ALTERATIONS IN ERYTHROCYTE MEMBRANE STRUCTURE INDUCED BY THERAPEUTIC AND PHARMACOLOGICAL AGENTS

By

Denise Lora Mazorow

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ABSTRACT

ALTERATIONS IN ERYTHROCYTE MEMBRANE STRUCTURE INDUCED BY THERAPEUTIC AND PHARMACOLOGICAL AGENTS

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Many compounds are known to affect erythrocyte membrane physical structure. In this study, evidence is given, that many of these compounds may modulate the membrane via the cytoskeletal matrix in intact cells.

Addition of 1 to 1000 prostaglandin molecules per cell were shown to change membrane structure as detected by electron spin resonance spectroscopy (ESR). It is unlikely that the membrane changes were the result of simple membrane lipid-lipid interactions. It is proposed that prostaglandins have specific receptors inducing the structural changes. Furthermore, glucose prevented the structural changes induced by prostaglandins. It is believed that the extrinsic membrane proteins are linked either directly or indirectly to each other forming the cytoskeletal matrix and through specific interactions are bound to integral membrane proteins. Thus, changes in cytoskeletal structure would obviate changes in overall membrane structure.

Chlorpromazine, a drug known to have specific lipid interactions was added to erythrocyte suspensions. Dose response curves of membrane structural changes upon addition of chlorpromazine obtained by both ESR and hypotonic hemolysis studies were in agreement. At 10^{-5} M, chlorpromazine induced maximal disordering and maximal protection to hypotonic hemolysis. Upon further addition of chlorpromazine, it is believed that all the protein binding sites become saturated and chlorpromazine begins to enter the lipid matrix with increasing doses, the drug binds to membrane lipid, until acting as a detergent, it disrupts the membrane.

Ingestion of aspirin and indomethacin, like addition of chlorpromazine and prostaglandins also induce membrane structural changes at therapeutic concentrations as detected with ESR. The degree and time course of membrane changes from erythrocytes taken from male and female subjects after ingestion of either aspirin or indomethacin were different for men and women, and for women were dependent on the menstrual cycle. In vitro addition induced no structural changes in erythrocyte membranes. Therefore, the changes in membrane structure after drug ingestion must be induced by metabolic products.

Finally, even alterations in the anionic milieu can apparently induce membrane alterations. Band 3, which is the anion port is attached to the cytoskeletal matrix. Thus changes in ion content can also induce membrane structural changes.

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Background

A. General Erythrocyte Membrane Structure

Erythrocytes have been the subject of study since 1878 when Herman Nasse (1) reported that the plasma chloride concentration decreased when erythrocyte suspensions were exposed to increasing partial pressures of carbon dioxide. Since then the distributions and flux rates of anions involved with the erythrocyte have been intensively studied. The chloride-bicarbonate exchange is very rapid with a half time of 0.3 sec. (2, 3). Phosphate and sulfate have much slower rates of exchange (4-6).

Later, interest was shown in the erythrocyte membrane itself. Indications are that the phospholipid classes are asymmetrically distributed in specific halves of the bilayer. This evidence has mostly been obtained from studies using specific phospholipases (7-11). Purified phospholipase added to whole cells converts about 50% of the phosphatidycholine (PC) to lysolethicin. All the PC can be hydolyzed by the same amount of phospholipase A_2 when added to leaky ghosts. Two-thirds of the PC is in the outer monolayer if one calculates the amount of PC degraded in whole cells versus that degraded in ghosts. Using purified sphingomylinase in a similar manner, it has been determined that 80-85% of the sphingomyelin is located in the outer monolayer. Trinitrobenzenesulfate (TNBS), a probe for amino phospholipids (12), does not permeate the erythrocyte membrane (13). Fluorodinitro-

benzene (FDNB), which easily passes through the membrane (13, 14), is also a probe for amino phospholipids. The results using FDNB and TNBS indicate that 80 to 90% of the total phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located in the inner monolayer of membrane. These data indicate that the major phospholipid components in the outer monolayer are choline containing phospholipid (PC and sphingomyelin) and the major phospholipid components of the inner monolayer are the amino phospholipids (PE and PS). Another demonstration of the asymmetric distribution of the membrane phospholipids is that PC and sphingomyelin can be exchanged from one membrane to another; whereas PS and PE can not (15-18).

Unfortunately, it is not known how or where cholesterol is located in the membrane even though approximately half the lipid on a molar basis is cholesterol (19). However, it is known that cholesterol is relatively easy to extract from erythrocyte membranes under conditions that do not remove any of the other lipids (20-22). Cells with a cholesterol:phopholipid ratio near 2 have an increased surface area and become broad, flattened cells. The membrane lipids of these cells have restricted motion as seen using the fluorescent probe 12-(9-anthroyl) stearic acid (23).

Like the phospholipids the erythrocyte membrane proteins are also asymmetric (24). The ghost membrane is composed of 8 major polypeptide chains when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (25). Along with the 8 major polypeptides there are 4 major glycopeptides as seen by periodic acid-Schiff's reagent staining (PAS) (26, 27). These four PAS-staining bands represent the major source of sialic acid found in the human red blood cell.

Singer and Nicolson (24) developed the approach of classifying the proteins as either intrinsic or extrinsic, extrinsic proteins being those which can be removed from ghost membranes by altering the ionic strength or the pH of the media. The extrinsic proteins correspond to the SDS-PAGE protein bands 1, 2, 4, 5, and 6. The intrinsic proteins; therefore, correspond to SDS-PAGE protein bands 3, 7 and PAS protein bands 1, 2, 3, and 4.

The extrinsic proteins, which are approximately 40% of the total, are believed to lie totally in the cytosol. The major evidence for this is that none of these polypeptides are degraded when whole cells are treated with proteolytic enzymes (25, 28, 29). However, when leaky ghosts are treated with the proteolytic enzymes, each of these proteins is degraded.

Bands 1 and 2 are the spectrin polypeptides. Band 1 (α subunit, 240,000 daltons) and band 2 (β subunit, 220,000 daltons) can self-associate to form the spectrin tetramer $(\alpha\beta)_2$ with a molecular weight of 920,000 daltons (30). It is now fairly clear that spectrin dimers and tetramers can form long, filamentous polymers (31).

Band 2.1, now also known as ankyrin, binds one mole per mole spectrin heterodimer (32). Ankyrin binds the spectrin heterodimer to the membrane by also binding to the integral protein band 3 (33-35).

Actin, which is band 5 has a molecular weight of 45,000 daltons. The spectrin tetramer, arranged by head to tail association of heterodimers (31), binds actin to the tail ends. The complexes when formed in the presence of band 4.1, have a higher viscosity and are better able to survive sedimentation through sucrose than those without band 4.1 (30, 36, 37). This complex is extremely sensitive to micromolar

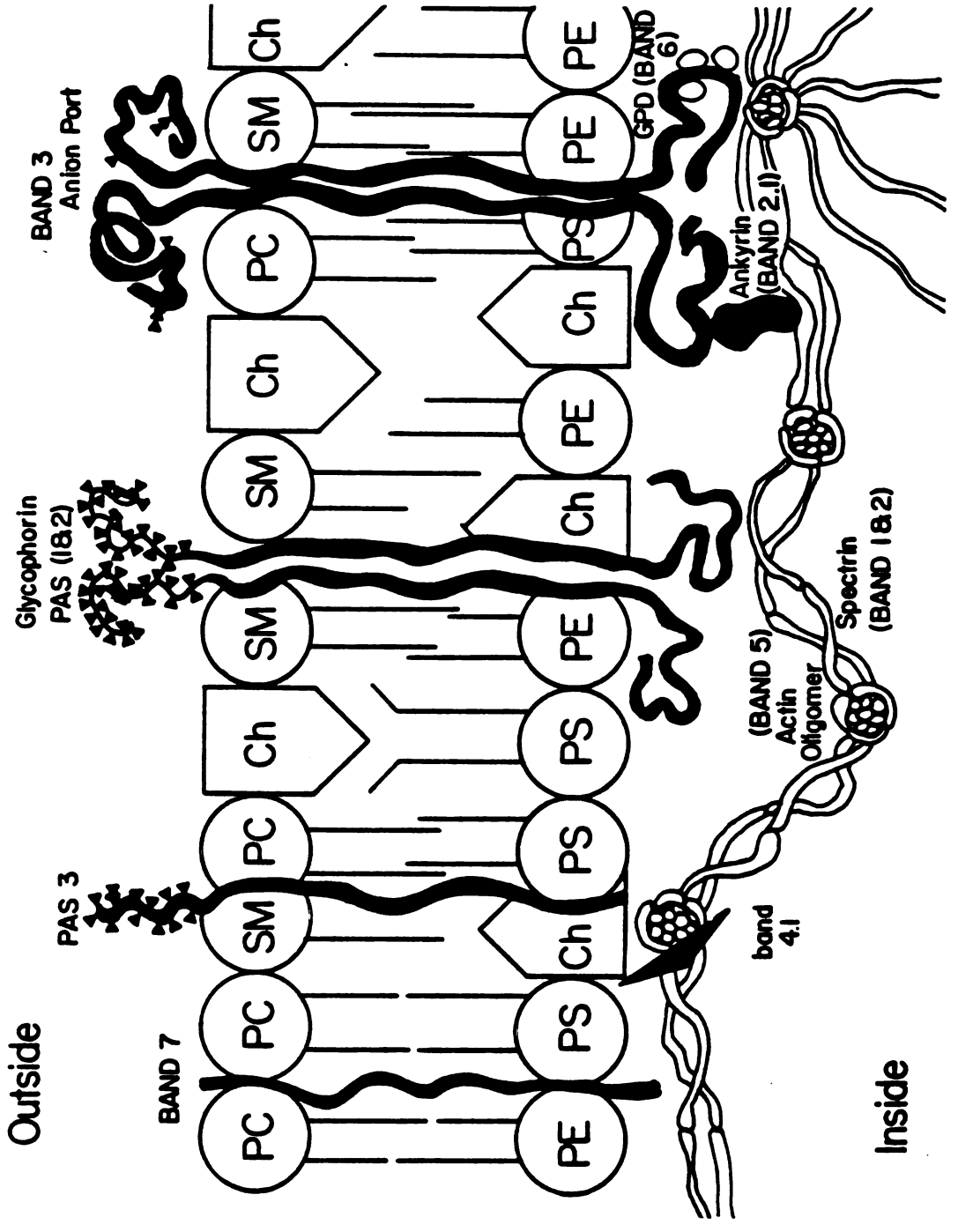
concentrations of calcium (36). Furthermore, it has been found that only in the presence of band 4.1 do spectrin-actin gels become thixotropic (36), and thus may play a role in erythrocyte membrane deformability: Band 4.1 along with ankyrin helps to hold the cytoskeletal matrix to the membrane and both may be important in modulating erythrocyte shape changes.

The last of the extrinsic proteins, band 6, is the monomeric form of glyceraldehyde-3-phosphate dehydrogenase (38, 39). The polypeptide appears to be associated with the cytoplasmic portion of band 3 (40).

Of the integral membrane proteins band 3 and PAS 1 are the most common and both appear in the membrane as dimers (41). Band 3 appears to be composed of 5-8% carbohydrate on a dry weight basis (42, 43). The sugars in this carbohydrate are mostly mannose, galactose, and N-acetylglucosamine (1:2:2) with trace amounts of fructose and glucose. PAS-1 and PAS-2 are believed to be interconvertible forms of glycophorin-A (44) and represent 75% of the sialoglycopeptide (44). The portion of the glycophorin/dimer located on the cytoplasmic side of the membrane is believed to be in close association with spectrin. When erythrocytes are incubated with antispectrin antibodies, a large shift in the glycophorin distribution on the cell surface is reported to occur (45). Little is known of the other integral membrane protein, band 7.

Other important proteins are known to exist in the erythrocyte membrane but not in large enough numbers to clearly be seen on gels. These include the Na-K ATPase and Ca-Mg ATPase. To visualize the placement of the erythrocyte membrane components see Figure 1. Both

Figure 1. A schematic of the major erythrocyte membrane components. The outer monolayer of the membrane contains the phospholipids sphingomyelin (SM) and phosphatidylcholine (PC). The inner monolayer contains the phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE). Cholesterol (Ch) is distributed approximately equally in both monolayers. The intrinsic proteins are the anion port, Band 3, which is a dimer and Band 7. The glycoproteins are glycophorin (PAS 1 and 2), which is also a dimer and PAS 3. Spectrin is shown as a heterodimer (Bands 1 and 2). The actin oligomer (Band 5) is attached to the ends of the spectrin heterodimer. Band 4.1 attaches the spectrin-actin complex to the membrane. The exact placement for this attachment is not known. Ankyrin, also known as band 2.1, attaches spectrin to Band 3. Band 6, the monomeric form of glyceraldehyde-3-phosphate dehydrogenase (GPD), is attached to the cytosolic tail of Band 3.


Outside
Inside
BAND 7
PAS 3
Glycophorin
PAS (1&2)
BAND 3
Anion Port
band
4.1
(BAND 5)
Actin
Oligomer
Spectrin
(BAND 1&2)
Ankyrin
(BAND 2.1)
GPD (BAND
6)
PC
SM
Ch
PS
PE

the lipids and proteins are placed in their relative positions, but the concentrations are not necessarily correct.

B. BAND 3

Band 3, which comprises 25% of the membrane protein, is believed to occur as a dimer. It has a molecular weight between 88,000 and 105,000 daltons (25). The function of band 3 in the membrane is, apparently, to act as a passive port for anion flux. The most compelling evidence for band 3 involvment in anion transport is that covalent labelling of band 3 with a variety of compounds, most notably the stilbene derivatives, will inhibit anion flux (46-48). 4,4'-diisothiocyano 2,2'stilbene disulfonate (DIDS) was first used by Cabantchik and Rothstein (47, 48). Approximately 300,000 copies of [^{3}H] DIDS were reported bound to a single erythrocyte and this binding was almost exclusive to band 3. Furthermore, DIDS binding to the membrane inhibited anion exchange. Experiments in which the disulfonic stilbenes (DIDS and 4-acetamido-4'-diisothiocyano-2,2'-disulfonic acid stilbene [SITS]) have been incorporated into resealed ghosts (49, 50) or have been reacted with inverted sealed vesicles (51) have not inhibited anion flux or bound to band 3. Thus, like the asymmetry exhibited in membrane architecture, DIDS and SITS bind only to the external side of the cell.

The model for anion flux that best fits the data is that of a ping-pong model. This ping-pong model simply proposes that one anion is translocated before the counterion binds and is transported. The ping-pong model predicts that if cells are suspended in solutions with differing internal and external calcium concentrations, the half- saturations concentrations for extracellular calcium flux will be reduced when the intracellular chloride is reduced (52). The prediction has

proven true. An essential feature of the ping-pong model is that there are two forms of the unloaded carrier, one facing in and one facing out. It is the existence of these two forms along with the fact that anions are generally in equilibrium across the membrane (53, 54) which accounts for the strange behavior of calcium just described.

Band 3 probably exists as a dimer in the membrane. Band 3 when extracted in Triton X-100 is a dimer (35). Almost quantitative cross-linking of reactive sulfhydryl groups can be accomplished by reducing crosslinking from the cytoplasmic side (41, 56). Fluorescent labelling of cross-linked band 3 doesn't affect its rotational mobility (57). Counting the number of particles seen by freeze-fracture electron microscopy yields about half the number of band 3 monomers one would expect (25, 28).

The question has been raised on the importance of integrity of band 3 as a dimer for its function. Evidence suggests that the dimeric conformation is not needed to allow anion flux. Dixon plots for several aromatic sulfonic acid inhibitors are linear (59, 60), which indicates that one inhibitor reacts with one monomer. Further evidence suggests that one DIDS molecule binds to one band 3 monomer (61).

A more complete discription of anion exchange can be found in several review articles (62, 63). Also of interest in this thesis are how certain drugs and hormone like compounds interact with the erythrocyte membrane. The next several sections are devoted to giving some background information on these compounds and their interactions.

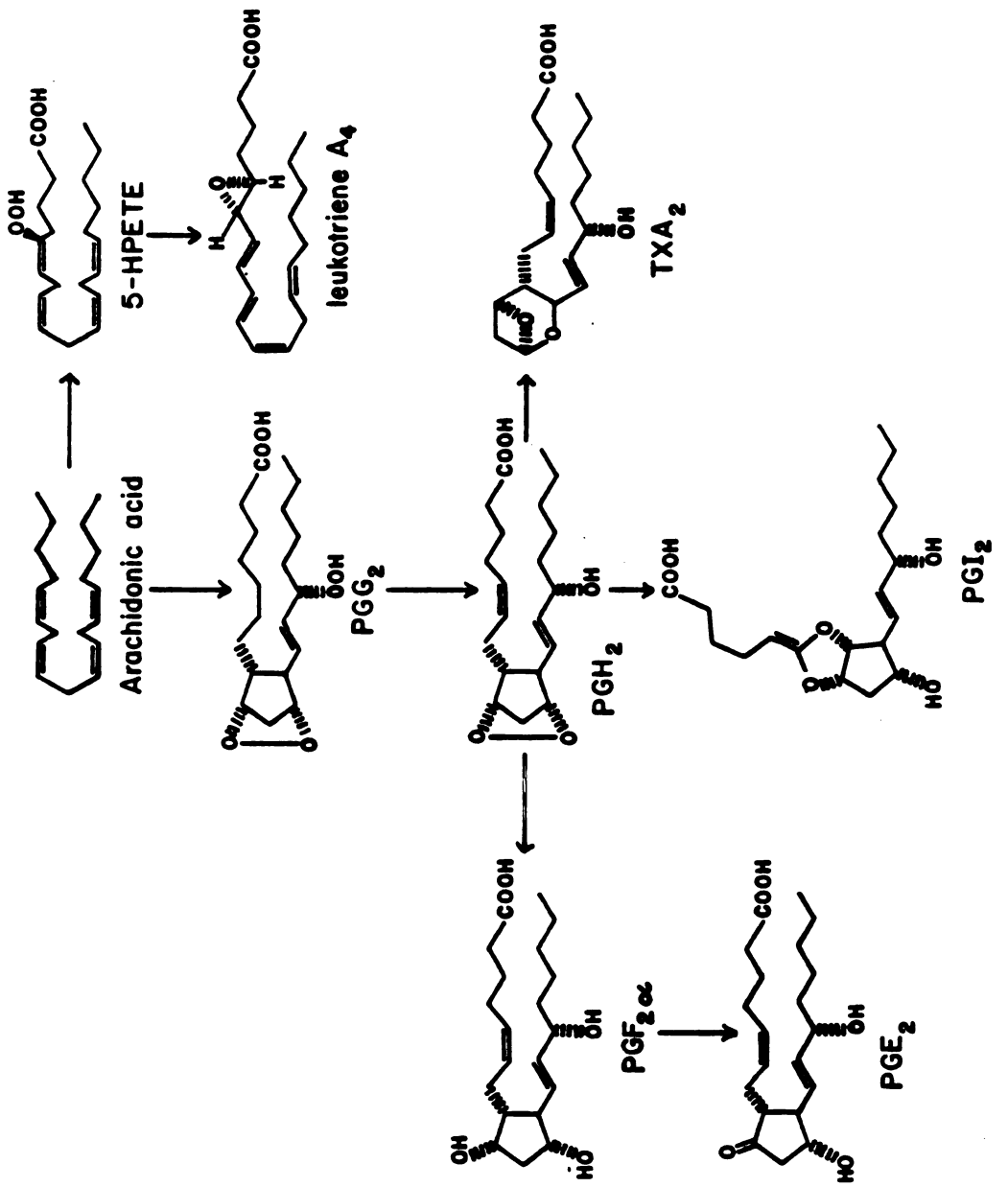
C. PROSTAGLANDINS (GENERAL)

The prostaglandins (PGs) are a group of oxygenated fatty acids which metabollicaly originate from the fatty acid arachidonic acid. They are one of the most biologically potent compounds known and can act either as bioregulators or can take part in pathological states. Release of arachidonic acid can be affected by hormones directly or indirectly (64), inflammatory or immunological stimuli (65), calcium ionophores (66), ultraviolet light (67), tumor promotion agents (68), and mechanical agitation (69). The arachidonic acid is converted to the endoperoxide intermediate PGG_2 by the enzyme cyclooxygenase (70). From PGG_2 the next step is determined by the tissue under consideration and the tissue enzyme content. For example, platelets will convert the PGG_2 primarily to thromboxane A_2 (TXA_2), whereas the aorta will synthesize prostacyclin (PGI_2) (Figure 2). PGE_1 and PGE_3 are much less common than the other prostaglandins and are synthesized from eicosanoic acids other than arachidonate.

Cis 5, 8, 11, 14 eicosatetraenoic acid (arachidonic acid) is the only eicosanoic acid converted enzymatically to active thromboxanes and prostaglandins. TXA_2 has a half life in biological tissues of seconds and is the most potent of the known arachidonic acid derivatives. Its function is to contract the aorta and to trigger platelet aggregation (71). PGI_2, the natural antagonist of TXA_2, relaxes aortic tissue and prevents the aggregation of platelets (72). The action of non-steroidal anti-inflammatory drugs regulates the synthesis of all prostaglandins since they inhibit cyclo-oxygenase activity.

An alternate pathway for oxygenation of arachidonic acid is via the enzyme lipoxygenase (73). The primary products of this pathway are the hydroperoxyeicosatetraenoic acids (HPETES). The substitution (5-HPETE,

Figure 2. Both the leukotriene pathway and the prostaglandin (PG) pathway begin with arachidonic acid. Arachidonic acid is converted to PGG_2 by the enzyme cyclo-oxygenase. PGG_2 is further converted to PGH_2. PGH_2 can be further converted to $PGF_{2\alpha}$, PGI_2 (prostacyclin), or thromboxane A_2 (TXA_2). This conversion is tissue dependent. $PGF_{2\alpha}$ under the proper conditions can be converted to PGE_2. An alternate pathway for the oxygenation of arachidonic acid is via lipoxygenase to the leukotriene pathway. An example of this oxygenation is the conversion of arachidonic acid to 5-hydroperoxyeicosatetraeonic acid (5-HPETE) to leukotriene A_4. The placement of oxygenation and resulting leukotriene is tissue dependent.


Arachidonic acid
5-HPETE
leukotriene A_4
PGG_2
PGH_2
TXA_2
PGI_2
$PGF_{2\alpha}$
PGE_2

12-HPETE) depends on the cell type synthesizing the compound. The class of compounds from this pathway are called leukotrienes (Fig. 2) (74).

When it became known that indomethacin and aspirin both inhibited prostaglandin synthesis (75) many laboratories began to study the anti-inflammatory role of prostaglandins. Glucocorticoids will also suppress prostaglandin synthesis (76) through a complex set of cellular responses. In general, the glucocorticoids will react with a specific membrane receptor which can alter protein synthesis. This results in decreased amounts of esterified fatty acid released.

D. PROSTAGLANDIN MEMBRANE RECEPTORS

In 1974, Smigel and Fleisher (77) reported techniques for measuring and characterizing prostaglandin binding to membrane isolates. They determined that the PG receptor was in the plasma membrane fraction of rat liver isolates and that this receptor was of a single type for binding the E type prostaglandin. Other PGE_1 binding sites were studied, most of which were in the plasma membrane fraction (77). PGE type receptors have been found in non-pregnant human myometrium (78), skin tissue (79, 80), bovine corpus luteum (81), human erythrocytes (82-84), and frog erythrocytes (85).

To demonstrate that the prostaglandins were binding to a protein receptor, (80), trypsin, pronase, or boiling were used to show a marked reduction of PGE_2 and PGF_2 binding. A second study showed that upon exposing rat skin to UV light there was loss of PGE_2 binding capability (79). This loss could be prevented by addition of a protein sulfhydryl oxidizing agent or by a lipid anti-oxidant. The authors

suggested that the UV light initiated reduction of needed disulfide groups and peroxidation of lipids which are needed in PG-receptor binding.

Human platelet membranes have been reported to have two distinct receptors, one for PGI_2 and another for PGD_2 (86, 87). It was shown that binding PGI_2 to platelets induced a large increase in cAMP. But, if PGE_1 and PGE_2 were added, PGI_2 stimulation of cAMP production was inhibited. Platelets are not the only system in which PGE can affect enzyme activity or substrate production. Addition of PGE_1 to normal or transformed fibroblasts in culture resulted in an activation of adenyl cyclase (88). In these studies, PGE_1 binding was dependent upon calcium.

These results indicate that prostaglandins have specific membrane receptors. Furthermore, these receptors are likely to be polypeptides and appear to affect the regulation of other membrane events.

E. PROSTAGLANDIN EFFECTS ON ERYTHROCYTES

Very low doses of prostaglandins (1 to 10 molecules per cell) have been reported to dramatically affect erythrocyte membrane strucutre as well as whole cell properties such as deformability. The prostaglandin binding to the erythrocyte membrane must induce an amplification. One to ten molecules of a fatty acid derivative could not induce generalized membrane structural changes unless this amplification occurred.

PGE_2, when added to human erythrocytes, caused a decrease in whole cell deformability (82). Kury et al (83), using blood from only male subjects repeated the work of Allen and Rasmussen (82). They also showed that PGE_2 not only caused a decrease in deformability, but

also caused an increase in membrane order parameter (electron spin resonance study, ESR). Further studies with PGE_1, a PGE_2 antagonist, showed that PGE_1 caused an increase in cell deformability and a decrease in membrane order. The maximal effect for either PGE_1 or PGE_2 occurred at 10^{-11} M prostaglandin. This was approximately 10 prostaglandin molecules per cell. A requirement for the membrane perturbation with either PGE_1 or PGE_2 was the presence of calcium. Therefore, like the study with cultured fibroblasts, calcium is needed for PGE_1 to initiate not only adenylcyclase activity (88) but also to induce erythrocyte membrane structural changes.

