M-AM-Sym1 STRUCTURE AND FUNCTION OF ACETYLCHOLINE RECEPTOR AND SODIUM CHANNELS. S. Numa, University of Kyoto, Japan.

M-AM-Sym2 CLONING OF Ca^{2+} ATPases. David H. MacLennan*, Christopher J. Brandl*, Bozena Korczak*, Angel Zarain-Herzberg*, and N. Michael Green+. *Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6. +National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

In order to gain insight into the structure and function of Ca^{2+} ATPases of sarcoplasmic reticulum, we cloned cDNA encoding two Ca^{2+} ATPases. Neonatal rabbit muscle cDNA libraries contained nearly equal proportions of mRNAs encoding fast-twitch and slow-twitch/cardiac forms of the Ca^{2+} ATPase. These Ca^{2+} ATPases are encoded in two genes which, in humans, are located on separate chromosomes. In rabbit muscle the fast-twitch transcript is alternatively spliced at the 3' end of the coding region in a developmentally regulated fashion. The neonatal transcript encodes an extended, highly charged carboxyl-terminus whereas the adult transcript contains one additional exon and encodes a protein ending in the uncharged residue, Gly. The slow-twitch/cardiac transcript does not appear to be alternatively spliced.

The analysis of our sequence led to a model of the enzyme in which three cytoplasmic domains make up a headpiece structure. The transmembrane segment consists of ten alpha-helices. Five of these extend on the cytoplasmic side into alpha-helices which make up the stalk sector of the structure. We propose that the high affinity Ca^{2+} binding sites are made up on more than one alpha-helical stalk segment. We also suggest that the energy generated in the headpiece is utilized in rotating one or more of the stalk segments to carry bound Ca^{2+} into a channel formed in the interior of the stalk. This rotation would lead to disruption of the high affinity Ca^{2+} binding sites and to deposition of the Ca^{2+} into a channel connected to luminal spaces.

M-AM-Sym3 ON THE FUNCTIONAL CORRELATES OF STRUCTURE IN TRANSMEMBRANE IONIC CHANNELS.
Robert M. Stroud. Department of Biochemistry & Biophysics, University of California, San Francisco, CA 94143-0448.

Structural studies of the actylcholine receptor from Torpedo californica reveal changes in organization of components, and conformational changes associated with channel opening and desensitization. Mechanisms for channel gating are discussed in the light of experimental results pertaining to contents of the ionic channel and location of the ligand binding sites. Progress in the crystallography of ACh receptors will be described. A second ionic channel, that of a colicin molecule whose structure is being determined indicates that the soluble form of the protein is alpha-helical. The low resolution structure indicates parameters important to channel definition, and the amino acid sequence suggests mechanisms for channel formation. Three-dimensional image reconstructions of the acetylcholine receptor suggests mechanisms of association with cytoskeletal components inside the cell. Structural analysis of other transmembrane proteins and their complexes, including bacteriorhodopsin, a light-driven proton pump, and of a Bacillus thuringiensis toxic protein complex are presented in attempts to focus on characteristics that define different aspects of transmembrane conducting proteins of different functions. Energy calculations are used to test possible mechanisms for conductivity by channels.

M-AM-Sym4 CALCIUM CHANNELS: FUNCTIONAL INSIGHTS AND STRUCTURAL IMPLICATIONS. R.W. Tsien, C.D. Benham, A.P. Fox, P. Hess, J.B. Lansman, D. Lipscombe, E.W. McCleskey, D.V. Madison & R.L. Rosenberg. Dept. Physiol. Yale Univ. Sch. Med., New Haven CT 06510.

We will review patch clamp studies characterizing multiple types of Ca channels and mechanisms of selectivity, permeation, and modulation that allow some inferences about Ca channel structure.

Three broad classes of Ca-permeable channel: Voltage-activated, receptor-operated, and "leak" channels show differences in conductance, gating and pharmacology, but the possibility of broad structural similarities remains. Within the category of voltage-activated Ca channels, T-, N- and L-type channels are expressed in different combinations in various muscle cells and neurons. Neurochemicals can selectively modulate one type of Ca channel while exerting no effect or the opposite effect on another type (e.g. Madison et al. this volume).

What makes a Ca channel a Ca channel? The high selectivity of L-type Ca channels for Ca or Ba over Na or K can be attributed to a single-file pore containing binding sites with high affinity for Ca and low affinity for monovalent cations. With high external Ca, ion fluxes of $>10^6$ s^{-1} are achieved because simultaneous occupancy causes ion-ion interactions (electrostatic repulsion?) that greatly promote the escape of bound Ca into the cell. Individual ion-pore interactions can be detected as discrete interruptions in drug-prolonged unitary currents carried by another, faster fluxing ion species; the primary rate of entry of Ca or Cd ions approaches that expected for diffusional control. L-type Ca channel pores of skeletal or cardiac muscle are functionally symmetrical and permeable to ions as large as TMA (~5 A diameter). Other kinds of Ca channels may function by similar mechanisms but differ in properties of their high-affinity Ca binding sites.

M-AM-A1 CROSS-BRIDGE KINETICS DURING LENGTHENING IN "TENDON-FREE" SEGMENTS OF INTACT FROG SINGLE MUSCLE FIBRES. F. Colomo, V. Lombardi and G. Piazzesi (Intr. by G. Cecchi). Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, 50134 Firenze, Italy.

The mechanism underlying the force response to the lengthening of tetanized muscle fibres has been studied by determining the characteristics of force transients following step length changes applied during constant velocity stretches. Experiments were performed at low temperature (4-5 °C). Ramp and step length changes were imposed at one tendon end of the fibres and tension was measured at the other end. The actual length change undergone by a selected segment (0.6-1.8 mm long) of the fibres was measured by means of a "striation follower". During slow lengthening (<0.1 μm/s per half-sarcomere), apart from a substantial shift to left, the relation between speed of early recovery (phase 2 of Huxley and Simmons transient) and amplitude of the step exhibited the same shape as that determined at the tetanus plateau. With faster lengthening (0.5-1 μm/s per half-sarcomere) the whole tension transient turned out to be faster so that phase 2 became practically indistinguishable from the rest of the transient; the slope of the relation between speed of recovery and step amplitude was reversed so that the recovery was faster for step stretches than for step releases.

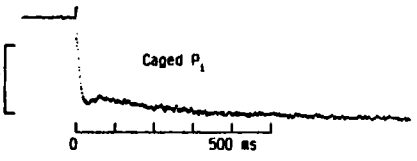
The transients obtained during slow lengthening can be satisfactorily interpreted in terms of Huxley and Simmons theory. Tension recovery during fast lengthening is interpreted in terms of the two state model of A.F. Huxley assuming (i) that the predominant rate of recovery is determined by the sum of the mean values for attachment and detachment rate constants and (ii) that cross-bridge cycling activity is characterized by very fast attachment and detachment rates.

M-AM-A2 BIREFRINGENCE TRANSIENTS INDUCED BY CAGED-ATP PHOTOLYSIS IN DEMEMBRANATED RABBIT MUSCLE FIBRES. Irving, M.,[2] Peckham, M.[2] & Ferenczi, M.A.[3] [2]Department of Biophysics, King's College London, 26-29 Drury Lane London WC2B 5RL. [3]N.I.M.R. Mill Hill, London, NW7 1AA.

The birefringence of relaxed muscle is higher than that of either rigor or isometrically contracting muscle, probably because crossbridges are more aligned with the filament axis in the relaxed state (Irving & Peckham (1986) J.Physiol. 377, 95P). We have investigated the kinetics of such transitions using rapid photogeneration of ATP. Single psoas fibres (sarcomere length 2.9μm) were initially in a Mg rigor solution containing 5mM caged-ATP, ionic strength 0.1M, pH7.0, 15°C. These conditions minimise optical path length and lattice spacing changes on ATP release (Ferenczi et al, this meeting). In the absence of Ca^{2+}, ATP release initiated a birefringence increase with two components. The first had a half time of 10-20ms; the second had a time course similar to the final force relaxation, complete in about 0.5s. During the first phase the force was roughly constant or rose slightly but stiffness (measured by applying 0.3ms length steps) fell to about half the rigor value, also with a half time of 10-20ms. Following a length step applied at 20ms or later there was some rapid force recovery, indicative of actively force-generating crossbridges. In the presence of Ca^{2+}, birefringence increased for about 10ms, then decreased as active force developed. The fast birefringence increase (with or without Ca^{2+}) may reflect crossbridge detachment to take up an orientation more parallel to the fibre axis. The subsequent birefringence decrease in the presence of Ca^{2+} may be due to reattachment in a more perpendicular orientation. It seems likely that such reattachment also occurs to some extent (and transiently) in the absence of Ca^{2+}. (Supported by the Muscular Dystrophy Association of America and the Royal Society).

M-AM-A3 **MECHANICAL TRANSIENTS INITIATED BY PHOTOLYSIS OF CAGED P_i DURING ACTIVE SKELETAL MUSCLE CONTRACTIONS.** [+]J.A. Dantzig, [*]J.W. Lacktis, [*]E. Homsher & [+]Y.E. Goldman. Depts. of Physiol., Schools of Med., [+]Univ. of Penna., Phila., PA 19104 and [*]UCLA, Los Angeles, CA 90024

The step of the cross-bridge cycle leading to a force-generating state is thought to be release of inorganic phosphate (P_i) from AM.ADP.P_i (Hibberd et al. Science, 228:1317, 1985). This hypothesis predicts that a sudden increase in the P_i concentration within an isometrically contracting fiber will cause a reducton of force. When 0.3-1 mM P_i is liberated by photolysis of caged P_i (1-(2-nitro)-phenylethyl phosphate) in rabbit glycerinated fibers during steady isometric contraction ($[Ca^{2+}]$>30 μM, 20 °C, Fig. 1), tension decreases rapidly ($t_{1/2}$=5-10 ms), recovers slightly and finally decreases again to a lower steady value ($t_{1/2}$ of final phase=200-500 ms). The force decline is not caused by the pulse of energy or photolysis by-products because photolysis of caged AMP does not effect tension and photolysis of caged ADP causes a tension increase. At lengths greater than 2.5 μm, the rate of the fast decline is independent of sarcomere length and the amplitude scales with isometric tension. Muscle stiffness (500 Hz) decreases during the fast decline suggesting net cross-bridge detachment, but stiffness remains constant during the slow force decline, suggesting a redistribution among force-bearing cross-bridge states. The amplitude of the fast phase is larger if P_i is released during the onset of contraction, suggesting that the number of cross-bridges in states near the P_i release step is greater in the pre-steady state. Supported by NIH grants HL15835 to PMI and AM30988 to E. H.



M-AM-A4 MICROSCOPIC AND WAVELENGTH DEPENDENT FLUORESCENCE POLARIZATION FROM 1,5-IAEDANS LABELED MYOSIN SUBFRAGMENT 1 DECORATING MUSCLE FIBERS IN THE PRESENCE AND ABSENCE OF MgADP.

Katalin Ajtai and Thomas P. Burghardt, CVRI, U.C., San Francisco, San Francisco, California 94143.

The microscopic and wavelength dependent fluorescence polarization signal from 1,5-IAEDANS labeled subfragment 1 of myosin (S-1) decorating muscle fibers in rigor and in the presence of MgADP is measured. Using microscopic fluorescence polarization we sample a small volume from the fiber ($\sim 0.1 um^3$) and detect a higher degree of angular order than reported previously. From this data we show that the probe angular distribution from fibers in rigor is quantitatively different from that present when MgADP is bound to S-1. Using wavelength dependent fluorescence polarization we vary the wavelength of the excitation light and thereby change the direction that the probe absorption dipole makes with a reference frame fixed in S-1. From this data we show that the binding of MgADP to S-1 causes an angular reorientation of S-1 relative to the actin filament. The difference between the angular distribution of probes for the rigor vs MgADP states cannot be accounted for by the addition of random probes. The microscopic fluorescence polarization experiment suggests that the earlier attempts with 1,5-IAEDANS to distinguish rigor and a MgADP probe angular distribution failed because fiber inhomogeneity over larger observation volumes made the subtle differences between the distributions insignificant. The wavelength dependent experiments indicate that the probe dipole orientation in S-1, at the typically used wavelengths, is not ideal for detecting the rigor to MgADP angular transition. This research was supported by U.S.P.H.S. HL-16683 and the Muscular Dystrophy Association. Dr. Ajtai's present address is: Department of Biochemistry, Eötvös Loránd Univ., VIII Puskin utca 3, 1088 Budapest Hungary.

