Th-Pos126

CHARACTERIZATION OF THE HISTIDINES OF APOCYTOCHROME b_5 BY NMR SPECTROSCOPY

C. D. Moore, O. N. Al-Misky, and J. T. J. Lecomte, Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

Upon removal of the heme prosthetic group, the soluble fragment of cytochrome b_5 loses compactness and some secondary structure. We are interested in describing the structural properties of the apoprotein of cytochrome b_5, in particular those of the vacant binding site. Preliminary ^{1}H NMR results have revealed the presence of a stable core in the apoprotein.[1] This region extends beyond the boundaries of the second hydrophobic core of the holoprotein (as defined in the solid state structure[2]) and is located in the β-sheet. In rat liver cytochrome b_5, it includes the four histidines which do not participate in heme binding. To probe the environment of these four residues we have examined their pK_a values, accessibility to Cu(NTA)$^-$ ions, and reactivity towards carboxymethylation. It was found that the pK_as are unaffected by heme removal; this confirmed native structural integrity. The Cu^{2+} titration demonstrated that His 15 and His 80 are buried, whereas His 26 and His 27 are highly accessible. After two days of the carboxymethylation reaction, three His were modified in the holoprotein and a corresponding number in the apoprotein. The three modified His of the holoprotein, as identified by NMR spectroscopy, are His 26, His 27, and His 15.

The presence of a stable core in the apoprotein and inspection of the X-ray structure of the holoprotein have suggested the organization of some elements of structure into a rudimentary heme binding site.[1] The data from the aforementioned titrations allowed for a comparison of the two pocket histidines in the apoprotein. Their pK_a differ by 0.32 unit (±0.04) and they exhibit distinct extent of broadening in the presence of Cu^{2+}. These observations, along with the number and intensity of the cross peaks for these two residues in NOESY spectra, implied that His 39 is less accessible to solvent than His 63.

[1]Moore, C. D. & Lecomte, J. T. (1990) *Biochemistry 29*, 1984-1989.
[2]Mathews, F. S., Argos, P., & Levine M. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 387-395.

Th-Pos127

ELECTRON TRANSFER REACTIONS: HEME PROTEINS AS REDUCING AGENTS. John P. Harrington and Rodgers Hicks, Department of Chemistry, University of South Alabama, Mobile, Alabama. The activity of heme proteins as reducing agents for the reduction of Fe(III) from biologically important (ATP,citrate) and non-biological (EDTA, NTA) complexes have been investigated. Electron transfer processes involving iron in aqueous systems are complicated by the nature of the complexing agents which are necessary for the solubility, transport, and delivery of iron in vivo. Our study examines and compares the ability of several heme proteins (hemoglobin A and S, cytochrome c) to reduce Fe(III)-complexes under different pH, ionic strength, and temperature conditions. Analysis of kinetic spectral data obtained from the visible region (700-490 nm) has permitted a determination of the reducing potential of these heme proteins. Differences in the kinetics of Fe(III)-complex reduction were determined from the rate of heme protein oxidation [Fe(II) -->Fe(III)] coupled to the formation of a Fe(II)-tris (2,2'bipyridine) complex under aerobic and anaerobic conditions. The observed redox events indicate that (1)each of the heme proteins studied is capable of transferring an electron to the Fe(III)-complexes leading to the release of reduced iron; (2)the rates of Fe(III)-complex reduction is dependent on the nature of the complex, pH, ionic strength, and temperature; (3)at pH 7.5 the reduction of Fe(III)-ATP and Fe(III)-NTA > Fe(III)-EDTA and Fe(III)-citrate; (4)the rates of reduction of the Fe(III)-complex are altered by the ratio of Fe(III) to complexing agent; and(5)this mechanism especially as it relates to Fe(III)-ATP and initially reported by Eguchi and Saltman (J.Biol.Chem. 259, 14337,1984) is important in the mobilization and utilization of iron within cellular environments.

Th-Pos128

EXAFS DETERMINATION OF HEME LIGAND ATOMS IN BOVINE CYTOCHROME b561. Ute M. Kent*, Patrick J. Fleming*, Irit Sagi#, Mark R. Chance#. Departments of *Biochemistry and Molecular Biology and #Chemistry, Georgetown University, Washington, DC.

Analysis of amino acid sequence similarity of bovine cytochome b561 with other cytochromes has shown a possible similarity to the four-helix family of cytochromes. Residues known to be important for heme binding in bacterial cytochrome b562 and c' are conserved in the mammalian cytochrome b561. A structural model of cytochrome b561 based on transmembrane topological information, which is consistent with the consensus sequences, would place the heme in bis-histidine coordination rather than between His and Met as suggested by others (Perin et al., (1988) EMBO J. 7, 2697). We have found no evidence for a pH titratable spectral band consistent with iron-methionine ligands (Myer, Y.P. and Bullock, P.A. (1978) Biochemistry 17, 3723). We have begun an analysis of the EXAFS of iron in cytochrome b561 to determine the identity of the heme ligands and to characterize these coordination bonds in different redox states of the cytochrome. Here we present preliminary EXAFS data consistent with nitrogen, but not sulfur as the atoms liganded to heme iron.

Supported by USPHS grant GM 27695 (to PJF) and Petroleum Research Fund grant 23257-G3 (to MRC).

Th-Pos129

DEFORMATIONAL TAUTOMERS OF FREE BASE PORPHYRIN AS A SENSOR OF EVENTS IN THE POCKET OF HEME PROTEINS Judit Fidy, J. Zollfrank[1], J. Friedrich[1], E. Anni and J. M. Vanderkooi, Department of Biochemistry & Biophysics, School of Medicine, University of Pennsylvania, Philadelphia PA 19104 and [1]Institut fur Physikalische Chemie, Johannes Gutenberg-Universitat, D-6500 Mainz, Germany.

The absorption and emission properties of mesoporphyrin IX (MP) substituted horse radish peroxidase were examined using the high resolution techniques of fluorescence line narrowing and hole burning. Cryogenic trapping and photochemical conversion permitted the stabilization of particular pyrrole tautomeric forms of MP from among the inhomogeneous distribution of protein conformations. At least four tautomeric forms were identified through their 0-0 transition energies. Their characteristic 0-0 energies were 16302, 16202, 16100 and 16000 cm^{-1} and the width of the inhomogeneous distribution was 50-60 cm^{-1}. These species can interconvert under irradiation, and they revert in the dark. Ground state barrier distributions for the reversion reaction were found around 9, 20, 30 K and above 100 K. Spectral diffusion and temperature cycling experiments revealed a different pattern for each form and behavior that contrasted from that expected for glassy matrices. It appears that whereas the protein resembles a glass in the over-all inhomogeneous distribution, the protein exhibits non-glass-like behaviour in that particular deformational forms of the porphyrin are stabilized and that there are discrete energy barriers between the "conformational substates". The conclusion that the porphyrin fluorescence senses dynamical events in the heme pocket was corroborated by observing changes when the protein was changed (as by altering pH) or by comparison of MP fluorescence in other heme proteins, such as MP cytochrome *c* peroxidase. (Supported NSF grants DMB 88-15723, DMB87-16796 and the Deutsche Forschungsgemeinschaft).

Th-Pos130

INTERACTION OF Mn^{2+} WITH ERYTHROCROORIN OF GLOSSOSCOLEX PAULISTUS. Maria Helena Tinto, Hidetake Imasato, Marcel Tabak, Janice Rodrigues Perussi. Instituto de Física e Química de São Carlos, USP, C.P. 369, 13560, São Carlos, SP, Brasil.

The erythrocruorin of Glossoscolex paulistus is a giant extracellular hemoglobin of molecular weight $3.1x10^6$ daltons. It has been demonstrated that divalent cations act as the modulators on oxygenation properties of this hemoglobin, Mg^{2+} and Ca^{2+}, both increase the oxygen affinity and the cooperativity. In the present work EPR spectroscopy studies of Mn^{2+} aqueous solution showed that the number of Mn^{2+} interaction sites is high, about 20 per heme. Scatchard plot analysis suggest that either negative cooperativity occurs upon binding of Mn^{2+} or at least two different classes of Mn^{2+} sites with association constants in the range $6.5x10^9M^{-1}$ and $1.2x10^3M^{-1}$ are observed. The number of Mn^{2+} interaction sites was reduced when the hemoglobin was incubated previously in the presence of Ca^{2+} and Mg^{2+}. On the other hand adding Ca^{2+} or Mg^{2+} displace Mn^{2+} only partially, showing again that there are two possibilities to explain the experimental data: 1 there is an effective competition of diamagnetic ions with Mn^{2+} for the same sites or 2. The interaction of these cations are not specific and the first higher affinity sites when occupied modify the protein conformation, changing the characteristics of binding of the other sites (lower affinity). The effect of protein concentration upon binding of Mn^{2+} is also very dramatic: below 30 micromolar the number of binding sites is considerably reduced. Different classes of sites for divalent ions could be related to the two roles of divalent ions as structural linkers stabilizing the macroprotein and as modulators of oxygen affinity and cooperativity.

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Th-Pos131

A HIERARCHY OF GLOBIN COMPLEXES IN THE GIANT INVERTEBRATE HEXAGONAL BILAYER HEMOGLOBIN AND CHLOROCRUORIN.
Aziz N. Qabar, Pawan K. Sharma, Serge N. Vinogradov and Joseph S. Wall, Biochemistry Department, Wayne State University, Detroit MI 48201 and Brookhaven National Laboratory, Upton, NY 11673.
The giant, 60S hexagonal bilayer (M_r ca. 3,600 kDa) chlorocruorin (Chl) of Eudistylia vancouverii and hemoglobin (Hb) of Lumbricus terrestris are highly cooperative oxygen-binding proteins which consist of several heme-containing chains of ca. 17 kDa and two or more chains of ca. 30 kDa which contain little or no heme. Both molecules can be dissociated by urea at neutral pH to produce globin complexes whose subunit composition has been defined by SDS PAGE and whose molecular masses have been determined by gel filtration and by scanning transmission electron microscope (STEM) mass measurements of unstained, freeze-dried specimens. The Chl dissociates into two particles of ca. 200 kDa and 65 kDa, respectively, each consisting of at least two kinds of globin chains of ca. 17 kDa. The Hb dissociates into ca. 200 kDa, 50 kDa and 17 kDa subunits. Since the Hb contains four different globin chains (I - IV) of known amino acid sequence, and since the chain composition of the three subunits is I+II+III+IV, II+III+IV and I, respectively, the ca. 200 kDa aubunit is likely to be a dodecamer of globin chains (calculated M_r = 209 kDa). Although no sequence information is available for the Chl chains, it appears likely that the ca. 200 kDa and the 65 kDa particles are dodecamers and tetramers, respectively, of globin chains of ca. 17 kDa. The quaternary structure of both molecules can be interpreted to consist of twelve dodecameric globin complexes linked by non-heme containing ca. 30 kDa chains to form the hexagonal bilayer structure observed by STEM. The resolution of these structures into hierarchies of globin complexes allows a direct investigation of the relationship between the cooperativity of ligand binding and the level of quaternary structure. Supported in part by NIH Grant DK38674.

Th-Pos132

MODIFICATION OF THE POLARITY OF THE DISTAL HEME POCKET OF HEMOGLOBIN. MUTANT E11(67)VAL--THR.
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Dept. Biochem., U.MD. Med.S., Baltimore MD and Dept.Chemistry., Temple U.,Philadelphia PA
We have investigated the relevance of the hydrophobic character of the heme pocket to oxygen affinity by constructing a mutant Hb in which the β67Val was replaced by the isosteric threonine. The oxygen equilibrium curve of this Hb was biphasic, with part of the hemes endowed with higher and part with lower oxygen affinity than in native human Hb. Cooperativity was present only in the low affinity part of the curve with a value of n=1.5. These data could be interpreted to represent the characteristics of a hemoglobin where the communication between the subunits was severely hampered, whereby the high oxygen affinity represents the oxygen affinity of the α chains, and the low oxygen affinity that of the mutant β-chains. From the Bohr effect it can be calculated that one proton/heme is released by the β67Thr mutant Hb as compared to 0.5 in native Hb. Molecular modeling performed by K.Fidelis (U.Maryland.CARB) shows that an H-bond may be established between the β67Thr and β63His which places the threonine 3 A° from the Ne Hist, 3.25 A° from the carbonyl at β63 and 3.68 A° from the heme. In oxyhemoglobin the presence of oxygen introduces steric constraints which hinder the formation of this H-bond. It could be speculated that, in this mutant hemoglobin, β63Hist has becomed a Bohr effect group. Alternatively the threonine could introduce a water molecule in the distal pocket hich could coordinate to the heme iron. However the absorption spectra of the native and mutant Hbs in the liganded and unliganded form were identical indicating the absence of water coordination to the iron.

Th-Pos133

SUBUNIT DISSOCIATION OF Lumbricus terrestris HEMOGLOBIN BY HIGH PRESSURE. R.E. Hirsch, J.P. Harrington, and S.F. Scarlata. Albert Einstein Coll. Med., Bronx, N.Y.; Univ. of South Alabama, Mobile, AL; Cornell Univ. Med. Coll., New York, N.Y. We recently studied the intrinsic fluorescence of the dodecameric hemoglobin (3.8kD) of Lumbricus terrestris and concluded (1)different subunit species result from acid vs. alkaline dissociation; (2)H-bonds are important in maintaining the integrity of the native molecule (Biophys. J. 57:237a). To further explore dissociation in Lumbricus terrestris Hb (LtHb) and the nature of the subunit bonds, high pressure effects on the steady-state fluorescence emission and light scattering properties were studied. Increasing hydrostatic pressure on oxy LtHb, equilibrated at pH 7.2, results in shifts of 1600 cm^{-1} in the center of spectral mass with simultaneous 12-fold decrease in light scatter. Data best fit to a subunit order of 12, as expected. Pressure dissociation measured by light scattering generally matched that of the higher-ordered G. paulistis Hb (Silva et al. JBC 264:15863) but the subunit dissociation is higher (96) and spectra are different suggesting structural differences compared to LtHb. Pressure studies of LtHb at pH 9.1 show little change in scatter intensity and emission, where pressure end points match those of pH 7.2, implying almost complete dissociation to its fundamental subunits at alkaline pH. The p(1/2) seen by fluorescence is about 300 bars lower than the scatter data indicative of Trp(s) microenvironmental changes prior to dissociation. Atmospheric spectra at pH 5.3 and 7.2 were the same; but high pressure studies had little effect at pH 5.3 in contrast to pH 7.2 suggesting no significant dissociation under acidic conditions. As controls, oxy HbA, known to dissociate into dimers, exhibited a small decrease in intensity with increased pressure whereas Carp Hb and Mb (which do not dissociate) did not exhibit any significant fluorescence changes. We conclude LtHb is fully dissociated into its fundamental subunits under alkaline conditions and H-bonds are fundamental in maintaining subunit integrity. (Supported by NIH Grants GM39924 (SFS); RO1 DK41253 & N.Y.C. Heart Affiliate (REH); USA Travel Grant (JPH)).

Th-Pos134

CONFORMATIONAL DISTRIBUTIONS OF HUMAN ALPHA APOHEMOGLOBIN UPON ASSEMBLY WITH HUMAN BETA HEMOGLOBIN. Shawn M. O'Malley and Melisenda J. McDonald, Biochemistry Program, Department of Chemistry, University of Lowell, Lowell, MA 01854.

The recent steady-state fluorometric method of Grysinski et al. (Biophys. J. 54:577-586, 1988) was used to recover the assumed gaussian distribution parameters for human α apohemoglobin ([α^o] = 5 μM) and its complex with human β hemoglobin ([β^A] = 5 μM) in 0.05 M potassium phosphate buffer, pH 7 and 5°. The distance measured was from the intrinsic tryptophan donor (α 14) to the acceptor probe 4-phenylazophenyl maleimide at cysteine (α 104). To simplify comparison both the Förster distance, R_o (-25.6 Å), and the Stern-Volmer dynamic quenching constant, K_D (-2.42 M^{-1}), were assumed to be the same for α^o in the absence and presence of β^A. The efficiency of energy transfer was 0.82 for α^o and in the presence of β^A was 0.62. Successive additions of acrylamide quenching agent resulted in a decrease in the efficiency of transfer for α^o and $\alpha^o\beta^A$ of 19% and 30%, respectively. The quenched efficiencies (E^Q) and Förster distances (R_o^Q) were analyzed by a least-squares program which determined the (X_R^2) goodness of fit for assumed distribution parameters: average distance $\bar{r}$ and half-width, hw. Preliminary quenching data for α^o showed a X_R^2 of 0.5 for $\bar{r}$ = 17 Å and hw = 14.8 Å; whereas, quenching data for $\alpha^o\beta^A$ yielded a X_R^2 of 4.9 for an $\bar{r}$ = 24.2 Å and hw = 14.2 Å. The increase in $\bar{r}$ is in part due to additional quenching from the heme moiety of β^A. Comparison of distribution half-widths suggest a decrease in α^o flexibility upon assembly with β^A. This application of the recovery of distribution parameters may provide a method of evaluating the role of conformational flexibility in hemoglobin assembly. Supported by NIH Grant 38456.

Th-Pos135

STABILITY OF CROSSLINKED HEMOGLOBINS. Thao Yang, Qun-Ying Zhang, He Huang, Susan M. Wojnicki, Thomas D. Corso, Doris Ranum, Sonali Dave, Frank L. White and Kenneth W. Olsen; Department of Chemistry, Loyola University of Chicago, 6525 N. Sheridan Rd., Chicago, IL 60626.

Human Hb A has been stabilized by a variety of single and double crosslinks. The change in the transition temperature compared to that of Hb A (ΔT_m) was measured by thermal denaturation of methemoglobins in 0.01 M MOPS, pH7, containing 0.9M guanidine to prevent precipitation. Hemoglobin crosslinked by bis(3,5-dibromosalicyl) fumarate between the two Lys β82's or Lys α99's had ΔT_m's of 16°C. The succinate and glutarate analogs were not as effective as the fumarate diaspirin in crosslinking deoxyHb A between the Lys α99's but worked well with oxyHb A. The resulting β82 crosslinked hemoglobins were slightly less stable than the fumarate crosslinked protein. Difluorodinitrobenzene crosslinked hemoglobin showed only monomers and dimers in equal amounts on SDS gels, but a more detailed analysis indicated that there were multiple sites of modification. These samples had two ΔT_m's of 6°C and 20°C, indicating the complexity of the crosslinking. Hb A crosslinked with the trilinker tris(β-chloroethyl)amine had a ΔT_m of 8°C, but the corresponding crosslinker, bis(β-chloroethyl) methylamine, did not raise the T_m of Hb A. Zero-length crosslinking with carbodiimide or disuccinyloxalate produced only intra-subunit crosslinks and a ΔT_m of 2°C. The Hb A treated with dimethylpimelimidate (DMP) had a ΔT_m of 15°C. The β82 and α99 crosslinked oxyHb A's were further treated with DMP to produce double crosslinked hemoglobins, both of which had ΔT_m's of 19°C. Polymeric hemoglobins produced by modification with DMP or glutaraldehyde had more complex denaturations. These results should improve the design of stabilized crosslinked hemoglobins as blood substitutes. (Supported by a grant from the American Heart Association of Metro. Chicago.)

Th-Pos136

PORPHYRIN-IRON AND MAGNESIUM-IRON HYBRID HEMOGLOBINS: ABSENCE/WEAKENING OF THE Fe-His BONDS FAVORS A *SUPER-T* STRUCTURE WITH EXTREMELY LOW OXYGEN AFFINITY.

FUJII, Motoyasu, ZHOU, Yu-xiang, and YONETANI, Takashi. Dept. of Biochemistry & Biophysics, Univ. of Pennsylvania, Philadelphia, PA 19104-6089

Porphyrin-Fe and Mg-Fe hybrid hemoglobins A (HbA), in which the protohemes (Fe) in either the α- or β-subunits were substituted with protoporphyrins IX (PP) or Mg[II]-protoporphyrins IX (Mg), i.e., $\alpha(PP)_2\beta(Fe)_2$ or $\alpha(Fe)_2\beta(PP)_2$ and $\alpha(Mg)_2\beta(Fe)_2$ or $\alpha(Fe)_2\beta(Mg)_2$. have been prepared. The oxygen equilibrium properties of the two Fe[II]-containing subunits in these hybrid tetramers have been measured. These hybrids exhibited low oxygen affinity with reduced cooperativity. These effects were more pronounced in the β(Fe)-containing hybrids, namely, $\alpha(PP)_2\beta(Fe)_2$ and $\alpha(Mg)_2\beta(Fe)_2$, which showed an extremly low oxygen affinity with substantially diminished cooperativity, Bohr effect and organic phosphate effect. These observations indicate that when the Fe-*proximal* His bonds are broken/weakened, especially in the α-subunits, or the structural constraint induced by the Fe-*proximal* His bonding in the α-subunits is removed/weakened, HbA favors a *Super-T (extreme low-affinity)* structure with diminished allosteric effects regardless of its ligation state. This implies that the Fe-*proximal* His bonding, particularly that in the α-subunits, prevents *deoxy* Hb A moving into the energetically more stable *Super-T* state and keeps *deoxy* HbA in a less stable or *constrained T (low-affinity)* state which can readily shift to a *R (high-affinity)* state upon ligation and maintains allosteric effects such as cooperativity, Bohr effect, and organic phosphate effect. Spectroscopic determinations of the quaterary/tertiary strctures of these hybrids are in progress. Scientific and practical implications of this new interpretation of these three different affinity states of HbA in relation to their quaternary/tertiary structures will be discussed. (Supported by NHLBI HL14508 grant).

Th-Pos137

ANALYSIS BY SINGULAR VALUE DECOMPOSITION OF THE VISIBLE SPECTRAL TRANSITION ON OXYGEN BINDING TO HEMOGLOBIN. Kim D. Vandegriff, Yvonne C. Le Tellier, Richard I. Shrager¶, and Robert M. Winslow. Blood Research Division, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129 and ¶National Institutes of Health, Bethesda, MD 20892.

Equilibrium oxygen binding to human hemoglobin is being measured by rapid-scanning spectrophotometry. Each spectral scan, over the range from 650 to 450 nm, is collected in 200 milliseconds, and 10 scans are averaged for each increment in oxygen partial pressure. Deoxyhemoglobin is oxygenated in a 1-cm cuvette fitted with an oxygen electrode by floving 100% oxygen over the solution. Oxygen equilibrium curves show normal cooperativity and are fit to the Adair equation for analysis of equilibrium binding constants. The absorbance matrix is evaluated by singular value decomposition (SVD) to determine the rank of the component contribution to the visible-spectral transition. For human hemoglobin Ao and hemoglobin crosslinked between the α(99)Lys residues, there are two primary components, corresponding to deoxy- and oxyhemoglobin, and one minor component that has 1/1000 the contribution of the major components. This minor spectral component arises at ~50% saturation with a peak at 570 nm and a half-width of ~7 nm. Since the αα-crosslinked hemoglobin does not dissociate to dimers, it can be ruled out that this transition arises from hemoglobin dimerization. Further studies are being carried out to determine the source of this optical component. The magnitudes of the components from SVD show that the visible optical spectra are determined by only the two major species and that the minor component plays an insignificant role. As a result, the optical transition in the visible region of the spectrum during hemoglobin oxygenation is linear with respect to hemoglobin saturation.

Th-Pos138

REGULATION OF HEMOGLOBIN ACTIVITY BY WATER MOLECULES, M.F.Colombo, D.C.Rau, and V.A.Parsegian, LBM-NIDDK-NIH, Bethesda, MD 20892, USA, Intro. by M.M.Garner

The ability of proteins to regulate biological activity is frequently accomplished through the control of the equilibrium between conformational states. The binding of small molecule with different strength and numbers to differents conformations are known to regulate this equilibrium between states. These conformational changes also affect differences in protein hydration. The binding or release of water should also be considered a potential regulator of biologically activity.

In this work, we have investigated the dependence of oxygen affinity of Hemoglobin (Hb), stripped of inorganic phosphates and ions, on water activity or osmotic pressures, varied with several different small molecules, sucrose, stachyose, polyethylene-glycol 150 and 400. In solutions with different concentrations of these solutes, we observe that O2 affinity of Hb decreases with decreasing water activity, up to osmotic pressures corresponding to about 50 atmospheres. When these data are analysed by Gibbs-Duhem relationships, assuming only water and protein direct interactions, a number of extra water molecules that bind to Hb during the transition from the deoxy T state to the oxy R state can be determined. Consistently, we find that aproximately 70 water molecules bind to Hb upon the change structure going from deoxy to oxy form. This number is independent of the nature of the solute used. We conclude that the effect of water activity on the oxygen affinity can be rationalized only considering the linkage between oxygen binding with the change in the protein hydration that follow the structural transition from T (deoxy) to R (oxy) strucuture upon oxygenation.

Th-Pos139

AUTOXIDATION: A PROBE FOR CONFORMATION CHANGES IN HEMOGLOBIN. A. Levy, O.O. Abugo and J.M. Rifkind. Molecular Dynamics Section, Laboratory of Cellular and Molecular Biology, NIH/NIA, Gerontology Research Center, Baltimore, Maryland.

Numerous studies have been directed at understanding the factors which control autoxidation and provide for the stabilization of oxygenated hemoglobin. Although the rates of autoxidation for hemoglobin are generally low, subtle changes in the ligand pocket can produce appreciable changes in these rates of autoxidation. In many cases the changes in autoxidation are more dramatic and easier to observe than the changes in ligand binding properties. In this way it has been possible to use autoxidation to:

1) Observe an intermediate quaternary conformation.
2) Detect conformational changes which occur during dissociation into dimers.
3) Detect conformational change associated with membrane binding.

These conformational changes are thought to modulate the functional oxygen binding properties of hemoglobin. Insights into the nature of these conformational changes are suggested by the particularly dramatic effects on autoxidation.

Th-Pos140

HEMOGLOBIN INTERACTION WITH SONICATED AND EXTRUDED VESICLES, C. C. LaBrake and Leslie W.-M. Fung, Department of Chemistry, Loyola University of Chicago, Chicago, IL 60626.

Human hemoglobin (Hb) has been shown to accumulate in the red blood cell membrane, under certain pathological conditions, as oxidized Hb, either hemichromes or Heinz bodies. Thus the interactions of Hb with the inner leaflet phopholipids of the erythrocyte membrane are of importance in understanding the effects of different membrane components on the stability of Hb. A solution of sonicated or extruded bovine phosphatidylserine (BPS) vesicles and pure Hb served as a simple model system for studying the interactions of Hb with the phospholipids of the inner leaflet of the red blood cell membrane. Two parameters were used to quantitate the interactions of Hb with the vesicles: 1) the initial rate of Hb oxidation, and 2) the extent of vesicle fusion. At pH 7.4 and 37 °C, we found that the oxidation rates of Hb were about 10 times faster in the presence of extruded vesicles than without the vesicles. Furthermore, the Hb oxidation rates with sonicated vesicles were about another 10 times faster than those with extruded vesicles. Using GC-MS, TBA and conjugated diene assays, we found only limited lipid peroxidation in the extruded and sonicated vesicles used. Vesicle fusion began about 30 min after incubation with Hb at 37 °C. The extent of fusion after 20 hours incubation was compared amongst the different systems studied. The effects of oxyHb, COHb and the addition of BHT to vesicles were also studied.

Our results show that lipid-induced Hb oxidation is probably not due to lipid peroxidation, but due to the lipid surface, suggesting that surface curvature and hydrophobicity may play an important role in Hb-lipid interaction.

(Supported by NIH and Loyola University of Chicago.)

Th-Pos141

NITROSYL HEMOGLOBIN - SPIN - LATTICE RELAXATION MECHANISM. George Bemski, Marília P.Linhares*, Léa El-Jaick and Eliane Wajnberg. Centro Brasileiro de Pesquisas Físicas. *Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil.

E.P.R. spectra of nitrosyl hemoglobin studied between 7.5K and 104K are complex and consist of at least three superposed signals with different relaxation characteristics. They dominate at different temperatures and microwave power. Using continuous saturation technique we have obtained the half-saturation power $P_{1/2}$. The relaxation rate T_1^{-1}, of the component dominating at low temperatures and low microwave power follows an Orbach exponential dependence with a characteristic energy of $28cm^{-1}$. This result evidences a low lying energy state for this component. Since one does not expect in the proposed energy level diagram such small difference in energy between eletronic states, we proposed that this energy refers to a transition between two conformations of the liganded heme, produced by different Fe-N-O angles.

The two components which dominate the spectra at high microwave power but at different temperature ranges have spin relaxation times which differ by about an order of magnitude. T_1 is about $6.3x10^{-5}s$ at 7.5K for the low temperature component and $6.3x10^{-6}s$ at 104K for the third component. The high $P_{1/2}$ values of these two components do not allow to obtain their temperature dependence.

Th-Pos142

An NMR study of the role of distal amino acids in the steric tilt of bound ligand in Sperm Whale Myoglobin.

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The role of protein structure in the selective binding of O_2 over CO in myoglobins (Mb) has been a subject of much debate. CO binds in a linear fashion in Fe-protoporphyrin whereas it is found to be tilted in Mb. It has been proposed that E7(His) and E11(Val), two highly conserved amino acids in the heme pocket, play a major role in reducing the binding of CO by steric interactions. NMR studies from this lab (Emerson & La Mar,(1990)Biochemistry, 29, 1556) have shown that it is possible to determine the orientation of cyanide in solution from determining the magnetic axes of Mb cyanide (MbCN) and it was also proposed that the orientation of the bound ligand determines the major magnetic axes. FeCO and FeCN are isostructural and hence it was proposed that MbCN can serve as a good model for the orientation of CO in MbCO. Chemical shifts calculated from the magnetic axes have shown that a few assignments of the protons of amino acids in the heme pocket (F8 His, FG3 His, FG5 Ile, CD1 Phe) can give a qualitative picture of the orientation of the ligand, not only with respect to the extent of the tilt but also the direction of orientation of the ligand. In order to study the steric effects in the distal pocket, we have measured the necessary chemical shifts in E7(His→Gly, Val), E11(Val→Ala, Ile), and CD3(Arg→Gly) mutants. The observations suggest that reduced steric bulk changes the *direction of the tilt* to a large degree and the *extent of tilt* to a less degree. In light of the above observations, the proposal that steric factors are the primary determinant of the orientation of bound ligand has to be reconsidered.

Th-Pos143

^{1}H NMR STUDY OF THE HEME POCKET STRUCTURE OF PARAMAGNETIC Fe-CHLORIN MYOGLOBINS

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Iron chlorins, which are heme porphyrins with a single reduced pyrrole ring, exist in naturally occurring 'green heme' enzymes. Very little is known about their molecular structure and relation to enzymatic function. We have studied the structure of the heme pocket of paramagnetic iron chlorin enzymes by NMR. This technique is well suited to a study of paramagnetic systems because of the well-resolved hyperfine shifted resonances outside of the intensely overlapped diamagnetic region where most of the amino acid residue chemical shifts lie. Here we use Fe pyropheophorbide *a*, and 2-desvinyl Fe pyropheophorbide *a*, iron derivatives of chlorophyll *a*, reconstituted into myoglobin as simpler models of more complex naturally occurring enzymes. We report the first successful assignments by NMR using the nuclear Overhauser effect (NOE), deuterium labelling, and T1 measurements of the paramagnetic, low spin ferric (metcyano) and high spin ferrous (deoxy) forms of these green chlorin myoglobins. In the reconstituted metcyano myoglobin forms we find the hyperfine chemical shifts to be very similar to those in the ferric biscyano pyropheophorbide complexes. This implies that the perturbed, rhombic orbital ground state of a chlorin is *not* altered when incorporated into the protein and that the pattern of the hyperfine shifted resonances is more a reflection of the site of the saturated pyrrole rather than of the axial and peripheral contacts between the chlorin and protein. Like its metcyano form, the paramagnetic deoxy state of these green hemoproteins give NMR spectra with a large chemical shift spread, 65ppm to -40ppm. In particular, we find the exchangeable proton of the proximal histidine, His F8, to occur significantly shifted to higher field by 8-12ppm compared to native deoxy myoglobin. Since green hemoproteins need not necessarily contain a *chlorin* prosthetic group, this last observation suggests its use as an important probe for identification of chlorin versus porphyrin heme pocket structure.

Th-Pos144

TWO-DIMENSIONAL INFRARED SPECTROSCOPIC INVESTIGATION OF HORSE MYOGLOBIN.

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We use two-dimensional infrared spectroscopy (2D IR) to examine intramolecular interactions between functional groups in horse myoglobin. The myoglobin molecule is immobilized in a polyacrylamide gel matrix. This is accomplished by first attaching myoglobin to an N-hydroxysuccinimide (NHS) active ester via reaction of the terminal amine groups of the solvent exposed myoglobin lysine side chains with the NHS active ester. Next, we establish a cross-linked network between the myoglobin-ester complex and the polyacrylamide gel, thus immobilizing the myoglobin.

We perturb the molecular system with a macroscopic stimulus in the form of a small external oscillatory strain. We examine the time-dependent intensity and vibrational frequency changes of iron-ligand vibrational modes as well as protein ligand interaction modes. The resulting two-dimensional IR spectrum permits the correlation of interacting modes, especially those at the active site, in response to an external macroscopic stimulus at the protein surface. This information establishes a communication network between functional groups involving the heme moiety and the myoglobin surface.

Th-Pos145

X-RAY STRUCTURES OF HEMOGLOBIN-ALKYL ISOCYANIDES

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The structures of human hemoglobin with bound methyl-, ethyl-, n-propyl- and n-butyl isocyanides have been solved at 2.1Å by x-ray crystallography. The alkyl isocyanides, used as steric probes of the ligand binding site, illustrate the role that distal amino acid side chains play in regulating the different ligand affinities of alpha and beta chains.

In the α-chain heme pocket, the distal histidine does swing slightly away from the ligands when compared to the oxyhemoglobin structure, but three other conserved residues (Leu-B29, Phe-CD1 and Val-E11) appear to be unaffected. The alkyl tails of the ligands lie parallel to the plane of the heme group. In β-chains, the imidazole ring of the distal histidine rotates away from the bound ligands by approximately 30° about its Cβ-Cγ bond . Leu-B29 is pushed away from the heme group by about 0.5Å. The alkyl tails of the ligands lie at an approximately 45° angle from the plane of the porphyrin ring. The flexibility of the β-chain heme pocket helps to explain why most of the alkyl isocyanide molecules escape to the solvent after photolysis of the iron-ligand bond and why the association and dissociation rate constants for alkyl isocyanide binding to β-subunit are large compared to those for α-subunits (Reisberg, P.I., and Olson, J.S., 1980, J. Biol. Chem. 255, 4151-4158).

The proximal side of the heme pocket of the isocyanide complexes shows differences compared to that of oxyhemoglobin. In particular, the iron-His(F8) is shorter in the oxy complex which causes greater movement of the F-helix when oxygen binds to deoxyhemoglobin. The longer iron-His(F8) bond in the alkyl isocyanide complexes suggest a structural cause for the smaller extent of cooperativity observed for the binding of these ligands.This work was supported by USPHS GM-3569, NIH AR-40252, W.M. Keck Foundation, and Welch Foundation C-612 and C-1142. KAJ is supported by an NIH training grant.

