Spin-Labeled Membranes

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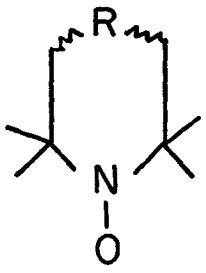
In the next few minutes I shall present a very brief discussion of what the spin-label technique is, and how it may be used to study biological membranes.[1, 2] A "spin label" is a synthetic organic paramagnetic free radical. Most spin

[1] For recent reviews, see: Hamilton, C. L., and H. M. McConnell, 1968, *in* Structural Chemistry and Molecular Biology, A. Rich and N. Davidson, editors, W. H. Freeman and Co., San Francisco; and Griffith, O. H., and A. S. Waggoner, 1969, *Accounts Chem. Res.* **2**:17.

[2] For recent papers on spin-label studies of membranes see: Hubbell, W. L., and H. M. McConnell. 1968. *Proc. Nat. Acad. Sci. U. S. A.* **61**:12; Keith, A. D., A. S. Waggoner, and O. H. Griffith. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **61**:819; Sandberg, H. E., and L. H. Piette. 1969. *Biophys. Soc. Annu. Meet. Abstr.* **9**:A-178; and Gendel, L., M. G. Goldfield, V. K. Koltovev, E. G. Rozantzev, and V. I. Suskina. 1968. *Biofizika.* **13**:1114.

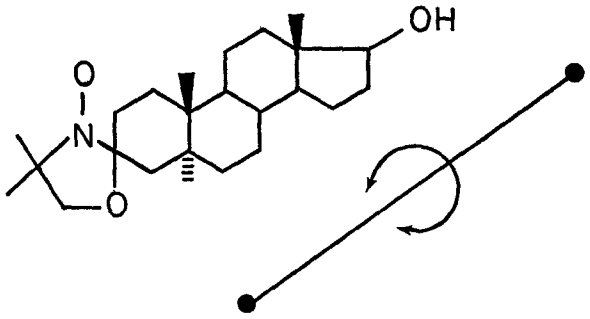
labels that have been used for biological studies are nitroxide radicals, and have the following general formula:



The label has an odd electron that is centered almost exclusively on the NO group. The nitrogen atom is bonded to two tertiary carbon atoms. The odd electron exhibits a magnetic hyperfine interaction with the nitrogen nucleus, and this interaction has both isotropic and anisotropic components. Because of this hyperfine interaction the paramagnetic resonance spectrum of the label depends on its environment: (*a*) If the label has a fixed orientation in space, then the resonance spectrum depends on this orientation. (*b*) If the label undergoes a rotational motion, the resonance spectrum depends on the rate and anisotropy of this motion. The spectra are particularly sensitive to motions with correlation times in the range 10^{-10} — 10^{-7} sec.

The group R serves to direct the label to a specific site in a biological system. The label may be attached to such sites by covalent bonds, by hydrophobic bonds, or by a combination of interactions characteristic of substrate-enzyme complexes. The literature on this technique is already extensive, so there is no time to review it here. Instead I shall only mention one of our more recent results that bears on the subject of membrane structure, and transport through membranes.

Mr. Wayne Hubbell and I have observed the paramagnetic resonance of the following steroid spin label in a number of biological membranes.



This molecule is the *N*-oxyl-4,4-dimethyloxazolidine derivative of the steroid 5α-androstan-3-one-17β-ol. In this molecule the paramagnetic nitroxide