PGE_1 not only affects lipid membrane bulk properties as indicated by the studies of Kury et al (83) and Allen and Rasmussen (82), but it induces protein structural changes. PGE_1 and PGF_2 both reportedly cause changes in the circular dichroism (CD) absorption spectrum of washed erythrocyte ghosts between 190 nm and 250 nm (84). These changes indicate changes in membrane protein α-helical and β-sheet content. These changes in CD absorption spectra were blocked by the PGE_1 antagonists 7-oxa-13-prostynoic acid (89).

PGE_1 and PGE_2 not only alter erythrocyte membrane lipid and protein structure, they also affect hemolysis. PGE_1 is reported to protect erythrocytes from hypotonic hemolysis, while PGE_2 has the opposite effect; i.e., it is lytic (90). This agrees with ESR and deformability data already presented. Although PGE_1 and PGE_2 appear to protect erythrocytes from mechanical lysis, the degree of protection seems to be dependent on whether the erythrocytes were drawn from male or female subjects (84). Erythrocytes drawn from male volunteers were more easily lysed than cells drawn from female volunteers; however, the

degree of protection from lysis given by PGE_1 was more pronounced for cells from men. Calcium did not alter PGE affects on lysis. The other studies presented earlier indicated a calcium requirement for a PG induced effect (82, 83). Perhaps the prostaglandins allow leakage of calcium into the cell. Increased internal levels of calcium may induce specific secondary reactions, which would reflect the need for calcium in some cases and not in others.

F. ASPIRIN

Both aspirin and indomethacin are cyclo-oxygenase inhibitors and thus prostaglandin synthesis inhibitors. The effects of indomethacin and aspirin are apparently ubiquitous, since prostaglandins are ubiquitous.

Therefore, it is not surprising that aspirin affects not only platelets (70, 91) and arterial walls (92) but also erythrocyte membrane structure (93). Aspirin added to heparinized whole blood will become deacetylated. Approximately 80% of the acetate is unbound and can be dialyzed out. 10% of the aspirin was bound to plasma proteins, 90% of which was to albumin. 0.41% of the aspirin was bound to the erythrocyte membrane. The aspirin and/or the labelled acetate presumably passes through the membrane since spectrin can become acetylated. Along with spectrin, acetylation of 5 or 6 other peptides also has been reported (93); the acetylation was temperature dependent.

It is also known that men have longer bleeding times than women. After aspirin ingestion, the bleeding times for both women and men are increased. However, the increase is larger for men. Interestingly,

PGE_2 will activate the secondary reactions which reduce bleeding prior to inhibition of prostaglandin synthesis by aspirin.

Even though aspirin has long been known to affect bleeding times of men more than that of women little work has been done to determine why. It is known that one hour after aspirin ingestion women have higher aspirin levels (1.6 µg/ml) in the plasma than do men (1.0 µg/ml)(95). However, the differential absorption of aspirin between men and women doesn't afffect platelet synthesis of TXB_2. 650 mg aspirin causes a total inhibition of thromboxane release for both men and women. It was thought that the differences in bleeding times between men and women was the result of differing PGF_2 circulating in plasma. However, the PGF_2 levels have been shown to be the same for both men and women (96).

Estradiol and/or estrogen levels may be the cause for the differences in the effects of aspirin in men and women. Aspirin plasma levels are lower for women taking estrogen based contraceptives. Aspirin plasma levels and half-life return to normal 3 to 5 months after stopping estrogen contraceptive intake. This indicates that increased levels of estrogen will ultimately change aspirin absorption (97). Estradiol (98), when applied to the aorta, enhanced prostacyclin production, but when applied to platelets did not affect thromboxane production. This increased ratio of PGI_2/TXB_2 may be one of the reasons why pre-menopausal women have a lower incidence of ischemic heart disease (99).

G. CHLORPROMAZINE EFFECTS ON MEMBRANE STRUCTURE

Chlorpromazine (CPZ) is believed to be able to pass through the erythrocyte membrane (100-102). Evidence suggests that CPZ can then bind to the bulk membrane lipids or to membrane proteins on the cytoplasmic side (103, 104).

The lipid binding properties of CPZ are fairly well established. CPZ preferentially binds to PC, PS, and phosphatidyl inositol (105), which, for the most part, are inner monolayer lipids. The amount of CPZ bound to dipalmityl phosphatidyl choline liposomes increased dramatically above the phase transition at pH 7.4 (106). Also, CPZ quenches perylene fluorescence. Perylene is known to be situated in the hydrocarbon region of the membrane (106). High concentrations (0.2-1 mM) of CPZ were shown not to affect lipid head group movement of ghosts (ESR study), but using 12-doxyl stearate, it was determined that the molecular motion in the lipid core was increased (107).

CPZ is known to modulate other membrane mediated events. 23 μM CPZ will change erythrocytes from discocytes to stomatocytes (108, 109) and eventually to spherostomatocytes (109) inducing a concomitant decrease (9-10%) in electrophoretic mobility (108) and in suspension viscosity at high shear rates (110).

CPZ, along with other phenothiazines, binds nonspecifically to calmodulin (111). Calmodulin, a calcium modulating protein, is known to regulate Ca^{2+}-Mg^{2+} ATPase activity (112-116). ^{125}I-labelled calmodulin can be derivatized with methyl-4-azidobenzoimidate (117). This azido calmodulin has been shown to activate the Ca^{2+}- Mg^{2+} ATPase in the erythrocyte membrane (118) and to photochemically cross-link with calmodulin binding proteins (118). Binding of this labelled calmodulin is calcium dependent (118). It has been shown to bind to

Ca^{2+}, Mg^{2+} ATPase and to a protein which is 230,000 daltons, about the same size as spectrin (118).

CPZ has been shown to decrease the Na^{+}, K^{+}-ATPase activity in erythrocytes (119). If the Na^{+}, K^{+}-ATPase inhibitor ouabain, is added to cells prior to CPZ, CPZ reportedly does not protect against lysis (119). Also, the ATP content of CPZ treated cells has been shown to drop to 10% of that of untreated cells (104), and membrane protein dephosphorylation of an 180,000 daltron protein and several proteins in the 60,000-80,000 dalton rage is induced (104).

By binding to calmodulin, CPZ could alter spectrin and/or Ca^{2+}-Mg^{2+} ATPase conformation. If CPZ alters spectrin structure it would modulate the entire cyto-skeletal matrix. If CPZ binds to the calmodulin controlling Ca^{2+}-Mg^{2+} ATPase, it would modulate an important intrinsic membrane protein. At micromolar concentrations, addition of CPZ induces vast shape changes. This is indicatiave of vast changes in the cyto-skeletal matrix. As the CPZ concentration in increased, the binding sites would become saturated and CPZ would begin to act like a detergent. CPZ displays a biphasic dose dependent curve of its affects on hypotonic hemolysis (120, 121). As the concentration of CPZ is increased above 23 μM (maximal protection from lysis), the drug's calmodulin binding sites may become saturated, and the CPZ may then enter the lipid matrix. If the dose of CPZ is further increased, the hypotonic hemolysis studies show hemolysis greater than 100% (121). This indicates that at high concentrations, CPZ is detergent like, since it solubilizes the membrane bound hemoglobin.

In conclusion, at low concentrations CPZ appears to interact with or affect the protein cytoskeletal matrix to alter erythrocyte shape.

As the concentration of CPZ is increased, it becomes detergent-like, and finally lytic.

H. Objectives

In this thesis presentation, I show that several compounds affect erythrocyte membrane structure. These changes were observed by two different techniques. The major technique used was electron spin resonance (ESR) spectroscopy. I used the fatty acid spin probe 5-doxyl stearate. This technique (ESR) gives information on the spin probe mobility and the general environment around the free electron. The other technique was hypotonic hemolysis. Hypotonic hemolysis is one procedure used to determine the fragility of erythrocytes. Using these two techniques, I looked at how various compounds affected erythrocyte membrane structure on a molecular level (ESR studies) and on the level of the whole cell (hypotonic hemolysis studies).

The concentration of perturbant needed to induce membrane structural changes range from picomolar levels for PGE_1 and PGE_2 up to the millimolar range for the anionic perturbants such as HEPES and phosphate. In this thesis presentation I wish to show that alteration of even "minor" components of the membrane can induce an overall membrane structural change.

In conclusion, I would like to advance the theory that changes in membrane lipid order can be instigated by structural changes in the cytoskeletal matrix. Also, I believe the converse is true; that changing the conformation of one of the components of the cytoskeletal matrix will not only affect the rest of the matrix (see fig. 1), but also the overlying membrane.

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CHAPTER II

Title: Alterations in the Human Erythrocyte Membrane Induced by Anions. I. Anions Affect Membrane Physical Structure.

Authors: Denise L. Mazorow, Alfred Haug, and Estelle J. McGroarty

ABSTRACT

Fresh whole human erythrocytes were used to study the effects of ions on the erythrocyte membrane properties. Various anionic agents affect the human erythrocyte membrane structure as determined using electron spin resonance (ESR) probing techniques. Membrane order parameter was increased with certain anionic agents, the degree of perturbation dependent on the anion and the anion concentration. Control studies were performed in a physiological bicarbonate buffer. N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) was found to be the most perturbing of the anions used with phosphate less pertrubing and sulfate least perturbing. Our results suggest that anions may effect their changes of membrane order by partially or totally blocking the anion port. To substantiate this, the anion port blocking agents 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene and 4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonic acid were used. Both blocking agents affected membrane phase transition temperature. Blocking the anion port may be indirectly inducing a change in membrane structure. The calcium ionophore A23187 was shown to increase membrane order. This ionophore allows Ca^{2+} ion influx into the erythrocyte which presumably induces cross-linking of the phosphatidylserine head groups and in turn increases membrane order. Addition of dimethylsulfoxide induced a large increase in order parameter. Ouabain, a Na-K ATPase inhibitor decreased the phase transition. Thus, we found that

those compounds which affect membrane protein structure appear to affect membrane lipid structure as well.

INTRODUCTION

One of the critical functions of the erythrocyte is to facilitate O_2/CO_2 exchange in the periphery. The passive exchange of anions, most specifically chloride and bicarbonate across the erythrocyte plasma membrane is believed to occur via a transmembrane protein known as band 3 or the anion channel (1). Band 3 represents more than 25% of the total membrane protein and has a molecular weight between 88,000 and 105,000 daltons (3). Within the membrane this protein apparently forms a dimer (4). There are five sulfhydryl groups in band 3 which can be labelled by N-ethylmaleimide (5). The C terminal end, 35,000 daltons by chymotryptic cleavage, is mostly located outside the cell and has two of the sulfhydryl groups (6). A 9,000 dalton segment of this 35,000 dalton piece traverses the membrane (6). It is also the segment which contains the two sulfhydryl groups. The other three sulfhydryl groups are located in the segment situated on the cytoplasmic side of the membrane (5, 7).

Band 3 is reportedly involved in anion transport. Several anion transport inhibitors including the stilbene derivatives 4-acetamido-4'-diisothiocyano-2,2'-disulfonic acid stilbene (SITS) and 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS) bind to sulfhydryl groups of band 3 (8-10). Changes in band 3 structure can be monitored with DIDS fluorescence (11), and changes in anion flux can be monitored using $^{35}SO_4^{2-}$ and $^{36}Cl^-$ (12,13). DIDS binding to band 3

has been shown to alter the amount of hemoglobin bound to band 3 (14) and to alter band 3 structure (15).

Recent work on the mechanism of anion transport across the erythrocyte membrane via band 3 suggests that influx and efflux of anions are separate events (16), and a ping-pong model best describes the mechanism of anion transport (17, 18). It has been suggested that the individual units of the band 3 dimer transport anions independently (19) with the primary event being anion efflux (19).

In this study we show that changing the anionic milieu from that found in situ alters the erythrocyte membrane structure. Membrane structural changes were induced by changes in the buffer or by inhibiting ion transport.

MATERIALS AND METHODS

Freshly drawn blood was obtained by venapuncture from at least two healthy individuals for each run. Only non-smokers who had taken no alcohol or medication for at least 48 hours prior to venapuncture were used as donors. Anticoagulation was effected by defibrination. Cells were centrifuged and the buffy coat was removed using standard procedures. The erythrocytes were washed three times in 300 millosmols (mOsm) NaCl pH 7.4, and packed to a hematocrit of 70 ± 1. A 1 ml aliquot of the packed cells was placed into a test tube in which 6 to 8 microliters of a 30 mM ethanolic solution of 5-doxyl stearate (5-DS) had been evaporated. The preparation was gently mixed for 2 minutes. The 5-DS labelled cells were added to 40 ml of the salt solution being tested and incubated for 10 minutes at 37°C. The salt solutions used were: a modified Earles' salt solution containing 145 mM NaCl, 5 mM KCl, 0.7 mM $MgSO_4$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, and 22 mM $NaHCO_3$ at pH 7.4; a phosphate buffer (pH 7.0 and 7.4) containing 145 mM NaCl, 5 mM KCl, 0.7 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose, 3.5 mM NaH_2PO_4 and 1.5 mM Na_2HPO_4; a phosphate buffer with 0.5 mM carbonic acid (PO_4/HCO_3 buffer), pH 7.0 and pH 7.4; a sulfate buffer containing Earles' salts plus 4 mM sulfate; a N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) buffer containing Earles' salts without $NaHCO_3$ and 30 mM HEPES; an Earles' salts solution with 15 mM HEPES and 11 mM $NaHCO_3$ ($HEPES/HCO_3$ buffer); filtered serum (proteins larger than 10,000

daltons were removed with ultrafiltration using an amicon filter UM 10) pH 7.4; and an unbuffered 300 mOsm NaCl pH 7.4. All cells were incubated for 10 minutes at 37°C in the presence of perturbants in the modified Earles' salt solution. The cells were packed to a hematocrit of 70 ± 1 and were placed in a quartz cuvette for electron spin resonance (ESR) analysis. All ESR experiments were carried out with a Varian century E line ESR spectrometer. An external calibrated thermistor probe was used to monitor the temperature dependent spectral parameters over the range of 20°C to 48°C. All temperature dependent ESR parameters were shown to change reversibly in this range.

Hypotonic Hemolysis.

Hypotonic hemolysis studies were performed in both a phosphate buffer and an Earles' salt buffer under a variety of conditions. The solutions were prepared by diluting an isotonic solution (300 mOsm) with double distilled water to the desired hypotonicities. The solutions studied were: phosphate buffer, pH 7.4, 155 mOsm; Earles' salts without HCO_3^-, 110 mOsm; Earles' salts with and without HCO_3^-, 126 mOsm; Earles' salts with HCO_3^-, 150 mOsm; phosphate buffer without calcium 165 mOsm; and Earles' salts without calcium, 160 mOsm. The osmolarity of the solutions was determined using an Advanced Instruments osmoter model 3R. A 10% (v/v) solution of erythrocytes was placed in 5 ml of hypotonic buffer such that the final concentration of cells was 0.1%. These cells were incubated for seven minutes in hypotonic buffer and returned to isotonicity by addition of the necessary amount of 4 M NaCl. The cells were centrifuged and the degree of lysis was

determined by the amount of absorbance at 543 nm in the supernatant solution. 100% lysis was determined by placing the cells in double distilled water and 0% lysis was determined by reading the degree of lysis for cells placed in a 300 mOsm solution. Percentage lysis was taken as the difference between these two values.

ESR Spectral Analysis.

In order to determine the relative fluidity of the erythrocyte membrane in the different buffer systems, the membrane of the intact cell was labelled with 5-DS. 5-DS incorporates into the lipid phase of the membrane in such a way that the unpaired electron of the nitroxide radical is situated close to but shielded from the aqueous phase. Spin labelled preparations were placed in the resonance cavity of an ESR spectrometer. In this fashion the erythrocyte membrane preparation was exposed to microwaves of a constant frequency while held in a linearly varying magnetic field corresponding to the spin states of the unpaired electron. The spectra obtained are displayed as the first derivative of the microwave absorption plotted in gauss against an increasing magnetic field. The distance betwen the low field and high field absorption peaks, the hyperfine splitting parameter ($2T_{||}$), is a measure of the largest energy difference between the spin states of the unpaired electron. With increasing temperatures, intermolecular influences upon the unpaired electron tend to average, resulting in smaller values of $2T_{||}$ which reflect more fluid environments. $2T_{||}$, therefore is related to the viscosity of the environment for which the probe is reporting. Large values of $2T_{||}$ indicate more fluid environments.

The order parameter, S, measures the deviation of the observed signal from that of a uniform orientation of the probe. For a uniformly oriented sample S=1; for a random sample S=0. Order parameter changes of less than 1% are considered to be biologically insignificant (20, 21).

Computer Analysis.

The data were analyzed by an iterative least squares program (22). Brifely, a B-spline (23) was used to provide a smooth fit for the ESR data and points of inflection were used to group data. Regression lines were calculated for each group and then plotted. This analysis allowed the determination of break points. Such breaks in the temperature dependence of $2T_{11}$ and S have been correlated with lipid phase separations or lipid phase transitions from gel to liquid crystalline lipid states (24).

RESULTS

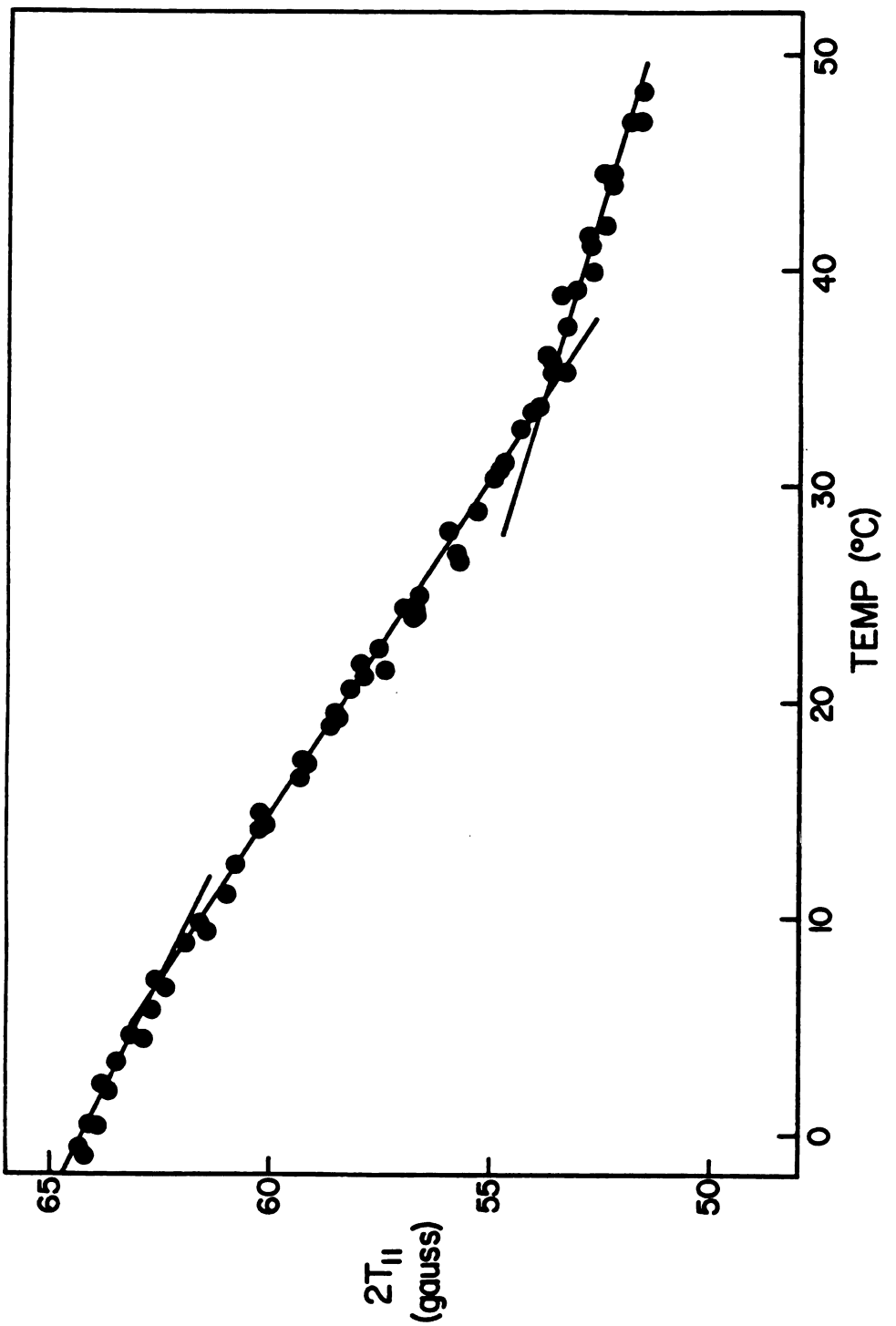
ESR spectra from cells labelled with 5-DS showed little free probe signal (Figure 1). Spectral symmetry of the low field peak indicates that the majority of the spin label was located in a single uniform environment over the temperature range examined (20°C-48°C). The hyperfine splitting parameter, $2T_{11}$, decreased linearly as a function of increasing temperature (Figure 2a, 2b) with a discontinuity occurring between 23°C and 39°C depending on the buffer in which the cells were suspended. Spectra recorded above 12°C permitted the determination of the order paramter S. When S was plotted as a function of temperature, a break similar to that observed with $2T_{11}$ was detected (Figure 3). The transition temperatures measured using S and $2T_{11}$ were nearly the same and may indicate protein-lipid structural changes in the erythrocyte membrane.

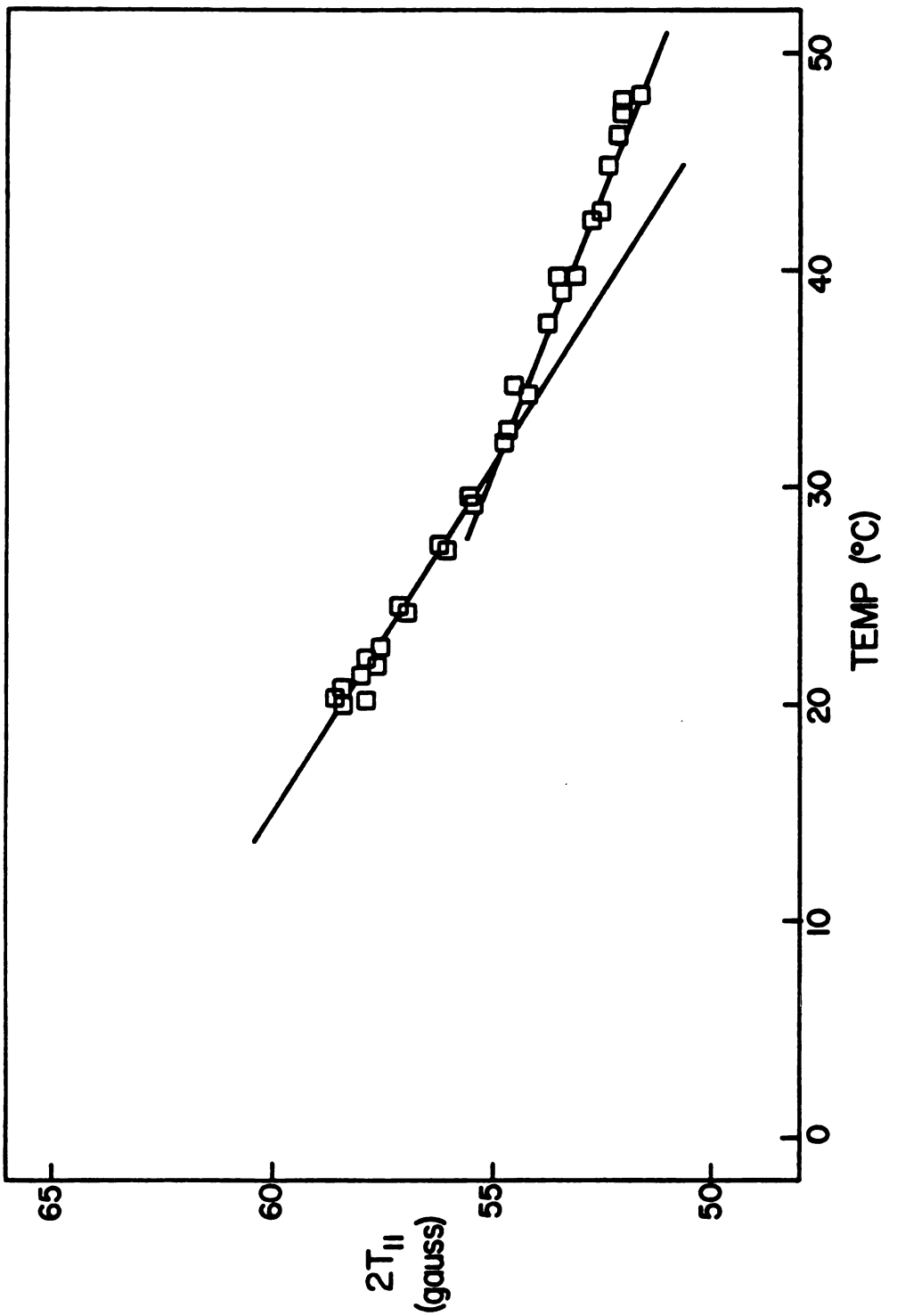
Our results indicated first, that the presence of specific anions alter the interactions in the human erythrocyte membrane. Secondly, we found that the ionic distribution across the erythrocyte membrane altered lipid-lipid and lipid-protein interactions. Finally we found that different buffer systems affect the fragility of the erythrocyte as seen by hypotonic hemolysis.