Th-Pos146

DISCONTINUOUS HEAT RELEASE AT SUCCESSIVE STEPS OF OXYGENATION IN HUMAN AND BOVINE HEMOGLOBIN AT PH 9.0. E. Bucci, C. Fronticelli, Z. Gryczynski. Biochemistry, Un. of MD at Baltimore,21201

We have measured the temperature dependence of the oxygen binding isotherms of human and bovine hemoglobin at pH 9.0 in 0.1 M borate buffer. In both hemoglobins the ionization of the Bohr protons is finished at this pH, therefore their heat does not interfere with the measurements. Two sets of curves have been obtained which have been analyzed either singularly or whith global procedures for estimating the enthalpy changes of subsequent steps of oxygenation. The following table reports data obtained from global analyses of the binding isotherms measured at several temperatures between 10° and 40° C, at different wavelength between 440 and 580. They are the enthalpy in Kcal/heme at 25° of the intrinsic affinity constants of each step of oxygenation of human and bovine hemoglobin.

Step		1	2	3	4
Human	H	-12.6	-27.7	0.5	-22.9
Bovine	H	0.8	-35.8	23.0	-20.5

The standard deviations are less than 10% of the estimated values. The data indicate that in human hemoglobin the reaction with oxygen is enthalpy driven for steps 1,2 and 4, while it is entropy driven for step 3. In bovine hemoglobin this phenomenon is even more evident, both steps 1 and 3 are entropy driven. Global analyses, using fixed values of enthalpy at each oxygenation step, failed to be consistent with the van't Hoff plots of the intrinsic affinity constants obtained from local analyses of individual curves. The discontinuous distribution of heat at subsequent steps of oxygenation suggests that the T to R transition in hemoglobin is not a monotonic process and involves conformations with novel characteristics.

Th-Pos147

SECONDARY STRUCTURAL STUDIES OF PROTEIN KINASE C BY CIRCULAR DICHROISM. Jyotsna Shah, Mary T. Walsh and G. Graham Shipley, Biophysics Department, Boston University School of Medicine, Boston, MA 02118.

The phospholipid- and Ca^{2+}-dependent protein kinase C (PKC) has been implicated as a pivotal regulatory element in signal transduction, cellular regulation, and tumor promotion. In vitro activation of PKC requires the interaction of the enzyme with Ca^{2+}, phosphatidylserine (PS) and diacylglycerols (DAG) / phorbol esters [e.g. phorbol myristate acetate (PMA)]. Here we report the effect of these activators on the secondary structure of PKC. PKC was isolated and purified from bovine brain [Niedel et al., Proc. Natl. Acad. Sci. 80, 36-40 (1983) and Wolf et al., J. Biol. Chem. 260, 15718-15222 (1985)]. Circular dichroism (CD) was used to study the secondary structure of protein kinase C (PKC) in 10 mM Tris/HCl buffer (pH 7.5) and to examine the conformational changes in the protein, resulting due to the presence of its regulatory cofactors (e.g. Ca^{2+}, PS and PMA). Computer analysis of the predicted secondary structure based on CD-data showed that PKC maintains a highly ordered structure containing 36.0% α-helix, 57.1% β-sheet and 6.9% β-turn. PKC displays a minor conformational change upon addition of Ca^{2+}. Addition of phosphatidylcholine (PC) vesicles did not show any significant change in the CD profile. However, a large change is observed in negative ellipticities on adding phosphatidylserine vesicles in presence of Ca^{2+} and analysis of CD-data showed the presence of 22.0% α-helix, 64.5% β-sheet and 13.5% β-turn. The protein does not experience further significant changes in conformation on adding PMA. The secondary structure of PKC was also predicted from the primary sequence by using Chou & Fasman algorithm. Mean hydrophobicities and hydrophobic moments were also determined. From our results we conclude that PKC undergoes a conformational change upon binding with its activators and these altered conformational properties may be helpful in understanding the mechanism by which PKC interacts with membrane surfaces.

Th-Pos148

EXTENDED LEFT-HANDED HELICAL CONFORMATION AS THE SECONDARY STRUCTURE TYPE OF POLYPEPTIDE CHAIN A.Adzhubei, F.Eisenmenger, N.Esipova, V.Lobachev, A.Makarov, V.Tumanyan, (Intro. by Prof. J.T.Yang) Institute of Molecular Biology, Acad.Sci.USSR Moscow 117984, Vavilov str.32, USSR

The left-handed helical conformation that is the basic structure of collagen is found to be quite common for amino acid residues in peptides and globular proteins. The CD criterion for testing of the left-handed helix in peptides was elaborated using X-ray diffraction and CD study of synthetic oligopeptides as well as collagen. In aqueous media the left-handed helix is the preferable conformation for polypeptide hormones of the anterior lobe of the pituitary gland, β-endorphin, histone H5 and H1 fragments. The temperature dependences of CD characteristics and partial heat capacity allow to consider left-handed helix as weakly cooperative. The analysis of the Brookhaven Data Bank revealed that the distribution of residues in the B-region of Ramachandran plot is bimodal. Along with the β-structure maximum there exists the maximum corresponding to the extended left-handed helix. This was verified by the criterion of absence of interchain hydrogen bonds; 19.1% of residues in the sample belong to this conformation in comparison with 19.6% of β-structure residues. The residues in the left-handed conformation in globular proteins do not form continuous clusters but are distributed along the chain. This conformation is characterized by specific hydration and is stabilized with the decrease of temperature. The left-handed helix can be treated as highly mobile, its conformational transition to 3/10 helix is shown to take place in hinge region of Ig G.

Th-Pos149

INTERACTION OF BOVINE BRAIN S100a WITH A DISTINCT CALMODULIN BINDING DOMAIN. [1]H. N. Parikh, [2]P.L. Pingerelli and [1]H. Mizukami. [1]Division Of Regulatory Biology and Biophysics, Dept of Biological Sciences, Wayne State Universtiy, Detroit, MI 48202. [2]Calbiochem Corporation, La Jolla, CA 92037.

S100a is an acidic, calcium binding, heterodimeric protein which exposes a hydrophobic domain upon binding calcium. Additionally, S100a has been shown to interact with a number of calmodulin antagonists including trifluoperazine (TFP) in a Ca^{2+}-dependent manner. In order to investigate the similarities between the hydrophobic domains of S100a and calmodulin (CaM), the interaction of S100a with a synthetic 20 residue peptide, that identifies a calmodulin binding domain (CDP) for calcium/CaM-dependent protein kinase II activation (Payne et al, 1988, JBC 263:7190), and with TFP were investigated using circular dichroism (CD) and nuclear magnetic resonance (NMR). CD ellipticity changes at 292 nm, corresponding to the single trp residue of S100a, were used to monitor peptide binding with and w/o Ca^{2+}, while far-UV CD was used to investigate secondary structural changes. ^{19}F-NMR chemical shift and line width changes of TFP were utilized to estimate CBD competitiveness for the hydrophobic domain of S100a. Upon addition of CBD, an upfield chemical shift of the TFP/S100a solution suggests that the calmodulin binding domains compete for the same hydrophobic site as TFP. Results suggest that CBD and TFP interact with S100a in a fashion similar to CaM.

Th-Pos150

THE pH-DEPENDENT CONFORMATIONAL CHANGES OF A TGFα-PSEUDOMONAS EXOTOXIN HYBRID PROTEIN

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TGFα-PE40 is a chimeric protein created by fusing transforming growth factor α (TGFα) with a truncated Pseudomonas exotoxin molecule (PE40), devoid of domain Ia of the exotoxin. This protein has shown a high degree of cytotoxic activity against A431 human epidermoid carcinoma cells that are rich in epidermal growth factor (EGF) receptors (Siegall, et al., J. Biol. Chem. 264, p. 14256, 1989). We have examined conformational changes of TGFα-PE40 induced by changes in pH using fluorescence and circular dichroism (CD) spectroscopy. The binding of the fluorescent probe, 2-(toludinyl)-naphthalene sulfonate (TNS), to TGFα-PE40, was studied by measurements of fluorescence spectra and anisotropy. At concentrations of 2μM TGFα-PE40 and 22μM TNS, the interaction was markedly enhanced with decreasing pH, and no significant binding of TNS to the protein was detected above pH 5. A guanidine denatured sample of TGFα-PE40 failed to bind TNS even at pH 3.0, suggesting that binding required a specific conformation of the folded protein. The far UV CD spectra suggested no appreciable change in the secondary structure between pH 3.2 and 7.1. The folded structure of TGFα-PE40 appeared to consist of a large fraction of β-sheet, a relatively small fraction (10-16%) of helix and a considerable amount of disordered structure. Our data suggest that, at low pH, one or more hydrophobic faces of the protein are exposed. This observation is similar to earlier findings on diphtheria toxin and and P. exotoxin. These data indicate that the PE40 portion of TGFα-PE40 retains its mechanistically important conformational characteristics. It has been suggested that the low pH form is required for translocation of the toxin molecule across the endosomal membrane into the cytoplasm.

We thank Dr. Allen Oliff of Merck Sharp & Dohme Research Laboratories for encouraging our work on this exciting protein.

Th-Pos151

SECONDARY STRUCTURE DETERMINATION OF OSPA PROTEIN FROM THE LYME DISEASE SPIROCHAETE, BORRELIA BURGDORFERI, USING VACUUM UV CIRCULAR DICHROISM AND THERMAL GEL ANALYSIS. L.L. France, J. Kieleczawa, J.J. Dunn, G. Hind and J.C. Sutherland. Biology Dept., Brookhaven National Laboratory, Upton, NY 11973.

The secondary structure of a major outer surface protein (ospA) of Borrelia burgdorferi, the causative agent of Lyme disease, was determined by measuring the circular dichroism (CD) spectrum to 175 nm[1]. Five classes of secondary structure were revealed: α-helix, 11%; anti-parallel β-sheet, 27%; parallel β-sheet, 9%; β-turns, 21%; and "other" structures (including random coil), 34%. The native conformation of ospA appears to be highly stable (although it contains no disulfide bridges) and is also resistant to trypsinization[2] (although its lysine content is 16%). The secondary structure, as measured by CD, shows little change from pH 3 to pH 11, indicating hydrogen-bonding does not play a primary role in its stability. Chemical denaturation by different concentrations of guanidinium-HCl (zero to 2 M) indicates that unfolding is a cooperative process, with little change in the secondary structure for [Gdn-HCl] < 0.75 M. At [Gdn-HCl] = 0.75 M, the CD spectrum indicates a large fraction of random coil present. At [Gdn-HCl] > 1.0 M, almost all secondary structures, other than random coil, are absent. The protein refolds to its native conformation after thermal denaturation (at 100°C) for periods ≤ 15 minutes. Thermal gel analysis indicates that the pathway of unfolding is temperature-dependent. Furthermore, CD measurements confirm that when the protein is thermally denatured at 80°C, the refolded secondary structure is different from that which results from thermal denaturation at 70°C and at 100°C.

[1]Johnson, W.C. (1990) Proteins: Structure, Function, and Genetics 7, 205-214.
[2]Dunn, J.J., Lade, B.A. and Barbour, A., submitted.

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Th-Pos152

CONFORMATIONAL STUDY OF α-LACTALBUMIN USING VIBRATIONAL CIRCULAR DICHROISM

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The conformation of the globular milk protein, α-lactalbumin, as a function of pH, $CaCl_2$, and added propanol concentration has been studied using vibrational circular dichroism (VCD). These results are compared to parallel studies, redone here, using resolution enhanced fourier transform infrared absorption and electronic circular dichroism. The VCD signals with increasing propanol concentration are consistent with a transformation from an extended structure to a helical form, which, at our current predictive level, appears to be from predominantly a β-sheet to an α-helical conformation. α-Lactalbumin is a strong Ca^{++} binding protein, however, no significant changes in the VCD spectra are seen upon addition of $CaCl_2$ to the Ca^{++}- free protein.

VCD and other spectral data for α-lactalbumin are compared to that of lysozyme due to their high sequence homologies. These proteins in D_2O or pH 7.6 buffer solution are in fact quite different in terms of VCD. The VCD of lysozyme in buffer resembles that of a transformed α-lactalbumin in 30% propanol.

Th-Pos153

THERMODYNAMIC LINKAGE STUDIES OF SALT BINDING AND SOLUBILIZATION OF BOVINE AND CAPRINE CASEINS. Adela Mora-Gutierrez[1], Harold M. Farrell, Jr.[2], and Thomas F. Kumosinski[2] (Intro. by Peter L. Irwin), [1]Cooperative Agricultural Research Center, Prairie View A&M University, Prairie View, TX 77446, and [2]USDA, Eastern Regional Research Center, Philadelphia, PA 19118

The linkage between solubility and salt binding in bovine and caprine whole casein has been studied as a function of divalent cations and ionic strength (KCl). Solubility profiles were determined at two temperatures (1 and 24°C), and the data were analyzed according to a model describing the sequential precipitation (salting-out) and resolubilization (salting-in) of caseins in terms of thermodynamically linked salt binding and solubilization. This approach allows determination of the sequential salt binding constants, K_1 and K_2, for bovine and caprine whole casein and the number of moles of salt bound per mole of protein, n and m. For conditions, K_1 was greater than K_2. However, K_1, was significantly larger for caprine whole casein than for bovine whole casein. The differences in K_1 values can be accounted for mainly by differences in caprine casein content of the $alpha_{s1}$-component. These results suggest that the affinities of phosphorylated sites for cation binding makes the caprine whole casein more sensitive to cation precipitation than its bovine counterpart. The observed solubility profiles of caprine $alpha_{s1}$-casein are consistent with the increased sensitivity to salt of caprine whole casein.

Th-Pos154

CALCIUM-INDUCED COLLOIDAL STABILITY OF CAPRINE WHOLE CASEIN AND THE RELATIONSHIP OF $ALPHA_{S1}$-CASEIN CONTENT TO PRECIPITATION AND RESOLUBILIZATION: A THERMODYNAMIC LINKAGE ANALYSIS. Adela Mora-Gutierrez[1], Harold M. Farrell, Jr.[2], and Thomas F. Kumosinski[2] (Intro. by Shu-I Tu), [1]Cooperative Agricultural Research Center, Prairie View A&M University, Prairie View, TX 77446, and [2]USDA, Eastern Regional Research Center, Philadelphia, PA 19118

Calcium-induced colloidal stability profiles for two caprine whole caseins, low and high in $alpha_{s1}$-casein, were analyzed by employing non-linear regression analysis and a model based on Wyman's theory of thermodynamic linkage. The colloidal instability and subsequent stability profile of these milk proteins by calcium at various concentrations could be described quantitatively by the corresponding binding constants and solubility parameters: K_1, S_1, and n, respectively, for colloidal instability; K_2, S_2, and m, for subsequent stability. Comparison of the K_1 values showed significant differences between both caprine whole caseins. This difference is suggested to be caused by the binding of Ca^{2+} ions to phosphorylated serines located on the $alpha_{s1}$-casein molecule. The less colloid stability of the caprine whole casein with high $alpha_{s1}$-casein content (as quantified by K_1) strongly indicated that significant interaction occurs between phosphorylated sites and the metal ion.

Th-Pos155

TRANSDUCTION OF LIGAND BINDING ENERGY INTO THERMAL STABILIZATION OF PROTEINS. Narinder Singh and Harvey F. Fisher, U. of Kansas Medical Center and VA Medical Center, 4801 Linwood, Kansas City, MO 64128.

We have previously shown (Adv. Enzymol. 61, 1 (1988)) that the formation of glutamate dehydrogenase (GDH) complexes involves a two-state predenaturational $E \rightleftharpoons E'$ transition in the protein component with a $\Delta H° = -20$ kcal/mol. Each complex is characterized by a temperature, T_o, at which $K = \frac{[E']}{[E]} = 1$. The interaction energies between the various ligands have been interpreted in terms of the modulation of $E \rightleftharpoons E'$ step by the ligands. Using this two-state model, we are now able to link this transition to the thermal denaturation step for GDH and its complexes. The ligand-induced changes in the $E \rightleftharpoons E'$ interconversion of the protein component affect the thermal stability of the enzyme. Data obtained from binding experiments and differential scanning calorimetry (DSC) show that the ligand binding energy is directly transduced into the protein structure, thus, affecting the melting temperature (T_m) and the enthalpy of denaturation ($\Delta H°_d$) of GDH-complexes.

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Th-Pos156

THERMODYNAMIC STUDIES OF THE DENATURATION OF BPTI AND ITS DISULFIDE MUTANTS

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We have characterized the thermally induced denaturation of wild type bovine pancreatic trypsin inhibitor (BPTI) and a series of BPTI mutants using calorimetric and spectroscopic techniques. Specifically, we have studied mutants in which each of the three cystine crosslinks has been replaced by amino acid substitutions at positions 5-55, 14-38, or 30-51. Our calorimetric measurements on this series of proteins allow us to characterize the thermodynamic contributions of each disulfide bridge to the stability and the melting behavior of BPTI. Circular dichroism (CD) also was used to characterize the conformational state(s) as well as the melting properties of each BPTI mutant. To evaluate the influence of pH, as well as to expand the temperature range available for study, the spectroscopic and calorimetric measurements were performed at both pH7 and pH3. The resulting data will be presented and interpreted in terms of structural models for BPTI.

Th-Pos157

POSITION OF DISULFIDE BONDS AND THE EFFECT OF REDUCTION ON CELLULASE E2 FROM *THERMOMONOSPORA FUSCA*.

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The breakdown of cellulose into useable products can be achieved through the use of cellulases. The thermophilic bacterium, *Thermomonospora fusca*, produces and excretes several cellulases into the culture medium. Five of these proteins have been purified and characterized, and the structural genes for four have been cloned and used to overproduce the enymes in *Streptomyces lividans*. The DNA sequence of the gene for *T. fusca* endoglucanase E2 shows that this enzyme contains four cysteines scattered throughout the protein. Using differential DTT reduction and iodoacetamide treatment, these four cysteine residues were found to participate in two disulfide bonds. Enzymatic activity was determined for the reduced and carboxymethylated enzyme. Neither full reduction of the disulfides nor complete carboxymethylation of the cysteines destroys enzymatic activity, but the activity of these modified enzymes is substantially reduced compared to the native enzyme. It may be that these disulfides maintain a stable active site structure. To determine the location of these disulfide bonds, the protein was cleaved with cyanogen bromide under conditions where the disulfide bridges remained intact. The peptides obtained were separated using an HPLC system equipped with a "disulfide analyzer" which employs the reagent disodium 2-nitro-5-thiosulfobenzoate. Fractions containing disulfides were analyzed by SDS-PAGE under reduced and non-reduced conditions. These same fractions were reduced and separated using HPLC and amino acid analysis of the peptides was used to determine which cysteine containing peptides were bound together. One disulfide linkage joins Cys (81) and Cys (126), while the other joins Cys (233) and Cys (268). This research was supported by Grant #PG02-84ER13233 from the Department of Energy.

Th-Pos158

PHYSICAL EVIDENCE FOR A NONESSENTIAL DISULFIDE BOND IN THE LAMB PROTEIN OF *ESCHERICHIA COLI* OUTER MEMBRANE

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LamB protein, the outer membrane component of the maltose transport system in Escherichia coli and the receptor for bacteriophage λ, is a trimer made up of three identical subunits. Each monomer contains two cysteines. In previous studies we obtained indirect evidence for the presence of a disulfide bond in LamB protein and we proposed that it plays a role in the unusual thermal stability of the trimer.

Site directed mutagenesis was used to convert Asp^{36} to Met, which allowed CNBr cleavage between the two cysteines. CNBr fragments were analyzed with two-dimensional polyacrylamide gel electrophoresis, under non-reducing conditions along the first dimension and reducing conditions along the second. The two N-terminal fragments of this D36M mutant protein migrated as a single 13K peptide in the first dimension, but separated into 9K and 4K fragments along the second. These results provide direct physical evidence for an intrasubunit disulfide bond between Cys^{22} and Cys^{38}.

The role of the disulfide bond was studied with mutagenized LamB proteins in which one or both Cys residues were converted to Ser. The disulfide is not required for transport function, since these purified mutant LamB proteins exhibit normal uptake of sugars in proteoliposomes. However, they exhibit a sharp loss of thermal stability. Finally, the disulfide bond must not be between subunits of the LamB trimer since reversible dissociation of trimer is achieved by low pH or denaturants in the absence of reducing agent.

This work was supported by NIH grants AI20727 and AI00800 and NSF grant DMB-8413868.

Th-Pos159

THE ROLE OF GLYCINE RESIDUES IN TRANSMEMBRANE PROTEIN STRUCTURES.

John F. Hunt, Yale University.

The distribution of glycine residues has been examined in a data base consisting of sequences of predicted membrane-spanning α-helices. It is shown that in sequences where glycine occurs more than once, the glycine residues tend to occur on the same face of the helix. This result suggests that there may be an important role for glycine in stabilizing protein structure within the phospholipid bilayer. A model for structural interactions involving glycine is inferred from the known three dimensional structure of the photosynthetic reaction center in conjunction with simple molecular modelling. Specifically, backbone atoms at these residues may participate in hydrogen bonds that would would be sterically restricted in the presence of any bulkier side-chain.

Th-Pos160

DISSOCIATION AND STRUCTURAL STABILITY OF THE MAJOR IMPORTED SUBUNITS IN YEAST CYTOCHROME C OXIDASE

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The structural stability of the major imported subunits of yeast cytochrome c oxidase has been investigated by high sensitivity differential scanning calorimetry. For these experiments, subunits IV, V, and VI were isolated from the intact enzyme by a mild treatment with GuHCl. Passage of a linear GuHCl gradient through an octyl sepharose column to which the intact yeast enzyme has been bound, results in the selective dissociation of subunits IV, V, and VI at concentrations ranging between 1.2 and 2M GuHCl. The hydrophobic core of the enzyme (subunits I, II, and III) can subsequently be eluted with a buffer solution containing 3% Triton X-100. In isolation, subunits IV and VI, and to a lesser extent subunit V , are readily soluble in aqueous solutions, exhibit folding/unfolding transitions centered between 34 and 45 °C, and characterized by van't Hoff enthalpies on the order of 50 kcal/mole. The transition temperatures of the isolated subunits are approximately 10-15 °C lower than those found with the intact enzyme indicating that the imported subunits are structurally stabilized by their association with the bulk of the enzyme. It is estimated that intersubunit interactions following association to the bulk of the enzyme contribute 3-4 kcal/mole to their Gibbs free energy of stabilization. The membrane reconstituted hydrophobic core of the enzyme (depleted of major imported subunits) lacks electron transfer activity and undergoes a very broad, poorly cooperative thermal denaturation transition with a very shallow maximum centered at 37 °C (about 23 °C below that of the intact enzyme). These results strongly suggest the existence of mutually stabilizing cooperative interactions between the major imported subunits and the hydrophobic core of the enzyme, and that these interactions are necessary for proper assembly of the enzyme into a functional complex.

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Th-Pos161

EFFECT OF ALANINE SUBSTITUTION ON THE STABILITY OF MYOGLOBIN

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Alanine has been found to have high helix forming propensity in isolated peptides and occurs with high frequency in α-helices of known proteins. We studied the helix promoting effect of Ala on the stability of a globular protein by replacing low helix propensity residues with Ala in helical positions of synthetic sperm whale myoglobin. Three of these mutant proteins are:

1. Val 66 (E-helix)
2. Val 66, Glu 59 (E-helix)
3. Ile 142. (H-helix)

Val66 and Glu59 are both external residues. Ile142 is an internal residue, involved in intrahelix packing. Temperature-dependent CD and absorption changes were used to monitor thermal denaturation of the four proteins in basic pH. From our measured Tm and ΔH we estimated the ΔΔG for thermal denaturation of the proteins, using the wild type protein as a reference.

	Tm	ΔH(kcal/mole)	ΔΔG
wild type	82.8	169	-
Ala66	84.3	191	-3.1
Ala66,59	80.7	166	2.4
Ala142	79.5	126	9.6

The results suggest that replacements of helix destabilizing amino acids in helical positions can result in higher protein stability provided internal packing and external charge distributions are not perturbed.

This work was supported by a NIH grant GM40746.

Th-Pos162

EFFECTS OF COVALENT CARBOHYDRATE ON PROTEIN STRUCTURE EQUILIBRIA IN OROSOMUCOID.

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The high amount of carbohydrate (48%) compared to peptide in orosomucoid (OMD) allows significant contributions to the physical properties by the glycans. The carbohydrate is all external to the peptide core and accounts for a fairly high concentration, up to 2M, of sugar residues at the protein surface. Epitope mapping of OMD has shown significant masking of the surface, as expected, by the steric bulk of the glycans (Villalobos, A., et al. (1989) Biophys. J. 55:No. 2, Pt. 2, p. 533a). Reports of protein stabilization by polyols (Lee, L. L-Y., and Lee, J. C. (1987) Biochemistry 26:7813) and glycerol (Gekko, K. and Timashoff, S. (1981) Biochemistry 20:4667) indicate that moderate (10-30%) concentrations of carbohydrate can significantly affect the conformational equilibrium of a protein. The contribution of carbohydrate concentration by the N-linked glycans is within the range where these effects are seen.

Two subpopulations of orosomucoid differing in glycan size and total carbohydrate amount were isolated by concanavalin-A chromatography. Stability properties of the con-A fractions were studied and the differences between protein subpopulations of different carbohydrate amount compared using polyethylene glycol and desialylation as probes to define the varying thermal properties. UV thermal melts gave a higher Tm for the subpopulation containing less carbohydrate, and increasing Tm for either con-A fraction with removel of sialic acids. Addition of polyethylene glycol-400 (31% w/w) stabilized both subpopulations similarly and maintained their relative stabilities. The stability data indicate that through steric and electrostatic repulsions the glycans prohibit the protein core from reaching lower energy equilibria, and do not contribute stabilization through a polyol effect.

Th-Pos163

ANTIBODY - PROTEIN BINDING STUDIED BY H-EXCHANGE AND 2D NMR

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The exchange of the peptide hydrogen in a protein with hydrogens in water is controlled by local unfolding motions that transiently expose a given amide hydrogen to the exchange catalyst, OH^-. One expects these motions to be perturbed by protein - protein binding. In order to further our understanding of antibody - antigen interactions, as well as to further our understanding of protein motions, we have used 2D NMR to measure the exchange rates of cytochrome c amide hydrogens in the protein complexed with the monoclonal antibody E3. Exchange at approximately 10 of 46 measured hydrogen positions is slowed by factors of 13 to 40 (residues # 29,32,38,42,52,59,60,64,79,80) in the complex as compared with the rate measured in the free protein. Given the known dissociation constant for the complex of $\sim 10^{-7}$, it is apparent that equilibrium dissociation is necessary in order for these residues to exchange. Four other residues (19, 33, 74, 75) are slowed by factors of 3 to 7.

Two effects are apparent in these results. Many of the slowed hydrogens appear to be slowed by direct protection by the antibody. These can be used, along with knowledge from chemical protection studies, to delineate the epitope. In addition, some residues appear to be slowed indirectly as a result of one or more mode of opening being closed by antibody binding. This information helps to delineate the cooperative opening units of the protein.

Th-Pos164

BPTI MUTANTS F22A, Y23A AND F45A: STUDIES ON THEIR STRUCTURES, STABILITIES AND INTERNAL MOBILITIES

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Single site mutants of bovine pancreatic trypsin inhibitor (BPTI) in which phe22, tyr23 and phe45 are changed to alanine were made by site-directed mutagenesis. In wild type BPTI, about two thirds of the 58 amino acids are in the secondary structure associated with the hydrophobic core. The remaining third are independent of the rest of the molecule. The side chains altered in our mutants reported here are the most highly conserved in the ß-sheet of the hydrophobic core. Proton hydrogen exchange rates in wild type BPTI indicate that residues 22, 23, and 45 are located in the most rigid region of the protein. In all three mutants, the average secondary structure is very similar to wild type, as shown by circular dichroism (CD) at 28°, pH 7. Trypsin binding activity is retained in all three variants. Wild type BPTI is very resistant to proteolysis; the half time for its degradation by thermolysin at 39° is >72hr. Under the same conditions, the half times for F22A, Y23A, and F45A are 48 hr, 30 min, and 30 min respectively. In reverse phase HPLC, F22A elutes about the same as wild type, but Y23A and F45A elute much later, indicating that they interact more strongly with the hydrophobic resin. Preliminary proton NMR experiments with F22A and F45A show that the chemical shifts of most backbone NH resonances are changed as compared to the wild type.

Th-Pos165

THE COUPLING OF CATALYTICALLY RELEVANT STRUCTURAL FLUCTUATIONS IN SUBTILISIN BPN' TO SOLUTION VISCOSITY AS REVEALED BY TRITIUM EXCHANGE AND INHIBITOR BINDING

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It is of considerable interest to ascertain whether structural fluctuations in proteins play a role in chemical reactions taking place in the protein matrix. The working hypothesis, based on this premise, is that the observed rate constants will be subject to the influence of micro-viscosity derived from molecular collisions with the solvent. In order to test this hypothesis, we have measured the viscosity dependence of tritium exchange of a specific group of hydrogens in subtilisin BPN'. A consistent and rather small group of hydrogens was isolated using the inhibitor, phenylboric acid. The viscosity dependence of the outexchange was then examined at pH 6.5 in the presence of glycerol and ethylene glycol. The exchange rates were corrected to be compared at identical hydroxyl ion and water activity. The salient observation was the strikingly similar viscosity coupling behavior when compared to the deacylation step of thioester hydrolysis catalyzed by the same enzyme. Despite the chemical dissimilarity of the two reactions, the very similar viscosity coupling behavior, when combined with the well established role for structural fluctuations in hydrogen exchange, implies a similar role of structural fluctuations in subtilisin BPN' catalysis.
(Supported by NSF-8704740)

Th-Pos166

ELECTROSTATIC EFFECTS IN *E. COLI* ASPARTATE TRANSCARBAMYLASE: CALCULATION OF ELECTROSTATIC POTENTIALS.

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A large body of experimental evidence indicates that electrostatic effects are central to function and energetics in aspartate transcarbamylase. Catalytic parameters [Pastra-Landis *et al.*, J. Biol.Chem. 253:426 (1978)], Hill coefficients for L-Asp and succinate [*ibid*; Knier *et al.*, Biochemistry 17:784 (1978)], and regulation by CTP and ATP [Thiry *et al.*, J. Mol. Biol. 125:515 (1978)] are highly pH dependent. Thermodynamic studies have defined linkages between proton binding and binding of substrates, competitive inhibitors, nucleotides and assembly [Allewell *et al.*, Biochemistry 18:3008 (1979); McCarthy *et al.*, Proc. Natl. Acad. Sci. USA 80:6824 (1983)]. Numerous ionizable groups involved in ligand binding and protein-protein interactions have been identified in the crystal structure [Kim *et al.*, J. Mol. Biol. 196:853 (1987)].

Static solvent accessibility modified Tanford Kirkwood (TK) theory has been used to model electrostatic effects in assembly and ligand binding and has shown that the pK values of many ionizable groups change by as much as 3 pH units [Gilackin *et al.*, Proteins 5:66 (1989)]. Since the pK changes extend to regions remote from the sites of interaction, it was proposed that electrostatic interactions may transmit information between binding sites.

We have begun to compare results obtained by TK theory and other methods. Of several numerical solutions to the Poisson-Boltzmann equation [Orttung *et al.*, Ann. N. Y. Acad. Sci. 303:22 (1977); Warwicker & Watson, J. Mol Biol. 157:671 (1982); Klapper *et al.*, Proteins 1:47 (1986)], the finite difference Poisson-Boltzmann (FDPB) method has been most widely used to calculate electrostatic effects in molecules of arbitrary size and shape. Preliminary calculations emphasize the importance of critically considering interactions between ionizable groups in assigning charges. Supported by NIH grant DK 17335.

Th-Pos167

THEORETICAL STUDIES OF COLLAGEN

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Collagen is the most abundant protein in the human body, and is obviously of great chemical interest and importance. Clinical studies include the phenotyping of both lethal and non-lethal perinatal forms of the collagenous disease *osteogenesis imperfecta*. We are trying to propose a theoretical model of type I collagen for investigating the role of structure in the differences of lethal and non-lethal forms of *osteogenesis imperfecta*. These models are defined by the amino acid sequence obtained from the phenotyping of affected and non-affected persons with *osteogenesis imperfecta*.

The structure of collagen is a triple helix with strands typically composed of the sequence Yyy-Pro-Gly where Yyy is any amino acid. This triple helical conformation observed in the collagen group of proteins is related to the presence of large numbers of imino residues (proline and hydroxyproline) and is derived from the stereochemical properties of these residues. The triple helix is stabilized by increasing numbers of these residues. Hydrogen bonds are usually considered to be a major factor in the formation and stability of protein conformation, however, imino residues are not hydrogen bond donors. We have examined the role of water in the stabilization of the triple helical polypeptide $(Pro\text{-}Pro\text{-}Gly)_X$ using molecular mechanics and molecular dynamics. Preliminary results indicate that the waters stay tightly associated with the triple helix and provide stabilization energy in the form of hydrogen bonds. Having developed a reasonable model of $(Pro\text{-}Pro\text{-}Gly)_X$, we have substituted known sequences of amino acids to model type I collagen with subsequent site mutations to model the differing forms of *osteogenesis imperfecta*.

Th-Pos168

A STRUCTURAL MODEL OF THE GLYCOPROTEIN OROSOMUCOID CONSTRUCTED BY HOMOLOGY WITH THE LIPOCALIN FOLD.

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Attempts to crystallize orosomucoid (McPherson, A., et al. (1984) BBRC 124:619) and so determine its structure have not yet yielded a definitive structure. Recognition of its sequence homology with the lipocalins (alpha-2u-globulin superfamily) allows a preliminary structural model to be built by a computational approach. The human retinol binding protein (RBP) structure (Newcomer et al. (1984) Embo. J. 3:1451-1454) was used as the initial homologous framework. A starting sequence alignment was performed in a refinement cycle initially using the lipocalin sequence alignment of Cowan et al. (Proteins: Structure, Function, and Genetics (1990) 8: 44-61) and improving the alignments with linear least squares structural overlays on a molecular graphics terminal. The alignment priority is given to the regions of highest homology. The segments 14-29 and 105-118 of RBP correspond most closely with 15-30 and 102-114 of orosomucoid, respectively. Additionally 2 homologous disulfide bridges occur between the two structures. The cystine 4:160 in RBP is similar to 5:147 in OMD and the cystine 70:174 corresponds to 72:165 in orosomucoid. Once a "best alignment" was reached the backbone structure was energy minimized by steepest descents. Side chains were then built sequentially by atom, proximal to distal, with minimization between steps. The resulting structure was subjected to molecular dynamics minima testing with heating to 500°C, cooling, and reminimization. Complex N-linked oligosaccharide structures were built up from existing structural fragments and placed at their respective asparaginyl attachment points. The resulting structure will serve as a preliminary model of this glycoprotein.

Th-Pos169

TRANSITION STATE THEORY OF TRYPTOPHAN-47 ROTATIONAL ISOMERIZATION IN VARIANT-3 SCORPION NEUROTOXIN.

