# STRUCTURAL STUDIES ON HUMAN ERYTHROCYTE SPECTRIN

by

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## ABSTRACT

Spectrin, the major structural protein of the human erythrocyte membrane, has been characterised by electron microscopy after low angle shadowing. In its simplest form, spectrin exists as a heterodimer composed of two polypeptide chains, Band 1 (240, 000 $M_r$) and Band 2 (220,000 $M_r$). When visualised in the electron microscope, the heterodimers appeared as long flexible molecules of mean length 97nm. The two constituent polypeptide chains were observed to lie in parallel and to run the entire length of the molecule. Heterodimers associate "head to head" without measurable overlap to form tetramers of mean length 194 nm. Band 1 has been selectively eluted from erthrocyte ghosts in 6M urea, suggesting that the membrane attachment occurs predominantly on the Band 2 polypeptide. Four hyrbidoma cell lines, which secrete monoclonal antibodies against spectrin, have been developed by fusion of spleen cells from immunized mice with myeloma cell lines. Three of these cell lines have been shown to secrete IgG antibodies, whereas the fourth secretes an IgM. The location of the binding sites, on spectrin, of the monospecific IgGs has been determined by using them to directly label the individual spectrin subunits, after separation on analytical SDS/polyacrylamide gels and transfer onto nitrocellulose filters. All three IgGs were found to recognise antigenic determinants on Band 1 only. By labelling tryptic peptides of spectrin separated on SDS gels it has been demonstrated that at least two of the IgGs bind to different sites on the Band 1 polypeptide, the relative locations of which have been determined. Two of the IgGs have been purified from ascites fluid by ion exchange chromatography on DEAE cellulose, and complexes of these antibodies with spectrin dimers and tetramers have been examined in the electron microscope after low angle shadowing. One of these antibodies

(718/a) appears to bind close to the proximal terminus ('head') of the spectrin dimer involved in tetramer formation, while the second (717) binds about 20-30nm from this same terminus. Because spectrin has been implicated in the disease hereditary spherocytosis (HS), a comparative study has been made between normal erythrocytes and those from HS patients : particular emphasis has been placed on the electron microscopic appearance of the cell membranes after freeze etching, on their protein content and on the ease of extraction of spectrin from their membranes. In six out of eight patients so far examined, membrane abnormalities have been apparent, but no consistent pattern has yet emerged confirming the suggestion that several distinct haemolytic anaemias make up the syndrome described as hereditary spherocytosis.

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ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| BSA | Bovine Serum albumen |
| BSS | Basic salt solution |
| CFA | Complete Freunds Adjuvant |
| D.MEM | Dulbecco's Modified Eagle Medium |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra acetate (disodium) |
| FCS | Foetal Calf Serum |
| FMMP | Formyl methionyl sulphone methyl phosphate |
| HAT | Hypoxanthine, Aminopterin, Thymidine |
| HS | Hereditary spherocytosis |
| IFA | Incomplete Freunds Adjuvant |
| NCS | New born Calf Serum |
| NP-40 | Nonidet P-40 |
| PBS | Phosphate buffered saline |
| PEG | Polyethylene glycol |
| RAM | Rabbit anti mouse IgG |
| SDS | Sodium dodecyl sulphate |
| SP2/0 | SP2/0-Ag-14 myeloma cell line |
| TEMED | NNN'N' tetramethyl ethylene diamine |
| Tris | 2 hydroxymethyl, 2 amino propan-1,3-diol |
| X-63 | X-63-Ag-8 myeloma cell line |
| 20pB | 20 milliosmolar phosphate buffer (Na) pH 7.6 |
| 310pB | 310 milliosmolar phosphate buffer (Na) pH 7.6 |

# CHAPTER I

## INTRODUCTION

### 1. The Development and Role of The Human Erythrocyte

With the exception of some lymphoid cells, the origins of all human blood cells may be traced to a single cell type in the haematopoietic tissue of the bone marrow. Upon suitable stimulation, this 'pluripotent' stem cell will differentiate to give rise to either erythroid or myeloid cell lines. Under the influence of the hormone erythropoietin the stem cell will undergo erythroid development (Keele and Neill, 1971).

The earliest erythroid cell which can be identified in the bone marrow is the proerythroblast. This is a large nucleated cell of diameter 15-20μm, which although capable of dividing without differentiating further, apparently does so only under stress conditions. In the presence of vitamin $B_{12}$ and folic acid, the proerythroblast undergoes three days of development, involving repeated mitosis, to form initially normoblast type cells and finally reticulocytes (Keele and Neill, 1971). During the normoblast period, much of the protein found in the mature erythrocyte, including haemoglobin, is synthesised (Harns and Kellermeyer, 1974). Maturation to the reticulocyte is characterised by a gradual reduction in cell diameter to 7-10μm and finally by extrusion of the nucleus. After this latter event, the cell may be released into the peripheral circulation. The reticulocyte phase lasts a further four days, during which time the cell adopts its characteristic

biconcave appearance and all remaining intracellular membranes are lost. Consequently for the whole of its 120 day life span the red cell must rely entirely upon glycolysis to satisfy its limited energy requirements. In this respect, the erthrocyte is unique among mammalian cells.

The major function of the human erythrocyte is the carriage of oxygen in the form of oxyhaemoglobin from the lungs to the peripheral tissues where it is required, and of carbon dioxide in the form of bicarbonate ($HCO_3^-$), from the tissues to the lungs. Cellular sequestration of haemoglobin confers two important benefits. Firstly, it provides an environment which protects the haemoglobin from oxidative damage. Secondly, it allows the concentration of haemoglobin to be sufficiently high to meet the stringent metabolic requirements of mammalian cells without causing the blood to be intolerably viscous. In fact, whole blood, containing 45% erythrocytes (100ml of packed cells contains about 35g of haemoglobin) has only twice the viscosity of plasma (Keele and Neill, 1971).

Allison (1960) estimated that during the lifespan of a single erythrocyte it will go through the cycle of oxygenation/deoxygenation about 160,000 times. It will consequently make about 320,000 passages through microcapillaries, many of which are only 3μm in diameter (Weed and LaCelle, 1969), less than half that of the erythrocyte. To withstand such continual stress, the erythrocyte plasma membrane must be both highly deformable and durable. This has been ably demonstrated by Weed and LaCelle (1969) who showed that a fresh erythrocyte could be drawn, without damage, into a micropipette only 2.85μm in diameter by a negative pressure of only 4mm of water. On this basis alone it may be appreciated that the red blood cell plasma membrane is a highly specialised structure, and certainly not simply a 'bag' for the containment of haemoglobin.

## 2. The Red Blood Cell in Membrane Research

For several years the erythrocyte has been a preferred cell type for the study of biomembranes. The reasons for this are quite straightforward. Firstly, large quantities of erthrocytes, human or otherwise, may be obtained very easily. Secondly, the mammalian erythrocyte contains no intracellular membrances, and as such enables one, with very little effort, to prepare completely pure plasma membrane preparations. Such purity is a prerequisite for biochemical and structural investigations.

These advantages, however, must be tempered by consideration of certain disadvantages. From the previous section it will be appreciated that although the erythrocyte is a relatively simple cell, it is nevertheless very highly specialized. Much of this specalization being reflected in the organization of its membrane.

Haemoglobin free membranes of erythrocytes produced by hypotonic lysis are normally referred to as 'ghosts'. A most striking feature of these ghosts is that they faithfully retain the shape of the original cell, despite their total lack of cytoplasm. Hence, the cell shape, at least under normal circumstances is determined by the membrane alone, which must therefore be regarded as being rather atypical of plasma membranes in general.

A problem may also arise from the fact that erythocyte preparations are heterogeneous with respect to age, containing cells from one day old to 120 days old. Very little is known about the aging process in erythrocytes, although it has been noted that there is a gradual loss of surface carbohydrate including sialic acid (Cohen et al., 1976; Baxter and Beeley, 1978) and also of $K^{\oplus}$, acetylcholinesterase, 2,3, diphosphoglycerate, adenosine triphosphate, cholesterol and phospholipids (Cohen et al. 1976). It may therefore be necessary in many investigations to fractionate young and old cells.

Danon and Marikovsky (1964) and Murphy (1973) have found that this may be accomplished by centrifugation since aging of erythrocytes is also accompanied by an increase in the mean cell density.

However, with these reservations in mind, the assertion of Bretscher (1973) that the general principles of membrane structure may be elicited from the red cell, still remains valid today. Certainly, the erythrocyte membrane will very likely be the first in which the molecular architecture is known in satisfactory detail.

## 3. Membrane Composition and General Organization

### a) Introduction

The modern view of membrane organization which originated in the fluid mosaic model of Singer and Nicolson (1972), has, as a cornerstone proposals made half a century earlier by Gorter and Grendel (1925) and Danielli and Davson (1935) that membrane lipid is organized in a continuous bilayer. These early models, however, were unable to cope with the disposition of membrane protein and failed to acknowledge the dynamic nature of biomembranes.

Without any previous knowledge of membrane structure and composition, it is possible to predict that the red blood cell membrane (and almost any other biomembrane) must be assymmetrical, at least with respect to some of its components. The membrane separates two compartments of quite different chemical composition. Maintenance of such a difference by a semipermeable membrane would not be possible unless the membrane itself possessed some form of polarity. It is now known that all the major structural components of the membrane are arranged assymmetrically (reviewed by Bretscher, 1973; Rothman and Lennard, 1977).

b) Carbohydrates

The two major components of the human erythrocyte membrane are lipid and protein, which account for approximately 40% and 52% of the membrane mass respectively. (Dodge et al., 1963; Rosenberg and Guidotti, 1968). The balance of about 8% consists of carbohydrate, most of which is linked to protein. A small quantity, about 8% of the total carbohydrate,is found in glycolipids (Winzler, 1969; Sweeley and Dawson, 1969). All carbohydrates located on the red cell membrane are found to be restricted to the outer surface of the cell (Steck, 1972a). This, in view of the large quantities of sialic acid present, endows the cell with a net negative charge.

c) Lipids of the Erthrocyte Membrane

The predominant forms of lipid present in the membrane are phospholipids and unesterified cholesterol. These are present in nearly equal proportions, the cholesterol phospholipid ratio being about 0.8:1 (Turner and Rouser, 1970). Glycolipids represent only a minor population (Sweeley and Dawson, 1969). The phospholipid species and their proportions are reproduced, in Table 1.1, from Turner and Rouser (1970).

TABLE 1.1 Major phospholipid Species of the erythrocyte membrane

| Phospholipid | | Percentage of Total |
|---|---|---|
| phosphatidyl Ethanolamine | (PE) | 27 |
| phosphatidyl choline | (PC) | 28 |
| sphingomyelin | (Sph) | 25 |
| phosphatidyl serine | (PS) | 14 |
| phosphatidic acid | (PA) | 2 |
| phosphatidyl inositol | (PI) | 1 |
| Lysophosphatidyl choline | (LPC) | 1 |

The phospholipids and cholesterol are organized into a continuous bilayer with their polar head groups oriented at the surface of the membrane and in contact with the aqueous medium while the acyl 'tails' form a hydrophobic phase at the bilayer core. This has been demonstrated by both X-ray diffraction (Wilkins et al., 1971) and by electron spin resonance spectroscopy using amphiphlic spin labels (Hubbell and McConnell, 1969). Cholesterol is buried in the bilayer, intercalated between the lipid tails, possibly hydrogen bonded by its β3 hydroxyl group to the carbonyl oxygen of the fatty acyl groups of the phospholipids (Huang, 1977). The role of cholesterol in the membrane appears to be to abolish the sharp, /temperature dependent gel to liquid crystalline phase transition which phospholipids undergo, by interfering with the packing of the hydrocarbon tails (Oldfield and Chapman, 1971). Rothman and Engelman (1972) have suggested that cholesterol may also have the effect of equalising the differences between fatty acids of different chain lengths and saturation.

d) Disposition of Membrane phospholipid

Phospholipids are not randomly distributed across the bilayer. Bretscher (1972) showed that when intact red cells were treated with formyl methionyl sulphone methyl phosphate (FMMP) containing $^{35}S$, only a small proportion of the amino phospholipids (PE and PS) were labelled. However, if leaky ghosts, in which the cytoplasmic surface of the membrane was accessible, were treated with this reagent PE and PS were readily labelled, indicating that these two lipids are found mainly on the cytoplasmic surface of the membrane.

Likewise it seems that choline phospholipids (PC and Sph) are found predominantly in the outer half of the bilayer as demonstrated by Turner et al.

(1958) and Ibrahim and Thompson (1965) using phospholipases from various snake venoms on intact cells and ghosts. Cholesterol is found on both sides of the bilayer (Blau and Bittman, 1978) but is possibly more abundant on the outer half (Fisher, 1976).

While being able to diffuse and rotate relatively freely in the plane of the membrane, phospholipids are not readily able to 'flip-flop' across the bilayer. In symmetrical model membranes, the half life of phospholipds on one side of the bilayer may be measured in days (Kornberg and McConnell, 1971) although in intact red cells this may be reduced to about 7 hours (Rousselet et al., 1976). It has been suggested that this increase in the translocations rate may be due in part to the asymmetry lipid distribution across the cell membrane since De Kruijff and Baker (1978) have demonstrated that in assymetric vesicles, the half life on one side of the bilayer may be as little as 30-40 minutes. Proteins also affect the rate of transbilayer exchange. De Kruijff et al. (1978) have shown that glycophorin, a major red cell protein, may enhance the rate of 'flip-flop', at least in model membranes, by up to two orders of magnitude. In the red cell membrane, another protein, spectrin, may act to stabilise the lipid asymmetry, since oxidative damage to this molecule results in an accelerated translocation rate (Haest et al. 1978).

Clearly it is thermodynamically unfavourable to 'flip' a polar head group across the hydrophobic domain of the bilayer. However, as 'flip-flop' does occur at a slow but finite rate there must be a mechanism to maintain lipid asymmetry in biological membranes; most likely by returning lipids to their correct side. Unfortunately at present, there are few details of how such a mechanism might operate.

As a final comment on membrane lipid asymmetry, Cullis and Grathwohl (1977) have found that membrane proteins may induce asymmetry in the plane of the bilayer. Some proteins, it seems, may associate preferentially with particular phospholipid species, thereby reducing their random distributions.

e) Proteins of The Red Cell Membrane

The application of sodium dodecyl sulphate polyacrylamide gel electrophoresis to the study of the human erythrocyte, gave a remarkably simple picture of the protein components of the plasma membrane (Fairbanks et al., 1971). When gels were stained for protein with coomassie blue, only about ten major bands were apparent. On staining for carbohydrate using the periodic acid-Schiff (PAS) method, only three major bands could be seen, none of which were found to correspond to bands which stained with coomassie blue.

These PAS bands are normally referred to as PAS 1, 2 and 3 in order of decreasing molecular weight. PAS 1 and 2 correspond to the major sialoglycoprotein, glycophorin A. PAS 1 is in fact a dimer of PAS 2 not dissociated by SDS (Marton and Garvin, 1973). PAS 3 is a minor glycoprotein - glycophorin B (Furthmayr, 1978a). Further details of these components (molecular weight etc.) is given in Table 1.2 (reproduced from Lux and Glader, 1980). Nomenclature of the bands is that of Steck (1974).

f) Disposition of Membrane proteins

Bender et al. (1971) found that if intact cells were treated with pronase, a broad spectrum proteolytic enzyme, only protein running in the band 3 region of gels underwent extensive proteolysis (large amounts of sialic acid were released at the same time). Furthermore, they found that the most prominent proteolytic fragment, although still membrane bound, was available for labelleling by sulphanilic acid diazonium salt, a membrane impermeable reagent. When they treated leaky ghosts with pronase all the membrance protein underwent extensive degradation. The inference was that only a limited amount of protein was exposed at the external surface of the cell.

Steck et al. (1971) performed a similar series of experiments,

TABLE 1.2 Major Erythrocyte Membrane Proteins

| Band No. | Mol. Wt. | Identification | Approx proportion % | Polypeptides per ghost | Oligomeric state (where known) | Oligomers per ghost |
|---|---|---|---|---|---|---|
| 1 | 240000 | Spectrin | 25 | 200000 | Tetramer | 100000 |
| 2 | 220000 | | | 200000 | | |
| 2.1 | 200000 | Ankyrin (Syndein) | 5 | 100000 | Monomer | 100000 |
| 3 | 93000 | Anion Exchange Protein | 25 | 1000000 | Dimer | 500000 |
| 4.1 | 78000 | – | 4-5 | 200000 | Monomer | 200000 |
| 4.2 | 72000 | - | 4-5 | 200000 | | |
| 5. | 43000 | Actin | 4-5 | 400000 | >Monomer? | <200000?† |
| 6 | 35000 | G3PD* | 4-5 | 500000 | Tetramer | 120000 |
| 7 | 29000 | - | 4-5 | 500000 | | |
| 8 | 23000 | - | 1 | 150000 | | |
| GP-A (PAS-2) | 31000 | Glycophorin A | 1-2 | 400000 | Dimer ? | 200000? |
| GP-B | 24000 | Glycophorin B | 0.5 | 100000 | Dimer ? | 50000 |

* G3PD = glyceraldehyde-3-phosphate dehydrogenase

† Oligomeric state of Actin is a matter of some controversy

using a selection of proteolytic enzymes on resealed ghosts and on inside and right side out vesicles derived from red cell ghosts. They unfortunately misinterpreted their original data. However, on subsequent clarification (Kant and Steck, 1972; Steck 1972a) they were able to show that only PAS staining bands and band 3 could be degraded when sealed right side out vesicles were used, whereas when leaky ghosts or inside out vesicles were used, all the proteins were degraded. The use of resealed vesicles had the advantage that only one surface at a time was exposed to proteolytic enzymes. Thus, from these results, it was possible to conclude that the PAS proteins and band 3 spanned the membrane, all other proteins being located on the inner surface.

These results were in excellent agreement with those obtained by chemical labelling studies (Hubbard and Cohn, 1972; Bretscher 1971a; Phillips and Morrison, 1970). Bretscher (1971b, 1971c) used FMMP to label both intact cells and ghosts, and then showed by peptide mapping of bands cut from gels, that both band 3 and glycophorin showed different patterns of of labelling in the two situations. This showed conclusively that different portions of both these proteins were exposed at the two surfaces of the membrane.

These experiments indicate quite clearly that there is gross asymmetry in the distribution of protein across the red cell membrane. About 70% is found on the cytoplasmic /side of the bilayer. This preponderance of material on the inner cell surface has recently been demonstrated, by X-ray and neutron scattering, on regularly stacked ghosts, cross linked at their external surfaces with poly (L-Lysine) (McCaughan and Krimm, 1980).

Such polarity of the membrane becomes quite obvious when erythrocytes are examined by freeze-etch electron microscopy. In this method of specimen preparation, membranes are cleaved along the centre of the bilayer, thereby

exposing the hydrophobic core, and any proteins which penetrate the membrane. These show up in a freeze-etch replica as small particles, about 8.5nm in diameter, on the smooth lipid fracture face. (For a review of the fracture process, and evidence for intra membrane splitting, see Branton, 1971; Branton and Deamer, 1972. For interpretation of results, see Branton and Kirchanski, 1977). Pinto da Silva and Branton (1970) noted that when red blood cell ghosts were fractured, most of the 8.5nm intra membrane particles made visible were located on the protoplasmic fracture face (PF-nomenclature of Branton et al., 1975). Only a small population of particles was located on the external fracture face (EF , Branton et al., 1975). This particle asymmetry is illustrated in Figure 1.1 and will be discussed in more detail in subsequent sections. The true surfaces of the membrane may be visualised by lowering the level of the surrounding ice by sublimation (etching). When this is done, a similar asymmetry of surface material is seen (Figure 1.1) in agreement with the chemical evidence demonstrating protein asymmetry reviewed above.

It is apparent from these studies that the proteins of the red cell membrane fall into two major classes according to how they associate with the lipid bilayer; whether they span the bilayer or whether they are simply attached at one surface. Those proteins which span the bilayer, (in the red cell membrane these are band 3 and glycophorin), are normally referred to as integral proteins (Singer and Nicholson, 1972), although if generalized to other cell types, one would also classify as integral, proteins which penetrate the hydrophobic core of the bilayer but which may not be exposed at both membrane surfaces. Inevitably, proteins which fall into this category contain large hydrophobic domains, are water insoluble and are usually extremely difficult to remove from the membrane without actually dispersing the lipid bilayer. The remaining proteins, which do not appear to penetrate the bilayer and which have a less tenacious association

Figure 1.1

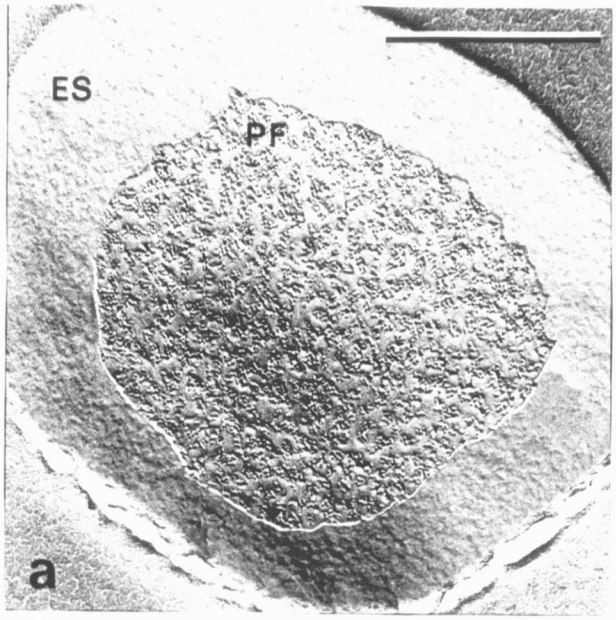
(a) Erythrocyte ghost

(b) Inside out veshicle derived from an erythrocyte ghost.

Both specimens were freeze fractured and then etched for 30s at -100°C.

Note the unequal distribution of particles between the external and protoplasmic fracture faces (EF and PF). The true protoplasmic surface (PS) appears to be much more textured than the true external surface (ES), indicating the presence of more protein material attached to the hydrophilic surface of the lipid bilayer.

Magnification: x 54,000, Bar = 0.5μm



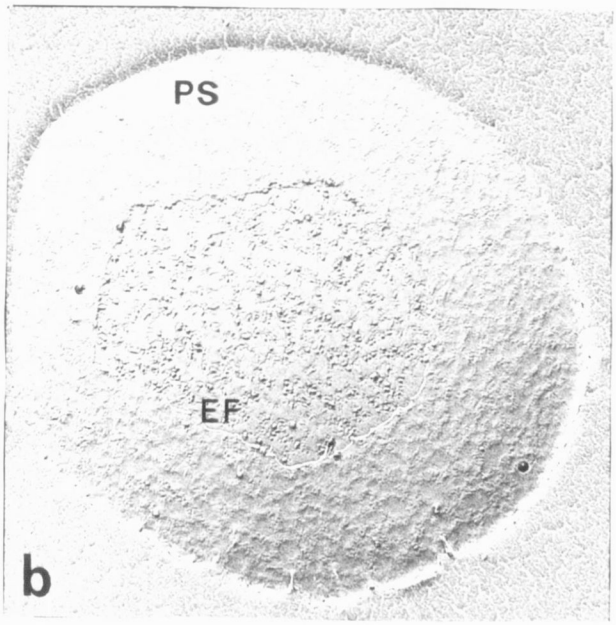




FIGURE 1.1

with it are termed peripheral or extrinsic proteins. In the red blood cell, all the peripheral proteins are found on the cytoplasmic surface.

As will become clear in later sections, at least 50% of the peripheral proteins of the red cell membrane are engaged in a structural role and constitute a protein meshwork forming the submembrane cytoskeleton; a structure which is intimately involved in the maintenance of the cell's normal biconcave shape and which bestows upon the cell membrane its remarkable visco-elastic properties. It has been well established that this protein network makes extensive contacts with the integral proteins, which appear in freeze-etch replicas as the intra membrane particles, and is able to influence their motions in the plane of the membrane. It is these interactions between the various membrane proteins which I now intend to discuss at greater length.

## 4. The Integral Membrane proteins of the Red Blood Cell

### a) Glycophorin

Glycophorin is the best characterised of all the proteins of the red cell membrane. The complete amino acid sequence has been determined (Tomita and Marchesi, 1975) and it shows the molecule to be divided into three functional domains. The N terminal region, which is located at the external surface of the membrane is very polar and contains a large quantity of carbohydrate which accounts for 60% of the molecular mass. This carbohydrate consists of sixteen oligosaccharide units attached to the protein at serine or threonine residues (and at one asparagine). A polar C terminal domain is located on the cytoplasmic surface of the membrane and is separated from the glycosylated N terminal region by a highly hydrophobic sequence of 23 amino acids which is just long enough to span the membrane as a single $\alpha$ helix.

The function of glycophorin is uncertain. Individuals have been known which completely lack glycophorin and suffer no ill effects. The only consequence being that their erythrocytes carry only 50% of the normal amount of sialic acid and do not express the MN blood group antigens. (Tanner and Anstee, 1976; Tanner et al., 1976; Gahmberg et al., 1978; Furth Mayr, 1978b).

b) Band 3

Band 3 runs as a diffuse, heterogeneous (Rose, 1976) glycoprotein band on SDS gels, its leading edge migrating at a molecular weight of 93,000 daltons. The major component is an anion exchange protein, responsible for the physiologically important membrane permeability to $HCO^-_3$ ions and the associated 'chloride shift'. (Ho and Guidotti, 1975; Rothstein et al., 1975; Cabantchik and Rothstein, 1974). Minor components in the band 3 region have been identified as $Na^+/K^+$ATPase (Avruch and Fairbanks, 1972), and acetylcholinesterase (Nidday et al., 1977). Steck (1972b) and more recently, Yu and Steck (1975) have provided evidence that band 3 exists in the membrane as a non-covalently linked dimer. More detailed structural studies (reviewed by Steck, 1978) have shown that unlike glycophorin, the N terminus of band 3 (a 38,000 dalton fragment) is on the cytoplasmic surface, while a 17,000 dalton fragment crosses the membrane, probably several times (Drickamer, 1977). The C terminal domain, carrying all the carbohydrate is located on the outer surface (Steck et al. 1976).

Band 3 is thought to be the major component of the 85Å intra membrane particles exposed by freeze etching on the P face (Yu and Branton, 1976). It has also been suggested that the small population of E face particles may be predominantly glycophorin (Edwards et al., 1979) although this has yet to be confirmed. The preferential partitioning of band 3 suggests a very strong association with the inner half of the bilayer, and possibly with material underlying it.

c) Lateral Mobility of the Integral Proteins

Both band 3 and glycophorin are able to undergo translational motion in the plane of the bilayer. This was demonstrated by Fowler and Branton (1977) who labelled both of these proteins, in intact cells, with fluorescein. After fusing these labelled cells, using Sendai virus or polyethylene glycol, with unlabelled cells, they found that the hybrids gradually became uniformly fluorescent, indicating that the labelled proteins were able to redistribute themselves in the plane of the membrane. However, the diffusion coefficient which they estimated for this process was in the order of $10^{-12}$-$10^{-11}$ $cm^2/s$ (at $23^oC$), which is about two orders of magnitude less than that calculated for rhodopsin (at $20^oC$) in retinal rod cells by Poo and Cone (1974), suggesting that there were some constraints acting on the integral proteins of the red cell membrane. This is also reflected in the rotational diffusion of band 3 about an axis perpendicular to the plane of the membrane. Cherry et al. (1976) measured the relaxation time for fluorescence depolarization of eosin labelled band 3 to be about 0.5ms. Again this differs by a factor of about 200 from the 20μs obtained for the relaxation time of retinal rhodopsin (Cone, 1972).

5. The Peripheral Membrane Proteins

a) Introduction

Yu et al. (1973) showed that if ghosts were dispersed in solutions containing the detergent Triton X-100, most of the membrane lipids and integral proteins were solubilized, leaving behind a residue consisting mainly of bands 1,2,5, 4.1 and variably 2.1. Electron micrographs of these residues showed a dense anastomosing network of filamentous material

(Hainfield and Steck, 1977; Sheetz, 1979a,b; Sheetz and Sawyer, 1978). It is this residue which constitutes the submembrane cytoskeleton. That these skeletons are intimately involved in control of cell shape is quite clear from the fact that they always retain the shape of the ghosts from which they are prepared (Lux et al., 1976; Lux and John, 1977; Lux, 1979). The submembrane cytoskeleton and its components will be discussed in more detail in the following sections.

The peripheral proteins which are not associated with these Triton residues are bands 4.2, 6 and 7. Band 6 has been identified as a glycolytic enzyme, glyceraldehyde 3 phosphate dehydrogenase (Tanner and Gray, 1971). Yu and Steck (1975) showed that its binding site is on the cytoplasmic domain of band 3 from which it is easily dissociated by 155mM sodium chloride or 1 mM NADH (one of its products). Since it is not associated with membranes prepared by isotonic lysis, its presence on ghosts prepared by hypotonic lysis may be fortuitous, although its ability to bind in a saturable manner as a tetramer to a cytoplasmic site on band 3 is also shared by aldolase, another glycolytic enzyme (Strapazon and Steck, 1976).

The functions of bands 4.2 and band 7 are unknown. Like band 6, band 4.2 has its binding site on the N terminal domain of band 3 (Yu and Steck, 1975). Its association, however, is considerably stronger, requiring protein perturbants to displace it (Steck and Yu, 1973). Band 7 is also very tightly associated with the membrane and can only be solubilised with detergent (Yu et al. 1973), indicating that it might penetrate the bilayer to some extent.

b) Bands 1 and 2 - 'Spectrin'

'Spectrin' was the name given to a high molecular weight protein by Marchesi et al. (1969) which was found in low ionic strength extracts from human red cell ghosts (Marchesi and Steers, 1968). They found that

this protein accounted for about 20% of the total membrane protein. Both Marchesi et al. (1969) and Juliano et al. (1971) concluded that spectrin must have an important structural or membrane stabilizing role since its extraction resulted in intense fragmentation of the ghosts. Nicholson et al. (1971) confirmed, by immunoferritin labelling, that the protein had a close association with the inner surface of the erythrocyte ghosts a result subsequently confirmed on whole cells by Ziparo et al. (1978).

Mazia and Ruby (1968) independently extracted a similar low ionic strength fraction from bovine ghosts which they originally called 'Tektin' (the name has since been dropped in favour of Spectrin). Clarke (1971) provided a partial characterization of spectrin, the dominant species in this extract. She found that it consisted of two very large polypeptides, bands 1 and 2, of molecular weight approximately 240,000 and 220,000 daltons respectively, which were present in equimolar amounts. Cross linking studies suggested that the molecule, at least in its simplest form, was a 460,000 dalton $\alpha$-$\beta$ heterodimer, containing one copy of band 1 and one of band 2. Similar conclusions were arrived at by Hulla and Gratzer (1972), Steck (1972b) and by Aizawa et al. (1977). Clarke (1971) also noted that spectrin was unusual in that it was soluble in 80% ethanol, suggesting the presence of hydrophobic regions, although its water solubility is high.

A great deal of confusion arose out of early protein chemical investigations on spectrin. Dunn and Maddy (1973) suggested that the two large subunits of spectrin were themselves composed of a number of much smaller polypeptide chains whose associations were not disrupted by SDS. This has since been shown not to be the case (Hulla, 1974; Pinder et al., 1976; Dunn et al., 1978), but merely reflects the susceptibility of spectrin to digestion by endopeptidases, and to the fact that the protein may absorb to some gel chromatography resins. Several other groups (Knuffermann et al., 1973; Fuller et al., 1974) reported the

existence of multiple N termini for spectrin. Again, however, this can almost certainly be ascribed to protease activity. Hudson and Ralston (1978) used metal catalysed transamination to demonstrate that there was only one N terminus per 250,000 daltons of protein.

More recently, Dunn et al. (1978) and Anderson (1979) have provided evidence from peptide maps, that the two chains, bands 1 and 2, have significant sequence homology. In fact Fuller et al. (1974) showed that both bands 1 and 2 have similar amino acid compositions. The amino acid composition of whole spectrin, determined by Marchesi et al. (1969), Fuller et al. (1974) and Shotton et al. (1979) indicates a large number of acidic residues consistent with spectrin's low isoelectric point of pH 4.8 (Clarke, 1971; Elgsaeter and Branton, 1974).

From the results of partial tryptic digests, Anderson (1979) has demonstrated that one or both terminal regions of band 2, in the native protein is folded into a series of trypsin resistant domains. These domains have an apparent molecular weight of about 8,000 daltons. However, the trypsin sensitive regions between these domains seem to be available only at low ionic strength. At physiological salt concentrations, a completely different fragmentation pattern is seen.

Physical and structural studies on spectrin will be reviewed in subsequent sections.

c) Band 5

The only other major species in low ionic strength extracts from erythrocyte ghosts is band 5 (Elgsaeter and Branton, 1974). This has since been shown to be erythrocyte actin (Tilney and Detmers, 1975; Sheetz et al., 1976). Erythrocyte actin appears to be very similar in all respects to muscle actin, in its ability to form double helical filaments which may be decorated with heavy meromyosin (Tilney and Detmers, 1975) and to stimulate the myosin ATPase (Sheetz et al., 1976).

Iso-electric focussing indicates that the erythrocyte contains predominantly β actin, the form normally associated with non muscle cells (Nakashima and Beutler, 1979). Tilney and Detmers (1975) found no evidence for the existence of F action in the intact red cell.

d) Band 4.1

Pinder et al. (1975) were the first to demonstrate an in vitro association between spectrin and actin (rabbit skeletal muscle). They showed that a crude spectrin extract, containing spectrin, actin and a small amount of band 4.1, was able to stimulate the polymerisation of actin under conditions where it would normally exist in the G form. They later found that these mixtures were able to form a gel, (Pinder et al., 1977), indicating cross linking of the actin filaments. A similar stimulated polymerisation of actin was found by Cohen et al. (1978) using inside out vesicles derived from erythrocyte ghosts. When examined in the electron microscope, actin filaments appeared to radiate out from these vesicles. The polarity of the filaments, as shown by decoration with heavy meromyorin was the opposite of those extending from muscle Z bands and from microvilli (Mooreber and Tilney, 1975) indicating that the membrane was merely presenting nucleation sites with growth at the distal ends of the filaments.

Attempts to repeat these experiments using spectrin purified by gel filtration chromatography on sepharose 4B were unsuccessful. (Pinder et al., 1979, Cohen and Branton, 1979). Only in the presence of band 4.1 was polymerisation and gel formation found to occur (Pinder et al., 1979, Fowler and Taylor, 1980). This clearly pointed to the existence of ternary complexes between spectrin, actin and 4.1. The obvious conclusion was that band 4.1 mediated interactions between actin and spectrin which could provide the basis for the assembly of a cytoskeleton and that these

interactions in vitro were mimicking those which occurred in the red cell membrane. However, Brenner and Korn (1979) have recently provided evidence that purified spectrin can interact directly with F actin although it cannot stimulate the polymerisation of G actin..

## 6. Association betwen Cytosketal and Integral Proteins

A most important question to be answered is how the cytoskeleton interacts with the lipid bilayer and integral membrane proteins. Elgsaeter and Branton (1974) provided the first indication that spectrin could influence the mobility of integral membrane proteins, in the plane of the membrane. They found that pH induced aggregation of the intra membrane particles, visualised by freeze etching, would only occur following removal of spectrin and actin from ghosts (pre-treatment). Elgsaeter et al. (1976) later demonstrated that such particle aggregation only occurred under conditions which would normally cause spectrin in solution to precipitate. They proposed that aggregation was caused by a two dimensional micro precipitation of the residual spectrin on the membrane and suggested that under normal circumstances, spectrin and actin form a meshwork beneath the membrane which limits the mobility of the integral proteins. This concept was further strengthened by the fact that intra membrane particles in crude band 3 reconstituted phospholipid vesicles could be made to aggregate under standard conditions, provided that crude spectrin-actin was first added to the system. (Yu and Branton, 1976). Final confirmation for such an association between spectrin and intra membrane particles was provided by Shotton et al. (1978) who labelled pretreated ghosts with ferritin conjugated anti spectrin and were able to show that the intramembrane particles and spectrin co-aggregated.

Bennett and Branton (1977) found that spectrin exhibits saturable, high affinity binding to inside out, but not right side out spectrin depleted vesicles. This was shown to be diminished if the vesicles were pretreated with low concentrations of either trypsin (Bennett and Branton, 1977) or chymotrypsin (Bennett, 1978). This loss of binding was accompanied by the appearance in solution of a 72,000 dalton fragment, which itself was able to inhibit binding of spectrin to non digested vesicles (Bennett, 1978) and was therefore presumed to contain the membrane attachment site for spectrin. Fowler and Bennett (1978) subsequently showed that incubation of leaky ghosts with this 72,000 dalton fragment caused a controlled dissociation of spectrin from the membrane and a concomitant increase in mobility of fluorescein isothiocynate labelled integral membrane proteins (70% of label was on band 3 and glycophosin A).

The origin of this 72,000 fragment has since been shown by immunochemical techniques (Bennettand Stenbuck, 1979) and by peptide mapping (Luna et al., 1979; Yu and Goodman, 1979).to be band 2.1. Sheetz (1979) has recently provided evidence that as well as binding to spectrin, 2.1 also binds to band 3, thereby providing a link between the cytoskeleton and the integral proteins. In view of its role, Bennettand Stenbuck (1979) have suggested the name 'ankyrin' for band 2.1.

## 7. Control of Cytoskeletal Functions

The question of how the various interactions of the proteins within the cytoskeleton is controlled, is at present both controversial and highly confusing. There is little doubt that ATP depletion causes erythrocytes to become echinocytic, less deformable (Weed et al., 1979, Weed and LaCelle, 1969) to accumulate intracellular calcium and

to show diminished spectrin extractability (Lux et al., 1978). Ghosts derived from such cells exhibit the same abnormal shape and decreased deformability, but may be returned to a normal state by incubation with Mg-ATP (Weed et al., 1969; Sheetz and Singer, 1977; Birchmeier and Singer, 1977). Sheetz and Singer (1977) noted that the return to normal could be accellerated by incubating the ghosts with divalent (not Fab) anti spectrin indicating the involvement of an increase in cross linking of the cytoskeleton.

Band 2 of spectrin is known to be multiply phosphorylated at several sites located within a terminal 8,000 dalton domain (Anderson, 1979). This phosphorylation is brought about by a cyclic AMP independent membrane bound spectrin kinase (Avruch and Fairbanks, 1974; Avruch et al., 1976; Fairbanks et al., 1976; Fairbanks et al., 1978; Hosey and Tao, 1977a, 1977b, 1977c) and is reversed by a phosphoproteinphosphatase (Fairbanks et al., 1976, Graham et al. 1977). However, it is now clear that this phosphorylation/dephosphorylation equilibrium cannot be correlated with the ATP reversible shape changes, (Anderson and Tyler, 1980) nor with any of the interactions which spectrin is known to undergo with other membrane proteins (Anderson and Tyler, 1980). Whatever mechanisms are involved in these ATP dependent shape changes, they do not involve large scale rearrangements of the cytoskeleton. Ziparo et al., (1978) showed that echinocytic processes on cells appeared to contain a normal distribution of spectrin. Lorand et al., (1979) have reported a calcium activated transglutaminase to be present in the membrane. This enzyme catalyses the formation of $\gamma$ glutamyl- $\varepsilon$lysine cross bridges producing membrane stiffening. However, all available evidence suggests that this process is irreversible and is more likely to contribute to the overall aging of erythrocytes rather than to these reversible, short term shape changes.

## 8. Spectrin Structure

### (a) Oligomeric States

As a basis for any complete understanding of how spectrin functions in the erythrocyte membrane, detailed information regarding shape and size of the molecule is obviously of prime importance. Many workers including Marchesi et al. (1969), Tilney and Detmers (1975), Kirkpatrick (1976) and Shotton (personal communication) have shown by negative staining that spectrin is capable of forming aperiodic fibrous aggregates, especially in the presence of divalent cations. These results suggest that spectrin molecules are elongated and are able to interact extensively with one another, but provide little in the way of specific molecular details.

The most important recent contribution to our understanding of spectrin structure has been provided by Ralston and his co-workers (Ralston, 1975; Ralston, 1976a; Ralston et al., 1977) who have shown that spectrin can be extracted from ghosts as a mixture of stable oligomers. The 460,000 dalton $\alpha$-$\beta$ heterodimer is the predominant species when spectrin is extracted at 37°C, whereas in 4°C extracts a 920,000 dalton $\alpha_2$-$\beta_2$ tetramer is found, consisting of two copies of band 1 and two of band 2. These two forms may be resolved by gel filtration chromatography on sepharose 4B at physiological ionic strength. At low ionic strength the two species appear as a single peak eluting at the void volume of the column. At the high salt concentration the heterodimer and tetramer exhibit sedimentation coefficients of 9S and 12S respectively. These values explain the differences in sedimentation coefficient observed between the spectrin preparations of Ralston (1975) and Gratzer and Beaven (1975) who used low temperature and high temperature extraction protocols respectively.

Ralston et al. (1977) noted that the tetramer would dissociate to the heterodimer during incubation of pure samples at 37°C. Ungewickell and Gratzer (1978) later showed that this dissociation is reversible, and that interconversion between the two oligomeric states only occurs above 29°C, obeying simple equilibrium thermodynamics. At low ionic strength the equilibrium strongly favours dimer formation. These findings suggest that it is the tetramer which is the normal association state of spectrin on the membrane since it is the major species found in 4°C extracts. Presumably in vivo, the tetramer is somehow stabilized. This conclusion is supported by the recent results of Ji et al. (1980) and Liu and Palek (1980) which indicate that at least some of the spectrin is present on the membrane in the form of the tetramer.

(b) Structural Studies on Spectrin

The most detailed structural investigations on spectrin so far undertaken have been confined to hydrodynamic and light scattering studies. These have lead to a number of conflicting deductions concerning the shape and conformation of the spectrin molecule (reviewed by Marchesi, 1979). The essential details of these investigations is reproduced in Table 1.3 and will be discussed fully in later chapters.

(c) Purpose of these investigations

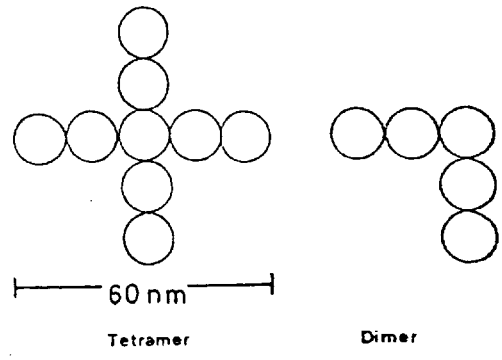
In an attempt to clarify this bewildering situation, I have undertaken a detailed structural study of spectrin, in collaboration with my supervisor, David M. Shotton, employing unidirectional and rotary low angle shadowing to allow direct visualization of spectrin dimers and tetramers by electron microscopy. As an extention of this investigation I have initiated a project design to map the spectrin

Table 1.3 Physical Studies of Isolated Spectrin

(adapted from Marchesi, 1979)

| Source | Conclusion |
|---|---|
| Clark, 1971 | Axial Ratio 45:1 |
| Schechter et al., 1976 | Hydrodynamic studies carried out in deoxycholate<br>$S_{20W}$ Monomer 5.55<br>$S_{20W}$ Dimer 6.05<br>Molecular weight of dimer ∿500 000<br>Calculated dimensions assuming cylindrical Rod<br>Monomer 2.4 x 83nm<br>Dimer 2.4 x 166nm<br>Spectrin considered to be Myosin like. |
| Ralston, 1976b | $S_{20W}$ of Heterodimer 8.55) under physiological<br>$S_{20W}$ of Tetramers 12.05) conditions<br>Molecular weight of Tetramer ∿1 000 000<br>Hydrodynamic properties interpreted as being due to a highly expanded conformation, but nevertheless symmetrical, with a Stokes radius of 20nm. Not Myosin like. |
| Kam et al., 1977 | $S_{20W}$ Heterodimer 9.55) under physiological<br>$S_{20W}$ Tetramer 12.55) conditions<br>Molecular weight of dimer from hydrodynamic and Light Scattering Studies ∿ 480 000<br>Radius of gyration of dimer, by light scattering ≲ 8 nm<br>Tetramer and Dimer only moderately Asymmetric.<br>Dimers associate side by side to form Tetramer.<br>Molecule is not Myosin like. |
| Elgsaeter, 1978<br>Mikkelsen, and Elgsaeter, 1978 | Light Scattering and electro-birefringence study.<br>Contour length of dimer from electro-optic study calculated to be not less than 40-50nm<br>Contour Length of dimer from classical Light Scattering calculated as 140 nm.<br>Radius of Gyration 40nm.<br>Conclusion: Heterodimers are long very flexible rods |

Table 1.3 (continued)

| | |
|---|---|
| Ralston, 1978 | Presented model based on previous hydrodynamic studies.<br>Symmetrical cross shaped structure suggested for tetramer (below)<br>Calculated hydrodynamic parameters in good agreement with values found for spectrin experimentally.<br>Each circle represents a 100 000 dalton globular domain of radius 6nm<br><br> |

molecule using monoclonal antibodies. The rationale behind this will be discussed in Chapter 7.

## 9. Hereditary Spherocytosis

In addition to these structural investigations on spectrin, I have also undertaken a comparative study of erythrocytes from normal individuals and those from individuals suffering from hereditary spherocytosis (H.S.) a congenital haemolytic anaemia, employing both freeze etch electron microscopy and protein chemical techniques. It has often been suggested that H.S. results from a membrane abnormality (Jacob et al., 1971; Greenquist and Shohet, 1974; Gomperts et al., 1973; Hayashi et al., 1974), indeed there is some evidence that the primary defect might reside on spectrin (Sheehy and Ralston, 1978). If this could be demonstrated to be the case, as well as being medically relevant, observations of the membrane in disease might provide further details of the mechanisms of its normal function.

## 10. Presentation of Thesis

This investigation falls naturally into two parts; an electron microscope study and an immunochemical labelling study. I have therefore preserved this division in the layout of my thesis. Chapter 2 contains all methods for spectrin purification and all aspects of electron microscopy. This is followed by three results chapters concerning hereditary spherocytosis (Chapter 3), low angle shadowing of Spectrin (Chapter 4) and Selective extraction

of band 1 from erythrocyte ghosts (Chapter 5). The low angle shadowing and band 1 extraction results are discussed in Chapter 6 while those of the H.S. study are discussed in Chapter 3. The second half of this investigation concerning the development and use of monoclonal antibodies against spectrin is introduced in Chapter 7. All methods required for the production and use of the monoclonal antibodies are detailed in Chapter 8. Results are given in Chapter 9 and are discussed fully in Chapter 10. Chapter 11 contains conclusions to be drawn from this study.

# CHAPTER 2

## MATERIAL AND METHODS I: Membrane and Spectrin Preparations, Low Angle Shadowing and Electron Microscopy

### 1. Preparation of Haemoglobin Free Erythrocyte Ghosts (Dodge et al., 1963)

A 20ml volume of freshly drawn blood from a healthy adult volunteer was mixed with 3ml of citrate anti coagulant (Handbook of Clinical Laboratory Data, 1968, The Chemical Rubber Co., Cleveland, Ohio., p.386).

Anti Coagulant Solution

| | |
|---|---|
| TriSodium citrate | 2.20g |
| Citric Acid | 0.80g |
| D Glucose (anhydrous) | 2.20g |
| Distilled Water to | 100ml |

The blood was suspendedby vortex mixing in 15 volumes of ice cold 310mOsM NaCl and centrifuged in 50ml tubes at 3000 rpm (1000 x g av) for 10 minutes in a Sorvall SS-34 rotor precooled to $4^{o}C$. The supernatant, and where possible, the 'buffy coat' of leukocytes were carefully aspirated. The erythrocytes were given a second wash in 30 volumes of cold 310mOsM NaCl and a final wash in the same volume of 310 PB. The cells were spun down, as before, in the SS-34 rotor and the supernatant discarded. They were lysed in 30 volumes of ice cold 20pB pH 7.6 with vortex mixing and then stirred gently

on ice. After 15 minutes the plasma membranes (ghosts) were centrifuged at 16,000 rpm (32,000 x g av) for 10 minutes in the SS 34 rotor to form a loose fluid pellet, and the supernatant aspirated. Care was taken to completely remove the small sticky pellet underlying the loose ghost pellet, which consisted chiefly of white blood cells. The ghosts were given two more washes in ice cold 20 PB pH 7.6, by which time they were virtaully haemoglobin free and pearly white in colour.

2. Extraction of Spectrin and Actin

a) $37^{o}$C Extraction (after Fairbanks et al., 1971)

Haemoglobin free ghosts were given two further washes in 30 volumes of ice cold extraction buffer containing

100 µM EDTA

10 µM DTT (Sigma)

Titrated to pH 8.0 or 9.5 with dilute ammonia.

After finally pelleting the ghosts at 32,000 x g as before, they were transferred, as a 50% suspension, to 10ml polycarbonate Beckman ultacentrifuge tubes and disrupted by sonicating for 10s at the maximum power setting of a MSE Sonifier using a titanium microprobe. The centrifuge tubes were then placed in a $37^{o}$C water bath. The contents of the tubes were mixed by inversion every 5 minutes. After 30 minutes, the vesicles were again sonicated, as before, and centrifuged at 50,000 rpm (160,000 x g av) for 2 h in a Beckman type 65 fixed angle rotor at $4^{o}$C.

b) $0^{o}$C Extraction

Packed ghosts in 20 pB pH 7.6 were sonicated as described above and transferred to a flaccid dialysis bag, which was placed in 200

volumes of ice cold extraction buffer. The ghosts were dialysed for a total of 40 h with occasional mixing of the contents of the dialysis bag, and three changes to fresh extraction buffer at 4, 16 and 24 hours. After dialysis, the vesicles were again sonicated and spun down in the type 65 rotor as described earlier.

Following either extraction protocol, the clear colourless supernatant containing spectrin and actin was removed from the centrifuge tubes, taking care not to disturb the pelleted membrane fragments. In general, a volume of about 10-12ml of supernatant (crude spectrin extract) with a protein concentration of about 0.8-1.2 mg/ml, was obtained from 20ml blood, and was used without delay for subsequent experiments.