M-AM-A5 THE CAUSE OF INTENSITY INCREASE OF THE 59 Å ACTIN LAYER-LINE ON X-RAY DIFFRACTION PATTERN FROM CONTRACTING FROG MUSCLE

Y.Maéda, EMBL at DESY, Hamburg and D.Popp, Max Planck Institute for med. Research, Heidelberg, Fed.Rep.Germany

The 59 Å layer-line (59 L.L.) is the most prominent reflexion originating from the thin filament structure. The present study has been designed to distinguish between two possible explanations of the intensity increase of the reflexion on contraction - binding of the myosin heads or conformation changes of actin subunits.

X-ray diffraction patterns were recorded from live sartorius or semitendinosus muscle of frog (mostly at a temperature of 3-4 ^{o}C) at the beam-line X-33 either with a fast linear detector (J.Hendrix et al.,1982) or on the "Imaging Plates" (J.Miyahara et al.,1986).

The extent of the intensity increase is linearly related to the sarcomere overlap, at least from 50 to 10 % of the full overlap, whereas the intensity increase of the outer part of the second layer line which has been assigned to the tropomyosin shift is as high as 60 % at 10 % overlap. The time course of the intensity increase of 59 L.L. is much faster than those of the equatorial reflexions and the myosin layer-lines (also K.Wakabayashi et al.,1985) and almost identical to the outer part of the second layer-line. At a higher temperature, 59 L.L. increases less, whereas the equatorial reflexions, which have been assigned to the S1 binding, change more. 59 L.L. shows almost no shift of the intensity profile on activation (also K.Wakabayashi et al.,1985).

These results suggest that conformational changes of actin subunits induced by small number of S1 binding cause the intensity increase of 59 L.L.

Supported partly by MDA.

M-AM-A6 TIME-RESOLVED CROSSBRIDGE MASS MOVEMENT FROM X-RAY DIFFRACTION OF ACTIVE FISH MUSCLE.

J.J. Harford*, J.M. Squire* and Y. Maeda+, *Biophysics Section, Blackett Laboratory, Imperial College, London SW7. +EMBL Outstation, DESY, Notkestrasse 85, Hamburg.

Bony fish muscle has a "crystalline" myosin crossbridge lattice and its X-ray diffraction pattern can be relatively simply interpreted in terms of a model for the resting configuration of the myosin heads (Harford & Squire, 1986, Biophys.J. 50, 145-155). Using the low-angle facility at DESY time-resolved low-angle X-ray diffraction studies of contracting bony fish muscle have enabled the active state of vertebrate muscle to be investigated in greater detail than before (Huxley et al, 1982, J. Mol.Biol., 158, 637-684). Acquisition of the intensity changes to 5ms time-resolution of six equatorial reflections (10,11,20,21,30 and 22) from short tetanic contractions (300ms) have allowed the equatorial projection of the crossbridge mass movement during contraction to be analysed in detail. The 10,11 and 21 equatorial reflections decrease in intensity when the muscle contracts whereas the 20, 30 and 22 increase. The time-courses of the decrease of the 10 intensity, and of the strongest reflection (the 11 row-line) on the very well sampled 1st myosin layer line (at 429A), lead the increase in tension by 15-20ms and return to their resting values with very similar time-courses to that of tension; the myosin heads must return rapidly to their resting helical configuration. Electron density maps have been computed by Fourier synthesis using the first five equatorial reflections, assuming the phases +,+,-,+,+ (Squire & Harford, 1984; in Contractile Mechanisms in Muscle, ed. Pollack & Sugi, 221-236 Plenum N.Y.). These show the equatorial projection of A-band density at 10ms intervals during the rising phase of tension. The time course of the mass transfer to the thin filaments leads the tension by 10-15ms. Thus it is shown directly that crossbridge movement to the thin filaments precedes force generation. (Supported by MRC programme grant).

M-AM-A7 THE EFFECT OF LOW IONIC STRENGTH UPON THE TROPOMYOSIN LAYER LINES IN RELAXED MUSCLE, Brian Collett[†¶] and Richard Podolsky[†], [†]NIAMS, NIH, Bethesda, MD, 20892, and [¶]Physics Dept., Hamilton College, Clinton, NY, 13323.

Rapidly equilibrating actomyosin crossbridges form in relaxed rabbit psoas fibers at low ionic strength (Brenner, Schoenberg, Chalovich, Greene, and Eisenberg, PNAS, 79, 7288, 1982). The degree to which tropomyosin (TM) movement on the thin filament is associated with ionic strength changes and crossbridge formation was studied in small bundles of fibers using 2-dimensional X-ray diffraction. At sarcomere length 4.0 μm, where thin and thick filaments do not overlap, the intensities of the "TM reflections" on the second (II) and third order actin layer lines were unaffected by changes in ionic strength (20 to 120 mM), which indicates that ionic strength does not change the position of TM on the thin filament. On the other hand, at sarcomere length 2.5 μm, where the thick and thin filaments do overlap, lowering the ionic strength from 120 mM to 30-40 mM caused the intensity of II to increase, suggesting that crossbridge formation is associated with TM movement. The intensities of the actin layer lines at 51 and 69 Å were not intensified under these conditions and parallel mechanical experiments showed no increase in muscle stiffness, indicating that rigor crossbridges were not the cause of the TM movement. These observations show that low ionic strength does not, in itself, cause TM movement, but that low ionic strength coupled with the presence of rapidly equilibrating actomyosin crossbridges does produce such movement even though the fiber remains relaxed.

M-AM-A8 CORRELATION BETWEEN X-RAY DIFFRACTION STUDIES ON CONTRACTING MUSCLE AND ELECTRON MICROSCOPE OBSERVATIONS USING RAPID FREEZING TECHNIQUES. H.E. Huxley, A.R. Faruqi and S. Whytock, MRC Laboratory of Molecular Biology, Cambridge, England.

Previous X-ray diffraction studies have shown that a strong meridional ∿143Å reflection is still present in contracting muscle and that its spacing is unaltered by changes in sarcomere length. This periodicity can therefore provide a good internal standard of length, so that any axial distortions of A-band dimensions during processing for electron microscopy can be corrected. Rapid freezing of specimens during contraction by contact with a liquid nitrogen-cooled copper block permits much more reliable fixation of contracting structures than was possible previously, and a strong axial periodicity of approximately 143Å is visible in freeze-substituted material, as already found by Tsukita and Yano (Nature 317, 182-184, 1985). We have used this procedure to demonstrate constancy of A-band length in contracting muscle without the uncertainties arising from limited light-microscope resolution or possible effects of direct chemical fixation.

An important question concerning molecular changes during contraction is whether a part of the cross-bridge structure can be demonstrated to attach to a specific site on actin i.e. does 'marking' of the actin helical structure occur? Part of the observed increase in intensity of the 59Å actin layer line may be due to activation itself, but another part of the increase seems to be dependent on crossbridge attachment and is reduced at higher shortening velocities. We find little sign in X-ray patterns from contracting frog muscle of a 360-380Å labelled first actin layer-line; however, like Tsukita and Yano, we have observed strong labelling of this spacing in electron-micrographs of rapid-frozen contracting rabbit muscle.

M-AM-A9 INFLUENCE OF INORGANIC PHOSPHATE (P_i) AND RELATED COMPOUNDS ON CONTRACTION OF SKINNED MUSCLE FIBERS. T.M. Nosek, K.Y. Fender & R.E. Godt; Dept. of Physiology & Endocrinology; Medical College of Georgia; Augusta GA 30912.

It is well known that P_i depresses both maximal force and calcium sensitivity of the contractile apparatus of striated muscle. Dawson et al. (Biophys. J. 49: 268a, 1986) suggested that the effective form of P_i is $H_2PO_4^{-1}$. As the pK of the reaction: $H^+ + HPO_4^{-2} <==> H_2PO_4^{-1}$ is ca. 6.8 we could test this notion by varying the concentrations of these ionic forms by altering total [P_i] and pH (6-7.25) and observing the effects on chemically skinned rabbit psoas fibers. Factoring out the effect of altered pH on force, we find that depression of maximal Ca^{2+}-activated force due to P_i correlates nicely with [$H_2PO_4^{-1}$] but not with [HPO_4^{-2}] or total [P_i]. Variation of calcium sensitivity by P_i, however, correlates with total [P_i] rather than [$H_2PO_4^{-1}$] or [HPO_4^{-2}]. The effect of P_i on maximal force can be explained inasmuch as P_i appears to reverse the putative force-producing step of the cross-bridge cycle, i.e. P_i release (Hibberd et al., Science 228: 1317, 1985). Given this mechanism, our data imply that it is $H_2PO_4^{-1}$ that is released during the power stroke of the cycle. Depression of maximal force by some compounds similar to P_i followed the potency sequence: phenyl phosphate < P_i < arsenate << vanadate. Preliminary evidence indicates that, as with P_i, the effective form of arsenate is $H_2AsO_4^{-1}$. Nitrate had no specific effect whereas methylphosphonate significantly enhanced maximal force. (Supported by NIH/AM-31636 and MCGRI).

M-AM-A10 THE INHIBITION OF MUSCLE CONTRACTION BY THE PRODUCTS OF ATP HYDROLYSIS. E. Pate, K. Franks, G. Luciani, and R. Cooke, Dept. of Biochem. and Biophys., and the CVRI, University of California, San Francisco, CA 94143, and Dept. Mathematics, WSU, Pullman, WA 99164.
We have used a permeable fiber preparation (rabbit psoas muscle) to measure the effects of ADP, phosphate and protons on the mechanics of contraction. These data provide information on both the kinetics of the actomyosin interaction and on the process of muscle fatigue. Increasing phosphate from 3 to 20 mM does not affect the maximum contraction velocity (Vmax) or the ATPase activity, but decreases the isometric tension (Po) by approximately 30%. Changing pH from 7 to 6 decreases Po by 30% and both Vmax and ATPase activity by 40%. Previous work has shown that increased ADP elevates tension and is a competitive inhibitor of Vmax, with a Ki of 200-300 μM. These data are consistent with models of cross-bridge kinetics in which ADP is released at the end of the power-stroke preceeding ATP binding, and phosphate is released within the power-stroke in a step involving rapid equilibrium between states. The inhibition by protons is more complex, and may involve less specific effects on protein structure. During fatigue of muscle, ATP is known to remain approximately constant at 4 mM, ADP to increase from 20 to 100 μM, phosphate from 3 to 20 mM, and protons from 0.1 to 1 μM. Our data show that the inhibition of Po that occurs during fatigue can be explained by the increased levels of phosphate and protons. The inhibition of fiber Vmax and ATPase activity can be explained by the increase in protons. The increase in ADP during fatigue plays little role in the inhibition of contraction.
Supported by grants from the USPHS HL32145 and AM30868 to RC and NSF DCB8641373 to EP.

M-AM-A11 THE EFFECTS OF ATPγS ON MUSCLE CROSS-BRIDGE ORIENTATION A.J. Baker and R. Cooke, Dept. of Biochemistry/Biophysics and the CVRI, Univ of California, San Francisco, CA. 94143
One plausible assumption of the cross-bridge theory of contraction is that the orientation of the cross-bridge head changes relative to the fiber axis during the powerstroke. However, this assumption is unsupported by an analysis of EPR spectra of spin labels attached to cross-bridges in contracting fibers. The use of non-hydrolysable analogues of ATP allows closer examination of a more restricted set of cross-bridge states than those of the full cycle. We have examined the effect of ATPγS on the spectra of maleimide spin probes in muscle fibers. ATPγS is known to be hydrolysed slowly and therefore when complexed with myosin will exist predominantly as M.ATPγS. This myosin-nucleotide complex is thought to form a weak bond with actin that is a candidate for a state at the beginning of the power-stroke. In agreement with this hypothesis ATPγS was found to support very little tension in fibers. Permeabilised rabbit psoas muscle fibers, labeled with maleimide spin-label and mounted in the spectrometer, were perfused with solutions containing ATP or ATPγS. In the presence of EGTA, ATP causes the probes to become isotropically disoriented, indicative of heads detached from actin. ATPγS results in a similar spectrum but with some fraction of probes in an oriented configuration. Addition of calcium causes a slight increase in the fraction of oriented probes, without any change in angle. The resultant spectra closely resemble those observed in actively contracting fibers where ATP is being hydrolysed. Thus the configuration of cross-bridges with bound nucleotide is not altered by the hydrolysis step.
Supported by USPHS AM 30868 and a fellowship from the AHA.

