Biochemical Pharmacology, 1965, Vol. 14, pp. 343–349. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECTS OF INCREASING NUCLEOPHILICITY ON TRANSMEMBRANE EFFLUX*

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(*Received* 29 *January* 1964; *accepted* 1 *June* 1964)

Abstract—Increasing the free base concentration of the tertiary analogs of choline by serially decreasing the hydrogen ion concentration of solvent over a pH range of 7·3–9·3 has shown that the free-base form of these analogs is capable of accelerating ^{86}Rb efflux of metabolically inhibited frog sartorius muscle. Control studies indicate membrane changes above pH 8·3. Dissociation energies do not confirm binding of effective substrates to tissue components.

THE observation that dimethylaniline reduces the acetylcholine-induced acceleration of rubidium efflux from iodoacetamide–cyanide-treated muscles, whereas aniline and the trimethylphenylammonium cation are ineffective in this respect,[1] leads to the speculation that the greater nucleophilic character of dimethylaniline may allow this compound to compete with acetylcholine for receptor binding sites in the muscle membrane. A further observation from the same laboratory is that dimethylaminoethyl acetate (pK_a 8·3) is more effective than acetylcholine in increasing the rubidium efflux from metabolically-inhibited muscle at elevated pH.[2]

The present study was initiated to test the ability of the free-base form of some aminoethanols to effect transmembrane ion flux in muscle tissue in which the "Na–K pump" had been inhibited. On the premise that receptor binding sites may exist in muscle which preferentially complex with compounds of nucleophilic character, serially increasing the free-base concentration of substituted aminoethanols might lead to increased binding by a receptor substance and hence possibly change membrane permeability characteristics. In addition to testing the generally accepted mechanism that mainly compounds with electrophilic centers interact with membrane substance(s) in initiating ionic flux changes in conductive tissue,[3,4] an attempt has been made to demonstrate that a given receptor substance may represent an ionic state and that it is not always a fixed chemical or physical entity that will interact in a very specific way with a substrate of given charge, charge distribution, or configurational adaptability.

MATERIALS AND METHODS

Paired frog sartorius muscles were incubated for 2 hr at 20° in an isomolar buffered Ringer's solution containing 4 mM glucose, 21 mM sucrose, and a trace of ^{86}Rb. After the incubation period the tissues were washed with either a buffered Ringer's

* These studies were supported in part by United States Public Health Service Grant B-2772 and the Colorado Heart Association.

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solution (the control solution) or a 2×10^{-3} M substrate–buffer–Ringer's solution. A 30-min equilibration period was allowed before assay of the tissues. Isotope desaturation rates were obtained by radiometric assay of the tissues with counts being taken at zero time and every 30 min thereafter for 120 min. The effectiveness of a given substrate was measured by the rate of isotope efflux as compared to the rate of efflux of muscles in the control solution. The isotopic rubidium ion with a half-life of 18·7 days was used as the tracer ion in preference to ^{42}K, which has a half-life of only 12·4 hr. Nightingale[5] has reported that ^{86}Rb has a hydrated radius which differs from that of ^{42}K by less than 0·05 Å and Fried *et al.*[6,7] confirmed that the two ions are very similar chemically and show similar affinities for the "carrier of potassium." Kunin *et al.*[8] found no difference in the manner in which ^{86}Rb and ^{42}K were handled by the kidney and more recently, Becker[9] has suggested the possible usefulness of ^{86}Rb as a tool for studying cation transport in the lens.

The free-base concentration of the substrates was increased by serially decreasing the hydrogen ion concentration of the solvent solution over a pH range of 7·3–9·3.

The substrates tested in the study include choline, 2-aminodimethylethanol (pK_a 9·11), 2-aminomethylethanol (pK_a 9·3) and 2-aminoethanol (pK_a 9·46). The effectiveness of each substrate was determined at five pH values, increasing the value by 0·5 unit through the experimental range. The bathing solution, which had a final osmolarity of 0·235, contained iodoacetamide (IAA), 5×10^{-4} M, and sodium cyanide (NaCN), 5×10^{-4} M; the Ringer's solution contributed 80% by volume and the buffer solution 20% by volume of the total osmolarity.