group is rigidly attached to the steroid nucleus, so that rotational motion of the nitroxide group signifies a rotational motion of the whole molecule. We have found that when label is incorporated in the walking leg nerve fiber of the Maine lobster (*Homarus americanus*), it shows a paramagnetic resonance spectrum that can be interpreted in terms of a rotational diffusion of I about its long molecular axis with a correlation time in the order-of-magnitude range 10^{-7} – 10^{-8} sec. This anisotropic thermal motion is thought to arise from a combination of circumstances: (*a*) the molecule I is approximately cylindrical about the long axis, as indicated by space-filling models; (*b*) the polar-OH group of the steroid is "anchored" at a polar surface of the membrane; and (*c*) this polar surface is relatively rigid—space-fixed—perhaps due to a surface coating of protein, as in the Davson-Danielli model. Mr. Hubbell has carried out a number of experiments that support this picure: (*a*) the "bent" sterioid label derived from 5β-androstan-3-one-17β-ol shows no evidence for this motional anisotropy; (*b*) the steroid having the same shape as I but no —OH group in the 17 position shows no motional anisotropy; and (*c*) the spectra of I in phospholipid suspensions also shows no strong motional anisotropy.

The high motional freedom of I and other steroid labels in membranes is considered to lend some plausibility to models of transport that involve the rotation and/or translation of carriers through membranes. Labels such as I are also useful probes for studying conformational changes in the phospholipid regions of membranes.

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Discussion from the Floor

Dr. Alan Steinbach (Albert Einstein College of Medicine, New York): When you consider a system involving receptor activation by chemicals like acetylcholine, you always run into the question of whether you are looking at a competition or accentuation of effect that depends on the kinetics of reaction rather than equilibrium effects. This is especially true when you are measuring at relatively long times compared with the rates of reaction involved.

For example, although local anesthetics decrease postsynaptic response at the neuromuscular junction, they do not act as competitive inhibitors of the acetylcholine receptor. They don't activate the receptors either. What they seem to do is act *after* acetylcholine has changed the receptor conformation. Actually, that's an extrapolation; I should say they act after the acetylcholine has activated the receptors. That is, they seem to act on an already modified site. I wonder if Dr. Karlin, would like to speculate about whether this sort of effect could have accounted for some of his observations.

Dr. Karlin: I'm not sure of the question. What effect specifically?

Dr. Steinbach: I'll try to reduce it to the essentials. Acetylcholine reacts with the receptor and the rate coefficients for the reaction are probably about 10 sec^{-1}. This is a very fast reaction, at least that is my feeling and I think the feeling of other people who have studied receptor activation. Basically the measurements that you make when you are changing solutions along an electroplax are relatively slow compared with the reaction rate. This raises questions concerning the interpretation of so-called inhibition or activation mechanisms, and the kinetics of the reaction.

For example, if you compare the effects of acetylcholine with the effects of methane sulfonyl choline chloride, you find an apparent "steady state" difference in effectiveness. It turns out that this is mainly a difference in the rates of reaction of the two activators: this becomes clear in looking at responses to rapid iontophoretic applications of chemicals.

Once you begin to worry about the kinetics of reaction, and the possibility that chemicals may change receptor activation by altering activation rather than just eliminating it, the situation gets much more complex in terms of the interpretation of the results.

Dr. Karlin: I've stated my reasons for believing that the covalent blocking reactions are occurring at the active site and not at some other location. I do believe that the covalently attached inhibitors decrease the rate at which an activator binds to the active site. Measurements of the rate of onset of depolarization indicate that this rate might decrease four- or fivefold following just reduction of the receptor. I do not see how this affects the main conclusion that the covalently attached inhibitors and activa-

tors are actually reacting near the active site and are directly interacting with the active site.