M-AM-A12 EFFECT OF DIFFERENT METAL-NUCLEOTIDE COMPLEXES ON ACTOMYOSIN KINETICS IN FIBERS AND IN SOLUTION. K. Burton & J. Sleep. MRC Cell Biophysics Unit, King's College, London WC2B 5RL
The rate of force recovery after a period of muscle shortening at low load and a rapid restretch has been reported to correlate with acto-S1 ATPase activity (Brenner & Eisenberg, PNAS 83:3542, 1986). We have explored this correlation further by substituting Mn^{+2} or Ni^{+2} for Mg^{+2} and ITP for ATP in solutions in which glycerinated rabbit psoas fibers were activated. Concentrations (mM): ATP, 5; ITP, 10; 2 mM excess divalent cations; @ 10°C, 0.2 M ionic strength. Isometric fiber tension, rate of force redevelopment, maximum velocity of shortening, and maximally actin-activated S1-ATPase rates (EDC crosslinked acto-S1) were measured. Tension was reduced in solutions of MnATP, NiATP, and MgITP to about 70%, 70%, and 50%, respectively, of that in MgATP. The maximum velocity of shortening was reduced to 70%, 25%, & 13%. The rate of force recovery after a period of unloaded shortening increased to 130% in MnATP, but decreased in NiATP and MgITP to about 20%. The rate of acto-S1 ATPase was slightly lower than the rate of force recovery and increased to 135% in the presence of MnATP. The ATPase rate was reduced by NiATP and MgITP to 25% and 12%, respectively, of the MgATP value. Our finding of a similar effect upon acto-S1 ATPase activity and the rate of force redevelopment of these treatments supports the idea that the rate of transition to the force-generating state in the fiber ("f" of A. F. Huxley, 1957) corresponds to the rate-limiting step of the crossbridge cycle in solution. We also observe that acto-S1 ATPase activity does not necessarily correlate to the maximum velocity of shortening and hence probably not to the rate of detachment ("g").

M-AM-B1 IONIC CHANNEL DISTRIBUTIONS IN DEMYELINATED FROG NERVE. Peter Shrager, Dept. of Physiology, Univ. of Rochester Medical Center, Rochester, NY 14642.

We have used the loose patch clamp to explore the distribution of Na^+ and K^+ channels in single demyelinated axons. Frog sciatic nerves were demyelinated by injection of 1% lysolecithin. One to 8 days later the nerve was dissociated with collagenase and demyelinated axon segments were voltage clamped. Experiments were run with two variables: distance from the patch site to neighboring nodes, and the number of days post-injection (P.I.). Demyelinated zones, accessible for patch clamping, could be seen as early as 1 day P.I. Na^+ currents, as well as K^+ currents, were recorded from most of these zones, and maximum peak inward currents appeared independent of distance along the internode. Plotting maximum peak currents vs. days P.I. shows no clear increase over time (up to 8 days). Near the edge of the demyelinated region nodes of transition are often found, with the paranodal myelin on one side loosened and 'blistered' while that on the other side has a normal appearance. Large inward currents, up to 5 nA, have been recorded from patches at these sites. The results suggest that Na^+ channels are present in internodal membranes of normal fibers, and that at least up to 6 days P.I. steep gradients in channel density remain at nodes of Ranvier. Stimulation of the nerve at a region proximal to a node of transition resulted in an all-or-node biphasic (outward, then inward) patch current. At 1-4 days P.I. conducted signals elicit only small outward currents at some internodal demyelinated zones even though Na^+ currents are seen with step depolarizations to the clamp. This technique provides a means of judging conduction at defined loci in isolated axons.

Supported by Grant RG-1774 from the National Multiple Sclerosis Society & NIH Grant NS-17965.

M-AM-B2 ARE GLIAL Na^+ CHANNELS IN MYELINATED FIBERS STRATEGICALLY LOCATED FOR GLIA-TO-AXON TRANSFER? S.Y. Chiu & W. Schwarz (Intr. by Bruce Spalding). Dept. of Neurophysiology, Univ. of Wisconsin, Madison, WI 53706 and Max-Planck-Institut fur Biophysik, Frankfurt. FRG.

Recent patch-clamp studies on axon-associated Schwann cells from adult rabbits (Chiu, 1987, J. Physiol., in press) have shown that myelination affects glial Na^+ channels; Na^+ currents, which are detected in undifferentiated Schwann cells lacking myelin, become virtually undetectable once differentiation occurs and myelin is elaborated. Does myelin sheath simply conceal Na^+ channels located at the innermost turn of a Schwann tongue adjacent to an axon? Internodal segments (100μm) from single rabbit myelinated fibers were voltage-clamped with the method of Nonner (1969), and lysolecithin was used to acutely disrupt the myelin sheath. At 30-50 minutes, a large internodal K^+ current, similar to that observed in frog internodes, was revealed. In addition, an internodal TTX-sensitive Na^+ current, kinetically similar to nodal Na^+ currents, was consistently revealed. The Na^+ current increased with membrane capacity, and could reach 90 nA for a 100-μm internodal segment. Interestingly, the observed membrane capacity at the time of maximal Na^+ current was about 10 times too large to be explained by axonal membrane in the recording pool alone. One explanation is that the detergent-like lysolecithin induced membrane fusion between the innermost turns of Schwann cell and the axon it ensheathed. This observation raises the question of whether the observed internodal currents were due to uncovering of axonal channels, or to glial-originated channels incorporated during demyelination. Of the 200 concentric rings of Schwann membrane wrapped around a myelinated axon, are Na^+ channels strategically located in the innermost ring, as if ready for a glia-to-axon transfer? Supported by NS-23375 (NIH) and RG-1839 (NMSS)

M-AM-B3 **GATING CHARGE IMMOBILIZATION AFTER REMOVAL OF NA-CHANNEL INACTIVATION.** Joëlle Tanguy, Jay Z. Yeh and Toshio Narahashi. Lab. Neurobiologie École Normale Supérieure, Paris, France and Dept. of Pharmacol., Northwestern Univ. Med. Sch., Chicago, IL 60611.

Gating charge immobilization was studied in voltage-clamped internally-perfused squid axons at 8-10° C before and after removal of Na-inactivation by three agents: batrachotoxin (BTX), chloramine-T (CT) and pronase, which have been shown to suppress Na inactivation by different mechanisms. The kinetic and steady-state properties of the charge immobilization were determined by using a double-pulse protocol applied from a holding potential of -80 mV: the total charge movement (Q_{ON}) was measured during a test pulse to +20 mV applied 1 ms after the conditioning pulse (CP). In control (h-gate intact axon), as CP duration was lengthened (CP = +20 mV), Q_{ON} decreased with a single exponential time course (tau = 1.35 ± 0.02 ms (n = 2)), and reached 40% of its initial value (no CP applied). After removal of inactivation by pronase or CT, no decrease in Q_{ON} was observed, indicating that the gating charge was no longer immobilized. In contrast with pronase and CT, the removal of inactivation by BTX (4 μM) was not accompanied by removal of the gating charge immobilization: 1) lengthening CP duration decreased Q_{ON} to about 50% of its initial value with a single exponential time course (tau = 0.31 ± 0.08 ms (n = 8)), 2) increasing CP amplitude decreased Q_{ON} with a voltage dependency similar to that observed in control and 3) a 30 ms CP to +20 mV shifted the Q_{ON}-V curve by about 40 mV (at its midpoint) in the hyperpolarizing direction as seen in control. The rapid onset of immobilization and its uncoupling from Na inactivation in the BTX-modified channel suggest that BTX binding might transform the inactivated state (I) into a second open state (O*) by: 1) preventing the closing of the h-gate and 2) inducing a fast conformational change of the h-gate receptor responsible for the gating charge immobilization. Supported by NIH grants GM-24866 and NS-14144.

M-AM-B4 EFFECT OF TEMPERATURE ON SODIUM CHANNEL STATES STABILIZED BY DELTAMETHRIN. Kevin Chinn and Toshio Narahashi. Dept. Pharmacol., Northwestern Univ. Med. Sch., Chicago, IL 60611.

Previous whole cell and single channel studies of Na^+ channels from mouse neuroblastoma N1E-115 cells at 11°C have shown that the pyrethroid deltamethrin stabilizes several Na^+ channel states including 1) an open state, 2) a closed or inactivated state, 3) a substate, 4) a flickering state, and 5) a state in which channels opened with some delay after terminating a depolarizing pulse (post pulse channels) (Chinn and Narahashi J. Physiol. in press). The purpose of this study was to determine if increasing the temperature from 11°C to 21°C where channel activity was increased would reveal more deltamethrin-stabilized channel states. In addition to the states observed at 11°C two new states at -30 mV and -100 mV respectively were found at 21°C. At 11°C the decay of modified whole cell current at -30 mV could be fit by a single exponential while at 21°C the current decay was best fit by the sum of two exponentials. This was reflected at the single channel level at -30 mV in two ways. First, whereas at 11°C the decay of the summed average of single channel currents was fit by a single exponential, at 21°C this decay was fit by the sum of two exponentials. Second, whereas at 11°C the open time histogram was fit by a single exponential, at 21°C this histogram was fit by the sum of two exponentials indicating that more than one open state may exist. At -100 mV at 11°C post pulse channels had amplitudes similar to those of channels opened during the pulse which remained open at -100 mV (tail channels). At 21°C two types of post pulse channels were found. One had amplitudes similar to those of tail channels. The other had amplitudes 28% that of tail channels. To summarize, raising the temperature allows more deltamethrin-modified Na^+ channel states to be resolved. Supported by NIH grant NS 14143.

M-AM-B5 OUTWARD CURRENTS THROUGH SINGLE SODIUM CHANNELS OF FROG SKELETAL MUSCLE. Mauricio Ortiz and Joseph Patlak. Dept. of Physiology, University of Vermont, Burlington, Vt 05405.

We have conducted a preliminary exploration of outward unitary sodium currents under conditions of reverse Na gradient in internally dialyzed, cut-open segments of Rana pipiens sartorius muscle fibers. Patch-clamp recordings were made at 10°C in the on-cell configuration. The intracellular dialysis solution was, (in mM): 100 NaF, 5 HEPES; the pipette solution, was (in mM): 90 CsCl, 10 NaCl, 3 KCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES). The membrane was held at -120 mV and pulsed to test potentials of -20 to +100 mV at 1 Hz. The patches had between 10 and 100 sodium channels. The single-channel conductance was 10-13 pS in the linear portion of the i-V curve, but the currents saturated at potentials positive to +30 mV. Single channel currents in the saturating region were much noisier than the currents at less positive potentials, suggesting rapid ionic blockade of the channel under these conditions. The number of channels, N, and the single channel current, i, during the fast transient currents were determined with ensemble fluctuation analysis. The open probability, p_o, was determined as $p_o = I/Ni$. In three of four patches analyzed in this manner, the peak open probability decreased systematically with increasing depolarizations above +10 mV, suggesting an increase in the rate of inactivation from the closed state at those potentials. Two modes of late sodium channel activity (prolonged bursts, and isolated brief openings) were observed, as described previously (Patlak & Ortiz, 1986, J Gen Physiol 87:305-326). For potentials positive to +20 mV, the probability that each channel functions in one of these modes was not significantly different from that observed in the previous study at more hyperpolarized potentials. We conclude that open probability during late currents is not strongly potential dependent.

