NORMAL AND ABNORMAL RED CELL MEMBRANES
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Contributions of Lipid to Red Cell Membrane Form and Stability. I. Molecular Arrangements and Dynamics

472 STRUCTURAL ORGANIZATION AND DYNAMICS OF PHOSPHOLIPIDS IN RED CELL MEMBRANES, L.L.M. van Deenen, Laboratory of Biochemistry, University of Utrecht, The Netherlands.

This paper reviews the possibilities and limitations of the use of phospholipases in the determination of the topographical distribution of phospholipids and their metabolizing systems in biomembranes. In mammalian erythrocyte membranes the choline-containing phospholipids, sphingomyelin and lecithin are predominantly concentrated at the exterior region whereas phosphatidylserine and phosphatidylethanolamine are located preferentially at the inner lipid monolayer of the membrane. A similar non-random distribution of phospholipids was detected in the plasma membrane of platelets. A relationship is suggested to exist between phospholipid asymmetry of blood cells and blood coagulation.

Current studies on the interaction of various intrinsic and extrinsic membrane proteins with monolayers and bilayers of phospholipids may contribute to the understanding of the asymmetric architecture of the red cell membrane.

The major pathways for the renewal of red cell phospholipids are (i) the exchange of these molecules between plasma lipoproteins and the erythrocyte, (ii) the acylation of mono-acyl phosphoglycerides. The exchange process occurs primarily at the exterior region of the lipid bilayer, whereas the incorporation of fatty acids into phosphoglycerides appeared to be located at the interior side of the erythrocyte membrane. In addition a temperature-dependent translocation of lecithin species between the two pools or sides of the erythrocyte membrane has been reported.

Studies on model systems revealed a number of factors which may induce or facilitate transmembrane movement of phospholipids.

473 TOPOLOGY OF AMINO-PHOSPHOLIPIDS IN THE RED CELL MEMBRANE. G. V. Marinetti and R. C. Crain, Department of Biochemistry, University of Rochester, Rochester, N.Y. 14642

The topology of phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the erythrocyte membrane, inner mitochondrial membrane and retinal rod disc membrane was determined by use of the following covalent chemical probes: trinitrobenzenesulfonate (TNBS), fluorodinitrobenzene (FDNB), methylacetimidate (MA), isethionylacetimidate (IMA) and difluorodinitrobenzene (DFDNB). The effect of time of reaction, temperature, probe concentration, buffer composition and pH were investigated (1-4). With mitochondria but not with red cells, buffer composition influences the activity of membrane-bound phospholipases which can degrade the dinitrophenyl and trinitrophenyl derivatives of PE. TNBS and IMA are impermeable probes under appropriate conditions whereas MA, FDNB and DFDNB are permeable probes. Both DFDNB and MA cross-link phospholipids to phospholipids, phospholipids to proteins and proteins to proteins. The results show that PE and PS are localized on the inner surface of the red cell membrane but on the outer surface of the retinal rod disc membrane. Approximately 70% of the PE is localized on the inner surface of the inner mitochondrial membrane. Cross-linking probes indicate that PE and PS occur as clusters in the red cell membrane. PE and PS are cross-linked to spectrin in the red cell membrane and possibly to rhodopsin in the retinal rod disc membrane. K^+-valinomycin forms a complex with TNBS and converts it from a non-penetrating to a penetrating probe. The penetration of TNBS induced by K^+-valinomycin was determined by the increased labeling of PE and hemoglobin. Rb^+ and Cs^+ can substitute for K^+. This permits the use of the same probe to sense both surfaces of the red cell membrane. The effect of interacting red cells first with MA and then with TNBS or FDNB indicate that these probes sense different populations of PE and PS molecules. Some PE and PS molecules do not react with MA but do react with TNBS or FDNB. Some PE and PS molecules are not accessible to any of these probes and are presumed to be tightly associated with membrane protein. Therefore the red cell membrane has heterogenous domains of PE and PS. Some of these domains appear to be involved in the transport of anions and cations. MA does not influence K^+ or inorganic phosphate (Pi) leak in red cells. FDNB markedly inhibits PI leak but enhances K^+ leak. TNBS inhibits Pi leaks and has little effect on K^+ leak. Pretreatment of cells first with MA to saturate available amino groups does not influence the effects of FDNB on K^+ or Pi leak and gives only slight protection against the effects of TNBS. These results suggest an asymmetric arrangement for anion and cation transport in the red cell.

This work was supported by NIH grant HLB 02063.

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474 INTERACTIONS OF INFLUENZA AND PARAINFLUENZA VIRUSES WITH CELL PLASMA MEMBRANES, F. R. Landsberger and D. S. Lyles, The Rockefeller University, New York, N.Y. 10021. Enveloped viruses acquire a membrane during their assembly by budding at the plasma membrane of the host cell. The composition of the viral lipid bilayer is largely determined by that of the host cell plasma membrane, whereas the proteins of the virion are virus specific. Associated with the glycoproteins which form the projections on the surface of influenza virus are hemagglutination and neuraminidase activities. Parainfluenza viruses also exhibit these activities and in addition have a glycoprotein F which is involved in hemolysis, fusion of the viral envelope with cell membranes, and the fusion of cell membranes with each other. Initial events of enveloped virus infection involve virus attachment to the cell surface probably followed by fusion of the viral envelope with the cell membrane. Attachment of influenza and parainfluenza viruses or lectins to erythrocytes causes a change in the lipid bilayer fluidity of the plasma membrane of avian erythrocytes, which contain microtubules, but not of mammalian erythrocytes, which lack microtubules. The effect of agglutinins with reduced valence on the structure of the avian erythrocyte membrane indicates that the cross-linking of receptors is obligatory for the observed structural change in the plasma membrane upon adsorption of influenza and parainfluenza virions. In the intact erythrocyte, the phosphatidylcholine and phosphatidylethanolamine derivative spin labels exist in membrane regions which are different in their fluidity. Hemolysis induced by Sendai virus, a parainfluenza virus, reduces this heterogeneity in the fluidity of the erythrocyte bilayer. The rate of the Sendai virus hemolysis induced change in the erythrocyte bilayer is less than or equal to the rate of fusion of the lipid bilayers of Sendai virions and erythrocytes. However, the rate of fusion of virus with erythrocytes is independent of the presence of hemolytic activity. For Sendai virions with full hemolytic activity, envelope fusion appears to be the rate-limiting step in virus-induced hemolysis.

Contributions of Lipid to Red Cell Membrane Form and Stability. II. Structural and Functional Consequences of Alterations in the Lipid Bilayer

475 "BILAYER BALANCE" AND THE REGULATION OF RED CELL SHAPE CHANGES, N. Mohandas, A. Greenquist, and S. B. Shohet, Cancer Research Institute, Hematology Division, University of California, San Francisco, CA 94143.

The bilayer couple hypothesis of Sheetz and Singer suggests that the asymmetrical distribution of phospholipids between inner and outer leaflets of the membrane may explain drug induced cell shape changes. To explore the role of the spatial distribution of a natural membrane phospholipid in the modification of cell shape, human RBC were incubated in buffered saline with glucose and 70 μmol/l of the echinocytic agent ^{14}C lysolecithin (LPC). Total membrane LPC was determined after saline washes, while inner leaflet LPC was determined after 4 g% albumin washes which removed LPC from the outer leaflet. Within 5 min the initial discocytes were transformed into echinocytes (uniformly spiculated spherocytes) containing 160 nmol LPC/ml RBC in the outer half of the bilayer, and no LPC in the inner half. After 7.5 hr of incubation these echinocytes were transformed back into discocytes; outer layer LPC remained unchanged at 160nmol/ml RBC but, surprisingly, the inner layer LPC increased to only 20 nmol and only 30 nmol phosphatidyl choline was formed by acylation. Albumin washing of these discocytes produced stomatocytes (cup shaped invaginated cells), with the 20 nmol LPC/ml RBC remaining in the inner leaflet and none in the outer leaflet. Such cells could then be re-converted into either discocytes or echinocytes with graded second additions of LPC to the outer layer. Hence different echinocytogenic (LPC outside), and stomatocytogenic (LPC inside) effects occurred with differing localizations of this agent. Further, these results indicate a differential concentration dependence for cell shape with LPC in the inner and outer halves of the bilayer, and suggest that a simple membrane expanding effect on each layer is insufficient to explain the production of these morphologic changes.

476 INCREASED MEMBRANE CHOLESTEROL AND DECREASED MEMBRANE FLUIDITY IN HUMAN RED BLOOD CELLS, Richard A. Cooper, Hematology-Oncology Section, Department of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104.

Cholesterol and phospholipid are the two major lipids of the red cell membrane. Phospholipid serves to solubilize cholesterol. Increases in the cholesterol/phospholipid (C/P) of both red cell membranes and lipoproteins have been studied under three conditions: first, spontaneous increases in vivo have been observed in patients with severe liver disease (1); second, similar increases in vivo have been induced by the administration of cholesterol enriched diets to rodents, and we have recently observed similar changes in dogs (2); third, increases in membrane cholesterol in vitro can be induced by enriching the C/P of the lipoprotein environment by the addition of cholesterol-phospholipid dispersions (liposomes) with a C/P of >1.0 (3). In each case, there is a close relationship between the C/P of the plasma environment and the C/P of the red cell membrane. In vivo, the C/P mole ratio of red cell membranes ranges from a normal value of 0.9-1.0 to values which approach but do not reach 2.0. In vitro, cholesterol enrichment of red cells may proceed to values which approach 3.0 (4). The C/P of red cell membranes directly influences membrane lipid fluidity, as assessed by the rotational diffusion of hydrophobic fluorescent probes such as diphenyl hexatriene (DPH) (4). A close correlation exists between increases in red cell membrane C/P and decreases in membrane fluidity over the range of membrane C/P from 1.0 to 2.0; however, little further change in fluidity occurs when membrane C/P is further increased to 2.0-3.0. Cholesterol enrichment of red cell membranes is associated with the transformation of cell contour to one which is redundant and folded, and this is associated with a decrease in red cell filterability in vitro (3). Circulation in vivo in the presence of the spleen further modifies cell shape to a spiny, irregular (spur) form (5), and the survival of cholesterol-rich red cells is decreased in the presence of the spleen. Although active Na-K transport is not influenced by cholesterol enrichment of human red cells, several carrier-mediated transport pathways are inhibited. We have demonstrated this effect for the co-transport of Na + K (6) and similar results have been obtained by others in studies of organic acid transport and the transport of small neutral molecules such as erythritol and glycerol. Thus red cell membrane C/P is sensitive to the C/P of the plasma environment. Increasing membrane C/P causes a decrease in membrane fluidity, and these changes are associated with a reduction in membrane permeability, a distortion of cell contour and filterability and a shortening of the survival of red cells in vivo. 1. Cooper, R.A. et al. J. Clin. Invest. 51:3182, 1972. 2. Cooper, R.A. et al. Clin. Res. 25: 454A, 1977. 3. Cooper, R.A. et al. J. Clin. Invest. 55:115, 1975. 4. Cooper, R.A. et al. Biochemistry, in press. 5. Cooper, R.A. et al. New Eng J Med 290:127, 1974. 6. Wiley, J.S. and Cooper, R.A. Biochim. Biophys. Acta 413:425, 1975.

477 CALCIUM-MEDIATED POLYPHOSPHOINOSITIDE PHOSPHODIESTERASE ACTIVITY AND MICROVESICULATION OF HUMAN ERYTHROCYTES, David Allan, Department of Experimental Pathology, University College Hospital Medical School, University Street, London WC1E 6JJ, and Robert H. Michell, Department of Biochemistry, University of Birmingham, PO Box 363, Birmingham, B15, United Kingdom.

An ionophore-induced increase of the intracellular Ca^{2+} concentration within human erythrocytes activates a phosphodiesterase specific for polyphosphoinositides and leads to the accumulation of 1,2-diacylglycerol and phosphatidate in the cell membrane (1-4). Associated with the rise in diacylglycerol content, the cells become echinocytic and microvesiculate with the loss of up to 20% of the cell lipid as small (1000Å) membrane-bounded spheres containing haemoglobin (5,6). These microvesicles are enriched in diacylglycerol but depleted of several major membrane proteins, particularly spectrin and actin.

Very similar changes to those produced by Ca^{2+} -ionophore have been observed in cells stored in ACD under normal blood bank conditions (7), and this suggests the possibility that aging in normal erythrocytes leads to a progressive loss of the capacity to maintain the usual low intracellular Ca^{2+} level, resulting in activation of polyphosphoinositide phosphodiesterase and a gradual loss of membrane material as microvesicles.

Some evidence indicates that hereditary spherocytosis also involves a failure of Ca^{2+} homeostasis, giving rise to lipid and shape changes analogous to those seen in aged or ionophore-treated normal cells

These observations suggest the possibility that Ca^{2+} -activated breakdown of polyphosphoinositides and the consequent production of 1,2-diacylglycerol may control the membrane fusion event which leads to microvesiculation.

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478 RELEASE OF SPECTRIN- FREE VESICLES FROM HUMAN ERYTHROCYTES DURING ATP-DEPLETION IS ONLY ONE MODE OF ERYTHROCYTE AGING, Hans U. Lutz, Department of Biochemistry, ETH, Federal Institute of Technology, Zürich, Switzerland.

Human erythrocytes incubated without glucose at 37°C release vesicles that contain the integral membrane proteins but lack spectrin (1). Vesicle release is dependent upon ATP-depletion. Vesicles with a similar protein composition have been found in plasma from outdated whole blood stored under blood bank conditions (2). - A method has been developed to purify vesicles originating from erythrocytes starting from platelet-rich plasma using filtration and adsorption of vesicles to antibodies (against M/N bloodgroup determinants) reversibly coupled to glas beads. Plasma from blood stored over 10 days contains vesicles that are depleted of spectrin but retain the integral membrane proteins and an additional protein band (app. mol w. 140000) not present in vesicles isolated from washed erythrocytes depleted of ATP. On the other hand plasma of freshly drawn blood does not reveal detectable amounts of spectrin-free vesicles containing all integral membrane proteins. However, a minute mass of vesicles (30-50 ug protein per unit of blood) can be recovered with a different protein composition. The major protein component of these vesicles is a sialoglycoprotein that has a slightly lower app. mol. weight than glycophorin. This protein component does not represent a contamination by platelet membranes. Acetylcholinesterase is one of the minor proteins present in these vesicles (70 umoles/ h.mg protein in vesicles). Whether the prominent protein band is partially identical with glycophorin is not yet established. Although these vesicles can be purified using erythrocyte specific antibodies and their composition is reminiscent of those components known to be decreased in old erythrocytes (sialic acid, acetylcholinesterase, lipids), contaminants from other blood cells could not yet be excluded entirely.

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Peripheral Membrane Proteins: Role in Red Cell Membrane Shape and Stability.

I. Chemistry

479 PHYSICAL-CHEMICAL STUDIES OF SPECTRIN,
Gregory B.Ralston, Department of Biochemistry,University of Sydney, Sydney,N.S.W., 2006, Australia.

The high molecular weight protein, spectrin, has been implicated in the maintenance of the stability and shape of the erythrocyte membrane (1). A clear knowledge of the structure, shape and size, and interaction behaviour of this protein is essential for an understanding of its functional role. Although spectrin is believed to associate with erythrocyte actin in the presence of salt (2), purified spectrin does not appear to undergo salt-dependent self association (3). The two major association states, dimer and tetramer, are stable in moderate salt concentrations, and do not dissociate or associate further (3). These two association states have been characterized by means of physical-chemical techniques. Both states appear to be highly expanded, but relatively symmetrical structures, quite distinct from the rod-like structure of myosin (4). In both the tetramer and dimer, spectrin appears to contain about 60% α-helical conformation. The dimer, however, may contain several percent less helical content than the tetramer. In low ionic strength solutions, the tetramer undergoes a temperature-dependent dissociation to the dimer (5), accompanied by changes in the circular dichroism spectrum. Spectrin has not been observed in the electron microscope under negative contrast in our laboratory, but others have observed a three-lobed structure in purified spectrin with the aid of high resolution shadowing techniques. Such a structure is consistent with the hydrodynamic properties. The apparent Stokes' radius of 200 Å, determined from the hydrodynamic studies, indicates that a monolayer of spectrin, perhaps cross-linked with erythrocyte actin, is able to cover the entire cytoplasmic surface of the erythrocyte membrane, and leads to a plausible model for the functional role of spectrin.

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480 EVIDENCE FOR A REPEATING STRUCTURE IN SPECTRIN, James M. Anderson, The Biological Laboratories, Harvard University, Cambridge, MA 02138

Native spectrin has trypsin susceptible sites spaced at a constant molecular weight interval. SDS-PAGE gels of trypsinized spectrin show a ladder of Coomasie blue staining bands spaced at 8,000-9,000 dalton intervals from the intact polypeptides, band 1 (240,000 daltons) and band 2 (220,000 daltons) down to about 100,000 daltons. Fragments containing ^{32}P-phosphate, incorporated exclusively into band 2 by an endogenous kinase, were found at 17,000 dalton intervals below intact band 2. We interpret these results as indicative of a repeated quaternary structure.

481 PHOSPHORYLATION AND DEPHOSPHORYLATION OF SPECTRIN, Grant Fairbanks, Joseph Avruch*, Judith E. Dino, Carol Graham* and Kathleen Bober, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545 and *Diabetes Unit, Massachusetts General Hospital, Boston, MA 02114.

Spectrin polypeptide 2 (MW 215,000) is a major substrate for protein kinase-mediated phosphorylation in intact human erythrocytes and isolated membranes. Spectrin phosphorylation requires Mg^{++}, is unresponsive to cAMP and, in isolated membranes, exhibits its maximum rate in isotonic media (1-4). Because the factors controlling spectrin phosphorylation in the circulating red cell are unknown, we are studying the properties of the kinase(s) and phosphatase(s) involved, with emphasis on (i) subcellular localization, (ii) modes of binding to spectrin and to other sites on the membrane and (iii) effects of potential regulatory factors on enzyme activity and localization.

