*Biochimica et Biophysica Acta*, 941 (1988) 141–149
Elsevier



BBA 74014

# Electron spin resonance and biochemical studies of the interaction of the polyamine, spermine, with the skeletal network of proteins in human erythrocyte membranes

Joseph W. Wyse and D. Allan Butterfield

*Department of Chemistry and the Center of Membrane Sciences, University of Kentucky, Lexington, KY (U.S.A.)*

(Received 14 December 1987)

Key words: Spermine; Spectrin; Skeletal protein; ESR; Erythrocyte membrane; Spin labeling; (Human)

**Spermine (*N*,*N*′-bis(aminopropyl)-1,4-butanediamine) is a polyamine thought to be important in several cell regulatory processes. Previous studies had shown that spermine prevented the lateral diffusion of transmembrane proteins in human erythrocyte ghosts (Schindler et al. (1980) Proc. Natl. Acad. Sci. USA 77, 1457–1461). In this paper, we present results of studies on the effect of spermine on erythrocyte membranes by employing electron spin resonance spin-labeling techniques in conjunction with spin labels specific for skeletal proteins, bilayer lipids or cell-surface sialic acid of the membrane and by employing SDS-polyacrylamide gel electrophoresis analysis of extracted spectrin and Triton shells. The major findings are: (1) spermine significantly decreases the segmental motion of protein spin-label binding sites ($P < 0.0001$), which are predominantly on cytoskeletal proteins; (2) addition of spermine leads to a significant increase in the rotational motion of spin-labeled terminal sialic acid residues ($P < 0.001$), most of which are located on glycophorin A, a result which may be secondarily caused by spermine-induced aggregation of cytoskeletal proteins and the cytoplasmic pole of this transmembrane sialoglycoprotein; (3) spermine completely inhibits the low-ionic strength extraction of spectrin, the major protein of the skeletal network which is attached to the bilayer proteins by two or more connecting proteins; (4) pretreatment of ghosts with spermine followed by Triton extraction resulted in the retention of significantly increased amounts of Band 3 and other skeletal and bilayer proteins including Bands 4.2, 6 and 7 in Triton X-100 shells relative to that of control-treated ghosts. These results suggest that spermine acts both to increase protein–protein interactions in the cytoskeletal protein network and to bridge skeletal and bilayer proteins and are discussed with reference to possible molecular mechanisms by which spermine may influence cell functions.**

## Introduction

Polyamines such as spermine (*N*,*N*′-bis(aminopropyl)-1,4-butanediamine) are thought to be important in several cell and membrane phenomena such as cell replication, growth and differentiation, although the molecular mechanisms by which these positively charged molecules exert their influence are unknown [1,2]. Consistent with the widespread importance of polyamines in cell and membrane physiology, recent evidence suggests

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, *N*-α-*p*-tosyl-L-lysyl chloromethyl ketone; MAL-6, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl; 5-NS, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; [$^{15}$N]Tempamine-$d_{17}$, 2,2,6,6-[$^{2}H_{12}$]tetramethyl-4-amino[$^{2}H_5$,$^{15}$N]piperidine-1-oxyl, 5P8, 5 mM sodium phosphate (pH 8.0).

Correspondence: D.A. Butterfield, Departments of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0055, U.S.A.

that spermine: (a) may act as an intracellular messenger to mediate rapid, $Ca^{2+}$-dependent alterations in membrane permeability to $Ca^{2+}$, transport of amino acids and hexoses, and endocytotic shape changes in kidney proximal tubules upon stimulation by testosterone or isoproterenol [3,4]; (b) is essential for the breakdown of the blood-brain barrier in brain edema following cryogenic injury [5]; (c) is oxidized by polyamine oxidase to aminoaldehydes which mediate intraerythrocytic death of *Plasmodium falciparum* [6,7]; (d) may inhibit iron uptake in rabbit reticulocytes by affecting the endocytic cycle of the transferrin receptor [8]; (e) binds to lipids leading to decreased membrane fluidity in bean leaves [9], increased order of erythrocyte membranes [10] and increased aggregation of phosphatidylserine vesicles [11]; (f) is elevated in erythrocytes in some types of cancer [12–14]; (g) stimulates $\beta$-hemoglobin chain synthesis to a greater extent than that of the $\alpha$-chain, resulting in a decreased synthetic $\alpha/\beta$ ratio [15]; (h) administration leads to increased production of antibody-forming spleen cells and increased polyamine oxidase activity in mice antigenically stimulated with sheep red blood cells and in mice with tumors [16].

Spermine has also been demonstrated to inhibit the lateral mobility of transmembrane glycoproteins [17]. The translational mobility of the major transmembrane proteins of the erythrocytes, Band 3 and glycophorin A, was completely halted by the addition of approx. 1 mM spermine to membranes but mobility was unaffected by spermine addition to intact cells [17]. In addition, the membrane shear modulus in intact cells and membranes was increased by spermine [18]. These results were interpreted as evidence that spermine interacted with skeletal proteins, thereby decreasing the ability of transmembrane glycoproteins to diffuse in the plane of the bilayer and decreasing membrane deformability by stabilization of the skeleton.