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Transition state theory is cast in a form suitable for modeling isomerization reactions by adiabatic mapping in a multidimensional space of participant degrees of freedom. This theory is specialized for rotational isomerization of the indole side chain of tryptophan-47 of variant-3 scorpion neurotoxin. An adiabatic mapping over $\chi^1 \times \chi^2$ torsion space defines transformation equations from this two dimensional participant space into the Cartesian atom coordinates and defines the internal energy as a function of the participant space. If the number of free protein atoms in the adiabatic mapping is appropriately restricted, both the transformation equations and energy function are sufficiently smooth to define a participant space Hamiltonian. The internal energy function has minima at $\chi^2 \approx \pm 90$ degrees that are separated by a barrier of about 10 kcal/mole. These χ^2 values correspond to the crystallographic and new rotational isomers identified in our previous combination thermodynamic perturbation and umbrella sampling study of the Helmholtz free energy as a function of the $\chi^1 + \chi^2$ reaction coordinate. Both of these minima are further split in the χ^1 direction. The transition state theory isomerization rates between all minima are calculated from the participant space Hamiltonian. The eigenvectors of the Hamiltonian at the transition states define isomerization reaction coordinates. Further analysis of individual nonbonded energy terms of the adiabatic mapping suggests which additional degrees of freedom must be included in the participant space to improve these eigenvector reaction coordinates. The effects of amino acid mutations neighboring tryptophan-47 are determined by comparing the adiabatic mappings and transition state theory rates before and after mutating glutamates 2 and 49 and tyrosines 4, 40 and 42 to alanine.

Th-Pos170

ANALYSIS OF THE SPECIFICITY OF THE INTERACTION BETWEEN THE TRANSMEMBRANE DOMAINS OF GLYCOPHORIN A.

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There is an increasing body of evidence suggesting that specific interactions between transmembrane α helices of integral proteins are important both structurally and functionally. In the cases of bacteriorhodopsin and the photosynthetic reaction centre they are clearly involved in defining the tertiary structure of the proteins. Similar interactions also appear to be involved in the oligomerisation of integral membrane proteins with single membrane spanning domains. This has been shown to be true for human glycophorin A and for interaction between TCRα and CD3δ in the T-cell receptor complex. It may also be important in the mechanism of signal transduction across membranes by growth factor receptors such as EGF-R and p185^{neu}.

We have constructed a chimeric protein for the study of such helix-helix interactions. Fusion of the transmembrane domain of glycophorin A (GpA) to the C-terminus of staphylococcal nuclease causes this protein to associate reversibly and specifically in SDS to form a dimer, in a manner identical to that previously described for GpA. A synthetic peptide with sequence identical to that of the transmembrane domain of GpA disrupts dimers of the chimera, with the resultant formation of a peptide - protein heterodimer. Peptides corresponding to the transmembrane domains of other similar proteins (*e.g.* glycophorin C, EGF-R) have no such effect despite similar amino acid composition and charge arrangement.

We have embarked upon a mutational analysis of the detailed specificity of this interaction. Using oligonucleotide directed mutagenesis to randomise the codon at selected positions in the transmembrane domain of the chimeric protein, we have identified a large number of mutants which prevent its association. The sensitivity of different positions to conservative substitutions varies greatly. Our initial strategy has been to mutate a stretch of residues corresponding to two turns of the α helix, in order to determine whether there is a helical periodicity in this variation. Results of the mutagenesis studies will be discussed.

Th-Pos171

STRUCTURAL AND FUNCTIONAL DOMAINS OF THE DnaA PROTEIN OF *E. coli*. W-S Zhu and HI Eberle. Dept. of Biophysics Univ. Rochester School of Med. Rochester, NY 14642

The DnaA protein is essential for the initiation of DNA synthesis at the *Escherichia coli* chromosomal origin of replication (*oriC*). It is know to bind as a multimer to a specific sequence, called the "DnaA box", that occurs in 3-4 copies at *oriC*, in the origins of other replicons and and in the 5' and coding regions of some *E. coli* genes. It may function in initiation of replication by activation of transcription through the *oriC* region, or by creating or trapping some special conformation of DNA at *oriC*. In addition to DNA-binding activity, DnaA is known to bind ATP and to slowly hydrolyze it to ADP. It has been reported to bind to membrane acidic phospholipids, which are thought to be important for release of ADP from the DnaA-ADP complex and for restoring DnaA to an active state where it can bind another ATP molecule. There is also genetic evidence that indicates that DnaA associates with other proteins involved in replication. Thus, from the work of other laboratories, DnaA is thought to have at least five functional domains: (1) DNA-binding, (2) ATP-binding, (3) membrane phospholipid-binding, (4) DnaA-DnaA subunit interacting and (5) DnaA-dissimilar subunit interacting. In order to locate the structural domains for these functions within the DnaA protein, we have amplified subregions of the *dnaA* gene by PCR using primers which contained transcriptional and translational sequences at the 5' end in addition to the homologous *dnaA* sequences at the 3' ends. The fragments produced by PCR were then used to produce the corresponding peptides by *in vitro* transcription and translation, and the peptides tested for certain of the proposed functions. The DNA-binding domain and the ATP-binding domain were found to reside in unique peptides, whereas several of the peptides appeared to show activity for some of the other functions. The locations of these structural and functional domains will be discussed.

Th-Pos172

RANDOM MUTAGENESIS OF THE N AND C-TERMINAL HELIX INTERFACE OF CYTOCHROME c

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Cytochromes c possesses N and C terminal helices that combine to form a helix pair which is one of the common structural motifs found in proteins. In addition, the formation and association of these helices plays a pivotal role in the folding of cytochromes c. To gain an understanding of this type of tertiary structure, all possible variants at two invariant positions in the helix interface have been made using random mutagenesis. A library of amino acid substitutions at the invariant amino acids gly-6 and phe-10 in the N-terminal helix has been constructed in a yeast shuttle phagemid which is used for expression of cytochrome c in yeast. When yeast which lack all genomic cytochrome c are transformed with the phagemid, selection for functional and nonfunctional variants of cytochrome c can be achieved by growing the yeast on nonfermentable carbon sources. Presently, sixteen functional variants of iso-1-cytochrome c have been observed in the library. Several of the variant proteins confer a temperature sensitive phenotype to the yeast harboring them. Preliminary characterization exemplifies the type of degeneracy inherent in the folding of proteins. This work was supported by the NIH (GM42501) and PRF (21597-G).

Th-Pos173

EXAMINATION OF THE PHYSIOLOGICAL SIGNIFICANCE OF MUTATIONS TO THE *CYC1* GENE OF THE YEAST *SACCHAROMYCES CEREVISIAE*

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Using molecular genetic techniques it is possible to study modified proteins both *in vitro* and *in vivo*. However, the physiological significance of modified proteins are to a large degree ignored. Cytochromes *c* contain a group of evolutionarily invariant residues of which Phe 82 is a member. Position 82 variants in iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae* are well studied *in vitro*, but the physiology of yeast containing the variant proteins is not known. All of the amino acid substitutions at position 82 of iso-1-cytochrome *c* have been made. Single copies of the mutant genes have been introduced into a yeast strain, both on a single-copy plasmid and by direct integration into the genome. The recipient strain is deleted in the gene for iso-1-cytochrome *c*, *CYC1*. Many of the mutant alleles have been recovered and the sequence confirmed. Iso-1-cytochrome *c* will tolerate many different amino acid substitutions at position 82 *in vivo*. Supported by grants from the NIH (GM42501) and the PRF (21597-G).

Th-Pos174

THE RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION OF CYTOCHROME c THROUGH THE STUDY OF TEMPERATURE SENSITIVE VARIANTS

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Random mutagenesis was employed to generate temperature sensitive (ts) variants of iso-1-cytochrome c from the yeast Saccharomyces cerevisiae. The specific aim of this work is to produce variant proteins whose unique dynamical changes as a function of temperature can be studied using NMR spectroscopy. A 36-mer oligonucleotide complementary to codons 43 to 54 was designed and synthesized such that a small percentage of the three non-complementary bases were introduced at each position. This part of the sequence was chosen because the hydrogen bonding in this region has been implicated in both the stability and the electron transfer mechanism of cytochromes c. The frequency of mutations in the library produced by this family of mutagenic oligonucleotides was shown to follow the expected Poisson distribution. The plasmid-borne mutated genes were introduced into a yeast strain which lacks cytochrome c. Growth on the non-fermentable carbon source glycerol at different temperatures revealed 22 ts mutants. They are able to grow at ambient temperature and 30 °C, but fail to grow at 37 °C. The plasmids containing the mutated alleles were rescued from the yeast and the amino acid substitutions that result in the ts and cyc1 phenotype were deduced by DNA sequence analysis. The results indicate that the ts phenotype may be brought by several factors including hydrogen bonding and electrostatics as well as certain changes of glycine in turns. Clearly, NMR studies will be extremely useful for understanding the molecular basis of ts variants. This work was supported by the NIH (GM42501) and the PRF (21597-G).

Th-Pos175

EFFECT OF POST-TRANSLATIONAL MODIFICATIONS TO MYELIN BASIC PROTEIN ON ITS INTERACTIONS WITH LIPID. J.M. Boggs and M.A. Moscarello, Research Inst., Hospital for Sick Children, Toronto, Ont. Canada M5G 1X8

Myelin basic protein (MBP) exhibits charge microheterogeneity due to phosphorylation, deamidation, and deimidation of arginine. These charge isomers are termed C1, C2, C3, C4,...C8 in order of decreasing positive charge and increasing modification. C2 and C3 differ from C1 primarily by deamidation and phosphorylation. In C8, the least positively charged isomer, 6 of the 19 Arg have been converted to citrulline. This isomer is the predominant one in myelin from infants and its proportion decreases with age during development. Electrostatic interactions of these isomers with acidic lipids, as determined by aggregation of vesicles, decreases with decreasing positive charge, i.e. in the order C1>C2>C3>C8 (1). In vitro phosphorylation of C1 or C2 also decreases electrostatic interaction of the protein with acidic lipids. MBP also shows evidence of hydrophobic interactions with lipids. It has a perturbing effect on the fatty acid chains and can be labeled by the hydrophobic photolabel TID (2). Phosphorylation reduces the ability of C1 to induce leakage of lipid vesicles suggesting less penetration of hydrophobic residues into the bilayer (3). We have investigated the effect of these post-translational modifications on the hydrophobic interactions of this protein with lipid by comparing the effect of the charge isomers on the motion of a fatty acid spin label and their labeling by TID from the lipid bilayer. The motional restriction of a fatty acid spin label labeled at the 12th carbon was greatest for C1 and decreased in the order C1>C3>C8 while hydrophobic photolabeling by TID decreased similarly C1≥C3>C8. The difference between C1 and C3 is consistent with the decrease in hydrophobic interactions caused by phosphorylation while the difference between C1 and C8 suggests that tight electrostatic interactions are necessary for the hydrophobic interactions with lipid to occur.

1. Cheifetz & Moscarello, Biochemistry 24 (1985) 1909.
2. Boggs, Rangaraj & Koshy, BBA 937 (1988) 1.
3. Cheifetz, Boggs & Moscarello, Biochemistry 24 (1985) 5170.

Th-Pos176

MI AND MII STATES OF RHODOPSIN IN RECOMBINANT MEMBRANES DEMONSTRATE SIMPLE pH AND TEMPERATURE DEPENDENT EQUILIBRIA. Nicholas J. Gibson and Michael F. Brown. *Department of Chemistry, University of Arizona, Tucson, AZ 85721.*

A growing body of research confirms an important influence of **lipid-protein interactions** with regard to membrane protein function. The lipid acyl chain length and degree of polyunsaturation, as well as lipid headgroup size and charge, all appear to affect the ability of the membrane protein rhodopsin to undergo the MI-MII transition.[1] In order to further investigate these interactions, rhodopsin was incorporated into vesicles of varying lipid composition. The extent of the MI-MII transition, for both recombinants and native rod outer segment membranes, was measured as a function of pH and temperature, using **flash photolysis** techniques. The MI and MII states for each system were found to coexist in a simple acid-base equilibrium; the pK_a's depended on the lipid composition.[2] The equilibria could be driven in either direction by varying pH or temperature, in accord with Le Chatelier's Principle. These results make possible a detailed interpretation of the **MI-MII equilibrium** in terms of thermodynamic parameters. Furthermore, they demonstrate that the likelihood of significant subpopulations of nonfunctional rhodopsin in recombinant membranes is minimal. [1]T.S. Wiedmann *et al.* (1988) *Biochemistry* 27:6469-6474. [2]N.J. Gibson and M.F. Brown (1990) *Biochem. Biophys. Res. Commun.* **169**:1028-1034. Supported by NIH Grant EY03754.

Th-Pos177

EFFECTS OF MODIFICATIONS IN PROTEIN STRUCTURE ON THE SPONTANEOUS INCORPORATION OF BACTERIORHODOPSIN INTO PHOSPHOLIPID BILAYERS. Martin Teintze and Zhi-jun Xu, Department of Medicine and Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021

Bacteriorhodopsin and many other integral membrane proteins rapidly and spontaneously insert into preformed unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) and other PCs without the use of detergents or sonicaton. Bacteriorhodopsin is incorporated in only one orientation, so that it pumps protons into the vesicles in response to light. We have investigated the insertion of bacterio-opsin and fragments or mutants thereof into ULVs of DMPC in order to determine which structural features of the protein mediate incorporation into the bilayer and determine the orientation of insertion. Bacterio-opsins lacking one or more of the transmembrane domains were prepared by proteolysis or by transforming Halobacterium halobium mutants lacking a functional bacterio-opsin (bop) gene with plasmids containing a bop gene with the appropriate sequences deleted. Other changes in the bacteriorhodopsin structure were made by inserting or replacing sequences in the cloned gene and expressing the mutant bop genes in H. halobium. The effects of the changes in structure on the protein's ability to incorporate into phospholipid bilayers and its orientation in the membrane were studied.

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Th-Pos178

VARIATION OF PHOSPHOLIPID HEADGROUP COMPOSITION MODULATES THE FORMATION OF METARHODOPSIN II

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Following photoexcitation, the integral membrane protein rhodopsin thermally decays to opsin and free retinal, passing through a series of spectrally defined intermediates. The transient equilibria established between the intermediates meta I and meta II is of primary interest, since production of meta II has been correlated with activation of the G-protein. Previous studies have shown that the K_{eq} for the meta I⇔meta II equilibrium is dependant upon the composition of the lipid bilayer surrounding rhodopsin. The native disk lipids are primarily highly unsaturated phospholipids of head group composition 42% phosphatidylcholine, 38% phosphatidylethanolamine and 16% phosphatidylserine. Recent studies have shown that increasing sn-2 unsaturation of phosphatidylcholine vesicles enhances meta II production. However, a high degree of unsaturation does not appear, in itself, to account for the level of meta II production in the native disk membrane. To examine the role of phospholipid head group variation upon the level of meta II production, equilibrium spectral measurements were performed upon a series of rhodopsin/phospholipid reconstituted membranes of the following headgroup composition: pure phosphatidylcholine; phosphatidylcholine/phosphatidylethanolamine (1:1); and phosphatidylcholine/phosphatidylserine (1:1). The acyl chains, sn-1, 16:0 and sn-2, 18:1, were invariant in all of the phospholipids in this study. Values of K_{eq} were calculated at 15°, 25° and 37°C. Inclusion of POPE increases the K_{eq}'s approximately twofold above those measured in the pure POPC system, while inclusion of POPS increases the K_{eq}'s by a factor of three. The headgroup induced differences among the equilibrium constants are diminished as the temperature is increased. These results demonstrate that variation in phospholipid head group composition is capable of modulating meta II production and suggests that the enhanced production of meta II in disk membrane, relative to pure PC bilayers, is at least in part due to the presence of PS and PE in the disk membrane.

(Supported by NIH grant EY00548)

Th-Pos179

MODULATION OF INTEGRAL MEMBRANE PROTEIN CONFORMATION BY LIPID ACYL CHAIN UNSATURATION: THE META I⇔META II EQUILIBRIUM IN RHODOPSIN

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The integral membrane protein rhodopsin, which is a prototypical G-protein activating receptor, undergoes its activating conformational change concurrent with photointermediate conversion from metarhodopsin I (meta I) to metarhodopsin II (meta II). Studies reported here extend our previous results, which demonstrated a direct correlation between the meta I to meta II equilibrium constant, K_{eq} and a parameter derived from DPH fluorescence depolarization measurements, f_v[1]. Our current studies emphasis a comparison of rhodopsin in disk membranes and rhodopsin reconstituted into DMPC and PAPC bilayers. We find that K_{eq} correlates in a linear fashion in all three systems, however, the slope of the correlation line increases in the following order: DMPC<PAPC<disk membrane. The correlation data for DMPC agrees with previous data obtained for rhodopsin reconstituted into bilayers formed from egg PC, with and without cholesterol, and demonstrates that the correlation holds both above and below the T_m for DMPC. The slope of the PAPC correlation line is 2.5x greater than that for systems containing predominantly or exclusively saturated or singularly unsaturated acyl chains in the sn-2 position. These results establish the increased permissiveness of bilayers containing lipids with polyunsaturation in their sn-2 acyl chains relative to those containing more saturated acyl chains in this position, and demonstrate a functional role for the polyunsaturation in the disk membrane. Although the PAPC system approaches the level of meta II production of the disk membrane, the slope of the correlation line for the native membrane is 4x that of the PAPC line. The further enhancement of the native membrane may be due to either the higher levels of unsaturation, head group effects, or the topological asymmetry of the components of the disk membrane. (Supported by NIH grant EY00548.)

[1]Mitchell, D. C., M. Straume, J. L. Miller, and B. J. Litman. 1990. *Biochemistry* 29: 9143-9149.

Th-Pos180

FOURIER TRANSFORM INFRARED SPECTROSCOPIC STUDY OF Ca^{2+}- AND MEMBRANE-INDUCED CONFORMATIONAL CHANGES OF BOVINE PROTHROMBIN AND PROTHROMBIN FRAGMENT 1.

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Fourier transform infrared (FTIR) spectroscopy was used to monitor secondary structural changes associated with binding of bovine prothrombin and prothrombin fragment 1 to acidic lipid membranes. The conformations of prothrombin and prothrombin fragment 1 were examined in the presence of Na_2EDTA, 5mM $CaCl_2$, and $CaCl_2$ plus membranes containing 1-palmitoyl-2-oleoyl-3*sn*-phosphatidylcholine (POPC) in combination with either bovine brain phosphatidylserine (bovPS) or 1,2-dioleoyl-phosphatidylglycerol (DOPG). The widely reported Ca^{2+}-induced conformational change in bovine prothrombin fragment 1 was properly detected by our procedures, although Ca^{2+}-induced changes in whole prothrombin spectra were too small to be reliably interpreted. Binding of prothrombin in the presence of Ca^{2+} to procoagulant POPC/bovPS small unilamellar vesicles produced an increase in ordered secondary structures (2% and 3% increases in α-helix and ß-sheet, respectively) and a decrease of random structure (5%) as revealed by spectral analysis on both the raw and deconvolved data and by difference spectroscopy. Binding to POPC/DOPG membranes, which are less active as procoagulant membranes, produced no detectable changes in secondary structure. In addition, no change in prothrombin fragment 1 secondary structure was detectable upon binding to either POPC/bovPS or POPC/DOPG membranes. This indicates that a membrane-induced conformational change occurs in prothrombin in the non-membrane-binding portion of the molecule, part of which is activated to form thrombin, rather than in the membrane-binding fragment 1 region that is released during thrombin formation. This result is entirely consistent with our recent calorimetric analysis of shifts in prothrombin denaturation profiles associated with binding to acidic lipid membranes (Lentz, Wu, Sorentino, & Carleton; submitted). The possible significance of this conformational change is discussed in terms of differences between the procoagulant activities of different acidic lipid membranes. *Supported by USPHS (SCOR) Grant HL-26309.*

Th-Pos181

MEMBRANE BINDING INDUCES LIPID-SPECIFIC CHANGES IN THE DENATURATION PROFILE OF BOVINE PROTHROMBIN: A SCANNING CALORIMETRIC STUDY.

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Prothrombin denaturation was examined in the presence of Na_2EDTA, 5mM $CaCl_2$, and $CaCl_2$ plus membranes containing 1-palmitoyl-2-oleoyl-3*sn*-phosphatidylcholine (POPC) in combination with either bovine brain phosphatidylserine (PS) or 1,2-dioleoyl-phosphatidylglycerol (DOPG). Heating denaturation of prothrombin produced thermograms showing two peaks, a minor one at ~59 °C previously reported (Ploplis, Strickland, & Castellino *Biochemistry 20,* 1981, 15) to correspond to denaturation of the fragment 1 region, and a main one at ~57-58 °C, reportedly due to denaturation of the rest of the molecule (prethrombin 1). The main peak was insensitive to the presence of 5mM Ca^{2+} while the minor peak was shifted to higher temperature (T_m ~65°C). Sufficient concentrations of POPC/bovPS (75/25) large unilamellar vesicles to guarantee binding of 95% of prothrombin resulted in an enthalpy loss in the main endotherm and a comparable enthalpy gain in the minor endotherm accompanying an upward shift in peak temperature (T_m ~73 °C). Peak deconvolution analysis on the prothrombin denaturation profile and comparison with isolated prothrombin fragment 1 denaturation endotherms suggested that the change occasioned by POPC/PS vesicles reflected a shift of a portion of the enthalpy of the prethrombin 1 domain to higher temperature (T_m ~77 °C). The enthalpy associated with this high-temperature endotherm increased in proportion to the surface concentration of PS. By contrast, POPC/DOPG (50/50) membranes shifted the prethrombin 1 peak by 4 °C to a lower temperature and the fragment 1 peak by 5 °C to a higher temperature. The data lead to a hypothesis that the fragment 1 and prethrombin 1 domains of prothrombin do not denature quite independently and that binding of prothrombin to acidic-lipid membranes disrupts the interaction between these domains. It is further hypothesized that PS- containing membranes exert the additional specific effect of decoupling the denaturation of two subdomains of the prethrombin 1 domain of prothrombin. *Supported by USPHS (SCOR) Grant HL-26309.*

Th-Pos182

BINDING KINETICS OF FLUORESCENTLY LABELED BOVINE PROTHROMBIN FRAGMENT 1 AT PLANAR MODEL MEMBRANES MEASURED BY TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY.

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In the blood coagulation cascade the mechanism by which prothrombin is converted to the active serine protease, thrombin, involves acidic membrane surfaces and calcium ions. The fragment 1 domain, residues 1-156 at the N-terminus of prothrombin, contains the calcium-dependent, acidic lipid binding region of the protein.

Total internal reflection fluorescence microscopy (TIRFM) has been used to investigate the membrane binding properties of fluorescein-labeled bovine prothrombin fragment 1 (F-bF1). Light scattering measurements demonstrated that F-bF1 bound to small, unilamellar phosphatidylserine/phosphatidylcholine vesicles with an apparent dissociation constant (≈ 1 μM) similar to that of unmodified protein (≈ 0.6 μM). Also, the calcium-induced tryptophan fluorescence quenching was equivalent for labeled and unlabeled bF1. Negatively charged supported planar model membranes were formed by fusing phosphatidylserine/phosphatidylcholine (25/75, mol/mol) vesicles at quartz surfaces. TIRFM measurements under equilibrium conditions showed that F-bF1 bound to the planar membranes with an apparent dissociation constant (≈ 2 μM) approximately equal to that for vesicles. Fluorescence photobleaching recovery measurements indicated that the apparent dissociation kinetic rate was ≈ 0.2 sec^{-1}. The dependence of the dissociation rate on the F-bF1 solution concentration, the solution concentration of calcium, and the surface density of phosphatidylserine is under investigation.

This work was supported by NSF-PYI grant DCB-8552986 (NLT) and NIH-HL-20161 (RGH).

Th-Pos183

CHARACTERIZATION OF THE INTERACTION BETWEEN BOVINE SOLUBLE DOPAMINE *BETA*-HYDROXYLASE AND PHOSPHATIDYLSERINE.

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Purified, bovine, soluble dopamine *beta*-hydroxylase (DBH) has been shown to reconstitute onto preformed phosphatidylserine (PS) containing vesicles (Taylor and Fleming (1989) *J. Biol. Chem.* **264**, 15242-15246). This interaction has been further investigated using the technique of 90 degree light scattering and the method of analysis according to Nelsestuen and Lim (1977) *Biochemistry* **16**, 4164-4171. These studies have confirmed the pH dependent and Ca^{2+} independent nature of the interaction. In addition, we have used different mole % PS vesicles to determine the PS dependence of the binding. Preliminary evidence indicates that a minimum surface density of 15 mole % PS is required for binding at pH 5.0. The limiting factor in binding is probably the protein packing on the membrane surface since similar amounts of DBH are bound to 30 mole % PS containing vesicles. The limit of saturation binding suggests a specific orientation of the bound DBH.

Th-Pos184

PULMONARY SURFACTANT PROTEIN SP-C AND PACKING ARRANGEMENTS IN MONOLAYERS OF DIPALMITOYLPHOSPHATIDYLCHOLINE. J. Perez-Gil, K. Nag and K.M.W. Keough, Department of Biochemistry and Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland, Canada, A1B 3X9.

SP-C is an extremely hydrophobic protein of 35 amino acid residues found in pulmonary surfactant. Epifluorescence microscopy was used to study the packing arrangements in monolayers of dipalmitoylphosphatidylcholine (DPPC) with 0, 2, 6, 10 and 50% (w/w) of porcine SP-C. Monolayers contained 1 mol % of the fluorescent probe NBP-PC. SP-C was found to be palmitoylated using a number of chemical tests. During compression of monolayers of DPPC the condensed phases exclude the probe and appear as dark domains against a background of fluorescent expanded phase. The size of the condensed domains varies with the surface pressure and packing density of the lipid. SP-C caused a dramatic reduction in the size of these domains and an increase in their number at all packing densities of DPPC. The maximum areas also decreased as a function of protein concentration. The mean distance between domain centres decreased dramatically up to a protein concentration of 6%. This distance changed only slightly with compression at any given concentration of protein. The distances between domain boarders converged to a limiting value with compression. These results suggest that SP-C provides nucleation sites for domain formation. The total area of condensed phase increased as a function of SP-C concentration from 0 to 6% but decreased at 10% protein. This suggests an additional change in packing arrangements above 6% protein. Protein aggregation may become an important factor effecting the domain distribution at 10% and higher concentrations. (Supported by MRC Canada and NATO.)

Th-Pos185

INTERACTION OF SYNTHETIC PULMONARY SURFACTANT PROTEIN SP-B WITH DIPALMITOYLPHOSPHATIDYLCHOLINES. J. Perez-Gil[1], G. Simatos[1], J. Stewart[1], K. Sarin[2], C.H. Baxter[3], D.R. Absolom[3], M.R. Morrow[4] and K.M.W. Keough[1,5]. Departments of Biochemistry[1], Physics[4] and Pediatrics[5], Memorial University of Newfoundland, St. John's, Newfoundland, Canada, A1B 3X9; Abbott Laboratories[2], Product Development Divison, Chicago, Illinois; and Ross Laboratories[3], Columbus, Ohio.

SP-B is a hydrophobic protein of 78-79 residues found in pulmonary surfactant and thought to be involved in promoting the rapid adsorption of lipid from bilayers into monolayers at the air-water interface. Synthetic human SP-B was incorporated into DPPC liposomes at 10% by weight. Circular dichroism studies of sonicated vesicles above and below the DPPC phase transition suggested that the protein contained about 45% α-helix and that this decreased very slightly, if at all, as the lipid was melted. Preliminary differential scanning calorimetric studies indicated that the 25% of the protein reduced the enthalpy change of transition substantially but the transition temperature was essentially unaffected. ^{2}H-nmr spectra of perdeuterated DPPC-d_{62} containing SP-B indicated that the first moments of the spectra were barely altered by the protein. These results suggest that interaction between SP-B and DPPC must occur in such a way that the average order of the chains is not perturbed. This might happen if the interaction of SP-B with DPPC was confined to the head group region of the molecule. (Supported by MRC and NSERC, Canada, Ross Laboratories, and NATO.)

Th-Pos186

THE ROLE OF PALMITIC ACID IN PULMONARY SURFACTANT: ENHANCEMENT OF SURFACE ACTIVITY AND PREVENTION OF INHIBITION BY BLOOD COMPONENTS. Amanda M. Cockshutt & Fred Possmayer (Intro. by Alan Groom). Dept. of Biochemistry, Univ. of Western Ont., London ON, N6A 5A5.

Pulmonary surfactant, a complex mixture of lipids and proteins produced in the lung, serves to reduce the surface tension at the air-liquid interface of the alveolus. The surface activity of two surfactant preparations used in clinical trials for the treatment of respiratory distress syndrome, Lipid Extract Surfactant (LES) and Survanta, was measured during adsorption and dynamic compression using a pulsating bubble surfactometer. At low surfactant phospholipid concentrations Survanta reduces surface tension at minimum bubble radius faster than LES: however LES obtains a lower surface tension with continued pulsation. Addition of surfactant-associated protein A (SP-A) to LES greatly enhances the surface activity, whereas a similar addition to Survanta has no effect. Blood components inactivate surfactants in vitro and in vivo. Addition of fibrinogen to Survanta or LES causes an increase in surface tension when measured in the absence of calcium. When assayed in the presence of calcium, no inhibition of Survanta is observed. Albumin and alpha-globulin strongly inhibit Survanta and LES at physiological serum concentrations both in the presence and absence of calcium. Lysophosphatidylcholine (lyso-PC) also inhibits the surface activity of Survanta and LES.

LES and Survanta are similar in composition, however the latter is supplemented with palmitate and triglycerides. The role of palmitic acid in the surface activity of Survanta was examined by adding palmitic acid to LES. At low phospholipid concentrations addition of palmitic acid (10% w/w of the surfactant phospholipid) greatly enhances adsorption of surfactant lipids to the interface and surface activity during dynamic compression. Maximal enhancement of surface activity by palmitic acid is observed only at concentrations above 7.5% (w/w of the surfactant lipid). LES supplemented with palmitic acid is more resistant to inhibition by fibrinogen, albumin, α-globulin and lyso-PC than LES alone, however the counteraction of blood protein inhibition is not as pronounced as is observed with SP-A.

Th-Pos187

Diphtheria toxin structure and lipid interaction: a study with phospholipid and protein monolayers.

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To study the structural change of diphtheria toxin induced by low pH and its influence on the interaction with membrane lipids, protein and lipid monolayers were formed and characterized. Diphtheria toxin at neutral and acid pH forms stable monolayers, whose surface pressure increase curves allow an estimation of the apparent molecular area of 2,950 A^2/molecule at pH 7.4 (corresponding to a radius of 30,6 A) and of 3,450 A^2/molecule at pH 5.0 (radius of 33,2 A).

Diphtheria toxin at pH 7.4 does not insert in phospholipid monolayers, while at pH 5.0 it penetrates in the lipid layer with a portion of apparent molecular area of 2,100 A^2/molecule (corresponding to a radius of 26 A).

The low-pH driven lipid interaction of the toxin is favoured by the presence of acidic phospholipids, without an apparent requirement for a particular class of negative lipids. Surface negative charges cause the involvement of a larger number of toxin molecules without an effect on the pH-dependence profile. The diphtheria toxin mutants CRM 45 and CRM 197 already at neutral pH are capable of hydrophobic interaction and cause an increase of surface pressure with a further increase upon acidification.

These results are discussed in terms of a model for the translocation of diphtheria toxin across membranes that reconciles all the available evidence on the penetration of diphtheria toxin into cells.

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Th-Pos188

THE EFFECT OF LUNG SURFACTANT APOLIPOPROTEINS B/C ON THE CHEMICAL SHIFT ANISOTROPY OF SN-2-[1-13C]-DPPC.
Timothy Wiedmann, University of Minnesota, Department of Pharmaceutics, Minneapolis, MN, and Amir Salmon, Department of Biophysics, Boston University School of Medicine, Boston, MA.
Nuclear magnetic resonance spectroscopy has been used to investigate the effect of the lung surfactant apolipoproteins B/C on dipalmitoylphosphatidylcholine to address the mechanism by which the adsorption rate of phospholipids from the bulk to the air/water interface is enhanced. Apolipoproteins B/C were isolated from bovine lung and separated from associated lipids by lipophilic sephadex column chromatography. The 13C chemical shift anisotropy of DPPC was determined as a function of temperature. Previous workers (Wittebort et al., 1982) have concluded that the observed magnitude of the chemical shift anisotropy of the carbonyl group of the sn-2 acyl chain in pure DPPC is a result of rapid rotation about an axis along the length of the phospholipid both in the gel and liquid crystalline state. The orientation of the carbonyl group with respect to the axis of diffusion however undergoes an apparent 24° shift in passage from the gel to liquid crystalline state, with the intermediate, rippled (P)state composed of an exchange between these two orientations. The presence of physiological concentrations of SP B/C reduced the width of the anisotropy of DPPC below but had no effect on lipids at or above the main phase transition temperature. This suggests that SP B/C has a general effect on the entire assemby of lipids. The temperature of the onset of the orientational change is lowered indicating a portion of the lipids are affected by the lung surfactant apolipoproteins.

Wittebort et al., Biochemistry 21: 3487 (1982)

Th-Pos189

CORRELATION OF PHOSPHOLIPID STRUCTURE WITH FUNCTIONAL EFFECTS ON THE NICOTINIC ACETYLCHOLINE RECEPTOR. Anil Bhushan and Mark G. McNamee, Department of Biochemistry and Biophysics, University of California, Davis, CA 95616.

Fourier transform infrared spectroscopy is used to characterize specific interactions between negatively charged lipids, such as phosphatidic acid, and the purified nicotinic acetylcholine receptor from *Torpedo californica*. Negatively charged lipids have been previously shown to have a higher binding affinity for the receptor and are essential for receptor-mediated ion translocation in reconstituted membranes. The ionization state of the phosphate group is monitored using bands arising from the phosphate headgroup region 1250-950 cm^{-1} of the infrared spectrum of phosphatidic acid. The ionization state of of the phosphate group of phosphatidic acid is dramatically perturbed on incorporation of receptor and the apparent pK_a is shifted to lower values. The functional activity of the receptor was determined by a manual assay of carbamylcholine-dependent influx of $^{86}Rb^+$ into reconstituted receptor containing vesicles. The pH dependence of the ionization state closely parallels the pH dependence of the ion channel functional response over the pH range 6-8. If the receptor is reconstituted with negatively charged phospholipids that do not change their ionization state over this range, the functional response is independent of pH. The relative protein secondary structure, measured over the same pH range, using the amide 1 band in the infrared spectra, showed changes over the range of the ionization of phospatidic acid. Thus, lipids such as phosphatidic acid interact specifically with acetylcholine receptor and can serve as allosteric effectors for membrane protein function. (Supported by NIH grant Grant NS 13050 to MGM)

Th-Pos190

FLUORESCENCE STUDIES OF ANNEXIN-MEMBRANE INTERACTIONS. Paul Meers, Castle Hematology Laboratory, Boston University School of Medicine, Boston, MA.

The Ca^{2+}-dependent interactions of annexins with membrane phospholipids were studied using fluorescence methods. Intrinsic tryptophan fluorescence was used to to monitor the single residues in human recombinant annexin I (lipocortin I) and V (lipocortin V). Binding of these proteins to phosphatidylserine/phosphatidylcholine (PS/PC) vesicles was accompanied by a large increase in fluorescence emission intensity and small red shift, indicating conformational change. The fluorescence of tryptophan 186 in membrane-bound annexin V was quenched when nitroxide derivatives of PC were used in the same vesicles. Quenching was strongest when the nitroxide was at the 5 position of the sn-2 acyl chain and progressively weaker when the quencher was at the 12 or 16 position. Therefore this tryptophan in the third consensus sequence of annexin V makes close contact with the phospholipids and is localized at the aqueous-membrane interface. By contrast, the single tryptophan in the N-terminal region of annexin I was not quenched by any nitroxide derivatives, indicating that this part of the protein is sequestered from the phospholipids.

