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Immunoreactive forms of erythrocyte spectrin and ankyrin in brain

By V. Bennett, J. Davis and W. E. Fowler
Department of Cell Biology and Anatomy, Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore, Maryland 21205, U.S.A.

Polypeptides immunologically related to erythrocyte spectrin and ankyrin have been detected in brain. The cross-reacting proteins include soluble as well as membrane-associated forms. A class of soluble cross-reacting polypeptides have been identified as high molecular mass microtubule-associated proteins (MAPS). MAP1, a group of polypeptides of molecular mass *ca.* 370 kDa contains a component that cross-reacts with anti-ankyrin IgG. MAP2, a polypeptide of molecular mass 300 kDa cross-reacts with anti-spectrin IgG, with the shared antigenic sites localized to the α chain of spectrin. The functional basis for structural homology between MAP1 and ankyrin may involve association with tubulin, since erythrocyte ankyrin binds to microtubules polymerized from pure brain tubulin. Spectrin did not associate with microtubules, but does have in common with MAP2 the ability to bind to actin (Brenner & Korn 1979; Sattilaro *et al.* 1981) and the shape of a flexible rod as visualized by rotary shadowing (Shotton *et al.* 1979; Voter & Erickson 1981). Immunoreactive forms of spectrin and ankyrin are also present in membrane fractions. A homologue of spectrin which constitutes 3% of the total membrane protein has been purified from low ionic strength extracts of membranes. This protein contains two non-identical polypeptide chains of molecular masses of 260 and 265 kDa, binds to F-actin, and displaces binding of erythrocyte spectrin to erythrocyte membranes. The brain protein has been visualized by rotary shadowing as an extended rod-like molecule 195 nm in length. These studies indicate that the organization of proteins in the membrane–cytoskeleton complex of erythrocytes has direct relevance to other types of cells, and suggest the existence of families of proteins related to spectrin and ankyrin.

Introduction

The cytoplasm of eukaryotic cells contains a meshwork of filaments assembled in a complex three-dimensional arrangement (Heuser & Kirschner 1980). The major filament systems consist of microtubules, actin filaments and intermediate filaments, and these structures are associated with each other as well as with cell membranes. For example, microtubules interact with actin filaments (Griffith & Pollard 1978) and intermediate filaments (Runge *et al.* 1981). Microtubules also associate with secretory granules (Sherline *et al.* 1977) as well as with other membranes (Moskalewski *et al.* 1977; Bhattacharyya & Wolff 1975).

The protein linkages that allow these filament systems to be integrated with each other and cell membranes are not understood. Studies with the erythrocyte membrane may provide insight into this problem, since the organization of the major proteins in this relatively simple structure has been elucidated (reviewed by Branton *et al.* (1981) and Bennett (1982)). Erythrocytes contain a cytoskeleton on the inner surface of the plasma membrane. This cytoskeleton is composed of spectrin, which is associated independently with actin, a 78 kDa polypeptide, and other spectrin molecules (Ungewickell *et al.* 1979; Tyler *et al.* 1979; Brenner & Korn 1979; Fowler & Taylor 1980; Ungewickell & Gratzer 1978). Spectrin is associated with the membrane by binding to another protein, ankyrin (Bennett & Stenbuck 1979*a*, 1980*a*; Luna *et al.* 1979;

Yu & Goodman 1979). Ankyrin is in turn associated with the cytoplasmic domain of band 3 (Bennett & Stenbuck 1979*b*, 1980*b*; Hargreaves *et al.* 1980), which is a major membrane-spanning protein.

Immunoreactive forms of ankyrin have been detected by radio-immunoassay in many types of tissues (Bennett 1979). The proteins cross-reacting with ankyrin have been localized by immunofluorescence in cultured cells in a microtubule-like pattern during interphase as well as mitosis (Bennett & Davis 1981). Spectrin was not detected in cultured cells by radioimmunoassay (Hiller & Weber 1977), but cross-reactivity has been observed in other tissues by different methods (Sheetz *et al.* 1976; Goodman *et al.* 1981). The purpose of this paper is to review recent studies from our laboratory which have identified several proteins in brain that share antigenic determinants and functional homology with erythrocyte ankyrin and spectrin.

Microtubule-associated forms of ankyrin and spectrin

Two high molecular mass microtubule-associated proteins (MAPs) have been demonstrated to cross-react with affinity-purified antibodies against erythrocyte ankyrin and spectrin respectively (Bennett & Davis 1981; Davis & Bennett 1982). Both preparations of antibodies cross-reacted with multiple polypeptides in crude extracts of brain (figures 1 and 2). In each case, microtubule protein isolated by repeated cycles of polymerization and depolymerization was greatly enriched in only one of the cross-reacting polypeptides. Antibody against ankyrin cross-reacted with a polypeptide molecular mass of *ca.* 370 kDa in the region of MAP1, while antibody against spectrin cross-reacted with a polypeptide of molecular mass *ca.* 300 kDa that comigrated with MAP2. Relatively little is known about MAP1, which has not been purified and contains more than one polypeptide. MAP2, however, has been isolated (Herzog & Weber 1978; Kim *et al.* 1979) and is known to form projections extending from microtubules polymerized *in vitro* (Murphy & Borisy 1975; Kim *et al.* 1979).

The cross-reactivity of MAP2 and anti-spectrin antibody has been characterized in more detail (Davis & Bennett 1982). Pure MAP2 cross-reacted with antibody after electrophoretic transfer from SDS–polyacrylamide gels to nitrocellulose (figure 2) and also was immunoprecipitated by anti-spectrin antibody. The immunoreactivity involved the major component of MAP2 rather than a minor contaminant since peptide maps of MAP2 and the polypeptides immunoprecipitated by antibody were nearly identical. Spectrin contains two subunits of molecular masses 260 and 225 kDa (α and β respectively) that have distinct amino acid sequences and different functions (Speicher *et al.* 1980; Morrow *et al.* 1980). The antigenic determinants shared by MAP2 and spectrin reside in the α subunit of spectrin, because binding of ^{125}I-labelled MAP2 to anti-spectrin IgG was displaced by the α subunit and not the β subunit. The homology between MAP2 and the α subunit is only partial because MAP2 displaced binding of ^{125}I-labelled α subunit to anti-spectrin antibody by a maximum of 20%. Furthermore, peptide maps of MAP2 and the α subunit were dissimilar.

The surprising finding of shared antigenic sites between erythrocyte membrane proteins and brain microtubule-associated proteins suggested the possibility of functional homology as well. Partly purified MAP1 and pure MAP2 did not displace binding of spectrin or ankyrin to erythrocyte membranes. Erythrocyte ankyrin did associate with microtubules polymerized from pure brain tubulin (figure 3). The binding of ankyrin was saturable at a ratio of 1 mol ankyrin per 4 mol tubulin dimer, and binding was displaced by brain MAPs. The apparent

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K_d for ankyrin was 2–4 μM with 10 μM tubulin. Ankyrin also promoted the polymerization of pure tubulin, provided that the tubulin was near its critical concentration.

The fact that ankyrin associates with microtubules suggests that ankyrin may have evolved from a MAP. Mature human erythrocytes lack tubulin, but tubulin is present as a membrane-associated marginal band in mammalian erythroblasts and primitive circulating foetal erythrocytes (Van Deurs & Behnke 1973). Furthermore a protein similar to MAP2 has been identified in the marginal band of nucleated erythrocytes (Sloboda & Dickersin 1980). Thus ankyrin in erythrocyte precursor cells and nucleated erythrocytes may bind to tubulin and maintain the

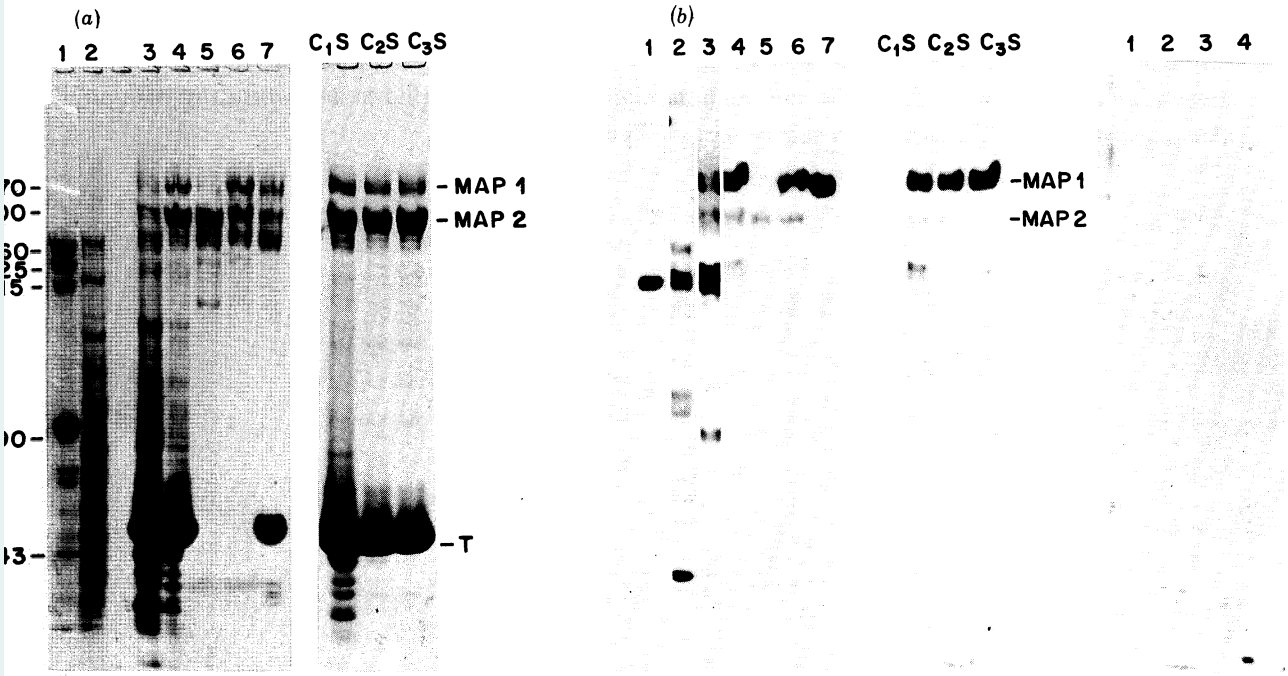

FIGURE 1. Identification of immunoreactive ankyrin polypeptides in SDS–polyacrylamide gels with affinity-purified anti-ankyrin antibody. Lanes: 1, pig erythrocyte ghosts; 2, HeLa cells; 3, pig brain extract, 4, microtubule pellet from pig brain; 5 and 6, MAP fractions; 7, MAP fraction 6 co-polymerized with pure tubulin; and C_1S-C_3S, microtubule pellets after repeated cycles of assembly and disassembly. Proteins were electrophoresed on a 3.5–17 % exponential polyacrylamide gel (Fairbanks *et al.* 1971) and either stained with Coomassie blue (*a*) or electrophoretically transferred to nitrocellulose (Towbin *et al.* 1979). Immunoreactive polypeptides were identified by overlaying the nitrocellulose with 1 μg ml^{-1} anti-ankyrin antibody or preimmune antibody and then ^{125}I-labelled protein A. An autoradiogram was then prepared. Pig brain cerebral cortex was homogenized (1 g ml^{-1}) in a buffer containing 0.32 M sucrose, 50 mM 1,4-piperazine-diethanesulphonic acid (PIPES), 1 mM Na ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5 mM dithiothreitol, 200 μg ml^{-1} $PhMeSO_2F$, pH 7.0, and centrifuged for 30 min at 200 000 *g*. The supernatant was adjusted to 3 M glycerol, 0.1 mM GTP, 0.5 mM $MgCl_2$ and 1 mM ATP, incubated for 45 min at 37 °C, and recentrifuged. The pellets were depolymerized at 0 °C in 10 mM Pipes, 1 mM Na EGTA, 0.2 mM dithiothreitol, pH 6.4, and centrifuged. MAP proteins were isolated from the supernatant (lane 4) by phosphocellulose chromatography (0.4 M NaCl step gradient in resuspension buffer) and resolved into two fractions (lanes 5 and 6) by gel filtration on Sepharose (C1)4B in 1 M NaCl, 10 mM sodium phosphate, 1 mM Na EGTA, 1 mM NaN_3 and 0.2 mM dithiothreitol, pH 7.4. Sample as in lane 6 was incubated with pure tubulin (figure 3) under polymerizing conditions, layered over a sucrose barrier gradient (200 g l^{-1}) and centrifuged for 20 min at 200 000 *g*. The pellet, containing microtubules and associated proteins, was dissolved in SDS (lane 7). Microtubules were subjected to three cycles of assembly and dissasembly; the supernatants of cold-depolymerized and centrifuged pellets are in lanes C_1S, C_2S, and C_3S, corresponding to one, two and three cycles, respectively. Molecular masses (numbers at the left in kilodaltons) are based on known mobilities of erythrocyte proteins; extrapolation from these values gave estimates of molecular masses of MAP1 and MAP2. (From Bennett & Davis 1981).