3. Purification of Spectrin By Gel Filtration Chromatography

a) Low Ionic Strength Separation of Spectrin from Actin

Up to 12ml of supernatant were loaded directly onto a 400ml bed volume K26/100 column (pharmacia) fitted with flow adaptors, and packed with sepharose cl 4B (pharmacia) and eluted at 30ml/hr with a low ionic strength buffer containing

10μM DTT
100μM EDTA
1mM Tris-Hcl pH 8.0

b) High Ionic Strength Fractionation of Spectrin Dimers and Tetramers (Ralston, 1975, 1976a)

The crude spectrin extract from ghosts was dialysed overnight at $0^{o}C$ against a high ionic strength column buffer (see below), centrifuged at 50,000 rpm in the Beckman type 65 rotor for 45 minutes

to remove any aggregated material and then applied to a 400ml bed volume Sepharose CL 4B column as in Section 4a, above. The sample was eluted at 4°C and at a flow rate of 30ml/h in the same high salt buffer. High Salt Column Buffers:

(i) 130 mM KCl
20 mM NaCl
1 mM EDTA
0.5mM Sodium azide
10 µM DTT
10 mM Tris-HCl pH 7.4

(ii) 155 mM Ammonium Acetate
100 mM EDTA
10 µM DTT
pH to 7.4 with ammonia.

Buffer (i) was used to approximate the intra-cellular concentrations of monovalent ions. Buffer (ii), which was volatile, was employed when the eluant fractions were to be used directly for low angle shadowing.

4. Polyacrylamide Gel Electrophoresis

The procedures employed were those described by Fairbanks et al., (1971) with the minor modifications of Steck and Yu (1973).

(a) Materials

| | |
|---|---|
| Acrylamide | (Eastman-Kodak Limited) |
| N.N' Methylene bisacrylamide | (Eastman-Kodak Limited) |
| TEMED | (Eastman-Kodak Limited) |

| | |
|---|---|
| Ammonium persulphate | (Eastman-Kodak Limited) |
| SDS - High purity | (Pierce and Warriner UK Ltd) |
| SDS - Low purity | (Hopkins and Williams) |
| Coomassie Brilliant Blue R-250 | (Biorad Laboratories Limited) |

(b) Stock Solutions

X10Tris Buffer

400 mM Tris Base

200 mM Sodium Acetate

20 mM EDTA

pH to 7.4 with acetic acid

Acrylamide/Bis acrylamide (Aca/bis)

Acrylamide 40%

N N' Methylene Bis acrylamide 1.5%

Before use, 3 heaped teaspoonfulls of Amberlite Monobed MB-1 Mixed bed resin(BDH) and 3 teaspoonfulls of activated charcoal were added to 500 mL of Aca/bis stock solution and stirred for five minutes. The suspension was then filtered through Whatman No. 1 filter paper and stored at $4^{o}C$ in a brown glass bottle.

(c) Preparation of 5% Acrylamide Tube gels

Composition

| | |
|---|---|
| 50% glycerol | 10 ml |
| Aca#Bis | 6.25ml |
| x 10 Tris buffer pH 7.4 | 5ml |
| 10% SDS (High purity) | 1ml |
| Distilled water to | 45ml |

The solution was mixed by inversion

| | | |
|---|---|---|
| 10% | Ammonium persulphate | 0.65ml |
| 0.5% | TEMED | 3 ml |
| Distilled water | to | 50ml |

The solution was mixed by inversion. degassed on a water pump and immediately poured to a height of 8 cm into acid washed glass tubes, 10cm in length and with an internal diameter of 5mm which had been dried after rinsing in a solution of 1% 'photoflo' (Kodak). The gel solution was then overlaid with a 0.02% solution of SDS (low purity). The photoflo aided subsequent removal of the gels from the tubes.

(d) Sample preparation

Samples for electrophoresis were mixed at a ratio of 4:1 with denaturation buffer (below) in 2ml pyrex test tubes which were placed within in a boiling water bath for 2 minutes or a 37°C water bath for 30 minutes. Denatured samples which were not to be used immediately were stored frozen at -20°C and reheated before subsequent use.

| Denaturation Buffer | Amount | Final concentration |
|---|---|---|
| SDS (high purity | 2.5g | 5 |
| Sucrose (AR) | 25 g | 50 |
| 1m Tris-HCl pH 8.0 | 2.5ml | 50mM |
| 50mM EDTA pH 8.0 | 5.0ml | 5mM |
| DTT | 1.23g | 160mM |
| Bromophenol blue (BDH) | 10mg | 0.02% |
| Distilled water to | 50ml | |

(e) The Electrophoresis Run

Twelve tubes were placed in the electrophoresis apparatus and the upper and lower compartments were filled with a running buffer

containing:

| | |
|---|---|
| x 10 Tris buffer | 100ml |
| 10% SDS (low purity) | 20ml |
| Distilled water to | 1000ml |

Samples, from 10-200µl, were layered onto the surface of the gels and electrophoresed at 5mA per gel for 2½h without cooling.

(f) Staining of Gels

Gels were fixed and stained overnight at room temperature in the following solution.

| | |
|---|---|
| Coomassie Blue R-250 | 0.025% |
| propan-2-ol | 25% |
| Glacial Acetic Acid | 10% |

The gels were destained for 8 h in 10% propan-2-ol, 10% acetic acid and then stored prior to photography in 7% acetic acid.

(g) Non-denaturing gels

3.5% acrylamide non denaturing gels were prepared and run as described above except that SDS was omitted from all solution and electrophoresis was performed at 4°C employing a current of 2mA per tube. Non-denatured samples were applied to the gels in a buffer containing 10mM Tris, 1mM EDTA, 10% glycerol, 0.005% Bromophenol blue, pH 8.0.

5. Low Angle Shadowing

Purified tetramer and dimer preparations which had eluted from the ammonium acetate column at 1.5 and 1.8 times the column's void volume, respectively, were diluted with 155mM ammonium acetate, 100µM EDTA

and 10μM DTT (pH 7.4) and gently mixed with glycerol at 0°C, to give a final solution containing 70% glycerol and spectrin concentration of 10-20 μg/ml. Similarly, rabbit skeletal muscle myosin (a gift from Dr. G. Offer) in 1M ammonium acetate pH 7.2 was diluted to the same glycerol and protein concentrations. Myosin was used as a control for the spectrin shadowing because of its distinctive molcular shape (Elliott et al., 1976; Elliott and Offer, 1978). Each protein solution was sprayed at room temperature using a laboratory aerosol sprayer (Shandon) with a Freon-12 propellent to form a cloud of droplets, some of which were caught on a surface of freshly cleaved mica. The protein was not sprayed directly onto the mica, instead the mica was passed through the mist out of which the large droplets had already settled, ensuring that only the smallest droplets were collected onto the mica surface. As an alternative method, during later experiments, the inlet tube of the aerosol sprayer was replaced with a blue disposable pipette tip containing the sample to be shadowed, which was sprayed directly at a piece of mica from a distance of about 40cm. In this way sample volumes as small as 5-10μl could be handled with ease. The sections of mica (about 5 x 4mm) were placed side by side on the uncooled rotary specimen stage of a Balzers (Balzers A.G. Liechtenstein) BAF 301 freeze etch apparatus adapted for low-angle shadowing, using double side scotch tape for attachment. The droplets were evaporated to dryness by evacuating the specimen chamber for about 5 minutes to a pressure of $2.10^{-6}$ mbar. Then the dried down protein molecules were shadowed with a platinum/carbon mixture (95% pt, w/w; density 17.4g/cm$^3$) and coated with a supporting film of carbon, 10nm thick, using Balzers electron bombardment guns and a quartz crystal film thickness monitor (Moor, 1971). A 9° shadow angle and a mean shadow thickness of 0.78nm ($1.4\ 10^{-6}$g pt/c mixture deposited per cm$^2$) were used for unidirectional shadowing, while a 6° shadow angle and a mean shadow thickness of 1.07 nm ($1.9\ 10^{-6}$g pt/c per

$cm^2$) with a specimen rotation speed of 120 rpm were employed for low angle rotary shadowing. The shadow thickness, d (in nm), was calculated from the change in frequency detected by the quartz crystal thin film monitor using the formula shown below:

$$d = \frac{\Delta f}{k} \cdot \frac{\sin\alpha}{\sin\beta} \cdot \frac{L_1^2}{L_2^2}$$

where $\Delta f$ is the crystal frequency change (mHz)

$k$ is the material constant (Hz/nm) = 100 for pt/c

$L_1$ source to specimen distance

$L_2$ source to monitor distance

$\alpha$ angle between gun axis and specimen plane

$\beta$ angle between gun axis and the normal to the plane of the quartz crystal

(Taken from Balzers A.G.(Liechtenstein) technical manual BB800048BE)

The shadow replicas so formed were carefully floated onto distilled water and broken into 2-3mm diameter fragments. These were picked up on bare 400 mesh copper electron microscope grids which had been made hydrophilic by brief immersion in alcoholic sodium hydroxide (30g NaOH in 30ml distilled water, made up to 250ml with a solute ethanol).

The specimens were examined in a Philips EM301 electron microscope fitted with a eucentric goniometer stage. The microscope was operated under conventional conditions at 80kV using a 50µm objective aperture. Astigmatism was corrected beforehand using holey formvar/carbon film. Stereo pairs were obtained by tilting the specimen stage through angles of plus and minus $12^o$ (total tilt, $24^o$) about an axis through the plane of the grid.

6. Preparation of Tropomyosin Magnesium Tactoids

(Caspar et al., 1969)

0.2ml of a solution of rabbit skeletal muscle tropomyosin (a gift from Dr. J. Kendrick-Jones) in 100mM NaCl, 10mM imidazole-Hcl pH 7.0, at a concentration of 9.2 mg/ml, was diluted, by the addition of 100 mM NaCl to 1.8 ml. This solution was dialysed overnight against 100 volumes of a dialysis medium containing 50 mM $MgCl_2$, 50mM Tris-Hcl pH 7.6, during which time the tropomysin associated into needle shaped tactoids which were visible under the phase contrast microscope .

7. Measurement of Molecular Lengths

Electron micrographs were taken of low angle shadowed spectrin and myosin at magnifications between 22,000X and 59,000X (standardly at 33,351). These magnifications were calculated by photography, under the same electron optical conditions, of magnesium tactoids of rabbit skeletal muscle tropomyosin negatively stained with 1% aqueous uranyl acetate. Such negatively stained tactoids have been shown to have an extremely regular axial repeat of 39.5 $\pm$ 0.4 nm. Dried fibres of unstained tactoids similarly exhibit X-ray diffraction to the 14th order of a 39.6 $\pm$ 0.6 nm axial period. (Caspar et al., 1969). A 39.5 nm period was assumed for these measuremtns. Subsequent measurements made on other photographs of magnesium tactoids which were taken as controls during photography of the spectrin and myosin preparations varied from the calibration set by not more than 0.6%.

Images of the tropomyosin tactoids and of the low angle shadowed spectrin and myosin molecules were projected from electron micrographs, using a Leitz projector fitted with a 90mm lens, onto the digitising surface of a Hewlett-Packard 9864A digitiser on line to a 9821A calculator

and 9862A plotter, which were used to measure lengths, calculate statistics and plot histograms of the resulting values. The optical magnification was 21.04 diameters, which varied by less than 1% over the entire digitizing surface. These values were determined by directly measuring the projected image of an optical grating (British American Optical Limited). The total magnification of molecules projected onto the digitizer was 701,705 (for e.m. Mag. of 33,351).

To determine mean molecular lengths, only images of molecules which lay isolated, extended and clearly visible were measured. The shape of the molecule was carefully followed from the first visible platinum grain at one end of the molecule to the last grain at the other end. In the case of myosin tails, measurement was to the junction of the tail with the more distal of the two heads. Not less than 100 molecules were measured for each length determination (for both unidirectional and rotary shadowed molecules) and histograms were plotted at intervals of approxiamtely 1/20 of the mean molecular lengths.

8. Spectrin/Actin Depletion of Ghosts: Pretreatment

(Elgsaeter and Branton, 1974)

Fresh, washed ghosts from normal and H.S. erythrocytes were resuspended in 10 volumes of 20 PB pH 7.6 containing 0.5mM Sodium azide and placed in a 37$^{0}$ waterbath. After 22h the ghosts were centrifuged for 10 minutes in the Sorval SS-34 rotor at 16,000 rpm. The supernatant was discarded and the packed ghosts used immediately for subsequent intra membrane particle aggregation experiments. Samples for gel electrophoresis were taken where appropriate.

9. Intra membrane particle aggregation conditions

(Elgsaeter et al., 1976).

(a) pH 5.5 Aggregation

Pretreated ghosts were suspended in 40 volumes of 20pB pH 5.5 on ice. The suspension was spun down at 16,000 rpm in the SS-34 rotor for 10 minutes and the supernatant aspirated. The ghost pellet was again resuspended in 20 pB pH 5.5 and incubated on ice for 30 minutes. The suspension was spun down as before and the pellet frozen for freeze etch electron microscopy.

(b) Protamine sulphate induced aggregation

Pretreated ghosts were resuspended in 10 volumes of 20pB pH 7.6 containing protamine sulphate (Sigma Chemical Company) at a concentration of 0.1 mg/ml (about 0.3 mg per mg of membrane protein) and incubated on ice for 30 minutes. The ghosts were then pelleted and frozen for freeze etch electron microscopy.

10. Freeze Etch Electron Microscopy

A small volume (1-2µl) of sample, typically a 50% suspension of unfixed, unglycerinated ghosts, was carefully placed on a gold or copper, Balzers freeze-etch specimen support ('hat') using a drawn out pasteur pipette. The specimen was frozen by rapid immersion in Freon 22 cooled to its freezing temperature with liquid nitrogen.

Freeze fracturing or freeze etching was carried out using a Balzers BAF 301 freeze etch apparatus fitted with a Meissener trap and electron bombardment guns (Moor, 1971). Well established techniques were employed (Fisher and Branton, 1974) with the exception that the supporting carbon film was deposited from an angle of $70^{o}$ with the specimen stage rotating at 120 rpm instead of the more conventional deposition from $90^{o}$ with the specimen stationary. I found that using

this method I could obtain significantly stronger replicas without losing any resolution as one might do if one simply deposited a thicker carbon supporting layer. In general, specimens were fractured with a microtome and then etched for 30 s at -100°C. Precise conditions, however, are given in the results sections

Replicas were floated on to distilled water and then transferred onto a cleaning solution containing 70% sodium hypochlorite, 30% 10mM NaOH for 30 minutes. The replicas were given three 5 minute washes of distilled water and then picked up on bare 460 mesh hexagonal electron microscope grids (Polaron) treated with alcoholic NaOH. Specimens were routinely examined in a Philips E.M. 301 as described in Section (5) or in a Hitachi H-600 electron microscope operated at 75kV with a 50μm objective apperture.

10. Selective Extraction of Band 1 from Ghosts

Ghosts were extracted either by a 30 minute incubation at 37°C in 10 volumes of extraction medium or by dialysis at 0°C against 200 volumes of extraction medium. Precise conditions and composition of media used are given in Results Chapter 5.

# CHAPTER 3

## THE ROLE OF THE CYTOSKELETON IN HEREDITARY SPHEROCYTOSIS

### 1. Introduction

#### (a) The Incidence of Hereditary Spherocytosis

Hereditary Spherocytosis (HS) is an important inherited disorder characterised by osmotically fragile, partially spherical erythrocytes which are selectively filtered out of the circulation by the spleen and destroyed, but which survive almost normally following splenectomy. Among persons of English or northern European descent HS is the most common of the hereditary haemolytic anaemias now recognised, affecting one out of every 5,000 individuals (Young, 1955). The disease appears to be inherited as an autosomal dominant trait; i.e. the disease is expressed in heterozygotes. There have so far been no confirmed reports of homozygotes, leading to suggestions that such a condition may not be compatible with life (reviewed by Dacie, 1960).

There are, however, anomalies in the inheritance of HS. Young et. al. (1951(a)) and Young (1955) have reported several patients whose parents were without any haematologic abnormalities, a finding confirmed by many other groups. They also noted that there was a marked shortage of affected siblings of propositi. Instead of the 50% incidence which one would expect from simple mendelian genetics, the actual figure was only 24%. Furthermore, the disease may vary enormously in severity (Dacie, 1960); in many cases it may be diagnosed early in

infancy, whereas in others it may escape notice until much later in life, often only being identified during routine examinations associated with other complaints. These findings have been interpreted to indicate that the HS gene may have variable penetrance or expression such that the most mildly affected individuals could really be described as HS carriers.

(b) Clinical Findings and the Evidence for an Erythrocyte Defect

The clinical manifestations of HS have been extensively reviewed by Dacie (1960). In short, the disease is first recognised as a haemolytic anaemia accompanied by jaundice and enlargement of the spleen, the haemolytic process being non-autoimmune (the red cells show negative in the Coombs test for anti erythrocyte γ-globulin). The spleen is the primary site of accelerated red cell destruction in HS and the clinical symptoms of the disease are found to be greatly reduced or even abolished following splenectomy.

Cross transfusion studies by Dacie and Mollison (1943) and by Young et. al. (1951(b)) indicate that it is the red blood cells and not the spleen which contain the primary defect in HS and which results in their premature destruction by the spleen. Normal cells transfused into HS patients show normal survival whereas HS cells transfused into normal non splenectomised individuals are rapidly removed from the circulation. HS cells transfused into splenectomised normal individuals show lifespans significantly nearer normal.

The hallmark of HS is the presence in the circulation of spherocytic red blood cells. However, the degree of spherocytosis may vary considerably (Young et. al., 1951(a) ) and in many patients may represent only a slight increase in the mean cell thickness with a corresponding reduction in mean diameter. Mackinney et. al. (1964) have reported that in 20-25% of cases the typical hyperchromic microspheres

may be completely absent. In virtually all cases, however, the reticulocyte count is abnormally high (Mackinney et. al., 1964). According to Young (1955) precursor bone marrow cells and reticulocytes do not exhibit any abnormal shapes and varying degrees of sphering only occur once the cell reaches the circulation.

The most consistent feature of HS, however, is the fragility of the red blood cells. Fresh HS red cells usually, but not always, show an increase in osmotic fragility over normal cells. This osmotic fragility is found to be considerably accentuated following a 24h incubation in serum at 37$^{o}$C, quite distinct from any other haemolytic anaemia. Young et al. (1951(a)) have pointed out that in the absence of spherocytes in the circulation diagnosis of HS may be made on the basis of osmotic fragility after incubation alone.

Not only are HS red cells osmotically fragile, they also exhibit increased mechanical fragility over their normal counterparts. When stressed by such methods as gently rotating in a flask with glass beads (reviewed by Young, 1955) HS cells show a greater tendency to fragment and lyse. The mechanical fragility may be associated with the decreased deformability of HS red cells, as judged by their abilit- to enter micropipettes of varying diameters, observed by LaCelle and Weed (1969). Such a decrease in deformability is almost certainly the basis of HS cells being specifically filtered out by the spleen as they may be unable to traverse the micro-fenestrations of the splenic circulation and as a consequence are trapped and destroyed.

(c) The Cellular Basis of Hereditary Spherocytosis

Table 3.1 summarises most of the abnormalities which have so far been identified. It is a reasonable conclusion that the cellular defect in HS resides in the membrane in view of the cell's fragility and rigidity. No metabolic abnormality or haemoglobin defect

have been reported. An increase in glycolysis and ATP turnover has been noted in some cases but this may be attributed to a secondary requirement for additional $Na^+$ pumping (Jacob and Jandl, 1964) due to increased permeability to this ion.

The view that the membrane in HS erythrocytes is defective is supported by the observations of Reed and Swisher (1966) who showed that following a 24h incubation at 37°C, lipid content of cells was reduced although lipid composition was normal, indicating a loss of membrane by fragmentation. This is consistent with the fact that HS red cells show a reduction in surface area, slightly reduced volume and hence an increased mean corpuscular haemoglobin concentration i.e. they are hyperchromic (Young et. al., 1951(a); Mackinney et. al., 1964). Cooper and Jandl (1969) later showed that HS erythrocyte lipid was reduced by up to 20-25% but that the surface deficit could not be accounted for by lipid loss alone, suggesting that the membrane could be fragmenting internally as well as externally. However no internal vesicles have so far been identified in HS red blood cells (Schrier et. al., 1974). Another interesting, although inexplicable, phenomenon associated with HS is that there is a deficiency of long chain fatty acids (greater than 20 carbons) in sphingomyelin, phosphatidyl choline and phosphatidyl serine (Kuiper and Levine, 1972). Whether such a deficit could be accounted for by preferential loss of membrane fragments rich in these components is uncertain.

(d) The Role of Membrane Proteins in HS

There have been a number of observations pointing to a membrane protein defect as being the primary cause of HS (see Table 3.1). Many of the reported anomalies, however, seem to be entirely unrelated and peculiar to single cases since they have not been found in HS patients studied by other groups. Two such observations are decreased

TABLE 3.1

Cellular Abnormalities Observed in H.S.

| Observations | Reference |
|---|---|
| (1) Haemoglobin | |
| a. Increased mean corpuscular haemoglobin concentration | Mackinney et al.(1962) |
| (2) Metabolism | |
| a. Increased ATP turnover | |
| b. Increased glycolysis | Jacob and Jandl (1964) |
| (3) Membrane | |
| a. Wrinkled and pitted membrane surface | Nozawa et al. (1974) |
| b. Increased membrane loss during ATP depletion | Reed and Swisher (1966) |
| c. Decreased phospholipid and cholesterol | " |
| d. Decreased long chain fatty acids | Kuiper and Livine (1972) |
| e. Increased $Na^{+}$ permeability | Jacob and Jandl (1964) |
| f. Decreased $Ca^{2+}$ ATPase | Feig and Guidotti (1974) |
| g. Decreased ghost protein phosphorylation | Greenquist and Shohet (1974, 1976) |
| h. Increased membrane bound haemoglobin and catalase | Allen et al.(1977) |
| i. Increased membrane skeletal dissociation by urea | Wolfe and Lux (1978) |
| j. Decreased cation induced aggregation of solubilized proteins | Jacob et al.(1971) |
| k. Absence of band 4.2 | Hayashi et al.(1974) Nozawa et al.(1974) |
| l. Inextractable spectrin | Sheehy and Ralston (1978) |

membrane $Ca^{2+}$ ATPase activity (Feig and Guidotti, 1974) and decreased memebrane protein phosphorylation (Greenquist and Shohet, 1974, 1975).

Several protein abnormalities have been reported which may be mimiced in other complaints suggesting that they may be secondary effects. Allen et. al. (1977) demonstrated increased membrane binding of catalase in HS, but found that it also occurred in metabolically stressed normal cells. Hayashi et. al. (1974) found band 4.2 to be completely absent or significantly reduced in 15 cases of HS, a result also seen in another patient before splenectomy by Nozawa et. al. (1974), but found that the severity of the disease could not be correlated with the degree of band 4.2 loss. Iida et. al. (1976) later showed that band 4.2 was also reduced or absent in cases of biliary obstruction, a complaint, like HS, resulting in jaundice, but that the band reappeared following surgical relief. Gomperts et. al. (1973) also noticed lack of a single membrane protein in HS. However they were not using SDS gels so this protein cannot be identified in the conventional terminology (Steck 1972), although it is presumed to be band 4.2.

Most data points towards the erythrocyte membrane cytoskeleton or associated proteins being the site of the defect. The first two pieces of evidence are merely circumstantial but do indicate a structural as opposed to a metabolic defect. The fact that the disease is apparentlyinherited as a dominant trait argues that the lesion is located on a structural protein. If it was a purely enzymic protein which was defective one would expect the disease to be recessive since the parental gene producing normal enzyme would mask the expression of the defective one. If, however, the lesion was located in a cytoskeletal protein which could be incorporated into macromolecular assemblies but which was either unable to function normally or which resulted in incomplete assembly of the cytoskeleton, due to a defective binding site for instance, one would expect dominant inheritance since non

functional cytoskeletons would result, even in the presence of normal protein. The second indication that a structural protein might be defective stems from the long established fact that HS red cell membranes are abnormally fragile and prone to fragmentation. It is worth emphasising that intense fragmentation of ghosts occurs following disruption of the spectrin-actin network during low ionic strength extraction of ghosts. Furthermore, Greenquist and Shohet (1978) have described an HS-like condition in mice in which spectrin is genetically absent. Obviously this bears no relationship to the human disease but it does serve to demonstrate that a cytoskeletal abnormality can result in the characteristic symptons of HS.

Further data which pointed to a defect in a cytoskeletal component was provided by Jacob et. al. (1971) who found that solubilized, but unfractionated and unfortunately uncharacterised membrane proteins from HS cells aggregated less in the presence of cations than the same extracts from normal cells. The major components of these extracts were almost certainly spectrin and actin, but the composition compared to low ionic strength extracts used in this investigation are not clear. Almost certainly they contained additional components.

More convincing evidence for a cytoskeletal involvement stems from the work of Sheehy and Ralston (1978) and of Wolfe and Lux (1978). The former found that in two HS cases spectrin was completely inextractable, a condition which could be mimiced in normal cells by warming to $50^{o}C$ at which temperature spectrin is heat denatured. The latter group have discovered that Triton skeletons of HS ghosts may be solubilized by urea at concentrations much lower than those required to solubilize normal Triton skeletons.

In order to further investigate the role of membrane proteins, and in particular, the cytoskeletal proteins in HS, I decided to compare

the behaviour of normal and HS red cell membranes by freeze etch electron microscopy, following the aggregation of intra-membrane particles using the standard pretreatment (spectrin-actin depletion) and particle aggregation conditions described by Elgsaeter and Branton (1974) and Elgsaeter et al. (1976). It was considered likely that a specific cytoskeletal lesion which significantly modified protein interactions in the spectrin-actin network or even between the network and integral proteins, might manifest itself in changes in the characteristics of the pattern of intra-membrane particle aggregration. As well as these ultrastructural studies, comparisons were also made of total ghost protein by SDS gel electrophoresis, spectrin-actin extractability at low ionic strength and the quantity of spectrin tetramer, relative to dimer extracted from the ghost membranes at $0^{o}C$.

## 2. Results

### (a) General Information

Blood from eight individuals with HS have been examined. Four of these were unrelated while the remainder represented two generations from a single family. The four unrelated cases showed no consistent membrane protein abnormalities although in each instance the cells showed slight sphering and were, according to the clinical findings, abnormally fragile. A typical wet field (310 NaCl) of normal and HS (patient JL) erythrocytes is shown in Figure 3.1. The cells from the other seven patients were essentially similar to those in Figure 3.1(b), most cells appearing biconcave with a small proportion of cells lacking the usual central depression (arrowed). The significance of the spurred cells seen in Figure 3.1(b) is uncertain.

Figure 3.1

Normal (a) and HS (b) erythrocytes photographed using Nomarski differential interference optics. Cells are present in both preparations which lack the central depression (arrowed).

Magnification, x 800. Bar = 25 m.

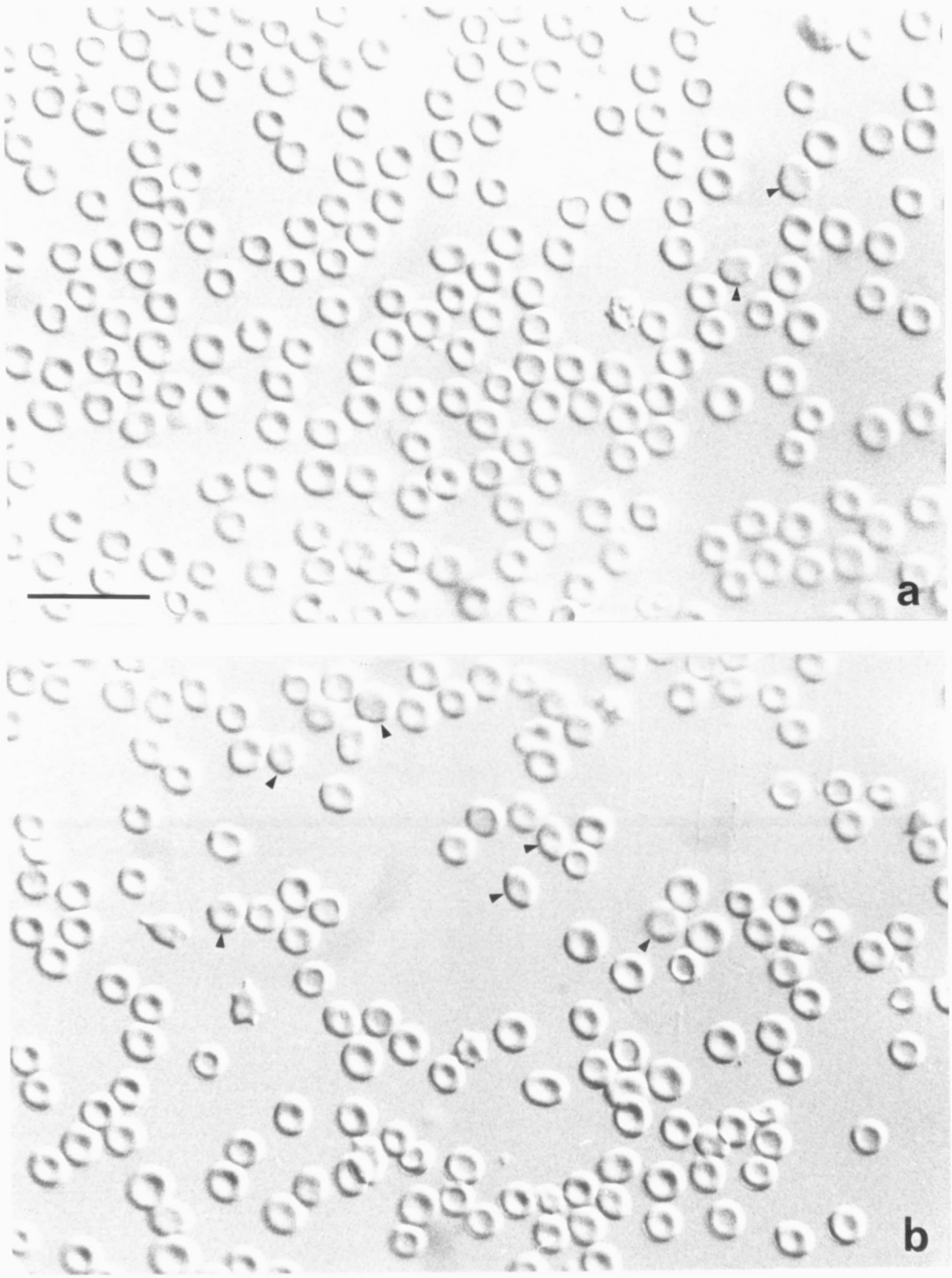



FIGURE 3.1

(b) Results in Four Unrelated HS Cases (patients JL, BA, DW, JD)

All four cases had undergone splenectomy several years prior to the collection of blood samples. In none of these cases was there any known family history of HS or other haematologic disorders. However, one patient (JL) had a daughter who had presented with symptoms suggestive of HS, although diagnosis was not confirmed. Ghosts prepared from these four sets of HS erythrocytes showed protein compositions, as judged by SDS gel electrophoresis, indistinguishable from that of normal ghosts (results not shown). Furthermore, all showed normal spectrin-actin extractability under ghost pretreatment conditions (Elgsaeter and Branton, 1974) and under the normal low ionic strength high pH extraction protocol at $0^{o}C$ (results not shown). One of these patients (JD) however, showed a noticeable reduction in the amount of spectrin tetramer relative to dimer present in a $0^{o}C$ extract compared with normal ghosts (Figure 3.2. More detailed results concerning the fractionation of spectrin dimers and tetramers from normal ghosts are found in the following Chapter.).

Fresh ghosts from each of these four cases, as visualized by freeze etch electron microscopy, exhibited morphologies which were indistinguishable from each other and from fresh, normal control preparations (one of which is shown in Figure 3.3(a) ). However, the appearance of the intra-membrane particles, after pretreatment and aggregation was found to vary quite considerably among the four HS cases. Ghosts from patients DW and JD exhibited particle aggregation which was identical with that seen in normal controls (as in Figures 3.3(b) and 3.4(a) ). However, the other two, JL and BA, differed quite substantially from normal.

Figure 3.2

Elution profiles of $0^{o}C$, spectrin/actin extracts of normal (N) and and HS ghosts (J.D.) from a 2.5 x 90 cm sepharose C14B column run at high ionic strength in the non volatile column buffer (130mM KCl, 20mM NaCl, 1mM EDTA, 10μm DTT, 10mM Tris-HCl pH 7.4). Composition of the samples applied to the column were identical as judged by SDS gel electrophoresis (not shown). Note the lack of both a tetramer peak (T) and large void volume peak (V) in the HS sample. Heterodimer peak is denoted by D.

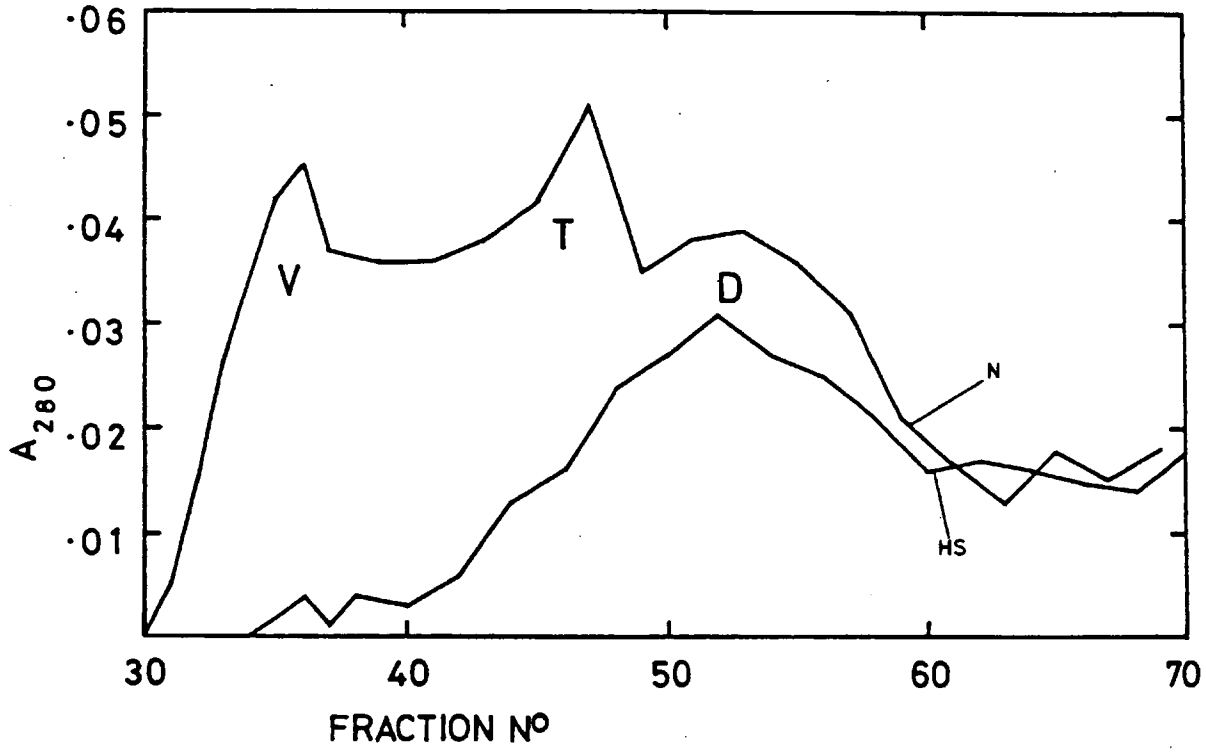



FIGURE 3.2

Figure 3.3(b) and (c) shows two typical protoplasmic fracture faces from a normal ghost (3.3(b) ), and an HS ghost, from patient JL (3.3(c) ), which were incubated on ice for 30 minutes at pH 5.5, following pretreatment, in order to promote particle aggregation. The normal ghost shows moderate aggregation with only a small proportion of particles remaining isolated, whereas the HS ghost, which was prepared at the same time as the normal specimen shows a rather greater degree of aggregation. Although the degree of aggregation in the standard assay may vary slightly between separate experiments run on different days such variation is not observed (at least in the case of normal ghosts) when several different samples are prepared at the same time using the same set of solutions. Another very interesting feature of the HS sample is the large proportion of particles which are not associated with the aggregates, suggesting a decrease in the interactions between the residual spectrin and actin on the inner membrane surface and the integral proteins or intra membrane particles. However, this is the only specimen in which I have ever observed this phenomenon; certainly in normal cells one always sees aggregation of close to 100% of the particles.

Figure 3.4 is of normal and HS (patient BA) ghosts in which particle aggregation has been induced with protamine sulphate (following pre-treatment) which tends to have more pronounced effects than the pH 5.5 treatment (compare Figures 3.3(b) and 3.4(a) ). The degree of aggregation exhibited by the HS ghosts is, however, considerably more intense than that of normal ghosts, giving rise to very large areas of particle-free lipid, the extent of which I have never seen in any normal preparations treated in the same way. Note in this case that there are few non-aggregated particles.

It is apparent from these results that these four unrelated HS cases exhibit differing cellular abnormalities. Indeed cells from

Figure 3.3

Comparison of the degree of pH 5.5 induced intramembrane particle aggregation between normal and HS (patient JL) ghosts.

(a) Freeze etch electron micrograph of normal, fresh ghosts.

(b) Freeze etch micrograph of normal ghosts which had been pretreated (incubated at 37°C for 18 h in 20pB) and then incubated on ice for 30min in 20pB pH 5.5. prior to freezing.

(c) Freeze etch micrograph of HS ghosts from patient JL treated as in (b).

All three specimens were fractured and then etched for 30s at -100°C.

Note the increased aggregation in the HS ghost and also the large number of particles not found in aggregates. Only protoplasmic fracture faces are shown.

Magnification, x 50,000. Bar = 0.5μm.

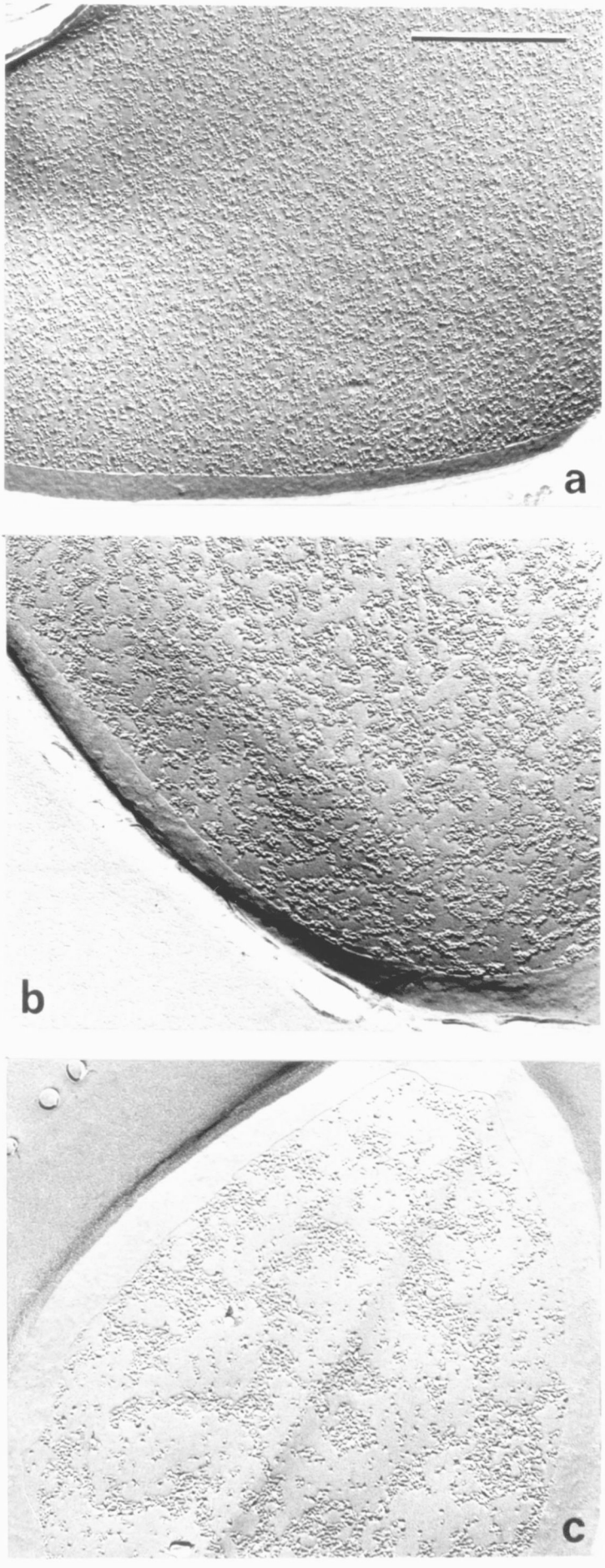



FIGURE 3.3

Figure 3.4

Comparison of the degree of intra membrane particle aggregation induced in normal and HS ghosts by protamine sulphate.

(a) Freeze etch electron micrograph of a normal ghost in which particle aggregation had been induced by incubation on ice for 30 minutes in 20pB containing protamine sulphate at a concentration of 0.1 mg|ml, following pretreatment (see text and previous figure).

(b) HS ghosts (from patient BA) treated as (a). Note the intense particle aggregation in the HS sample

Specimens were freeze fractured and then etched for 30s at $-100^{o}C$.

Magnification, x 50,000. Bar = 0.5μm.

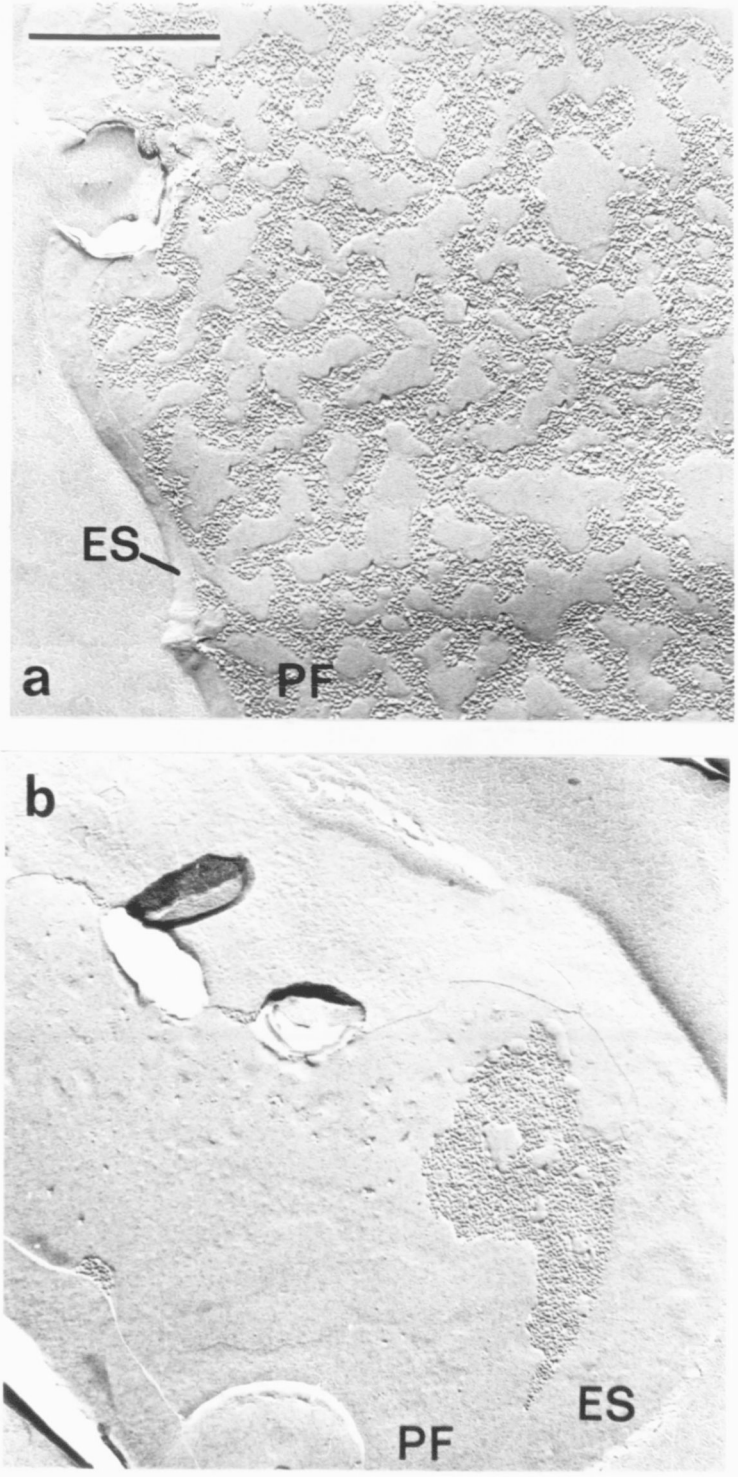



FIGURE 3.4

one of these patients (DW) appeared essentially normal in this investigation, despite the clinical findings that the cells possessed the fragility characteristic of HS.

The next four cases which I intend to review are all related and, not surprisingly, exhibit virtually identical membrane abnormalities. These abnormalities are, however, somewhat different from those of the previous four unrelated cases.

(c) Results from Four Related HS Cases

The members of this HS family which were affected were the father (FS) and all three offspring (AS, SS, JS). There was, however, no evidence for the incidence of HS in any previous generations on the paternal side of the family. The maternal side of the family was likewise uniformly free from any known haematological disorders. Further details of the four patients are given in Table 3.2.

T A B L E 3.2

| Patient | Sex | Age | Splenectomy | Age at splenectomy |
|---|---|---|---|---|
| FS | M | 34 | Yes | 10 |
| AS | M | 10 | Yes | 4 |
| SS | F | 8 | Yes | 8 |
| JS | F | 8 | No | - |

The three who had undergone splenectomy (Table 3.2) appeared physically normal showing no evidence of anaemia. JS, however, was both anaemic and jaundiced. Of particular interest was that SS and JS were non identical twins, SS having undergone splenectomy only 3 months prior to the collection of blood.

(d) Gel Results

Figure 3.5 shows SDS gels of normal (N) and HS ghosts. The ghosts in Set A were prepared from small volumes of blood within 2 h of collection, and used for freeze etch electron microscopy. Those in Set B were prepared from the remainder of the cells, which had been stored overnight on ice in citrate anticoagulant, and were used to observe spectrin-actin extractability at $0^{o}C$ in the 0.3mM phosphate extraction buffer (Chapter 2). In addition to extracting spectrin and actin, this buffer may also elute a small quantity of Band 4.1 (see Chapter 4 for further details). The gels of ghosts all appeared to be essentially normal except for increased haemoglobin retention in some cases, particularly in the stored samples. This haemoglobin retention was accompanied by an increase in the intensity of Band 2.3 and also of a minor band in the band 4.5 region (arrow). Band 2.1 showed a concomittant decrease in intensity suggesting that these phenomena might be in some way related to proteolytic activity. The significance of these observations, however, is unclear since they appeared in both normal and HS samples. In addition to these findings, Band 4.2, in patient JS, appeared to show a slight decrease in intensity with respect to Band 4.1, most evident in gel set A. As shown in Figure 3.6, spectrin-actin extractability at $0^{o}C$ was identical in all cases with the exception that the spectrin extracted from the ghosts of patient JS showed evidence of proteolysis, despite the fact that all the samples were prepared at the same time on ice. Note also, that haemoglobin initially retained by the ghosts is extracted under these conditions of high pH and low ionic strength. In all four cases, there was no obvious departure from normal in the amount of spectrin tetramer relative to dimer extracted from the membranes (results not shown).

Figure 3.5

SDS (polyacrylamide gels of normal (N) and HS ghosts from the four related cases. The gels are identified with each patient's initials. Samples in gel set A were prepared soon (2 h) after collection while those of set B were prepared after storing the blood on ice overnight for 18 h in citrate anticoagulant. Note the increased haemoglobin retention in some cases, accompanied the increase in intensity of Band 2.3 and also of a band running in the 4.5 region (arrow). All loadings were 10µl.

Figure 3.6

Comparison of spectrin/actin extractability at $0^{o}C$ in 0.3mM phosphate buffer pH 7.6 from normal (N) and HS ghosts. (A) in each case is the spectrin/actin extract (50µl) while (B) is the extracted membranes (20µl). The patients are identified by their initials. Note that spectrin and actin extractability appear to be normal in each case while there is some variability both between normal and HS and among the HS cases in the extraction of low molecular weight material in the Band 7 to Band 8 region.

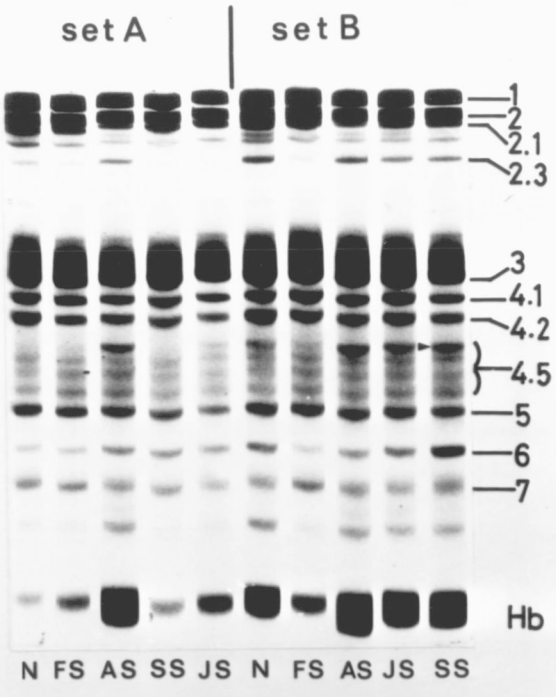



FIGURE 3.5




FIGURE 3.6

The limited spectrin extraction of ghosts involved in pre-treatment, prior to intramembrane particle aggregation presented a rather difrerent picture. As shown in Figure 3.7 only normal ghosts exhibited any release of spectrin and actin. It would therefore appear that, in these cases of HS, spectrin is slighly less extractable than normal, a fact which may only be recognised under these very mild extraction conditions (37$^{o}$C overnight incubation in 20pB pH 7.6). In addition to this reduction in spectrin extractability, all the pretreated HS ghosts showed a reduction in the amount of band 4.2. However, band 4.2 did not appear in the supernatants, consequently this loss may have been due to proteolysis (protease inhibitors were not included) or to the formation of cross linked complexes not dissociated by SDS or DTT.