Figure 1. Electron spin resonance spectra of human erythrocytes labelled with 5-doxyl stearate. The spectra were taken at the temperatures indicated. Absence of free probe is indicated by the lack of a major absorption signal at points indicated by arrows. Symmetry of high and low field peaks reveals that the probe is in a single environment.

Figure 2a. The temperature dependent change in the hyperfine splitting parameter ($2T_{\parallel}$) of erythrocytes labelled with 5-doxyl stearate and suspended in Earles salts.

Figure 2b. The temperature dependent change in the hyperfine splitting parameter ($2T_{\parallel}$) of erythrocytes labelled with 5-doxyl stearate and suspended in Earles salts containing 10^{-7} M A23187.


65
60
55
50
$2T_{II}$
(gauss)
0
10
20
30
40
50
TEMP (°C)


65
60
55
50
$2T_{11}$
(gauss)
0
10
20
30
40
50
TEMP (°C)

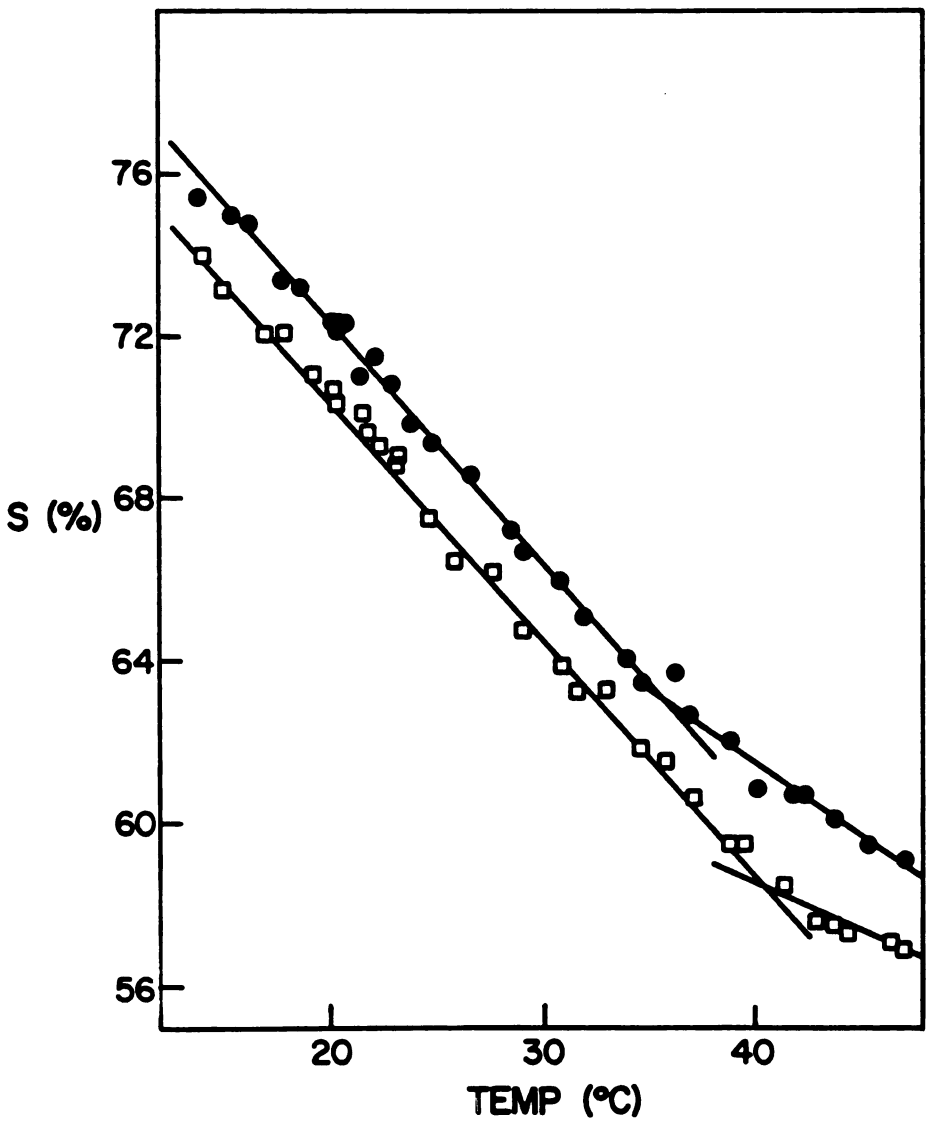


Figure 3. The temperature dependent change in the order parameter (S) of erythrocytes labelled with 5-doxyl stearate and suspended in Earles salts (●—●) or in Earles salts with 10^{-7} M A23187 (□—□).

Anion Perturbation.

Filtered serum, which is close to whole serum in ionic composition was considered as a control solution for resuspending cells. Whole serum was not used since the serum albumin sequestered most of the spin probe. Any variations in cell structure from that of cells in filtered serum were considered to be a perturbation of the erythrocyte membrane. Since the structure of cells in modified Earles' salts was essentially indistinguishable from that of cells in the filtered serum (Table 1), the Earles' salts was used as a standard salt solution. The membrane phase transition of cells in Earle's salts as observed by $2T_{11}$ vs. temp and S vs. temp was at 37°C (Figure 2A, 3). Cells in Earle's salts and the Earles' salts with glucose were also essentially identical in their physical properties (Table 1). The phase transition temperature of cells in isotonic NaCl was reduced by several degrees (Table 1). Phosphate buffer at pH 7.0 and pH 7.4 in the presence of bicarbonate (approx. 0.5 mM) and at pH 7.0 in the absence of calcium induced membrane perturbation. The phosphate buffer at pH 7.0 with or without calcium perturbed the erythrocyte membrane. Phosphate buffer, pH 7.0, slightly ordered the membrane and this was about the same change as seen in phosphate buffer at pH 7.4 (Table 1, Figure 4a, 4b). the presence of bicarbonate in the solution caused significant ordering of the membrane relative to phosphate buffer alone at pH 7.0 but disordered slightly at pH 7.4 (Figure 4a, 4b).

30 mM HEPES also significantly ordered the erythrocyte membrane at room temperature (24°C) and at physiological temperature (37°C) as did a solution of 15 mM HEPES and 11 mM bicarbonate in Earles' salts. An

Table 1

Effects of buffers on erythrocyte membrane phase transitions

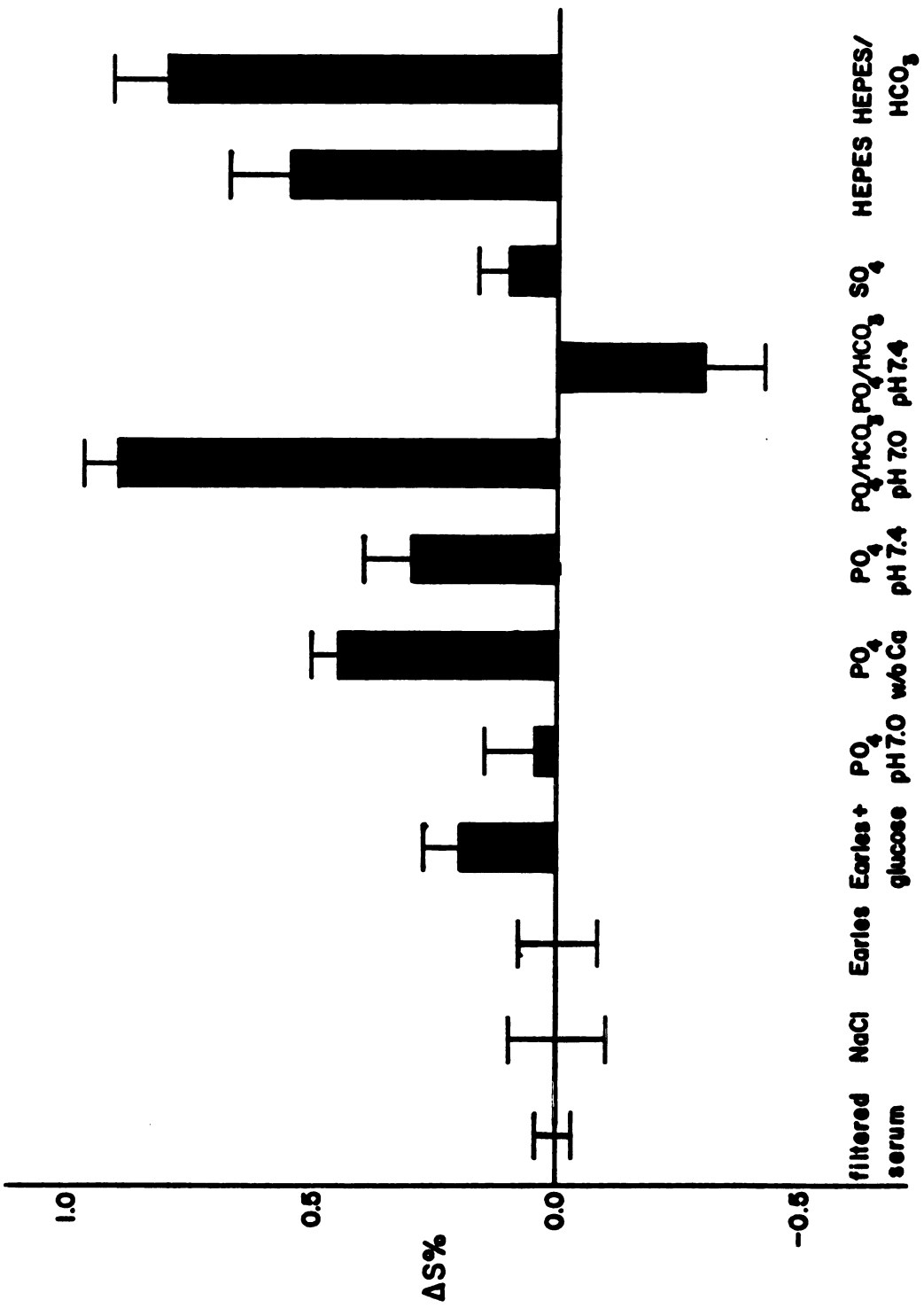
Suspending Buffer	Phase transition temperature determined using $2T_{11}$	S	Average
filtered serum	37.08	37.4	37.24
NaCl (unbuffered)	33.11	34.38	33.45
Earles' modified salts	38.39	35.77	37.08
Earles w/glucose	35.8	36.22	36.01
PO_4 pH 7.0	35.0	38.8	36.9
PO_4 pH 7.4	37.2	36.2	36.7
PO_4/HCO_3 pH 7.0	30.40	31.2	30.8
PO_4/HCO_3 pH 7.4	29.25	30.38	29.82
SO_4 in Earles	33.33	31.24	32.29
HEPES	33.61	32.50	33.06
HEPES/HCO_3^-	31.72	30.53	31.13

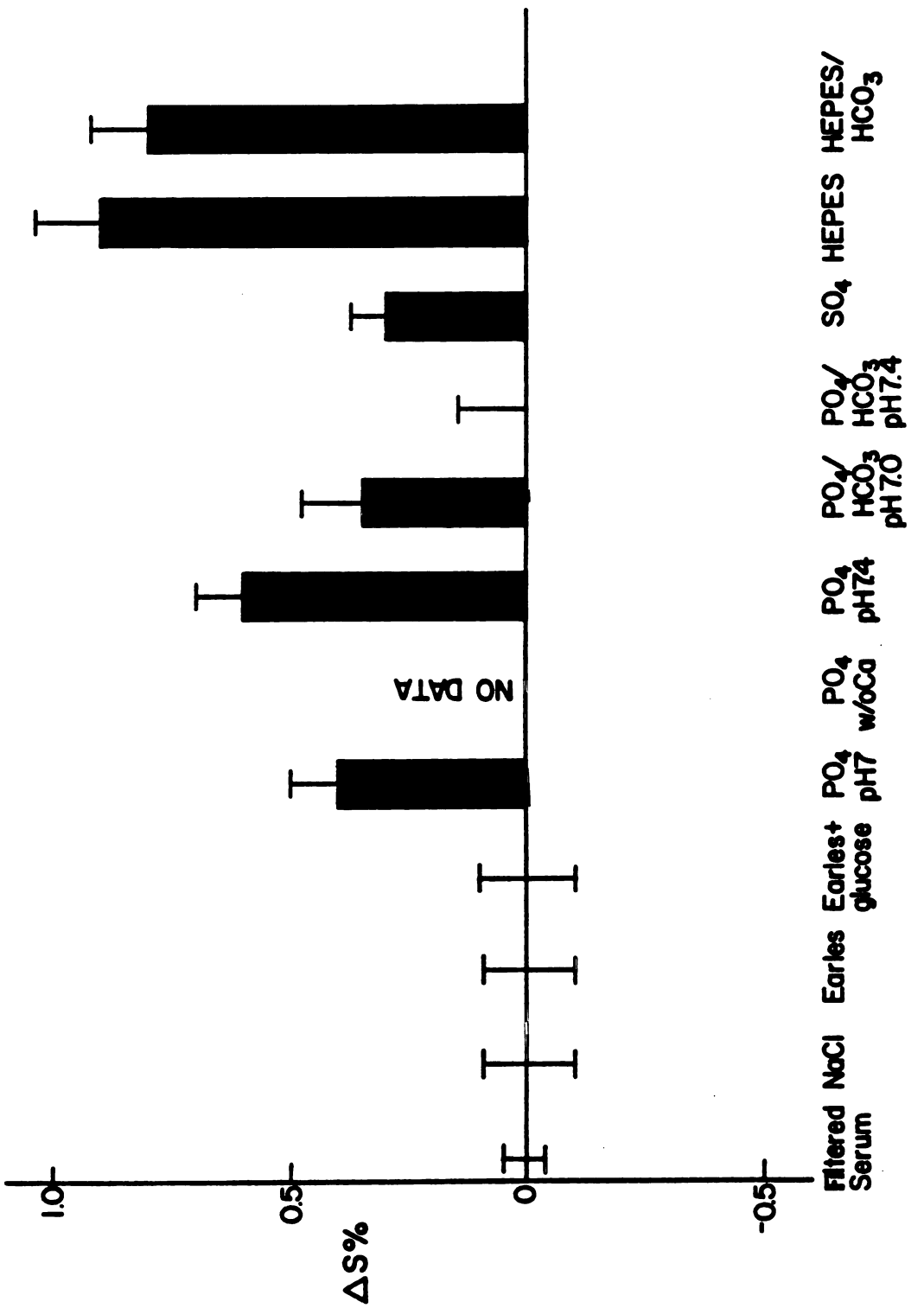
Table 2

Effects of perturtants in modified Earle's salts on erythrocyte membrane phase transitions

Perturbant	Phase transition temperature determined using $2T_{11}$	S	Average
modified Earles' salt	38.39	35.77	37.08
Ouabain 10^{-5} M	29.24	32.97	31.11
Ouabain 10^{-6} M	33.26	37.5	35.38
A23187 10^{-7} M	32.38	32.16	32.27
A23187 10^{-8} M	27.45	24.33	25.87
A23187 10^{-9} M	37.88	23.15	30.52
.028 M DMSO	37.27	37.47	37.37
DIDS 10^{-5} M	No transition	No transition	No transition
SITS 10^{-5} M	35.78	35.78	35.78

Figure 4. Change in order parameter of cell labelled with 5-doxyl stearate and suspended in various buffers compared to cells suspended in filtered serum. Membrane order was measured at 24°C (a) and 37°C (b).


1.0
0.5
0.0
-0.5
ΔS%
filtered serum
NaCl
Earles
Earles + glucose
PO_4 pH 7.0
PO_4 w/o Ca
PO_4 pH 7.4
PO_4/HCO_3 pH 7.0
PO_4/HCO_3 pH 7.4
SO_4
HEPES
HEPES/ HCO_3


1.0
0.5
0
-0.5
ΔS%
NO DATA
Filtered Serum
NaCl
Earles
Earles+ glucose
PO4 pH7
PO4 w/oCa
PO4 pH7.4
PO4/ HCO3 pH 7.0
PO4/ HCO3 pH 7.4
SO4
HEPES
HEPES/ HCO3

increase of sulfate ions from 0.7 mM to 4 mM in the Earles' salts solution caused minimal changes in membrane order (Figure 4a, 4b).

The phase transition of membranes from cells in Earles' salts, Earles' salts with glucose, and phosphate buffer pH 7.0 and pH 7.4 were not significantly different from that of cells in filtered serum. The phase transition temperature was lower compared to control when cells were in unbuffered NaCl, SO_4 in Earles, HEPES buffers, and the phase transition temperature was further reduced when bicarbonate was added to either the phosphate buffer or the HEPES buffer (Table 1).

Ion Gradient Perturbation.

The perturbants ouabain, 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS), 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS), A23187, and dimethylsulfoxide (DMSO) (Table 2, Figure 5a, 5b) directly or indirectly alter the ion environment or membrane structure of the erythrocyte. Ouabain inhibits the Na^+/K^+ ATPase which in turn causes an alteration in internal to external ratios of Na^+/K^+ levels. Both DIDS and SITS bind to and inhibit the erythrocyte anion port and the Ca^{2+} ionophore A23187 allows Ca^{2+} to enter the erythrocyte. DMSO is known to solubilize membrane protein components (25).

Ouabain at 10^{-5} caused slight changes in membrane order at both 24°C and 37°C and a very dramatic shift in the phase transition temperature from 37.2 to 31.1°C. The changes in lipid order induced with 10^{-6} M ouabain were small and the phase transition temperature was less affected at the lower temperature.

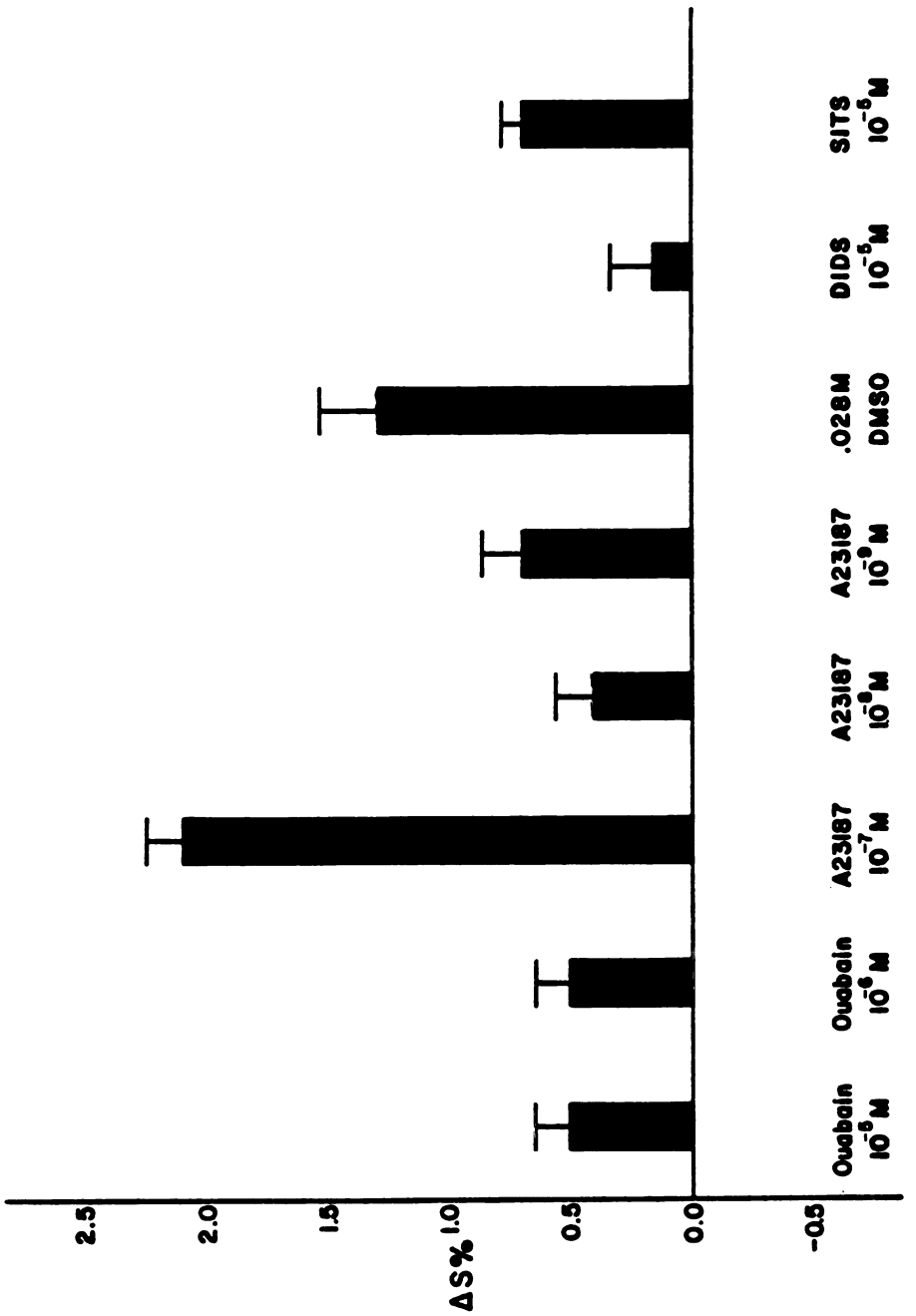
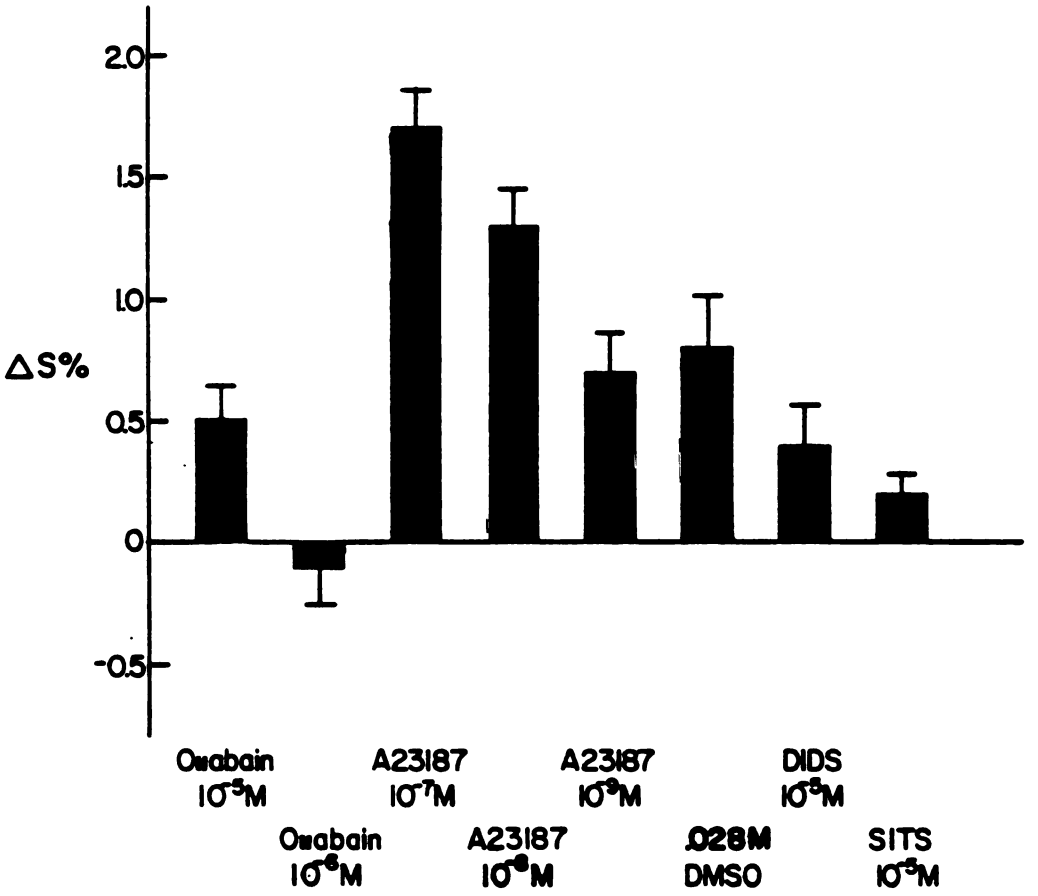


Figure 5. Change in order parameter of cells labelled with 5-doxyl stearate and suspended in Earles salts containing different perturbants compared with cells suspended in Earles salts alone. Membrane order was measured at 24°C (a) and 37°C (b).


2.0
1.5
1.0
ΔS%
0.5
0
-0.5
Ouabain
10⁻⁵M
Ouabain
10⁻⁶M
A23187
10⁻⁷M
A23187
10⁻⁸M
A23187
10⁻⁹M
.028M
DMSO
DIDS
10⁻⁵M
SITS
10⁻⁵M

When the calcium ionophore A23187 was added to erythrocytes at final concentrations of 10^{-7} M, 10^{-8} M, and 10^{-9} M, membrane order increased dramatically. At 24°C, A23187 at 10^{-7} M induced an increase in membrane order which was greater than 2% (Figure 3). At 37°C, the increase in order was 1.7% (Figure 3). The degree of perturbation induced by A23187 decreased with decreasing concentration. The shift in phase transition temperature induced by A23187 was dose dependent, with the maximal decrease occurring at 10^{-8} M. However, at 10^{-7} M (Fig. 2B) and 10^{-9} M, A23187 still significantly decreased the phase transition temperature (32.3°C and 30.6°C respectively). The increase in order induced by 10^{-7} M A23187 was 0.89% (data not shown) at 24°C in the absence of Ca^{2+}.