In a previous publication from our laboratory [10], an initial study of the influence of spermine on the physical state of membrane proteins, lipids and cell-surface sialic acid residues was presented. Additional spin-labeling investigations and SDS-PAGE analysis given in the present report provide further support for the idea that spermine interacts with skeletal proteins in human erythrocyte membranes resulting in both increased skeletal–skeletal and skeletal–bilayer protein interactions [10]. In particular, it is suggested that spermine bridges skeletal proteins to the major transmembrane protein, Band 3, as well as to other proteins.

## Materials and Methods

Spermine, phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), *N*-$\alpha$-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and 2-mercaptoethanol were obtained from Sigma. All electrophoresis reagents were electrophoresis grade from Bio-Rad, United States Biochemicals Corporation, or Eastman Chemicals. The spin labels,, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) and 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-NS), were obtained from Aldrich, while the perdeuterated $^{15}$N-spin label, 2,2,6,6-[$^{2}H_{12}$]tetramethyl-4-amino[$^{2}H_{5}$,$^{15}$N]piperdine-1-oxyl ([$^{15}$N]Tempamine-$d_{17}$), was obtained from Merck Stable Isotopes.

Blood was obtained from healthy human volunteers by venipuncture into heparinized tubes, immediately placed on ice and processed within 30 min of collection. Intact cells were isolated by centrifugation at 4°C at $600 \times g$ and subsequent resuspensions and washings in phosphate-buffered saline (150 mM NaCl and 5 mM sodium phosphate, pH 8.0). The erythrocytes were washed three times with phosphate-buffered saline and the buffy coat was carefully removed. Erythrocyte ghost membranes were obtained by hypotonic lysis with 5P8 (5 mM sodium phosphate, pH 8.0) employing 1 vol. of cells to 20 vol. of 5P8, and subsequent centrifugation at 4°C and $27\,000 \times g$. The ghosts were resuspended in ice-cold 5P8 and this process was continued until the membranes were free of residual hemoglobin. Protein content was estimated by the method of Lowry et al. [19].

Erythrocyte membranes were spin labeled with MAL-6 [20–22], 5-NS [20], or Tempamine [23,24] as previously described in publications from our laboratory. ESR spectra were recorded on a Varian E-109 ESR spectrometer equipped with computer acquisition and analysis capabilities. Spectrometer parameters are given in the legends of Figs. 1 and 2.

Spermine solutions were prepared in 5P8 at known concentrations of 0.1–9.0 mM. The pH of spermine solutions of 1.2–9.0 mM was adjusted to 8.0 by adding a known volume, $V_o$, of 1 M HCl. The error introduced in the concentration of spermine was typically less than 1%. A control solution was prepared by adding the volume $V_o$ of a 0.5 M NaCl solution to a volume of 5P8 equivalent to the volume of spermine solution prior to HCl addition. The resulting concentration of NaCl in the control solution is typically 8–10 mM. Serial dilution of a 1.2 mM spermine solution (pH 8.0) was used to prepare low-concentration spermine solutions in 5P8 in the concentration range 0.1–1.0 mM. The pH remained at 8.0 in the latter cases.

4 vol. of ghosts in 5P8 kept at 4°C were allowed to equilibrate at room temperature for 15 min. 1 vol. of spermine solution (final spermine concentration 0.02–1.8 mM) or of the control solution, previously warmed to room temperature, was added to the 4 vol. of spin-labeled ghosts for 30 min prior to ESR studies, spectrin removal, or Triton X-100 extraction.

In order to remove spectrin from the membrane, ghosts were washed once in spectrin-removal buffer (SR buffer: 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK and 0.1 mM TPCK and 0.1 mM 2-mercaptoethanol, pH 7.6) by centrifugation at $27\,000 \times g$ at 4°C. The supernatant was aspirated and the ghosts were resuspended to twice their original volume in ice-cold SR buffer. The extraction proceeded for approx. 40 h at 4°C after which the residual membrane pellet was then packed by centrifugation for 1 h at $27\,000 \times g$ and 4°C. The supernatant was carefully removed in order to avoid contamination by any protein in the pellet and stored at 4°C for protein and polyacrylamide gel electrophoresis (PAGE) analysis if they were to be performed on the same day. Otherwise, all samples were frozen in liquid nitrogen and stored at −10°C until used. PAGE was performed according to the method of Laemmli [25] employing an 11 cm resolution gel of 10% acrylamide and a stacking gel of 3.5% acrylamide. Electrophoresis was carried out at room temperature with 2.5 mA per gel tube. Gels were stained in 0.5% Coomassie blue and scanned at 580 nm with an ISCO gel spectrophotometric gel scanner.

