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LIPID MECHANISM AND ACETYLCHOLINESTERASE FUNCTION

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Abstract

The mechanism of acetylcholine-induced ion channels is described in detail. On the basis of thermodynamic phase diagrams, the free energy coupling, membrane excitation, and permeability fluctuations appear as a consequence of first principles of chemomechanical coupling, hydrodynamic excitations, and thermal fluctuations. The theory is outlined and compared with the observations of proton-induced conformational changes, adiabatic membrane current pulses, and proton-induced ion channels in pure phospholipid bilayers. Local control of permeability and electromotive force across asymmetrically protonated phospholipid membranes is discussed. Enzymatic hydrolysis of acetylcholine thus induces ion channels in agreement with the requirement of a specific receptor mechanism. The mechanism is chemiosmotic and offers a solution to the controversy about localised versus delocalised energy coupling at ATPases.

"Es ist wohl unnötig zu betonen, dass ich mit vielen Ideen nicht einverstanden bin. Aber ich bin in den Haber Colloquien erzogen worden, in denen Controversen als ein wesentliches Element wissenschaftlichen Fortschritts betrachtet wurden."

David Nachmansohn 14.11.1981

Protonic mechanism of membrane transport

Although the chemical basis of nerve activity has been established and molecularly characterised for the cholinergic system by Nachmansohn, Silman, Changeux, and others, the physical mechanism of the transport processes across biological membranes has not yet been identified despite considerable effort.

Molecular Basis of Nerve Activity

Such situation could arise from erroneous a priori hypotheses. One such hypothesis, implicit in most membrane models, is that of an inert lipid bilayer, and the consequent assignment of all elements of the transport system to autonomous proteins. The latters are not measurable autonomously, since any membrane transport is observed in the presence of the associated lipid bilayer. Although it is evident that specific proteins induce specific transport across biological membranes, it is impossible to exclude that the protein activities control specifically a lipid mechanism of transport across the bilayers.

All membranes are constituted by a lipid bilayer. Therefore, any consequence of any protein activity on the physical properties of the lipid monolayers by necessity takes part in the function of the proteins. The lipid properties imply free energy coupling, propagating excitations, and conductivity fluctuations, as derived from first principles below and demonstrated directly experimentally. Local enzymatic proton sources such as acetylcholinesterase (AChase) and ATPases inevitably imply, with certain probability values, coupling of chemical to osmotic free energy as well as induction of specific ion channels, on the basis of the same lipid phase diagrams.

On the other side, the hypothesis of inert lipid membranes requires to assume very different protein mechanisms for free energy coupling, receptor function, voltage-induced excitation, distinguished by postulating, respectively, intrinsically chemiosmotic enzymes, non-catalytic receptors, voltage-dependent channel proteins. Each of such postulated protein mechanisms has, in physical detail, remained completely mysterious.

The assumption of a non-catalytical receptor of ACh violates causality because catalytic hydrolysis has a faster relaxation time than the physiological response; instead, the slow response has to follow, and therefore to be caused by, enzymatic hydrolysis. I have earlier predicted that AChase activity is sufficient to increase the permeability of the lipid component of nerve membranes.

In vitro, the catalytic mechanism has been established in collaboration with Silman. It makes use of the local decrease in pH at AChase as described by Silman and Karlin; it is also compatible with the observation by Podleski and Changeux that physiological responses to ACh are subject to pH buffers. The mechanism of the ion channel induction was identified: ion channels of the same kinetic relaxation times appear at corresponding pH in the absence of proteins across the pure phospholipid bilayers. The lipid component was shown to be capable to produce the ion channel conductivities reported for various lipid-protein membranes.

Although the induction by specific proteins is obvious, the assumption of autonomous protein channels for ion transport must therefore be considered inobservable.

The falsification of inert lipid bilayer hypotheses demands for a membrane theory on the basis of the physical properties of lipids, and of the specific control functions of proteins. I shall outline protonic control functions; the obvious generalisation to control by voltage, calcium, or surface pressure is not attempted.

Active ion transport

Catalytic protons exert a two-fold function on the membrane:

On the one side, protonation of the phospholipid surface influences the thermal fluctuations in the fluid-crystalline order of the polymeric hydrocarbon chains. This proton receptor function suffices to induce ion channels in monolayer states of large compressibility and large consequent lipid area and bilayer thickness fluctuations of the membrane. In particular, such fluctuations diverge at a critical point and become extremal at the phospholipid pK.