The enzyme phosphorylating spectrin 2 in isolated membranes is observable as a cAMP-independent, salt-stimulated casein kinase. This enzyme is selectively extracted with 0.5 M NaCl (4). Salt-extracted membranes exhibit unimpaired phosphorylation of components 2.1 and 4.5 in the cAMP-stimulated endogenous reaction (2) but are depleted in their capacity to phosphorylate spectrin 2. The latter function is partially restored when the system is reconstituted by addition of extract. Casein kinase in the cytosol normally accounts for about two-thirds of the total cellular activity. The cytosol enzyme resembles the membrane-associated enzyme in its sedimentation properties in high salt (MW ca. 50,000), but has not been characterized otherwise in our laboratories. Only 15-25% of the membrane-associated casein kinase is eluted with spectrin at low ionic strength. The enzyme is recovered bound to spectrin, but is wholly dissociated from the complex by 0.15 M NaCl, which is the optimal concentration for spectrin 2 phosphorylation in the intact membrane (1). These results suggest that the casein kinase is heterogeneous in intrinsic properties or has multiple binding sites on the membrane.

In our hands, dephosphorylation of [^{32}P]spectrin in white ghosts is extremely slow (1,5). Addition of diluted cytosol augments turnover significantly, and over 98% of the total cellular [^{32}P]spectrin phosphatase is recovered in the hemolysate supernatant and washings. The phosphatase is inhibited by ATP and 2,3-DPG at physiologic concentrations, as well as by various other anions; like the kinase, it is unaffected by cyclic nucleotides (5).

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Peripheral Membrane Proteins: Role in Red Cell Membrane Shape and Stability. II. In Situ Relationships

482 MACROMOLECULE MOBILITY AND PROTEIN INTERACTIONS IN THE ERYTHROCYTE MEMBRANE, Daniel Branton, Cell and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Ma 02138.
The lateral mobility of the major transmembrane proteins of the erythrocyte is restricted by protein-protein interactions. These interactions involve both spectrin, actin and other cytoplasmic surface proteins and are being investigated using both electron microscopical and biochemical methods.

483 ROTATIONAL DIFFUSION OF BAND 3 IN THE RED CELL MEMBRANE.
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Slow rotational motion of macromolecules may be investigated using triplet state probes. Following flash excitation with linearly polarized light, rotational diffusion is measured by observing the decay of dichroism of transient absorbance changes (1,2). The method has been applied to the human erythrocyte membrane labelled with the probe eosin isothiocyanate (3). Most of the probe is attached to band 3. The decay of the absorption anisotropy exhibits two components, one decaying with time constant ~ 0.5 ms and one time-independent. This result is consistent with rotation of band 3 which is confined to an axis normal to the plane of the membrane. Self aggregation of band 3 may contribute to the time-independent component. Removal of spectrin does not change the rotational motion of band 3 at pH 7.6, although immobilisation of the protein occurs with spectrin depleted samples at pH 5.4. This latter effect correlates with the aggregation of membrane particles seen in freeze-fracture electron micrographs. The above results have been confirmed using a different probe, iodoacetamidoeosin. Investigations of the inhibitory effects of the two probes on anion transport indicate that they in part occupy different binding sites.

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484 CONCANAVALIN A INHIBITION OF RED CELL MEMBRANE SHAPE CHANGE AND ATPase ACTIVITIES, Michael P. Sheetz, Suzanne Jackowski, and David Sawyer, Physiology Department, University of Connecticut Health Center, Farmington, Connecticut 06032.

We have observed that the binding of Concanavalin A to the external surface of human erythrocyte membranes inhibits both the ATP dependent shape change and ATPase activities of the membranes. Inhibition is detectable with less than 5,000 Con A molecules bound per cell and is maximal at concentrations which do not saturate membrane binding. The addition of Con A to intact cells before membrane isolation also inhibits both membrane functions. The shape change activity in ghosts from cells depleted of ATP by incubation with 20 mM KF was also inhibited by Con A. This suggests that the cellular energy state does not affect Con A's ability to alter membrane activities. Treatment of intact cells with trypsin before ghosting does not alter Con A binding but does abrogate the lectin's effects on both the shape change and ATPase activities. Analysis of the various membrane ATPase activities has shown that Con A binding inhibits the Mg^{++} ATPase and the Na^{+}, K^{+} ATPase but not the Ca^{++}-stimulated Mg^{++} ATPase. These effects are not due to a rise in c-AMP, c-GMP or Ca^{++} levels. Also, no changes in membrane kinase or phosphatase activities were detected with Con A binding. Total membrane phosphorylation and dephosphorylation were measured as well as the phosphorylation and dephosphorylation of the specific proteins. This leads us to suggest that Con A binding causes an undetermined structural change which results in the inhibition of the membrane shape change and ATPase activities.

Peripheral Membrane Proteins: Role in Red Cell Membrane Shape and Stability. III. Membrane Abnormalities

485 FORMATION OF γ-GLUTAMYL-ε-LYSINE BRIDGES BETWEEN MEMBRANE PROTEINS BY A Ca^{++}-REGULATED ENZYME IN INTACT ERYTHROCYTES, L. Lorand, Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, IL 60201.

A rise in the intracellular concentration of Ca^{2+}-ions (to about 0.3 mM or higher, using 10-20 μM ionophore A23187, Eli Lilly, at 37° for periods of about 30 min to 18 hr) in human erythrocytes causes the formation of high molecular weight membrane protein polymers ($Mw > 10^6$), cross-linked by γ-glutamyl-ε-lysine side chain bridges. Cross-linking involves proteins at the cytoplasmic side of the membrane (band 4.1, spectrin and band 3 materials) and the reaction is catalyzed by the intrinsic transglutaminase. This enzyme is regulated by Ca^{2+}-ions and it exists in a latent form in normal cells (Lorand et al., Proc. Natl. Acad. Sci. 73, 4479, 1976). The protein polymer, isolated from the membranes of Ca^{2+}-loaded intact human red cells, is heterogeneous in size and contains as many as 6 moles of γ-glutamyl-ε-lysine cross-links per 100,000 g of protein.

Primary amines (exemplified by cystamine, aminoacetonitrile, glycine methylester or histamine), which compete against the ε-lysine cross-linking functionalities, inhibit polymerization of membrane proteins in intact human erythrocytes. Amines (tested for aminoacetonitrile using scanning electronmicroscopy) also prevent the Ca^{2+}-induced shape change, from discocyte to echinocyte, from becoming irreversible, and they inhibit (as tested for histamine, using the "micropipette" method) loss of membrane deformability. Thus, the irreversible loss of membrane deformability and the irreversible fixing of shape changes in human erythrocytes caused by the intracellular accumulation of Ca^{2+}-ions, seem to be related to the transamidase-catalyzed de novo production of γ-glutamyl-ε-lysine cross-links which are responsible for the formation of large molecular weight membrane protein polymers.

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486 *HEMOLYTIC ANEMIAS ASSOCIATED WITH DEFICIENT OR DYSFUNCTIONAL SPECTRIN*, Samuel E. Lux, Barbara Pease, Mary Beth Tomaselli, Kathryn M. John, and Seldon Bernstein, Div. of Hematology-Oncology, Children's Hospital Medical Center, Boston, MA and Jackson Laboratories, Bar Harbour, ME.

Current evidence suggests that spectrin, actin and Band 4.1 form a protein meshwork which laminates the inner surface of the red cell (RBC) membrane. This "membrane skeleton" can be isolated by treatment of RBCs or ghosts with Triton X-100 and is likely a major determinant of RBC membrane shape, strength and flexibility. To test this postulate we are comparing membrane skeletons and skeletal proteins of congenitally fragile, rigid or misshapen RBCs with normal. Hereditary elliptocytosis (HE) is a dominant trait characterized by >20% elliptical RBCs. We observed that all hereditary elliptocytes form elliptocytic ghosts and membrane skeletons, which implies the HE shape is due to a defective skeletal component(s). Since HE skeletons were quantitatively normal on SDS gels, we sought a qualitative defect in spectrin, the major skeletal constituent. We first tested thermal denaturation (precipitation at 10^5 g for 60 min) of spectrin using a sensitive thermoelectric heater. Chromatographically pure spectrin dimers from 6 normals contained 2 subcomponents: 65% ± 3%(SE) were heat labile, denaturing at 49.0 ± 0.3°C and 35 ± 3% were heat stable to at least 54°C. The explanation for this heterogeneity remains to be determined. Denaturation of the first heat labile subcomponent coincided with membrane fragmentation and spherocytosis. Unlike normal, spectrin from 7/7 HE patients (3 kindreds) was entirely heat labile and abnormally heat sensitive (denatured at 48.0 ± 0.1°C, $p<0.0005$). In these patients RBC fragmentation also occurred at 48°C. Spectrin and RBCs from 2/2 HE patients in a fourth kindred denatured normally. These observations re-emphasize the intimate correlation between spectrin and RBC shape, suggest that defects in the membrane skeleton may be a common feature of HE and indicate that at least 2 genetically distinct forms of HE exist. HE patients with heat sensitive spectrin must have a molecular abnormality of spectrin which alters its conformational stability.

We are also studying RBC membranes of normoblastic (nb/nb), hemolytic (ha/ha), spherocytic (sph/sph) and jaundice (ja/ja) mouse mutants. These mutants all have a severe, recessive, hemolytic anemia. RBCs show marked membrane budding, fragmentation and spherocytosis, suggesting membrane instability. Ghosts spontaneously vesiculate and are variably spectrin deficient: ja/ja ghosts have no detectable spectrin, sph/sph ghosts have no Band 1 but traces of Band 2, ha/ha ghosts have 25-30% and nb/nb ghosts 45-50% of both Bands 1 and 2. The clinical severity of the 4 mutants correlates with the degree of spectrin deficiency. The cause of this deficiency remains to be determined. Preliminary studies exclude defective membrane binding and suggest that increased spectrin proteolysis is occurring. These mutants prove that spectrin is a critical determinant of membrane structural integrity and provide a unique opportunity to test, in intact RBCs, putative functions of spectrin.

487 ABNORMAL MEMBRANE PROTEIN ORGANIZATION IN ATP DEPLETED AND IRREVERSIBLY SICKLED RED CELLS, J. Palek, St. Vincent Hosp., U. Mass. Med. School, Worcester, MA 01604.

The spectrin actin submembrane network may participate in control of red cell (rbc) shape and deformability which depends on rbc ATP and Ca levels. Employing membrane protein crosslinking (XL) and a two dimensional polyacrylamide gel electrophoresis, we investigated the nearest membrane protein neighbors in (1) rbcs undergoing ATP depletion and/or Ca accumulation and (2) irreversibly sickled cells (ISCs) which exhibit a permanent membrane deformation and have an increased Ca and decreased ATP content.

(1) Echinocyte (E) formation produced by anaerobic rbc ATP depletion or by Ca^{2+} (0.1 mM) + ionophore A23187 was associated with membrane attachment of 4.5 (catalase) and globin. After XL of intermolecular protein -SH groups in ATP depleted rbc ghosts by catalytic oxidation with $CuSO_4$ + O-phenanthroline, a $>1 \times 10^6$ daltons polymer (P) was produced which was absent in fresh rbcs. After P cleavage by dithiothreitol (DTT) reduction, the P constituents were separated in the 2nd dimension and subjected to densitometric scanning. The P was selectively enriched in spectrin while bands 3 and actin were less prominent than in normal rbc membranes. Similar P was found in untreated ghosts of aerobically ATP depleted rbc due to spontaneous intermolecular disulfide couplings. P formation under both conditions was prevented by maintenance of ATP and was decreased after a subsequent ATP repletion. A similar P was produced in an- or aerobically ATP depleted rbcs (but not fresh rbcs) by non-reducible XL of other protein groups, such as amino groups by glutaraldehyde. Formation of spectrin enriched P in ATP depleted, Ca enriched rbcs after XL of their protein -SH or $-NH_2$ groups suggests a rearrangement of spectrin to closer contacts which may contribute to E formation. In addition, Ca^{2+} introduction at conc. >0.5 mM into fresh rbc by A23187 produced an irreversible P containing both spectrin and band 3, which others attributed to their endogenous XL catalyzed by Ca^{2+} stimulated rbc transglutaminase. Both aerobically ATP depleted E forming spontaneously a reducible P and Ca (>0.5 mM) enriched rbc containing a nonreducible P failed to restore biconcave shape after a subsequent ATP repletion and Ca extrusion suggesting that membrane protein polymerization contributed to a fixation of cells in E shape.

(2) ISCs separated on a discontinuous stractan gradient resembled ATP depleted, Ca enriched rbc in attachment of 4.5 and globin. However, they failed to exhibit the P both spontaneously or after XL with catalytic oxidation or glutaraldehyde. When ISCs were further depleted in ATP or enriched in Ca^{2+} by A23187, they transformed into E and this was accompanied by P formation. Thus, ISC formation is not related to Ca^{2+}, ATP dependent spectrin rearrangement or formation of permanent crosslinks between the adjacent membrane proteins. However, further progressive Ca accumulation and ATP depletion in ISCs leads to their transformation into E which resemble in vivo spiculated ISCs occasionally found in the densest ISC fraction. The latter cells may represent an end stage ISC lesion resulting in ISC removal.

Integral Membrane Glycoproteins: Mediators of Transmembrane Actions. I. Transport

488 STRUCTURAL STUDIES ON RED CELL BAND 3 PROTEIN, Steck, T. Dept. of Biochem. & Med. Univ. of Chicago, Chicago, IL 60637

Band 3, the predominant 95,000-dalton polypeptide and purported anion transport protein of the human erythrocyte membrane, was cleaved into large fragments representing its outer-surface (38K), membrane-spanning (17K), and cytoplasmic-surface (40K), regions. The former two peices are glycosylated and are hydrophobic in both composition and behavior. The 40K cytoplasmic polypeptide is readily released into solution by proteolysis. It is distinctly polar in composition and is far more immunogenic than the two integral segments. It contains binding sites for both glyceraldehyde 3-P dehydrogenase (G3PD) and aldolase; certain segments of this fragment are potent inhibitors of aldolase catalytic activity. The cytoplasmic pole of band 3 can be identified in freeze-etch electron micrographs, particularly when decorated with G3PD. Neither the binding of the two glycolytic enzymes nor the proteolytic excision of the 40K polypeptide significantly alters anion (i.e., pyruvate) transport in inside-out membrane vesicles. A 22K tryptic fragment from the inner-surface donain has an unreactive amino-terminus, suggesting that the blocked N-terminus of band 3 is oriented toward the cytoplasm. A similar, N-terminal 23K S-cyanylation fragment was prepared, further cleaved with trypsin and cyanogen bromide, and the composition of its sub-fragments established. A partial amino acid sequence analysis of this 23K fragment will be presented.

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489 A MODEL FOR THE ACTION OF THE ANION EXCHANGE PROTEIN OF THE RED BLOOD CELL
A. Rothstein, P.A. Knauf, S. Grinstein, Y. Shami and S. Ship, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada.

The kinetics of anion transport are consistent with an equilibrating mobile carrier model in which the anion-transport-site complex can move across the membrane in either direction, but in which the movement of the "unloaded" carrier is very slow. Binding of anions to a second site, the modifier, results in non-competitive inhibition of transport. Studies using inhibitory chemical probes capable of covalent reaction have led to the identification of band 3 (95,000 daltons) as the anion transport protein and have also helped to determine that a 17,000 dalton transmembrane segment is essential. The disulfonic stilbene, DIDS (4,4'-diisothiocyano-2,2'- stilbene disulfonate) and its reduced form (H_2DIDS) are potent non-penetrating compounds that interact with 1 to 1 stoichiometry with band 3 to produce virtually complete inhibition. DIDS reduces the capacity of band 3 to bind Cl^- (measured by NMR). In short experiments in the cold, while the reactions of H_2DIDS are still reversible, the inhibition involves competition with Cl^-, apparently for the transport (carrier) site. DIDS does not, however, have any effect at the cytoplasmic face of the membrane in inside-out vesicles, so the transport site must be accessible to DIDS only from the outside. The photoreactive probe, NAP-taurine (N-(4-azido-2-nitrophenyl)-2-amino ethyl sulfonate), acts in the dark as a substrate. It also inhibits Cl^- transport from either side of the membrane, but with quite different properties. On the outside it is a high affinity inhibitor with behaviour consistent with interaction at the modifier site. On the inside, on the other hand, it is a low affinity inhibitor which appears to act on the transport site.These and other results suggest that the modifier site is accessible only from the outside, but that the transport site is accessible from either side because it is mobile relative to an anion diffusion barrier. This assumption was further tested by demonstrating that the transport sites could be "recruited" to one side or the other. For example, interaction of DIDS with the transport site on the outside reduces the irreversible binding of NAP-taurine to band 3 sites on the inside. The kinetic data, the studies with probes, and the studies of the structure of band 3 and its arrangement in the membrane are consistent with a model in which a 17,000 dalton segment of band 3 forms a channel through the lipid, providing a pathway for anion transfer. Since the flux is a non-conductive obligatory exchange,the channel must be blocked by a diffusion barrier. It is proposed that anion-loaded transport sites can oscillate rapidly across this barrier in the protein channel by a local conformational change so that they alternately become accessible to the inside and outside. The rate of the conformational shift would be influenced by interactions of anions with the modifier site. (The study was supported by Medical Research Council (Canada) Grants #MT4665 and #MA5149).

490 STUDIES OF THE D-GLUCOSE TRANSPORTER FROM HUMAN ERYTHROCYTES. Peter C. Hinkle and David C. Sogin, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N. Y. 14853.

The reconstitution and purification of the D-glucose transporter from human erythrocytes has been described (1-3). A Triton X-100 extract of NaCl-washed ghosts was chromatographed on DEAE-cellulose yielding a single band on SDS-polyacrylamide gel electrophoresis (PAGE) with apparent molecular weight of 55,000. When the protein was incorporated into liposomes of soybean phospholipids or phosphatidylcholine plus phosphatidylethanolamine it catalyzed specific D-glucose permeability with K_m = 2.2 mM and V_{max} = 1 μmole/min. mg protein. The band was unusually broad on both continuous and discontinuous PAGE. The trailing edge of the band ran slower than the leading edge on a second electrophoresis. Amino acid analysis indicated a different composition from other red cell proteins and a calculated minimum polypeptide molecular weight of about 45,000. Carbohydrate analysis showed glucosamine 3.7%, galactose 3%, mannose 1.2%, fucose 0.5% and glucose 0.25%. N-terminal amino acid determination by Dansylation showed only aspartic acid.