DIDS and SITS are both anion port blocking agents although DIDS is more specific for anion port binding. The level of DIDS and SITS added was sufficient to block all available anion port sites in the cell sample. Although DIDS and SITS at 10^{-5} M did not induce any appreciable changes in membrane transition temperature, SITS at 10^{-5} M did induce a slight increase in membrane order at 24°C. DIDS did not induce a change in the phase transition temperature, but caused an elimination of the phase transition. SITS, in contrast, induced a slight decrease in the phase transition temperature.

Dramatic increases in order parameter of the probe were seen upon the addition of DMSO to the erythrocyte suspension, but no changes in phase transition temperature were seen.

Hypotonic Hemolysis Studies.

The degree of lysis depended on the buffer and ions present. The presence of bicarbonate in the Earles' salts solution increased the erythrocytes' fragility. In the absence of bicarbonate 50% lysis occurred at 110 mOsm (pH 8.2) whereas in the presence of bicarbonate 50% lysis occurred at 150 mOsm (pH 7.4). Eleven percent of the cells suspended in 126 mOsm Earles' salts solution without bicarbonate lysed while 93% lysed in the same solution containing bicarbonate. In the phosphate buffer, pH 7.6, 50% lysis was achieved with 155 mOsm phosphate. When calcium was deleted from the buffers, the osmolarity of the buffers needed to be raised by 10 mOsm to maintain 50% lysis, i.e., cells in phosphate without calcium were 50% lysed at 165 mOsm and in Earles' salts (with bicarbonate) without calcium were 50% lysed at 155 mOsm.

DISCUSSION

Membrane integrity is vital in normal cell function. We have evidence which suggests that changing the anion environment affects the binding properties of Prostaglandins E_1 and E_2 (26). We suggest that agents which alter membrane structure may alter or disrupt the proper hormone binding (26) and/or enzyme (27) activity within the erythrocyte membrane. We have noted that both anions and membrane perturbants such as anesthetics (24) alter membrane structure and some of these changes may result from alterations in the ionic environment of the membrane.

Changes in membrane lipid order reflect changes in lipid domain structure (27) while alterations in the temperature of the membrane phase transition may reflect changes in membrane protein-lipid interactions (24). Perturbations in ion transport across the membrane have been shown to influence membrane order (28), membrane phase transition temperature (29), and protein conformation (30). Ouabain, acting extracellularly, (31) is a specific inhibitor of the Na^+/K^+ ATPase, and we have shown that this inhibitor induces a decrease in phase transition temperature without changing the membrane order. In the presence of ouabain the normal physiological ratio of K^+_{in}/ Na^+out cannot be maintained due to passive flux (32). Since the Na^+-K^+ ATPase is closely associated with erythrocyte membrane protein band 3 (33) alterations in the anion transport in the presence of ouabain may

occur which are associated with the structure or activity of the Na^+-K^+ ATPase. The shift in phase transition induced by the presence of ouabain may be due to protein conformational changes of the anion port-Na^+-K^+ ATPase complex. Conversely, the shift may result from lipid-lipid interactions induced by the inhibitor which occur as a result of ion inbalance. A similar shift in phase transition temperature was seen with the addition of the anion port blocking agent SITS. SITS induced a small decrease in the phase transition temperature while causing a small change in membrane order. The blocking agent DIDS induced no appreciable change in membrane order, but its alteration of the membrane phase transition was most unusual: no transition was observed. In all of these cases, upon the addition of specific inhibitors, membrane ion transport was altered and a change in the phase transition occurred. These alterations in phase transition temperature may reflect conformational changes in the protein-lipid configuration which regulates the ionic homeostasis.

The calcium ionophore A23187, when added in the presence of calcium induced marked alterations in both the phase transition temperature and in membrane order. Since the increase in membrane order was dependent on ionophore concentration, it is presumable that higher concentrations of ionophore allowed more calcium to enter the erythrocyte. The ordering could be due to Ca^{2+} induced cross-linking of phosphotidylserine head groups (34), Ca^{2+} cross-linking of proteins forming heteropolymers (35), or Ca^{2+} induced cross-linking of band 3 with spectrin (36). Membrane ordering due to high levels of calcium could also be due to changes in phospholipid phosphorylation (37). The decrease in phase transition temperature in the presence of the ionophore

was also dose dependent. This alteration may reflect restructuring of intrinsic membrane proteins (38).

High concentrations of anions are thought to disrupt the charge distribution across the erythrocyte membrane (39). This disruption could occur in two ways. First anions may alter either the lipids within the inner or outer monolayer of the cell (much as calcium perturbs the inner monolayer of the erythrocyte); or secondly, large anions may "block" the anion port and thus alter the transmembrane distribution of anions. Chloride ions traverse the membrane several orders of magnitude faster (40) than either sulfate or phosphate ions; whereas, phosphate and sulfate have similar rates of flux (40). At physiological temperatures chloride transport is relatively independent of pH but sulfate and phosphate flux depends on the pH (40). We have shown that membrane structural changes occur in the erythrocyte in phosphate buffer when the pH is changed from pH 7.0 to pH 7.4. In phosphate buffer the membrane was less ordered at the lower pH. Changes in anion transport rates have been shown to occur when specific anions are raised above the physiological concentration. Examples include chloride inhibition of phosphate (41) and sulfate transport (40, 42); sulfate inhibition of phosphate (43, 44) and chloride transport (40); chloride and bicarbonate inhibition of chloride transport (44, 45) and phosphate inhibition of sulfate and chloride exchange (46). The buffer HEPES, also an anion, is known to decrease the stability of red cell ghosts relative to phosphate buffer (47). This decrease in stability may be due to alterations of the transmembrane anionic environment. In our studies membranes of erythrocytes in HEPES, were more

ordered and had a lowered phase transition temperature compared to cells in Earles' salts.

The decrease in phase transition temperature induced by anion perturbation across the membrane may be the result of alteration in the lipid protein matrix caused by conformational changes in band 3 (30, 24). Although the phosphate buffer, pH 7.4, induced no change in either membrane order or phase transition, phosphate in the presence of bicarbonate disordered the membrane relative to membranes in phosphate, at either pH 7.0 or pH 7.4. This alteration in membrane structure may relate to the Ca^{2+}-Mg^{2+} ATPase. It has recently been shown that H_2PO_4 eliminates the B_2 transition (29) of band 3, which involves the cytoplasmic portion of the protein, detected with microcalorimetry. This transition is reported to be related to the anion port structure and is associated with a cytoplasmic protein (29). Anomalous changes in band 3 conformation were also reported in the presence of high phosphate by Ginsburg (30). We saw a reduction in both phase transition and in lipid order when both phosphate and bicarbonate were present. Phosphate alone may inactivate the Ca^{2+}-Mg^{2+} ATPase and the presence of bicarbonate may reactivate the ATPase (48). The presence of bicarbonate in HEPES buffer may induce the same changes.

Calcium may help to stabilize the erythrocyte membrane. If calcium is present in the buffer calcium may leak in at the beginning of lysis and help stabilize the membrane by cross-linking the membrane structural proteins (e.g. spectrin). We noted that bicarbonate destabilized the membrane to hypotonic hemolysis. This may be an effect of pH, but it is possible that destabilization is also related to the bicarbonate effect on the Ca^{2+}-Mg^{2+} ATPase. Erythrocytes in phosphate buffer

buffer and Earles' salts with bicarbonate require approximately the same osmolarity to achieve 50% lysis.

The erythrocyte membrane appears to exist in a state of flux. The phase transition temperature was shown to occur at body temperature, 37°C. Thus, the membrane appears to exist in the metastable transition state of gel + liquid crystalline to liquid crystalline (39). This state may allow for enhanced transport across the membrane. In addition, our evidence suggests that bicarbonate destabilizes the red cell membrane and may further facilitate an ion exchange across the membrane.

These anions and perturbants all change the ionic milieu of the erythrocyte membrane from physiological conditions. The induced changes alter the overall conformation of the erythrocyte membrane lipids and/or proteins. Alterations in erythrocyte membrane structure may be amplified by changes in ion transport, in the hormone receptor structure binding (26) and in membrane enzyme activity (50).

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CHAPTER III

Alterations in the Human Erythrocyte Membrane by Anions. II. Anions Affect Prostaglandin E_1 and E_2 Receptors

Authors: D.L. Mazorow, A. Haug, and E.J. McGroarty

ABSTRACT

Fresh whole human erythrocytes drawn only from female subjects were used to study the effects of prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), and the synthetic prostaglandin 16,16 dimethyl PGE_1 (di-Me PGE_1) on erythrocyte membrane properties. These prostaglandins affect erythrocyte membrane structure as determined using electron spin resonace probing techniques. The effects of prostaglandins on erythrocyte membrane structure were examined in several different buffer systems. Each of the prostaglandins induced an increase in membrane order when the erythrocytes were suspended in a physiological bicarbonate buffer (Earles'). When calcium was not added to the buffer the membrane ordering induced by PGE_1 was not affected, but the membrane ordering induced by PGE_2 was eliminated. Neither PGE_1 nor PGE_2 induced significant membrane ordering in an Earles' buffer containing glucose. Erythrocytes suspended in a phospahte buffer containing both glucose and calcium at either pH 7.0 or pH 7.4 were not significantly perturbed by PGE_1 or PGE_2. However, if 0.5 mM bicarbonate was not added to phosphate buffer, pH 7.0, the erythrocytes became dramatically disordered in the presence of either PGE_1 or PGE_2 at room temperature. Erythrocytes added to phosphate buffer pH 7.4 with 0.5 mM bicarbonate became ordered in the presence of both PGE_1 and PGE_2. Erythrocytes exposed to prostaglandins in a N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid buffer (HEPES), pH 7.4, became only minimally disordered

in the presence of prostaglandins. However, if 0.5 mM bicarbonate was added to the HEPES buffer (p 7.4), the erythrocytes became disordered in the presence of prostaglandins. Thus, we found that prostaglandins can alter erythrocyte membrane structure and that the degree of alteration can be modulated by different buffer components.

INTRODUCTION

Prostaglandins are a class of compounds derived from arachidonic acid, which are extremely biologically active and, generally, have a very short half-life. The techniques to characterize and measure prostaglandin binding were first developed using rat liver fractions (1). It was shown that the majority of the prostaglandin binding sites were located in the plasma membrane and that there existed a single binding site for the E type prostaglandins. Since then, E type receptors have been found in several different tissues. Prostaglandin E (PGE) receptors have been reported in the non-pregnant human myometrium plasma membrane (2), both the plasma membrane and the smooth endoplasmic reticulum from both rat and human skin (3, 4), the plasma and nucleus membranes of the bovine corpus luteum (5, 6), human platelet membrane (7), normal and transformed fibroblasts (8), frog erythrocytes (9), and human erythrocytes (10-15).

The function of the prostaglandin receptor is known in some of these tissues, but in others it has not yet been determined. Prostaglandins are believed to bind to protein receptors (3-5, 11, 16). In platelets, addition of PGI_2, resulted in a large increase in cAMP levels (17, 18). Prostaglandins, likewise, reportedly activate the adenyl cyclase of normal and transformed fibroblasts (8).

In other tissues, such as the human erythrocyte, the role of prostaglandin receptors is not yet clear. In this study, we investigated

the erythrocyte membrane structural changes induced by the E type prostaglandins under a variety of buffer conditions.

MATERIALS AND METHODS

The prostaglandins were a gift from Dr. M. Ruwart of the Upjohn Company. The 5-doxyl stearate (5-DS) was purchased from Molecular Probes.

The blood was obtained from non-smoking female volunteers and collected without a tourniquet. The erythrocytes were washed and packed in the buffers as described earlier (Chapter 2, Methods) and incubated in either prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), or the synthetic prostaglandin 16, 16 dimethyl PGE_1 (di-Me-PGE_1) for 30 minutes at 37°C and repacked to a hematocrit of 70 ± 1 (Chapter 2, Methods).

The prostaglandins were initially dissolved at millimolar concentrations in 100% ethanol and serially diluted in the appropriate buffer to the desired concentrations.

All other procedures and techniques are the same as described in Methods, Chapter 2.

RESULTS

EARLES BUFFER

PGE_1, PGE_2 and diMe-PGE_1 significantly ordered the erythrocyte membrane at room temperature (24°C). Maximal ordering occurred at 10^{-9} M for PGE_1, 10^{-8} M for PGE_2, and 10^{-10} M for diMe-PGE_1. At 37°C PGE_1 induced significant ordering of the erythrocyte membrane but PGE_2 and diMe-PGE_1 did not (Figure 1A, 1B).

The absence of calcium in the suspending medium (Earles without calcium) reduced the membrane structural changes induced by PGE_1 by between 30% and 55% at 24°C and by 0% to 50% at 37°C. PGE_2 induced no erythrocyte membrane structural changes in the absence of calcium at 24°C. However, the slight changes induced by PGE_2 at 37°C were not affected by the absence of calcium (Figure 1A, 1B). Neither PGE_1 nor PGE_2 induced any membrane structural changes in the presence of glucose.

PHOSPHATE BUFFER

In phosphate buffer pH 7.0 neither PGE_1 nor PGE_2 induced any significant membrane structural changes in the erythrocyte membrane at the concentrations studied. PGE_1 in phosphate buffer, pH 7.4, induced a disordering of the erythrocyte membrane at 24°C, but PGE_2 in

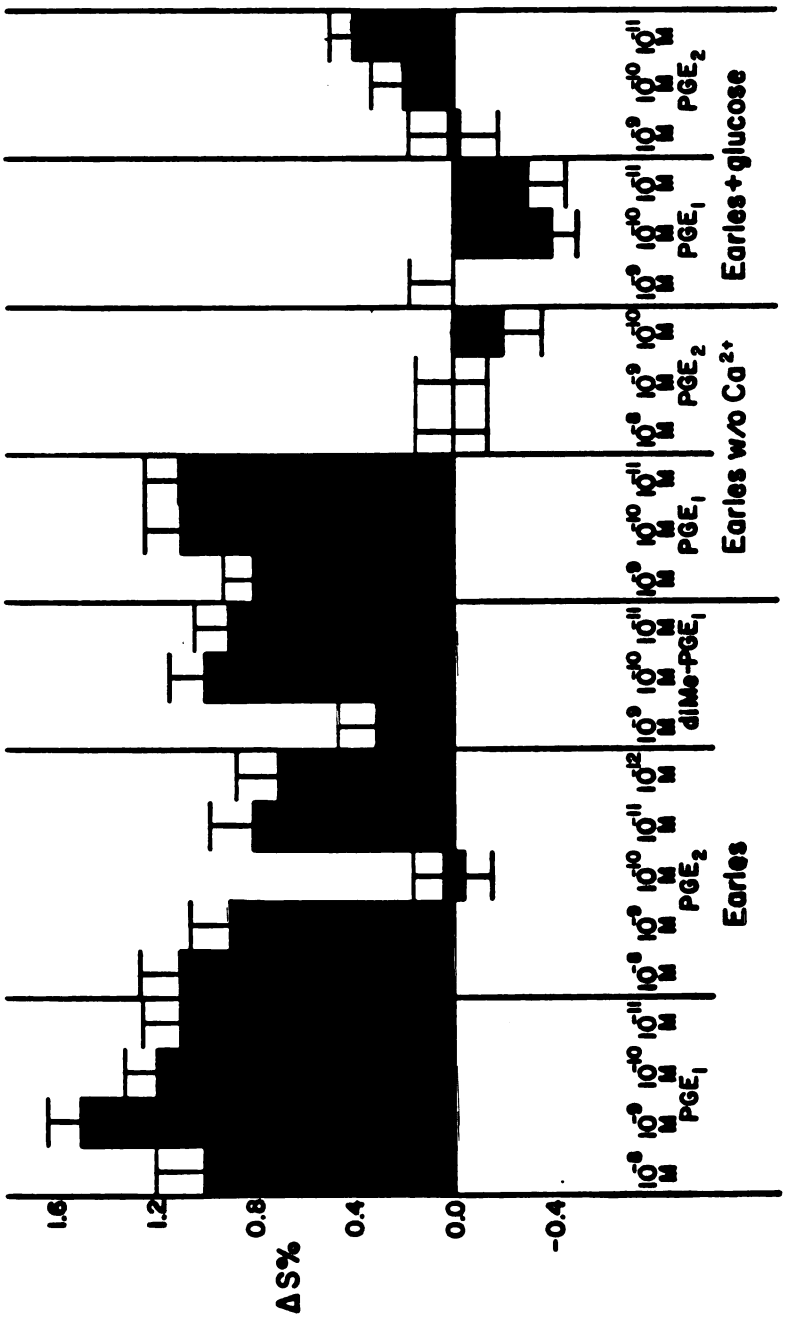
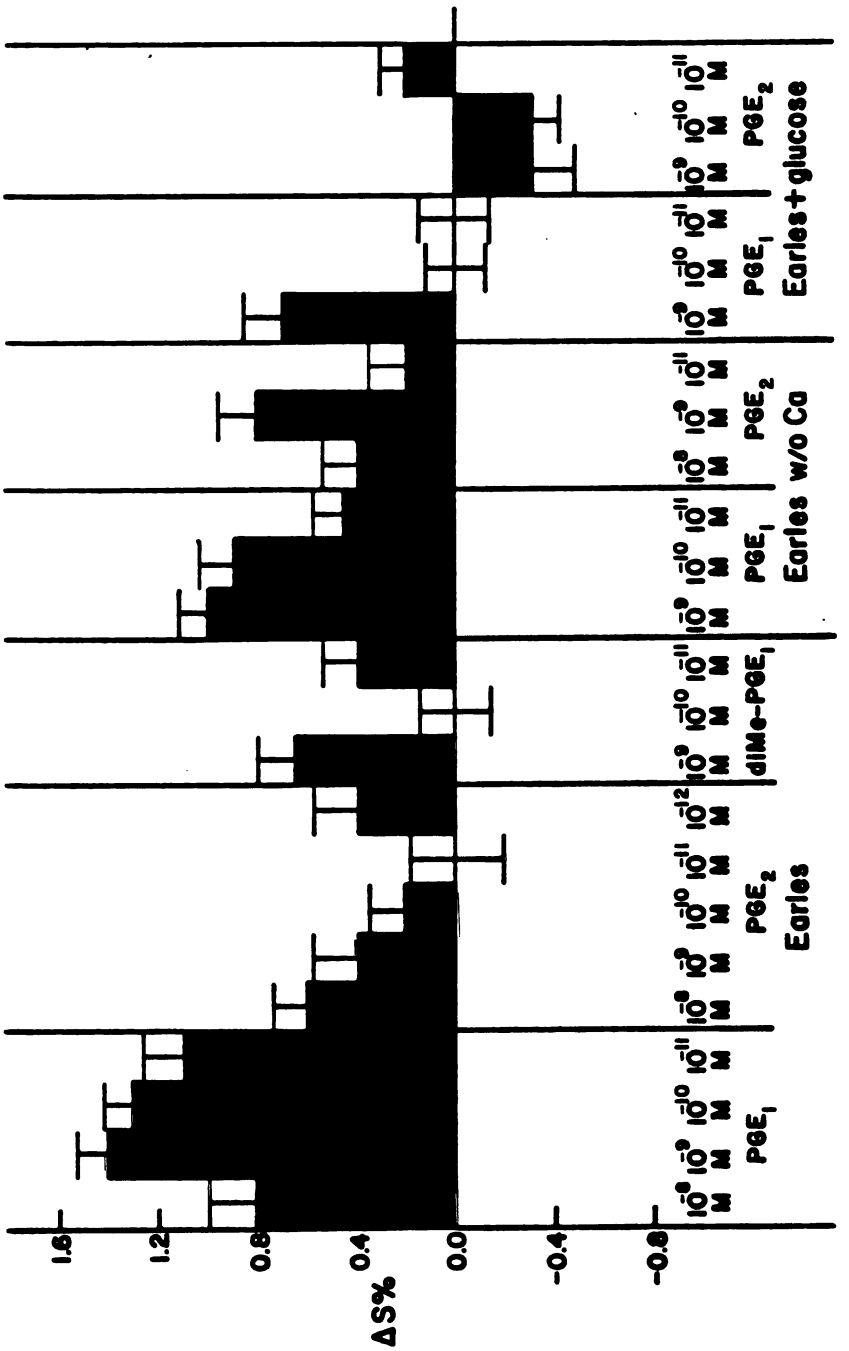


Figure 1. Percent changes in order parameter (ΔS%) of human erythrocytes from female volunteers in the presence of 10^{-8} M - 10^{-12} M prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), and prostaglandin 16, 16 dimethyl PGE_1 (di-Me PGE_1) suspended in Earles' salts, Earles salts without Ca^{2+}, and Earles salts plus glucose A. at 24°C, and B. at 37°C.


ΔS%
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
PGE1
PGE2
Earles
diMe-PGE1
Earles w/o Ca
Earles+glucose

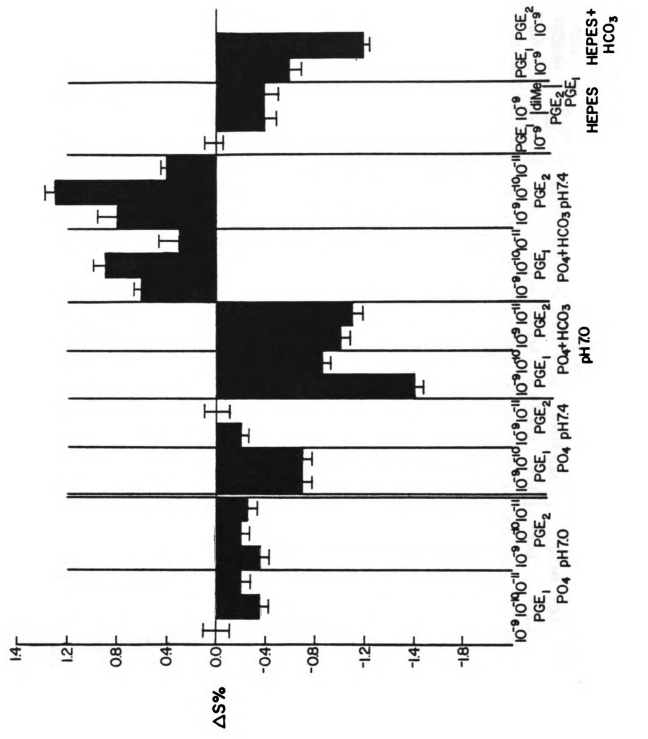
the same buffer induced no structural changes in the erythrocyte membrane.

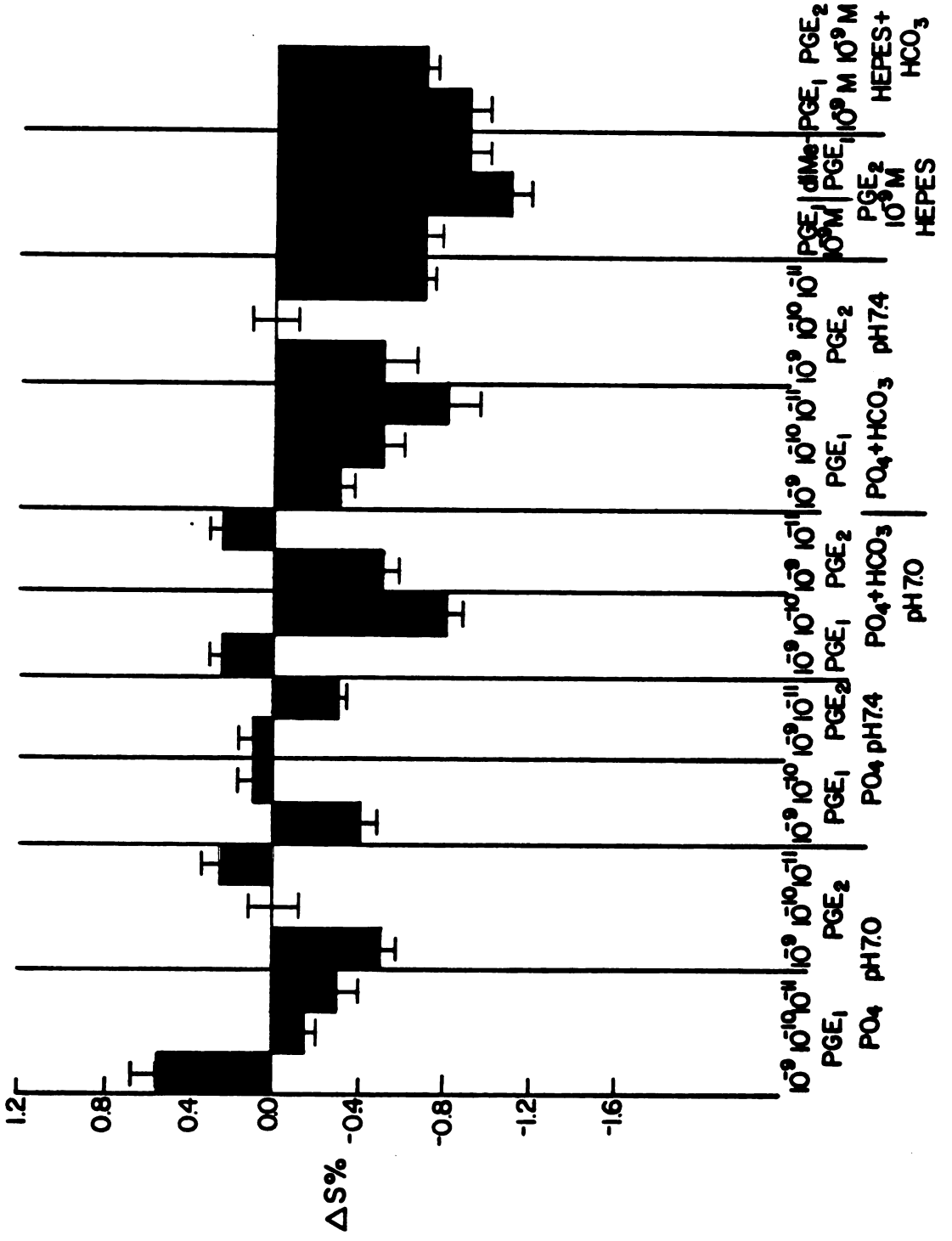
When PGE_1 or PGE_2 was added to phosphate buffer, pH 7.0 containing bicarbonate (PO_4/HCO_3) a disordering of the erythrocyte membrane was seen at 24°C with a less significant disordering at 37°C (Figure 2A,B). When PGE_1 or PGE_2 was added to erythrocytes suspended in phosphate buffer, pH 7.4, with bicarbonate (PO_4/HCO_3 pH 7.4), the membrane became more ordered at 24°C and less ordered at 37°C.

