THE CHANGES IN THE NERVE MEMBRANE AND THE ROLE OF CHOLINESTERASE IN THE CONDUCTIVE PROCESS

by

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In a number of recent publications[1-3] it has been shown that the active surface of acetylcholinesterase contains two different groups:

1. Anionic sites, represented by univalent negative charges, which attract the positive centres in substrate or inhibitor.

2. The esteratic site, which attaches itself to the ester group or any other related, strongly electrophilic group in substrate or inhibitor.

Quaternary ammonium salts combine with the anionic sites through Coulombic forces, whereas neutral esters attach themselves exclusively to the esteratic site. It has been found, however, that the hydrolysis of diacetin or ethyl acetate is competitively inhibited by quaternary ammonium salts[4]. This must be due to occupation of the esteratic site and its surroundings by the alkyl chains of the quaternary salts. Such groups are attached to a protein surface by dispersion (VAN DER WAALS) forces. These forces then should play a major role in the combination enzyme—quaternary salt. To test this conclusion the following experiments have been carried out*.

METHODS

Purified acetylcholine esterase from the electric organ of *Electrophorus electricus* was used. The dilution applied hydrolyzed 4.5 μmoles/ml/hour, when $3.3 \cdot 10^{-3}$ M acetylcholine was the substrate. Diacetin, $4.3 \cdot 10^{-1}$ M, served as neutral substrate; double the enzyme concentration as before was required to hydrolyze 3 μmoles/ml/hour. Enzyme and inhibitor were incubated in a Warburg vessel at room temperature for ½ hour, then the substrate was tipped in and the rate of hydrolysis measured at 23° by the manometric method. The buffer used was 0.1 M bicarbonate at a pH 7.2. The gas phase consisted of 95% air and 5% CO_2. For all inhibitors the concentration producing 50% inhibition (c_{50}) was determined graphically.

RESULTS

In Table I two groups of inhibitors are shown: In the first group all four substituents on nitrogen were enlarged. Here the inhibitory effect increases up to the tetrapropyl compound, but the tetrabutyl derivative is again less effective. This phenomenon must be ascribed to the limited space available around the active surface.

* A preliminary note on the present investigation has been published in *Biochim. Biophys. Acta*, 8 (1952) 352.

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TABLE I

INHIBITORY EFFECT OF QUATERNARY AMMONIUM SALTS ON VARIOUS ESTERASES

Compound	c_{50} for cholinesterase		c_{50} for liver esterase
	Substrate: Ach	Diacetin	Substrate: Diacetin
Tetramethylammonium iodide	$1.5 \cdot 10^{-2}$ M	$5 \cdot 10^{-3}$ M	No inhibition up to $5 \cdot 10^{-1}$ M
Tetraethylammonium bromide	$3 \cdot 10^{-3}$ M	$1 \cdot 10^{-3}$ M	No inhibition up to $5 \cdot 10^{-1}$ M
Tetra-*n*-propylammonium iodide	$1.5 \cdot 10^{-4}$ M	$2 \cdot 10^{-4}$ M	No inhibition up to 10^{-1} M
Tetra-*n*-butylammonium iodide	$3 \cdot 10^{-4}$ M	$7 \cdot 10^{-4}$ M	No inhibition up to $2 \cdot 10^{-2}$ M
Trimethyl *n*-propyl ammonium bromide	$3 \cdot 10^{-3}$ M		
Trimethyl *n*-butyl ammonium bromide	$2 \cdot 10^{-3}$ M		
Trimethyl *n*-pentyl ammonium bromide	$1.5 \cdot 10^{-3}$ M		
Trimethyl *n*-heptyl ammonium bromide	$6 \cdot 10^{-4}$ M		

The second group comprises derivatives of trimethylamine with only the fourth alkyl substituent increasing in length. Here a continuous increase of the inhibitory effect is observed up to seven carbon atoms*. No crowding effect is found in this series. It should be noted that in all cases under investigation the inhibition has been shown to be competitive. The degree of inhibition is of the same order of magnitude, whether a cationic or neutral ester is used as substrate. No effect was found against liver esterase. Thus it becomes clear that quaternary ammonium salts are directed towards the active surface by Coulombic forces, but their affinity for the enzyme is determined mainly by dispersion forces, which are a function of molecular size.

