ELECTRICAL ACTIVITY IN ELECTRIC TISSUE*

I. THE DIFFERENCE BETWEEN TERTIARY AND QUATERNARY NITROGEN COMPOUNDS IN RELATION TO THEIR CHEMICAL AND ELECTRICAL ACTIVITIES

by

MARIO ALTAMIRANO, WALTER L. SCHLEYER, CHRISTOPHER W. COATES AND DAVID NACHMANSOHN

Department of Neurology, College of Physicians and Surgeons, Columbia University, and the New York Zoological Society, New York (U.S.A.)

INTRODUCTION

Electric organs have been used since 1937 by NACHMANSOHN and his colleagues in studies of the chemical mechanisms underlying the generation of bioelectric potentials; the sequence of energy transformations has been analysed, acetylcholine has been integrated into the pathways of intermediary metabolism and various chemical and electrical events have been correlated[1-4]. From these and a great variety of other observations much evidence has accumulated supporting the view that acetylcholine plays an essential role in the generation of propagated electric potentials along animal cells. The picture which has emerged, assumes that acetylcholine is stored in an inactive bound form. Stimulation releases the ester which combines with a receptor protein. This reaction determines the complex changes in the ion permeability of the cell membrane that are generally accepted to occur during the action potential. The hydrolysis of acetylcholine by acetylcholinesterase permits the acetylcholine receptor to return to its resting condition and this initiates the recovery. The synthesis of acetylcholine is effected by choline acetylase in presence of other necessary constituents. Two of the proteins reacting with acetylcholine directly, *i.e.* choline acetylase and acetylcholinesterase, have been isolated and extensively studied *in vitro*. In contrast, our knowledge about the postulated receptor protein is limited to deductions made from observations on intact cells.

The investigations of the molecular forces acting between the two enzyme proteins and acetylcholine and related compounds have revealed a considerable difference in their behavior towards quaternary nitrogen compounds and their tertiary analogues[5,6]. The rate of hydrolysis and synthesis, *i. e.* the functional activities, are strongly altered

* This work was supported in part by research grants from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army (Contract No. DA-49-007-MD-37), the United States Public Health Service, Division of Research Grants and Fellowships of the National Institutes of Health (Contract No. RG-1901 (B-400)) and by the Atomic Energy Commission (Contract No. AT(30-1)-1503).

References p. 282.

when the substrate has a fourth alkyl group, although the binding forces between the proteins and the small molecule are not greatly changed by the presence of that group. Only a limited number of interactions between a molecule, such as acetylcholine, and a protein are possible, none of which are strong. A strong interaction is realized only by the summation of relatively weak forces. The summation is a maximum when the small molecule has a specific structure determined by the structure of the protein site. Consequently all proteins which interact strongly with acetylcholine must be similarly constituted at the binding site. The enzymes may thus serve as models for the receptor. Studies with the enzymes thus suggest that the binding of various tertiary and quaternary compounds by the acetylcholine receptor should not differ very greatly but that the functional consequences of this binding, *i.e.* a change in electrical potential of the cell membrane, may be very different.

The development of microtechniques has made possible the introduction of electrodes inside the cells, thus permitting direct measurements of the potentials across resting and active membranes. During the last two years such methods have been applied to the study of the transmembrane potentials of the electroplaque of *Electrophorus electricus*[7,8]. One important result of these studies has been the demonstration of a propagated spike similar to the action potentials of nerve and striated muscles that can be elicited either by direct or nerve stimulation. In contrast to some axons and striated muscles, the active membrane of the electroplaque, as will be shown in this paper, is markedly affected by quaternary nitrogen derivatives. It offers, therefore, a favorable material for studying the difference between the action of tertiary and that of quaternary compounds upon the electrical potentials of the cell.

Whereas in studies of the enzyme proteins in solution the reaction of one component is followed, in intact cells all the proteins are present in close vicinity. The active tertiary and quaternary nitrogen derivatives are structurally related to acetylcholine and therefore they will have affinity to both receptor and esterase. Since binding to either member will in theory block the propagated spike, it is essential to be able to distinguish between these two different causes of electrical failure. This difficulty has been overcome by the development of a procedure to measure the esterase activity of the intact electroplaque when failure of the propagated electrical potentials occurs. In those cases in which block of the electrical response was caused by combination with the receptor, the difference between the effects of tertiary and quaternary nitrogen derivatives upon this protein could be observed. The results of these investigations will be described in this and the following papers. It must be emphasized that this paper does not deal with block of the synaptic transmission, but of the propagated action potential along the cell. These observations will be discussed in the light of the knowledge obtained with the proteins of the system in solution.

METHODS

(a) *Electrical recordings.* The single layered preparation of the Organ of Sachs was dissected and mounted as previously described[7]. The Ringer's solution had the same composition as that used in the previous experiments. The compounds to be studied were dissolved in this solution to give the desired concentration. The temperature varied between 22 and 24° C.

The stimuli were rectangular pulses of controlled amplitude; their duration varied from 0.1 to 0.3 msec. The direct stimulation of the cell was done by means of two stainless steel needles completely insulated except at the tips and placed across the studied electroplaque. The cell was directly excited only when the stimulating current was flowing "outwards" through the innervated

References p. 282.

membrane. (Figs. 1 to 4). Whenever the current was going "inwards" (opposite polarity), the small nerve terminals were stimulated, thus the electroplax were excited through synaptic processes (Fig. 2, A, B, C, and D). However, in the majority of the experiments, whole intercostal nerve trunks were excited by means of small platinum wires, positioned under the nerves as described previously (Figs. 1, 3 and 4).

The electrical activity was recorded by means of paired saline-filled micropipettes, of the type described by LING AND GERARD[9]. One microelectrode was introduced inside the cell through the non-innervated membrane and the other was placed just outside the innervated membrane in front of the internal electrode. The former was inserted and withdrawn for each series of measurements. In experiments carried out over prolonged periods of time, where the resting potential and the electrical activity were determined on 20 to 60 different occasions, sometimes focal damage of the membrane occurred. In that case the electrodes were displaced laterally a few microns and a region with a normal membrane could always be reached (Fig. 4; compare C and C′ with D′).

