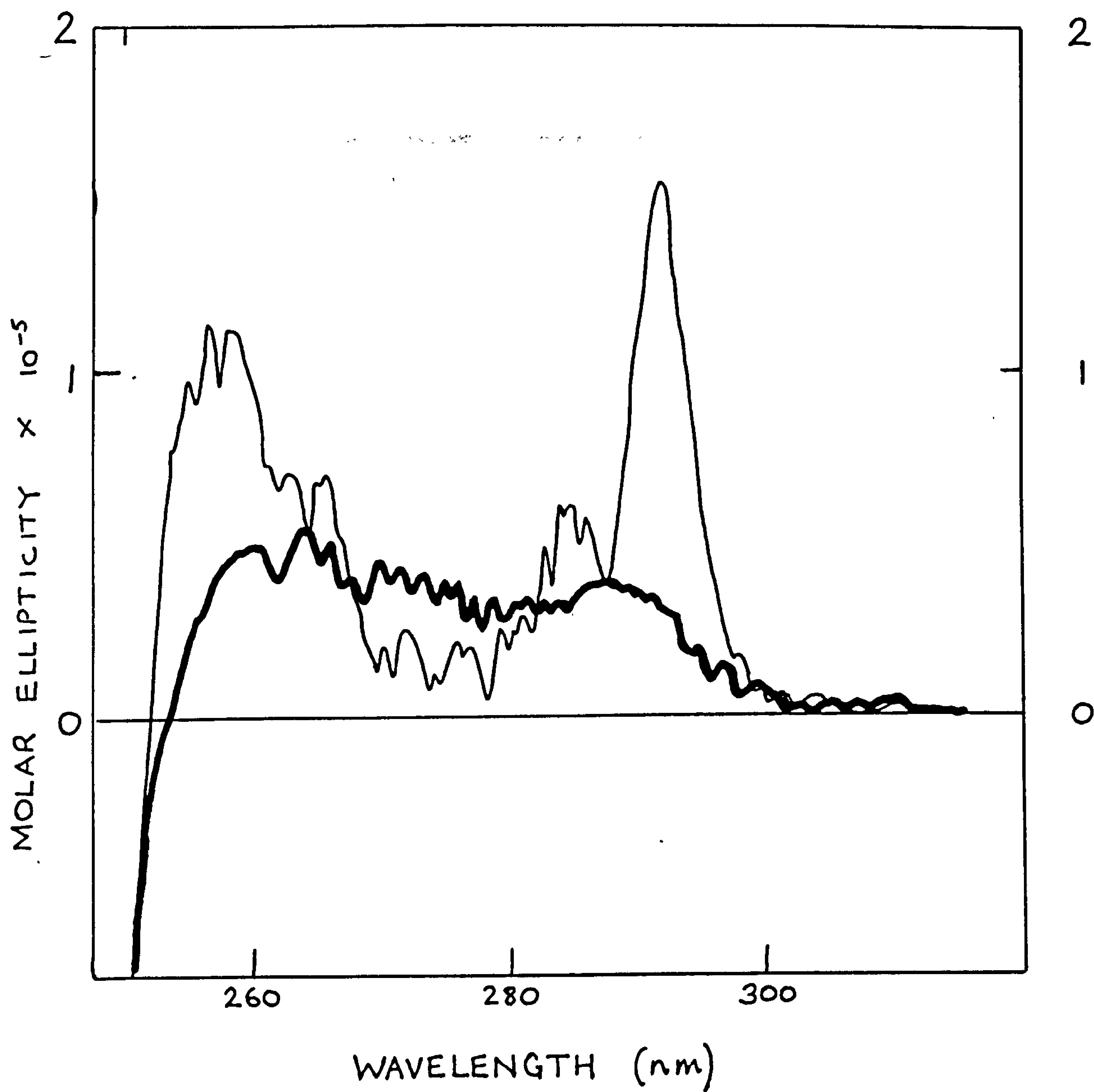



Figure 3. 8.

The near ultra-violet circular dichroism spectrum of spectrin dimer. (———) native spectrin; (▬▬▬) spectrin in 7M urea.

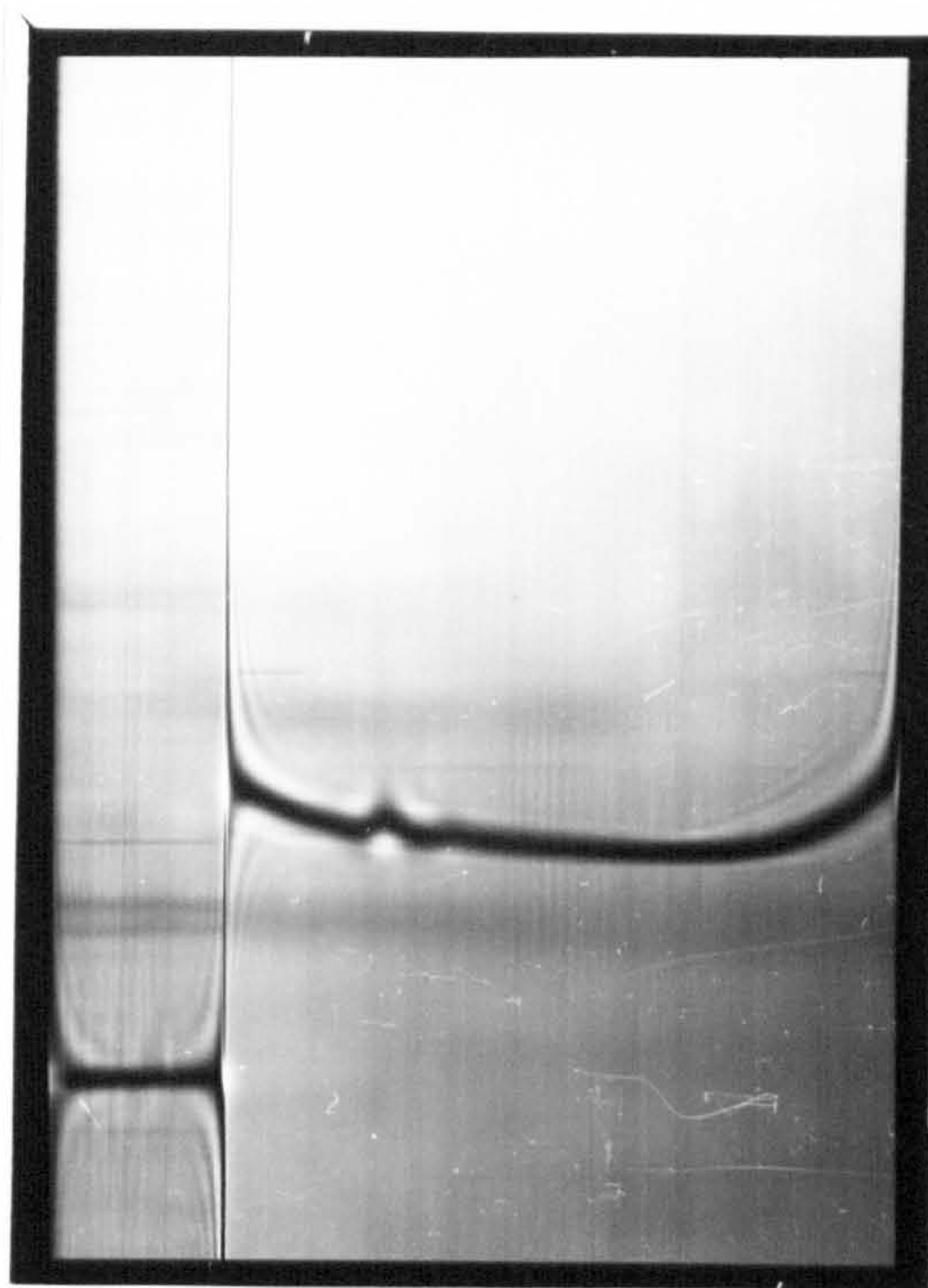
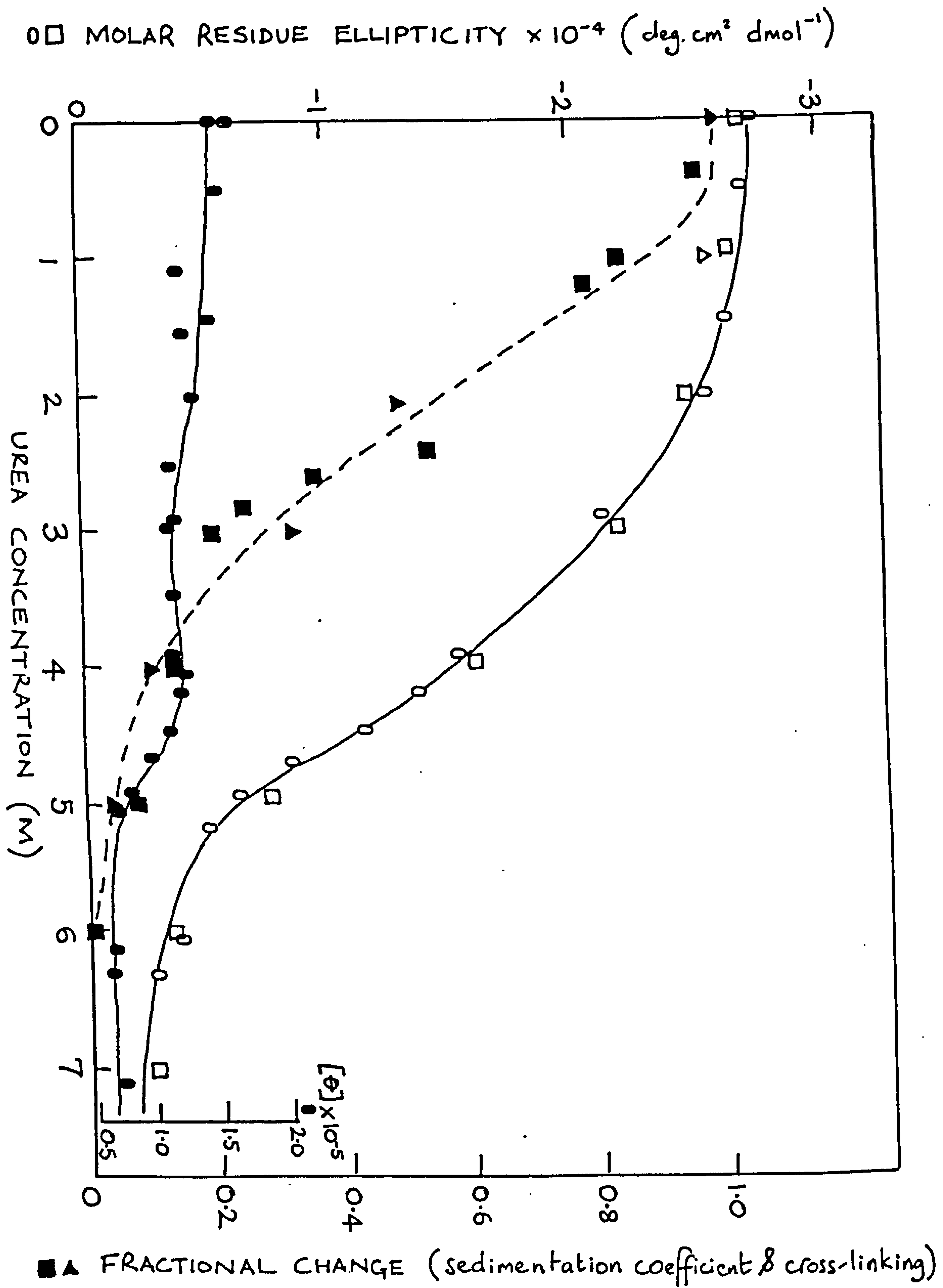


Figure 3.9.

The sedimentation velocity profile of spectrin, renatured from 7M urea.



○□ MOLAR RESIDUE ELLIPTICITY × 10⁻⁴ (deg. cm² dmol⁻¹)
0
-1
-2
-3
0
1
2
3
4
5
6
7
UREA CONCENTRATION (M)
[Φ] × 10⁻⁵
0.5
1.0
1.5
2.0
0
0.2
0.4
0.6
0.8
1.0
■▲ FRACTIONAL CHANGE (sedimentation coefficient & cross-linking)

Figure 3. 10.

The unfolding of spectrin dimer in urea solution: molar residue ellipticity at 222nm. O and □ represent experiments with spectrin concentrations of 1 and 0.1 mg/ml respectively, (left-hand ordinate); molar ellipticity, referred to dimers, at 292 nm (●, inner right-hand ordinate); fractional changes in sedimentation coefficient, $\underline{s}_{20,w}$ ( ▲ ) and degree of chemical cross-linking with dimethylsuberimidate ( ■ ) (right-hand ordinate). The buffer used for cross-linking was triethanolamine.

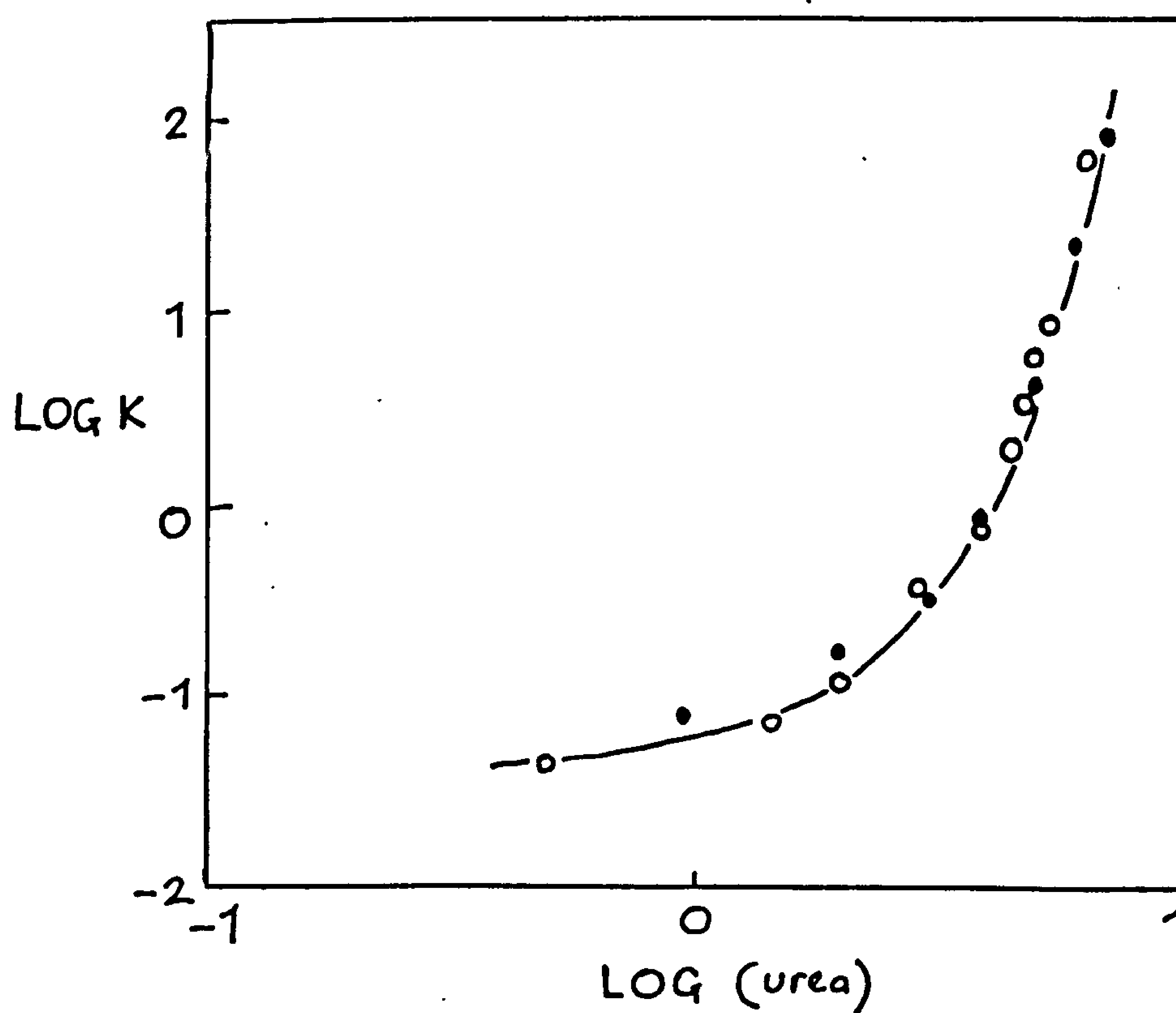



Figure 3.11.

A plot of the logarithm of the equilibrium constant for unfolding (K) versus the logarithm of the denaturant concentration for spectrin in urea.