(e) Freeze-Fracture Results

Because of this inability to extract spectrin and actin during pretreatment of the HS ghosts, one would not expect to see aggregation of the intra-membrane particles using the mild pH 5.5 protocol, since Elgsaeter and Branton (1974) and Elgsaeter et. al. (1976) showed that particle aggregation depended upon the prior removal of these proteins. Suprisingly, however, significant aggregation was observed in all the HS cases equal to or even greater than that seen in the normal control (Figure 3.8). The fracture faces of the fresh HS and normal ghosts appeared to be identical. However, in the case of JS, the patient who had yet to undergo splenectomy, cross-fractured fresh ghosts were occasionally found to contain larger numbers of small vesicles. An example of this is shown in Figure 3.9. Presumably these vesicles arose by internal fragmentation of the membrane, which would be consistent with the findings of Cooper and Jandl (1969) that the surface area deficit of HS red cells cannot be accounted for by lipid loss alone. It has not yet been determined whether this fragmentation is peculiar to ghosts

Figure 3.7

Pretreatment of normal and HS ghosts. Gels A to E are of material extracted from ghosts by an 18 h pretreatment incubation at 37°C in 20pB. Loadings are all 100µl. The samples A to E are normal and then from HS patients FS, AS, SS and JS respectively. Gels F to J are of the corresponding pretreated ghosts (loadings, 20µl). Only spectrin and actin are extracted from normal ghosts (A) under these conditions. Note the loss and 'smearing out' in Band 4.2 in the HS membranes (G-J).

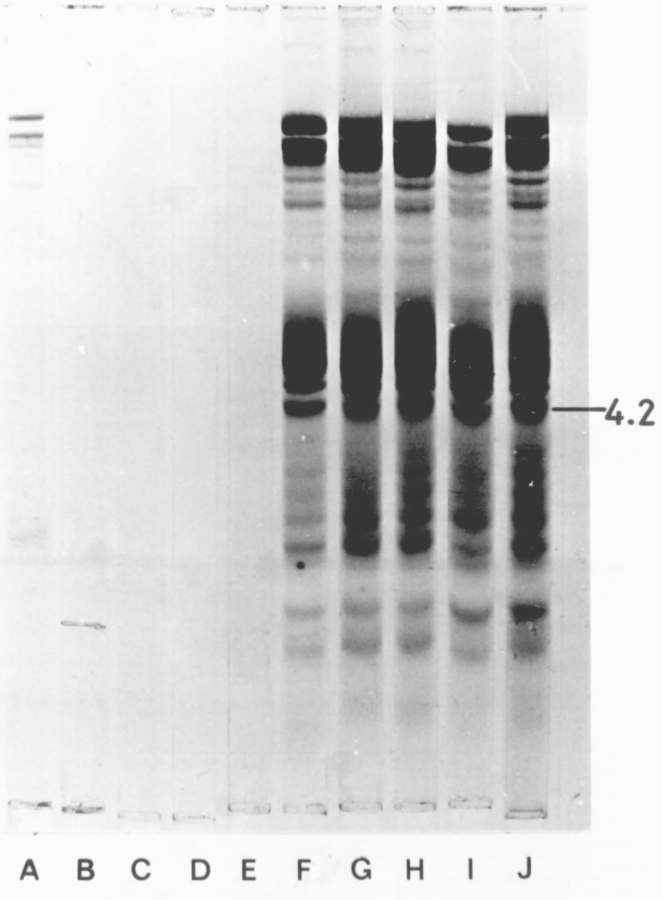



FIGURE 3.7

Figure 3.8

Freeze etch electron micrographs of fresh ghosts (A) and pretreated ghosts after pH 5.5 particle aggregation (B) from normal (N) and from the four related HS individuals. Note that aggregation of the intramembrane particles has occurred in the HS samples.

All magnifications x 50,000. Bar = 0.5μm

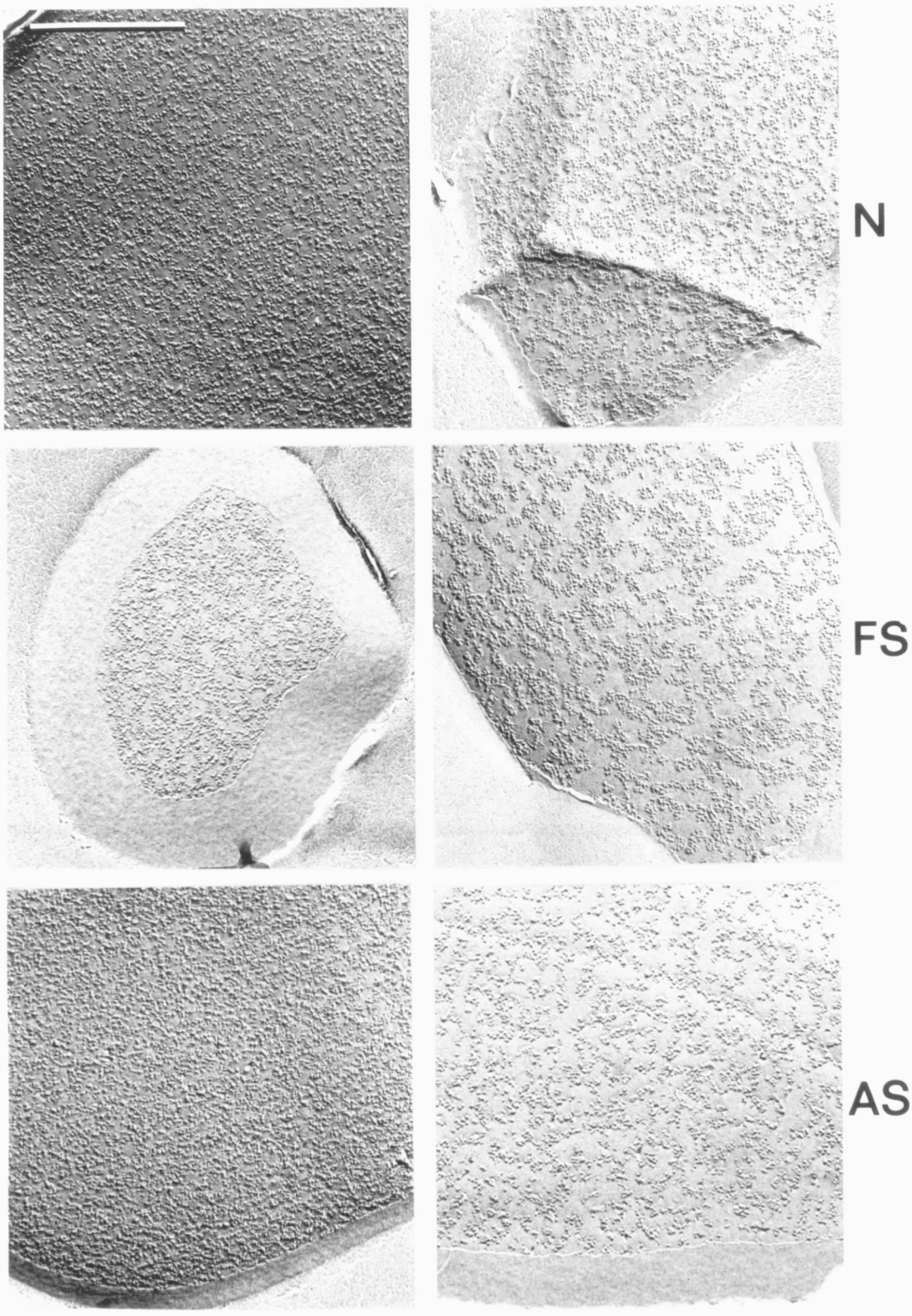



FIGURE 3.8

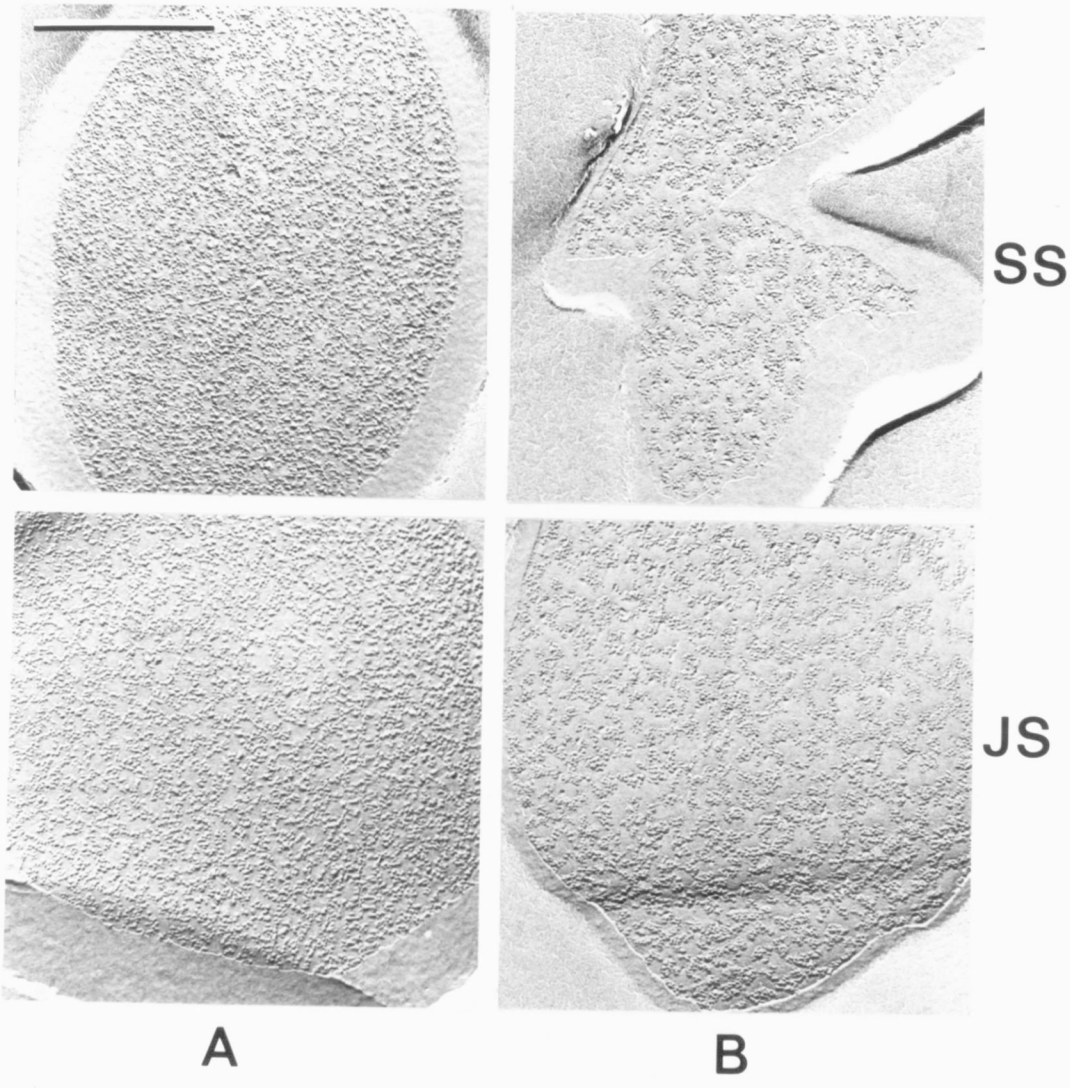



FIGURE 3.8 cont.

Figure 3.9

Cross fractured ghost from patient JS which appears filled with small vesicles. Note the large inside out vesicle towards the top of the micrograph.

Magnification, x 23,000



FIG. 3.9

or whether it also occurs in intact cells.

## 3. Discussion

The results presented here show no consistent pattern of membrane abnormalities associated with HS which could be interpreted as arising from a single molecular defect. All but patient DW exhibit features suggestive of a cytoskeletal lesion. In fact the findings that little spectrin tetramer appeared in the $0^{o}C$, low ionic strength extract of ghosts from patient JD and that spectrin was less extractable in the four related patients is indicative of spectrin being defective in these five cases.

Ghosts from six out of the eight patients showed particle aggregation greater than expected, indicating less cross linking of the spectrin-actin network which would be expected to allow increased micro-precipitation of the residual spectrin on the inner surface of the membrane. How this could be achieved in the case of the four related patients where the spectrin-actin network was apparently not disrupted during pre treatment is uncertain. One possibility, however, is that cross linking could be reduced by the dissociation of spectrin tetramers on the membrane to form heterodimers. This might have occured during the $37^{o}C$ pretreatment incubation.

The slight reduction in the amount of Band 4.2 present in the ghosts of patient JS is also difficult to interpret. However, since this was not apparent in the other affected members of her family and since she was suffering from jaundice, it presumably has the same origins as the Band 4.2 loss in other conditions associated with jaundice seen by Iida et. al. (1976). The changes in Band 4.2 in the four related cases following pretreatment cannot, as yet, be explained.

In view of the variability of these findings it is unwise to present any firm conclusions regarding any single molecular defect which might result in HS. Nevertheless, the balance of evidence suggests a cytoskeletal disorder, which is consistent with the fragility and stiffness of HS erythrocytes. However, it is not difficult to imagine that malfunction of any one of a number of erythrocyte cytoskeletal proteins could produce the same overall effects. It is therefore probable, in the light of this study and the many others which have been performed (Table 3.1), that the clinical condition known as hereditary spherocytosis may in fact be a manifestation of any one of a number of molecular defects.

# CHAPTER 4

## SPECTRIN PURIFICATION AND LOW ANGLE SHADOWING

### 1. Extraction and Purification of Spectrin

Both the 37°C and the 0°C extraction methods employed during this study were effective at removing spectrin and actin from ghosts. Although the 37°C extraction protocol was found to be rather more efficient and also very convenient in reducing the purification time of spectrin it was very often found that the crude spectrin extract contained a component migrating at the position of band 2.1 on SDS polyacrylamide gels (results not shown). This band was thought to be a proteolytic fragment of spectrin as band 2.1 is only known to be eluted from ghosts under high salt conditions (Tyler et al., 1979). In view of this, spectrin was routinely extracted at 0°C.

The spectrin extraction procedures used here were very similar to those employed by other groups. I found, however, that inclusion of 100μM EDTA in the spectrin extraction buffer was essential to avoid aggregation of the extracted material, regardless of the temperature of extraction. If ghosts were extracted in 0.3mM sodium phosphate buffer, pH 7.6, without the chelating agent present, a large amount of band 4.1 appeared in the crude extract. This is illustrated in Figure 4.1. Gel (c) is of crude spectrin extraction in EDTA, while gel (g) is a crude extract in 0.3mM sodium phosphate, pH 7.6. Both extractions were performed at 0°C.

Figure 4.1

Spectrin extraction and purification. Sodium dodecyl sulphate/polyacrylamide gels showing two 0°C extractions of spectrin and actin from fresh erythrocyte ghosts. (A) 10µl of fresh ghosts; (B) 10µl of ghost membranes, resuspended to their original volume in water, which had been extracted in 100µM EDTA, 10µM DTT, pH 9.5; (C) 20µl of the extract (D) 50µl of a sample taken from the void volume peak of a sepharose Cl 4B column loaded with (C) and run at high salt in the KCl/NaCl/Tris buffer (Figure 4.2c); (E) 10µl of fresh ghosts; (F) 10µl of ghost membrances extracted in 0.3mM phosphate buffer pH 7.6; (G) 20µl of the extract; (H) 20µl of a sample from the void volume peak of a sepharose Cl 4B column loaded with (G) and run at high salt as above. Note the presence of band 4.1 (arrow) in (G) and band 4.1 and actin (band 5) in (H).

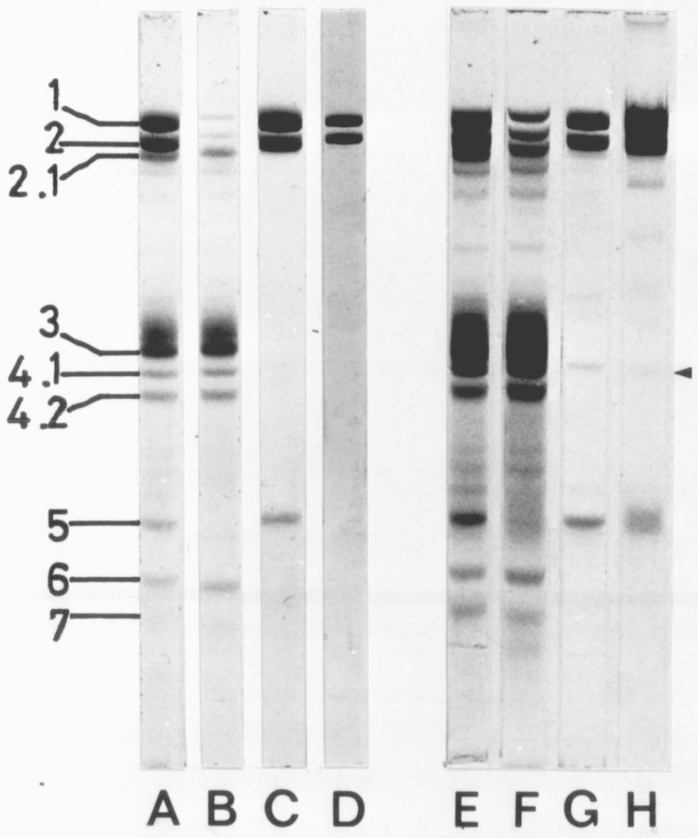



FIGURE 4.1

When these two extracts were fractionated on sepharose Cl 4B in the non volatile KCl/NaCl/Tris buffer, marked differences in the amount of material eluting at the void volume were apparent (Figure 4.2). The 0.3mM phosphate extracted material exhibited an extremely large void volume peak. SDS gels (Figure 4.1(h) ) showed that this peak contained, in addition to spectrin, most, if not all, of the extracted band 4.1 and some of the actin. EDTA extracted material showed a relatively small void volume peak (Figure 4.2(c)) containing exclusively spectrin (Figure 4.1(d)).

Crude spectrin extracts fractionated in the non volatile high salt buffer on sepharose Cl 4B gave results very similar to those obtained by Ralston et. al. (1977). The 37$^{o}$C extracted material was found to contain exclusively spectrin in the form of heterodimers eluting at approximately twice the void volume (Vo) of the column (Figure 4.2(b)). The 0$^{o}$C extracted material, on the other hand, contained predominantly spectrin tetramers which eluted at roughly 1.5 times the void volume (Figure 4.2(a,c)). The sedimentation coefficients of material eluting in the tetramer and heterodimerpeaks were 12.5s and 8.9s respectively (D.M. Shotton, 1978, personal communication).If a 0$^{o}$C extract was incubated for 30 minutes at 37$^{o}$C, prior to fractionation, partial conversion of tetramers to dimers took place (Figure 4.2(d)).

Spectrin tetramers from 0$^{o}$C extracts showed a marked tendency to dissociate into heterodimers when fractionated in the volatile ammonium acetate column buffer (Figure 4.3(b)). This occurred in spite of stringent precautions to ensure that the preparation remained in the temperature region of 0$^{o}$C-4$^{o}$C at all times. This was a surprising result in view of the finding of Ralston (1976) that high salt stabilises the tetramer and that conversion of tetramer to dimer, in non-volatile salts, was prevented by low temperature (Ungewickell and Gratzer, 1978). As expected, 37$^{o}$C extracted material, when fractionated in ammonium acetate, gave only the heterodimer peak (Figure 4.3(a)).

Figure 4.2

Elution profiles from sepharose Cl 4B in non-volatile buffer at physiological ionic strength (130 mM KCl , 20mM NaCl, 1mM EDTA, 0.5mM sodium azide, 10μM DTT, 10mM Tris. HCl, pH 7.4). (a) Extract from fresh erythrocyte ghosts made at 0°C using the 0.3mM phosphate extraction buffer. Note the large void volume peak. The non-aggregated spectrin elutes predominantly as the tetramer at about 1.5 times the void volume. (b) Extract from fresh ghosts made at 37°C using the EDTA extraction buffer. The majority of the spectrin is in the heterodimer form. (c) Extract from ghosts at 0°C using the EDTA extraction buffer showing spectrin to be mainly in the form of tetramers (note the reduced void volume peak when compared with (a) ). (d) 0°C, EDTA extracted from ghosts, warmed to 37°C for 30 minutes prior to loading of the column. Note the substantial conversion of tetramers to heterodimers.

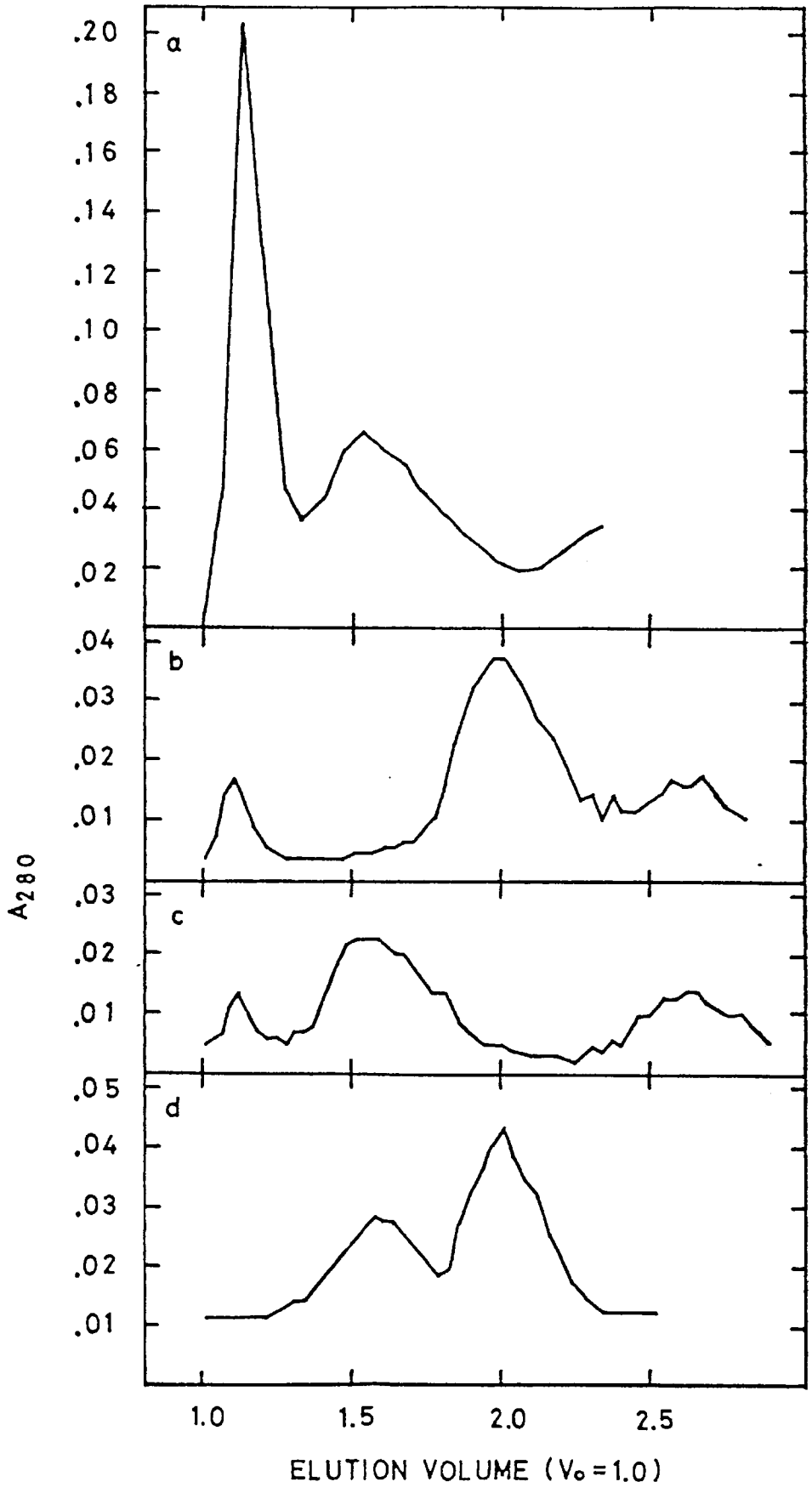

a
b
c
d
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
.04
.03
.02
.01
.03
.02
.01
.05
.04
.03
.02
.01
A280
1.0
1.5
2.0
2.5
ELUTION VOLUME (Vo = 1.0)

FIGURE 4.2

Figure 4.3

Elution profiles from sepharose Cl 4B in volatile buffer at physiological ionic strength (155mM ammonium acetate, 100μM EDTA, 10 μM DTT, ph 7.4). (a) Spectrin purified on sepharose Cl 4B from a 37°C EDTA extract of fresh ghosts. As in non volatile buffers, this 37°C extract contains predominantly heterodimers. (Compare with Figure 4.2b). (b) 0°C EDTA spectrin extract from fresh ghosts. Note that in this volatile column buffer much of the spectrin is in the form of heterodimers (compare with Figure 4.2C).

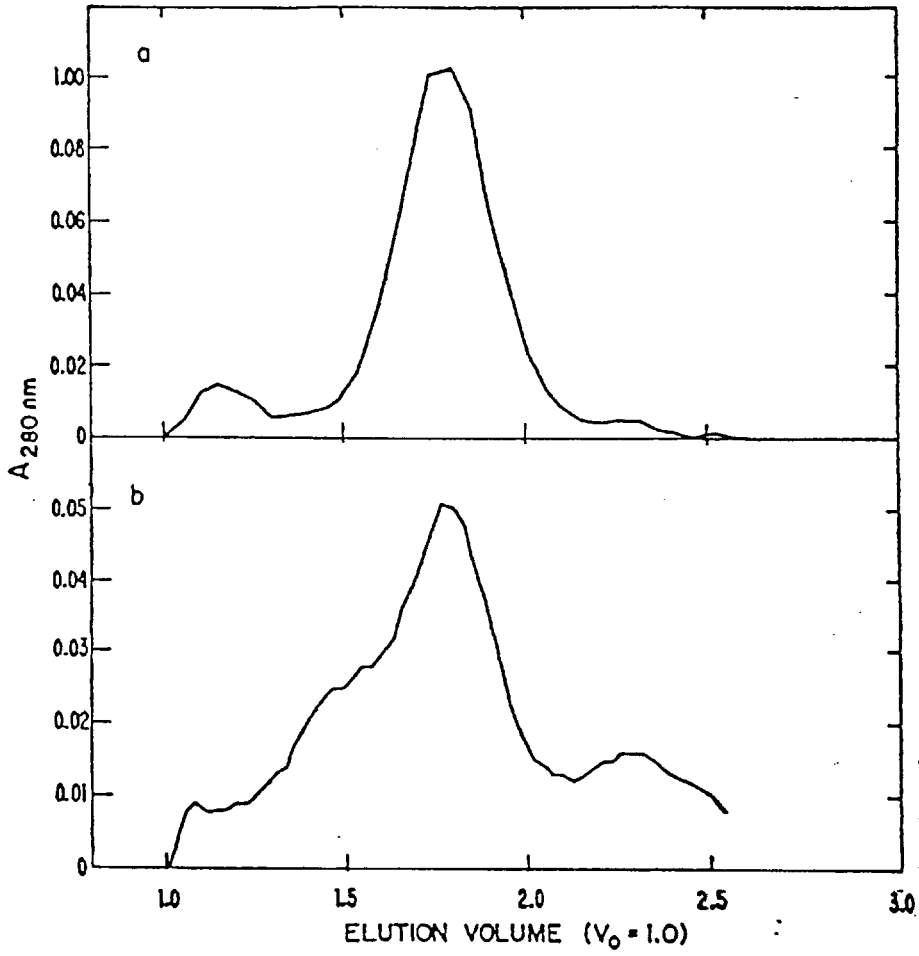

a
b
1.00
0.08
0.06
0.04
0.02
0
0.05
0.04
0.03
0.02
0.01
0
$A_{280\,nm}$
1.0
1.5
2.0
2.5
3.0
ELUTION VOLUME ($V_0$ = 1.0)

FIGURE 4.3

The reasons for this dissociation are unknown and no attempt was made to investigate it further. However, there appears to be no significant structural rearrangement of the molecule as the hydrodynamic and chiroptical properties of spectrin appear to be identical in both volatile and non-volatile buffer systems (D.M. Shotton, 1978, personal communication). It was, however, a very convenient phenomenon, as it allowed both spectrin tetramer and heterodimer preparations, which could be used directly for low-angle shadowing, to be obtained from a single unwarmed, unproteolysed low temperature extract. It was also noted that spectrin tetramers, heterodimers and actin, all eluted slightly closer to the void volume of the sepharose 4B colum when in the volatile ammonium acetate buffer than when in the non volatile KCl/NaCl/Tris buffer. The reasons for this are again unknown.

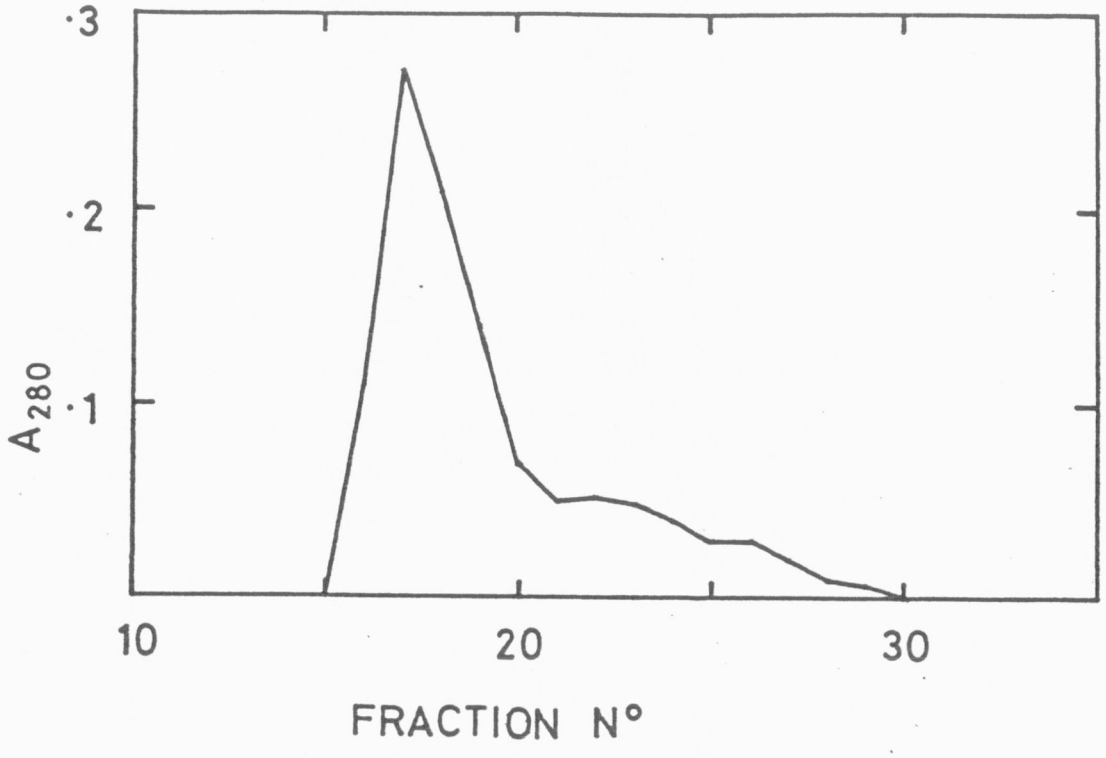
Spectrin, purified at low ionic strength, which eluted free from actin at the void volume of the sepharose 4B column (Figure 4.4) was not used for any structural studies. This method of purification was, however, useful in obtaining spectrin at sufficiently high a concentration to be employed for immunological purposes, which will be discussed at length in later chapters.

## 2. Myosin- Low Angle Shadowing

The technique of low angle shadowing has been used successfully to elucidate the strcuture of a number of biological macromolecules, notably collagen (Hall and Doty, 1958), fibrinogen (Hall and Slayter, 1959) and myosin (Slayter and Lowey, 1967; Lowey et. al., 1969; Elliott et. al., 1976; Elliott and Offer, 1978). Myosin was chosen as a control for this electron microscopic investigation of spectrin as its characteristic double headed morphology would provide a useful check on the resolution of the shadowing technique and furthermore, because a redetermination of its tail length would test the accuracy of the measurement protocol.

Figure 4.4.

Fractionation of spectrin and actin at low ionic strength. Above is shown the elution profiele of a $0^{o}C$ EDTA extract of fresh ghosts from a sepharose Cl 4B column equilibrated in 100μM EDTA, 10μM DTT, 1mMTris HCl, pH8.0. The void volume (Vo) is at fraction 15. The SDS/polyacrylamide gels are as follows. (A) 50μl of the $0^{o}C$ low ionic strength ghost extract which was applied to the column. (B) 50μl sample from the void volume peak (fraction 18) which contains pure spectrin. (C) 100μl sample from the actin peak (band 5) at fraction 22.



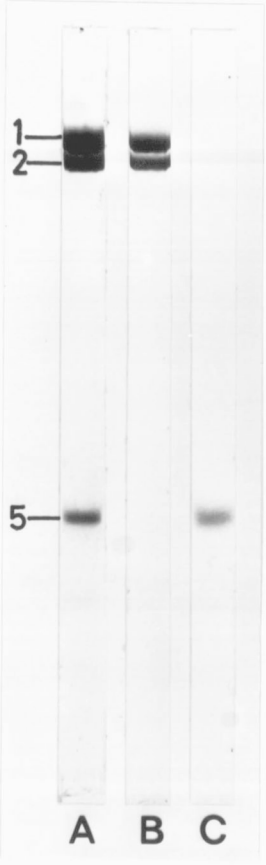




FIGURE 4.4

Figure 4.5 shows both unidirectionally (4.5(a) and rotary (4.5(b)) shadowed rabbit skeletal muscle myosin molecules. Its double headed structure, with uniform tail is clearly in evidence. None of the molecules showed any tendency to align, indicating that there was little or no shear on drying. The presence of non volatile components (100μM EDTA, 10μM DTT) in the shadowing buffer caused no loss in resolution. In fact, all non volatile material appeared to be confined to a narrow ring at the periphery of each droplet and a deposit at the centre of the droplet. In subsequent experiments, I found that larger clear areas containing well defined protein molecules could be obtained using NaCl at final concentrations as high as 50mM. This is in contrast to the assertion of Slayter (1976) that 100μM was the upper limit for non volatile components. More recently, Tyler and Branton (1980) have shown that provided glycerol was present, large amounts of non volatile materials such as KCl (200mM), Triton X-100 (up to 0.2%) and sucrose (up to 10%) could be tolerated in the shadowing buffer without loss of resolution.

Measurement of myosin tail lengths gave an uncorrected mean value of 147nm±13nm (S.D.) for 106 unidirectionally shadowed molecules (Table 4.1). The overall distribution of these lengths is shown in Figure 4.6(a)). This value is in good agreement with that published by Elliott et. al. (1976) of 150 ± 20 nm also for unidirectionally shadowed molecules. Elliott et. al., (1976) reported a skewing of their distribution towards shorter lengths which they concluded was a consequence of (a) fragmentation of tails during spraying and (b) apparent foreshortening due to some tails drying raised up in loops off the mica surface rather than fully extended.

The histogram shown here for unidirectionally shadowed myosin (Figure 4.6(a)) appears to exhibit a similar effect.

Figure 4.5.

Myosin molecules after (a) unidirectional and (b) rotary shadowing. These and subsequent electron micrgraphs of low angle shadowed molecules are printed with normal rather than reversed contrast, platinium grains appearing black, and shadows white. The micrographs of unidirectionally shadowed molecules are printed with the shadow direction from below.

Magnification: x 100,000, Bar = 100nm.

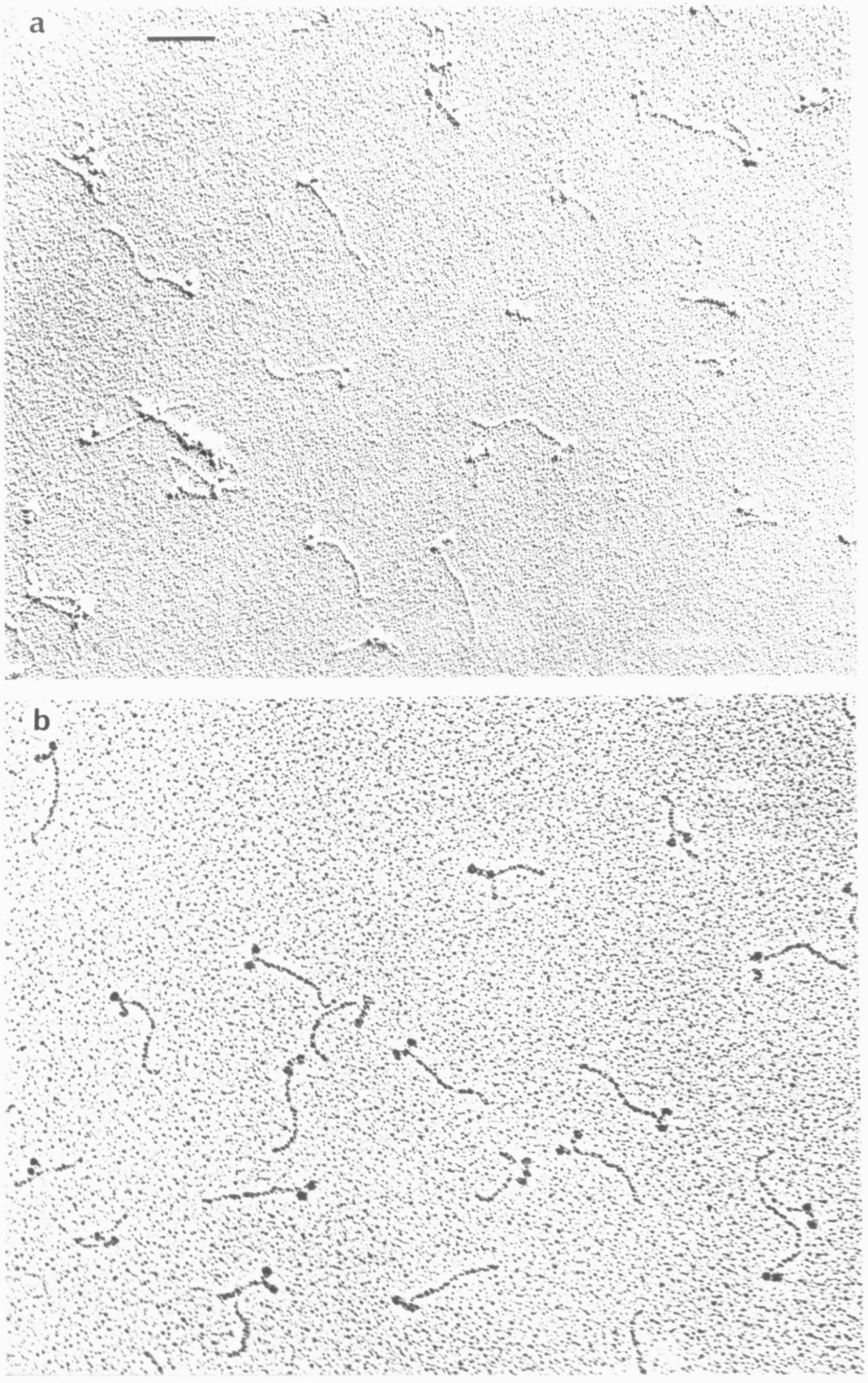



FIG. 4.5

Figure 4.6.

Histograms showing the distribution of molecular lengths after ((a) to (c)) unidirectional and ((d) to (f)) rotary showing. (a) and (d) myosin tails; (b) and (e) spectrin heterodimers; (c) and (f) spectrin tetramers. Histograms are plotted in nm, at intervals of approx. 0.05 of the molecular lengths.

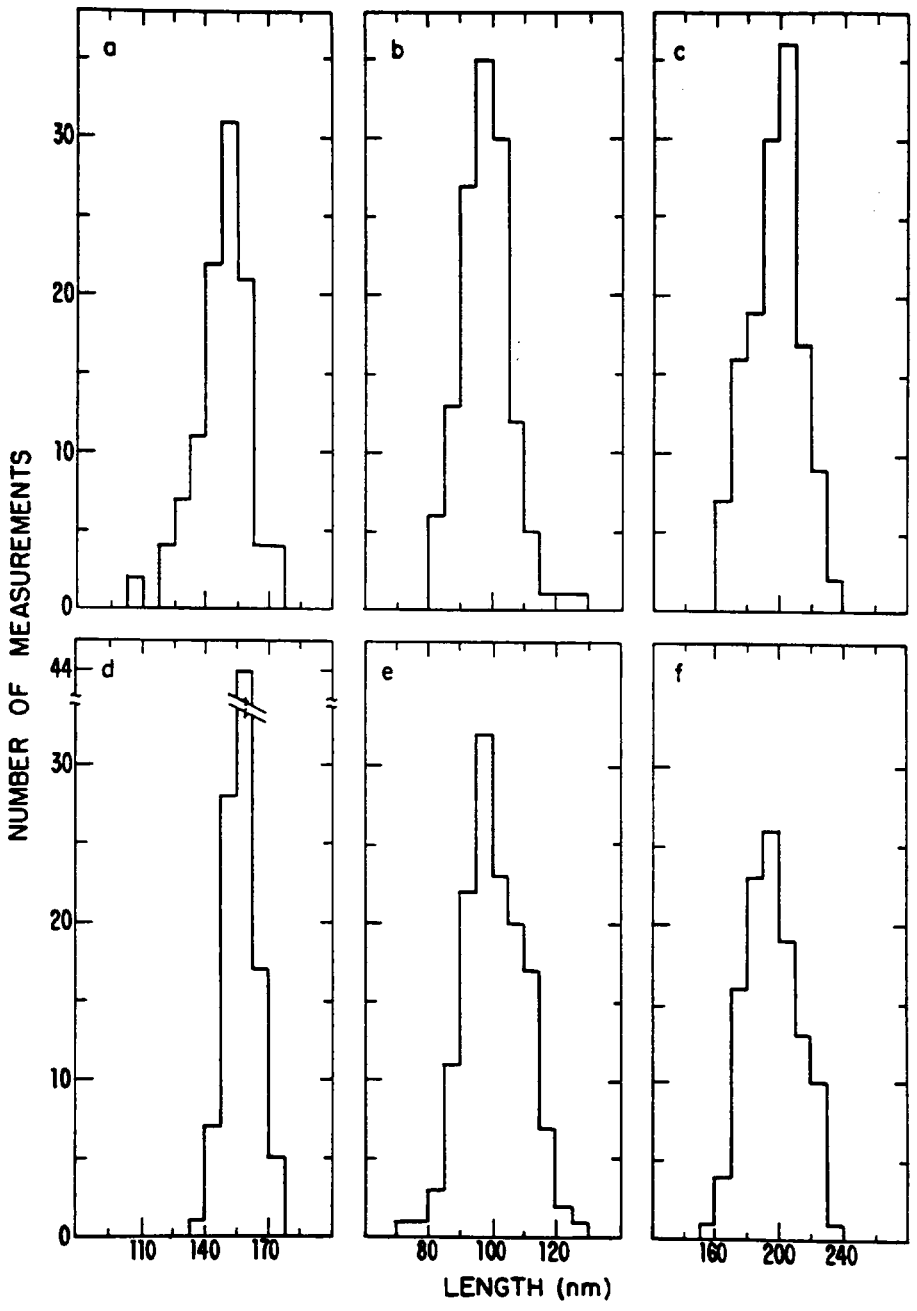

a
b
c
d
e
f
NUMBER OF MEASUREMENTS
0
10
20
30
44
110
140
170
80
100
120
160
200
240
LENGTH (nm)

FIGURE 4.6

T A B L E 4.1 Observed Molecular Length Measurements

| PROTEIN | SHADOWING TECHNIQUE | MEAN LENGTH(nm) | STANDARD DEVIATION(nm) | NO. OF MEASUREMENTS |
|---|---|---|---|---|
| Myosin tail | U | 147 | ± 13 | 106 |
| Myosin tail | R | 157 | ± 7 | 103 |
| Spectrin Heterodimers | U | 98 | ± 8 | 131 |
| Spectrin Heterodimers | R | 101 | ± 10 | 140 |
| Spectrin Tetramers | U | 197 | ± 15 | 136 |
| Spectrin Tetramers | R | 196 | ± 17 | 113 |

Rotary shadowed myosin exhibited a value for mean tail length of 157nm ± 7nm (S.D.) from a sample of 103 molecules. I consider that this value is a more accurate estimate of tail lenth than that obtained for the unidirectionally shadowed myosin, as the junction between the tail and the heads was much more easily distinguished in rotary shadowed molecules. In unidirectionally shadowed myosin preparations the junction was often obscured by the shadow thrown by one of the heads. This increase in accuracy of measurement was indicated by the very much smaller standard deviation associated with the rotary shadowed molecules, ± 7 nm compared to ± 13 nm. The length distribution of these measurements is shown in Figure 4.6(d).

It is quite apparent from these rotary shadowing results that the foreshortening proposed by Elliott et al., (1976) is not as significant as had first been though; although, as shown in the stereoelectron micrographs of Figure 4.7, myosin tails may indeed dry raised up in loops off the mica surface. I conclude, therfore, that the lower mean length and greater scatter for the unidirectionally shadowed molecules is a direct

Figure 4.7

Stereo electron micrographs of rotary low angle shadowed myosin molecules. Note how the tails of some of these molecules (e.g. in (a) ) loop up off the flat mica surface. The great flexibility in the junction between the heads and the tails of these molecules is also evident in (b).

These micrographs were taken employing a specimen tilt of + and - $15^{o}$ (total tilt, $30^{o}$).

Magnification, x 170,000, Bar = 100nm.

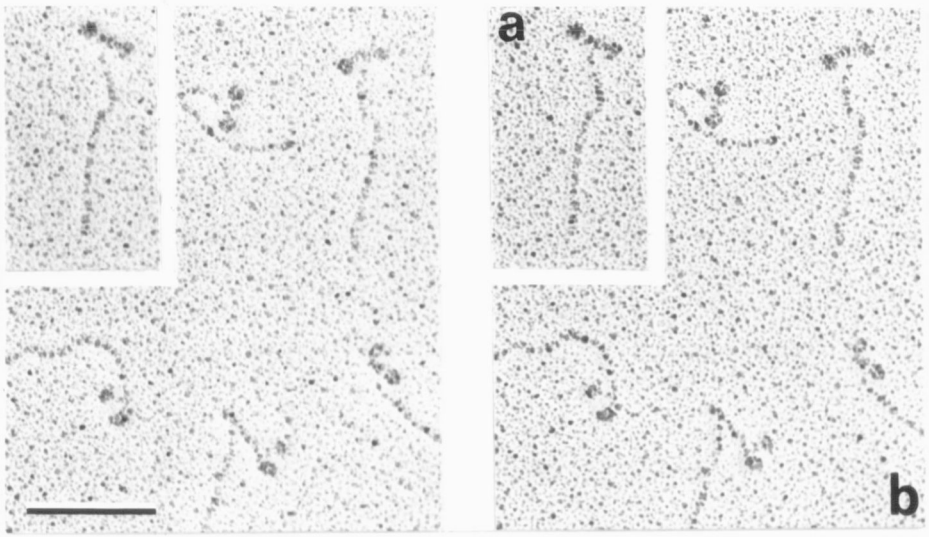



FIGURE 4.7

consequence of the inherent difficulties in obtaining accurate measurements and that the value obtained from rotary shadowed myosin molecules is a more reliable estimate of tail length. The value obtained here, is in fact in excellent agreement with that of 156(± 5) nm reported by Elliott and Offer (1978) for the tail length of rotary shadowed skeletal muscle myosin.

3. Spectrin Heterodimers - Low Angle Shadowing

In view of the fact that the shadowing method used here, involving spraying and drying at room temperature in the presence of high concentrations of glycerol, produced images of myosin comparable to those obtained by the more elaborate low temperature drying techniques of Elliott et. al. (1976) and Elliott and Offer (1978), it was considered unnecessary to use these more complex procedures to examine spectrin. Figure 4.8 shows typical fields of unidirectionally and rotary shadowed spectrin heterodimers sprayed in and dried from ammonium acetate and glycerol at room temperature. They appeared as long flexible molecules exhibiting a variety of conformations which could be reproduced quite consistently in different shadowing experiments. In their most extended form they appeared as compact rods. The majority, however, tended to lie as curved 's' or 'c' shapes or collapsed upon themselves.

Many of the molecules appeared to lie partially unfolded and could quite clearly be seen to be composed of two strands which extended the entire length of the heterodimer. This was most obvious in the rotary shadowed preparations. The simplest interpretation is that these two stands represent the two subunit polypeptides of the spectrin heterodimer, band 1 and band 2, and that in the native molecule these two associate side by side. Characteristic morphologies of hetero-

Figure 4.8

Spectrin heterodimers after (a) unidirectional and (b) rotary shadowing.

Magnification x 100,000 Bar = 100nm.




FIG. 4.8

Figure 4.9

Spectrin heterodimers after unidirectional low angle shadowing. In this figure and in Figures 4.10, 4.12 and 4.13, images have been selected to show the typical variety of curved, helical and partially unfolded configurations which the molecules adopt. In many cases, the two strands which comprise each heterodimer molecule are clearly visible (arrows).

Magnification, x 170,000. Bar = 100nm.

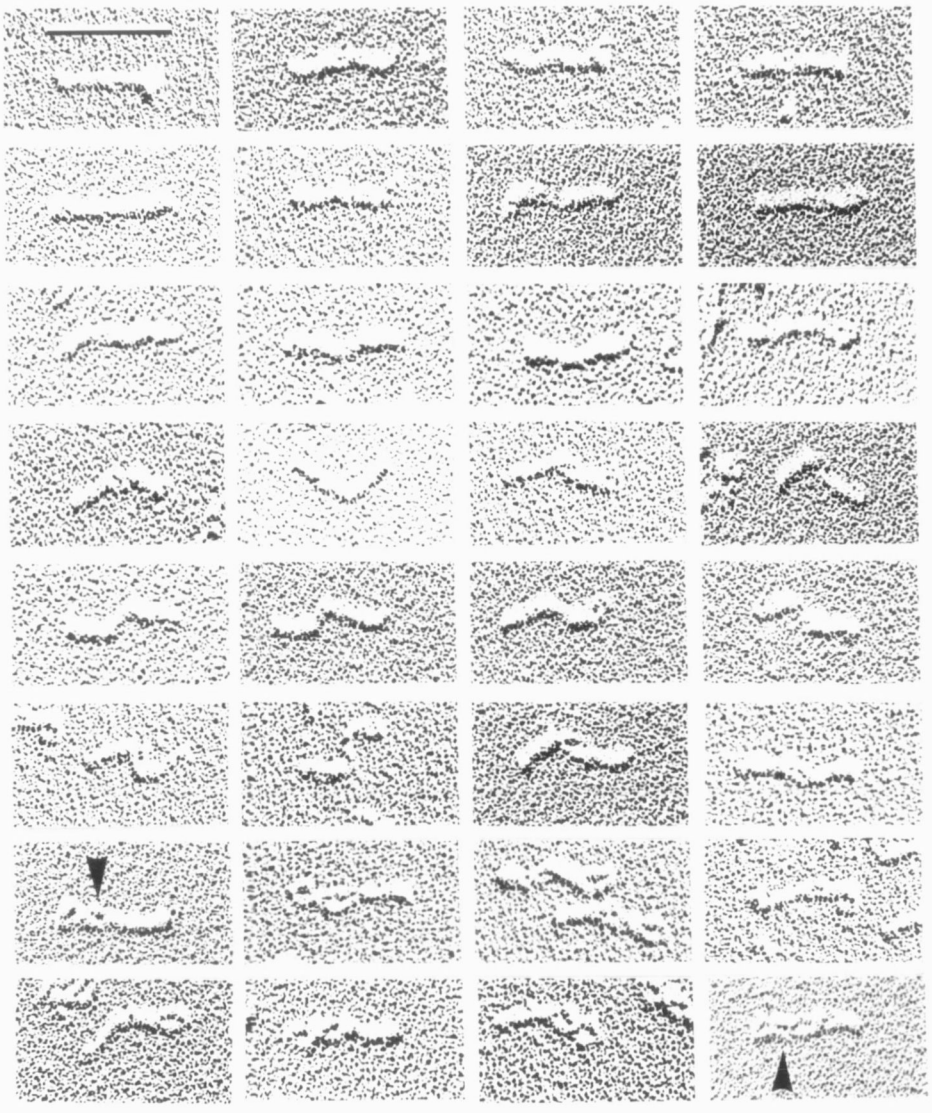


FIG. 4.9

Figure 4.10

Spectrin heterodimers after rotary shadowing. Note the close association of the two individual strands of each heterodimer (arrows) at the termini of the molecules. Separation of the strands at either terminus (as shown bottom right) is rarely seen.