The cell potentials were amplified by a differential amplifier directly coupled and led to a cathode ray oscillograph for photographic recording. A more detailed analysis of the techniques will be presented elsewhere.

(b) *Chemical determinations.* Freshly excised monocellular layers of electroplax of the Organ of Sachs were cut into segments of six laterally adjacent compartments. Six segments, physically as similar as obtainable, constituted a single experiment, three segments serving as control and three being exposed to a compound under investigation. The substrate was ethyl monochloroacetate; its molar concentration in the external medium in two series of otherwise identical experiments was 0.03 and 0.01 respectively. The inhibitors, described below, were added to the medium and tissue prior to their action for approximately 20 min before the measurement of enzyme activity was begun. The concentration of inhibitor used was that which produced block between 20 and 30 min. The hydrolytic activity of the tissue was determined by the usual Warburg manometric technique, at 24° C and at pH 7.4.

RESULTS

I. Effects upon electrical potentials.

The compounds selected were the salts of acetylcholine and its tertiary analogue, dimethylaminoethyl acetate (DMEA), carbamylcholine, decamethonium, *d*-tubocurarine, prostigmine and its tertiary analogue referred to as tertiary prostigmine (T.P.), eserine, procaine and di*iso*propyl fluorophosphate (DFP).

(a) *Quaternary nitrogen compounds.* Quaternary nitrogen compounds block the spike elicited directly or by nerve stimulation, but simultaneously most of them depolarize the membrane. A representative selection of the data is given in Table I. A more detailed analysis of the sequence of events that takes place will be found in a subsequent paper.

Acetylcholine must be tested in presence of eserine, since it is rapidly hydrolyzed by the esterase. Without eserine the concentrations required are very high, of the order

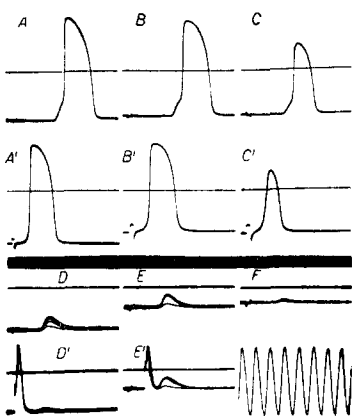

Fig. 1. Effect of Carbamylcholine. A, B, C, D, E and F: Stimulation of the electroplax by the nerve. A′, B′, C′, D′ and E′: Direct stimulation. A,A′: Control. B,B′: 6 min after the addition of 10 μg/ml of carbamylcholine to the Ringer's solution. The resting potential and the spike height are already decreased. The resting potential in B is greater than in B′. The former was measured at the beginning of a short series of observations and the electrode was probably coming out when B′ was determined. C,C′: after 18 min. D, D′: after 23 min. Only post-synaptic potentials can be obtained (stimulation at 25/sec). E, E′: after 35 min (stimulation: 25/sec). F: after 53 min. Resting potential very much decreased. In all the figures, the straight horizontal line corresponds to zero potential difference between the recording electrodes where both are just outside the electroplaque membrane. When one electrode is inside the cell, the value of the resting potential is measured by the distance between the two lines. Calibration for all recordings: 1000 cycles and 100 mV.

References p. 282.

TABLE I

ACETYLCHOLINE AND RELATED COMPOUNDS WITH DEPOLARIZING ACTION

The figures indicate the potential differences in millivolts, preceded by the time (min) of exposure of the compounds. Con. = Control; potential before exposure. Beg. = beginning, Adv. = advance, Fin. = final effect upon the potential. All time indications refer to the beginning of the experiment: time = O. ACh and DMEA, where added after eserine, were dissolved in a solution containing the same concentration of eserine as before (indicated in the Table).

					Action potential							
	Resting potential				*Indirect stimulation*				*Direct stimulation*			
Concen. µg/ml	*Con.*	*Beg.*	*Adv.*	*Fin.*	*Con.*	*Beg.*	*Adv.*	*Fin.*	*Con.*	*Beg.*	*Adv.*	*Fin.*
					Acetylcholine (ACh) + Eserine (Es)							
100 at 86′	76	—	—	86′:70	130	22′:106	—	80′:84	126	22′:98	—	80′:76
1000	70	4′:44	29′:14	184′:6	84	—	—	4′:0	76	—	—	4′:0
Es 25 at 26′	66	—	—	26′:62	120	—	—	26′:114	106	—	—	26′:102
10 ACh	62	23′:56	18′:10	24′:10	114	23′:78	—	5′:0	102	23′:50	—	4′:0
Es 25 at 19′	74	—	—	18′:70	150	—	—	18′:148	150	—	—	18′:146
Es + 1 ACh at 78′	74	—	—	56′:74	150	—	—	56′:144	150	—	—	56′:136
5 ACh	74	49′:62	65′:60	72′:44	150	23′:138	65′:56	78′:0	150	23′:124	—	65′:0
					Carbamylcholine (Carch.)							
100	76	5′:66	8′:54	49′:6	80	—	7′:30	8′:0	90	1′:82	65′:30	8′:0
100	70	3′:52	—	40′:0	98	3′:80	—	4′:0	96	3′:80	—	4′:0
10	70	6′:64	23′:64	55′:16	144	4′:136	18′:98	23′:0	140	4′:130	18′:90	23′:0
10	64	20′:58	—	54′:10	120	20′:66	—	22′:0	116	—	—	20′:0
1 at 77′	79	—	—	76′:76	140	32′:120	—	76′:110	140	32′:122	—	76′:110
10	79	4′:70	13′:42	43′:6	110	5′:108	—	13′:0	110	5′:104	—	13′:0
					Dimethylaminoethyl acetate (DMEA) + Eserine (Es)							
Es 25 at 14′	70	—	—	23′:72	130	—	—	23′:120	134	—	—	23′:110
100 DMEA	72	4′:40	—	40′:10	120	—	—	4′:0	110	—	—	4′:0
Es 25 at 38′	70	—	—	36′:72	140	—	—	36′:145	150	—	—	36′:144
50 DMEA	72	20′:54	25′:34	72′:20	140	12′:132	20′:114	25′:0	144	12′:139	20′:112	25′:0
					Decamethonium							
100	60	3′:56	5′:50	14′:16	106	2′:100	3′:78	5′:0	110	2′:90	3′:64	5′:0
10	72	7′:60	9′:50	30′:4	134	2′:110	8′:68	9′:0	136	2′:110	8′:70	9′:0
					Prostigmine							
5000	68	2′:60	3′:54	25′:18	110	—	—	3′:0	112	—	—	3′:0
2500	72	2′:67	5′:52	50′:14	124	2′:94	4′:32	5′:0	124	2′:96	4′:18	5′:0
500	78	15′:36	—	43′:12	130	5′:116	8′:94	15′:0	126	5′:108	—	8′:0
500	76	3′:56	11′:30	29′:12	—	—	—	—	120	3′:112	7′:82	11′:0