HEPES BUFFER

Erythrocytes were suspended in N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) buffer and mixed with either PGE_1, PGE_2, or diMe-PGE_1. At 24°C only minimal membrane structural changes were seen, but at 37°C each of the three prostaglandins induced membrane disordering. Both 10^{-9} M PGE_1 and 10^{-9} M PGE_2 induced a disordering of the erythrocyte membrane when suspended in a mixture of HEPES and bicarbonate buffer ($HEPES/HCO_3$) at both 24°C and 37°C (Figure 2A,B).

Figure 2. Percent changes in order parameters ($\Delta S\%$) of human erythrocytes from female volunteers in the presence of 10^{-9} - 10^{-11} M prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), and prostaglandin 16, 16 dimethyl PGE_1 (di-Me PGE_1) suspended in phosphate buffer, pH 7.0 (PO_4 pH 7.0); phosphate buffer, pH 7.4 (PO_4 pH 7.4); phosphate buffer with 0.5 mM bicarbonate pH 7.0 (PO_4+ HCO_3 pH 7.0); phosphate buffer with 0.5 mM bicarbonate, pH 7.4 (PO_4 + HCO_3 pH 7.4), HEPES buffer, and HEPES buffer pH 7.4 (HEPES); and HEPES buffer with 11 mM bicarbonate pH 7.4 (HEPES + HCO_3) A. at 24°C and B. at 37°C.


ΔS%
1.4
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-1.4
-1.8
PGE1 PGE2
PO4 pH7.0
PO4 pH7.4
pH7.0
PO4+HCO3 pH7.4
dMe
HEPES
HEPES+ HCO3


ΔS%
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-1.6
PGE1
PO4 pH7.0
PGE2
HEPES+ HCO3

DISCUSSION

Results presented here indicate that the amount and degree of structural changes induced within the erythrocyte membrane by prostaglandins is dependent upon the buffer used, the energy level of the cells (glucose present), the presence of calcium, the pH, and bicarbonate in a buffer.

Several studies have shown that the presence of very small amounts of prostaglandins affect erythrocyte whole cell properties such as protection from hemolysis. Kury et al (14) have shown that erythrocytes drawn from male volunteers were made less deformable and the membrane more ordered by PGE_2. The increase in order parameter was dependent on PGE_2 concentration with the maximal increase at 10^{-11} M. Conversely, PGE_1 was shown to have the opposite effect (14). It disordered the erythrocyte membrane and increased whole cell deformability with a maximal affect also at 10^{-11} M. In addition, their study reported that calcium was needed in the buffer for the PGE_1 and PGE_2 induced perturbations. Even though PGE_1 caused an increase and PGE_2 caused a decrease in whole cell deformability, the relative apparent viscosity (viscosity ratio blood/plasma) of the erythrocyte was not altered by the presence of either PGE_1 or PGE_2 (13) in phosphate buffer, pH 7.0.

It has been shown that the presence of either PGE_1 or PGE_2 alters the circular dichroism (CD) adsorption spectrum of washed

erythrocyte ghosts (14). Both prostaglandins reportedly cause a decrease in the mean residue ellipticity at 37°C when observed between 250 nm and 190 nm. The CD spectral changes, however, were not biphasic as a function of prostaglandin concentration.

In other studies erythrocytes in an isotonic solution which were shaken more than 200 times per minute showed either 6% (cells from females) or 10% (cells from males) lysis (15). 10^{-9} M PGE_1 or PGE_2 reduced lysis of cells from both male and female subjects, but the degree of protection was greater for erythrocytes drawn from male subjects. The presence of calcium did not affect protection resulting with PGE_1.

Our results show that at room temperature in an Earles buffer PGE_1, PGE_2, and diMe-PGE_1 each induce an ordering of the erythrocyte membrane. Contrary to the results of Kury et al (14) the absence of calcium did not dramatically affect the degree of structural alterations induced by PGE_1, but was needed for the PGE_2 induced membrane changes. PGE_1 and PGE_2 may initiate some calcium leakage which is protective to cells against lysis. Cells from male subjects are more susceptible to mechanical lysis and may also be more deformable. The calcium requiring PGE_1 induced changes seen by Kury and co-workers may reflect the use of erythrocytes only from male subjects. One reason that we did not see a PGE_1 initiated calcium effect on erythrocyte structure may be because we used erythrocytes only from female volunteers. Cells from females may not be as sensitive to a calcium leakage as from men.

When we repeated the experiments of Kury et al (14), with the erythrocytes suspended in phosphate buffer, the membrane structure was

not affected by PGE_1 or PGE_2 (glucose present). At pH 7.4, PGE_1 induced a decrease in membrane order but PGE_2 induced no change. When either PGE_1 or PGE_2 was added to erythrocytes suspended in phosphate buffer pH 7.0 containing 0.5 mM bicarbonate a dramatic membrane disordering was induced. When the pH of the solution was increased to 7.4, a dramatic membrane ordering was seen which was dependent on the concentration of either PGE_1 or PGE_2. At 37°C, the membrane structural alterations induced by PGE_1 or PGE_2 are less pronounced in all cases except for cells in phosphate buffer with bicarbonate pH 7.4, where both PGE_1 and PGE_2 induce a slight membrane disordering. Bicarbonate added to a HEPES buffer system also induced the same type of changes. The bicarbonate appears to cause an increase in the membrane structural changes induced by the prostaglandin.

The presence of bicarbonate in either phosphate or HEPES buffers appears to induce a magnification of the structural changes induced by prostaglandins. It is possible that the prostaglandin receptor is situated near the band 3 protein and reflects band 3 conformational changes. If the presence of prostaglandins helps to regulate transport processes, the additional presence of bicarbonate may accentuate these changes.

Band 3 is probably not the only protein involved in the prostaglandin induced effect. Changes in CD absorption spectra upon addition of prostaglandins indicate protein conformational changes. Band 3 is bound to the cytoskeletal matrix via ankyrin (19-21). So, small changes in band 3 structure may be reflected as large changes in the cytoskeletal matrix. This could account for much of the prostaglandin effect. The cytoskeletal matrix is also extremely sensitive to small

fluctuations in calcium levels (22). If PGE_1 and/or PGE_2 induce calcium leakage, this could be reflected as large conformational changes in the cytoskeletal structure.

Our data suggests that in an Earles' buffer membrane structural changes induced by prostaglandins are most pronounced in the presence of calcium; however, changes induced by PGE_1 do not appear to depend on the presence of calcium. Energy depleted cells would be especially sensitive to prostaglandin induced structural changes if the cytoskeletal matrix is involved. It would take energy to restore the cytoskeletal matrix to its unperturbed state.

Having repeated the experiments of Kury et al (14) we saw no structural changes induced by either PGE_1 or PGE_2. The major difference between their methodology and ours was that they used male subjects and we used female subjects. Taniguchi et al (15) have shown that the degree of protection given by PGE_1 from mechanical lysis is more dramatic for cells drawn from men. Also preliminary data from our laboratory indicates that aspirin, a prostaglandin synthetase inhibitor (23), when ingested induces opposite erythrocyte membrane structural changes in men and women.

In conclusion, we have shown that in an energized cell, neither PGE_1 nor PGE_2 induce any detectable changes in membrane order. If bicarbonate is added to the suspending media, a change in membrane order occurs. Finally, the alterations in membrane order induced by either PGE_1 or PGE_2 may be dependent on the sex of the donor.

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23. Mazorow, D.L., Haug, A., Bull, R., and McGroarty, E.J. (Chapter 4) Effects of aspirin, indomethocin, and sodium salicylate on human erythrocyte membranes as detected with electron resonance spectroscopy.

CHAPTER VI

Title: Effects of aspirin, indomethacin, and sodium salicylate on human erythrocyte membranes as detected with electron spin resonance spectroscopy.

Authors: D.L. Mazorow, A. Haug, R. Bull, and E.J. McGroarty

ABSTRACT

Electron spin resonance spectroscopy was used to determine the structural changes in human erythrocyte membranes prior to and at intervals following ingestion of either 10 grains acetylsalicylic acid, 10 grains sodium salicylate, or 50 mg indomethacin by both male and female subjects. Analysis of erythrocytes from female subjects indicated a time-dependent disordering of the membrane over the eight hour period following aspirin ingestion while the cells of male subjects showed a slight membrane ordering over the same time period. Erythrocytes drawn from females at the beginning of the menstrual cycle showed the greatest amount of membrane disordering at one hour following aspirin ingestion, but by eight hours, the membrane structure had returned to that of control. The time dependent changes in membrane structure from females in the middle of the menstrual cycle displayed a biphasic disordering of the membrane. Ingestion of indomethacin induced a slight membrane ordering in both male and female subjects over the times examined. Ingestion of sodium salicylate by either men or women did not induce significant changes in erythrocyte membrane order. Washed erythrocytes when mixed with salicylate, aspirin, or indomethacin were either identical to control cells or slightly more ordered. This study suggests that aspirin-induced alterations in membrane structure may depend upon steroid hormone levels.

INTRODUCTION

Ingestion of aspirin (acetylsalicylic acid) reportedly affects many diverse physiological processes including histamine release (1), platelet aggregation (2, 3, 4), broncho constriction (1, 5), renal transplant rejection (6), and renal proteinuria (7). These effects are likely to be induced by irreversible aspirin inhibition of the enzyme cyclo-oxygenase, which is involved in the synthesis of the prostaglandins and the thromboxanes (8-10). Indomethacin is believed to work in a manner similar to aspirin, but its inhibition of the cyclo-oxygenase is reversible (11-13). Salicylate is believed to bind reversibly to the cyclo-oxygenase, but unlike aspirin or indomethocin, it does not inhibit the enzyme (11-12).

Levels of estradiol and estrogen or other steroid hormones may modulate the affect of aspirin. Aspirin increases the bleeding time in men more so than in women (14), and women absorb more aspirin than men do (15). The incidence of ischemic heart disease among pre-menopauasal women is much lower than among men and post-menopausal women (16). Low dose aspirin therapy has been implicated as an antithrombotic agent (17,18). Endogenous levels of estrogen are believed to maintain a more favorable ratio of prostacyclin to thromboxane which prevents platelet aggregation (19).

Specific low dosages of aspirin enhance the prostacyclin to thromboxane ratio by inhibiting platelet throboxane production with less of an effect on vasculature prostacyclin production (3, 20-27).

In this study we have analyzed the effects of aspirin, sodium salicylate, and indomethacin ingestion on the physical structure of the human erythrocyte membrane. We have shown that the effects of aspirin are different for men and women, and among women are dependent upon the menstrual cycle. In addition we analyzed changes in membrane structure induced by aspirin, sodium salicylate, and indomethacin addition to cells suspended either in bicarbonate buffer or in filtered serum. Our results indicate that differential effects of aspirin on the erythrocytes of women may require metabolic reaction or physical association with serum components and that the effects of aspirin on erythrocyte membrane structure appear to be dependent upon the menstrual cycle.

METHODS

Freshly drawn blood was obtained from healthy non-smoking, consenting individuals. The donors had not taken any medication or alcohol for at least 48 hours prior to ingestion of aspirin, sodium salicylate, or indomethacin. Female subjects were not taking oral contraceptives. Erythrocytes were drawn prior to, 1/2 hour, 1 hour, 2 hours, 5 hours, and 8 hours following ingestion of 10 grains (648 mg) of aspirin. Cells were also drawn prior to, 1 hour, and 2 hours following ingestion of 10 grains of sodium salicylate; and prior to, 1 hour, 5 hours, and 8 hours following ingestion of 50 mg indomethacin. Studies using female subjects were performed both at the beginning and between the middle and end of their menstrual cycle.

Anticoagulation was effected by defibrination. Cells were centrifuged, the buffy coat was removed, and the erythrocytes were washed three times in 300 imOsm NaCl, pH 7.4, and once in an Earle's salt solution or in filtered serum. The Earle's salts contained 116.4 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM NaH_2PO_4, 1.8 mM $CaCl_2$, and 26.2 mM $NaHCO_3$. Filtered serum was prepared by filtering human serum through an Amicon filter UM10 (10,000 dalton cut-off). The cells were packed in fresh Earle's salts or filtered serum to a hematocrit of 70 ± 1. A 1.0 ml aliquot of the packed cells was placed into a test tube which contained 0.2 µmoles of the electron spin resonance (ESR) probe 5-doxyl stearate. For in vitro studies, 22 µg/ml

of aspirin, 74 µg/ml of sodium salicylate, or 2 µg/ml of indomethacin was added to either the Earle's salt solution or the filtered human serum. 1 ml of packed cells was added to 39 ml of the drug-containing solutions, incubated at 37°C for 30 minutes and repacked. Packed cells were placed in a quartz cuvette for ESR probing studies. All ESR experiments were carried out with a Varian Century line ESR spectrometer, model E-112, equipped with a variable temperature controller. An external calibrated thermistor probe was used to monitor the temperature of the sample.

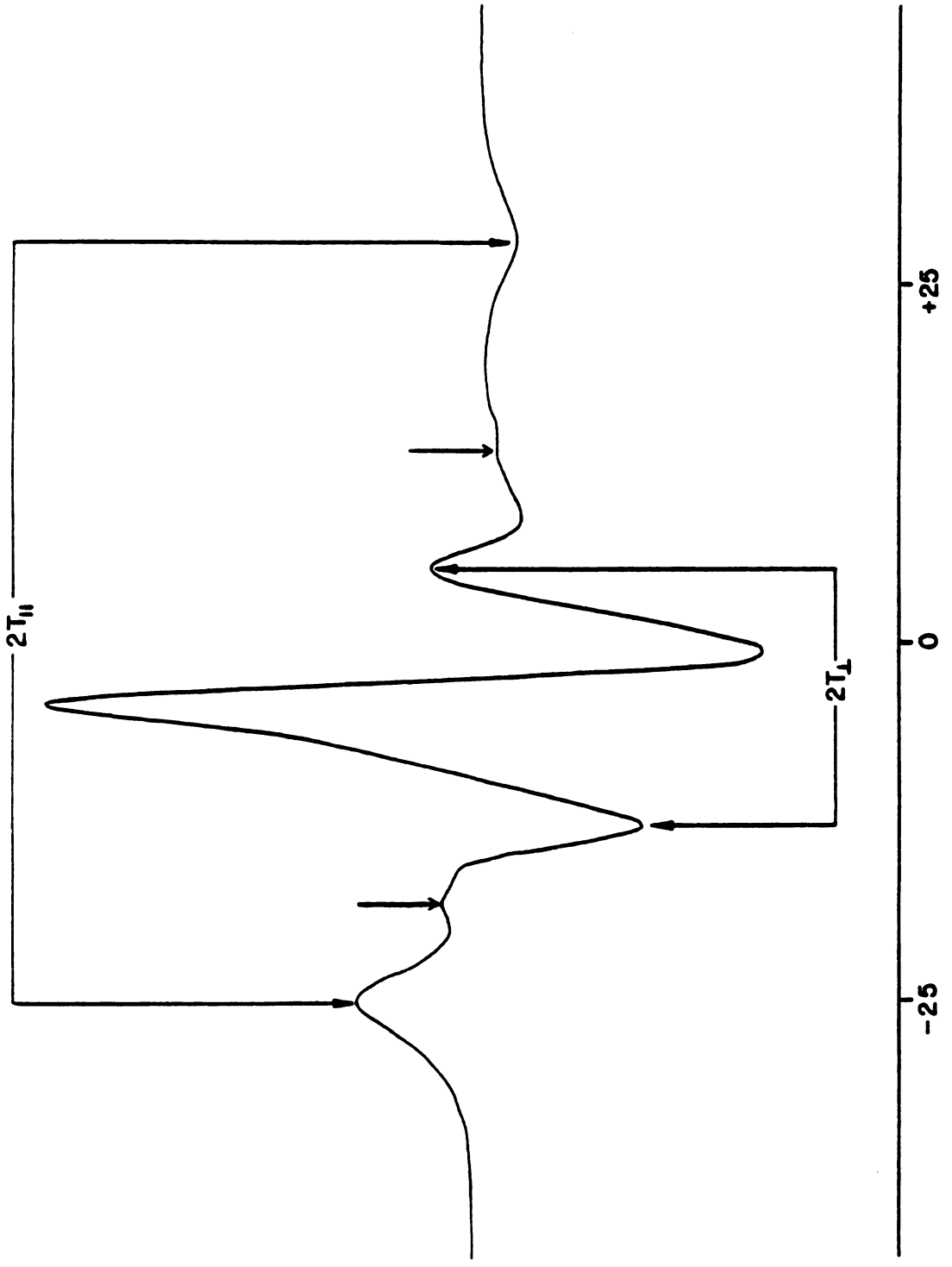
The data were analyzed by an iterative least squares program (28). Briefly, a B-spline was used to provide a smooth fit for the ESR data and points of inflection served for grouping data. Regression lines were calculated for each group and then plotted. This analysis allowed the determination of break points in the linear temperature dependent spectral parameters. Such breaks in the temperature dependence of the hyperfine splitting parameter and order parameter have been correlated with lipid phase separations or lipid phase transitions from gel-to-liquid crystalline lipid states (28, 29).

The relative fluidity of the erythrocyte membrane from cells drawn at different times following aspirin, sodium salicylate, or indomethacin ingestion was determined by labelling intact cells with 5-doxyl stearate. 5-doxyl stearate incorporates into the lipid phase of the membrane in such a way that the unpaired electron of the nitroxide radical is situated close to but shielded from the aqueous phase. Spin labelled preparations were placed in the resonance cavity of an ESR spectrometer. In this fashion the erythrocyte membrane preparation was exposed to microwaves of a constant frequency while held in a linearly

varying magnetic field corresponding to the spin stages of the unpaired electron. The ESR spectra are displayed as the first derivative of the microwave absorption plotted against an increasing magnetic field in gauss (Figure 1). The distance between the low field and the high field absorption peaks, the hyperfine splitting parameter ($2T_{||}$), is a measure of the largest energy difference between the spin states of the unpaired electron. With increasing temperature, intermolecular influences upon the unpaired electron tend to average, resulting in smaller values of $2T_{||}$ which reflect more fluid environments. $2T_{||}$, therefore, is related to the micro-viscosity of the environment from which the probe is reporting. Large values of $2T_{||}$ indicate less fluid environments.

The order parameter, S (calculated using $2T_{||}$ and $2T_{\perp}$) measures the deviation of the observed signal from that of a uniform orientation of the probe. For a uniformly oriented sample S=1; for a random sample S=0. Order parameter is an indication of lipid acyl chain orientation.

Figure 1. Electron spin resonance spectrum of human erythrocytes labelled with 5-doxyl stearate. The spectrum was taken at 37°C and the scan range was 100 gauss. Absence of free probe is indicated by the lack of a major absorption signal at points indicated by arrows. Symmetry of high and low field peak reveals that the probe was present in a single environment in the membrane.