Magnification, x 170,000, Bar = 100nm.

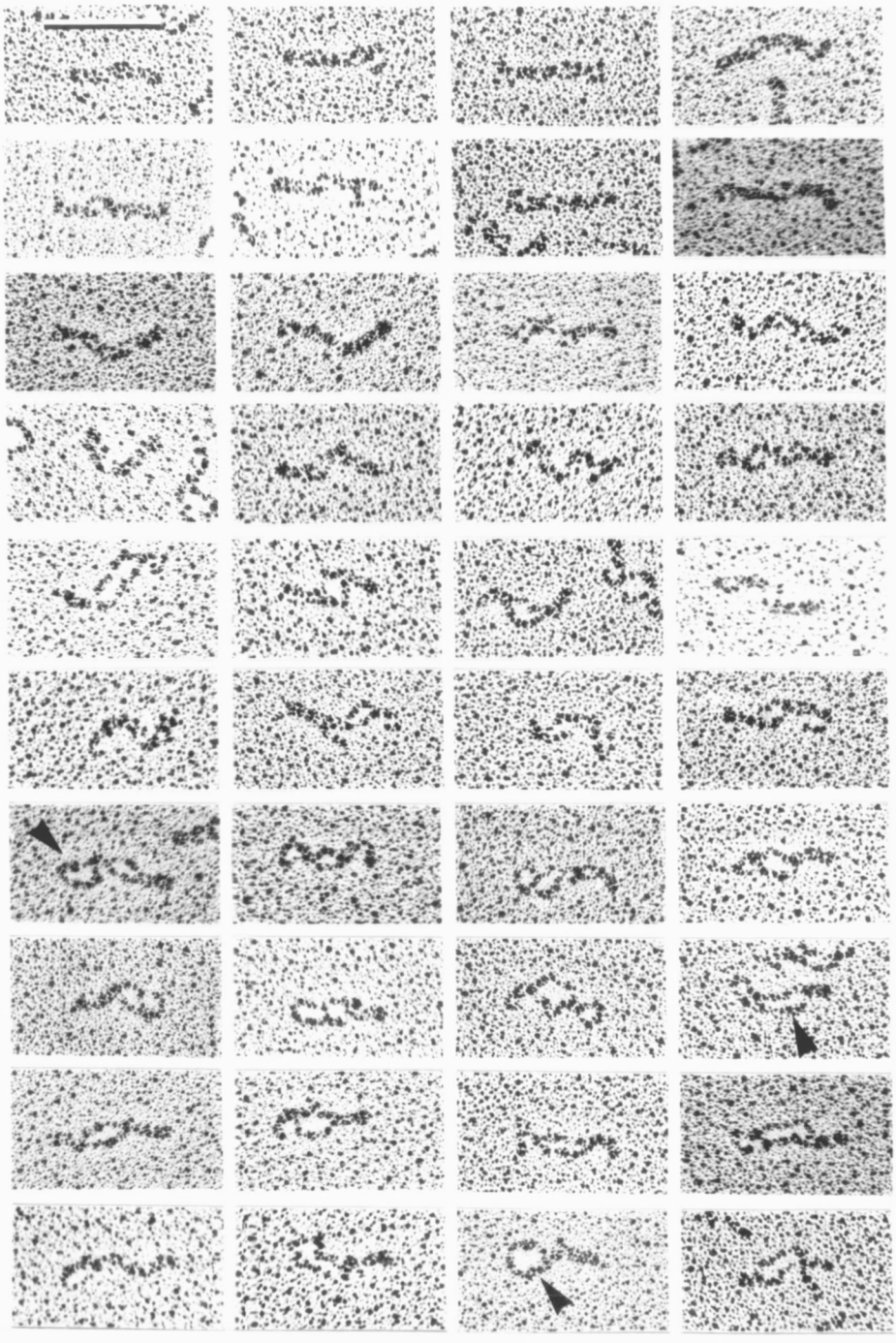


FIG. 4.10

dimers are shown in Figure 4.9 and 4.10.

The two strands appeared to be very loosely associated. In unidirectionally shadowed preparations they could often be seen to be twisted around each other in a loose double helix (Figure 4.9). However, both right and left handed helices could be seen suggesting that this coiling was a rather non specific phenomenon, merely reflecting the highly flexible nature of the two strands.

The most consistent feature of the association between the two subunits was seen in rotary shadowed preparations. Even in molecules where the two strands lay separated for most of their lengths, it was extremely rare to see separation at either of the ends of the molecule (such as is shown in the bottom right hand micrograph of Figure 4.10), indicating strong binding in these terminal regions.

In molecules where the two strands were clearly separated, measurements were made by tracing the digitizer cursor along the best resolved of the two. From a sample of 131 unidirectionally shadowed molecules, the mean uncorrected length was determined as 98nm $\pm$ 8 nm (S.D.) while the value for a sample of 140 rotary shadowed heterodimers was found to be 101 nm $\pm$ 10 nm (S.D.)(Table 4.1). The respective histograms for these length determinations are found in Figures 4.6(b) and (c). The close agreement between these two values is in sharp contrast with the myosin results, confirming that the disagreement in this case was due to the peculiarities of myosin's shape and the difficulties involved in carrying out precise measurements which resulted from it.

## 4. Spectrin Tetramers - Low Angle Shadowing

Unidirectionally and rotary shadowed spectrin tetramers are shown in Figure 4.11. Length measurements gave an uncorrected value of 197 nm $\pm$ 15 nm (S.D.) for a sample of 136 unidirectionally shadowed molecules and a value of 196 nm $\pm$ 17 nm (S.D.) for 113 rotary

Figure 4.11

Spectrin tetramer molecules after (a) unidirectional and (b) rotary shadowing.

Magnification x 100,000, Bar = 100nm

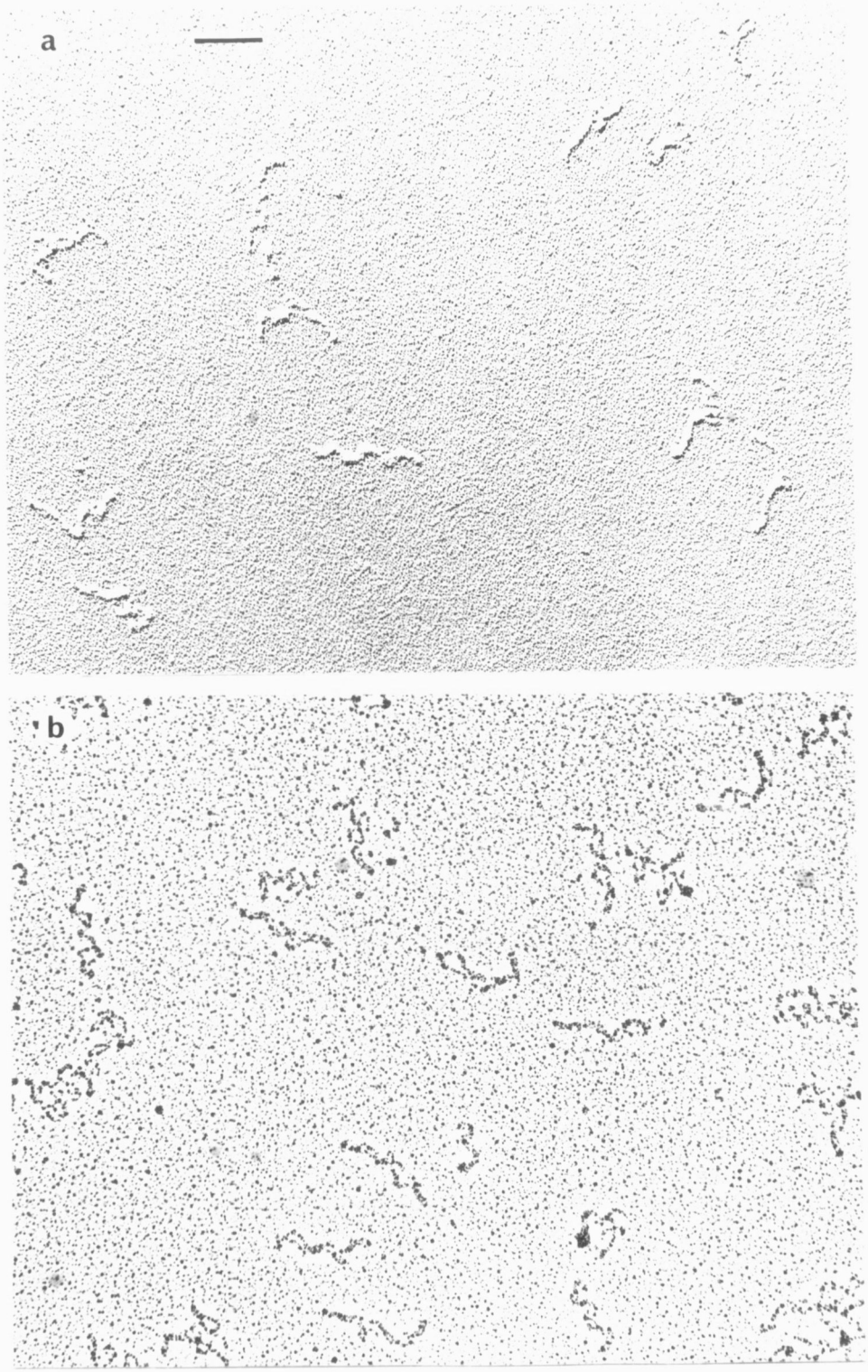



FIG. 4.11

Figure 4.12

Spectrin tetramer molecules after unidirectional shadowing.

Magnification, x 170,000  Bar = 100nm.

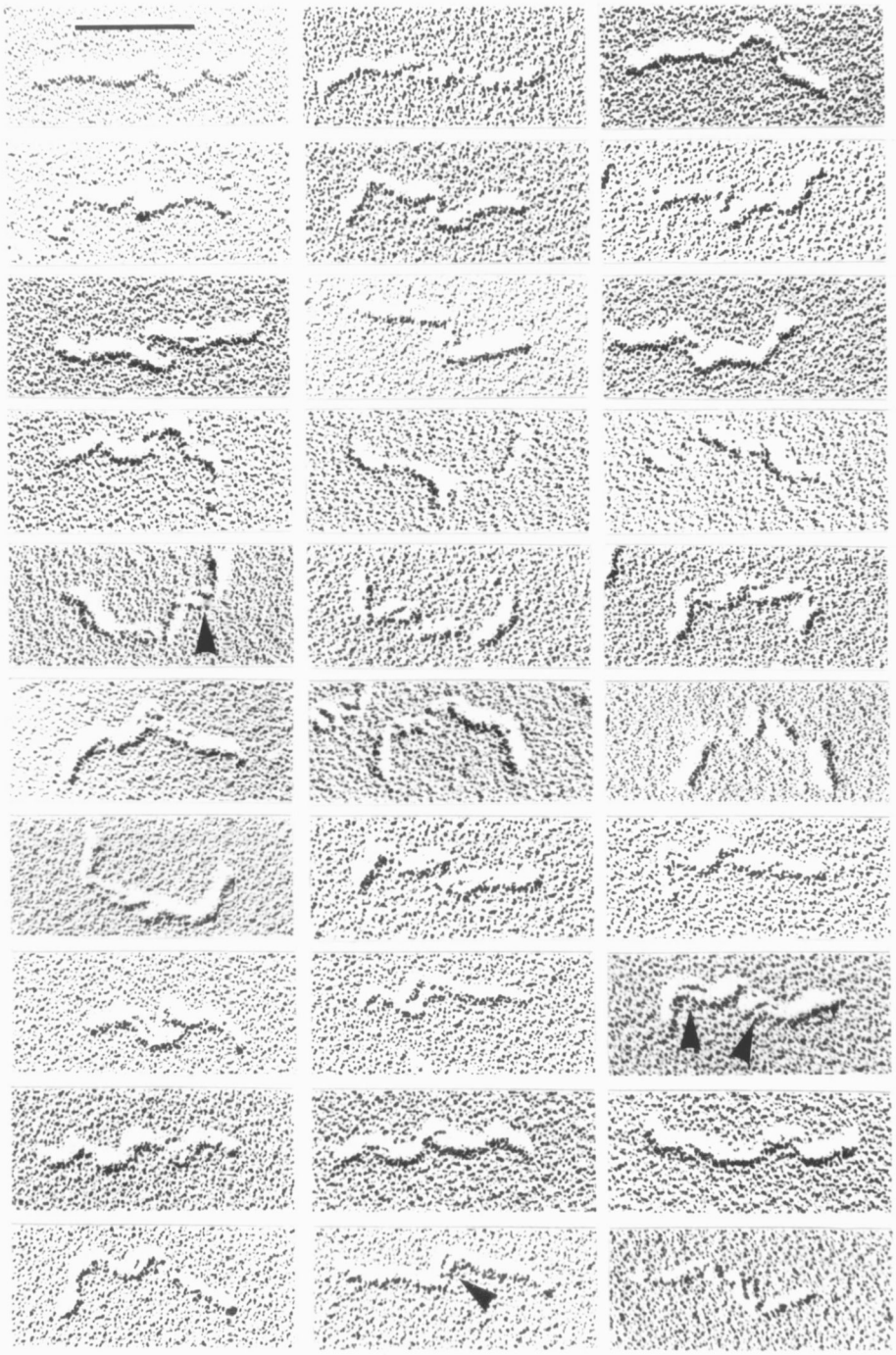


FIG. 4.12

Figure 4.13

Spectrin tetramers after rotary shadowing. Note the individual strands (arrows) and their frequent separation at the midpoint of the molecules (e.g. bottom row).

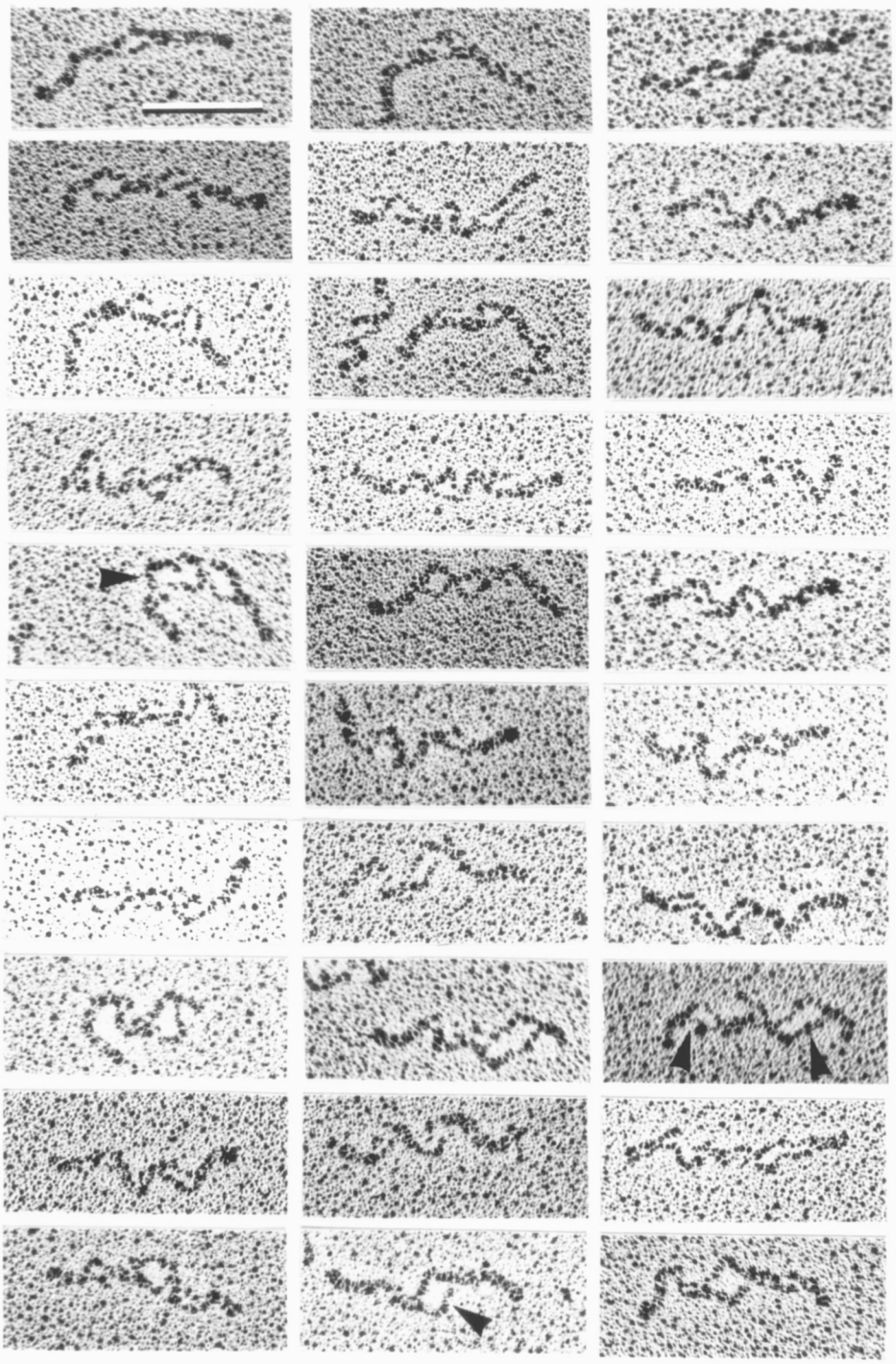


FIG. 4.13

shadowed molecules (Table 4.1). The respective distributions are given in Figure 4.6(c) and (f). It is apparent that spectrin tetramers are formed by end to end association of two heterodimers without measurable overlap. In all tetramer preparations, a small number of heterodimers was always found to be present. Presumably this was due to the effects of ammonium acetate which has already been shown to cause dissociation of tetramers to heterodimers.

Typical morphologies of the spectrin tetramers are shown in Figure 4.12 and 4.13. As with the heterodimers, two strands could be resolved, which often appeared to be loosely coiled in both right and left handed helices (Figure 4.12). A notable feature of the tetramers, which was especially clear in rotary shadowed preparations, was that in the central region of the molecule where it would be expected to find the termini of the two constituent heterodimers, the two strands were often seen to be separated (as in Figure 4.13, bottom row, centre). It would,of course, have been expected to find the strands firmly linked in this region. Presumably in the tetramer binding between sister strands of a single heterodimer becomes replaced by interdimer binding.

Many tetramers, seen in the two dimensional projection of an electron micrograph appear sinusoidal (Figures 4.12 and 4.13 row 9, centre). The use of stereo photography, however, shows that such molecules are, in fact, coiled and loop up off the mica surface in super helices (Figure 4.14). However, as with the coiling of the subunits about each other, these super helices are both left and right handed, indicative of the highly flexible nature of the molecule.

Since Harris et al. (1978) have suggested that sonication may cause degradation of spectrin it was felt important to demonstrate that control preparations of both heterodimers and tetramers, in which sonication was avoided during initial extraction of spectrin, gave images identical to those already seen. Such a preparation is shown in Figure 4.15.

Figure 4.14

Stereo electron micrographs of rotary low angle shadowed spectrin tetramers. Note how some of the molecules have adopted a very regular helical conformation. This is particularly evident in (c) and (e). A spectrin hexamer (arrow) is also seen in (a). Each stereo pair was obtained using a specimen tilt of + and - $12^{o}$ (total tilt = $24^{o}$).

Magnification, x 170,00, Bar = 100nm.

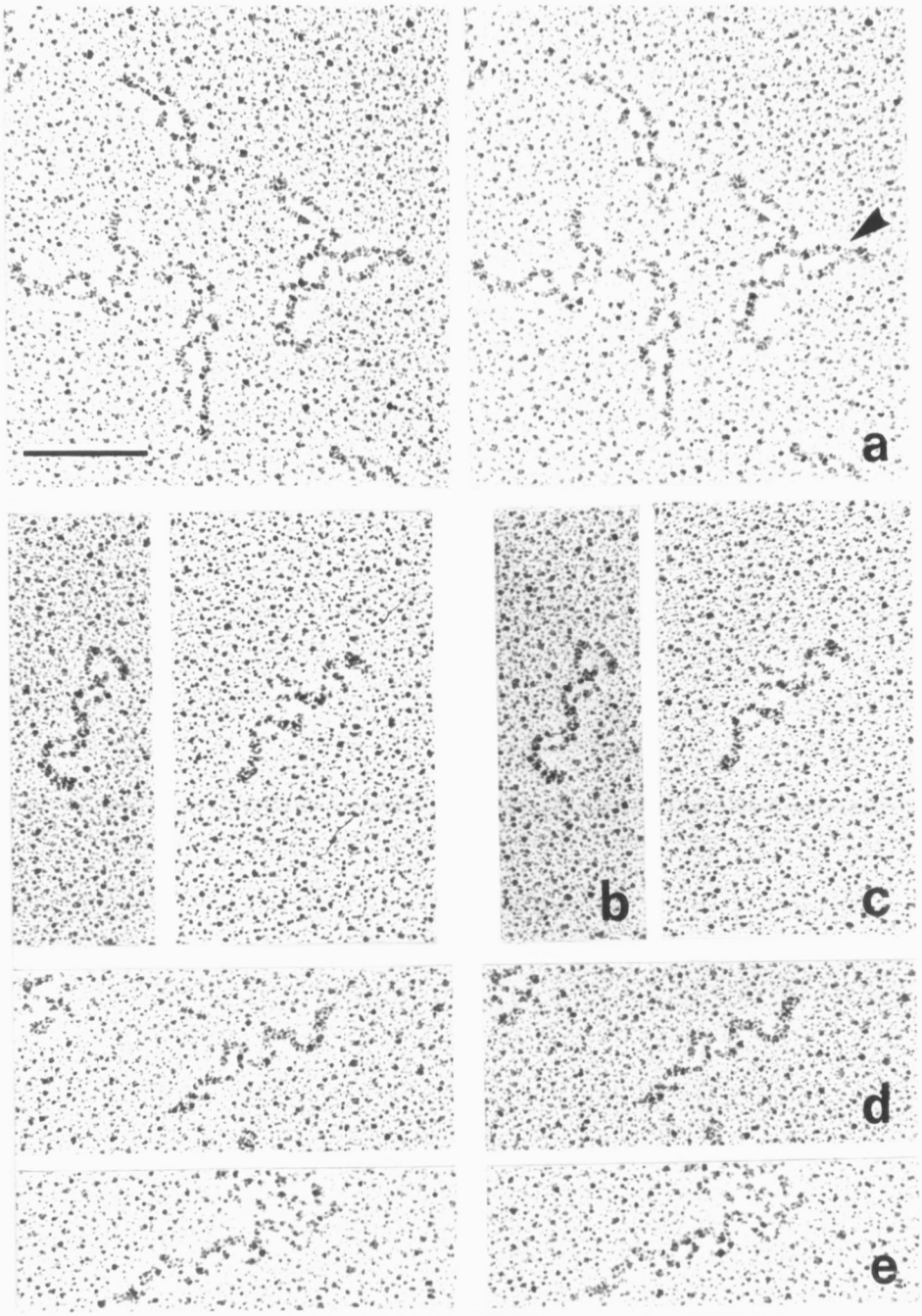



FIGURE 4.14

Figure 4.15

Rotary shadowed specimens of (a) spectrin tetramers and (b) spectrin heterodimers prepared without sonication. These are indistinguisable from sonicated specimens. (compare with Figures 4.8 and 4.11).

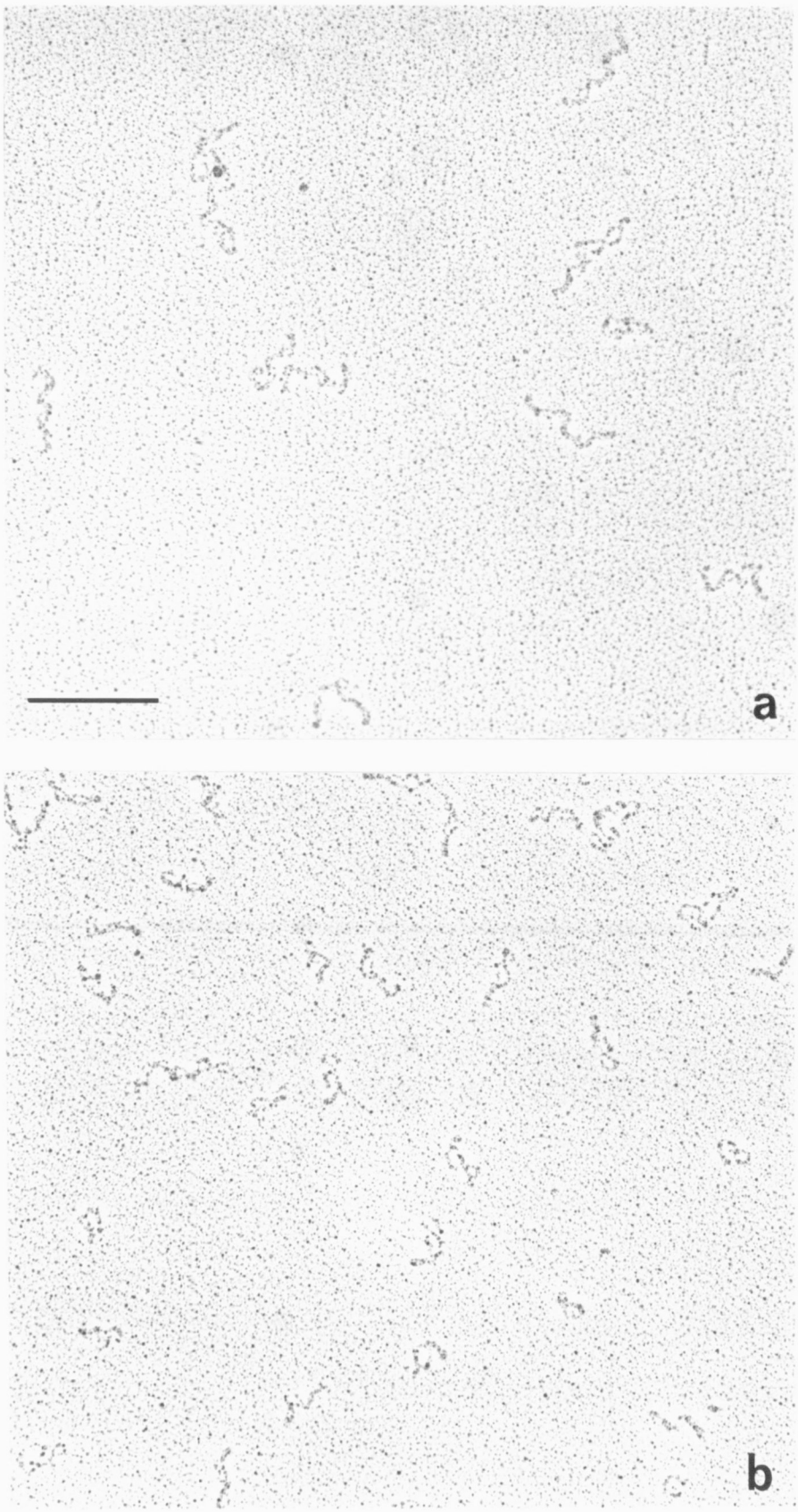



FIGURE 4.15

Thus, any possibility that these images were of molecules damaged or altered in some way by sonication has been ruled out.

5. Estimation of True Molecular Lengths - Correction for Shadow Thickness

A major source of error in determining the lengths of protein molecules after low angle shadowing is the uncertainty concerning the correction to apply for the presence of the shadow material, in this case Pt/C, which results in the observed length exceeding the actual length by a small value. Slayter (1976) determined experimentally the correction to be -5nm for molecules unidirectionally shadowed with $2 \times 10^{-6} g/cm^2$, Pt/C and -2.5nm for molecules rotary shadowed with the same amount of Pt/C. The shadow thicknesses used in this investigation were $1.4 \times 10^{-6} g/cm^2$ (unidirectional) and $1.9 \times 10^{-6} g/cm^2$ (rotary). The corrections applied were thus -3nm for unidirectional shadowing and -2 nm for rotary shadowing. For myosin an additional arbitrary correction of +1 nm was included to compensate for the foreshortening of some myosin tails as described by Elliott et. al. (1976).

As discussed earlier, it was felt that values obtained from rotary shadowed myosin more accurately reflected the length of the myosin tail. After due correction, a value of 156 nm $\pm$ 7 nm (S.D.) was obtained, in good agreement with that previously determined by Elliott and Offer (1978). For spectrin heterodimers and spectrin tetramers the mean of the corrected values for unidirectionally and rotary shadowed preparations was taken. For heterodimers this gave a mean contour length of 97nm. For spectrin tetramers, a figure of 194 nm was obtained.

## 6. Diameter of the Extended Spectrin Molecule

Estimates of molecular thickness from shadowed images of elongated molecules are extremely unreliable because of the inherent difficulties in accurately correcting for the shadow thickness which is of the same order of magnitude as the thickness of the molecule itself. Measurements of length on the other hand are not subject to such severe errors as, at least in the case of spectrin, the molecular length is about 100-200 times the shadow thickness and reasonable corrections, proportionately much smaller, may be made, as described above.

It was, however, possible to obtain a reasonable estimate of the diameter of the spectrin molecule by making a direct visual comparison between spectrin and myosin molecules which had been shadowed at the same time, side by side on the specimen stage during the same experiment, and hence had received exactly the same amount of platinum/carbon. When molecules photographed under identical conditions were compared in this way, spectrin heterodimers and tetramers appeared to be considerably broader than the myosin tail, which consists of two supercoilded $\alpha$ helical polypeptides. It was occasionally found that two myosin molecules would associate via their heads and lie on the mica surface with their tails close together or coiled around each other (Figure 4.16). Comparison of spectrin heterodimers and tetramers with these 'four headed' myosin pairs showed the spectrin molecules to be only slightly broader than the two myosin tails. Each of the two single polypeptide strands from a single spectrin molecule were found to be slightly narrower than the two myosin tails but broader than a single tail (Figure 4.16). As the diameter of the two supercoiled $\alpha$-helices which form the tropomyosin molecule has been determined to be approximately 2.0nm ( Cohen, C. and Phillips, G., unpublished results ) this suggests that an individual polypeptide strand from a spectrin molecule has a diameter of between 2-3 nm. The

Figure 4.16

Rotary shadowed images of (a) single myosin molecules, (b) pairs of entwined myosin molecules, (c) spectrin tetramers and (d) spectrin heterodimers. All the molecules were shadowed in the same experiment, receiving identical exposure to the stream of platimum vapour. The shadow thickness on all the molecules can thus be assumed to be the same. (Mean pt shadow thickness, 0.78nm) so that direct visual comparisons of molecular size is possible (see text).

Magnifications all x 170,000. Bar = 100nm.

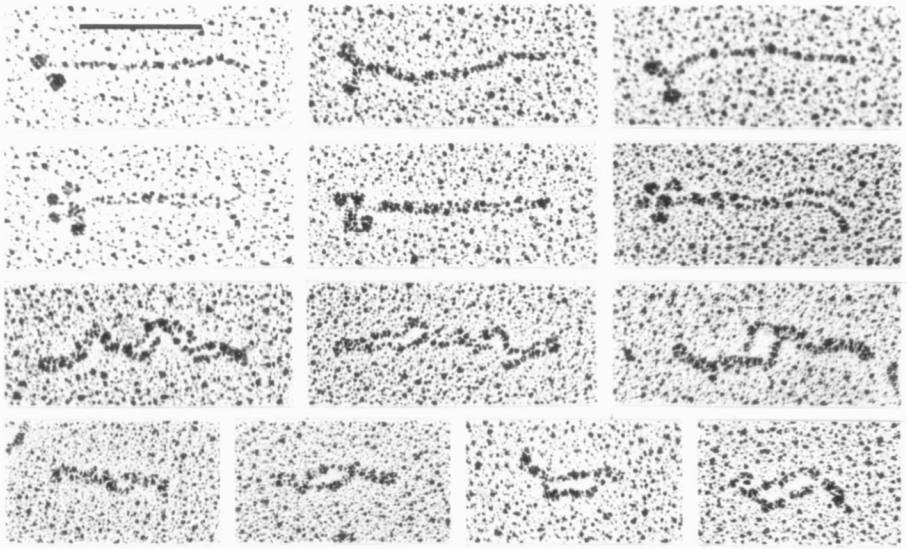


FIG. 4.16

diameter of the complete heterodimer and tetramer must therefore be in the region of 4-6 nm.

## 7. Higher Oligomers of Spectrin

With the exception of large aggregates often containing actin and band 4.1, which elute at the void volume of sepharose 4B columns run at high salt, the existence of oligomeric forms of spectrin larger than the tetramer has not so far been reported. In this investigation no linear oligomers larger than the tetramers were seen. Occasionally what appeared to be linear octamers were identified, but these were so infrequent that they may be reasonably accounted for as chance juxtapositions of two spectrin tetramers.

A non linear spectrin hexamer was, however, identified. Examples of these hexamers is shown in Figure 4.17(a). One is also seen in stereo in Figure 4.14(a). These structures were initially identified as a minor species in the tetramer peak of $0^{o}C$ crude spectrin extracts fractionated on sepharose 4B at high salt and were assumed to be an artefact. However, even in dilute, well spread preparations they remained a consistent feature. Recently it has been demonstrated in this laboratory that the structures show a peak in frequency of occurrence which lies approximately halfway between the void volume peak and the tetramer peak (A.E. Burnip, 1980, personal communcation). Analysis of the arm lengths of these structures indicates that each arm is a spectrin heterodimer (A.E. Burnip, 1980, personal communication). It has not yet been determined what fraction of the total $0^{o}C$ extracted spectrin these hexamers represent. However, it should be emphasised that the proportion of hexamers must be very low to have escaped notice before now. Certainly non-denaturating polyacrylamide gels of crude spectrin, spectrin tetramers and spectrin dimers give no indication of the existence of this

Figure 4.17

(a) Typical examples of spectrin "hexamers" after rotary low angle shadowing. For comparison a spectrin tetramer is shown bottom centre while two tetramers lying across each other are shown bottom right.

Magnification, x 170,000. Bar = 100nm.

(b) Non denaturing polyacrylamide gels (3.5% acrylamide) loaded with, on the left, 10µg of column purified spectrin tetramer (T), and on the right, 10µg of spectrin heterodimer (D), also column purified. The large amount of high molecular weight material remaining at the top of each gel cannot be accounted for by spectrin hexamers since electron microscopy indicates that at best, they would constitute no more than 10% of the total population of spectrin molecules. Much of this material must therefore result from non specific aggregation. Gels were run at 4°C at 2mA per tube for 8 h.

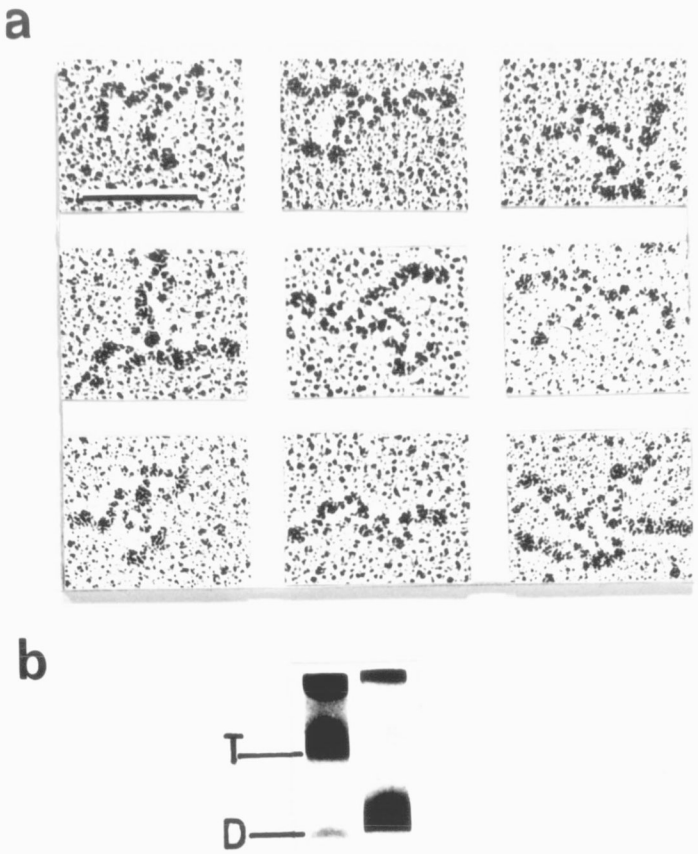



FIGURE 4.17

discreet higher oligomeric state, although some high molecular weight material does remain on the top of 3.5% gels loaded with spectrin tetramers (Figure 4.17(b)). It is therefore premature to asssign any physiological significance to spectrin hexamers. Nevertheless, their existence does impose a number of constraints on any models to describe the modes of association of spectrin heterodimers to form tetramers, and provides a number of clues as to how this might take place. These considerations will be covered in more detail in Chapter Six.

Effects of Glycerol on Molecular Structure as Viewed by Low Angle Shadowing

Glycerol was first introduced into the low angle shadowing technique by Elliott et. al. (1976) who used it at a concentration of 30% (v/v) in their low temperature drying protocol as a cryoprotectant. I found, however, that inclusion of glycerol in the shadowing buffer, for the room temperature drying used in this investigation was vital if interpretable and reproducible images of spectrin were to be obtained. Tyler and Branton (1980) have recently stated that glycerol is necessary for the removal of all non volatile salts to the centre of each droplet, and that it prevents the fields of proteins from being obscurred by these non volatile components. In my view, however, the role of glycerol is considerably more complex than this.

Omission of glycerol was found to cause a dramatic reduction in the shadow profile of spectrin tetramers (Figure 4.18 (c,d) ). Myosin, also was found to be very sensitive to glycerol concentration. Figure 4.18(b) shows rabbit skeletal muscle myosin which was shadowed as described earlier but in the absence of glycerol. Both myosin heads, like the spectrin, appear to be collapsed, whereas the tail retains at least some of its original structure. A possible explanation for this phenomenon is that glycerol might affect the rate at which intra molecular water is lost.

Figure 4.18

The effects of glycerol on the appearance of molecules after low angle shadowing.

Myosin molecules dried from a volatile buffer (155mM ammonium acetate, 100μM EDTA, 10μM DTT) containing (a) 70% glycerol and (b) 0% glycerol. Note the collapse of the heads (arrows) in the absence of glycerol. (c) and (d) show spectrin tetramers similarly dried from 70% and 0% glycerol respectively. Note the collapsed, low profile structures (arrows) seen in (d).

Magnification, x 100,000, Bar = 200 nm.

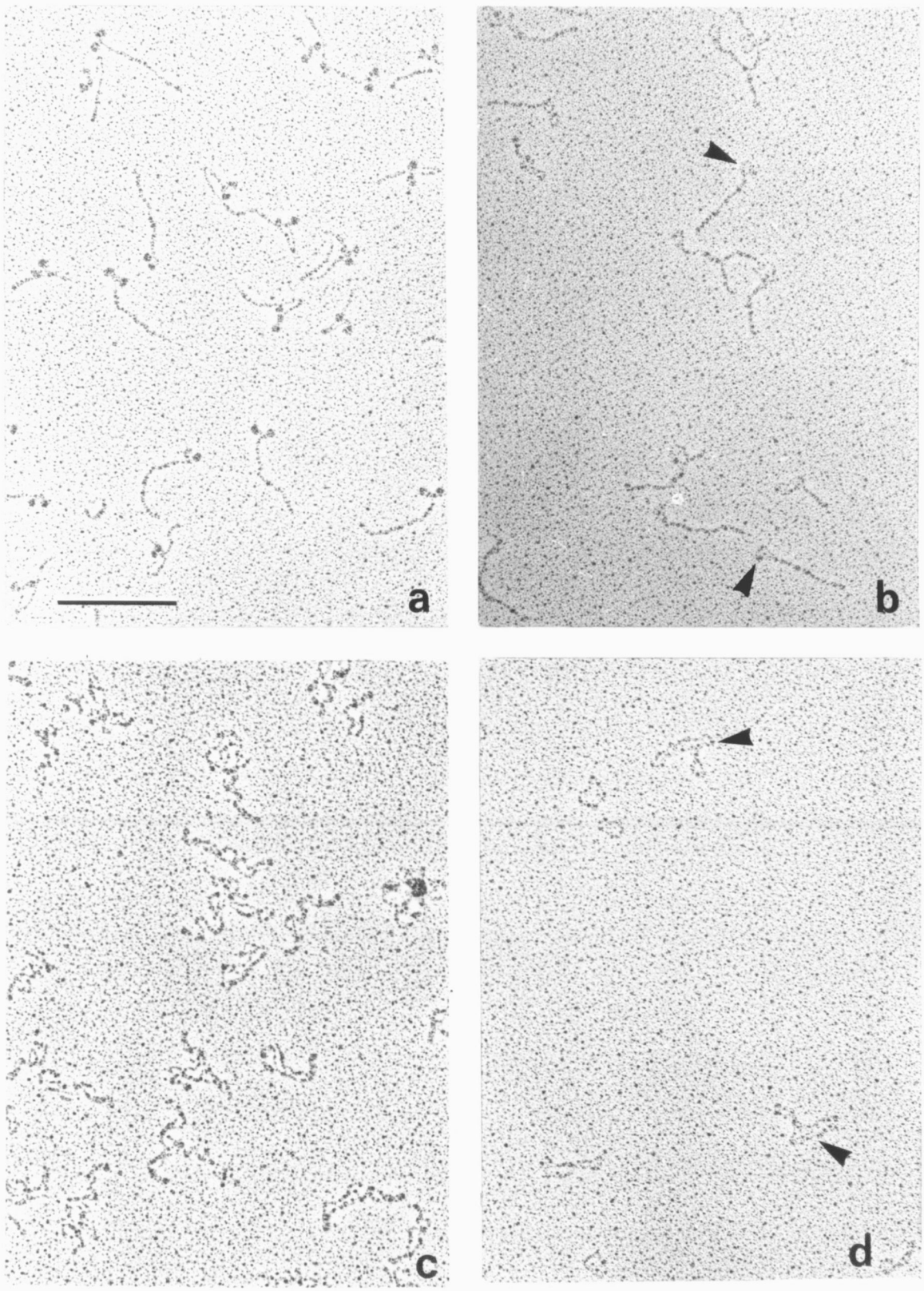



FIGURE 4.18

All soluble proteins have water molecules incorporated via hydrogen bonds into their tertiary and quaternary structure. Loss of this water during shadowing would be expected to have a serious destabilising effect on the protein structure which might result in the collapse or 'flattening' of the protein, as seen with the spectrin and with the myosin heads. Such a water loss, however, would not be expected to have quite the same effect on the two supercoiled $\alpha$ helices of the myosin tail. The $\alpha$ helix is a very rigid structure where all the hydrogen bonding groups of the polypeptide backbone are internally satisfied. It would therefore be expected to withstand the drying process without the same loss of structural integrity. In my experience, reduction of the glycerol concentration below 50% v/v causes deterioration of the images of shadowed molecules; at least in the case of spectrin and myosin.

There is little doubt that glycerol radically affects the drying process. Certainly during drying in the presence of glycerol there appears to be a very large accompanying fall in ionic strength. This conclusion was reached after low angle shadowing rabbit skeletal muscle F-actin ( a gift from M.J. Dickens ). It is well documented that F-actin dissociates to form G actin at low ionic strength. When F-actin was dried and shadowed from 155 mM ammonium acetate, 70% (v/v) glycerol, no filaments could be seen, but only individual actin monomers (not shown). Only in the absence of glycerol and in 1M ammonium acetate could F actin be identified (Figure 4.19(a)). Addition of glycerol to 30% resulted in dissociation of the filaments. Often rows of monomers could be seen where filaments must have dissociated where they lay on the mica surface (Figure 4.19(b)). Such dissociation would only be expected to take place at a salt concentration below about 50mM (Spudich and Watt, 1971). Brief fixation with 0.2% glutaraldehyde for 5 minutes was found

Figure 4.19

The apparent effects of glycerol on ionic strength during drying of molecules from volatile buffers prior to low angle shadowing.
(a) F actin in 1M ammonium acetate pH 7.4
(b) F actin in 1M ammonium acetate pH 7.4 containing 30% glycerol.
(c) F actin, in 60m MKCl, 5mM sodium phosphate pH 7.5, at a concentration of 1mg|ml fixed for 10 minutes with 0.2% glutaraldehyde, then diluted x 50 with 1M ammonium acetate pH 7.4 containing 30% glycerol and low angle shadowed. (d) F actin, used in the previous experiments, negatively stained with 2% uranyl acetate in distilled water.

Magnification, x 100,000. Bar = 200nm.

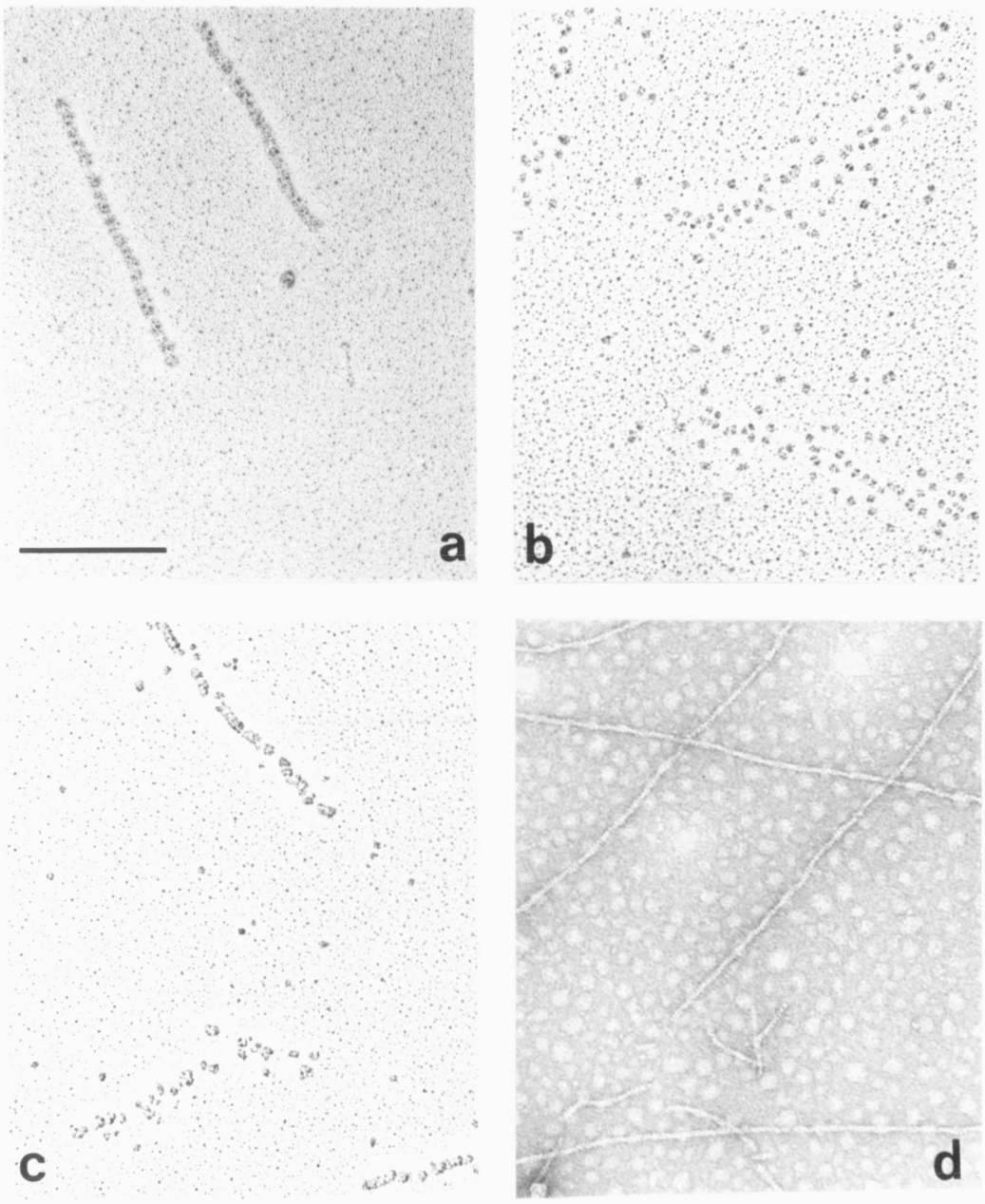



FIGURE 4.19

to limit depolymerisation (Figure 4.19(c). Presumably in the presence of glycerol volatile solutes (i.e. ammonium acetate) are lost considerably faster than the solvent itself actually evaporates.

# CHAPTER 5

## Selective Extraction of Band 1 from Erythrocyte Ghosts

### 1. Introduction

The effects of protein pertubants on red blood cell ghosts has, in recent years, only received limited attention. Steck (1972c) showed that guanidine hydrochloride in 5mM sodium phosphate buffer at pH 8.0 was able to cause the release of most of the peripheral membrane proteins from red cell ghosts leaving behind lipid vesicles containing predominantly band 3, band 7 and the PAS staining proteins. These remaining components could only be solubilized by further treatment with detergents. Similar results were obtained using other protein perturbants and protein modifying agents such as organic mercurial compounds, lithium di iodosalicylate and acid anhydrides, again at pH 8.0 (Steck and Yu, 1973). To date, no attempts have been made to observe the effects of such reagents under alternative conditions of pH and ionic strength.