of 1 milligram per ml. Eserine itself in 500 μg per ml blocks conduction, but in a small concentration, 25 μg per ml, it has no inhibitory effect under the conditions of our experiments. With this concentration of eserine, acetylcholine in 10 μg per ml produces

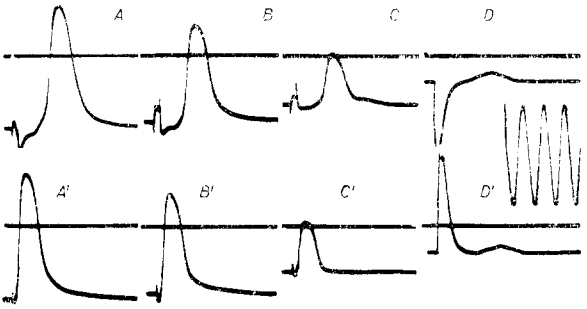

Fig. 2. Effect of Prostigmine. A, B, C, and D: Indirect Stimulation (see methods). A', B', C' and D': Direct stimulation. A, A': Control. B, B': 1 min after the addition of 2500 μg/ml of prostigmine. The magnitude of the resting potential and spike are already decreased. C, C': after 2 min: the small spikes illustrated were the last obtained. D, D': after 9 min: both types of stimulation produced only post-synaptic potentials. Resting potential strongly reduced.

block and rapid depolarization. With 5 μg per ml the action is slow and with 1 μg per ml no effect was observed within an hour. It is possible however, that with higher eserine concentrations lower acetylcholine concentrations are effective. Carbamylcholine is not split by the esterase. It can, therefore, be applied without eserine. Decamethonium and prostigmine were also found to depolarize the membrane.

The only quaternary nitrogen compound among those tested which does not depolarize is *d*-tubocurarine, but it can block the propagation of the spike (see Table II) along the membrane. After concentrations adequate to produce the latter effect, carbamylcholine even in 5 $\times$ the minimal effective dose (50 μg/ml) is not able to produce depolarization.

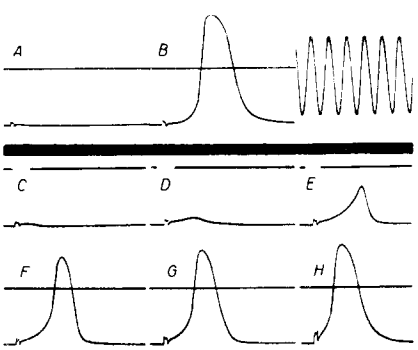

Fig. 3. Procaine. Electroplaque directly stimulated. A–B: control; in A largest local response obtained without spike. C to H: stimulation with increasing strength, 44 min after addition of 200 μg/ml of procaine. Same resting potential but the action potential is not propagated. (see text).

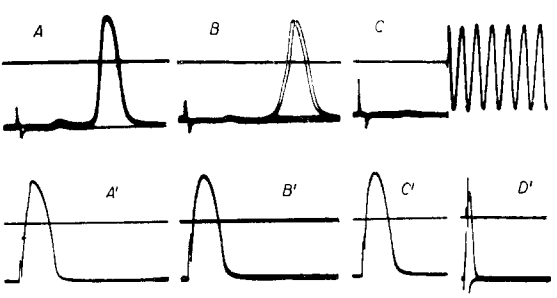

Fig. 4. Effect of T.P. A, B, and C: Nerve stimulation. A', B', C' and D': Direct stimulation. A, A': Control. A single nerve volley did not elicit a spike, thus Fig. A is a multiple exposure of a repetitive stimulation at 50/sec. B, B': 2 min after addition of 1000 μg/ml of T.P. to the Ringer. The spikes illustrated in B were the last obtained by nerve stimulation. C, C': 7½ min afterwards. The propagation of the spike was blocked after 15 min (see text). D': after 176 min. Activity nearly completely blocked. Resting potential same as the control, in spite of the addition of 10 μg/ml of carbamylcholine 124 min before.

References p. 282.

TABLE II

COMPOUNDS WHICH BLOCK PROPAGATION WITHOUT DEPOLARIZATION

Abbreviations as in Table I. Carbamylcholine was dissolved in a solution containing the compound added before at same concentration.

