PHARMACOLOGICAL REVIEWS
Vol. 18, No. 3

Printed in U.S.A.

# THE MUTUAL DISPOSITION OF CHOLINORECEPTORS OF LOCOMOTOR MUSCLES, AND THE CHANGES IN THEIR DISPOSITION IN THE COURSE OF EVOLUTION

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## I. CHOLINORECEPTORS OF STRIATED MUSCLES OF HIGHER VERTEBRATES

### *A. The structure of the cholinoreceptive unit*

The concept of "receptors" was put forward by Paul Ehrlich, who defined a receptor as "that combining group of the protoplasmic molecule to which a foreign group, when introduced, attaches itself" (61). Ehrlich deduced that receptor is only a small part of the molecule (2).

In the course of studies on the action of poisons on various cells, especially the action of nicotine and curare on the neuromuscular junction, J. N. Langley came to the conclusion that "neither the poisons nor the nervous impulse act directly on the contractile substance of the muscle but on some accessory substance. Since this accessory substance is the recipient of stimuli which it transfers to the contractile material, we may speak of it as the receptive substance of the muscle" (105). Langley had come to this conclusion long before the chemical theory was generally accepted, and his own investigations were an important contribution to this theory (106–108). After the discovery of cholinergic transmission the term "cholinoreceptor" appeared. The investigations of the evolution of muscles led A. G. Ginetsinsky to the concept of "cholinergic receptive substance" (71). In the literature the terms receptor, cholinoreceptor, and cholinoreceptive site are often used in different meanings. In this review, we will use the term "cholinoreceptor" (or "the receptor") to refer to the protein molecule which is able to interact with one molecule of acetylcholine. There are reasons to consider the cholinoreceptor as a protein molecule (21, 60, 104, 129–131, 167–170a, 185), but this point of view is not generally accepted (41, 42).

We know very little about the structure of cholinoreceptors. Barlow (12) assumed that the active surface of the receptor has two active points, like that of cholinesterase (fig. 1). One of them, the anionic point, interacts with the cationic head of acetylcholine. The role of the anionic point might be played, for instance, by a carboxylate-anion of a dicarboxylic amino acid (aspartic, glutamic), or an

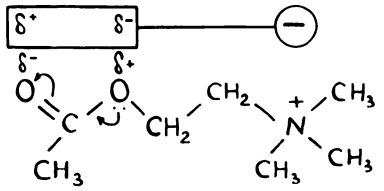
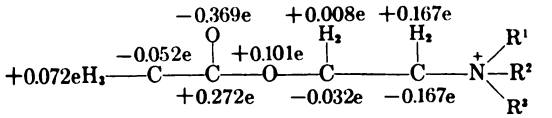



FIG. 1. Scheme of cholinoreceptor.

anion of some phosphoric acid derivative, or anionic groups of sulfonic and substituted sulfuric acids, such as cysteic acid or sulfuric acid esters (37), or the mercapto-group of cysteine (168, 169).

The second active point of the receptor is a "dipole arrangement" (12), adapted for dipole-dipole interaction with the highly polarized acetylcholine ester group. This point resembles the esteratic point of cholinesterase but is not identical with it. In the receptor this point has no esteratic function and is not acylated. It is not quite correct therefore to call it an "esteratic" point. It would be better to call it simply an *esterophilic* point, as it has an affinity for the ester group of acetylcholine (89).

The scheme of the interaction of the acetylcholine ester group with the receptor esterophylic point suggests the presence of a $\delta+$ charge on the acetylcholine ester oxygen (12). This suggestion is confirmed by the calculation of the electric charge distribution in the acetylcholine molecule (145) (the calculation does not take account of the inductive effect of the radicals at the nitrogen):




When the distance between the two dipoles is minimal (about 3 Å), the antiparallel dipoles are attracted as strongly as in the case of the pairs of opposite charges interacting independently. Therefore in the interaction of the receptor with acetylcholine the contribution of the ester group of acetylcholine ($\delta-$ = 0.369 e; $\delta+$ = 0.101 e) is about 50 % as compared to that of the cationic head of acetylcholine (+ 1 e). Certainly this is true only after the acetylcholine molecule is already adsorbed on the receptor. It is clear that the role of the cationic head is much more important in the process of the approach of acetylcholine to the receptor and its orientation on the receptor's active surface; the ionic forces decrease inversely to the square of the distance, and the forces of dipole-dipole interaction decrease inversely to the 4th power of the distance.