The erythrocyte skeletal network of proteins and a small amount of some integral proteins ('Triton shells') can be extracted from ghosts as a unit employing the non-ionic detergent Triton X-100 [26]. 'Triton shells' were extracted from 1 vol. of ghosts pretreated with spermine or control by incubation with 5 vol. 0.5% Triton at 0°C for 20 min and centrifuged at $27\,000 \times g$ for 30 min at 4°C. The supernatant was carefully removed leaving a gelatinous pellet consisting of skeletal proteins and attached transmembrane proteins as described by others [26]. The supernatant was stored at 4°C for protein and PAGE analysis if they were performed the same day, or frozen in liquid nitrogen and stored at −10°C until used. PAGE was performed according to method of Laemmli [25] employing a 4% stacking gel and a 10% acrylamide slab resolution gel and was carried out at room temperature with 25 mA per slab gel. The gel was stained with Coomossie blue and scanned at 580 nm with an ISCO spectrophotometric gel scanner.

## Results

Spectrin, the major skeletal protein of the erythrocyte membrane largely exists in a tetrameric state of aggregation of two heterodimers consisting of $\alpha$- and $\beta$-spectrin [27]. Spectrin and the skeletal network of proteins are thought to be attached to transmembrane bilayer proteins in at least two ways: spectrin is held to a fraction of transmembrane Band 3 molecules via Band 2.1 [27,28] and is also attached via Band 4.1 to glycophorin A, the major transmembrane sialoglycoprotein [28].

In order to determine whether the physical state of skeletal membrane proteins was altered by spermine, the protein-specific spin label, MAL-6, was used. MAL-6 binds primarily to cysteine SH groups of membrane proteins [20]. Under labeling conditions employed in our laboratory 70–90% of MAL-6 is covalently bound to spectrin and the cytoplasmic pole of Band 3 [20,21]. Others have confirmed this location of MAL-6 in erythrocyte ghosts by protein extraction and immunological techniques [29,30]. A typical spectrum of MAL-6 attached to membrane proteins is shown in the top spectrum of Fig. 1. The $M_I = +1$ low-field

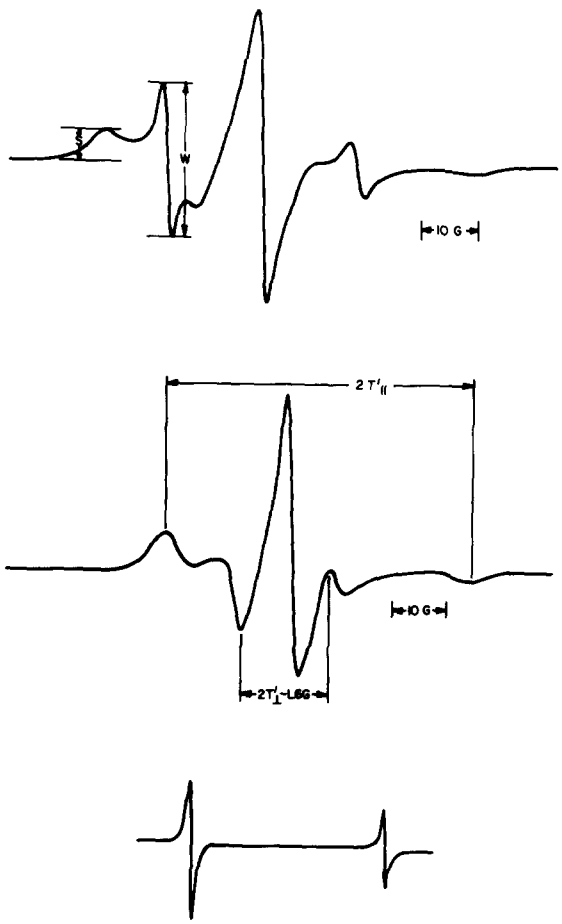


Fig. 1. Typical ESR spectra of MAL-6- (top), 5-NS- (middle) and [$^{15}$N]Tempamine-$d_{17}$- (bottom) labeled erythrocyte ghosts. Relevant spectral parameters from which the W/S ratio and order parameter are calculated from the spectra of MAL-6 and 5-NS, respectively, are indicated. In the case of the spectrum of [$^{15}$N]Tempamine-$d_{17}$ the rotational correlation time is calculated by well-established equations [24] employing the single crystal anisotropic $T$-tensor values of 2,2,6,6,-[$^{2}H_{12}$]tetramethyl-4-maleimido[$^{2}H_{5}$,$^{15}$N]piperidine-1-oxyl [41]. Instrument settings: MAL-6, 100 G scan width, 0.32 G modulation amplitude, and 16 mW power incident on the resonant cavity; 5-NS, 100 G scan width, 0.32 G modulation amplitude, and 18 mW power; [$^{15}$N]Tempamine-$d_{17}$ 40 G scan width, 0.32 G modulation amplitude, and 16 mW power.

resonance lines of this spectrum are illustrated in Fig. 2. The spectrum of MAL-6 demonstrates the presence of at least two different classes of membrane protein SH binding sites for this spin label, discernable by their motion: those that are strongly immobilized (S sites) and those that are weakly immobilized (W sites). W sites are located in a highly polar environment as judged by the electron-nuclear hyperfine coupling constant of MAL-6 bound to erythrocyte membranes [20]. Based on ascorbate decay kinetics, S sites are likewise in a polar environment, locations consistent with various reports noted above that nearly all the spin label is found on spectrin and the cytoplasmic pole of Band 3 [20,29,30].