A second fluorescence method relies on the ability of bound annexins to decrease the lateral mobility of phospholipids. Annexin binding to phospholipid vesicles increased the fluorescence of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine quenched by itself or by rhodamine B-PE. It also increased the monomer and decreased the excimer fluorescence of 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphocholine incorporated into PS vesicles. The magnitude of this response increased monotonically with bound protein concentration. Annexin V binding isotherms could be modeled assuming that the protein bound as monomers that require approximately 59 phospholipid molecules. Binding was completely reversible by chelators and by the polycation, spermine indicating it is largely controlled by ionic interactions. Annexin V bound most strongly, as measured by competition, to phosphatidate, phosphatidylglycerol and PS but did not bind to phosphatidylinositol at 100 μM free Ca^{2+}. Binding was very rapid, such that it would reach completion before aggregation of vesicles in most cases. Exchange between labeled and unlabeled vesicles was slow on the time scale of minutes.

Th-Pos191

ALAMETHICIN-MEMBRANE INTERACTIONS AND VOLTAGE-GATING MECHANISM OF ALAMETHICIN CHANNEL

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Although the voltage-dependent alamethicin channel is one of the best characterized ion channels, for a long time there is no agreement as to which model best describes all the experimental data. While the barrel-stave configuration is accepted by most investigators as a good description of the conducting state of alamethicin, there are conflicting reports on its nonconducting state--in the absence of an applied field, some found alamethicin molecules on the membrane surface, but others found them incorporated in the hydrophobic core of the membrane. This problem is now resolved by the discovery of a phase transition of alamethicin in membrane. Using the method of oriented circular dichroism (Wu, Huang & Olah 1990, Biophys. J. 57: 797), we found that the state of alamethicin in membrane varies with lipid/peptide ratio L/P and the chemical potential of water μ. If L/P exceeds a critical value, all peptide molecules are bind parallel to the membrane surface. If L/P is below the critical value, all peptide molecules insert perpendicularly into the membrane when μ is high; when μ is low, alamethicin is again on the membrane surface. In a typical conduction experiment, alamethicin molecules are partitioned between the aqueous phase and the lipid phase; in the lipid phase, the L/P ratio is such that all alamethicin molecules are on the membrane surface in the absence of a field. When an electric field is applied, it is those surface peptide molecules (rather than those in the aqueous phase) which will probabilistically turn into the membrane to form channels.

Th-Pos192

FREE ENERGY OF ALAMETHICIN CHANNEL FORMATION AND THEORY OF ALAMETHICIN PHASE TRANSITION

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In the accompanying poster, a phase transition of alamethicin in membrane is described. Since this is a macroscopic cooperative phenomenon, there must be interactions between peptide molecules in membrane. We believe that an alamethicin channel fits into a bilayer by matching its (exterior) hydrophobic and hydrophilic regions with those of the surrounding lipids; and this fitting causes some deformation in the configuration of the bilayer. The range of such a deformation can indeed be long (many nanometers; see Huang, 1986, Biophys. J. 50:1061); and within that range, the fitting of another channel into the bilayer is facilitated. Thus membrane-mediated peptide-peptide interactions are possible. We will discuss the free energy of channel formation, which explains why the state of alamethicin depends on the chemical potential of water. We will also present a statistical model which can qualitatively explain the observed phase diagrams.

Th-Pos193

THE AGGREGATION STATE OF MEMBRANE BOUND ALAMETHICIN: STUDIES USING SPIN-LABELED DERIVATIVES.

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Two spin-labeled derivatives of the voltage-dependent, ion-conductive peptide alamethicin were synthesized and used to examine the free energy of binding and state of aggregation of this peptide in lipid vesicles. One derivative was spin labeled at the C-terminus and the other, a leucine analogue, was spin labeled at the N-terminus. Both of these analogues were active in lipid vesicle systems, and produced a non-linear current-voltage behavior similar to that of unlabeled "native" alamethicin. In methanol, both the C and N terminal labeled peptides were monomeric. In aqueous solution, the C-terminal derivative was monomeric at low concentrations, but aggregated at higher concentrations with a critical concentration of 23 μM. In the membrane, the C-terminal label was associated with the membrane-aqueous interface. This was clearly indicated by the paramagnetic enhancement of the ^{13}C T_1 relaxation rates of the membrane lipid. In fully hydrated oriented bilayers, the EPR spectra also provide evidence that the peptide assumes more than one orientation.

When the membrane binding of the C-terminal derivative was examined using EPR, it exhibited a cooperativity seen previously for native alamethicin. At low peptide concentrations, the free energy of binding becomes more negative with increasing peptide. However, this cooperativity was not the result of an aggregation of the peptide in the membrane. When the spectra of either the C or N-terminal labeled peptide were examined over a wide range of membrane lipid to peptide ratios, no evidence for aggregation could be found and the peptides remained monomeric under all conditions examined. Since electrical measurements on this peptide provide strong evidence for an ion-conductive aggregate, the ion-conductive form of alamethicin likely represents a very minor fraction of the total membrane bound peptide. This finding has important implications for future structural work on the ion-conductive form of this peptide.

Th-Pos194

Interaction of zinc and cadmium with the pig intestinal brush-border membrane. A ^{31}P-NMR study.

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^{31}P-NMR experiments on intact pig small intestine brush-border membrane vesicles (BBMV) and detergent solubilized membranes gave direct insights on the phospholipid (PL) organization and their interactions with zinc and cadmium ions. Various endogenous PL were identified from well resolved BBM micelle spectra. These experiments revealed a strong interaction of Zn^{2+} and Cd^{2+} with the negatively charged phosphatidyl-inositol and -serine. In BBM micelles, a progressive time-dependent PL degradation occured in the absence of ions and indicated the presence of active phospholipases. A preventive zinc effect against PL alteration was observed whereas cadmium has the opposite influence. ^{31}P spectra of BBMV were carefully characterized. Both zinc and cadmium neither affected the PL bilayer structural organization nor induced the appearance of other PL structures such as micelles, inverted micelles, small vesicles or hexagonal phases. A degradation of PL, monitored by the increase of the inorganic phosphate (P_i) signal, also occured in vesicles but to a lesser extent than in micelles. Most of resulting P_i ions were located in the intravesicular milieu. A 2/3 internal, 1/3 external PL asymmetry was observed in the absence or presence of ions.

Th-Pos195

LOCAL CONTROL OF ANTIBODY BINDING TO HAPTEN PRESENTING INTERFACES: STERIC AND ELECTROSTATIC INTERACTIONS

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The binding of an antibody to membrane-bound (DNP) haptens depends critically on the relative arrangement of the hapten and the membrane, and on electrostatic interactions. In order to characterize the geometric requirements for specific binding, the membrane surface and the position of a hapten with respect to the interface have been systematically varied. The binding of labeled antibodies has been studied by means of fluorescence microscopy on monolayers at the air/water interface.

With monolayers of DMPC (doped with a hapten substituted fatty acid), unspecific antibody binding has been shown to predominate over specific binding due to electrostatic interactions. Due to the bulky choline head group the minimum spacer length between the surface anchored fatty acid and the hapten is 10 Å. Still, at high surface pressures, the bound antibody is detached because of steric interference with the lipid head groups. No unspecific binding is observed to monolayers of cholesterol, which carries a small dipole moment and presents a small hydrophilic part to the aqueous phase. As the spacer length d is increased (varied from 0 to 10 Å) the hapten protrudes more deeply into the water subphase and binds progressively better to the antibody. Two-dimensional phase separation has been observed in the system of protein-bound lipid and cholesterol. At low π, mixed monolayers of cholesterol and DMPC separate into two fluid phases with preferential antibody binding to the cholesterol-enriched phase. For $\pi > 7.5$ mN/m neither specific nor unspecific binding is observed. This is, again, attributed to steric hindrance by the large choline head groups and to the reduced electrostatic interaction of the protein with the interface.

Th-Pos196

STUDIES OF PARATHYROID HORMONE 1-34 IN SOLUTION AND ITS INTERACTIONS WITH MODEL LIPIDS: STEADY STATE AND TIME RESOLVED FLUORESCENCE STUDIES.

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Parathyroid hormone is an 84 residue hormonal peptide which is involved in the regulation of the level of serum calcium.The interest in this peptide/protein is stimulated by the possibility that it may have therapeutic use in the treatment of osteoporosis since there is some evidence that it may be critically involved in bone development and repair in mammals. It has been reported that the N-terminal fragment 34 residues also has significant biological activity. Preliminary studies have indicated that both PTH nor the 1-34 peptide are structurally heterogeneous in solution. Advantage has been taken of the single tryptophan residue at position 23 to study the structure, dynamics, and interactions of the 1-34 N-terminal peptide under a variety of solution conditions and in complexes with model lipid systems. The data show that there is a significant effect of pH on the structure of the peptide in solution. On interaction with lipids the environment of the single tryptophan residue is perturbed probably because it is now located in the bilayer interior.

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Th-Pos197

LATERAL DIFFUSION IN AN ARCHIPELAGO: SHIFTS IN THE PERCOLATION THRESHOLD

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Lateral diffusion of membrane components may be obstructed by the presence of immobile species. When the concentration of immobile species reaches the percolation threshold, long-range lateral diffusion is blocked. The value of the percolation threshold is well-known for randomly distributed point obstacles on regular lattices, but correlations in the positions of point obstacles can shift the threshold. Here the percolation threshold is calculated for various types of obstacles on the square and triangular lattices, including dimers, trimers, and cluster-cluster aggregates. One goal of this work is to see how low a percolation threshold can be obtained for random structures. The results are applied to recent measurements of lateral diffusion of fluorescent lipid analogues during lateral phase separations of lipid mixtures [Vaz, Melo, and Thompson. 1990. *Biophys. J.* 58:273-275]. In lateral diffusion measurements by fluorescence photobleaching recovery, a mobile species may be trapped in domains small enough that the species appears immobile. Monte Carlo calculations yield an estimate of the fraction of label that is trapped purely as a result of the geometry of the obstacles.

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Th-Pos198

DYE EFFLUX RATIO METHOD FOR MEASURING MEMBRANE POTENTIAL OF MITOCHONDRIA.

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Traditional methods for determining the mitochondrial membrane potential, Ψ_m, rely on measuring the relative equilibrium partition of amphiphilic cationic compound between the suspending medium volume and the inner mitochondrial volume of respiring mitochondria (mito). The partition is quantified by separation of mito containing radiolabeled amphiphile by centrifugation of the equilibrated mixture. The mito volume must be independently determined by sucrose exclusion measurement and assumptions made as to the non-Ψ_m dependent binding of amphiphile. Presented here is an homogeneous method for measuring Ψ_m based on measuring relative cationic dye efflux rates from mitochondria which does not require independent measurements or assumptions. Efflux is quantified from time course of dye fluorescence intensity and is initiated either by simple dilution (+) of equilibrated dye + respiring mito suspensions (whence Ψ_m exists during efflux) or by addition of excess CCCP (–) to collapse Ψ_m during efflux. The ratio, α, of the dye efflux time constants, τ^-/τ^+, obtained under these conditions of initiation is related to Ψ_m by $\alpha=\Psi_m C[1-\exp(-\Psi_m C)]$, where C =0.0391 mV^{-1} is a constant. The method correlates well with [^{14}C]-TPP radiometric partition method giving $\Psi_m = -158 \pm 11$ mV (n=8) as compared to -163 ± 13 (n=8) for the classical radiometric method. Correction for the -20 mV residual Ψ_m in the presence of excess CCCP gives an estimate of Ψ_m identical to the radiometric method.

Th-Pos199

PHOTORELEASE OF LIPOSOME CONTENTS BY DYE-MEDIATED LOCALIZED HEATING INDUCED BY PICOSECOND OR NANOSECOND LASER EXCITATION.

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Our data demonstrates for the first time that the contents of liposomes can be released by pulsed, laser-induced localized heating of micron or sub-micron diameter vesicles containing a dye. Most experiments have used xanthene dye solutions encapsulated inside the liposome where fluorescence is dimer-quenched, but successful release was also obtained using the FDA-approved dye methylene blue, which is membrane-associated. We have shown that the results obtained with these preparations depend on several interdependent variables, including composition and size of the lipid bilayer vesicle, type and concentration of dye, initial temperature, and laser excitation parameters.

With 50 mM sulforhodamine encapsulated in 4.5 micron diameter liposomes, essentially complete release of the contents can be achieved with a single 8 ns pulse at 532 nm from a doubled Nd-Yag laser (ca. 0.5 J/cm^2). Experiments with picosecond pulses deposit thermal energy with minimal thermal diffusion; the irradiation of these liposomes with a single 25 ps pulse at 532 nm suggest that the kinetics of liposome breakage is on the order of the lifetime of fluorescence (<10 ns). The fluorescence lifetime of free dye is 4 ns, but results for the dye in liposomes requires three components (0.03 - 3 ns), providing data on the quenching rates of encapsulated dye. Model calculations based on the data are consistent with a photoinduced heating mechanism.

Th-Pos200

TRANSBILAYER FLIP-FLOP OF DOXYLSTEARIC ACID NITROXIDES IN LIPOSOMES

by Jeu-Ming Yuann and Philip D. Morse II, Department of Chemistry, Illinois State University, Normal, IL 61761

Doxylstearic acid nitroxides have been used for over 20 years to study the fluidity of cell membranes. However their rapid reduction and lack of knowledge about their distribution in cells have made their use difficult in living systems. Recently, we showed that 5-doxyl stearic acid (5DS) distributes throughout all membranes in certain cells (1). This distribution occurs within 1-2 minutes.

Our current work studies the rate of transbilayer flip-flop of the doxylstearic acid nitroxides in unilamellar dipalmitoylphosphatidylcholine (DPPC) liposomes. We use flavin mononucleotide (FMN) and EDTA to photoreduce the nitroxides and follow signal intensity with time. Control experiments with CAT1, an impermeable water-soluble nitroxide, trapped inside the liposomes, show that FMN or reducing equivalents produced during the photochemical reaction do not cross the liposome membrane over the time course of the experiment (5 minutes). We trapped FMN and EDTA inside the liposomes to measure the flip rate of 5DS from the outer to inner leaflet of the DPPC bilayer. 5DS is reduced by FMN with a half-time of 7 seconds when FMN and EDTA are on both sides of the membrane.

Our results show that 5DS flips from the inner to the outer leaflet with a half-time of about 5.2 sec while the upper limit of the rate of flip from the outer to the inner leaflet is 24 seconds or faster. Thus it is clear that 5DS moves from one side of the bilayer to the other much faster than phospholipids can. 5DS attains its rapid distribution in cells by its rapid lateral diffusion (1) and trans-bilayer flip flop.

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(1) Nettleton, D.O., Morse, P.D., II, Dobrucki, J.W., Swartz, H.M., and Dodd, N.F.J. *Biochim. Biophys. Acta* **944**(1988) 315-320.

Th-Pos201

INTERMIXING OF 1-PALMITOYL-2-OLEOYL-*SN*-GLYCERO-3-PHOSPHOCHOLINE WITH NATURAL GALACTOYSYLCERAMIDES AND HOMOGENEOUSLY *N*-ACYLATED GALACTOSYLCERAMIDES BEARING ONE OR TWO DOUBLE BONDS: A MONOLAYER STUDY.

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Homogeneously *N*-acylated galactosylceramides (GalCer) having one or two double bonds in their acyl chains have been synthesized and characterized at the argon-water interface using a Langmuir film balance. Both surface pressure (π) and surface potential (ΔV) were measured at 24°C as a function of molecular area. Monolayers of *N*-oleoyl GalCer (C18:1$^{\Delta 9}$ GalCer), *N*-eicosenoyl GalCer (C20:1$^{\Delta 11}$ GalCer), and *N*-eicosadienoyl GalCer (C20:2$^{\Delta 11,14}$ GalCer) form liquid-expanded films. In contrast, bovine brain GalCer, with its heterogeneous array of fatty acids, forms liquid-condensed films. The mixing behavior of each GalCer sample with POPC was measured and analyzed using classical approaches (e.g., surface pressure-composition phase diagrams, molecular area-composition diagrams) as well as by an equation of state for liquid-expanded monolayers [Brockman & Smaby, (1990) *Langmuir* (submitted)]. The results indicate that C18:1 GalCer, C20:1 GalCer, and C20:2 GalCer are ideally miscible with POPC over the entire composition range. In contrast, binary mixtures of bovine brain GalCer and POPC display nearly ideal mixing behavior below 30 mol% GalCer, but mix non-ideally at higher GalCer compositions. Analysis of the fatty acyl chain composition of bovine brain GalCer reveals an abundance of saturated and 2-hydroxy long-chain fatty acids (C22-C26). These results show that fatty acyl composition is a critical parameter governing the mixing behavior of GalCer with phospholipids. (Supported by NIH HL08214 and Hormel Foundation)

Th-Pos202

THE USE OF LAURODAN AS A PROBE OF MEMBRANE SURFACE PROPERTIES.

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In lipid bilayers laurodan has a fluorescence emission spectra consisting of two overlapping components. They appear to be due to a combination of a species fluorescing with a maxima around 440 nm and another at around 500 nm. The fluorescence emission in chloroform shows a single emission at 440 nm, while in methanol the emission is at 500 nm. This suggests that in membranes the fluorophore may exist either in a hydrophobic environment, corresponding to emission at 440 nm and at a position in the bilayer below the phospholipid head group region, or alternatively in the head group region itself near the membrane surface where the emission is around 500 nm.

It was found that the relative emission of the two locations or the proportion of fluorophores residing in the regions differed according to the lipid type. Further, addition of divalent cations or reduction in temperature favored the hydrophobic disposition of the fluorophore. In contrast, interaction with the extrinsic membrane protein phospholipase A_2 favored the hydrophillic disposition of the fluorophore.

In addition to steady state spectra the fluorescence decay at the red edge (>470 nm) was also investigated. Analysis of the fluorescence decay as arising from two components was found to be a reasonable approximation, one fluorescence lifetime being around 3-4 ns and the other of around 1 ns but with a negative pre-amplitude indicative of solvent relaxation around the excited state fluorophore dipole, assuming a two state relaxation model. The solvent relaxation time, i.e the fluorescence lifetime associated with the negative pre-amplitude, was used to investigate a range of different lipid bilayer conditions including those above. In summary the data provide evidence that the fluorophore laurodan may be useful as a probe of membrane properties, particularly those at the membrane-aqueous interface.

Th-Pos203

FLUORESCENT MEMBRANE PROBES INCORPORATING THE PYRROMETHENE-BORONDIFLUORIDE (BODIPY™) CHROMOPHORE

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The spectroscopic properties of a new series of membrane probes in which a BODIPY chromophore forms a segment of the 2-acyl chain of phosphatidylcholine are presented. When incorporated as a low mole fraction component in liposomes, these fluorescent phospholipids retain the principal characteristics of the parent chromophore: green fluorescence emission with high quantum yield, extensive spectral overlap and low environmental sensitivity. The absolute fluorescence quantum yield is typically 0.9 - 0.7 which is approximately 2.5 times that of comparable visible emitting membrane probes based on the NBD chromophore. This fluorescence output advantage is increased to about 10 fold for excitation at 488 nm and narrow emission bandpass conditions typical of microscope filters, primarily due to the position and strength of the lowest BODIPY absorption band. The spectral overlap results in a calculated Förster energy transfer radius (R_o) of about 57 Å. Consequently, increasing fluorescence depolarization and quenching are observed as the mole fraction of BODIPY phosphatidylcholine in liposomal membranes is increased. Low environmental sensitivity is manifested by retention of high quantum yield emission in aqueous dispersions of BODIPY fatty acids which requires the use of phospholipid derivatives to obtain membrane specific fluorescence data free of aqueous background contributions. The response of BODIPY probes to phospholipid phase transitions is examined and is found to be indicative of nonrandom fluorophore distribution in the gel phase. It is concluded that the spectroscopic properties of BODIPY phospholipids are primarily suited to applications in optical imaging of model and cellular membrane systems.

Th-Pos204

UNUSUAL PRESSURE DEPENDENCE OF THE LATERAL MOTION OF PYRENE-LABELED PHOSPHATIDYLCHOLINE IN POLAR LIPID E ISOLATED FROM SULFOLOBUS ACIDOCALDARIUS.

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The effects of pressure and temperature on the lateral motion of 1-palmitoyl-2-[(10-pyrenyl)decanoyl]-phosphatidylcholine (Pyr10PC) in liposomes composed of archaebacterial polar lipid E (PLE) isolated from Sulfolobus acidocaldarius have been examined. The excimer to monomer fluorescence intensity ratio (E/M) was measured isothermally in the pressure and temperature ranges of 0.001 - 1.5 kbar and 16 - 60°C. At PLE/Pyr10PC = 50, E/M decreases monotonically with pressure at 60.4°C. At 38.3°C, the result is biphasic. E/M decreases with pressure up to 1 kbar and then slightly increases with pressure. At 16.8°C, E/M remains virtually unchanged up to 0.7 kbar, and then increases with pressure. At a lower PLE/Pyr10PC ratio (500:1), a similar result was obtained. In comparison, the E/M of Pyr10PC in other lipid dispersions, including diphytanoylPC, C(18):C(10)PC, DMPC, DPPC, and POPC, does not show a high pressure/low temperature anomaly but instead showed a much more pronounced decrease with pressure. The anomaly has been interpreted in terms of the three excited state model recently developed by Sugar et al. (1990). The low pressure and temperature sensitivity in lipid mobility found in PLE liposomes may explain why archaebacteria live in harsh environments.

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Th-Pos205

Epifluorescence Microscopy and Spectroscopy Studies of Phophatidylcholine Monolayers

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The molecular packing in monolayers of dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was studied by using the fluorescent dye, merocyanine 540 (MC-540), which has been applied to differentiate lipid packings in cell membranes. Conditions for phase separation in these monolayers were defined by pressure/area (π-A) isotherms. Within the range where the LE/LC phases coexist in DPPC monolayer, low level fluorescence microscopy revealed discoid domains of lower fluorescence intensity. These domains expanded with increasing surface pressure. Under the same conditions of phase separation in DPPC, the π-A isotherms of DOPC and POPC showed the existence of only one phase down to 12°C, and no fluorescent domains were observed. Fluorescence spectra of MC-540 labeled monolayers were recorded at different structural states. Two emission peaks were observed. An 572 nm peak was present during all conditions. An 584 nm emission peak, typically representing the dye molecules in lipid, appeared only within the area/molecule range of 40-65 $Å^2$. The intensity of this peak diminished with increasing surface pressure, while the intensity ratio of 584 nm/572 nm peaks increased. No spectral shift of these peaks was observed. The results were explained by the location and orientation of the dye, with respect to the monolayer at various structural states. The usefulness of MC-540 as a probe for lipid packing will be discussed.

Th-Pos206

FLUORESCENT ASSAY OF MONOACYL- AND DIACYLPHOSPHOLIPASE A ACTIVITY ASSOCIATED WITH RABBIT INTESTINE BRUSH BORDER MEMBRANES.

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Phospholipids labeled with 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) were used to continuously monitor phospholipase activity associated with purified rabbit intestine brush border membrane vesicles (BBMV). Phospholipase activity was tested with two labeled molecules: N-(NBD) monopalmitoylphospatidylethanolamine (N-NBD-PPE) and 1-myristoyl-2-[6-(NBD amino)caproyl] phosphatidylcholine (M-C_6-NBD-PC). Addition of either of the probe molecules to BBMV (probe concentration 1% of BBMV phospholipid concentration) resulted in a rapid increase in fluorescence ($t_{1/2}$<1-2 s) indicative of their partitioning out of the quenching aqueous environment into the hydrophobic environment of the membrane. This rapid increase was followed by a slow decrease of fluorescence to near zero ($t_{1/2}$~5-10 min). Aliquots of the mixture were removed at timed intervals, extracted, and separated by thin-layer chromatography. The fluorescence decay correlated with the hydrolysis of N-NBD-PPE to N-NBD-glycerolphosphoryl-ethanolamine and the hydrolysis of M-C_6-NBD-PC to 6-(NBD amino)caproic acid and two other unidentified products. Since NBD fluorescence is highly quenched in an aqueous environment, hydrolysis of the hydrophobic NBD-labeled phospholipids to more water-soluble products resulted in the observed decrease in fluorescence. This activity was not inhibited by the presence of 2 mM Na_2EDTA, but was inhibited by sulfhydryl reagents. Supported by NIH grants GM32342 and DK40642.

Th-Pos207

SUBMICELLAR CONCENTRATIONS OF TAURODEOXYCHOLATE SELECTIVELY EXTRACT MONOACYLPHOSPHOLIPIDS FROM PHOSPHOLIPID VESICLES.

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We examined the ability of submicellar concentrations of the bile salt, taurodeoxycholate (TDC), to extract phospholipids from unilamellar vesicles. Vesicles were prepared to contain 3% by mole of either the monoacylated fluorescent probe, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-monomyristoylphosphatidylethanolamine (N-NBD-MPE) or the diacylated probe N-NBD-DMPE together with 3% by mole of N-Rhodamine-PE to quench the fluorescence of the NBD group. Vesicles suspended at 100 µM at 10°C were placed in a stopped flow device that was mixed with an equal volume of the TDC solutions. The TDC concentration [TDC] of the solution was increased and the fluorescence change monitored with time. [TDC]'s below 300 µM selectively increased the fluorescence of the N-NBD-MPE vesicles while not increasing the fluorescence of the N-NBD-DMPE containing vesicles. This result indicates the increase in fluorescence is due to the actual extraction of the fluorophore from the membrane by the added TDC and not simply a partitioning of TDC into the bilayer with a resultant separation of the NBD and rhodamine fluorophores. As the [TDC] was raised from 0 to 300 µM the amount of N-NBD-MPE extracted from the vesicles rose to 0.6% with no effect on the vesicles containing N-NBD-DMPE. This result was consistent with a calculation of N-NBD-MPE interaction with TDC based on their measured interaction in buffer (Shoemaker & Nichols, Biochemistry 29:5837, 1990). There was no change in the rate constant of removal of N-NBD-MPE (15.0 ± 1.5 s^{-1}) as a funtion of [TDC]'s under 300 µM. Between 400 and 700 µM TDC sufficient bile salt partitioned into the phospholipid bilayer to effect a nonspecific increase in fluorescence of both the N-NBD-MPE and N-NBD-DMPE containing vesicles while raising the [TDC] to values greater than 700 µM converted the vesicles to mixed bile salt-phospholipid micelles as indicated by a simultaneous decrease in light scattering. Similar results were obtained with probes containing acyl chains of 12 to 18 carbons. Supported by USPHS grants DK40641 and GM32342.

Th-Pos208

MEMBRANE PENETRATION DEPTH OF FLUORESCENT MOLECULES BY THE USE OF SPIN-LABELED PHOSPHOLIPIDS: CALIBRATION OF ACCURACY.

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We have previously, by determining the distance dependence of fluorescence quenching by spin labels, derived expressions that allow determination of membrane penetration depth.

We here present results which establish experimentally that one can obtain depths accurate to around 2 angstroms. This was done by comparing the results of the spin-label method to the fluorescence quenching obtained from lipids labeled with bromines at different positions along one of their fatty acyl chains. Bromines are well suited to the calibration work presented here because they are short range quenchers and their positions in membranes have been determined by X-ray diffraction. Our method was calibrated using fluorophores located at three depths in the membrane. A fatty acid with a carbazole group located at the 11 position on the acyl chain was used. Upon ionization of the carboxyl the carbazole group moved to a shallower location in the membrane by around two angstroms. This gave two deep calibration points in the membrane. Tryptophan octyl ester provided a third membrane location at a shallow depth in the membrane. Supported by NIH grant GM31986.

Th-Pos209

LIPID DIFFUSION IN SUPPORTED BILAYERS: APPLICATION OF PYRENE-PHOSPHOLIPID PROBES AND QUANTITATIVE FLUORESCENCE MICROSCOPY.

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Excimer forming pyrene-phosphatidylcholines (pyPC), incorporated in small unilamellar (SUV) and multilamellar (MLV) vesicles of a variety of phosphatidylcholines, have been shown to accurately report changes in diffusivity, as well as other thermodynamic properties, of the host membrane (Biophys.J. 1990, 57:281). The values of the lateral diffusion coefficients (D) in liquid-crystalline phase DMPC bilayers obtained by the excimeric technique were shown to agree reasonably well with those obtained by other investigators using the fluorescence photobleaching recovery (FPR) technique: however, in gel phase DMPC bilayers the lateral mobility of the excimeric probes was only moderately lower than in the liquid-crystalline phase, while D measured by FPR decreased by at least two orders of magnitude. It was proposed that a possible explanation for this discrepancy might reside in the difference in characteristic diffusion length scales between the two techniques (microns for FPR, nanometers for excimeric probes). To test this hypothesis and allow a more direct comparison with the FPR results, we have now extended our previous studies of lateral diffusivity by the excimer formation technique to glass-supported phospholipid bilayers. Quantitative fluorescence microscopy is used to obtain excimer-monomer fluorescence ratio images of the pyPC labeled membrane. Results of experiments on a variety of phospholipid matrices and as a function of temperature will be presented.

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Th-Pos210

EFFECT OF WATER PENETRATION ON LAURDAN FLUORESCENCE IN PHOSPHOLIPID VESICLES. R.M. Rusch,* T. Parasassi♦ and E. Gratton.* **Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, 1110 W. Green Street, Urbana, IL 61801, ♦Istituto di Medicina Sperimentale, CNR, Viale Marx 15, 00137 Rome, Italy.*

Laurdan is a fluorescent membrane probe whose excitation and emission spectra are different in the gel and in the liquid crystalline phases. The fluorescence emission spectrum for Laurdan in the liquid crystalline phase is red-shifted compared to the gel phase emission spectrum. The excitation spectrum is blue-shifted in the liquid crystalline phase compared to the gel phase. This spectral sensitivity to the physical state of the membrane arises from dipolar relaxation processes in the membrane region surrounding the Laurdan molecule.

Experiments in phospholipid vesicles with different polar head groups show that the polar head group does not affect Laurdan fluorescence. pH titration of the vesicles from pH 4 to 10 shows that the charge of the head group also does not affect the fluorescence of Laurdan. It is concluded that water molecules in the phospholipid bilayer are responsible for the dipolar relaxation behavior of Laurdan. In the gel phase, these water molecules are largely immobilized. But in the liquid crystalline phase these water molecules are instead mobile and can reorientate around the Laurdan excited-state dipole during the few nanoseconds of the excited-state lifetime. (Supported by NIH grant RR03155 and CNR).

Th-Pos211

EPR STUDY OF THE INTERACTION BETWEEN Fe^{3+} - PROTOPORPHYRIN IX AND SPIN LABELS IN LIPID BILAYERS. Tais H. Schmitt and Shirley Schreier (Intro. by L. M. Gierasch) Laboratory of Molecular Biophysics, Institute of Chemistry, Universidade de S. Paulo, C.P. 20780, 01498, S. Paulo, Brazil

The concentration of free heme increases under a variety of abnormal conditions. Addition of hemin (Fe^{3+} - protoporphyrin IX) causes lysis of erythrocytes and of parasites such as Plasmodia and Trypanosomae. Making use of a spin labels, previous work from our laboratory has shown that hemin binds to lipid bilayers, decreasing the degree of molecular order. This result correlates well with hemin-induced increased membrane permeability. In contrast, studies of lipid peroxidation did not exhibit a correlation between the latter and the two previous processes. Moreover, hemin - induced membrane disorder and increased permeability were still observed in the presence of an antioxidant. In the present work, analysis of hemin binding to bilayers was performed making use of EPR spectra of lipid spin labels containing the nitroxide moiety at different depths in the bilayer. The spectra indicated a magnetic interaction between the hemin iron and the nitroxide leading to a loss of spectral intensity. The extent of the interaction was depth-dependent, suggesting that the heme is located close to the water-membrane interface, at physiological pH. At acidic pH, the interaction is selectively enhanced, being larger for labels located more deeply in the bilayer. Experiments performed in aqueous medium indicate that the iron-nitroxide interaction is at least partly due to an exchange mechanism and suggest that hemin, which is strongly aggregated in water, undergoes dissociation into smaller aggregates upon binding to the bilayer.

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Th-Pos212

^{2}H NMR STUDIES OF THE INTERACTION OF ALPHA-TOCOPHEROL WITH A POLYUNSATURATED PHOSPHOLIPID IN A MODEL MEMBRANE. Stephen R. Wassall [1.], M. Alan McCabe [1.], William D. Ehringer [2.] and William Stillwell [2.], Departments of Physics [1.] and Biology [2.], Indiana University - Purdue University at Indianapolis, Indianapolis, IN 46205.

Structural roles within membranes, in addition to the generally accepted function as an antioxidant, have been proposed for α-tocopherol (the major constituent of vitamin E). In particular, it was hypothesized that the methyl groups on the phytyl side chain of α-tocopherol insert into "pockets" produced by double bonds in the polyunsaturated fatty acyl chains of phospholipids and thereby stablize the membrane. The current ^{2}H NMR studies directly address this hypothesis.

^{2}H NMR spectra were recorded for aqueous multilamellar dispersions of 80 mol% DPPC (dipalmitoylphosphatidylcholine)/20 mol% *sn*-1- [$^2H_{31}$] palmitoyl (16:0)-2-docosahexaenoyl (22:6) PC as a function of incorporation of α-tocopherol. Moment analysis of the spectra indicates that 0-10 mol% α-tocopherol produces only a slight increase in acyl chain order (2%) for the polyunsaturated phospholipid in the liquid crystalline state. Order parameter profiles constructed from depaked spectra support this conclusion. These observations compare with the situation in saturated membranes, where 10 mol% α-tocopherol increases order by almost 10%. Thus, instead of detecting membrane stabilization due to lipid-lipid interactions between the vitamin and polyunsaturated phospholipid, our results suggest that the presence of acyl chain polyunsaturation facilitates accomodation of α-tocopherol into the membrane with minimal perturbation to molecular order.

Th-Pos213

EFFECT OF A PHOSPHATIDYLGLYCEROLPHOSPHATE ANALOGUE ON MEMBRANE PROPERTIES USING FLUORESCENCE MEMBRANE PROBES. Michael Glazman, Burton E. Tropp* and Lesley Davenport, Department of Chemistry, Brooklyn College of CUNY, NY 11210 and Department of Chemistry, Queens College of CUNY*, NY 11367.

When 3,4-dihydroxybutyl-1-phosphonate (DBP) is taken up by *E.coli*, a phosphonate analogue of phosphatidylglycerolphosphate (PGP) appears in the plasma membrane (Tyhach et al., (1976) J.B.C. 251, 6717) with a concurrent reduction in levels of the naturally occurring phosphatidylglycerol (PG). The presence of this unnatural anionic phospholipid leads to abnormal responses of the organism to cold temperatures and the presence of Ca^{2+} or Mg^{2+}. In an attempt to understand the effects of this lipid on the membrane architecture we have synthesized pure PGP analogue *in vitro* using PGP synthase isolated from *E.coli*. Steady-state fluorescence emission anisotropy measurements of DPH labeled dipalmitoylphosphatidylcholine (DPPC) unilamellar vesicles (ULVs) containing PGP analogue or PG (0-30 mole%) indicate that the lipid analogue causes severe lipid disordering effects below the lipid phase transition temperature for DPPC (T_c=41°C). Corresponding studies using TMA-DPH labeled ULVs suggest that the analogue affects lipid packing in the acyl-chain region of the bilayer. Membrane permeability assays (using carboxyfluorescein liposome escape) show an increased 'leakiness' of bilayers containing PGP analogue or PG over ULVs containing no anionic phospholipids. Furthermore, addition of Ca^{2+} or Mg^{2+} (up to 15mM) to DPPC ULVs containing the PGP analogue results in facilitated bilayer fusion. Our fluorescence studies suggest that the phosphonate analogue of PGP appears to have significant effects on the physical state of model membrane systems. Control of certain enzymatic and permeability functions of the *E. coli* membrane can be seriously affected by changes in membrane fluidity and permeability. Present investigations are focussed on studies of altered membrane potential and heterogeneity of lipid packing resulting from the presence of the PGP analogue in the bilayer. (Supported in part by the Pet. Res. Fund, PSC-CUNY awards, AHA-NY Affiliate and NSF DMB9006044).