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position of the marginal band. Conversely, MAP1 will have some of the properties of ankyrin, such as binding sites for a membrane-bound analogue of band 3 or for a spectrin (see below). Such an activity of MAP1 could explain how microtubules interact with membranes (Bhattacharyya & Wolff 1975; Sherline *et al.* 1977; Stephens 1977; Moskalewski *et al.* 1977).

Spectrin, in contrast to ankyrin, did not associate with microtubules or affect the polymerization of tubulin. An activity shared by spectrin and MAP2 is association with actin. Spectrin associates with muscle actin (Brenner & Korn 1979), and a direct association between MAP2 and actin has been reported (Sattilaro *et al.* 1981). A further similarity between spectrin and MAP2 is the distinctive morphology of these proteins, which appear by rotary shadowing with platinum to be flexible rod-shaped molecules 200 nm in length for spectrin tetramers (Shotton *et al.* 1979) and 180 nm in length for MAP2 (Voter & Erickson 1981). Other high molecular mass actin-binding proteins such as filamin and macrophage actin-binding protein also exhibit a similar flexible rod morphology (Tyler *et al.* 1980).

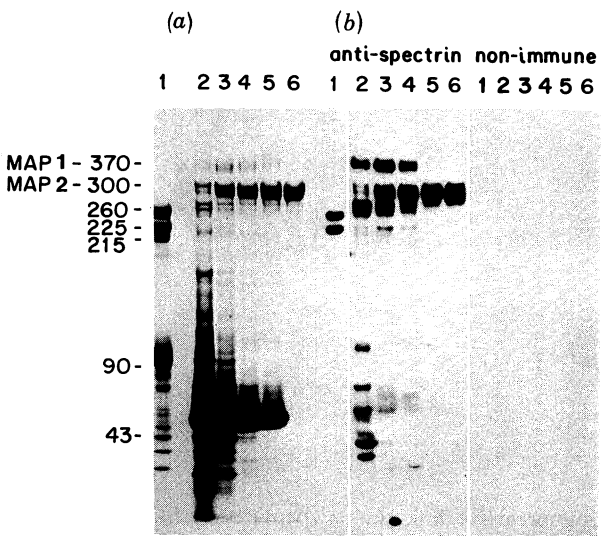

FIGURE 2. Identification of MAP2 from pig brain as an immunoreactive form of erythrocyte spectrin with affinity-purified anti-spectrin antibody. Erythrocyte ghosts (lane 1), pig brain extract (lane 2), microtubules isolated by repeated cycles of assembly and disassembly: C_1S (lane 3), C_2S (lane 4), C_3S (lane 5) and purified MAP2 (lane 6) were electrophoresed on SDS-polyacrylamide gels. Polypeptides were visualized by staining with Coomassie blue (*a*), and immunoreactive polypeptides were identified by electrophoretic transfer from polyacrylamide gels to nitrocellulose paper, followed by overlaying the nitrocellulose with antibody and then ^{125}I-labelled protein A (*b*) (figure 1). Microtubule proteins were prepared as described (figure 1). MAP2 was purified by the method of Kim *et al.* (1979) with the modifications of Sandoval & Weber (1980). Numbers at the left are molecular masses in kilodaltons. (From Davis & Bennett (1982).)

These considerations suggest the existence of a family of related proteins with the shared features of molecular masses of 260–300 kDa, the ability to bind to actin, and with the shape of an extended, flexible rod. An apparent difference between spectrin and the other actin-binding proteins is that spectrin contains a β subunit, which associates with ankyrin and thus allows spectrin to bind to the membrane (Calvert *et al.* 1980; Morrow *et al.* 1980). Filamin and macrophage actin-binding protein are homodimers, did not associate with erythrocyte membranes (Tyler *et al.* 1980), and presumably lack a β-type subunit. Furthermore, the homology between spectrin and MAP2 is localized to the α subunit. It is conceivable that MAP2 and the other

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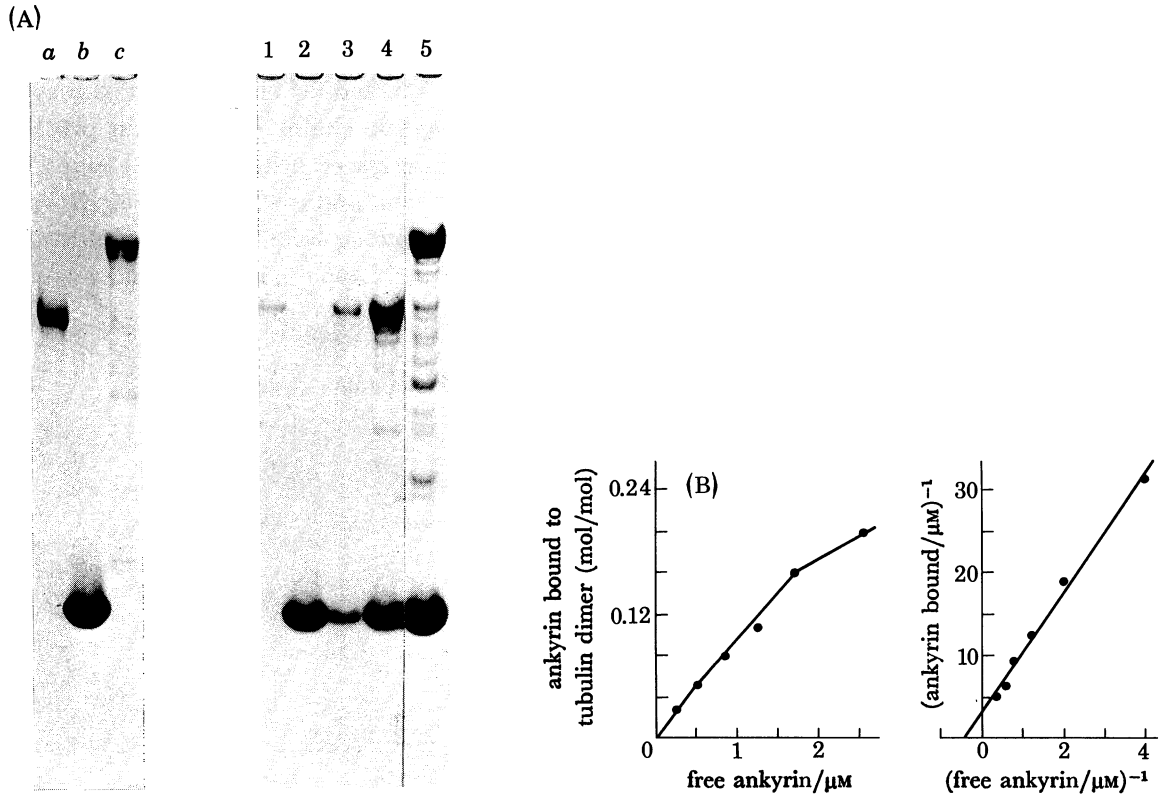

FIGURE 3. Association of erythrocyte ankyrin with brain tubulin.

(A) Brain microtubules (figure 1) after two cycles of assembly and disassembly were depolymerized and applied to DE-53 cellulose in a buffer containing 50 mM PIPES, 1 mM NaEGTA, 0.1 mM $MgCl_2$, 0.1 mM GTP and 0.1 mM dithiothreitol, pH 7.0. The column was washed with 10 volumes of buffer, and a fraction enriched in MAPS was eluted with 0.1 M KCl; the column was washed with 10 volumes of 0.2 M KCl, and tubulin was eluted with 0.4 M KCl. Erythrocyte ankyrin and purified tubulin dimer (dialysed against assay buffer) were centrifuged for 20 min at 200 000 *g* and then combined at 2 μM and 10 μM, respectively, in 5 % sucrose, 100 mM PIPES, 1 mM NaEGTA, 1 mM GTP, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol. After 30 min at 37 °C, the solutions were layered over 20 % sucrose in assay buffer, and microtubules were collected by centrifugation for 20 min at 200 000 *g* at 25 °C. The pellets were analysed on an SDS–polyacrylamide gel. Control lanes: (*a*) erythrocyte ankyrin; (*b*) pure tubulin; (*c*) MAP fraction. Pellet lanes: 1, ankyrin alone; 2, tubulin alone; 3, ankyrin and tubulin chilled to 0 °C after polymerization; 4, ankyrin and tubulin; 5, ankyrin and tubulin plus MAP fraction (400 μg ml^{-1}).

(B) Various concentrations of ankyrin were incubated alone or with 10 μM tubulin for 30 min at 37 °C, and microtubules were collected and analysed on an SDS–polyacrylamide gel. The relative peak areas were estimated by densitometry. The data are corrected for the amount of ankyrin sedimented in the absence of tubulin, which was less than 5 % of the value in the presence of tubulin. (From Bennett & Davis (1981).)

actin-binding proteins also have an analogue of the spectrin β chain but that this subunit is dissociated during the procedures employed to extract and purify these proteins. Such a protein might provide a linkage between membranes and actin filaments or microtubules.

MEMBRANE-ASSOCIATED ANALOGUE OF SPECTRIN

The particulate fraction from homogenates of brain contains two polypeptides of molecular masses *ca.* 265 and 260 kDa, which compose 3 % of the total membrane protein and cross-react with anti-spectrin antibody (figure 4). These polypeptides have been purified, and exhibit a number of properties indicating a strong resemblance to erythrocyte spectrin (Bennett *et al.*

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1982). The purified protein was able to associate with muscle actin in sedimentation assays, and cross-linked actin filaments as monitored by masurements of low-shear viscosity. The protein also contains a binding site for ankyrin because it competed for binding of erythrocyte spectrin to spectrin-depleted membranes. The two polypeptide chains of the brain protein represent two different subunits, because these polypeptides produced distinct peptide maps.