The ratio of the ESR spectral amplitude of the low-field weakly immobilized component to that of the strongly immobilized component (W/S ratio) has been shown repeatedly by us and others (reviewed in Refs. 20, 32, 33) to be a convenient and highly sensitive monitor of the conformation and organization of membrane skeletal proteins and is reflective of the segmental motion of protein binding sites. For example, we recently demonstrated, employing MAL-6 spin-labeling techniques, that the state of aggregation of spectrin (tetramer or dimer) greatly influenced the protein–protein interactions in the erythrocyte membrane skeletal network: the W/S ratio of MAL-6

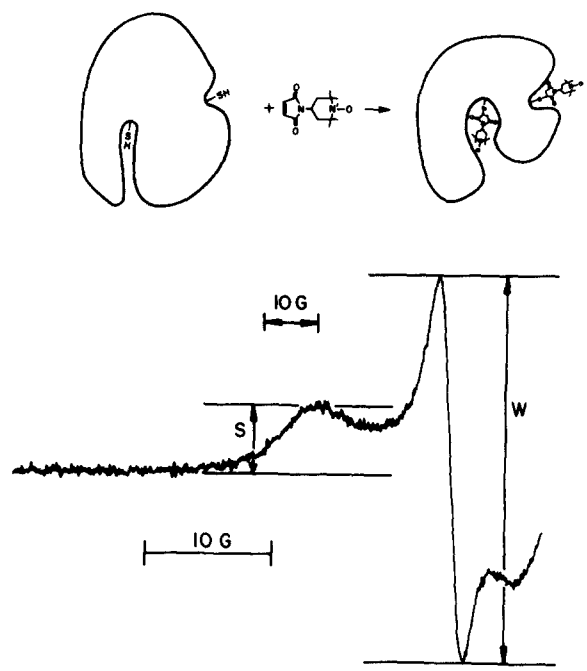


Fig. 2. Typical ESR spectrum of the low-field ($M_I = +1$) lines of MAL-6 covalently attached to membrane proteins. The spectral amplitudes of the low-field strongly and weakly immobilized spin label binding sites are given by S and W, respectively.

TABLE I

CONCENTRATION DEPENDENCE OF SPERMINE ON THE PHYSICAL STATE OF MEMBRANE PROTEINS IN HUMAN ERYTHROCYTE GHOSTS AS MONITORED BY THE W/S RATIO OF MAL-6

Mean ± S.D. of the W/S ratio values are presented. Each concentration of spermine was separately controlled to pH 8.0 and ionic strength as described in Materials and Methods. *P* values were calculated by a Student's *t*-test. n.s., not significant.

| Spermine (mM) | W/S ratio (% of control) | *N* | *P* |
|---|---|---|---|
| 0.00 | 100 | 2–17 | - |
| 0.02 | 98.5 ± 3.5 | 2 | n.s. |
| 0.06 | 97.5 ± 7.8 | 2 | n.s. |
| 0.20 | 70.5 ± 0.7 | 2 | < 0.02 |
| 0.60 | 58.5 ± 8.4 | 4 | < 0.001 |
| 1.20 | 56.2 ± 7.5 | 17 | < 0.0001 |
| 1.80 | 51.2 ± 5.4 | 4 | < 0.0001 |

could be reversibly cycled (normal–increased–normal) with the reversible cycling of tetrameric to dimeric to tetrameric spectrin on the erythrocyte membrane [10]. Additionally, we reported that polyphosphates, known to disrupt skeletal protein–protein interactions, increased the W/S ratio of spin-labeled erythrocyte membranes [22]. Others [31] have indicated that our MAL-6 spin-labeling method [20,21] yields highly reproducible spectra that can be used with confidence.

Table I shows that spermine (0.02–1.8 mM) reduced the W/S ratio of MAL-6 in a concentration-dependent manner. At low concentrations (0.02–0.06 mM), spermine caused no alteration in the physical state of skeletal proteins relative to control. However, over the concentration range of 0.6–1.8 mM, a reduction of nearly 50% in the W/S ratio was found (*P* ranges from $< 0.001$ to $< 0.0001$), suggesting that this polyamine produced a highly significant reduction in the segmental motion of spin-labeled binding sites. The half-maximal effect of spermine occurred at approx. 0.2 mM. These results are consistent with spermine causing an increase in protein–protein interactions in the skeletal network.