On the other side, asymmetric protonation of one surface of the bilayer only creates an electromotive force which drives ion transport across such channels.

By consequence, asymmetric protonation fluctuations cause directed proton transport across proton-induced ion channels. Phospholipid bilayers thus relate chemiosmotic and chemoreceptor phenomena. This relation is demonstrated experimentally, since both AChase and ATPase activity induce ion channels. The protonic receptor mechanism is "active" after Ussing, since the local electrochemical diffusion occurs even in the absence of global bulk asymmetry due to local asymmetric hydrolysis.

The establishment of a stationary active gradient across the membrane does require stationary enzyme activity, i.e. localisation of enzyme and substrate at the same side of the membrane. Therefore, at a cholinergic membrane, only ATPase activity is capable to establish such membrane potentials by the lipid mechanism, in contrast to AChase.

The opposite asymmetry of AChase localisation by consequence induces instationary and depolarising ion transport upon hydrolysis of applied ACh. The agreement with the experimental observations, and in particular transient overshoot of depolarising membrane excitation with reversal of its sign demonstrates the intrinsic relation of chemiosmosis and receptor functions.

Free energy coupling

Non-catalytic models of ACh receptor function admit dissipation of free energy useful for transport. Williams argued that free energy coupling, to avoid dissipation, has to make use of local, "energised" protons at the membrane surface. Eigen and deMaeyer discovered very high proton mobilities in water and ice according to which another mechanism is required in addition to store free energy. The discussion of surface proton mechanisms by Nagle and Morowitz, Haines, and Nicholls and Deamer is in this respect insufficient. The situation is further complicated by the fact that Mitchell's theory was clearly applicable although formulated in terms of aqueous protonic electrochemical potentials, and verified by the observed coupling of bulk gradient to chemical free energy.

How can protonic free energy be stored at the membrane surface?

This question is related to the present scientific controversy on local versus global chemiosmosis. It is also related to the problem how the protonic mechanism of ion transport by ACh could apply in the presence of neutral pH buffers in the global bulk medium.

Chemomechanical coupling to the phospholipids resolves this problem principally, applying for two-dimensional monolayers the one-dimensional polymeric muscle models developed by Katchalsky, Oplatka, and Kedem.

The reversible chemomechanical coupling between local enzymatic proton sources and associated protonable phospholipid monolayers stores the free energy which would, otherwise, drive protons into the bulk solution. Storage at the surface is due to the lipid thermodynamic transitions of long relaxation times, e.g. several msec according to Tsong, and Träuble and Eibl, in order to release protons from the phosphate groups. According to monolayer phase diagrams by Träuble and Teubner, the model lipid methyl phosphatidic acid (dimyristoyl) at the apparent pK stores above 0.1 kcal/mole protonation even for constant surface pressure. For adiabatic coupling, of course, efficiency is 1. Chemomechanical coupling above 1 kcal/mole phospholipid protonation is easily achieved not too far from the apparent critical point of onset of first order phase transitions.

The assumption of efficient free energy coupling thus leads to the hypothesis of specific boundary lipids near the enzymes which are driven by the catalytic activity toward the critical pK range. Examples for specific boundary lipids are phosphatidyl serine at Na^+ - K^+- ATPase, or phosphatidyl inositol at hydrophobic AChase, reported by Papahadjopoulos and Kimelberg and, respectively, by Futerman and Silman.

With respect to these properties of phospholipid bilayers, the controversy between Mitchell and Williams on the role of localised versus global protons resolves as follows:

enhanced proton activity and surface diffusion is a consequence of the negatively charged lipid phosphate groups. The semi-conductor like mobility mechanism of protons in the structured surface water leads to a proton specificity of the equilibrium Gouy-Chapman layer and of non-equilibrium propagating excitations. In thermal equilibrium between surface and bulk, the local proton activity is controlled by the globally applied electrochemical potentials. Enzymatically produced "energised" local protons delocalise rapidly along the surface only, and couple free energy chemiosmotically, by "protomotive" force, along the surface and eventually, due to membrane thickness fluctuations, across the bilayer to the other surface.

Critical osmosis

The protomotive force can only drive ion transport when permeable structures are induced by critical perturbation of the impermeable lipid order. The protons do so in the range of pK of the phospholipids, as observed. In case of asymmetric proton-sources, a proton-induced ion channel appears only in the presence of a protomotive force even if symmetry is observed of the global, neutral bulk pH.