Th-Pos214

THE EFFECTS OF GEL PHASE OBSTACLES ON THE TRANSLATIONAL DIFFUSION OF LIPIDS IN TWO-COMPONENT, TWO-PHASE BILAYER SYSTEMS: AN EXPERIMENTAL STUDY. Paulo F. Almeida and Thomas E. Thompson, University of Virginia, Charlottesville, and Winchil L.C. Vaz, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany.

As indicated by FRAP measurements, the binary lipid mixture Lignoceryl-Galactosyl Ceramide/DPPC exhibits an insular gel phase in the region of gel-fluid phase coexistence. By varying the temperature at constant composition, we measured the dependence of the diffusion coefficient of NBD-POPE on the fraction of gel phase. Since this probe partitions exclusively in the liquid phase, the gel domains present obstacles for its diffusion. We performed the same experiment using as a probe the membrane-spanning lipid NBD-MSPE (Vaz et al., Eur. Biophys. J. 12: 19-24, 1985) which has the same partitioning behavior but senses the gel domains in both monolayers. The results indicate that the gel domains are uncorrelated in the two monolayers of each bilayer.

We have also carried out similar FRAP measurements using the two types of NBD probes in DMPC/DSPC mixtures. In previous studies (Vaz et al., Biophys. J. 56: 869-876, 1989) we have shown that these mixtures exhibit a reticular gel phase in regions where both gel and fluid phases coexist. Comparison of the results obtained with the two types of NBD probes leads to the conclusion that the reticular gel domains are correlated in the two monolayers of each bilayer in this system.

We interpret the results in terms of percolation and hindered diffusion in an archipelago, and compare them with computer simulations (Saxton, Biophys. J. 52: 989-997, 1987; Biophys. J. 56: 615-622, 1989).

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Th-Pos215

THE EFFECT OF α-TOCOPHEROL ON PHOSPHOLIPID BILAYERS. William D. Ehringer[1], Stephen R. Wassall[2] and William Stillwell[1] Departments of Biology[1] and Physics[2], Indiana University-Purdue University at Indianapolis, Indianapolis, IN 46205.

It is proposed that α-tocopherol supports two main roles in membranes, that of an antioxidant and as structural component of membranes. Here we report the effect of α-tocopherol on phosphatidylcholine (PC) and phosphatidylethanolamine (PE) bilayers composed of either saturated or unsaturated acyl chains. Fluorescence polarization of a series of anthroyloxy stearic acid probes indicates that the vitamin decreases membrane fluidity in the liquid crystalline state for both saturated diacyl chain PCs and diunsaturated PCs, while fluidity of heteroacid PCs exhibit a mixed behavior. These results are confirmed by bleaching of α-tocopherol fluorescence by acrylamide and UV absorbance, which indicate that α-tocopherol preferentially interacts with unsaturated acyl chains. Laurdan emission studies of PC or PC/PE vesicles, indicate that the vitamin shifts the laurdan emission maxima indicating a possible head group preference as well. Lipid domain formation was investigated by NBD-PE fluorescence and compliments the findings of the laurdan emission studies These results are defining the role of α-tocopherol in phospholipid bilayers.

Th-Pos216

THEORY FOR MOLECULAR DYNAMICS OF MEMBRANE LIPID BILAYERS BASED ON DEUTERIUM NMR SPECTROSCOPY. Michael F. Brown. *Department of Chemistry, University of Arizona, Tucson, AZ 85721.*

How can one obtain experimental information regarding the configurational and collective dynamics of membrane lipids, and how are such properties implicated in biological functions? A useful approach is to combine deuterium (^{2}H) NMR relaxation measurements with theory to obtain new knowledge of the **molecular dynamics.**[1] In one model (i) the *static* electric field gradient (efg) tensor is modulated by reorientation of the C-^{2}H bond vector due to local or molecular motions. The principal axes of the efg tensor coincide with the frame of the C-^{2}H bond and the *static* principal values are known. Here the ability to predict the angular anisotropy of the **relaxation** for lipid bilayers containing specifically deuterated cholesterol is an important test.[2] The case of phospholipids with flexible acyl chains is less clear cut. Alternatively the *residual* efg tensor left-over from faster segmental motions can be modulated by slower motions (order fluctuations). A second model (ii) considers the relaxation to arise from order fluctuations due to molecular motions (*non-continuum model*). The slow motions involve rotations about molecular principal axes for which the moments of inertia are reduced by preaveraging due to more rapid segmental fluctuations.[3] Isomerization of the phospholipid acyl chains can also break the symmetry of the *static* efg tensor, yielding an asymmetric residual tensor that is further averaged by molecular rotations. Finally, the third model (iii) formulates relatively slow order fluctuations for simplicity in terms of motion of a local director axis (*continuum model*). For bilayers of phospholipids, earlier interpretations have focussed on the influences of segmental motions and collective order fluctuations. Current results indicate that **molecular features** may also need consideration to explain relaxation data in the MHz regime. [1]M.F. Brown *et al.* (1990) *Mol. Phys. 69*, 379. [2]M.F. Brown (1990) *Mol. Phys.*, in press. [3]M.F. Brown and O. Söderman (1990) *Chem. Phys. Lett. 167*, 158. Supported by NIH Grants GM41413, EY03754, and RR03529.

Th-Pos217

MOLECULAR DYNAMICS OF PHOSPHOLIPID BILAYERS VIA ^2H NMR SPECTROSCOPY: DETERMINATION OF INDIVIDUAL MOTIONAL SPECTRAL DENSITIES. Theodore P. Trouard, Judith A. Barry, S.C. Shekar, and Michael F. Brown (Intro. by J.F. Ellena). *Department of Chemistry, University of Arizona, Tucson, AZ 85721.*

Experimental investigations of molecular dynamics in lipid bilayers have rested heavily upon nuclear spin relaxation measurements and their interpretation using various dynamical models. Underlying the observable relaxation rates are **spectral densities** of motion $J_M(\omega)$, which are Fourier transform partners of the correlation functions describing the reorientational dynamics. Measurement of the spectral densities thus enables one to test more directly the applicability of proposed motional models. To determine the individual spectral densities $J_1(\omega_0)$ and $J_2(2\omega_0)$, where ω_0 is the Larmor frequency, spin-lattice and quadrupolar relaxation rates (R_{1Z} and R_{1Q}) were measured for 1,2-diperdeuteriomyristoyl-*sn*-glycero-3-phosphocholine in the lamellar phase at magnetic fields of 7.05 and 11.7 Tesla (^2H NMR Larmor frequencies of 46.1 and 76.7 MHz respectively). The derived spectral densities were dependent on the segmental **order parameters**, S_{CD}, of the acyl chain segments and followed a $|S_{CD}|^2$ functional dependence.[1] In addition, the values of $J_M(\omega)$ were **frequency dependent**, consistent with previous studies.[2] These results are consistent with the hypothesis that slow motions influence the relaxation in which the amplitude is roughly the same along the length of the lipid acyl chains, i.e. the slow motions modulate the residual electric field gradient tensor left-over from fast motions. [1]M.F. Brown (1982) *J. Chem. Phys.* 77, 1576. [2]M.F. Brown *et al.* (1990) *Mol. Phys.* 69, 379. Work supported by the NIH (GM41413, EY03754, and RR03529).

Th-Pos218

INTERMOLECULAR INTERACTIONS IN PHOSPHOLIPID SYSTEMS STUDIED BY DEUTERIUM NMR SPECTROSCOPY. Robin L. Thurmond, Mikael Jansson, Judith A. Barry, and Michael F. Brown (Intro. by D.E. Goll). *Department of Chemistry, University of Arizona, Tucson, AZ 85721.*

Deuterium (^2H) NMR spectroscopy is a powerful method for investigating average properties of lipid bilayers. We have used ^2H NMR to probe the effects of **intermolecular interactions** on the orientational order of 1-perdeuteriopalmitoyl-*sn*-glycero-3-phosphocholine (PaLPC-d_{31}) in four different aqueous systems: pure PaLPC-d_{31}, 1:3 PaLPC-d_{31}/dipalmitoylphosphatidylcholine, 1:1 PaLPC-d_{31}/palmitic acid, and 1:1 PaLPC-d_{31}/cholesterol. ^2H NMR spectra were obtained for these systems as a function of temperature.[1] Moment analysis of the lineshapes indicated that the first three bilayers underwent a main order-disorder phase transition at approximately the same temperature, and that the molecular order was almost identical in the low-temperature phases. The ^2H NMR spectra provide clear evidence for **axial rotation** of all-trans chains at low temperatures in each of the first three cases. However, in the L_α phase the segmental order parameters, S_{CD}, were found to differ appreciably among the different systems, in spite of the similar order-disorder transition temperatures. Using simple geometric arguments we have related the findings to the average **cross-sectional area** available per acyl chain, which in turn reflects a balance of attractive and repulsive forces within the bilayer.[2] [1]M. Jansson *et al.* (1990) *Chem. Phys. Lipids* 54:157. [2]R.L. Thurmond *et al.* (1990) *Biophys. J.*, in press. Work supported the NIH (GM41413, EY03754, and RR03529) and by a NIH postdoctoral fellowship (J.A.B.).

Th-Pos219

THE GEL-LIQUID CRYSTALLINE TRANSITION OF LIPID BILAYERS FOLLOWS CLASSICAL KINETICS WITH A FRACTIONAL DIMENSIONALITY OF APPROXIMATELY TWO. Qiang Ye and Rodney Biltonen. (Intro. by Gordon Rule) Biophysics Program, University of Virginia, Charlottesville, Virginia 22908

The relaxation kinetics of the gel-liquid crystalline transition of phosphatidylcholine multilamellar vesicles have been studied with volume-perturbation calorimetry. The temperature and pressure relaxations following a volume perturbation are used to monitor the trasition time course. Data collected in the time domain are converted into the frequency domain using Fourier series representations of the perturbation and response functions. Since a very small perturbation is imposed during the experiment, linear response theory is suitable for analysis of the relaxation process. The Laplace transform of the classical Kolmogorov-Avrami relation of transition kinetics can be used to describe the dynamic response in the frequency domain. In one-component systems, the relaxation process is better fit with the effective dimensionality of n=2 rather than n=1. It is also noted that the relaxation process with n>1 can not be well fit with a summation of multiple sub-processess with n=1. These results indicate that the gel-liquid crystalline transition of lipid bilayers folllows the classical Kolmogorov-Avrami kinetic theory with the effective dimensionality close to n=2, and that the common assumption of simple exponential decay (n=1) used in data analysis of most published work in the literature may not be valid. Therefore, one should be cautious when interpreting each nominal exponential decay as representing a specific step (e.g. nucleation) of the overall physical process. (Supported by National Institutes of Health GM37658 and Office of Naval Research N00014-88-k-0326.)

Th-Pos220

Ca^{++} BINDING TO PS MEMBRANES REVISITED. Joel A. Cohen and Deidre M. Bradford, Dept. of Physiology, Univ. of the Pacific, San Francisco, CA 94115.

Despite decades of experimentation, a precise description of the interaction of Ca^{++} with charged phospholipid membranes remains elusive. For isolated membranes containing phosphatidyl-serine (PS), it still has not been demonstrated whether the Ca:PS binding stoichiometry is 1:1 or 1:2 (or both), nor whether Ca^{++} competes with monovalent cations for membrane binding sites. Particle-electrophoretic charge reversal of PS vesicles at 80 mM Ca^{++} in 0.1 M monovalent salt is consistent with some 1:1 binding and K_a=12 M^{-1} if the binding is competitive. We have used a monazomycin conductance probe with PS BLMs to investigate the low [Ca^{++}] regime, where added Ca^{++} (1-100 uM in 0.1-1 M monovalent salts) just begins to produce a detectable shift (<1-5 mV) of membrane surface potential. The initial slope of surface potential vs. bulk [Ca^{++}] is a sensitive indicator of the type of adsorption occurring. Comparison of these slopes for 0.1 M and 1 M CsCl and NaCl discriminates among the possible adsorption isotherms, since in the first case very few binding sites are occupied by monovalent cations, and in the last case most are; hence the comparative amounts of Ca^{++} binding depend strongly on competition and stoichiometry. The initial slopes of surface potential shift vs. bulk [Ca^{++}] for the above solutions at pH 7.2, in mV/M units, are: 0.1 M CsCl $(7.8\pm0.8)\times10^5$, NaCl $(7.4\pm0.8)\times10^5$; 1.0 M CsCl $(2.9\pm0.5)\times10^4$, NaCl $(1.3\pm0.2)\times10^4$. These values are not explainable by simple Gouy-Chapman-Stern 1:1 competitive binding of Ca^{++} to PS. A complete analysis will be presented. (Supported by NIH GM35241)

Th-Pos221

KINETICS OF PRESSURE-INDUCED PHASE TRANSITIONS IN HYDRATED PHOSPHOLIPIDS FROM TIME-RESOLVED X-RAY DIFFRACTION MEASUREMENTS.

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Time-resolved x-ray scattering data were obtained during pressure-jump (P-jump) and pressure-ramping experiments on fully hydrated samples of 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), the latter with measurement of in-sample temperature. Pressures up to 3.06 kbar (45,000 psig) were applied to an x-ray "transparent" beryllium pressure vessel.

The lamellar liquid crystal/ripple ($L_\alpha/P\beta'$) phase transformation in DMPC is induced and reversed during pressure up- and down-ramping between 0 and 1.0 kbar at rates of ±40 bar/s. This is evidenced by changes in the wide- and low-angle x-ray scattering pattern directly relating structural changes to the measured temperature variations due to the heat (ΔH) of transition.

Following the application of a fast P-jump from 0 to 1.3 kbar (19,000 psig), completed in 2 ms, the simultaneous wide- and low-angle scattering pattern changes from the initial L_α to approach that of the $P\beta'$ phase within 33 ms, the frame-rate at which the diffraction pattern was recorded. The diffraction pattern continues to change gradually, especially in the low-angle region, over a period of several minutes possibly reflecting slow annealing of the $P\beta'$ phase. During rapid release to atmospheric pressure all changes are reversed, essentially stabilizing in less than 33 ms. These measurements are obtained with an in-sample equilibrium temperature of 37 °C. A side effect of the up/down P-jumps is a rapid +5 °C/–5 °C deviation of the temperature from equilibrium to which it returns within 3 s.

In the case of DHPE (in equilibrium at *ca.* 83 °C) where in-sample temperature was not measured, the P-jump induced L_α to $L\beta'$ phase transformation is evidenced by a wide-angle scattering peak at ca. 4.2 Å emerging on a time-scale of less than 33 ms. In a correlated experiment, the low-angle scattering, stemming from the (001) lamellar reflection, provides an estimate of the order of 0.15 s for the half-life ($\tau_{1/2}$) for phase interconversion. All reflections sharpen up gradually within the following 1.5 s and no further changes are seen.

During depressurization of DHPE, changes in the (00l) low-angle scattering associated with the $L\beta'$ to L_α transition are complete within 3 - 4 s. These changes clearly trail those in the wide-angle region which disappear in 1 s after the pressure drop. This disparate response of wide-angle and low-angle scattering implies that the chain packing order within the bilayer responds immediately to pressure whereas the lamellar stacking adjusts slowly to the pressure perturbation.

Finally, a measurement of the half-life of the inverted hexagonal (H_{II}) to L_α phase transition (with H_{II} initially in equilibrium at T=100°C following a P-jump from 0 to 3.06 kbar yields 20 ms < $\tau_{1/2}$ < 25 ms.

Th-Pos222

KINETICS OF THE BAROTROPIC $P\beta'/L_\alpha$ PHASE TRANSITION IN FULLY HYDRATED PHOSPHATIDYLCHOLINE MONITORED BY TIME-RESOLVED X-RAY DIFFRACTION (TRXRD).

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We present here the first study of the use of a pressure-jump to induce the ripple ($P\beta'$)/lamellar liquid crystal (L_α) phase transition in fully hydrated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC). The transition was monitored by TRXRD. Applying a pressure-jump from atmospheric to 11.3 MPa (1640 psig, 111.6 atm, 113 bar) in 2.5 s induces the L_α to $P\beta'$ phase transition which takes place in two stages. During the first 10 s after the pressure-jump, the lamellar repeat spacing increases from 66.0 ±0.1 Å (n = 4) to a maximum value of 70.3 ± 0.8 Å (n = 4) slightly *overshooting* the final equilibrium value of 68.5 ± 0.3 Å (n = 4) which it reaches gradually over the next 100-150 s. Wide-angle position and width equilibrate within the fist 20 s, which is also the estimated time for the heat of the transition to dissipate. We speculate that the slow changes following the overshoot corresponds to the accommodation of lipid and water layer thicknesses and the development of ripples in the $P\beta'$ phase. The reverse transition takes place following a pressure-jump in 5.5 s from 11.3 MPa to atmospheric pressure. Again, the transition occurs in two stages with the repeat spacing steadily decreasing from an initial value of 68.5 ± 0.3 Å (n = 3) to a minimum undershoot value of 66.6 ± 0.3 Å (n = 3) after 50 s and then increasing by approximately 0.5 Å over a period of 100 s. The transition temperature increases linearly with pressure up to 14.1 MPa in accordance with the Clapeyron relation, giving a dT/dP value of 0.285 °C/MPa (28.5 °C/kbar).

Th-Pos223

SIMULTANEOUS CALORIMETRY AND TIME-RESOLVED X-RAY DIFFRACTION FOR DIRECT CORRELATION OF STRUCTURAL AND THERMAL EVENTS IN LIPID SYSTEMS

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In many lipid systems, polymorphic and mesomorphic behavior is very much dependent on the thermal history of the sample. To establish uniquivocally the structural origin of endothermic and exothermic events in such systems, we have performed simultaneous calorimetry and time-resolved x-ray diffraction (TRXRD). To this end, thin-walled aluminum DSC crucibles (attenuation at 8 KeV, *ca.* 0.3) were modified slightly and used to contain the lipid samples and the calorimeter was mounted with the crucible oriented perpendicular to a synchrotron-derived focused, monochromatic (1.57 Å) x-ray beam for simultaneous calorimetry and TRXRD data collection. Measurements were made with 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC)/ water (30 wt% water), monoelaidin /excess water and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/ excess 2.5 M ethanol in water samples contained in hermetically sealed crucibles. TRXRD data were collected using an x-ray image intensifier/video system and a streak camera wherein x-ray sensitive film was translated continuously at rates of 4.1 - 6.4 mm/min behind a 3 mm wide slit. Calorimetry scanning rates varied from 1 to 5 °C/min in the temperature range 0 °C to 60 °C.

Calorimetry gives transition temperature and enthalpy change information. X-ray diffraction gives structural information at the level of hydrocarbon chain packing and mesophase long range order. Thus, simultaneous calorimetry and TRXRD measurements enable us to determine the relative contributions to the measured enthalpy change of the various structural rearrangements undergone during a phase transition. More importantly, this "methods-in-combination" approach facilitates the *direct correlation of structural and thermal events in the same sample under identical conditions of thermal history.* The information content of the data so derived far surpasses that available from either method used in isolation.

Th-Pos224

THE USE OF VARIABLE PERIOD X-RAY STANDING WAVES TO MONITOR STRUCTURAL CHANGES OCCURRING IN MODEL MEMBRANES

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Long period x-ray standing wave (XSW) measurements have been used previously to interpret structural rearrangements undergone during a phase transition in a model membrane with ångström resolution (Bedzyk *et al. Science* 241:1788, 1988; *Phys. Rev. Lett.* 62:1376, 1989). In the present study, a similar length scale ranging from 40 to 150 Å has been used without sacrificing resolution while addressing the question of whether the phase transition characteristics are sensitive to the number and identity of the lamellae in the membrane stack. The system chosen for examination consisted of an octadecyl thiol coated gold mirror on top of which a variable number (0 or 2) of ω-tricosenoic acid (C23) bilayers followed by a single, upper bilayer of zinc arachidate (C23) was deposited by the Langmuir-Blodgett technique. Ellipsometry gave zinc-to-gold surface separations of *ca.* 42 and 152 Å, respectively, based on total film thickness measurements. Variable period XSW measurements provided precise positional information on the zinc layer coherent position and fraction and were used to track the collapse of the heavy atom layer during a thermotropic phase transition. The two samples showed quite disparate pretransitional rearrangements, transition temperatures and apparent cooperativity and final, high temperature zinc distribution. However, in both cases the temperature-induced change was not reversed upon cooling to and subsequent storage at room temperature. The results suggest that the variable period XSW method might now be used to obtain heavy atom position not only in membranes but also in peripheral components such as membrane-associated antibodies and cytoskeletal and peripheral proteins.

Th-Pos225

CALORIMETRIC AND X-RAY DIFFRACTION STUDIES OF RYE GLUCOCEREBROSIDE MESOMORPHISM

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Glucocerebrosides (GlcCer) isolated from the leaves of winter rye (*Secale cereale* L. cv Puma) differ from the more commonly investigated natural and synthetic cerebrosides in that greater than 95% of the fatty acids are hydroxy acids which include C24:1(h), C22:1(h), C24:0(h) and C22:0(h). Isomers of the trihydroxy long chain base hydroxysphingenine ($t18:1^{8\ cis\ or\ trans}$) comprise 77% of the total long chain bases. Isomers of sphingadienine ($d18:2^{4\ trans,\ 8\ cis\ or\ trans}$) account for 17% of the total. The phase behavior of fully hydrated and dry rye leaf GlcCer was investigated using differential scanning calorimetry (DSC) and x-ray diffraction. On initial heating, aqueous dispersions of GlcCer exhibit a single endothermic transition at 56 °C and have an enthalpy change (ΔH) of 46 J/g. Cooling to 0 °C is accompanied by a small exothermic transition (ΔH = -8 J/g) at 8 °C. On immediate reheating, a broad exothermic transition (ΔH = -39 J/g) is observed between 10 °C and 20 °C in addition to a transition at 56 °C. These transitions are not reversible, and the exothermic transition rapidly diminishes when the sample is held at low temperature. Using x-ray diffraction it was determined that the endotherm at 56 °C represents a transition from a highly ordered lamellar crystalline phase (L_c) with a d-spacing of 57 Å and a series of wide-angle reflections in the 3-10 Å range, to a lamellar liquid crystalline (L_α) phase having a d-spacing of 55 Å and a diffuse wide-angle scattering peak centered at 4.7 Å. Cooling leads to the formation of a metastable lamellar gel phase (L_β) with a d-spacing of 64.0 Å and a single broad reflection at 4.28 Å. Subsequent warming to above 15 °C restores the original L_c phase Thus, rye GlcCer in excess water exhibit a series of irreversible transitions and gel phase metastability. In contrast to the hydrated lipid dry GlcCer undergo an initial heating endothermic transition at 130 °C which is ascribed to a transformation into the H_{II} phase from a two phase state characterized by the coexistence of phases with disordered (α) and helical (δ) type chain conformations but of unknown lattice identity. An exotherm at 67.5 °C observed upon subsequent cooling is of unknown origin. Since an undercooled H_{II} phase persists down to 19 °C, the exotherm may derive in part from an α-to-δ type chain packing conformational change especially under slow cooling conditions. Upon reheating from low temperatures to 65 °C a phase with a two-dimensional, primitive rectangular lattice and δ-like chain packing ($R\delta$ phase) in coexistence with the H_{II} phase emerges. With continued heating to 90 °C these coexisting phases give way to a phase with a two-dimensional, centered rectangular lattice and δ-like chain packing ($P\delta$ phase) which again coexists with the H_{II} phase. Above 130 °C, the $P\delta$ phase disappears and the sample converts completely to the H_{II} phase as observed upon initial heating. These results indicate that the mesomorphic behavior of rye leaf GlcCer is distinct from that of other cerebrosides.

Th-Pos226

POLYMORPHISM AND METASTABILITY OF FULLY HYDRATED MONOELAIDIN MONITORED BY SIMULTANEOUS CALORIMETRY AND TIME-RESOLVED X-RAY DIFFRACTION

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Problems associated with a pronounced thermal history dependent phase behavior can be overcome by performing simultaneous calorimetry and time-resolved x-ray diffraction (TRXRD) on a single sample. Such an approach has been used in the study of the polymorphism and metastability of fully hydrated monoelaidin (ME). Each simultaneous calorimetry/TRXRD measurement was made on a sample in a modified, hermetically sealed aluminum crucible. Scan rate was controlled to 5 °C/min in both heating and cooling directions in the temperature range 4 °C to 60 °C. Diffraction data were collected using a streak camera in which x-ray sensitive film was translated continuously at a rate of 4.1 mm/min behind a 3.175 mm slit. Analysis of the streak film data provides structural characterization and identification of the individual and coexisting phases. The nature of the phase transitions undergone is apparent from an examination of the film in the transition region.

ME/water samples were prepared at room temperature and stored at 4 °C for a week before measurement. The initial heating scan from 4 °C to 60 °C showed complex phase behavior. The cooling scan of samples which had been cycled thermally twice between 4 °C and 47 °C gave a single exotherm at 22 °C, which was assigned to an L_α + $Im3m(Q^{229})$-to-L_β transition. Upon immediate reheating from 4 °C, L_β-to-L_α and L_α-to-Im3m phase transitions were seen at 23 °C and 38 °C, respectively. Following a 6 min incubation at 4 °C, coexistence of an L_β and a lamellar crystalline phase was observed at the beginning of the heating scan. When the sample was held at 4 °C for 15 min, a lamellar crystalline phase alone, i.e., with no L_β present, was seen at the beginning of the heating scan which showed a complex phase behavior above 12 °C. Isothermal conversion from the L_β phase, which formed immediately upon cooling, to a lamellar crystalline phase was complete in 8 min at 4 °C. This lamellar crystalline phase was different from the one observed in the initial heating scan. The slow isothermal conversion from the L_β to the lamellar crystalline phase indicates L_β phase metastability.

Th-Pos227

TEMPERATURE-COMPOSITION PHASE DIAGRAM OF THE MONOPALMITOLEIN/WATER SYSTEM DETERMINED BY X-RAY DIFFRACTION

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A temperature-composition phase diagram of monopalmitolein in water was constructed using X-ray diffraction. Low- and wide- angle diffraction patterns of samples of fixed composition in the range of 0% to 53% water (w/w) were collected at the Cornell High Energy Synchrotron Source on X-ray sensitive film. Samples were incubated for approximately five minutes at temperatures ranging from 0°C to 120°C in 10°C steps in the heating direction only. Patterns from a lyotrope gradient sample were collected in real time as the sample was translated through the X-ray beam to ensure that narrow phase regions were not missed as a result of using a finite number of samples of fixed composition (Caffrey, M. 1989. *Biophys. J.* 55:47-52). The phases identified in the phase diagram include at least one lamellar crystalline phase, the lamellar liquid crystalline phase (L_α), the fluid isotropic phase, and two inverted cubic phases (Pn3m and Ia3d). The lamellar crystalline phase(s) is (are) present at compositions of less than 5% water (w/w) between 0°C and 20°C. The L_α phase is found in the composition range of 5% to 50% water (w/w) and up to a temperature of approximately 70°C. At compositions greater than 30% water (w/w) and at 10°C, the L_α and Ia3d phases coexist. The two cubic phases appear at compositions of approximately 30% water (w/w): Ia3d at lower temperatures and Pn3m at temperatures above approximately 50°C. All liquid crystalline character is lost above 110°C. Of interest is the absence of the inverted hexagonal phase which is seen in the related monoolein/water system. Based on the diffraction measurements, a pronounced dependence of the lattice parameter of the different phases was not apparent. However, with all of the liquid crystalline phases, a negative thermal lattice coefficient was observed.

Th-Pos228

X-RADIATION DAMAGE OF AQUEOUS PHOPHOLIPID DISPERSIONS MONITORED BY X-RAY DIFFRACTION

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Radiation damage of aqueous phospholipid dispersions upon exposure to synchrotron x-radiation at 7.9 keV (1.58 Å) is reported. In this study the effect of incident flux, aqueous phase composition, degree of lipid hydration, and lipid and mesophase identity on the severity of damage was evaluated using time-resolved low- and wide-angle x-ray diffraction. ESR measurements of spin-trapped intermediates generated during irradiation suggest a free radical mediated process.

The damaging effect of x-radiation was evident in the diffraction patterns as an increase in the diffuse scattering which was pronounced between the first and second order lamellar reflections and an overall decrease in the low- and wide-angle diffracted peak intensity. For example, the area of the (002) lamellar diffraction from fully hydrated DPPC at 30 °C in the $L_{\beta'}$ phase decays to 1/e of the original in 7 min with an accumulated dose of $6x10^{12}$ photons (7 MRad). No significant difference was observed in the decay pattern of this same sample with an identical accumulated dose when beam intensity was varied by a factor of 2.5. Of the fully hydrated lipid systems studied, DPPC was most sensitive to radiation damage while the effect lessens for a DPPC/DPPE (1:1 mol/mol) mixture and becomes imperceptible in the case of pure DPPE and DOPE. The addition of HEPES, Tris/HCl, or phosphate buffer or NaCl to the aqueous medium appears to lessen the degree of radiation damage. Radiation effects on DPPC in both $L_{\beta'}$ and L_α phases are considerably reduced when the lipid is water stressed. These results contribute to our understanding of the mechanism of radiation damage and the importance of structure and composition in determining radiation sensitivity.

Th-Pos229

LIPIDAT: A DATABASE OF LIPID PHASE TRANSITION TEMPERATURES AND ENTHALPY CHANGES - UPDATE AND DMPC DATA SUBSET ANALYSIS.

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The systematic study of the mesomorphic phase properties of synthetic and biologically derived lipids began some 30 years ago. In the past decade, interest in this area has grown enormously. As a result, there exists a wealth of information on lipid phase behavior, but unfortunately, these data have, until now, been scattered throughout the literature in a variety of books, proceedings and journals. The data have recently been compiled in a centralized database, LIPIDAT, with a view to providing ready access to same and to the appropriate literature. LIPIDAT consists of a tabulation of all known mesomorphic and polymorphic phase transition temperatures and enthalpy changes for synthetic and biologically-derived lipids in the dry and in the partially and fully hydrated states. Also included is the effect on these thermodynamic values of pH, and of salt and metal ion concentration and other additives such as proteins, drugs, etc. The methods used in making the measurements and the experimental conditions are reported. Bibliographic information includes complete literature referencing and list of authors. As of this writing, the database is current through June, 1989 and contains in excess of 7,000 records. Each record contains 28 fields.

An analysis of the fully hydrated DMPC data subset of LIPIDAT has been performed. The subset includes data collected over a 23 year period from 1967 to 1989 and consists of 694 records obtained from 331 articles in 55 different journals. The number of records per year rises steadily beginning in 1971, reaches a maximum of 89 records/year in 1977 and remains relatively constant at 60-70 records/year in the succeeding period. Journals making the greatest contribution to the DMPC subset include **Biochimica et Biophysica Acta, Biochemistry, Chemistry and Physics of Lipids** and the **Biophysical Journal**. These four journals account for 71% of the total records. The analysis shows that DSC, ESR, Fluorescence, NMR and Raman are the methods most commonly used for transition temperature determination and an interesting pattern emerges as to the place in time the different methods assume or loose popularity. The main, pre and subtransition have been described, respectively, in 24, 8 and 2 distinct ways in the literature, an observation that points to the urgent need for a universally accepted lipid phase notation. The distribution of main, pre and subtransition temperatures and enthalpies determined in the heating and cooling direction and their dependence on aqueous phase additive, scan rate and methods used will be described.

Th-Pos230

X-RAY STANDING WAVES: A MOLECULAR YARDSTICK FOR MEMBRANES USEFUL IN THE 0 - 1,000 Å RANGE

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The ability to obtain structural information with subångström resolution for Langmuir-Blodgett (LB) model membranes using long period x-ray standing waves (XSW) has been demonstrated previously (Bedzyk *et al.* 1988. *Science* 241, 1788). In this case, the atom layer of interest was situated 53 Å above the solid substrate surface. In the present study we wished to determine if the variable period XSW generated by an x-ray mirror during total external reflection could be used to locate precisely and accurately a heavy atom layer positioned several hundred ångströms above the mirror surface. Such a situation would prevail in a host of biologically relevant model systems. The sample series examined in this study consisted of an octadecyl thiol coated gold mirror on top of which a variable number (0, 2, 8, 14) of ω-tricosenoic acid (C23) bilayers followed by a single, upper bilayer of zinc arachidate (C23) was deposited by the LB technique. Ellipsometry gave zinc-to-gold surface separations of *ca.* 42, 152, 584 and 928 Å, respectively, based on total film thickness measurements. The zinc K_α fluorescence yield profiles from the sample set are in excellent agreement with the calculated XSW electric field distribution. More importantly, the results demonstrate that *the XSW field is well defined at close to a thousand ångströms above the mirror surface.* The numerical fitting of the data is in progress and a precision of ångströms is expected for both zinc atom layer mean position and width. The quality of these data demonstrate the enormous potential of XSW as structural probes in membranes and in thin film related phenomena.

Th-Pos231

A PARTIAL PHASE DIAGRAM OF MIXTURES OF TWO MAJOR ROD OUTER SEGMENT PHOSPHOLIPIDS. A ^{31}P NMR STUDY.

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The lipid composition of bovine rod outer segment (ROS) membranes is very unique, with docosahexaenoic acid (22:6) comprising about half of the fatty acids. It has been suggested that the ROS lipids in some way influence the function of the visual ROS protein rhodopsin. This work explores the possibility that the nature of the lipid-rhodopsin interaction is related to the same properties which dictate the phase diagram of the membrane lipids. The phase behavior of mixtures of two major constituents of the ROS, (16:0)(22:6)PC and (16:0)(22:6)PE, was monitored with ^{31}P NMR spectroscopy as a function of temperature, water content, and the relative concentration of the two lipids. In some of the mixtures an isotropic NMR signal occurs between the lamellar liquid-crystalline (L_α) and inverted hexagonal (H_{II}) phases, which presumably corresponds to an inverted cubic phase.

Supported by NIH Postdoctoral Fellowship EY06111 (J.A.B.).

Th-Pos232

POLYMORPHIC PHASE BEHAVIOR OF DOPE-DOPG MIXTURES.

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We have examined the phase diagram of dioleoyl phosphatidylamine-dioleoyl phosphatidylglycerol (DOPE-DOPG) mixtures containing minor amounts of DOPG by ^{31}P NMR spectroscopy and freeze fracture electron microscopy. At low mole% PG (<5), the phase diagram is very similar to that of DOPE alone, with a bilayer to inverted hexagonal phase transition at approximately 5°C. At PG contents ranging from 5 to 20 mole%, a complex ternary phase structure consisting of bilayer, inverted cubic and inverted hexagonal phases is observed over a wide temperature range. The fraction of inverted hexagonal phase decreases with increasing PG content, and with decreasing ionic strength. These results are readily explainable by formulations developed by Gruner and co-workers, where the tendency to form the inverted phase is shown to be modulated by the lipid spontaneous radius of curvature and the energy inherent in packing acyl chains (Gruner, S. M. Proc. Natl. Acad. Sci. 82, 3665, 1985). Electrostatic repulsion between negatively charged PG headgroups likely decreases the spontaneous radius of curvature, and results in changes in geometric structure of the inverted phase which hinder acyl chain packing. The observed phase structure is highly dependent on the details of sample preparation and history. Also, this system exhibits extreme metastability with temperature variation. The enhanced metastability in this system relative to DOPE suggests a higher energy barrier for intermediate formation necessary for phase transitions. This decreased rate of phase transitions may be a general property of multi-component systems.