Decreased values of the W/S ratio of MAL-6 caused by spermine could be due to either a redistribution of W sites to S sites with no change in linewidth or to direct changes in linewidth or to a combination of both effects. Detailed spectral analysis of the more narrow W peak indicated that no change in linewidth occurred upon addition of spermine relative to that in control MAL-6 spectra. It is, therefore, suggested that spermine induced a redistribution of W sites to S sites. Further, it should be noted that the pH was controlled at 8.0 and the ionic strength of spermine in these experiments was approx. 10-times less than the ionic strength needed to reduce the W/S ratio to the levels in Table I, as shown in previous studies from our laboratory [20,21]. In addition, there was no significant difference in the reduction of the W/S ratio by spermine at concentrations from 0.6 to 1.8 mM (Table I). Consequently, it is unlikely that the osmolarity or ionic strength of spermine is solely responsible for the large reduction in the W/S ratio implying that this polyamine decreases the segmental motion of spin-label binding sites on skeletal proteins.

Sialic acid of membrane glycoproteins (70% of the total sialic acid is on glycophorin A [34]) was specifically spin labeled with perdeuterated, [$^{15}$N]Tempamine-$d_{17}$ employing reductive amination methods as reported from our laboratory [24]. A typical spectrum of spin-labeled sialic acid in erythrocyte membranes is presented in Fig. 1 (bottom). The effect of 1.2 mM spermine was monitored by calculation of an apparent rotational correlation time, $\tau$, of spin-labeled sialic acid as described previously [24]. $\tau$ can be conceptualized as the time required for spin-labeled sialic acid to rotate through an angle of one radian [20,23,24]. The smaller the value of $\tau$, the faster the rotational motion of this spin-labeled carbohydrate. Spermine was found to increase the motion of sialic acid by nearly 40% ($P < 0.001$, Table II).

Since spectrin and the skeletal network are reportedly coupled to glycophorin A via Band 4.1 [28], it is conceivable that skeletal protein–protein interactions enhanced by spermine could secondarily induce changes in the physical state of sialic acid on the opposite side of the membrane. Alternatively, changes in the sialic acid environment caused by spermine could conceivably secondarily cause alterations in skeletal protein–protein interactions. In order to make a decision between these two alternatives, we treated intact cells with neuraminidase, an enzyme which selectively cleaves

TABLE II

EFFECT OF SPERMINE (1.2 mM) ON THE PHYSICAL STATE OF ERYTHROCYTE MEMBRANE PROTEIN, SIALIC ACID AND LIPIDS AS MONITORED BY ESR SPIN-LABELING TECHNIQUES

Values are means ± S.D. (number of samples). $P$ values were calculated by a two-tailed Student's $t$-test.

| | Control ($n$) | Spermine ($n$) | $P$ |
|---|---|---|---|
| Protein | | | |
| MAL-6-labeled (W/S ratio) | | | |
| Ghosts with sialic acid present | 5.43 ±0.54(15) | 3.09 ±0.63(15) | < 0.0001 |
| Ghosts with sialic acid removed | 4.99 ±0.43(6) | 2.86 ±0.26(5) | < 0.0001 |
| Sialic acid | | | |
| Tempamine labeled ($\tau$) (n.s.) | 0.301 ± 0.043(6) | 0.181 ± 0.033(6) | < 0.001 |
| Lipid | | | |
| 5-NS-labeled (order) | | | |
| Ghosts | 0.694 ± 0.005(11) | 0.704 ± 0.004(5) | < 0.001 |
| Spectrin-depleted ghosts | 0.694 ± 0.003(4) | 0.703 ± 0.003(4) | < 0.005 |
| Isolated lipids | 0.672 ± 0.003(4) | 0.685 ± 0.004(4) | < 0.005 |

terminal sialic acid, and spin labeled subsequently prepared ghosts with MAL-6. Spermine addition to these protein-specific labeled membranes with greater than 90% of the sialic acid removed caused an approx. 50% decrease in the W/S ratio (Table II), a result statistically indistinguishable from that observed for spermine added to MAL-6-labeled ghosts with sialic acid present. This finding suggests that spermine may alter the motion of sialic acid through a secondary effect resulting from decreased segmental motion of spin-label binding sites on skeletal proteins and subsequent altered skeletal–glycophorin A interactions.

The effect of spermine on the lipid bilayer was investigated employing the lipid-specific spin label, 5-NS [20]. 5-NS was used to determine the average relative orientation of phospholipids in the lipid bilayer by calculation of the order parameter, $S$, from experimental parameters denoted in Fig. 1 [20]. The order parameter was slightly but significantly increased by spermine addition to erythrocyte membranes, isolated lipids, and spectrin-depleted ghosts which may imply a small ordering effect of this polyamine on lipid orientation (Table II) [10].