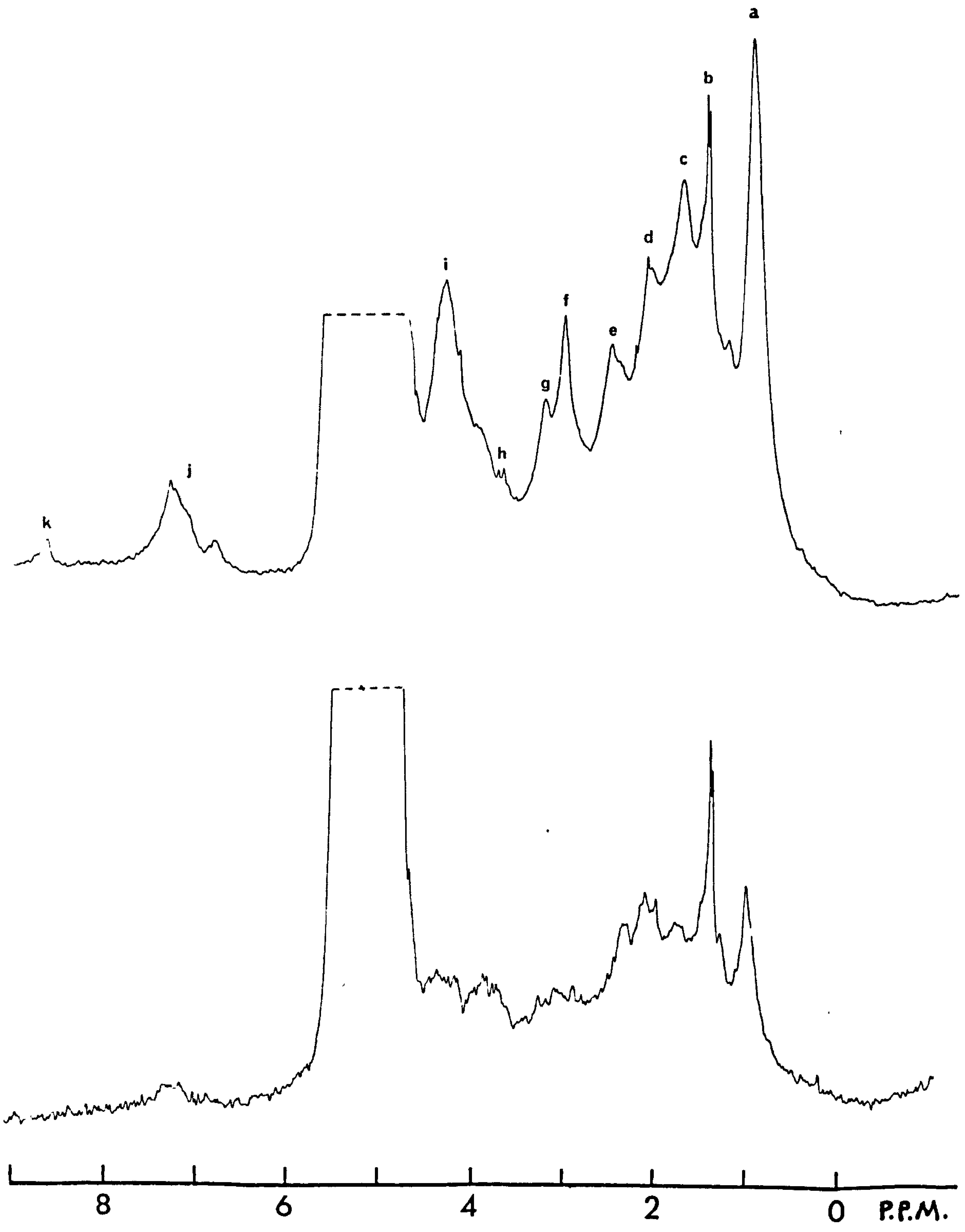

a
b
c
d
i
f
e
g
h
j
k
8
6
4
2
0
P.P.M.

Figure 3. 12.

Proton magnetic resonance spectra at 270 MHz of native spectrin in $^{2}H_2O$ solution ( lower trace ) and acid-denatured spectrin at the same concentration ( 5 mg/ml. ); 7500 free induction pulses were accumulated in either case. Raw data are shown, so that the intensities of the two spectra are not normalised relative to each other ( being referred to the $H^{2}HO$ signal, as the most intense resonance present ). The resonances indicated are : a-methyl groups of valine, leucine and isoleucine, b-methyl groups of alanine, ( threonine methyl intervening between a and b ), c and d - various aliphatic methylene groups, e-methylene groups, adjacent to carboxyl of glutamic acid ( protonated ) and glutamine ( shoulder ), f - $\varepsilon$- methylene of lysine, g - $\delta$-methylene of arginine h - $\delta$- methylene of proline, i - $\alpha$-carbon protons ( serine and glycine methylene groups between h and i ), j-tryptophan ring (with tyrosine upfield of these), k - $C_2$-protons of histidine. Note the prevalence of signals from non-polar side chains in the spectrum from native spectrin.

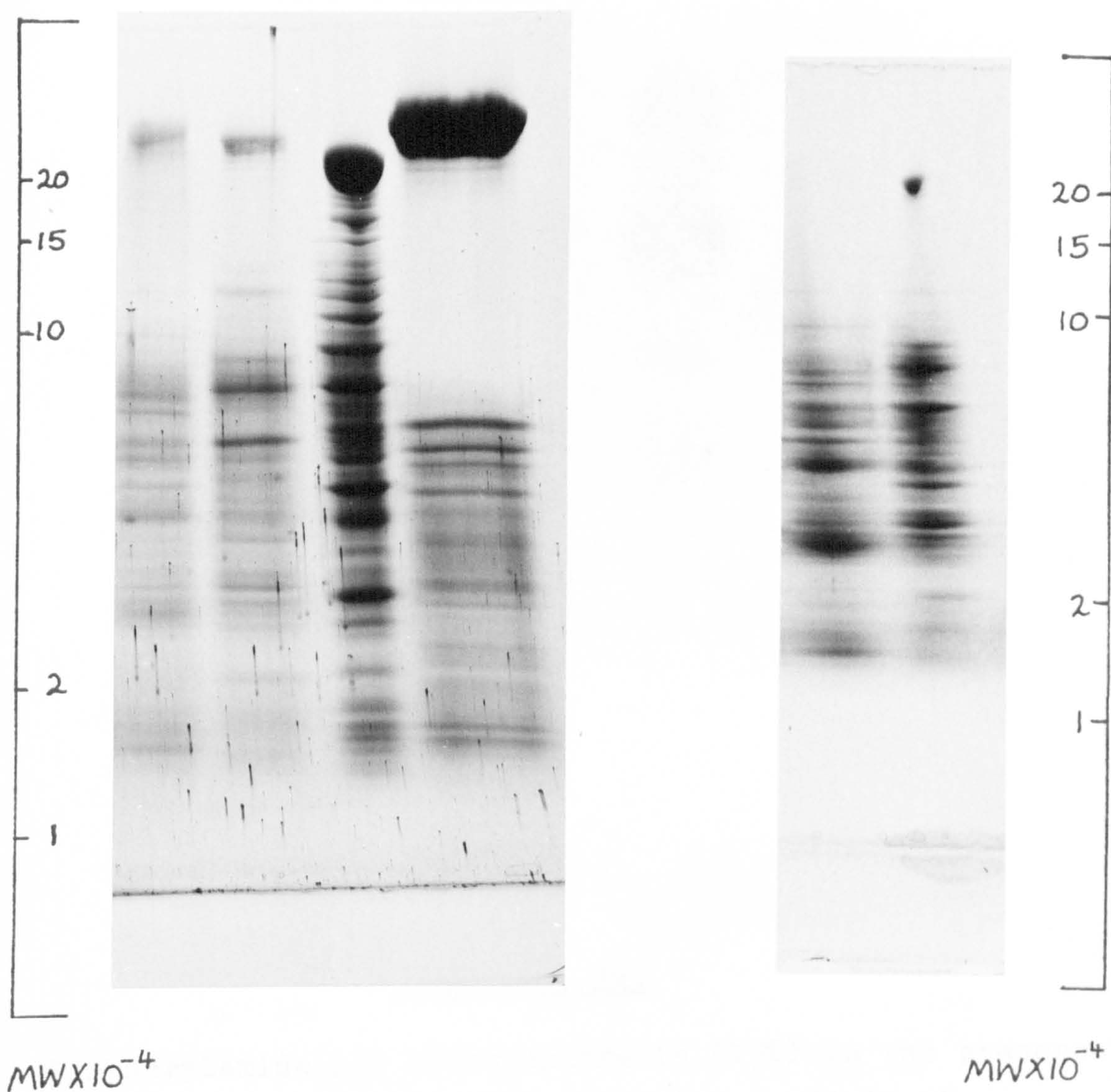



Figure 3. 13.

Polyacrylamide gel electrophoresis ( 5-20% gradient ), in the presence of SDS, of papain digestion products of ( left to right ): spectrin ( larger subunit ), spectrin ( smaller subunit ), skeletal muscle myosin, chicken gizzard filamin. The right-hand panel shows a separate experiment, with only the two spectrin subunits. The approximate molecular weight scales are based on marker proteins.

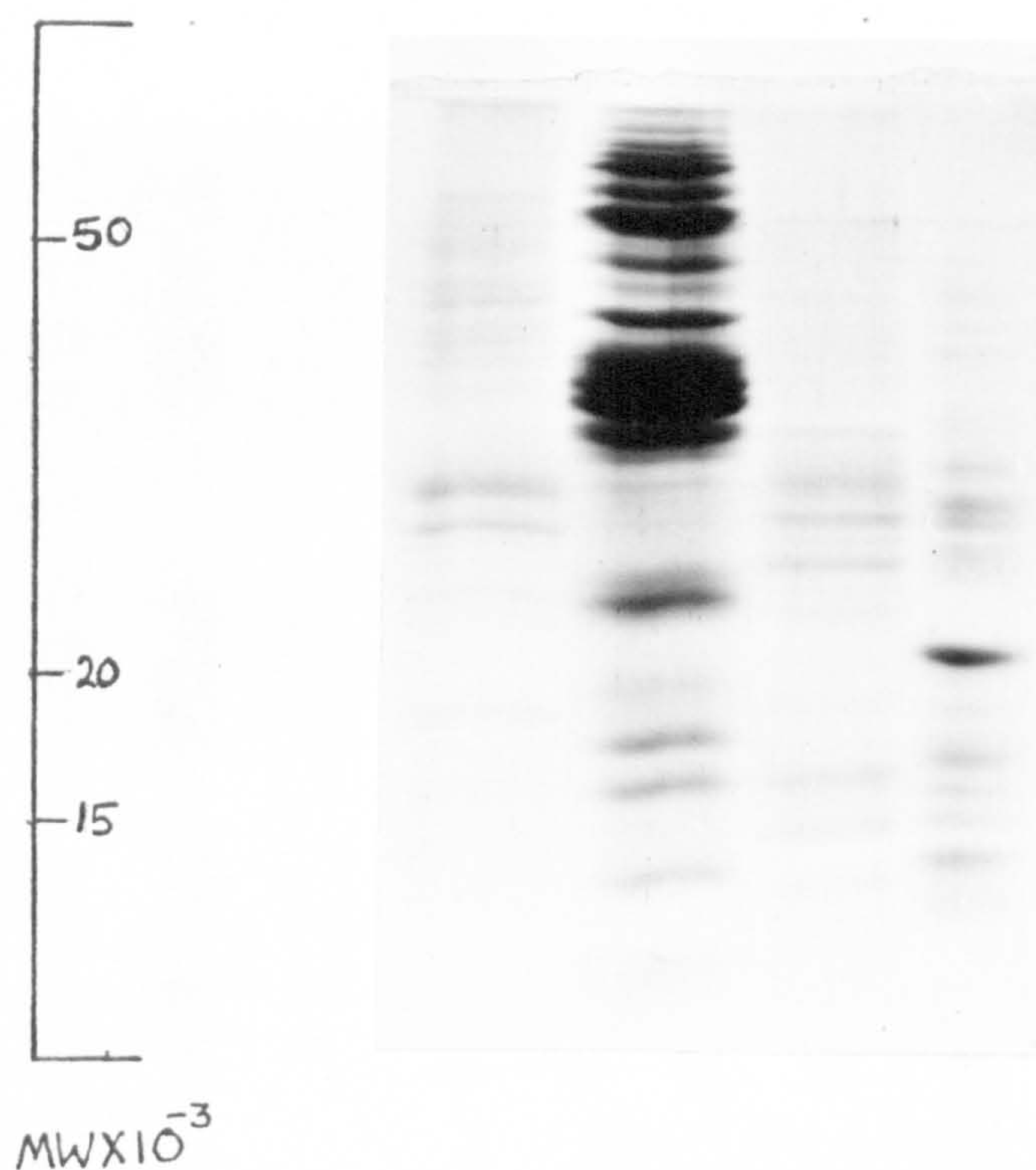



Figure 3. 14.

Polyacrylamide gel electrophoresis (15%) in the presence of SDS of cyanogen bromide digestion products of (left to right): spectrin (larger subunit), chicken gizzard filamin, spectrin (smaller subunit), skeletal muscle myosin. The approximate molecular weight scale is based on marker proteins.

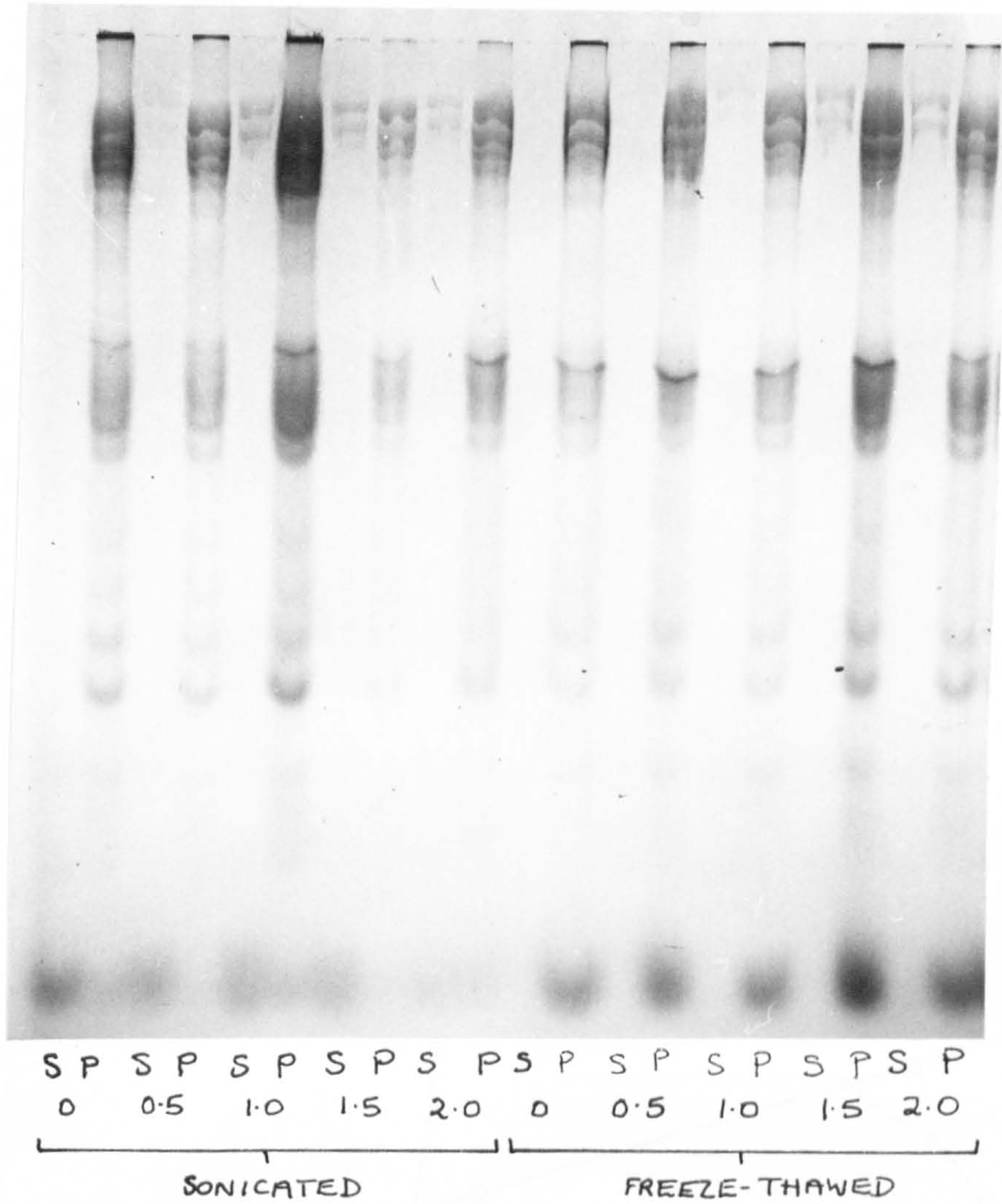



Figure 3. 15.

The extraction of spectrin from freeze-thawed and sonicated ghosts by increasing concentrations of urea. An SDS gel of the pellets (P) and supernatants (S) after extraction. The molarity of urea is indicated under each gel track.