The scheme of cholinoreceptor (fig. 1) seems to be correct for all types of receptor, including muscarinic and nicotinic ones. The general principles of the arrangement of cholinoreceptors are discussed in many books and reviews (4–7, 11–19, 31, 35–37, 41, 42, 47, 55, 59, 89, 90–93, 96, 101–103, 118, 126, 129–131, 137–141, 143, 148, 156, 160–162, 169, 178, 179, 181, 193).

### *B. The distance between the anionic points of adjacent cholinoreceptors: The "C-10 structure" and the "C-16 structure"*

*1. The "C-10 structure".* The occurrence of the sharp maximum of the curare-like activity in the polymethylene-bis-trimethylammonium series at the deca-compound (17, 142) was the main reason for the suggestion that the neighbouring receptors are located on the postsynaptic membrane in such a manner that the distance between their anionic points corresponds to the internitrogen distance in decamethonium (about 14 Å; "C-10 structure"). This suggestion is corrob-

orated by Schueler's data (159). He synthesized a linear polymer containing anionic groups ($-SO_2O^-$) located at a distance of 14 Å from each other. In fact, this polymer, which may be considered as a model of the cholinoreceptor, exhibited anticurare activity, *i.e.*, it was able to compete with the receptor for tubocurarine in the living organism. The suggestion about the "C-10 structure" originally was concerned only with the mutual disposition of the anionic points. There were no data on the mutual disposition of the esterophylic points in the same receptors. Several schemes of the receptor structure have been put forward. For instance in Waser's scheme (178, 181) the "C-10 structure" is regarded as a pore in the postsynaptic membrane with a diameter of about 14 Å.

The "C-10 structure" hypothesis proved to be very fruitful and stood the test of time. In the last 17 years, hundreds of myorelaxants (*i.e.*, drugs that interfere with neuromuscular transmission) with an interquaternary distance of about 14 Å were synthesized and several of them are now used in medical practice. Nevertheless, very soon after the first publications examples began to be published of highly potent synthetic myorelaxants in which quaternary nitrogen atoms were separated by a chain of 16 (about 20 Å) and not 10 atoms. Many of them surpass tubocurarine and decamethonium in potency (table 1). These findings cast doubt on the schemes which take into account only the 14 Å distance between the anionic points, and led to new suggestions about the structure of the receptor.

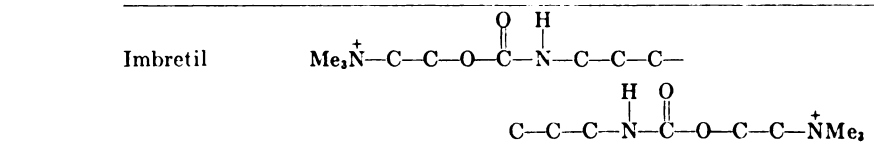
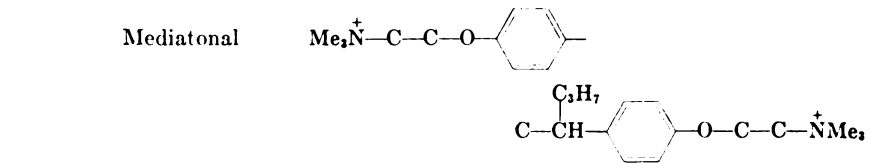
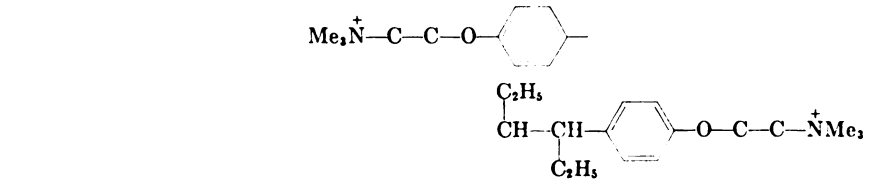
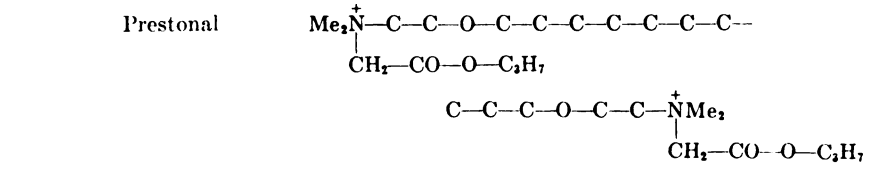
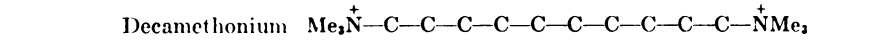
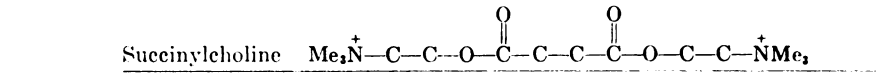
Before going any further we should like to say a few words about the possibilities of using the pharmacological approach to the investigation of this problem.