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Integral Membrane Glycoproteins: Mediators of Transmembrane Actions. II. Abnormalities of Membrane Barrier Function

491 HEREDITARY STOMATOCYTOSIS. W.C. Mentzer, G. Lam, B.H. Lubin, A. Greenquist and S.L. Schrier. Departments of Pediatrics and Medicine, University of California, San Francisco, CA 94143; Department of Medicine, Stanford University, Stanford, CA 94305. Hereditary stomatocytosis is a rare form of congenital hemolytic anemia in which the red cells are swollen and cup shaped instead of normal biconcave discs. Anion permeability is normal but cation permeability, measured with ^{24}Na and ^{42}K, is enormously increased in stomatocytes (Na^+ influx = 96-128 meq/L. cells/Hr., normal = 2.8 ± 0.3; K^+ efflux = 8.3-40.3 meq/L. cells/Hr., normal = 1.7). Active transport of both Na^+ and K^+ is increased 5-12 fold, but is nonetheless insufficient to prevent accumulation of Na^+ and loss of K^+. Total cell monovalent cation content ($Na^+ + K^+$) is increased as is cell water (704-777 ml/L. cells), size (MCV = 108-122 fl), and osmotic fragility. Cell deformability, assessed by filtration through 5 μ Nuclepore filters, is reduced. Possibly because of their rigidity (as is the case with hereditary spherocytes) stomatocytes are sequestered by the spleen where they are thought to undergo metabolic depletion with subsequent hemolysis. Unlike spherocytosis, splenectomy reduces but does not abolish hemolytic anemia in stomatocytosis. Electron microscopy, analysis of total membrane lipids or phospholipid ratios, and PAGE of SDS solubilized membrane proteins have revealed no abnormalities of membrane structure or composition. The amount of spectrin phosphorylation (at 1 hour) is only 20% of normal. Red ghost endocytosis is strikingly impaired. The cation permeability defect can be completely eliminated in vitro by incubation of stomatocytes with bifunctional imidoesters such as dimethyl adipimidate (DMA). Within 6 hours, active transport of cations restores the Na and K content of treated cells to normal. Cell water content, size, morphology, osmotic fragility, and membrane deformability also become normal. Red ghost endocytosis increases to normal but spectrin phosphorylation is not improved. The survival (T 1/2) in vivo of DMA treated, ^{51}Cr labeled stomatocytes infused into rats rendered tolerant to human erythrocytes by pretreatment with ethyl palmitate and cobra venom factor is double that of untreated stomatocytes. After reaction of ^{14}C-DMA with stomatocytes, radiolabel is found associated with several membrane amino phospholipids (TLC) and is widely distributed among membrane proteins (SDS-PAGE). Thus, the site of action of imidoesters is not yet defined.

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492 ERYTHROCYTE RECEPTORS FOR MALARIA MEROZOITES, Louis H. Miller, Florence M. McAuliffe, and James Johnson, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20014

The in vivo host specificity of malarial parasites is determined in large measure by the ability of the merozoite to invade erythrocytes of the species in vitro. The block in invasion may be caused by absence of receptors for attachment or for subsequent steps in invasion. It is known that Duffy negative erythrocytes are not invaded in vitro by Plasmodium knowlesi (1) and that individuals with Duffy negative erythrocytes are resistant to infection by P. vivax (2). The majority of blacks have this phenotype and are resistant to P. vivax.

Four questions will be discussed:

1) Is resistance the result of the FyFy genotype or a closely linked gene (3)?
2) If the Duffy blood group system is involved in invasion, what is the nature of the determinant?
3) Where does the Duffy blood group system function in the invasion sequence?
4) What are the erythrocyte determinants for P. falciparum (4)?

In addition, the mechanism by which merozoites enter within an invagination of the erythrocyte membrane will be discussed. It is known that malaria merozoites have limited motility and invade a nonphagocytic cell, the mature erythrocyte. Invasion, therefore, must be explained other than by a parasite pushing its way into a cell or by the cell ingesting the parasite.

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Integral Membrane Glycoproteins: Mediators of Transmembrane Actions. III. Membrane Antigens and Receptors

493 STRUCTURE AND ANTIGENIC RELATIONSHIPS OF VARIOUS GLYCOPHORINS IN RED CELL MEMBRANES, Heinz Furthmayr, Department of Pathology, Yale University, New Haven, Conn. 06510

The sialoglycoproteins of the human red cell membrane, glycophorin A and B, carry the antigens coded for by the MNSs locus. M or N antigenic activity is correlated with differences in the amino acid sequence of glycophorin A isolated from red blood cells of individuals that are homozygous for these antigens. The amino terminal amino acid sequence of A^M is ser-ser-thr-thr-gly and that of A^N leu-ser-thr-thr-glu. The sequence of glycophorin B is identical to glycophorin A^N in the first 23-25 residues regardless of the MN type of the cells, from which it has been isolated, and possesses serological N-activity. Antibodies to a C-terminal determinant of glycophorin A have been used to study genetic variants, which lack MN antigens. Although this antigenic deficiency is associated with the absence of glycophorin A in certain variants, the red cell membrane of others contains normal amounts of this protein.

*indicates glycosylation at these amino acid residues.

494 ABSENCE OF GLYCOPHORIN AND INCREASED BAND 3 GLYCOSYLATION IN HUMAN EN(a-) RED CELLS, Carl G. Gahmberg, Jorma Wartiovaara and Ismo Viranen, Department of Bacteriology and Immunology, and Department of Electron Microscopy, University of Helsinki, Helsinki, Finland.

The human En(a-) red cell is a rare recessive trait (1,2). It lacks the MN antigens and shows abnormal agglutination with lectins. Recently we and others have shown that the membrane of these cells lacks the major sialoglycoprotein, glycophorin (3 - 5). Interestingly there is instead more carbohydrate on the band 3 polypeptide but no or very little N-acetyl neuraminic acid in this protein. This indicates that during the biosynthesis of the membrane glycoproteins the cell can compensate for the loss of a major glycosyl transferase acceptor by adding more sugars to other available acceptors.

Because it is unclear whether the intramembrane particles seen on freeze-fracturing contain glycophorin, we have studied these particles of the En(a-) cells. Such particles are present in En(a-) cells in about the normal amount but we have now got evidence that their distribution is different as compared to normal cells. Under certain conditions they are more easily clustered than those of normal cells. The data indicate but do not prove that this is due to the absence of glycophorin from the En(a-) particles, and that glycophorin normally is a constituent of these particles.

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495 THE TRANSFERRIN RECEPTOR OF ERYTHROID CELLS, Philip Aisen, Hsiang-Yun Yang Hu, Adela Leibman and A.I. Skoultchi, Departments of Biophysics and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461.

Transferrin is a major, and possibly the only, source of iron for the biosynthesis of hemoglobin by the immature erythroid cell. The initial event in the delivery of iron from protein to cell is the binding of transferrin to specific receptors on the cell surface. These receptors are destroyed by proteolytic enzymes, and are lost as the red cell matures into a circulating erythrocyte no longer capable of hemoglobin synthesis.

Because of its accessibility in peripheral blood the reticulocyte is often used as a model of the transferrin-dependent, hemoglobin-synthesizing primitive red cell. When membranes of reticulocytes incubated with ^{125}I-labeled transferrin are solubilized in detergents, complexes of transferrin and one or more membrane components may be demonstrated by gel chromatography. These membrane-derived macromolecules bind transferrin in reversible, saturable and specific fashion, and probably represent the transferrin receptor of the plasma membrane.

A Triton X-100 solubilized macromolecular complex of transferrin and a membrane constituent can be isolated by gel chromatography from ghosts of rabbit reticulocytes previously incubated with transferrin doubly labeled with ^{125}I and ^{59}Fe. The hydrodynamic radius of this complex (in detergent) is close to that of ferritin, a spherical molecule with a molecular weight of 445,000. Also evident in the chromatogram is a peak corresponding to free transferrin (MW 80,000). The ^{59}Fe:^{125}I ratio in this peak is appreciably less than in the transferrin-receptor complex, confirming that iron-depleted transferrin binds to the receptor less strongly than iron-transferrin. On SDS electrophoresis the complex displays two glycoprotein subunits, of molecular weights 176,000 and 95,000, in addition to transferrin. Both subunits appear to cross-link to transferrin. They are also present in a transferrin-binding component of apparent molecular weight 350,000-400,000, isolated from membranes of non-incubated reticulocytes. The corresponding membrane fraction from mature erythrocytes shows similar subunits, but the 176,000 MW component fails to give a PAS stain for carbohydrate. We believe, therefore, that the primary transferrin receptor of the rabbit reticulocyte is comprised of two glycoprotein subunits, and that transferrin binding activity depends on the integrity of the carbohydrate.

The Friend erythroleukemic cell behaves like a transformed erythroid precursor cell arrested at an intermediate stage of development. When grown in media supplemented with dimethyl sulfoxide, the Friend cell differentiates into forms resembling orthochromatic erythroblasts, and develops the capability of hemoglobin synthesis. As differentiation proceeds, the transferrin-binding activity of the cells increases 3-5 fold. Gel filtration studies corroborate that this binding is due to specific transferrin-receptor complex formation, indicating that transferrin receptors are inducible by DMSO.

Degradation of Red Cell Membranes

496 MEMBRANE GLYCOPROTEINS IN ERYTHROCYTE SURVIVAL, John R. Durocher, Department of of Medicine, Pennsylvania Hospital, Phila., Pa. 19107.

The recognition process of senescent or altered erythrocytes by the reticuloendothelial system has not been clarified. Decreased erythrocyte deformability explains sequestration of abnormal cells in the microvasculature, but does not explain recognition of abnormal erythrocytes by macrophages. This recognition process probably involves superficial membrane changes. Ashwell et al have shown that certain desialylated radiolabelled glycoproteins are rapidly cleared from the circulation in animals. This accelerated clearance returns toward control levels after oxidation of the newly exposed galactosyl moieties with galactose oxidase or removal of galactose with β-galactosidase. Kay has presented evidence that desialylated human erythrocytes incubated with autologous serum in vitro and older density-separated human erythrocytes, with reduced sialic acid content, have increased binding of autologous IgG. Her findings indicate that IgG attaches to older human erythrocytes in vivo, and may mediate the recognition and phagocytosis of older erythrocytes by macrophages. Desialylation of animal and human erythrocytes by sialidase reduces sialic acid content without affecting cellular deformability. Desialylated ^{51}Cr-labelled erythrocytes are rapidly cleared from the circulation of rats and this destruction follows a 10% enzymatic reduction in membrane sialic acid, a reduction similar to that reported in older density-separated human erythrocytes. However, in splenectomized rats, greater than 50% of membrane sialic acid can be removed with only minor decreases in ^{51}Cr-erythrocyte survival. In contrast to studies of desialylated glycoproteins, galactose oxidase treatment of desialylated rat erythrocytes does not improve ^{51}Cr-erythrocyte survival. Furthermore, galactose oxidase treatment of intact rat erythrocytes does not affect cellular deformability, but markedly reduces ^{51}Cr-erythrocyte survival and promotes attachment of autologous immunoglobulins. Subsequent sodium borohydride reduction of the aldehyde returns erythrocyte survival toward control levels and reduces IgG binding. Mild periodate oxidation forms an aldehyde on erythrocyte membrane sialic acid moieties. Periodate-treated human and rat erythrocytes maintain normal cellular deformability, bind autologous immunoglobulins, and decreased ^{51}Cr erythrocyte survival occurs in rats. Both immunoglobulin binding and decreased survival are partially reversed by borohydride reduction. Finally, the immunoglobulins which bind to sialidase- or galactose oxidase-treated erythrocytes can be absorbed from autologous serum by repeated exposure to altered erythrocytes. Immunoglobulins from absorbed serum, however, will then bind to cells treated with the alternative modification. These findings suggest that erythrocytes can be destroyed by the reticuloendothelial system without changes in cellular deformability, and that naturally circulating IgG may mediate in the recognition process between altered erythrocytes and macrophages.

497 ROLE OF PHYSIOLOGIC AUTOANTIBODY IN THE REMOVAL OF SENESCENT HUMAN RED CELLS Marguerite M.B. Kay, Geriatric Center (11G) VA Wadsworth Hospital Center, Los Angeles California 90075.

Macrophages distinguish mature "self" from senescent "self" cells as reflected by their ability to phagocytize cells which have reached the end of their functional lifespan while sparing the mature cells. The mechanism by which macrophages make this distinction was investigated by using human RBC as a model system. Conditions simulating those encountered in situ were adhered to as closely as possible by using short term culture techniques and incubating with autologous cells and immunoglobulins (Ig). Previous studies in this laboratory suggested that macrophages phagocytize senescent cells by recognizing the IgG which are bound on their surface. The presence of IgG on senescent cells is only presumptive evidence that an immunological receptor-IgG binding has occurred and that the IgG is an autoantibody. Definitive evidence for the role autoantibodies play in the selective removal of senescent cells can be obtained first by dissociating the Ig from senescent cells and then by demonstrating their specific reattachment to homologous cells via the F ab region, leading to their selective destruction by macrophages (1). The following results were obtained when these experiments were performed 1) Ig eluted from senescent cells was shown to be IgG free of other Igs by immunodiffusion, immunoelectrophoresis, and polyacrylamide gel electrophoresis; 2) The eluted IgG reattached to old but not young autologous and allogeneic cells via the Fab region as determined by SIEM, and initiated their phagocytosis by macrophages; 3) the phagocytosis inducing ability of the eluted IgG could be removed by absorption with RBC aged in vitro but not by absorption with freshly isolated young RBC. Evidence suggests that the IgG is directed against altered membrane molecules. Thus, the IgG which attaches to senescent RBC appears to be a physiological autoantibody as it may contribute to homeostasis by promoting elimination of aged red cells.

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498 ENDOCYTOSIS IN ERYTHROCYTES AND THEIR GHOSTS, Stanley L. Schrier and Britta Hardy, Department of Medicine, Stanford University School of Medicine, Stanford, Ca. 94305

Endocytosis can be induced in intact erythrocytes, in resealed red ghosts, and in white "hemoglobin free" ghosts and the phenomena can serve as models for the study of pinocytosis and phagocytosis. Intact erythrocytes would presumably represent the most physiologic preparation for study, but in order to understand the molecular events that occur in endocytosis, and the role of membrane associated proteins, the white ghost is a preferred preparation. However, the assumption that there is a common basic mechanism for all forms of erythrocyte endocytosis may not be correct. In intact human neonatal erythrocytes, endocytosis occurs spontaneously while in adult human erythrocytes, it requires the addition of pharmacologic concentrations of agents like Primaquine, Hydrocortisone, Vinblastine, Chlorpromazine and Vitamin A. Even in this group of drug-induced endocytosis, different mechanisms may exist. Chlorpromazine and Vinblastine endocytosis can proceed even when ATP levels are markedly reduced. Primaquine endocytosis, however, is uniquely dependent on persistence of ATP. There are other differences in drug-induced endocytosis: Primaquine endocytosis is enhanced by the ionophore (A23187) mediated entry of Ca into erythrocytes, while Vinblastine endocytosis is inhibited in such circumstances. In contrast, endocytosis in resealed red ghosts seems to be an energized phenomenon requiring Mg-ATP and Ca. The supposition is that a Ca, Mg-ATPase is involved, although it may not be the Ca efflux pump related ATPase. In white ghosts, endocytosis was initially induced by the addition of Mg-ATP. We can now produce endocytosis by exposure of white ghosts to 0.1 mM EDTA, or by controlled trypsin digestion. The resulting endocytic vacuoles, whether produced by Mg-ATP, EDTA, or trypsin, when separated and harvested and subjected to polyacrylamide gel electrophoretic analysis are in each case spectrin-free, while the remaining ghosts have normal PAGE patterns. These observations indicate that there is a common path for white ghost endocytosis involving the displacement of spectrin, or its extraction, or digestion, thereby allowing spectrin-free portions of the membrane to appear. Since exocytic vesicles produced by ATP depletion of erythrocytes are also spectrin-free, it seems that a prior event for either endocytosis or exocytosis is the establishment of spectrin-free areas in the membrane. This hypothesis was tested by resealing rabbit antispectrin antibody into the white ghosts and then attempting to induce endocytosis by adding Mg-ATP, 0.1 mM EDTA, or trypsin. The antispectrin antibody completely inhibited all forms of endocytosis whereas the BSA and rabbit IgG controls did not. Following the proposed creation of spectrin-free areas, the membrane decision as to whether there will then be invagination or evagination probably reflects the known asymmetry of membrane proteins and phospholipids. Since ATP usually produces endocytosis, and ATP depletion produces exocytosis, invagination probably involves phosphorylation of a cytosol facing membrane polypeptide(s).

Membrane Lipid Organization and Dynamics in Normal and Abnormal Red Cells

499 THE EFFECT OF OZONE ON THE MEMBRANE AND METABOLISM OF HUMAN ERYTHROCYTES, B. A. Freeman and J. B. Mudd, Dept. of Biochem., Univ. of Calif., Riverside, CA 92521.
Ozone is an ubiquitous component of photochemical air pollution. In vitro exposures of human erythrocytes to ozone resulted in a decrease of osmotic fragility after low dosages followed by an increase after higher dosages. High concentrations of ozone caused no change in erythrocyte phospholipid, fatty acid or cholesterol composition, even though these lipids are oxidizable by ozone when not in a biological membrane. Ozone is capable of crossing cell membranes. In intact erythrocytes and right side out vesicles, acetylcholinesterase, marking the outside of the membrane and glyceraldehyde-3-phosphate dehydrogenase, marking the inner face of the membrane, were both inactivated by ozone. Ozone had no effect on the activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase and glutathione peroxidase in erythrocytes exposed to ozone. The metabolism of glucose through the pentose phosphate shunt was stimulated in a dose-dependent manner in ozone treated erythrocytes. The increased pentose phosphate metabolism provided the reductive power necessary to re-reduce oxidized glutathione and glutathione-protein mixed disulfides caused by ozone exposure.

500 ENHANCED AGGLUTINATION OF ALL THE ERYTHROCYTES WHEN ONLY HALF THE CELLS ARE TRYPSINIZED. J. A. Gordon and C. A. Kuettner, Univ. Colo. Med. Ctr., Denver, CO 80262.
Enhanced lectin-mediated agglutination following alteration of cells by transformation or protease treatment is well documented. Recent evidence suggests that the enhanced agglutinability of erythrocytes treated with proteolytic enzymes, neuraminidase or both may be the result of reduced surface charge, loss of sterically hindering peptides, and/or increased lectin-cell affinity constant. We wondered whether the alteration in only part of the cell population is sufficient to increase the agglutinability of the entire cell population. The mixed cell agglutination of erythrocytes mixed with either equal numbers of trypsinized cells or neuraminidased cells proceeds to an extent and at a rate similar to cell populations fully treated with either enzyme. This occurred at lectin concentrations of soybean lectin and con A which gave little or no agglutination of untreated cells. Residual tryptic activity (by assay) or the presence of soybean trypsin inhibitor ruled out carry-over proteolytic activity of the treated cell population to the untreated. Preliminary studies indicate an increase in total cell deformability of trypsinized cells. Increased flexibility of the trypsinized cell surface allowing an increased area of cell to cell contact, even with untreated cells, is our working hypothesis explaining the enhanced agglutination. In any case, our observations suggest that explanations of enhanced agglutination based primarily on the removal of surface polypeptides, surface charge, or changes in lectin-cell affinity of the total cell population must be revised. (Supported by grants CA-14313 and CA-15823 from the National Institutes of Health.)