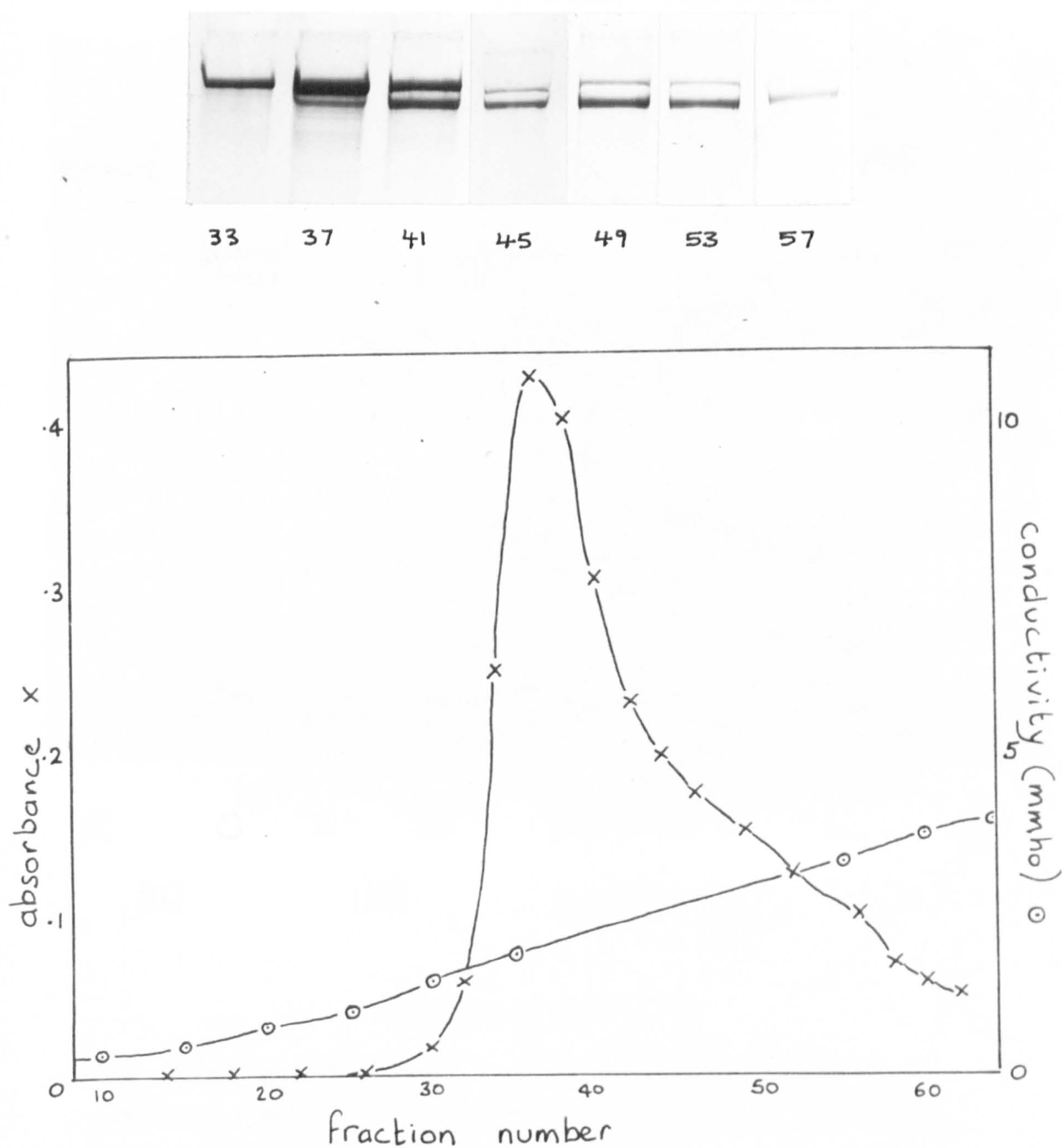
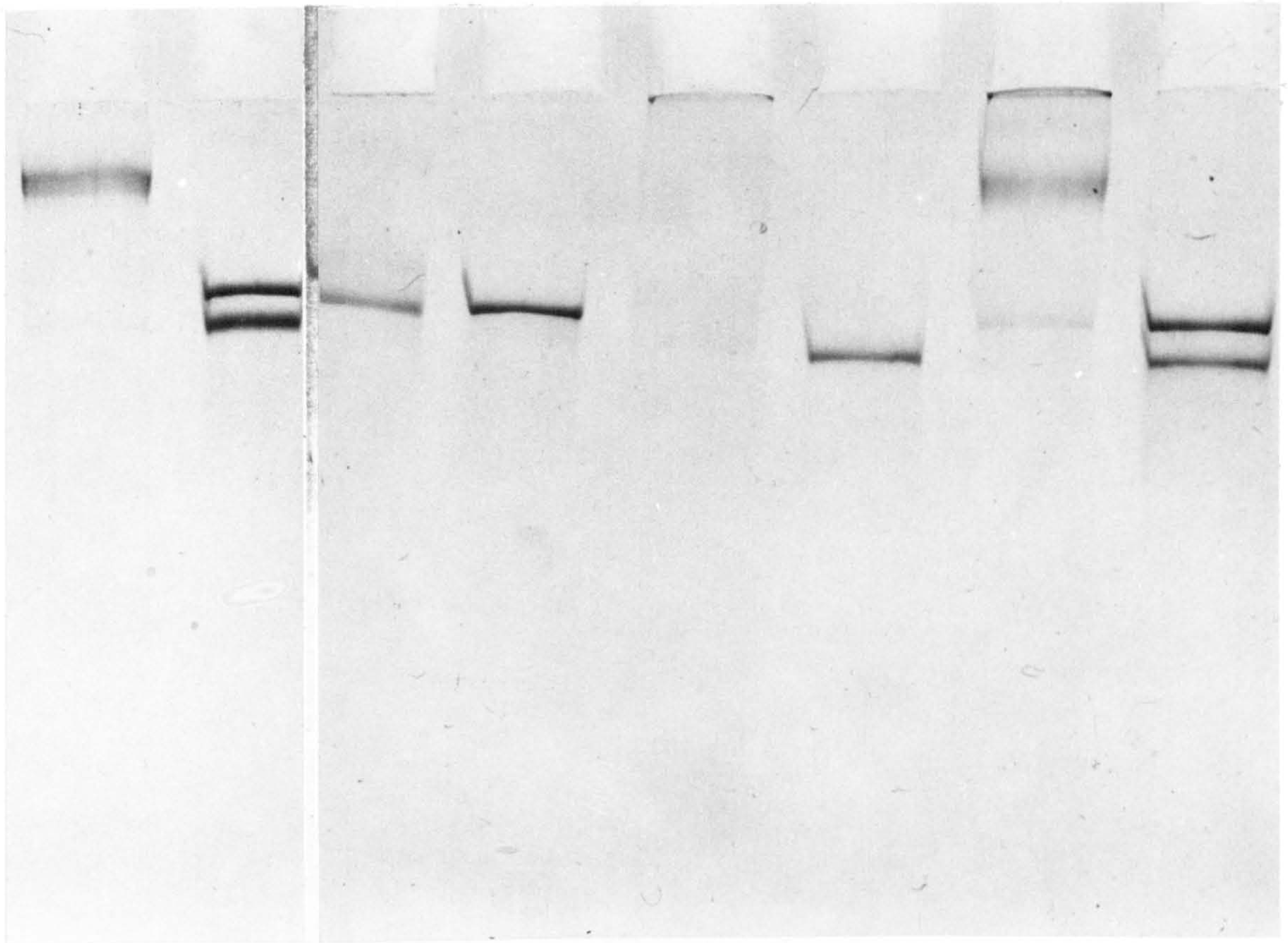



Figure 3. 16.

Separation of the spectrin subunits under denaturing conditions in hydroxyapatite. Elution profile using a phosphate gradient. An SDS gel of every fourth fraction from the column is shown.



X O X O X O X O

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

Figure 3. 17.

Gel electrophoresis in the presence of SDS of the purified subunits of spectrin and products of cross-linking with dimethylsuberimidate.

(a) Native dimer, (X) - cross-linked, (O) - untreated.

(b) Band 1.

(C) Band 2.

(d) A mixture of bands 1 and 2.

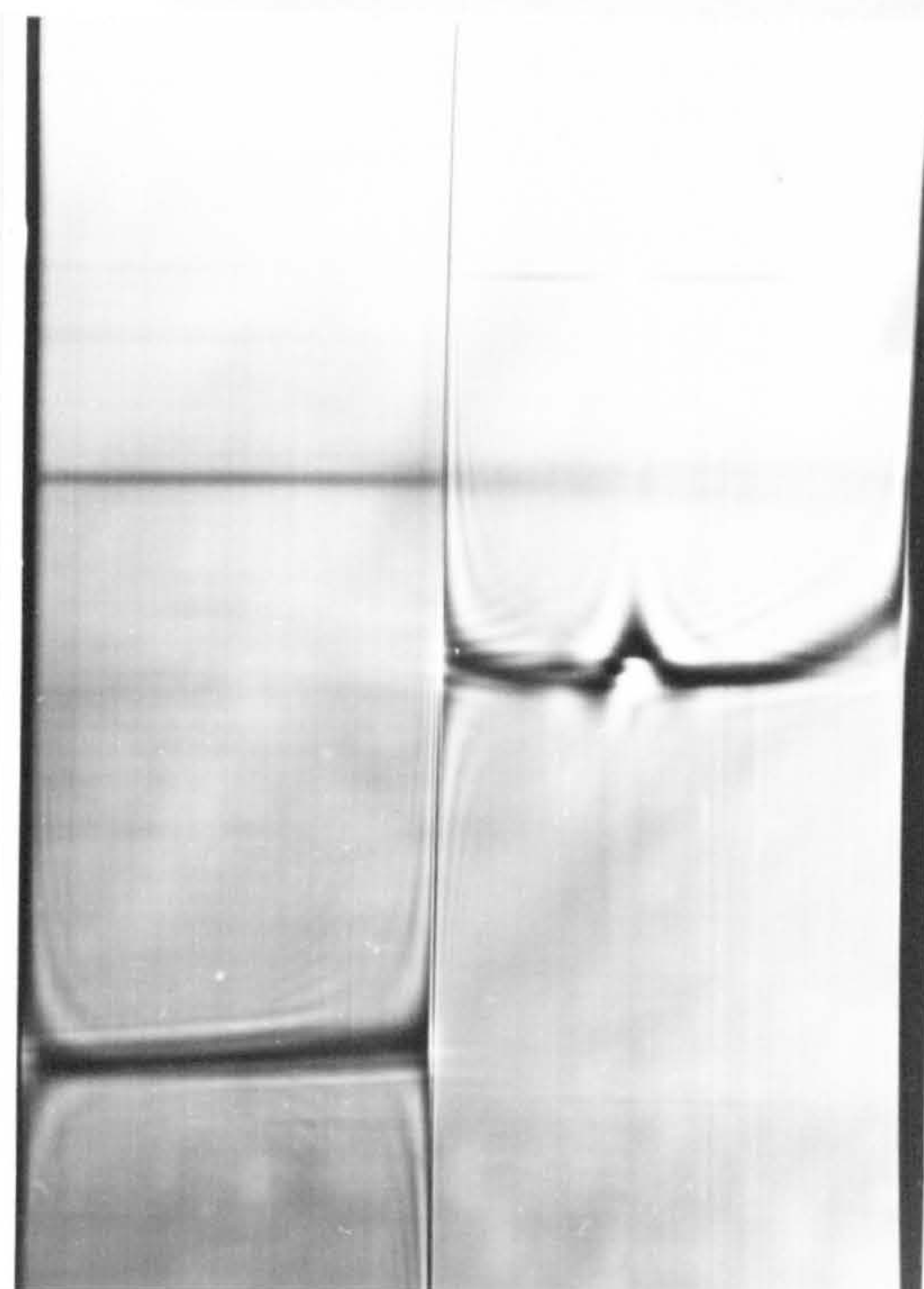


Figure 3. 18.

The sedimentation velocity profile of a mixture of renatured bands 1 and 2.

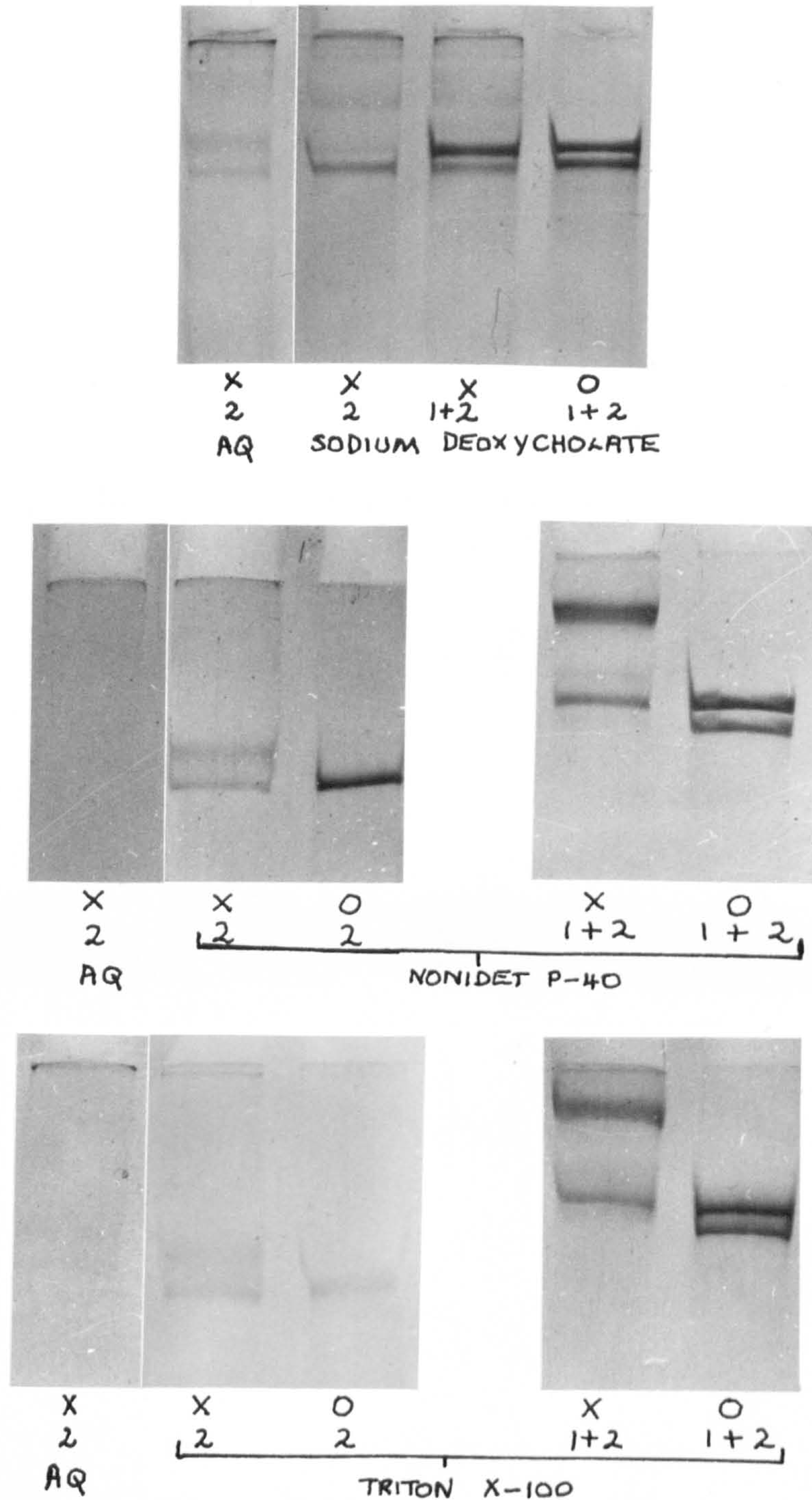
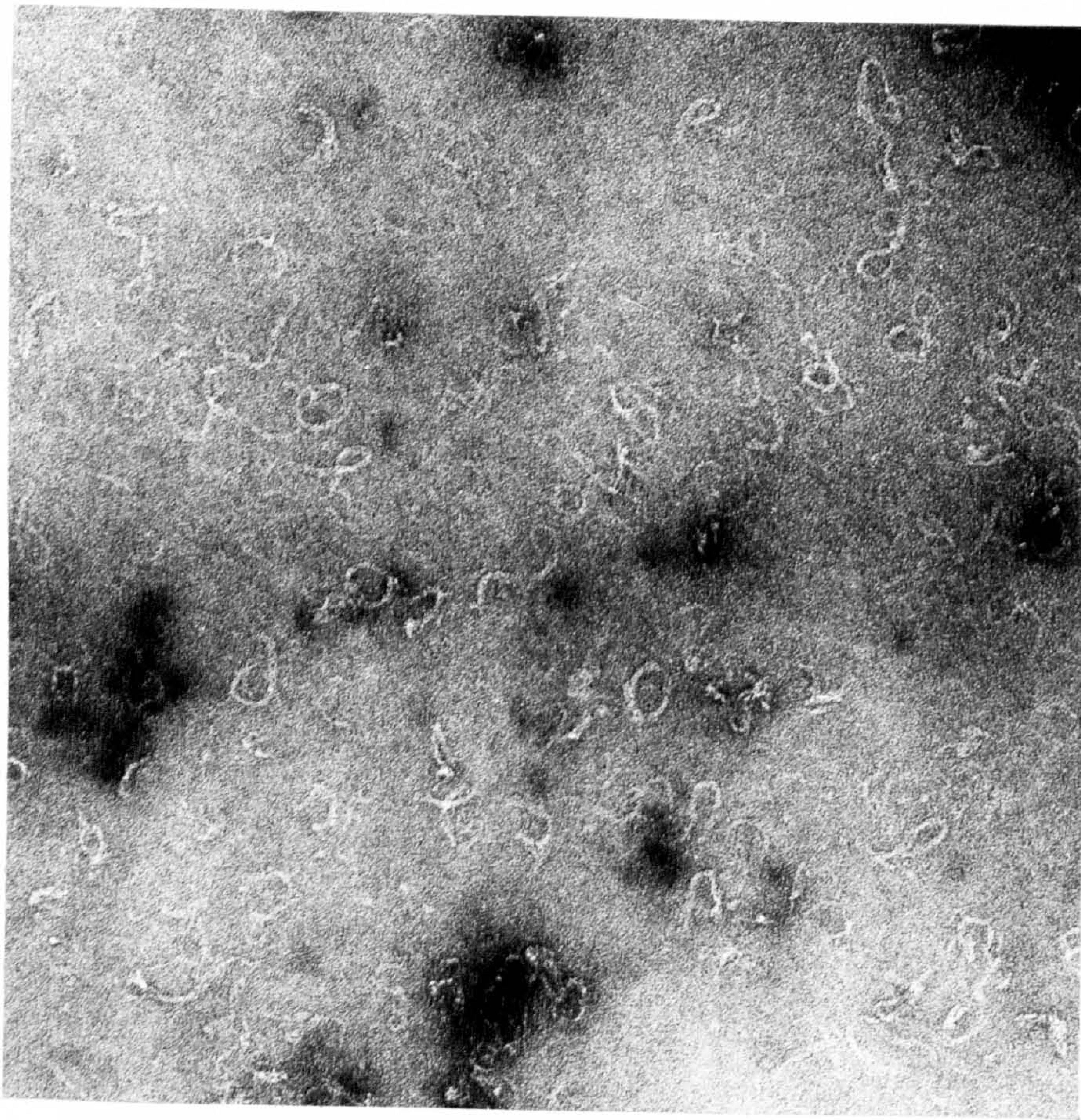
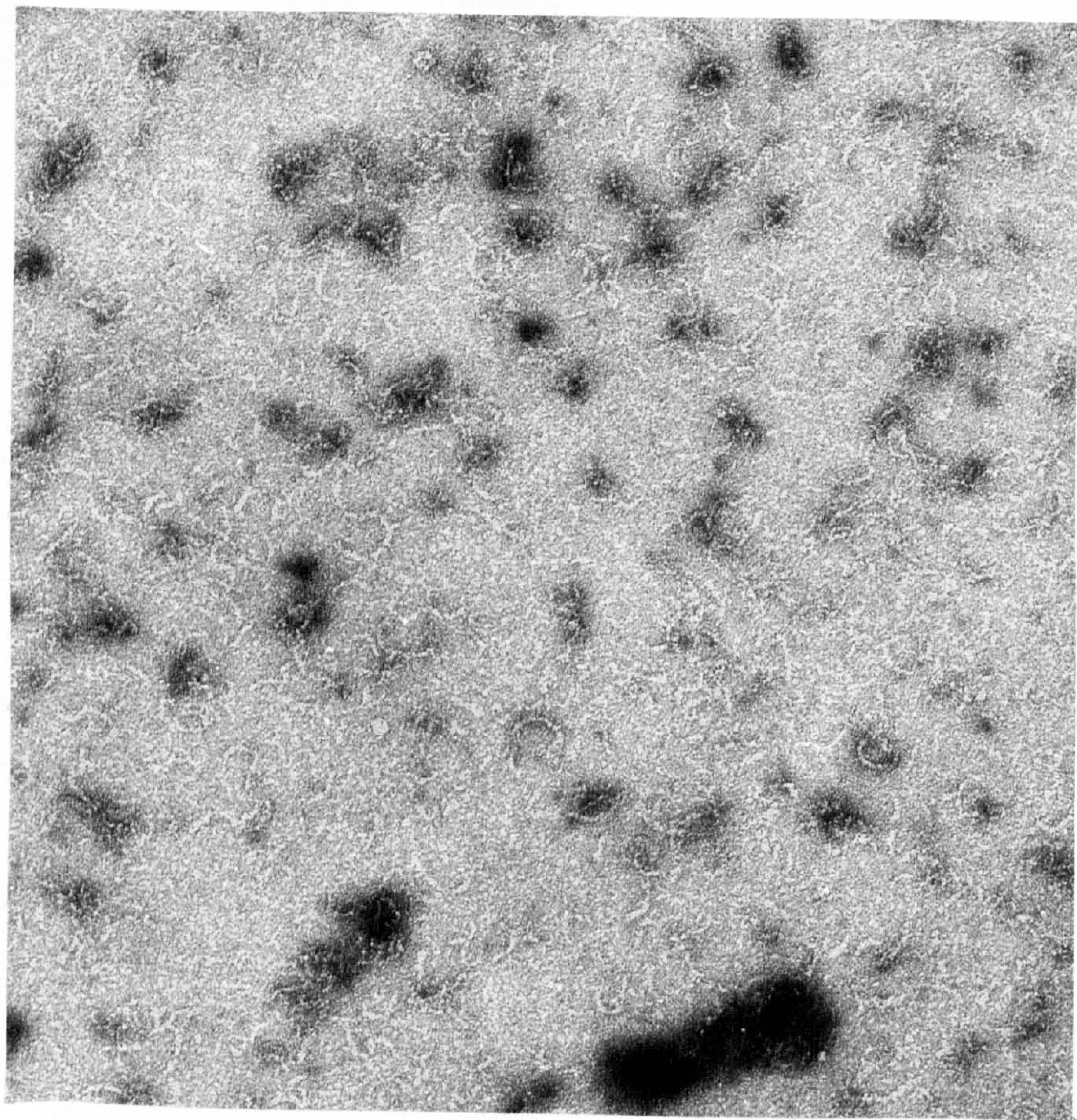