Choline chloride, obtained from Mann Research Laboratory, was acetone-washed and dried under vacuum over $CaCl_2$ at 39° before use. The aminodimethylethanol (DME) was redistilled once at 33 mm Hg and 53–55°, 2-aminomethylethanol (MME) was redistilled twice at 31 mm Hg and 85°, and 2-aminoethanol (EA) was redistilled once at 10 mm Hg and 73–73·5°.

Sorensen's sodium phosphate buffer system was used in the pH range 7·3–8·3, and a glycine–NaOH system was used in the experiments conducted at pH 8·8 and 9·3.

The Arrhenius equation was used to determine the apparent ΔH for the reaction of selected substrates at each pH value.

RESULTS AND DISCUSSION

Rubidium efflux was essentially linear for a period of 2 hr throughout the experimental range of the pH of the solvent solution; Fig. 1 is given as an example of this linearity.

The data obtained at pH 7·3 (Figs. 1 and 2) suggest that the substrates are effective, generally, according to their relative electrophilicity, although the efflux rates at this pH value do not follow a pattern that might be predicted on the basis of either the nucleophilic–electrophilic character of the substrate or the effects of the methyl substituents. The degree of solvation of EA may be a factor in accounting for its behavior in this series of experiments.

Two changes can be observed in the efflux pattern as the hydrogen ion concentration is decreased by 0·5 unit (Fig. 2): (i) the substrates are now effective in the reverse order of the magnitude of their pK_a values and in direct order of the number of methyl groups held by a given agent, and (ii) choline, which was only some 3%–4% more

effective than DME at pH 7·3, is now 11% more effective than its dimethyl analog; however, at pH 8·3 the difference in the rate of efflux between these two compounds returns to the pH 7·3 level. The difference in efflux rate between choline and DME at pH 7·8 is not due to an increase in the effectiveness of choline but largely to a decrease in the isotope desaturation rate in the presence of DME.

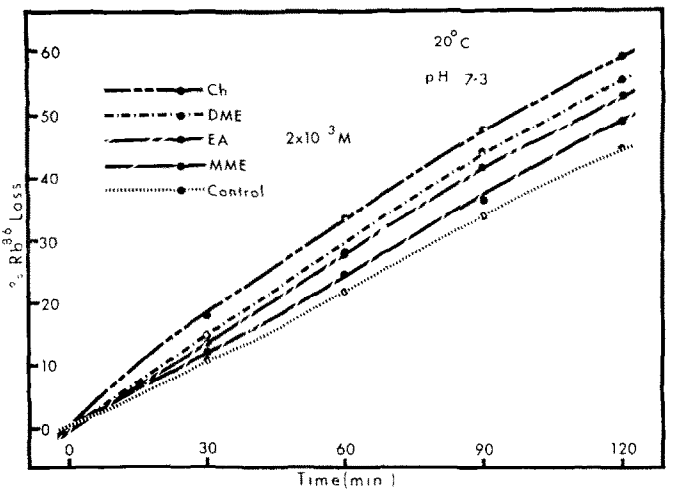

FIG. 1. Isotope efflux rates at pH 7·3. Each dot represents the mean average for 16 muscles. Standard error for each mean is <2·0%.

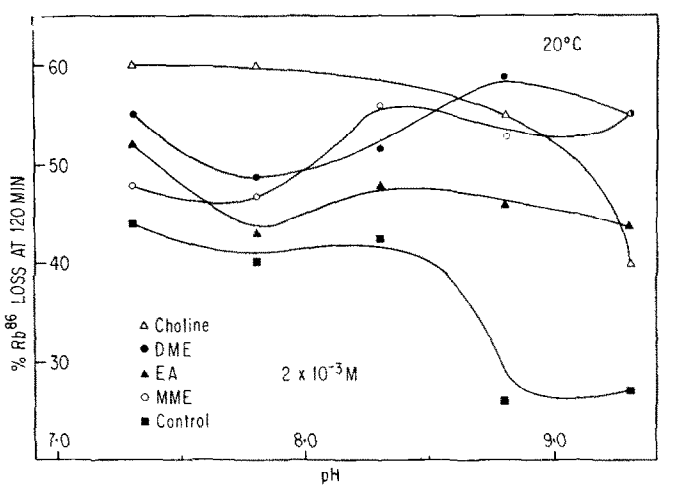

FIG. 2. Isotope loss at 120 min vs. pH, showing reduced efflux in presence of control solution above pH 8·3. Each dot represents the mean average for 16 muscles.