Concentr. μg ml	*Resting potential*			*Action potential*							
				Indirect stimulation			*Direct stimulation*				*Propag. blocked min*
	Con.	*Beg.*	*Fin.*	*Con.*	*Beg.*	*Fin.*	*Con.*	*Beg.*	*Adv.*	*Fin.*	
					Eserine						
1000	73	—	66′:74	136	6′:134	15′:0	142	—	15′:140	40′:130	24
500 at 43′	70	—	40′:72	—	—	—	120	—	—	40′:118	12
10 Carch.	—	73′:68	89′:60	—	—	—	120	73′:100	83′:100	89′:88	
					Tertiary analogue of Prostigmine						
3000 at 32′	76	—	30′:76	120	4′:116	8′:0	114	—	8′:110	18′:110	
20 Carch.	76	26′:68	67′:22	—	—	—	114	—	—	86′:0	
1000 at 52′	72		50′:70	120	2′:118	8′:0	117	—	—	50′:122	15
10 aarch.	—	—	124′:70	—	—	—	—	—	—	124′:104	
					Procaine						
1000 at 39′	72	11′:70	38′:72	160	11′:146	14′:0	154	14′:144	27′:150	36′:128	4
10 Carch.	72	—	111′:70	—	—	—	—	—	3′:114	44′:110	
200	70	—	125′:78	—	—	—	136	50′:130	70′:120	125′:116	44
					d-Tubocurarine						
5000 at 94′	62	—	135′:62	144	10′:138	15′:0	124	18′:120	—	90′:112	44
10 aarch.	62	—	41′:62	—	—	—	112	—	—	8′:108	
5000 at 134′	68	—	134′:62	126	4′:120	6′:0	121	—	6′:114	134′:118	118
50 Carch.	66	—	88′:66	—	—	—	121	—	—	114′:100	
1000	64	—	270′:66	—	—	—	96	—	—	270′:96	120
					DFP						
5000	82	15′:46	60′:14	132	—	2′:0	116	2′:108	4′:92	10′:10	2
100	74	—	76′:76	126	14′:112	44′:0	118	14′:110	44′:100	75′:128	
500 at 99′	71	—	93′:70	—	—	—	106	78′:92	—	96′:60	78
10 Carch.	70	12′:56	35′:8	—	—	—	60	—	—	12′:0	

In Figs. 1 and 2 the electrical recordings of carbamylcholine and prostigmine are shown to illustrate with two typical compounds of this series the characteristics of their blocking and depolarizing action. These two compounds have been selected because, as will be seen, their effect upon acetylcholinesterase activity is not identical.

(b) *Tertiary compounds.* The tertiary amines block conduction, but in contrast to the quaternary ammonium salts they do not depolarize the membrane with the exception of DMEA. A representative selection of the data is given in Table II. By block of conduction is meant block of the propagation of the spike along the electroplaque. Electrical activity with a magnitude even as high as the normal spike can be elicited by direct stimulation, but is not propagated (Fig. 3 and 4). The direct demonstration of this type of block has been obtained by the recording of the cell potentials by means of two intracellular electrodes. However, the time when it appears can also be accurately determined in experiments carried out with only one intracellular electrode. Details of these results will also be given in a subsequent paper of this series.

Like acetylcholine, its tertiary analogue requires the presence of eserine since it is otherwise too rapidly hydrolyzed by the esterase and produces an action only in extremely high concentrations, 5,000 to 10,000 μg per ml. With 25 μg per ml of eserine the concentration required in order to obtain a comparable effect is still about 10 times as high as that of the quaternary analogue (see Table I).

Procaine and eserine are known to block conduction without depolarizing the nerve fiber[10,11]. This is also true in the case of the electroplax, where they do not depolarize even when applied in high concentration. The tertiary analogue of prostigmine acts similarly. Small concentrations of carbamylcholine (10 μg/ml) do not produce depolarization after the block caused by these three tertiary nitrogen derivatives.

Figs. 3 and 4 show the similarity of the pattern of electric recordings obtained with procaine and the tertiary analogue of prostigmine. Here again two examples have been selected because of the difference in chemical action to be discussed later.

(c) *Diisopropylfluorophosphate (DFP).* This compound blocks the propagation of the spike along the electroplaque without depolarization (Table II). It reduces the resting potential only if very high concentrations are used. However, the incidence of complete block and the beginning of depolarization are separated by a significantly long period of time suggesting that at least two different processes are involved. When, for instance, 5,000 μg per ml were applied, block of propagation without depolarization occurred within two minutes, but only after 15 min was depolarization observed. In other experiments in which smaller doses of DFP were used, the addition of 10 μg of carbamylcholine at the time of complete block or before produced depolarization.

II. Effects on acetylcholinesterase activity.

Since the size of electroplax and, still more, the size of the compartment in which each is situated, varies greatly throughout the organ, variations in hydrolytic activity among individual segments had to be minimized. Preliminary experiments showed that it was best to relate activity to number of electroplax used regardless of wet or dry weight. By electing closely similar specimens for comparison of absolute activity within any single experiment, it was possible to obtain an average deviation (*a.d.*) of from 0 to 14% (average *a.d.* 7%) in a series of eleven experiments with a substrate concentration of 0.03 *M*, where each experiment involved a triplicate activity determination, as described above. For the same series of experiments the average values for the hydrolytic

References p. 282.

activity ranged from 5.0 to 14.4 micromoles per hour, with an average value for the series of 11.1 micromoles per hour and an *a.d.* of each experiment of 21%. Similarly, in a series of eight experiments at a substrate concentration of 0.01 *M*, the averages of each of the eight triplicate determinations ranged from 3.5 to 8.9 micromoles hydrolyzed per hour. The average for the series was 6.3 micromoles per hour, and the *a.d.* 24%. However, the *a.d.*'s within each experiment ranged from 0 to 15% and themselves averaged again 7% for this series. Consequently, comparison of activity in the presence of inhibitor and corresponding control was in each case confined within the appropriate experiment. Although the absolute velocities of hydrolysis varied considerably from experiment to experiment, the degree of inhibition, determined separately in each experiment, proved to be a reasonably reproducible quantity. At least two experiments (of three controls and three segments with inhibitor each) were performed for each inhibitor at a given concentration. The data of a typical inhibition assay are given in Table III. A summary of the results of all compounds tested together with the probable error are given in Table IV. It may be seen that the instances of sharply reduced enzymic activity are quite clearly distinguished from those of little or no inhibition.

TABLE III

TYPICAL ASSAY EXPERIMENT. THE HYDROLYTIC ACTIVITY OF SEGMENTS OF SIX ELECTROPLAX AFTER EXPOSURE TO THE TERTIARY ANALOGUE OF PROSTIGMINE

(T.P., 500 μg per ml. Ethyl monochloroacetate, 0.03 *M*).

	Initial velocity of hydrolysis (corrected for spontaneous hydrolysis) (micromoles split per h)	
	Controls	*In presence of T.P.*
Three spec.	12.6 13.6 10.5	1.4 1.0 1.0
Average value	12.2 ± 10%	1.1 ± 18%
		Residual activity 9% ± 2

The validity of the correlation of electrical events with acetylcholinesterase activity of intact cells evidently requires an assay of, if not all, at least a major portion of the enzyme in each cell.