Figure 3. 19.

The aggregation states of band 2 and mixtures of bands 1 and 2 in detergent solutions. X denotes species which have been exposed to dimethylsuberimidate, O, those which are untreated. AQ shows a sample of band 2 in aqueous solution, cross-linked and run on the same gel for comparison.



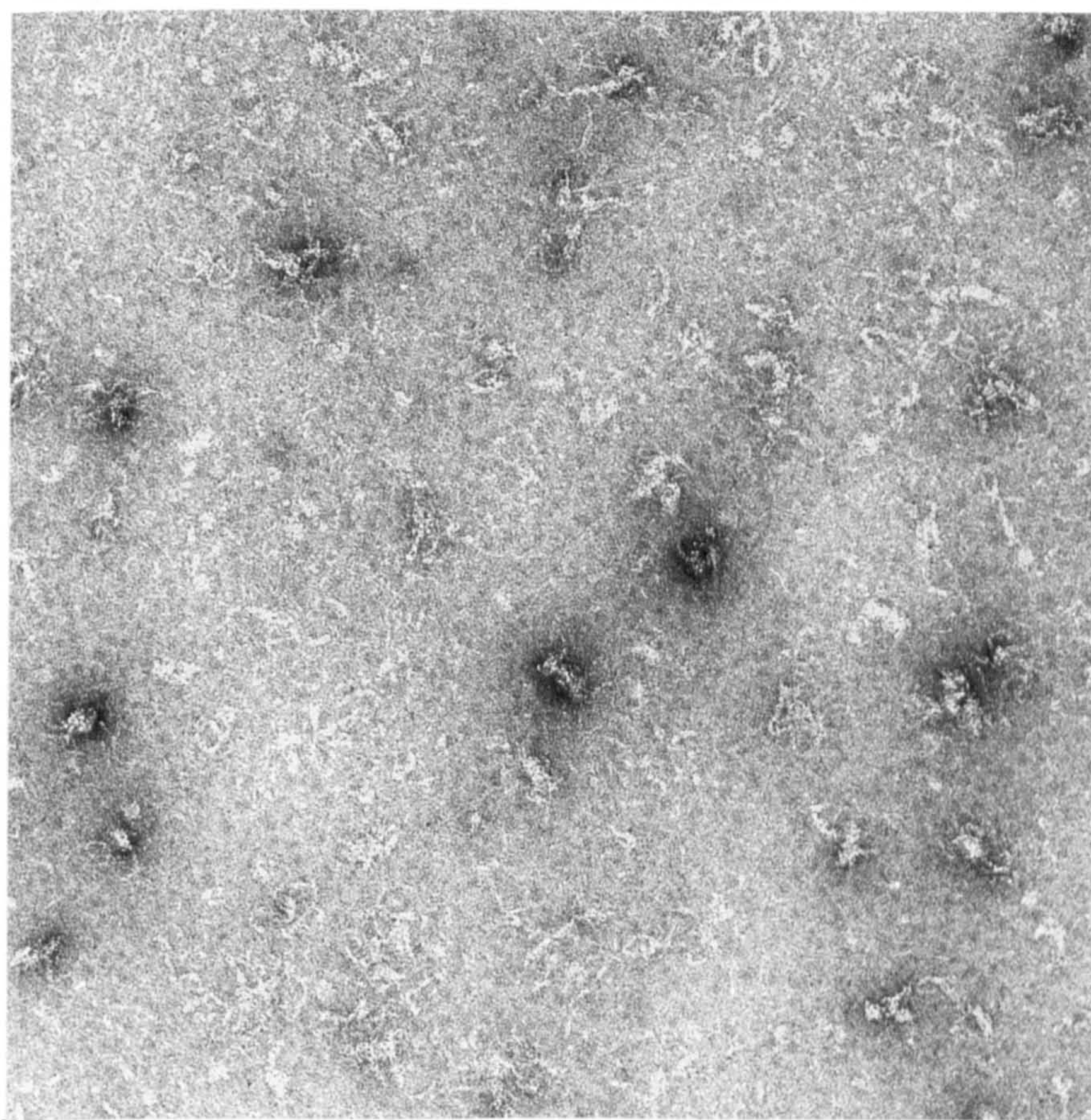
(a)



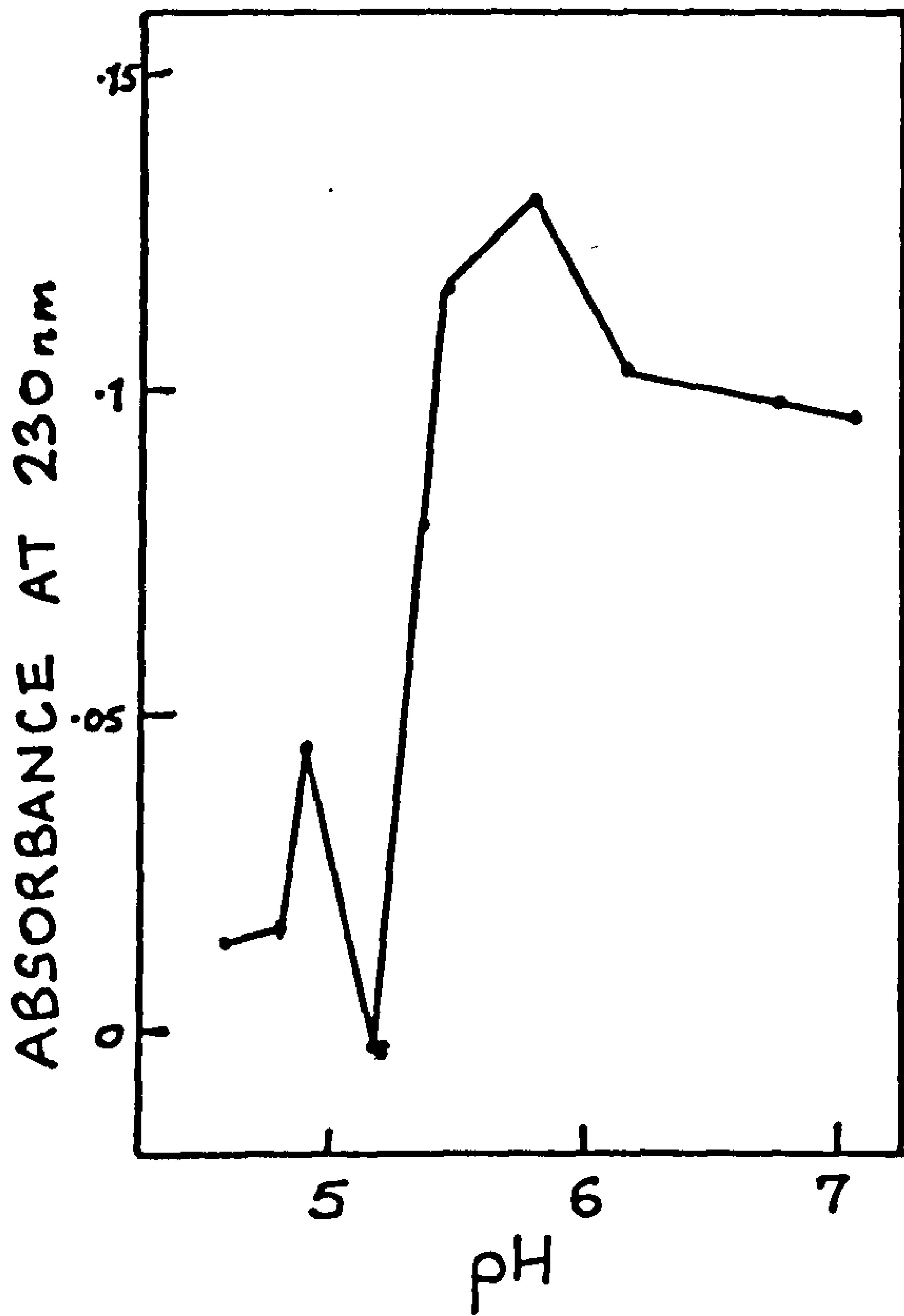
(b)

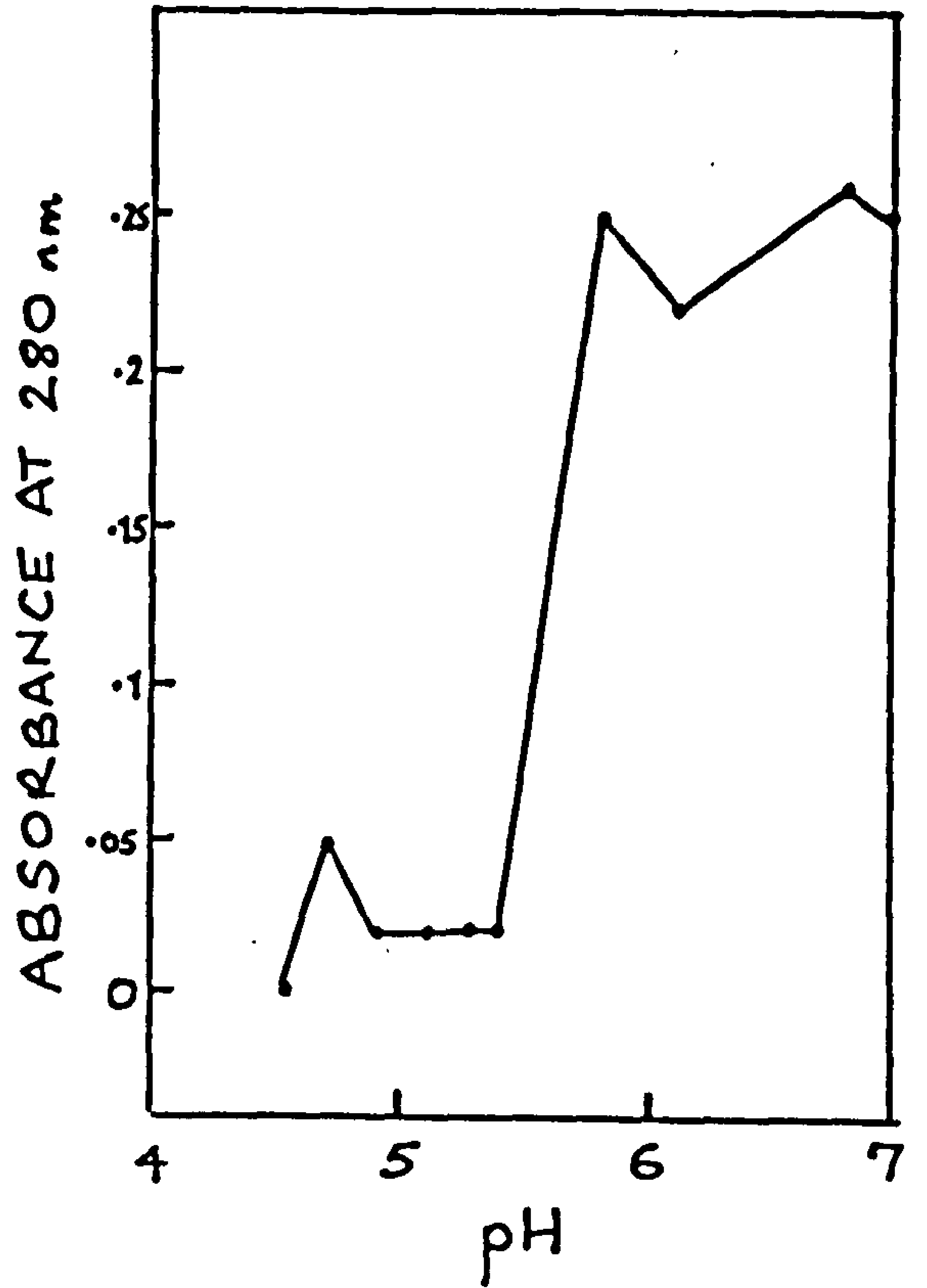
Figure 3. 20.

Electron micrographs (negatively stained) of (a) spectrin dimer; (b) purified band 1; (c) purified band 2. (Magnification X 175,000).



(c)



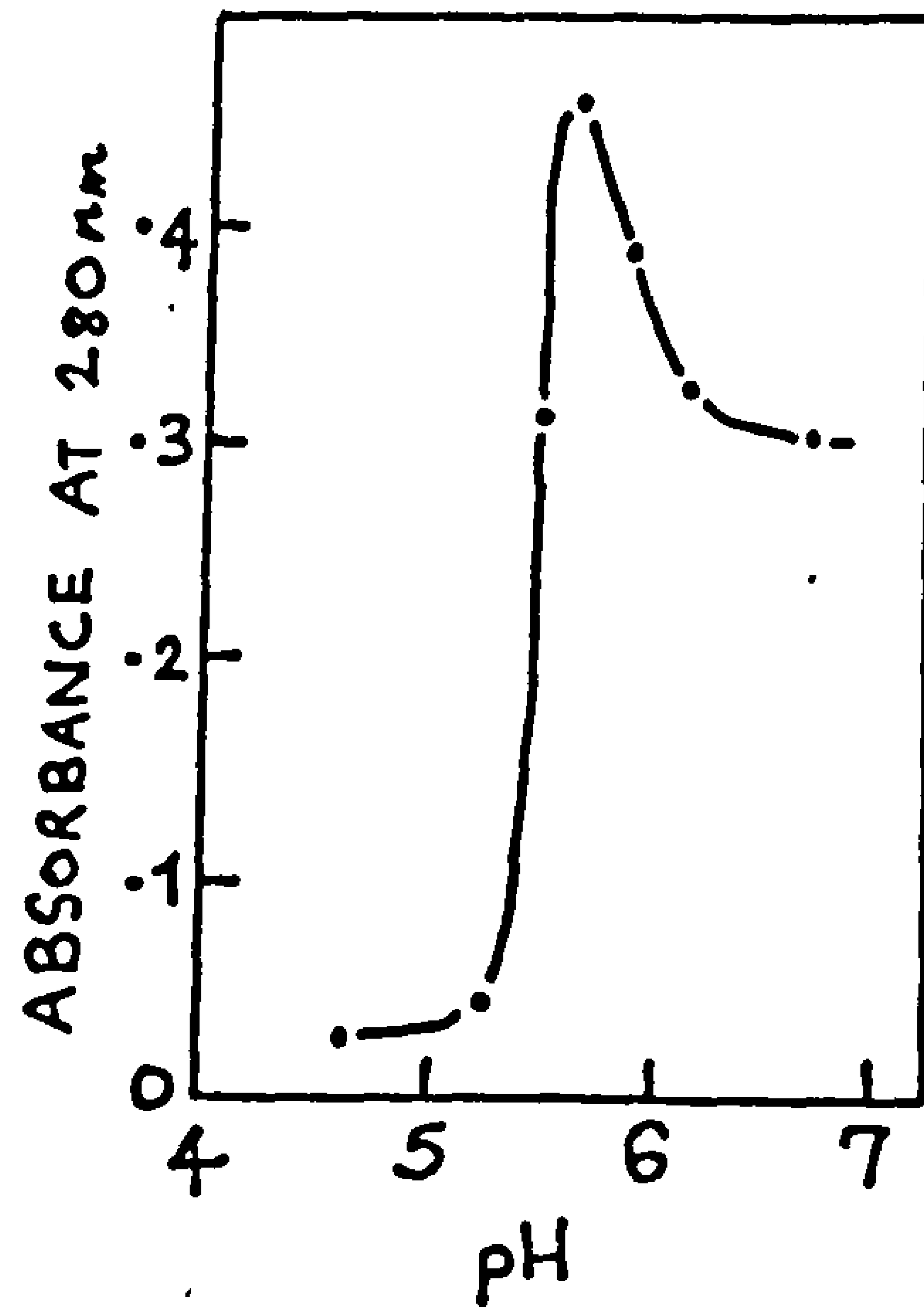




Figure 3. 21.