The phospholipid bilayer fluctuations define a very interesting thermodynamic system with respect to the third dimension of transport. It is in most states closed or impermeable, but conditionally open in a critical range only. Since conductivity fluctuations can be induced by asymmetric proton sources, as observed in collaboration with Silman, Nernst-Planck diffusion suffices to establish a non-equilibrium membrane gradient. When considering only the transport one-dimensionally, the lipid membrane appears as a Maxwellian demon, although it directly follows from the thermodynamic principles of fluctuations and diffusion.

The non-enzymatic side of the membrane becomes protonated, driven by catalysis which also induces the open state. The closed state in absence of catalytic protons stores the established osmotic gradient for, in principle, infinite times.

A testable prediction of critical osmosis is a lipid-dependent upper limit for the "static head" electrochemical potential difference which can be built up. It is achieved when the local protomotive force is zero even though the enzyme is actively inducing the critical pH range, say pK, of the phospholipids. Therefore, the reverse electrochemical potential built up must balance the local protomotive force. This value can be estimated from the pK observed in the absence of applied electrostatic potential differences in a monolayer system. If the bulk pH at the enzymatic side is 7, a pK of 6 gives ca. 60 mV, pK 5 ca. 120 mV, pK 2 ca. 300 mV which should never be exceeded for typical phospholipids crucially.

Desensitisation

With respect to the mechanism of ACh-induced ion transport, the prediction from phospholipid monolayer phase diagrams of reduction of fluctuation strength upon full protonation at very acid pH below the pK is of particular interest. Further production of protons has no effect any more on the low strength of thickness fluctuations in such states. A similar desensitisation of proton receptors has been observed in vivo by Gruol and by Krishtal et alii. Since the work of Katz and Thesleff, desensitisation of the physiological ACh receptor mechanism is well-known in cholinergic systems.

Again, the slow lipid relaxation times allow to observe a local membrane state such as full protonation even at rather neutral bulk pH reversibly.

The effect of Ca^{2+} on phospholipid bilayers is more complex, but basically mimicks the effect of protons. Ca^{2+} also produces the phenomenon of desensitisation at too high concentrations, but there is no experimental proof that this is achieved by the phospholipid properties.

Clearly, the observations in collaboration with Silman demonstrate that phospholipid bilayers represent proton receptors, capable of desensitisation. The proton-induced ion channel activity near the pK was observed to disappear or to be reduced dramatically upon further application of acid.

While desensitisation remains an obstacle requiring adjustable parameter models in the hypothesis of autonomous protein receptors, desensitisation naturally follows from the direct observation of a critical range of increased fluctuations of area and thickness, surrounded by states of lower fluctuation strength at both more neutral and more acid pH. This observation is made on monolayer surface pressure-area diagrams as well as, by measurement of membrane current, on bilayers made of synthetic lipids such as methyl phosphatidic acid.

Physiological receptor of acetylcholine

The lipid mechanism described above fulfills the requirements of the physiological receptor of ACh in vivo, i.e. in the presence of AChase activity and the absence of pharmacological modifications.

As described by Nachmansohn, Changeux, Neumann, and others, specific ligand binding does induce a conformational change, via a permeable state, into the desensitised state. Receptor coupling is allosteric, however, the channel subunit appears to be the lipid bilayer associated to the ACh-specific binding site.

Adiabatic excitation

Despite the rather slow release of protons from phospholipid surfaces, the local protons propagate rapidly along the two-dimensional surface according to hydrodynamic principles. The propagation velocity equals the adiabatic density derivative of surface pressure. From isothermal phase diagrams of Träuble and Teubner, velocities up to 20 m/sec can be roughly estimated. Clearly, slowing down is predicted at the apparent pK, even down to 0 m/sec at a critical point, since any local excitation will be of an effective mass corresponding to the diverging coherence length.

It is very interesting to note that sub-threshold excitations do not propagate, as already described half a century ago by Auger and Fessard. Above a critical threshold value, the voltage-induced depolarising pulse gradually increases in amplitude until it appears as an all-or-nothing action potential of defined propagation velocity. Propagation velocities of action potentials as well as of monolayer shock waves, described recently by Moebius et al., achieve maximal velocities in the order of several m/sec.

Propagation velocities may achieve optimal values in myelinated axons due to the insulation from viscous damping. Viscosity is essentially due to coupling to transversal excitation of the associated bulk aqueous medium in contact with the two-dimensional density pulse in the monolayers. As pointed out to me by H.W. Strube, in the worst case of complete coupling to the transversal modes, propagation of the excitation must cease due to dissipation after a few µm, allowing for almost adiabatic propagation of free energy associated to electrical signal in unmyelinated membrane systems for typical cellular dimensions only.