Following a suggestion by Professor Daniel Branton, I have employed another protein pertubant, urea, to selectively solubilise one of the subunits of spectrin while leaving the other firmly attached to the ghost membrane. It was argued that by extracting ghosts in high concentrations of urea under conditions of ionic strength and/or pH at which spectrin would not normally be eluted, it might be possible to release whichever subunits of spectrin had the weaker association with the membrane. To this end I have used a variety of combinations of pH, ionic strength, urea concentration and temperature to extract red blood cell ghosts. The results are summarised below.

## 2. Urea Extraction of Ghosts at High Ionic Strength

When fresh red blood cell ghosts were extracted at 4°C in isotonic phosphate buffered saline (PBS), pH 7.6 containing 3M or 6M urea the only protein to be extracted was a small amount of band 6 (less than 50%). At 6M urea, in addition to band 6 a trace amount of spectrin was also released, with both subunits present in equal amounts (results not shown). Both urea concentrations caused mild vesiculation of the ghosts as judged by phase contrast microscopy, although it was more pronounced at the higher concentration. Extraction at pH 5.5 and at 4°C in PBS containing 6M urea caused no protein release while a corresponding 37°C extraction resulted only in the appearance of band 6 plus a few unidentified minor components (possibly proteolytic fragments) in the supernatant (results not shown).

## 3. Urea Extraction of Ghosts at Low ionic strength and Low pH

Extraction of ghosts in 6M urea at low ionic strength and low pH (1mM Histidine HCl, pH 5.5-6.0) was found to selectively solubilize band 1 of spectrin while leaving band 2 and all other proteins bound to the membrane. As shown in Figure 5.1a, the pH of the extraction medium had a critical effect on the kinetics of protein release from ghosts; extraction of protein occuring only above pH 5.7 and increasing rapidly over a narrow pH range. The relationship between pH and quantity of protein released was not obviously linear, indeed the graph appears to show a plateau in the region of pH 5.9 to 6.3. The SDS gels of the extracted ghosts and supernatants (Figure 5.1b) show that at the lowest pH's the only polypeptide to be solubilised was band 1. Indeed up to pH 6.0-6.1 extraction of ghosts in urea provided an almost completely pure band 1 preparation. Above pH 6.1 significant quantities of actin and band 2 began to appear in the supernatant.

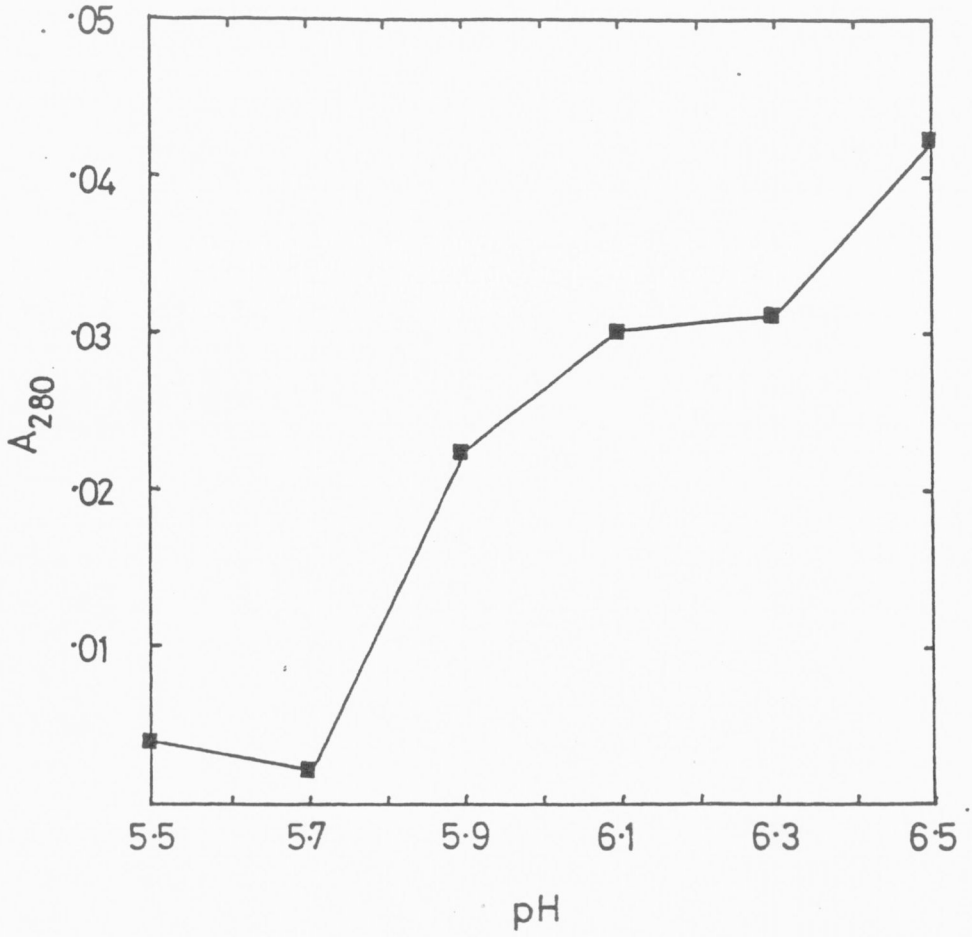
Figure 5.1

Effects of pH on protein release from ghosts in 6M urea at low ionic strength.

(a) six 1ml aliquots of fresh ghosts previously washed twice in 30 volumes of 20pB at a pH between 5.5 and 6.5 (see graph) were each mixed with 10 ml of an extraction medium containing 6M urea, 100μM DTT, 1mM Histidine-HCl adjusted to six different pH values from pH 5.5 in steps of 0.2pH units to pH 6.5. Following a 30 minute incubation at 37°C, the samples were centrifuged in a Beckman type 65 rotor for 1 h at 50,000 rpm. The absorbance at 280nm was recorded for each supernatant and gel samples were taken.

(b) SDS gels stained with coomassie blue showing the pellet (A) and supernatant (B) for each pH recorded on the graph above. Loadings were 20μl and 200μl respectively.

a


·05
·04
·03
·02
·01
$A_{280}$
5·5
5·7
5·9
6·1
6·3
6·5
pH

b

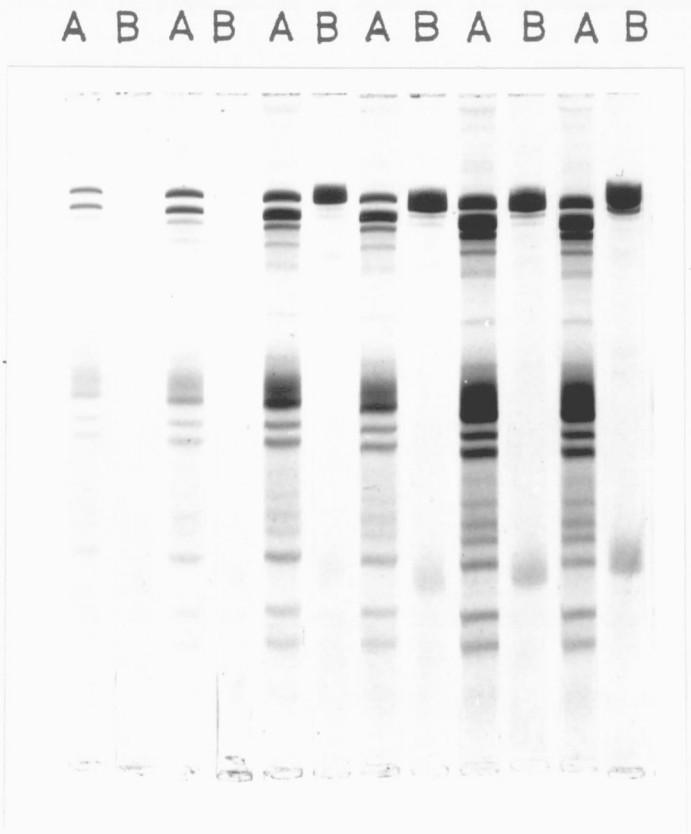

A B A B A B A B A B A B
5.5 5.7 5.9 6.1 6.3 6.5

FIGURE 5.1

The conditions used to selectively elute band 1 from ghosts, caused both fragmentation of the membranes and also intense intra membrane particle aggregation as viewed by freeze fracture (Figure 5.2a). However, such particle aggregation is not caused by band 1 extraction as it was also observed when band 1 extraction was almost completely prevented by the addition of NaCl to a final concentration of 155 mM to the band 1 extraction medium. This suggests that even when protein is not extracted, urea may be causing considerable disruption of the membrane cyto skeleton, since Elgsater and Branton (1974) and Elgsater et al. (1976) have previously demonstrated that pH - and salt - induced particle aggregation will only be appreciable after disrupting the cytoskeleton by a pretreatment during which spectin and actin are removed. No aggregation was seen in the presence of urea, if in addition to raising the ionic strength, the pH of the extraction medium was made slightly alkaline (pH 7.4). However under these conditions vesiculation nevertheless took place.

4. Low angle Shadowing of Band 1

Attempts to low angle shadow purified band 1 after extensive dialysis into the usual ammonium acetate shadowing buffer (Chapter 2) were only partially successful. Figure 5.3 show typical fields of rotary shadowed band 1 (unidirectional shadowing in this case provides no additional useful information). Extended, flexible, single stranded structures were consistent features of these preparations, although these varied in length quite considerably from about 50 nm to 80-90nm and as a consequence, no detailed length analysis was carried out. These band 1 preparations also invariably exhibited large amounts of amorphous or aggregated material, possibly indicating incomplete renaturation.

Figure 5.2

Erythrocyte ghosts extracted at 37°C in solutions containing 6M urea and then freeze fractured.

(a) ghosts extracted in 6M urea, 100μM DTT, 1mM Histidine HCl, pH 5.9. Under these conditions only Band 1 is released (see Figure 5.1).

(b) ghosts extracted in 6M urea, 155μM NaCl, 100μM DTT, 1mM Histidine HCl, pH 5.9. Under these conditions little or no protein is released from the ghosts.

(c) ghosts extracted in 6M urea, 155mM NaCl, 100μM DTT, 1mM Histidine HCl pH 7.4. Only band 6 is eluted under these conditions. All specimens were fractured at -110°C and shadowed without etching.

Magnification, x 50,000. Bar = 0.5μm.

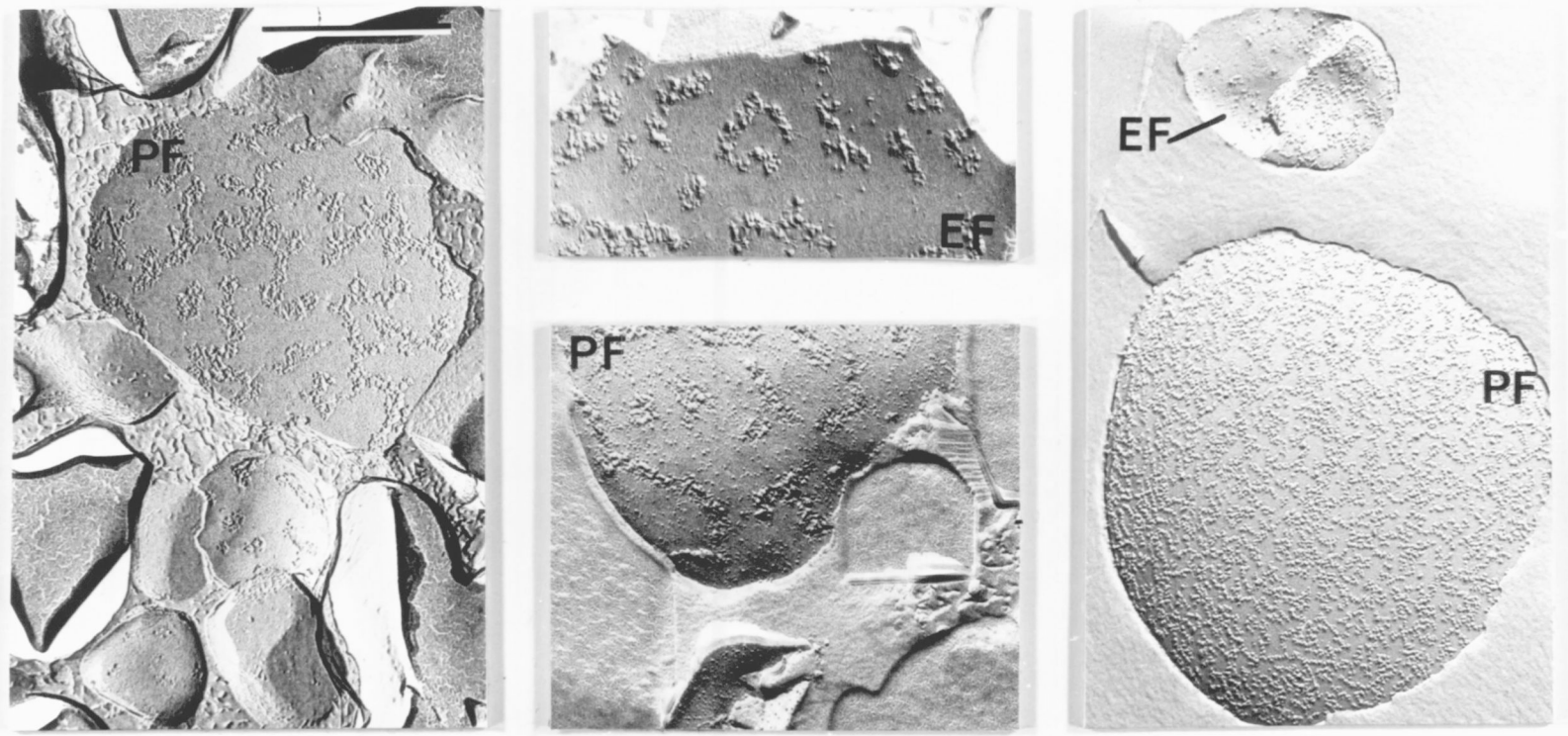



FIGURE 5.2

Recently, Calvert et al. (1930a) have successfully separated band 1 and band 2 by chromatography on hydroxyapatite in the presence of 7 M urea. They found that the isolated subunits may be largely renatured following dialysis first into 6M guanidine hydrocholoride and then into a physiological buffer, since circular dichroism measurements indicated that the α helix content of either subunit was similar to that found in the intact spectrin dimer. Indeed, I have obtained immunological evidence that at least some native structure may be returned to band 1 following removal of urea (Chapter 9).

5. Extraction of Band 2 from Band 1 Depleted Ghosts

Selective extraction of band 1 from ghosts raised the possibility of purifying band 2 by re-extraction of the band 1 depleted membranes at low ionic strength and high pH, in the absence of urea; conditions under which spectrin is normally solubilised. It was hoped that the material so extracted, would be enriched in band 2 which could then be separated from whole spectrin and actin by gel filtration chromatography. However, it was found that following band 1 extraction, band 2 became even more firmly attached to the membrane and that re-extraction at high pH and low ionic strength released predominantly band 1 (Figure 5.4). Even actin was found to be relatively inextractable under these conditions. This situation is, in fact, consistent with the recent finding of Calvert et al. (1980a) that pure band 2 is highly prone to aggregation. It seems highly likely that inability to remove band 2 after band 1 extraction could result from a microprecipitation of band 2 on the membrane.

Figure 5.3

Purified Band 1 (Figure 5.4(d)) after rotary low angle shadowing. Pure Band 1 was dialysed into the usual volatile buffer (155 mM ammonium acetate 10μm DTT, 100μM EDTA pH 7.4), glycerol added to 70% and then sprayed and shadowed as described in Chapter 2. Note the large amounts of amorphous material.

Magnification (a) x 100,000 (b)-(d) x 170,000
Bars = 100nm.

Figure 5.4

Re-extraction of Band 1 depleted membranes under conditions where spectrin would normally be extracted from fresh ghosts. SDS/ polyacrylamide gels were loaded as follows:

(a) 10μl of fresh ghosts

(b) 20μl of spectrin/actin extract eluted from fresh ghosts at $0^{o}C$ in 100μ MEDTA, 10μM DTT, pH 9.5.

(c) 20μl of spectrin/actin depleted ghost membranes

(d) 5μg of Band 1 extracted at $37^{o}C$ from fresh ghosts in 6M urea, 1mM Histidine 100μM DTT pH 5.9.

(e) 20μl of Band 1 extracted ghost membranes.

(f) 100μl of material extracted from Band 1 depleted membranes by dialysis at $0^{o}C$ for 40 h against 100μM EDTA, 10μM DDT pH 9.5.

continued/...

Figure 5.4 (continued)

Under these conditions spectrin and actin are normally readily eluted from ghosts (gel b).

(g) 20µl of Band 1 depleted membranes re-extracted as in (f).

Note in (f) that little actin or Band 2 is extracted.

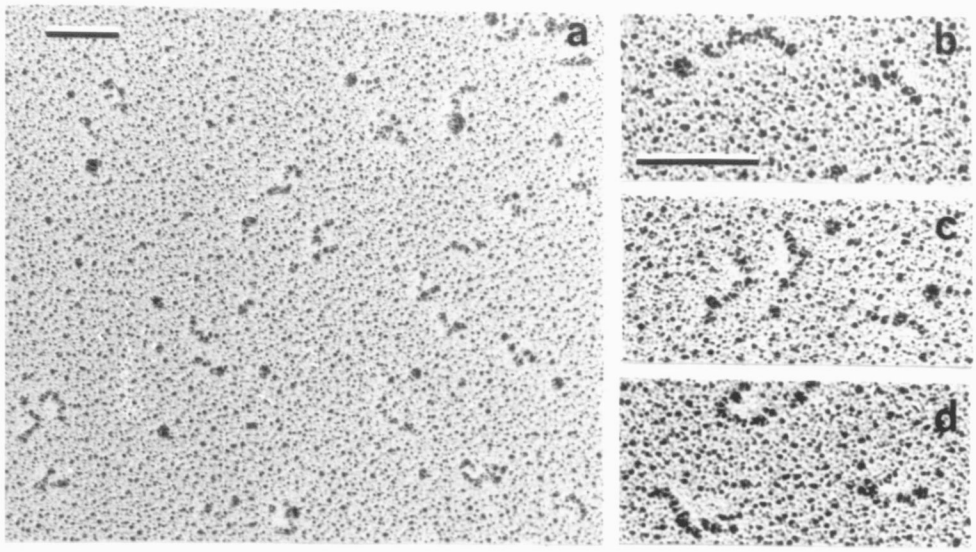



FIGURE 5.3

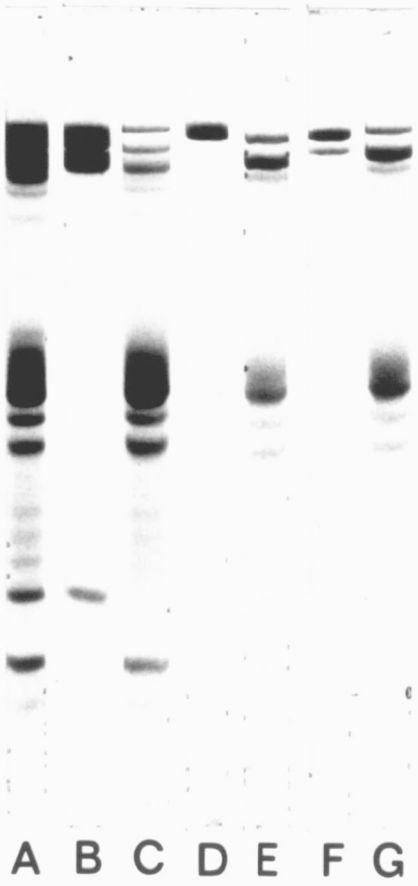



FIGURE 5.4

# CHAPTER 6

## DISCUSSION

### 1. Conformation of the polypeptide chains of spectrin

The measured lengths of 97nm for spectrin heterodimers and 194nm for spectrin tetramers are considerably shorter than one would expect if the two polypeptide chains of spectrin were to form a supercoiled α-helical rod, in the manner of tropomyosin or the myosin tail. The dimensions of several such dimeric α-helical protein molecules (α-proteins) have been determined. One of these is paramyosin which has a monomer molecular weight of 110,000 daltons and a length of 127.5nm (Cohen et. al., 1971). If the spectrin heterodimer were to adopt a similar tertiary structure its expected length would be well in excess of 250nm. Additional evidence against such a simple structure is that the width of the spectrin dimer is about 2.5 times that of a single myosin tail. Furthermore, while all known α-proteins are composed of two identical polypeptide chains, the spectrin molecule is composed of two dissimilar polypeptides. In addition, the molecule appears to be extremely flexible and the subunits are only loosely associated precluding the possibility of such a simple structure.

The spectrin molecule does, however, possess a remarkably high α helix content. This has been variably estimated to comprise about 60-70% of the molecule (Ralston, 1978). As argued above, this amount of α helix could not be contained in just a single element per subunit as it would impose a minimum length of about 180 nm on the heterodimer. This assumes a residue translation along the helix axis of 0.15nm and takes no account of supercoiling.

Taken as a whole, this evidence indicates that Band 1 and Band 2 possess a more complex and folded tertiary structure than the myosin tail. However, in view of the enormous size and flexibility of the spectrin heterodimer it seems highly unlikely that the two subunits could fold up to form a single well defined structure. Instead, I share the opinion of Anderson (1979) and Calvert et.al. (1980) that much of two subunits is folded into a series of relatively rigid globular domains, containing all the α-helix which are linked by short flexible sections of the polypeptide chain. Certainly, globular domains could accommodate this amount of α-helix since Kendrew et. al. (1960) demonstrated that myoglobin, a globular protein, also contained approximately 70% α-helix. Such a structure would allow the molecule to adopt the huge variety of quaternary configurations seen in the electron microscope and would account for the larger standard deviations recorded for spectrin length determinations compared with that of the rotary shadowed myosin tail with its precise supercoiled α helical geometry (Table 4.1).

This type of structure would certainly not be setting a precedent among protein molecules. It has been demonstrated by Highsmith et. al. (1977) using proton magnetic resonance spectroscopy, that subfragment -1 (S-1) of myosin is linked to the rest of the molecule by a region of polypeptide chain which has freedom of movement comparable to that of a random coil and accounts for about 22% of the mass of the S-1 moiety. DNA polymerase I of E. coli possesses two globular domains containing different enzymic functions linked by a trypsin sensitive segment of the polypeptide backbone. Following brief trypsinolysis the domains, which retain their enzymic activities, become completely separated and show no tendency to associate (Kornberg, 1974).

That spectrin does contain domains of some form is evident from the controlled trypsin digestion of spectrin performed by Anderson (1979a). His results indicate the existence of repeating 8,000 dalton

trypsin-insensitive units which resemble the whole spectrin molecule in having a high glutamic acid and leucine content. (Anderson, 1979b). Recently, Calvert et. al., (1980b) by following the urea denaturation of spectrin dimers by means of circular dichroism measurements, have shown that spectrin contains a number of independently unfolding globular domains as well as flexible non globular regions. Using high resolution proton magnetic resonance spectroscopy, these workers have demonstrated that the flexible regions account for about 20% of the polypeptide chains and are highly hydrophobic in nature.

## 2. The Assembly of Spectrin Tetramers

The results shown in Chapter 4 suggest that the spectrin monomer polypeptides, Band 1 and Band 2, associate side by side to form the spectrin heterodimer and that the binding between the two polypeptides is strongest at their ends. Tetramer formation quite obviously only takes place by the end to end association of heterodimers. Furthermore both constituent polypeptides of each heterodimer must be involved in the assembly of the tetramer in view of the finding that binding between sister monomer strands of a single heterodimer is replaced by inter dimer bonding of the monomers at the mid point of the tetramer, i.e. at the dimer-dimer interface (Figures 4.12 and 4.13).

There are two ways in which two heterodimers could associate end to end to give rise to a spectrin tetramer; either head to head or head to tail. If the association was head to tail there is no reason to suppose that the tetramer would be the largest linear oligomeric species of spectrin. However, I have found no evidence for the existence of higher linear oligomers such as hexamers and octamers and none has been reported elsewhere. If on the other hand, association was head to head

the tetramer would logically be the largest linear species seen, which has so far proved to be the case. Higher oligomers could only then form in the presence of a second component capable of acting as a cross linking agent. Thus circumstantial evidence points in favour of head to head binding of heterodimers. This conclusion has been confirmed by Tyler et. al. (1979) who purified Band 2.1, the membrane attachment protein for spectrin, and used it to 'decorate' spectrin dimers. When these complexes were observed in the electron microscope after low angle shadowing, Band 2.1 could be quite clearly seen to bind about 20nm from one terminus of the dimer. When complexes with the tetramer were similarly examined, two Band 2.1 molecules could be seen binding in the central region of the tetramer about 40nm apart. Tetramer assembly must therefore involve head to head binding.

Such head to head association would be expected to involve reciprocal binding at two identical pairs of binding sites since both sub-units of each heterodimer are seen to take part in the formation of a tetramer. This is represented schematically in Figure 6.1. It is proposed that a binding site (A) on a Band 1 monomer can associate with a complementary site (B) on its sister Band 2 monomer or to a corresponding site (B) on the Band 2 of a second heterodimer. This association would make available site (B) on the first heterodimer and site (A) on the second which would then also be able to undergo a reciprocal association. Such a scheme for tetramer formation requires initially the breaking of two bonds before dimers may associate, which is consistent with the high activation energy required for tetramer formation observed by Ungewickell and Gratzer (1978) which they ascribed to local conformational adjustments at the dimer-dimer interface.

Further evidence for the proposal that tetramer formation requires a reciprocal exchange of inter dimer bonds stems directly from the identification of the non-linear spectrin hexamer. There is now

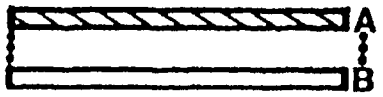
Figure 6.1

Hypothesis for head to head reciprocal binding interactions within spectrin tetramers. The hatched and empty bars represent Bands 1 and 2 respectely. The three dots respresents binding interactions between sister strands in a heterodimer (a) and between paired strands in a tetramer (b).

Figure 6.2

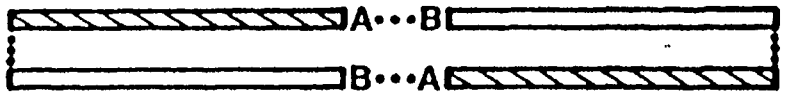
Proposed arrangement of subunits within the spectrin hexamer. A reciprocal exchange of bands is shown in (a) while non-reciprocal binding is shown in (b). Clearly the assoication mode shown in (b) could not give rise to a fully formed hexamer with all binding sites satisfied, indicating that association of dimers must involve a reciprocal banding pattern as shown in (a) and in Figure 6.1 (a) and (b).

HETERODIMER




a

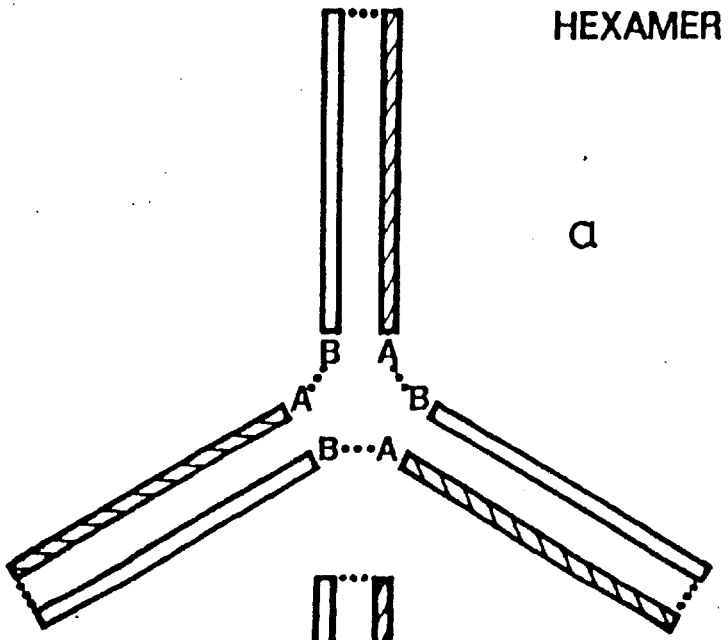
TETRAMER



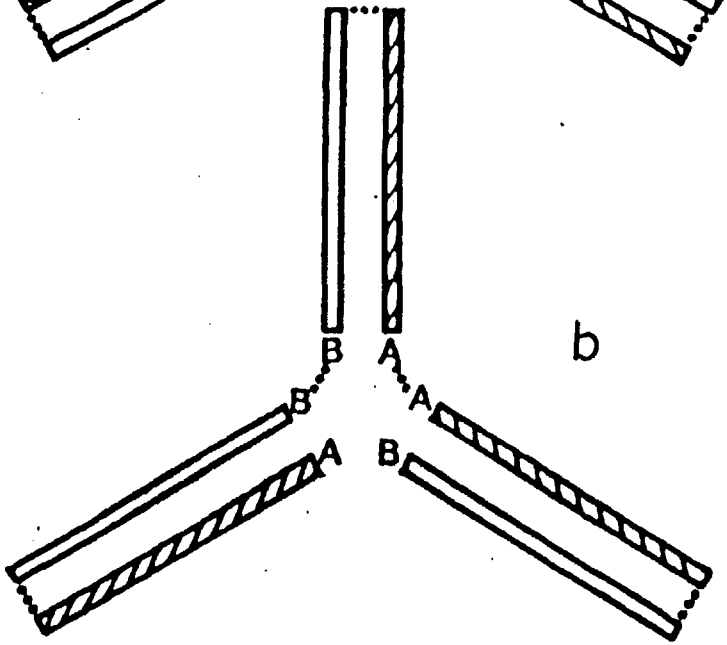

b

FIGURE 6.1

HEXAMER




a




b

FIGURE 6.2

little doubt that the hexamer does exist as a minor species in 0°C spectrin preparations since Tyler et. al. (1980b) have shown by low angle shadowing of spectrin complexed with ferritin conjugated Band 4.1, that all hexamers are symmetrically labelled. This would not be expected to occur if 'hexamers' simply appeared due to a chance juxtaposition of a tetramer and a dimer. If heterodimers were to associate in a non reciprocal manner with bonds being exchanged between like subunits at the dimer-dimer interface, there is no conceivable way that a hexamer could be assembled with all binding valencies satisfied. There would always be one bond left unformed (Figure 6.2a). Hexamer formation requires that dimers associate via a reciprocal exchange of bonds.

3. Mechanism of Hexamer Assembly

The formation of a spectrin hexamer from three heterodimers should be expected to occur in solution only at a very low frequency. The event must involve the near simultaneous cleavage of three intra dimer bonds, followed by association of the dimers. After the first inter dimer bond has been made, formation of a tetramer, rather than a hexamer must be highly favoured due to the proximity of the two remaining complementary binding sites in the now half formed tetramer. At this point the third dimer must engage one of these two binding sites or else tetramer formation will go to completion. The only alternative assembly mechanism is that a third dimer could be inserted after the transient breakage of one of the inter dimer bonds of a preformed tetramer.

Either way, however, it would appear to be rather unlikely that hexamers could arise as an artefact of spectrin preparation in view of the finding of Ungewickell and Gratzer (1978) that at 0-4°C spectrin heterodimers and tetramers are kinetically trapped and that there is no interconversion between the two. One would expect this situation to apply

equally to hexamers. It must be concluded therefore, that hexamers are present as a minor species on the red blood cell membrane.

## 4. The Reliability of Low Angle Shadowing as a preservative of Molecular Structure

I have shown in Chapter 4 that conditions chosen for low angle shadowing may seriously affect the structure of protein molecules. Consequently, interpretation of shadowing results must be performed with caution and where possible, in conjunction with other structural studies. Indeed, initial shadowing experiments on spectrin using a 1M ammonium acetate shadowing buffer sometimes gave rise to compact trilobed images of length 50nM (Shotton et. al. 1978, D.M. Shotton personal communication).

Negative staining and low angle shadowing of other proteins have produce encouragingly similar results. Fowler and Erickson (1980) have successfully used both techniques on fibrinogen, in each case obtaining characteristic tri lobed images. These workers, incidentally, used a high glycerol concentration (30%) in their shadowing buffer and noted that the images which they obtained deteriorated in the absence of glycerol. Takahashi (1978) successfully negatively stained rabbit skeletal muscle myosin, producing images very similar to those shown here and to those shown by Elliott et. al. (1976) and Elliott and Offer (1978).

So far it has not proved possible to produce negatively stained images of spectrin, of a quality comparable to those obtained for these other large molecules, although its use has suggested that spectrin has a flexible elongated strcuture (Ungewickell et. al. 1979). More detailed comparisons with images of spectrin produced by low angle shadowing are not possible. Consequently support for this low angle shadowing data must be obtained from other sources.

## 5. Spectrin In Solution

The view of spectrin, obtained in this electron microscopic investigation, as an elongated and highly flexible molecule is compatible with much of the physical data obtained for spectrin in solution (see Table 1.3). In several cases it is possible to unify some of these physical data in terms of the electron microscope results.

Ralston (1976a,b) using hydrodynamic techniques and Kam et. al. (1977) using laser autocorrelation spectroscopy measured the diffusion coefficients for spectrin tetramer and spectrin dimer as $1.1 \times 10^{-7} cm^2/s$ and $1.55 \times 10^{-7}$ $cm^2/s$ respectively. Such a difference in these values is quite consistent with the tetramer being formed by end to end association of heterodimers. Purely on the basis of his hydrodynamic data, Ralston (1976b, 1978) suggested that the spectrin tetramer was a highly expanded but basically symmetrical molecule. Such a view is not incompatible with my own electron microscope evidence as it seems reasonable to assume that this is exactly how an elongated and highly flexible molecule might appear in solution with a time averaged diameter considerably less than its contour length.

This view of spectrin as a flexible extended molecule is supported by the classical light scattering study of Elgsaeter (1978) which indicated a minimum length for the spectrin dimer of 76nm. A complementary electro-optic study (Mikkelsen and Elgsaeter, 1978) also supported this conclusion. Recently Calvert et. al. (1980b) pointed out that the light scattering results of Kam et. al. (1977) do in fact agree with those of Elgsaeter (1978), the original discrepancy (Table 1.3) arising out of a computational error.

Only the results of Schecter et.al. (1976) cannot be reconciled with the view that spectrin dimers and tetramers are expanded flexible

structures. However, this group studied spectrin in the presence of deoxycholate. The effects of this detergent on spectrin are not at all clear.

## 6. The Relationship of Spectrin to Myosin

It has been suggested that spectrin resembles myosin in structure and function, forming an actomyosin like complex on the cytoplasmic surface of the red blood cell membrane (Guidotti, 1972; Juliano, 1973). Such speculation was fuelled by reports of a non reciprocal immunological cross reactivity between anti serum against humam uterine smooth muscle myosin and human erythrocyte spectrin (Painter et. al., 1975). However, the results of this and other investigations have more recently shown spectrin and myosin to be structurally quite distinct.

## 7. The Assembly of Spectrin on the Erythrocyte Membrane

It is clear from the freeze etch studies of Elgsaeter et. al. (1976) and Shotton et. al. (1978) that spectrin is arranged in a network just beneath the cell surface. Using a novel tannic acid fixation regime Tsukita et. al. (1980) have recently been able to visualise this network by thin section electron microscopy. They found an anastomosing system of fibres, presumably spectrin, closely associated with the inner surface of the membrane. This was the first time such a network had been seen without first stripping off the lipid bilayer with detergent. The diameter of the fibres seen by these workers was about 9nm, which is about 50% greater than my own estimate for the width of the spectrin molecule. Presumably this discrepancy arises due to differences in the methods of preparation for electron microscopy.

As discussed in Chapter 1, all available evidence points to the spectrin tetramer as opposed to the heterodimer being the physiological species of spectrin on the membrane and that attachment, at least in part is mediated by Band 2.1, ankyrin. As demonstrated by Tyler et. al. (1979), the 2.1 attachment sites are located about 20nm on either side of the tetramer mid point. My data concerning selective urea extraction of Band 1 from ghosts indicates that it is Band 2 of spectrin which contains the major membrane binding site. This has been confirmed by Hsu et. al. (1980) who showed that purified Band 2 but not pure Band 1 could associate with inside out, spectrin depleted vesicles. Likewise, Calvert et. al. (1980a) have demonstrated a direct association of Band 2.1 with Band 2 but not with Band 1.

It should be noted, however, that although the relative stoichiometry of the erythrocyte membrane proteins (Table 1.2) would permit each spectrin tetramer to bind one copy of Band 2.1, there are five times as many Band 3 dimers (and also intra membrane particles) as there are Band 2.1 molecules. Thus unless spectrin can interact directly with Band 3 (or the intra membrane particles) only 20% of Band 3 dimers may be associated with the spectrin network at any one time. Certainly the spectrin tetramer is large enough to interact with several Band 3 dimers. Band 2.1 is almost certainly not the sole means of attachment of spectrin to the membrane. The finding of Calvert et. al. (1980b) that spectrin contains highly hydrophobic segmentally mobile sequences suggests that it may be able to interact directly with phospholipids. This suggestion is supported by evidence from several quarters. The fact that spectrin is only dissociated from the ghost membrane at low ionic strength is indicative that hydrophobic interactions must be broken. Mombers et. al. (1977) and Sweet and Zull (1970) have shown that spectrin will associate with model bilayers and Haest et. al. (1978) find that phospholipid "flip-flop" rate is greatly accelerated in cells where spectrin has been damaged.

A curious aspect of erythrocyte development is that spectrin synthesis occurs very early on during the normoblast period, long before the erythrocyte adopts its biconcave shape and is virtaully complete before Band 3 synthesis and Band 2.1 synthesis is initiated (Chang et. al., 1976; Eisen et. al. 1977). However, it is not yet known at what stage assembly of spectrin onto the membrane begins. If this was found to be prior to the synthesis of these other two proteins, it would provide conclusive evidence for alternative modes of membrane attachment in vivo.

## 8. Actin in the Red Blood Cell Membrane

As spectrin tetramers are formed by head to head association of heterodimers, assembly of a submembrane network requires at least one other component to act as a cross linking agent. It is now clear that at least in vitro that F actin may form a cross linked complex with spectrin tetramers in the presence of Band 4.1 (Pinder et. al., 1975; 1977; 1979; Cohen and Branton, 1979; Fowler and Taylor, 1980; Ungewickell et. al. 1979). Brenner and Korn (1979), however, have shown that 4.1 may not be required in this complex formation with preformed F actin, but only for stimulated G actin polymerization. It has been shown by Sheetz (1979) that Triton X-100 cytoskeletons of erythrocytes are dissociated by DNA ase I, an enzyme which is known to form complexes with G-actin and to dissociate F actin (Hitchcock et. al., 1976). This strongly supports the view that actin is involved in cross linking in vivo.

Band 4.1 binds directly to spectrin in the absence of actin. Tyler et. al. (1979, 1980b) have used purified Band 4.1 and ferritin-avidin: biotin-4.1 complexes to directly label spectrin heterodimers and tetramers for observation in the electron microscope after low angle shadowing. They found that the 4.1 molecule binds specifically at one terminus of the

heterodimer and at both termini of the tetramer. Calvert et. al. (1980a) have shown that both Band 1 and Band 2 are required for Band 4.1 binding. It is to be expected that actin attaches in the same region (this has recently been confirmed by Cohen et. al., 1980, manuscript in preparation).

Simply on the basis of my own experience of spectrin extraction, I would expect Band 4.1 to bind to at least one other membrane component. Extraction of all the actin and more than 90% of the spectrin in EDTA at low ionic strength, leaves all the Band 4.1 firmly attached to the membrane. Thus, Band 4.1 could provide spectrin with an additional link to the bilayer or to integral proteins.

Whether the actin of the red cell membrane is polymerised or not is still uncertain. Direct electron microscope examination of the membrane has not so far yielded an answer, although Marchesi and Palade (1967) noted the appearance of actin like filaments after trypsin treatment of ghosts. Cohen and Branton (1979) also showed that the cytoplasmic surface of the membrane possessed sites which were able to 'seed' actin polymerisation in the same manner as short segments of F actin.

It seems likely, although unproven, that an actin monomer would contain only one spectrin binding site. Thus if actin were to cross link spectrin it would have to be at least dimeric. Table 1.2 shows that for each spectrin tetramer on the red cell membrane there are about four actin monomers. So in theory the actin could exist as short filaments at least four monomers long and still be able to cross link all the spectrin tetramers. Of course stretches of F actin three or more monomers long would be able to provide foci for more than two spectrin molecules to attach, thereby producing a network truly cross linked in two dimensions. Spectrin hexamers would have the same effect although there is no evidence to suggest that they have any physiological relevance. Chemical cross linking studies (Kiehm and Ji, 1977; Huang and Richards, 1977) have indicated that at least actin dimers or trimers may be present on the

membrane.

## 9. The Existence of Spectrin on other cell types

Spectrin appears to be ubiquitous to erythrocytes. Pinder et. al. (1978) have identified a high molecular weight spectrin like protein in the erythrocytes of a marine invertebrate, *Terbella lapidaria*. In non erythroid cells, spectrin appears to be uniformly absent. The only possible exception being the sperm cells of the echinoderm *Thyone briareus* which possess a protein with two subunits of molecular weight similar to Bands 1 and 2, and is associated with actin (Tilney, 1976). However, it has yet to be conclusively identified as spectrin or even spectrin-like.

Other high molecular weight proteins which interact with actin, but which are not related to myosin, have been identified. Hartwig and Stossel (1975) isolated 'actin binding protein' from rabbit alveolar macrophages. A second such protein, filamin, was identified in chicken gizzard smooth muscle and in several non-muscle cell types (Wang et. al., 1975; Wang, 1977; Wang and Singer, 1977). Both of these proteins have striking similarities with spectrin, and hydrodynamically the two are indistinguishable from spectrin heterodimers. (Hartwig and Stossel, 1975; Wang, 1977). Actin binding protein and filamin do, however, differ from spectrin in that they are both homodimers and do not form tetramers. Furthermore, filamin, at least, shows no immunological cross reactivity with spectrin (Wang et. al., 1975, B.E. Burke, unpublished observations) and has a quite dissimilar amino acid composition (Wang, 1977). Comparisons of these proteins after low angle shadowing indicate that like spectrin heterodimers, filamin and actin binding protein appear double stranded but that they differ from spectrin in so far as the strands are associated only at one end of the molecule (Tyler et. al., 1980a; B.E. Burke, unpublished observations). It is therefore clear

that these two very closely related proteins are quite distinct from spectrin.

# CHAPTER 7

## Development of Monoclonal Antibodies Against Human Erythrocyte Spectrin: Introduction

### 1. Justification for Producing Monoclonal Antibodies Against Spectrin

In the preceding chapters I have dealt mainly with gross structural features of spectrin, making little mention of any structural differentiation within this enormous molecule. Indeed, with the exception of the finding that one or both of the subunits of spectrin may contain a series of globular domains (Andersson, 1979; Calvert et al., 1980b) linked by flexible, hydrophobic regions of the polypeptide chain (Calvert et al., 1980b), information regarding the sub-structure of spectrin heterodimers and tetramers is virtually non existent. There are several reasons for this state of affairs. Firstly, the size of the molecule precludes any large scale sequence analysis, at least by conventional protein chemical techniques; it seems very likely that when the amino acid sequence is elucided it will be obtained from the spectrin messenger RNAs using recombinant DNA technology. Secondly, the molecule has not yet been induced to form ordered aggregates which would be suitable for diffraction techniques. This would seem to be yet another reflection of the flexibility of the molecule and the enormous number of conformations which it may adopt. Thirdly, it has not so far proved possible to dissect spectrin into a limited number of functional fragments using proteolytic enzymes as has been done for other large protein molecules such as Myosin (Lowey et al., 1969) and immunoglobulins

(Porter, 1959). Clearly spectrin does contain several major functional sites: the band 4.1 binding site, / the band 2.1 binding site, and the two reciprocal binding sites on the heterodimer involved in tetramer assembly, all of which have been localised by electron microscopy (Tyler et al., 1979). However, it has not been possible to isolate any of these sites for closer inspection. In view of these problems most recent work has been devoted to studying the interaction of spectrin with other membrane proteins.

A fundamental question which has not, as yet, been answered is whether the chemical termini of band 1 and band 2 correspond to the physical termini, as seen in the electron microscope. If they do, this raises yet another question as to whether the two polypeptides lie in a parallel or anti-parallel conformation i.e. are both C termini found at one end of the molecule and both N termini at the other or is there one C terminus and one N terminus at either end? Should it turn out that the chemical and physical termini are not coincident one would obviously like to determine just how the polypeptide chains are disposed within either subunit.

In an attempt to answer these and other questions, I decided to undertake an immunological dissection of the spectrin molecule. It is ultimately hoped to use antibodies against specific functional domains of spectrin as immunoabsorbants in order to purify single proteolytic fragments which might then be more amenable to intensive protein chemistry. These same antibodies could be used, in the first instance, to label intact spectrin molecules prior to low angle shadowing in order to map the molecule at the electron microscope level; spectrin is certainly large enough for this to be feasible. In this manner it should be possible to correlate chemical and structural data. For instance, a specific antibody against the phosphorylated region of band 2 could be used to locate this region with respect to other known functional sites such as the 2.1 binding site or the dimer-dimer interface.

To carry out such an investigation it is necessary to use extremely pure antibodies. For this reason, simply immunising a rabbit or mouse with spectrin or fragment of spectrin and obtaining an immune serum is not good enough, as the animal will produce a broad spectrum of antibody directed against many antigenic sites upon the spectrin molecule. For instance, it has been estimated that a mouse has a potential repertoire of 1-5 x $10^7$ different antibody molecules (Kreth and Willaimson, 1973; Köhler, 1976) and in fact a single antigenic determinant may be recognised by as many as 1000 - 8 000 of these molecules (Kreth and Williamson, 1973; Köhler, 1976). Normally, however, for each determinant, only 5-10 different antibody species may be expressed and these probably represent a random sample from all the possible molecules which could have been selected. Clearly, for a molecule as complex as spectrin, the immune system of a mouse may recognise a large number of antigenic determinants. The animal may therefore mount a complex immune response involving tens or maybe even hundreds of species of antibody molecules. Such a heterogeneous population would be effectively useless for any detailed structural work on spectrin.

Another problem related to the heterogeneity of the immune response is that it is impossible to elicit a second identical response in another animal since the antibodies expressed in the first animal appear to be just a random selection from a pool of many thousands. For these reasons in order to obtain a truly monospecific antibody which may be reproducibly obtained in large quantities it is necessary to resort to lymphocyte fusion methods to produce antibody secreting hybrid cell lines which may be grown continuously in culture (Köhler and Milstein, 1975). Such a cell line is derived from a single antibody secreting cell, consequently the antibody which it produces is monoclonal. A monoclonal antibody is therefore completely homogeneous showing a single specificity

against a single antigenic determinant. Furthermore, it may be obtained reproducibly, since once the antibody secreting cell line is established, it may be grown virtually indefinitely or stored frozen for use at any time in any laboratory.

Since their development by Köhler and Milstein (1975), monoclonal antibodies have become major tools in immunology for dissecting complex populations of cell surface antigens (Williams et al., 1977; Barnstable et al., 1978; Lemke et al., 1978; Silver et al., 1980). More recently, however, their use has attracted much wider attention such as serological typing of viruses (Koprowski et al., 1977; Lubeck et al., 1980), differentiation between tissue cell types (Barnstable, 1980), dissection of the acetylcholine receptor (Moshly-Rosen et al., 1979; Gomez et al., 1979; Tzartos and Lindstrom, 1980) and differention between collagen subtypes (Linsenmeyer et al., 1979; Linsenmeyer and Hendrix, 1980). There have, however, as yet been no reports of monoclonal antibodies being used to map a single soluble protein molecule nor has the complex between an antigen and a monoclonal antibody been visualised in the electron microscope.

## 2. Rationale of Monoclonal Antibody Production:

### a) Introduction:

Although monoclonal antibodies afford great advantages over antibodies from immune serum in terms of homogeneity and reproducibility they have the disadvantage that their production requires considerable time and effort. Ideally, to make a monoclonal, what one would like to do is to immunise a mouse or rabbit with the appropriate antigen, pick out useful antibody secreting cells and then grow up single clones of these antibody secreting cells in culture. This, however, is not possible as the cells which can be identified as secreting a specific antibody are incapable of further division. It is therefore necessary

to use more sophisticated methods to obtain cells which may both divide and secrete antibodies.

Essentially, the way this is done is to fuse an antibody secreting cell with a cell adapted to growth in culture, thereby producing a hybrid cell which is capable of growing continually in vitro while retaining the ability to secrete its specific antibody. Of course the mechanics of the process are considerably more complex than this suggests and will be discussed in detail shortly.

The actual cells used in such a fusion are isolated spleen cells from an immunised mouse (Strain Balb/c) and a Balb/c myeloma cell line. The choice of a myeloma line, as opposed to another cell line of non-lymphoid origins is of considerable significance. Being a cancer of B lymphocytes, the myeloma is itself capable of antibody secretion, which is an important consideration since Coffino et al. (1971) showed by fusing the antibody secreting myeloma MPC-11 with the 1T mouse fibroblast line that immunoglobulin secretion is surpressed in hybrids between lymphoid and non-lymphoid cells. Furthermore, it appears that the myeloma may specifically fuse with B cells since T cell hybrids are not seen (Köhler and Milstein, 1976).

b) Hybrid Cell Selection

The mouse myeloma cell lines employed in monoclonal antibody production are almost all derived from the Balb/c myeloma strain MOPC-21 adapted to culture by Horibata and Harris (1970). These MOPC-21 cells (also known as P3) secrete an IgG ($\gamma_1,\kappa$) antibody of unknown specificity. Köhler and Milstein (1975) isolated a variant of MOPC-21 known as P3-X-63-Ag8 (abbreviated to X-63) which, like the original line, secreted the IgG ($\gamma_1,\kappa$) antibody but which was capable of growing in medium containing 20µg/ml of a purine analogue, 8-azaguanine which under normal circumstances would be incorporated into the DNA and kill the cell.

X-63, unlike MOPC-21 was also unable to survive in medium supplemented with hypoxanthine, aminopterin and thymidine (so called HAT medium). This inability of the X-63 myeloma to grow in HAT medium yet to be unaffected by 8-azaguanine is due to a metabolic efficiency in that the cells lack the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT), which normally salvages purine nucleotides and purine analogues, such as 8-azaguanine, for re-use. No revertants of X-63 have ever been identified, consequently the defect is considered to be due to a gene deletion.