Precipitation curves for band 1, band 2 and spectrin dimer. The curve for dimer is taken from Gratzer and Beaven (1975).

Figure 3. 22.

The sedimentation velocity profile of band 1.

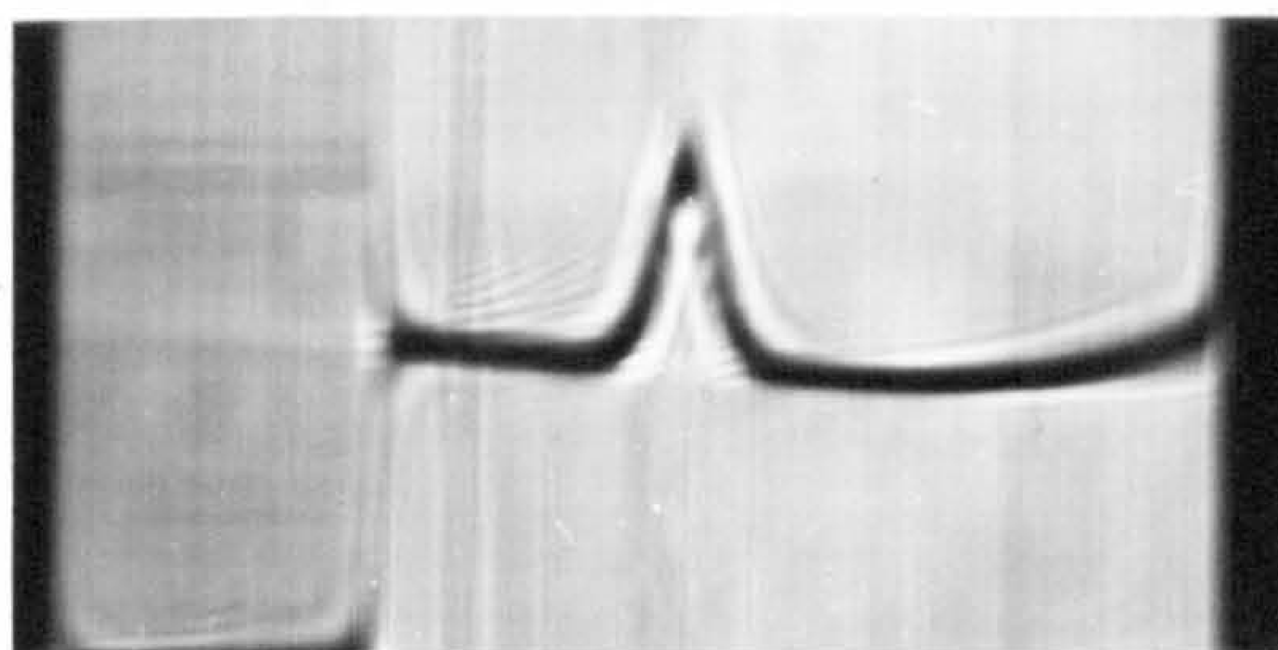


Figure 3. 23.

The near and far ultra-violet spectra of band 1.

·3

·2

·1

ABSORBANCE

210 230 250 270

WAVELENGTH nm.

·03

·02

·01

260 280 300 320

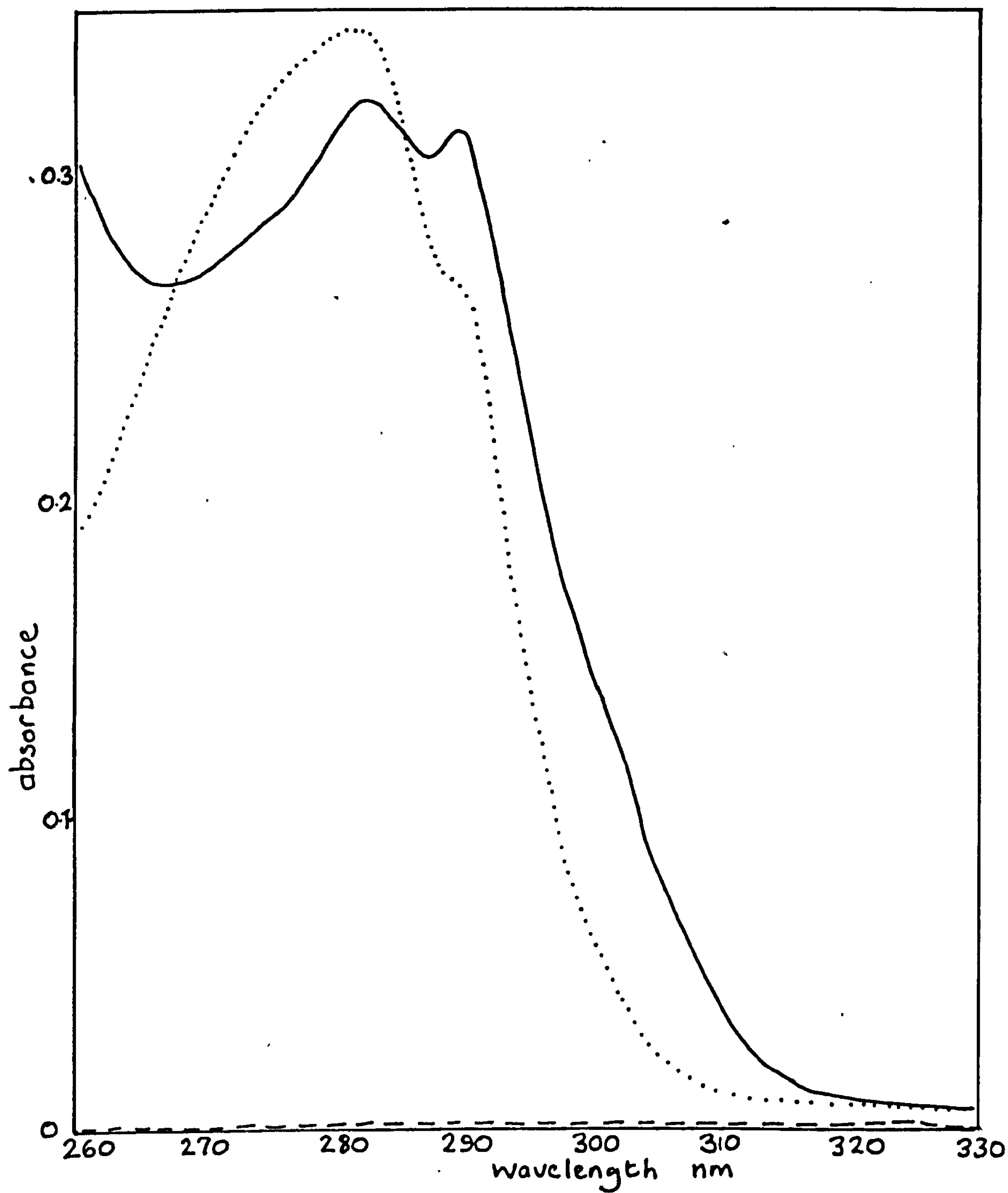
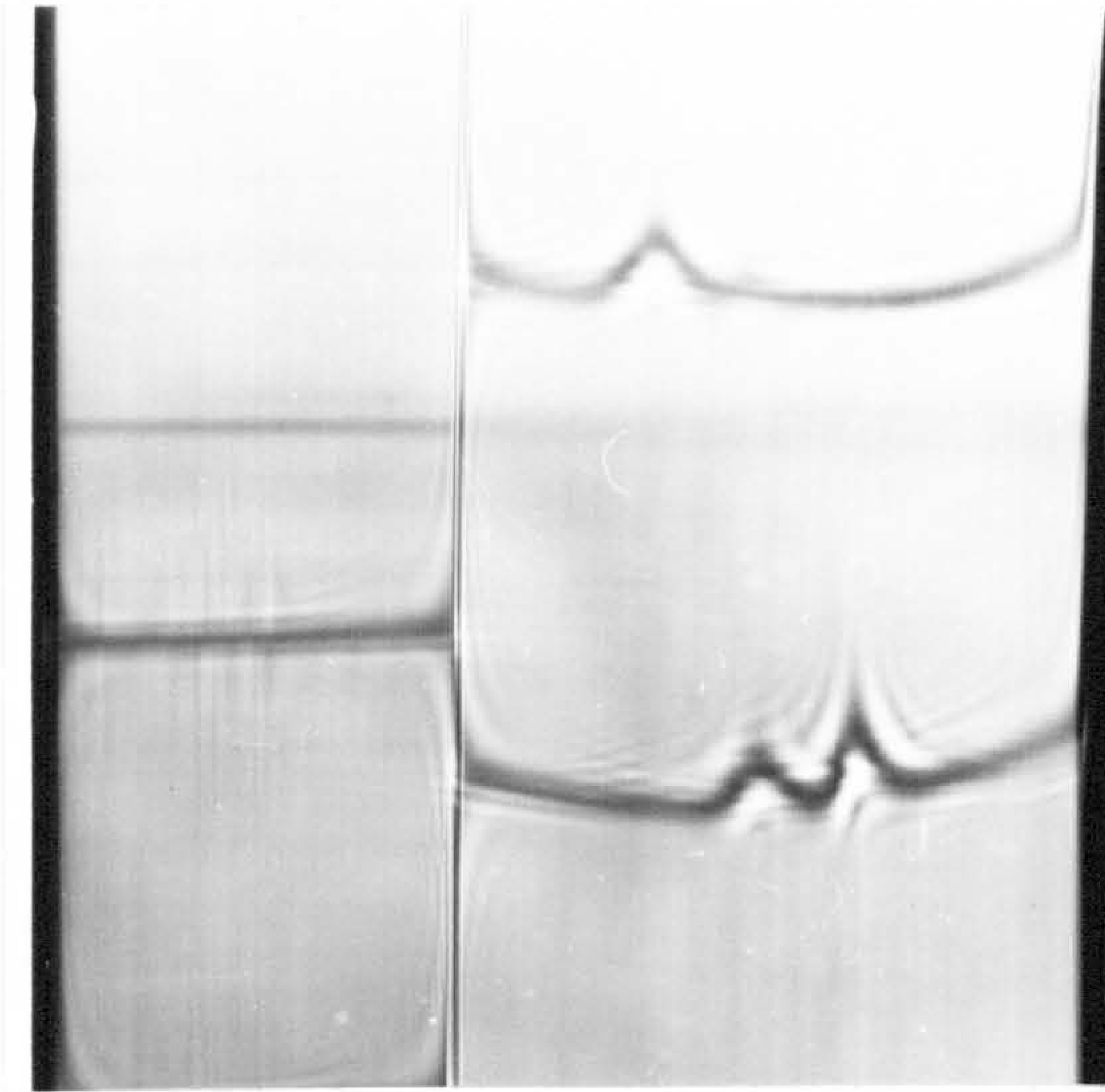
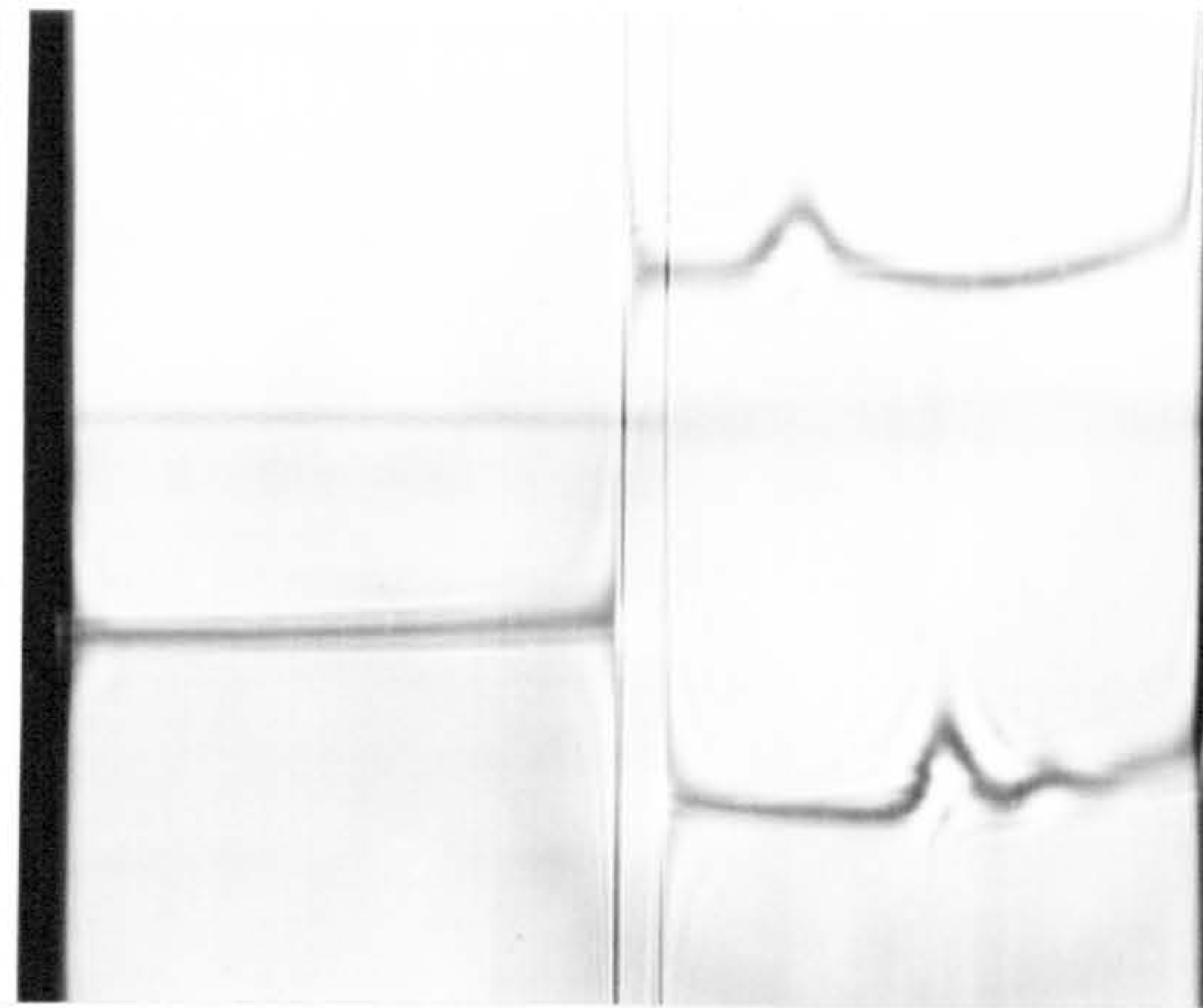



Figure 3. 24.

The near ultra-violet spectrum of spectrin dimer in the presence (———) and absence (.......) of 0·1N sodium hydroxide.



(a)



(b)

Figure 3. 25.

Sedimentation velocity profiles of band 1 (upper trace), and spectrin (lower trace) after incubation in conditions of ionic strength and temperature which favour (a) the formation of spectrin tetramer from dimer, (b) the formation of spectrin dimer from tetramer.