Preliminary experiments had shown that both acetylcholine and DMEA are unable to serve as substrates for the assay because of their failure to reach more than 15% of the enzyme when the cell is intact. This is in contrast to the permeability of spider crab nerves to DMEA. DMEA was used successfully by WILSON AND COHEN[11] to correlate acetylcholinesterase activity and conduction of nerve impulses in intact axons. Ethyl monochloroacetate appeared to be suitable as substrate. However, in the course of establishing this it became clear that the tissue contains another enzyme hydrolyzing ethyl cloroacetate, though not acetylcholine. A detailed analysis of the hydrolytic activity of whole electroplax toward the former substrate will be presented in the following paper. With respect to acetylcholinesterase the results may be summarized as follows:

References p. 282.

1. Using ethyl chloroacetate, it becomes possible to assay about two thirds of the total acetylcholinesterase in the intact cell. About one-third remains unassayed in this method.

2. The contribution of this enzyme to the total hydrolytic acitvity, shown in Table IV, is blocked more or less completely by eserine, prostigmine, T.P. and DFP at their critical concentration. T.P. and DFP have in addition inhibited substantial portions of the other enzyme, and eserine a small portion of it. Procaine, carbamylcholine and decamethonium have little or no effect on either enzyme at the concentrations which produce block of propagation. It was possible to inhibit the acetylcholinesterase activity with greatly increased amounts of decamethonium and procaine, but not of carbamylcholine (see Table IV).

TABLE IV

RELATIONSHIP BETWEEN THE EFFECTS OF SOME COMPOUNDS ON THE CONDUCTING MEMBRANE OF ELECTROPLAX AND ON THE HYDROLYTIC ACTIVITY OF INTACT CELLS

The electroplax were taken from the Sachs organ of *Electrophorus electricus*. All the agents used block propagation, some with and some without depolarization. Ethyl monochloroacetate was used as substrate in 0.03 and 0.01 *M* concentration. Segments of 6 cells were exposed to the compounds for about 30 min before addition of substrate from sidearm. The smallest concentrations employed are those which cause block of propagation in that period of time. 25° C. pH 7.6.

Compound	*Conc. µg per ml*	*% remaining activity*	*Probable error* *
non-depolarizing			
T.P.	500	9	2
T.P.	1000	4	1
Eserine	500	28	7
Procaine	500	91	11
Procaine	10000	51	4
depolarizing			
Prostigmine	300	51	6
Decamethonium	10	86	11
Decamethonium	2000	60	5
Carbamylcholine	10	99	30
Carbamylcholine	200	70	13
Carbamylcholine	2000	83	12
DFP	300	10	4

* Initial velocity of hydrolysis relative to controls. Average values at the 2 substrate concentrations.

3. The concentration of inhibitor (eserine and prostigmine were tested) required to inhibit more or less completely the *assayable* portion of the cellular acetylcholinesterase is considerably smaller than the concentration required to cause block of propagation or depolarization.

4. Ethyl chloroacetate and eserine appear to penetrate quite readily to the assayable portion of the cellular enzyme. The penetration of prostigmine to this portion is approximately 10%; that of decamethonium and carbamylcholine is small.

References p. 282.

DISCUSSION

A. The first problem was to distinguish experimentally the action upon the receptor from that upon the enzyme. The existence of a receptor has long been postulated by many investigators. An excellent discussion may be found in CLARK's review[13]. Recently the question has been raised whether or not the receptor and the esterase are identical (see *e.g.* ZUPANCIC[14]).

An experimental distinction has now been achieved by testing the effect upon the electrical potentials in relation to esterase activity in the intact electroplaque. It should be emphasized, that the evaluation of the esterase activity in intact cells is the only permissible method if the enzyme activity is to be related to changes in electrical potentials in presence of reversible inhibitors. It is surprising that far-reaching conclusions have been drawn on the basis of experiments in which the esterase activity was evaluated after homogenization of the tissue[15]. It has been stressed previously that only a very small fraction of many inhibitors may penetrate into the cell and especially to the active site. Homogenization without washing brings the enzyme into contact with an extremely large excess of inhibitor and makes the determination meaningless. On the other hand it is not permissible to wash out reversible inhibitors, since this process removes the inhibitor and restores activity. With irreversible inhibitors, for instance DFP, the use of homogenized tissue for assaying intracellular activity is permissible provided, however, that the excess of the compound can be removed by washing.This is sometimes possible, but is by no means always the case.

Use of ethyl chloroacetate as the substrate made it possible to assay the major portion of acetylcholinesterase of intact electroplax, but still not the total activity. However, it seems reasonable to assume that all the compounds tested encounter the same penetration barrier and that none of them penetrates better to the unassayed than to the assayed enzyme of the electroplax. It may then be concluded that if a compound at the concentration causing block of electrical activity leaves the assayable enzyme activity virtually untouched, the unassayable enzyme will also be virtually untouched. Its action, therefore, cannot be attributed to the inhibition of acetylcholinesterase. On the other hand, if the assayable enzyme is nearly completely inhibited we are left in ignorance as to the degree of inhibition in the remaining one-third of the total enzyme.

Our data show that carbamylcholine blocks propagation of the spike and depolarizes the membrane when the enzyme activity is very little affected or not at all. The K_I of this compound with acetylcholinesterase is $2 \cdot 10^{-4}$; in view of this relatively low affinity and the low concentration at which block and depolarization are produced, it could have been anticipated as has now been experimentally established, that the effect cannot be attributed to the action upon the enzyme. It has been shown previously with crab nerve fibers that even inhibition of 60–70% of the enzyme does not cause block of conduction[12]. The same is true with regard to the electroplaque as may be deduced from experiments with eserine and prostigmine described in the following paper. Therefore the block obtained with carbamylcholine must be attributed to the reaction with another cell constituent referred to as the acetylcholine receptor. At the present state of our knowledge it is not possible to ascertain whether receptor and esterase are different proteins, but the experiments show that at least the active sites are different. The data obtained with decamethonium are very similar to those obtained with carbamylcholine and the same considerations may be applied.