M-AM-B6 A MACROSCOPIC CHARACTERIZATION OF USE-DEPENDENT ION CHANNEL BLOCKADE

C. F. Starmer, A. O. Grant, D. L. Packer (Intr. by Charles Tanford)
Duke University Medical Center, Durham, NC 27710.

Many agents that block Na, K and Ca channels exhibit voltage sensitive apparent binding affinities. We consider channel proteins in either of two classes of conformations: U_o with an accessible binding site, and U_c with a hidden or inaccessible binding site. For many ion channels the transition rates between channel conformations are voltage sensitive so that changes in membrane potential produce changes in the fraction of channels with accessible sites. Channels exposed to blockers, D, result in blocked channels, B. When the conformation equilibration time is small compared with the blockade equilibration time, the overall interaction between drug and unblocked channels, U, can be described by $U + D \underset{gl}{\overset{fk}{\rightleftharpoons}} B$ where f reflects the voltage dependent fraction of channels with accessible binding sites and g reflects the fraction of bound channels where unbinding can occur. With such a system, binding affinity appears to vary with membrane potential, and reflects the voltage sensitive mixture of channels in bindable and unbindable conformations. With periodic stimulation, use-dependent blockade associated with the nth pulse is described by

$$b_n = b_{ss} + (b_o - b_{ss})e^{-n\lambda} \text{ where } \lambda = \lambda_a t_a + \lambda_r t_r,\ \lambda_a = f_a kD + g_a l,\ \lambda_r = f_r kD + g_r l$$

$$b_{ss} = a_\infty + \gamma(r_\infty - a_\infty) \text{ and } \gamma = (1-e^{-\lambda_r t_r})/(1-e^{-\lambda}),\ a_\infty = f_a kD/(f_a kD + g_a l),\ r_\infty = f_r kD/(f_r kD + g_r l)$$

t_a, t_r, λ_a, λ_r, a_∞, and r_∞ are the activated and rest time intervals, mixture binding rates and equilibria. The theoretical linear relations between apparent uptake rate, λ, and mixture dependent rates and between steady state block and mixture dependent equilibria provide a means for estimating mixture specific rate constants and a novel test of the consistency with the model. Supported by NIH grant HL32994.

M-AM-B7 PERMEATION PATHWAY OF POTASSIUM IONS IN SINGLE TOXIN-MODIFIED SODIUM CHANNELS IS ASYMMETRIC. Sarah S. Garber, Dept. of Biochemistry, Brandeis University, Waltham, MA 02254: present address; Dept. of Neurobiology, Stanford University, Stanford, CA 94305.

Single Na^+ channels from rat skeletal muscle were inserted into planar lipid bilayers in the presence of either 200nM batrachotoxin (BTX) or 50uM veratridine (VT). In order to investigate mechanisms of ion permeation through these channels, bi-ionic reversal potentials (Vr) were measured with a single ionic species on one side of the channel, and an equal concentration of another ionic species on the other. The value of the Vr (corrected for liquid junction potentials) measured in BTX-modified Na^+ channels with an ionic composition of opposite physiological orientation, 200mM NaOAc or NaCl facing the inside and 200mM KOAc or KCl facing the outside, was -57±4 mV. When the Vr is measured in a more physiological orientation with 200mM KOAc (inside) and 200mM NaOAc (outside) of the channel, the value is decreased to 35±5 mV. Preliminary results also indicate that the Vr measured with 200mM RbOAc, outside (≥-88 mV) is also greater than that measured with 200mM RbOAc, inside (33±5 mV). The Na^+/K^+ Vr measured for VT-modified Na^+ channels, however, was independent of the orientation of the Na ions with respect to the channel (+/-29±4 mV). The values of the Vr's are also independent of ionic strength over a ten-fold range (50-500mM) of concentration. These data indicate that toxin-modified Na^+ channels may be occupied by only a single ion at a time. The bi-ionic reversal potential measurements, together with the outward rectification of the conductance in pure KOAc solutions in both BTX- and VT-modified Na^+ channels (in contrast to the linear conductance in pure NaOAc solutions; Garber and Miller, J. Gen. Physiol. In Press), suggest that less permeant ions (ie. K^+, Rb^+) must cross a higher energy barrier when entering Na^+ channels from the extracellular side than when entering from the intracellular side of the channel. The difference in the height of the entry barriers for the less permeant ions, however, is greater when BTX is present as the modifier of the Na^+ channel. (Supported by NIH grant GM31768)

M-AM-B8 THE EFFECTS OF LOW EXTERNAL CALCIUM CONCENTRATION ON SODIUM CHANNELS CLOSING KINETICS IN SQUID GIANT AXONS. Clay M. Armstrong and W. Bamrungphol. Department of Physiology, University of Pennsylvania, PA 19104-6085.

The effects of low external [Ca] on Na channels closing kinetics were studied in squid giant axons. K channels were functionally eliminated by perfusing internally and externally with K-free solutions for 30 min at the beginning of each experiment. In addition, 7mM Ba was included in the internal perfusing solution (225mM K-glutamate, 50mM KF, sucrose, Hepes). Na channels kinetics were not significantly affected by either manipulation. The following observations were made when external [Ca] was lowered from the control level of 50mM. 1) Closing time constants, as determined by repolarizing the membrane to -70 mV after a short pulse (0.5 to 1.0 ms), were little changed in 20 or 10mM external Ca. 2) Below 5 to 8mM Ca, closing time constants increased steeply with decreasing [Ca]; a plot of time constants vs [Ca] is approximately hyperbolic. 3) When Ca was lowered to 3mM, Na current amplitude initially increased, then decreased progressively and disap - peared. Our results support the hypothesis that the presence of external Ca is essential for Na channel closing. With [Ca] below the required level, there is less tendency for the channels to close and increased probability of inactivation or 'slow inactivation'; thus the current magnitude decreases. There is no evidence that Na channels lose their selectivity in low [Ca]. In contrast, previous reports on squid neurons have shown that K channels lose both their ability to close and their selectivity in low external [Ca].

M-AM-B9 SOME NA CHANNELS MUST OPEN BEFORE THEY CAN INACTIVATE. L. Goldman. Dept. of Physiology, School of Medicine, Univ. of Maryland, Baltimore, Md. 21201.

Delay preceding development of Na inactivation (two-pulse method) has properties of Na activation (Goldman and Kenyon, J. Gen. Physiol. 80:83, 1982). In previous pooled data studies individual variation obscured agreement between inactivation delay and Na activation kinetics. I find τ_{delay} and τ_3 (slowest conditioning pulse activation time constant) agree closely when compared in the same axon. Experiments were on internally perfused Myxicola from Plymouth or Roscoff which have a longer delay in inactivation than North American specimens, in 1/2 Na ASW, with external 2 mM 3,4-diaminopyridine and in some cases internal 40 mM TEA, under R_S compensation, and using TTX subtraction. Kinetic parameters were extracted by a computer non-linear least squares fit of a sum of exponentials to both current records and conditioning pulse data. In one experiment a three exponential fit to a conditioning pulse record at 2 mV yielded 4.70, 0.491 and 0.126 ms for τ_h, τ_3 and τ_2. Two pulse determination in this axon gave 4.68 and 0.493 ms for τ_c and τ_{delay} for a ratio of τ_{delay}/τ_3 of 1.004. Mean τ_{delay}/τ_3 over a range of -27 to 2 mV, both Cs and K perfused, and with and without eternal Zn was 1.067. The slowest Na activation and delay time constants are essentially identical suggesting delay is generated by activation. For some channels inactivation gates close only subsequent to a precursor process which is channel opening. Others may inactivate in parallel directly from resting state(s). Alternatively, very similar molecular machinery has been constructed twice for each channel, once for activation and again for inactivation pathways. Supported by USPHS Grant NS-07734 and Bressler Research Fund, Univ of Md.

M-AM-B10 ADJACENT INTERVAL ANALYSIS OF FAST CHLORIDE CHANNELS IN CULTURED RAT SKELETAL MUSCLE ALLOWS EXCLUSION OF SEVERAL CLASSES OF KINETIC SCHEMES. A. L. Blatz and K. L. Magleby. Dept. of Physiology and Biophysics, University of Miami School of Medicine, Miami, FL 33101.

Unconditional distributions of open and shut interval durations obtained from single channel currents provide information about the number and mean dwell times of conformational states of a channel molecule, but contain little information about the transitional relationships between the various states. To determine the transitional pathways we examined the durations of adjacent open and shut intervals, as in McManus & Magleby (Biophys. J., 1985, 47:137a) and McManus, Blatz, & Magleby (Nature, 1985, 317:625-627). The mean open intervals and distributions of open intervals adjacent to shut intervals of specified durations were obtained. The time constants of the two exponential components of the distribution of open intervals were relatively independent of the adjacent shut interval, whereas the area of the slow open component decreased and the area of the fast open component increased as the specified shut interval durations increased. Several models which gave identical descriptions of the unconditional distributions of open and shut intervals, but which had differences in transition pathways between the states, were examined with adjacent interval analysis. Since these models all described the unconditional distributions of experimental intervals as well as theoretically possible, distinctions between the models could not be made on the basis of the unconditional distributions. Predicted means and distributions of adjacent intervals for these models were determined by simulation, taking into account filtering and missed events. Some of these models could be excluded because they did not describe the experimentally observed means and distributions of adjacent intervals. Supported by NIH and the Muscular Dystrophy Assoc.

M-AM-B11 **CHLORAMINE-T ALTERS BIREFRINGENCE RESPONSE IN *LOLIGO* AXONS.**
David Landowne. Department of Physiology and Biophysics. University of Miami. 33101

Segments of squid giant axons were perfused internally with a CsF, Cs glutamate solution and externally with 20% Na sea water. They were mounted in an optical pathway between crossed crystal polarizers and voltage-clamped with an axial carbon fiber electrode. The intensity of light passing through a 3 mm length of axon and the polarizers was monitored with a photo-diode. The voltage-clamp currents and the changes in photo-current were digitized (12 bit, 10 µsec) and signal-averaged (typically 1024 sweeps).

There are two major phases of the birefringence response to a depolarizing pulse that is long compared to the time course of the sodium current. (*J. Membr. Biol.* **88**, 173-185) At the beginning of the pulse there is a rapid decrease in birefringence of about 30 ppm, with kinetics which are similar to the fast 'gating' polarization currents, followed by a slower decrease in birefringence of about 2 ppm/msec. External treatment with the protein oxidizing agent chloramine-T (1 mg/ml) reduces the slow decrease to 21% ± 16% of before treatment values (N=5). The sodium currents were prolonged indicating the removal of sodium inactivation as seen by Wang, Brodwick & Eaton (*J. Gen. Physiol.* **86**, 289-302). Internal perfusion with the reducing agent Na metabisulfite (1 mg/ml) protected both the optical and the electrical responses. These results suggest there is a structural or conformational change of the sodium channel protein associated with the inactivation process

M-AM-B12 DEMONSTRATION OF Na-Ca EXCHANGE ASYMMETRY IN SQUID OPTIC NERVE VESICLES.
Madalina Condrescu, Andrés Gerardi and Reinaldo DiPolo. Centro de Biofísica y Bioquímica, IVIC, Apartado 21827, Caracas 1020A, Venezuela.

Classical models of the Na-Ca exchange are based on a reversible and symmetric carrier system in which Na ions are required at the *trans* face of the membrane (with respect to Ca ions) in order to translocate calcium, and vice versa. Recently, DiPolo and Beaugé (BBA, 854:298, 1986) have shown in dialyzed squid axons that the reverse mode of the exchange only operates in the presence of internal Ca^{2+}, suggesting the asymmetry of the carrier. In the present study we explored this hypothesis in squid optic nerve membrane vesicles (10% inside-out, 67% rightside-out), by controlling the intravesicular Ca^{2+} concentration with EGTA. Fractionated membranes were resuspended in a medium containing: a) 200 mM NaCl, 100 mM KCl, 2 mM EGTA or b) 200 mM NaCl, 100 mM KCl, 2mM EGTA and different concentrations of $CaCl_2$ (in order to obtain intravesicular ionized Ca in the range comprised between 10^{-6}-10^{-4} M). Membranes were stored frozen at -70°C. The assay of the Na-Ca exchange activity was performed by diluting the vesicles 20 fold in a medium containing 1 mM $^{45}CaCl_2$ and 100 mM KCl, in the presence of 5 µM valinomycin. The vesicles containing internal calcium (at 100 µ M Ca^{2+}) exhibited about 3 fold increase in the initial rate of $^{45}Ca^{2+}$ uptake compared to vesicles containing EGTA. This activatory effect of intravesicular calcium on the reverse mode of the Na-Ca exchange showed an apparent $K_{\frac{1}{2}}$ of 20 µM. The increase in $^{Ca2+}$ uptake elicited by intravesicular calcium is not due to Ca-Ca exchange since this component is inhibited in the presence of 200 mM NaCl and is independent of the intravesicular Ca^{2+} concentration. Our results indicate that the Na-Ca exchange present in squid optic nerve vesicles is not symmetric. (Supported by CONICIT S1-1144 and Fundación Polar).