One of the most striking similarities between the brain protein and erythrocyte spectrin was the appearance of these proteins after low-angle rotary shadowing with platinum (figure 5). The brain protein was an extended rod with average dimensions of 195 nm in length and 4–6 nm

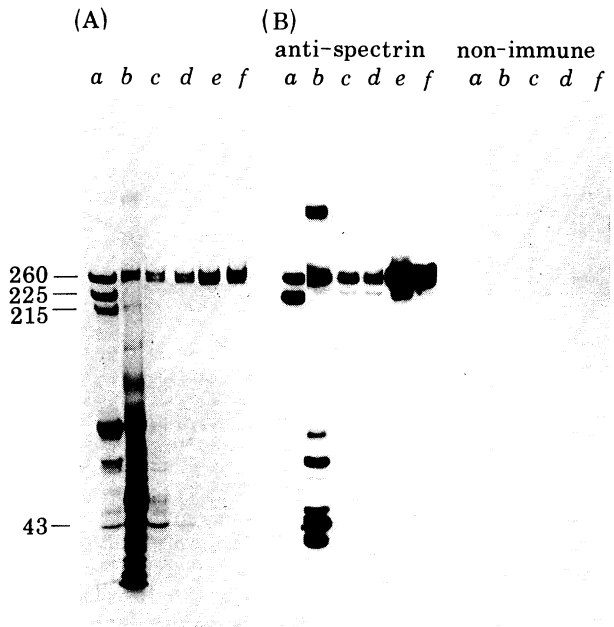

FIGURE 4. Purification from brain membranes of a protein that shares antigenic determinants with erythrocyte spectrin. 125 g frozen grey matter from pig brain was homogenized in 800 ml 0.32 M sucrose, 2 mM NaEGTA, 200 μg ml^{-1} $PhMeSO_2F$, 0.015 % (by volume) diisopropylfluorophosphate, 3 μg ml^{-1} pepstatin A, pH 7.0. The 900 g supernatant of this suspension was pelleted for 25 min at 30000 *g* to collect the crude membrane fraction. This fraction was depleted of myelin by centrifugation for 40 min at 30000 *g* in 0.8 M sucrose, which causes the myelin to float while other membranes are pelleted. The myelin-depleted membranes were washed successively by centrifugation for 25 min at 30000 *g* with homogenization buffer, followed by 10 mM sodium phosphate, 2 mM NaEGTA, 50 μg ml^{-1} $PhMeSO_2F$, pH 7, and finally 2 mM NaEGTA, 50 μg ml^{-1} $PhMeSO_2F$, pH 7. The washed membranes (1.6 g protein) were extracted for 60 min at 37 °C in 800 ml 0.2 mM NaEGTA, 0.5 mM dithiothreitol, 50 μg ml^{-1} $PhMeSO_2F$, pH 7. The suspension was pelleted for 30 min at 200000 *g* and the supernatant (120 mg extracted protein) was adjusted to 45 % saturation with solid ammonium sulphate. The precipitated proteins were dialysed overnight against 1 M urea, 20 mM glycine, 2 mM NaEGTA, 1 mM NaN_3, 0.2 mM dithiothreitol, and centrifuged for 3 h at 200000 *g*. The supernatant was applied to a Sephacryl S400 gel filtration column equilibrated with urea buffer. Fractions containing the 260 kDa polypeptides eluted near the void volume. The protein was precipitated with ammonium sulphate, dialysed extensively against 0.2 mM NaEGTA, 0.5 mM NaN_3, 0.2 mM dithiothreitol, pH 7, and centrifuged for 90 min at 200000 *g*. The supernatant was fractionated on linear sucrose gradients (100–300 g l^{-1}) in dialysis buffer with a Beckman VTi50 rotor (14 h at 36000 rev. min^{-1}). The gradient fractions with the purest protein (at about a sedimentation rate of 11*S*) contained 1.2 mg of protein. An additional 2 mg of less pure protein was recovered from adjacent fractions. The fractions at various stages of purification were analysed by SDS-polyacrylamide gel electrophoresis. Polypeptides were visualized by staining with Coomassie blue (A) and polypeptides cross-reacting with spectrin (B) were identified as in figure 2. Samples are: (*a*) pig erythrocyte ghosts; (*b*) pig brain membranes; (*c*) low ionic strength extract of brain membranes; (*d*) soluble protein recovered from ammonium sulphate precipitation of sample (*c*); (*e*) pooled fractions from gel filtration; (*f*) pure protein from sucrose gradients. The numbers at the left are molecular masses in kilodaltons.

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in width (estimated without compensation for the additional mass of platinum). Occasionally, the rods open in the middle to reveal two parallel chains. The chains otherwise are closely opposed or even helically entwined along the length of the molecule. Pig erythrocyte spectrin tetramers were slightly shorter with an average length of 175 nm, but otherwise were quite similar to the brain protein. One significant difference between the two proteins in these preparations is that, on the average, the brain protein was straighter and there were fewer instances of separation of individual chains than is true with erythrocyte spectrin. The purified brain protein was a stable tetramer, since the molecular mass calculated from preliminary hydrodynamic parameters (R_S = 22 nm, $S_{20,w}$ = 11 S) is about 1 MDa. An important similarity between these proteins is that oligomers larger than tetramers were observed only rarely. Thus it is likely that the dimers have a polarity and are associated head-to-head, as was originally suggested for erythrocyte spectrin by Shotton *et al.* (1979). Otherwise, with a non-polar or a head-to-tail association, polymerization could occur to form extended filaments.

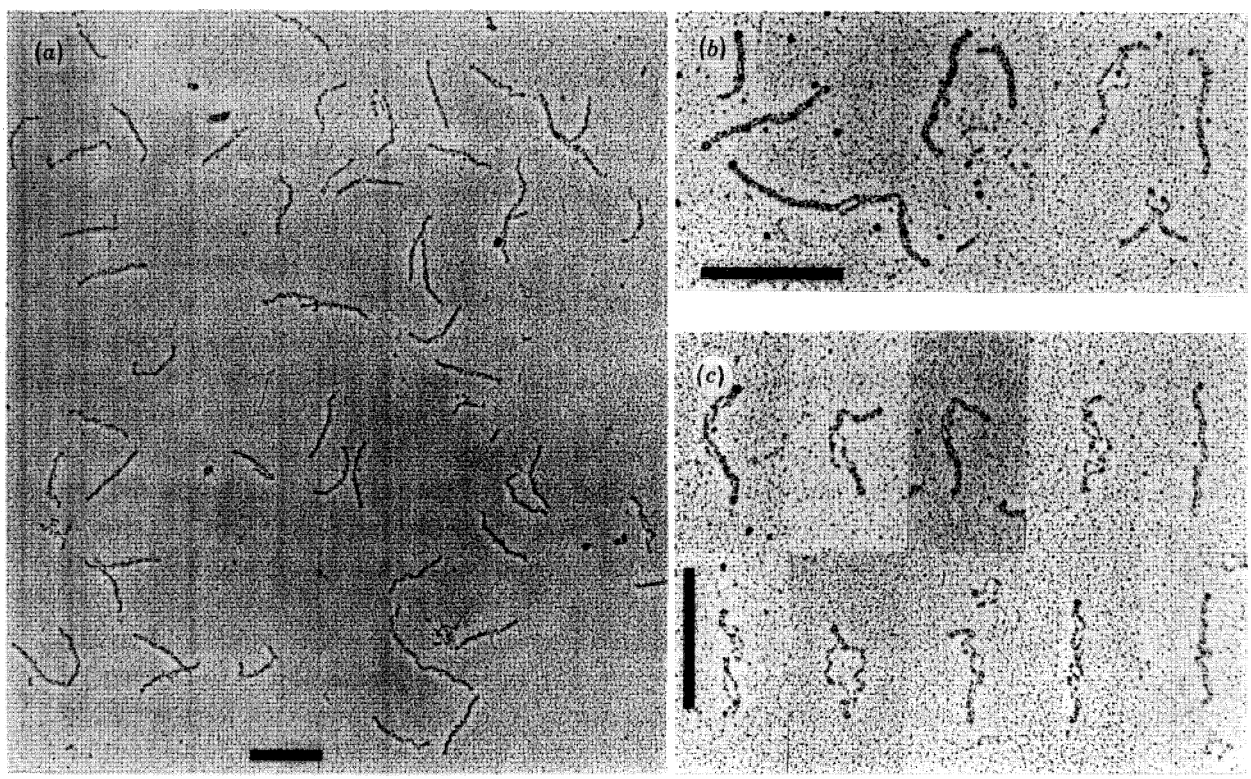

FIGURE 5. Electron micrographs of rotary-shadowed brain spectrin ((*a*), (*b*) and top row of (*c*)) and erythrocyte spectrin (bottom row of (*c*)).

(*a*) Representative field of purified brain spectrin molecules that were diluted to 10 μg ml^{-1} in 100 mM ammonium formate (pH 7), mixed with one-third volume of glycerol, sprayed onto mica, dried *in vacuo* at room temperature and rotary-shadowed with platinum at an angle of 1 in 10. Bar = 200 nm. (Fowler & Erickson (1979); Shotton *et al.* (1979).)

(*b*) Brain spectrin molecules from a different, less pure preparation, treated with 1 M NaBr for several days before dialysis against 100 mM ammonium formate and rotary-shadowing as described in (*a*). The field on the left includes an 'octomer' and two 'dimers', as well as two 'tetramers'. The two-stranded character of the molecules is seen more clearly in the field on the right and in (*c*). Bar = 200 nm.

(*c*) Selected brain spectrin molecules are shown in the top row and erythrocyte spectrin tetramers in the bottom row. These molecules were selected to demonstrate structural similarities between the two spectrin species, such as their two-stranded, double-helical nature. Bar = 200 nm.

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Polypeptides of nearly identical molecular mass to the brain protein were evident on SDS–polyacrylamide gels of plasma membrane fractions in tissues other than brain, including liver, testes, kidney and heart (not shown). These polypeptides also cross-reacted with anti-spectrin antibody. The subcellular localization of this protein was examined in liver, and the cross-reacting 260 and 265 kDa polypeptides were found almost exclusively in the plasma membrane fraction (figure 6). The microsomes, mitochondria, nuclei and soluble fractions all contained polypeptides cross-reacting with anti-spectrin antibody, but none were of molecular mass 260 and 265 kDa. It thus appears that a close analogue of spectrin is a ubiquitous component of membranes. If this is true, then we would also expect to find analogues of ankyrin and band 3 in these membranes. In fact, a polypeptide in liver membranes of molecular mass 200 kDa cross reacts with anti-ankyrin antibody, and two polypeptides of molecular mass *ca.* 140 kDa react with anti-band 3 antibody (figure 6). The function of these proteins remains to be studied, but their presence is consistent with a band-3–ankyrin–spectrin linkage, as occurs in erythrocytes.

The brain analogue of spectrin composes a significant amount of the total membrane protein, and it would be surprising if this protein had not been noticed before. One of the early studies to report such a protein described partial purification of an actin-associated protein from synaptosomal membranes with a molecular mass of 240 kDa on SDS–polyacrylamide gels (Berl *et al.* 1973). This protein was named 'stenin' and was thought at the time to be the brain equivalent of myosin. More recently, an actin-binding protein containing two polypeptides of molecular masses *ca.* 240 and 235 kDa has been partly purified from brain and has been named brain actin-binding protein (Shimo-Oka & Watanabe 1981). This protein fraction contained an ATPase activity and also activated actomyosin ATPase activity, which may explain myosin-like features of 'stenin'. An axonally transported protein with two polypeptide chains of molecular masses *ca.* 250 and 240 kDa has been isolated from the particulate fraction of brain, and demonstrated to bind to actin (Levine & Willard 1981). This protein was named 'fodrin' and was found by immunofluorescence to be localized in the cortical cytoplasm of cultured fibroblasts, neurons and intestinal epithelial cells. Antibody against fodrin did not form a precipitin reaction with spectrin in Ouchterlony immunodiffusion tests (Levine & Willard 1981). This result does not, however, rule out weak or partial cross-reactivity between the proteins.

It is possible that the same protein has been discovered several times and named differently by each group of investigators. We would suggest that, in view of the similarity to erythrocyte spectrin, this protein be referred to as brain spectrin. The related protein in liver would be named liver spectrin, and so on. The relation between brain spectrin and spectrins in other tissues is not known, but it is likely that these proteins will be related to each other more closely than to the spectrin in erythrocytes.

The function of this family of membrane-associated actin-binding proteins has not been studied. However, by analogy with erythrocytes, these proteins may couple actin filaments to integral membrane proteins. The spectrins may also be involved in specialized structures such as zonula adhaerens and postsynaptic densities. It is of interest that filaments of the same diameter as spectrin molecules have been observed adjacent to membranes of postsynaptic densities and that these filaments appeared to associate with intramembrane particles and actin filaments (Gulley & Reese 1981).