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Th-Pos233

THE THERMOTROPIC PHASE PROPERTIES OF A HOMOLOGOUS SERIES OF SYNTHETIC 1,2-DI-O-ACYL-3-O-(β-D-GALACTOPYRANOSYL)-*SN*-GLYCEROLS.

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We have synthesized a homologous series of saturated 1,2-di-O-*n*-acyl-3-O-(β-D-galactopyranosyl-*sn*-glycerols with acyl chain lengths ranging from 10 to 20 carbon atoms and have investigated their physical properties using DSC, X-ray diffraction and FTIR spectroscopy. The DSC results show a complex pattern of phase behaviour, which in a typical preheated sample consists of a lower temperature, moderately energetic L_β/L_α phase transition and a higher temperature, weakly energetic BL/NBL phase transition. On annealing at a suitable temperature below the L_β/L_α phase transition, the L_β phase converts to an L_c phase which may undergo a highly energetic L_c/L_α or L_c/H_{II} phase transition at very high temperatures on subsequent heating. The nature of these phase transitions has been confirmed by both FTIR spectroscopic and X-ray diffraction measurements. The transition temperatures of all of these events are higher than those seen in the corresponding α- and β-glucosyl glycerols. In contrast, the L_β/L_α and BL/NBL phase transition temperatures of the β-galactosyl glycerols are lower than those of the corresponding diacyl PE's. The rate of conversion from the L_β to the L_c phase in the β-galactosyl glycerols is slightly faster than that seen in the α-glucosyl glycerols and much faster than that seen in the corresponding β-glucosyl glycerols. A comparison of the first order spacings for the analogous phases in the three glycolipids is revealing. Those of both β-linked glycolipids are similar and are greater than the comparable values obtained for the α-glucosyl glycerol. This suggests: (1) that there is a difference in the headgroup orientation between the α- and β-anomers; (2) that the headgroup orientations of the β-glucosyl and -galactosyl glycerols may be similar; (3) that the differences in the phase behavior of the β-anomers is dependent on hydration and H-bonding in the headgroup and interfacial regions.

Th-Pos234

SOLUBILIZATION OF EGG PHOSPHATIDYLCHOLINE VESICLES BY ALKYL GLUCOPYRANOSIDES (C_n-G).

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The C_n-G is one of the best detergent for protein solubilization and is often used for vesicle reconstitution. The behavior of the C6- to C12-G compounds in solution (distilled water and NaCl 145 mM, Hepes 10 mM, pH 7.4) were studied by ANS fluorescence dequenching, surface tension and 90 light scattering measurements. At room temperature, C_n-G, with n from 6 to 9, form homogeneous solutions even above their critical micelle concentrations (CMC), while those with n from 10 to 12 are insoluble. However, the dependence of the CMC on the carbon chain length was determined, and it was shown that the presence of salt in solution decreases the CMC of these non-ionic detergents. The mechanism of Egg Phosphatidylcholine (EPC) vesicles solubilization by C8-G which has been extensively studied (1, 2) and begins to be understood, is compared to those induced by other alkyl glucopyranosides, C7-G and C9-G. The solubilization processes were followed by turbidimetry and fluorescence resonance energy transfer (FRET) measurements. The limits of the vesicular and micellar phases were deduced from the experiments. In particular, the molar $[C_n\text{-G}]/[lip]$ ratio in the mixed aggregates (vesicles saturated with detergent or mixed micelles) and the $[C_n\text{-G}]_{monomer}$ in equilibrium with these mixed aggregates were determined. The saturation of vesicles occurs at a $[C_n\text{-G}]/[lip]$ ratio of 1.3, 1.4 and 2.6 for C7-, C8- and C9-G respectively, while the respective monomer concentration in equilibrium with these aggregates are 50.3, 16.9 and 5.5 mM. The corresponding molar ratios for the mixed micelles are 2.1, 2.7 and 5 for C7-, C8- and C9-G respectively with monomer concentration in equilibrium of 52.4, 17.8 and 6.1 mM. An appropriate analysis of the FRET experiments gave the evolution of the partition coefficient of the detergent all along the solubilization process.

(1) : Ollivon et al., (1988), Biochem., 27, 1695-1703.
(2) : Paternostre et al., (1988), Biochem., 27, 2668-2677.

Th-Pos235

MOLECULAR RECOGNITION OF OPTICAL ISOMERS (+)- AND (-)-CARVONE BY PHOSPHOLIPID MONOLAYERS.

[1]Vitaly Vodyanoy, [2]Suram Pathirana, [1]Lawrence J. Myers, and [2]William C. Neely. [1]*Institute for Biological Detection Systems, College of Veterinary Medicine,* [2]*Department of Chemistry, Auburn University, AL 36849.*

The effects of odorants (+) and (-)-carvone on surface properties of 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) monolayers were studied with a Wilhelmy surface balance (KSV 2200, KSV-Chemicals, 0-100, 0.05 mN/m). Surface pressure as function of surface area was measured in the temperature range 10-35 ^{0}C in the presence and absence of 5 mM of the odorants in the subphase. Thermodynamic values of free energy (ΔG), entropy (ΔS), and enthalpy (ΔH) of monolayer compression were calculated as function of temperature, surface pressure and surface area.[1] Our data indicate that the DPPC monolayer with (-)-carvone absorbs twice as much heat as DPPC monolayer with (+)-carvone when compressed at 25-30 ^{0}C. Under the same conditions, DPPC monolayers with (-)-carvone undergo a higher entropy change than DPPC monolayers with (+)-carvone. High values of ΔH and ΔS for DPPC monolayers with (-)-carvone compared to ΔH and ΔS of the monolayers with (+)-carvone may be related to findings of Leitereg et al.[2] who reported that the (-)-carvones are substantially stronger odorants than the (+)-carvones on a threshold basis. This possibility of an important role of phospholipid components of olfactory receptor membrane in olfaction does not necessarily conflict with hypothesis concerning chemospecific protein receptor sites, but does offer an added dimension to the complex question of olfactory detection.

Supported by: U.S. Army DAAL03-90-G-0009 (VV); Office of Naval Research N00014-90-J-1515 (WCN)

1. H. Ito, T.H. Morton, V. Vodyanoy (1989) Thin Solid Films, 180, 1-13.
2. T.J. Leitereg, D.G. Guadagni, J. Harris, T.R. Mon, R. Teranishi (1971) Nature, 230, 455-456

Th-Pos236

POLYMERIZABLE THIN FILMS: MISCIBILITY OF DIACETYLENIC PC'S AND SHORT-CHAIN SATURATED PC'S

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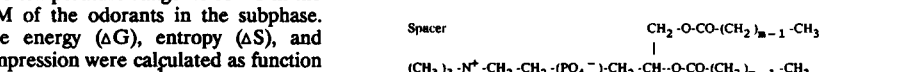
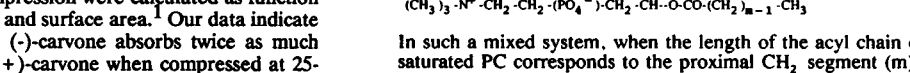
We have used L/B methods to investigate the miscibility of short-chain saturated PC lipids (spacers) with long-chain polymerizable diacetylenic PC's, $DC_{m,n}PC$.





In such a mixed system, when the length of the acyl chain on the saturated PC corresponds to the proximal CH_2 segment (m) of the diacetylenic lipid, the polymerization is enhanced [Singh et al.]. Earlier diffraction data with (m=8) multibilayers had indicated coexistence of a short (40 Å) repeat lattice and a long (69 Å) repeat lattice. To determine whether this coexistence was due to a lateral phase separation or had developed as an artifact of the multilayer preparation, we investigated the phase behavior of Langmuir films of the mixed lipid system as a function of composition. The data indicate that the two lipids are miscible over a fairly wide range of composition, and that the mixture is nonideal at low π's (5-30 dyne/cm), but nearly ideal at higher π's (35-40 dyne/cm). The monolayer thin films are polymerizable, and the degree of polymerization appears to be enhanced by spacer lipids. Structure experiments with Langmuir- Blodgett deposited multilayers of mixed lipids are currently underway.

Supported by NSF grant #CTS-8904938 (DGR).

Th-Pos237

ASSAY FOR LONG CHAIN UNBOUND FREE FATTY ACIDS WITH FLUORESCENTLY-LABELED INTESTINAL FABP. A.M. Kleinfeld, R.T. Ogata, and G.V. Richieri. Medical Biology Institute, La Jolla, CA 92037.

A fluorescently modified form of the intestinal fatty acid binding protein (I-FABP) has been developed to determine levels of unbound free fatty acid (FFA) The cDNA for I-FABP was cloned from a neonatal rat intestinal library and the amino acid sequence was found to be identical with that obtained by Lowe, *et al.* (1987, *J. Biol. Chem. 262*:5931). Recombinant protein was expressed to high levels in E. coli using the pET-11 expression system, and protein purification was done essentially as in Lowe, et al. Addition of μM levels of FFA quenched the native protein's tryptophan fluorescence by up to 40% at ~20μM total FFA. This result, together with the crystal structure of I-FABP (Sacchettini, *et al.* (1988, *J. Biol. Chem. 263*:5815), suggested that the region around Trp_{83} would be sensitive to FFA binding. Threonine residues flanking Trp_{83} point away from the FFA binding pocket and we chose to modify these residues in order not to perturb seriously FFA binding. Site directed mutagenesis was used to introduce a cysteine residue (no cysteine occurs in the native protein) at either Thr_{82} or Thr_{84}. Expression and purification of the mutated protein was done using the same procedures as for the native protein. The mutated protein was derivatized with the fluorescent thiol reagent ACRYLODAN. In preliminary studies, addition of long chain FFA to this modified protein caused a monotonic spectral change that was isosbestic with increasing FFA concentrations. These changes occur in the same concentration range as those observed in the native protein and suggest that the mutagenesis and fluorescent modification did not significantly alter the FFA binding characteristics of the protein. More importantly, these results indicate that the ratio of fluorescence emissions at 460 and 530nm may provide a direct measure of the concentration of unbound FFA. This work was supported by grants BE-11 and 89-139, from ACS and AHA, respectively and by LIDAK Pharmaceuticals.

Th-Pos238

CONFORMATIONAL CHANGES IN BAND 3 BY *IN SITU* INHIBITOR/CROSSLINKING AGENTS. M.M. Batenjany and H. Mizukami. Division of Regulatory Biology and Biophysics, Department of Biological Sciences, Wayne State University, Detroit, MI 48202.

Band 3 is one of the most highly represented integral proteins found in the human erythrocyte membrane. The protein exists as dimeric and tetrameric forms *in situ* which may be functionally interchangeable. Band 3 transports a wide variety of mono- and divalent anions and various inhibitors/crosslinking agents have been found.

Band 3 and it's membrane domain (B3MD) were studied after the cytoplasmic or exofacial exposure of red cells to a number of inhibitors, in conjunction with BS^3 and some diimidate crosslinkers of differing lengths (C_4-C_8). The different conformational states of Band 3 and B3MD (i.e. the putative outward or inward facing forms) were studied using far-UV and near-UV circular dichroism (CD), SDS-PAGE, and nondenaturing PAGE. Additionally, we followed these changes in stripped erythrocyte ghosts, Lubrol-PX solubilized Band 3 forms, and after ion-exchange chromatography.

DIDS (exofacial) or PITC (cytoplasmic) treatment show distinct changes in the near-UV CD spectra of Band 3 and B3MD. The near-UV CD spectra of B3MD also show noticeable changes after exofacial treatment with pyridoxal-5'-phosphate. Both Band 3 and B3MD show little change in secondary structure after treatment with inhibitors or crosslinking agents. Crosslinking of B3MD by the C_8 diimidate agent was similar in intact ghosts, Lubrol-PX solubilized B3MD and after ion-exchange chromatography. The correlation among CD spectra, protein aggregation states, crosslinked products, and inhibitor treatment is reported. This project was funded by NIH grant HL16008.

Th-Pos239

EFFECTS OF THE GENERAL ANESTHETIC HALOTHANE ON LIPID ORDER AND BAND 3 ROTATIONAL DYNAMICS AND FUNCTION IN HUMAN ERYTHROCYTES. C.E. Cobb, S. Juliao, & A.H. Beth, Mol. Physiol. & Biophysics, Vanderbilt Univ., Nashville, TN 37232. We have previously reported (Cobb, et al., *Biochemistry, in press)* that incorporation of diethyl ether into *intact* human erythrocyte membranes preferentially fluidized lipids at the lipid-water interfaces of the bilayer as reported by spin labeled fatty acid probes, increased rotational motion of spin labeled band 3, and had no significant effect on the kinetic or thermodynamic properties of sulfate / chloride exchange. We have performed similar experiments with the general anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane), using sufficiently low concentrations (0 - 1%, v/v) to minimize changes in erythrocyte morphology detectable by light microscopy and to prevent hemolysis from exceeding 5%. Measurement of membrane lipid order in *intact* human erythrocytes by EPR spectroscopy (via the introduction of stearic acid spin labeled in the 5- or 16- position) indicates that halothane decreases lipid ordering in the midzone of the bilayer as well as near the surface of the membrane at 20° C. The membrane fluidizing effect of halothane is reversible upon removal of the anesthetic by washing the cells. Saturation transfer EPR has been employed to examine the rotational dynamics of band 3 labeled with the membrane impermeant bifunctional spin label BSSDA. In contrast to diethyl ether, halothane decreases the rotational motion of band 3 in the same concentration range used for the spin labeled fatty acid experiments. However, like ether this effect is fully reversible upon removal of halothane. The rate of sulfate / chloride exchange is slightly inhibited by halothane. The molecular basis for the decreased rotational mobility of band 3 is under active investigation. The present results, when compared with those of earlier studies on diethyl ether, as well as studies underway with other general anesthetics, indicate that the anesthetics all fluidize erythrocyte membrane lipids to some extent, but their effects on band 3 motion and function are variable. *Supported by HL34737 & RR04075.*

Th-Pos240

INFLUENCE OF NONIONIC SURFACTANT ON THE VISCOELASTIC RESPONSE OF PACKED SUSPENSIONS OF SICKLE CELLS. Bruce E. Coffey, Roger Tran-Son-Tay, and Robert M. Hochmuth. Mechanical Engineering and Materials Science. Duke University, Durham, N.C. 27706

We have studied the effects of a nonionic, copolymer surfactant (RheothRx®, Burroughs Wellcome Co.) on the rheology of packed suspensions of washed normal, sickle, and density separated sickle cells in an attempt to see what influence the surfactant may have on cell-cell interactions. The rheological properties of packed suspensions of cells are dependent upon many factors including cell rigidity, cell shape, and cell-cell interactions such as adhesion. While it has been established that sickle cells are more adherent to cultured vascular endothelium than are normal cells (Hebbel, 1980, J. Clin. Invest., 65: 154-160), the relative importance of cell-cell interactions in determining the response of packed cell suspensions is not known. RheothRx (MW 8400) is composed of a hydrophobic polyoxypropylene center, flanked on either side by hydrophilic polyoxyethylene. This surfactant inhibits abnormal hydrophobic interactions by binding to hydrophobic surfaces with its central region and covering these areas with its flexible hydrophilic end chains. Thus, by comparing the rheological properties of control suspensions with those of suspensions with surfactant added, influences due to any abnormal hydrophobic interactions between cells in the suspension can be seen. Measurements of steady-state viscosity as well as dynamic viscous and elastic moduli were made at 25°C with a magneto-acoustic ball microrheometer developed in our laboratory. For normal red cells, the results indicate no difference between the control and suspensions with surfactant. Similar results were obtained for sickle cells, except for those suspensions that were very viscous. In this case, the addition of RheothRx significantly reduced the loss (viscous) and storage (elastic) moduli, apparently by decreasing abnormal hydrophobic interactions between cells. Results for density-separated sickle cells are also discussed.
(supported by NIH grants HL 23728 and HL28391)

Th-Pos241

Mg INHIBITION OF KCl COTRANSPORT IN LK SHEEP RED BLOOD CELLS REQUIRES THE PRESENCE OF ATP.
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The effect of Mg on Cl-dependent K efflux was investigated in control and ATP depleted low K (LK) sheep red blood cells. Internal Mg was varied using the ionophore A23187 and controlling extracellular free Mg concentration with EDTA. ATP depletion was achieved by incubating the cells 4 hours in the presence of deoxy-D-glucose.

Mg depletion caused a significant activation of K efflux. The level of stimulation, however, was dependent on the order of treatment. Thus, activation was maximal when the cells were first depleted of Mg, and in the presence of ATP. Any further ATP manipulation had no effect. In contrast, when the cells were first depleted of ATP, the stimulation associated with subsequent Mg removal was significantly lower. These data strongly demonstrate the requirement of ATP for the Mg effect. Moreover, increasing the total cellular Mg concentration from 0 to 1200 µmol/liter original cell (which constitutes the normal Mg level of LK sheep red cells), induced a 70% inhibition of K efflux in controls and a 30% inhibition in ATP-depleted cells.

In the absence of Mg, the K efflux failed to exhibit the volume sensitivity characteristic of the Cl-dependent K fluxes: all fluxes were identical at the osmolarities tested between 240 mOsM and 440 mOsM.

Finally, the inhibition of RbCl influx by Mg did not require the presence of internal K or Cl. The data suggest that Mg does not interfere with the internal binding sites for K or Cl. (Supported by a Grant-in-Aid from the American Heart Association.)

Th-Pos242

HUMAN ERYTHROCYTE SHAPE CHANGE INDUCED BY CHANGES IN BUFFER PH IS MOST DEPENDENT ON CHANGES IN CYTOPLASMIC PH. M.M. Gedde and W.H. Huestis, Department of Chemistry, Stanford University, Stanford, CA 94305.

Human erythrocytes, normally smooth biconcave discs, are known to undergo stomatocytosis (cupping) in low pH buffers and echinocytosis (crenation) in high pH buffers at room temperature. Glaser (J. Membrane Biol. 66: 79-85, 1982) reported that changes in membrane potential cause external pH-induced shape change. However, changes in buffer pH strongly affect not only membrane potential, but also cytoplasmic pH, cell water, and cell chloride, and Glaser's design did not separate the effects of these other parameters. Here, cells were prepared in a broad range of combinations of cell pH, potential, water, and electrolyte by varying buffer pH, Cl^-, and osmolality and by changing cell K^+ using the ionophore nystatin. Cell water content was measured gravimetrically, cell pH was measured in freeze-thaw lysed cell pellets, and potential was calculated from cell and buffer pH's; cell electrolyte was the sum of cell K^+ and Cl^- activities, with cell Cl^- calculated from buffer Cl^- and membrane potential. Multivariate regression analysis generated a well-conditioned model of the dependence of cell shape on the four parameters.

Red cell shape was most strongly dependent on cytoplasmic pH: in general, normal pH cells were discs, low pH cells were stomatocytes, and high pH cells were echinocytes. Low and normal pH cells varied little from the stomatocytic or disc shape as cell water, electrolyte, and potential varied widely. However, the degree of echinocytosis at high pH was affected by the other three parameters. Echinocytosis was greater when cells were shrunken, and was relieved when cell water content was high. High pH cells were protected against echinocytosis by very high electrolyte activities, while low electrolyte activities intensified the crenation, but this effect was apparent only when membrane potential was around 0 mV. The results indicate that pH-induced shape change is a product of several interacting factors that are dominated by internal pH, and that the electrical environment of the membrane has a role in maintenance of cell shape. The mechanisms of these effects are being investigated. (Supported by NIH HL 23787.)

Th-Pos243

SHAPE VARIATION & DENSE CELL FORMATION IN VIVO OF SICKLE ERYTHROCYTES IN RELATION TO PAINFUL CRISIS K. Horiuchi, N. Hijiya, J. Ohata, M.J. Stephens, *T.G. Gabuzda, E. Schwartz, T. Asakura, *The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine and *Thomas Jefferson Medical College, Philadelphia, PA.*

Changes in morphology and the percentage of dense cells in red blood cells from two patients with homozygous sickle cell disease were studied weekly or biweekly over a period in which painful crises occurred. Sickle cells (SS cells) were separated into two fractions; less dense fraction (density<1.11) and dense fraction (d≥1.11) using Percoll-Hypaque density gradient. Morphology of oxygenated and density-separated sickle cells was studied by an automated image analysis system using two shape factors: circular shape factor (CSF) and elliptical shape factor (ESF). CSF is sensitive to indentation and elongation while ESF is sensitive only to elongation. We found that the morphology of SS cells in the dense fraction was statistically deformed or elongated more than that of cells in the less dense fraction. We also found that morphology of oxygenated SS cells in the less dense fraction from both SS patients was almost round and did not change much over several months, irrespective of painful crises. In contrast, mean CSF and ESF values for cells in the denser fraction decreased gradually for 3 to 4 months before crisis in each patient. After the crisis, both CSF and ESF values increased, indicative of a relatively rounded shape followed by a gradual decrease again. One of the patients had a second crisis after this decrease. To further characterize these changes, we used a new parameter, (1-CSF)x(dense fraction) and (1-ESF)x(dense fraction), for weighting the degree of deformation. The calculated values tended to increase before the painful crises and to drop after the crises during each of the two crisis episodes. In conclusion, the morphology of oxygenated SS cells seems to be correlated with the dense cell formation, and the degree of deformation appears to be related to painful crisis in patients with sickle cell disease. *(Supported in part by NIH HL 44640 and HL 38635).*

Th-Pos244

INTERACTION OF CHARYBDOTOXIN (ChTX) WITH Ca-ACTIVATED K CHANNELS OF HUMAN RED BLOOD CELLS.
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ChTX is known to inhibit with high affinity the Ca-activated K channel ($P_{K(Ca)}^G$) of human red blood cells. We studied the binding characteristics more fully by using a highly purified preparation of ChTX (see J. Biol. Chem. 264: 20902, 1989) kindly provided by M.L. Garcia. We optimized the assay system to measure Ca-induced net K loss from washed, energy-depleted red cells (pretreated with 50 μM DIDS) by suspension at 3-4% hematocrit in a low ionic strength medium containing 0.05 mM KCl, 10 mM NaCl, 5 mM HEPES, 248 mM sucrose, 0.1% BSA, 80 μM $CaCl_2$ ± ChTX. K loss, measured with a K-electrode over 10 min at 37°C., was initiated by the addition, to a final concentration, of 400 nM A23187. A linear relationship between ChTX concentration and % inhibition of $P_{K(Ca)}^G$ was found with a K_I of 200-400 pM, with essentially complete block at 2 nM. The % inhibition seen at 2 min preincubation (at 23°C.) with a specific concentration of ChTX, prior to assay at 37°C., was the same when preincubation was extended to 90 min. The inhibition by ChTX was not reversible by washing the cells over extended periods of time. Cells partially inhibited by ChTX could be further inhibited by addition of fresh ChTX to the suspension medium. The supernatant from partially inhibited cells, while not eliciting further inhibition of those particular pre-exposed cells, could inhibit $P_{K(Ca)}^G$ of new (not previously exposed) cells. This latter finding provided a bioassay for estimating the inhibitory potency of ChTX remaining in supernatant that had been consecutively exposed to a known number of red cells. The concentration of ChTX in the supernatant of each consecutive exposure was determined from the dose-response curve referred to above. This approach yielded a maximum number of ChTX binding sites approximating 1400 per red cell. This result (presuming that binding sites represent K channels) differs by 2 orders of magnitude from those estimates based on single channel conductance. (This work was supported by NIH grants HL-09906 and DK-07259.)

Th-Pos245

LACK OF SIGNIFICANT FIXED CHARGE EFFECTS ON ANION CONCENTRATION NEAR THE EXTERNAL-FACING TRANSPORT SITE OF THE HUMAN ERYTHROCYTE BAND 3 ANION TRANSPORT PROTEIN. Si-qiong Liu and Philip A. Knauf. Dept. of Biophysics, Univ. of Rochester Sch. of Med., Rochester, NY 14642.

Previously we reported that the dissociation constant for iodide binding to the outside-facing transport site, K_{o}^{I}, increases with external pH (pH_o). In theory, this could be due to direct titration of groups involved in substrate binding (substrate binding site model, SBS), or to titration of positive fixed charges, which increase the anion concentration in a local region near the transport site (fixed charge model, FC). Both models predict linear Dixon plots of 1/Cl flux versus $[I_o]$, as observed. The FC model, however, predicts that at low pH_o and low external anion concentrations, a plot of the ID50 (concentration required to half-inhibit Cl flux) for external iodide versus $[Cl_o]$ should be a straight line which passes through the origin. In contrast, for the SBS model, the ID50 versus $[Cl_o]$ plot has a positive y-intercept equal to K_{o}^{I}. In fact, at pH 7.2 the line which best fits the data crosses the ordinate at 2.0 mM, which is significantly different from zero ($p<.001$), contrary to the FC model. Other data which appear to favor the FC model can be equally well explained by the SBS model. Thus, it does not appear that there is a sufficiently high concentration of fixed positive charges in the region surrounding the external substrate binding site to significantly affect the anion concentration in this region. The binding site itself probably contains at least two positive charges: the SBS model which best fits the pH_o dependence data has two titratable groups, one with pK 8.7 which may be lysine and one with pK 11.1 which may be the arginine that reacts with phenylglyoxal at high pH_o. (Supported by NIH Grant DK27495.)

Th-Pos246

DISACCHARIDE BINDING INDUCES OSCILLATIONS IN HUMAN RED CELL GLUCOSE TRANSPORTER. Agnes Janoshazi, Gabriela Kifor and A. K. Solomon, Biophysical Laboratory, Harvard Medical School. Boston, MA 02115.

We have extended our studies of glucose transport protein interactions with band 3 to include short-term and long-term effects on tryptophan fluorescence (ex 280 nm; em 332 nm) of the transporter. Binding kinetics depend sensitively on maltose concentration in the 1-10 mM range. At 1 mM maltose the rate of fluorescence enhancement is $k = 5.8 \pm 0.7$ sec^{-1}; 2μM DBDS (4,4'-dibenzamido-2,2'-stilbene disulfonate) increases the rate to 10.6 ± 1.6 sec^{-1}, providing further evidence of a band 3/glucose transporter link. The slower (100 - 150 sec) biphasic kinetic effects of disaccharides, seen at higher concentrations (75 mM), are related to the specific sugar conformation, showing that this fluorescence enhancement is specific to the glucose transport protein. Both 1:4 α-D-pyranosyl sugars such as maltose and 1:4 β-D-pyranosyls such as cellobiose exhibit biphasic kinetics and a DBDS effect, indicating that the disaccharide site does not discriminate between the anomers. Sucrose, which is a β-D-furanosyl with a 1:2 linkage between the two sugars, does not show any DBDS effect. Trehalose, which has an unusual 1:1 linkage, exhibits neither biphasic kinetics nor a DBDS effect. Long term studies of 75 mM maltose binding show a damped anharmonic oscillation extending over 15 min. Arrhenius plots show that the activation energies of the two principal terms governing the oscillations are: damping factor, 9.5 ± 2.8 kcal mol^{-1} deg^{-1} and angular frequency factor, 7.6 ± 0.9 kcal mol^{-1} deg^{-1}, consistent with an important role for H-bonds in maltose binding. If the binding site snaps shut as soon as a maltose enters, vibrations could be elicited in the membrane system. 2μM DBDS increases the damping factor activation energy to 38.9 ± 5.2 kcal mol^{-1} deg^{-1}, showing that DBDS binding to its site on band 3 exercises an important effect on the conformation of the glucose transport protein. (Supported in part by the Squibb Inst for Med Res, the Council for Tobacco Res and the Amer Heart Assoc).

Th-Pos247

MEMBRANE SKELETON INHIBITION OF GRAMICIDIN AND LIPID LATERAL DIFFUSION IN ERYTHROCYTE MEMBRANES CAN QUANTITATIVELY EXPLAIN THE GRAMICIDIN-INDUCED INCREASE IN BOTH K^+ LOSS RATE AND LIPID FLIP RATE. Roderick D. MacGregor and C. Anthony Hunt. University of California, School of Pharmacy, San Francisco, CA 94143-0446

A statistical-thermodynamic model of solute binding into a membrane is developed to explain unique erythrocyte membrane properties. The lateral diffusion of both lipid and membrane-bound solute is limited in this model to discrete zones. Surface areas of vesicles produced by hypotonic hemolysis of fresh human erythrocytes, and areas delineated by the membrane skeleton, are both about 3×10^5Å^2, suggesting that the membrane skeleton interacts sufficiently with membrane lipid to determine the areas of hypotonically-produced vesicles. We test the hypothesis that the membrane skeleton corrals membrane zones by predicting the concentration dependence and the effects of gramicidin (10^{-10} - 10^{-5} M) binding into erythrocyte membranes. Using an experimentally derived lipid-only corral zone area of 2.5×10^5Å^2 and no adjustable parameters, the rate of potassium loss from erythrocytes is calculated and found to agree with published data[1]. From the probability distribution that a zone contains 0, 1, 2, . . . gramicidin molecules per bilayer leaflet, the time and gramicidin concentration dependence of tracer lipid flip from outer to inner membrane leaflet induced by gramicidin aggregates[1] is successfully predicted, further supporting the hypothesis. Functionally corralled membranes are a considerable deviation from the fluid mosaic model.

1. J. Classen et al. (1987): *Biochemistry* 26:6604.

Th-Pos248

FLUORESCENCE POLARIZATION ANALYSIS AND LIPID COMPOSITION OF THE ERYTHROCYTE MEMBRANE FROM HEALTHY DOGS AND LABRADOR RETRIEVERS WITH HEREDITARY MUSCULAR DYSTROPHY. J.R. Mehta, K.G. Braund, G.A. Hegreberg, and V. Thukral (Introduced by Dr. P. K. Sarathy). Neuromuscular Laboratories of Scott-Ritchey Research Program, College of Veterinary Medicine, Auburn University, AL 36849, and Department of Veterinary Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99163

The molecular pathology of muscular dystrophy is believed to be expressed at the plasma membrane level. In this study, erythrocyte membranes and their liposomes were prepared from clinically normal dogs and Labrador Retrievers with hereditary muscular dystrophy. The "static" and "dynamic" components of fluidity of each membrane were then assessed by steady-state fluorescence polarization techniques using limiting hindered fluorescence anisotropy and order parameter values of 1,6-diphenyl-1,3,5-hexatriene (DPH) and fluorescence anisotropy values of DL-2-(9-anthroyl)-stearic acid and DL-12-(9-anthroyl)-stearic acid, respectively. Membrane lipids were extracted and analyzed by thin-layer chromatography and gas chromatography. The results of these studies demonstrated that the lipid fluidity of erythrocyte membranes and their liposomes prepared from dystrophic dogs had significantly lower "static and dynamic components of fluidity" than control counterparts. Analysis of the composition of membranes from dystrophic dogs presented no significant changes in cholesterol to phospholipid ratio; however, the double bond index was altered and the ratio of saturated fatty acyl chain/unsaturated chains (w/w) was increased. Altered fatty acid composition, such as decreased levels of linoleic (18:2) and arachidonic (20:4) acids and increased levels of palmitic (16:0) and stearic (18:0) acids, were also observed in the membranes of dystrophic animals. These associated fatty acyl alterations could explain, at least in part, the differences in membrane fluidity between dystrophic and control dogs. These findings suggest a generalized membrane defect in Labrador Retriever dogs with hereditary muscular dystrophy.

Th-Pos249

A SPIN LABEL MARKER FOR THE ERYTHROCYTE UREA TRANSPORTER. Lidia Mannuzzu, Mario Moronne, and Robert Macey. University of California, Berkeley, CA 94720.

A pCMB-nitroxide was used to study the facilitated transport system for urea in the human red cell membrane. The rate of urea transport inhibition by mercurial reagents (pCMB, pCMB-nitroxide, pCMBS and chlormerodrin) increases with their hydrophobicity; the mercurial spin label is the most powerful. The specificity of pCMB-nitroxide binding to the urea inhibition site is increased by using resealed ghosts, and by incubating ghosts with NEM before pCMB-nitroxide treatment. Subsequently, cysteine is used to remove additional non-specifically bound mercury without affecting urea transport inhibition. When this procedure is used, 2.5 μM pCMB-nitroxide fully inhibits urea transport, with no effect on transport of water or glucose. Full restoration of urea transport is achieved using the sulfhydryl reagents DTT and mercaptoethanol, which are more hydrophobic than cysteine. The amount of inhibitor bound to ghosts incubated with 1-10 μM pCMB-nitroxide was evaluated by cold-vapour atomic absorption measurement of mercury. Scatchard plots of these data gives a $K_{diss} = 0.4$ μM. The number of sites labeled when urea transport is > 95% inhibited is ≤ 50,000/ghosts. The ratio of V_{max} to n (where V_{max} is the maximal urea transport rate measured by Karan and Macey [BBA 1024, 271-277, 1990], and n is the number of labeled sites when urea transport is fully inhibited) gives a turnover of about 2×10^6/sec. at 22°C.

These data are consistent with the following conclusions: 1) the mercurial binding site for urea transport inhibition is hydrophobic or must be accessed through a hydrophobic path, 2) the number of labeled sites is at least 20 times smaller than the number of band 3s per cell, but similar to the number of Jk antigens measured by Masouredis et al. (Blood 56, 969-977, 1980), and 3) the high turnover number supports a channel mechanism for urea transport. Supported by NIH HL 20985.

Th-Pos250

HYDROGEN PEROXIDE TREATMENT DAMAGES THE PHOSPHOLIPID TRANSLOCATING AND SEQUESTERING PROPERTIES OF NORMAL HUMAN ERYTHROCYTES

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The effect of oxidative damage on transbilayer phosphatidylserine (PS) distribution in erythrocytes was examined by morphology assay (D.L. Daleke and W.H. Huestis, Biochemistry 24, 5406-5416, 1985) and protein kinase C (PKC) activation. Treatment of cells with H_2O_2 had no effect on the steady-state distribution of endogenous PS (as evidenced by a lack of PS dependent PKC activation). However, a morphological assay demonstrated that the rate of exogenous PS translocation was diminished by prior exposure of cells to H_2O_2. In addition, the quantity of PS sequestered in the membrane inner monolayer at steady state decreased. Exclusion of oxygen from cell samples by CO pretreatment has been previously reported to prevent oxidation linked spectrin-hemoglobin cross-linking (L.M. Snyder, *et al.*, J. Clin. Invest. 76, 1971-1977, 1985). We have found that CO pretreatment also diminishes the inhibitory effects of H_2O_2 on PS distribution. These findings are consistent with the oxidative sensitivity of the PS translocator; but also suggest that oxidation decreases available cytofacial PS anchorage sites. Despite these effects, the oxidatively compromised erythrocytes have ATP levels and translocator activity adequate to maintain normal distribution of endogenous PS. (Supported by NIH HL 23787 and by a William and Flora Hewlett Grant of Research Corporation. MSM is a NSF fellow.)

Th-Pos251

THE SALICYLIC ACID DERIVATIVE 3,5-DIIODOSALICYLIC ACID INHIBITS HL60 CELL ANION EXCHANGE IN A NONCOMPETITIVE MANNER. Diego Restrepo[1], David J. Kozody[2], Laurie J. Spinelli[2], Brenda Diefenbach[2], Ruthanne B. Snyder[1] and Philip A. Knauf[2]. [1]Monell Chem. Senses Center, Philadelphia, PA 19104 and [2]U.of Rochester, School of Medicine, Rochester, NY 14642.