*2. The possibilities of the pharmacological approach to the investigation of receptors.* The contradictions and obscurities in the present conceptions about the structure of receptors reflect certain defects inherent in the chemico-pharmacological method. Indeed, any minute change in the drug structure which is undertaken to study one property of its molecule inevitably alters many other properties of the same molecule. For example, if one changes the internitrogen distance in a molecule of a bisquaternary agonist, the resulting change in activity may depend not only on that change in the distance, but on alterations in the solubility, absorbability, electronic charge distribution, or other physicochemical properties. These complex changes can alter the fit of the molecule to the receptor, its efficacy as well as its affinity (7, 8, 156, 160). Alterations in the capacity to be adsorbed by nonspecific receptors (silent receptors, acceptors, sites of loss) can also become a source of serious error (24, 25, 28, 162).

When two antagonists are compared, many additional difficulties arise. The blocking effect can be reached by various mechanisms: by depolarization as well as by antagonism of depolarization. The action of depolarizing agents can be complicated by desensitization phenomena (15, 96) and by various rates of transition from the phase of pure depolarization block to that of a "mixed" block. Besides, the activity ratio of two myorelaxants can vary greatly depending on the choice of the test object (88, 186–189). A classical example is that

TABLE 1

*The potencies of myorelaxants with different interquaternary distances (23)*

| | | | Rabbit Head-drop dose, mg/kg Intravenously |
|---|---|---|---|
| Imbretil |  | $2Br^-$ | 0.034 |
| Mediatonal |  | $2I^-$ | 0.25 |
| |  | $2I^-$ | 0.018 |
| Prestonal |  | $2Br^-$ | 0.075 |
| Decamethonium |  | $2I^-$ | 0.1 |
| Succinylcholine |  | $2Cl^-$ | 0.15–0.2 |

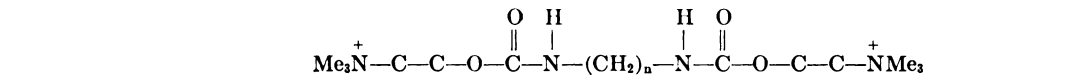
decamethonium in the cat muscle is 10 times stronger than tubocurarine, while in the rat muscle it is 31 times weaker. In other words, the activity ratio changes more than 300-fold (142).

Despite these difficulties, we are convinced that it is not hopeless to study the disposition of active groups in cholinoreceptive membranes by comparing the activities of compounds having different structures. If the relationship is deduced from the activity ratio of two substances in one test-object, the probability of error is indeed very high. If the relationship holds good for various series of compounds and for various test-objects, it acquires high reliability even though certain discrepancies exist which are as yet difficult to explain.