In summarizing the results obtained between pH 7·3 and pH 8·3, it appears that either an electrophilic center is being preferentially bound by the tissue membrane substance, and secondary effects are being promoted by the ethanol moiety of the substrates, or perhaps primary binding may occur via the anionic oxygen, since electrophilicity is being serially reduced in the nitrogen head. The substrates show a definite tendency

to promote increased membrane permeability in the reverse order of their pK_a values within this pH range; thus the membrane receptor seems to prefer electrophilic agents under these experimental conditions.

A pronounced change in the rate of ion efflux occurs as the pH of the solvent is raised above 8·3. Indeed, the control tissue at pH 8·3 seems to undergo a transition. Figure 3 reveals that above this pH value the rate of ^{86}Rb efflux seems to increase markedly, reaching a maximum in the presence of a given substrate at pH 8·8. It

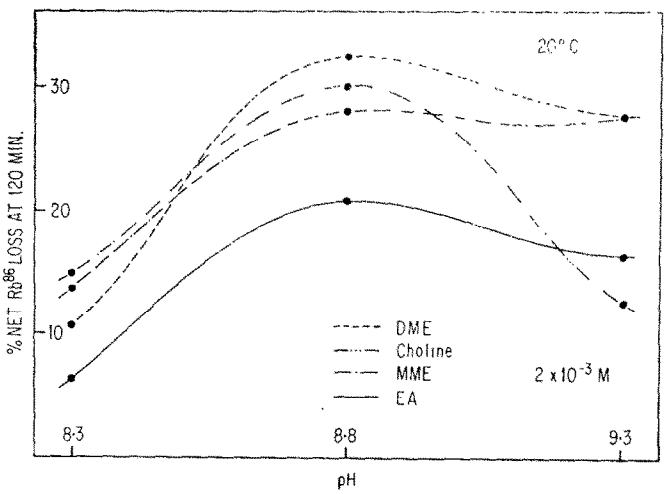

FIG. 3. Net isotope loss at 120 min from pH 8·3 to 9·3.

should be pointed out, however, that at this pH the permeability, in tissues washing in the control solution, has decreased by approximately 20% (Fig. 2); thus the values shown in Fig. 3 depict relative increases in efflux and not absolute values. The behavior of tissues bathed in control solution above pH 8·3 seems to be evidence of changes in membrane characteristics. It would be tempting to conclude that the muscle membrane has been damaged by the increased alkalinity, i.e. the plasma membrane architecture irreversibly disrupted, but if this were true one would expect substrates to be ineffective in changing efflux rate or rather not to differ in effect from changes noted in the presence of the control solution. While the efflux rate of the control tissues has diminished by some 20% between pH 8·3 and 8·8, it can be seen that the rate of efflux in the presence of choline changes only slightly in this range, and the other substrates actually significantly increase isotope efflux above pH 8·3. These observations seem to indicate that above pH 8·3 there occur definite changes in the membrane receptor substance of muscle (electrical or structural or both) and that an entirely different substrate–receptor interaction may exist than appeared at lower pH values.

The results obtained at pH 9·3 are of special interest when these values are compared to those obtained at pH 7·3. At the lower pH value choline was the most effective agent and MME the least effective, whereas at pH 9·3 choline shows the least efficacy, and the monomethyl derivative is equal in efficacy to the dimethyl derivative, and the two substrates emerge as the most effective agents at pH 9·3. This seems an especially

pertinent observation in view of the fact that choline presumably does not chemically change with changing pH. The greater effectiveness of DME and MME as compared to EA at this pH may be due to the fact that the pK_a value of DME has been exceeded and that of MME reached, whereas the pK_a value of EA (9·46) has not been attained.

The evidence that the muscle membrane becomes less permeable above pH 8·3 to the positive rubidium ion, is at first unchanged by choline, and then shows reduced permeability in the presence of this substrate, and becomes more permeable to basic substrates which are approaching their pK_a values leads to the postulation that the receptor entity (protein?) has unfolded or undergone other configurational changes to some extent above this pH value, thereby exposing positive charges for interaction or binding with substrates. That binding actually did occur between substrates and tissue membrane in all instances under these experimental conditions cannot be substantiated by the dissociation energies obtained for the reactions.