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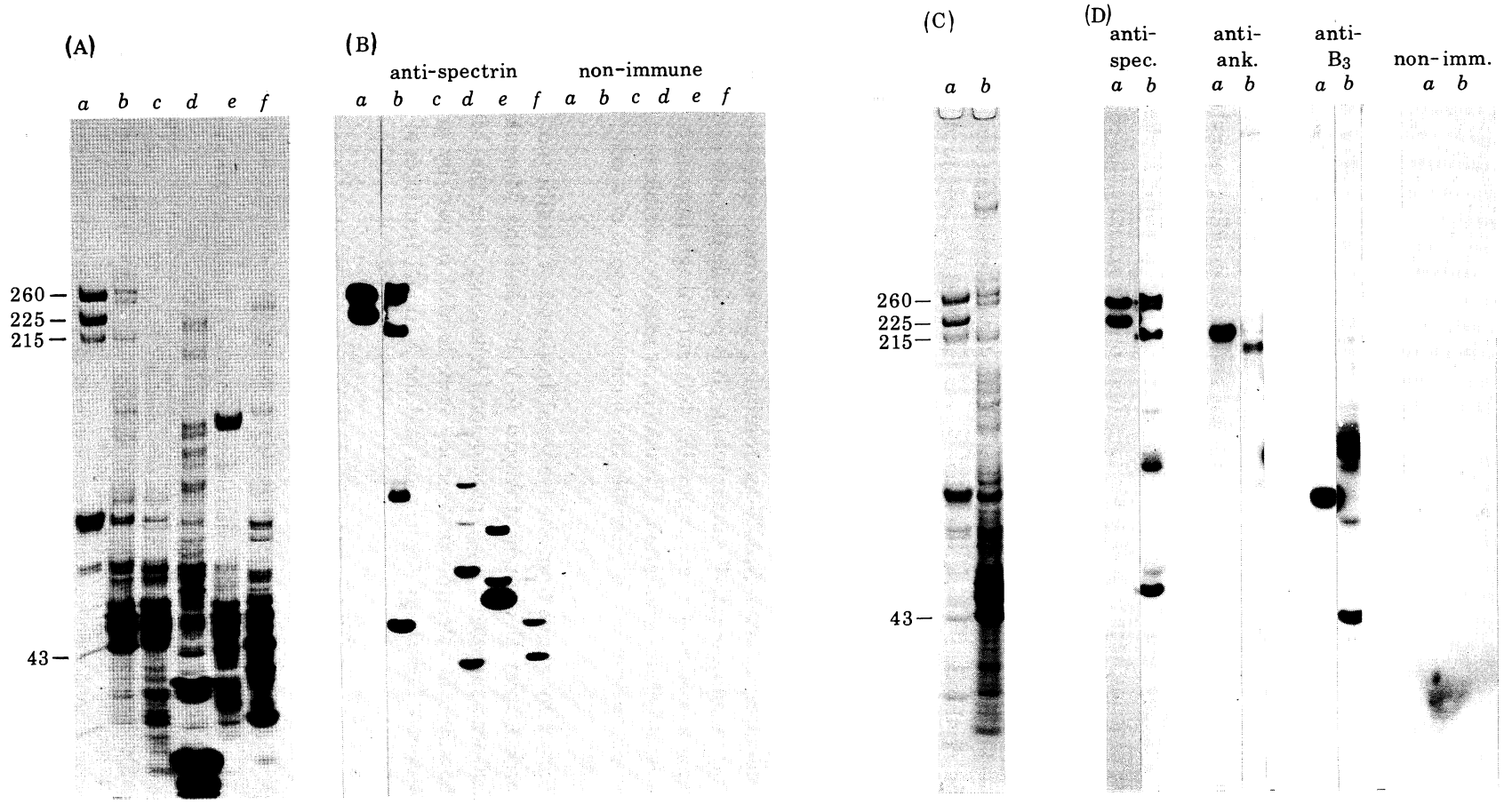

FIGURE 6. Immunoreactive forms of erythrocyte spectrin, ankyrin and band 3 are present in liver plasma membranes. Liver plasma membranes from blanched livers were isolated by two steps of upward flotation through 1.42 M sucrose essentially as described (Toda *et al.* 1975) with modifications in that the buffers contained 7.5 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.5, and protease inhibitors (200 μg ml^{-1} $PhMeSO_2F$ and 0.01 % (by volume) diisopropylfluorophosphate). Mitochondria were isolated as described (Fleischer & Kervina 1974) with the same buffer and protease inhibitors as above. The total microsomal and soluble fractions were isolated by pelleting for 45 min at 200 000 *g*, the 25 000 *g* supernatant from liver homogenized in 0.25 M sucrose, 7.5 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.5, and protease inhibitors. The microsomal pellet was washed once in 7.5 mM sodium phosphate, 1 mM NaEGTA, pH 7.5. Nuclear membranes were prepared essentially by the procedure of Dwyer & Blobel (1976) with the modifications that 0.5 mM Na EGTA and protease inhibitors were included in the buffers, and that the nuclei were digested with RNase as well as DNase. The samples were analysed on SDS–polyacrylamide gels. Polypeptides were visualized by staining with Coomassie blue (A, C), and immunoreactive polypeptides (B, D) identified as in figures 1 and 2. Lanes are (*a*) erythrocyte ghosts; (*b*) plasma membranes; (*c*) microsomal membranes; (*d*) nuclear envelopes; (*e*) mitochondria; (*f*) soluble proteins. Affinity-purified antibody against band 3 was prepared against a 43 kDa proteolytic fragment of this polypeptide (Bennett & Stenbuck 1980*b*). The numbers at the left of the gels stained with Coomassie blue are molecular masses in kilodaltons.

Conclusion

The studies outlined here demonstrate the presence of antigenic determinants shared by erythrocyte membrane proteins and several brain proteins, including a membrane-associated protein very similar to spectrin. These results suggest the existence of two broadly defined families of structural proteins. (1) High molecular mass actin-binding proteins with an extended rod-like structure. These proteins can attach to membranes if a β-type subunit is present, as with spectrins. Some members, such as MAP2 lack a β subunit and are associated with microtubules and actin filaments. Other proteins such as filamin and actin-binding protein from macrophages are associated only with actin filaments. (2) Ankyrin-like proteins that bind to the β subunit of spectrins, to microtubules, and to membrane-associated band 3 molecules. Little is known about these proteins, such as which binding functions are actually shared by the various proteins.

The extent of structural homology as determined by immunoreactivity is on the order of 20 %, which is much less than normally observed with families of proteins. It should be kept in mind that these proteins are unusually large, and that a homologous region can be as large as 60 kDa. Moreover, the erythrocyte proteins probably are the most deviant members of these families, since the functional constraints in these specialized cells are different from those in more complex cell types.

Future work will be focused on determining in detail how proteins in the ankyrin and spectrin families are integrated with each other, membranes and structural protein systems. For example, it is possible to devise several mechanisms for linkage of actin and microtubules to membranes: membrane–spectrin–actin–MAP2–microtubule; membrane–spectrin–MAP1–microtubule–MAP2–actin; membrane–MAP1–microtubule. It is possible that one or more of these linkages occurs in the same region of a cell, or possibly different linkages prevail in specialized regions or at various stages of differentiation. Ultimately, it may be possible to define in molecular terms the associations required to assemble the basic framework of a cell.

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References

Bennett, V. 1979 Immunoreactive forms of human erythrocyte ankyrin are present in diverse cells and tissues. *Nature, Lond.* **281**, 597–599.

Bennett, V. 1982 The molecular basis for membrane–cytoskeleton association in human erythrocytes. *J. cell. Biochem.* **18**, 49–65.

Bennett, V. & Davis, J. 1981 Erythrocyte ankyrin: immunoreactive analogues are associated with mitotic structures in cultured cells and with microtubules in brain. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7550–7554.

Bennett, V., Davis, J. & Fowler, W. E. 1982 Brain spectrin – a membrane-associated protein related in structure and function to erythrocyte spectrin. *Nature, Lond.* (In the press.)

Bennett, V. & Stenbuck, P. 1979*a* Identification and partial purification of ankyrin, the high affinity membrane attachment site for human erythrocyte spectrin. *J. biol. Chem.* **254**, 2533–2541.

Bennett, V. & Stenbuck, P. 1979*b* The membrane attachment protein for spectrin is associated with band 3 in Human erythrocyte membranes. *Nature, Lond.* **280**, 468–473.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Bennett, V. & Stenbuck, P. 1980*a* Human erythrocyte ankyrin. Purification and properties. *J. biol. Chem.* **255**, 2540–2548.

Bennett, V. & Stenbuck, P. 1980*b* Association between ankyrin and the cytoplasmic domain of band 3 isolated from the human erythrocyte membrane. *J. biol. Chem.* **255**, 6424–6432.

Berl, S., Puszkin, S. & Nicklas, W. J. 1973 Actomyosin-like protein in brain. *Science, Wash.* **179**, 441–446.

Bhattacharyya, B. & Wolff, J. 1975 Membrane-bound tubulin in brain and thyroid tissue. *J. biol. Chem.* **250**, 7639–7646.

Branton, D., Cohen, C. M. & Tyler, J. 1981 Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell* **24**, 24–32.

Brenner, S. L. & Korn, E. D. 1979 Spectrin–actin interaction: phosphorylated and dephosphorylated spectrin tetramer cross-link F-actin. *J. biol. Chem.* **254**, 8620–8627.

Calvert, R., Bennett, P. & Gratzer, W. 1980 Properties and structural role of the subunits of human spectrin. *Eur. J. Biochem.* **107**, 355–361.

Davis, J. & Bennett, V. 1982 Microtubule–associated protein 2, a microtubule-associated protein from brain, is immunologically related to the α subunit of erythrocyte spectrin. *J. biol. Chem.* **257**, 5816–5820.

Dwyer, N. & Blobel, G. 1976 A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* **70**, 581–591.

Fairbanks, G., Steck, T. L. & Wallach, D. F. H. 1971 Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry, Wash.* **10**, 2607–2617.

Fleischer, S. & Kervina, M. 1974 Biomembranes, part A. *Methods Enzymol.* **31**, 15.

Fowler, V. & Taylor, D. L. 1980 Spectrin plus band 4.1 cross-link actin: regulation by micromolar calcium. *J. Cell Biol.* **85**, 361–376.

Fowler, W. E. & Erickson, H. P. 1979 Trinodular structure of fibrinogen. Confirmation by both shadowing and negative stain electron microscopy. *J. molec. Biol.* **134**, 241–249.

Goodman, S., Zagon, I. S. & Kulikowski, R. 1981 Identification of a spectrin-like protein in nonerythroid cells. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7570–7574.

Griffith, L. & Pollard, T. 1978 Evidence for actin filament–microtubule interaction mediated by microtubule-associated proteins. *J. Cell Biol.* **78**, 958–965.

Gulley, R. L. & Reese, T. S. 1981 Cytoskeletal organization at the postsynaptic complex. *J. Cell Biol.* **91**, 298–302.

Hargreaves, W., Giedd, K. N., Verkleij, A. & Branton, D. 1980 Reassociation of ankyrin with band 3 in erythrocyte membranes and in lipid vesicles. *J. biol. Chem.* **255**, 11965–11972.

Herzog, W. & Weber, K. 1978 Fractionation of brain microtubule-associated proteins: isolation of two different proteins which stimulate tubulin polymerization *in vitro*. *Eur. J. Biochem.* **92**, 1–8.

Heuser, J. E. & Kirschner, M. W. 1980 Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* **86**, 212–234.

Hiller, G. & Weber, K. 1977 Spectrin is absent in various tissue culture cells. *Nature, Lond.* **266**, 181–183.