*3. Potency in the series of biscarbaminoylcholines.* Among the data which caused doubts of the existence of "C-10 structure" one fact was of great importance, namely, that in the biscarbaminoyl series the compound having 10

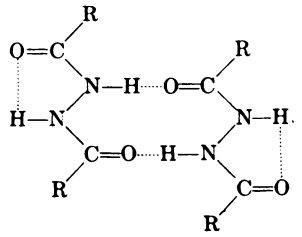
TABLE 2

*Relative curare-like potencies in the series of biscarbaminoylcholines. Rabbit; head-drop dose; intravenous. The potency of imbretil is taken as 100 (13)*



| n | $\overset{+}{N}\cdots\cdots\overset{+}{N}$ | Relative potency |
|---|---|---|
| 0 | 10 | 0.1 |
| 1 | 11 | 5.1 |
| 2 | 12 | 2.9 |
| 3 | 13 | 4.1 |
| 4 | 14 | 11 |
| 5 | 15 | 24 |
| 6 (imbretil) | 16 | 100 (0.034 mg/kg intravenously) |
| 7 | 17 | 117 |
| 10 | 20 | 38 |

atoms between the quaternary nitrogen atoms (dicholine bicarbamate) was 1000 times weaker than the compound which had a chain of 16 atoms between the quaternary nitrogens (imbretil, see table 2) (29, 44, 45). However, the low potency of biscarbaminoylcholine, with a chain of 10 atoms between the quaternary nitrogens, can be explained by the presence of the N—N bond in the central part of biscarbaminoylcholine molecule. The diacylated derivatives of hydrazine are known (189a) to form stable complexes of the form.

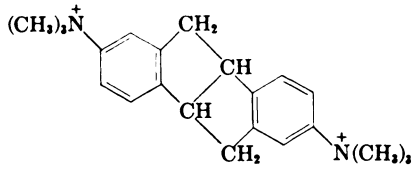


This change in the structure could certainly hinder reaction with the receptor molecule. Table 2 shows that even the incorporation of one methylene group between the carbaminic nitrogens increases the potency 50-fold, though in this case there are already 11 and not 10 atoms between the quaternary nitrogens.

It is interesting to note that the inclusion of one more methylene group between the carbaminic nitrogens again slightly lowers the potency (12 atoms between the quaternary nitrogens), but with inclusion of further methylene groups the potency increases once more and reaches a maximum with 16 or 17 atoms. Thus two maxima can be observed in the biscarbaminoyl series. with 11 and with 16 or 17 atoms between the quaternary nitrogens.

*4. The flexibility of the molecule.* The existence of the "C-10 structure" is also confirmed by a considerable activity of a myorelaxant (I) with an absolutely

rigid structure and the distance between the quaternary nitrogens about 14 Å (6, 165).



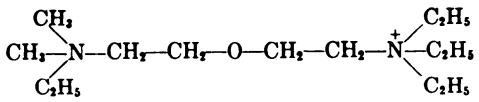

(I)

One of the explanations of the high potency displayed by the compounds in which the distance between the quaternary nitrogens is greater than 14 Å might be that a longer molecule can bend in such a way that its cationic heads would react with the anionic points of the same "C-10 structure." But then it would be difficult to understand why the second maximum is observed only when about 16 atoms are present between the nitrogens while the presence of 12 or 13 atoms results in very low potency. Why are many substances with the interquaternary distance of about 20 Å much more potent than decamethonium, tubocurarine, and other preparations with the distance of 14 Å?

It is necessary to bear in mind that, notwithstanding the flexibility of the polymethylene chain, by no means all possible configurations actually appear in the substance. This is due to the instability of most conformations. The spectra of normal paraffins indicate that these substances contain only a trans-isomer (a stretched one) and one coiled isomer with the rotation of the terminal $CH_3$ group about the terminal C—C bond (173). A stretched isomer should predominate still more in the polymethylene bisquaternary compounds, as they carry terminal charges of the same sign. It was possible to prove this for the crystalline state by studying the X-ray diffraction pattern. The investigation of the crystalline structures of dibromide hydrates of hexa- and decamethonium showed that both chains are centrisymmetrical and are in a completely stretched state (109). Similar investigation of crystalline pentamethonium diiodide (33) showed that within one layer the distance between the anions is 5.61 to 7.7 Å. This agrees well with Gill's conclusions (69) about the distance between the anionic points of cholinoreceptive membranes in autonomic ganglia (6.0 to 7.8 Å).