Dr. Nachmansohn: May I add the following comment to Dr. Steinbach's question. There is a fundamental difference between the action of a compound released within the membrane and the same compound applied to the membrane from the outside. Within the membrane, or other cellular structures, reactions are organized, as we know for instance from the electron transfer chain or from the fatty acid synthesis, etc. We have learned in the last 2 decades that the same systems in solution, even when complete, do not work. Therefore, it is usually difficult and frequently even impossible, to extrapolate from reactions in solution to those in membranes. This has been repeatedly stressed by several speakers at this Symposium. Only occasionally may we encounter special conditions, where we are able to mimic to some extent the effects of certain compounds acting in a membrane by applying them from the outside. This is, for instance, the case with acetylcholine at the level of many, although not all, synaptic junctions: since at these points the excitable membranes are in general poorly protected, and for good biological reasons, we can reproduce to some extent the action of the physiological signal by applying acetylcholine to the junction from the outside. The same series of reactions is initiated and leads to the depolarization as in the biological process. But even in this case there are several marked discrepancies between the biological events taking place in the membrane and the response to the acetylcholine applied to the junction. First, the amounts of acetylcholine applied must be 10^6 or more times than those actually released in the intramembranous process. Secondly, a potent inhibitor of ACh-esterase must be added in order to get a reaction; physiologically, of course, there is no inhibitor present. Finally, the biological event takes place in a few millionths of a second, which is very much faster than the reactions by external application. It is difficult to apply to the events in the membrane the kinetic aspects studied in solution. The similarity of the action of ACh and nerve stimulation was the basis of the theory of neurohumoral transmission proposed several decades ago. The marked discrepancies became only known after decades of analysis of the process on a molecular level just as it took decades to demonstrate that ACh-receptor and -esterase are present in the excitable membranes of axons and act there in the same way as in the pre- and postsynaptic membranes at the junction. The difference is that in the axons the membrane proteins are protected by structural barriers, making it impossible for most ligands to reach them, except where these barriers offer inadequate protection, as for instance at the Ranvier nodes or after they have been reduced by chemical treatment, by detergents or enzymes, etc.

Let us return now to the problem of local anesthetics as antimetabolites. These compounds do not react like ACh with the receptor protein. ACh and a few congeners, such as carbamylcholine, are receptor activators; they produce a conformational change of the receptor protein. Local anesthetics are receptor inhibitors; they do not produce a change. They act in a way comparable with competitive enzyme inhibitors: they "fit" into the active site without affecting the protein, but prevent the substrate to react with the enzyme. The assumption that local anesthetics act as antimetabolites is based on the striking resemblance of their structure to that of acetylcholine. By very small substitutions it is possible to follow step by step the transformation of the mole-

cule from a receptor activator into an inhibitor: by the first substitution the strength as an activator is reduced; then, in a second step, the molecule, benzoylcholine, is structurally and functionally an intermediary form; finally, by a very small addition, an amino group on the ring, the molecule becomes a receptor inhibitor and a typical local anesthetic. It still strikingly resembles the main features of ACh, but it prevents its reaction with the receptor. This is exactly what is considered as an antimetabolite. The concentration of inhibitor is high, at least that applied on the outside, when compared with the ACh released within the membrane; therefore, even if the affinity of ACh to the protein is higher than that of the inhibitor, due to their high concentration the ACh released is unable to react with the active site. This results in the blocking of electric activity of the excitable membrane, whether at the junction or in the axon. However, the effects of antimetabolites on activity may vary to some extent according to the preparation used. The extraordinary and unique features of the monocellular electroplax preparation discussed before make it possible to measure directly the effects of ligands on the receptor protein and obtain quantitative evaluations difficult to achieve with other preparations.

As repeatedly emphasized, manifestations of a chemical reaction may greatly vary on cellular level even if on the molecular level the basic mechanism is the same. The differences of manifestation have, in the past, led many biologists to assume fundamental differences of mechanisms and chemical forces not only in the field under discussion, but in many other cell mechanisms, while actually the differences are due to variations of structure and organization. In this respect the advent of biochemistry and molecular biology was instrumental in demonstrating the similarities on a molecular level.

Dr. Maurice Feinstein (State University of New York, Downstate Medical Center): I'd like to object to the interpretation of the universal blockade of excitable tissues by local anesthetics as proof of the existence of an acetylcholine receptor involved in conduction, on the following basis: You're assuming that it is a competitive blockade. Now, 2 yr ago I reported in *Nature* an investigation of the mode of action of the blocking of acetylcholine by local anesthetics in five or six different tissues; these were all muscles, smooth or striated muscles, all of which were sensitive to acetylcholine. In every case the dose-response curves, whether log dose-response curves or plotted in an analogous way to the Lineweaver-Burk plots, all show that the local anesthetics act as noncompetitive inhibitors. That is, you cannot completely overcome the inhibitory action of the local anesthetic by increasing the concentration of the agonist, which is acetylcholine.