501 THE EFFECT OF COMPLEMENT ON MEMBRANE LIPID ORGANIZATION. Agustin P. Dalmasso, Elda B. Ciavedoni and Ronald P. Mason, Univ. of Minnesota, V.A. Hospital, Minneapolis, MN 55417
To investigate the role of membrane (M) fluidity in the production of complement (C) lesions, sheep red cells were lysed hypotonically and ^{3}H-Dextran 20 was introduced into the M ghosts during resealing. M ghosts were treated with antibody and rabbit or human C. This resulted in a significant increase in M rigidity as measured by changes in mobility of the M-incorporated spin label 5-doxylstearate. Thus, rabbit C caused 4.9% increase in $T_{//}$ and 19.0% decrease in Δ_1. The change was found to be produced by C9. Pretreatment of the M ghosts with increasing concentrations of glutaraldehyde (GA) caused increments in M rigidity. M pretreated with low GA concentrations (0.025%) still underwent further increment in rigidity upon reaction with C. In contrast, at a GA concentration of 0.25% there was no C-induced modification in the mobility of the spin label. The number of C lesions as evaluated by uptake of ^{125}I-C8 either did not change or decreased slightly in GA treated M. However, the C-induced release of ^{3}H-Dextran 20 increased 2-3 times by pretreatment with GA, indicating that the C transmembrane channel attains a larger size in GA treated M. The results demonstrate that the initial fluidity of the M plays no major role in its susceptibility to damage by C and that C-induced changes in M fluidity are not essential in the resulting cytotoxic reaction.

502 THE EXCHANGEABILITY OF HUMAN ERYTHROCYTE CHOLESTEROL, Yvonne Lange and Josephine D'Alessandro, Biophysics Section, Boston University School of Medicine, Boston, MA 02118. The removal from human erythrocytes of cholesterol (mass) and of ^{3}H-cholesterol which had been introduced into the erythrocytes by exchange was studied. It was found that the exchange of cholesterol between erythrocytes and plasma and the net movement of cholesterol out of the cells into plasma of lowered free cholesterol content are characterized by the same rate constant. From this it was shown that cholesterol molecules which participate in exchange and cholesterol molecules which can be removed from the membrane are in the same pool. This led to a novel method of determining what fraction of red cell cholesterol is available for exchange. Erythrocytes which had been labelled with ^{3}H-cholesterol by exchange were incubated with sonicated phospholipid vesicles giving rise to a net movement of cholesterol out of the cells. The cholesterol molecules removed from the membrane came from the pool which was labelled by exchange. The specific activity of the cholesterol taken up by the vesicles was greater than the specific activity of the cholesterol in the erythrocytes, indicating the presence of erythrocyte cholesterol molecules which were not labelled by the exchange process. From these studies it was concluded that approximately 90% of the cholesterol in human erythrocytes is exchangeable. Since it is extremely unlikely that 90% of erythrocyte membrane cholesterol is located at the outer half of the membrane bilayer, this confirms that transmembrane movement of cholesterol occurs in the human erythrocyte as has been shown previously (Lange, Y, Cohen, C.M. and Poznansky, M, 1977, Proc. Nat'l. Acad. U.S. 74, 1538). The reason for the nonexchangeability of 10% of human erythrocyte cholesterol is under investigation.

503 X-RAY SCATTERING FROM RED CELL MEMBRANES, J. Stamatoff, T. Bilash, and Y. Ching, Bell Laboratories, Murray Hill, New Jersey 07974

X-ray scattering studies of suspensions of red cell membranes have been performed using a new high flux, high brightness X-ray system with a stable position sensitive detector. The apparatus makes possible accurate recording of patterns from hemoglobin-free membranes in concentrated dispersions at higher resolution than previously obtained. More importantly, these patterns may be recorded from samples in suspension (avoiding the use of high g centrifugation).

We have used heavy atom stains (including one which is specific for membrane protein) to determine the thickness of the lipid bilayer region of the membrane. Analysis of the heavy atom distribution also provides information about protein distribution and the consequences of spectrin removal.

504 FACTORS INFLUENCING THE HYDROLYSIS OF RED BLOOD CELL MEMBRANE PHOSPHOGLYCERIDES BY BEE VENOM PHOSPHOLIPASE A_2, S.A. Singal and L. Huey, Medical College of Georgia, Augusta, GA 30901

The action of highly purified bee venom phospholipase A_2 on the hydrolysis of phosphoglycerides and hemolysis of red blood cells has been studied. Results are in general agreement with those of Martin et al. (Biochemistry 14, 5400 (1975)) who used the basic phospholipase from Agkistrodon halys blomhofii. At pH 7.4, 10 mM Ca^{++}, 60% of phosphatidylcholine (PC) and 8 percent of phosphatidylserine (PS) were hydrolyzed by the bee enzyme with no evident hemolysis. At pH 7.4, 40 mM Ca^{++} and pH 8.0, 40 mM Ca^{++}, PC, PS and phosphatidylethanolamine (PE) were completely hydrolyzed. This was associated with extensive hemolysis. However, the initial stages of hydrolysis of PS and PE occurred prior to onset of hemolysis. 5 mM glucose prevents the effects associated with elevated pH and/or [Ca^{++}], restricting PC hydrolysis to 60 percent, and also prevents the lowering of intracellular ATP occurring under these conditions. Enzyme concentration did not influence the time of onset of hemolysis. However, the time of addition of enzyme, Ca^{++} or glucose did influence the onset time. Influx of Ca^{++} occurred when intracellular ATP was lowered.

505 ROLE OF BOUND WATER IN MEMBRANE STRUCTURE: FLUORESCENCE AND INFRARED STUDIES
Allan S. Schneider and C. Russell Middaugh, Sloan-Kettering Institute for Cancer Res. and Cornell University Graduate School of Medical Sciences

Bound water is a major component of biological membranes and is required for the structural stability of the bilayer and the mixing of cholesterol and phospholipids. It has also been postulated to be involved in aqueous transport, membrane fusion and mobility of membrane proteins and lipids. We have measured the fluorescence emission of membrane bound ANS and the infrared spectra of membranes, both as a function of hydration. ANS fluorescence is sensitive to polarity and fluidity of the membrane aqueous interface, while infrared absorption is sensitive to the hydrogen bonding and vibrational motion of water and membrane proteins and lipids. The results provide: a) evidence of increasing rigidity of the membrane aqueous interface with removal of loosely bound water, b) an identification of several classes of membrane bound water in terms of their infrared OH stretching frequencies, c) an estimate of protein exposure to the aqueous environment via H_2O-D_2O exchange, and d) an indication of hydration dependent conformational changes in specific membrane components. Further characterization of bound water should allow its incorporation into current models of membrane structure and give insight into the relevance of membrane hydration to cell surface function.

506 ACTION OF COBRA VENOM PHOSPHOLIPASE A_2 TOWARD LIPIDS OF ERYTHROCYTE MEMBRANES, Marina Adamich and Edward A. Dennis, Department of Chemistry, University of California at San Diego, La Jolla, California 92093

Studies on the disposition of lipids in surfaces of erythrocyte membranes utilizing phospholipases have been interpreted to suggest that phospholipids in these membranes are asymmetrically arranged. For such studies, it is important that pure enzyme be used and that the specificity of the enzyme be known. In our laboratory, a single form of phospholipase A_2 from cobra venom (Naja naja naja) was purified to homogeniety and was found to have a monomer molecular weight of about 11,000 [Deems, R.A. and Dennis, E.A. (1975) J. Biol. Chem. 250, 9008-9012]. This enzyme has been used to investigate the specificity of phospholipase A_2 toward various phospholipids in mixed micelles with the nonionic surfactant Triton X-100 [Roberts, M. F., Otnaess, A-B., Kensil, C.A., and Dennis, E.A. (1978) J. Biol. Chem. 253, in press]. It was found that the enzyme prefers phosphatidylcholine and that phosphatidylethanolamine is a poor substrate for the enzyme. It is important to know whether this enzyme is active toward erythrocyte membranes and whether the observed specificity is similar to that determined with mixed micelles. We have now examined the action of the homogeneous phospholipase A_2 toward intact human erythrocytes and ghost membranes, toward these membranes solubilized with Triton X-100, and toward extracted erythrocyte membrane lipids in mixed micelles with Triton. We will report on these findings, and on their relevance to membrane phospholipid asymmetry.

Peripheral Membrane Proteins in Normal and Abnormal Red Cells

507 CONTROL OF THE INTERACTION OF SPECTRIN AND ACTIN BY PHOSPHORYLATION. Jennifer C. Pinder, MRC Cell Biophysics Unit, 26-29 Drury Lane, London WC2B 5RL.

Spectrin isolated from fresh normal red blood cells by conventional methods causes polymerisation of muscle G-actin in solvents which normally depolymerise F-actin. Exogenous nucleotide is not essential. Electron microscopy of the polymer shows bundles of actin filaments which decorate with S1 and SDS-PAGE of the pellet obtained after centrifugation shows actin and smaller amounts of spectrin. Control of this polymerisation is shown to depend on phosphorylation of the smaller spectrin subunit. This specific phosphorylation may be achieved on spectrin in solution using a preparation of an endogenous cAMP-independent phosphoprotein kinase from erythrocyte ghosts. Phosphorylated spectrin causes rapid actin polymerisation whereas the activity is lost after dephosphorylation with bacterial alkaline phosphate. Extraction of erythrocyte ghosts with Triton X100, leaves a fibrous protein network, comprising about 60% spectrin and actin. The addition of ATP and kinase to a 20 mg/ml suspension of cytoskeletal extract leads to the rapid formation of a stiff gel; if DNAase I is added at actin equivalence to the in vitro system, no gelation ensues. Muscle actin and erythrocyte actin are not identical, they may be distinguished by 2D-PAGE migrating as α-actin and β-actin respectively. There is no evidence that gelation involves actin polymerisation rather than an association between spectrin and actin monomers. We suggest that actin and spectrin form a complex which undergoes morphological changes mediated by spectrin phosphorylation.

508 QUANTITATION OF TOTAL AND 32P LABELED SPECTRIN PHOSPHATES, H. William Harris Jr., L. C. Wolfe and S.E. Lux, Children's Hos. Med. Cent. Boston, MA 02115

The number and distribution of phosphorylated residues in spectrin may help to control red cell shape and deformability. Protein bound phosphate measurements of A-15 column chromatography purified (greater than 98%) spectrin from fresh red cells by a microphosphomolybdate assay yield 6.9±0.8(n=9) moles of phosphate per 460,000 spectrin dimer(Bands 1+2). This number is reduced to 4.0±0.3(n=8) per spectrin dimer after denaturation of purified spectrin in 6M guanidine HCl or 8M urea. Incubation of intact erythrocytes with 32P orthophosphate and quantitation of spectrin 32P label at equilibrium(30-32 hrs.) by simultaneous determination of SDS gel protein and ATP specific activity results in 2.5±0.1(n=9) moles of 32P per spectrin dimer. The 32P label of spectrin is phosphoserine or phosphothreonine as determined by pH stability studies. Trypsinization converts all of the 32p label into low molecular weight peptides. Concomitant with the loss of phosphate, urea or guanidine denaturation of purified spectrin releases 1-2% of its 32P label. The nature of this material is currently under investigation. We conclude that isolated spectrin from fresh red cells contains 4 phosphorylated sites, 2 or 3 of which possess significant turnover rates in the mature red cell under in vitro incubation conditions. In addition, approximately 3 moles of phosphorus are noncovalently associated with each spectrin dimer but represent only a small fraction of the total 32P label present. The multiplicity of spectrin phosphorylation sites suggests that spectrin's interaction with other red cell components may be controlled by a complex interplay between these sites and not simply phospho and dephospho forms. Phosphorylation defects may represent both qualitative and quanitative changes in spectrin phosphate distribution.

509 ERYTHROCYTE TROPONIN INHIBITOR-LIKE PROTEIN: ISOLATION AND PARTIAL CHARACTERIZATION, Jonathan Maimon and Saul Puszkin* -- Department of Pathology, Mount Sinai School of Medicine of The City University of New York, 100th Street & Fifth Avenue, New York, NY 10029

Human erythrocytes contain a modulatory protein which binds calcium and has phosphodiesterase activating activity. From a human red cell lysate, we have isolated a protein with an apparent molecular weight of 24,000 daltons. This protein was purified by DEAE-cellulose chromatography and concentrated by ammonium sulfate precipitation. On SDS-acrylamide disc electrophoresis this protein co-migrated with troponin inhibitor (TN-I) isolated from striated muscle. The erythrocyte TN-I-like protein was found to inhibit actin-activated magnesium ATPase activity of muscle myosin. Furthermore, it partially inhibited muscle myosin calcium and potassium-EDTA ATPase activity. When introduced into a mixture of muscle myosin-actin-troponin-C (TN-C), the erythrocyte TN-I blocked TN-C modulatory effects. Excess erythrocyte-TN-I showed again inhibition of myosin actin interaction. Binding experiments performed with myosin coating the surfaces of polystyrene latex particles showed the erythrocyte inhibitor sedimenting with myosin as determined by elution of coated proteins on SDS acrylamide gel electrophoresis. The presence of an inhibitory protein in erythrocyte may be significant for the modulation of contractile protein activity. Its presence in the cytoplasm of red cells probably represents a controlling mechanism for the modulator proteins recently described in erythrocytes.

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* S.P. is an Established Investigator of the A.H.A.

510 FILTERABILITY AND MORPHOLOGY OF RED BLOOD CELLS (RBC) FROM VITAMIN E-DEFICIENT LEAD-POISONED VS. IRON-DEFICIENT RATS. O.A. Levander, V.C. Morris and S.O. Welsh, Nutrition Institute, U.S. Dept. Agriculture, Beltsville, MD 20705

Vitamin E deficiency and lead poisoning interact to produce an increased hemolytic anemia and splenomegaly in rats. Filterability through polycarbonate membranes of RBC from E-deficient (-E) rats decreased during incubation in tris-buffered saline and lead poisoning (+Pb) accelerated this decline. The decreased filterability of RBC from -E+Pb rats was related to the extent of RBC lipid peroxidation and was prevented by feeding the synthetic antioxidant, N,N'-diphenyl-p-phenylenediamine (DPPD). Filterability of RBC from -E+Pb rats was decreased by lowering the pH of the incubation medium from 7.4 to 6.6, an acidity typical of the spleen. Incubation with H_2O_2 decreased the filterabiity of RBC from -E non-poisoned rats and concurrent lead poisoning intensified this effect. Older (denser) RBC from -E+Pb rats lost their filterability much faster during incubation in vitro than younger RBC. RBC from -E+Pb rats were sphero-stomatocytes after losing their filterability, whereas RBC from +E-Pb rats retained filterability and discocytic shape. Iron-deficient non-poisoned (-Fe) rats are also characterized by anemia, splenomegaly and decreased filterability of RBC incubated in vitro. As with RBC from -E+Pb rats, the impaired filterability of RBC from -Fe rats was decreased further by lowering the pH of the incubation medium to 6.6. But filterability of RBC from -Fe rats was not affected by incubation with H_2O_2, was not related to lipid peroxidation, and was not accompanied by formation of sphero-stomatocytes. These studies demonstrate two different mechanisms by which nutritional status influences RBC filterability and suggest new approaches to the problem of RBC deformability.

511 MEMBRANE ALTERATIONS IN IRREVERSIBLY SICKLED CELLS (ISC'S): ULTRASTRUCTURAL, MEMBRANE PROTEIN AND RHEOLOGIC CORRELATIONS, L.S. Lessin, J. Kurantsin-Mills, H.B. Weems. The George Washington University Medical Center, Washington, D.C. 20037

The ISC functions as an initiator of microvascular occlusion and a determinant of hemolytic rate in sickle cell disease. ISC's are sickle erythrocytes which retain bipolar elongated shapes despite reoxygenation and owe their biophysical abnormalities to acquired membrane alterations. Our studies of ISC membranes are summarized as follows: Structural: (1) Freeze-etched membranes of both in vitro produced and in vivo isolated ISC's show microbodies fixed to the internal (PS) surface obscuring spectrin filaments. (2) Membrane particles on the PF surface aggregate over regions of subsurface microbodies. (3) TEM sections of D.A.B. treated ISC ghosts show microbodies to contain hemoglobin. (4) SEM and freeze-etching show that membrane-hemoglobin interaction in ISC's enhances membrane loss by microspherulation. Biochemical (1) Membrane-bound hemoglobin is 5X greater in vivo ISC's than non-ISC's and increases during ISC production, paralleling depletion of ATP, increase in cellular Ca++ and reversal of Na+ & K+ gradients. (2) SDS-PAGE gel analysis of ISC membranes show increases in macroaggregated proteins in the pre-Band-1 regions, Band 8 and globin. Rheologic: (1) Micropipette elastimetry of ISC's shows 4X increase in membrane rigidity ("P") vs. controls & 7X decrease in cell deformability ("PT"). (2) Pressure-flow filtration at ambient pO2 and temperature across Nuclepore filters shows a 3-5 fold increase in relative resistance (Rr) of sickle cell blood. (3) Both Rr and dP/dt (rate of pressure rise) correlate (r=0.89 & 0.94) with ISC content of sickle blood. (4) SEM study filtration shows pore plugging to correlate with dP/dt and to be principally due to ISC's.

512 INTERACTION OF LOCAL ANESTHETICS WITH NORMAL AND SICKLE CELL MEMBRANES, Vijay K. Kalra and Richard F. Baker, Department of Biochemistry and Microbiology, University of Southern California, School of Medicine, Los Angeles, California 90033.