Aminopterin, a folate analogue (Figure 7.1) blocks the main pathways of the denovo synthesis of thymidylic acid and purine nucleotides by inhibiting the enzyme dihydrofolate reductase which catalyses the formation of tetrahydrofolate, a cofactor required in both of these pathways (see Figure 7.1). An aminopterin blockade of a cell, however, need not be lethal if thymidine and hypoxanthine are included in the medium, since thymidine may be converted to thymidylic acid by thymidine kinase (T K Figure 7.1) whilst hypoxanthine may be used to form inosinic acid (IMP), the common purine nucleotide precursor, by the enzyme HGPRT. However, since X-63 lacks HGPRT, it is not able to synthesise purine nucleotides from hypoxanthine and thus cannot survive in the presence of aminopterin in HAT medium. It should be noted that a cell which lacks TK but not HGPRT would also be unable to survive in HAT medium. One such is P1-BU1, also of MOPC-21 origin.

The relevance of this sensitivity to HAT medium is that it provides a very straight forward method for the selection of hybrid cells (Littlefield, 1964). The antibody producing normal spleen cells are incapable of growth in culture but possess the gene to synthesise HGPRT, whereas X-63 is HGPRT negative but is adapted for growth in culture medium lacking aminopterin. Thus in HAT medium, only spleen cell/

Figure 7.1

The biochemistry of the selection of hybrid cells using "HAT" medium.

(a) The structures of dihydrofolic acid and aminopterin, a folate analogue. Di hydrofolic acid is the normal substrate of dihydrofolate reductase required in the formation of tetra hydrofolic acid ($FH_4$) which is used as a co-factor in the synthesis of purine nucleotides and deoxythymidylic acid. Aminopterin selectively inhibits dihydrofolate reductase.

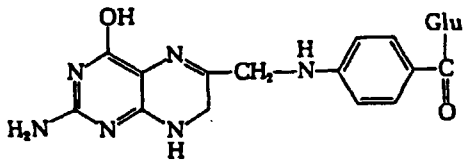
(b) The step in the synthesis of dTMP which requires tetrahydrofolate.

(c) and (d) The two steps in the synthesis of inosinic acid (IMP), the common purine precursor, which require tetrahydrofolate.

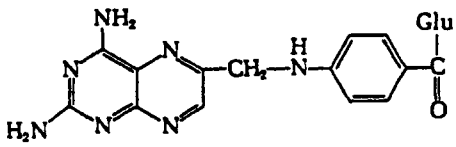
(e) The alternative pathway to IMP utilising exogenous hypoxanthine and involving the enzyme hypoxanthine guanine phosphoribosyltransferase.

(f) The formation of dTMP from exogeneous thymidine by the enzyme thymidine kinase.

a

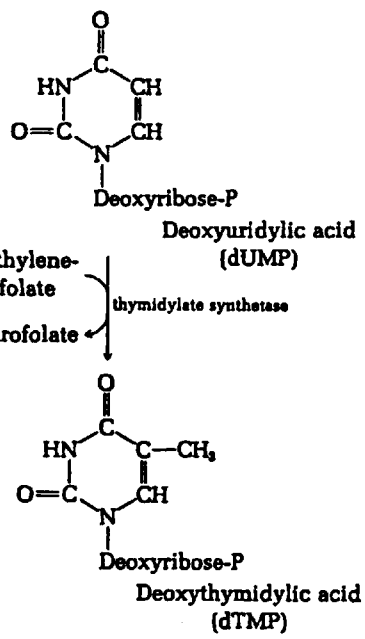


Dihydrofolic acid
(normal substrate)

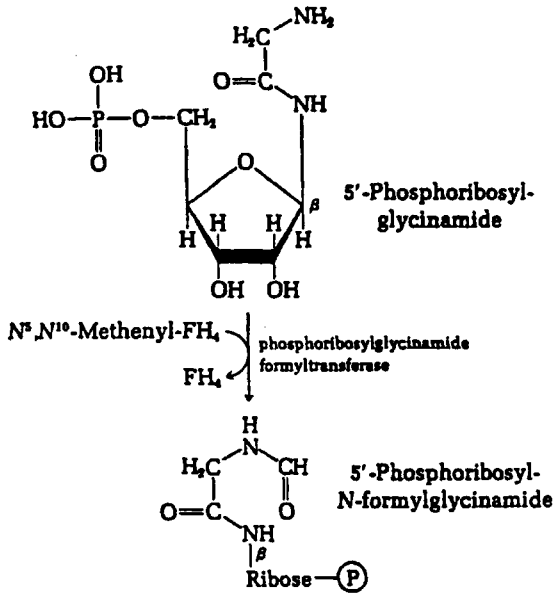


Aminopterin

b




c




d

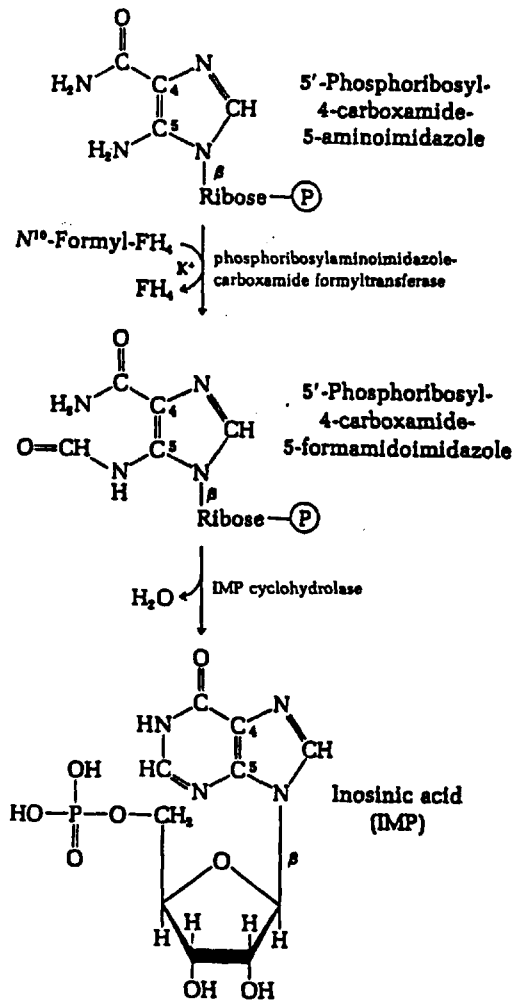



FIGURE 7.1

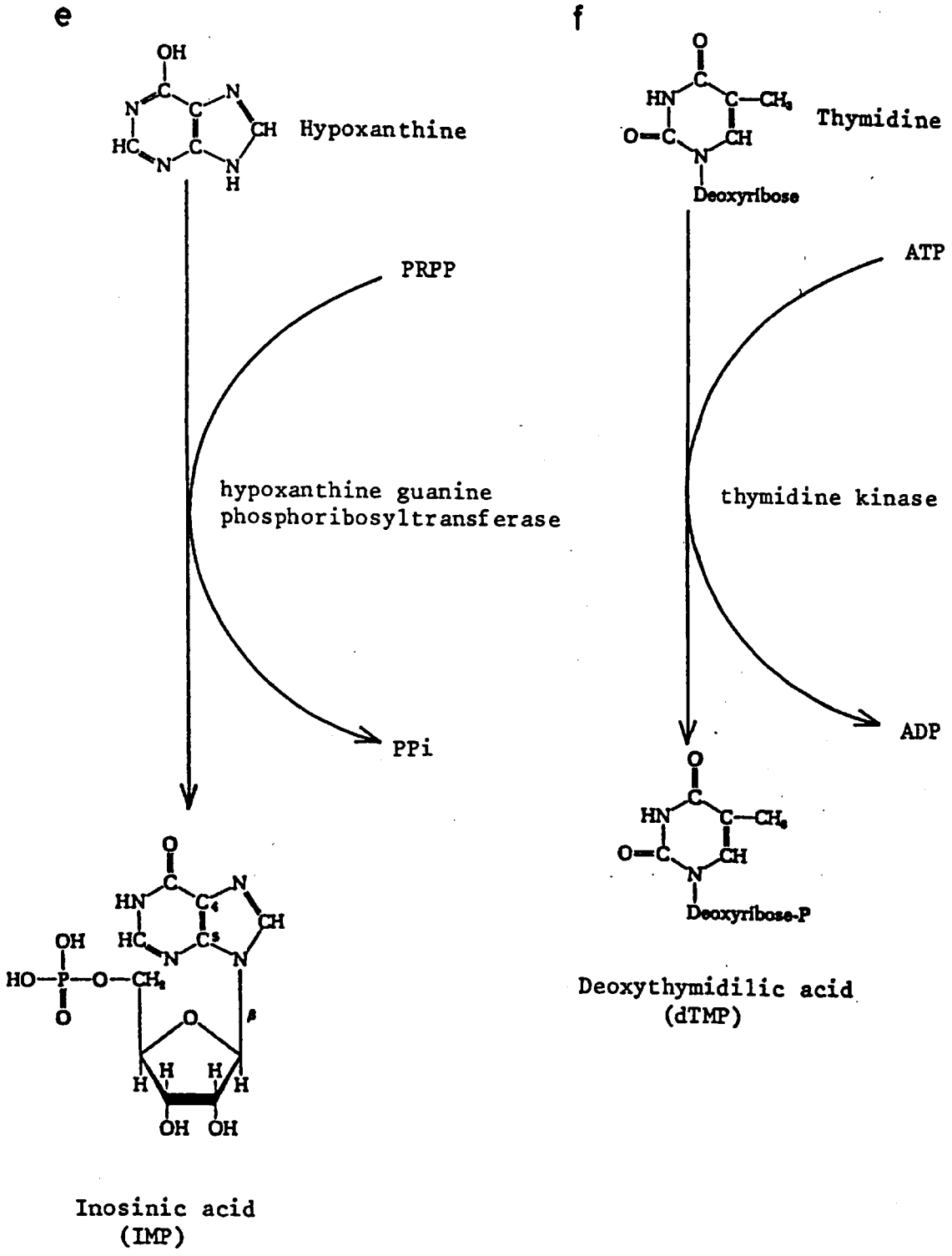



FIGURE 7.1 cont.

myeloma hybrids can survive, as the spleen cell provides the HGPRT necessary for purine nucleotide synthesis in the presence of aminopterin, whilst the myeloma provides the ability to grow in culture. Unfused myelomas are killed by the medium, while unfused spleen cells die anyway.

3. Outline of Methodology for Monoclonal Antibody Production

A schematic outline of the procedures used to develop a monoclonal antibody is given in Figure 7.2. $10^8$ spleen cells obtained aseptically from a suitably immunised mouse are fused with $10^7$ myeloma cells by brief exposure to polyethylene glycol (PEG Galfre et al., 1977; Gefter et al., 1977). The suspension of fused cells, in non selective medium, is plated out in 48-72 2ml cultures and allowed to grow for 24 h after which hybrid selection is initiated by the addition of an equal volume of HAT medium to each individual culture. Addition of HAT results in the death of at least 95% of all the cells. However, within about a week to 10 days, individual clones of hybrid cells may be seen to be developing in most of the small cultures; typically, each culture may contain four or five single clones. About three weeks after fusion, when the cultures are greater than 50% confluent, they must be screened for specific antibody secreting cells. In this investigation both radio immune assay and fluorescence microscopy were used for this purpose. Clearly, the screening method used will depend very much upon the nature of the antigen under scrutiny, although whatever technique is chosen it should be both rapid and suitable for screening a large number of samples.

Once a specific antibody secreting culture has been detected it is necessary to pick out, and grow up, clones of the antibody secreting cells since many of the cells in the culture may not be secreting antibody

Figure 7.2

A schematic outline of the procedures used to make a monoclonal antibody. Further details are given in the text.

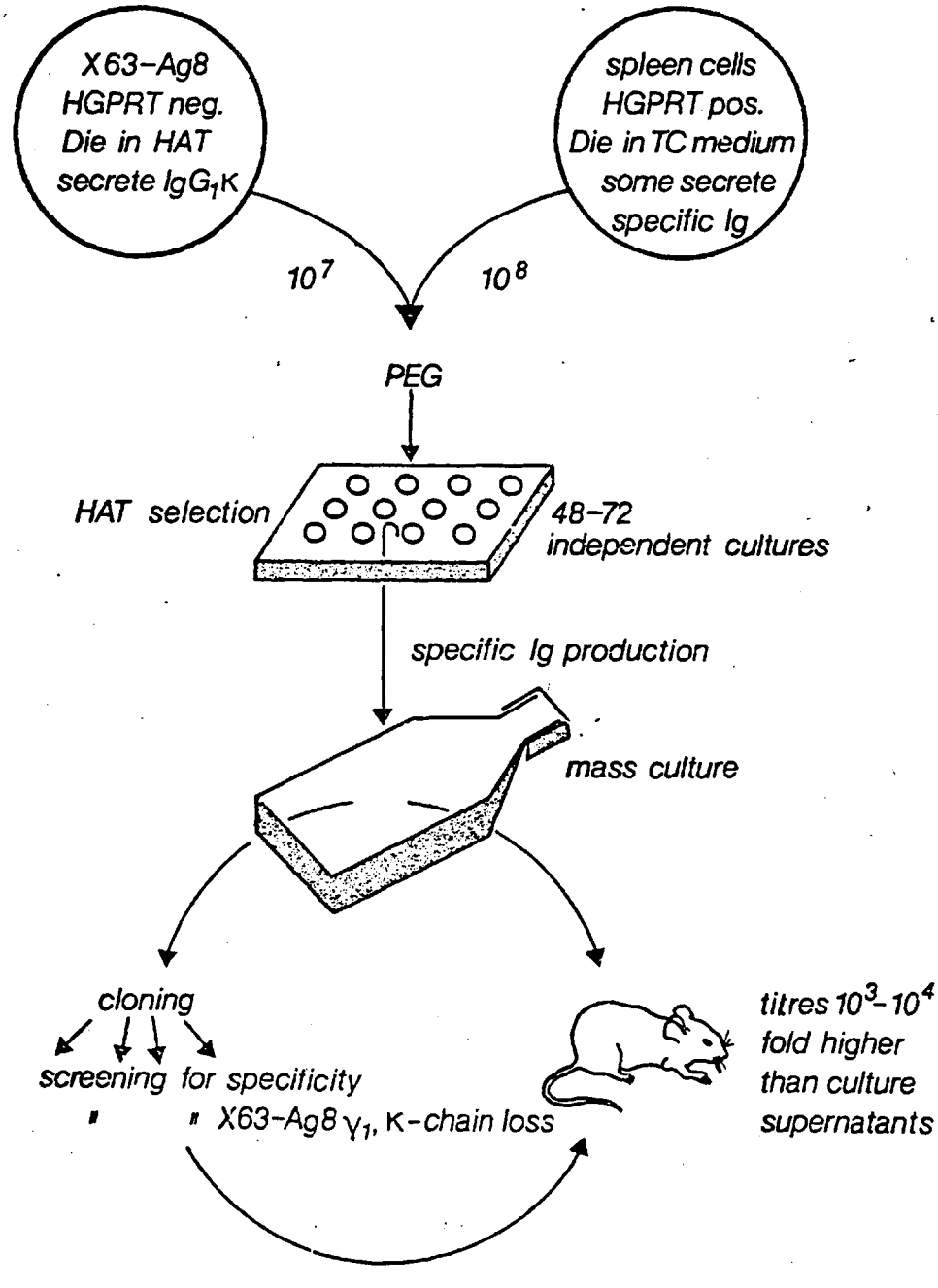

X63-Ag8
HGPRT neg.
Die in HAT
secrete IgG1K
spleen cells
HGPRT pos.
Die in TC medium
some secrete
specific Ig
10^7
10^8
PEG
HAT selection
48-72
independent cultures
specific Ig production
mass culture
cloning
screening for specificity
" " X63-Ag8 γ1, K-chain loss
titres 10^3-10^4
fold higher
than culture
supernatants

FIGURE 7.2

or else they may be secreting antibody of unknown specificity. This selection of clones may be performed by suspending the cells from a specific antibody secreting culture, at a suitable dilution, in soft agar (Cotton et al., 1973). The agar is of such a consistency that it does not prevent the cells from dividing and growing but is sufficiently firm to keep single clones of cells growing in discreet clumps. After about ten days of growth, at a suitably low cell concentration (see Chapter 8) it is possible to pick out balls of cells from the agar, each of which should be a single clone. Each clone may then be grown up in an individual 2ml culture for about three weeks and then retested for specific antibody production. This procedure of testing for antibody production and then cloning must be performed several times (at least twice and preferably four times) until a stable antibody secreting cell line is established.

The reason why this repetitive cloning is necessary is that the hybrid cells continually eject chromosomes until a stable number is arrived at. Although this chromosome loss is most pronounced immediately after fusion it may occur for several months (reviewed in Current Topics, Microbiology and Immunology 81). The result is that in uncloned cultures there is invariably a steady decline in antibody production, which may be due to loss of either antibody coding genes or to loss of the genes involved in the maintainance of the stability of malignant growth. Because of this inherent instability, at least in the early stages, it is good practice to freeze down samples of antibody secreting cells at every phase in the operation as an insurance against mishaps later on.

Once a single clone of antibody secreting hybrid cells has been isolated, it is possible to obtain the monoclonal antibody direct from the spent tissue culture supernatant medium. However, the antibody

concentration here is rarely more than a few μg per ml, mixed with a huge excess of other serum protein from the calf serum with which the medium is supplemented. Although such culture supernatant medium is useful for many diagnostic purposes such as fluorescence microscopy and direct labelling of protein separated on polyacrylamide gels, it is tedious to obtain high concentrations of antibody from this source. However, if the cells are readapted from HAT to normal culture medium and then grown in the peritoneal cavities of Balb/c mice as ascites tumours large amounts of monoclonal antibody may easily be obtained. The hybrid cells are not rejected since they are of Balb/c origin. Because of the huge density of cells which develops in the peritoneium (more than $10^8$/ml) the ascitic fluid so obtained may contain monoclonal antibody at concentrations as high as 5-10mg/ml.

## 4. The Use of Other Myeloma Cell Lines in Monoclonal Antibody Production

The Balb/c myeloma X-63-Ag 8 was the first myeloma to be used for monoclonal antibody production (Köhler and Milstein, 1975). However, it has the disadvantage in that it retains the ability of its parent line, MOPC-21 to secrete the Ig G ($\gamma_1$, k) antibody. Thus in the initial screening of hybrids, one must not only select for cells which secrete the desired monoclonal antibody, but also, where possible, for cells which have lost the ability to secrete the X-63 antibody, otherwise the hybrid cells may secrete hybrid antibodies if the specific monoclonal antibody is also an IgG (Cotton and Milstein, 1973; Köhler and Milstein, 1976; Köhler and Shulman, 1978). As an IgG contains two heavy chains and two light chains, randomisation of the assembly of two different IgG molecules results in 10 different hybrid IgG molecules being secreted,

and only 1/16 of these is the bifunctional specific antibody. Mixing of heavy chains between immunoglobulin classes does not occur (Köhler and Shulman, 1978), for instance in a hybrid cell secreting the X-63 IgG and a specific IgM one does not see $\mu$ and $\gamma$ heavy chains combined. Neither does one see mixing of specificities. Shulman and Köhler (1978) showed that no scrambling of variable and constant regions between the two antibody molecules occurred.

Several other 8-azaguanine reistant Balb/c myeloma cell lines are now used. One of these, MPC-11-XU5-6TG, also secretes its own antibody, an IgG$\gamma$2bk, and therefore creates the same problems as X-63 in forming hybrid molecules. However, two further, potentially more useful myelomas have been derived from X-63. The first is P3-NS1-1-Ag4-1 (abbreviated to NS1) which has lost the ability to synthesise the X-63 heavy chain and consequently only makes the k light chain which is not secreted but degraded intracellularly, except in hybridoma lines when it may combine with heavy chains of spleen cell origin and be secreted (Köhler et al., 1976; Köhler and Milstein, 1976). The second myeloma cell line, SP2/0-Ag-14, was itself derived from a fusion between Balb/c spleen cells and X-63 (Shulman et al., 1978). This cell line produces no antibody at all but still supports antibody synthesis in culture when fused with an antibody secreting spleen cell. Sp2/0-Ag-14 (abbreviated to Sp2/0 is thus a preferred cell line for the production of monoclonal antibodies.In this investigation I have used both the X-63 and SP2/0 myelomas.

# CHAPTER 8

## METHODS FOR MONOCLONAL ANTIBODY PRODUCTION

### 1. PREPARATION OF TISSUE CULTURE MEDIUM

#### (a) Dulbecco's Modified Eagle Medium (DMEM)

Single strength DMEM, lacking both sodium bicarbonate and sodium pyrurate, was made up in 10 l quantities in double distilled water (ddw) from powdered medium obtained from Gibco-Biocult Limited (Cat. No. 430-2100). To each 10 l batch, 37g of $NaHCO_3$ (anhydrous) and 1.1g of sodium pyruvate were added and the pH adjusted to 7.1. The medium was immediately sterilized by filtration through a 154mm diameter, 0.22μm pore size millipore filter (Millipore Limited) under nitrogen pressure. The medium was collected in 400ml aliquots in sterile 500ml bottles and stored at $4^{o}C$, in the dark prior to use. From each fresh batch of medium, two bottles were selected at random and incubated at $37^{o}C$ for 48h as a sterility test. If neither bottle showed signs of growth of microorganisms they were both used straight away.

When liquid medium was stored for more than one month L-glutamine (BDH) was added prior to use. After 1-2 months storage 5ml of 200mM glutamine stock (sterile filtered) was added. After 2-6 months, 10ml of glutamine stock was included. The sterile 200mM glutamine stock solution was stored at $4^{o}C$ for not more than one week.

(b) Puck's Saline $D_1$ x 20 Stock

| | | |
|---|---|---|
| NaCl | | 80g |
| KCl | | 4g |
| $Na_2HPO_4.7H_2O$ | | 0.45g |
| ($Na_2HPO_4$.Anhydrous) | | (0.37g) |
| $KH_2PO_4$ | | 0.3g |
| phenol red | | 12mg |
| ddw | to | 500ml |

(c) 1 x $D_1$G Basic Salt Solution (BSS)

| | | |
|---|---|---|
| $D_1$ x 20 stock | 5ml | |
| 10% glucose | 1ml | |
| ddw | 94ml | pH to 7.2-7.4 |

The solution was sterile filtered and stored at $4^oC$.

(d) Aminopterin x 100 Stock (A)

1.76mg of aminopterin (Sigma) were added to about 90ml of ddw. The aminopterin was then solubilized by the addition of 0.5ml of 1M NaOH. After adjusting the volume to 99.5ml, the solution was neutralized by the addition of 0.5ml of 1M HCl. The solution was then sterile filtered and stored in the dark at $-20^oC$.

(e) Hypoxanthine/Thymidine x 100 Stock (HT)

It was found most convenient to dissolve the hypoxanthine and thymidine together.

| | | |
|---|---|---|
| Hypoxanthine | (BDH) | 136.1mg |
| Thymidine | (Sigma) | 38.7mg |
| double distilled water | to | 100ml |

The solution was heated for 1h at 45-50$^{o}$C in order to ensure that the hypoxanthine dissolved. The x 100HT stock was then sterile filtered and stored at -20$^{o}$C. On thawing the solution was warmed briefly at 45$^{o}$C to redissolve the hypoxanthine.

(f) Myeloma Medium

Complete Myeloma medium was prepared from the following constutuents

400ml (1 bottle) DMEM

5ml penicillin/streptomycin (Gibco) 10,000 units/ml (pen/strep)

Final concentration, 100 units/ml

75ml Foetal or new born calf serum (Gibco) (FCS or NCS)

Final concentration, about 15% (v/v)

Calf serum was heat inactivated by incubation at 56$^{o}$C for 30min prior to addition to medium.

(g) Hybridoma Medium

400 ml DMEM

5 ml pen/strep

100 ml Heat inactivated FCS or NCS, final concentration 20% (v/v)

(h) HAT medium

500 ml Hybridoma Medium

5 ml HT stock

5 ml Aminopterin stock

(i) HT medium

500 ml Hybridoma Medium

5 ml HT stock

(j) Freezing Medium

Dimethyl sulphoxide (DMSO; BDH, AnalaR) was added dropwise, with shaking to ice cold culture medium, of the same type as that in which the cells to be frozen were growing, to give a final concentration of 10% (v/v). It was unnecessary to take steps to sterilise the DMSO since it is so toxic it remains sterile by itself. The medium was stored at $4^{o}C$ prior to use.

(k) PEG Fusion Medium

Polyethyleneglycol (M.Wt. 1500, BDH) was melted by either autoclaving or boiling for 30 min. in a water bath. It was then cooled to $50^{o}C$ (at which temperature it is still liquid) and mixed with an equal volume of serum free DMEM. The resulting 50% PEG solution was stored frozen. Prior to use, the pH was adjusted to give a slightly alkaline solution, (as judged by colour, the solution should be pink) by leaving the bottle uncapped in a laminar flow sterile hood for a short period of time.

2. MAINTENANCE OF MYELOMA CELL LINES

All operations involving the handling of cells and/or tissue culture media were carried out under sterile conditions in a laminar flow hood.

Both X-63-Ag-8 and Sp2/0-Ag-14 were grown in 90mm tissue culture petri dishes (Nunc) containing 10ml of myeloma medium, in a $37^{o}C$ humidified incubator with a 5% $CO_2$ atmosphere. The cells were passaged by transferring 1ml of cell suspension from a confluent plate (containing about $10^{6}$ cells/ml) to a plate containing 9ml of fresh myeloma medium. SP2/0 require passaging approximately every second day whereas X-63, which was slighly slower growing could be left for about three days.

Neither cell line was kept growing continuously, but were stored frozen (below), samples only being thawed out about two to three weeks prior to a fusion.

3. STORAGE OF CELLS

(a) Freezing of cells

Approximately $10^7$ cells in their normal growth medium were spun down at 500xg for 5 min., the supernatant discarded and the pellet placed on ice. The pellet was then resuspended in 1.5ml of ice cold freezing medium containing 10% DMEM and transferred to a cold, sterile, 2ml screw top, plastic freezing vial (Sterilin). The sealed vial was wrapped in cotton wool and placed in a styrofoam box, with approximately 1cm thick walls, which was then deposited in a -70°C freezer. After 24h the vial was transferred to a liquid nitrogen refrigerator.

(b) Thawing of cells

A vial was removed from liquid nitrogen and the cells thawed as quickly as possible in a 37°C water bath. As soon as the contents of the vial had liquified, the outside was sterilised by rinsing with 70% ethanol and the cells transferred to a 50ml tube (Falcon 2070F) containing cold growth medium. The cells were centrifuged for 5 min. at 500xg and the supernatant discarded. The pellet was given a second wash in cold medium and then divided up into about four or five 2ml cultures and placed in a 37°C humidified incubator with a 5% $CO_2$ atmosphere. After about 24h the cells were transferred to 90mm dishes and grown as normal. Typically 50-70% of cells survived freezing and thawing.

## 4. PURIFICATION OF RABBIT ANTI MOUSE IgG (RAM) FOR RADIO-IMMUNE ASSAY

### (a) Immunisation of Rabbits with mouse IgG

New Zealand white rabbits were injected with normal mouse IgG (Miles-Yeda) according to the following regime.

Day 1: 100µg IgG in 100µl PBS emulsified 1:1 with complete Freunds adjuvant (CFA, Gibco), injected sub scapular, side 1.

Day 7: 100µg IgG in 100µl PBS emulsified 1:1 with incomplete Freunds adjuvant (IFA, Gibco) injected sub scapular, side 2.

Day 28: 10µg IgG in 100µl PBS intravenous. Rabbits were bled 7-10 days later after which they were boosted intravenously as before every 28 days and bled about one week after each boost.

### (b) Purification of Antibodies by DEAE Chromatography

(Adapted from Hudson and Hay, 1976)

Anti-serum was centrifuged for 15 min. at 15,000 rpm and at 4°C in a Sorvall SS-34 rotor. To the cleared supernatant, an ice cold saturated ammonium sulphate solution (adjusted to pH 7.5 to 7.8 with NaOH) was added slowly with constant stirring, on ice, until a final concentration of 40% ammonium sulphate was reached. The solution was stirred for a further period of about 20 minutes and then centrifuged at 10,000 rpm for 10 min in the SS-34 rotor. The supernatant was discarded and the IgG containing pellet dissolved in a minimum volume of ice cold, 10mM potassium phosphate buffer, pH 7.8 (10KP). If the solution was found to be slightly pink, it was brought back to the original serum volume with 10KP and the ammonium sulphate precipitation step repeated. The redissolved pellet was dialysed for 48 h against three changes of 200 volumes of 10KP at 4°C and then applied to a DE52 (Whatman) column equilibrated in 10KP. 1ml of packed DE52 was used for every 10mg of protein. The column was eluted with 10KP and all

fractions with an $A_{280}$ greater than 10% of the peak value were pooled (peak I). Washing with 10KP was continued until the $A_{280}$ of the effluent had fallen to below about 0.05. The column was then eluted with 10KP containing 50mM NaCl and a second peak (peak II) collected. These peaks, which both contained pure IgG, were pooled, dialysed against PBS at 4°C and then stored frozen at -20°C. A small sample was checked for anti mouse IgG activity by double immuno diffusion (Ouchterlony and Nilsson, 1973). Since DEAE purified RAM gave sufficient sensitivity in the radioimmune assay it was not considered necessary to affinity purify it.

5. IODINATION OF RABBIT ANTIMOUSE IgG

Two methods were used for protein iodination. Initially the Bolton and Hunter reagent, N succinimidyl 3-(4-hydroxy, 5- $^{125}I$ iodophenyl) propionate (Bolton and Hunter, 1973) was employed. However, this was found to give very variable results, consequently for later work the Chloramine T method was used (Hunter and Greenwood, 1962). All operations were carried out behind lead bricks in the fume hood of the radiation laboratory.

(a) Iodination using the Bolton and Hunter Reagent

1mCi of Bolton-Hunter reagent (Amersham) was dried down in its vial, the benzene solvent being evaporated by a gentle stream of nitrpgen. To this was added 20µl of 100 mM borate buffer pH 8.5 at 0°C (composition given below) followed immediately by 20µl of RAM in PBS at a concentration of 1mg|ml. The mixture was incubated on ice for 15 minutes with continuous agitation after which 0.5ml of 0.2M glycine in 100mM borate buffer pH 8.5 was added and incubation continued for a further 5 minutes. Finally 0.5ml of 0.25% (w/v) gelatin in PBS was added and the mixture desalted on a 10ml Bio-gel p-10 column (Bio-rad) equilibrated in 0.25% (w/v) gelatin in PBS. The labelled protein fractions were collected and diluted in PBS containing 2% BSA and 2% sodium azide (both w/v) to give an activity of 100,000 cpm

per 100µl and stored in a plastic tube in a lead container.

The 100mM borate buffer was prepared as follows. $H_3BO_3$ was dissolved in 0.2M KCl to a concentration of 0.2M. 50ml of this solution was brought to pH 8.5 with 0.2M NaOH (about 23ml). The solution was then made up to 100ml with distilled water to give a final borate concentration of 100mM.

(b) Iodination using the Chloramine T method

Before carrying out the iodination several grams of Zerolit FF (iP) quaternary ammonium ion exchange resin (BDH) was washed in 3I0 NaCl and then packed to form a column in a 2ml plastic disposable syringe. Through this was passed 1ml of normal rabbit pre-immune serum, to prevent non specific binding of radio-labelled immunoglobulin to the column. All excess liquid was then pressed out using the syringe plunger, leaving the syringe packed with damp resin.

100µl of RAM in PBS at a concentration of 1mg|ml was diluted with 100µl of 0.05M sodium phosphate buffer, pH 7.5 and added to 1mCi of carrier free Na $^{125}I$ (Amersham) in the NaI shipping vial. To this was added 50µl of chloramine T (BDH) at a concentration of 2mg|ml in 0.05M sodium phosphate buffer, pH 7.5 and the solution mixed. After 1 min, the reaction was quenched by transfer of the mixture, using a piston pipette, to a 1ml plastic Eppendorf tube containing 200µl of sodium meta-bisulphite at a concentration of 2.4 mg|ml in the 0.05M phosphate buffer.

The labelling mixture was passed slowly through the zerolit column under gentle pressure and collected in a plastic Eppendorf tube (1ml). The column was then washed with 600µl of 3I0 NaCl, all of which was pressed through into the same tube using the syringe plunger. Free $^{125}I^-$ was retained on the column whereas the labelled protein passed directly through. The labelled protein solution, approximately 1ml, was mixed with 1ml of PBS

containing 2% (w/v) BSA, 2% (w/v) sodium azide (PBS/BSA/Azide) and stored at 4°C. A small sample (2-3µl) was always taken, diluted x 100 with PBS/BSA/azide and the protein precipitated by the addition of an equal volume of 6% trichloroacetic acid. When the precipitate was centrifuged out, never more than 3% of the total radioactivity was found in the supernatant. Incorporation of radioactivity into protein was usually in the order of 90%.

## 6. IMMUNISATION REGIME FOR MICE

Three week old Balb/c mice (male or female) were injected intraperitoneally with about 40µg of spectrin (originally column purified at low salt) in PBS emulsified with an equal volume of CFA. The volume injected varied from about 100µl to 400µl according to the spectrin concentration. After three weeks the mice were boosted with a further 40µg of spectrin, again intraperitoneally, but in incomplete Freunds's adjuvant (Gibco). Three days after this boost, spleens were take for fusion. It was found most convenient to give the boost early on a Friday morining and then to carry out the fusion on the following Monday morning.

## 7. PREPARATION OF CELLS FOR FUSION

### (a) Mouse Spleen Cells

Usually it was possible to obtain enough cells from a single mouse to carry out one fusion. Three days after its booster injection, a Balb/c mouse was killed by cervical dislocation. The whole animal was sterilised by brief immersion in absolute methanol and then transferred to a sterile dissecting board in a laminar flow hood where the spleen was carefully removed. Particular attention was paid to freeing the spleen from as much fat and connective tissue as possible. The spleen was placed in a sterile, plastic bacteriological petri dish containing 10ml of BSS and the cells gently teased from the capsule using the flat end of a stainless steel spatula.

Clumps of cells were further dispersed by trituration in a 10ml pipette. The cell suspension was then transferred to a sterile 15ml conical bottom plastic tube (Sterilin) where the large aggregates were allowed to settle out for 2-3 minutes, after which the supernatant was decanted into a second similar tube and centrifuged for 5 min at 500xg(av). The supernatant was discarded and the pellet of single cells resuspended in 10ml of BSS. At this stage a small sample of cell suspension (20µl) was mixed with 1ml of 0.2% Trypan blue (BDH) in 155mM NaCl containing 1% glacial acetic acid, and the number of viable cells counted using a haemocytometer. Viability should be better than 95%. The remaining cell suspension was centrifuged again at 500xg(av) for 5min and the pellet resuspended in 10ml of slightly alkaline (as judged by colour) serum free DMEM to give a final concentration of at least $2 \times 10^7$ cells per ml.

(b) Mouse peritoneal macrophages

A Balb/c mouse (unimmunised) was carefully killed and sterilised as before. After transfer to the sterile hood the skin over the abdomen was removed, taking great care not to penetrate the peritoneum. The peritoneal cavity was then flushed out with 5ml of ice cold, sterile 0.34 M sucrose in double distilled water, rapidly injected using a 5ml disposable syringe fitted with a 21 gauge needle. With practice, about 4.5ml of fluid could be recovered from the peritoneum, containing about $3 \times 10^6$ cells. Fluid from several mice was pooled in a 50ml Falcon 2070F tube and spun down for 10 minutes at 500g(av). The pellet was resuspended in complete hybridoma medium to give a minimum concentration of $5 \times 10^6$ cells per ml and kept on ice until required.

## 8. FUSION OF MOUSE SPLEEN CELLS WITH MYELOMA CELLS

(Galfre et al., 1977).

$10^7$ optimally proliferating myeloma cells (X-63 or SP2/0) in myeloma medium were spun down for 5 min at 500 x g(av) and resuspended in 10ml

of serum free DMEM. The myeloma cells were mixed with $10^8$ spleen cells in a 50ml conical bottom plastic tube (Falcon 2070F) and the volume made up to 50ml with serum free DMEM, after which the cells were spun down together for 5 min at 500xg (av). The supernatant was carefully withdrawn and the pellet loosened by tapping the bottom of the tube and warmed to 37°C. 0.7ml of 50% PEG fusion medium at 37°C was added slowly over a period of 15s while gently stirring the pellet with the tip of the pipette. The cell suspension was then incubated for a period of 45s at 37°C after which 2ml of serum free DMEM at 37°C was added dropwise over the next 2 min with constant agitation. During the following three minutes a further 13ml of 37°C, serum free DMEM were added, also with continuous agitation. (Total addition time for 15ml = 5 min.). The fused cells were spun down for 5 min at 500xg(av) and the supernatant discarded. The pellet was gently resuspended in 10ml of complete hybridoma medium (37°C) and about $5.10^6$ peritoneal macrophages added as feeder cells. The volume was made up to 50ml with hybridoma medium at 37°C, and 1ml aliquats were distributed into each well of two 24 place Costar tissue culture trays (Costar Inc., Cambridge, Mass., Agents A.R. Horwell) which were then placed in a 37°C humidified $CO_2$ incubator. After 24h, selection for hybrids was initiated by the addition of 1ml of HAT medium to each well. Then, on each of the following 3 days and later at 2 day intervals, 1ml of medium was removed from each well and replaced with 1ml of fresh HAT medium. About two weeks or more after fusion, HAT medium was replaced by HT medium.

About three weeks post fusion when large clones of hybrid cells had developed and the wells were more than 50% confluent, each 2ml culture was tested for specific antibody production (below). Samples of cells from antibody positive cultures were frozen down, while other samples were taken for cloning in soft agar.

9. SCREENING CULTURES FOR ANTI SPECTRIN PRODUCTION

(a) Solid phase Radioimmune Assay (Catt and Tregear, 1967; Stocker and Heusser, 1978).

A fast, solid phase radioimmune assay (RIA) was used for initial screening of cultures for specific antibody production. 100µl of spectrin (column purified at low ionic strength) at a concentration of about 1mg|ml in PBS were pipetted into each well of a 96 place, non-sterile, 'V' bottom Cooke M25 PVC flexible microtitration plate (Cooke Laboratory Products; Agents, Dynatech Ltd.). The plate was covered in Nescofilm and allowed to stand in a 37°C incubator for 2 hours. During this time a small quantity (1%) of the spectrin was absorbed to the plastic surface. At the end of the incubation the spectrin solution was removed and was either discarded or used to coat further microtitration plates. After removal of the spectrin solution each well was filled completely with PBS/BSA/azide, the plate covered in Nescofilm and incubated for a further 30 minutes at 37°C. During this step, all remaining protein binding capacity of the plastic was blocked off. The PBS/BSA/azide was aspirated and 100µl of cell culture supernatant was pipetted into each well. Each supernatant was used at three different concentrations; undiluted, diluted x 3 with PBS and diluted x 10 with PBS. The plate was covered with Nescofilm and incubated for at least 2 hours at 37°C, after which time the supernatants were removed and the plates washed thoroughly three times in PBS/BSA/azide. 100µl of $^{125}$I-RAM in PBS/BSA/azide, containing not less than 20,000 cpm, were added to each well, and the plate again incubated at 37°C for a minimum of 2 hours (this incubation could be carried out overnight). At the end of this final incubation, the radioactive antibody was carefully aspirated and the microtitration plate given one wash in PBS/BSA/azide and then 5 washes in PBS.

The washed microtitration plate was fitted firmly onto a hard plastic 96 well plate (Sterilin) and the top of the labelled plate sliced off with a hot wire so that each of the labelled wells was released. Each well was then counted for 1-5 min. in an LKB-Wallac RAK $\gamma$ counter. In this way 100 or more cultures could be tested in about 24 hours. Anti-spectrin positive culture supernatants gave counts, in general, about ten times background, although this varied between each batch of microtitration plates used. The method was not suitable for precise quantitation although it was useful for comparative purposes.

(b) Fluorescence Microscopy

This method was not used for initial screening of culture supernatants since I considered it to be not as reliable as the RIA. However, it was extremely quick and convenient for evaluating the usefulness of ascitic fluids (below) or supernatants from cloned cells. A small quantity, about 1ml, of freshly drawn blood in citrate anticoagulant was given two washes in 30 volumes of PBS. The red blood cells were then suspended at a concentration of 0.2% (v/v) in PBS and 20ml aliquots of the cell suspension poured into several 90mm plastic tissue culture petri dishes. The dishes were left standing at room temperature for the cells to settle out, which took about 20-30 minutes. At the end of this period a monolayer of red blood cells was fixed to the plastic by very carefully replacing the PBS with a 1:1 mixture of acetone and methanol (at room temperature). After 2 minutes fixation the acetone/methanol was removed and the plate air dried. This fixation protocol had the advantage that it produced a non wettable layer of cells which was essential for screening large numbers of samples, as it allowed many discrete droplets to be placed on the monolayer without mixing. The surface of the cell coated plate was ruled into 5mm squares and in the centre of each square was placed a single 3µl drop of sample solution (PBS diluted ascitic fluid or tissue culture supernatant). The

plate was then left in a humid atmosphere for 30 min. at room temperature after which time it was flooded with PBS and then given three 5 min PBS washes. The plate was then flooded with the second layer antibody, fluorescein conjugated RAM (Miles), suitably diluted and left for a further 30 min. To keep the volume of RAM required to a minimum coverslips were placed over the sample area. The plate was finally given a further three 5 min. PBS washes and then coverslips mounted over the samples on PBS containing about 25% (w/v) polyvinyl alcohol (Gelvatol, Monsanto Corporation). The samples were examined under epi illumination using a Zeiss fluorescence microscope fitted with a x 40 oil immersion objective and a Zeiss 35mm camera loaded with Kodak professional Ektachrome 200. Samples containing anti-spectrin antibodies gave very distinctive surface type fluorescence images of the red blood cells. Blank samples showed no background fluorescence.

(10) CLONING OF HYBRID CELLS IN SOFT AGAR (Goffino and Scharff, 1971; Cotton et al., 1973).

30mls of double distilled water containing 2.5% purified agar (Oxoid) were sterilised by placing in a boiling water bath for 30 minutes, after which it was transferred to a 45°C water bath. 117ml of culture medium (HAT, HT or hybridoma medium) were mixed with 3ml of 10 times concentrated DMEM (Flow Laboratories, Liquid Medium) and also placed in the 45°C water bath. When the two solutions had equilibrated at this temperature they were mixed to give a 0.5% solution of agar in single strength DMEM (plus serum etc.). 10ml of this solution was rapidly poured into each of ten 90mm bacteriological petri dishes and allowed to set for 20 minutes at room temperature. The remaining 50ml were transferred to a 37°C water bath. Hybridoma cells in their appropriate culture medium were next mixed with the 37°C, 0.5% agar solution at a ratio of 1:1. 2ml of this hybridoma-agar mixture was quickly pipetted dropwise over the entire surface of the 0.5% agar layer in each of the dishes to give a thin layer of 0.25% agar in DMEM

containing the cells with a 'feeder' layer underneath. The cell concentration was adjusted before mixing with the agar to give approximately 5-10,000 cells per plate. The plates were then placed in a 37°C incubator (humidified plus 5% $CO_2$) for about 7-10 days, after which time individual clones were visible to the naked eye. It was important not to let the clones get too large, otherwise cells in the centre of the clumps became starved of medium and died off.

When the clones had grown up to be easily visible (16-32 cells) 50 or so were picked at random out of the agar and each single clone was placed in an individual well of two or three 24-well Coster trays with 1ml of culture medium and allowed to grow up. After 2-3 days 1ml of medium was added to each well after which they were fed as normal by removing half the culture medium and replacing it with fresh medium. Picking of clones out of the agar was most easily accomplished using a pasteur pipette fitted with a teat, the barrel of the pipette having been briefly warmed over a bunsen burner. Contraction of air inside the pipette drew individual clones into the tip surrounded by a plug of agar; it was then simple to expel the clone into a Costar tray well. After 2-3 weeks of growth, clones were ready to be tested for anti spectrin activity. Following their first cloning, cells were usually adapted from HT medium into hybridoma medium.

## 11. INDUCTION OF ASCITES TUMOURS

Very high antibody titres could be obtained by growing monoclonal antibody secreting hybridomas as ascites tumours in Balb/c mice. Three week old mice (male or female) were primed with an intra-peritoneal injection of 200µl of pristane (2, 6, 10, 14 tetra methyl pentadecane, Aldrich) as described by Koprowski et al. (1977). After 10-14 days, $10^7$ - $10^8$ cells, adapted to growth in hybridoma medium were injected intra-peritoneally in 0.5ml of sterile PBS or serum free DMEM. After a further 10-14 days, the peritoneal cavities of mice showing obvious tumour development were drained.

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Usually 2-3ml could be obtained per mouse, and each could be drained several times. The fluid so collected, which contained extremely high concentrations of cells (more than $10^7$ per ml) was centrifuged at 500xg(av) for 5 minutes and the antibody rich supernatant stored frozen. When these samples were thawed for use, fibrin clots formed which were removed by centrifugation at 19,000 rpm in a Sorvall SS-34 rotor for ten minutes. Very often, ascites did not form following the first injection and instead, the cells embedded to give large solid masses. To overcome this problem, the softest parts of solid tumours were dissected out aseptically and dispersed in PBS, whereupon the cells were reinjected into further pristane primed mice. Usually after 2-3 such in vivo passages, ascites tumours formed. The tendency of hybridomas to form solid tumours varied greatly from cell line to cell line. The antibody content of ascitic fluid was evaluated by RIA or fluorescence microscopy. Polyacrylamide gel electrophoresis was also performed as the antibody concentration was normally high enough to easily distinguish the heavy and light chains of the monoclonal immunoglobulin.

12. CHROMOSOME PREPARATION (Seabright, 1971)

Twice cloned hybrid cell lines were further characterised by counting the chromosome complement. 5ml of culture medium containing about $10^6$ optimally proliferating cells were transferred to a 6ml sterile tube (Falcon, 2003F). They were arrested in mitosis by adding colchicine (Sigma) to the culture medium at a final concentration of 10µg/µl and incubated for 2 hours at 37°C after which the cells were spun down at 500xg(av) for 4 minutes and resuspended in 5ml of 75mM KCl. The cells were incubated in this solution for 15 minutes at room temperature, centrifuged again and then fixed by resuspending them in 5ml of methanol/glacial acetic acid (3:1 v/v). After a 10 minute incubation at room temperature, the cells were given two washes in fixative and then prepared for microscopy.

Acid washed, glass microscope slides which had been soaked for 24hours in ether/ethanol (1:1v/v) were cooled for about 10s by placing them across the open top of a styrofoam container full of liquid nitrogen. Four or five drops of cells in fixative were placed on the slides which were then partially air dried at room temperature and then fully dried by warming over a small spirit lamp. Slides were directly stained using 0.68% Giemsa in methanol-glycerol (BDH) and examined using a Leitz Ortholux microscope. Groups of chromosomes were photographed on Ilford FP4 using a Leitz 35mm camera and the numbers of chromosomes counted on photographic enlargements.

13. GEL ELECTROPHORESIS (Laemmli, 1971)

(a) Stock solution

Acrylamide/BisAcrylamide (Aca/Bis)

| | |
|---|---|
| Acrylamide | 30% |
| N,N, Methylene bisacrylamide | 0.8% |

The solution was pretreated with amberlite resin and charcoal as described in Chapter 2.

(b) Preparation of Analytical gel

Composition for 10% gel (7.5% in brackets)

| | |
|---|---|
| Aca/Bis | 16.7ml (12.5ml) |
| 1.5M Tris. Hcl pH 8.8 | 12.5ml |
| Distilled water to | 49.5ml |

The solution was mixed by inversion and degassed.

| | |
|---|---|
| 10% SDS (High purity) | 0.5ml |
| TEMED (Neat) | 25µl |
| 10% Ammonium persulphate | 150µl |

The solution was mixed and then poured to a height of 12.5cm into a conventional slab gel apparatus consisting of 18 x 18cm glass plates separated by 1.5mm thick perspex spacers and sealed with a solution of 1.5% agarose, 0.1% SDS. The gel solution was carefully overlaid with a 0.02% solution of SDS and left 30 min to polymerise.

(c) Preparation of 3% Stacking gel

| | |
|---|---|
| Aca/Bis | 1ml |
| 0.5M Tris. HClpH 6.8 | 2.5ml |
| Distilled water to | 9.8ml |

The solution was mixed by inversion and degassed

| | |
|---|---|
| 10% SDS (High purity) | 100µl |
| TEMED | 5µl |
| 10% Ammonium persulphate | 100µl |

The solution was mixed and a small quantity (1ml) used to rinse the top surface of the analytical gel and was then discarded. The remaining solution was poured onto the top of the gel and a perspex sample application comb slotted into place. The gel was then left to polymerise for a further 30 min.

(d) Denaturation Buffer

| | |
|---|---|
| Glycerol | 2ml |
| 0.5M Tris. HClpH6.8 | 2.5ml |
| 10% SDS (high purity) | 4ml |
| Bromophenol Blue | 0.002% |
| DTT | 200mM |
| Distilled water to | 10ml |

Samples for electrophoresis were mixed 1:1 with this buffer and placed in a boiling water bath for 2 minutes.

(e) The Electrophoresis Run

The slab gel, with bottom spacer and comb removed was clamped in place in a conventional gel tank and the upper and lower resevoirs filled with a running buffer containing

| | |
|---|---|
| Glycine | 14.4g |
| Tris | 3g |
| SDS | 1g |
| Distilled water to | 1 L |

Appropriate volumes (up to 100µl) were layered into each slot and were electrophoresed at 40V for 18 hours (without cooling). The gel was stained and fixed in coomassie blue/iso-propanol/acetic acid as described in Chapter 2. For autoradiography, stained gels were equilibrated in 7% acetic acid containing 1% glycerol and then dried down onto a piece of card using a Bio-rad gel dryer. Films and exposure conditions are given in following sections.

## 14. ANALYSIS OF $^{14}$C LEUCINE LABELLED MONOCLONAL ANTIBODIES ON SDS POLYACRYLAMIDE GELS

(a) Materials

$^{14}$C Leucine (Amersham, Specific antivity 250µCi/µMol) 50µCi/ml

Leucine free DMEM (Gibco-Biocult) containing 100 units/ml pen/strep

Foetal calf serum dialysed extensively against PBS to ensure removal of all leucine.

These components were used to make up DMEM containing 5% foetal calf serum and 5µCi/ml $^{14}$C Leucine. Where necessary L glutamine and HAT or HT were added at the appropriate concentrations (Section 8.1).

(b) Incubation of Cells with Radioactive Medium

About 2 x $10^5$ cells were pipetted into a sterile capped 6ml polystyrene tube (Falcon 2003F) in their normal growth medium and centrifuged for 5 min. at 500 xg(av). The supernatant was carefully aspirated and replaced with 200µl of the $^{14}C$ Leucine containing medium. The cells were gently resuspended and the loosely capped tube placed in a humidified 37°C $CO_2$ incubator for 18 hours. Following this incubation the cells were again spun down and SDS gel sample taken from the supernatant. 80µl of denatured sample were run per track on a 10% polyacrylamide gel. Stained, dried down gels were autoradiographed at room temperature using Fuju RX NIF X-ray film and an exposure time of 1-2 days. Autoradiographs were developed using a Kodak X-OMAT X-ray film processor.