M-AM-C1 ADDITIVE MUTATIONS AND THE PERTURBATIONS OF STABILITY IN SEQUENCE VARIANTS OF T4 LYSOZYME

Wayne J. Becktel, John A. Schellman and Walter A. Baase
Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

We observe a scaler relationship between the changes in free energies of denaturation and the melting temperatures for single and multiple mutants of bacteriophage T4 lysozyme. At the melting point of a reference protein, the relationship is:

$$g_0 = \Delta S_0 \, \Delta T$$

where g_0 is the isothermal free energy difference at Tm_{ref}, ΔS_0 is the entropy of denaturation of the reference protein and ΔT the melting temperature difference. This relationship is extended to compare single and multiple mutants of T4 lysozyme. When the change of stability observed in the multiple mutant is the linear sum of the single site changes, the following relationships both apply:

$$T_{ab} = T_a + T_b - T_{ref} \qquad \text{and} \qquad g_{ab} = g_a + g_b$$

where a and b refer to different point mutations taken relative to a reference protein. Examples are given for combinations of destabilizing mutants, of stabilizing mutants and destabilizing mutants, and of stabilizing mutants. The relationship is valid for changes in g_0 as great as 10 kcal/mole and differences in melting temperature of 25 deg C. The error between the measured and predicted free energy difference is 5 to 10%. T4 lysozymes differing by as many as six point mutations have been successfully compared by this method.

M-AM-C2 Structural and Functional Mapping of Human Erythropoietin. Sylvia Lee-Huang, Dept. of Biochemistry, NYU Medical School, N.Y., N.Y. 10016

Site-directed mutagenesis and chemical modifications are used to study the structural and functional relationships of human erythropoietin (Ep). The effect of specific alternations was studied by in vivo exhypoxic polycythemic mouse assay (physiological activity), in vitro mouse bone marrow assay (target cell/receptor recognition), RIA and immunoblotting. The mutant and modified Ep proteins were isolated and purified, their activities were compared to that of the native molecule. Delation of Ala^1 results in a significant decrease (90%) of in vivo bioactivity, whereas no effect on in vitro activity and antibody binding affinity was detected. These results suggest that the N-terminal alanine of Ep is probably not involved in the active and antigenic sites of the hormone, but it is important for the in vivo stability and physiological activity of Ep. Cys→Ser mutation at Cys^7 or Cys^{29} causes 75-85% loss in all activities, indicating that intact disulfide bridges are essential for full activity. Mutants isolated from Tyr^{51}→Phe substitution possess about 60% of the erythropoietic activity and full immunological activity. Phosphorylation of native Ep protein causes a 30 to 40% decrease in bioactivity and over 65% loss of antibody binding. Iodination of Ep with unlabelled NaI results in complete loss of bioactivity yet full retention of antibody binding ability. These results suggest that tyrosine residue(s) are critical to erythropoietic activity but not to antigenic activity. Serine and/or threonine residue(s) are likely to be associated with antigenic sites. These studies provide useful information in the structural and functional mapping of the Ep protein as well as in the development of Ep analogs. Supported by NIH grants HL 21683 and HL 30862.

M-AM-C3 UNFOLDING PATHWAY OF APOMYOGLOBIN : A MULTI-STEP PROCESS.

Ettore BISMUTO and Gaetano IRACE
Cattedra di Chimica e Propedeutica Biochimica - I Facoltà di Medicina e Chirurgia - Via Costantinopoli 16 - 80138 Napoli - Italy.

The guanidine-induced unfolding of apomyoglobin has not been found to be a simple two-step process but consistent with the occurrence of at least two molecular events. The first event, observed at low denaturant concentration, involves the occurrence of a molecular intermediate, which has lost the ability to bind the heme but still retains a considerably high degree of structural organization. The molecular properties of the intermediate form produced by low guanidine concentration resemble rather closely those possessed by the acid denatured form of the protein. The disarrangement of the heme binding site, induced by low guanidine concentration or by acids, has been found to involve conformational changes occurring in the two structural domains identified in the myoglobin molecule, i.e. segments 1-79 and 80-146. The high degree of cooperativity of the molecular event leading to the formation of the intermediate form has been documented by fluorescence studies performed on the ANS*(or TNS) - apomyoglobin conjugate. The lifetime analysis of the bound chromophore (determined by multifrequency phase and modulation fluorometry) as well as the emission maximum remains unchanged in the same range of denaturant concentration which makes the protein unable to bind the chromophore.

M-AM-C4 A UNIFYING MODEL OF THE THERMODYNAMICS OF LIGAND BINDING TO PYRIDINE NUCLEOTIDE LINKED DEHYDROGENASES. Narinder Singh and Harvey F. Fisher, VA Medical Center and Department of Biochemistry, Kansas University Medical Center, Kansas City, MO 64128.

We propose a unifying model to account for a large group of thermodynamic phenomena exhibited by the pyridine nucleotide linked dehydrogenases and their various complexes. These phenomena include the anomalous energetic parameters of the glutamate dehydrogenase-reduced coenzyme binary complex; the large negative enthalpies of interaction and heat capacities of interaction for both positively and negatively interacting systems; the variations in heat capacities among the various dehydrogenase complexes; and the significant effects of the formation of such complexes on the thermal stabilities of the enzymes.

The model assumes only that the enzyme can exist in either of two physically distinguishable states; that the ΔH° between these two states is very large, is temperature independent, and is the same for all forms of the enzyme (free enzyme and enzyme in any binary or ternary complex); that the ΔG°'s of the two-state equilibria differ from one enzyme form to another. We present evidence in support of this theory, and show that this very simple model can produce all of the phenomena described above in a quantitatively consistent manner and can make experimentally testable predictions for a given dehydrogenase system. The nature of the two physical states of the enzyme will also be discussed.

M-AM-C5 SURFACE FREE ENERGY AS THE POTENTIAL IN OLIGOMERIC EQUILIBRIA: PREDICTION AND MEASUREMENT OF HEMOGLOBIN DISAGGREGATION CONSTANT AND USE OF MODEL FOR GATED CHANNELS. Martin Blank and Lily Soo, Dept. of Physiology and Cellular Biophysics, Columbia University, 630 W. 168 St., New York, NY 10032

The disaggregation of hemoglobin tetramers into dimers as a function of pH has been measured osmotically, and the equilibrium constant calculated. In the alkaline range, the equilibrium constant varies with the pH, and its logarithm is a linear function of the charge on the molecule. Since aggregation-disaggregation reactions appear to involve changes at the interfaces of the subunits rather than in the internal structure, it was assumed that the total free energy change in such reactions could be evaluated in terms of the surface free energy change (i.e., calculated from the area and surface charge density). This assumption leads to the correct prediction of the disaggregation constant.

The surface free energy model of oligomer association appears to be useful as a model for the opening and closing of oligomeric channel structures in membranes, and we have used it for the voltage gated channels of excitable membranes, in conjunction with the surface compartment model (which includes ionic processes in the electrical double layer regions at charged surfaces). The results of these calculations show that: (1) the ionic currents in excitable membranes can be described by electrodiffusion theory, (2) the selectivity of an ionic channel is due to the kinetics of channel opening, and (3) the transient ion concentraion changes due to oscillating electric fields can stimulate ion pump enzymes.

M-AM-C6 FREE VOLUME DIFFUSION OF PROTEINS IN AQUEOUS SOLUTIONS AND IN LIPID BILAYERS. Timothy J. O'Leary, Division of Biochemistry and Biophysics, Center for Drugs and Biologics, FDA, Bethesda, MD 20892.

A free volume diffusion theory has been developed for protein diffusion in aqueous solution and in lipid bilayers. Diffusion is assumed to proceed by a three step mechanism in which first a region devoid of protein is formed by statistical density fluctuations, then a protein molecule diffuses into this void, and finally the void is closed by diffusion of other protein molecules. When no obstructing molecules are present, the protein molecules are assumed to diffuse as if in dilute solution. No diffusion takes place if a protein's path is obstructed by another molecule. The overall self diffusion coefficient D_{self} is then the product of the self diffusion coefficient at infinite dilution D_o and the probability of vacancy formation. This probability is given by the Cohen and Turnbull expression $P = \exp[-\gamma\rho V_H/(1-\rho V_H)]$, where ρ is the number density of protein molecules V_H is a hydrodynamic volume and γ is an adjustable parameter. The theory accurately describes the concentration dependence of human and lumbricus terrestris hemoglobin diffusion in aqueous solution, and mimics Monte Carlo calculations for the concentration dependence of protein diffusion in lipid bilayers.

M-AM-C7 **PROGRESS TOWARD A COMPUTATIONAL MOLECULAR BIOLOGY**

Paul A. Bash , U. C. Singh, Robert Langridge, Peter Kollman, Dept. of Pharmaceutical Chemistry, University of California, San Francisco 94143

Thermodynamic perturbation theory implemented with molecular dynamics is described together with its application to problems in drug and protein design. The perturbation method allows one to transform or "mutate" a system between any two states that can be suitably parameterized. This method is applied to the protein thermolysin complexed with phosphonamidate and phosphonate ester inhibitors. The atomic structure of these protein-inhibitor complexes has been determined (Matthews et. al.) and they are nearly identical, yet their binding constants differ by three orders of magnitude (Bartlett and Marlowe). We have calculated the difference between the free energy of binding of these inhibitors with a result of 4.2 ± 0.54 kcal/mol which compares remarkably well with the experimental value of 4.1 ± 0.1. In preparation for the use of this method in protein design, we simulated the free energies of solvation for all classes of amino acids. Calculated values are all within about 10% of relevant experimental data and provides the necessary experience to apply these methods to protein design. An example of its use on a site-specific mutant in dihydrofolate reductase and a prediction of the effects of such a change in trypsin will be presented. The resultant close correspondence between calculated and experimental values are very encouraging and lead us to believe that this technique will provide a significant advance toward a rational approach to both drug and protein design.

M-AM-C8 NEW METHOD FOR CALCULATION OF HYDRATION ENTHALPIES FOR ARBITRARY POLAR OR CHARGED MOLECULES.

Alexander A. Rashin, Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029.

A new general method for calculation of hydration enthalpies for arbitrary polar or charged molecules is presented. The method is based on a classical macroscopic description of the solvent and the assumption that the total enthalpy of hydration is the sum of the enthalpy of electrostatic interactions of the molecule with the solvent represented as dielectric and the hydration enthalpy of a nonpolar molecule forming the same cavity in the solvent. The electrostatic contribution to the enthalpy is calculated using a new reaction field treatment of the solute-solvent interactions and an objective self-consistent definition of the boundary between the solute and the solvent. Quantum chemical or semi-empirical treatments are used for the description of the charge distribution of the solute. An empirical relation based on correlation of cavity surface areas with hydration enthalpies of nonpolar molecules serves on the evaluation of the nonpolar contribution to the hydration enthalpy. The method has been applied to calculations of hydration enthalpies of a number of polyatomic molecules, both neutral and charged, The calculated values agree within a few percent with the available experimental values and with the results of unbiased Monte Carlo simulations.

This work has been supported by a Fellowship from the Revson Foundation and by NSF grant DMB-8519273.

M-AM-C9 HYDRODYNAMIC AND BIOPHYSICAL STUDIES ON SINGLE CHAIN AND TWO CHAIN UROKINASE. Grant H. Barlow, Susan L. Firestone, and Victor Gurewich.
Bioprocessing Research Center, U. of Texas Health Science Center, Houston TX, and Vascular Laboratory, St. Elizabeth Hospital, Tufts U. School of Medicine, Boston MA.