Now, this is not true in the case of atropine or curare. You can shift the log dose-response curve several orders of magnitude with atropine or curare without decreasing the maximum response. And in this type of system tetracaine consistently produced a very marked decrease of the maximum responses attainable with acetylcholine.

Secondly, many other agonists were capable of producing activation of these tissues, apparently *not* due to an interaction with acetylcholine receptor. For example, histamine, serotonin, and norepinephrine. If you use concentrations of these agonists producing equal degrees of activation of the muscle tissue, you produce approximately the same degree of inhibition of all of them with a local anesthetic such as tetracaine.

Now, to explain this you'd have to assume either that the receptors are all the same, or very closely the same at their active sites. Or rather that there is a secondary step,

which takes place in every case after drug reacts with receptor, leading to muscle contraction, and that this step *is* blocked by local anesthetics.

Now, in your own remarks this morning you raised the question of calcium controlling permeability. We have shown in many tissues, that the local anesthetics act as competitive inhibitors of calcium-binding or calcium permeation through cell membranes. I would suggest that such inhibitory effects on ionic permeability are possibly the more correct interpretation of local anesthetic action. That is that acetylcholine, reacting with the receptor, does produce a disturbance in the structure of the membrane, leading to an increase in permeability to ions. But this secondary effect, that results from reaction of acetylcholine with receptor, changes the membrane structure in an area through which ions flow across the membrane. That is the active site, and not the acetylcholine receptor itself, at which the local anesthetic reacts and thereby shuts off an ionic current.

Now, I think actually within your own laboratory that some of your own work shows the same thing. If I'm not mistaken the most detailed work on the action of tetracaine in the electroplax shows that as you increase the concentration of tetracaine, you produce greater and greater inhibition of the maximal response to carbamylcholine or acetylcholine. And that as you further increase the dose of tetracaine you depress this plateau of maximal response continually, which is exactly the situation you expect with a noncompetitive inhibitor and not at all what occurs with a competitive inhibitor.

Dr. Nachmansohn: In your comments you ignore again the basic structural difference between the excitable membrane at the level of synaptic junctions and in nerve and muscle fibers. As my associates and I have shown time and again over a period of 25 yr, and as I just mentioned in my reply to Dr. Steinbach's questions, in fibers the excitable membrane is well protected by structural barriers against ACh, curare, and other related compounds, at junctions poorly or not at all. This has been demonstrated unequivocally with a great variety of chemical methods, sometimes combined with electron microscopy. It is useless to repeat it again and again and I have no intention to convince everybody. I was once asked whether there is in my opinion a teleological explanation for it. It is not difficult to see the necessity for it. When Rubinstein plays the piano, many thousands of fibers, each about one meter long, are active, leading messages from his brain to the tip of his fingers. This is a very large surface area of excitable membrane and, if unprotected, many compounds would readily reach the membrane and, since it is according to definition excitable, there would be continuous interference with the messages and the precision and elegance of his movements would suffer. We would be so jittery if our axonal membranes would be unprotected, that nobody would be able to drive a car. Only where impulses have to cross a nonconducting gap of a few hundred A, i.e. at synaptic junctions, a minimum of outside structure is present, thus facilitating the ion movements carrying the message from one cell to the other.

What has this to do with your comments on the mode of action of the local anesthetics? Since ACh, when applied to the preparation from the outside, reaches the excitable membrane at the junctions only, the classical picture of competitive action between ACh and local anesthetics can be obtained only there. This has been demonstrated with tetracaine and other local anesthetics at the junction of the electro-

plax. In the conducting membrane, of axons or electroplax, only the tetracaine reaches the ACh-receptor protein. It competes with the ACh within the membrane. This inhibition cannot be overcome by ACh from the outside. In the muscles which you have used, ACh again can only reach the excitable membrane at the junction but not in the fiber, whereas the local anesthetics reach the entire membrane. Therefore, there is apparently, but not actually, a contradiction to the competitive nature of these compounds. On the other hand, if you use curare or atropine, these compounds reach the membrane also at the junctions only; therefore, with these compounds you get the classical picture of competitive action between them and ACh.