In a recent publication (Restrepo et al, Am. J. Physiol. 257:C520, 1989) we postulated that anion exchange takes place in a simultaneous manner in the HL60 cell. According to this model, following binding of suitable anions to intra and extracellular sites, translocation of bound anions occurs simultaneously. We have used the anion exchange inhibitor 3,5-diiodosalicylic acid (DIS) to test this simultaneous model of anion exchange. We found that DIS inhibited Cl/Cl exchange in the HL60 cell in a reversible manner. The concentration of DIS that inhibited Cl_o-dependent ^{36}Cl efflux (J) by 50% (ID_{50}) increased slightly as a function of extracellular chloride concentration (ID_{50} extrapolated to zero Cl_o is 18.6±2.1 uM and the slope of the dependence of ID_{50} on $[Cl_o]$ is 0.08±0.04 uM/mM, n=17 at 60 mM $[Cl_i]$). This amounts to a 1.6-fold increase in ID_{50} when $[Cl_o]$ is increased from 0 to 150 mM, which is much lower than the over 30-fold increase expected for a competitive inhibitor (K_{app} for Cl_o is 4.5mM). Similarly, the ID_{50} for DIS inhibition increased slightly as intracellular chloride was changed from 2 to 60 mM (slope 0.13±0.04 uM/mM and y-intercept 17.6±0.7 uM, n=7). This change was smaller than what would be expected for an inhibitor competitive with Cl_i. Replots of the slope of the Dixon plot (1/J vs [DIS]) as a function of either $1/[Cl_o]$ or $1/[Cl_i]$ could be fit with straight lines with non-zero x-intercepts, confirming the noncompetitive nature of the interaction of DIS with Cl_i and Cl_o. These results can be explained within the frame of the simultaneous model of anion exchange.

This work was supported by NIH grants GM42495, HL18208 and DK27495.

Th-Pos252

CARBONIC ANHYDRASE INTERACTION WITH BAND 3 IN HUMAN RED CELL GHOSTS. Michael R. Toon, Gabriela Kifor and A. K. Solomon, Biophysical Laboratory, Harvard Medical School. Boston, MA 02115.

When dansyl sulfonamide (DANSA), a fluorescent analog of acetazolamide (ACTZ), binds to crystalline beef carbonic anhydrase (CA), the fluorescence is enhanced (ex 330 nm; em 470 nm). In the range of [DANSA] from 5 - 10 μM, τ for uptake is 0.15 - 1 sec and can readily be measured by stopped-flow spectrofluorimetry. Chen and Kernohan (J Biol Chem 242, 5815,'67) have shown that DANSA binding inhibits CA activity noncompetitively with K_I = 0.4 μM. Our K_D of 0.13 μM for DANSA binding to commercial CA agrees reasonably well with their value of 0.25 μM. Since hemoglobin absorbance precludes intact cell measurements, we have studied white ghosts reconstituted with bovine CA. These ghosts bind DNSA with an on rate constant, $k_1 = 0.46 \pm 0.01\ \mu M^{-1}\ sec^{-1}$, slower than $k_1 = 0.55 \pm 0.01\ \mu M^{-1}\ sec^{-1}$ for free CA, consistent with CA binding to the cytoskeleton. ACTZ can displace all the bound DANSA from reconstituted CA showing that DANSA binds specifically to the inhibitory site of CA. Freezing and thawing the ghosts opens holes large enough to let CA escape; CA can be easily washed out of ghosts when the cytoskeleton is disrupted. Treatments which preserve the cytoskeleton, such as washing with isotonic salts, remove no more than 35% of the CA, and the remainder can only be removed by washes at lower ionic strength, or with 0.1 N NaOH, which attack the cytoskeleton. When red cells are covalently reacted with DIDS before reconstitution with CA, k_1 is linearly dependent on the fraction [moles DIDS bound/mole band 3] up to binding fractions of 16 to 23% where the k_1 effect saturates. This finding suggests that each CA molecule interacts with a band 3 tetramer, similar to the interaction between ankyrin and the cytosolic pole of band 3. Control experiments show no effect of DIDS on DANSA binding to free CA. [Supported in part by the Squibb Inst for Med Res, The Council for Tobacco Res and the American Heart Assoc].

Th-Pos253

HUMAN ERYTHROCYTE SHAPE REGULATION: ROLE OF AMINOPHOSPHOLIPID TRANSLOCATION IN DITHIOTHREITOL-DEPENDENT STOMATOCYTOSIS. Hoai-Thu N. Truong, David L. Daleke, and Wray H. Huestis. Chemistry Department, Stanford University, Stanford, CA 94305.

The echinocyte to discocyte shape recovery of metabolically depleted erythrocytes is compromised by sulfhydryl reducing agents. In the presence of dithiothreitol (DTT) and sugars, crenated cells recover normal discoid shape transiently, but then develop the invaginations and intracellular inclusions of stomatocytes. DTT-dependent stomatocytosis is not accompanied by alterations in cell ATP, spectrin phosphorylation or phosphoinositide metabolism (Truong et al., Blood 67, 214 (1986)). DTT also induces stomatocytosis during the shape recovery of erythrocytes crenated by Mg^{2+} depletion or Ca^{2+} loading. The effect of DTT on outer to inner monolayer transport of aminophospholipids was examined by monitoring shape changes induced by dilauroylphosphatidylserine (Daleke and Huestis, J Cell Biol 108, 1375 (1989)). Stomatocytosis induced by the transport of this exogenous lipid to the membrane inner monolayer is accelerated and exaggerated by DTT. The effect of DTT on DLPS translocation is reversible. Thus, cell crenation by methods that involve cellular metabolic processes (ATP or Mg^{2+} depletion, Ca^{2+} loading) develop a redox related defect, which makes shape recovery sensitive to reducing agents. The aminophospholipid translocator may be involved in the stomatogenic effect of DTT during the shape recovery of such cells. [This work was supported by research grants from the NIH (HL32836) and the USPHS (R01 HL 32839)].

Th-Pos254

Dipole and Surface Potentials in Erythrocyte Ghosts. Mario M. Moronne, and Robert I. Macey, Lawrence Berkeley Laboratory and the Dept. of Molecular and Cell Biology, University of California, Berkeley, CA, 94720.

Dipole and surface potentials of human erythrocyte ghosts were determined using electron spin resonance (ESR). Previously, the inner leaflet surface potential (Ψ_{sl}) was estimated from theory to be -60 to -70 mV at physiological ionic strength (Heinrich et al, 1981, Acta. Biol. Med. Germ. 40: 765-770). Adapting the method of Sunberg and Hubbel (1985, Biophys. J. 49: 553-62), the cationic spin label CAT12 was used to determine Ψ_{sl} in intact erythrocyte ghosts from the probe partition equilibrium distribution in cells treated with and without tetraphenylborate anion (TPB^-). In the absence of TPB^-, CAT12 is very slowly permeant and effectively binds only to the outer membrane leaflet. Following addition of low concentrations of TPB^- (0.2-0.3 μM), CAT12 is rapidly transported to the inner surface establishing a new equilibrium dependent on the interfacial potential. With this approach, an estimate of -40 mV is obtained for Ψ_{sl} (KCl 150 mM, pH 7.4). This value indicates substantial net surface charge, but suggests that some charges are screened by cytoskeletal proteins.

The erythrocyte dipole potential (Ψ_μ) was estimated from a comparison of the permeability of TPB^- and its positively charged analog tetraphenylarsonium (TPA^+). The permeability of TPB^- was determined from its distribution coefficient, ß (measured by electrode), and its translocation rate constant, k_i, estimated from analysis of CAT12-TPB^- facilitated transport. Transport of the spin label is limited by the return flux of TPB^-. Using a simple carrier model, titration of the initial flux vs. [CAT12] gives a k_i of 86 sec^{-1}. Combined with $ß=3.3x10^{-3}$ cm^{-1} the permeability for TPB^- is calculated to be 0.28 cm/sec at 22°C. In the same cells, the TPA^+ permeability was $1.3x10^{-6}$ cm/sec yielding an estimate of +173 mV for Ψ_μ after accounting for effects of Ψ_{si}. This value is significantly smaller than Ψ_μ estimated for planar membranes of phospholipids (Andersen et al, 1976), but is consistent with the smaller effects of dipole reagents such as phloretin on hydrophobic ion conductances in erythrocytes. (Supported by NIH grant GM18819).

Th-Pos255

EFFECT OF PHLORETIN ON UREA AND HYDROPHOBIC ION TRANSPORT IN HUMAN ERYTHROCYTES. Lenore Yousef, Mario Moronne, Bret Sherman, Kip Mihara, and Robert Macey. California State University, Fresno, CA 93740 and University of California, Berkeley, CA 94720.

Phloretin binds to membrane proteins and lipids; it inhibits urea transport and is presumed to decrease membrane dipole potentials (inferred from its effects on hydrophobic ion permeability in planar lipid bilayers - Andersen et al., J. G. Physiol. 67:749-771, 1976). We investigate the relation between these phenomena through a comparative study of the concentration and pH dependence of phloretin effects on urea transport and hydrophobic ion permeability (TPA^+) in red cell membranes. These studies require careful attention to binding of phloretin to hemoglobin. To establish a given phloretin concentration, cells were washed repeatedly with the same solution; alternatively, experiments were performed on resealed ghosts. Failure to heed this precaution leads to interpretations of permeabilities and binding constants which are in error by an order of magnitude.

At pH 6.0 and at low phloretin concentrations, the inhibition of urea exchange by phloretin fits a competitive model, with a K_i around 1 μM. This is consistent with Jennings and Solomon's (J. G. Physiol. 67:381-397, 1976) estimate of phloretin binding to membrane proteins. Urea transport inhibition in the presence of > 7 μM phloretin is larger than can be accounted for by the competitive model evaluated at 0, 2.5 μM and 5.0 μM phloretin. This greater inhibition might be due in part to phloretin induced changes in the membrane dipole potential. This is consistent with our results showing that phloretin stimulates TPA^+ permeability by a factor of 2 at 10 μM, rising to a factor of 20 at 160 μM. It is also consistent with results showing that phloretin effects on both urea and TPA^+ permeability increase with decreasing pH. This indicates that the active species is the neutral form rather than the ion, a condition that is necessary (but not sufficient) if dipole effects are involved. Studies in progress address the question of changes in TPA^+ stimulation at lower phloretin concentrations.

Supported by NIH HL 20985 and GM18819.

Th-Pos256

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO ERYTHROCYTE MEMBRANE PROTEINS OF BIPOLAR ILLNESS SUBJECTS AND NORMAL CONTROLS. **Yatian Zhang, M.D., Ph.D., Department of Lithium/Manic Depressive Studies, New York State Psychiatric Institute, New York, New York 10032**

Two membrane abnormalities in erythrocyte membranes of bipolar illness subjects were known, namely, an increase in the concentration of the enzyme system which moves calcium out of the cell and a large increase in ankyrin (2.3). In the present study, four fusions were done by using two populations of human erythrocyte ghosts which were prepared from blood collected in acid-citrate-dextrose solution; in one group from each population the membranes were also treated with sodium dodecyl sulphate and B-mercaptoethanol. Although the use of total membrane proteins produces a large number of antibodies, antibodies to ankyrins were selected out at an early stage by screening with pure ankyrins.I have fused spleen cells isolated from BALB/C female mice injected with human (bipolar illness subjects or normal controls) erythrocyte membrane protein suspensions with a mouse myeloma cell line, SP2/0-Ag14, in order to obtain hybridoma cells which secrete monoclonal antibodies (McAbs) to various membrane proteins. Hybridomas secreting McAbs to human membrane proteins were screening by using horseradish peroxidase-IgG conjugate dot blotting method. Their relative specificities for ankyrins or other peptides were established by Western blotting and enzyme-linked immunosorbent assay (ELISA). Three new sets of McAbs were generated and found to be strongly reactive with either, (1) all polypeptides of erythrocyte membranes; (2) only erythrocyte ankyrins; (3) polypeptides other than ankyrins. In addition, preliminary results are available for some McAbs revealing cross-reactivity with brain ankyrins. This study suggests that monoclonal antibodies against human erythrocyte membrane proteins will be a very useful tool to study the structure and function of the ankyrin isoforms as well as to identify ankyrins in brain. These findings should add significantly to our understanding of the biochemical substrates of bipolar illness and could lead to studies that may improve the methods of diagnosis and treatment. (This research was supported in part by the NARSAD Young Investigator Award and by NIMH grant MH-36946.)

Th-Pos257

WATER CHANNEL mRNA EXPRESSION IN XENOPUS OOCYTES: EVIDENCE THAT THE RED CELL WATER CHANNEL IS NOT BAND 3. *R. Zhang, S.-T. Tsai, S. Alper, and A.S. Verkman. U.C.S.F. and Harvard.*

Indirect evidence has suggested that the mercurial-sensitive pathway for water transport in red cells is the anion exchanger band 3. We tested this hypothesis using a Xenopus oocyte expression assay for water channel mRNA (Zhang, Logee & Verkman, J. Biol. Chem. 265:15375-15378, 1990). Osmotic water permeability (P_f) measured in defolliculated oocytes 72 h after injection with water or 50 ng mRNA from brain, muscle, liver or several cultured cell lines was (3.5-4) x 10^{-4} cm/s (10°C) and not inhibited by mercurials. Injection of 50 ng of mRNA from rabbit reticulocyte increased P_f to (18 ± 2) x 10^{-4} cm/s. P_f was inhibited to <5 x 10^{-4} cm/s rapidly by $HgCl_2$, slowly by pCMBS, and had an activation energy of 4 kcal/mol, similar to results in native rabbit red cells. Several lines of evidence indicate that the expressed water channel is not band 3: a) Injection of pure mRNA coding for band 3 transcribed from the cDNA clone increased oocyte Cl exchange >5-fold without effect on P_f. Cl exchange was inhibited by the stilbene DNDS but not by $HgCl_2$, whereas P_f was inhibited by $HgCl_2$ but not by DNDS. b) Injection of reticulocyte mRNA together with an anti-sense DNA probe against the band 3 message inhibited band 3 expression by >95% but did not affect P_f. c) Expression of size-fractionated reticulocyte mRNA gave a 2-2.5 kb size for water channel mRNA, much less than the 4-4.5 kb size for Cl exchange. Although definitive identification of the red cell water channel awaits elucidation of the cDNA and protein sequence, these data provide strong evidence that band 3 is not the water transporter.

Th-Pos258

NO SYNTHETASE ACTIVITY OF HUMAN KERATINOCYTES.
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Human keratinocytes derived from an epidermoid squamous cell carcinoma (SCC-13) possess NO synthetase activity. Irradiation with UVB (100 mJ/cm^2) results in a marked increase in NO and cGMP formation. The rate of NO release increases four times above baseline during the first 10 min after irradiation and continues at a slower rate for over 60 min. The rate of cyclic GMP formation by SCC-13 cells increases slowly during the first 10 min after irradiation and much faster during the following 50 min. By contrast, the cytosolic lysate shows a parallel increase of both NO and cyclic GMP for 10 min after irradiation followed by a rapid decline below basal levels. In the presence of PGI_2 and LTD_4, this decline was not observed and NO and cylic GMP increases for an additional 60 min. The NO formed by the NO synthetase of SCC-13 cells stimulates cyclic GMP production in human endothelial cells (HEC) in culture. This was shown by placing the SCC-13 and HEC in two chambers separated by a thin teflon membrane which permits only gas diffusion. Cyclic GMP production by HEC was not stimulated when untreated SCC-13 were present in the adjacent chamber but was stimulated several folds when either irradiated SCC-13 cells or buffer gassed with NO was present. These results suggest UVB activated NO synthetase in keratinocytes with subsequent activation of endothelial cell cyclic GMP production by NO. This could explain vascular relaxation responses that accompany UVB-induced erythema in skin.
Supported by NIH AR39750.

Th-Pos259

^{2}H-NMR STUDIES OF EPIDERMAL LIPID MEMBRANE MODELS.
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The human epidermal permeability barrier is thought to reside in the uppermost layer (the stratum corneum), in an unusual lipid domain located between the cells. The major lipid classes present are ceramides, cholesterol, and free fatty acid. We have performed ^{2}H-NMR studies of an equimolar mixture of bovine brain ceramide, cholesterol, and perdeuterated palmitic acid, and contrasted the results with an equivalent mixture in which sphingomyelin was substituted for ceramide. All samples were hydrated in excess NaCl 150 mM,HEPES 100 mM, EDTA 4 mM with pH adjusted to 7.4 or 6.2. As expected, the sphingomyelin mixtures gave rise to lineshapes consistent with the liquid crystalline bilayer phase being present over the temperature range 20-75°C. In contrast and under the same conditions, the ceramide mixtures showed polymorphic thermotropic phase behavior that was sensitive to pH. Spectra consistent with gel phase bilayers were detected at pH 6.2 at 20 and 37°C, but were accompanied by coexisting signals interpreted as representing liquid crystalline and "solid" palmitic acid phases. Thus under the conditions that allowed formation of gel phase bilayers, other phases were also experienced by the probe, and we conclude that phase separation occurred.

At 50°C both types of mixtures gave rise to liquid crystalline spectra, and the order profiles derived from these were virtually identical, implying that under these conditions the presence of the phosphorylcholine headgroup was irrelevant to hydrocarbon order. Above 60°C and at pH 7.4, an inverted hexagonal phase was observed for the ceramide model, whereas at pH 6.2 an isotropic signal was found .

One hypothesis that follows from these results is that the removal of sphingomyelin headgroups, as occurs in vivo in stratum corneum formation, may allow the formation of bilayer phases that are not liquid crystalline.

Th-Pos260

MAGNETIC RESONANCE STUDY OF THERMAL PHASE TRANSITIONS ASSOCIATED WITH HYDRATION OF MAMMALIAN STRATUM CORNEUM.
Selwyn J. Rehfeld[1] and William Z. Plachy[2], [1]Dept of Dermatology, University of California, and Vet. Adm. Med. Ctr. San Francisco 94121, and [2]Dept of Chem. and Biochem., San Francisco State University, San Francisco, California 94132.

Proton NMR and spin label EPR techniques were employed to determine the phase transitions and partitioning of water in mammalian stratum corneum (SC). The pd di-t-butylnitroxide spin probe was found to partition between aqueous and hydrocarbon regions of the SC lipid bilayers. Two distinct broad ^{1}H NMR peaks were observed in hydrated SC, one of an aqueous phase and the other originating from a lipid acyl region. Thermal phase transitions associated with the acyl region of the lamellar lipid phases were observed with both NMR and EPR. The transition temperatures seen with these techniques were in agreement for both dried and hydrated SC. The NMR lipid line width decreased and coincidentally the spin probe rotational times decreased with increasing hydration and temperature indicating an increase in the rotational motion of the lipid acyl chains. Further, the polarity of the microenvironment of the spin probe in the acyl chains remained non-polar between 0 and 60°C for all samples. These spectral data clearly show that 1) hydration increased the acyl chain motion but did not result in a significant change in the lipid phase transitions; and 2) the water concentration is very low in the lipid acyl region. A model is proposed for water penetration in the SC which is consistent in part with a general bilayer model previously proposed by Franks and Lieb (J Mol Biol. 141: 43-61, 1980).

Th-Pos261

THE RELATIONSHIP OF TRANS-GAUCHE CONFORMATIONAL CHANGES IN STRATUM CORNEUM LIPIDS TO WATER PERMEABILITY:
M.Francoeur & R.Potts, Pfizer Inc., Groton, CT, B. Ongpipattanakul & R.Burnette, Univ. of Wisconsin, Madison, WI: **The regulation of water loss through mammalian skin is a poorly understood but crucial process in maintaining viability. Extensive evidence from many laboratories have demonstrated that the extracellular lipids present in the outermost layer of the skin, viz., stratum corneum (SC) govern the water permeability. We have evaluated, on a molecular level, the relationship between SC water permeability and the formation of alkyl lipid gauche conformers using Fourier transform infrared spectroscopy (FTIR). The results show a strong correlation between the C-H stretching frequency and water permeability at several temperatures (20-90°C). In fact, the correlation holds both above and below the endogenous SC lipid phase transition. Consequently, these results indicate that the formation of gauche conformers and water permeability share the same functional dependence on temperature; and thus, suggest that water transport occurs through transient free-volume spaces along the lipid alkyl chains. This premise is consistent with 2 hypotheses reported in the literature, one involving the propagation of "kinks" and the other involving stochastic "jumping" from hole to hole. To our knowledge, this is the first experimental evidence directly linking water permeation to the number of gauche conformers in a lipid membrane system.**

Th-Pos262

PHASE SEPARATION OF OLEIC ACID IN THE STRATUM CORNEUM LIPIDS. Boonsri Ongpipattanakul,[1,2] Michael L. Francoeur,[2] Russell O. Potts[2], and Ronald R. Burnette[1]. [1]School of Pharmacy, University of Wisconsin, Madison, WI 53706. [2]Pfizer Central Research, Groton, CT 06340.

The interaction of perdeuterated oleic acid with lipids in porcine stratum corneum (SC) was investigated by Fourier transform infrared spectroscopy. Studies on the changes of the symmetric CH_2 vibration as a function of temperature indicated that oleic acid was able to induce two distinct effects on the endogenous lipid alkyl chains. One was the lowering of the phase transition temperature (Tm), and the other was the increased disorder of lipid alkyl chains at temperatures above Tm. Separate studies on the lipids extracted from porcine stratum corneum also suggested that both effects were dependent on the amount of oleic acid incorporated in the sample. However, oleic acid did not appear to significantly affect the relative trans-gauche conformation of SC lipids at physiological temperatures. In addition, changes in lipid fluidity alone could not explain the skin permeability enhancement by oleic acid. Evidence from the CD_2 stretching frequency demonstrated the coexistence of liquid oleyl chains and solid SC lipids at physiologically relevant temperatures. Based on the CH_2 scissoring vibration, there was no indication of major alteration in the two-dimentional arrangement of endogenous lipid alkyl chains, which further substantiated the poor miscibility between oleic acid and SC lipids at physiological temperatures. We, therefore, believe that the phase separation of liquid oleic acid in the SC lipids could create permeable defects at the phase boundary, similar to those used to explain the increased Na^+ permeability of lipid vesicles which are phase-separated.

Th-Pos262A

EVALUATION OF EPIDERMAL CELL CULTURE FUNCTION AND STRUCTURE BY CONFOCAL MICROSCOPY. Christopher Cullander*, Richard H. Guy*, Robert Hinz*, D. Bommannan*, Patrick Bilbo†, Cindy Nolte†, Leon M. Wilkins†, and Nancy L. Parenteau††. Intro. by Russell O. Potts††. *Depts. of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco, CA ,†Organogenesis, Inc., Cambridge, MA, and ††Cygnus Research Corporation, Redwood City, CA.

We have used laser-scanning confocal microscopy (LSCM) to optically section organotypic epidermal cell cultures and visualize functional and structural changes created by modifications in culture conditions. The cultures are studied without prior fixation or embedding, so that lipids, which tend to be lost during processing for light or electron microscopy, are retained. Testskin™ cultures grown using two different protocols were stained with Nile Red (in 75% glycerol), which fluoresces after binding to non-polar lipids. Type I cultures (C1) typically contained large non-fluorescent (dark) regions, with large and small lipid droplets (LD) in a thin 'stratum corneum' ('SC'). The Type II cultures (C2) lacked the dark areas and had a thick, stratified 'SC' filled with punctate fluorescence, which may represent staining of lamellar bodies (LB) as well as small LD. In addition, the shape and intercellular staining of patches of C2 superficial cells resembled that of corneocytes in vivo, suggesting the presence of the intercellular lipid lamellae characteristic of terminally differentiated keratinocytes. Taken together, these features indicated enhanced development in C2. TEM of cultures post-fixed with $OsK_3Fe(CN)_5$ found lamellar sheets as well as cytoplasmic and extruded LBs in C2 but not in C1. HPTLC chromatograms showed increased levels of acyl and glucosyl ceramides in C2 relative to C1, but attenuated total reflectance Fourier transform infrared spectroscopy scans of both cultures yielded essentially identical results. More keratohyalin granules appear in the upper stratum spinosum of C2 than in C1, increasing in number and size in the granular layer. The lowered permeability to water of the C2 is possibly due to an increase in diffusional path length ('tortuosity') through the culture rather than the partial formation of a true barrier. LSCM is a rapid and simple method of evaluating intact cultures. The technique has proven useful for inter- and intra-sample comparisons, and can suggest directions for more detailed analyses.

Supported by the CAAT, HD27839, and Cygnus Research Corp.

Th-Pos262B

ELUCIDATION OF STRATUM CORNEUM BIOPHYSICAL PROPERTIES *IN VIVO* USING FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR). Richard H. Guy[1,2], D. Bommannan[2], Vivien H.W. Mak[1,3], Naruhito Higo,[1,4] and Russell O. Potts[5]. Departments of Pharmacy and Pharmaceutical Chemistry[1], and Graduate Group in Bioengineering[2], University of California, San Francisco; Central Research Division, Pfizer, Inc., Groton, CT[3]; Hisamitsu Pharmaceutical Co., Inc., Japan[4]; Cygnus Research, Redwood City, CA[5].

The stratum corneum (SC) is the outermost and the least permeable layer of mammalian skin. The accessibility of the SC renders the membrane amenable to study *in vivo* in man by attenuated total reflectance (ATR)-FTIR. Using this technique, we have conducted the following investigations: (1) During sequential removal of the SC by adhesive tape-stripping, FTIR has revealed that the extracellular lipids are more ordered as one proceeds deeper into the SC. As expected, hydration of the membrane increases, as revealed by the shifts in amide I and II absorption frequencies. (2) Oleic acid, which is a known enhancer of drug delivery across the skin, shifts the observed C-H stretching frequencies to higher wavenumbers, suggesting an overall increase in lipid disorder. Spectroscopic quantification of the enhancer level in the SC has also been achieved. (3) Transport of model penetrants into and through the SC has been monitored successfully. In particular, advantage has been taken of compounds with intense IR absorbance in regions of the spectrum where the SC is essentially transparent. As a result, unique and novel observations of skin permeation enhancement *in vivo* have proved possible.

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Th-Pos263

CHLORIDE CHANNEL REGULATION IN T84 CELLS MEASURED BY FLUORESCENCE OF ENTRAPPED SPQ.

Yong-Xiong Wang andA.S. Verkman (Intro. by A.C. Chao). U.C.S.F.

The T84 intestinal cell line contains a regulated apical membrane Cl channel and has application as a model system to examine the physiological role of the cystic fibrosis gene product, CFTR. To study Cl transport, cells were loaded with the fluorescent Cl indicator SPQ by a hypotonic loading procedure (Verkman, Am. J. Physiol. 259:C375-C388, 1990). Intracellular Cl activity was measured continuously by quantitative microfluorimetry. The SPQ leakage and photobleaching rates were <10%/hr. In paired experiments in which isethionate was substituted for solution Cl, addition of chlorophenylthio-cAMP (CPT-cAMP, 0.5 mM) increased the initial rate of cell Cl efflux by >8-fold. The increase was blocked by the Cl-channel blocker diphenylamine-2-carboxylate (1 mM). In the absence of CPT-cAMP, >90% of Cl efflux was blocked by furosemide (0.5 mM), indicating the presence of a Na/K/2Cl symport mechanism. The activation of apical Cl conductance by a series of agonists was examined from the rates of Cl efflux upon isethionate substitution in the presence of furosemide. Cl conductance was strongly activated by cAMP-agonists and by 4-bromo-A23187 (1 uM) in the presence, but not in the absence of external calcium. Histamine (1 mM) caused a transient increase in Cl conductance. There was no effect of kinase C activation by phorbol myristate acetate (1 uM) on baseline or cAMP-stimulated Cl conductance. These results establish the methodology for measurement of Cl transport in T84 cells and demonstrate stimulation by cAMP and Ca signaling pathways.

Th-Pos264

WITHDRAWN

Th-Pos265

HALIDE PERMEABILITY OF 20 pS ANION CHANNELS IN THE APICAL MEMBRANES OF AIRWAY EPITHELIAL CELLS. M.A. Wilk-Blaszczak, A.S. French, and S.F.P. Man, Departments of Physiology & Medicine, University of Alberta, Edmonton, Canada.

Apical membranes of epithelial cells contain at least four types of anion channels. A rectifying channel of ~45 pS has been most thoroughly studied, but we have previously found a channel of ~20 pS linear conductance to be the commonest channel in human nasal airway epithelia. The relative contributions of different anion channels to total apical membrane anion conductance are unknown, but might be estimated from knowledge of their relative halide permeabilities, since several different components of whole-cell epithelial anion conductance, with different halide permeability sequences, have been identified. Relative halide permeability could also be used to test theories of permeation through the 20 pS channel. Available data suggests a single ion binding site model, but this should be tested by measuring mixed anion conductances.

The inside-out single channel patch clamp technique was used to characterize anion channels in apical membranes of nasal epithelial cells from normal humans. Cells were obtained from resected nasal turbinates and maintained in primary culture on plastic dishes at 36±1°C. Pipets contained 150 mM choline chloride, because cation channels of airway epithelia are impermeable to choline. Current-voltage relationships were measured with bath solutions containing 150 mM sodium chloride, bromide, iodide and fluoride, and fitted with the Goldman-Hodgkin-Katz current equation. Only channels with linear conductances in the range of 15-30 pS in symmetric 150 mM chloride solutions were used for further study. Preliminary data show that the channel is relatively unselective amongst the halides, but the probable permeability sequence is $Cl^- > Br^- > I^- > F^-$.

Supported by the MSI Foundation, the Alberta Lung Association, and the Canadian Medical Research Council.

Th-Pos266

CURRENT FLUCTUATION ANALYSIS OF CHLORIDE CHANNELS IN THE APICAL MEMBRANES OF EPITHELIAL CELLS. M. Duszyk, A.S. French, and S.F.P. Man, Departments of Physiology & Medicine, University of Alberta, Edmonton, Canada.

Apical membranes of epithelial cells contain at least four major groups of chloride channels with conductances of approximately 300, 45, 20, and 10 pS. The 45 pS channel rectifies in symmetric chloride solutions and has been reported most frequently, while the 20 pS channel has been found in large numbers in some cells. However, the relative contributions of the different channel types to total chloride conductance are not established. In particular, the numbers of small channels are not well known and probably underestimated. Whole cell patch clamp experiments have shown a mixture of rectifying and linear current-voltage relationships, again suggesting that non-rectifying channels of unknown size could play a significant role.

Whole cell patch clamp experiments were performed on human airway epithelial cells and MDCK cells. Cultures were plated at low densities to allow recordings from single isolated cells. Current-voltage relationships for chloride currents were approximately linear in most experiments and stable for periods up to 30 minutes.

To identify the size of ion channels carrying the current, whole cell recordings of 20-40 seconds duration were made at each holding potential. Current records were filtered by an active filter to a bandwidth of 0-500 Hz, sampled at 1 KHz, and processed in sections of 512 s duration by fast Fourier transformation and power spectrum estimation, followed by fitting with Lorentzian functions plus $1/f^n$ noise. Data were well-fitted by single Lorentzian functions, indicating that a single class of channels carry most of the current. Channel size was estimated from a simple conductance model with symmetric chloride solutions or by a model including the Goldman-Hodgkin-Katz current relationship for asymmetric solutions.

Supported by the Alberta Heritage Foundation for Medical Research and the Canadian Medical Research Council.

Th-Pos267

EFFECT OF SULFHYDRYL REAGENTS ON H^+ TRANSPORT AND Cl^- FLUX IN RABBIT RENAL CORTICAL ENDOSOMES. Shirley A. Hilden,* and Nicolaos E. Madias. Division of Nephrology, New England Medical Center, Boston, Massachusetts.

Endosomes contain a vacuolar H^+ ATPase, a Cl^- conductance, and a Ca^{2+}/H^+ exchanger. N-ethylmaleimide (NEM), a sulfhydryl compound, is a known inhibitor of the H^+ ATPase. We have previously shown that the sulfhydryl reagents, mersalyl, aldrithiol, p-chloromercuribenzoic acid (PCMB), and p-chloromercuriphenyl sulfonic acid (PCMBS) also inhibit endosomal H^+ transport (at 10^{-5}-10^{-6} M) (Fed. Proc. 46:272, 1987). In the present studies, NEM, mersalyl, aldrithiol, and PCMBS had no effect on pH gradient dissipation, but PCMB decreased the pH gradient faster than control. In the absence of ATP, PCMB (10^{-4}M) stimulated endosomal $^{36}Cl^-$ uptake, particularly in the presence of an inside-alkaline pH gradient (pH_i = 7.6, pH_o = 5.5). This result was not an effect of PCMB on the Cl^- conductive pathway. The less permeable PCMBS did not stimulate $^{36}Cl^-$ uptake. Dithiothreitol prevented the effects of PCMB. The effects of PCMB were concentration dependent. ATP-dependent $^{36}Cl^-$ uptake was decreased by the addition of PCMB. Finally, PCMB had no effect on $^{45}Ca^{2+}$ uptake.

These results suggest that modification of sulfhydryl groups with PCMB activates a Cl^-/OH^- exchanger that is functional both in the presence and absence of ATP. This endosomal transporter is similar to the PCMB-activated Cl^-/OH^- exchanger described in rabbit renal brush-border membranes (J. Membr. Biol. 112:59, 1989).

Th-Pos268

CHLORIDE CONDUCTANCES IN HUMAN NASAL EPITHELIAL CELLS. R. Grygorczyk. Merck Frosst Centre for Therapeutic Research, Kirkland, Que., Canada.

Single-channel and whole-cell conductances were studied in brushed human nasal epithelial cells using the patch-clamp technique. On a single-channel level four different chloride channels could be identified based on their conductive properties: 30 pS outwardly rectifying; 5 pS, 18 pS and 200-400 pS with linear I-V's. During whole-cell recordings at room temperature a swelling induced chloride current was observed. It exhibited inactivation at depolarizing voltages (> +80 mV), was inhibited by IAA-94 (10^{-4} M) and the underlying single-channel events showed outward rectification. Similar inactivation and inhibition by IAA-94 was observed for the rectifying 30 pS chloride channel in excised membrane patches. Under non-swelling condition and pipette free calcium buffered to 20 nM a chloride current was observed with markedly different kinetics. An activation of the current was seen for depolarizing voltages (>+50 mV) and inactivation for voltages negative to the cell's resting potential. Addition of ionomycin in the presence of 1 mM calcium in the bathing media increased the current significantly without changes to its kinetics. Peak tail currents measured after a preconditioning voltage pulse of +100 mV exhibited a linear I-V. Linear I-V was also observed for single-channel events as resolved in these experiments. It is concluded that a rectifying chloride channel underlays the swelling induced whole-cell conductance, but this channel is not directly involved in the chloride conductance activated by calcium.

Th-Pos269

DUAL ACTION OF CARBACHOL ON CHLORIDE SECRETION IN COLONIC EPITHELIAL CELLS (T84). Cz. Grygorczyk and R. Grygorczyk (Intr. by M. Glavinovic). Merck Frosst Centre for Therapeutic Research, Kirkland,Que. Canada.