Studies were carried out to determine the effect of metabolic depletion and EGTA on the formation of irreversibly sickled cells in vitro. Deoxygenated SS cells incubated for 24 hrs at 37° with and without glucose and EGTA exhibited no significant differences in the rate of filtration through a 5μm Nucleopore filter. The percentage of ISC formation were similar under the above conditions. These results suggest that metabolic state and Ca^{+2} levels in SS cells are not the prime determinants of ISC formation. Studies by Baker et al. (BBRC 75, 381, 1977) have shown that procaine and p-aminobenzoic acid influence the deformability and Ca^{++} uptake in metabolically depleted AA cells and the time rate of formation of ISC in vitro. Photoaffinity derivatives of procaine and p-aminobenzoic acid have been used to characterize the site of interaction of these drugs in the membranes of AA and SS cells. Data will be presented which show that chloropromazine affects the red cell shape without invoking Ca^{++} displacement. The role of local anesthetics in mediating cell shape changes is not necessarily related to Ca^{++} displacement from the membrane (Supported by grant from NIH-HL-15162).

513 PHYSICAL CONTACTS BETWEEN SPECTRIN AND BAND 3 IN HUMAN ERYTHROCYTE MEMBRANE, S.C. Liu and J. Palek, St. Vincent Hospital and University of Massachusetts School of Medicine, Worcester, Ma. 01604.

Spectrin and actin are thought to interact with other erythrocyte membrane components and to affect cell shape and viscoelastic properties. We have examined the nearest neighbor arrangement of these proteins in the membrane by coupling the adjacent intermolecular sulfhydryl groups and analyzed the complexes with two-dimensional gel electrophoresis. At physiological pH (7.4) and isotonic conditions, cross-linkings between spectrin-actin and band 3 (i.e. 1+3, 2+3, 1+5, and 3+5) were observed after mild oxidation of ghosts with oxygen-saturated Tris buffer or with $CuSO_4$/O-phenanthroline in phosphate buffer. Similar cross-linkings have previously been demonstrated in ghosts incubated in hypotonic Tris-acetate buffer at pH 4.0-5.5 (S.C. Liu, G. Fairbanks, and J. Palek, Biochemistry 16:4066, 1977). The amount of 1+3 complex produced by oxidation at pH 7.4 depended on ionic strength and divalent cations. The yield of this complex gradually diminished as the NaCl conc. decreased from 90 mM to 30 mM. In addition, Mg^{2+} or Ca^{2+} at 1 mM stimulated 1+3 formation in ghosts incubated in the presence of low salt (0.5 mM phosphate buffer, pH 7.4). The data suggest (1) structural linkage between the spectrin-actin network and band 3 in the erythrocyte membrane and (2) the degree of their contacts is in part governed by divalent cations and electrostatic interactions.

514 ERYTHROCYTE SHAPE AND DEFORMABILITY. J.E. Smith, N. Mohandas, M.R. Clark, S.B. Shohet. Div. Hematology, Univ. of Calif., San Francisco; Dept. Path., Kansas State Univ.

Abnormally shaped erythrocytes such as spherocytes, sickle cells, and acanthocytes have been associated with decreased deformability and a shortened erythrocyte life span. We have investigated the relationship between shape and deformability by studying three similarly shaped elongated cells: non-hemolytic human elliptocytes, irreversibly sickled cells (ISCs), and normal llama erythrocytes. Properties studied included cell deformability (measured with an ektacytometer), morphologic and osmotic alterations induced by anionic and cationic phenothiazines, spectrin extractability, and membrane protein kinase activity. The human elliptocytes were normally deformable and reacted similarly to discocytes: membrane-active drugs produced uniformly spiculated echinocytes and sphero-stomatocytes with unipolar endovesicles. They also had normal casein kinase activity and normal spectrin extractability. ISCs were undeformable and produced an uneven and random distribution of spicules and endovesicles, suggesting localized membrane lesions. In contrast to the elliptocytes, they had normal casein kinase activity and, as shown previously by Lux et al, the extractability of their spectrin was reduced. Llama cells were virtually undeformable and demonstrated very limited morphologic response to drugs in spite of normal drug uptake. These cells had no casein kinase activity, and had significantly reduced spectrin extractability.

These observations indicated that similarly shaped red cells need not be associated with similar mechanical properties of their membranes. They also suggest that erythrocyte deformability and morphologic changes induced by membrane-active drugs may be modulated by the state of spectrin aggregation and casein kinase activity.

515 MYOSIN-LIKE ATPASE ACTIVITY IN SPECTRIN PREPARATIONS. Francis H. Kirkpatrick, Thomas E. Milligan, Mary L. Sweeney & Paul L. La Celle, Dept. Radiation Biology & Biophysics, U. of Rochester School of Med. & Dent., Rochester, N.Y. 14642. We have previously reported stimulation of a spectrin-associated ATPase by $CaCl_2$. This ATPase is preferentially activated by 1 M KCl or NH_4Cl. NaCl and other monovalent cations are inactive. The K^+-ATPase is inhibited by micromolar levels of ionized Mg^{2+}, and calcium above 10 mM reduces ATPase activity to the level seen with calcium alone. The ratio of activities at optimal NH_4, K, Ca and Mg is approximately 2:1:1:0.3. This pattern of activity is similar to that shown by myosin. However, we are unable to obtain stimulation of the Mg-ATPase activity with actin (from rabbit muscle) under a variety of conditions including those which cause stimulations of muscle myosin ATPase. Actin also inhibits the NH_4 or K ATPase activity even in the absence of Mg. We have established that the Ca or K-ATPase is not due to a phosphatase-kinase couple, to a non-specific phosphatase, or to an AMP or cAMP producing enzyme plus a pyrophosphatase. Hence, we conclude that the activity is due to a myosin-like enzyme. The range of specific activities of skeletal myosins is wide enough that the activity could be due to 100% or 0.1% of the protein present. We can remove up to 3/4 of the spectrin and retain 90% of the activity, but are unable to determine whether the activity is due to spectrin polypeptides or to erythrocytic myosin. In either event the myosin-like activity has a number of unusual properties. Supported by NIH and DOE (Report No. UR-3490-1268).

516 RESEALING WHITE RBC GHOSTS WITH AND WITHOUT ADDED HEMOGLOBIN: USE OF ESR AND OXYGEN DISSOCIATION CURVES TO ASSAY RESEALING, P.D.Morse II, D.Simpson, H.Mizukami, D.Luszczakoski, and R.Palazzo. Dept. of Biology, Wayne State University, Detroit, Michigan 48202

Methods currently available for resealing red cell ghosts allow a substantial amount of hemoglobin to remain. We report here a method to remove hemoglobin and still reseal the cells.

Red cells and ghosts are prepared essentially by the method of Steck and Kant (Methods in Enzymology 31:172, 1975). Washed red cells are lysed in 30 vols. of 5 mM PO_4 buffer (pH 8) and sedimented at 40,000 g. This is repeated 3-4 times to obtain white ghosts. Ghosts are resealed in 150 mM NaCl at 37°C for 1 hour. Assay by glyceraldehyde-3-phosphate dehydrogenase indicates complete resealing. However, the ghosts are not resealed to small ions. The spin label TEMPAMINE (2,2,6,6-tetramethyl-piperidine-N-oxyl-4-amine) freely crosses red cell membranes (Morse, P.D.II, BBRC 77:1486,1977). When TEMPAMINE and 80 mM $K_3Fe(CN)_6$ are added to a fresh suspension of resealed ghosts, only a broadened TEMPAMINE signal is seen. This indicates interaction between TEMPAMINE and $Fe(CN)_6^{3-}$ and demonstrates that $Fe(CN)_6^{3-}$ is inside the ghosts. Addition of TEMPAMINE and $Fe(CN)_6^{3-}$ to the resealed ghosts after incubating overnight at 0°C yields an unbroadened TEMPAMINE signal. This indicates that TEMPAMINE is inside the cell, but $Fe(CN)_6^{3-}$ is not. Thus, exposure to 0°C is required to reseal white ghosts to $Fe(CN)_6^{3-}$.

If hemoglobin (30 mg/ml) is resealed into the ghosts, O_2 dissociation curves with and without $Fe(CN)_6^{3-}$ are similar to those of hemoglobin in solution. This also confirms sealing of the cells to $Fe(CN)_6^{3-}$.

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517 PARTITIONING OF HEMOGLOBIN IN MEMBRANES FROM CA^{+2}-LOADED HUMAN ERYTHROCYTES, J.K. Haynes, F.-Z. Shiao, M. Deveau and H. Patthey, Meharry Med. Col., Nashville, TN. 37208
Ca^{+2}-loaded human erythrocytes are abnormally shaped and rigid cells which may serve as models for pathologic erythrocytes such as irreversibly sickled cells. Membrane preparations from Ca^{+2}-loaded cells retain hemoglobin which may be involved in the altered cell properties. This investigation further clarifies the nature of the association between hemoglobin and membranes. Ultrastructural examination reveals that membrane preparations contain, in addition to ghosts, electron dense vesicles. Centrifugation in sucrose density gradients resolved the preparation into a pure vesicle fraction and a second fraction composed predominantly of whole ghosts. When the two fractions were assayed for hemoglobin, 50% of the hemoglobin was contained in the vesicle fraction while the remainder was associated with the fraction composed chiefly of whole ghosts. Since the latter fraction was contaminated with a few vesicles, unfractionated membrane preparations were stained with diaminobenzidine (DAB) in order to determine if hemoglobin was associated with whole ghosts. Some ghosts were observed to contain DAB reaction product along their cytoplasmic surfaces. This reaction is not due to catalase since it was not inhibited by 50mM aminotriazole which abolished the peroxidatic activity of catalase. Published data indicate that high intracellular levels of Ca^{+2} cause erythrocytes to extrude vesicles containing hemoglobin which is not bound to the vesicle membrane. Coupled with the analyses reported here, these data indicate a need for caution before concluding that hemoglobin present in membrane preparations is bound to membranes. The cytochemical observations reported here provide the first definitive evidence that hemoglobin is bound to membranes from Ca^{+2}-loaded cells.

518 PEPTIDE MAPPING OF THE SPECTRIN POLYPEPTIDES. Alun H. Maddy, Richard B. Kemp and Michael J. Dunn, Department of Zoology, University of Edinburgh, Edinburgh, EH9 3JT, SCOTLAND. The spectrin polypeptides P1 and P2 from the EDTA extract of ox erythrocyte membranes have been isolated in pure state by preparative polyacrylamide gel electrophoresis in the presence of SDS. Amino acid analysis of the two polypeptides show that both contain the characteristically high leucine and glutamate contents of the parent unfractionated extract. The two polypeptides were labelled with ^{125}I by Chloramine T catalysis and ^{14}C by radioactive iodoacetate and hydrolysed with pig trypsin. Peptide maps of the digests were prepared on TLC silica gel plates by chromatography and electrophoresis. Autoradiographs of maps prepared from both ^{125}I and ^{14}C labelled polypeptides showed that P1 and P2 share many common peptides while also having peptides unique to themselves. It is concluded that the two major polypeptides of spectrin are similar to each other but not identical.

519 SPECTRIN CONTROLS THE LATERAL MOBILITY OF INTEGRAL MEMBRANE PROTEINS IN HUMAN ERYTHROCYTES. V. Fowler, The Biol. Labs., Harvard Univ. and V.Bennett, Dept. Molec. Biol., Wellcome Research Lab.
Interactions between spectrin and the inner surface of the human erythrocyte membrane have been implicated in the control of lateral mobility of the integral membrane proteins. We report here that incubation of "leaky" erythrocytes with a water soluble proteolytic fragment containing the membrane attachment site for spectrin achieves a selective and controlled dissociation of spectrin from the membrane, and increases the rate of lateral mobility of FITC-labelled integral membrane proteins (>70% of label in band 3 and PAS-1). Mobility of membrane proteins is measured as an increase in the percentage of uniformly fluorescent cells with time after fusion of fluorescent with non-fluorescent erythrocytes by Sendai virus. The cells are permeable to macromolecules since virus-fused erythrocytes lose most of their hemoglobin. The membrane attachment site for spectrin has been solubilized by limited proteolysis of inside-out erythrocyte vesicles and has been purified (V.Bennett, J. Biol. Chem., in press). This 72,000 dalton fragment binds to spectrin in solution, competitively inhibits association of ^{32}P-spectrin with inside-out vesicles with a K_i of $10^{-7}M$, and causes rapid dissociation of ^{32}P-spectrin from vesicles. Both acid-treated 72,000 dalton fragment and the 45,000 dalton cytoplasmic portion of band 3 which also was isolated from the proteolytic digest have no effect on spectrin binding, release or on membrane protein mobility. The enhancement of membrane protein lateral mobility spectrin from these vesicles provides direct evidence that the interaction of spectrin with protein components in the membrane restricts the lateral mobility of integral membrane proteins in the erythrocyte.

520 SPECIFIC ASSOCIATION OF ACTIN WITH THE CYTOPLASMIC SURFACE OF THE RED CELL MEMBRANE, Carl M. Cohen, Pamela L. Jackson and Daniel Branton, Cell and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, MA. 02138

We have used purified rabbit skeletal muscle actin,chemically tritiated to high specific activity,to investigate the association of actin with the human red cell membrane. The labeled actin is identical to unmodified actin in its ability to polymerise and to activate HMM - ATPase. Using sealed and unsealed red cell vesicles we have shown that actin binds in significant quantities to the cytoplasmic but not the external membrane surface. Almost no actin binding is detected to inside out vesicles which have been stripped of spectrin and endogenous actin by low ionic strength incubation. However, when membrane extracts containing primarily spectrin and small amounts of red cell actin are added back to stripped vesicles under conditions which promote reassociation of spectrin with the cytoplasmic membrane surface, subsequent binding of labeled actin is enhanced almost 40 fold. Further, spectrin in solution can compete for and completely abolish actin binding to spectrin-containing unsealed membranes, suggesting that actin binding may be the result of the formation of an actin-spectrin complex. This interaction appears to be specific since it is abolished by mild heat denaturation of acceptor membranes and of the added spectrin extract and is highly dependent upon the ionic composition and nucleotide content of the medium.

521 PARTICIPATION OF SPECTRIN IN VIRUS-INDUCED FUSION AND MOBILITY OF INTRAMEMBRANE PARTICLES OF HUMAN ERYTHROCYTES: K. Sekiguchi and A. Asano, Inst. for Protein Res.,Osaka Univ., Suita, Osaka 565 JAPAN

Conditions were established for extensive fusion of human erythrocyte ghosts by HVJ(Sendai virus). Afinity-purified antispectrin antibody incorporated into the ghosts was highly inhibitroy for the virus-induced fusion at 1 4 mg/ml. Monovalent F_{ab} fragments of the antibody at similar concentrations were without effect on the reaction.

Since spectrin was reproted to regulate movement of surface glycoproteins, distribution of intramembrane particles(IMP) in erythrocyte membrane was studied. As already reproted by Bachi and Howe, the virus was found to induce clustering of IMP of the fused cells. Therefore, IMP distribution of the fused ghosts was compared with that of antibody-inhibited samples by freeze-fracture electron microscopy. Although clustered distribution of IMP was observed with fused ghosts, the antibody-treated samples showed random distribution. Thus, immobilization of spectrin by the antibody may be responsible for the immobilization of IMP and also inhibition of the cell fusion.

Since clustering of surface glycoprotein was reported to occur by the addition of antispectrin antibody alone to the ghosts at hypotonic conditions, we have studied the effect of antibody on IMP distribution at hypotonic conditions and of the resealed ghosts used for fusion reaction(in isotonic saline containing BSA). Almost no clustering of IMP was observed by the antibody(1 4 mg/ml)in the resealed ghosts, whereas some clustering of IMP was occured under hypotonic conditions at lower antibody concentrations(1.2 1 mg/ml). Thus, apparent movability of spectrin on the membrane seems to be higher at low ionic strength that in isotonic salts.

522 LOW DENSITY LIPOPROTEIN-INDUCED ALTERATIONS OF THE ERYTHROCYTE MEMBRANE. David Y. Hui and Judith A.K. Harmony. Chemistry Dept. Indiana University, Bloomington, In. 47401.

Intact erythrocytes incubated in the presence of low density lipoprotein (LDL) undergo a time-dependent morphologic transformation from biconcave discs to spherocytes within 4 hrs. The level of ATP in the cells incubated with LDL is similar to that of the control cells. No shape change is observed when erythrocytes are incubated with high density lipoproteins (HDL). Furthermore, the LDL-induced morphologic alteration is inhibited by HDL. The disc to sphere transition appears to be correlated with phosphorylation of membrane proteins. When control erythrocytes are incubated with ^{32}p, the label is linearly incorporated into membrane proteins, primarily component 2 of spectrin. However, in the presence of LDL, ^{32}p uptake is linear for only 2 hrs, then levels off to a value 60% of that obtained for the control cells. This time period coincides with the first observation of morphologic alteration. When erythrocyte ghosts are incubated with LDL, the activity of protein kinases is not altered; however, phosphatase activity is enhanced 2-fold. The addition of 10 μM ATP, which inhibits phosphatase activity in control experiments, has no effect on LDL-stimulated phosphatase activity. LDL also induces changes in the osmotic sensitivity of intact erythrocytes. 42% of erythrocytes incubated with LDL hemolyze in 0.525 M NaCl compared to 55% of control cells. These studies suggest that the morphology of erythrocytes is intimately related to the phosphorylation and dephosphorylation of spectrin. Furthermore, normal cellular function may depend on the concentration and composition of the circulating lipoproteins.

523 AGE DEPENDENT MEMBRANE PHOSPHORYLATION IN NORMAL AND SICKLE CELL ERYTHROCYTES, James Dzandu and Robert M. Johnson, Wayne State University, Detroit, MI 48201.
Human erythrocyte membrane proteins and lipid(s) are phosphorylated when washed intact cells are incubated with [^{32}P] phosphate (inorganic). When erythrocytes are fractionated according to their density, younger cells incorporated more radio-label into their membranes than older cells. During 18-24 hr incubations at 21° C, sickle cells incorporated significantly more radio-label into their membranes than normal human erythrocytes. ATP levels remained constant during the incubation and the specific activity of ATP increased to the same level in both normal and sickle cells. 37 $\pm$ 5 percent of label was found in a TCA insoluble fraction while lipids account for 63 $\pm$ 5 percent of ^{32}P incorporated. Irreversibly sickled cell rich fractions incorporate more label into their membranes than either reversibly sickled or normal cells. Coomassie blue stained profiles of normal, SS, ISC-rich and RSC membrane proteins solubilized in SDS and separated on polyacrylamide gradient slab-gels are similar. Autoradiography of dried gels show labelling in Bands 2, 3, and 4.5. These studies indicate that intact sickle cells autophosphorylate their membranes more than normal erythrocytes, and that irreversibly sickled cells are more readily labelled than either reversibly sickled cells from the same patient, or than young normal erythrocytes. Supported by NIH grant HL-15793 and the Detroit Sickle Cell Center.