15. TRYPSIN DIGESTION OF SPECTRIN AT LOW IONIC STRENGTH (Anderson, 1979).

Crude spectrin at a concentration of about 1mg|ml in a solution containing 10mM Tris, 2mM $CaCl_2$ pH 7.5 was exposed at room temperature to trypsin (Sigma 2 x recrystallized) at a ratio of 200µg of spectrin to 1µg of trypsin. The reaction was stopped after 10 minutes by denaturation for electrophoresis.

16. IN SITU LABELLLING OF POLYACRYLAMIDE GELS WITH MONOCLONAL ANTIBODIES (after Burridge, 1976)

Crude spectrin was run on a 7.5% SDS polyacrylamide gel such that band 1 and band 2 were well resolved. Electrophoresis was terminated when the tracker dye had reached the bottom of the gel which was then briefly fixed for 2 hours in a solution containing:

46% Water

46% Methanol

8% Glacial acetic acid (v/v)

The gel was placed in solution 'A' containing

0.15M NaCl

0.05M Tris. HCl pH 7.4

0.1% Sodium azide

The gel was allowed to equilibrate in this solution for at least 4 hours with several changes. To label the gel with monoclonal anti spectrin it was immersed in spent culture medium from a clone of antibody secreting cells, and left at room temperature for 12 hours. To remove unbound antibody, the gel was soaked in solution 'A' for 3 days with frequent changes of solution. The gel was next treated with $^{125}$I-RAM by placing it in a flat humidified box and then layering on the $^{125}$I-RAM in a minimum volume of PBS/BSA/azide such that it was evenly covered with about $2 \times 10^5$ cpm/cm$^2$. The gel was incubated in this way for 12 hours at room temperature and then washed for 3 days in PBS to remove all unbound radioactivity. The gel was finally fixed and stained, dried down and autoradiographed. The film used, Fuji RX NIF was presensitized using an electronic flash unit and then exposed at -70°C with an image intensifying screen for 24 hours (Laskey and Mills, 1977).

17. LABELLING OF PROTEIN BLOTS FROM GELS USING MONOCLONAL ANTIBODIES (Towbin et al., 1979; Bowen et al., 1980)

This method for immunologically detecting bands on gels was used as an alternative to that described in the previous section. Either ghosts, crude spectrin or tryprinised spectrin were run out on a 7.5% polyacrylamide SDS gel. Often it was found convenient to run one sample at a time on a gel polymerised

without a comb in place so that the sample was layered across the entire width of the gel, which could later be cut into strips for use with different culture supernatants. Following electrophoresis, the gel was equilibrated for 3 hours, with shaking, in a renaturation buffer containing:

4 M urea
50mM NaCl
2mM EDTA
0.1mM DTT
10mM Tris HCl pH 7.0

It is important to note that the gel was not fixed at any stage. Following equilibration, the gel was sandwiched between the two sheets of nitro-cellulose (Sartorius Membrane Filter, 0.1μm pore size) which had been wetted in transfer buffer containing:

50mM NaCl
2mM EDTA
0.1mM DTT
10mM Tris. HCl pH 7.0

This nitrocellulose filter/gel sandwich was placed between two foam rubber pads, also wetted in transfer buffer, and clamped together between two stainless steel screens using bulldog clips (Bowen et al. 1980). The whole assembly was immersed in 5l of transfer buffer for 48 h (changed once after 18h) during which time most of the protein diffused out of the gel and onto the nitrocellulose filters. After transfer, one of the nitrocellulose filters was stained for protein in a solution containing:

| | |
|---|---|
| 0.1% | Amido black (Bio-rad) |
| 43 % | Methanol |
| 10 % | Glacial acetic acid |
| 47% | Water |

After 15 minutes in this solution, the filter was destained for 1 hour in three changes of 90% methanol, 2% acetic acid, 8% water (Schaffner and Weissman, 1973). The second filter was used for antibody labelling. All remaining protein binding capacity of this filter was blocked off by immersing it for 2 hours in PBS/BSA/azide at room temperature. In later experiments a more efficient blocking solution was used, namely, PBS containing 1% gelatin and 0.01% Nonidet P-40 (NP-40, BDH) also at room temperature. Longitudinal strips from blocked filters were separately immersed in about 50ml of cell culture supernatants containing monoclonal antibody and incubated for 2 hours at 37°C. The strips were given three 5 minute washes in blocking solution and then overlaid with $^{125}$I-RAM at 2 x $10^5$ cpm/cm$^2$ in a minimum volume of either PBS/BSA/azide, or more recently PBS containing 1% gelatin and 0.05% NP-40. Incubation with labelled antibody was for 2 hours at room temperature. The strips were finally washed in the blocking solution for five minutes and then 3 x 5 minutes in PBS, after which they were thoroughly air dried and autoradiographed, using presensitized film and intensifying screen for 6-8 hours at -70°C.

# CHAPTER 9

## RESULTS: MONOCLONAL ANTISPECTRIN PRODUCTION

### 1. The Fusion Process

A total of seven fusions have been carried out between mouse myeloma cell lines and spleen cells from mice immunized with spectrin. The fusion technique employed in this investigation (Galfre et al., 1977) was found to give fairly varied results. In some experiments as few as 20 wells out of the 48 initially plated out contained viable hybrid cells whereas in others, all 48 wells contained HAT resistant, rapidly proliferating cells. This variability was probably a reflection of the difficulties involved in controlling the fusion process. The fusion method used here, involving a brief 1 min. exposure to a high concentration of PEG has the disadvantage that it is very sensitive to such factors as rate of addition of PEG to the cells and to the efficiency of mixing which may be very difficult to reproduce between fusion experiments. Gefter et al. (1977) have devised a protocol involving spinning the cells down in the presence of lower concentrations of PEG than have been used here but giving the cells a much longer 8 min. exposure, which in their hands gives consistently efficient fusions. It is intended to evaluate this technique for the future production of antispectrin secreting hybridomas.

The addition of macrophages as 'feeder' cells to cultures soon after fusion was found to be extremely beneficial to the developing hybrid cells. In the 24 h. following HAT selection, more than 95% of the input of $2.10^6$ cells per well died. The cell debris so formed was removed within a few days by the macrophages, after which the only other cells seen were

hybridomas. Figure 9.1 shows a typical culture about 2 weeks after a fusion using the X-63 myeloma line. The round, bright cells are rapidly dividing hybridomas, while the elongated, flattened cells (arrowed) are non-dividing macrophages, which soon become diluted out. There is no evidence of the enormous number of dead cells which were initially present in the well. The supernatant from a culture such as this would be ready for testing for specific antibody production within about 2-3 days.

My limited data suggests no difference in the ability of either X-63 or SP2/0 to undergo fusion with spleen cells to form viable hybrids. However, it was clear to me that hybrids formed from fusions involving SP2/0 were rather more resiliant than X-63 hybrids, in that they were able to survive much greater overcrowding of cultures and that they invariably showed rather higher survival rates following thawing from 10% DMSO.

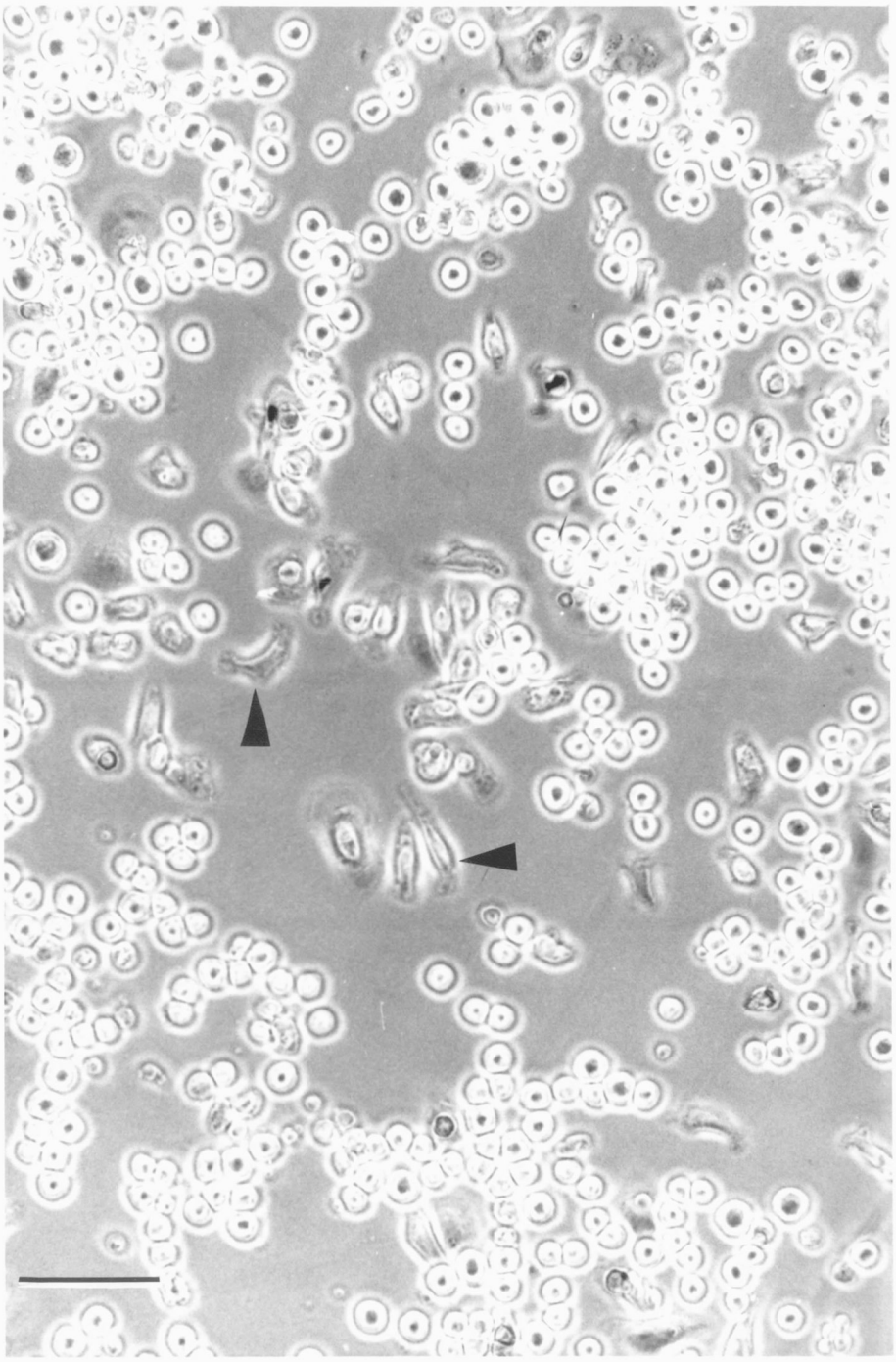
## 2. Isolation of Specific Antibody Secreting Cell lines

Of the seven fusions carried out, three were performed using the X-63 myeloma cell line and four using the SP2/0 line. A total of 11 wells were identified by RIA as containing anti-spectrin secreting cells, giving approximately one positive well out of every 30. Nine of these wells contained cells of SP2/0 origins whilst the remaining two contained X-63 hybrid cells. Only two of the SP/0 wells yielded stable cell lines; namely Spec-SP2-706 and Spec-Sp2-717 (abbreviated to 706 and 717 respectively). Two out of the seven remaining wells originated from the same fusion as 717, however anti-spectrin activity was rapidly lost from these. A similar situation was seen with four more wells which were identified in the 706 fusion. The seventh positive SP2/0 well was obtained in a third fusion where anti spectrin activity was detected

Figure 9.1

Phase contrast micrograph of a typical culture of hybrid cells about 2 wk after a fusion using the p3-X63-Ag 8 myeloma cell line. The round, bright cells are rapidly dividing HAT resistant hybridomas, while the elongated, flattened cells (arrowed) are mouse peritoneal macrophages.

Magnification = x 190. Bar = 100μm.



by RIA for 2-3 months. However, it proved impossible to clone any of the cells from this culture as they invariably died when plated out in soft agar or when they were extensively diluted. This culture was consequently abandoned, no attempt being made to investigate further the antibody class or its specificity. The two anti-spectrin positive, X-63 hybrid containing wells were both obtained from a single fusion and one stable cell line was isolated from each. These have been termed Spec-X-63-718/a and Spec-X-63-718/b (abbreviated to 718/a and 718/b). As will be shown later, each hybrid cell line secretes a quite distinct antibody.

All four hybrid cell lines have now been recloned twice and in the case of 706 this has been performed three times. After their second cloning the four cell lines all showed greater than 95% anti spectrin positive wells indicating that a single uncontaminated anti spectrin secreting clone had been picked during the first cloning procedure in each case. 717 and 718/a gave about 80% and 50% anti-spectrin positive wells after their first cloning in soft agar suggesting that the anti-spectrin secreting cells were predominant in their respective wells following HAT selection. 706 and 718/a on the other hand showed only about 5% anti-spectrin positive wells at this stage.

Both SP2/0 hybrids, 706 and 717 have been karyotyped. They both exhibit a chromosome complement of about 80 compared with the normal number of chromosomes of 40 for mouse somatic cells (Griffen, 1966) and 73 for the SP2/0 cell line (Shulman et al., 1978). Thus they have both shed a considerable number of chromosomes since fusion and have now presumably reached a stable number. Of the two X-63 hybridomas a chromosome count has only been performed on 718/a giving a value of about 72 which is not a great deal more than the X-63 myeloma complement of 65 chromosomes. The constant difference of 7 chromosomes between the X-63 and SP2/0 hybridomas and the X-63 and SP2/0 myelomas is probably

purely coincidental since Koprowski et al. (1977) have reported an X-63 hybridoma as possessing 92 chromosomes.

3. Antibody Classes Secreted by the Hybrid Cells

The $^{14}C$ Leucine labelled products secreted by the hybridomas were analysed on polyacrylamide SDS gels. Figure 9.2a shows the coomassie blue staining pattern of labelled culture supernatants (5% serum). For comparative purposes, labelled supernatants from already characterised cell lines secreting antibodies against tri-nitrophenol and sheep red blood cells were also run (from Drs. G. Köhler and C. Milstein). These are in tracks A to F. Samples from my own cell lines are on tracks G to K. Track C is a labelled supernatant from an X-63 myeloma culture containing the IgG($\gamma_1\kappa$) antibody. The slight difference in pattern between the standard and test samples, particularly at high molecular weight, is simply due to different serum being used to supplement the DMEM. The standards were all labelled in horse serum while the test samples were labelled in foetal calf serum. Tracks G and H are 706 second and third clones respectively, while tracks I to K are 717, 718/a and 718/b respectively.

Figure 9.2b shows the corresponding autoradiograph of Figure 9.2a. A most striking feature of this is the very limited number of polypeptides secreted by the hybrid cells. Most show a single immunoglobulin heavy chain and a single light chain. The pair of heavy chain bands seen in the IgG($\gamma_{2b}$) with anti sheep red cell activity run on track F is due to a difference in glycosylation, the protein component of the two bands being identical (Köhler et al., 1978).

It is apparent from Figure 9.2b that clone 706 secretes an IgM anti spectrin whereas the other three clones, 717, 718/a and 718/b all secrete IgG antibodies with $\gamma_1$ heavy chains. Their light chains are,

Figure 9.2

Analysis of $^{14}$C-Leucine labelled hybridoma culture supernatants by SDS/polyacrylamide gel electrophoresis. (a) coomassie blue stained gel of culture supernatants of 9 different monoclonal antibody secreting cell lines. Each track was loaded with 80ul of denatured sample (described below).

(b) Autoradiograph of (a) showing the secreted products. The tracks are as follows. A and B, IgM with anti phosphoryl choline specificity; C, IgG, $\gamma_1$k secreted by X-63 myeloma; D, IgM with anti sheep red blood cell specificity; E, IgM with anti tri nitro phenol specificity; F, IgG $\gamma_2$b with anti sheep red blood cell specificity; G and H are both IgH with anti spectrin specifity, produced by second and third clones respectively, of the 706 cell line; I, 717, IgG $\gamma_1$ anti spectrin; J, 718/a IgG $\gamma_1$ anti spectrin (note the double light chains, Lc); K, 718/b IgG $\gamma_1$ anti spectrin. Tracks I-K are all from uncloned cultures. The arrow in (a) marks the position of the $\gamma_1$ immunoglobulin heavy chains. In (b) the immunoglobulin heavy chain positions are denoted by $\mu$ and $\gamma$. Lc refers to the light chains. (Samples A-F were obtained from Dr. G. Köhler, Basel Institute for Immunology)

Figure 9.3

Analysis of culture supernatants by SDS gel electrophoresis of twice recloned 717 and 718/a hybridomas. Only the autoradiograph is shown. Tracks A and B are from two x 2 subclones of the 717 anti spectrin secreting cell line, while tracks C and D are from two similar sub clones of the 718/b cell line. Hc denotes the immunoglobulin heavy chains while Lc refers to the light chains. Note that the 718/b cultures still secrete the X-63 k light chain (upper of the two).

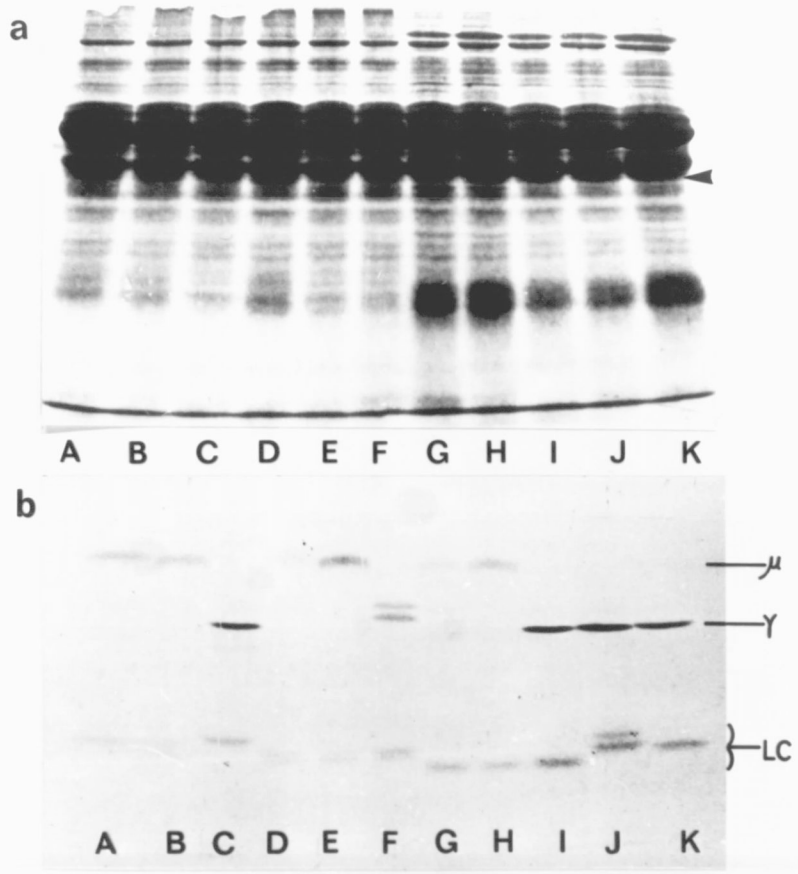



FIGURE 9.2

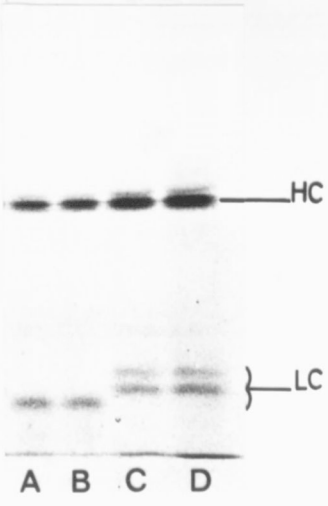



FIGURE 9.3

however, clearly varied and must therefore be derived from quite distinct cell lines. 718/a (track J), in addition to its light chain with anti spectrin specificity also secretes the original X-63 (MOPC-21) light chain, which is the higher molecular weight of the two (compare with track C). It was intially thought that the presence of this extra light chain might have been due to a contaminating cell type. However, this is now known not to be the case as twice recloned 718/a also exhibits the X-63 light chain (Figure 9.3). It is uncertain whether 718/a still synthesises the X-63 $\gamma_1$ heavy chain as it would be masked on the autoradiograph by the anti spectrin $\gamma_1$ heavy chain. It is quite possible, however, that the X-63 heavy chain is no longer made since Köhler (1980) has found that hybrid cells secreting more than one antibody may undergo preferential heavy chain loss (but not light chain loss). Clone 718/b has lost the ability to secrete the X-63 immunoglobulin and shows no X-63 light chain on the gel (Figure 8.2b). It is considered to be extremely unlikely that the X-63$\gamma_1$ polypeptides are still being made as extra heavy chains, in the absence of sufficient light chains have never been seen to be either secreted or even synthesised by a hybrid cell.

4. The Specificity of the Monoclonal Antispectrin Immunoglobulus

(a) Reactivity with pure Band 1

Using Band 1 of spectrin, purified as described in Chapter 5, as a substitute for whole spectrin in the radio-immune assay it was found that the antibodies produced by clones 717, 718/a and 718/b all recognised this subunit. The 706 monoclonal antibody gave unequivocably negative results. It was, however, considered essential to determine whether any of these antibodies would recognise sites on band 2 in view of the findings of Dunn et al. (1978) and Anderson (1979) that the two subunits of spectrin

may possess some sequence homology. Since I had no reliable method for purifying band 2 on a large enough scale to use in the RIA, it was decided to employ electrophoretic separation of the two subunits of spectrin on an analytical scale and then to directly label with monoclonal antibody, either the gel itself, or nitro-cellulose filters onto which protein from the gel had been blotted.

(b) Direct labelling of gels

Figure 9.4 shows three strips cut from a gel initially loaded with crude spectrin and which have been labelled directly with the culture supernatants, from the three hybridoma clones secreting IgG anti spectrin, according to the method of Burridge (1976). It is apparent that the antibody produced by clones 717 and 718/a specifically label band 1 but not band 2. Under the conditions of the experiment, however, the 718/b antibody labels neither subunit. The 706 antibody also failed to label either of the subunits (results not shown) although this was to be expected, since being an IgM of molecular weight probably about 900,000 (a monoclonal IgM of abnormally low S value has been reported by Green et al., 1980) it would be unable to penetrate a 7.5% polyacrylamide gel to any great extent.

(c) Labelling of Ghost proteins blotted from SDS gels onto Nitro-cellulose filters

The efficiency of transfer of protein from gels onto nitro-cellulose filters, using the technique of Bowen et al. (1980), was found to decrease quite considerably with increasing molecular weight. Ghost proteins run on 7.5% polyacrylamide gels (Figure 9.5F) only showed complete transfer at molecular weights below about 72,000 daltons (Figure 9.5d and e). Bands 1 and 2 only exhibited limited transfer over a period of 48 hours, although quite sufficient was in fact adsorbed onto the filter to give an

Figure 9.4.

Analysis of the specificities of the three Ig G anti-spectrin antibodies according to the direct gel labelling method of Burridge (1976). A refers to the coomassie blue stained, antibody labelled gel while B is the corresponding autoradiograph. Gels were initially loaded with 50µl of crude spectrin/actin extract. Further details are given in the text. 717 and 718/a antibodies both show labelling of Band 1.

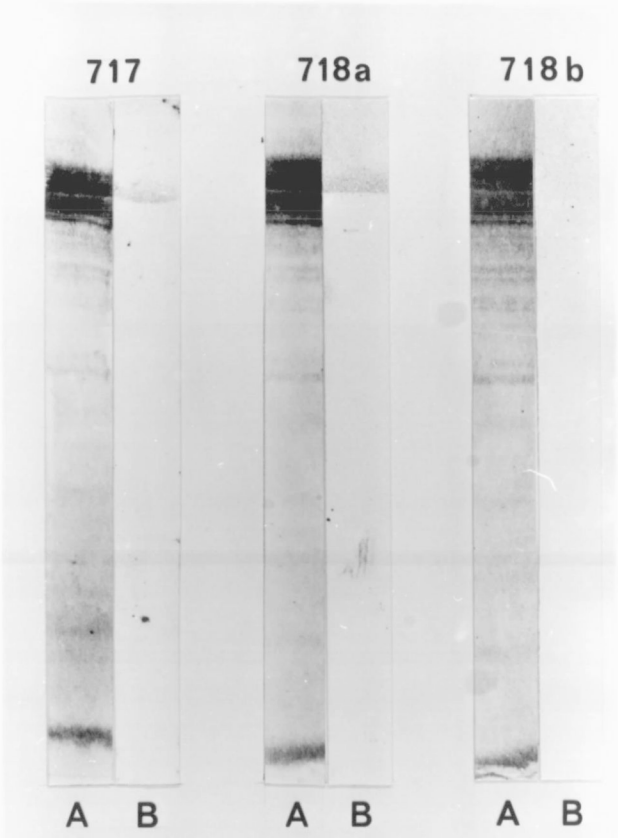



FIGURE 9.4

Figure 9.5

Detection of antigens transferred from SDS polyacrylamide gels onto nitrocellulose filters. Total ghost proteins were separated on SDS gels blotted onto nitrocellulose filters and these labelled with the three IgG monoclonal anti spectrin anti bodies: (A) autoradiograph of filter labelled with 717 IgG; (B) autoradiograph of filter labelled with 718/a IgG; (C) autoradiograph of filter labelled with 718/b IgG; (D) amido black stained filter after transfer of protein. (E) Gel stained with coomassie blue after blotting. (F) Gel stained with coomassie blue prior to blotting (note how Band 4.1 is resolved into two components using the Laemmli gel system. This is also seen in Figure 9.6).

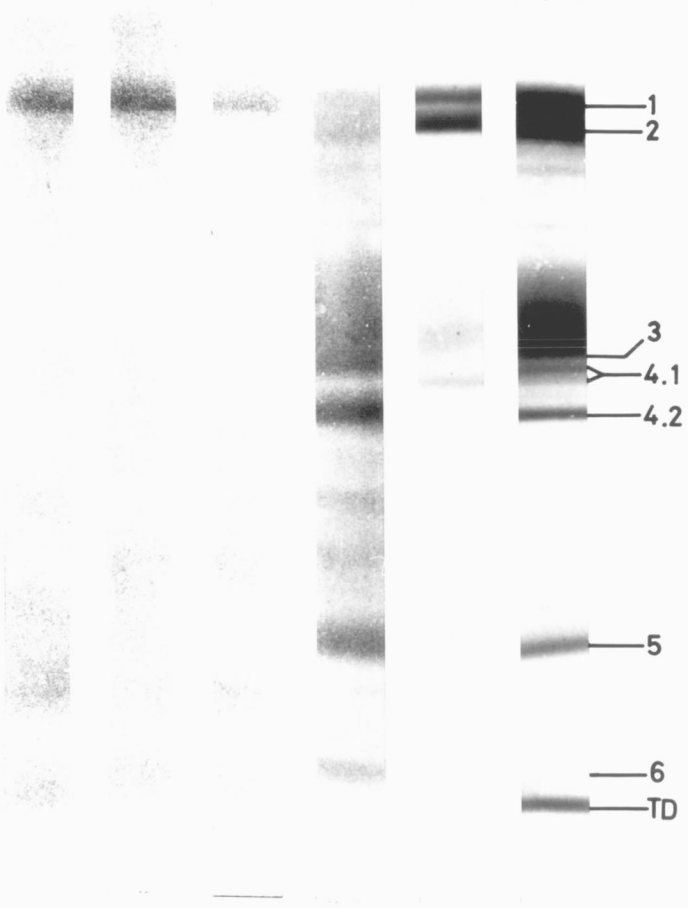



FIGURE 9.5

adequate signal when labelled with monoclonal anti spectrin and $^{125}$I-RAM (below). Band broadening due to diffusion was found not to be a serious problem at high molecular weight (compare Figure 9.5d, e, f). The relevance of this to the blotting technique will be discussed in more detail in the next chapter.

Autoradiographs of filters absorbed with electrophoresed ghost proteins and labelled with the three IgG anti-spectrin antibodies (Figure 9.5a-c) showed, as in the direct gel labelling method that 717 and 718/a antibodies specifically recognised band 1 (Figure 9.5 a,b). However, this technique also detected the binding of the 718/b antibody to band 1 (Figure 9.5c). No labelling by the 706 IgM was observed (results not shown) in spite of the fact that using this technique there is no size limitation on the labelling antibody. It is important to appreciate that this blotting method differs fundamentally from the direct labelling technique in that it allows at least some renaturation of the proteins separated by electrophoresis. This is a consequence of the fact that to directly label a gel, the proteins must be first fixed to prevent their diffusion out of the gel during the course of the experiment (up to 1 week), whereas in the blotting technique one actually requires the proteins to be free to diffuse, so fixation is not carried out. Furthermore, prior to blotting, the gel is taken through a urea step as an aid to renaturation from SDS. These results could therefore be interpreted to indicate that the 718/b antibody recognises some specific secondary or tertiary structure while the 717 and 718/a antibodies may only recognise a specific primary structure regardless of the conformation of the protein.

## 5. Labelling patterns of Trypsin Digested Spectrin

In order to further define the antibody binding sites on band 1 and to determine whether or not the monoclonal antibodies recognised different

antigenic determinants, the blotting technique was used to observe the labelling patterns of partial tryptic digests of spectrin. The results of such an experiment, using the 717, 718/a and 718/b antibodies as probes is shown in Figure 9.6. During the 10 min. room temperature trypsin digestion of crude spectrin, bands 1 and 2 were almost completely degraded giving rise to a complex pattern of fragments (Figure 9.6b,f), all of which were transferred, at least to some extent, onto the nitro cellulose filter (Figure 9.6g. With each of the three monoclonal antibodies, the labelling pattern seen on the autoradiograph of the filter (Figure 9.6 c-e) was much simpler than the fragmentation pattern on the gel. In the case of 717 and 718/b the patterns seen were indistinguishable. However this does not necessarily mean that they recongnise the same antigenic determinant since the smallest fragment which they both labelled had a molecular weight in the region of about 50-60,000. Thus the two antibodies could be binding up to about 500 amino acids apart. The 718/a antibody, on the other hand, gave a completely different labelling pattern indicating a unique specificity; only about 50% of the labelled bands being common to the other two antibodies. The smallest fragment which is labelled by both the 717 (or 718/b) and 718/a antibodies has a molecular weight of about 100,000 and hence the binding sites of the two antibodies must lie within about 900-1000 amino acids of each other (about 40% of band 1). The antibodies exclusively label different high molecular weight bands. The 718/a antibody labels a band migrating at approximately 170,000 daltons, while the 717 (or 718/b) antibody labels a band at about 130,000 daltons. Taken together, these results are not compatible with either antibody binding close to a chemical terminus of the band 1 polypeptide. The further localization of the binding sites, and the consequences will be discussed more fully in the next chapter.

Figure 9.6

The labelling pattern of tryptic fragments of spectrin by the three monoclonal IgGs. Spectrin was briefly digested with trypsin as described in the text. The fragments were separated by SDS gel electrophoreris, blotted onto nitrocellulose filters and labelled with the 717, 718/a and 718/b monoclonal antibodies. (a) red blood cell ghosts stained with coomassie blue (b) tryptic digest of spectrin stained with coomassie blue (c) autoradiograph of filter onto which the separated fragments had been blotted and which was then labelled with the 717 antibody (d) autoradiograph of filter labelled with the 718/a antibody (e) Autoradiograph of filter labelled with the 718/b antobody (f) gel stained with coomassie blue prior to blotting (g) Amido black stained filter after transfer of protein (h) gel stained with coomassie blue after blotting.

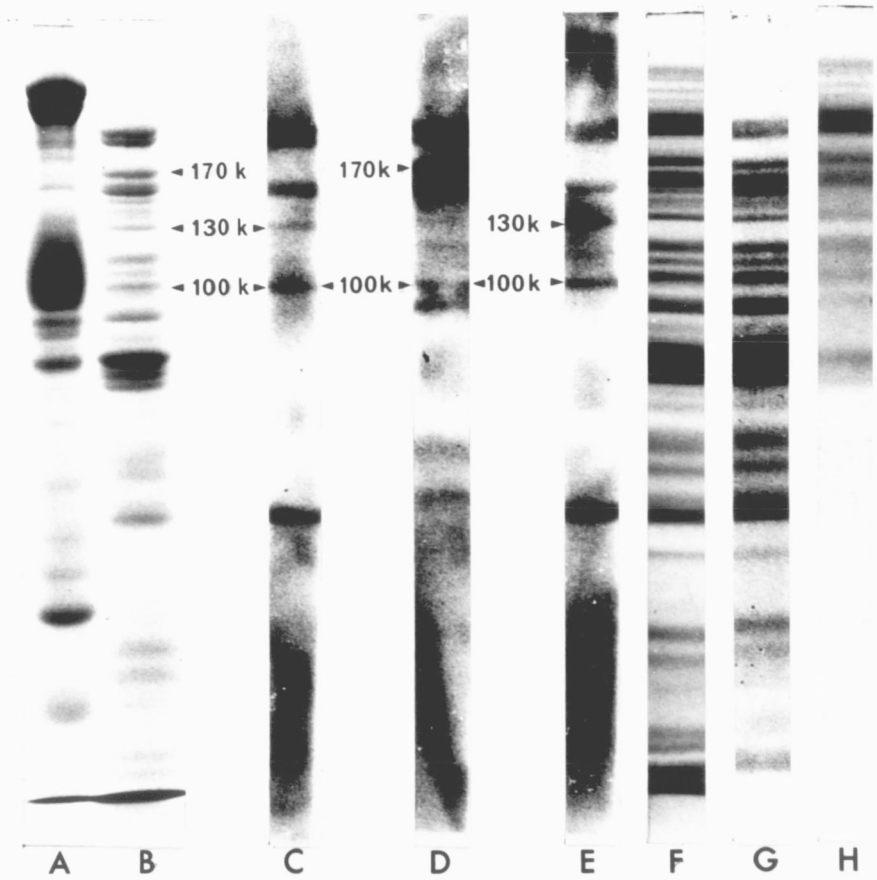



FIGURE 9.6

## 6. Purification of 717 and 718/a Antibodies

### (a) Production of Ascitic Fluid

It is apparent from Figure 9.2 that the monoclonal antibodies secreted by hybridema cell lines constitute only a very minor protein component of the cell culture supernatants which contain mainly added serum protein. Although these supernatants were used successfully for such purposes as gel labelling experiments, fluorescence microscopy or binding experiments involving RIA, they provided poor starting material for purification of monoclonal antibody for electron microscopy, since the concentration was so low. As a consequence of this, it was therefore necessary to grow the hybridomas as ascites tumours in mice which provided large amounts of antibody in the ascitic fluid.

Both 717 and 718/a have been successfully propogated as ascites in mice after initial passages as solid tumours. Subsequent passages of 718/a consistently formed ascites while 717 continued to form about 50% solid tumours. Priming of mice with pristane proved to be essential if the injected cells were to 'take'. The IgM secreting clone 706 only rarely grew as an ascites tumour even after repeated passages in mice, and in serveral cases where X1 and X2 706 clones were injected no antibody was detected in the ascitic fluid. No attempts have so far been made to grow the 718/b cell line in this manner.

The enormous concentration of antibody in an ascitic fluid is portrayed quite vividly in Figure 9.7. The two gel tracks in Figure 9.7a show on the right, ascitic fluid produced by 717 and on the left, fluid from a 706 clone which failed to make antibody. On the 717 track, the $\gamma_1$ heavy chains form the second most prominent band after serum albumen, but are essentially absent from the 706 track. The 717 light chains are located just behind the tracker dye and are likewise absent from the 706 track.

Figure 9.7

Analysis of ascites fluids on SDS/polyacrylamied gels stained with coomassie blue. (A) comparison of ascites fluids produced by 706 cells which failed to produce any antibody and 717 cells which produced large quantities of IgG. Note the very prominent $\gamma_1$ heavy chain (HC) band in 717 which is absent in 706.

(B) comparison of 717 and 718/a ascites fluids. Note the distinctive differences in the light chain (LC) bands, in particular the higher molecular weight X-63 light chains in 718/a.

All loadings were 5µl.

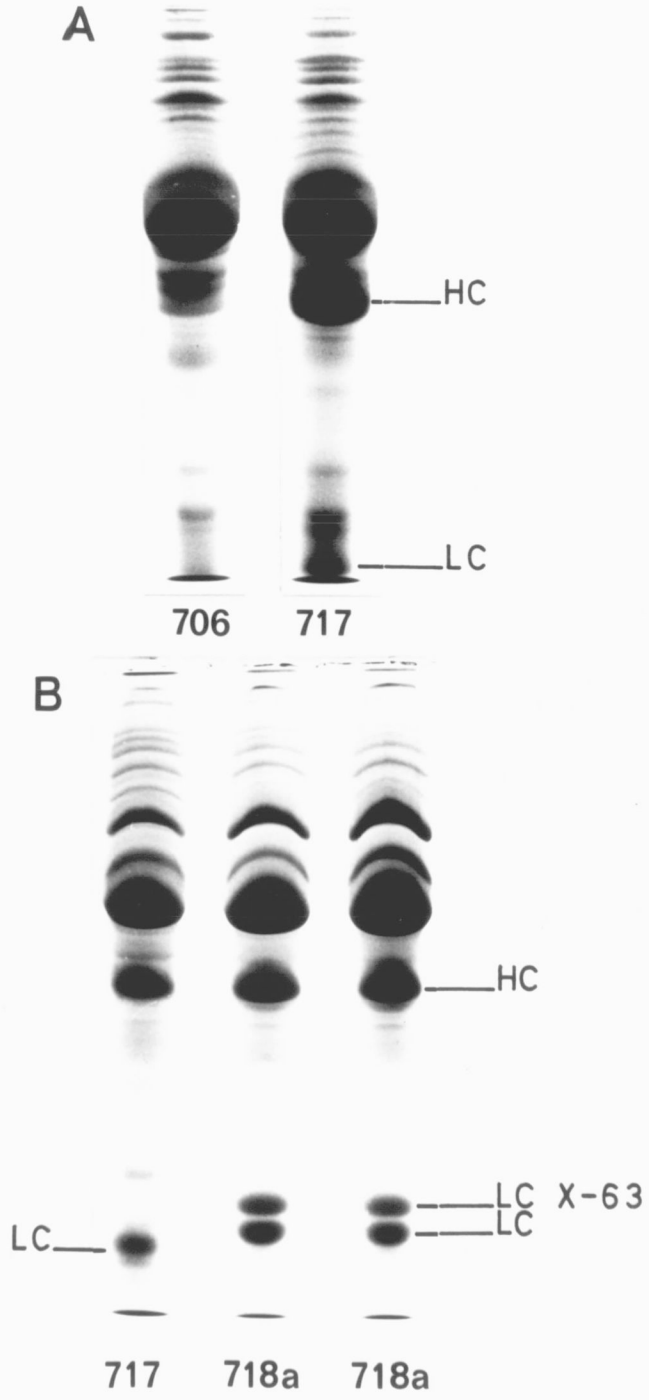



FIGURE 9.7

The position of both bands was verified by comparison with the autoradiograph of a $^{14}C$ Leucine labelled 717 supernatant run at the same time. (This gel was only run for 12h hence the proximity of the light chain band and the dye front.)

Figure 9.7b is a comparison of the composition of ascitic fluids produced by the 717 and 718/a cell lines. The two 718/a ascitic fluids are easily recognised by their characteristic set of double light chains which form very prominent bands, whereas the 717 ascitic fluid shows only the single higher mobility light chain. The $\gamma_1$ heavy chain of each antibody again features as a very heavily staining band. Taking account of the volume of sample loaded onto the gel and the intensity of staining of the bands, I would estimate that the concentration of monoclonal IgG in these ascitic fluids was in the region of 5-10 mg/ml.

Titres of culture supernatants and ascitic fluids are compared in Figure 9.8. Samples were serially diluted and then radio-immune assayed. Differences in the maximum counts bound between the two graphs was due to different batches of cooke microtitration plates being used in each case. Naturally, each supernatant-ascites pair were assayed on the same plate. Precise details of the protocol used are given in the figure legend. Figure 9.8a shows that for the counts bound in the 718/a ascites test to drop to the same level as the maximum counts observed with the 718/a supernatant required a dilution of approximately $2^{10}$. However, the 718/a ascites was diluted x 4 prior to use, giving a total dilution of $2^{12}$ indicating a difference in antibody concentration between the ascitic fluid and the culture supernatant of approximately 3000-4000 times. The comparison between the 717 ascites and supernatant indicates a similar difference in concentration of about x 1000.

As described in Chapter 8, fluorescence microscopy was also employed in screening ascites fluids. Figure 9.9 is a fluorescence micrograph

Figure 9.8

Solid phase radio immune assay of serially diluted samples of ascites fluid (black squares) and spent tissue culture supernatant (black circles) produced by (a) 718/a cell line and (b) the 717 cell line. Open circles and squares are the respective blanks. For further details see the text.

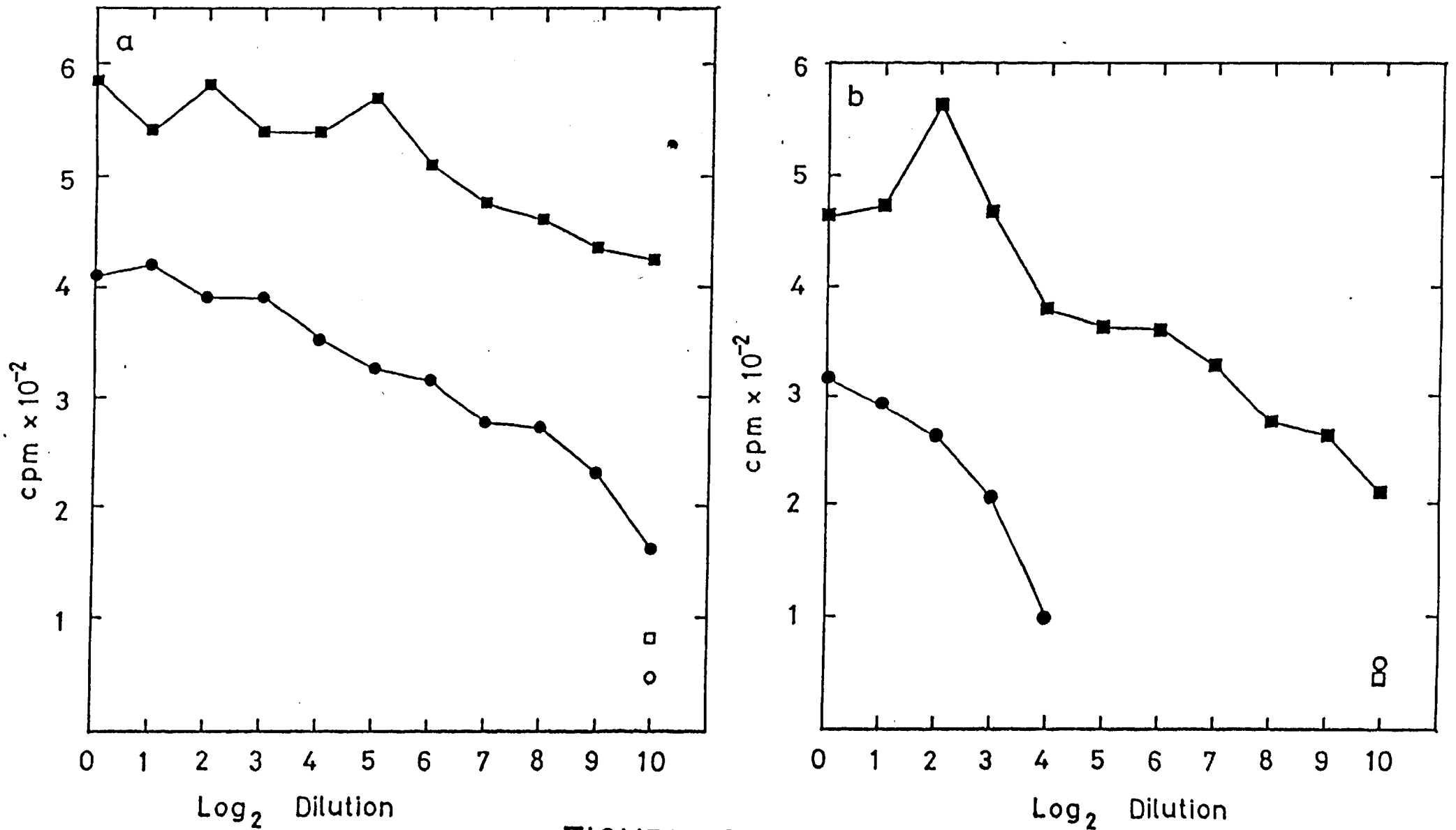



FIGURE 9.8

Figure 9.9

Fluorescence micrograph of human erythrocytes fixed and stained, as described in the text, with ascites fluid (1/100 dilution) from the 718/a cell line. The entire field as shown is covered by a monolayer of cells, but only those within the 718/a antibody droplet fluoresce. The edge of the droplet is seen in the bottom half of the field, the cells being deformed in the region of the meniscus.

Magnification x 400.

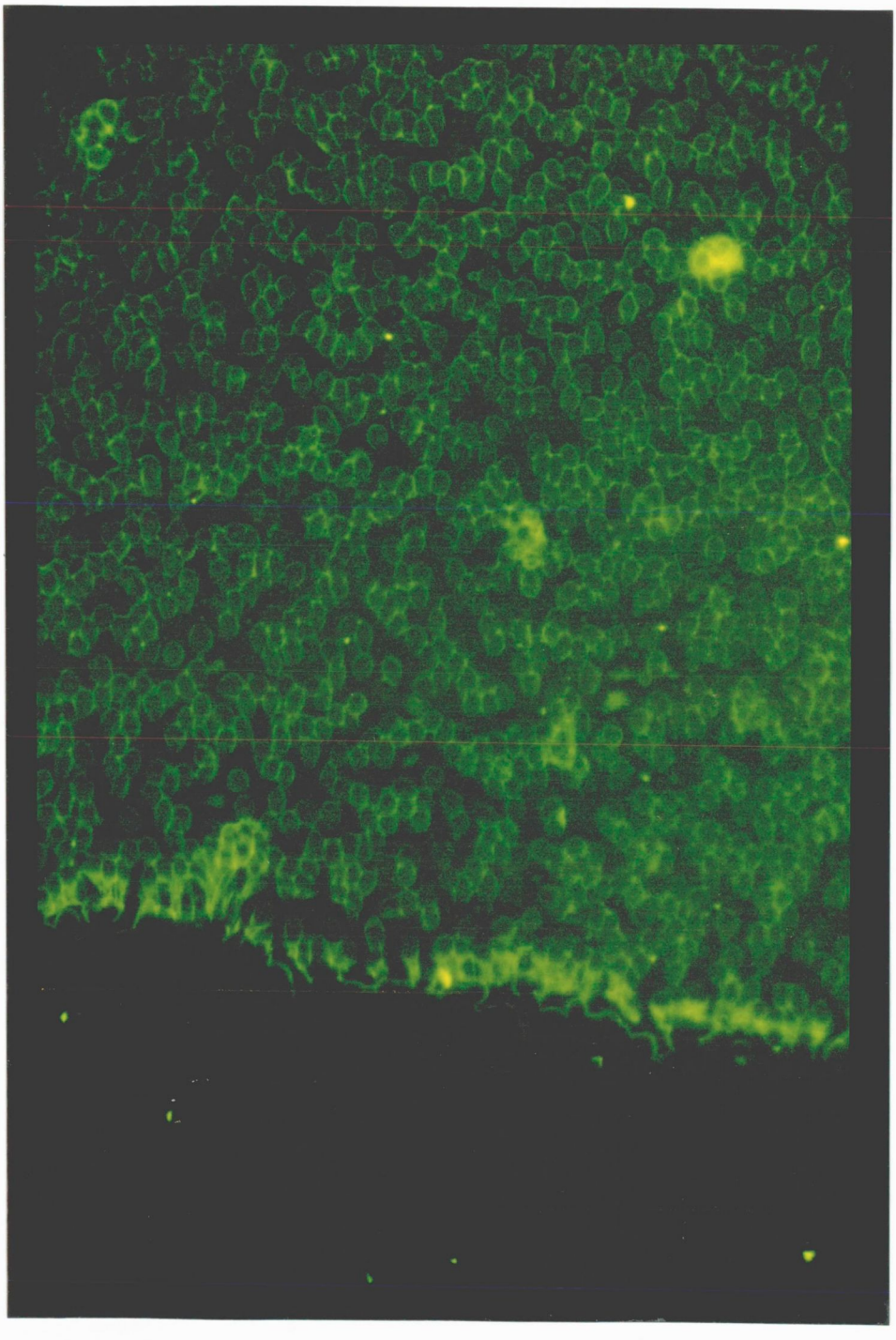


FIG. 9.9

using x100 diluted ascitic fluid as the first antibody layer. The field shown is completely covered by a monolayer of fixed red blood cells, but only those within the first layer antibody droplet are visible. (The entire plate was flooded with the second layer antibody, fluorescein-RAM). This distinctive fluorescence pattern with each cell appearing as a bright ring was still apparent when ascites fluids were diluted up to 10,000 times.

(b) Isolation of 717 and 718/a antibodies from Ascitic fluids

The most obvious way to purify the monoclonal anti spectrin from ascitic fluids is by affinity chromatography. This was attempted with both the 717 and 718/a antibodies using either spectrin conjugated sepharose 4B or spectrin conjugated ultrogel AcA22 (LKB). However, after applying either ascitic fluid, I found that it was not possible to elute the antibody in any useful quantity using conventional methods such as low pH or high concentrations of magnesium. It was therefore necessary to resort to alternative tactics to purify these antibodies.