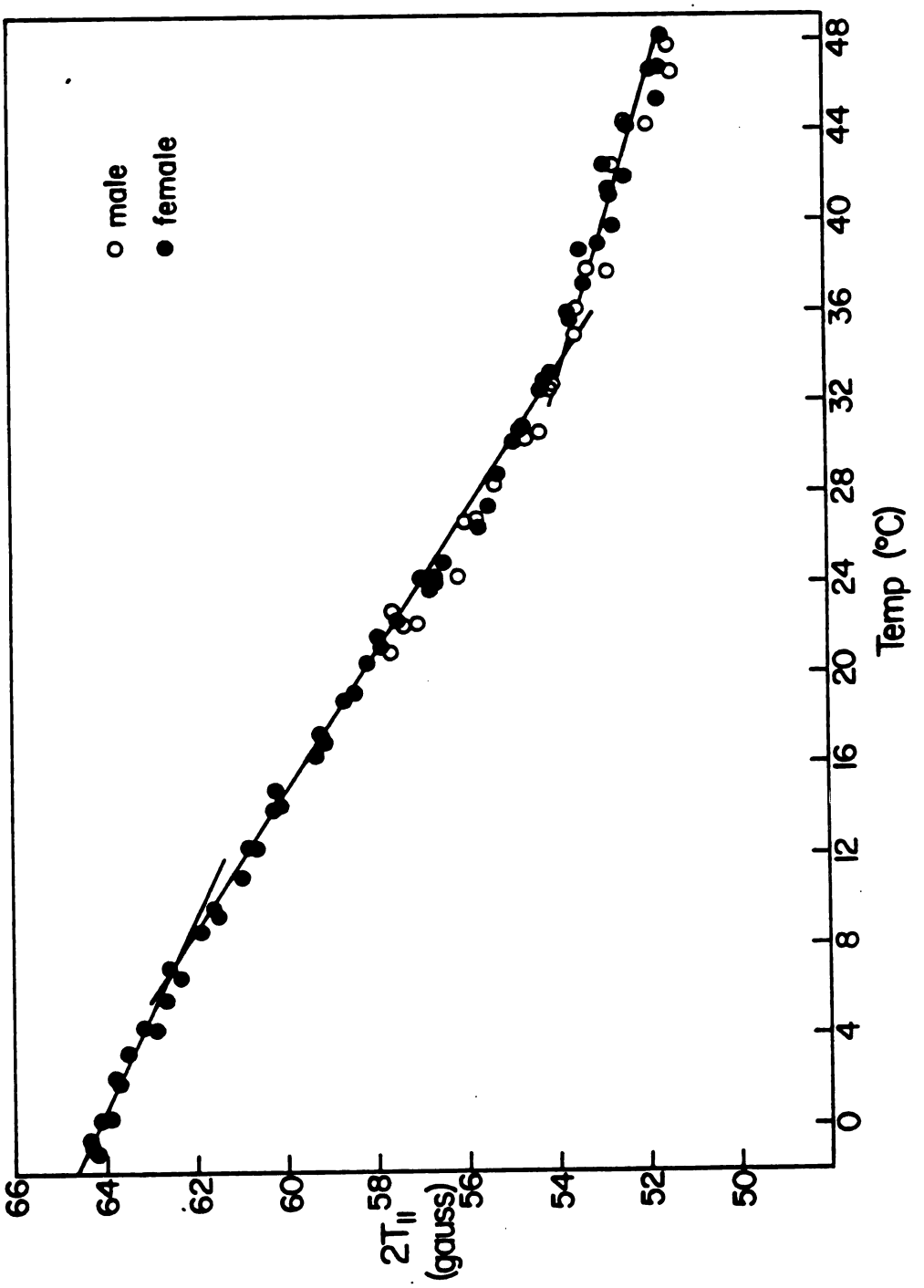

$2T_{\parallel}$
$2T_{\perp}$
+25
0
-25

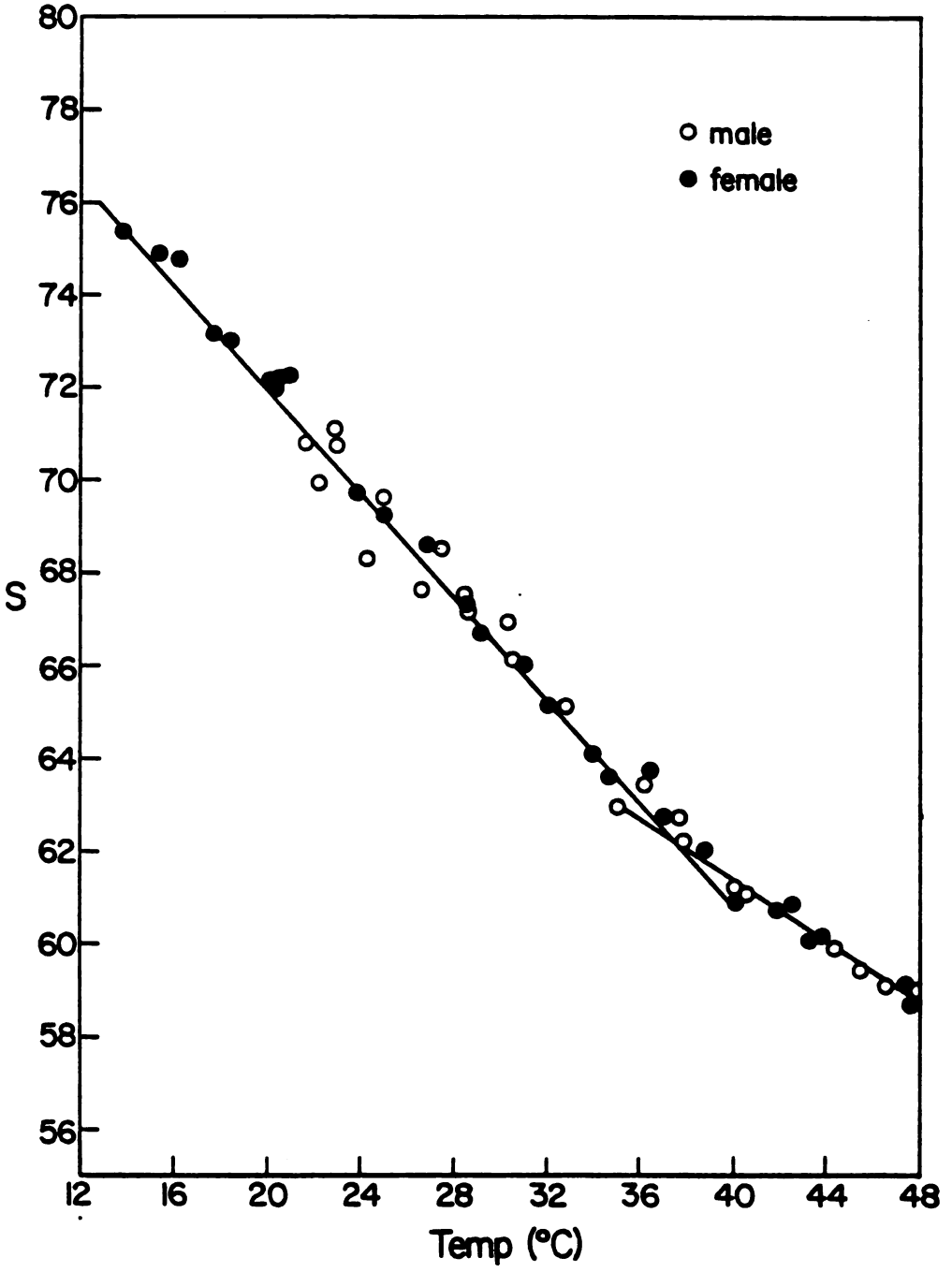
RESULTS

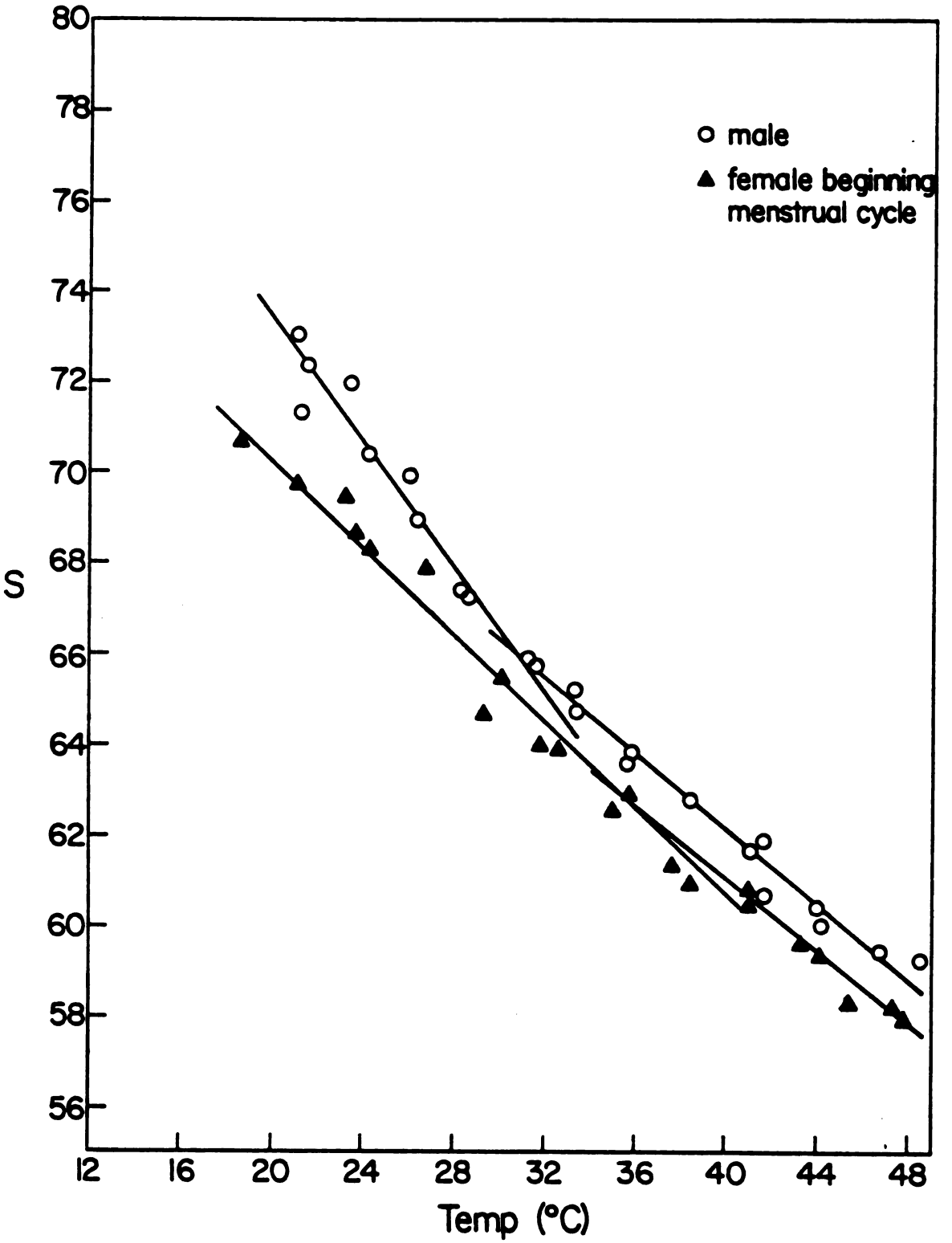
Erythrocyte membrane structure of cells drawn prior to and at different times following consumption of aspirin, sodium salicylate, or indomethacin was measured using the ESR spin probe 5 doxyl stearate. The spectral parameter S, was measured to determine structural changes in the erythrocyte membrane. The temperature dependence of the spectral parameters as plotted in Figure 2 were analyzed in terms of linear components using a linear regression program (28). The changes in membrane order parameter were compared to that of erythrocytes from donors prior to ingestion of the drug and the differences were computed. Spectral order changes of less than 0.5% are not considered significant.

The probed erythrocytes drawn from both male and female subjects appeared structurally identical prior to ingestion of any medication (Figures 2A and B). One hour after ingestion of aspirin, the erythrocytes from women at the beginning of the menstrual cycle were disordered to the maximum extent seen after ingestion, and the erythrocytes from men were maximally ordered at the same time point (Figures 2C and 3). The time dependent changes in erythrocyte membrane structure in men and women following the ingestion of aspirin is shown in Figures 3A and 3B. Erythrocyte membranes from men were more ordered following ingestion of aspirin, and the membranes remained ordered throughout the time span studied (Figure 3). Membranes of erythrocytes drawn from

Figure 2. Erythrocytes were drawn from male (open circles) and female (closed circles) donors. Spectral parameters $2T_{11}$ (hyperfine splitting parameter) and S (order parameter) were plotted versus temperature (°C). A. Temperature dependence of membrane structural parameters of erythrocytes from male and female subjects prior to ingestion of any drug. B. Temperature dependence of membrane order of male and female subjects prior to ingestion of any drug. C. Temperature dependence of erythrocyte membrane structure of cells drawn from female subjects at the beginning of the menstrual period and from male subjects 1 hour after ingestion of aspirin.


o male
• female
66
64
62
60
58
56
54
52
50
$2T_{\parallel}$ (gauss)
0
4
8
12
16
20
24
28
32
36
40
44
48
Temp (°C)


80
78
76
74
72
70
68
66
64
62
60
58
56
S
12
16
20
24
28
32
36
40
44
48
Temp (°C)
male
female


80
78
76
74
72
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68
66
64
62
60
58
56
S
12
16
20
24
28
32
36
40
44
48
Temp (°C)
male
female beginning menstrual cycle

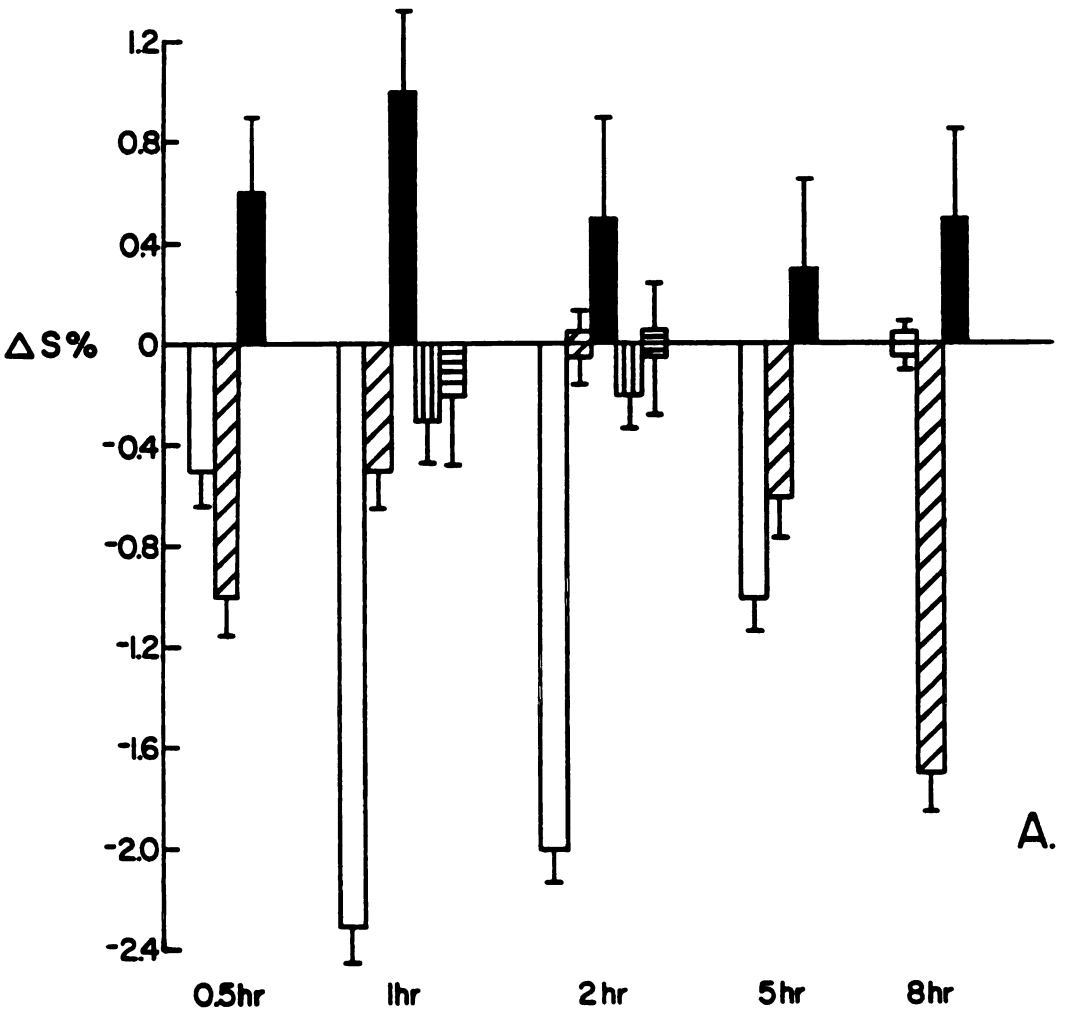
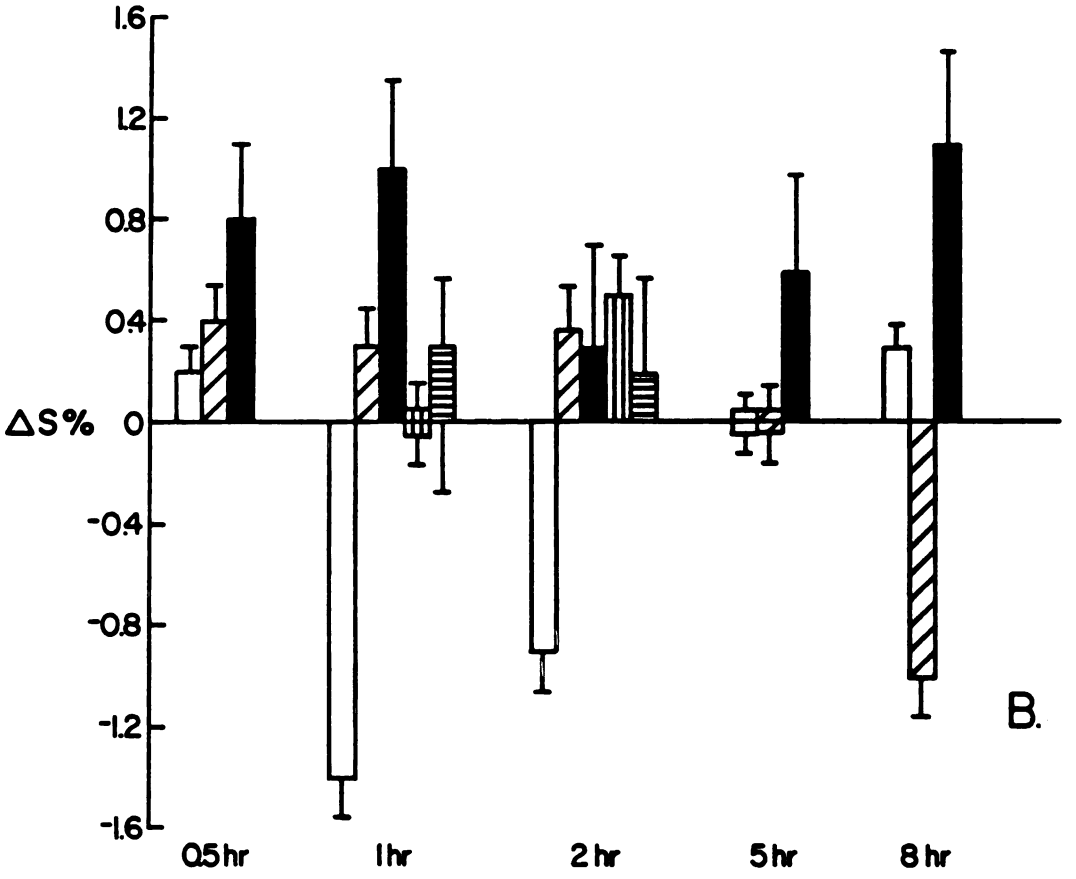


Figure 3. Time related changes in order parameter of erythrocytes from female subjects at the beginning of the menstrual cycle (□), from female subjects at the middle to end of the menstrual cycle (▨), and from male subjects (■) following aspirin ingestion. Time related changes in order parameter of erythrocyte membranes from female subjects at the beginning of the menstrual cycle (▥) and from male subjects (▤) following ingestion of sodium salicylate, A. measured at 24°C or B. measured at 37°C.


1.6
1.2
0.8
0.4
ΔS%
0
-0.4
-0.8
-1.2
-1.6
0.5hr
1hr
2hr
5hr
8hr
B.

women at the end of their menstrual cycle became disordered following ingestion of aspirin (Figure 3). However, if aspirin was ingested by the female subjects at the beginning of the menstrual cycle the maximum membrane disordering occurred at 1 hour with little or no membrane disordering at 30 minutes and 8 hours (Figure 3).

Following ingestion of indomethacin, erythrocytes from men were maximally ordered after 1 hour. For women, erythrocytes drawn at the beginning of the menstrual cycle were maximally ordered in membrane structure 1 hour following ingestion of indomethacin and remained ordered over 5 hours after ingestion of indomethacin (Figure 4). Membranes of erythrocytes from women at midestrous became ordered (at 37°C) by 1 hour after ingestion of indomethacin and remained ordered for at least 2 hours. In contrast, the membranes of erythrocytes from men or women drawn either 1 or 2 hours following ingestion of sodium salicylate were identical to control (Figure 3).

In vitro addition of aspirin to washed erythrocytes resuspended in either Earle's salt or filtered serum caused no changes in membrane order when measured at room temperature, but at 37°C, the membranes of erythrocytes from both men and women were slightly more ordered than control erythrocytes (Table 1). The in vitro addition of indomethacin to washed cells from either men or women induced no significant changes in membrane structure. Likewise, in vitro addition of sodium salicylate induced essentially no changes in cells from women. However, the membranes of erythrocytes from men when analyzed in an Earle's salt solution were significantly ordered at 37° (Table 1).

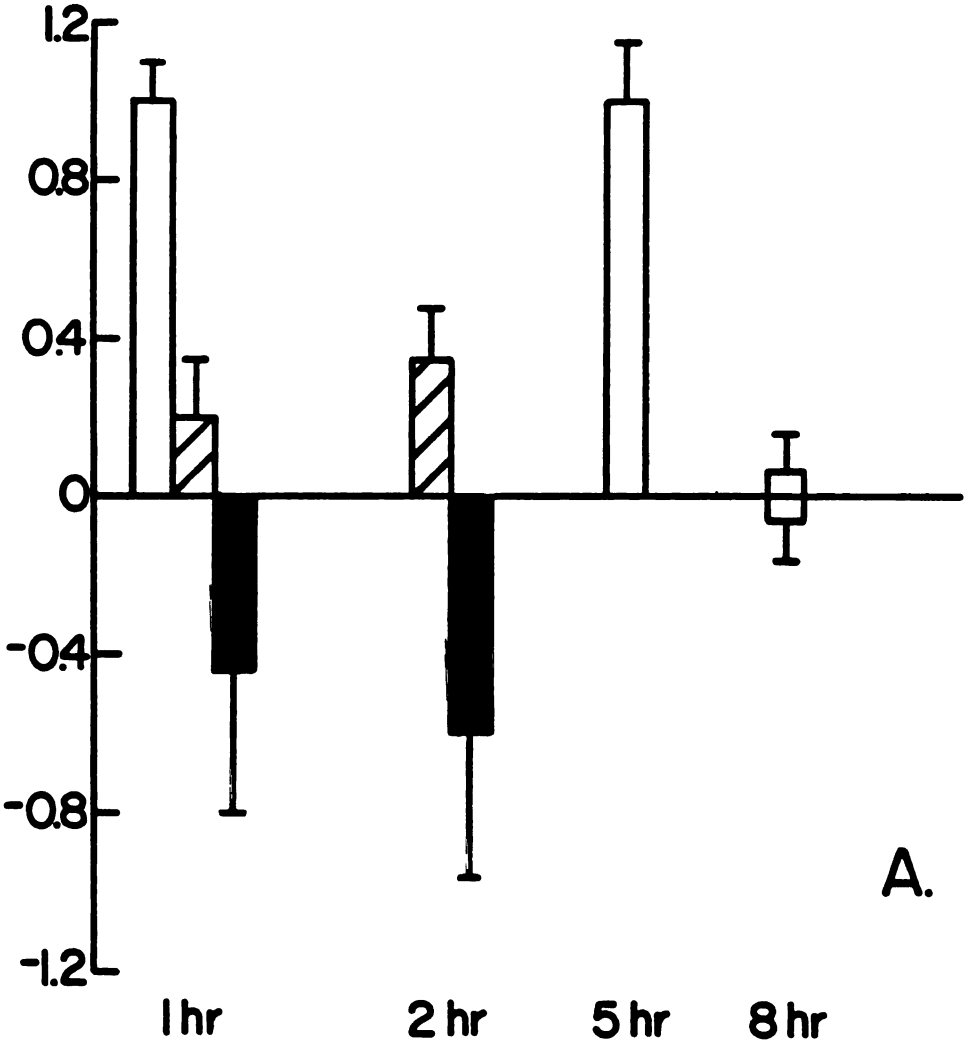


Figure 4. Time related changes in order parameter of erythrocytes from female subjects at the beginning of the menstrual cycle (□), from female subjects at the middle to end of the menstrual cycle (▨), and from male subjects (■) following ingestion of indomethacin, measured either at A. 24°C, or at B. 37°C.

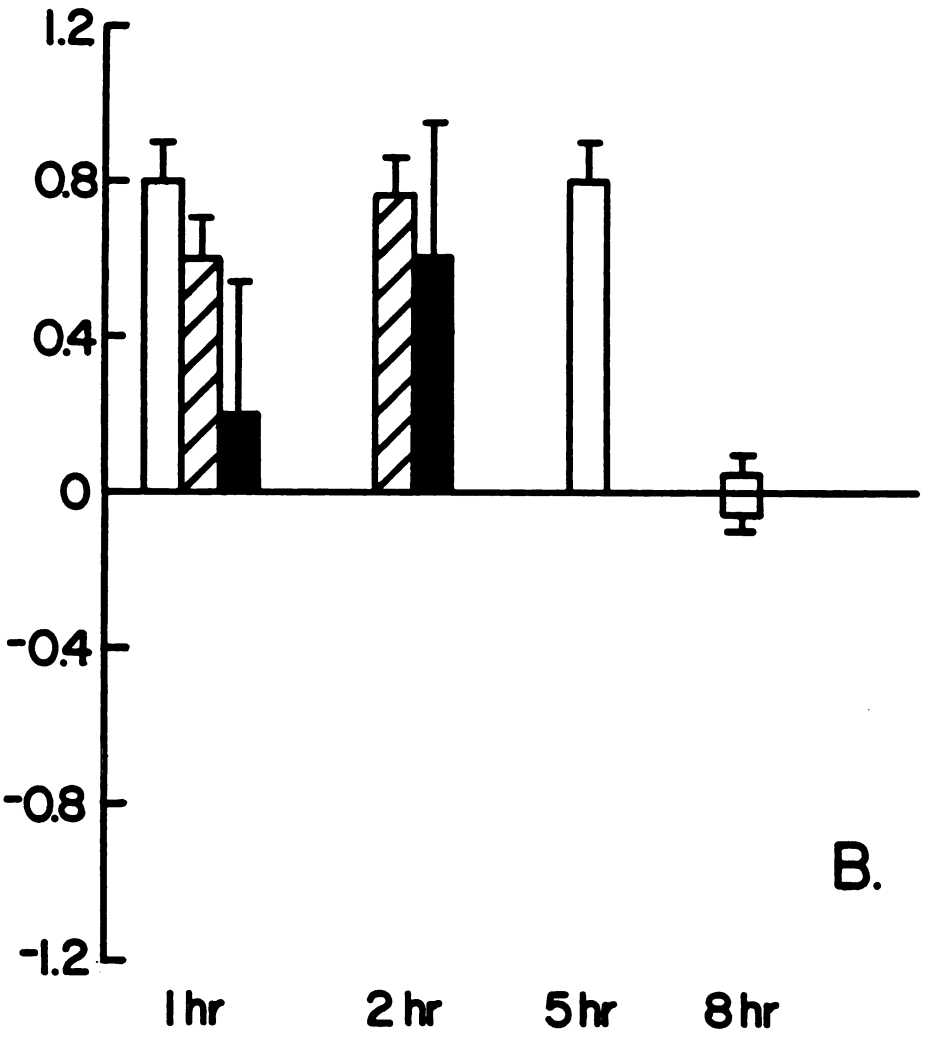

1.2
0.8
0.4
0
-0.4
-0.8
-1.2
1hr
2hr
5hr
8hr
B.

Table 1. Changes in order parameter of erythrocytes drawn from males and females and suspended in either Earle's salts or serum ultrafiltrate containing either aspirin, sodium salicylate, or indomethacin.

Drug	Donor	Incubation temperature	Earles Changes in order(S)%	filtered serum
Aspirin	female	24°	0.2 ± 0.1	0.2 ± 0.15
		37°	0.85 ± 0.11	0.2 ± 0.15
	male	24°	0.15 ± 0.31	-0.5 ± 0.37
		37°	0.6 ± 0.33	0.7 ± 0.37
Sodium salicylate	female	24°	0 ± 0.15	-0.1 ± 0.13
		37°	0.0 ± 0.25	-0.15 ± 0.15
	male	24°	-0.2 ± 0.35	0.2 ± 0.34
		37°	1.0 ± 0.35	0.35 ± 0.35
Indomethacin	female	24°	0.2 ± 0.1	-0.3 ± 0.15
		37°	0.0 ± 0.05	-0.15 ± 0.17
	male	24°	0.0 ± 0.25	0.0 ± 0.25
		37°	0.0 ± 0.25	0.35 ± 0.32

DISCUSSION

Results presented here indicate no detectable differences in erythrocyte membrane structure between cells drawn from men and women prior to aspirin, sodium salicylate, or indomethacin ingestion (Figure 2A and B). After ingestion of aspirin, time related changes in membrane structure occurred in both men and women (Figures 2C, 3A, 3B). For men the erythrocyte membrane became more ordered than the control. In contrast, the erythrocyte membrane of cells from women became less ordered than that of cells prior to aspirin consumption. For women, the degree of change appeared to be somewhat dependent upon when in the menstrual cycle the study was done. As can be seen in Figure 2B, 1 hour after ingestion of aspirin, membrane order is dramatically different between women (at the beginning of the menstrual cycle) and men. For men, the erythrocyte membranes became more ordered than the control. The differences in effects of aspirin seen between men and women and among the same women at varying times in the menstrual cycle is probably caused by differences in amounts of steroid hormones and/or prostaglandins prior to aspirin ingestion.