Kim, H., Binder, L. J. & Rosenbaum, J. L. 1979 The periodic association of MAP2 with brain microtubules *in vitro*. *J. Cell Biol.* **80**, 266–276.

Levine, J. & Willard, M. 1981 Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* **90**, 631–643.

Luna, E., Kidd, G. & Branton, D. 1979 Identification by peptide analysis of the spectrin-binding protein in human erythrocytes. *J. biol. Chem.* **254**, 2526–2532.

Moskalewski, S., Thyberg, J., Hinek, A. & Friberg, U. 1977 Fine structure of the Golgi complex during mitosis of cartilaginous cells *in vitro*. *Tissue Cell* **9**, 185–196.

Morrow, J. S., Speicher, D. W., Knowles, W. J., Hsu, C. J. & Marchesi, V. T. 1980 Identification of functional domains of human erythrocyte spectrin. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6592–6596.

Murphy, D. B. & Borisy, G. G. 1975 Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly *in vitro*. *Proc. Acad. Natn. Sci. U.S.A.* **72**, 2696–2700.

Runge, M. S., Laue, T. M., Yphantis, D. A., Lifsics, M. R., Saito, A., Altin, M., Reinke, K. & Williams, R. C. 1981 ATP-induced formation of an associated complex between microtubules and neurofilaments. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1431–1435.

Sandoval, I. V. & Weber, K. 1980 Different tubulin polymers are produced by microtubule-associated proteins MAP2 and tau in the presence of guanosine 5′-(α, β methylene) triphosphate. *J. biol. Chem.* **255**, 8952–8954.

Sattilaro, R. F., Dentler, W. L. & LeCluyse, E. L. 1981 Microtubule-associated proteins (MAPs) and the organization of actin filaments *in vitro*. *J. Cell Biol.* **90**, 467–473.

Sheetz, M. P., Painter, R. G. & Singer, S. J. 1976 Relationship of the spectrin complex of human erythrocyte membranes to the actomyosins of muscle cells. *Biochemistry, Wash.* **15**, 4486–4491.

Sherline, P., Lee, Y.-C. & Jacobs, L. 1977 Binding of microtubules to pituitary secretory granules and secretory granule membranes. *J. Cell Biol.* **72**, 380–389.

Shimo-Oka, T. & Watanabe, Y. 1981 Stimulation of actomyosin Mg^{2+}-ATPase activity by brain microtubule-associated protein fraction. High-molecular-weight actin-binding protein is the stimulating factor. *J. Biochem., Tokyo* **90**, 1297–1307.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Shotton, D., Burke, B. E. & Branton, D. 1979 The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopic studies. *J. molec. Biol.* **131**, 303–329.

Sloboda, R. D. & Dickersin, K. 1980 Structure and composition of the cytoskeleton of nucleated erythrocytes. I. The presence of microtubule-associated protein 2 in the marginal band. *J. Cell Biol.* **87**, 170–179.

Speicher, D. W., Morrow, J. S., Knowles, W. J. & Marchesi, V. T. 1980 Identification of proteolytically resistant domains of human erythrocyte spectrin. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5673–5677.

Stephens, R. E. 1977 Major protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry, Wash.* **16**, 2047–2058.

Toda, G., Oka, H., Oka, T. & Ikeda, Y. 1975 Subfractionation of rat liver plasma membrane: uneven distribution of plasma membrane-bound enzymes on the liver cell surface. *Biochim. Biophys. Acta* **413**, 52–64.

Towbin, H., Staehelin, T. & Gordon, J. 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4355.

Tyler, J. M., Anderson, J. M. & Branton, D. 1980 Structural comparison of several actin-binding macromolecules. *J. Cell Biol.* **85**, 489–495.

Tyler, J. M., Hargreaves, W. & Branton, D. 1979 Purification of two spectrin-binding proteins: biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5192–5196.

Ungewickell, E., Bennett, P. M., Calvert, R., Ohanian, V. & Gratzer, W. B. 1979 *In vitro* formation of a complex between cytoskeletal proteins of the human erythrocyte. *Nature, Lond.* **280**, 811–816.

Ungewickell, E. & Gratzer, W. 1978 Self-association of human spectrin: a thermodynamic and kinetic study. *Eur. J. Biochem.* **88**, 379–385.

Van Deurs, B. & Behnke, O. 1973 The microtubule marginal band of mammalian red blood cells. *Z. Anat. EntwGesch.* **143**, 43–47.

Voter, W. A. & Erickson, H. P. 1981 Visualization of microtubule associated protein (MAP2) by electron microscopy. *J. Cell Biol.* **91**, 331*a*.

Yu, J. & Goodman, S. 1979 Syndeins: the spectrin-binding proteins of the human erythrocyte membrane. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2340–2344.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

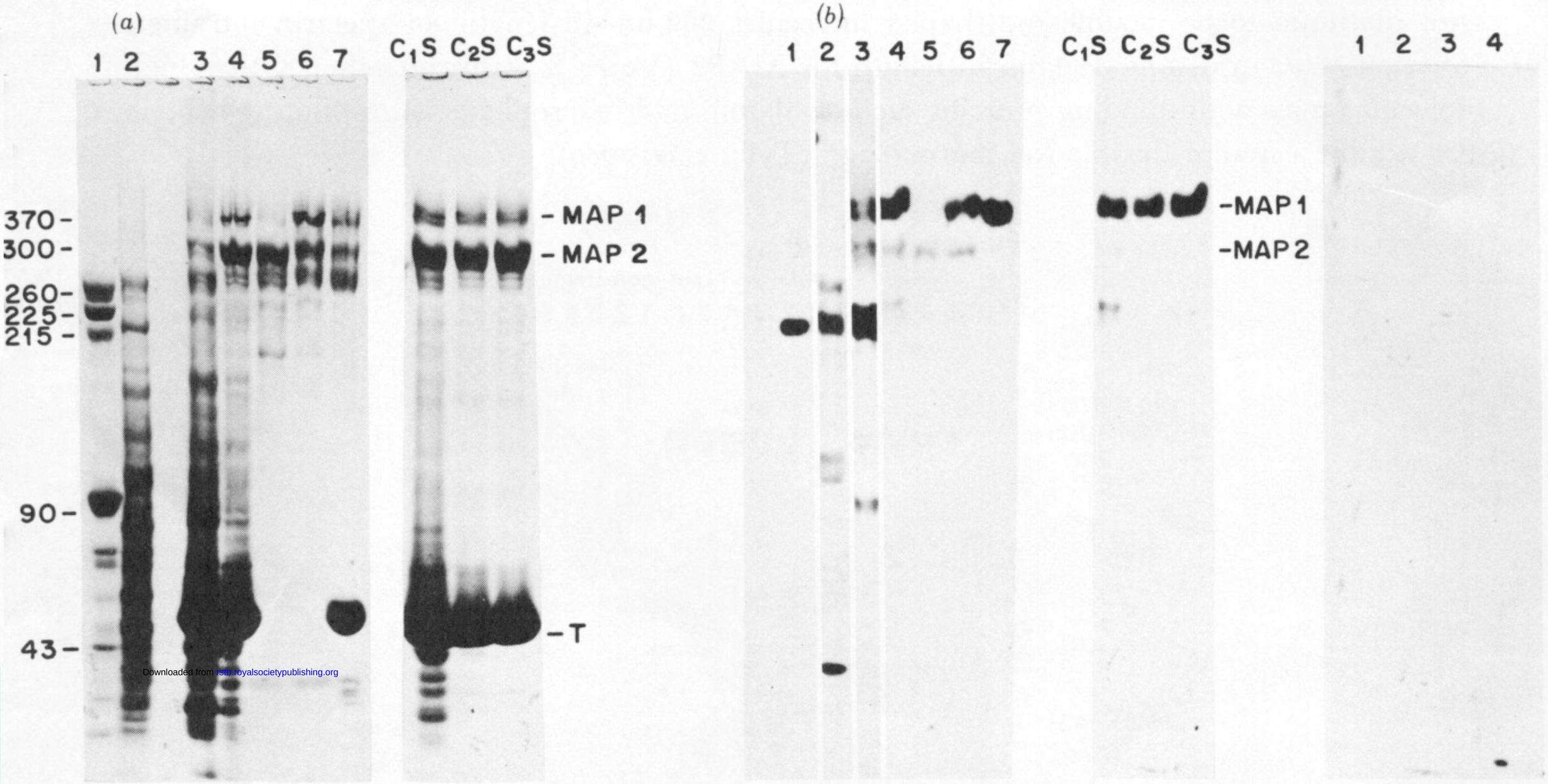

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FIGURE 1. Identification of immunoreactive ankyrin polypeptides in SDS–polyacrylamide gels with affinity-purified anti-ankyrin antibody. Lanes: 1, pig erythrocyte ghosts; 2, HeLa cells; 3, pig brain extract, 4, microtubule pellet from pig brain; 5 and 6, MAP fractions; 7, MAP fraction 6 co-polymerized with pure tubulin; and C_1S-C_3S, microtubule pellets after repeated cycles of assembly and disassembly. Proteins were electrophoresed on a 3.5–17 % exponential polyacrylamide gel (Fairbanks *et al.* 1971) and either stained with Coomassie blue (*a*) or electrophoretically transferred to nitrocellulose (Towbin *et al.* 1979). Immunoreactive polypeptides were identified by overlaying the nitrocellulose with 1 μg ml^{-1} anti-ankyrin antibody or preimmune antibody and then ^{125}I-labelled protein A. An autoradiogram was then prepared. Pig brain cerebral cortex was homogenized (1 g ml^{-1}) in a buffer containing 0.32 M sucrose, 50 mM 1,4-piperazine-diethanesulphonic acid (PIPES), 1 mM Na ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5 mM dithiothreitol, 200 μg ml^{-1} $PhMeSO_2F$, pH 7.0, and centrifuged for 30 min at 200 000 *g*. The supernatant was adjusted to 3 M glycerol, 0.1 mM GTP, 0.5 mM $MgCl_2$ and 1 mM ATP, incubated for 45 min at 37 °C, and recentrifuged. The pellets were depolymerized at 0 °C in 10 mM Pipes, 1 mM Na EGTA, 0.2 mM dithiothreitol, pH 6.4, and centrifuged. MAP proteins were isolated from the supernatant (lane 4) by phosphocellulose chromatography (0.4 M NaCl step gradient in resuspension buffer) and resolved into two fractions (lanes 5 and 6) by gel filtration on Sepharose (C1)4B in 1 M NaCl, 10 mM sodium phosphate, 1 mM Na EGTA, 1 mM NaN_3 and 0.2 mM dithiothreitol, pH 7.4. Sample as in lane 6 was incubated with pure tubulin (figure 3) under polymerizing conditions, layered over a sucrose barrier gradient (200 g l^{-1}) and centrifuged for 20 min at 200 000 *g*. The pellet, containing microtubules and associated proteins, was dissolved in SDS (lane 7). Microtubules were subjected to three cycles of assembly and dissasembly; the supernatants of cold-depolymerized and centrifuged pellets are in lanes C_1S, C_2S, and C_3S, corresponding to one, two and three cycles, respectively. Molecular masses (numbers at the left in kilodaltons) are based on known mobilities of erythrocyte proteins; extrapolation from these values gave estimates of molecular masses of MAP1 and MAP2. (From Bennett & Davis 1981).