Carbachol (CCh) a muscarinic agonist may under certain conditions exhibit both a stimulatory and an inhibitory action on chloride secretion in T-84 cells. Serosal application of CCh (10^{-4} M) to a T-84 cell monolayer mounted in an Ussing chamber caused a small and transient increase in short circuit current (I_{sc}); typically from 1.3 ± 0.6 uA/cm^2 (basal level) to 4.7 ± 1.1 uA/cm^2 (stimulated). This CCh activated current declined back to the basal level within 5 to 10 min. The effect of carbachol was greatly potentiated in cells pretreated with 8-Br-cAMP (10^{-3} M) or agents that act by increasing intracellular levels of cAMP (forskolin 10^{-5} M, VIP 10^{-8} M). Under such conditions the I_{sc} peaked sharply within 1-2 min from 15.3 ± 6.0 uA/cm^2 to 68.3 ± 4.9 uA/cm^2. This was followed by a biphasic decay to a level well below I_{sc} seen prior to application of CCh. This may indicate that in addition to a transient stimulation an inhibitory process was also triggered. In a similar experiment, if the addition of CCh was proceeded by the addition of ionomycin (10^{-6} M) only an inhibition of I_{sc} was observed. The response to CCh was reduced in high potassium (50 mM) media, however, Ba^{2+} (1 mM, a K^+ channel blocker) was without effect. A number of putative epithelial chloride channel antagonists affected the cAMP stimulated I_{sc} from both apical and basolateral side indicating nonspecificity of their action. DPC, IAA-94 and flufenamic acid (10^{-4}) inhibited while DIDS (serosal, 10^{-4}) transiently stimulated I_{sc}. All these agents except for IAA-94 had no effect on the subsequent response to CCh. It has been suggested that the different effects of carbachol on I_{sc} are related to a dual mode of action of protein kinase C on Cl channels which depends upon the intracellular calcium concentration. Calcium dependent K channels insensitive to Ba may also be involved in mediating the response to CCh. This study further indicates that currently available chloride channel antagonist may also affect secretion via mechanisms different from a direct block of Cl channels.

Th-Pos270

SINGLE CHANNEL AND WHOLE-CELL Cl^- CURRENTS IN ISOLATED FROG SKIN EPITHELIAL CELLS. J. F. Garcia-Diaz (Intro. by C. Cornwall), Dept. Physiol., Boston Univ. Sch. Med., Boston, MA 02118.

Cells from frog (*R. pipiens* and *R. catesbiana*) skin were isolated by collagenase and trypsin treatment and kept in primary cultures up to 3 days. Patch pipettes for whole-cell recordings contained 117 mM K, 24 mM Cl, ATP and EGTA. Currents were elicited by voltage steps of 200 ms from a holding potential of -60 mV. Since currents were not time dependent, they were subsequently evoked by applying a voltage ramp of 100 mV/s from -160 mV to 100 mV. With NaCl Ringer in the bath outward currents were twice as large as with Na gluconate, without changes in inward currents. Addition of the anion channel blocker diphenylamine-2-carboxylate (DPC, 1 mM) to the NaCl solution reversibly inhibited outward currents to the level observed with gluconate. The Cl current component, obtained as the difference between the currents in the absence and presence of the blocker, exhibited a strong outward rectification with reversal at -41±4 mV (n=3), close to the Cl equilibrium potential (-39 mV). This Cl component was observed in 70 % of the cells tested. For single channel recordings the pipette contained 112 mM Cl. A Cl channel was observed on a few occasions, activating upon excision and brief strong depolarization. IV curves showed strong outward rectification with conductance 48 pS at 0 mV in symmetrical 112 mM Cl. Selectivity for Cl against gluconate was at least 10. Kinetic analysis showed two open and at least two closed states. Open time constants and open probability increased markedly with depolarization. The Cl channel is likely of basolateral origin, since most isolated cells derive from deeper epithelial layers and contain membranes with basolateral properties. The similarity of the IV relations of the single channel and the Cl-dependent component of whole-cell current suggests that the Cl channel is responsible for this component. Its physiological function is probably related to the regulation of cell volume, but not to the transepithelial movement of Cl that takes place in frog skin *in vivo*. Supported by USPHS DK 39214.

Th-Pos271

POLARIZED Cl^- CHANNELS IN CULTURED RENAL CELLS (MDCK).

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Mature MDCK cells (3 or more days in culture at confluence) exhibit a Cl^- conductance of 2.65 ± 0.45 (n = 25) nS, which may be inhibited by 72% by DPC and by 45% by SITS. Patch clamp studies show that it is carried through 43 pS Cl^- channels distributed polarizedly on the basolateral side, and that exhibit the typical voltage-sensitivity of outward rectifiers. Cl^- conductance do not decrease upon trypsination. It increases in newly plated cells, reaching 4.87 ± 0.41 (n = 36) nS one day later (a 83% increase), and decreases to control values by the second day. Since this increase coincides with a peak of cell proliferation, we investigated whether Cl^- channels are associated to the cell cycle. Yet blockade of Cl^- currents with SITS or with DPC does not reduce the incorporation of $[^{14}C]$-thymidine in synchronized cells. Conversely, prevention of mitosis with thymidine does not modify the amplitude of Cl^- currents 24 hrs after plating.

Th-Pos272

REGULATION OF CHLORIDE CHANNELS IN NORMAL AND CYSTIC FIBROSIS DERIVED EPITHELIAL CELL LINES.

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LCBG, NIDDK, and CIPCB, NIDR, NIH, Bethesda, MD, Ben Gurion University and Dept. of Biol. Chem., The Hebrew University of Jerusalem, Israel.

Chloride transport systems play important roles in epithelial physiology. Indeed, impairment in Cl channel regulation is believed to be the basis for many of the symptoms of cystic fibrosis. To understand the various mechanisms responsible for the regulation of epithelial Cl^- channels we have studied the effect of putative modulators on Cl^- efflux in human tumor epithelial cell lines HT_{29}, T_{84} and PANC-1 derived from non-CF patients and CF-PAC-1 derived from a CF patient. Different patterns of response of Cl^- secretion to various factors were observed in these cells. One pattern of response was seen in HT_{29} cells, where IBMX was found to be better than forskolin in activating Cl^- efflux although specific inhibitors of phosphodiesterase had little effect on Cl^- fluxes. The cells also exhibited a very fast regulatory volume decrease in response to hypotonic shock which was slowed down by monensin. An exactly opposite pattern was observed in T_{84} cells. The PANC-1 pancreatic cell line seemed to resemble the HT_{29} pattern of Cl^- channel modulation. However, in CF-PAC-1, a pancreatic line derived from a CF patient, Cl^- fluxes were not activated either by forskolin or by IBMX. However, a specific antagonist of A_1 adenosine receptors was able to cause stimulation of Cl efflux, especially in conjunction with cAMP analogs. We conclude that different modes of Cl^- channel regulation are available in otherwise ostensibly similar epithelial cell lines. This observation might have therapeutic implications for disorders of Cl transport regulation.

Th-Pos273

SYNTHESIS OF IMPROVED Cl-SENSITIVE FLUORESCENT INDICATORS FOR CELL STUDIES. *Joachim Biwersi, Nazih Farah, Ming Li, R. Ketcham and A.S. Verkman. U.C.S.F.*

Quinolinium-based compounds have been used as Cl-sensitive fluorescent indicators in intact cells and cell-free membrane fractions. To improve Cl sensitivity and for conjugation via nucleophilic reaction, the compounds 6-methoxy-N-[2-aminoethyl] quinolinium dichloride (MEQ) and 6-methoxy-N-[4-aminobutyl] quinolinium dichloride (MBQ) were synthesized. MEQ and MBQ were highly water soluble, fluorescent (excitation peak 310-385 nm; emission 420-500 nm), and strongly quenched by Cl by a collisional mechanism. When protonated, the Stern-Volmer constants (K_{Cl}) for quenching of MEQ and MBQ by Cl were 354 M^{-1} and 272 M^{-1}, respectively, much higher than K_{Cl} for the reference compound SPQ (118 M^{-1}, 6-methoxy-N-[3-sulfopropyl] quinolinium). MEQ and MBQ were conjugated with neutral dextran (Mr 65,000) activated by cyanogen bromide to give indicator:dextran mol ratios of 5-20. The dextrans were stable in aqueous solution and had the same fluorescence spectra as the original indicators. K_{Cl} at pH 7.4 were 132 M^{-1} (MEQ-dextran) and 237 M^{-1} (MBQ-dextran). For use as an impermeant, pH-independent indicator of cytoplasmic Cl, the MEQ/MBQ derivative 6-methoxy-N-[3-trimethylammoniumpropyl] quinolinium dichloride (TMPQ) was synthesized. K_{Cl} for quenching by Cl was 365 M^{-1}; TMPQ was used in cultured fibroblasts and T84 cells for Cl transport studies. These results indicate that greatly increased Cl-sensitivity can be obtained by N-substitution of quinoline with an alkyl chain containing a nearby positive charge. For studies in living cells, TMPQ is a useful intracellular Cl-indicator and the substituted dextrans are impermeant fluid-phase markers of endocytosis.

Th-Pos274

APICAL CHLORIDE CHANNELS IN TIGHT MONOLAYERS OF A COLON CARCINOMA CELL CLONE (HT-29/B6). H. Fischer, K.M. Kreusel, U. Hegel, and W. Clauss (Intro. by T.E. Machen). Inst. of Veterinary Physiology, and Inst. of Clinical Physiology, FU Berlin, 1000 Berlin 33, FRG.

The HT-29/B6 clone is distinguished from its parent line (HT-29) by its ability to form tight monolayers when seeded on permeable support. Transepithelial measurements in Ussing chambers revealed Cl secretion when stimulated with forskolin or cAMP. Confluent tight monolayers were used to characterize apical Cl channels by means of the patch-clamp technique. Cell layers were patched from the apical side only. When cells were seeded on cover slips we occasionally observed K channels in patches. However, on permeable support Cl channels were seen exclusivly, suggesting that polarization of the cell layers depends on the permeability of the support. Cl channels were investigated in cell-attached and in excised mode after stimulating the cell sheets with dibutyryl cAMP (0.1 mM). The observed Cl channel was of the rectifying type with a conductance in the positive voltage scale of 72±6.3 pS and 25±2.6 pS on the negative side (mean±SEM, n=13). Channel's open probability increases with the holding potential. Both conductance and kinetic properties of the channel were similar in either cell-attached or excised recording mode, suggesting that this channel is not affected by calcium from the intracellular side. Excised channels were blocked by the known Cl channel blockers 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), and 4,4'-dinitro-2,2'-stilbenedisulfonate (DNDS). NPPB (1 and 10 uM) caused a rapid flickery appearance of the channel current, significantly decreasing the open propability of the channel. At concentrations of 0.1 mM the block was totally, within a frequency range of 1 kHz. DNDS (0.1 mM) caused a rapid flickerblock of Cl current without closing the channel completely. Both blockers were completely reversible. The characteristics of this Cl channel are very similar to the socalled ORDIC channel described in other tight Cl secreting epithelia. We propose that this Cl channel is the site of Cl secretion in colonic crypt cells.

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Th-Pos275

ACTIVATION OF AN AMILORIDE-BLOCKABLE SODIUM CHANNEL BY LOW LUMINAL OSMOLARITY IN RENAL EPITHELIAL CELLS.

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To clarify the effect of altering osmolarity on Na transport in renal distal nephron cells, we used patch clamp techniques to examine Na channels in the apical membrane of a renal distal nephron cell line (A6) cultured on permeable collagen films for 10 ~ 14 days under the open circuit conditions. A reduction of osmolarity in the luminal solution to 120 mOsm (60 mM NaCl) from 240 mOsm (120 mM NaCl) increased the open probability (P_o) of individual Na channels. However, the same reduction of serosal osmolarity had no significant effect on P_o. The change in P_o is not due to a change in the apical Na concentration since P_o did not change when 120 mM luminal Na was replaced with 60 mM Na and 60 mM N-methyl-D-glucamine keeping Cl concentration and osmolarity normal. Further, the change in P_o is not due to a change in the apical Cl concentration since P_o also did not change when 120 mM luminal Cl was replaced with 60 mM Cl and 60 mM gluconate keeping Na concentration and osmolarity normal. These observations imply that decreases in the osmolarity of the luminal solution increase the open probability of individual sodium channels but that changes in Na and Cl concentration do not. Supported by research grants from the Natinal Kidney Foundation to Y. Marunaka and NIH to D. C. Eaton.

Th-Pos276

EFFECTS OF GTPγS, GDPβS, AND ATP ON THE EPITHELIAL NA CHANNEL IN A6 CELLS

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Patch clamp experiments were done to investigate the effects of GTP-binding proteins (G-proteins) and ATP on the amiloride-blockable, epithelial Na channel (single channel conductance, 4-5 pS) from apical membranes of confluent A6 cells (a renal cell line derived from Xenopus kidney) which were cultured on permeable collagen films. After making cell-attached apical-membrane patches which contained Na channels, we excised the patches to make inside-out patches. In most inside-out patches, Na channel activity rapidly decreased or disappeared and never recovered spontaneously. However, when we applied ATP (1-2 mM), GDPβS (100-200 μM), or both to the cytoplasmic surface of the patch membranes, Na channel activity increased to levels similar to the levels found in cell-attached patches. GDP (500 μM - 1 mM) produced the same effect as GDPβS but GTPγS (100-200 μM) did not restore channel activity. In a few cases when Na channel activity was maintained in inside-out patches, GTPγS applied to the cytoplasmic surface of the patches inactivated the Na channels. GTPγS also inactivated Na channels which had recovered in inside-out patches after ATP or GDP treatment. From these results we think that there are at least two mechanisms to regulate the epithelial Na channel activity. One of them is an ATP-dependent pathway, e.g., modulation of the channel protein directly by ATP or via phosphorylation. The other is the regulation of channel opening by G-proteins, e.g., G-protein in a GTP-bound, activated state closes the epithelial Na channel.

Th-Pos277

FROG-SKIN CURRENT OVERSHOOT: EFFECT OF TIME IN LOW APICAL [NA] BEFORE THE NA JUMP.

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At the 1989 meeting [Biophys.J. 55, 159a (1989)] we reported an experiment that gives further insight into the mechanism of the short-circuit-current overshoot following a sudden jump in the [Na] of the apical bathing solution. We varied the incubation time at low [Na] before the jump. A 5-second incubation gives a peak current less than half of what you get with 300 seconds (=infinity); 30 s gives 75%; 120 s is as good as infinite. The model [Biophys.J.57, 88a (1990)] postulating a virtual "compartment" at the intracellular/distal end of the sodium channel shows good agreement with the shape of the current-vs-time curves [Abstracts International Biophys. Congress, Vancouver, B.C., Aug. 1990, p.326], adjusting the five time constants to achieve the best fit. The same theory with the same five values describes the inhibition of the current peak at short incubation times. Typical values of those parameters: compartment filling time = 40 s, compartment leakage time = 12 s, channel-closing time = 10^3 s, apical-solution mixing time = 26 ms, unstirred-layer diffusion-delay time = 350 ms. Channel opening in low [Na] is not needed to explain the experiment.

Th-Pos278

VOLTAGE-DEPENDENT CURRENTS IN CULTURED HUMAN RETINAL PIGMENT EPITHELIAL CELLS.

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Whole-cell currents in cultured human retinal pigment epithelial (RPE) cells were studied using the patch-clamp technique under voltage-clamp conditions. RPE cells were obtained from human donors (age range from fetus to 80 years old). Cells were cultured in DMEM medium with 15% FBS and 1 ng/ml bFGF. For recording, single cells were enzymatically dissociated from confluent monolayers of primary through passage 4 cultures.

Two types of voltage-dependent inward currents were observed. A transient inward current was seen when the cell membrane was depolarized above -40 mV from a holding voltage of -90 mV. The maximum amplitude occurred when the cell was depolarized to -10 mV. Its kinetics mimicked that of the traditional Na^+ current of excitable cells. TTX (200 to 500 nM) completely blocked this current. Another inward current was recorded when the cell was hyperpolarized beyond -80 mV (holding at -60 mV). Its I-V curve was linear from -80 mV to -120 mV. Beyond -120 mV, however, the current showed a time-dependent, voltage-dependent inactivation. This inward current was blocked by 2 mM of either Ba^{2+} or Cs^+, and was probably carried by K^+.

Two types of outward currents were also recorded. A transient outward current was activated when the cell was depolarized above -10 mV. The kinetics of this current resembled the A-current found in neurons. This current was inhibited by 5 mM 4-AP. The other current was an outwardly rectifying current, which appeared when the membrane was depolarized above 0 mV. Its relatively slow activation could be fit reasonably well with single exponential curves. TEA (20 mM) greatly reduced this current, while Ba^{2+} or Cs^+ had no significant effects.

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Th-Pos279

INWARDLY RECTIFYING POTASSIUM CURRENT IN MAMMALIAN LENS EPITHELIAL CELLS

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Lens potassium conductance is essential for the maintenance of lens volume and transparency. Recent work has identified three major potassium currents in lens, 1) an outwardly rectifying, 2) an inwardly rectifying, and 3) a calcium-activated current. We present here a study of the lens inward rectifier using whole-cell and single channel patch clamp techniques. The current is strongly inwardly rectifying. The voltage about which rectification occurs depends on the external potassium concentration. Internal magnesium is not necessary for rectification. In physiological saline, a time dependent decrease in current during sustained hyperpolarization is seen. This "droop" is due to a voltage dependent block by sodium. The inward rectifier is also effectively blocked by external cesium or barium but not by TEA or 4-AP. The lens inward rectifier has a single channel conductance of 33 pS (measured on-cell with 150 mM potassium in the pipette). The single channel current-voltage relationship is linear in the inward direction, but no outward current was measurable. The inward rectifier in lens may be involved in setting resting voltage.

Th-Pos280

WHOLE CELL CURRENTS IN ISOLATED NECTURUS GASTRIC OXYNTIC CELLS.

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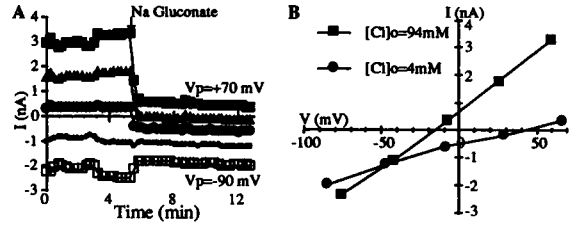
Necturus gastric mucosa transports Cl actively across the gastric glands which are composed almost entirely of acid and enzyme secreting oxyntic cells. Single channel studies on Necturus oxyntic cells have shown that the basal lateral membrane possesses multiple K selective channels but no observable Cl channels while the apical membrane has Cl channels but no observable K channel. To relate channel properties to the conductance of the intact cell we have investigated the nature of membrane currents across the plasma membrane of the resting oxyntic cells using the whole cell technique. When measured in amphibian Ringer's solution, with a pipette solution containing K 110mM, Cl 42mM, Gluconate 70mM, Mg 1mM, EGTA 1mM, ATPTris 4mM, GTP 0.1mM and Hepes 10mM, pH 7.3, oxyntic cells have a reversal potential of -16 ±2mV, a membrane resistance of 47 ±3MΩ, and a membrane capacitance of 76 ±3pF which give a specific membrane resistance of 3.3 ±0.2KΩ cm² (n=83, ±s.e.m). Replacement of external Cl ($[Cl]_o$=4mM) with gluconate increased the resistance (+60% Fig.A), and shifted the reversal potential by +50mV (Fig.B). The putative Cl channel blocker NPPB at 35μM inhibited reversibly and slowly ($t_{1/2}$≈ 3min) 80% of whole cell current with no change in the reversal potential. The addition of 10mM Ba^{2+} to the ringer solution inhibited rapidly 25% of the current without affecting the reversal potential while 10mM TEA inhibited no more than 10% of the outward current. In combination NPPB and Ba^{2+} eliminated more than 95% of the whole cell current. This shows that a major fraction of the current is due to Cl transport, presumably across the apical membrane and the remaining conductance is due to the presence of a smaller K current across the basal lateral membrane in these resting cells. An increase in $[K]_o$ (with normal or low $[Cl]_o$) from 6 to 90mM in the bathing solution increased fourfold the conductance which was blocked either by Ba^{2+} or NPPB but not by TEA 20mM . We conclude that chloride is responsible for the main resting conductance; the K conductance of the oxyntic cell is enhanced by external K; NPPB is not entirely Cl transport selective.



Th-Pos281

SEROTONIN (5-HT) INHIBITS K^+ CHANNELS IN THE APICAL MEMBRANE OF MOUSE CHOROID PLEXUS.

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The highest density of serotonin (5-hydroxytryptamine) receptors ($5\text{-}HT_{1C}$) in the brain is located at the choroid plexus and it has been proposed that 5-HT may modulate cerebrospinal fluid (CSF) secretion. We have previously shown that 5-HT activated Cl^- channels in the apical membrane of mouse choroid plexus. In the present study, we have examined the effect of 5-HT on the apical membrane K^+ channels. When the pipette solution contained 140 mM KCl and the tissue was bathed in a NaCl Ringer solution, cell-attached patches on the apical membrane of the intact choroidal epithelium revealed a K^+ channel in 30% of the patches. This channel had a linear I-V relationship, a conductance of 18 ± 2 pS (mean ± SEM, n=10) and the reversal potential (V_p) was -53 ± 2 mV (n=10). When the pipette solution contained 280 mM KCl, the reversal potential shifted to -68 ± 3 mV (n=3), indicating that these channels were highly selective for K^+. The channels were activated by depolarizing membrane potentials. Channel open probability (P_o) increased from 8.7 ± 2.0 x 10^{-2} (n=5) at V_p = 0 mV to 4.3 ± 0.3 x 10^{-1} (n=5) at V_p = -80 mV. Addition of 1μM 5-HT to the bath solution in cell-attached patches reduced P_o 100-fold from 1.5 ± 0.5 x 10^{-1} (n=3) to 1.2 ± 0.1 x 10^{-3} (n=3) at V_p = 0 mV. In the presence of 1 mM mianserin, a specific blocker of the $5\text{-}HT_{1C}$ receptor, 5-HT (1μM) had little effect on the K^+ channel. We conclude that $5\text{-}HT_{1C}$ inhibits the apical K^+ channel, and thereby modulate CSF secretion.

Th-Pos282

POTASSIUM CHANNEL-DEPENDENT MEMBRANE POTENTIAL OSCILLATIONS IN OXYNTIC CELLS.

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Several classes of secretagogue sensitive K^+ channels have been identified in the basolateral membranes (BM) of Necturus oxyntic cells (OCs). Activation of these channels contributes to the stimulation induced hyperpolarization of BM potential (V_b) and increase in its conductance (G_b). Spontaneous hyperpolarizations of V_b of resting OCs impaled in the intact gastric mucosa have been observed previously (Demarest and Machen. 1985. Am. J. Physiol.249:C535). Isolated OCs were used to investigate the possible relationship between these membrane potential (V_m) oscillations and BM K^+ channels. Isolated OCs impaled with microelectrodes exhibited V_ms as large as -60 mV and oscillations of similar amplitude (-14 ± 2 mV) and frequency (1 per 124 ± 8 s) to those observed in the intact epithelium. In addition, input resistance (R_i) declined by 24 ± 5 % during the hyperpolarizations. Amplitude and frequency decreased progressively during stimulation of the OCs with cAMP (10^{-3} M) resulting in the complete absence of oscillations in V_m of fully stimulated OCs. In resting OCs 0.5 mM Ba^{2+}, which depolarized V_m and increased R_i, completely blocked the spontaneous hyperpolarizations of V_m and decline in R_i. Conclusion: Brief episodes of spontaneous activation of K^+ channels are responsible for the oscillations in V_m and R_i observed in resting OCs. These channels may be identical to those responsible for the steady state increases in OC V_b and G_b in the intact mucosa that are induced by stimulation. (Supported by NIH grant DK38664)

Th-Pos283

SENSITIVITY OF CAPILLARY HYDRAULIC CONDUCTIVITY (L_P) TO EXTRACELLULAR CALCIUM (Ca^{2+}) AND MEMBRANE POTENTIAL. R-S Zhang and V H Huxley. Dept. Physiology, U. Missouri, Columbia, Mo. 65212

Elevation of capillary hydraulic conductivity (L_p) in frog mesenteric microvessels was well correlated with an increase in free intracellular calcium (Ca^{2+}) using ionomycin to set intracellular Ca^{2+} (He et al., FASEB J. 3:A1388, 1989). In cultured calf pulmonary arterial endothelium, cytosolic free Ca^{2+} was decreased when the membrane was depolarized in high potassium (K^+) solutions (John et al., Tissue & Cell 19:733, 1987). Moreover, Ca^{2+} content in frog mesenteric microvessels decreases during exposure to high K^+ solutions and increases in low K^+ solutions (He et al., FASEB J. 4:A835, 1990). Based on the notion that a rise in cytosolic Ca^{2+} leads to a widening of the interendothelial clefts, thus increasing water conductivity, we hypothesized that L_p will fall below basal levels in 1) nominally Ca^{2+}-free or 2) high K^+ solutions, and 3) will rise during exposure to low K^+ solutions.

These hypotheses were tested in *in situ*, singly perfused capillaries of frog mesentery. Repeated paired measurements of L_p were made under control and test conditions using the modified Landis technique. As anticipated, L_p decreased significantly ($p < 0.05$) from the control values under nominally Ca^{2+}-free conditions (to 73% of the control, n=8) as well as in high K^+ (10x) conditions (to 44% of the control, n=14). Furthermore, L_p increased by 2-fold above the control level in low K^+ (1/10x) solutions (n=9). These data are consistent with our original hypotheses.

(NIH HL 42528; VHH is an AHA Established Investigator).

Th-Pos284

OPTICAL MEASUREMENT OF OSMOTIC WATER TRANSPORT IN CULTURED CELLS: EVALUATION OF THE ROLE OF GLUCOSE TRANSPORTERS. *Miriam Echevarria and A.S. Verkman. University of California, San Francisco.*

Water transport across plasma membranes can occur by solubility/diffusion in the lipid bilayer and/or through specialized proteins. Recently it has been proposed that Na-independent glucose transporters in J774 macrophages are water channels (Fischbarg et al, PNAS 86:8397, 1989). Light scattering was used to measure osmotic water permeability (P_f) in monolayer cultured cells and applied to examine the role of glucose transporters in the water pathway. J774 macrophages were grown on glass coverslips and mounted in a channel-type perfusion chamber for rapid fluid exchange (<1 s) without cell detachment. Relative cell volume was measured by 45° light scattering using an inverted microscope; measurement accuracy was validated by confocal imaging microscopy. In response to a decrease in perfusate osmolality from 300 to 210 mOsm, cells swelled at an initial rate of $(0.91 \pm 0.1) \times 10^{-2}$ (n=20, 23° C), corresponding to a water permeability of $(70 \pm 8) \times 10^{-4}$ cm/s. The initial rate of cell swelling was linearly related to osmotic gradient size and was strongly temperature dependent (activation energy 9.5 Kcal/mol). Phloretin (20 uM) and cytochalasin B (2.5 mg/ml) inhibited uptake of ^{3}H-methylglucose by >90%, but did not affect water transport significantly in paired experiments in which P_f was measured before and after inhibitor addition. Also, the red cell water transport inhibitors $HgCl_2$ (50 uM) and pCMB (1 mM) did not influence P_f. These results provide evidence that glucose and water transport inhibitors do not influence water transport in J774 cells. In J774 macrophages, the major component of the osmotic water flow across the plasma membrane occurs by the lipid bilayer and not through glucose transporters.

Th-Pos285

DIFFERENTIAL EXPRESSION OF SINGLE CAPILLARY BARRIER PROPERTIES (L_p, P_d AND σ) BY ALBUMIN AND PLASMA. V H Huxley and F E Curry. Dept. Physiology, U. Missouri, Columbia, MO. 65212 and Dept. Human Physiology, U. California, Davis, Davis CA 95616

We tested the hypothesis that frog plasma, but not albumin, confers the charge selectivity of the blood-tissue exchange barrier. This hypothesis was assessed by measuring hydraulic conductivity (L_p), solute permeability (P_d) and solute reflection coefficient (σ) to an anionic solute, α-lactalbumin, in individually perfused exchange microvessels of the frog mesentery. Perfusion of capillaries with frog plasma followed by bovine serum albumin (BSA) did not alter L_p ($L_p^{Plasma}/L_p^{BSA} = 1.2 \pm 0.2$ (mean ± SEM); n=18, 10 mg/ml total protein). By contrast, $P_d^{\alpha\text{-lactalbumin}}$ was lower during plasma perfusion ($(P_d^{Plasma}/P_d^{BSA})^{\alpha\text{-lactalbumin}} = 0.3 \pm 0.1$; n=10). The solute reflection coefficient (σ) to α-lactalbumin was 0.69 ±0.03 in frog plasma and 0.49 ±0.06 during BSA perfusion (n=8). The increase in barrier resistance to the transfer of an anionic protein and the lack of plasma effect on transcapillary water movement are consistent with the notion that plasma confers a charge selectivity at the transvascular barrier by component(s) which are not present during, or are removed by, perfusion with albumin alone. Further studies are required to determine whether the actions of plasma are exclusively electrostatic.

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Th-Pos286

HYPOTONIC CONDITIONS ELEVATE INTRACELLULAR CA^{2+} IN TRACHEAL EPITHELIAL CELLS. Michael J. Sanderson and Ellen R. Dirksen. Department of Anatomy and Cell Biology, University of California, Los Angeles, CA, 90024. (Intro. by G. Zampighi).

The activity of the apical Cl^- and basolateral K^+ channels is important in the control of fluid transport across the airway epithelium. Although the activity of these channels is Ca^{2+}-dependent, the role of Ca^{2+} in the control of trans-epithelial fluid transport is not well understood. Earlier we found that mechanical stimulation increases $[Ca^{2+}]_i$ of ciliated epithelial cells (CC) cultured from the rabbit trachea and suggested that this response was transduced by a stretch-activated channel. From these results we postulate that cell swelling or stretching, induced by hypotonic saline, would lead to an elevation in $[Ca^{2+}]_i$. Imaging of fura-2 fluorescence was used to measure $[Ca^{2+}]_i$. When cells were exposed to a control solution containing 130 mM NaCl, 5 mM KCl, 25 mM HEPES, 2 mM free Mg^{2+}, 1 mM free Ca^{2+} and 1 mM EGTA, pH 7.3, no change in $[Ca^{2+}]_i$ was observed. Changing to an isotonic solution containing 65 mM NaCl and 130 mM sucrose did not significantly change $[Ca^{2+}]_i$. However, exposure to a hypotonic solution (no sucrose) did increase $[Ca^{2+}]_i$ in many cells in less than 45 sec. An increase in ciliary activity was also observed in the ciliated cells. Although cell swelling was not clearly observed, an expansion of the extracellular space occurred, as seen by the formation of pockets between cells, presumably filled with fluid. To determine the role of extracellular Ca^{2+} in this response cells were washed with isotonic Ca^{2+}-free saline. Interestingly, oscillations in $[Ca^{2+}]_i$ were observed in many cells under these conditions. Changing to a low NaCl (65 mM), Ca^{2+}-free solution made isotonic with sucrose produced little further change in $[Ca^{2+}]_i$. However, a low NaCl, Ca^{2+}-free hypotonic solution again produced an increase in $[Ca^{2+}]_i$. These results suggest that cell swelling induces a release of Ca^{2+} from intracellular stores and that an elevation of $[Ca^{2+}]_i$ may be involved in the osmoregulatory mechanism of CC cells. Supported by Smokeless Tobacco Research Council, Inc., Cystic Fibrosis Foundation and NIH grant HL40144.

Th-Pos287

ROLE OF MEMBRANE FLUIDITY IN ADAPTATION OF Na-Pi COTRANSPORT IN PHOSPHATE-DEPRIVED RENAL TUBULAR CELLS. M. Levi, P. Wilson, and J. Cooper. U of Texas Southwestern Med. Ctr. and VAMC, DALLAS, TX.

Previous studies in the rat have shown that renal adaptation to dietary phosphate (Pi) deprivation is characterized by an increase in proximal tubular apical membrane sodium gradient - dependent Pi transport (Na-Pi cotransport), which is associated with an increase in apical membrane fluidity (Molitoris et al, Am J Physiol 1985, Levi et al,Am J Physiol 1989). The purpose of the present study was to determine if MCT cells grown in culture, a continuous cell line derived from the mouse proximal tubule, also adapt to Pi deprivation by increasing membrane fluidity. Compared to MCT cells maintained in a 2mM Pi medium, MCT cells maintained in a 0mM Pi medium had an increase in the initial linear rate of Na-Pi cotransport (5.9±0.3 vs 2.8±0.2 nmol Pi/mg protein/4 min, $p<0.001$).Kinetic studies of Na-Pi cotransport as a function of extracellular [Na] and/or [Pi] demonstrated that the adaptive increase in Na-Pi cotransport was mediated by an increase in the Vmax (21.6±1.6 vs 9.4±0.7 nmol Pi/mg protein/4 min in 2 mM Pi, $p<0.001$) and no alterations in the affinities for Na (23.9±1.6 vs 20.7 ±1.5 mM Na in 2mM Pi, p=NS) or Pi (276±30 vs 262±28 uM Pi in 2mM Pi, p=NS), or the stoichiometry of Na-Pi cotransport ($2Na^+$:1 $HPO_4^=$). The increase in the Vmax of Na-Pi cotransport was paralleled by a decrease in the cell membrane fluorescence anisotropy of diphenylhexatriene, i.e. an increase in cell membrane fluidity (r_{DPH}, 0.206±0.001 vs 0.214±0.001 in 2mM Pi, $p<0.001$). The results of our study therefore indicate that an increase in membrane fluidity is an important mechanism in the adaptive increase in the Vmax of renal cellular Na-Pi cotransporter in response to Pi deprivation.

Th-Pos288

EXOGENOUS ATP STIMULATES GOBLET CELL SECRETION: DIRECT OBSERVATIONS ON LIVING, ISOLATED CANINE TRACHEAL EPITHELIUM. C. William Davis, Maria L. Dowell, and Michael Van Scott. University of North Carolina School of Medicine, Chapel Hill, NC 27599.

Living goblet cells, in cultured explants of canine tracheal epithelium mounted in a miniature Ussing chamber, were visualized by Normarski microscopy. Goblet cells were identified by the presence of large (0.6-2.5 um) intracellular vesicles which presumably contained mucin. ATP (10^{-4}M) perfused mucosally stimulated granule secretion in a biphasic manner. A burst of secretion, with an initial rate of 23.8 +/- 6.1 (mean +/- SE, n = 10 cells) degranulation events/min (DE/min), lasted less than 1 min. The frequency distribution of the intervals between degranulation events during this period was exponential. By 9 min the rate had slowed an order of magnitude to 2.3 +/- 0.3 DE/min. ATP perfused serosally elicited a slower, monotonic secretion. The initial rate of 2.0 +/- 1.1 DE/min (n=8) was not different from the final rate induced by a mucosal challenge. There were two other major differences between mucosal and serosal challenges in goblet cell secretion: the mucosal challenge, (a) lead to a much more rapid onset of secretion (35 sec versus 3.8 min), and (b) cells were largely depleted of granules after 10 min. Finally, we tested whether cells challenged first from the serosal side would respond to a subsequent ATP challenge from the contralateral side. After a 10 min primary serosal challenge and a 25 to 30 min washout, ATP was added mucosally. The effects were again biphasic and similar to a primary mucosal challenge: the initial rate of granule release was 19.7 +/- 5 DE/min (n=11). We conclude that exogenous ATP causes goblet cell degranulation, and that the response is highly asymmetric. This latter result could be due to different purinergic receptor sub-types on the two sides of the epithelium, and/or may portend an interesting intracellular control mechanism.