Aspirin is known to inhibit prostaglandin synthesis by acetylating the enzyme cyclo-oxygenase which is involved in one of the first steps in its synthesis (8). Therefore, following ingestion of a normal dose of aspirin (648 mg) most if not all prostaglandin synthesis in the vasculature is inhibited (3, 12, 2-22, 30-34). It is also known that erythrocytes have receptors for prostaglandins of the E type (35-37,°),

and receptors for either estrogen (38) or estradiol (39). It is possible that either the prostaglandin and the steroid hormone receptor are related or they interact within the membrane.

Several studies have shown a relationship between estradiol levels and prostaglandin production. Estradiol is known to increase prostacyclin production in the aorta (19), and estrogen reportedly increases prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) production (40). Both uterine estradiol and $PGF_{2\alpha}$ concentrations increase during pregnancy (11) as do plasma estradiol levels (42). These reports indicate a correlation between estradiol and prostaglandin production. Thus, aspirin and indomethacin inhibition of prostaglandin synthesis may be modulated by endogenous levels of estradiol. This may account for differences in erythrocyte membrane structure following ingestion of aspirin and indomethacin at different times in the menstrual cycle.

Cyclo-oxygenase, the site of action of these drugs, has an active site and an allosteric site (11, 12, 31). It is known that pretreatment of the enzyme with either sodium salicylate (11, 12, 31) or indomethacin (11, 12) will prevent the irreversible inhibition of cyclo-oxygenase by aspirin. Indomethacin reversibly (12) inhibits the cyclo-oxygenase. Our studies showed that ingestion of sodium salicylate did not significantly alter membrane structure of erythrocytes from either men or women. The erythrocyte membrane structure has been shown to be altered by prostaglandins (35-37,°). In vitro addition of sodium salicylate, aspirin, or indomethacin to cells in a bicarbonate buffer did not affect membrane structure. Thus, specific enzymatic or physical interactions must occur with the cells within the vasculature

°Chapter 3

following consumption of the drug. Ingestion of aspirin induced dramatic membrane changes in the erythrocytes drawn from both men and women over the 8 hour time span examined. Aspirin as well as indomethacin are in the blood stream 30 minutes after ingestion (43) and their effects were followed for several half-lives. Aspirin has a half-life of 20 to 30 minutes (43) and indomethacin has a half life of 4 hours (43).

To determine if inhibition of cyclo-oxygenase was the cause for the membrane structural changes in men and women after aspirin ingestion, indomethacin and sodium salicylate were given orally to male and female volunteers. Indomethacin, also a cyclo-oxygenase inhibitor (11, 12, 31), should upon ingestion result in changes in erythrocyte membrane structure similar to that of aspirin. Sodium salicylate, which is not a cyclo-oxygenase inhibitor (11, 12, 31), should not induce erythrocyte membrane changes.

Indomethacin and aspirin did not induce the same structural changes in erythrocyte membrane structure, but the membrane changes induced by indomethacin and aspirin were different for men and women. Other studies show that indomethacin can modulate the erythrocyte membrane and estradiol levels in ways different from aspirin; and the differences observed in membrane order following ingestion of aspirin or indomethacin may not be solely prostaglandin related. The ability of these two drugs to protect erythrocytes from heat-induced lysis is different (44, 45) with only indomethacin having a protective effect. Also, indomethacin will prevent ovulation of ovariectamized rats if injected into the anterior hypothalamus while aspirin will not (46). Indomethacin may inhibit synthesis of membrane active compounds other

than prostaglandins. Furthermore, indomethacin may not saturate the receptors as does aspirin, or aspirin may affect enzymatic systems other than the cyclo-oxygenase.

Our data indicate that structural changes induced in erythrocytes by cyclo-oxygenase inhbitors may be modulated by prostaglandin and/or estradiol levels. Both indomethacin and aspirin induce changes in erythrocyte structure which in women appear to be menstrual period modulated. Sodium salicylate, which is not an inhibitor of prostaglandin synthesis, induces very little structural change in erythrocyte membranes.

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CHAPTER V

Chlorpromazine Induced Changes in Human Erythrocyte Membrane Structure

Authors: D.L. Mazorow, A. Haug, and E.J. McGroarty

ABSTRACT

Fresh whole human erythrocytes were used to study the effects of chlorpromazine and chlorpromazine-methiodide on erythrocyte membrane properties. The effects of these two phenothiazines on membrane properties were determined using electron spin resonance probing techniques and hypotonic hemolysis. Chlorpromazine induced a biphasic membrane disordering which was maximal at 2.0×10^{-5} M. This is the same concentration at which chlorpromazine maximally protected erythrocytes from hypotonic hemolysis. Chlorpromazine methiodide did not induce any membrane disordering nor did it protect erythrocytes from hypotonic hemolysis at any of the concentrations examined. We present a hypothesis to explain the biphasic effects seen with chlorpromazine.

INTRODUCTION

Studies have shown that chlorpromazine (CPZ) protects erythrocytes from hypotonic hemolysis (1), and induces erythrocyte shape changes (2-5). The membrane expansion resulting from CPZ addition has been questioned (6), and CPZ reportedly induces no change in cell volume (7). The binding of CPZ to the cell is freely reversible (8,9), but the lower the concentration of plasma, the more CPZ goes into the cell (8).

To better understand the site of action of CPZ on the erythrocyte membrane we have investigated the dose dependence of CPZ and its quanternary ammonium analog chlorpromazine methiodide (CPZ-MeI) in protecting the erythrocyte from hypotonic hemolysis. In addition, we have analyzed the dose dependent membrane structurel alterations induced by CPZ and CPZ-MeI using electron spin resonance spectroscopy.

MATERIALS AND METHODS

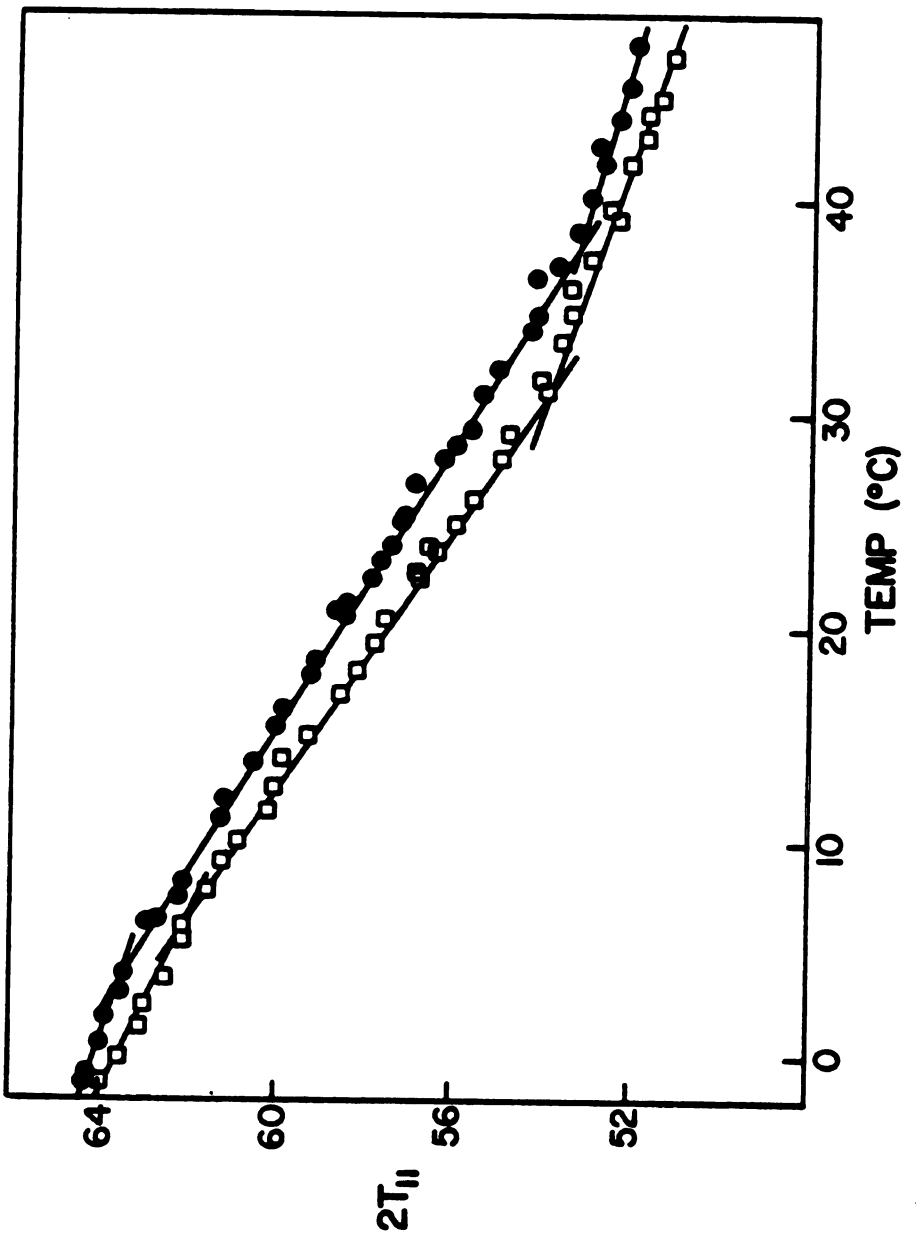
Freshly drawn blood was obtained from consenting healthy non--smoking individuals. The donors had not taken any medication or alcohol for at least 48 hours prior to venapuncture.

Anticoagulation was effected by defibrination. Cells were centrifuged, the buffy coat was removed, and the erythrocytes were washed three times in 300 imOsm NaCl pH 7.4, and once in filtered (Amicon filter UM 10, 10,000 dalton cutoff) human serum, in Earle's salts (145 mM NaCl, 5 mM KCl, 0.7 mM $MgSO_4$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, and 22 mM $NaHCO_3$) at pH 7.4, or in Earle's salt solution without calcium. The cells were packed in Earle's salts, Earle's salts without calcium, or filtered serum to a hematocrit of 70±1. A 1.0 ml aliquot of the packed cells was placed into a test tube which contained 0.2 μMoles of 5-doxyl stearate (5-DS). After gentle mixing for one minute the cells were resuspended into 40 ml of Earle's salts, Earle's salts without calcium, or filtered serum containing either 1×10^{-6} M, 2×10^{-5} M, or 5×10^{-5} M CPZ (gift of Smith, Kline, and French, Inc.). Cells were incubated at 37°C for 20 minutes and centrifuged. Packed cells (hematocrit 70±1) were placed in a quartz cuvette for electron spin resonance (ESR) analysis. All ESR experiments were carried out with a Varian century 3 line ESR spectrometer model E-112, equipped with a variable temperature controller. An external calibrated thermistor probe was used to monitor temperature of the sample.

The data were analyzed by an iterative least squares program. Briefly a B-spline was used to provide a smooth fit for the ESR data and points of inflection served for grouping data. Regression lines were calculated for each group and then plotted. This analysis allowed the determination of break points. Such breaks in the temperature dependence in the hyperfine splitting parameter ($2T_{11}$) and order parameter (S) have been correlated with lipid phase separations or lipid phase transitions from gel-to-liquid crystalline states (11).

Hypotonic hemolysis studies were performed in an Earle's salt solution at 100 mOsm. The solution was prepared by diluting an isotonic solution (300 imOsm) with double distilled water to the desired hypotonicity. The osmolarity of the solution was determined with an Advanced Instruments osmometer model 3R. A 10% (v/v) solution of erythrocytes was placed in 5 ml of hypotonic buffer such that the final concentration of cells was 0.1%. These cells were incubated for seven minutes at room temperature in hypotonic buffer and returned to isotonicity by addition of the appropriate amount of 4 M NaCl. The cells were centrifuged and the degree of lysis was measured from the absorbance at 543 nm of the supernatant solution. 100% lysis was achieved by placing the cells in double distilled water, and 0% lysis was defined as the degree of lysis of cells suspended in a 300 imOsm solution. Percentage lysis was taken as the difference between these two values. Protection from hypotonic hemolysis by CPZ and CPZ-MeI was analyzed at concentrations between 10^{-2} M and 10^{-7} M.

Figure 1. The temperature dependent changes in the hyperfine splitting parameters ($2T_{11}$) of erythrocytes labelled with 5-doxyl stearate suspended in Earles salts (-●-●-) or in Earles salts with 2×10^{-5} M chlorpromazine (-□-□-).


64
60
56
52
$2T_{11}$
0
10
20
30
40
TEMP (°C)

RESULTS

ESR spectra from erythrocytes labelled with 5-DS showed little free probe signal (data not shown). Spectral symmetry of the low field peak indicated that the majority of the spin label was located in a uniform environment over the temperature range examined (-2°C to 48°C). The hyperfine splitting parameter, $2T_{11}$, decreased linearly as a function of increasing temperature (Figure 1) with discontinuities occurring at 3°C to 5°C and in the range of 31°C to 41°C, depending upon the concentration of CPZ or CPZ-MeI added. Spectra recorded above 12°C permitted the determination of the order parameter, S. When S was plotted as a function of temperature, a break similar to that observed with $2T_{11}$ was detected (Figure 2). The control erythroctyes were shown to be more rigid (Figure 1) or more ordered (Figure 2) than erythrocytes suspended in solution containing 2×10^{-5} M CPZ. The ESR studies showed a maximal decrease in membrane fluidity ($2T_{11}$) at 2×10^{-5} M (data not shown). A similar dose dependent decrease in membrane order, S, was also detected (Figure 3A,B). Our results indicate that similar CPZ dose dependent changes occurred in membrane structure and protection from hypotonic hemolysis with the maximum protection from lysis occurring at 1.5×10^{-5} M CPZ (Figure 3C). At concentrations greater than 10^{-4} M, CPZ became lytic (Figure 3C). CPZ-MeI did not protect erythrocytes from hypotonic hemolysis at concentrations between 10^{-7} M and 10^{-3} M, but neither was it lytic

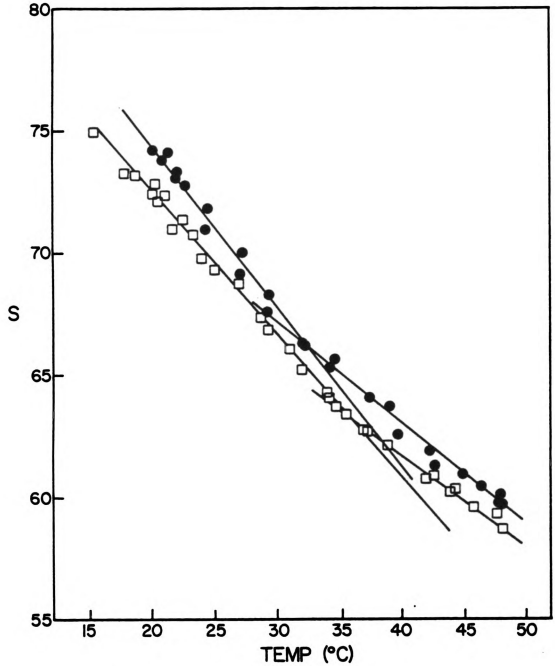
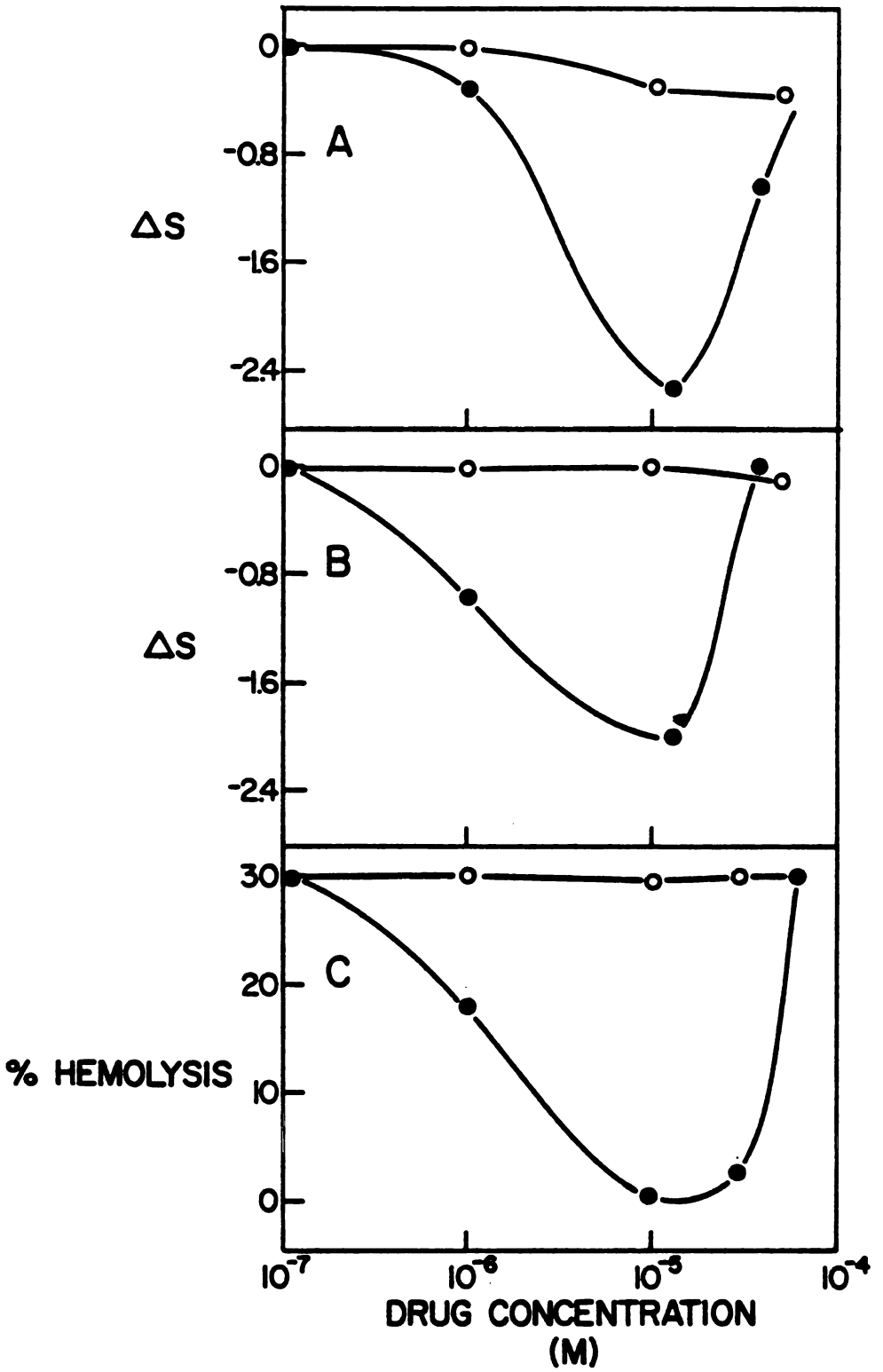


Figure 2. The temperature dependent change in the order parameters (S) of erythrocytes labelled with 5-doxyl stearate and suspended in Earles salts (-●-●-) or in Earles' salts with 2 x 10^{-5} M chlorpromazine (-□-□-).

Figure 3. Chlorpromazine (-●-●-) or chlorpromazine methiodide (-o-o-) induced concentration dependent changes in A. order parameter (S) at 24°C, B. order parameter (S) at 37°C, C. hemolysis in hypotonic Earles buffer at 24°C.


0
-0.8
-1.6
-2.4
A
ΔS
0
-0.8
-1.6
-2.4
B
ΔS
30
20
10
0
C
% HEMOLYSIS
10^{-7}
10^{-6}
10^{-5}
10^{-4}
DRUG CONCENTRATION
(M)

these concentrations (Figure 3C). It also did not change membrane fluidity (data not shown) or membrane order (Figure 3A,B) at any of the concentrations tested.

Erythrocytes suspended in Earle's salts without calcium containing 10^{-5} M CPZ were labelled with 5-DS. At 24°C and 37°C the cells were dramatically disordered (data not shown). Cells suspended in filtered serum and incubated in the presence of CPZ did not appear to be sensitive to changes in pH between 7.0 and 8.5.

DISCUSSION

Our analysis of membrane structure of erythrocytes in the presence of CPZ suggests that CPZ stabilizes the membrane to lysis by inducing membrane structural changes. This alteration in membrane structure does not appear to be calcium dependent (data not shown).

It has been reported that CPZ passes through the erythrocyte membrane and binds to the inner monolayer (3), while the quaternary ammonium analog can not penetrate the membrane (9,12). In addition it has been reported that CPZ preferentially binds phosphatidylcholine, phosphatidylserine, and phosphatidylinositol (13) phospholipids which are found in the inner monolayer of the membrane. The amount of CPZ that can bind to dipalmityl phosphatidylcholine liposomes reportedly increases dramatically above the phase transition at pH 7.4 (14). CPZ also has been shown to quench perylene fluorescence in erythrocytes, indicating that CPZ must be situated in the hydrocarbon core region of the membrane where perylene localizes (14). Finally, high concentrations (0.2-1 mM) of CPZ reportedly do not affect lipid head group movement in red cell ghosts (ESR spin labelled phospholipid studies) but molecular motion in the lipid core is increased (15). These studies support the theory that CPZ is able to interact with the membrane lipids, preferably the core region and preferentially with the amino phospholipids.

CPZ, however, may not associate solely with the lipids of the erythrocyte membrane, but it may also interact with specific proteins. CPZ has been shown to decrease Na-K ATPase activity in red blood cells (16). In addition, if ouabain, a Na-K ATPase inhibitor, is added to the cells prior to treatment with CPZ, CPZ reportedly does not protect against lysis (16). Furthermore, cellular ATP content can be induced by CPZ to drop to 10% of that in untreated cells, and protein dephosphorylation is induced (17). Not only does CPZ reduce cellular ATP content, it also reportedly displaces membrane bound calcium. Although the data is not conclusive, CPZ may affect glucose transport (18,21).

Gross cellular changes are induced by CPZ. 23 μM CPZ will change erythrocytes from discocytes to stomatocytes (9), and eventually to spherostomatocytes (5) along with a concomitant decrease (9-10%) in both electrophoretic mobility (9) and suspension viscosity at high shear rates (5).

Our results support those of Elferink (12) which indicate that CPZ-MeI does not enter or pass through the erythrocyte membrane. CPZ-MeI did not affect either hypotonic hemolysis or membrane structure as seen by ESR spectral analysis. If CPZ-MeI does not pass through the membrane or if it binds only to the outer monolayer, it will not alter the membrane the same way as does CPZ.

We propose that at low concentrations CPZ passes through the membrane and acts on the cytoskeletal matrix. CPZ, along with other phenothiazines, is known to bind to calmodulin (22,25). Calmodulin is reported to interact with the cytoskeletal matrix via spectrin (24-27)

and to interact with Ca^{2+}-Mg^{2+} ATPase and to alter its activity (22,23). Thus, we propose that CPZ interactions with calmodulin affects the cyto-skeletal matrix inducing erythrocyte shape changes and changes in membrane lipid structure. It has been reported that if ankyrin (band 2.1) is absent from erythrocyte ghosts, CPZ can not induce shape changes (5). Ankyrin is bound to both band 3 and the spectrin-actin oligomer (28-30).

At high concentrations, CPZ which is fairly lipophilic, will act as a detergent. We propose that once the optimal receptor concentration is surpassed, CPZ will begin to destabilize the membrane (Figures 3A, B, C) and as the concentration is increased it can even solubilize the hemoglobin off the lysed membrane (Figure 3C).

In conclusion, ESR and hypotonic hemolysis results suggest that CPZ affects the erythrocyte membrane perhaps by interactions with the cyto-skeletal matrix. CPZ-MeI does not appear to affect membrane structural properties. This lack of membrane perturbation could be related to the inability of CPZ-MeI to cross the erythrocyte plasma membrane. If CPZ does alter membrane structure via the cytoskeletal matrix, then it is possible that low dose treatment with CPZ could ameliorate hemolytic disorders in which membrane rigidification occurs.

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CHAPTER VI

Concluding Statements

CONCLUSIONS

We have shown that addition of very small amounts of prostaglandins (10^{-9} M to 10^{-12} M) can induce very large membrane structural changes in intact erythrocytes. The number of molecules present vary from 1 per cell (10^{-12} M) up to 1,000 per cell (10^{-9} M). It is unlikely that the changes in membrane structure induced by the prostaglandins are the result of just membrane lipid interactions. It is more likely that prostaglandins have specific receptors which induce the structural changes. In support of this thought, we found that the presence of glucose prevented the structural changes induced by prostaglandin. With glucose present, the cells presumably were able to react to the presence of prostaglandin and modulate its affects on membrane structure. Upon addition of bicarbonate to phosphate buffer at either pH 7.0, or pH 7.4, or to HEPES buffer, red cell membrane structural changes were enhanced. Bicarbonate, by acting upon band 3, may accentuate membrane structural changes. We believe that the membrane structural changes are mediated by changes in the cytoskeletal matrix. It appears that all the extrinsic proteins are linked either directly or indirectly to each other and through specific interactions the complex is bound to the membrane. Thus, any change in cytoskeletal structure would obviate a change in overall membrane structure.