References p. 282.

Procaine also blocks the propagation of the spike virtually without inhibition of acetylcholinesterase; it has a low affinity to the enzyme. Therefore, this compound too must combine with another constituent of the membrane in order to produce such effect. Procaine does not depolarize the membrane, but since it antagonizes the depolarizing action of carbamylcholine and since the important features of the chemical structure of both compounds are similar, it may be suggested that it combines competitively with the same receptor as the latter compound.

No determinations of the enzyme activity were carried out at the incidence of block by acetylcholine but its chemical structure and its effect upon the electrical potentials are essentially similar to those of carbamylcholine. The dissociation constant of acetylcholine and acetylcholinesterase (K_m) is $4.5 \cdot 10^{-4}$ [16]. It is true that in high concentrations this physiological substrate has an inhibitory effect, but the concentration required for 50% inhibition is $3 \cdot 10^{-2}$ *M*. On the other hand, acetylcholine exerts powerful biological actions in *extracellular* concentrations of 10^{-7} or less (heart, frog muscle). The low affinity of *d*-tubocurarine to acetylcholinesterase is also well known. Thus the block of conduction produced by this compound must also be — and has usually been — attributed to its combination with the receptor. Moreover, *d*-tubocurarine antagonizes the depolarizing action of acetylcholine and carbamylcholine, an effect demonstrated to be a competitive antagonism for the same receptor[17]. The fact that the electroplaque is blocked only by high concentrations of *d*-tubocurarine, may be due to a low permeability of the cell membrane for this substance. There is evidence that this factor is important in other cells; in axons such a permeability barrier to quaternary nitrogen compounds has been experimentally established[18, 19].

In contrast to the compounds discussed so far, eserine and T.P. block conduction with a concomitant inhibition of the assayable cholinesterase. The experiments here reported do not indicate the maximal degree of acetylcholinesterase inhibition compatible with propagation of the impulse in the electroplaque, because only about two-thirds of the total enzyme has been assayed. The two compounds block propagation at a concentration in excess of that required to inhibit all the assayable fraction of acetylcholinesterase.

Since we are left in ignorance concerning the activity of the remaining one-third of the enzyme it is uncertain whether block has been produced by enzyme inhibition or by receptor inhibition. Quite possibly conduction failure in these cases is caused by both mechanisms. That combination with the receptor has occurred at the time of block is clearly indicated by the failure of carbamylcholine to depolarize the membrane at this time.

Prostigmine blocks the propagation of the spike and simultaneously depolarizes the membrane. The assayable acetylcholinesterase at the incidence of block is inhibited as in the case of eserine and T.P. Moreover, it is known from many other observations[20], that prostigmine, in low concentrations, depolarizes rapidly in contrast to eserine. Therefore, the rapid depolarization of the electroplaque strongly supports the assumption of a primary action upon the receptor before a critically low level of acetylcholinesterase activity has been reached, all the more since the essential features of the structure of prostigmine are similar to those of acetylcholine and carbamylcholine.

Additional evidence for the existence of two or more different sites is offered by the observations with DFP. This alkylphosphate has a strong inhibitory action upon acetylcholinesterase which in contrast to that of eserine and prostigmine is irreversible. DFP

References p. 282.

is a competitive inhibitor reacting with the same site as acetylcholine[16]. The enzyme attacks the ester in a nucleophilic substitution reaction[20]. A P-bond is broken and the resulting intermediary complex is a phosphorylated enzyme instead of the physiologically acetylated enzyme. The enzyme may be reactivated only with special treatment, by adding nucleophilic agents which dephosphorylate the enzyme[21,22].

At the incidence of block of propagation the assayable esterase is completely inhibited. There is, however, no depolarization. On addition of carbamylcholine a rapid depolarization occurs. It is difficult to see how this effect could be explained by action upon the same site. TOMAN, WOODBURY AND WOODBURY[11] have described experiments with nerve fibers in which they obtained block by exposure to DFP without depolarization. This agrees with the present observations. The observations show, moreover, that the action of DFP cannot be attributed to acetylcholine "poisoning", as is believed by some investigators, since the presence of an excess of acetylcholine would certainly produce depolarization; the irreversible action may be attributed to an inactivation of the esterase.

B. We turn now to the second problem about which new information has been obtained by the data presented. The distinction between the action upon the receptor and that upon the esterase makes it possible to interpret the effect of tertiary and quaternary nitrogen compounds upon the receptor and to analyze it in the light of the observations on the isolated enzyme proteins of the sytem in solution. Two different types for response have been distinguished: the quaternary derivatives, with the exception of *d*-tubocurarine, cause depolarization; in contrast, the tertiary compounds, with exception of DMEA, produce only block. Apparently all combine with the same receptor. This seems to indicate that we have to deal with two phases of action, as in the case of the enzyme proteins in solution; there too is a sharp distinction between binding and a second phase which is associated with functional activity[13]. It has been pointed out repeatedly[4,6] that the presence of the fourth alkyl group makes the ammonium group chemically less reactive. Therefore, an interpretation based only upon the chemical reactivity of the compounds is not sufficient. Since quaternary methylated nitrogen groups have a tetrahedral structure, *i.e.*, a more or less spherical shape, it has been suggested that the full functional activity may require a reshaping of the protein so as to engulf the quaternary group[4]. A rearrangement of acidic or basic groups by a change in protein configuration may well account for the transitory change in permeability to sodium ions during the action potential. On the other hand, the tertiary compounds are bound to the protein but are not able to produce the change in configuration responsible for the permeability change. They may antagonize by competition with the binding of acetylcholine to the receptor and block the propagation of the impulse by this mechanism. They may inhibit the depolarizing action of the carbamylcholine in the same manner.