TABLE II

EFFECTIVE RADIUS OF VARIOUS UNIVALENT IONS

I. *Alkali metal ions*

Ion	Crystal Radius (A)*	Hydrated Radius (A)**
Li	0.60	2.31
Na	0.95	1.78
K	1.33	1.22
NH_4	—	1.21
Rb	1.48	1.18

II. *Quaternary ammonium ions*

Ion	Diameter of unhydrated ion (A)***
Tetramethylammonium	4.6
Tetraethylammonium	7.2
Tetra-*n*-propylammonium	9.0 (approx.)
Trimethyl *n*-pentyl ammonium	9.5

* According to PAULING, *The Nature of the Chemical Bond*, 1944, p. 346.
** From mobilities; quoted from KRESSMAN AND KITCHENER[5].
*** Calculated according to bond lengths given by PAULING, *loc. cit.*, p. 160 etc.

* Ammonium salts with longer alkyl chains are difficult to purify by crystallization.

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The relationship of quaternary ammonium ions to the active surface of cholinesterase parallels their behaviour to cation-exchange resins. There it has been shown[5] that in contrast to univalent metal ions the ammonium salts increase their affinity to the negative sites of the resin with increasing molecular size (as long as the available space permits their accommodation). It is to be noted that quaternary ammonium ions are practically unhydrated, whereas the effective radius of metal ions is determined by the number of bound water molecules (see Table II). Therefore the affinity of univalent metal ions to a cation exchanger increases with decreasing effective radius. We have made an analogous observation with choline esterase: K^+ and NH_4^+ are better inhibitors than Na^+, although no significance can be attributed to the absolute figures, which were found experimentally (the concentrations required are so high that the ionic strength of the solutions is no longer comparable to the standard conditions[6]).

One conclusion can be drawn from our results: Cholinesterase behaves towards cations like a cation exchanger. Therefore when the enzyme is built into the nerve membrane, the latter will acquire properties of a "permselective" membrane[7].

DISCUSSION

In order to evaluate the role of a cation exchanger present in the nerve membrane, it will be useful to recall the fundamental phenomena observed during the conductive process, which require explanation:

1. The resting state is characterized by a disequilibrium in the internal and external ion concentrations (*e.g.* in the giant axon of the squid K^+ is 20–40 times higher in the axoplasm than in the extracellular fluid, whereas for Na^+ the ratio $c_{inside} : c_{outside}$ is about 1:7)[8].

2. In the resting state the permeability of the membrane is higher for K^+ than for Na^+[9].

3. During passage of the action current this specific permeability breaks down, *i.e.* the exchange of Na^+ ions increases now much more than that of K^+, and an "equilibration" of ionic concentration starts, although the magnitude of the effect during a single stimulation does not change the ionic concentrations to a measurable degree[10].

4. During the falling phase of the action potential the resting conditions are progressively re-established. The recovery of the resting potential requires not more than a few milliseconds and in special cases only a fraction of one millisecond[11].

The first phenomenon, the ionic disequilibrium between axoplasm and extracellular fluid, is not specific for the nerve fibre. It is apparent in all living cells and dependent on their metabolic functions[12]. Therefore this problem will not be discussed here further.

The second fact finds a simple explanation in the observation that cholinesterase acts as a cation exchanger, due to its characteristic anionic sites*. The nerve membrane can now be characterized by its selectivity coefficient K_D:

$$K_D = \left(\frac{n_1}{n_2}\right)_{resin} \cdot \left(\frac{n_2}{n_1}\right)_{solution}$$, where n_1, n_2 = molar concentration of different ionic species.

In order to change the selectivity, as required by the third phenomenon, it is necessary to change K_D. It has been shown[13] that this coefficient is a function of the

* This does not exclude that other components of the membrane structure may also contribute to its ion-exchange properties.