The importance of Ca^{++} in excitability is widely, if not generally, accepted, as I mentioned in my lecture, in which I also discussed certain features of the properties of the Ca^{++} ions, which may make them important in the processes changing permeability. Local anesthetics may, of course, react with many molecules. There seems to be an interaction with phospholipids and some investigators see in this reaction the basis of the effects of local anesthetics on membranes. The problem, however, is not whether local anesthetics are able to react with other molecules, but which particular reaction is actually responsible for the block of electrical activity. If one assumes, as the Cambridge group does, that all that happens during electrical activity are ion movements across the excitable membrane, and if one denies that any chemical reactions are involved in this process, obviously one will attribute the effects of local anesthetics to a reaction with Ca^{++} ions or phospholipids, etc. However, if one assumes that proteins must play an essential role in the control of ion fluxes, as in all known cellular mechanisms, then we must look for the specific signal and a specific protein recognizing the signal and initiating and controlling the sequence of events leading to the ion movements. In the theory proposed, the signal is ACh and the receptor the protein recognizing it. When chemical compounds, such as local anesthetics, which so strikingly resemble ACh, block the action of the signal, it seems to me a strong evidence for the competitive nature of their action. Much depends on the basic notions and attitude; i.e., whether one denies any role of protein in the permeability changes or accepts the theory proposed. When I visited Alan Hodgkin in Cambridge a few years ago and showed him the chemical structure of the local anesthetics and our data, he was unimpressed and replied that it is well known that local anesthetics are general toxic agents. I was struck when I then visited Adrian, how impressed he was by the similarity of the chemical structure of ACh and the local anesthetics and how he realized the implications. Many misunderstandings are due to differences in background and fundamental notions. Last year I visited Luzatti and asked him whether he had tested the effect of Ca^{++} ions on the formation of phospholipids. I pointed out that the release of these ions by the conformational change of the receptor protein may affect the conformation of phospholipids and thus form part of the amplification process initiated by the signal. He had indeed found such an effect, but added that ACh has the same effect. It turned out, however, that Ca^{++} ions act in very low, whereas ACh acts only in very high concentration. ACh is, of course, as ionized as Na^{+} or K^{+} ions. While the effects of ACh and Ca^{++} ions may have some similarities, especially in different concentrations, due to their ionic character, the question arises which of them is biologically responsible for the conformational changes of the phospholipids. In view of the high con-

centrations of ACh and the low of the Ca^{++} ions required for the effect, and in view of the fact that ACh appears only in very low amounts in the membrane whereas Ca^{++} ions are abundant it appears more likely that the latter are the ions responsible.

Dr. Feinstein: I won't argue the question about whether there is a difference due to action on the surface or inside the membrane, I'll grant you there is no question about it. The only thing I can't understand is why, if there is an acetylcholine receptor that responds to acetylcholine added from the outside, and it can be blocked by certain things, why its behavior should be different than one inside? Why in one case inhibition would be competitive and in the other noncompetitive?

But, more to the point, a question about the signal and the response to that signal. In the adrenal medulla, Douglas and Kanno have shown, by putting microelectrodes into the medullary cells, that they can get a depolarization when they add acetylcholine. And that this depolarization apparently is composed of ionic current involving sodium and calcium.

Now, if you have predominantly sodium there, you don't affect the depolarization very much when you add tetracaine. However, if you substitute all the sodium with calcium, you block the depolarization due to acetylcholine completely with tetracaine.

And the other interesting thing is that whether the extracellular cation is predominantly calcium or predominantly sodium with some calcium, when you add tetracaine you always block secretion of catecholamines. Whether the secretion is evoked by acetylcholine or by calcium itself, without any acetylcholine, it can be blocked by tetracaine.