524 SPECTRIN BINDS TO THE INNER SURFACE OF THE HUMAN RED CELL MEMBRANE VIA ASSOCIATIONS WITH BAND 4.1-4.2 AND 3, D. Litman, J.H. Chen and V.T. Marchesi, Department of Pathology, Yale University, New Haven, CT 06510
Purified spectrin binds to inside-out red blood cell ghost vesicles with a K_a of greater than 10^7 and a Hill coefficient of approximately 1.0. This binding is salt dependent and saturable and is reversible under conditions which promote the extraction of natural spectrin from erythrocyte membranes. The isolated form of band 2 of spectrin binds to inside-out vesicles and competes with native spectrin for the same high affinity binding sites. Isolated band 1 of spectrin is inactive.

Antibodies directed against the cytoplasmic segment of band 3 and antisera raised against purified bands 4.1-4.2 bind to inside-out vesicles and inhibit the binding of spectrin to these membranes. Antibodies directed to the cytoplasmic segment of glycophorin A also bind to inside-out vesicles but do not influence spectrin binding. Detergent purified bands 4.1-4.2 form specific complexes with purified spectrin. The stoichiometry and characteristics of this binding suggest that the association between spectrin and the 4.1-4.2 complex has features in common with the association between spectrin and the inner surface of the red cell membrane.

These results suggest that spectrin binds to the 4.1-4.2 polypeptides on the cytoplasmic surface of the red cell membrane in close proximity to the cytoplasmic portion of band 3.

Membrane Transport in Normal and Abnormal Red Cells

525 PLASMA MEMBRANE Ca^{++} TRANSPORT: STIMULATION BY A PROTEIN ACTIVATOR FROM RED BLOOD CELLS OR BOVINE BRAIN. F.L. Larsen, T.R. Hinds and F.F. Vincenzi, University of Washington, Seattle, WA 98195.
The red blood cell (RBC) contains a membrane bound (Ca^{2+}+Mg^{2+})-ATPase which is associated with Ca^{2+} transport. Crude RBC hemolysate has been shown to activate (Ca^{2+}+Mg^{2+})-ATPase activity (Bond and Clough, BBA 323:592, 1973) and to increase Ca^{2+} uptake (Macintyre and Green, Fed. Proc. 36:271, 1977) into inside-out vesicles. These effects are presumably due to an acidic activator protein which is present in the RBC and which resembles the phosphodiesterase (PDE) activator found in other cells. The present study examined the effects of purified RBC (Ca^{2+}+Mg^{2+})-ATPase activator, isolated from human RBCs, and purified PDE activator, isolated from bovine brain, on Ca^{2+} transport into inside-out vesicles. In the presence of Mg^{2+} (1 mM), ATP (1 mM) and Ca^{2+} (.1 mM), ^{45}Ca (3.671 x 10^5 cpm/μmole) is transported into vesicles at a rate of 7.77 nmoles Ca^{2+}·mg prot.$^{-1}$·hr^{-1} at room temperature. Addition of purified ATPase activator protein (0.9 μg/ml) increased uptake to 34.8 nmoles Ca^{2+}·mg prot.$^{-1}$·hr^{-1}. PDE activator (0.6 μg/ml) increased uptake to 17.4 nmoles Ca^{2+}·mg prot.$^{-1}$·hr^{-1}. No Ca^{2+} uptake occurred, either in the presence or absence of activator, unless ATP was present. Addition of the Ca^{2+} ionophore, A23187 (4 μM) after 90 min resulted in the rapid loss of 90% of the accumulated Ca^{2+} for vesicles incubated with ATP and a gain in Ca^{2+} for vesicles incubated without ATP. La^{3+} (0.1 mM) inhibited uptake rapidly and completely. Results provide further evidence that the two proteins are closely related and show that these proteins may be involved in regulation of plasma membrane Ca^{2+} transport. Supported by USPHS grants AM-16436, GM-00109 and a Muscular Dystrophy Association grant.

526 MODULATOR BINDING PROTEIN INHIBITS ACTIVATION OF RBC MEMBRANE (Ca^{2+}+Mg^{2+})-ATPase, Frank F. Vincenzi and Beat U. Raess, University of Washington, Seattle, WA 98195. A cytoplasmic protein in the human red blood cell (RBC) which has been shown to selectively activate RBC membrane (Ca^{2+}+Mg^{2+})-ATPase may be involved in regulation of the plasma membrane Ca^{2+} pump. Activation occurs in a concentration dependent fashion. Half maximal activation was obtained with 0.46 µg, and maximal with 10.8 µg cytoplasmic activator mg^{-1} membrane protein. RBC cytoplasmic protein is similar to a Ca^{2+}-dependent modulator protein (activator) of cyclic AMP phosphodiesterase (PDE). Protein modulator from bovine brain produces activation of (Ca^{2+}+Mg^{2+})-ATPase (Gopinath and Vincenzi, BBRC 77:1203, 1977) and RBC cytoplasmic activator activates PDE (Jarrett and Penniston, BBRC 77:1210, 1977). Wang and Desai (JBC 252:4175, 1977) described a modulator binding protein (MBP) from bovine brain. MBP prevents activation of PDE by protein modulator by binding to the calcium-protein modulator complex. Dr. Wang kindly provided a sample of MBP. MBP inhibited activation of RBC membrane (Ca^{2+}+Mg^{2+})-ATPase by RBC cytoplasmic activator but did not decrease basal activity. An approximately 10-fold shift of the concentration-effect curve of the activator was obtained with 28.7 µg MBP mg^{-1} membrane protein. Results show that both the protein modulator of PDE and the RBC cytoplasmic activator protein are sensitive to MBP. Results are compatible with the suggestion that the proteins are closely related. Possible role(s) of MBP in RBC function, if any, remain to be determined. Supported by NIH Grants GM07270, AM16436 and by a grant from the Cystic Fibrosis Foundation.

527 INFLUENCE OF AN ENDOGENOUS PROTEIN ACTIVATOR ON THE PROPERTIES OF(Mg^{2+}+ Ca^{2+})-ATPase IN RED CELL MEMBRANES PREPARED BY DIFFERENT PROCEDURES, Sidney Katz, Basil D. Roufogalis, Amiram D. Landman and Larry Ho, University of British Columbia, Vancouver, B.C., V6T 1W5. Controversy exists as to the properties, number and function of(Mg^{2+}+Ca^{2+})-ATPase(s) of red cell membranes. Erythrocyte ghosts prepared by three different procedures showed (Mg^{2+}+ Ca^{2+})-ATPase activities differing in specific activity and in affinity for Ca^{2+}. Ghosts prepared by extensive EDTA wash (Katz and Blostein, 1975) had lower maximum activities and Ca^{2+} affinity than ghosts prepared by a step-wise hemolysis procedure (Quist and Roufogalis 1975) or by hemolysis in 20 mM imidazole (Farrance and Vincenzi 1977). The differences in Ca^{2+} affinity were abolished at 2 mM ATP, whereas the Vmax differences were not. Ghosts prepared by step-wise hemolysis showed both high and low Ca^{2+} affinity (Mg^{2+}+ Ca^{2+})-ATPase activities at 2 mM ATP, but only a high Ca^{2+} affinity activity at ATP concentrations from 2-200 µM (6.4 mM $MgCl_2$). The expression of the low Ca^{2+} affinity (Mg^{2+} + Ca^{2+})-ATPase also required Mg/ATP ratios greater than 1:1. Removal of soluble proteins from ghosts by low ionic strength extraction (1 mM Tris-0.1 mM EDTA) at pH 6.5 or pH 8.0 resulted in a lowering of both the Ca^{2+} affinity and the maximum activity. This effect was associated with removal of a low molecular weight endogenous protein activator. The (Mg^{2+} + Ca^{2+})-ATPase activity of all three preparations was stimulated by the addition of protein activator. It is suggested that the differences in properties of the (Mg^{2+} + Ca^{2+})-ATPase prepared by these different procedures are due to the state of the enzyme as determined by the degree of association of a protein activator and the Mg^{2+} and ATP concentrations utilized. (Supported by the MRC of Canada, the B.C. Heart Foundation, and the Cystic Fibrosis Foundation of Canada)

528 ON THE QUESTION OF THE IDENTITY OF HIGH-AFFINITY CA^{2+} -ATPASE AND CA^{2+} DEPENDENT PHOSPHATASE OF HUMAN ERYTHROCYTE MEMBRANES, H. Uwe Wolf, Abt. Pharmakol. Toxikol. Univ. Ulm, D-7900 Ulm/Donau, and Reinhardt Zecher, Inst. Biochem. Univ. Mainz, D-6500 Mainz, GFR.
Investigations on membrane-bound high-affinity Ca^{2+}-ATPase and Ca^{2+}-dependent p-nitrophenyl-phosphatase of human erythrocyte membranes led to the conclusion that these two enzymes might be identical in part or totally (A. F. Rega et al. Biochem. J. 136 (1973) 185). In contrast to these results, we could not detect any phosphatase activity in membrane-bound and purified ATPase preparations. In order to confirm our hypothesis of non-identity of these two enzymes, we tried to isolate the phosphatase activity. Solubilization from the membrane was achieved by 1o % Tween 2o and 2oo mM K^+ at pH 7.o and 0° C. The phosphatase activity could be partially purified (61o fold) to 3.8 U/mg protein by chromatography on DEAE-Sepharose 6B-CL using a KCl-gradient of 2o - 2oo mM. SDS gel electrophoresis of the active fractions yielded three bands with MW in the range of 2o - 7o ooo Daltons. During chromatography of the solubilized crude material on Sephadex G-2oo the active fractions showed a MW of ca. 7o ooo Dalton. No bands were found in the MW range, in which the bands of ATPase appear, i. e. 115 - 145 ooo Dalton (H. U. Wolf et al., Acta biol. med. ger. (1977), in press). These results confirm our hypothesis of the non-identity of high-affinity Ca^{2+}-ATPase and Ca^{2+}-dependent p-nitrophenyl-phosphatase of human erythrocyte membranes.

529 ULTRASTRUCTURAL STUDIES OF THE CYTOPLASMIC SURFACE OF THE HUMAN RED CELL MEMBRANE
Ronald S. Weinstein, Jena K. Khodadad and Theodore L. Steck, Department of Pathology, Rush Medical College, and Department of Biochemistry, University of Chicago, Chicago, Ill.

Previous biochemical studies have demonstrated the presence of several peripheral proteins, and hydrophilic domains of the integral membrane proteins, at the cytoplasmic surface of human red cell membranes. In this study, we have selectively removed portions of these constituents and have examined the consequences by freeze-fracture, electron microscopy. The cytoplasmic surface (PS surface) of unextracted ghosts bears a loose granulofibrillar array (PS array). The subjacent membrane, as viewed through the interstices of the network is smooth. The PS network appears intact when ghosts are converted to right-side-out (RO) and inside-out (IO) vesicles. Two lines of evidence suggest that Band 3 protein is a major component of the PS network: (a) the array can be decorated with rabbit muscle G3PD, which has been shown to bind exclusively to Band 3 protein molecules; and (b) both trypsin and chymotrypsin digestion of IO vesicles, known to excise the cytoplasmic pole of the Band 3 protein, remove the granulofibrillar elements of the PS array without further reducing the amount of residual spectrin. Following proteolysis, a novel class of particles, retaining the distribution of the PS array, is revealed. These PS particles are present, although relatively inconspicuous, in the PS array of undigested membranes. They have the same topographical distribution as EF-face IMP although they are less numerous. The data suggests that Band 3 molecules and previously undescribed PS particles are constituents of a spectrin-free array which can be visualized at the cytoplasmic surface of isolated membranes.

530 CALCIUM-ANESTHETIC INTERACTIONS IN THE ERYTHROCYTE MEMBRANE, Philip S. Low and John A. Rogers III, Purdue University, West Lafayette, IN 47907.

Local anesthetics are found to displace bound Ca^{++} from the cytoplasmic side of the erythrocyte membrane at anesthetic concentrations which correlate both with their anesthetic potencies in nerves and also with their abilities to inhibit anion exchange across the erythrocyte membrane. Moreover, there exists a linear relationship (negative slope) between μg-atoms of Ca^{++} displaced by the anesthetic and the rate constant for anion exchange in the presence of different concentrations of local anesthetic. The relationship not only holds for cationic anesthetics (e.g., procaine, tetracaine, dibucaine) and for neutral anesthetics (e.g., benzyl alcohol), but also for quaternary amine analogues of the local anesthetics which reportedly are incapable of traversing the membrane of an intact erythrocyte. These observations suggest that Ca^{++} displacement is not due to a simple charge repulsion mechanism.

The possibility that the displaced internal free Ca^{++} might be involved in the inhibitory effect of local anesthetics on anion exchange across the erythrocyte membrane was investigated. Using Ca^{++} EGTA buffers it was found that free internal Ca^{++} is a potent inhibitor of anion exchange with a K_I = 7 μM. However, in the presence of EGTA and Ca^{++} ionophore (A23187), tetracaine retains the ability to inhibit anion exchange. Thus, tetracaine does not appear to require Ca^{++} to block anion exchange and must therefore function by some other mechanism.

531 GENETIC POLYMORPHISM IN THE CYTOPLASMIC DOMAIN OF BAND 3, H. Köhler, M. Singh, T.L. Steck and J. Murashige, La Rabida Institute & Depts of Path & Biochem, U of Chicago, Chicago, Illinois 60649

The 23,000 dalton amino-terminal fragment from the cytoplasmic domain of the major human red cell membrane protein, Band 3, was generated by S-cyanylation and purified in high yield. 23K fragments from several donors were compared with respect to their amino acid composition, composition and partial sequence of their tryptic peptides and antigenic differences in a radio-immuno assay. We found that the amino acid compositions of 23K fragment and of its tryptic peptides derived from different individuals were not identical. For example, the sequence of a pentapeptide isolated from tryptic digests of two different 23K fragments is as follows: Glu-Glu-Leu-Leu-Arg and Met-Glu-Ala-Ala-Arg respectively. The amino acid differences among the 23K fragments and their tryptic peptides were found to be restricted to a few specific residues, notably Glx and Leu. Finally, the 23K fragments from different donors generated different inhibition profiles in a solid-phase RIA which tested the binding of ^{125}I-labeled 23K fragment to rabbit anti-23K antisera. The demonstration of structural differences in the 23K polypeptide backbone and of antigenic variation present in the same fragment prepared from different donors indicates genetic polymorphism. Further protein-chemical and immunological studies on Band 3 from selected donors should clarify the genetic basis for this polymorphism. (Supported by GM 22722 and ACS BC-95E,F).

532 BAND 3 ANTIBODIES AND ANION TRANSPORT IN THE RED BLOOD CELL MEMBRANE, B.J. England and T.L. Steck, Dept. of Biochemistry, University of Chicago, Chicago, IL 60637.

Band 3, the predominant membrane-spanning polypeptide in human red cells, was purified by a new procedure involving selective solubilization and anion-exchange chromatography in the presence of 0.5% Triton X-100 - 0.03% SDS. Rabbit antisera were prepared against band 3 in Triton X-100; attempts to generate antibodies to band 3 in SDS were unsuccessful. Anti-band 3 antibodies were not absorbed by intact red cells, but were readily removed by ghosts, as judged by Ouchterlony analysis. These antisera also strongly agglutinated inside-out membrane vesicles and precipitated two major fragments derived from the cytoplasmic surface domain of band 3. Band 3 antisera thus appear directed primarily against the cytoplasmic pole of this molecule. (Related studies have shown that a dominant specificity in antisera to whole ghosts is also directed against the cytoplasmic-surface domain of band 3. The anti-serum against ghosts also was a potent hemagglutinin, whereas antisera to band 3 were not. Since the agglutinin but not band 3-precipitating antibody could be absorbed by intact cells, the agglutinin was not directed against band 3.) Resealed ghosts were prepared so that both surfaces were exposed to control or immune sera at high concentration. Phosphate transport both into and out of the resealed ghosts as well as chloride efflux were not altered by anti-body treatment. Pyruvate influx was also not inhibited, although nonimmune sera increased pyruvate flux over the serum-free control. It is concluded that these antisera against band 3, the purported anion-transport protein of the red cells, do not alter anion transport significantly. [Supported by Amer. Cancer Society Grant BC-95E and USPHS Grant AI 452.]

533 KINETICS OF PASSIVE ANION TRANSPORT ACROSS THE HUMAN ERYTHROCYTE MEMBRANE, J.M. Salhany and Jhan C. Swanson, The Univ. Nebr. Med. Ctr., Omaha, NE, 68105.

Steady state kinetics are measured for dithionite ($S_2O_4^{=}$) flux into resealed human erythrocyte ghosts containing methemoglobin. Methemoglobin reduction is observed with dithionite permeation being rate determining. At low (6 mM) *cis* and *trans* sulfate, the kinetics show non-linear double reciprocal patterns characteristic of *substrate activation followed by substrate inhibition*. The kinetic patterns change with the concentration of *cis* or *trans* co-anion. Increasing the *cis* co-anion produces competitive-like inhibition and also eliminates substrate inhibition. The *trans* co-anion *stimulates* dithionite flux; does not change the substrate inhibition at high dithionite, but prevents substrate activation at low dithionite. These effects occur with both permeable (sulfate) and non-permeable (citrate) co-anions, and therefore, may suggest that anion transport through the erythrocyte membrane is under some type of "allosteric" control.

534 CATECHOLAMINE ACTIVATED SODIUM-POTASSIUM CO-TRANSPORT IN DUCK RED CELLS. Thomas J. McManus and William F. Schmidt, III, Duke University, Durham, N.C. 27710.

Catecholamines activate a specific pathway for sodium and potassium across the avian red cell membrane in which these ions participate as co-passengers on a carrier type of facilitated diffusion mechanism. Sodium enters the cell taking external potassium with it, and potassium leaves the cell taking internal sodium with it. This pathway is insensitive to ouabain, but blocked by furosemide. Coupling between the electrochemical gradients of the co-ions can readily be demonstrated. Thus, systematic variation of external and internal ion concentrations in the presence of ouabain reveals that the cell can accumulate potassium against its gradient if external sodium is optimally elevated, and can extrude sodium against its gradient if internal potassium is optimally high. Potassium appears to ride in against its gradient by utilizing the force driving sodium into the cell. Similarly, sodium can be "pumped" out of the cell by coupling with the downhill movement of potassium. Rubidium can substitute for potassium and lithium can substitute for sodium in these net movements. If cells are prepared (nystatin technique) which contain more sodium than potassium, and then incubated in a high potassium medium, the addition of catecholamine actually promotes a net extrusion of potassium from the cells against its electrochemical gradient. Variation of external chloride and pH reveals that the system is very sensitive to the chloride ratio, and therefore by inference to the membrane potential. This suggests that the carrier is charged at some point in its transport. A thermodynamic approach has been developed which predicts that the carrier moves in response to the *net* driving force urging sodium and potassium across the membrane. Predictions from this theory fit the data for a wide variety of experimental conditions.