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degree of cross—linking within the matrix of the ion exchanger. Usually the network of a polymer molecule is fixed by the method of its synthesis and therefore K_D is constant for a given preparation. However in a protein molecule the specific structure is dependent mainly on hydrogen bonds and the whole network is thus subject to changes produced by variation of pH. This pH dependence expresses itself in the change of all physical properties, including the ion-exchange ability. In this connection it is significant that the anionic sites of cholinesterase also show a dependence of their degree of dissociation on the pH[14].

We wish therefore to advance a new hypothesis regarding the conductive process in the nerve membrane. When the action current flows across the membrane, in the first line a shift of hydrogen ions occurs due to the extraordinarily high mobility of H^+. Thereby the concentration and activity of H^+ in the hydrated parts of the membrane is changed. Simultaneously its hydrogen—bonded network and its selective permeability breaks down. (It is to be noted that pH changes in the membrane, as in any disperse phase, are not necessarily linked to corresponding changes in the surrounding medium. Much higher pH changes are possible in the membrane than are encountered physiologically in the intracellular or interstitial fluid[15]). A somewhat similar explanation was already considered by BLINKS[16], in order to account for the conduction phenomena in certain algae.

The most surprising fact in the conductive process is the great speed with which the resting potential is re-established. This is in contrast with the behaviour of artificial membranes which require minutes for readjustment of their potential[17]. Even in the membrane of *Nitella flexilis* the recovery of the normal potential after stimulation extends over several seconds[18].

It is clear that in the nerve membrane the resting potential is re-established long before the ion-exchange processes are finished. Therefore the recovery process must be dependent on a chemical change in the membrane itself. A special mechanism must be available to recover the resting conditions through readjustment of the original concentration and activity of H^+ in the hydrated membrane phase. This could be done by an *appropriate* buffer, which would not prevent the original "acidification" required by the breakdown of selective permeability, but would react within a measurable, though very short time. The usual buffers would not serve this purpose. It is necessary to look for a fast chemical "buffering" reaction, catalyzed by an enzyme. In the nerve membrane a suitable system with a sufficiently high reaction velocity is represented by the acetylcholine—cholinesterase system. This is so far the fastest enzymic reaction known in animal cells, since one mole of enzyme can split $2 \cdot 10^7$ moles of substrate per minute. According to HESTRIN[19] the equilibrium between the ester and its hydrolytic products

$$\text{acetylcholine} + H_2O \rightleftarrows \text{acetic acid} + \text{choline}$$

is shifted to the left with decreasing pH. Thus under more acid conditions more ester will be formed by the enzyme, whereas in a more alkaline environment hydrolysis will be favored. Taking into account the dissociation constant of acetic acid, $pK_a = 4.7$, it is clear that the assumed buffer system will work best around pH 5, but will be rather poor at pH 7–8. However, as pointed out above, large pH changes are possible in the membrane without concomitant large changes in the surrounding medium.

The mechanism, as supposed in the present paper, is in contradiction to several now accepted views, regarding the changes in the nerve membrane during conduction.

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a. HODGKIN AND KATZ[9] assumed that due to the high permeability of the resting membrane for K^+ ($P_K : P_{Na}$ is calculated to be about 25:1) the resting potential is defined practically exclusively by the equation

$$E = \frac{RT}{F} \ln \frac{K^+_{outside}}{K^+_{inside}}$$

whereas during conduction the permeability for Na^+ becomes so much higher than for K^+, that the peak of the action potential is now dependent on the ratio $Na^+_{inside} : Na^+_{outside}$, which would explain the "overshoot". However, according to our view, K_D can only approach the value 1, *i.e.* both ions will finely possess equal affinity to the negative sites of the exchanger. Actually from the measurements of KEYNES[20] it is evident that K^+—exchange is also very much increased during activity. We conclude therefore that the measured potentials do not represent a shift from a potassium to a sodium electrode, but reflect the changes of hydrogen ion in the membrane.

b. Current theories separate the site of action of acetylcholine from the place of its destruction. Thus NACHMANSOHN[21] assumes, that Ach is first liberated from its bound form, then acts on a "receptor" protein to produce depolarization and a change of membrane permeability, and finally undergoes splitting by cholinesterase. Its resynthesis is effected by choline acetylase.