Msec relaxation times have been observed in pure lecithins near the phase transition temperature with high activation energies (60 kcal/mole) by Tsong, using Eigen and deMaeyer's temperature jump technique. 100 msec relaxation time of vanishing activation energy had also been observed. Hill reported similar time scales for rapid "local potentials" and, respectively, slow "threshold relaxations";most remarkably, Hill also discovered small microdegree temperature changes associated to, and of the same shape as, the action potential. Iwasa and Tasaki confirmed these observations in axons and detected reversible changes in membrane pressure and area, too.

Msec membrane current pulses are also routinely observed in pure phospholipid bilayers. As demonstrated below, these pulses as well as the ion channels only require the presence of phospholipids and protons. Propagating density pulses in lipid monolayers therefore represent a physically defined mechanism for propagating electrostatic potential changes associated to the other thermodynamic variables temperature, surface pressure, and so forth. These other variables have, however, no place in a priori electrostatic equivalent circuits. Moreover, these surface variables have no meaning in terms of single protein molecules.

Ion specificity

How could ion-specific transport such as of Na^+ as opposed to K^+ arise across lipid ion channels by likewise inspecific electrochemical diffusion?

Thermodynamically, ion specificity can only be achieved at the expense of free energy. It is therefore intrinsically related to the problem of active ion transport. Passive channel models cannot, as shown by Hille, achieve ion specificity merely by specific binding sites because such a mechanism uses free energy from the applied potential and thus hinders diffusion.

There is no need for a priori ion-specific channels since transport requires both a permeable structure and a thermodynamic driving force. Since the ion channels can kinetically be directly attributed to the rather inspecific lipid bilayer, the specificity of biological membrane transport arises by control of the electromotive forces created by chemiosmotic free energy coupling.

In particular, specific ions which activate catalysis induce ion channels in the presence of specific electromotive forces only. Generalising critical osmosis, discussed above, to Na^+- activated ATPase, it is found theoretically that the electrostatic component of the protomotive force created only in the local presence of Na^+ inavoidably drives specific H^+- Na^+- cotransport toward the non-enzymatic side of the membrane.

Other ions which do not directly activate the enzyme, such as K^+ which is also present in large concentration generally, are not specifically driven by the local chemiosmotic mechanism. Since, however, the ion channels per se are passive and inspecifically uncoupling in principle, the reverse global electromotive force built up does drive ions in the corresponding direction. For example, Na^+- ATPase, which establishes as described a reverse global potential difference positive at the non-enzymatic side, indirectly thus drives reverse K^+ transport toward the enzymatic membrane side. In particular, K^+ approaches by this mechanism an equilibrium potential equal, but opposite in sign, to the electrostatic component of the membrane potential built up by Na^+- ATPase. Though, K^+ appears to be actively driven by the hydrolysis of ATP.

This ion-specific mechanism is clearly testable by the predicted specificities and directions which are due to the enzyme activation and localisation. It represents a crucial test of the theory that these specificities as well as these directions agree with those observed for H^+- ATPases following Mitchell, Schatz and Ernster, for Na^+- K^+- ATPase following Skou, Glynn and Karlish, and also for Ca^{2+}- activated ATPase, which transports Ca^{2+} toward the non-enzymatic side according to Fleischer. AChase, which is not activated by either of those specific ions, should only induce uncoupling of the specific gradients established by Na^+- K^+- ATPase, i.e. immediate Na^+- influx and delayed K^+- efflux after depolarisation of the electrostatic component of the membrane potential. Again, the specificities and directions crucially test the proposed mechanism.

A particularly interesting prediction following from the properties of critical osmosis is active proton transport toward the non-catalytic side by any hydrolase of sufficient activity to induce ion channels at acid phospholipid pK. It were falsified if Na^+- K^+- ATPase, as generally assumed, would not transport H^+. Ken-Dror, Schnaiderman and Avi-Dor found such H^+ transport in the predicted direction. That this active H^+- Na^+- cotransport was not observed previously may derive from the fact that proton activities are very low usually, as compared to Na^+ and K^+; therefore, large potential differences, i.e. logarithmic changes in proton activity, can be achieved by comparatively small and eventually inobserved proton fluxes. Still, these forces drive by their electrostatic component Na^+ and K^+ transport until the static head or equilibrium potentials described above.