535 ANION EXCHANGE: EVIDENCE FOR A SEQUENTIAL REACTION MECHANISM AND A SINGLE TRANSPORT SITE, R. B. Gunn and O. Froehlich, Dept of Pharm. and Physiol.Sci., Univ. Chicago, Chicago, IL 60637.

We have measured the stimulation of chloride efflux from human red cells through the anion exchange mechanism into acetate solutions by external bromide. The initial chloride efflux was nearly all in exchange for external bromide since efflux into acetate alone was very much slower (Gunn, et al. J.G.P. 65: 731, 1975) and the stimulation by bromide was inhibited by furosemide and phloretin. The plot of the reciprocal of the bromide stimulated chloride efflux vs. the reciprocal of external bromide was a straight line when Br_{out} = 1.5 to 15mM. The maximum flux (V_{max}) was 330 mM/(kg cell solids·min) and the bromide concentration at which the flux was half maximum ($K_{1/2}$) was 10mM when Cl_{in} = 118mM. Above 30mM Br_{out} the flux decreased as does the self-exchange of halides but at higher concentrations. When cell chloride was reduced by substitution with acetate (a non-competitive inhibitor), the bromide stimulated initial chloride efflux was reduced and the extrapolated V_{max} and $K_{1/2}$ were both reduced as expected if the molecular mechanism is a sequential reaction of a single site with alternating access to the inner and outer solutions as proposed in the titratable carrier model. These findings are inconsistent with a simple simultaneous heteroexchange of bromide and chloride.

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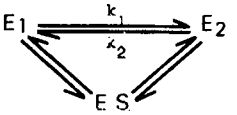
536 A FLUORESCENT PROBE FOR THE URIDINE TRANSPORT SYSTEM IN HUMAN RED BLOOD CELL, Ruth Koren, Esther Shohami, The Institute of Life Sciences, Biophysics Section, The Hebrew University, Jerusalem, Israel.

A fluorescent derivative of 6-mercaptoguanosine, N-dansyl(amino-ethyl-6-mercaptoguanosine (DAMG), has been synthesized, and found to be a strong inhibitor of the uridine transport system of the red blood cell, ($K_i \sim 0{,}3\mu M$). The emission spectrum of this compound has peaks at 400 and 500 nm and only the latter is sensititve to the environment. A suspension of red blood cell membrane fragments with low light scattering was used in a detailed study of the fluorescence of the probe when bound to membranes. Direct binding measurements of the probe to the membrane showed the existence of a tight binding site, with a dissociation constant of the same order of magnitude as the inhibition constant. Binding of probe and substrate are not mutually exclusive, but the fluorescence and affinity of the bound probe are sensitive to the presence of uridine. The nature of the emission spectrum of the bound probe suggests that it penetrates into the bilayer region of the membrane. A comparison between the kinetic and direct binding results indicate that the inhibitor acts by reducing the "mobility" of the carrier, not loaded with substrate, i.e. increasing the Roo = $1/k_1 + 1/k_2$ in the one complex model of a simple carrier, described below.



537 SIDE-DEPENDENT EFFECTS OF NAP-TAURINE ON CHLORIDE EXCHANGE, P.Knauf, S.Ship, W.Breuer, L.McCulloch and A.Rothstein, Res Inst, Hosp for Sick Children, Toronto, Ont. Canada.

The photo-affinity reagent, NAP-taurine, acts in the dark as a substrate and a reversible inhibitor of the human red cell anion exchange system (Cabantchik et al, 1976, BBA 455: 526). At 0°C permeation is minimal, so that the reversible effects of internal and external NAP-taurine on chloride-36 exchange at 0°C can be compared. External NAP-taurine is a potent inhibitor of chloride exchange, with a K_i of about 20 µM. The effects of chloride concentration on K_i were much smaller than would be expected if external NAP-taurine were acting at the substrate site of the anion exchange system, but were consistent with the interaction of external NAP-taurine with the modifier site, a second lower-affinity chloride binding site of the transport system (Dalmark, 1976, J.Gen.Physiol. 67: 223). When NAP-taurine was present inside cells or resealed ghosts it was a far less potent inhibitor with a K_i of about 840 µM. Based on the variation in K_i with chloride concentration, internal NAP-taurine appears to act at the substrate site. It can be concluded that the high affinity site is only accessible to NAP-taurine from the outside. Since NAP-taurine is converted by light to a highly reactive nitrene, internal or external NAP-taurine can be used to label functionally distinct sites of the transport system. In particular, when red cells are exposed to external NAP-taurine in the light, the chloride sensitive labelling is found primarily in a 65,000 dalton fragment of the band 3 protein. This fragment therefore appears to contain the modifier site of the transport system as well as the substrate site. (Supported by Medical Research Council (Canada) Grants #MA5149 and MT4665).

538 PATHOLOGICAL CONDITIONS EFFECTING DECREASED ERYTHROCYTE MEMBRANE PERMEABILITY TO WATER, William R. Galey and Mary J. Gillon, Dept. of Physiol., Univ. of New Mexico, School of Medicine, Albuquerque, New Mexico 87131.

Studies of erythrocyte membrane osmotic water permeability (Lp) have been conducted on cells from patients with McLeod Syndrome and cystic fibrosis. It has been determined that the water permeability is decreased in both conditions. However, Na^+ and k^+ transport across rbc membrane in both conditions have been shown to be normal.

Studies of membrane lipid composition in McLeod cells show no qualitative or quantitative deviation from normal rbc membranes. On the other hand, the abnormal antigentic reactivities and the bizarre shapes of these cells suggest a possible membrane protein abnormality.

Experiments on erythrocytes from cystic fibrotic patients have shown decreased membrane linolenic acid content. Membrane osmotic water permeabilities of red cells from a single cystic fibrotic patient on a dietary supplement of linolenic acid are normal. This suggests that the low Lp associated with the condition may be due to the decreased membrane fatty acid.

Although recent studies by Sha'afi et.al. and Macey et.al. suggest membrane proteins to be the controllers of Lp, our studies suggest that either membrane component may be responsible for causing decreased water permeability. (Supported in part by the Office of Naval Research Grant No. N00014-76-C-0815)

539 IS THERE A MOBILE ANION TRANSPORT SITE IN BAND 3 PROTEIN OF THE RED BLOOD CELL MEMBRANE? S.Grinstein, L.McCulloch, A. Rothstein, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada.

The disulfonic stilbene, DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) inhibits anion transport by binding to the transport site located in band 3 (Shami et al, this meeting). It was found to be impermeant and to act only from the outside. NAP-taurine, a photoaffinity probe (Cabantchik et al, Biochim.Biophys.Acta (1976) 455,526-537) has been shown to react with the transport site when it is present at the inside of the membrane (Knauf et al, this meeting). DIDS was used to sequester the transport sites at the outer surface and NAP-taurine to titrate the number of sites available at the inner surface. In inside-out vesicles (IOV) prepared from DIDS treated cells, the binding of NAP-taurine at the cytoplasmic face (outside in IOV's) was substantially reduced compared to controls (no DIDS), most of the reduction occurring in band 3. Appropriate controls indicated that NAP-taurine had not penetrated into the intravesicular space, so that the two probes were reacting on opposite sides of the membrane. This trans-membrane effect can be explained on the basis that the transport site in band 3 moves across the diffusion barrier by a conformational change in the protein, so that the transport site alternately faces outward and inward.(This study was supported by Medical Research Council (Canada) Grant #MT4665)

540 IDENTIFICATION OF THE Cl^- TRANSPORT SITE OF HUMAN RED BLOOD CELLS BY KINETIC ANALYSIS OF THE INHIBITORY EFFECTS OF A CHEMICAL PROBE. Y.Shami, P.A. Knauf, A. Rothstein. Research Institute, The Hospital for Sick Children,Toronto,Ontario,Canada.

H_2DIDS, the dihydro analog of DIDS (4,4'- diisothiocyano stilbene 2,2'-disulfonic acid) can interact covalently with membrane sites resulting in an irreversible inhibition of anion exchange. The irreversible reaction is, however, preceded by reversible binding which produces an equivalent inhibitory effect. The kinetic analysis was, therefore, done under conditions where the irreversible interaction was minimal (0^oC and short exposure to H_2DIDS). Under these conditions the addition of albumin to the medium reversed the inhibition to the level of the control flux. Increasing the Cl^- concentration of the cells and the medium equally (by the nystatin technique) increased the K_i for H_2DIDS from 0.24μM at 150mM Cl^- to 1.09μM at 600mM Cl^-, with double reciprocal plots indicating competition between Cl and H_2DIDS. A Hunter and Downs plot of $I(1-i)/i$ vs Cl^- concentration revealed that the dissociation constant for Cl^- at the inhibitory site is 68mM, in good agreement with the value of K_s (65mM to 67mM) reported for Cl^- interaction at the transport site. If, as seems probable, the covalent labelling of H_2DIDS occurs at the same site as the reversible binding, H_2DIDS can be used as a covalent label for the transport site. The specific localization of H_2DIDS in the band 3 protein thus indicates that this protein participates directly in anion exchange.
(Supported by Medical Research Council (Canada) Grants #MA5149 and #MT4665)

541 THE SODIUM-POTASSIUM ATPASE AND FRIEND CELL DIFFERENTATION. D. Mager and A.Bernstein, Ontario Cancer Institute, Toronto, Ontario M4X 1K9.

Friend erythroleukemic cells can be induced to differentiate in culture along the erythroid pathway by treatment with certain chemical agents. While the expression of several markers, characteristic of erythroid differentiation, has been extensively studied in these cells, relatively little is known about the early events which precede the overt expression of differentiation in this system. We have made several experimental observations relevant to the study of the early stages of Friend cell differentiation. First, ouabain, a specific inhibitor of the NA^+/K^+ ATPase, induces Friend cells to differentiate. Secondly, the initial rates of ouabain-sensitive $^{86}Rb^+$ uptake and of amino acid uptake are decreased early after the addition of several different inducing agents. This decreased transport of ^{86}Rb (used as a K^+ analog) does not result in any significant change in internal K^+ ion concentration since, although there is a decrease in total K^+ ion content early after induction, there is a parallel decrease in cell volume. Studies with variant Friend cell clones which do not synthesize hemoglobin indicate that these non-inducible clones can be divided into two classes - those that demonstrate the early transport changes and those which exhibit only very small changes in transport. Thus, measurements of membrane transport early after induction may be useful in the phenotypic characterization of non-inducible Friend cells. Finally, we have observed that incubation of Friend cells in medium containing high (80-100 mM) K and low (50-70mM) Na^+ induces these cells to differentiate. These observations suggest that early changes in transport, or inhibition of the Na K ATPase, may be an important early event in the induction of Friend cell differentiation. (Supported by grants from the MRC and NCI of Canada)

542 EFFECTS OF ABNORMAL CATION TRANSPORT ON DEFORMABILITY OF DESICCYTES. M.R, Clark, N. Mohandas, S.B. Shohet. Cancer Res. Inst. and Div. Hemat., Univ. Calif., San Francisco.

Glader et al described a disorder called desiccytosis, in which a specific K permeability defect produced cell water loss. Reduced cell deformability was also reported. Using blood from two patients with apparent desiccytosis, we have attempted to learn whether reduced deformability was the result of increased hemoglobin concentrations (MCHC) or intrinsic membrane rigidity.

To obtain cell populations of progressively increasing MCHC, cells were separated on discontinuous stractan gradients. Size distributions of the fractions were heterogeneous and did not correlate with MCHC. With increasing MCHC, a progressive decrease in deformability as measured by an ektacytometer was evident. Fractions with MCHC 38 g/dl were totally undeformable in isotonic medium. Reduction of suspending medium osmolality to 180 mosm/kg allowed the red cells of these patients, with the exception of the most dense fraction, to deform normally. 40% of the cells in the most dense fraction (4% of total cells) were undeformable even in hypotonic medium.

Normal cells were artificially adjusted to the same cation concentrations and MCHCs as the patient subpopulations, using nystatin in buffers of appropriate ion compositions and osmolalities. These artificial desiccytes showed the same dependence of deformability upon MCHC as did the patient cells, including a residue of undeformable cells in the most dehydrated sample, even in 150 mosm/kg medium. The reason for this small residual undeformability is not yet clear. These observations suggest that the reduced deformability of desiccytes is regulated primarily by cell water content rather than by intrinsic membrane rigidity.

543 CHEMICAL CHARACTERIZATION OF A MEMBRANE-SPANNING SEGMENT OF BAND 3, J.J. Koziarz, H. Köhler, and T.L. Steck, Dept. Biochem., Univ. of Chicago, Chicago, IL 60637

Band 3, the purported mediator of anion transport and the predominant polypeptide of the human erythrocyte membrane, was cleaved by chymotrypsin at both membrane surfaces to generate a 17,000 dalton (17K) fragment. The primary structure of this membrane-spanning segment is of particular relevance to an understanding of anion translocation. The aqueous insolubility of this segment and its subfragments necessitated the exploration of novel analytical and preparative approaches, which will be described. The 17K segment was purified in sodium dodecyl sulfate (SDS) by preparative polyacrylamide gel electrophoresis in an apparatus of our own design. While the fragment was rich in nonpolar residues, notably PHE (8.3 mole %), LEU (9.5%), and ILE (6.9%), a significant fraction of the residues were found to be polar: LYS (3.7%), ARG (5.3%), ASX (6.7%), and GLX (10.2%). The C-terminal amino acid was tyrosine, as demonstrated by carboxypeptidase A digestion in SDS. The presence of carbohydrate was demonstrated both by galactose oxidase-catalyzed labeling with ^3H-$NaBH_4$ and by gas chromatography. Tryptic and cyanogen bromide digestion in the presence of detergent yielded peptide maps with the appropriate number of spots. The fraction of polar and nonpolar residues did not substantially differ among the various cyanogen bromide peptides. Chemical variation was not observed among the 17K preparations derived from different donors nor was there evidence for heterogeneity within a single source.

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Integral Membrane Proteins in Normal and Abnormal Red Cells

544 SOLUBILIZATION AND RECONSTITUTION OF THE GLUCOSE-SENSITIVE CYTOCHALASIN B RECEPTOR FROM HUMAN ERYTHROCYTE MEMBRANES, Michael A. Zoccoli, Stephen A. Baldwin, and Gustav E. Lienhard, Dept. Biochem., Dartmouth Med. Sch., Hanover, N.H. 03755.

Erythrocyte membranes were largely depleted of protein bands 1,2,5 and 6 and of glucose-insensitive cytochalasin B receptors by washing first with 0.1 mM EDTA, pH 8, at 37° and then with 0.5 M NaCl-5 mM Tris·HCl, pH 7.4, at 3°. Equilibrium dialysis with [^{3}H]cytochalasin B (CB) showed that the depleted membranes (DM) possess a single set of high affinity sites, characterized by a dissociation constant of 1.3×10^{-7} M. D-Glucose, but not L-glucose, competitively inhibited the binding of CB to DM; the dissociation constant for D-glucose was 19 mM. The CB receptor was solubilized by suspending DM in 0.5% Triton X-100 in 1 mM EDTA-1 mM dithiothreitol-10 mM Tris·HCl, pH 7.4, and centrifuging twice at 100,000 g for 1 hr. Upon removal of the detergent from the supernatant with BioBeads SM2, CB binding was recovered with a dissociation constant and sensitivity to D-glucose almost identical to those for the binding to DM. It was necessary to remove the Triton to obtain binding, since Triton is itself a competitive inhibitor of CB binding, with a K_i of 8×10^{-5} M. Under the above conditions about 50% of the CB receptor was solubilized and reconstituted. Gel filtration of the Triton supernatant on Sepharose 4B equilibrated with 0.05 or 0.1% Triton yielded a single peak of binding activity in a complex with a Stokes radius of 6.8 nm. We are in the process of estimating the molecular weight and protein content of this complex, by sedimentation in H_2O and D_2O sucrose gradients, according to the method of Clarke (J. Biol. Chem. 250, 5459 (1975)). Supported by grant GM 22996 from the National Institutes of Health.

545 ERYTHROCYTE MEMBRANE ALTERATIONS IN HUNTINGTON'S DISEASE, D. A. Butterfield and W. R. Markesbery, Depts. of Chemistry and Neurology, University of Kentucky, Lexington, Ky. 40506

Biophysical and biochemical studies have been performed on erythrocyte membranes from patients with the neurological disorder Huntington's disease (HD). HD, inherited as an autosomal dominant trait, is characterized clinically by progressive choreiform movements and dementia, pathologically by a marked loss of small neurons in the neostriatum, and biochemically by decreased levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and decreased activities of several enzymes in the basal ganglia of the brain. Using the spin labeling technique we have observed alterations in the physical state of membrane proteins in erythrocytes in HD, while that of the lipids is unaffected. Scanning electron microscopic studies showed an increased number of stomatocytes in unmanipulated erythrocytes. Biochemical studies have revealed an increased amount of sialic acid with no alteration in several other membrane constituents and an increased activity of the Na,K-stimulated ATPase in HD red cell membranes. Spin label investigations of the effect of GABA on erythrocyte membranes suggest a differential response in control and HD ghosts. The alterations in the physical state of erythrocyte membranes which are outside the central nervous system suggest that HD may be associated with a generalized cell membrane abnormality. Supported in part by grants from the Muscular Dystrophy Association of America, the Research Corporation, and the Graduate School of the University of Kentucky.

546 ACTIVATION OF THE ADENYLATE CYCLASE SYSTEM OF INTACT TURKEY ERYTHROCYTES AFTER LIMITED PROTEOLYSIS. M.L. Lacombe & J. Hanoune, INSERM U-99, Hôp. H. Mondor, F 94010 Créteil.

We have previously shown that mild proteolysis activates the adenylate cyclase (A.C.) system of rat liver plasma membranes (J. Biol. Chem., 252, 2039-2045, 1977); this activation was paralleled by the proteolysis of a high molecular weight protein (FEBS Lett., 77, 159-163, 1977). The present experiments were aimed at further elucidating the mechanism of this effect in a less complex system, possessing a highly sensitive A.C., namely turkey erythrocytes.