To gain further insight into the interactions of spermine with skeletal and bilayer proteins, SDS-PAGE analysis of the effect of spermine on spectrin extraction and Triton extraction was performed. Spectrin and several other proteins are easily removed from control membranes by the spectrin extraction procedure described in Materials and Methods (Fig. 3A) and spectrin is concomitantly lost from the residual pellet of control ghosts (Fig. 3B). In spermine-treated ghosts, however, the situation is quite different. No detectable spectrin, actin or Band 4.1 (three of the components of the erythrocyte skeletal network) are extracted in the presence of spermine (Fig. 3C) under the low temperature, low ionic strength conditions described above. The residual pellet still contained these proteins (Fig. 3D) (compare with the trace of untreated ghosts subjected to PAGE in a separate experiment (Fig. 3E)).

The above results suggested that spermine may be blocking the release of spectrin by bridging this protein to other proteins in membrane. We wondered if these other proteins were skeletal or bilayer proteins. It is known that the spectrin extraction procedure used in these experiments releases not only spectrin, predominantly in the tetrameric form, but also a high-molecular-weight complex consisting of spectrin, actin and Band 4.1 from control ghosts [35] and that the non-ionic detergent, Triton X-100, can be used to extract all cytoskeletal proteins and a fraction of bilayer proteins, such as Band 3 [26]. Consequently, the idea that spermine enhanced not only skeletal protein–protein interactions but also skeletal–bilayer protein interactions was tested employing

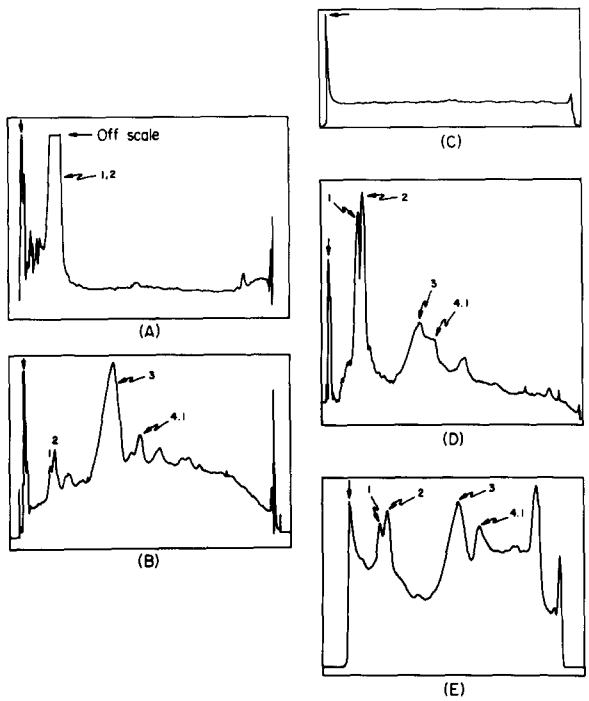


Fig. 3. The supernatant of ghosts exposed to SR buffer in the presence of 2-mercaptoethanol or the membrane residue after spectrin extraction treatment were subjected to SDS-PAGE under reducing conditions. Prior to the spectrin extraction treatment, the ghosts had been treated with the following solutions: A and B, 5P8; C, D, 1.8 mM spermine in 5P8. A and C represent PAGE gel profiles of the supernatant following spectrin extraction, B, D of the residual membrane pellet. E is a gel profile of untreated control ghosts. Protein designation: 1 and 2, spectrin; 3, Band 3; 4.1, Band 4.1. The arrows on the left of each scan indicate the top of the gel.

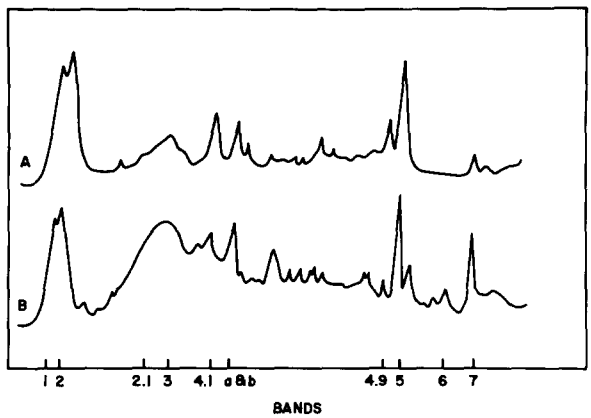


Fig. 4. SDS-PAGE gel profiles of residual pellets of Triton X-100 extraction of control (A) or spermine-treated (B) ghost membranes. The 'Triton shells' were subjected to electrophoresis as described in Materials and Methods and the gels were scanned at 580 nm. Protein band definition: 1 and 2, spectrin; 2.1, ankyrin; 3, Band 3; 4.1a and b, Band 4.1a and Band 4.1b; 4.9, Band 4.9; 5, actin; 6, Band 6; and 7, Band 7.