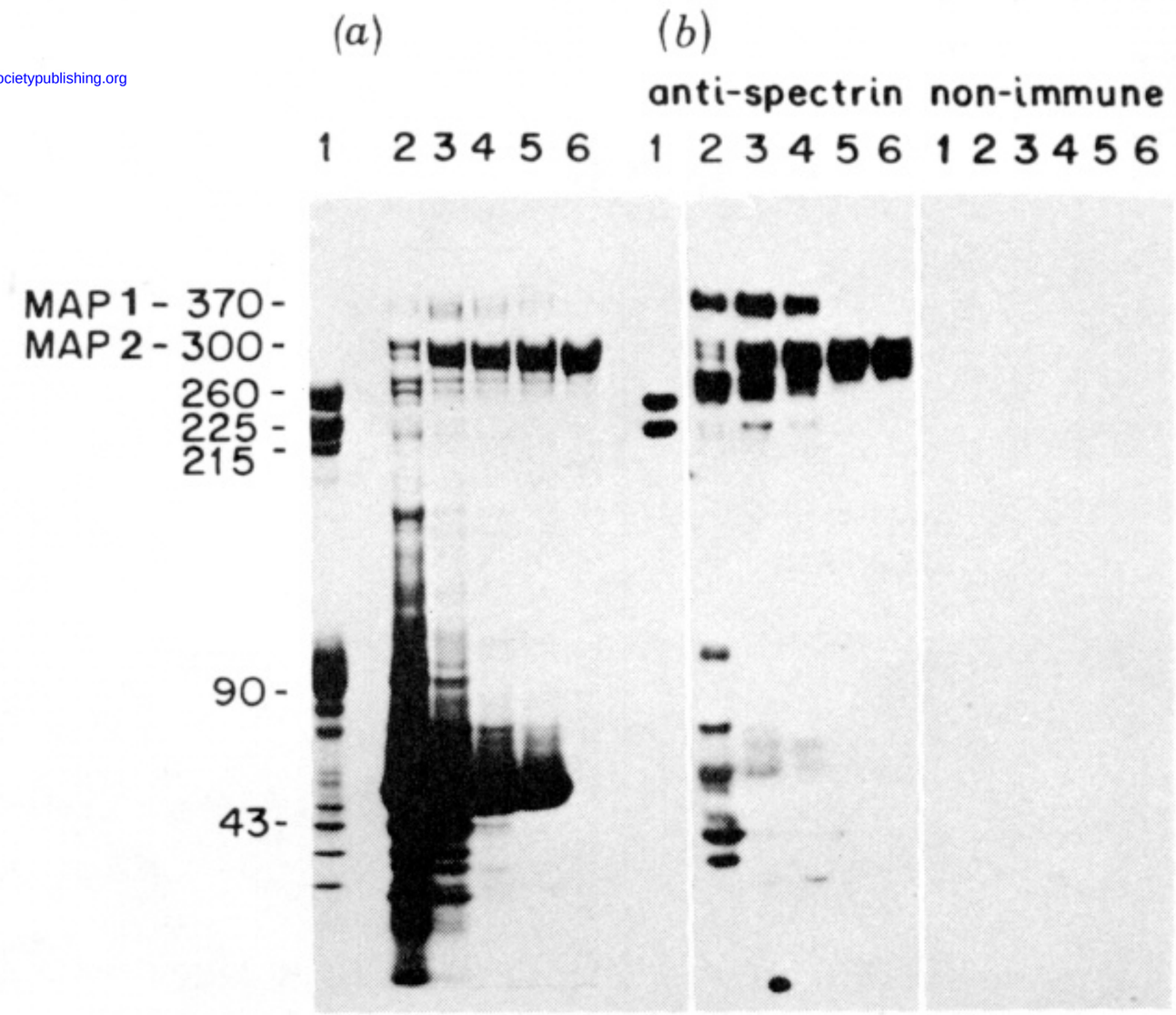
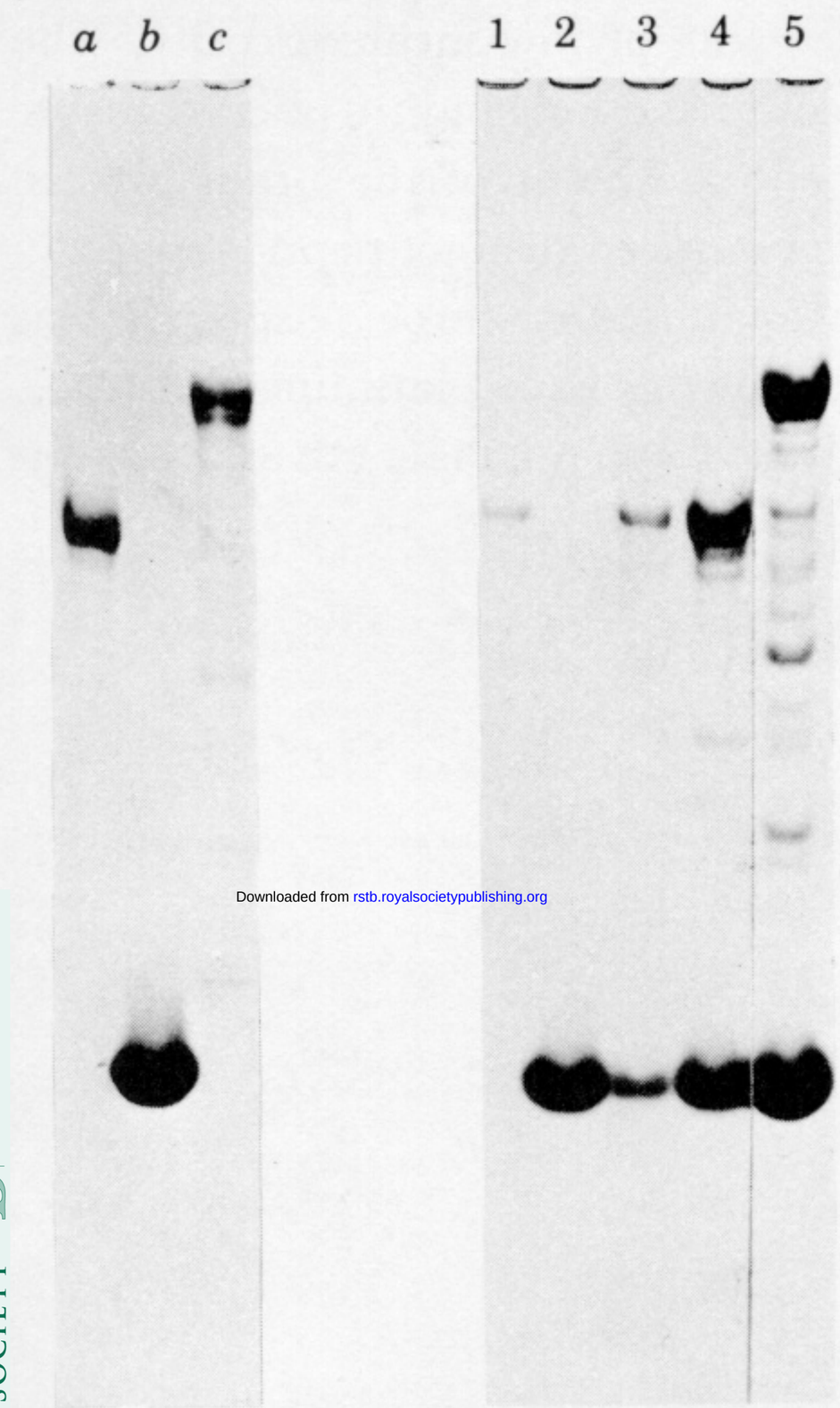


FIGURE 2. Identification of MAP2 from pig brain as an immunoreactive form of erythrocyte spectrin with affinity-purified anti-spectrin antibody. Erythrocyte ghosts (lane 1), pig brain extract (lane 2), microtubules isolated by repeated cycles of assembly and disassembly: C_1S (lane 3), C_2S (lane 4), C_3S (lane 5) and purified MAP2 (lane 6) were electrophoresed on SDS-polyacrylamide gels. Polypeptides were visualized by staining with Coomassie blue (*a*), and immunoreactive polypeptides were identified by electrophoretic transfer from polyacrylamide gels to nitrocellulose paper, followed by overlaying the nitrocellulose with antibody and then ^{125}I-labelled protein A (*b*) (figure 1). Microtubule proteins were prepared as described (figure 1). MAP2 was purified by the method of Kim *et al.* (1979) with the modifications of Sandoval & Weber (1980). Numbers at the left are molecular masses in kilodaltons. (From Davis & Bennett (1982).)



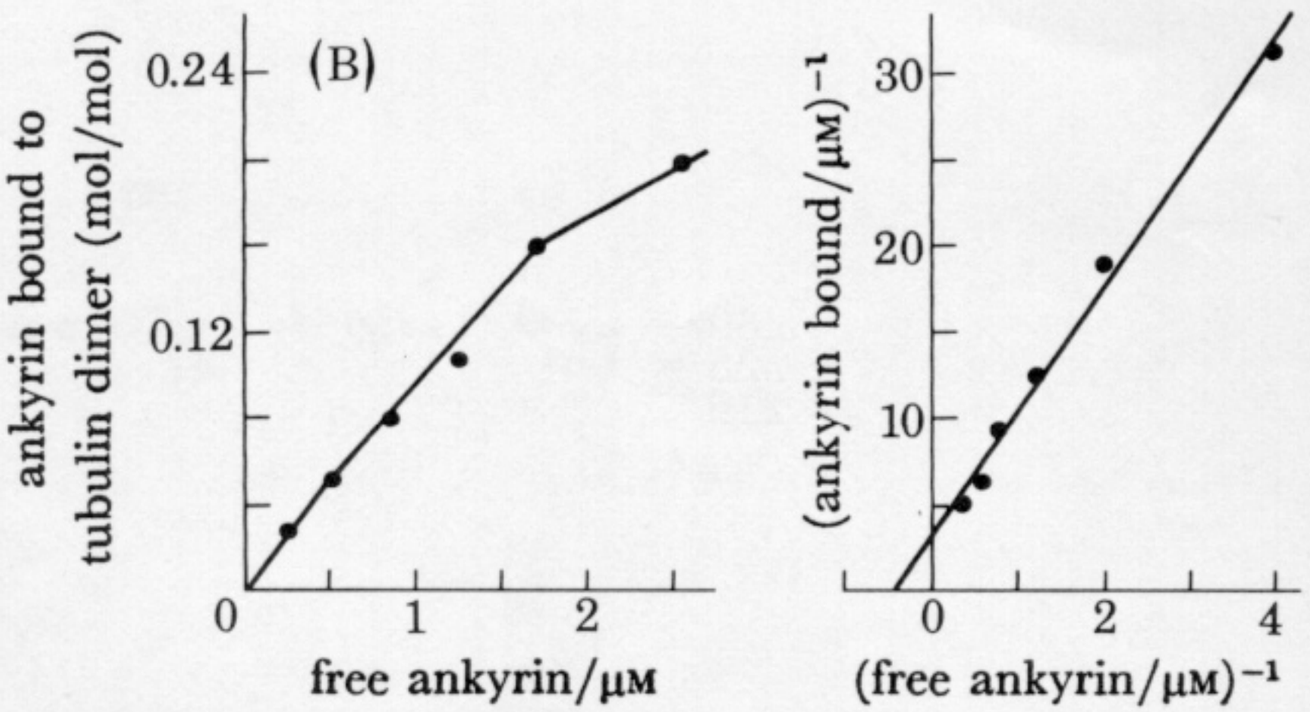


FIGURE 3. Association of erythrocyte ankyrin with brain tubulin.

(A) Brain microtubules (figure 1) after two cycles of assembly and disassembly were depolymerized and applied to DE-53 cellulose in a buffer containing 50 mM PIPES, 1 mM NaEGTA, 0.1 mM $MgCl_2$, 0.1 mM GTP and 0.1 mM dithiothreitol, pH 7.0. The column was washed with 10 volumes of buffer, and a fraction enriched in MAPS was eluted with 0.1 M KCl; the column was washed with 10 volumes of 0.2 M KCl, and tubulin was eluted with 0.4 M KCl. Erythrocyte ankyrin and purified tubulin dimer (dialysed against assay buffer) were centrifuged for 20 min at 200 000 *g* and then combined at 2 μM and 10 μM, respectively, in 5 % sucrose, 100 mM PIPES, 1 mM NaEGTA, 1 mM GTP, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol. After 30 min at 37 °C, the solutions were layered over 20 % sucrose in assay buffer, and microtubules were collected by centrifugation for 20 min at 200 000 *g* at 25 °C. The pellets were analysed on an SDS–polyacrylamide gel. Control lanes: (*a*) erythrocyte ankyrin; (*b*) pure tubulin; (*c*) MAP fraction. Pellet lanes: 1, ankyrin alone; 2, tubulin alone; 3, ankyrin and tubulin chilled to 0 °C after polymerization; 4, ankyrin and tubulin; 5, ankyrin and tubulin plus MAP fraction (400 μg ml^{-1}).