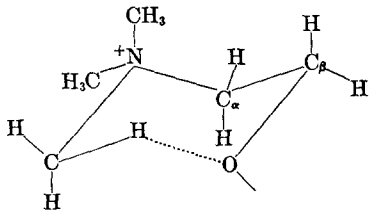
So in this situation acetylcholine reacts with the receptor, it is the signal. But what happens upon receipt of the signal is a change in permeability to calcium. And this is what is affected by the tetracaine in this case, not the signal itself.

Dr. Nachmansohn: It seems to me that I have sufficiently explained why ACh applied from the outside to nerve and muscle fibers cannot reach the receptor in the membrane except at junctions; tetracaine, however, is able to penetrate and therefore able to compete with the ACh released within the membrane for the receptor all along the fibers. It would be repetitious to start all over again.

As to the experiments which you mentioned on the adrenal medulla, there the situation is extremely complex. I am not prepared to discuss this question. Even isolated membranes are very complex systems; I am sure this audience will agree with this statement. But the interpretation of effects of drugs, applied to such a complex tissue as the adrenal medulla, in terms of molecular events, seems to me at present premature and nearly impossible.

Dr. Marfey: I am directing this question to Dr. Mautner. In his presentation, Dr. Mautner pointed out that oxygen and quaternary nitrogen atoms of the choline moiety of acetylcholine are in synclinal (*gauche*) conformation. Similar synclinal conformations between these two atoms of a choline moiety are found in L-α glycerophosphorylcholine (Abrahamsson, S., and I. Pascher. 1966. *Acta Cryst.* **21:**79.), L-α-glycerophosphorylcholine cadmium chloride trihydrate (Sundaralingam, M., and L. H. Jensen. 1965. *Science.* **150:**1035.), and in 2-aminoethanol phosphate (Krant, J. 1961. *Acta Cryst.* **14:**1146). Dr. P. Pauling (cf. Canepa, F. G., P. J. Pauling, and H. Sörum. 1966. *Nature.* **210:**907) has considered the possibility that hydrogen bonding between one of

the methyl groups on the quaternary nitrogen atom and the choline oxygen atom may be responsible for the observed synclinal conformation in acetylcholine. If this were the case, the resulting hydrogen-bounded structure of the choline moiety can be pictured as a six-membered ring in a chair form:



Dr. P. von Ragué Schleyer has studied hydrogen bonding involving C-H groups as proton donors (Allerhand, A., and P. von Ragué Schleyer. 1963. *J. Amer. Chem. Soc.* **85:**1715), but, to my knowledge, no data are available on hydrogen bonding of methyl groups attached to quaternary nitrogen atoms.

I would like to ask Dr. Mautner if he would be willing to comment on the possibility and significance of such a hydrogen-bonded structure in acetylcholine molecule.

Dr. Mautner: It's a very interesting comment. There is great need for more quantitative information about the strengths of such hydrogen bonds. All in all, I think it is unlikely that hydrogen-bonding interactions play a major role for one simple reason. The X-ray diffraction of choline, the hydrolysis product of acetylcholine, has also been carried out. And choline also is in the *gauche* conformation. Choline is the least effective compound as far as depolarization is concerned that is known. Of all the literally hundreds of onium compounds that have been tested for depolarizing activity in the electroplax, choline is unique because of its inactivity.

Cholinethiol, in which the oxygen of choline has been replaced by sulphur, is a very much more effective depolarizing agent than is choline, but a poorer hydrogen-bond former. If you start methylating and go to methoxycholine, methylthiocholine, and methylselenocholine, you get extremely potent depolarizing agents.

Now there is no way that I can see where outside the trimethylammonium portion of the molecule something like methylthiocholine is going to form a hydrogen bond effectively. It sounds very much as if what one is dealing with, at least in compounds like methylthiocholine, is a hydrophobic interaction and not a hydrogen-bonding interaction.