TABLE 1. APPARENT DISSOCIATION ENERGIES OF SUBSTRATE MEMBRANE COMPLEXES

pH	Substrate	Apparent ΔH^* (cal)
	Ringer-buffer solution (control)	7,944
7·3	Choline-Cl^-	10,199
	MME	15,257
	Ringer-buffer solution (control)	5,655
7·8	Choline-Cl^-	8,209
	DME	11,111
9·3	Ringer-buffer solution (control)	9,329
	Choline-Cl^-	30,183

* At 10°–20°.

The data in Table 1 indicate that binding and substrate efficacy are opposed, although, at best, little can be concluded from apparent ΔH values. Entropy studies may be much more revealing than dissociation energy studies in explaining the mode of action of the substrates. The greater affinity of purified acetylcholinesterase for the methylated derivatives of the ester of ethanolamine over the simple ester could not be explained on either the basis of the chemistry of the compounds or on the dissociation energies of the enzyme–substrate complexes.[10] Entropy values, however, eventually pointed to a molecular rearrangement whereby the enzyme is thought to encompass all of the methyl groups of acetylcholine. Results of the present study may well be due to molecular rearrangement of membrane constituents, induced by approaching substrates.

The behavior of the tissues in the presence of the control solution at elevated pH (20% reduction in isotope efflux rate) seems to rule out the possibility that changes in permeability are entirely a result of induced dipoles in the receptor *by substrates* in the free-base form. At lower pH values, decreasing the hydrogen ion concentration by a factor of 10 and increasing the concentration of the free-base form of a substrate from 1·5 to 13·5% (DME) did not result in appreciable changes in efflux rate. It appears that only after the receptor substance has apparently undergone electrical or structural changes at elevated pH, i.e. above pH 8·3, does it bind effectively with nucleophilic agents. There is the possibility that once the outer protein layer(s) of the

membrane has been disrupted, the agents may be acting according to their lipid-solubility properties. The breakdown in the continuity of the protein layer may expose lipid layers for penetration by substrates. Choline is insoluble in ether, EA is soluble in ether in the proportion 0·72 g/100 ml, and DME and MME are soluble in ether in all proportions. At pH 9·3, the order of efficacy of the substrates is in increasing order of their lipid solubilities if one makes the assumption that ether-solubility and lipid-solubility are synonymous.

The foregoing observations are offered to further substantitate a growing belief that the receptor substance of the membrane of conductive tissue does not represent an entity of fixed geometry or charge that interacts only with a molecule of corresponding geometry or charge. Friess[11] pointed out several years ago that some investigators are beginning to believe that a receptor is a state of polarization that exists on the approach of a polarizing agent, so that one can have infinite variety of receptor surfaces, depending on the mode of attack and polarizing field of the attacking agent. Koshland[12] demonstrated in 1959 that active sites on enzymes are flexible and adapt to their substrates and Karush[13,14] has proposed configurational adaptability for the bovine serum albumin molecule. Deformation of the "receptor" may have to follow binding of an agent to affect permeability characteristics and ion movement. Ariëns[15] has suggested the possibility of different agonist and antagonist receptor sites which may be very close together and which may interact. In the present study, the situation may be that the alcohol moiety of the substrates serves as antagonist to the agonistic positive nitrogen center. The dissociation energies indicate that a given substrate may be tightly bound in the membrane and not appreciably affect ion efflux. Yet this same substrate is potentially capable of affecting ion movement, as evidenced by the behavior of choline at pH 9·3 vs pH 7·3. That changing pH affects both the behavior of the substrate and the membrane has been implied in analyzing the results obtained in the ranges pH 7·3–8·3 and 8·3–9·3: in the range 7·3–8·3, decreasing the hydrogen ion concentration in the absence of substrate (controls) does not result in changes in ion efflux, but the addition of choline, for example, results in appreciable change in efflux rate even between pH 7·3 and 7·8. In the range 8·3–9·3, decreasing hydrogen ion concentration results in permeability changes in the membrane, and further changes in membrane characteristics seem to depend on the nature of a given substrate. Muscle may contain a component similar to the one (or ones) with which it is thought[16,17] drugs in general combine in exerting their effects on the conducting membranes of electroplax and squid axons, but there is no common experimental ground between the work on nerve and electric tissue and the present study.

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