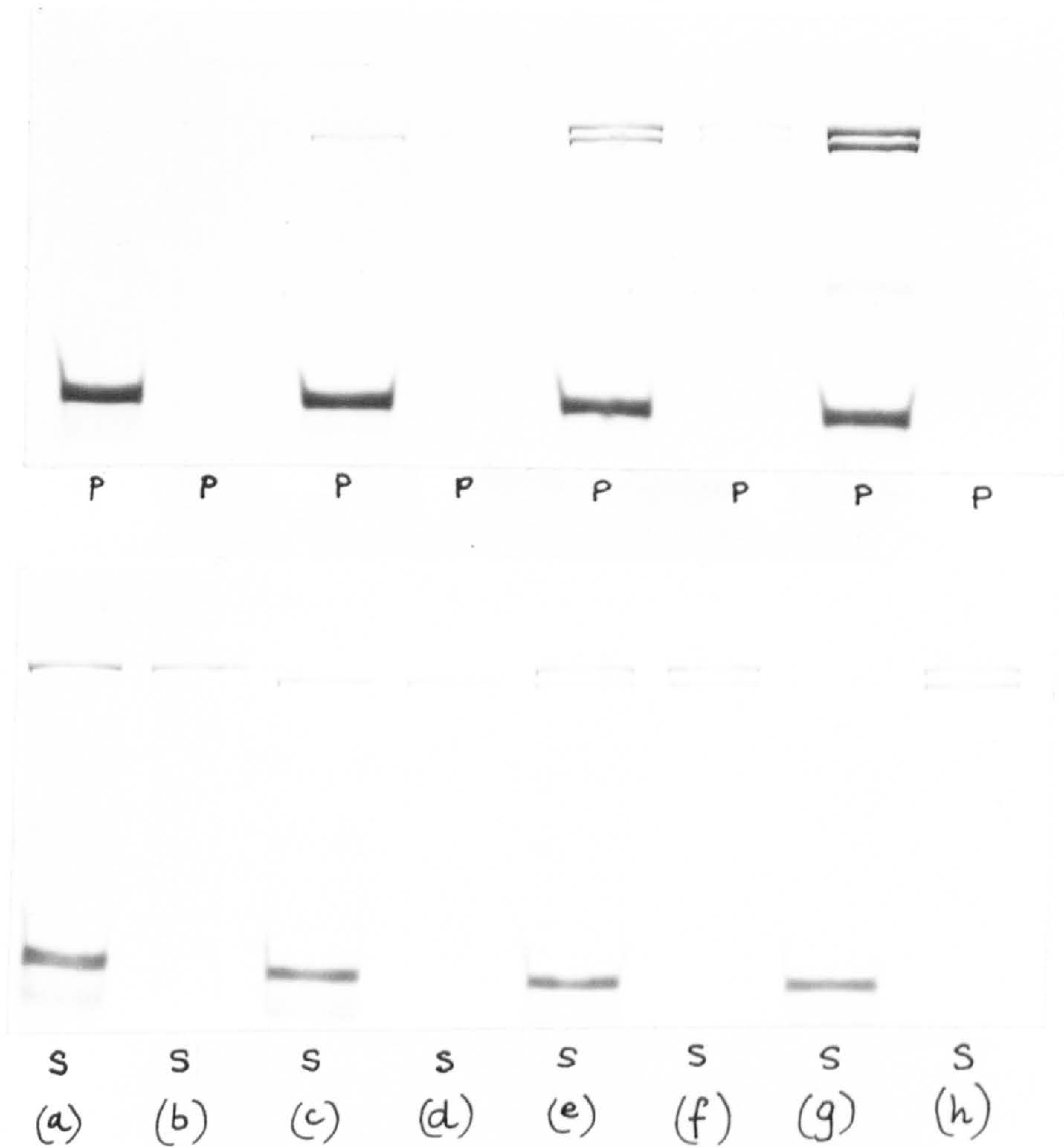



Figure 3. 26.

The binding of the spectrin subunits to 4.1 and F-actin. SDS gels of pellets (P) and supernatants (S) after centrifugation of mixtures of the proteins. Gel samples represent 20% of the pellet and 5% of the supernatant. The concentration of each spectrin subunit was 0.024 mg/ml, of dimer 0.048 mg/ml and of actin 0.18 mg/ml. The 4.1 concentration was about 0.04 mg/ml. In this experiment, almost 100% of the spectrin dimer, 35% of reconstituted dimer, 11% of band 2 and no band 1 bound to 4.1 and actin.

(a) Band 1, 4.1 and actin.
(b) Band 1 alone.
(c) Band 2, 4.1 and actin.
(d) Band 2 alone.
(e) Bands 1 and 2, 4.1 and actin.
(f) Bands 1 and 2.
(g) Spectrin dimer, 4.1 and actin.
(h) Spectrin dimer alone.

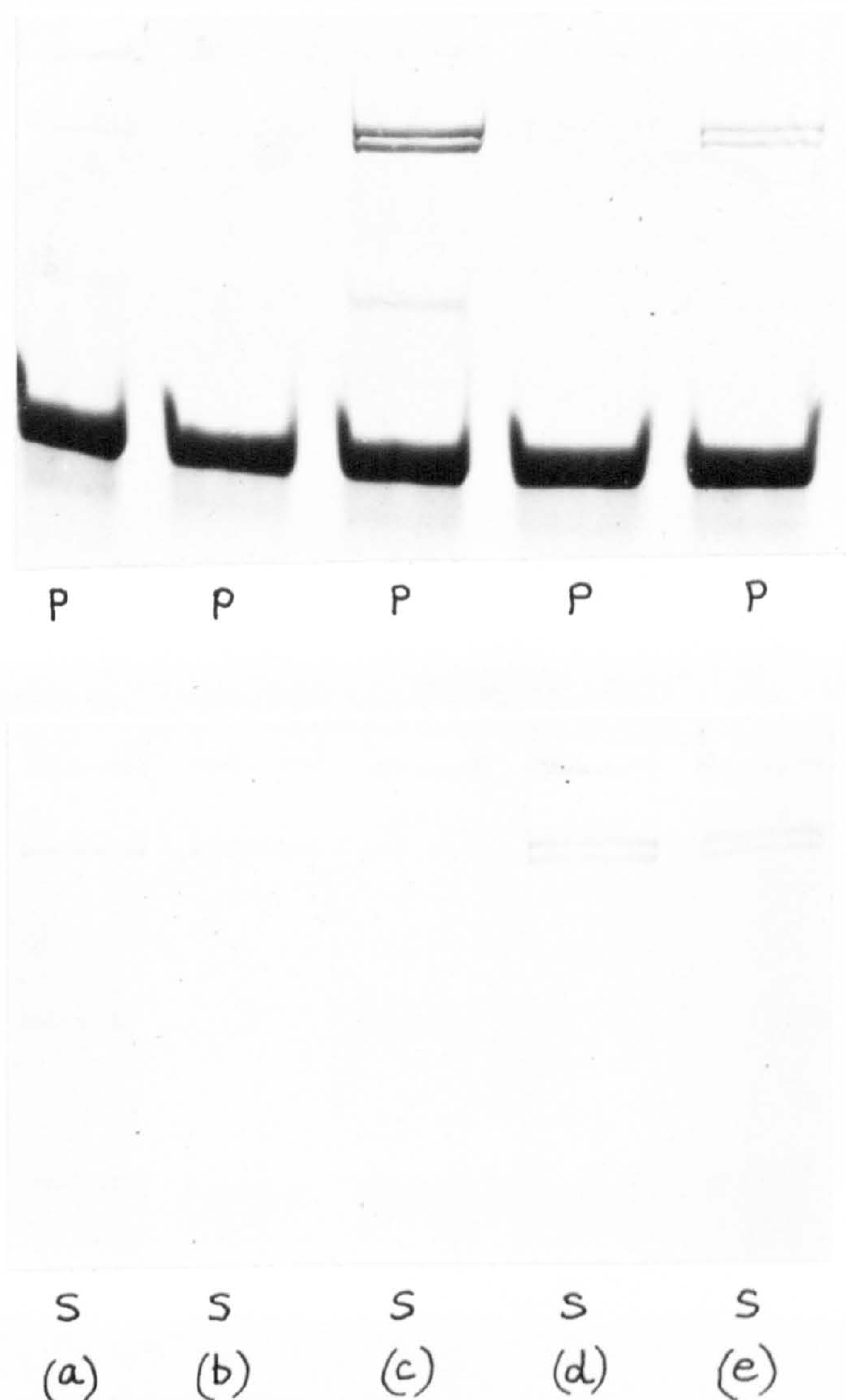
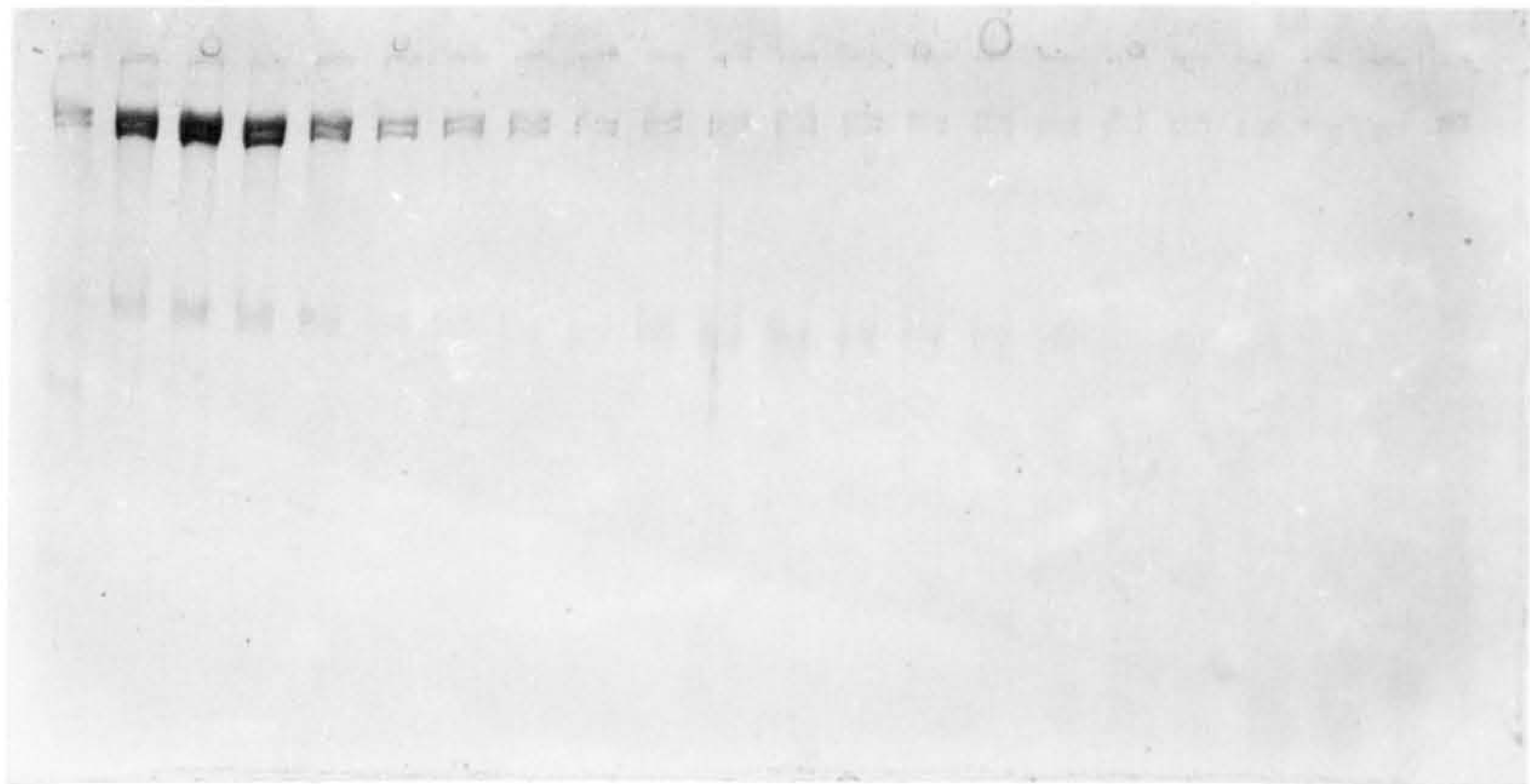



Figure 3. 27.

The binding of band 2 and spectrin dimer to 4.1 and F-actin. SDS gels of pellets (P) and supernatants (S) after centrifugation of mixtures of the proteins. Gel samples represent 20% of the pellet and 5% of the supernatant. The concentration of band 2 was 0.02 mg/ml, of spectrin dimer 0.04 mg/ml, and of actin 0.2 mg/ml. In this experiment, band 2 did not bind to 4.1 and actin.

(a) Band 2, 4.1 and actin. 0.016 mg/ml 4.1.
(b) Band 2 alone.
(c) Spectrin dimer, 4.1 and actin. 0.016 mg/ml 4.1.
(d) Spectrin dimer alone.
(e) Spectrin dimer, 4.1 and actin. 0.0016 mg/ml 4.1.

(a)



(b)

(c)

Figure 3. 28.

Polyacrylamide gels, containing SDS, of the fractions from sucrose gradients through which have been sedimented: (a) the ankyrin fragment alone; (b) a mixture of the ankyrin fragment and spectrin dimer; (c) a mixture of the ankyrin fragment and band 2. P denotes pellet. The fractions are numbered from the bottom of the gradients.

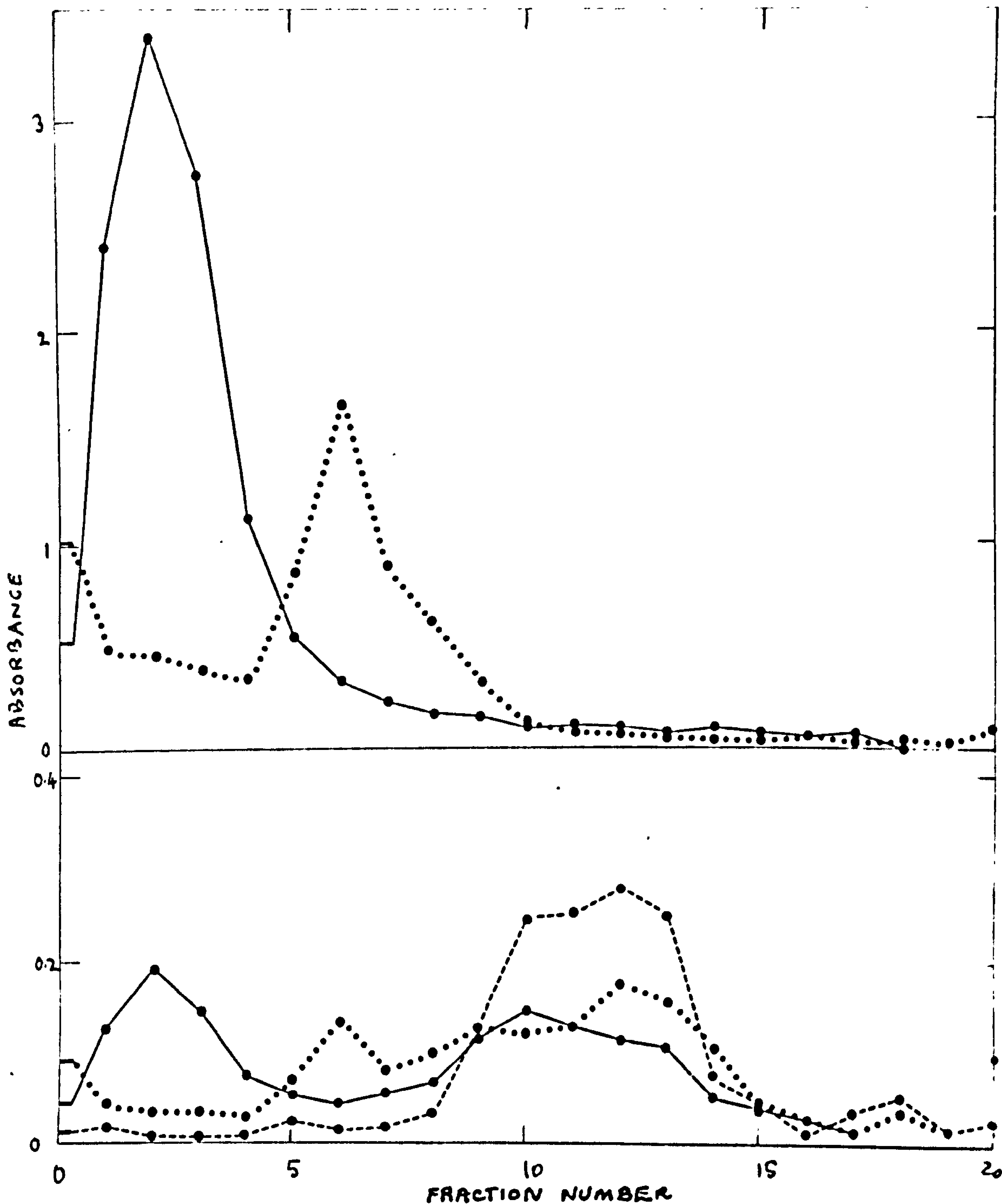



Figure 3. 29.

A graph showing the amounts of each protein in every fraction of the three sucrose gradients, as measured by densitometry. The upper panel shows the sedimentation of spectrin dimer (———), and purified band 2 (....) in the presence of the ankyrin fragment. The lower panel shows the sedimentation of the binding fragment alone (- - -), in the presence of spectrin dimer (———), and of band 2 (....). Binding is shown by the appearance of the fragment in the zone containing the dimer or band 2. Note that the fragment itself shows evidence of heterogeneity.

Figure 3. 30.

A graph showing the amount of ankyrin fragment in each fraction of two sucrose gradients, as measured by densitometry. (....)-the ankyrin fragment alone. (----)-the ankyrin fragment in the presence of band 1. Since the peak of the zone containing band 1 is in fraction 2, the distributions of the ankyrin fragment show that it does not bind to band 1.