A.C. activity in a particulate fraction from turkey erythrocytes was markedly increased by concentrations as low as 1 µg/ml of highly purified proteinases (papain, α chymotrypsin, thermolysin, subtilisin, and, to a lesser extent, elastase). Trypsin exerted only an inhibitory effect. Papain (20 µg/ml) promoted a 2- to 3-fold increase in NaF, isoproterenol (IPR) and IPR + Gpp(NH)p stimulated A.C. activities. The time course of the proteolytic action in the particulate fraction was biphasic: a rapid activation (less than 1 min) was immediately followed by a decrease in A.C. activity, probably due to a proteolytic degradation of the enzyme itself. In contrast, pretreatment of intact erythrocytes by papain for up to 60 min, followed by extensive washing, evoked a 2- to 3-fold increase in all A.C. activities, without any subsequent inhibition. Control experiments showed that papain did not permeate the membrane.

In conclusion, mild proteolysis of a component of the outer surface of the erythrocyte resulted in activation of an enzyme (A.C.) located at the cytoplasmic side of the erythrocyte membrane, suggesting that a transmembrane regulatory process may exist in addition to the normal hormonal activation.

547 IDENTIFICATION OF THE TRANSFERRIN RECEPTOR OF THE RABBIT RETICULOCYTE, Daniel P. Witt and Robert C. Woodworth, Dept. of Biochem., Univ. Vermont College of Medicine, Burlington, Vermont 05401

Rabbit reticulocytes were separated on the basis of density by isopycnic centrifugation in dextran gradients. This parameter was shown to correlate with the degree of maturation of the cells. Lactoperoxidase catalyzed iodination of cells from different fractions revealed an external membrane protein of MW 190,000 that was present only in reticulocytes. Subsequent studies with relatively homogeneous populations of early reticulocytes demonstrated that this species was not well labeled when rabbit transferrin was included in the reaction mixture. In addition, when steps were taken to clear endogenous transferrin from the membranes, the iodination was enhanced. These observations are consistent with the concept that transferrin can sterically block the lactoperoxidase catalyzed iodination of this membrane polypeptide by specifically interacting with it. It is concluded from this that the 190,000 d polypeptide is the primary receptor for transferrin in the reticulocyte membrane. Additional studies involving covalent modification of the membrane by photo affinity labeling have supported this conclusion.

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548 PURIFICATION OF A HUMAN ERYTHROCYTE SURFACE ANTIGEN. David W. Lehman and Carol Jones. Eleanor Roosevelt Institute for Cancer Research, University of Colorado Medical Center, B129, 4200 East Ninth Avenue, Denver, CO 80262.

The human red blood cell (HRBC) has antigens on its surface which are coded for by genes on chromosome 11 as shown by the presence of these antigens on Chinese hamster ovary-human fibroblast hybrid cells which contain a full complement of Chinese hamster chromosomes and only human chromosome 11. These antigens have been designated a_1 and a_3; a third antigen, a_2, is also coded by a gene on chromosome 11 but is not present on HRBCs.[1] Antisera, raised in rabbits or sheep against HRBCs, will kill the hybrid cell in the presence of complement from normal rabbit serum. Our assay system for the antigen during its purification is the ability of the antigen to bind specific antibody and thereby protect our standard hybrid cell from killing by antibody and complement.

Two approaches to the purification of these antigens are being pursued. 1) HRBC membranes are solubilized with lithium diiodosalicylate and fractionated by solvent extractions and phosphocellulose chromatography. 2) The antigen is released by the action of trypsin on intact HRBCs. Fractionation of the supernatant on a gel filtration column gives a peak which contains the antigen. Further purification of these materials is in progress. Preliminary characterization indicates the presence of a carbohydrate moiety since reaction with periodate destroys the inhibitory activity of the antigen preparation.

This research is supported by grants from NSF (PCM 76-80299) and the NCI, DHEW (CA-18734). DWL is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Fund.

549 The Interaction Between Glycophorin and Phospholipids in Reconstituted Systems; An ESR and NMR Study. Philip L. Yeagle, Alice Y. Romans and Charles M. Grisham. Department of Chemistry, University of Virginia, Charlottesville, Va. 22901, and Department of Pathology, Medical Center of the University of Alabama in Birmingham, Birmingham, Al. 35294.

Both the MN-glycoprotein from human erythrocytes and the hydrophobic fragment from the protein isolated with trypsin treatment, T(is), have been reconstituted in egg phosphatidylcholine bilayers at various phospholipid to protein ratios. Methyl esters of fatty acids spin labelled at position 12 were used to investigate the boundary layer of phospholipid around the portion of the protein buried in the membrane. Some of the lipid was immobilized by the protein as detected by ESR spectra of the spin label. In order to investigate the effect of the protein on the phospholipid headgroups, ^{31}P NMR spectra were obtained with the MN-glycoprotein, which revealed two classes of phospholipid environments, analogous to the effects deep in the bilayer detected with the ESR measurements.

550 AFFINITY PURIFICATION OF ACETYLCHOLINESTERASE FROM HUMAN ERYTHROCYTES AND ELECTRIC EEL, CHARLES K. CARTWRIGHT, GEORGE E. TILLER, A. SCOTT BROOKS, AND WILLIAM G. STRUVE, Dept. of Biochemistry, The University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38163.

We have developed an easy and reliable method for the synthesis of an acridinium-sephrose affinity resin for the purification of acetylcholinesterase. This enzyme has been purified from human erythrocytes and electric eel electroplax.

Eel acetylcholinesterase floats up a sucrose density gradient when sphingomyelin is added to the enzyme prior to centrifugation. Results are similar in the presence of low (.05M) and high (1M) NaCl. Other lipids tested do not float this enzyme.

Acetylcholinesterase purified from human erythrocytes differentially floats in the presence of a number of lipids. In the presence of high salt the preference of binding is Cardiolipin > Sphingomyelin > Dimyristoyl phosphatidyl choline. In the presence of low salt the order of preference is reversed.

Preliminary results from gel electrophoresis and amino acid analysis of the enzyme purified from electroplax indicate that a disulfide-linked collagen fragment may be co-purified with the eel enzyme thus altering its lipid binding properties.

551 PURIFICATION AND CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM HUMAN ERYTHROCYTE MEMBRANES, Evelyn Niday, Chi-Sun Wang and Petar Alaupovic, Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Human erythrocyte membranes were solubilized in 5% Triton X-100 and the acetylcholinesterase (ACHE) was isolated by affinity chromatography. Homogeneity was shown by the presence of a single band on SDS-PAGE and single precipitin lines between the enzyme and its antiserum on immunodiffusion and rocket, crossed and immunoelectrophoresis. A pI of 4.8 was demonstrated by isoelectric focusing. Results of amino acid, carbohydrate, lipid, phosphorus and fatty acid analyses indicated that ACHE is a lipoglycoprotein. The N-terminal amino acid was blocked. Phosphatidyl serine and cholesterol were demonstrated as the major lipid constituents and the sugar components were glucose, galactose, mannose, glucosamine, galactosamine and sialic acid. The subunit has an apparent molecular weight of 160,000 and is composed of two covalently linked polypeptides each with a molecular weight of 80,000. Comparative kinetic analyses using acetylthiocholine (ATCh) or acetylcholine (ACH) as substrate revealed qualitatively similar enzymic properties. ACHE activity has an *in vitro* pH optimum of 8.0 and is a linear function of enzyme concentration. The K_m and V_{max} values using ATCh were 150±30 μM and 610±100 U/mg, respectively; corresponding values using ACH were 200±20 μM and 425±60 U/mg. Results of the Hill plot showed the existence of either a single or multiple independent catalytic sites. Kinetic analyses indicated allostery as the mechanism of substrate inhibition. Antibodies to the enzyme inhibited its activity competitively indicating that the antigenic determinant(s) is at or very close to the active site. Agglutination of red cells by antibodies to acetylcholinesterase indicated that the enzyme is localized on the outer surface of the membrane.

552 EXTERNAL PROTEINS ON HEMOSPORIDIAL INFECTED MOUSE ERYTHROCYTES R.J. Howard and G.F. Mitchell, Hall Institute, Melbourne, Australia.

Demonstration of "agglutinogens" specific for Plasmodium or Babesia infected erythrocytes indicates that parasite dependent changes occur on the external membrane. This study examined whether changes in external proteins on mouse (BALB/C) erythrocytes follow infection with *Plasmodium berghei berghei, P. berghei yoelii,* or *Babesia rodhaini,* using several techniques of radiolabelling external membrane proteins. The following changes were observed.

(1) *P. berghei:* small decreases in Mol. wt. of some proteins; a large decrease in IO_4^- sensitive protein sialic acid, but neuraminidase still required to expose galactose oxidase sensitive Gal/GalNAc; no unique tyrosyl labelled proteins on purified schizont infected cells.
(2) *P. yoelii:* as for *P. berghei* plus new external membrane proteins.
(3) *B. rodhaini:* several new external membrane proteins (tyrosine and sialic acid labelled). A new 60,000 Dalton protein appears on erythrocytes early after infection and rapidly becomes a major tyrosine labelled external protein.

In no case was surface Ig detected and there was no difference between tyrosyl labelled surface proteins on cells from immunologically deficient (nude) or intact hosts.

553 A NEW TECHNIQUE FOR THE ISOLATION OF MEMBRANE SURFACE PROTEINS, Lawrence P. Wennogle and Howard C. Berg, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado, 80309

DNA is being used as a handle for the isolation of proteins accessible at the outer surface of the human erythrocyte. Such proteins often are present as minor components, not readily soluble in aqueous buffers. Polydeoxythymidylic acid is covalently linked to the surface of intact cells. Membranes are isolated, dissolved in SDS and passed through a column of polyriboadenylic acid - agarose.
Protein - polydeoxythymidylic acid complexes are selectively retained. The proteins are eluted by a nuclease and characterized. This technology is being used in a study of the orientation, molecular associations and structure of erythrocyte membrane proteins. In addition, it is possible to hybridize intact cells to polyriboadenylic acid - agarose via polydeoxythymidylic acid. This research has been supported by NSF Grant PCM75 08392.

554 HUMAN RED CELL MEMBRANE SIALOGLYCOPEPTIDES: ELECTROPHORETIC RESOLUTION AND IMMUNOCHEMICAL COMPARISON, C. Bron, D. Berthoud and M. Girardet. Institut de biochimie, Université de Lausanne, Switzerland.

Glycophorin preparations can be resolved into at least ten different bands as revealed by periodic acid Schiff staining or by autoradiography of ^{125}I-labeled proteins on 10 % polyacrylamide gels in 0.1 % SDS, using a discontinuous buffer system. In order to further investigate the structural relationship between these proteins, each band was eluted from gels and analysed by PAGE-SDS, peptide mapping and immunochemical methods using antibodies to whole glycophorin or separated glycopeptides. The results indicate that three glycopeptides PAS-2_a (Rf 0.52; app. MW 46,000), PAS-2_b (Rf 0.54; app. MW 42,000) and PAS-3 (Rf 0.75; app. MW 24,000) differing in their molecular size and antigenicity can form various aggregates yielding the complex electrophoretic pattern observed. The main band PAS-1 (Rf 0.22; app. MW 110,000) previously shown to be a dimer of PAS-2 also contains PAS-3 probably in its monomeric form. A molecular species with a mobility corresponding to PAS-4 (Rf 0.31; app. MW 85,000) is composed of a dimer of PAS-3 aggregated with the monomer of PAS-2_a. Material of the same composition as PAS-1 or PAS-4 is found in bands of higher molecular weight Rf 0.08 (app. MW 168,000) and Rf 0.09 (app. MW 150,000) respectively, thus suggesting a different Stokes radius of identical aggregates. In addition, PAS-3 also forms a dimer (Rf 0.48; app. MW 55,000). Finally, these data are consistent with the existence of two types of closely related sialoglycopeptides: glycophorin A or PAS-2_a which can be converted in PAS-1 and glycophorin B or PAS-2_b, which is detectable as a monomer only and does not form aggregates.

555 INTERACTION OF VIBRIO CHOLERAE NEURAMINIDASE (VCN) WITH THE SURFACE OF THE HUMAN ERYTHROCYTE, Frank J. Nordt, Robert J. Knox and Geoffrey V.F. Seaman, University of Oregon Health Sciences Center, Portland, OR 97201.

Others have reported that removal of sialic acid (NANA) from red blood cells (RBC) by VCN drastically reduces their life span and predisposes them to erythrophagocytosis in vitro by autologous macrophages. It has been assumed that the action of VCN results only in the removal of NANA from the cell surface. Our data shows that the reduction in electrophoretic mobility (u) of VCN treated RBC is a function of both the amount of NANA released and the concentration of VCN used. RBC incubated for 1 hr at 37°C with VCN (concentration range 1.5 to 360 Behringwerke Units/10^{10} RBC) lose 80 to 100% of the susceptible NANA at VCN concentrations > 14 Units/10^{10} RBC with concomitant decreases in u. Modification of the VCN treated RBC with formaldehyde (HCHO) increases their u from < 0.1 μm sec^{-1} $Volt^{-1}$ cm to ∿ 0.45 μm sec^{-1} $Volt^{-1}$ cm. The pH versus u profiles for the VCN-HCHO treated RBC display shifts to higher pH for the isopotential point and the appearance of an upward inflection in the pH 10-11 region suggestive of positive surface groups. The data indicate that after VCN treatment the RBC are no longer simply polyanionic as would be expected for simple removal of NANA. These observations along with the reported increase in the susceptibility of VCN treated RBC to acid treated serum lysis and agglutinability by Con-A indicates that VCN treatment may appreciably alter the surface structure of RBC in addition to removal of NANA. Thus NANA loss per se may not be the crucial determinant of RBC life span in the above mentioned studies. Supported by the National Institutes of Health, Research Grant HL 18284.

556 PURIFICATION AND CHARACTERIZATION OF NADH:(ACCEPTOR) OXIDOREDUCTASE FROM HUMAN ERYTHROCYTE MEMBRANES, Chi-Sun Wang and Petar Alaupovic,Laboratory of Lipid and Lipoprotein Studies,Oklahoma Medical Research Foundation,Oklahoma City,Oklahoma 73104

The NADH:(acceptor) oxidoreductase was isolated from human erythrocyte ghosts by a procedure including Triton X-100 solubilization, affinity chromatography on a NAD^+-Sepharose 4B column, $(NH_4)_2SO_4$ precipitation and isoelectric focusing. This preparation gave a single band on SDS-PAGE with an apparent molecular weight of 40,000 and a single precipitin line on double diffusion with its corresponding antiserum. The chemical composition indicated that the enzyme is a glycolipoprotein. Lipid moiety consisted of cholesterol as the main neutral lipid and sphingomyelin as the only phospholipid. The sugar analysis revealed the presence of fucose, mannose, galactose and glucosamine. All common amino acids were present including half-cystine and tryptophan. The optimal pH was 6.8. The K_m values for NADH and ferricyanide were 0.014 mM and 0.049 mM, respectively. A kinetic study showed that the reaction follows an ordered BiBi mechanism with the sequential binding of NADH and ferricyanide to form a ternary complex with the enzyme. Ferrocyanide was released as a reaction product prior to NAD^+. The enzyme activity was inhibited by increased concentrations of ferricyanide which competed with NADH for the binding sites and resulted in the formation of an inactive binary complex. We suggest that the substrate inhibition of the enzyme by ferricyanide plays an important role in the preservation of the NADH concentration in the cell. The agglutination of intact red cells by the antiserum to oxidoreductase indicated that its antigenic determinant is localized on the outer surface of the cell.

557 EFFECTS OF PH AND TEMPERATURE ON CONFORMATION OF SURFACE PROTEINS OF HUMAN RED CELLS. M. G. Luthra and D. A. Sears, Div. of Hematology, Dept. of Medicine, University of Texas Health Science Center, San Antonio, Texas 78284.

The conformation of the outer surface of human red cell membrane has been studied under various conditions using the impermeant probe [^{125}I] diazodiiodosulfanilic acid (DDISA). At least seven polypeptides were labeled by the reagent including each of the three extractable glycoproteins. The 43,000 molecular weight protein band contained two labeled species, one a glycoprotein. The extent and pattern of labeling were very sensitive to changes in pH and temperature. Total labeling increased with increasing pH and was greater at 4° and 37° Binding of the probe to the 90,000 molecular weight polypeptide and major glycoprotein were relatively increased with increasing pH and temperature while opposite effects were observed for the 43,000 molecular weight peptide(s). The pH effects on external membrane labeling were rapidly reversible. The labeling of either bovine serum albumin or membrane free hemolysate was reduced due to increase in pH. Similar results were obtained when leaky ghosts from human red cells were labeled at various pH without any selective effect on an individual protein. These data suggest that conformation of external red cell membrane proteins are effected by pH and temperature and can be probed by DDISA.

558 SEGREGATION OF GLYCOPHORIN-ENRICHED VESICLES FROM HUMAN ERYTHROCYTE MEMBRANES UPON TREATMENT AT LOW pH, Hans U. Lutz, Albert von Däniken, and Thomas Bächi*, Department of Biochemistry, ETH, Federal Institute of Technology, Zürich; Institute of Medical Microbiology, University of Zürich, Switzerland.

Intramembranous particles are known to aggregate when ghosts are incubated at pH 4.5 for 30 min at 37°C. When ghosts pretreated at pH 4.5 are washed with 0.1 mM EDTA (pH 7.4) and with 0.5 M NaCl in phosphate buffer 5 mM (pH 7.4) and then transferred to 5 mM phosphate buffer (pH 7.4), they segregate vesicles that contain almost exclusively glycophorin, lipids and glycolipids. The amount of glycophorin found in glycophorin-enriched vesicles recovered from dextran density gradients does not exceed 3% of the total glycophorin. However, the bulk of glycophorin seems to be segregated as well from other integral membrane proteins but not detached from the membranes because Triton X-100 (0.15%) preferentially extracts glycophorin from these membranes. The Triton X-100 extract obtained as the supernatant following centrifugation of the suspension for 1 hr at 100,000 g forms glycophorin-enriched vesicles during treatment with Bio Beads SM 2. The glycophorin-enriched vesicles thus prepared contain up to 90% of the total glycophorin in a purity approaching that of isolated glycophorin by other techniques with up to 380 µg sialic acid per mg protein. The method allows to isolate glycophorin-enriched vesicles without using ionic detergents or strong organic solvents and will be useful to study the functions of this protein. The observed segregation of glycophorin-enriched vesicles and the preferential extractability of the bulk of glycophorin imply that glycophorin is no longer associated with intramembranous particles when erythrocyte membranes have been treated at low pH.