It was not attempted to derive strict stoichiometries. Our mechanism implies stoichiometric catalysis, but diffusive transport across eventually uncoupling ion channels. Pietrobon, Azzone and Walz have described "slips" in active redox pumps which clearly falsify strict stoichiometries. So far, no contradiction arises, however, from these observations with respect to the lipid mechanism described here, although a more detailled analysis is required to settle this question.

Direct observation of the conformational transitions

In order to directly reconstitute the bilayer transport properties predicted from phospholipid monolayer surface pressure- area phase diagrams, two crucial elements of the theory appear particularly accessible: the conformational changes and the transport of protons.

In order to quantitatively control eventual correlations between bilayer conformational changes and area/thickness fluctuations to the induction of discrete ion channel conductivities, I measured the electrical capacitance of pure phospholipid bilayers. Marty and Neher have already reported in vivo membrane capacitance steps in states of ion channel activity. While these observations were made in the presence of vesicles and attributed to vesicle fusion, capacitance steps of similar magnitude do also appear in planar lipid bilayers made from two monolayers. Boheim, Hanke and Eibl reported a ca. 20% change in membrane capacitance at the thermal phase transition of myristoyl-stearoyl phosphatidyl choline to be associated by membrane current fluctuations.

Electrical capacitance clearly represents an order parameter of the lipid component of membrane and cannot be attributed to autonomous proteins. Therefore, the observation of capacitance steps in the presence of ion channel activity demonstrates the correlation of lipid order fluctuations to the induction of ion channels. The membrane conductivity per se does in contrast not represent an order parameter, but rather a phenomenon associated to its fluctuations.

I here report the induction by protons of stepwise changes in membrane capacitance, correlated to the induction of ion channels, of synthetic diphytanoyl phosphatidyl choline bilayers. Planar bilayers were made following Hanke, as described earlier, in 1 M KCl unbuffered at room temperature ca. 22°C. In addition to an applied +77 mV voltage, ±3.7 mV were superimposed by triangular AC voltage of 10 Hz.

Fig. a) demonstrates the membrane current under these conditions. The step in membrane current every 50 msec is proportional to the electrical capacitance and represents 60 pF, corresponding to 0.5 $\mu F/cm^2$. The capacitance was not crucially altered during successive titration of HCl asymmetrically to the + side of the bilayer, and no significant effects on membrane current were observed above pH 2.5 on this side. On the other side, pH 6.5 was approximately maintained throughout the experiment, in the non-conducting states. Conductivity was only 4 pS.

However, when pH 2.4 was achieved in this membrane, clearly, discrete changes in membrane capacitance were observed. Three such changes are shown in Figs. b) and c), amounting to ca. 50 % and 25 %. Other capacitance steps ranged from few % to above 100 %. They appeared spontaneously, but only near the apparent pK of the phospholipid used.

Fig. c) also demonstrates superimposed small membrane current steps. In order to unambiguously assign these current steps to the appearance of ion channels, controls with disconnected AC voltage generator were routinely undertaken. Fig. d) at +77 mV DC pH 2.4 of the same membrane as in c) does prove the appearance of ion channels which we already reported earlier.

Similar results were obtained with bilayers made of
- synthetic dimyristoyl methyl phosphatidic acid, generously provided by H.Eibl;
- heterogeneous soybean lecithin from Sigma;
- crude Torpedo electric organ membrane fractions which contained native AChase and ATPase.

Induction of ion channels and capacitance steps was always achieved by direct asymmetric acidification with HCl or, in the latter system, by enzymatic hydrolysis of ACh and, likewise, of ATP. ATP-induced ion channels could already be observed, in 0.1 M NaCl unbuffered, at asymmetric pH 5 produced by hydrolysis.

We have reported earlier that ACh-induced ion channels are induced by AChase even at buffered bulk pH 7.4. Kinetically, the capacitance steps, ion channel opening and closing, as well as the msec membrane current pulses appeared very similar, no matter whether they were induced enzymatically or by direct acidification of pure synthetic lipid bilayers.

Protonic control

Eigen and deMaeyer earlier predicted that biological membranes represent almost ideal conditions for protonic control systems. Träuble demonstrated the specific role of protons in controlling the thermodynamic phospholipid states. Control experiments demonstrated, again, that protons control both permeability and ion transport kinetically.