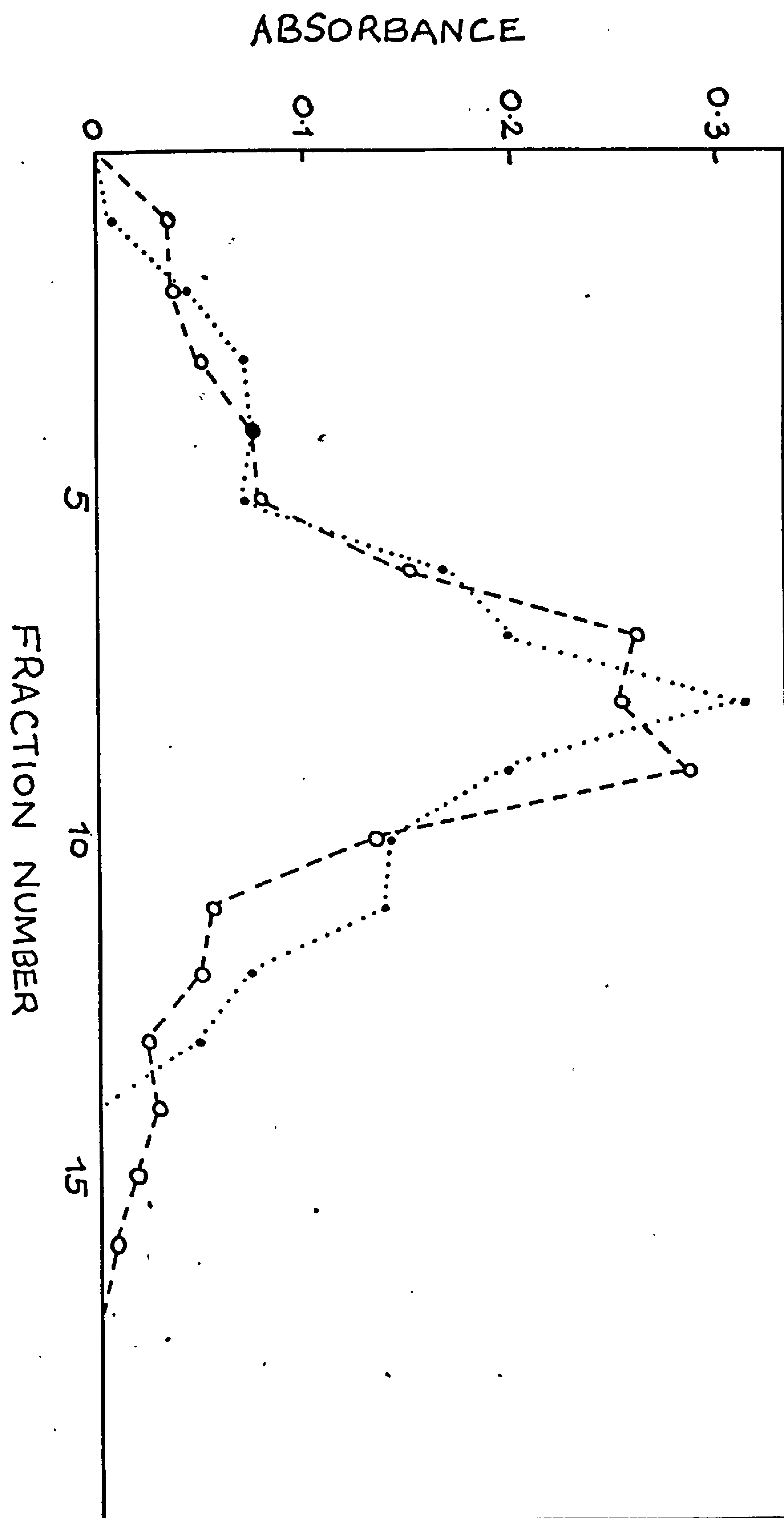

ABSORBANCE
0
0·1
0·2
0·3
5
10
15
FRACTION NUMBER

Table 3.1

A comparison between the digestion patterns of spectrin 1 (larger subunit), spectrin 2 (smaller subunit), myosin heavy chain and filamin. P(x) is the probability that the degree of similarity observed between the digestion patterns arises by chance.

| Polypeptides compared | Method: Papain digestion | Method: CNBR digestion | Assumed resolution between fragments: 0.3mm | Assumed resolution between fragments: 0.5mm | P (x) | P (x) <0.001 |
|---|---|---|---|---|---|---|
| Spectrin 1 with spectrin 2 | x | | x | | 0 | x |
| | x | | | x | 0.00001 | x |
| | | x | x | | 0.00001 | x |
| | | x | | x | 0.00046 | x |
| Spectrin 1 with myosin | x | | x | | 0.0817 | |
| | x | | | x | 0.2103 | |
| | | x | x | | 0.0382 | |
| | | x | | x | 0.1411 | |
| Spectrin with filamin | x | | x | | 0.2837 | |
| | x | | | x | 0.3010 | |
| | | x | x | | 0.0352 | |
| | | x | | x | 0.1533 | |
| Spectrin 2 with myosin | x | | x | | 0.1912 | |
| | x | | | x | 0.1359 | |
| | | x | x | | 0.0322 | |
| | | x | | x | 0.1258 | |
| Spectrin 2 with filamin | x | | x | | 0.3492 | |
| | x | | | x | 0.1612 | |
| | | x | x | | 0.0012 | x |
| | | x | | x | 0.0202 | |
| Myosin with filamin | x | | x | | 0.2006 | |
| | x | | | x | 0.1975 | |
| | | x | x | | 0.0067 | x |
| | | x | | x | 0.0607 | |

Allan, D. and Michell, R.H. (1976) Biochim.Biophys.Acta 455, 824-830.

Anderson, D.R., Davis, J.L. and Carraway, K.L. (1977) J.Biol.Chem. 252, 6617-6623.

Anderson, J.M. (1979) J.Biol.Chem. 254, 939-944.

Anderson, J.M. and Tyler, J.M. (1980) J.Biol.Chem. 255, 0000-0000.

Baker, R.F. (1967) Nature 215, 424-425.

Beaven, G.H. and Holiday, E.R. (1952) Adv. in Prot.Chem. VII. 319-386.

Bender, N., Fasold, H., Kenmoku, A., Middelhoff, G. and Volk, K-E. (1976) Eur.J.Biochem. 64, 215-218.

Bender, N., Fasold, H., Kenmoku, A., Middelhoff, G. and Volk, K-E., unpublished work.

Bennett, V. (1978) J.Biol.Chem. 253, 2292-2299.

Bennett, V. (1979) Nature 281, 597-599.

Bennett, V. and Branton, D. (1977) J.Biol.Chem. 252, 2753-2763.

Bennett, V. and Stenbuck, P.J. (1979a) J.Biol.Chem. 254, 2533-2541.

Bennett, V. and Stenbuck, P.J. (1979b) Nature 280, 468-473.

Birchmeier, W. and Singer, S.J. (1977a) J.Cell Biol. 73, 647-659.

Birchmeier, W. and Singer, S.J. (1977b) Biochem.Biophys.Res. Comm. 77, 1354-1360.

Birchmeier, W., Lanz, J.H., Winterhalter, K.H. and Conrad, M.J. (1979) J.Biol.Chem. 254, 9298-9304.

Bjerrum, O.J., Bhàkdi, S., Knüfermann, H. and Bøg-Hansen, T.C. (1974). Biochem.Biophys.Acta 373, 44-50.

Bloomfield, V., Dalton, W.O. and Van Holde, K.E. (1967) Biopolymers 5, 135-148.

Branton, D., Bullivant, S., Gilula, N., Karnovsky, M.J., Moor, H., Mühlethaler, K., Northcote, D.H., Packer, L., Satir, B., Satir, P., Speth, V., Staehlin, L.A., Steere, R.L. and Weinstein, R.S. (1975) Science 190, 54-56.

Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, R.D. (1977) Biochemistry 16, 3450-3459.

Brenner, S.H. and Korn, E.D. (1979) J.Biol.Chem. 254, 8620-8627.

Bretscher, M. (1971a) J.Mol.Biol. 58, 775-781.

Bretscher, M. (1971b) J.Mol.Biol. 59, 351-357.

Bretscher, M. (1971c) Nature New Biol. 231, 229-232.

Brotschi, E.A., Hartwig, J.H. and Stossel, T.P. (1978) J.Biol.Chem. 253, 8988-8993.

Bull, B. (1973) In: Red Cell Shape, pp. 115-124. (Bessis, M., weed, R.I. and Leblond, P.F., eds.). Springer Verlag.

Calissano, P., Monaco, G., Castellani, L., Mercanti, D. and Levi, A. (1978) Proc.Nat.Acad.Sci. U.S.A. 75, 2210-2214.

Carlsson, L., Nyström, L-E., Lindberg, U., Kannan, K.K., Cid-Dresdner, H., Lövgren, S. and Jörnvall, H. (1976) J.Mol.Biol. 105, 353-366.

Carlsson, L., Markey, F., Blikstad, I., Persson, T., and Lindberg, U. (1979) Proc.Natl.Acad.Sci. U.S.A. 76, 6 76-6380.

Castellani, L., personal communication.

Cecil, R. (1963) In: The Proteins, pp. 379-476. (Neurath, H., ed.) Academic Press.

Chen, Y-H. and Yang, J.T. (1971) Biochem.Biophys.Res.Commun. 44, 1285-1291.

Chen, Y-H., Yang, J.T. and Chau, K.H. (1974) Biochemistry 13, 3350-3359.

Clark, T.G and Merriam, R. (1978) J.Cell Biol. 77, 427-438.

Clarke, M. (1971) Biochem.Biophys.Res.Commun. 45, 1063-1070.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J.Biol.Chem. 252, 1102-1106.

Cohen, C. and Holmes, K.C. (1963) J.Mol.Biol. 6, 423-432.

Cohen, C., Szent-Gyorgyi, A.G. and Kendrick-Jones, J. (1971) J.Mol.Biol. 56, 223-237.

Cohen, C.M., Jackson, P.L. and Branton, D. (1978) J.Supramol.Struct. 9, 113-124.

Cohen, C.M. and Branton, D. (1979) Nature 279, 163-165.

Condeelis, J.S. and Taylor, D.L. (1977) J.Cell Biol. 74, 901-927.

Condeelis, J.S. (1979) J.Cell Biol. 80, 751-758.

Cooke, R. and Murdoch, L. (1973) Biochemistry 12, 3927-3932.

Cullis, P.R. and De Kruijff, B. (1979) Biochim.Biophys.Acta 559, 399-420.

Dancker, P., Löw, I., Hasselbach, W. and Wieland, Th. (1975) Biochim.Biophys.Acta 400, 407-414.

Davies, G.E. and Stark, G.R. (1970) Proc.Nat.Acad.Sci. U.S.A. 66, 651-656.

Davies, P.J.A., Bechtel, P. and Pastan, I. (1977) FEBS Letters 77, 228-232.

Davies, P.J.A., Wallach, D., Willingham, M.C. and Pastan, I. (1978) J.Biol.Chem. 253, 4036-4042.

Deuticke, B. (1968) Biochim.Biophys.Acta 163, 494-500.

Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem.Biophys. 100, 119-130.

Drabikowski, S. and Gergely, J. (1964)In: The Biochemistry of Muscle Contraction, pp. 125-134. Gergely, J., ed. Little Brown, Boston.

Dubbelman, T.M.A.R., de Bruijne, A.W. and van Steveninck, J. (1977) Biochem.Biophys.Res.Commun. 77, 811-817.

Dunbar, J.C. and Ralston, G.B. (1978) Biochim.Biophys.Acta 510, 283-291.

Dunn, M.J., McBay, W. and Maddy, A.H. (1975) Biochim.Biophys.Acta 186, 107-119.

Dunn, M.J., Kemp, R.B. and Maddy, A.H. (1978) Biochem.J. 173, 197-205.

Eaton, B.L., Kominz, D.R. and Eisenberg, E. (1975) Biochemistry 14, 2718-2742.

Ebashi, S., Endo, M. and Ohtsuki, I. (1969) Quart.Rev. Biophys. 2, 251-284.

Edidin, M. and Famborough, J. (1973) J.Cell Biol. 57, 27-37.

Edidin, M., Zagyanky, Y. and Lardner, T.J. (1976) Science 191, 466-468.

Edwards, H.E., Mueller, T.J. and Morrison, M. (1979) Science 203, 1343-1346.

Eisinger, J. and Blumberg, W.E. (1973) Biochemistry 12, 3648-3662.

Elgsaeter, A. and Branton, D. (1974) J.Cell Biol. 63, 1018-1030.

Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys.Acta 426, 101-122.

Elgsaeter, A. (1978) Biochim.Biophys.Acta 536, 235-244.

Evans, E.A., Waugh, R. and Melnik, L. (1976) Biophys.J. 16, 585-595.

Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.

Feo, C. and Mohandas, N. (1977) Nature 265, 166-168.

Fischer, T.M., Haest, C.W.M., Stöhr, M., Kamp, D. and Deuticke, B. (1978) Biochim.Biophys.Acta 510, 270-282.

Fischer, E.H., Heilmeyer, L.M.G. and Haschke, R.H. (1971) Curr.Top.Cell Regul. 4, 211-251.

Fischer, K.A. (1976) Proc.Natl.Acad.Sci. U.S.A. 73, 173-177.

Fowler, V. and Branton, D. (1977) Nature 268, 23-26.

Fowler, V. and Bennett, V. (1978) J.Supramol.Struct. 8, 215-221.

Fowler, V. and Taylor, D.L., unpublished work.

Fujii, T. and Tamura, A. (1979) J.Biochem. 86, 1345-1352.

Fuller, G.M., Boughter, J.M. and Morazzani, M. (1974) Biochemistry 13, 3036-3041.

Gabbiani, G., Chaponnier, C., Zumke, A. and Vassalli, P. (1977) Nature 269, 697-698.

Garrels, J.I. and Gibson, W. (1976) Cell 9, 793-805.

Gerritsen, W.J., Verkleij, A.J. and Van Deenen, L.L.M. (1979) Biochim.Biophys.Acta 555, 26-41.

Godfrey, J.E. and Harrington, W.F. (1970) Biochemistry 9, 886-908.

Goldman, R.D., Lazarides, E., Pollack, R. and Weber, K. (1975) Exp.Cell Res. 90, 333-344.

Graham, C., Avruch, J. and Fairbanks, G. (1976) Biochem. Biophys.Res.Commun. 72, 701-708.

Gratzer, W.B. and Beaven, G.H. (1975) Eur.J.Biochem. 58, 403-409.

Gratzer, W.B., personal communication.

Gratzer, W.B. and Beaven, G.H., personal communication.

Grazi, E. and Magri, E. (1979) FEBS Letters 104, 284-286.

Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108-4116.

Greenquist, A.C. and Shohet, S.B. (1975) In: Erythrocyte Structure and Function, pp. 515-531.(Brewer, G., ed.) Alan Liss.

Griffith, I.P. (1972) Biochem.J. 126, 553-560.

Hanson, J. (1966) Budapest Symposium on Muscle, p.99. (Ernst, E. and Straub, F.B., eds).

Hardy, B., Bensch, K.G. and Schrier, S.L. (1979) J.Cell Biol. 82, 654-663.

Hargreaves, W.R., Giedd, K.N. and Branton, D. (1979) J.Cell Biol. 83, 267a.

Harris, H.E. and Weeds, A.G. (1978) FEBS Letters 90, 84-88.

Harris, H.W. (1980) Ph.D. Thesis, Harvard University.

Hartwig, J.H. and Stossel, T.P. (1975) J.Biol.Chem. 250, 5696-5705.

Hartwig, J.H. and Stossel, T.P. (1976) J.Cell Biol. 71, 295-303.

Hartwig, J.H., Davies, W.A. and Stossel, T.P. (1977) J.Cell Biol. 75, 956-967.

Hartwig, J.H. and Stossel, T.P. (1979) J.Mol.Biol. 134, 539-553.

Heggeness, M.H., Wang, K. and Singer, S.J. (1977) Proc.Natl. Acad.Sci. U.S.A. 74, 3883-3887.

Hiller, G. and Weber, K. (1977) Nature 266, 181-183.

Hitchcock, S.E., Carlsson, L. and Lindberg, U. (1976) Cell 7, 531-542.

Hitchcock, S.E. (1977) J.Cell Biol. 74, 1-15.

Hochmuth, R.M., Worthy, P.R. and Evans, E.A. (1979) Biophys.J. 26, 101-114.

Hosey, M.M. and Tao, M. (1976a) Biochemistry 15, 1561-1568.

Hosey, M.M. and Tao, M. (1976b) Nature 263, 424-425.

Hosey, M.M. and Tao, M. (1977) Biophys.Biochim.Acta 482, 348-357.

Houk, T.W. and Ue, K. (1974) Anal.Bioch. 62, 66-74.

Hsu, C.J., Lemay, A., Eshdat, Y. and Marchesi, V.T. (1979) J.Supramol.Struct. 10, 227-239.

Hubbell, W.L. and McConnell, H.M. (1969) Proc.Natl.Acad.Sci. U.S.A. 64, 20-27.

Hudson, J.R. and Ralston, G.B. (1978) Biochim.Biophys.Acta 535, 169-177.

Hulla, F.W. and Gratzer, W.B. (1972) FEBS Letters 25, 275-278.

Hunter, T. and Garrels, J.I. (1977) Cell 12, 767-781.

Huxley, H.E. (1963) J.Mol.Biol. 7, 281-308.

Huxley, H.E. (1969) Science 164, 1356-1366.

Ji, T.H. and Nicolson, G.L. (1974) Proc.Natl.Acad.Sci. U.S.A. 71, 2212-2216.

Kam, Z., Josephs, R., Eisenberg, H. and Gratzer, W.B. (1977) Biochemistry 16, 5568-5572.

Kane, R.E. (1975) J.Cell Biol. 66, 305-315.

Kane, R.E. (1976) J.Cell Biol. 71, 704-714.

Kant, J.A. and Steck, T.L. (1973) J.Biol.Chem. 248, 8457-8464.

Katz, A.M. and Hall, E.J. (1963) Circulation Research 13, 187-198.

Kawahara, K. and Tanford, C. (1966) J.Biol.Chem. 241, 3228-3232.

Kawamura, M. and Maruyama, K. (1970) J.Biochem. 67, 437-457.

Kendrick-Jones, J., Szentkiralyi, E.M. and Szent-Györgyi, A.G. (1976) J.Mol.Biol. 104, 747-775.

Kiehm, D.J. and Ji, T.H. (1977) J.Biol.Chem. 252, 8524-8531.

Kirkpatrick, F.H., Woods, G.M., La Celle, P.L. and Weed, R.I., (1975) J.Supramol.Struct. 3, 415-425.

Kirkpatrick, F.H., Rose, D.J. and La Celle, P. (1978) Arch. Biochem.Biophys. 186, 1-8.

Klevens, H.B. and Platt, J.R. (1949) J.Chem.Phys. 17, 470-481.

Knüfermann, H., Bhakdi, S., Schmidt-Ulrich, R. and Wallach, D.F.H. (1973) Biochim.Biophys.Acta 330, 356-361.