The plasminogen activator, urokinase, has been shown to exist in both a single chain, poorly active form and a two chain form which has high activity. Hydrodynamic studies on the one and two chain urokinase forms show a small but reproducible difference in the sedimentation coefficient indicating a change in conformation during the transformation from a one chain to a two chain form. The change is in the direction of a more compact molecule. Isopycnic runs in cesium chloride also show a small difference in the buoyant densities which can be interpreted as a loss of bound water during the same transformation. The changes that occur are not nearly as significant as those seen in the conversion of the zymogen plasminogen to the protease plasmin. SDSPAGE studies on the conversion by the addition of low levels of plasmin show a large increase in the degraded form of the molecule at 31,000 daltons indicating that the Lys-Lys bond at positions 135-36 is broken as well as the Lys-Iso bond at positions 158-159. This observation would tend to indicate that plasmin is not the physiological enzyme required for this transformation.

M-AM-C10 2D-NMR STUDY OF SECONDARY STRUCTURE OF P401 IN SOLUTION
N. VASANT KUMAR[1], David E. Wemmer[2] and N.R. Kallenbach[1]
[1]Department of Biology, University of Pennsylvania, Philadelphia, Pa 19104
[2]Department of Chemistry, University of California, Berkeley, Ca 94720

Peptide 401 (or the mast cell degranulating protein, MCD) is a relatively small protein toxin found in bee venom. It consists of 22 amino acids and is highly basic in solution (5 lysines, 2 arginines, and no acidic residues). Its primary structure is homologous to apamin, another neurotoxin found in bee venom. We have studied the structure of P401 in solution by two-dimensional ^1H NMR spectroscopy at 500MHz. Almost all the backbone proton resonances have been assigned using the sequential assignment strategy and data from COSY (Correlated Spectroscopy), NOESY (Nuclear Overhauser Effect Spectroscopy) and RELAY (Relayed Coherence Transfer) experiments. A contiguous stretch of NOEs between consecutive amide protons (d_{NN}) defines a C-terminal alpha-helix involving residues 13 to 22. The N-terminal residues 1 to 5 are found to be in an extended conformation. These two structural elements are connected via a beta-turn, resulting in a structural fold very similar to that in apamin but with an added arm near the N-terminus.
(This work is supported by N I H grant GM-38161.)

M-AM-C11 LASER-EXCITED Eu^{3+} LUMINESCENCE AS A PROBE OF Ca^{2+} BINDING SITES: TIME RESOLUTION AND DIFFUSION ENHANCED ENERGY TRANSFER STUDIES OF CALMODULIN AND ITS TARGETS.
W. DeW. Horrocks, Jr., J. M. Tingey, C. B. Thompson and D. T. Cronce, Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

Laser-excited lanthanide ion, Ln^{3+}, luminescence techniques have been used in this laboratory to probe the Ca^{2+} binding sites of proteins including calmodulin, CaM, (Mulqueen *et al.*, Biochemistry 1985, 24, 6639). The four metal binding sites of calmodulin cannot be distinguished in the frequency domain of the excitation spectrum of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+}, but different classes of sites can be resolved using time resolution techniques. In D_2O solutions the excited state lifetimes of Eu^{3+} bound to calmodulin distinguish three classes of sites. The two tight Ln^{3+} binding sites (in domains I and II) have indistinguishable lifetimes, τ, of 2400 μs, while the weaker sites (in domains III and IV) have τ values of 1600 and 750 μs. The order of filling of the sites can be followed during the course of a titration with Eu^{3+} from the amplitude of each of the luminescent decay components. The accessibility of the various Eu^{3+} binding sites to the solvent is assessed by means of diffusion-enhanced energy transfer to small energy acceptor ions, eg. $[Co(NH_3)_6]^{3+}$ free in solution. All of the above experiments were carried out both in the presence and absence of the calmodulin antagonists melittin, trifluoperazine, and fluphenazine. (Supported by NIH grant GM23599 and NSF grant CHE-8504256.)

M-AM-C12 MOSSBAUER SPECTROMETRY OF LIPOXYGENASE.
M.O. Funk, R.T. Carroll J.F. Thompson,* R.H. Sands[+] and W.R. Dunham[+] (Intr. by M.L. Ludwig) Department of Chemistry, The University of Toledo, Toledo, OH 43606, U.S. Plant Soil and Nutrition Laboratory* USDA-ARS-NAA, Ithaca, NY 14853, Biophysics Research Division, The University of Michigan,[+] Ann Arbor, MI 48109

Isotopic iron has been incorporated into soybean lipoxygenase by a method to be described. The isoenzymes were found to occur in immature seeds and in cultured cotyledons. The lipoxygenases from soybeans, developing embryos and cultured cotyledons were found to be similar in apparent molecular weight, isoelectric point and peptide mapping. The *in vitro* biosynthesis of type-1 enzyme was demonstrated by the incorporation of radioactive iron-59: the iron to protein ratio was found to be 1.0. Using the tissue culture technique, iron-57 was successfully incorporated into two lipoxygenase isoenzymes. The iron in the enzyme as isolated from the cultured cotelydons without treatment by product hyperoxide was shown to be partially oxidized as determined by Mossbauer spectrometry. EPR spectrometry confirmed the presence of oxidized lipoxygenase in fresh tissues from immature seeds. It was also possible to obtain natural abundance Mossbauer spectra at protein concentrations of 2mM. The spectroscopic results will be interpreted in terms of the structure of the iron binding site of the lipoxygenase.

M-AM-D1 LOW TEMPERATURE SOLID-STATE ^{13}C-NMR STUDIES OF THE RETINAL CHROMOPHORE IN RHODOPSIN
S.O. Smith[1], I. Palings[2], V. Copié[1,3], D.P. Raleigh[1,3], J. Courtin[4], J.A. Pardoen[4], J. Lugtenburg[4], R.A. Mathies[2], and R.G. Griffin[1]. [1]Francis Bitter National Magnet Laboratory, and [3]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139. [2]Department of Chemistry, University of California, Berkeley, CA 94720. [4]Department of Chemistry, Leiden University, 2300 RA Leiden, The Netherlands.

Magic angle sample spinning ^{13}C-NMR spectra have been obtained of bovine rhodopsin regenerated with retinal prosthetic groups isotopically enriched with ^{13}C at C-5 and C-14. In order to observe the ^{13}C-retinal chromophore resonances, it was necessary to employ low temperatures (-15→-35°C) to restrict rotational diffusion of the protein. The isotropic chemical shift (130.3 ppm) and principal values of the chemical shift tensor of the ^{13}C-5 label indicate that the retinal chromophore is in the twisted 6-s-*cis* conformation in rhodopsin. This indicates that the conformation of the C_6-C_7 bond does not contribute to the opsin shift in rhodopsin, in contrast to bacteriorhodopsin which contains a planar 6-s-*trans* chromophore [Harbison et al., Biochemistry 24, 6955 (1985)]. The ^{13}C-14 isotropic shift (121.2 ppm) and shift tensor principal values show that the Schiff base C=N bond is *anti*. Furthermore, the ^{13}C-14 chemical shift is within the range of values (120-123 ppm) exhibited by protonated (C=N *anti*) Schiff base model compounds, indicating that the C=N linkage is protonated. The absence of a perturbed ^{13}C-14 chemical shift provides no support for the presence of an external protein charge near this position.

M-AM-D2 INFLUENCES OF PHOSPHOLIPID POLAR HEADGROUPS AND ACYL CHAIN POLYUNSATURATION ON PHOTOCHEMICAL FUNCTION OF RHODOPSIN IN RECOMBINANT MEMBRANES.
Timothy S. Wiedmann, Robert D. Pates, James M. Beach, Amir Salmon, and Michael F. Brown (Intr. by S. G. Frasier). Department of Chemistry and Biophysics Program, University of Virginia, Charlottesville, VA 22901.

We have continued our studies of the MI to MII conformational transition of rhodopsin which may be linked to visual function (1). Previous work from our laboratory and others has shown that in recombinants of rhodopsin with saturated phosphatidylcholines (PC), such as di(C14:0)PC, the MI to MII transition is essentially blocked on the time scale of visual phototransduction and does not display the absorbance changes characteristic of native retinal rod membranes (1,2). Using the magnitude of the MI to MII phototransient following a 20-30% bleaching flash as the criterion of photochemical activity, we have found that (i) full activity of rhodopsin may be reconstituted with the extracted native rod outer segment lipids; (ii) rhodopsin activity is enhanced by the presence of both phosphatidylethanolamine (PE) and docosahexaenoyl (C22:6ω3) acyl chains; and (iii) an equimolar mixture of PC and PE with 50% of the acyl chains being C22:6ω3 provides an environment corresponding to optimal photochemical activity. (1) J. M. Beach et al., Biophys. J. 45, 292a (1984). (2) P. A. Baldwin and W. L. Hubbell, Biochemistry 24, 2624 (1985). **Work supported by NIH Postdoctoral Fellowships (T.S.W. and J.M.B.), an American Heart Association Postdoctoral Fellowship (R.D.P.), an NSF Predoctoral Fellowship (A.S.), NIH Grants EY03754 and EY00255, the Jeffress Trust, and the Alfred P. Sloan Foundation (M.F.B.).**

M-AM-D3 A LOW CONDUCTANCE LIGHT-DEPENDENT CHANNEL OBSERVED IN CELL ATTACHED AND EXCISED PATCHES OF LIMULUS VENTRAL PHOTORECEPTORS.
J. Bacigalupo, J. Lisman, and E. Johnson. Dept Biol. Brandeis U., Waltham, MA and U. of Chile, Santiago, Chile.

We have previously reported (Nature 304:268) that light increases the frequency of both large and small single-channel events in cell-attached patches and that the larger events are due to channels having a conductance of 40pS. We now describe the properties of the smaller events. These are due to a 13pS channel (range 11-19pS) having a mean open time, voltage-dependence and reversal potential nearly identical to that of the 40pS channel. As with the larger channel, the smaller channel is directly activated by light and not by the light-induced depolarization. It remains unclear whether the smaller channel is a different state of the larger channel or an altogether different channel. After the patch is excised in ASW and transferred to an internal solution (high K^+, low Ca^{2+}(10^{-7}M)), spontaneous channel activity was usually observed, and often was greater than occurs in cell-attached patches during intense illumination. This channel activity did not require the application of a second messenger and could not be suppressed by ATP. However, this spontaneous activity could be nearly shut off with 10^{-5}M Ca^{2+} on the internal side. The channel seen in these excised patches is a 13pS channel that is similar to both light-dependent channels in its voltage dependence and mean open time which increases markedly at positive voltages. The high observed in excised patches suggests that the closed state of the 13ps channel requires a regulatory component that is lost or damaged with our excision conditions. The ability of Ca^{2+} to block channel activity suggests a site for the role of Ca^{2+} in light adaptation. Supported by N.S.F. BNS-8319331 and N.I.H. EY01496 and EY05802.

M-AM-D4 EVIDENCE FOR A FUNCTIONAL INTRACELLULAR H^+-Ca^{2+} LIGAND IN BALANUS PHOTORECEPTOR. H. Mack Brown, J. Burnham & J. Smolley. Dept. Physiology, Univ. of Utah, Salt Lake City, Utah.

Acidification of Balanus receptor cytoplasm by as little as 0.1 pH unit produces a clear desensitization of the receptor. pH changes of the same magnitude have been recorded with intracellular pH electrodes upon illumination of this cell which has raised the possibility that pH changes could contribute directly to desensitization of the cell during light. However, recent lines of evidence indicate that intracellular pH changes may mediate effects via intracellular Ca^{2+} changes. One such observation is that removal of extracellular Ca^{2+} produces a rapid onset of cellular alkalinization suggesting that a small Ca^{2+} leak in darkness contributes to cell acidity. Acidification of the cell with CO_2/HCO_3^- salines produces biphasic responses in both intracellular pH and intracellular Ca^{2+} recorded with intracellular pH and Ca^{2+} ion-selective electrodes. A pH change of ~0.1 pH unit raises Ca_i by 0.5 pCa in darkness. In a cell stimulated with brief, repetitive flashes of light, biphasic changes in the receptor potential are also observed under the same conditions. Injection of a Ca^{2+} buffer ~7 x 10^{-7}M transiently raised intracellular Ca^{2+} and transiently reduced the receptor potential. Thus, both small pH changes and Ca^{2+} changes exert similar effects on the receptor potential. These observations suggest that an intracellular H^+/Ca^{2+} ligand could play a key role in functional changes of the cell's response to light.