Triton X-100 extraction of ghosts pretreated with spermine or control as described in Materials and Methods. The resultant SDS-PAGE gel profiles of Triton pellets are shown in Fig. 4. Fig. 4A demonstrates that the residual proteins from control Triton shells include the skeletal proteins spectrin, Band 4.1a, 4.1b and actin and a small amount of the transmembrane protein, Band 3 as expected. However, the gel profile of the Triton shell of spermine-treated ghosts (Fig. 4B) indicates the presence of additional proteins which include: significantly more Band 3 (about 4-times as much relative to Band 2 compared to control, as determined by peak measurements) and Bands 4.2, 4.9, 6 and 7. We, therefore, conclude that spermine does bridge spectrin to bilayer proteins in human erythrocyte membranes.

## Discussion

Spermine was observed to cause a pronounced decrease in the segmental motion of spin-labeled skeletal proteins in human erythrocyte membranes (Table I). Approx. 16% of Band 3 molecules are reportedly linked to the $\beta$-subunit of spectrin by a 210 kDa, highly basic protein, termed ankyrin (or syndein) [27]. The basic nature of ankyrin plus its extreme sensitivity to trypsin [36] suggest that this protein is rich in positively charged lysine and/or arginine residues. Spectrin, however, is rich in negatively charged residues, aspartic and glutamic acid [37], as is the cytoplasmic portion of Band 3 [38]. Ankyrin presumably serves in part as an electrostatic bridge between spectrin and some Band 3 molecules. That the mode of interaction of spermine with the erythrocyte membrane is electrostatic was evidenced by our earlier findings that the effect of spermine on the physical state of membrane proteins determined by spin-labeling methods with MAL-6 could be abrogated by washing treated membranes with the high ionic strength buffer, phosphate-buffered saline [10].

Washings with 5P8, a low ionic strength buffer, did not abrogate this effect [10].

The bridging of Band 3 and possibly glycophorin A to spectrin by spermine would account for the observation by Schindler et al. [17] that in spermine-treated erythrocyte ghosts, transmembrane glycoprotein lateral diffusion no longer occurred and also would account for the results in Fig. 3 which show that spectrin cannot be extracted under the usual conditions in the presence of this polyamine. In addition, spermine pretreatment of ghosts subjected to Triton extraction resulted in the retention of significantly more Band 3 and additional bilayer and skeletal proteins, namely Bands 4.2, 4.9, 6 and 7 in the Triton shell compared to that of control-treated ghosts (Fig. 4). This result further supports the idea that spermine acts to increase protein–protein interactions in the cytoskeleton and to bridge skeletal and bilayer proteins. These results are also consistent with the finding that spermine decreased the deformability of erythrocytes, presumably due to skeletal stabilization [17,18].

Spectrin is also thought to be attached to glycophorin A through Band 4.1 [28]. In the present study, spermine added to the membrane produced a 40% increase in the motion of sialic acid, most of which is on this glycoprotein. There is but little known about the topology of glycophorin on the external membrane surface. Model calculations suggested that this sialoglycoprotein is hydrogen-bonded to the lipid bilayer head groups [39]. While the molecular details by which increased motion of sialic acid occurred upon addition of spermine (Table II) are not known, it is possible that spermine increased the aggregation of the cytoplasmic pole of glycophorin A with the skeletal network which would likely result in decreased segmental motion of spin-labeled skeletal proteins (as suggested by the decreased W/S ratio of MAL-6 (Table I)). This alteration in the conformation of glycophorin A would, presumably, then free sialic acid from putative steric or electrostatic constraints [39], thereby increasing its motion.

We found that spermine slightly increased the order of the lipid bilayer in erythrocyte membranes, in spectrin-depleted ghosts, and in isolated lipids (Table II) [10]. These results are consistent with the results from other laboratories indicating that spermine leads to increased aggregation of phosphatidylserine vesicles [11] and decreased bilayer fluidity of bean leaf membranes [9]. Since the small effect of spermine was found in lipids isolated from protein of the erythrocyte membrane (Table II), this ordering effect may be independent of the presence of protein, which would suggest that spermine may also bind directly to lipids, presumably by electrostatic interaction with polar or charged head groups.

Although molecular mechanisms are not yet known, the bridging of the skeletal network to transmembrane proteins suggested in the present study may be one mode by which polyamines influence cell regulatory processes such as differentiation [1,2], transport [3,4], information transfer across cell membranes [3,4] and diffusion of transmembrane proteins (e.g., receptor formation, endocytosis and mitogenesis). In addition, the reported increased levels of spermine in sickle cell erythrocytes [40] and certain tumors [12–14] raises the possibility that the skeletal network of proteins interacts abnormally under these pathological conditions.

## Acknowledgements

We gratefully thank Dr. Bennett T. Farmer, II for technical assistance. This work was supported in part by grants from NSF (RII-86-10671), DOD and the Tobacco and Health Research Institute.