(B) Various concentrations of ankyrin were incubated alone or with 10 μM tubulin for 30 min at 37 °C, and microtubules were collected and analysed on an SDS–polyacrylamide gel. The relative peak areas were estimated by densitometry. The data are corrected for the amount of ankyrin sedimented in the absence of tubulin, which was less than 5 % of the value in the presence of tubulin. (From Bennett & Davis (1981).)

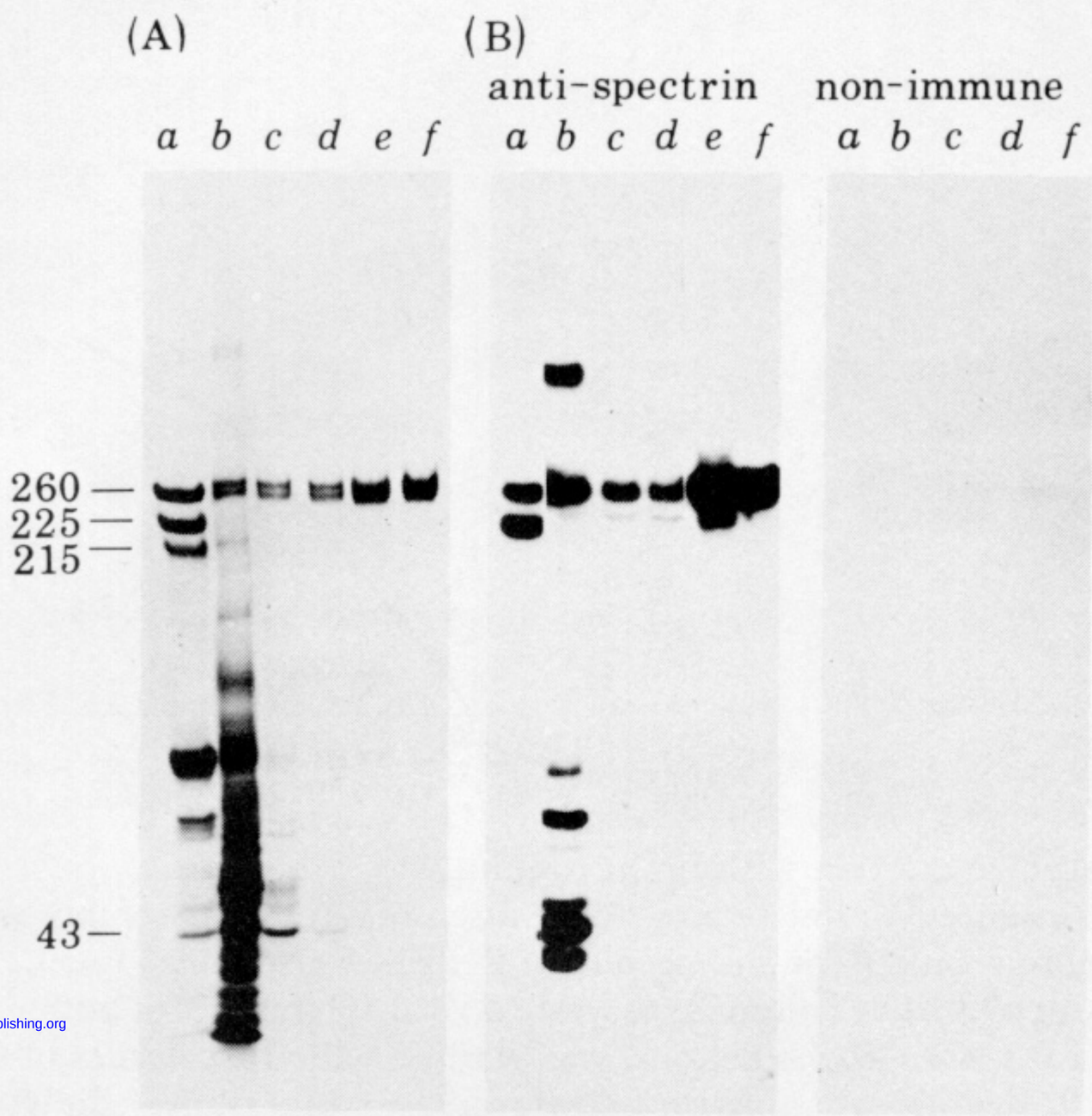
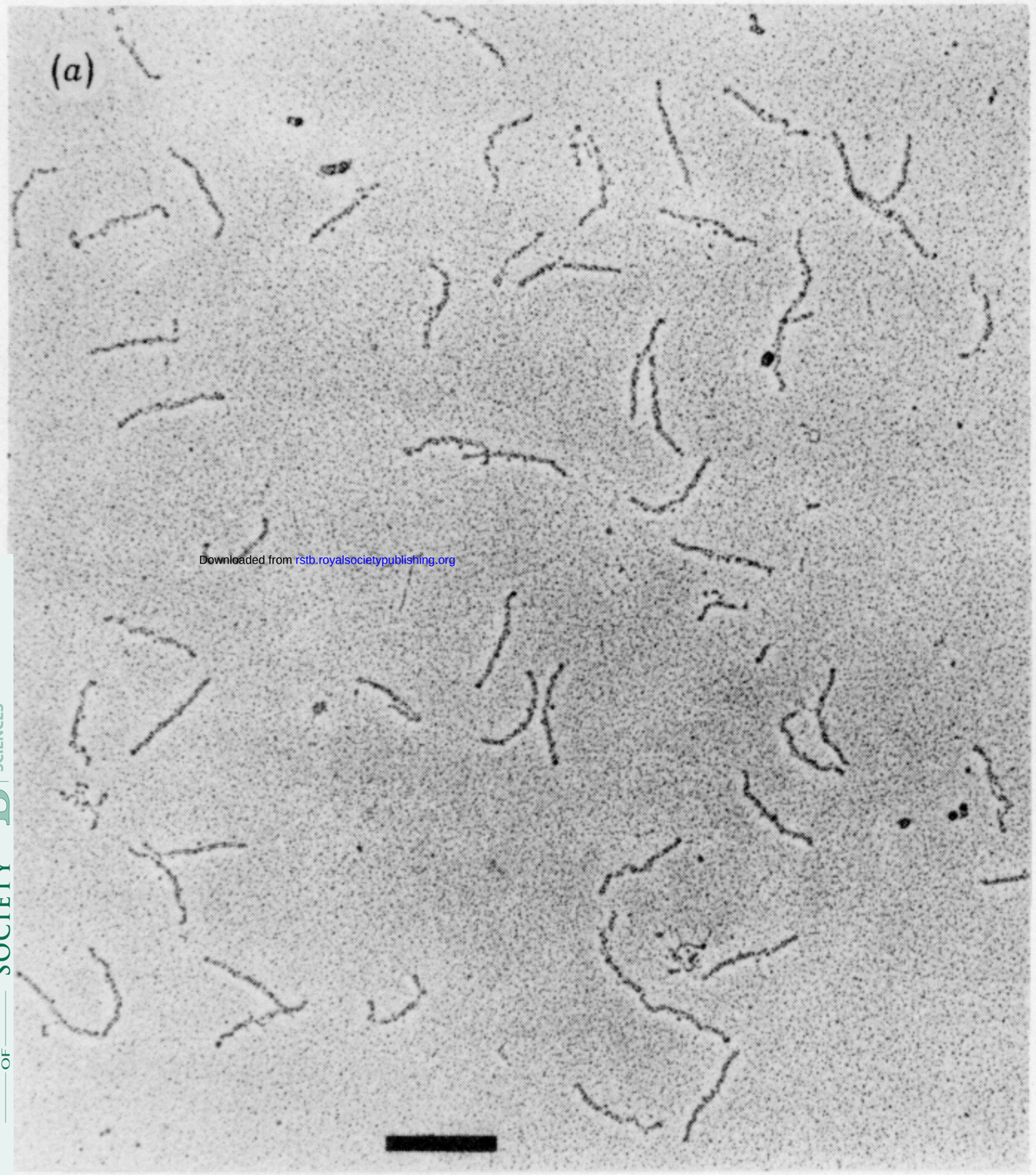
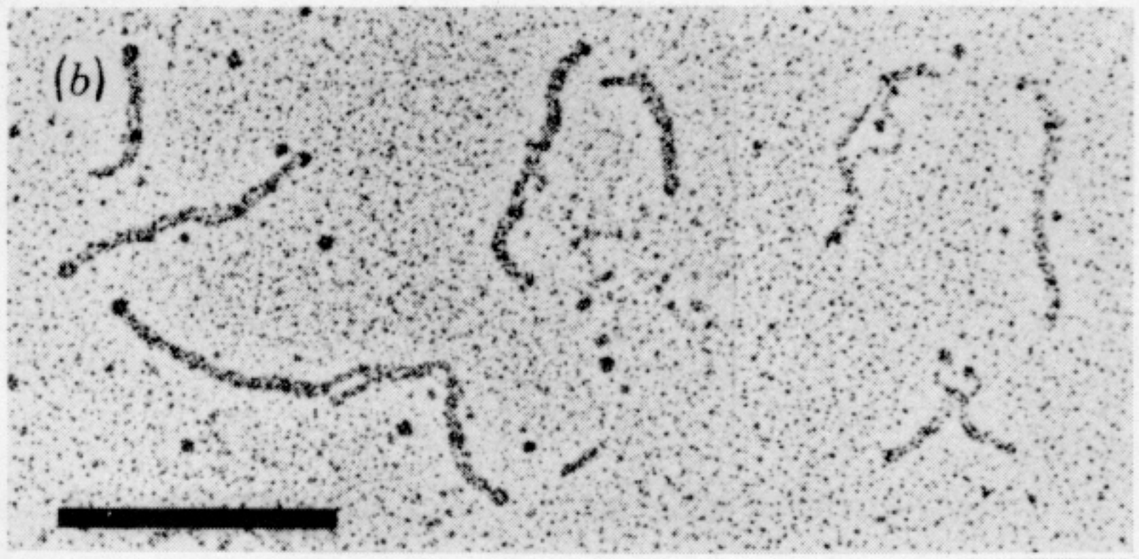