M-AM-D5 EVIDENCE OF TWO FUNCTIONALLY DIFFERENT MEMBRANE FRACTIONS IN RETINAL ROD OUTER SEGMENTS. Paul J. Bauer, Inst. f. Biol. Information Processing, KFA, D-5170 Jülich, FRG

We investigated the cGMP-sensitive cation conductance and the Na/Ca-exchange activity of bovine rod outer segments which were washed free of peripheral proteins (sRDM). After equilibrating sRDM in a Ca-containing buffer for 15 h at 2° C and subsequent removal of external Ca, the Ca-activity was monitored with arsenazo III. Addition of cGMP resulted in a Ca-release which at most was 22 % of the maximal Ca-release (i.e. when adding 1 µM A23187). Addition of Na instead of cGMP lead to a Ca-release which, again, saturated at 22-25 % of the maximal Ca-release. When the Na/Ca-exchangeable Ca-pool was partly depleted, the maximal Ca-release upon addition of cGMP was smaller than 22 % and vice versa: partial depletion of the cGMP-sensitive Ca-pool resulted in a reduction of the maximal Ca-release due to Na/Ca-exchange; however, the sum of the Ca-releases after cGMP- and Na-addition never exceeded 22-25 % of the maximal Ca-release. - When loading the cGMP-sensitive Ca-pool of sRDM with Ca by short incubation (30 sec) in 15 mM $CaCl_2$ and 0-40 µM cGMP followed by a quick removal of the external medium by centrifugation and resuspension of the pellet in Ca-free buffer, full depletion of the cGMP-sensitive Ca-pool lead to Ca-releases ranging from 7 % (0 µM cGMP) to 49 % (40 µM cGMP) of the maximal Ca-release. After the exhaustive cGMP-stimulated Ca-releases, the amplitudes of the ionophore-stimulated Ca-releases were constant for sRDM loaded at different cGMP concentrations. - We conclude that sRDM essentially comprise two membrane fractions: one fraction (enclosing ca. 20 % of the particulate volume) which contains the cGMP-sensitive channels as well as the Na/Ca-exchanging protein and another fraction (enclosing ca. 80 % of the particulate volume) which contains neither cGMP-sensitive channels nor Na/Ca-exchanging proteins.

M-AM-D6 COMPARISON OF LIGHT-SENSITIVE AND cGMP-ACTIVATED CONDUCTANCES OF ROD PHOTORECEPTORS AT THE SINGLE-CHANNEL LEVEL. Gary Matthews and Shu-Ichi Watanabe, Dept. of Neurobiology, SUNY, Stony Brook, NY 11794-5230.

Patch-clamp recordings from rod photoreceptors revealed light-sensitive channel activity. Individual light-sensitive channel events consisted of a burst of brief openings and closings, with an average burst duration of 0.78 ± 0.03 msec (mean ± s.e.; N=17). The average closed duration within a burst was 0.37 ± 0.02 msec, and the average duration of an individual opening was 0.18 ± 0.01 msec. After recording light-sensitive channel activity in the intact rod, the patch of membrane was excised in the inside-out configuration. Application of cGMP to the intracellular membrane face activated channels whose properties could then be compared directly with the light-sensitive channels recorded earlier in the same patch of membrane. For the cGMP-activated channel, the average burst duration was 0.85 ± 0.07 (mean ± s.e.; N=11) msec, the average closed duration within a burst was 0.38 ± 0.03 msec, and the average duration of an individual opening was 0.23 ± 0.01 msec. Histograms of burst duration, open duration, and closed duration for light-sensitive and cGMP-activated channels within the same patch were indistinguishable. The channel conductance was slightly higher in the excised patch than in the intact cell (24.0 ± 1.2 vs. 20.5 ± 1.1 pS). Voltage had little or no effect on the temporal parameters of either channel. The results demonstrate that the cGMP-activated channel and the light-sensitive channel of the rod photoreceptor are the same channel. (Supported by NIH Grant EY03821.)

M-AM-D7 INTERACTIONS OF CATIONS WITH THE CYCLIC GMP-SENSITIVE CHANNEL OF RETINAL RODS. A.L. Zimmerman and D.A. Baylor. Neurobiology Department, Stanford Medical School, Stanford, CA 94305.

We have studied conductance of ions through the cGMP-sensitive channels of salamander rod outer segments by measuring macroscopic current from inside-out excised patches. The cGMP-sensitive conductance was activated by exposing the inside surface of the patch to saturating cGMP.

With Na as the external cation and Cs the internal cation, and with low divalents, the reversal potential of the cGMP-induced current was 11 mV more positive at 130 mM salt than at 5 mM. The outward current carried by Na ions did not show the concentration dependence expected for saturation of a single ion binding site. These observations suggest that more than one ion may occupy the channel at a time.

In symmetrical solutions containing 130 mM Na and low divalents, addition of small amounts of Mg on the internal side of the membrane blocked the monovalent current as if by saturating a single site with a dissociation constant of 0.3 mM at 0 mV. Depolarization increased the apparent Mg affinity of this site and hyperpolarization lowered it. These observations indicate that the channel contains a binding site with a high affinity for divalent cations, and that the site is located within the transmembrane electric field.

The organic cation tetramethylammonium did not carry measurable current through the channel. This suggests that the pore through which ions pass is less than 6 Angstroms in diameter.

M-AM-D8 ION SELECTIVITY OF THE cGMP-DEPENDENT CONDUCTANCE FROM PHOTORECEPTORS. R.E. Furman and J.C. Tanaka*. Depts. of Neurology and Biochemistry & Biophysics*, Univ. of Pennsylvania, Philadelphia, PA.

Reversal potentials and conductance-voltage relations were determined in voltage-clamped, inside-out patches from Rana pipiens rod outer segments by exchanging solutions on the cytoplasmic side of the patch. Rods were patched in (mM) 120 NaCl, 10 HEPES, 0.1 EDTA, 0.1 EGTA. Test solutions substituted 120 mM monovalent cations or 60 mM divalents and 60 mM sucrose for Na. pH was adjusted to 7.3 with TMA, NH_4^+, or Tris. Currents were activated by 100 uM cGMP. Net currents at 90 mV ranged from 100-900 pA.

After corrections for junction potentials and leakage selectivity, reversal potential measurements for group Ia cations showed permeability ratios of 1.0 Na, 0.95 K, 0.90 Li, 0.88 Rb. Cs and choline currents were too small for reversal potential determinations. Conductance ratios at 90 mV were 1.0 Na, 0.83 Li, 0.37 K, 0.17 Rb, and <0.05 Cs and choline.

The divalent cations, Ca, Ba, Mg, showed conductances relative to Na of 0.18, <0.05, <0.05, respectively. Permeabilities relative to Na were 0.62 Ca, 0.35 Ba, and Mg was indeterminate.

All monovalent cations supporting cGMP-dependent current produced asymmetrical, highly non-linear IV curves with increasing rectification to either side of the reversal potential. At the cytoplasmic side the divalent cations as well as Cs completely or partially inhibited the inward Na currents from the extracellular side. Buffers examined had no overt effect on the rectification nor did changes in cGMP concentration over two orders of magnitude.

M-AM-D9 EFFECTS OF cGMP ON THE PHOTOTRANSDUCTION OF DETACHED ROD OUTER SEGMENTS. Shaul Hestrin and Juan I. Korenbrot. Department of Physiology, University of California Medical School, San Francisco, CA 94143.

Quantitative understanding of the role of cGMP in rod phototransduction requires experimental control of the cytoplasmic milieu of rod outer segments. Using patch-clamp pipettes we have found that control of the intracellular milieu can be better achieved in isolated outer segments than in intact rods. We have detached outer segments from the retina of the tiger salamander and studied their phototransduction properties while controlling the composition of the cytoplasm with a patch pipette containing GTP, ATP and cGMP. If cGMP is the transduction messenger it can be predicted that at cytoplasmic cGMP concentrations well above the Kd of the outer segment channels for cGMP, there should be a large dark current and a long delay between the actinic flash and the photoresponse. We found that these predictions are correct in isolated outer segments. In the presence of 5 mM cGMP in the pipette the dark-current was about 1200 pA (holding potential:-30 mV) and the response to a saturating flash (200-400 photons/u(2)) had a delay of 2-3 sec. It can also be predicted that the flash photosensitivity of the rod should decrease as the cGMP concentration increases. Indeed, we found that the photosensitivity in the presence of 0.2 mM cGMP was similar to that of intact rods, but was about 100 fold less in the presence of 5mM cGMP. The cytoplasmic level of cGMP does not appear to be in equilibrium with the cGMP in the patch pipette: addition of 100 uM IBMX to the bath resulted in a 2-4 fold enhancement of the dark current in the presence of 0.2 mM cGMP. There appears to be sufficient PDE activity in a dark-adapted rod to prevent complete equilibration between the pipette and the cytoplasm. The detached outer segment, thus, provides an improved preparation for a detailed analysis of the role of cGMP in phototransduction.

M-AM-D10 A MAGNESIUM COMPONENT IN THE DARK CURRENT IN RETINAL RODS. K. Nakatani, and K.-W. Yau, Howard Hughes Medical Institute and Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

The inward dark current which flows through the light-sensitive conductance in retinal rods has long been thought to be carried exclusively by Na^+. Recently, however, we have found that about 15% of this current is in fact carried by Ca^{++} (Yau and Nakatani, Nature 313, 579, 1985). We now report that another component of this current seems to be carried by Mg^{++}. Membrane current was recorded from an isolated toad rod with a suction pipet containing Ringer's solution while its outer segment was bath-perfused. Normal Ringer's contained (in mM): 110 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 1.6 $MgCl_2$, 5 TMA (tetramethylammonium)-HEPES, 5 glucose, pH 7.6. Upon replacing with choline all external permeant cations, except the 1.0 mM Ca^{++}, around the outer segment we observed a small inward Ca current in darkness. The Ca influx calculated from integrating this current agreed with the amount of Ca^{++} trapped in the outer segment as estimated from the subsequently reactivated sodium-calcium exchange in Ringer's. Upon repeating the experiment with 1.0 mM Ca^{++} and 1.6 mM Mg^{++} present externally we found this time that the charge influx through the light-sensitive conductance exceeded the amount of trapped Ca^{++}, thus indicative of a Mg component in the dark current. We estimated the ratio of Ca influx to Mg influx to be about 3:1. In other words, about 5% (or$\sim$1 pA) of the physiological dark current should be carried by Mg^{++}. The Ca and Mg influxes exhibited mutual inhibition. Furthermore, they both had an inhibitory effect on the Na influx. Thus, ion movements through the light-sensitive conductance are highly interactive. The order of preference of the channel for passing the three cations appears to be $Ca^{++} > Mg^{++} > Na^+$.

M-AM-D11 SINGLE cGMP-ACTIVATED CHANNEL ACTIVITY RECORDED FROM EXCISED CONE MEMBRANE PATCHES. L.W. Haynes and K.-W. Yau (Intro. by G. Yellen), Howard Hughes Medical Institute and Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

We and others[1-3] have previously reported that, in the absence of divalent cations, cGMP-induced single channel activity can be recorded from excised patches of rod outer segment plasma membrane. We now report that such activity can also be observed in cone membrane under similar conditions. Current was recorded in visible light from an excised, inside-out membrane patch from the catfish cone outer segment. The cells had been treated with hyaluronidase and collagenase prior to recording. Identical solutions (118 mM NaCl, 0.1 mM Na-EGTA, 0.1 mM Na-EDTA, and 5 mM Na-HEPES, pH 7.6) were present in the patch pipet and the bath. With cGMP in the bath, single-channel activity could be recorded whch resembled that observed in rod patches. At +30 mV, the prominent unitary current had an amplitude of 1.0 - 1.5 pA, corresponding to a conductance of 30-50 pS. A similar-sized unitary current of opposite polarity was observed at -30 mV. The channel openings occurred both singly and in bursts, with a mean channel open time of about 0.5 msec and a mean burst time of about 3 msec. Besides events of the above size there were also smaller events which were more difficult to resolve. The channel activity increased with both depolarization and the concentration of cGMP.

On the whole, the individual channel openings were briefer and larger in amplitude than those observed in rod patches. Whether collagenase (not used in the rod experiments) might have to do with this difference remains to be examined.

Reference: 1) L.W. Haynes, A.R. Kay and K.-W. Yau (1986) Nature 321: 66-70. 2) A.L. Zimmerman and D.A. Baylor (1986) Nature 321: 70-72. 3) G. Matthews (1986) PNAS: in press.