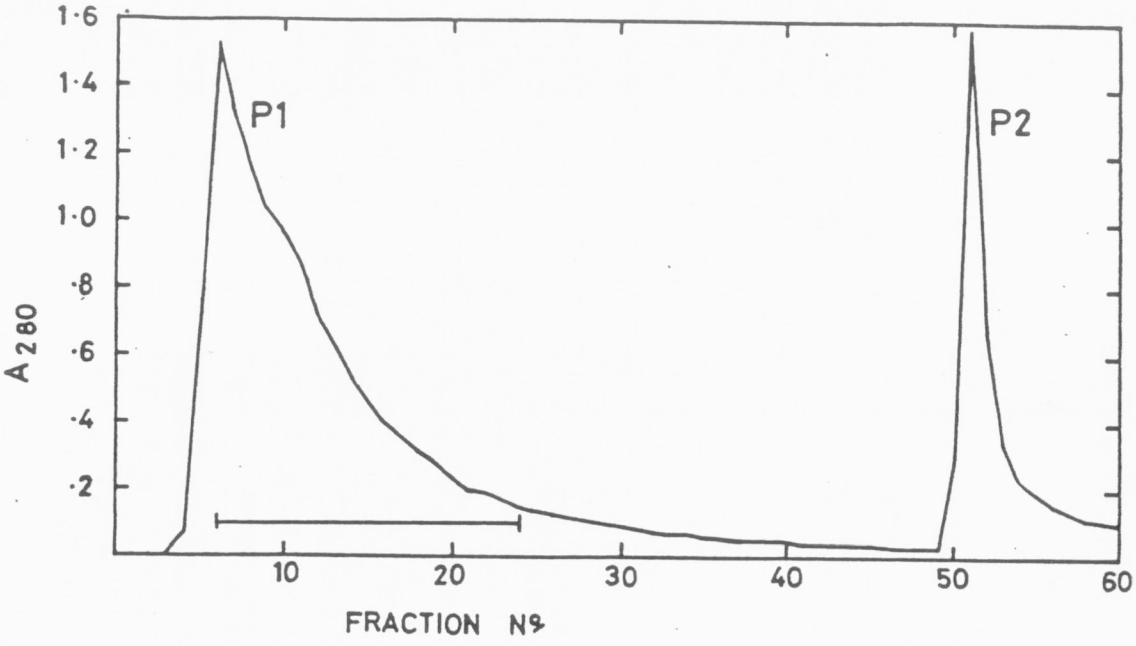
In view of the extremely large amounts of monoclonal IgG in the ascitic fluids I decided that a reasonable approach to obtaining pure anti-spectrin was to simply purify the entire IgG fracation. I considered that such a preparation would be enormously enriched in the monoclonal antibody (perhaps as high as 90-95% anti-spectrin). The IgG fraction from both 717 and 718/a ascitic fluids was therefore purified according to the method given in Section 8.4b using ammonium sulphate precipitation and ion exchange chromatography on DE52.

Purification of the 717 antibody is shown in Figure 9.10. As judged by RIA, the monoclonal antibody appeared to elute from the column almost entirely in Peak I (Figure 9.10a). This interpretation

Figure 9.10

Purification of the 717 monoclonal antispectrin from ascites fluid. The monoclonal antibody was precipitated from ascites fluid by addition of ammonium sulphate to 40% saturation, was purified by ion exchange chromatography on DE 52 (see the text). The elution profile is shown in (a). The first, unretarded, peak (P1) was eluted in 10mM potassium phosphate pH 7.8. The second peak (P2) was eluted by 10mM potassium phosphate, pH 7.8 containing 75mM NaCl. The bar in (a) indicates the location of most of the anti-spectrin activity. (b) shows the purification monitored by SDS gel electrophoresis. AF (loading, 30µl) is the 40% ammonium sulphate fraction applied to the column. The numbers refer to fractions off the DE 52 column (all loadings, 80µl). Note the intense heavy and light chain bands in P1. S refers to $^{14}$C-Leu labelled 717 culture supernatants (loading, 80µl). These last samples were included so that the heavy and light chain bands on the gel could be correlated with the known positions of the 717 heavy and light chains as identified on the appropriate autoradiograph (not shown).

a




b

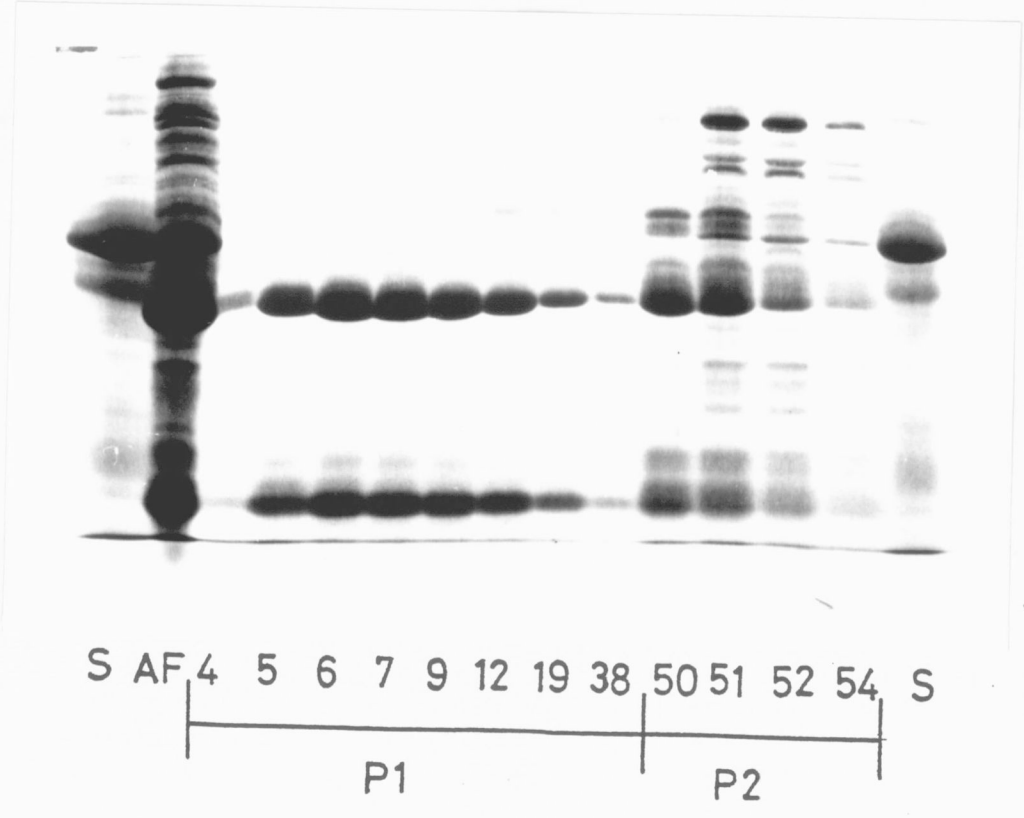
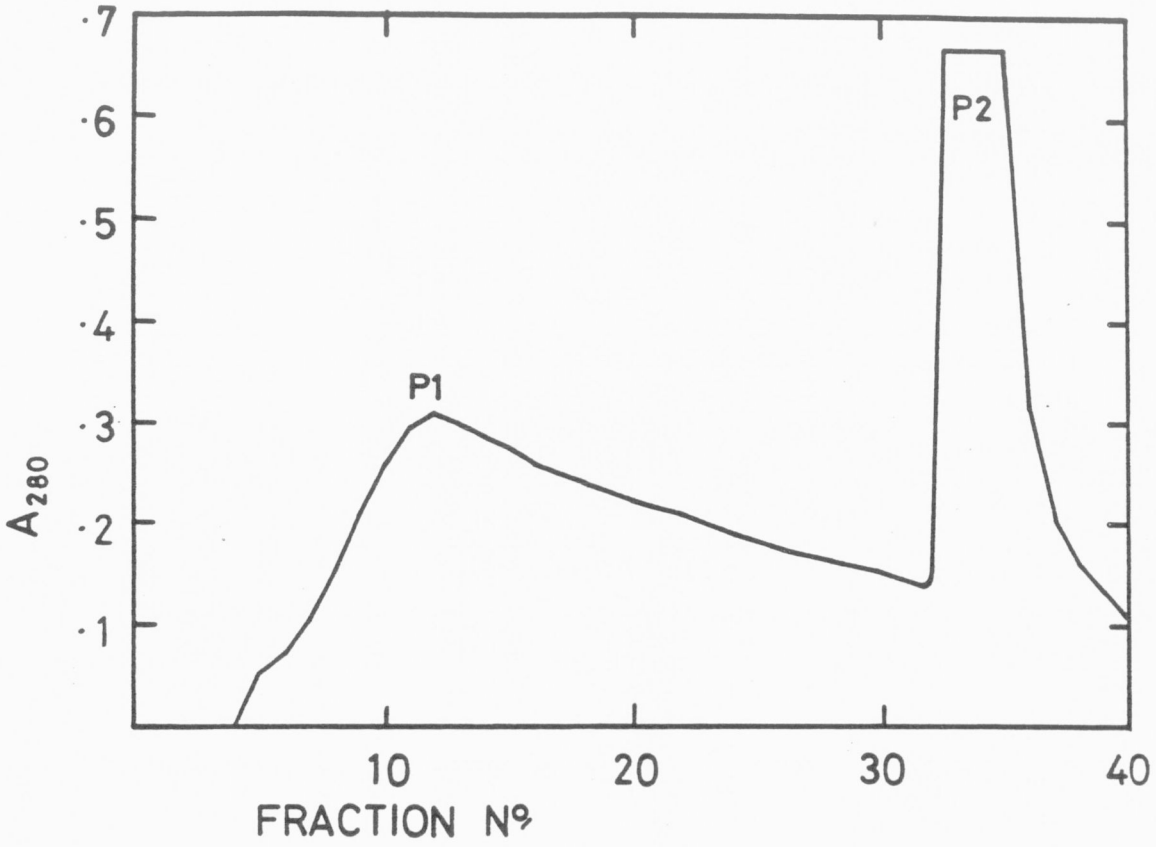



FIGURE 9.10

Figure 9.11

Purification of the 718/a antibody from ascites fluid. This was carried out as described for the 717 antibody (Figure 9.10). (a) is the elution profile of the 40% ammonium sulphate fraction of ascites fluid from the DE52 column. P1 is the unretarded peak. P2 was eluted in 10mM potassium phosphate pH 7.8 containing 75 mM NaCl. The purification was monitored by SDS gel electrophoresis (b). AF is the ammonium sulphate fraction applied to the column (10µl). The figures refer to fractions off the column. 8-20 are from P1 (loading, 100µl) while 33-35 are from P2 (loading, 30µl). S is $^{14}$C-Leu labelled 718/a culture supernatant (50µl) for identification of 718/a heavy and light chain bands by autoradiography. (Autoradiographs not shown).

A




B

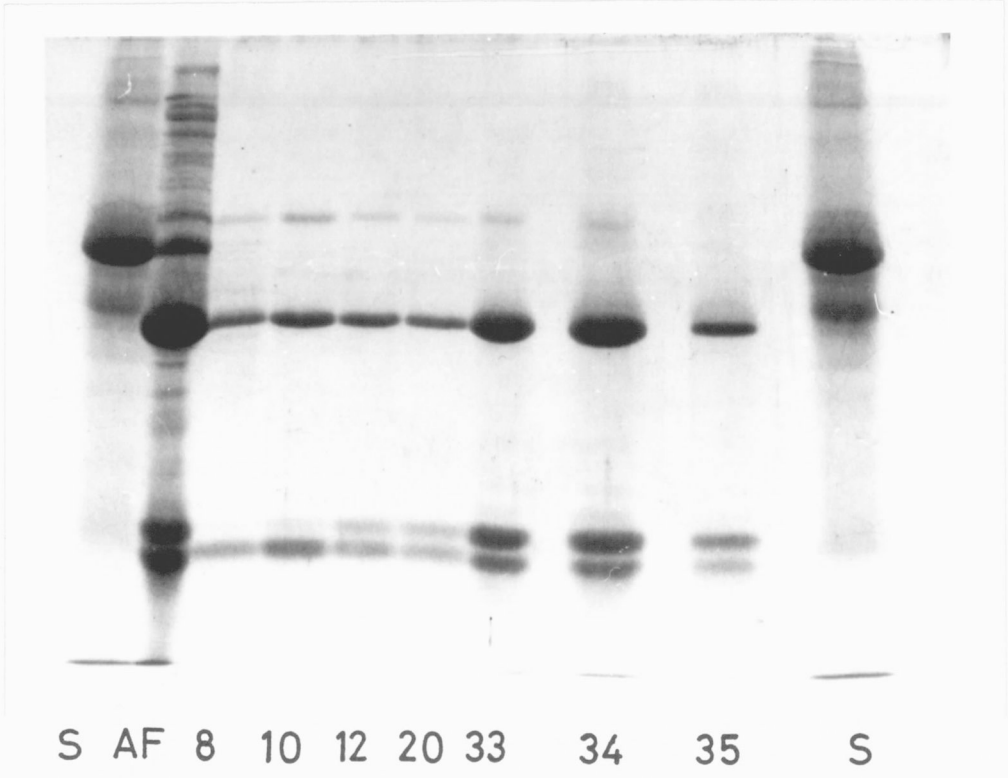



FIGURE 9.11

is confirmed by gel electrophoresis which showed this peak to contain a single heavy chain band and a single light chain band both of which were coincident with $^{14}C$ labelled 717 heavy and light chain bands (Figure 9.10a).

The purification of 718/a antibody gave rather different results. Anti spectrin activity was found in significant quantities in both peaks I and II (Figure 9.11a). Examination of fractions off the column run on SDS gels showed a separation of the two light chains made by this clone. The anti-spectrin light chain and a corresponding amount of $\gamma_1$ heavy chain appeared in Peak I (Figure 9.11b). Whereas both X-63 and anti-spectrin light chains appeared in Peak II. What appears to have happened, quite fortuitously, is that both the antibodies, hybrid or otherwise, containing the X-63 light chains were retarded by the column, whereas the non hybrid antibodies containing only the anti spectrin light chain passed directly through the column and appeared in Peak I, contaminated only by a small amount of IgM.

7. Low angle shadowing of Spectrin-Antibody complexes.

Attempts were made to visualise, by low angle shadowing, complexes of spectrin dimers and tetramers with the two antibodies in order to localise the antibody binding sites. Spectrin (dimers or tetramers) and DE52 purified monoclonal antibody were mixed, in PBS, with each component at a concentration of about 0.1mg/ml. The mixtures were left overnight at $4^{o}C$, diluted x 10 with 155mM ammonium acetate/70% glycerol pH7.4 and then sprayed and rotary shadowed as described in Chapter 2. The small amount of IgM contaminating each antibody preparation (figures 9.10b and 9.11b) was not considered to pose any serious problem as it never represented more than about 10% of the total protein and since IgM is pentameric it could not constitute more than 2% of the antibody

molecules seen in the electron microscope.

Figure 9.12a is of spectrin heterodimers labelled with the 718/a antibody. These images indicate that the antibody binds close to one end of the dimer (at least to within 10nm). Not many obviously cross linked spectrin dimers could be identified although these might have been expected after incubation with divalent anti spectrin IgG. The reasons for this are uncertain, although one possibility is that shear during spraying may have broken them apart since it would only require the breakage of one out of two possible bonds to effect separation. For comparison, to break apart a tetramer during spraying would require the simultaneous cleavage of two bonds. The 718/a antibody, when combined with spectrin tetramer gave images which were rather more difficult to interpret (results not shown). Most tetramers appeared to have no antibody bound. A few however, (less than 10%) showed what appeared to be antibody molecules bound to their mid point. None were identified with antibodies bound at their termini. It is therefore concluded that this antibody binds near to the dimer-dimer association region. It is possible that so few antibodies appeared bound to tetramers because of steric effects caused by the binding of a second dimer close to the antibody binding site. So far, the 717 antibody has only been used to label spectrin tetramers (Figure 9.13). The binding site for this antibody has been tentatively determined to lie about 20-30nm either side of the tetramer mid-point.

Figure 9.12

Spectrin heterodimers labelled with the 718/a monoclonal anti spectrin after rotary shadowing. Left hand two columns show spectrin heterodimers only; the central two columns are antibody molecules; the right hand two columns and the main field show antibody-spectrin heterodimer complexes.

Magnification all x 100,000. Bar = 100nm.

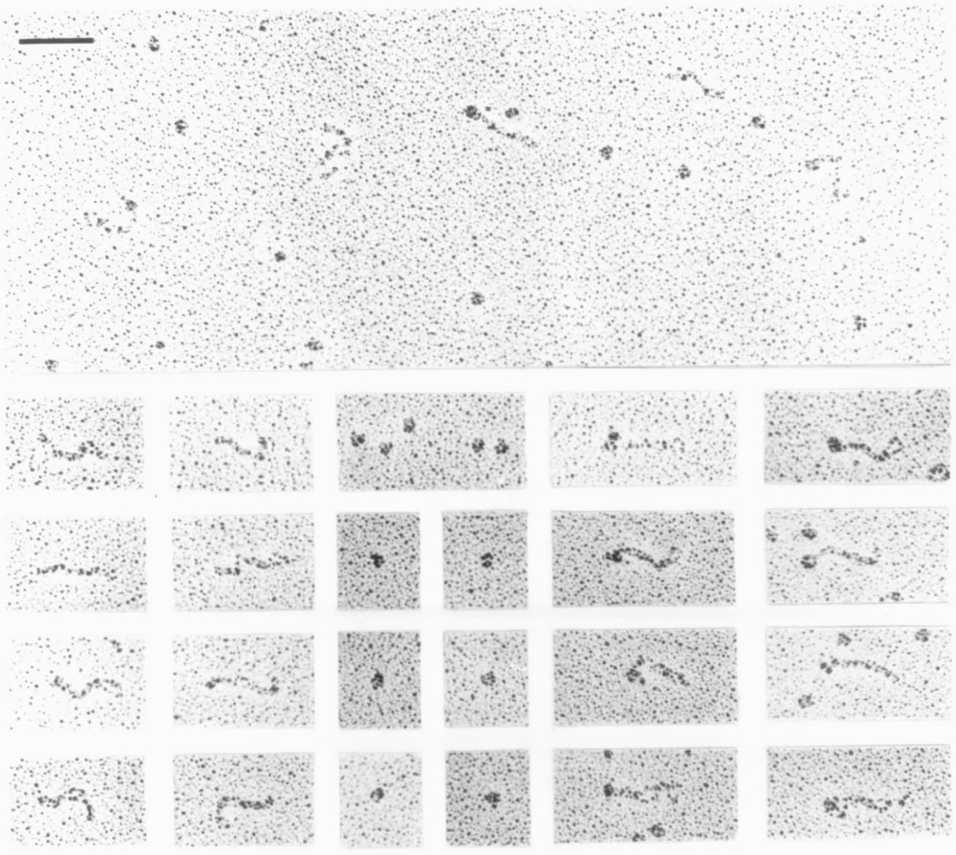


FIGURE 9.12

Figure 9.13

Spectrin tetramers labelled with the 717 monoclonal antibody after low angle shadowing. On the bottom left there appears to be two cross-linked tetramers. On the bottom right there is a tetramer apparently labelled at two positions, near to, but either side of the midpoint.

Magnification, all x 100,000. Br = 200nm.

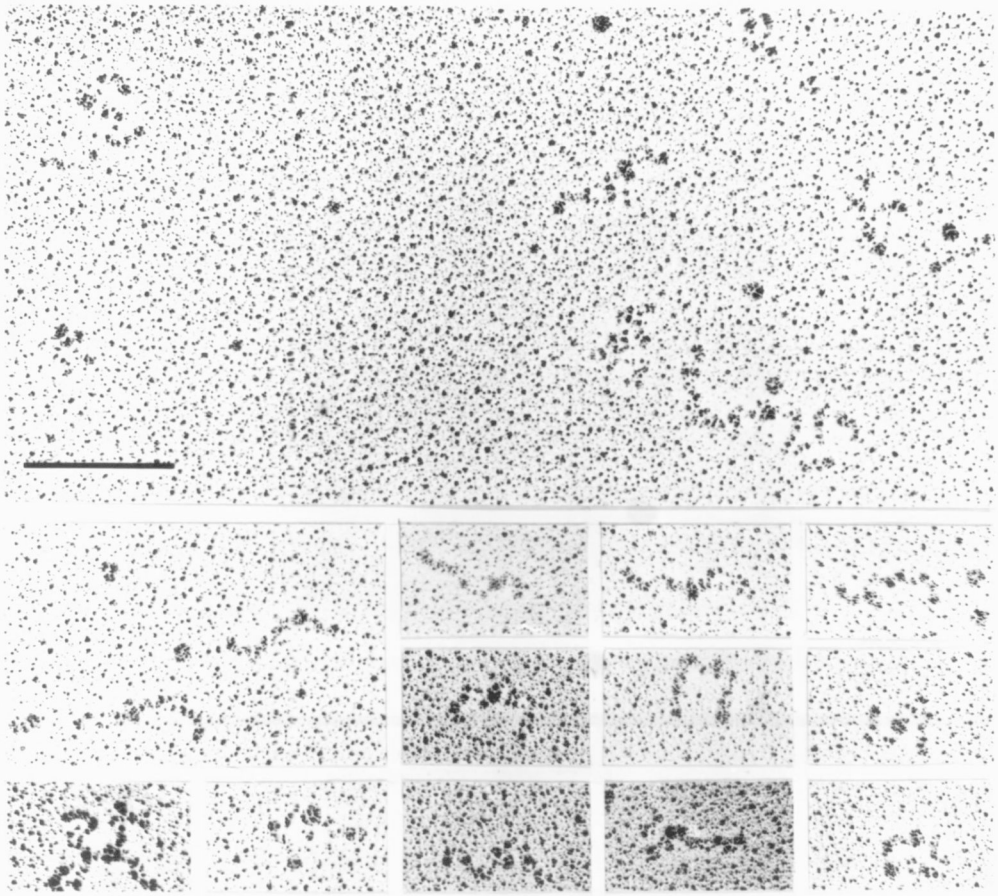


FIGURE 9.13

# CHAPTER 10

## DISCUSSION OF HYBRIDOMA WORK

### 1. Isolation of Anti Spectrin secreting Hybridomas

The value of 1:30 for the frequency of monoclonal anti spectrin positive cultures observed in the initial screening of wells three weeks post fusion compares quite favourably with the results of other groups raising monoclonal antibodies to various soluble antigens. Published frequencies range from one positive well out of 400 tested for type I collagen (Linsenmeyer et. al. 1979), through 1:40 for carcino-embryonic antigen (Accolla et. al. 1980) to 1:10 for estrophilin (Greene et. al. 1980). A crude estimate for the percentage of anti spectrin specific hybridomas present after fusion may be obtained as the product of the fraction of anti spectrin positive wells prior to cloning and the fraction of positive wells after the first soft agar cloning. This was found to give an average value, for all seven fusions, of approximately 1%, which is about an order of magnitude less than one would expect for a particulate antigen such as sheep red blood cells (Stähli et. al. 1980). This rather low frequency of antigen specific hybridomas, which seems to be common to most soluble antigens, appears to be related to the mouse immunisation regimes generally used. Stähli et. al. (1980) have recently found, using human chorionic gonadotrophin as the immunogen, that specific hybridoma frequences could be increased from 0.5% to 40% simply by adjusting their immunisation parameters. In particular, they find that by giving a succession of four daily boosts beginning 5 days before fusion, instead of the single boost three days prior to fusion, a significant increase in specific

hybridoma frequency may be obtained. These workers have also pointed out that the high frequencies of specific hybridomas could be correlated with the percentage of large blast cells in the spleen immediately prior to fusion but that serum antibody levels are neither a reliable guide to the timing of the fusion, nor to the suitability of the immunisation regime.

Although no difference was noted in the ability of X-63 and SP2/0 myelomas to fuse with spleen cells to form viable hybrids, my results show that the fraction of anti-spectrin positive wells from SP2/0 fusions was about 3.5 times the fraction from X-63 fusions. Stähli et. al. (1980) have noticed the same phenomenon and have ascribed it to the fact that X-63 hybridomas may synthesise two sets of heavy chains and two sets of light chains which may then be assembled in any combination,provided that both heavy chain sets are of the $\gamma$ class (Köhler and Shulman, 1978). As discussed in Chapter 7, such a random assoication would result in 10 different antibody species only 1/16 of which would be bivalent and antigen specific, the majority being monovalent or inactive. The solid phase RIA depends upon the continuous association of the antibody with the antigen (spectrin) for at least 4 h and during several washes. Stähli et. al. (1980) state that monovalent antibodies show shorter half lives of antigen-antibody complex and therefore may not remain attached to the antigen for the time course of the RIA. Hence, such antibodies would give negative results. Because of its shorter incubation periods, the fluorescence method of screening might detect these antibodies where the apparently more sensitive RIA is unable to. The advantage of using RIA, however, is that it should only detect high affinity antibodies under these circumstances.

## 2. The Immunological Detection of Protein Bands on Gels

The conventional techniques of immuno-precipitation have been used by several groups in order to examine the specificities of monoclonal antibodies raised against complex protein mixtures which could not be conveniently fractionated into their component polypeptides. For instance, Roehrig et. al. (1980) have produced monoclonal antibodies against structural proteins of Sindbis virus which they were then able to characterise by using them to immune-precipitate $^{35}S$ labelled viral proteins which were analysed on SDS gels. For many purposes, however, it may not be possible to do this. In the case of spectrin it would be necessary to carry out the specific precipitation of one $^{125}I$ labelled subunit in the presence of a powerful protein perturbant, such as 6M urea, in order to prevent the co-precipitation of the second subunit. However, it is rather unlikely that an antibody would remain bound under such circumstances. The same problems apply to trypsin digested spectrin, with the addition that only precipitated tryptic fragments containing tyrosine would be detected.

In view of these difficulties, it is quite clear that much of this investigation could not have been carried out were it not possible to directly label with monoclonal antibodies, polypeptides separated by SDS gel electrophoresis. Both labelling methods used here had their drawbacks. The direct labelling technique of Burridge (1976) was very lengthy while the blotting procedure of Bowen et. al. (1980) gave very high background radioactivity tending to obscure minor bands. Nevertheless, these two techniques were able to provide complementary information regarding the specificities of the labelling antibodies. This became clear in the ability of the protein blotting technique to detect the binding of the 718/b antibody to Band 1 (Figure 9.5) while the

direct labelling technique was unable to do so, yet still demonstrated the binding of the 717 and 718/a antibodies to the same subunit (Figure 9.4). Evidently the two techniques are able to differentiate between subclasses of antibodies. As discussed in Chapter 9, it is believed that this differentiation is a function of whether the antibody in question recognises a specific piece of sequence or a specific three-dimensional conformation of the polypeptide. It was concluded that the 718/b antibody must recognise some aspect of secondary or tertiary structure which is present in both the native spectrin molecule and in Band 1 renatured from SDS by way of urea, but not in fixed SDS denatured Band 1. Similarly, the 717 and 718/a antibodies must recognised structure present in native spectrin and in SDS denatured and renatured Band 1. It is a reasonable assumption, although by no means proven, that these two antibodies simply recognise unique amino acid sequences without any requirement for specific secondary or tertiary structure being preserved. Assuming these interpretations to be correct, the results are consistent with the findings of Litman et. al. (1980) and Calvert et. al. (1980a) that at least isolated Band 1 may retain some of its native structure, following renaturation from urea and SDS.

Only the specificity of the IgM produced by clone 706 remains undetermined. There can be no question of the iodinated rabbit anti mouse IgG being unable to recognise the IgM if present on the nitro cellulose, since the same iodinated antibody was used to detect the IgM anti-spectrin in the first place by RIA. One must therefore conclude that the IgM does not recognise either Band 1 or Band 2 after separation on SDS gels and blotting onto nitro-cellulose filters. There are only two possible explanations for this. Either the antibody recognises native structure which is not reformed following removal of SDS, or that <u>both</u> subunits

contribute to the binding site for the antibody, which is then unable to bind either subunit in isolation. Such a recognition is actually known to exist for Band 4.1 binding to spectrin (Calvert et. al. 1980a).

## 3. Improvements to the Protein Blotting Technique

Of the two techniques used in this investigation for in-situ labelling of proteins separated on SDS gels, the blotting method is probably the more versatile. Certainly it is the quicker of the two. The technique is strictly analogous to that devised by Southern (1975) for the blotting and in-situ hybridisation of nucleic acid fragments separated on gels and may soon become as widely used as more laboratories find the need to develop monoclonal antibodies against complex mixtures of antigens.

The blotting technique which I employed during this investigation was initially devised by Bowen et. al. (1980) for the detection of DNA binding proteins. However, they did note that it could be used to detect protein-protein interactions, although under these circumstances the technique gave a very high background radioactivity, which for many purposes was totally unacceptable. A very similar technique was developed by Towbin et. al. (1979) specifically for the immunological detection of proteins separated on SDS gels. However, instead of using passive diffusion, they effected transfer of proteins from the gel onto a single filter by electrophoresis. After transfer and prior to labelling they used 3% BSA to block additional protein binding capacity on the filter, which gave a significant decrease in background radiation (Bowen et. al. used only 0.02% BSA and 0.02% Ficoll). My own observations

indicated that the gelatin NP-40 mixture (see Methods, Chapter 8) was even more effective at blocking non specific binding. I also found that the best results were obtained when using freshly iodinated RAM.

There is little doubt, however, that there is still room for improvement in the technique. The more rapid electrophoretic transfer appears preferable to the passive diffusion method used here as it gives rise to less band broadening and all the protein is transferred into one filter. These effects must combine to give better resolution and an improved signal to noise ratio. It may also be advantageous to disregard nitrocellulose altogether in favour of substrates less prone to non specific adsorption. Chemically activated paper has been used with some success by several groups (Renart, 1979; Brûlet, 1980). This provides the benefit that once transfer has been performed and protein is covalently linked to the paper substrate, the remaining protein binding sites may then be chemically inactivated. Recently, Brûlet et. al. (1980) have produced technically excellent results using a monoclonal antibody to label intermediate filament proteins separated on a two dimensional gel and transferred electrophoretically onto diazobenzyloxymethyl paper which was subsequently blocked in ethanolamine/BSA.

## 4. The Antigenic Similarity of Band 1 and Band 2 of Spectrin

It is well accepted that Band 1 and Band 2 share some similarities in primary structure (Dunn et. al., 1978; Anderson, 1979) and yet they are apparently functionally quite distinct (Anderson, 1979; Litman et. al., 1980; Calvert et. al. 1980a). Little is known, however, regarding the immunological identity of these two very large polypeptides and of their structural relationship. Bjerrum et. al. (1975) claimed on the basis of

data obtained using crossed immuno-electrophoresis that Band 1 and Band 2 showed common antigenic determinants. They also suggested, however, that Band 2.1 was related immunologically to spectrin, although this would seem to be at odds with more recent structural and sequence analyses (Bennettand Stenbuck, 1979; Luna et. al., 1979; Tyler et. al., 1979). Sheetz et. al. (1976), on the other hand, having immunised rabbits with intact spectrin found that their antiserum would recognise only Band 1, indicating that it was rather more antigenic than Band 2.

Ziparo et. al., (1978) have employed preparative SDS gel electrophoresis to purify Band 1 and Band 2 in order to raise anti-sera against the two subunits. The affinity purified antibodies which they obtained, showed quite remarkable specificities. They found that their purified anti band 1 antibodies bound to determinants which are present on both Band 1 and Band 2, while their anti Band 2 antibodies reacted with specific determinants on Band 2 only. My own results, however, present a rather different picture, more in line with the results of Sheetz et. al. (1976), in showing Band 1 to be antigenically quite distinct from Band 2 since the three monoclonal antibodies which I have produced against Band 1 show no ability to recognise sites on Band 2 (or any other ghost protein) at least as detected by gel labelling and protein blotting techniques. These two sets of data serve as a striking example of the inconsistencies which may arise between different laboratories when antibody produced by two different animals are compared. Finally, there is only limited data from which to draw any conclusions as to whether Band 1 is more immunogenic than Band 2, although at first sight this would appear to be the case, in the light of my own results and those of Sheetz et. al. (1976).

## 5. Localisation of the 717 and 718/a antibody Binding Sites

Not only are the binding sites of the three monoclonal IgG antibodies unique to Band 1, but electron microscopy and the labelling of tryptic fragments of spectrin suggest that the antibodies secreted by the 717 and 718/a clones recognise unique sites on the Band 1 polypeptide. It appears quite possible that the 718/b antibody also recognises a single unrepeated site since it gives an identical pattern of labelling of trypsin digested spectrin to the 717 antibody (Figure 9.6).

The labelling patterns of the tryptic fragments of spectrin gives considerable information regarding the locations of the 717 (or 718b) and 718/a antibody binding sites (Figure 9.6). The findings that they exclusively label fragments of 130,000 and 170,000 daltons (130 and 170 kd) respectively but that both appear to label a common 100 kd fragment, can be interpreted in only one basic model which is shown in Figure 10.1. The assumption made here, is that both antibodies label the same 100 kd fragment, which is quite reasonable considering the very simple appearance of the two labelling patterns.

The two antibody binding sites are located on two tryptic fragments (of 130 kd and 170 kd) which because of their length must overlap in the intact Band 1 polypeptide. Obviously neither antibody binding site can be located within the overlap region, the size of which cannot be less than 60kd if the two fragments are to fit into a 240 kd polypeptide, and cannot be greater than 100kd since this is the size of the smallest fragment to which both antibodies bind. For simplicity, the special case of the minimum overlap in which one terminus of each fragment would contribute one terminus of the entire Band 1 polypeptide is shown in Figure 10.1. In view of the fact that the two binding sites are separated by no more than 100kd (see above), the 718/a site must be located more than 70kd, but less than 110kd from one terminus, while the 717 site must

Figure 10.1

Proposed locations of the binding sites for the 717 and 718/a antibodies on the Band 1 polypeptide, based on the data shown in Figure 9.6. A 170 kd tryptic fragment of Band 1 is exclusively labelled by 718/a while a 130 kd fragment is labelled only by 717. If these two fragments are to be fitted into the 240 kd Band 1 they must overlap. The special case of the minimum overlap is shown in the figure with two fragments each contributing to one terminus of Band 1. The minimum overlap of 60 kd is shown as the shaded region. Both antibodies appear to label a common 100 kd fragment. The two extreme positions of this 100 kd fragment is shown which define the limits of the two antibody binding sites since neither terminus of the 100 kd fragment may extend into the overlap region of the two larger fragments.

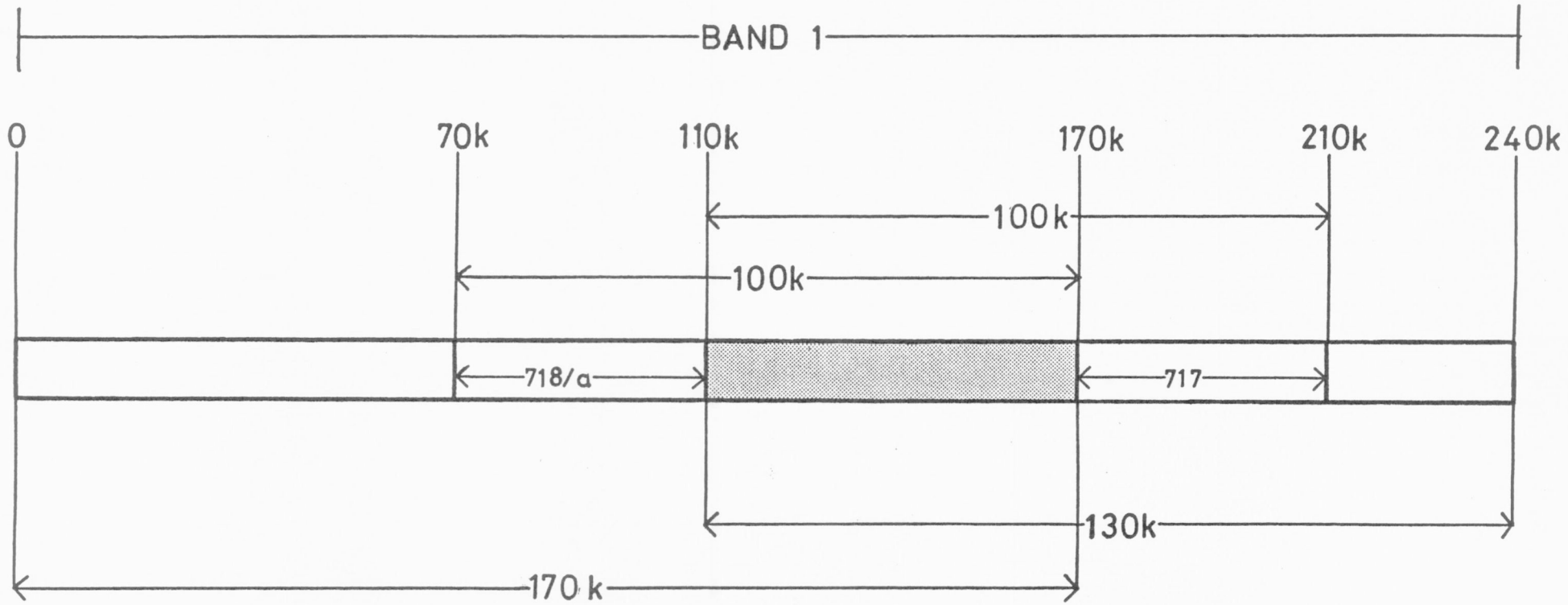



FIGURE 10.1

be more than 30kd but less than 70kd from the other terminus. These values are independent of how much the two fragments are made to overlap in the model (Figure 10.1).

## 6. Implications for the Substructure of Band 1

The data presented here indicate that the structure of Band 1 of spectrin may differ quite substantially from the 'beads on a string' type structure, involving a series of globular domains linked by flexible sections of the polypeptide chain, envisaged for Band 2 (Anderson, 1979; Calvert et. al., 1980b). The in-situ labelling of trypsin digested spectrin with the three IgG monoclonal antispectrin antibodies provides no evidence for the existence of repeating domains linked by trypsin sensitive segments of the polypeptide chain. Neither do the antibodies themselves appear to label repeating units.

An obvious consequence of a repeating domain type conformation of the Band 1 polypeptide (or any other) is that the chemical termini would logically be coincident with physical termini. However, the balance of evidence suggests this may not be the case for Band 1. Electron microscopy has shown the 718/a antibody to bind very close to one physical end of the spectrin heterodimer, whereas in situ labelling of tryptic fragments indicates that the antigenic determinant which this antibody recognises is located between 70 and 110kd from the nearest chemical terminus, assuming both 717c and 718/a antibodies label the same 100kd fragment. This suggests that the Band 1 polypeptide must possess a rather more complex tertiary structure than that which has been proposed for Band 2 (Anderson, 1979), in which the physical and chemical termini do not correspond. A further indication of this more complex substructure is the finding of Anderson (1979a) that Band 1, but not Band 2,

contains a relatively trypsin insensitive 80,000 dalton segment of the polypeptide chain with frictional properties similar to those of intact spectrin.

These findings are quite consistent with the facts that the two subunits of spectrin are functionally quite distinct (Anderson, 1979; Litman et. al., 1980; Calvert et. al., 1980a) and may be differentiated immunologically. However, how they may be reconciled with spectrin's obvious flexibility and apparent chemical uniformity (Anderson, 1979) must await further research. Clearly, it is now of considerable importance to verify that both the 717 and 718/a antibodies label the same 100kd polypeptide.

## Future Research

It should be appreciated that many of the results concerning the prdocution and use of monoclonal antibodies reported here are only of a preliminary nature. Nevertheless, it has been successfully demonstrated that by using monoclonal antibodies it is possible to immunologically dissect a large protein molecule and to correlate chemical and electron microscope data. Of the monoclonal antibodies so far produced, 718/a holds the most interest since its binding site on Band 1, as viewed by electron microscopy, appears to be very close to the end of the spectrin heterodimer involved in tetramer assembly. Already significant progress has been made towards localizing its binding site with respect to the chemical termini of the polypeptide chain. The information so far obtained may be further refined using a combination of other proteolytic enzymes, two-dimensional gel techniques and new monoclonal anti Band 1 antibodies with different binding specificities. Ultimately it should be possible to use the 718/a antibody to isolate a fragment of Band 1 which

might be able to interact specifically with isolated Band 2, or even with spectrin heterodimers, thereby mimicing tetramer assembly.

To further this line of research, however, it will be necessary to increase the library of monoclonal antibodies, specifically selecting for those which are suitable for in-situ labelling of protein blots; the 706 IgM would clearly not fall into this category. In particular, antibodies against Band 2 must be obtained since many of spectrin's interactions with other membrane components appear to reside on this subunit. Originally, it was thought that simply immunising mice with whole spectrin would produce a range of antibody against both subunits. The situation now, however, is not quite so clear, as it appears that Band 1 may be more immunogenic than Band 2. Consequently, if this is in fact the case, detecting an anti band 2 antibody could require the screening of a considerable number of cultures. Fortunately this problem may be avoided by adopting the more lengthy immunisation regime of Stähli et. al. (198L) and by immunising with Band 2 purified as described by Calvert et. al. (1980a). More efficient fusion and detection of antibodies may be achieved, employing the fusion protocol of Gefter et. al. (1977) with the Sp2/0 myeloma cell line. There is therefor no reason to suppose that several anti Band 2 antibodies cannot be obtained fairly rapidly.

The protein labelling technique, as well as being invaluable in determining the specificities and in localising the binding sites of monoclonal antibodies, could also be used specifically for detecting spectrin or spectrin like molecules using either monoclonal anti spectrin, or anti spectrin purified from immune serum as a probe. As discussed in Chapter 6, spectrin has not been identified in non erythroid cells. The use of the blotting technique would greatly simplify screening of

such cells for spectrin like proteins as one need only fractionate total cellular protein on SDS gels. A prime candidate for this type of investigation would, of course, be the sperm from Thyone briareus (Tilney, 1976) with its two high molecular weight polypeptides. Another interesting possibility would be to screen H.S. spectrin samples with monoclonal antibodies, in particular with those directed against functional sites. The detection of labelling differences would certainly show once and for all that, at least in those cases, the primary defect resides in the spectrin molecule.

# C H A P T E R 11

## CONCLUSIONS

Many of the conclusions to be drawn from this investigation have already been stated in earlier chapters. Instead of repeating these here, I would like to propose an up to date molecular model of the red blood cell membrane, incorporating both my own results presented in this dissertation and those obtained in many other laboratories. This model, shown in Figure 11.1, is an attempt to unify what is known regarding the numerous molecular interactions which take place in the membrane while preserving as much as possible the relative sizes and sense of scale of the individual components. This latter constraint on the model I consider to be of particular importance since many other membrane models have placed most emphasis on the lipid bilayer at the expense of the peripheral components. As shown in Figure 11.1, at least in the case of the red blood cell, these peripheral membrane components may extend a considerable distance from the lipid bilayer (scale is approximately 1cm equal to 5nm) and account for much of the membrane mass.

Membrane proteins in the model are labelled according to the nomenclature of Steck (1974), their relative sizes being calculated on the assumption that actin (band 5) is a 5nm diameter sphere with a molecular weight of 43,000 daltons. A and B in the model refer to glycophorin A and glycophorin B respectively. These two glycoproteins almost certainly traverse the lipid bilayer as a single segment of $\alpha$ helix (Tomita and Marchesi, 1975) the diameter of which has been exaggerated about four times in the model as an aid to clarity. The N terminal (extra cellular) domains of glycophorin A and B have been drawn as extended segments of polypeptide chain which are heavily glycosylated.

Figure 11.1

A model showing the arrangement of the major erythrocyte membrane polypeptides. All proteins are identified according to the nomenclature of Steck (1972). A and B refer to glycophorins A and B respectively. The lipid bilayer is represented by double tailed phospholipids and cholesterol (black rectangles) in approximately the correct molar ratio (1:0.8). No attempt has been made to differentiate between phospholipid species (or glycolipids). Further details are descibed in the text.

The scale is approximately 1cm to 5nm.

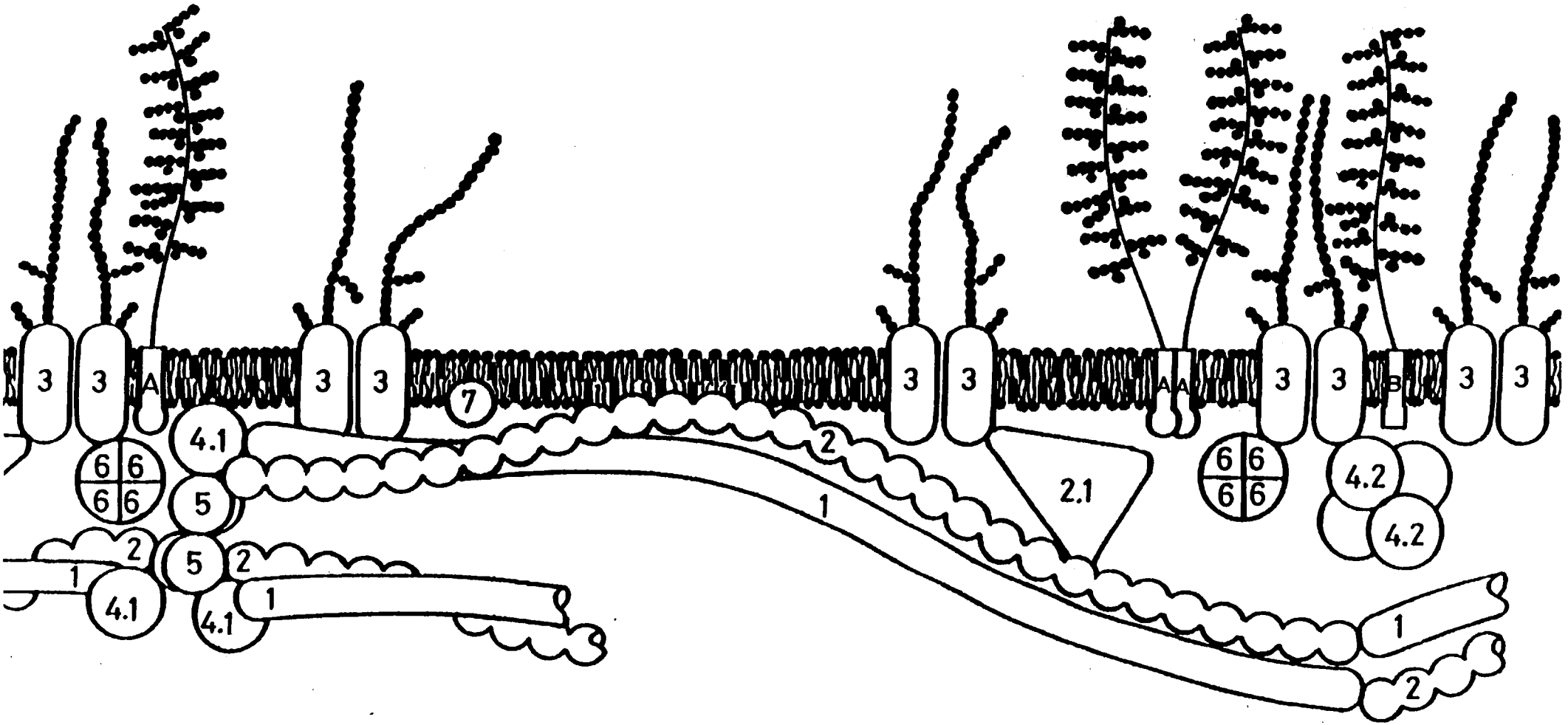



FIGURE 11.1

Carbohydrate moieties, found exclusively on the outside of the membrane, are represented by small filled circles, but no attempt has been made to differentiate between any of these. The arrangement of carbohydrate is according to the data of Gahmberg et al. (1976) and Tomita and Marchesi (1975).

Spectrin (bands 1 and 2) has been drawn such that the two subunits appear structurally distinct. The representation of band 2 has been designed to suggest a repeating domain type conformation as proposed by Anderson (1979) and Calvert et al. (1980b) with the primary and tertiary structures being colinear, while band 1 is represented by a uniform flexible rod without indicating any structural differentiation along its length, in view of my own findings that the folding of the band 1 polypeptide chain may be more complex than that envisaged for band 2. This should not, of course, preclude the possibility that band 1 may still be folded into several well defined domains. Band 2 is shown to interact with the lipid bilayer, although this is not meant to be taken as a literal representation of such an association but is more to serve as a reminder that spectrin does contain hydrophobic sequences (Calvert et al. (1978(b) ) and may indeed be able to associate specifically with phospholipids as indicated by the results of Sweet and Zull (1970), Mombers et al (1977) and Haest et al (1978).

Cross linking of several spectrin tetramers is shown to occur by means of a small segment of F actin containing four G actin units. The association of each spectrin tetramer with the actin is indicated to take place by way of both a direct interation between band 2 and a single actin monomer and an indirect interaction mediated by band 4.1. Brenner and Korn (1979) have shown that the direct interaction does occur, at least <u>in vitro</u> , although the sole involvement of band 2 is purely conjectural. Clearly, band 4.1 does associate with both actin

and spectrin (Pinder et. al., 1979; Fowler and Taylor, 1980; Tyler et al., 1979), and its association with spectrin has been shown to require both bands 1 and 2 (Calvert et al. 1980a) as indicated in the model. One of the 4.1 molecules is shown to be bound to the bilayer. Such an association is only inferred, since, as discussed in Section 6.8, it is expected that Band 4.1 should bind to at least one other membrane component as well as to actin and spectrin. Attachment of spectrin to integral membrane proteins (Band 3 dimer) is shown to occur indirectly through Band 2.1 ('ankyrin'). The overall shape of 2.1 is that described by Tyler et al., 1979, although its dimensions have been increased by about 20% over their published values in order to preserve the scale of 2.1 with respect to its mass. It should be noted that the majority of the integral membrane proteins are not assigned any special association with the cytoskeletal proteins, the relative numbers in these two protein classes being approximately that found in vivo.

The only other protein components shown in the model to be associated with Band 3 dimers are Band 6 (glyceraldehyde-3-phosphate dehydrogenase) and Band 4.2, both of which proteins are thought to exist in the red cell as tetramers (Yu and Steck, 1975; Steck, 1972b). In the case of the former, it is uncertain whether the association with Band 3 occurs within the intact red blood cell or whether it arises simply as an artefact of preparation of ghosts since it is easily eluted under isotonic conditions. Finally, Band 7 has been portrayed as an integral membrane protein despite the fact that it is normally classified as being peripheral. This decision was taken in view of the protein's extremely tenacious and apparently hydrophobic association with the bilayer (Yu and Steck, 1973; Yu et al., 1973). Band 7 is not known to associate with any other membrane proteins, nor is it known to form oligomers.

This model is a highly simplified representation of the red blood cell mebrane, since many other proteins whose assoications are not clear have not been included. In particular there is the series of components from band 2.2 to band 2.9 all of which appear to be structurally related to band 2.1 (Luna et al., 1979) and also band 4.9 which may be associated with the spectrin-actin-4.1 complex (Calvert et al., 1980a). Another component which has yet to be localised runs in the band 8 region of SDS/polyacrylamide gels and is thought to form the hollow cylinder like complexes (Ralston, 1979) originally described by Harris (Harris, 1968; Harris and Naeem, 1978). Clearly there are many questions to be answered regarding the function and location of these minor components before this model can be further updated.

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