FIGURE 4. Purification from brain membranes of a protein that shares antigenic determinants with erythrocyte spectrin. 125 g frozen grey matter from pig brain was homogenized in 800 ml 0.32 M sucrose, 2 mM NaEGTA, 200 μg ml^{-1} $PhMeSO_2F$, 0.015 % (by volume) diisopropylfluorophosphate, 3 μg ml^{-1} pepstatin A, pH 7.0. The 900 g supernatant of this suspension was pelleted for 25 min at 30 000 *g* to collect the crude membrane fraction. This fraction was depleted of myelin by centrifugation for 40 min at 30 000 *g* in 0.8 M sucrose, which causes the myelin to float while other membranes are pelleted. The myelin-depleted membranes were washed successively by centrifugation for 25 min at 30 000 *g* with homogenization buffer, followed by 10 mM sodium phosphate, 2 mM NaEGTA, 50 μg ml^{-1} $PhMeSO_2F$, pH 7, and finally 2 mM NaEGTA, 50 μg ml^{-1} $PhMeSO_2F$, pH 7. The washed membranes (1.6 g protein) were extracted for 60 min at 37 °C in 800 ml 0.2 mM NaEGTA, 0.5 mM dithiothreitol, 50 μg ml^{-1} $PhMeSO_2F$, pH 7. The suspension was pelleted for 30 min at 200 000 *g* and the supernatant (120 mg extracted protein) was adjusted to 45 % saturation with solid ammonium sulphate. The precipitated proteins were dialysed overnight against 1 M urea, 20 mM glycine, 2 mM NaEGTA, 1 mM NaN_3, 0.2 mM dithiothreitol, and centrifuged for 3 h at 200 000 *g*. The supernatant was applied to a Sephacryl S400 gel filtration column equilibrated with urea buffer. Fractions containing the 260 kDa polypeptides eluted near the void volume. The protein was precipitated with ammonium sulphate, dialysed extensively against 0.2 mM NaEGTA, 0.5 mM NaN_3, 0.2 mM dithiothreitol, pH 7, and centrifuged for 90 min at 200 000 *g*. The supernatant was fractionated on linear sucrose gradients (100–300 g l^{-1}) in dialysis buffer with a Beckman VTi50 rotor (14 h at 36 000 rev. min^{-1}). The gradient fractions with the purest protein (at about a sedimentation rate of 11*S*) contained 1.2 mg of protein. An additional 2 mg of less pure protein was recovered from adjacent fractions. The fractions at various stages of purification were analysed by SDS-polyacrylamide gel electrophoresis. Polypeptides were visualized by staining with Coomassie blue (A) and polypeptides cross-reacting with spectrin (B) were identified as in figure 2. Samples are: (*a*) pig erythrocyte ghosts; (*b*) pig brain membranes; (*c*) low ionic strength extract of brain membranes; (*d*) soluble protein recovered from ammonium sulphate precipitation of sample (*c*); (*e*) pooled fractions from gel filtration; (*f*) pure protein from sucrose gradients. The numbers at the left are molecular masses in kilodaltons.





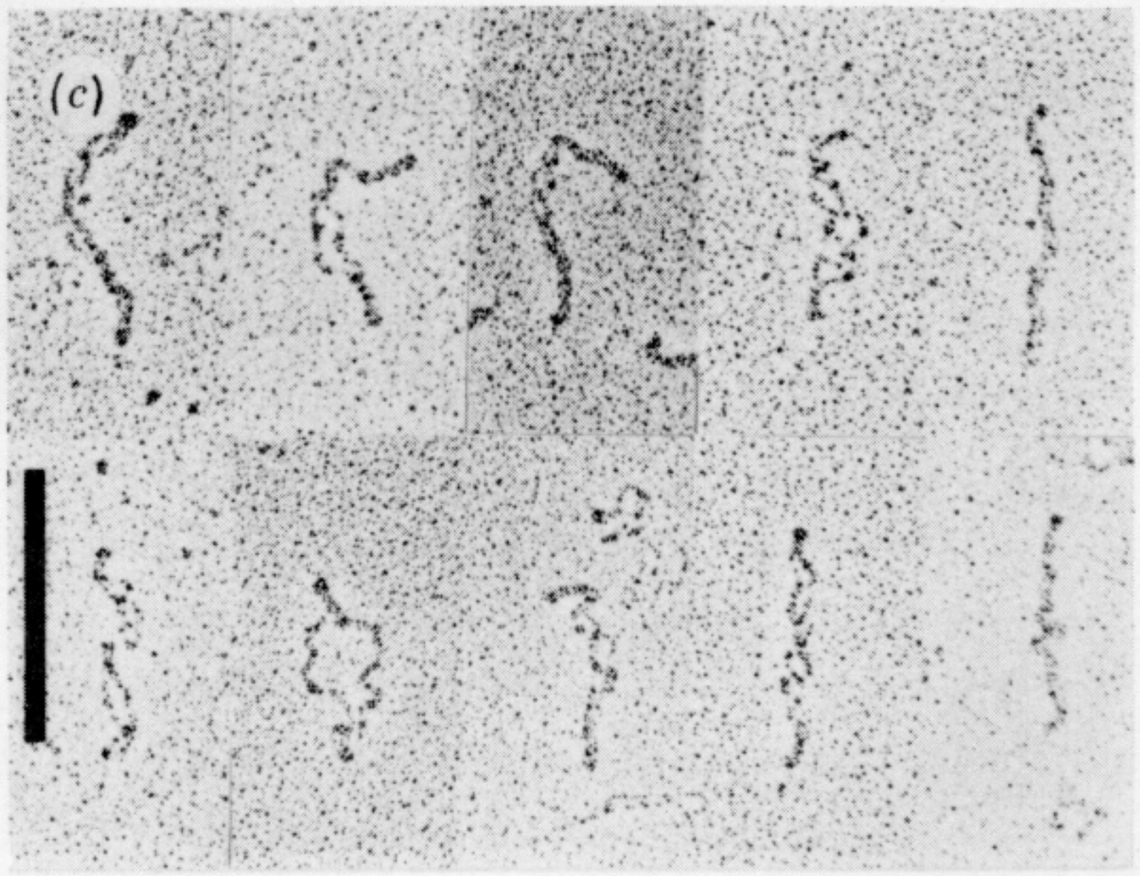


FIGURE 5. Electron micrographs of rotary-shadowed brain spectrin ((*a*), (*b*) and top row of (*c*)) and erythrocyte spectrin (bottom row of (*c*)).

(*a*) Representative field of purified brain spectrin molecules that were diluted to 10 μg ml^{-1} in 100 mM ammonium formate (pH 7), mixed with one-third volume of glycerol, sprayed onto mica, dried *in vacuo* at room temperature and rotary-shadowed with platinum at an angle of 1 in 10. Bar = 200 nm. (Fowler & Erickson (1979); Shotton *et al.* (1979).)

(*b*) Brain spectrin molecules from a different, less pure preparation, treated with 1 M NaBr for several days before dialysis against 100 mM ammonium formate and rotary-shadowing as described in (*a*). The field on the left includes an 'octomer' and two 'dimers', as well as two 'tetramers'. The two-stranded character of the molecules is seen more clearly in the field on the right and in (*c*). Bar = 200 nm.

(*c*) Selected brain spectrin molecules are shown in the top row and erythrocyte spectrin tetramers in the bottom row. These molecules were selected to demonstrate structural similarities between the two spectrin species, such as their two-stranded, double-helical nature. Bar = 200 nm.

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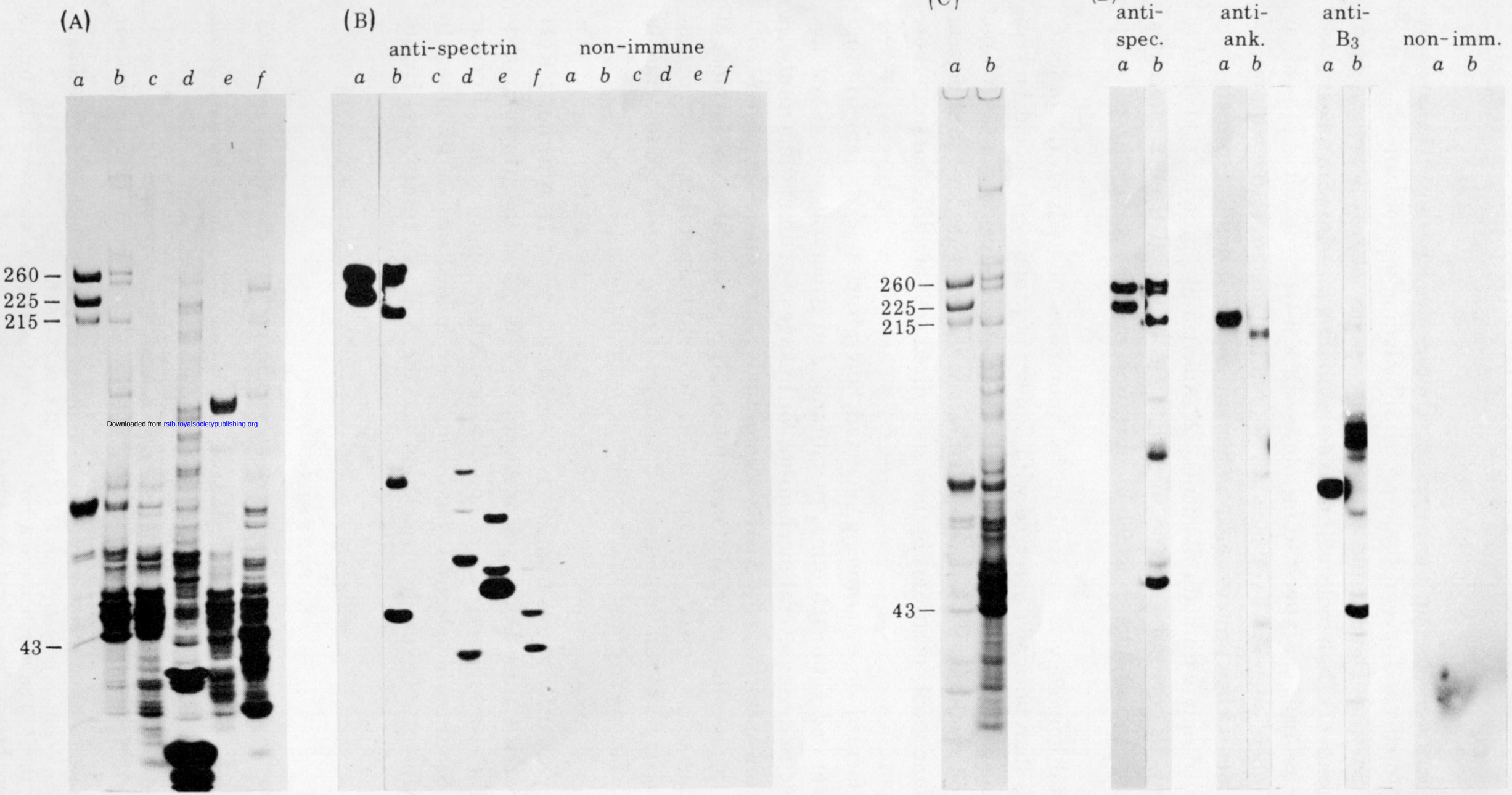

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FIGURE 6. Immunoreactive forms of erythrocyte spectrin, ankyrin and band 3 are present in liver plasma membranes. Liver plasma membranes from blanched livers were isolated by two steps of upward flotation through 1.42 M sucrose essentially as described (Toda *et al.* 1975) with modifications in that the buffers contained 7.5 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.5, and protease inhibitors (200 μg ml^{-1} $PhMeSO_2F$ and 0.01 % (by volume) diisopropylfluorophosphate). Mitochondria were isolated as described (Fleischer & Kervina 1974) with the same buffer and protease inhibitors as above. The total microsomal and soluble fractions were isolated by pelleting for 45 min at 200 000 *g*, the 25 000 *g* supernatant from liver homogenized in 0.25 M sucrose, 7.5 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.5, and protease inhibitors. The microsomal pellet was washed once in 7.5 mM sodium phosphate, 1 mM NaEGTA, pH 7.5. Nuclear membranes were prepared essentially by the procedure of Dwyer & Blobel (1976) with the modifications that 0.5 mM Na EGTA and protease inhibitors were included in the buffers, and that the nuclei were digested with RNase as well as DNase. The samples were analysed on SDS–polyacrylamide gels. Polypeptides were visualized by staining with Coomassie blue (A, C), and immunoreactive polypeptides (B, D) identified as in figures 1 and 2. Lanes are (*a*) erythrocyte ghosts; (*b*) plasma membranes; (*c*) microsomal membranes; (*d*) nuclear envelopes; (*e*) mitochondria; (*f*) soluble proteins. Affinity-purified antibody against band 3 was prepared against a 43 kDa proteolytic fragment of this polypeptide (Bennett & Stenbuck 1980*b*). The numbers at the left of the gels stained with Coomassie blue are molecular masses in kilodaltons.