In the case of DMEA the loss of the fourth alkyl group decreases the depolarizing activity, but does not abolish it. It requires a 10 times higher external concentration to obtain the same effect as with acetylcholine. This corresponds to the observations with the two proteins studied in solution. DMEA is split by acetylcholinesterase, but at 45% of the rate at which acetylcholine is split[5]. Dimethylethanolamine is acetylated by choline acetylase, but only at 8% of the rate at which choline is acetylated[6]. Consequently, in the interactions of each protein with the small molecule which resembles the physiologically active one very closely, it is seen that the presence of the third methyl

References p. 282.

(fourth alkyl) group is a prominent factor, but not the only feature determining the functional activity of the compound.

Other aspects of the chemical structure must be important. *d*-Tubocurarine, *e.g.* is also a quaternary nitrogen compound and does not depolarize. Besides being a very large molecule containing six rings, the two nitrogens are members of heterocyclic rings. The essentially spheric structure of the cationic portion of the molecule is thus lost. The difference between tertiary and quaternary compounds discussed in this paper is restricted to methylated nitrogen derivatives.

ACKNOWLEDGEMENTS

We wish to express our great appreciation to Dr. IRWIN B. WILSON for his valuable advice and especially for his suggestion to use ethyl chloroactate as a substrate. We are indebted to Dr. J. A. AESCHLIMANN of Hoffman La Roche for the supply of the tertiary analogue of prostigmine and to Dr. E. J. DE BEER of the Wellcome Research Laboratories for the suppply of decamethonium.

SUMMARY

1. The resting and action potentials of isolated electroplax of *Electrophorus electricus* have been recorded by means of intracellular electrodes, and their changes by compounds reacting specifically with the acetylcholine system have been studied.

2. A method has been developed which permits the assay of a major portion (about two-thirds) of acetylcholinesterase activity in intact electroplaques. The procedure is based upon the use of ethyl chloroacetate as the substrate.

3. The combination of these two methods made possible a correlation between the effect on the electrical potentials and that on the enzyme activity.

4. The compounds tested can be divided into two groups:

i. Those which do not significantly affect the enzyme. This group comprises carbamylcholine, decamethonium and procaine. Acetylcholine, dimethylaminoethyl acetate and *d*-tubocurarine, not tested here in relation to esterase inhibition, are regarded as belonging to this group.

ii. Those which depress the assayable acetylcholinesterase activity to a low level at the time block of propagation of the action potential along the cell occurs; eserine, DFP, prostigmine and its tertiary analogue belong to this group.

5. All the quaternary compounds tested except *d*-tubocurarine block the spike and simultaneously depolarize the electroplaque membrane. All the tertiary compounds, except dimethylaminoethyl acetate, block the propagation of the spike without depolarization.

6. DFP blocks the conduction of the spike without simultaneous depolarization.

7. The depolarization caused by carbamylcholine is antagonized by procaine, eserine, *d*-tubocurarine and the tertiary prostigmine analogue.

8. It is concluded that the compounds which do not depolarize, block propagation of the action potential by competition with acetylcholine for the same receptor. A similar mechanism underlies their antagonism to carbamylcholine. These results suggest that the combination of a substance with the acetylcholine receptor does not necessarily cause depolarization. The latter effect is apparently determined by a special change of the receptor attending the binding of a specific chemical structure, in which one important characteristic seems to be the presence of a methylated quaternary nitrogen group.

9. The results are discussed in connection with previous observations with the enzyme proteins of the system in solution.

RÉSUMÉ

1. Les potentiels de repos et d'action de l'électroplaque isolée d'*Electrophorus electricus* ont été enregistrés au moyen d'électrodes intracellulaires, et leurs modifications, sous l'influence de composés réagissant spécifiquement avec le système de l'acétylcholine, ont été étudiées.

References p. **282**.

2. Une méthode permettant le dosage d'une fraction importante (environ les deux tiers) de l'acétylcholinesterase dans les électroplaques intactes a été mise au point. Elle est fondée sur l'emploi du chloroacétate d'éthyle comme substrat.

3. L'utilisation conjuguée de ces deux méthodes a permis d'établir les relations entre les variations des potentiels électriques et celles de l'activité de l'enzyme.

4. Les substances essayées peuvent se répartir en deux catégories:

i. Celles qui n'affectent pas sensiblement l'enzyme. Ce groupe comprend la carbamylcholine, le décaméthonium et la procaïne. L'acétylcholine, l'acétate de diméthylaminoéthyle et la *d*-tubocurarine, dont l'action sur l'inhibition de l'estérase n'a pas été testée ici, sont considérées comme appartenant à ce groupe.

ii. Celles qui diminuent fortement l'activité acétylcholinestérasique dosable au moment où le blocage de la propagation du potentiel d'action le long de la cellule a lieu; l'ésérine, le DFP, la prostigmine et son analogue tertiaire font partie de ce groupe.

5. Tous les composés quaternaires essayés, à l'exception de la *d*-tubocurarine, bloquent la propagation du potentiel d'action et en même temps dépolarisent la membrane de l'électroplaque. Tous les composés tertiaires, à l'exception de l'acétate de diméthylaminoéthyle bloquent la propagation sans dépolarisation.

6. Le DFP bloque la conduction de l'onde sans dépolarisation simultanée.

7. La dépolarisation provoquée par la carbamylcholine est supprimée par la procaïne, l'ésérine, la *d*-tubocurarine et l'analogue tertiaire de la prostigmine.

8. On peut en conclure que les corps qui ne provoquent pas de dépolarisation, bloquent la propagation du potentiel d'action en entrant en compétition avec l'acétylcholine pour un même récepteur. Un mécanisme analogue est à la base de leur antagonisme avec la carbamylcholine. Ces résultats suggèrent que la combinaison d'une substance avec le récepteur de l'acétylcholine ne provoque pas nécessairement une dépolarisation. Ceci serait du à une modification particulière du récepteur liée à la fixation d'une structure chimique spécifique, dont un élément caractéristique important semble être la présence d'un azote quaternaire méthylé.

9. Les résultats sont discutés par référence à des observations antérieures portant sur les enzymes du système en solution.