Since we believe that the cytoskeletal matrix is involved in the structural changes seen upon addition of prostaglandins, we added

chlorpromazine (CPZ), a drug known to have specific interactions with lipids, to erythrocyte suspensions. The dose response curve of membrane structural changes upon addition of CPZ obtained both by ESR and by hypotonic hemolysis studies were in agreement. At 10^{-5} M, CPZ induced maximal membrane disordering and maximal protection to hypotonic hemolysis. We believe that upon further addition of CPZ, all the sites at which CPZ is able to bind proteins became saturated and that CPZ began to enter the lipid matrix. As the dose of CPZ was increased, the drug bound to membrane lipid until, acting like a detergent, it disrupted the membrane.

Prostaglandins and CPZ are not the only compounds which at therapeutic concentrations induce membrane structural changes. Upon ingestion of aspirin, the erythrocyte membranes from cells of both male and female subjects underwent structural changes. The degree and time course of the erythrocyte membrane changes for cells from women were dependent on when in the menstrual cycle the erythrocytes were drawn. The in vitro addition of aspirin induced no significant changes in red cell structure. Therefore, the changes in erythrocyte structure, seen upon the ingestion of aspirin must be induced by metabolic products. Aspirin, a prostaglandin synthetase inhibitor, may indirectly induce the erythrocyte membrane changes by creating an inbalance in the ratio of the various prostaglandins or by induction of compounds from the leukotriene pathway. These changes in membrane structure may be the result of a receptor directly or indirectly interacting with the cytoskeletal matrix and thus resulting in an amplification of the prostaglandin signal. Indomethacin, like aspirin, induced erythrocyte structural changes which were dependent upon whether the cells were

drawn from men or women, and when in the menstrual cycle the cells were drawn. Indomethacin, also an inhibitor of prostaglandin synthesis, probably induces membrane structural changes via the same pathway as does aspirin.

Finally, even an inbalance in the anionic milieu can apparantly induce membrane alterations. Band 3, which is believed to be the anion port, is attached to the cytoskeletal matrix via the ankyrin-spectrin complex. Thus, changes in anion flux or concentration gradients may also induce membrane structural changes via interactions with the cytoskeletal matrix. HEPES, the largest of the anions studied, induced the largest structural changes. The changes in membrane structure could be the result of HEPES blocking anion flux and thus creating an inbalance in the anionic gradient.

My results suggest that very small amounts of certain classes of compounds can induce very large membrane structural changes. These changes are most likely induced by an amplification of protein structural changes of the cytoskeletal matrix.

The most interesting questions raised by this thesis study are:
(1) Is the cytoskeletal matrix truly sensitive to these compounds and do changes in the cytoskeletal matrix reflect changes in whole cell structure?
(2) Are the induced membrane structural changes the result of an influx of calcium?
(3) Is there a difference in the erythrocyte prostaglandin receptors of men and women: Or, are the induced changes the result of differing kinds and levels of steroid hormones.

(4) Can administration of the prostaglandins and/or phenathiazines help regulate cell shape and viability in various hemolytic disorders? (5) As the erythrocyte ages, it undergoes various shape changes. If these shape changes can be regulated by the compounds discussed here or other similarly acting agents, can better techniques be developed to prolong blood shelf life?

APPENDIX

APPENDIX A

THE MODIFICATION OF HUMAN ERYTHROCYTE MEMBRANE STRUCTURE BY MEMBRANE STABILIZERS: AN ELECTRON-SPIN RESONANCE STUDY*

ABSTRACT

Membrane structure in intact human erythrocytes was analyzed by electron-spin resonance (ESR) spectroscopy. The spin probes 5-doxyl stearate and 5-doxyl stearate methyl ester revealed thermally-induced structural transitions in the membrane at 37°C and 15°C. The addition of propranolol, diazepam, chlorpromazine, or Pluronic F68 all caused a decrease in temperature of the upper transition, but did not markedly alter the temperature of the lower transition. In addition, diazepam caused a significant decrease in the ordering or packing of the membrane-lipid acyl chains. It is proposed here that the protection from hypotonic hemolysis that has been reported in the presence of these drugs is mediated by a structural rearrangement in the erythrocyte membrane involving a change in protein-lipid interactions.

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INTRODUCTION

Present approaches to the treatment of hemolytic disorders are limited to both in therapeutic principle and in effectiveness. They depend mainly on the use of agents such as corticosteroids, for which the mechanism of action is uncertain, or on the prolongation of survival of damaged cells by splenectomy. It is axiomatic that hemolysis results ultimately from structural failure in the erythrocyte membrane: attempts by Brewer (1) to diminish irreversible sickling by divalent ion substitution suggest that erythrocyte resistance to hemolysis may be modified therapeutically. The present study examines the hypothesis that there are topographical sites in the membrane that are especially critical with respect to hemolysis and that might be modifiable with therapeutic effect.

The rapid removal from the circulation of cells showing prehemolytic damage mandates an indirect approach to the problem; consequently, we have done an initial investigation in vitro on the structural perturbations of the erythrocyte membrane induced by several agents affecting the mechanical and osmotic resistance of red cells. It is anticipated that this may ultimately lead to the identification of susceptible membrane structures that are affected in common in diverse hemolytic processes.

Osmotic fragility and mechanical deformability of human erythrocytes are reportedly altered by low concentrations of a variety of membrane-stabilizing drugs (2). Such drugs have the potential to protect erythrocytes from hemolysis and to increase deformability in the microcirculation, provided that ancillary effects in other systems can be

held within acceptable limits. There is at present insufficient understanding of the action of these compounds to define the optimal molecular form for erythrocyte-related effects. Although protein-lipid interactions may be affected, alterations in membrane architecture have not been characterized in detail. One technique useful in studying membrane architecture involves incorporating electron-spin resonance (ESR) probes such as 5-doxyl stearate (5DS) into the lipid bilayer. The unpaired electron of the doxyl group absorbs microwave energy when the sample is inserted into a magnetic field. The spectrum of the absorption reflects the structure and fluidity of the membrane in the region of the probe. Since 5DS reportedly localizes in a lipid region that is closely associated with proteins and devoid of cholesterol in the human erythrocyte membrane (3,4), electron-spin resonance spectroscopy of erythrocytes labeled with 5DS should reveal structural information specifically related to protein-lipid interactions.

In this paper, we report that 5DS- and 5-doxyl stearate methyl ester (5DS-ME)-labeled human erythrocytes exhibit discontinuities in the temperature dependence of ESR spectral parameters. These temperature-induced changes in membrane structure correlate with other reported temperature-dependent phenomena of erythrocytes that are presumed to reflect membrane structural changes (5-9). We report that a variety of membrane stabilizing agents affect the temperature dependence of these measured spectral parameters. Thus, membrane stabilization may be the result of a structural rearrangement in the erythrocyte membrane predominantly involving a change in protein-lipid interactions.

MATERIALS AND METHODS

Preparation of Cells

Blood was obtained from healthy individuals by venipuncture; informed written consent was obtained from each donor. Anticoagulation was effected by defibrination. Using standard procedures, the buffy coat was removed and the cells were washed three times in 300 mOsm NaCl. To remove serum albumin and other potential binding substances of the spin probe, the reserve serum was filtered using an Amicon ultrafiltration apparatus and Diaflo ultrafiltration membrane Um10 (molecular exclusion 10,000 daltons). Cells were washed once in serum ultrafiltrate and packed in fresh ultrafiltrate to a hematocrit of 70 ± 2 ml/dl. The cells were analyzed within 18 hours of collection.

Electron-Spin Resonance Spectroscopy

The spin probes 5DS and 5DS-ME (Syva Corp., Palo Alto, California) were dissolved as a 30 mM solution in absolute ethanol. The labeling procedure used was described previously (10), except that the ethanol was evaporated prior to the addition of erythrocytes. Drugs were added following labeling. To standardize drug concentrations with respect to the stabilizing effect, concentrations were selected that caused approximately equivalent degrees of submaximal protection against hypotonic hemolysis (11,12). The final probe concentration was approximately 0.4 μmoles/mg of membrane protein. At this concentration, 5DS has been reported to minimally perturb erythrocytes (13) and to localize in lipid domains in close association with proteins (3,4).

All ESR studies were performed by standard methods as described previously (10). The spin labels used incorporate into the membrane in such a manner that the unpaired electron of the doxyl radical is situated close to, but shielded from, the aqueous phase. A typical spectrum of erythrocytes labeled with 5DS is shown in Figure 1. The distance between the low-field and high-field microwave absorption peaks, $2T_{\parallel}$ (the hyperfine splitting parameter) reports the rotational mobility of the probe and therefore the viscosity of the surrounding environment. With increasing temperatures, intermolecular influences upon the unpaired electron of the probe result in lower values of $2T_{\parallel}$ which reflect a more fluid environment. The hyperfine splitting parameter $2T_{\parallel}$ is therefore directly related to the viscosity of the environment from which the probe is reporting. High values of $2T_{\parallel}$ indicate rigid environments, while low values of $2T_{\parallel}$ indicate more flexible environments (14).

In studies reported here, the directly measured parameter, $2T_{\perp}$, could be determined in 5DS-labeled preparations above about 12°C. This parameter is used along with $2T_{\parallel}$ to calculate the order parameter S (14). The order parameter measures the deviation of the observed ESR signal from the case of a completely uniform orientation of the probe. For a uniformly oriented sample, S = 1; for a random sample, S = 0 (14).

The hyperfine coupling constant also calculated from the directly measured $2T_{\parallel}$ and $2T_{\perp}$ parameters is considered to reflect local polarity (15) and thus reflects the position of the probe within the membrane.

Data Analysis

The data ($2T_{\parallel}$ vs. temperature, S vs. temperature) were analyzed in terms of linear components by an iterative least-squares program to be described elsewhere (Brunder, D.G., Coughlin, R.T., McGroarty, E., in press). Briefly, a B-spline (a piece-wise set of polynomials that are smooth at the points of connection) was used to provide a fit for the ESR data and points of inflection were used to group data. Regression lines were calculated for each group and break points were determined. This analysis has been shown in other membrane systems to permit characterization of subtle changes (16). All ESR spectral parameters changed with temperature reversibly up to 48°C. For each set of data, blood samples were examined from at least two healthy, nonsmoking individuals.

RESULTS

Control Erythrocytes

ESR spectra of 5DS-labeled, intact erythrocytes showed little or no free probe in the supernatant, indicating that its site is predominantly in the cellular phase (Figure 1). The shape of the spectra indicated that the majority of the fatty acid label was in a single environment over the temperature interval examined (0-48°C). The hyperfine splitting parameter, $2T_{\parallel}$, decreased in a discontinuous fashion as a function of temperature, and a break point was determined to occur at 37°C (Figure 2a). Spectra recorded at cuvette temperatures above approximately 12°C allowed the determination of the order parameter, S. When S was plotted as a function of cuvette temperature, a similar discontinuity was

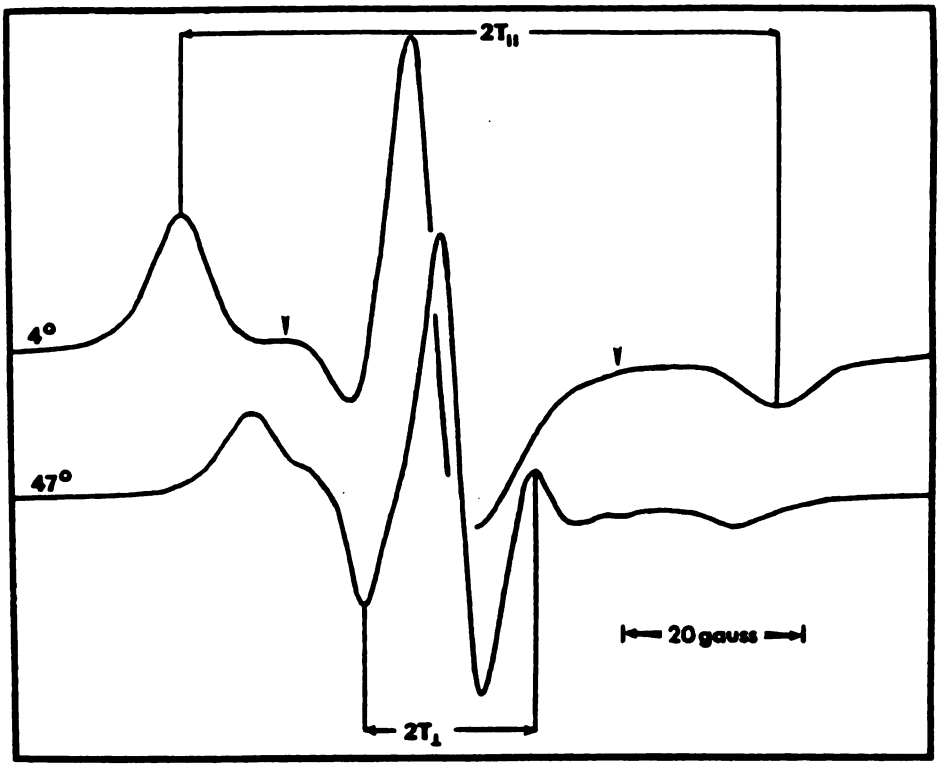

Figure 1. Electron-spin resonance of human erythrocytes labeled with 5-doxyl stearate.

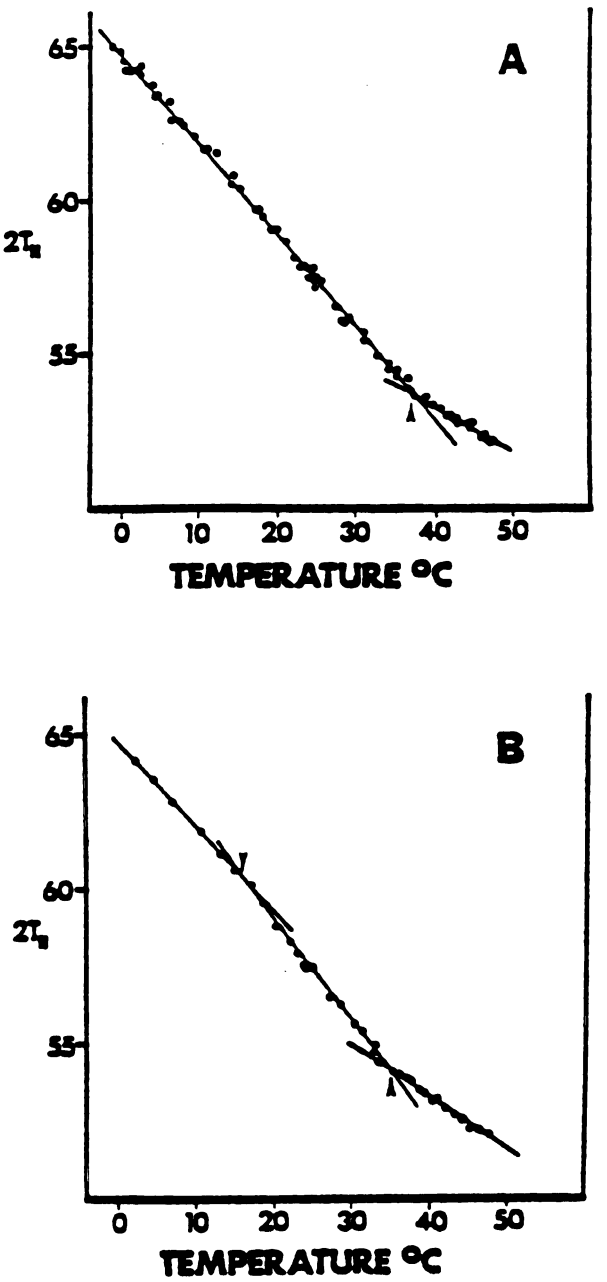

Figure 2. Hyperfine splitting parameter, $2T_{\parallel}$ (Gauss), as a function of temperature in erythrocytes labeled with 5-doxyl stearate in the absence of perturbants (A), and in the presence of 5×10^{-4} M propranolol (B). Arrows indicate the transition temperatures.

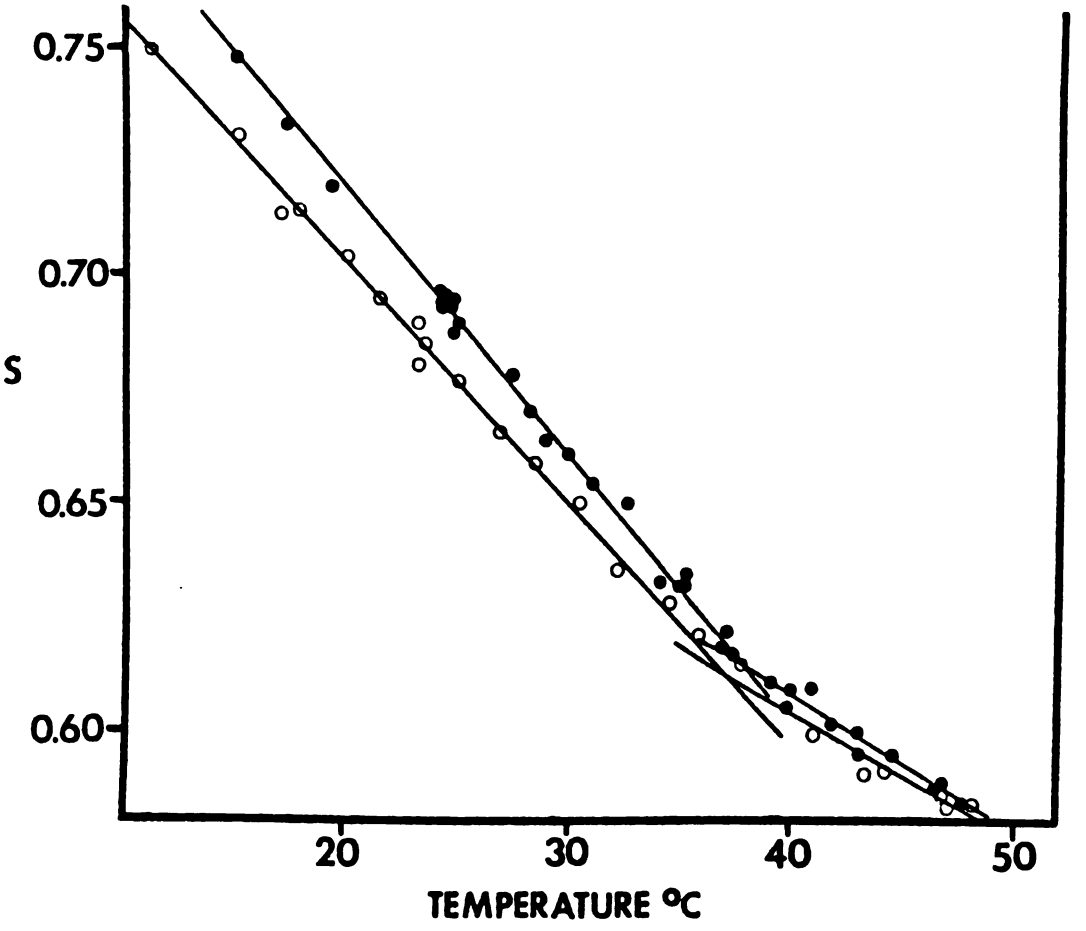

Figure 3. The temperature depence of the order parameter, S, in erythrocytes labelled with 5-doxyl stearate in the absence of perturbants (closed circles) and in the presence of 3 x 10^{-4} M diazepam (open circles).

observed (Figure 3). The transition temperature determined with the order parameter agreed quite well with that determined using $2T_{\parallel}$ and presumptively indicates a structural change in the erythrocyte membrane.

A second spin probe 5DS-ME was also used to analyze erythrocyte membranes. This uncharged probe causes less perturbation of the cells. Spectra of 5DS-ME-labeled cells allowed determination of the hyperfine coupling constant, which indicated that the probe was in an environment similar to that of 5DS-labeled cells at temperatures up to approximately 28°C. Above that temperature, the spectra changed in such a manner that the spectral parameters were difficult to measure.

In spectra of 5DS-ME-labeled cells recorded between 0°C and 28°C, $2T_{\parallel}$ was shown to decrease discontinuously with a break at approximately 15°C (data not shown). This discontinuity is presumed to reflect a second structural change in the erythrocyte membrane that occurs at lower temperatures than the transition detected with 5DS. Therefore, it appears that erythrocyte membranes exhibit two thermotropic transitions, one at 15°C and a second at about 37°C (Table 1).

Effects of Membrane Stabilizing Agents on Membrane Structure

To further characterize erythrocyte membrane-mediated phenomena, we analyzed the changes in membrane structure induced by compounds known to protect human erythrocytes from osmotic hemolysis, propranolol, diazepam, chlorpromazine (11), and pluronic polyols (polyoxypropylene-poly-oxy-ethylene condensates) (12). The addition of any of these drugs did not appreciably alter the position of the spin probes as determined by the hyperfine coupling constant. Propranolol (Sigma Chemical Corp.) when added to 5DS-labeled erythrocytes caused a slight decrease in the upper

transition temperature as measured using $2T_{\parallel}$ and S (Table 1). In addition, a low temperature transition could be detected with 5DS when propranolol was added (Figure 2b). The temperature of the lower transition detected with 5DS-ME was not significantly affected by the addition of propranolol (Table 1).

Similar studies were carried out with labeled cells in the presence of diazepam, chlorpromazine, or Pluronic F68. All of these agents reduced the upper transition temperatures but did not appreciably alter the lower transition temperature (Table 1). Furthermore, the addition of diazepam or chlorpromazine (but not Pluronic F68) permitted the detection of the low-temperature transition in 5DS-labeled cells. In addition, the presence of diazepam was shown to significantly alter the order parameter as indicated in Figure 3. The other perturbants induced only slight changes in the order parameter. The addition of diazepam caused a decrease in S by as much as 2% at low temperatures; changes greater than 1% in S are regarded as significant (17,18).

DISCUSSION

Numerous investigations employing a variety of techniques have reported temperature-dependent changes in erythrocyte membranes. Considering the diversity of approaches utilized, the temperatures at which changes were found to occur are remarkably similar to those reported here. Thus quenching of intrinsic tryptophan fluorescence by spin labels showed discontinuities at 15°C and 35°C (4). Laser-raman spectroscopy (5) and viscosimetry (6) revealed a discontinuity at approximately 18°C. A discontinuity in the susceptibility of human

at 37°C (8). Finally, the preservation of membrane lipid asymmetry by Mg^{+2} upon lysis has been shown to diminish above 18°C and disappear at about 40°C (9). These data support the contention that temperature-induced structural transitions occur in erythrocyte membranes and can be detected using spin-labeling techniques. Apparently, these transitions occur in local areas not influenced by cholesterol.

Data presented here indicate that there are two thermally induced structural transitions in human erythrocyte membranes that can be detected using the ESR probes described above; one of these is found at 15°C, and the other at 37°C. These transitions are presumed to be associated with changes in the conformation of either membrane proteins, phospholipids, or both. Two independent studies, one involving quenching of intrinsic tryptophan fluorescence (2,3), the other involving the determination of binding affinities (19), support the contention that the spin probe 5DS is closely associated with erythrocyte membrane protein. The high-temperature transition demonstrated in this study, therefore, appears to be associated with protein-lipid interaction.

In all cases, the addition of the membrane stabilizing drugs to the spin-labeled erythrocytes predominantly affected the high but not the low transition temperature. Since 5DS reportedly localizes in close proximity with membrane proteins and since lipids interact more strongly with proteins at elevated temperatures (20), it appears highly probable that these agents perturb protein-lipid interactions. In fact, stabilization of the erythrocytes by other drugs has been reported to require an intact membrane protein structure (21).

Addition of propranolol, chlorpromazine, and diazepam (but not Pluronic F68) to erythrocytes allowed for the determination of a

erythrocytes to benzyl-lysolecithin has been reported to occur at about 15°C (7). Transfer of phospholipid from hemagglutinating virus of Japan to erythrocyte membrane was reported to begin at about 19°C and saturate low-temperature transition using 5DS and caused hemolysis at high concentrations in isotonic saline (data not shown). Since Pluronic F68 did not cause hemolysis at high concentrations, it is probable that its structural interaction with the membrane has unique features that may be especially important to its effectiveness as an antihemolytic agent. Diazepam was shown to significantly alter membrane order. We are aware of at least one instance in which a drug-induced decrease in erythrocyte membrane order (17) can be correlated with a protein conformational change (22).

Propranolol, diazepam, chlorpromazine, and Pluronic F68 have been reported to protect erythrocytes against hypotonic hemolysis. Preliminary evidence (manuscript in preparation) indicates that membrane structural changes induced by some of these drugs are maximal at concentrations that cause maximal protection to hypotonic hemolysis. Thus, it is probable that these compounds affect specific membrane domains such as sites of lipid-protein interaction and that these domains are especially significant to cell fragility. The marked reduction of intact cell osmotic fragility and the increase in mechanical deformability that is brought about by an elevation in temperature, or by the presence of perturbants such as the drugs used in this study (17,23,24), might therefore be mediated by specific membrane structural changes. If the relevant changes, as suggested here, are related to lipid-protein interactions in the erythrocyte membrane, there is the possibility of developing more highly specific agents to modify these

structures in a controlled fashion. Such agents might find wide application in a variety of disease states in which red cell destruction or erythrocyte perfusion in the microcirculation are critical factors.

CONCLUSIONS

The human erythrocyte membrane was shown by biophysical probing techniques to undergo structural changes at 15°C and 37°C. Addition of membrane stabilizing drugs caused a decrease in the high-temperature transition but did not appreciably alter the structural change detected at lower temperatures. Furthermore, one of these drugs, diazepam, caused a significant disordering of the membrane lipids. It is proposed that these drugs are altering the membrane structure by changing the lipid-protein interactions within the membrane.

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