M-AM-D12 BLEACHING ADAPTATION OF ISOLATED CONE PHOTORECEPTOR CELLS. Gregor J. Jones, M. Carter Cornwall and Alan Fein. Department of Physiology, Boston University School of Medicine, Boston, MA 02118 and Laboratory of Sensory Physiology, Marine Biological Laboratory, Woods Hole, MA 02543.

Previous experiments on the adaptation to light of cone photoreceptor cells have been done in the presence of continuous and rapid photopigment regeneration, since, even in the isolated retina, cones are able to regenerate photopigment that is bleached by light. Isolated cone, cells, however, do not recover their photopigment after bleaching, as is well known from microspectrophotometry experiments. Therefore, we are studying adaptation in isolated cells to separate the effects of pigment bleaching and pigment regeneration. Cone cells were mechanically isolated from dark-adapted retinae of larval Ambystoma Tigrinum, and light-induced changes in membrane current recorded using external suction electrodes. Bleaching lights produced large changes in sensitivity of the cells in response to brief light flashes, with a shift in threshold of up to 4.5 log units of light intensity. There was an associated decrease in the amplitude of the saturating response to flashes, of up to 90%. Both changes were fast - the new levels were stable within a few seconds after the end of the bleaching light, and they were then maintained in the dark for at least 1 hr. Stepwise bleaching of the same cell produced a progressive reduction in sensitivity and response amplitude. Control values of threshold and maximum response amplitude were restored by reconstitution of photopigment using unbleached chromophore, by adding 11-cis retinal in phospholipid vesicles. In the presence of excess 11-cis retinal this recovery was complete within a few seconds. Supported by NIH grants EY01157 and EY03793.

M-AM-E1 IN-SITU FT-IR STUDIES OF PHOSPHOLIPID MONOLAYERS ON AQUEOUS SUBSTRATES.
Richard A. Dluhy, National Center for Biomedical Infrared Spectroscopy, Battelle-Columbus Laboratories, 505 King Avenue, Columbus, Ohio 43201.

Monomolecular films composed of synthetic phospholipids have been widely utilized as model systems in order to study the physical properties of biological membranes. Unfortunately, while insoluble monolayers at the air-water interface have been extensively studied as models for interfacial phenomena, knowledge of the detailed physical structure of these films has been lacking. In the past, a detailed molecular-level understanding of the structure and dynamics of monomolecular films was hampered by the limited ability of spectroscopic techniques to study low surface-area, flat interfaces. Recently, however, we have shown that it is possible to measure, in-situ, the infrared spectrum of a monomolecular film at the air-water phase boundary by an application of external reflection Fourier transform infrared spectroscopy. Theoretical calculations based on classical electromagnetic theory show that the optical properties of monolayers on water compare favorably with those of monolayers on reflective metals. Detailed examination of the spectra of 1,2-distearoyl-sn-glycero-3-phosphocholine that had been spread at the air-water interface shows that the monolayer acyl chains are in a rigid, mostly all-trans configuration with an average angular distribution of 35° from the surface normal. Spectra will also be presented of a monolayer film of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine at several points along its surface pressure/molecular area curve in order to follow the first-order thermodynamic phase transition of the film from its liquid-expanded to liquid-condensed states.

M-AM-E2 HEADGROUP AND CHAINLENGTH DEPENDENCE OF PHOSPHOLIPID SELF ASSEMBLY STUDIED BY SPIN LABEL ELECTRON SPIN RESONANCE. Martin D. King & Derek Marsh. Max-Planck-Institut fur biophysikalische Chemie, D-3400 Gottingen, W. Germany. (Intr. by James K. Zimmerman).
The critical micelle concentrations (CMCs) of a variety of spin-labelled phospholipids, 1-acyl-2-[4-(4,4-dimethyloxazolidine-N-oxyl)-valeryl]-sn- glycero-3-phospho- derivatives, have been determined by electron spin resonance spectroscopy. The rapidly tumbling monomers provide narrow line spectra clearly distinguished from the spin-spin broadened spectra of the micellar aggregates. For phosphatidylcholine (PC), ln (CMC) decreases linearly with the sn-1 chain's length as characteristic of the hydrophobic effect. The CMCs of the 1-lauroyl derivatives of the phospholipids in 0.15 M NaCl are: phosphatidic acid^{2-}, 0.77 mM; phosphatidic acid H^{-}, 0.13 mM; phosphatidylserine, 0.24 mM; phosphatidylglycerol (PG), 0.17 mM; PC, 0.10 mM; phosphatidylethanolamine (PE), 0.05 mM. For negatively charged phospholipids, CMCs decrease by one to two orders of magnitude on increasing the ionic strength to 2.0 M NaCl, with a characteristic Debye-Huckel type of screening. For the zwitterionic lipids, the CMCs for PC and PE decrease by a factor of approximately four on going from 0 to 2 M NaCl. The CMC of the 1-lauroyl derivative of phosphatidic acid increases by almost an order of magnitude on titration from pH 5 to pH 9, with pK_a of 7.4 (second deprotonation). These results demonstrate that the polar headgroup can also have a very marked influence on the energetics of self assembly. The rate of monomer-micelle collisions has been measured from the exchange broadening of the monomer signal as a function of the micelle concentration. The differences in rates correspond to the effects of the electrostatic surface potential for PG, to possible differences in the micelle size, n, and are consistent with a diffusion-controlled process.

M-AM-E3 HEAD-GROUP INTERACTIONS AMONG AMPHIPHILIC MOLECULES AT OIL/WATER INTERFACES.
Dirk Stigter and Ken A. Dill, Departments of Pharmaceutical Chemistry and Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143.

Pressure-area isotherms for amphiphile monolayers spread at an oil/water interface, yield information on head group interactions that may be extrapolated to membranes, vesicles, micelles. We discuss isotherms available in the literature on C18 sulfate and di-C18-phosphatidylcholine (PC). The interaction between sulfate head groups as a function of NaCl concentration is well explained by the electrical double layer theory (Davies), provided it is appropriately corrected for the self energy of the head group charges. Interactions among zwitterionic head groups such as PC are described in terms of the two dimensional second virial coefficient (B2) as a function of temperature. B2 depends on the hard core repulsion between PC head groups and the interactions of the P-N zwitterionic dipoles whose average orientations change slightly as a function of temperature.

M-AM-E4 IONIC EFFECTS ON THE POLAR HEADGROUP INTERFACE OF UNILAMELLAR PHOSPHOLIPID VESICLES STUDIED BY GEL CHROMATOGRAPHY, TRAPPED VOLUME, ANALYTICAL ULTRACENTRIFUGATION AND DYNAMIC DENSIMETRY METHODS. F. Bellemare and M. Fragata, Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières, Québec, Canada.

The effect of NaCl on the diameter of small, unilamellar vesicles constituted of phosphatidylcholine (PC) was determined by molecular sieve chromatography, trapped volume analysis with ferricyanide, sedimentation coefficient and specific volume of the phospholipids obtained by dynamic densimetry. The trapped volume determinations were carried out after elution of vesicles' preparations on a Sepharose 4B column. The sedimentation coefficients were obtained by analytical ultracentrifugation after sample dialysis and incubation with different NaCl concentrations. We found that only the dynamic densimetry procedure is sensitive enough to detect ionic strength effects on the vesicles' diameter. It is interesting to note that the results obtained with this method are consistent with the kinetic data of Lessard and Fragata (J. Phys. Chem. 90 (1986) 811). The effect of NaCl on the headgroup of the PC vesicles is explained by a salting-out mechanism. (This work was supported by grants from the N.S.E.R.C. Canada (A-6357), and the Fonds F.C.A.R. du Québec (EQ-3186)).

M-AM-E5 CO-SOLUBILITY IN BINARY CHOLESTERYL ESTER SOLIDS - AN ELECTRON DIFFRACTION AND DSC STUDY. D. L. Dorset, Electron Diffraction Dept., Medical Foundation of Buffalo, Inc., 73 High St., Buffalo, NY 14203.

Although the physical state of polydisperse cholesteryl ester mixtures has been shown to be an important factor in diseases where such deposits accumulate, it is difficult to predict rules for the occurrence of solid solutions or eutectic separations from the paucity of published phase diagrams for binary model systems. Accordingly, a combined electron diffraction and DSC study of a saturated cholesteryl ester series, from undecanoate to stearate, was carried out on various binary mixtures, which were originally well-mixed in the molten state and allowed to equilibrate for a week after recrystallization. Electron diffraction measurements of lamellar spacings were made on epitaxially crystallized fractions to observe deviation from Vegard's rule. The laurate is polymorphic forming either monolayer I or bilayer type crystal structures, and, accordingly, forms continuous solid solutions with either the undecanoate (monolayer I) or myristate (bilayer). The myristate also forms continuous solid solutions with other bilayer-forming esters with similar molecular volume, e.g. the pentadecanoate and palmitate; the myristate/stearate system is a eutectic of solid solutions. The myristate/undecanoate mixtures are totally immiscible because the components have different crystal structures. The overall behavior is consistent with rules given by Kitaigordskii for the formation of continuous solid solutions which require similar crystal structures and molecular volumes for the pure components. Research funded by a grant from the Manufacturers and Traders Trust Company.

M-AM-E6 DSC STUDIES OF THE VESICLE-MICELLE TRANSITION IN BILE SALT-LECITHIN MIXTURES. David Fame and Charles Spink, Chemistry Department, SUNY-Cortland, Cortland, NY, 13045.

Precision scanning calorimetry is used to examine mixtures of sodium taurocholate (TC) and dipalmitoylphosphatidylcholine (DPPC) in regions of composition and TC/DPPC mole ratios where micelles and vesicles form. In solutions of 5mg/ml DPPC and TC/DPPC mole ratios of 1-2.5 the DSC pattern corresponds to a typical micelle transition with low cooperativity and low enthalpy. Light scattering from the solutions is also low. On the other hand, at 0.7mg/ml DPPC and again TC/DPPC ratios from 1-2.5, sharp, higher temperature transitions are found, and light scattering is high. In these more dilute solutions the DSC thermograms become more complex. With increasing bile salt-DPPC mole ratio as many as four peaks emerge. At DPPC contents between 0.7 and 5.0 mg/ml the array of peaks changes, some of which decrease in proportion to light scattering. These peaks are associated with the vesicles, and by deconvoluting the overlapping transitions assignment of the micelle peak can be made in the mixtures. It is shown that small amounts of cholesterol ($\sim$4 mole percent) incorporated into the solutions change the thermal transitions significantly.

M-AM-E7 THE METASTABILITY OF CHOLESTEROL SUPERSATURATED MIXED MICELLAR BILE MODELS. D. Lichtenberg, S. Ragimova, A. Bar, N. Grushka, and S. Almog (Introducted by J.W. Ogilvie). Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 67798, Israel.

The maximal equilibrium solubility of cholesterol (CH) in mixtures of phosphatidylcholine (PC) and bile salts (BS) depends on the CH/PC ratio (R_c) and on the effective ratio (R_e) between non-monomeric bile salts and the sum (C_T) of PC and CH concentrations (Carey and Small, 1978; Lichtenberg et al., 1984). Contradistinctively, the concentration of BS required for solubilization of liposomes made of PC and CH does not depend on R_c (Lichtenberg et al., 1984). Thus for $R_c > 0.4$, solubilization of the PC-CH liposomes yields PC-CH-BS mixed micellar systems which are supersaturated with CH. In these systems, the mixed micelles spontaneously undergo revesiculation. The rate of the latter process depends upon R_c, R_e and C_T. For any give R_c and R_e, the rate increases dramatically with increasing C_T, reflecting the involvement of many mixed micelles in the formation of each vesicle. The rate also increases, for any given C_T and R_e, upon increasing CH to PC ratio R_c, probably due to the increasing degree of supersaturation, whereas a decrease in the degree of supersaturation brought about by elevation of cholate concentration (increased R_e, for constant R_c and C_T) enhances the revesiculation process. As expected, cholate concentration higher than that required for complete solubilization at equilibrium yields stable mixed micellar systems which do not undergo revesiculation, but for lower cholate concentrations decreasing the degree of supersatuation (by increasing [cholate]) results in faster revesiculation. This surprising result can be rationalized in terms of the structure of the mixed micelles.