## References

1 Jänne, J., Pösö, H. and Raina, A. (1978) Biochim. Biophys. Acta 473, 241–293.
2 Heley, O. (1981) Differentiation 19, 1–29.
3 Koenig, H., Goldstone, A. and Lu, C.Y. (1983) Nature 305, 530–534.
4 Koenig, H., Goldstone, A.D. and Lu, C.Y. (1983) Proc. Natl. Acad. Sci. USA 80, 7210–7214.
5 Koenig, H., Goldstone, A.D. and Lu, C.Y. (1983) Biochem. Biophys. Res. Commun. 116, 1039–1048.
6 Morgan, D.M.L., Bachrach, U., Assaraf, Y.G., Harrari, E. and Golenser, J. (1986) Biochem. J. 236, 97–101.
7 Rzepczyk, C.M., Saul, A.J. and Ferrante, A. (1984) Infect. Immun. 43, 238–244.
8 Loh, T.T., Chan, R.Y.Y. and Fong, W.F. (1985) Cell Biol. Int. Rep. 9, 143–150.
9 Roberts, D.R., Dumbroff, E.B. and Thompson, J.E. (1986) Planta 167, 395–401.
10 Farmer, B.T., Harmon, T.M. and Butterfield, D.A. (1985) Biochim. Biophys. Acta 821, 420–430.

11 Ohki, S. and Daux, J. (1986) Biochim. Biophys. Acta 861, 177–186.
12 Ota, D.M., Nishioka, K., Grossie, G. and Dixon, D. (1986) Eur. J. Clin. Oncol. 22, 837–842.
13 Ota, D.M., Nishioka, K., Foulkes, M. and Grossie, B. (1984) J. Clin. Oncol. 2, 1157–1164.
14 Moulinoux, J-P., Quemener, V., Larwul, J-J., LeCalve, M.L. and Roch, A-M., Toujas, L. and Quash, G. (1984) Int. J. Cancer 34, 277–281.
15 Ballas, S.K., Smith, E.D. and Marton, L.J. (1985) Biochem. Biophys. Res. Commun. 129, 549–552.
16 Boggust, W.A. and O'Connell, S. (1984) IRSC Med. Sci. 12, 265–266.
17 Schindler, M., Koppel, D.E. and Sheetz, M.P. (1980) Proc. Natl. Acad. Sci. USA 77, 1457–1461.
18 Ballas, S.K., Mohandas, N., Marton, L.J. and Shohet, S.B. (1983) Proc. Natl. Acad. Sci USA 80, 1942–1946.
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 Butterfield, D.A. (1982) Biol. Magn. Reson. 4, 1–28.
21 Butterfield, D.A. and Markesbery, W.R. (1981) Biochem. Int. 3, 517–525.
22 Wyse, J.W., Barker, R., Franco, R.S., Martelo, O. and Butterfield, D.A. (1987) Biochem. Biophys. Res. Commun. 144, 779–786.
23 Feix, J.B. and Butterfield, D.A. (1980) FEBS Lett. 115, 185–188.
24 Farmer, B.T., II and Butterfield, D.A. (1985) Analyt. Lett. 18, 555–561.
25 Laemmli, U.K. (1970) Nature 227, 680–685.
26 Sheetz, M.P. and Sawyer, D. (1978) J. Supramol. Struc. 8, 399–412.
27 Cohen, C.M. (1983) Sem. Hematol. 2, 141–158.
28 Bennett, V. (1985) Annu. Rev. Biochem. 54, 273–304.
29 Barber, M.J., Solomonson, L.P. and Eichler, D.C. (1985) Biochem. Biophys. Res. Commun. 127, 793–798.
30 Fung, L.W.M. and Simpson, M.J. (1979) FEBS Lett. 108, 269–273.
31 Barber, M.J., Rosen, G.M. and Rauckman, E.J. (1983) Biochim. Biophys. Acta 732, 126–132.
32 Butterfield, D.A. (1985) Membr. Fluidity Biol. 3, 161–255.
33 Butterfield, D.A. (1986) Crit. Rev. Neurobiol. 2, 169–240.
34 Marchesi, V.T. (1979) Sem. Hematol. 16, 3–20.
35 Sheetz, M.P. (1983) Sem. Hematol. 20, 175–188.
36 Jinbu, Y., Sata, S., Nakao, T. and Nakao, M. (1982) Biochem. Biophys. Res. Commun. 104, 1087–1092.
37 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101–122.
38 Kaul, R.K., Murthy, S.N.P., Reddy, A.G., Steck, T.L. and Kohler, H. (1983) J. Biol. Chem. 258, 7981–7990.
39 Stibenz, D. and Geyer, G. (1980) Folia Hematol. 107, 787–792.
40 Ballas, S.K., Mohandas, N., Marton, L.J. and Shohet, S.B. (1982) Fed. Proc. 41, 512.
41 Beth, A.H., Balasubramamian, K., Wilder, R.T., Venkataramu, S.D., Robinson, B.H., Dalton, L.R., Pearson, D.E. and Park, J.H. (1981) Proc. Natl. Acad. Sci. USA 78, 4955–4959.