ZUSAMMENFASSUNG

1. Die Ruhe- und Aktionspotentiale von isolierten elektrischen Zellen (electroplax) des elektrischen Aals *Electrophorus electricus* wurden mit intrazellulären Elektroden registriert und ihre Veränderungen durch Verbindungen, die spezifisch mit dem Azetylcholinsystem reagieren, untersucht.

2. Es wurde eine Methode entwickelt, die die Bestimmung des grösseren Anteils (ungefähr zweidrittel) der Azetylcholinesteraseaktivität in den intakten elektrischen Zellen gestattet. Das Verfahren beruht auf der Verwendung von Äthylchloracetat als Substrat.

3. Die Kombination dieser beiden Methoden macht es möglich, eine Beziehung zwischen der Wirkung auf die elektrischen Potentiale und auf die Fermentaktivität herzustellen.

4. Die untersuchten Verbindungen können in zwei Gruppen unterteilt werden:

i. Solche, die das Enzym nicht wesentlich beeinflussen. Diese Gruppe umfasst Carbamylcholin, Dekamethonium, und Procain. Acetylcholin, Dimethylaminoäthylacetat und *d*-Tubocurarin wurden in ihrer Beziehung zur Esterasehemmung nicht geprüft. Es wird angenommen, dass sie zu dieser Gruppe gehören.

ii. Solche, die die bestimmbare Azetylcholinesteraseaktivität stark senken, wobei gleichzeitig die Ausbreitung des Aktionspotientials entlang der Zelle blockiert wird. Eserin, DFP, Prostigmin und sein tertiäre Analog gehören zu dieser Gruppe.

5. Alle geprüften quaternären Verbindungen mit Ausnahme von *d*-Tubocurarin blockieren den Aktionsstrom und depolarisieren gleichzeitig die Membran der elektrischen Zelle. Alle tertiären Verbindungen, mit Ausnahme von Dimethylaminoäthylacetat, blockieren die Fortleitung des Aktionsstromes ohne Depolarisation.

6. DFP blockiert die Leitung des Aktionsstromes ohne gleichzeitige Depolarisation.

7. Procain, Eserin, *d*-Tubocurarin und das tertiäre Prostigminanalog wirken antagonistisch auf die durch Carbamylcholin verursachte Depolarisation.

8. Es wird geschlossen, dass die Verbindungen, die nicht depolarisieren, die Ausbreitung des Aktionsstromes blockieren, da sie mit Azetylcholin um den gleichen Receptor konkurrieren. Einem ähnlichen Mechanismus unterliegt ihr Antagonismus zu Carbamylcholin. Diese Ergebnisse lassen vermuten, dass die Bindung einer Substanz an den Azetylcholinrezeptor nicht notwendigerweise eine Depolarisation hervorruft. Letzterer Effekt ist offenbar bestimmt durch eine besondere Veränderung des Receptors, die die Bindung einer spezifischen chemischen Struktur, deren wichtigstes Merkmal die Anwesenheit einer methylierten quaternären N-Gruppe ist, begleitet.

9. Die Ergebnisse werden im Zusammenhang mit den früheren Beobachtungen an den Fermentsystemen in Lösung diskutiert.

References p. 282.

REFERENCES

1 D. NACHMANSOHN in E. S. G. BARRON, *Modern Trends of Physiology and Biochemistry*. Academic Press, New York, (1952) 229.
2 D. NACHMANSOHN, *The Harvey Lectures*, Academic Press, New York, 1953/1954.
3 D. NACHMANSOHN AND I. B. WILSON, *Advances in Enzymology*, 12 (1951) 269.
4 I. B. WILSON AND D. NACHMANSOHN, in *Ion Transport Across Membranes*, ed. by H. T. CLARKE, Academic Press, New York, 1954, p. 35.
5 I. B. WILSON, *J. Biol. Chem.*, 197 (1952) 215.
6 R. BERMAN, I. B. WILSON AND D. NACHMANSOHN, *Biochim. Biophys. Acta*, 12 (1953) 315.
7 M. ALTAMIRANO, C. W. COATES, H. GRUNDFEST AND D. NACHMANSOHN, *J. Gen. Physiol.*, 37 (1953) 91.
8 R. D. KEYNES AND H. MARTINS-FERREIRA, *J. Physiol.*, 119 (1953) 315.
9 G. LING AND R. W. GERARD, *J. Cell. and Comp. Physiol.*, 34 (1949) 383.
10 A. L. BENNETT AND K. G. CHINBURG, *J. Pharmacol. Exp. Therap.*, 88 (1946) 72.
11 J. E. R. TOMAN, J. W. WOODBURY AND L. A. WOODBURY, *J. Neurophysiol.*, 10 (1947) 429.
12 I. B. WILSON AND M. COHEN, *Biochim. Biophys. Acta*, 11 (1953) 147.
13 A. J. CLARK, *Handb. Experiment. Pharmakologie*, Vol. IV, W. HEUBNER AND J. SCHÜLLER Editors. Julius Springer, Berlin (1937).
14 A. O. ZUPANCIC, *Acta Physiol. Scand.*, 29 (1953) 340.
15 C. CHAGAS, A. COUCEIRO AND H. MARTINS-FERREIRA, *Compt. rend. soc. biol.*, 145 (1951) 246.
16 K. B. AUGUSTINSSON AND D. NACHMANSOHN, *J. Biol. Chem.*, 179 (1949) 543.
17 L. B. KIRSCHNER AND W. E. STONE, *J. Gen. Physiol.*, 34 (1951) 821.
18 TH. BULLOCK, D. NACHMANSOHN AND M. A. ROTHENBERG, *J. Neurophysiol.*, 9 (1946) 9.
19 M. A. ROTHENBERG, D. B. SPRINSON AND D. NACHMANSOHN, *J. Neurophysiol.*, 11 (1948) 111.
20 W. F. RIKER, *Pharmacol. Rev.*, 5 (1953) 1.
21 I. B. WILSON AND F. BERGMANN, *J. Biol. Chem.*, 185 (1950) 479.
22 I. B. WILSON, *J. Biol. Chem.*, 190 (1951) 111.
23 I. B. WILSON, *J. Biol. Chem.*, 199 (1952) 113.

Received August 20th, 1954