However, no other experimental fact about the physiological role of Ach is known besides the presence of a specific enzyme in the nerve membrane for its hydrolysis. The conductive process can be described in a simple way as follows: The action current across the membrane changes the permeability through a change in the concentration and activity of the hydrogen ions, these changes being much greater than and independent of the corresponding changes in the surrounding media. The enzymic buffer system Ach—cholinesterase re-establishes the membrane pH.

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SUMMARY

1. Quaternary ammonium ions inhibit the hydrolysis by acetylcholinesterase both of acetylcholine and of neutral esters in a competitive manner. The inhibitory effect increases in both cases with molecular size, up to a certain limit.

2. The behaviour of quaternary ammonium ions towards the anionic sites of the active surface resembles the relationship of these ions to cation exchange resins. Therefore the enzyme protein as part of the nerve membrane will impart to the latter the properties of a "permselective" membrane.

3. The selective permeability of the nerve membrane must be a function of the structure of the protein, thus changing with pH. The breakdown of the selective permeability during the conductive process is an expression of a structural change, produced by a shift of H^+ ions inside the membrane.

4. The hypothesis is forwarded that the length of the refractory period is dependent on a chemical reaction inside the nerve membrane, which leads to the re-establishment of the resting pH. An appropriate "buffer" system for this reaction can be found *e.g.* in the system Ach-cholinesterase, which is located in the nerve membrane and works with a sufficient speed.

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RÉSUMÉ

1. Les ions d'ammonium quaternaires empêchent l'hydrolyse, par l'acétylcholinesterase, aussi bien de l'acétylcholine que des esters neutres, de manière compétitive. Dans les deux cas, l'effet inhibiteur augmente avec le poids moléculaire jusqu'à une certaine limite.

2. Le comportement des ions ammonium quaternaires vis-à-vis des parties anioniques de la surface active ressemble à la relation entre ces ions et des résines échangeuses de cations. C'est ainsi que la protéine de l'enzyme faisant partie de la membrane nerveuse confère à cette dernière les propriétés d'une membrane "permsélective".

3. La perméabilité sélective de la membrane nerveuse doit être une fonction de la structure de cette protéine; elle doit donc varier avec le pH. Pendant le processus de la conduction, la perméabilité de la membrane cesse d'être sélective; c'est là l'expression d'un changement structural dû au déplacement d'ions H^+ dans la membrane.

4. Les auteurs émettent l'hypothèse suivante: la durée de la période réfractaire dépendrait d'une réaction chimique dans la membrane nerveuse, réaction qui conduirait au rétablissement du pH de repos. Comme système "tampon" approprié pour cette réaction l'on pourrait considérer p. ex. le système Ach-cholinestérase qui est situé dans la membrane nerveuse et qui agit avec une vitesse suffisante.

ZUSAMMENFASSUNG

1. Quaternäre Ammoniumionen hemmen in konkurrierender Weise die durch Acetylcholin-Esterase bewirkte Hydrolyse von Acetylcholin und auch von neutralen Estern. In beiden Fällen nimmt die Hemmwirkung bis zu einer gewissen Grenze mit dem Molekulargewicht zu.

2. Das Verhalten von quaternären Ammoniumionen zu den anionischen Gruppen der aktiven Oberfläche ist dem Verhalten dieser Ionen zu Kationen austauschenden Harzen ähnlich. Das Enzymeiweiss, welches ein Bestandteil der Nervenmembran ist, verleiht dieser die Eigenschaften einer "permselektiven" Membrane.

3. Die selektive Permeabilität der Nervenmembran muss eine Funktion der Struktur dieses Proteins sein, und sich also mit dem pH ändern. Das Verschwinden der selektiven Permeabilität während der Konduktion ist der Ausdruck einer durch H^+-Ionenverschiebung bedingten Strukturänderung in der Membran.

4. Die Verfasser stellen eine Hypothese auf, welche besagt, dass die Dauer der Refraktärperiode von einer chemischen Reaktion innerhalb der Membran abhängt, welche zur Wiederherstellung des Ruhezustand-pH's führt. Ein geeignetes Puffersystem für diese Reaktion könnte man z.B. in dem System Ach-Cholinesterase sehen, welches sich in der Nervenmembran befindet und mit genügender Geschwindigkeit wirkt.

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