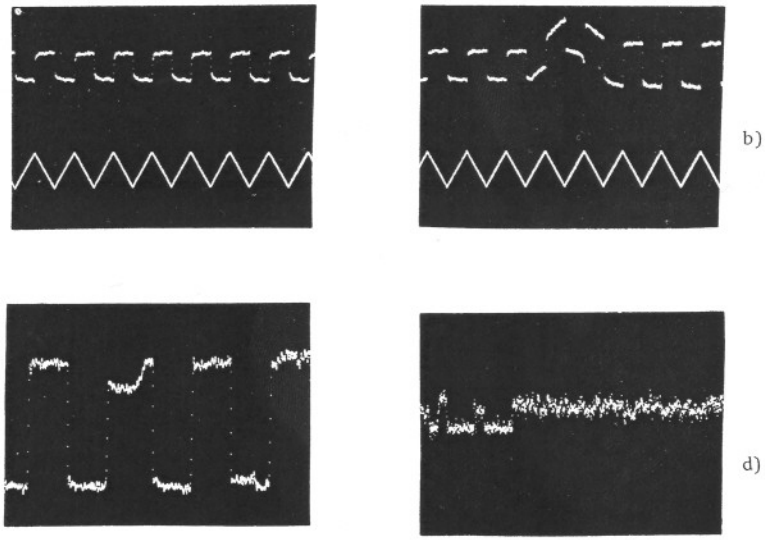
Capacitance and associated ion channels appeared in the absence of salt, and in the virtual absence of OH^- at acid pH 2. Controls in the absence of added HCl demonstrated current pulses of similar kinetic relaxation times in the virtual absence of Cl^-. Protons and protonable phospholipids were the only ions required.

The drop in pH from 6 to 5-4 at the non-titrated side during the conductive state clearly demonstrates proton transport across the membrane, since the electrostatic potential was kept constant. While other ions such as Na^+ and K^+ are obviously conducted across many ion channels, too, the similar relaxation times of current pulses and ion channel opening and closing in most lipid-protein membranes, and the appearance of phenomenologically very similar properties due to proton-phospholipid interactions clearly demonstrates the prediction to be valid for both receptor and transport function of the bilayers.

Interestingly, msec membrane pulses can, as expected for adiabatic excitations, in certain instances clearly be asymmetrically directed against the bulk-applied DC voltage. This is evident from the fact that, after reversal of the electrode positions, and the associated membrane potential, the current direction persists with respect to the membrane for at least several seconds. In the pure lipid membrane, the required free energy may derive from the initial condition, in particular from membrane curvature. The slow kinetics of such active protonic pulses has not yet been consistently determined.

Conclusion

Phospholipid bilayer membranes represent protonic control systems. From Langmuir thermodynamic phase diagrams, free energy coupling, propagation of adiabatic excitations, and area/thickness fluctuations in the monolayers are evident. Slow lipid relaxation times store local protons at the surface, which couple catalytic to osmotic free energy. ACh thus induces ion channel fluctuations by local surface hydrolysis. The responsible proton-induced phospholipid conformational transitions are directly observed as stepwise changes in the membrane capacitance. The lipid mechanism described appears evident and sufficient for specific protein-induced transport across biological membranes.



a) Diphytanoyl lecithin (Avanti) planar bilayer in 1 M KCl at +77 ± 3.7 mV. Acidification of the + side to pH 2.5 did not significantly alter the membrane properties. pH 6.5 non-titrated side. Top trace shows the capacitive membrane current response corresponding to 60 pF. Lower trace shows the applied voltage of 10 Hz frequency.
b) Same membrane as in a) but at pH 2.4. First discernible capacitance step from 60 to 90 pF.
c) Same as in b) showing two subsequent capacitance steps between 100 and 75 pF.
d) Same state as in c), but at DC voltage +77 mV. The ion channels on this trace correspond to 100 and 70 pS calculated conductivity. At a similar pH close to the pK of this lipid, unit channels as well as manifold channel sizes had already been observed in collaboration with Silman.

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"Das bedeutet aber nicht, dass ich mit Ihrer Interpretation übereinstimme. Ich habe vor meinen Mitarbeitern oft Hippokrates zitiert: "ἡ δὲ πετρα σφαλερή, ἡ δὲ κρίτος χαλεπή" (der Versuch ist trügerisch, die Interpretation schwierig). Ich habe viele Jahre gebraucht, um zu meiner Theorie zu kommen, ein wirkliches Ende gibt es nicht, wie es Einstein und Planck oft betont haben. Es ist durchaus möglich, Modelle herzustellen ohne Rezeptor-eiweiss, in der Ionenkanäle isoliert werden können, nur mit Hilfe von AcCh und AcChE und mit Lipiden!"

David Nachmansohn 12.9.1981

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