Korn, E.D. (1978) Proc.Natl.Acad.Sci. U.S.A. 75, 588-599.

Kury, P.G., Ramwell, P.W. and McConnell, H.M. (1974) Biophys.Biochem.Res.Commun. 56, 478-483.

La Celle, P.L., Kirkpatrick, F.H., Udkow, M.P. and Arkin, B. (1973) In: Red Cell Shape, pp. 69-78. (Bessis, M., Weed, R.I. and Leblond, P.F., eds.). Springer Verlag.

Laemmli, U.K. and Favre, M. (1973) J.Mol.Biol. 80, 575-599.

Lazarides, E. and Lindberg, U. (1975) Proc.Natl.Acad.Sci. U.S.A. 71, 4742-4746.

Lazarides, E. (1976) J.Supramol.Struct. 5, 531-563.

Lee, A.G., Birdsall, N.J.M. and Metcalfe, J.C. (1973) Biochemistry 12, 1650-1659.

Lee, A.G. (1977) TIBS 2, 231-233.

Lehrer, S.S. and Kerwar, G. (1972) Biochemistry 11, 1211-1217.

Lewis, M.S., Davies, P.J.A. and Pastan, I. (1978) Biophys.J. 21, 142a.

Lin, D.C. and Lin, S. (1979) Proc.Natl.Acad.Sci. U.S.A. 76, 2345-2349.

Lin, D.C., Tobin, K.D., Grumet, M. and Lin, S. (1980) J.Cell Biol. 84, 455-460.

Lindberg, U. (1967) Biochemistry 6, 335-342.

Liu, S.C., Fairbanks, G. and Palek, J. (1977) Biochemistry 16, 4066-4074.

Liu, S.C. and Palek, J., unpublished work.

Lorand, L., Siefring, G.E. and Lowe-Krentz, L. (1978) J.Supramol.Struct. 9, 427-440.

Lowey, S. (1971) In: Subunits in Biological Systems, pp. 201-259. (Timasheff, S.N. and Fasman, G.D., eds.) Dekker.

Luna, E.J., Kidd, G.H. and Branton, D. (1979) J.Biol.Chem. 254, 2526-2532.

Lutz, H.U., Liu, S-C. and Palek, J. (1977) J.Cell Biol. 73, 548-560.

Lutz, H.U., Von Däniken, A., Semenza, G. and Bächi, T. (1979) Biochim.Biophys.Acta 552, 262-280.

Lux, S.E., John, K.M. and Karnovsky, M.J. (1976) J.Clin. Invest. 58, 955-963.

Lux, S.E. (1980) In: Haematology of Infancy and Childhood (Nathan, D. and Oski, F.A., eds.) In press.

Lux, S.E., personal communication.

Marchesi, V.T. and Palade, G.E. (1967) Proc.Natl.Acad.Sci. U.S.A. 58, 991-995.

Marchesi, V.T. and Steers, E. (1968) Science 159, 203-204.

Martin, R.E. and Ames, B.N. (1961) J.Biol.Chem. 236, 1372-1379.

Martonosi, A., Gouvea, M.A. and Gergely, J. (1960) J.Biol. Chem. 235, 1700-1703.

Maruta, H. and Korn, E.D. (1977) J.Biol.Chem. 252, 8329-8332.

Maruyama, K. and Ohashi, K. (1978) J.Biochem. 84, 1017-1019.

McDonagh, J., Messel, H., McDonagh, R.P., Murano, G. and Blombäck, B. (1972) Biochim.Biophys.Acta 257, 135-142.

Mimura, N. and Asano, A. (1979) Nature 282, 44-48.

Mohandas, N., Greenquist, A.C. and Shohet, S.B. (1978) J.Supramol.Struct. 9, 453-458.

Mombers, C., Van Dijk, P.W.M., Van Deenen, L.L.M., De Gier. J. and Verkleij, A.J. (1977) Biochim.Biophys.Acta 470, 152-160.

Mombers, C., Verkleij, A.J., De Gier, J. and Van Deenen, L.L.M. (1979) Biochim.Biophys.Acta 551, 271-281.

Morrow, J., Speicher, D., Knowles, W., Hsu, J. and Marchesi, V. (1979) J.Cell Biol. 83, 272a.

Murphy, J.R. (1965) J.Lab.Clin.Med. 65, 756-774.

Nagai, R., Yoshimoto, Y. and Kamiya, N. (1975) Proc.Japan Acad. 51, 38-43.

Nakao, N., Nakao, T. and Yamazoe, S. (1960) Nature 187, 945-946.

Nakashima, K. and Beutler, E. (1978) Proc.Natl.Acad.Sci. U.S.A. 75, 3823-3825.

Nakashima, K. and Beutler, E. (1979) Proc.Natl.Acad.Sci. U.S.A. 76, 935-939.

Nelson, M.J., Ferrell, J.E. and Huestis, W.H. (1979) Biochim.Biophys.Acta 558, 136-340.

Nicholson, G.L., Marchesi, V.T. and Singer, S.J. (1971) J.Cell Biol. 51, 265-272.

Nicolson, G.L. and Painter, R.G. (1973) J.Cell Biol. 59, 395-406.

Nigg, E.A. and Cherry, R.J. (1979) Biochemistry 18, 3457-3465.

Norberg, R., Thorstensson, R., Utter, G. and Fagraeus, A. (1979) Eur.J.Biochem. 100, 575-583.

Offer, G. In: Topics in Biochemistry, pp. 623-671 (1974) Longman.

Ohnishi, T. (1962) J.Biochem. 52, 307-308.

Oosawa, S. and Kasai, M. (1971) In: Subunits in Biological Systems, pp. 261-322. (Timasheff,S.N. and Fasman,G.D., eds.) Dekker.

Ornstein, L. (1964) Ann.New York Acad.Sci. 121, 321-349.

Pederson, D.M. and Foster, J.F. (1969) Biochemistry 8, 2357-2365.

Penniston, J.T. and Green, D.E. (1968) Arch.Biochem.Biophys. 128, 339-350.

Perry, S.V. (1955) Meth.Enzymol. 2, 582-599.

Phillips, D.R. and Morrison, M. (1970) Biochem.Biophys.Res. Commun. 48, 284-289.

Pinder, J.C. and Gratzer, W.B. (1972) Eur.J.Biochem. 26, 73-80.

Pinder, J.C., Bray, D. and Gratzer, W.B. (1975) Nature 258, 765-766.

Pinder, J.C., Tidmarsh, S. and Gratzer, W.B. (1976) Arch. Biochem. Biophys. 172, 654-660.

Pinder, J.C., Bray, D. and Gratzer, W.B. (1977) Nature 270, 752-754.

Pinder, J.C., Ungewickell, E., Bray, D. and Gratzer, W.B. (1978) J.Supramol.Struct. 8, 439-445.

Pinder, J.C., Ungewickell, E., Calvert, R., Morris, E. and Gratzer, W.B. (1979) FEBS Letters 104, 396-400.

Pinder, J.C., personal communication.

Pinto da Silva, P. and Branton, D. (1970) J.Cell Biol. 45, 598-605.

Pollard, T.D. (1975) In: Molecules and Cell Movement, pp. 257-286 (Inoue, S. and Stephens, R.E., eds). Raven Press, New York.

Pollard, T.D. (1976a) J.Cell Biol. 68, 579-601.

Pollard, T.D. (1976b) J.Supramol.Struct. 5, 317-334.

Ponder, E. (1948) Hemolysis and Related Phenomena. Grune and Stratton.

Poo, M.M. and Cone, R. (1974) Nature 247, 438-440.

Portzehl, H., Caldwell, P.C. and Rüegg, J.C. (1964) Biochim. Biophys.Acta 79, 581-591.

Potter, J.D. (1974) Arch.Biochem.Biophys. 162, 436-441.

Puszkin, S., Maimon, J. and Puszkin, E. (1978) Biochim. Biophys.Acta 513, 205-220.

Quist, E.E. and Roufogalis, B.D. (1976) Biochem.Biophys.Res. Commun. 72, 673-680.

Ralston, G.B., Dunbar, J. and White, M. (1977) Biochim. Biophys.Acta 491, 345-348.

Ralston, G.B. (1978) TIBS 3, 195-198.

Ralston, G.B. and Dunbar, J. (1979) Biochim.Biophys.Acta 579, 20-30.

Reichstein, E. and Korn, E.D. (1979) J.Biol.Chem. 254, 6174-6179.

Reisler, E. and Eisenberg, H. (1969) Biochemistry 8, 4572-4578.

Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A. and Van Deenen, L.L.M. (1976) Eur.J.Biochem. 61, 53-58.

Rosenheck, K. and Doty, P. (1961) Proc.Natl.Acad.Sci. U.S.A. 47, 1775-1785.

Rosenthal, A.S., Kregenow, F.M. and Moses, H.L. (1970) Biochim.Biophys.Acta 196, 254-262.

Rossi Fanelli, A., Antonini, E. and Caputo, A. (1964) Adv. Prot.Chem. 19, 73-222.

Rothman, J.E. and Dawidowicz, E.A. (1975) Biochemistry 14, 2809-2816.

Rousselet, A., Guthmann, C., Matricon, J., Bienvenue, A. and Devaux, P.F. (1976) Biochim.Biophys.Acta 426, 357-371.

Sato, S.B., Yanagida, M., Maruyama, K. and Ohnishi, S. (1979) Biochim. Biophys.Acta 578, 436-444.

Schechter, N., Sharp, M., Reynolds, J.A. and Tanford, C. (1976) Biochemistry 15, 1897-1904.

Schekman, R. and Singer, S.J. (1976) Proc.Natl.Acad.Sci. U.S.A. 73, 4075-4079.

Schloss, J.A. and Goldman, R.D. (1979) Proc.Natl.Acad.Sci. U.S.A. 76, 4484-4488.

Seeman, P., Cheng, D. and Iles, G.H. (1973) J.Cell Biol. 56, 519-527.

Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967) Biochem. Biophys.Res.Commun. 28, 815-820.

Sharom, F.J., Barratt, D.G. and Grant, C.W.M. (1977) Proc. Natl.Acad.Sci. U.S.A. 74, 2751-2755.

Sheetz, M.P. and Singer, S.J. (1974) Proc.Natl.Acad.Sci. U.S.A. 71, 4457-4461.

Sheetz, M.P., Painter, R.G. and Singer, S.J. (1976) Bio-

chemistry 15, 4486-4492.

Sheetz, M.P. and Singer, S.J. (1977) J.Cell Biol. 73, 638-646.

Sheetz, M.P. and Sawyer, D. (1978) J.Supramol.Struct. 8, 399-412.

Sheetz, M.P. (1979) Biochim.Biophys.Acta 557, 122-134.

Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., Pastan, I. and Lewis, M.S. (1976) J.Biol.Chem. 251, 6562-6567.

Shotton, D., Thompson, K., Wofsy, L. and Branton, D. (1978) J.Cell Biol. 76, 512-531.

Shotton, D., Burke, B. and Branton, D. (1979) J.Mol.Biol. 131, 303-329.

Siefring, G.E., Aspotol, A.B., Velasco, P.T. and Lorand, L. (1978) Biochemistry 17, 2598-2604.

Singer, S.J. (1974) Ann.Rev.Biochem. 43, 805-833.

Smith, R.M. and Alberty, R.A. (1956) J.Am.Chem.Soc. 78, 2376-2380.

Spencer, M. (1978) J.Chromatog. 166, 435-446.

Stark, G.R., Stein, W.H. and Moore, S. (1960) J.Biol.Chem. 235, 3177-3181.

Stark, G. (1967) Meth.Enzymol. 11, 590-594.

Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255-257.

Steck, T.L. (1972) J.Mol.Biol. 66, 295-305.

Steck, T.L. (1978) J.Supramol.Struct. 8, 311-324.

Stossel, T.P. and Hartwig, J.H. (1975) J.Biol.Chem. 250, 5706-5712.

Stossel, T.P. and Hartwig, J.H. (1976) J.Cell.Biol. 68, 602-619.

Strickland, E.H. (1974) Crit.Rev.Biochem. 2, 113-175.

Strzelecka-Golaszewska, H. (1973) Biochim.Biophys.Acta 310, 60-69.

Sweet, C. and Zull, J.E. (1970) Biochem.Biophys.Res.Commun. 41, 135-141.

Szent-Gyorgyi, A.G. (1975) Biophys.J. 15, 707-723.

Tanford, C., Kawahara, K. and Lapanje, S.(1967) J.Am.Chem. Soc. 89, 729-736.

Tanford, C. (1968) Adv.Prot.Chem. 23, 269-282.

Taylor, D.L. and Condeelis, J.S. (1979) Int.Rev.Cytol. 56, 57-144.

Taylor, R.B., Duffus, W.P.H., Raff, M.C. and de Petris, S. (1971) Nature New Biol. 233, 225-229.

Tillack, T.W. and Marchesi, V.T. (1970) J.Cell Biol. 45, 649-653.

Tilney, L. and Mooseker, M. (1971) Proc.Natl.Acad.Sci. U.S.A. 68, 2611-2615.

Tilney, L. (1973) J.Cell Biol. 59, 109-126.

Tilney, L.G. (1975) J.Cell Biol. 64, 289-310.

Tilney, L.G. and Detmers, P. (1975) J.Cell Biol. 66, 508-520.

Tilney, L.G. (1976a) J.Cell Biol. 69, 51-73.

Tilney, L.G. (1976b) J.Cell Biol. 69, 73-89.

Toh, B.H. and Hard, G.L. (1977) Nature 269, 695-697.

Tökes, Z.A. and Chambers, S.M. (1975) Biochim.Biophys.Acta. 389, 325-338.

Tokuyasu, K.T., Schekman, R. and Singer, S.J. (1979) J.Cell Biol. 80, 481-486.

Tombs, M.P., Soutar, F. and Maglagan, N.F. (1959) Biochem.J. 73, 167-171.

Tonomura, Y. (1972) Muscle Proteins, Muscle Contraction and Cation Transport. University of Tokyo Press.

Triplett, R.B. and Carraway, K.L. (1972) Biochemistry 11, 2897-2903.

Turner, J.D. and Rouser, G. (1970) Anal.Biochem. 38, 423-436.

Tyler, J.M., Hargreaves, W.R. and Branton, D. (1979) Proc. Natl.Acad.Sci. U.S.A. 76, 5192-5196.

Ungewickell, E. and Gratzer, W.B. (1978) Eur.J.Biochem. 88, 379-385.

Ungewickell, E., Bennett, P.M., Calvert, R., Ohanian, V. and Gratzer, W.B. (1979) Nature 280, 811-814.

Ungewickell, E., personal communication.

Van Deenen, L.L.M. and De Gier, J. (1974) In: The Red Blood Cell, pp. 147-211. (Surgenor, D., ed.) Academic Press.

Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and Van Deenen, L.L.M. (1973) Biochim. Biophys.Acta 323, 178-193.

Verleij, A.J. and Ververgaert, P.H.J.T., (1978) Biochim. Biophys.Acta 515, 303-327.

Wallach, D., Davies, P.J.A. and Pastan, I. (1978) J.Biol. Chem. 253, 3328-3335.

Wang, K., Ash, J.F. and Singer, S.J. (1975). Proc.Natl.Acad. Sci. U.S.A. 72, 4483-4486.

Wang, K. and Richards, F.M. (1974) J.Biol.Chem. 249, 8005-8018.

Wang, K. (1977) Biochemistry 16, 1857-1865.

Wang, K. and Singer, S.J. (1977) Proc.Natl.Acad.Sci. U.S.A. 74, 2021-2025.

Weed, R.I., La Celle, P.L. and Merrill, E.W. (1969) J.Clin. Invest. 48, 795-809.

Weeds, A.G. and Taylor, R.S. (1975) Nature 257, 54-56.

Wegner, A. (1976) J.Mol.Biol. 108, 139-150.

Weidekamm E. and Brdiczka, D. (1975) Biochim.Biophys.Acta 401, 51-58.

Weihing, R.R. (1976) In: Cell Motility, pp. 671-684. (Goldman, R., Pollard, T. and Rosenbaum, J., eds.) Cold Spring Harbor Laboratory.

Weinstein, R.S. (1974) In: The Red Blood Cell, pp. 214-268. (Surgenor, D., ed.). Academic Press.

Wetlaufer, D.B. (1962) Adv. in Prot.Chem. 17, 303-390.

White, M.D. and Ralston, G.B. (1976) Biochim.Biophys.Acta 436, 567-576.

Wold, F. (1967) Meth.Enzymol. 25, 623-651.

Wolfe, L.C. and Lux, S.E. (1978) J.Biol.Chem. 253, 3336-3342.

Woodrum, D.T., Rich, S.A. and Pollard, T.D. (1975) J.Cell Biol. 67, 231-237.

Wu C-S.C. and Yang, J.T. (1976) Biochemistry 15, 3007-3019.

Yin, H. and Stossel, T.P. (1979) Nature 281, 583-586.

Yu, J., Fischman, D. A. and Steck, T.L. (1973) J.Supramol. Struct. 1, 233-247.

Yu, J. and Steck, T.L. (1975) J.Biol.Chem. 250, 9176-9184.

Yu, J. and Goodman, S.R. (1979) Proc.Natl.Acad.Sci. U.S.A. 76, 2340-2344.

Yutani, A., Yutani, K. and Isemura, T. (1969) J.Biochem. 65, 201-208.

Zeece, M.G., Robson, R.M. and Bechtel, P.J. (1979) Biochim. Biophys.Acta 581, 365-370.

Zwaal, R.F.A., Comfurius, P. and Van Deenen, L.L.M. (1977) Nature 268, 358-360.

Zweig, S. and Singer, S.J. (1979a) J.Cell Biol. 80, 487-491.

Zweig, S. and Singer, S.J. (1979b) Biochem.Biophys.Res. Commun. 88, 1147-1152.