*5. Are the two anionic points identical?* In regard to the relationship between the structure and the activity of ganglion-blocking bisquaternary compounds, in which the optimal chain length between the quaternary nitrogens is 5 or 6 atoms, a suggestion was advanced that only one of the cationic heads of the ganglion-blocking substance interacts with the receptor's anionic point, while the other is fixed by some group at the protein surface having a negative charge but not belonging to the active surface of the receptor (69, 83). The existence of potent ganglion-blocking substances containing only one cationic head (tetraethylammonium, dimecamine or dimethylaminoisocamphan, pempidine, *etc.*) is in favour of this point of view, which is also confirmed by the high potency of bisquaternary compounds with different radicals at the nitrogen atoms. For

example, compound (II)



(II)

was more potent than either of its symmetrical analogues (63, 69). For curare-like substances, however, compounds with one cationic head as a rule are of very low potency (in contrast to the ganglion-blocking compounds). Besides, no examples are known among the bisammonium curare-like agents of substances with different radicals at both quaternary nitrogens that are more potent than those with identical radicals (see, for example, 12, 178).

*6. Mutual disposition of anionic points.* Barlow (13) considered the possibility of the disposition of anionic points of the neighbouring receptors on the postsynaptic membrane at the angles of a rectangle (fig. 2A). The short side of the rectangle corresponds to the 10-atom chain, and the long side to the 16-atom chain. In the other variant (fig. 2B) the anionic points are located in the angles of a square; the side of the square corresponds to the 10-atom chain, and the diagonal to the 16-atom chain. However, in this article Barlow regarded these schemes as scarcely probable since if they were true, the representatives of the series of decamethonium and succinylcholine should also be expected to have a second maximum of activity when the distance between the nitrogens is increased to 16 atoms. At that time the complete series was not studied, and the incomplete data available suggested that this second maximum does not exist (13). In recent years new data appeared for these series of compounds.

*7. Potency in the series of polymethylene-bis-trialkylammonium compounds.* The second maximum of potency in the decamethonium series was revealed in further investigations by Barlow and Zoller (20). At present, the curare-like activity of polymethylene-bis-trialkylammonium compounds has been compared for methylene chain lengths of 3 to 21 (17, 20, 142). For all the test objects, in

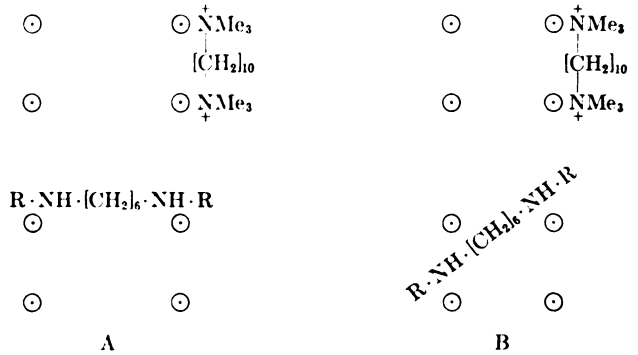



FIG. 2. Scheme of mutual disposition of anionic points in neighbouring cholinoreceptors [reproduced by permission of R. B. Barlow, Biochemical Society Symposium, No. 19 (13)].

particular for cat muscle and for the rat phrenic-diaphragm preparation, two sharp maxima of potency were revealed: the first at 9 or 10 methylene groups, and the second at 14 to 18 groups (on the average, 16 groups) (fig. 3). The second maximum is broader and less sharp but very strongly expressed. In particular, in the rat diaphragm the compound with 16 methylene groups proved to be 10 times more potent than decamethonium.

*8. Potency of the dicholinic esters of higher dicarboxylic acids.* In the succinylcholine series, dicholinic esters of higher dicarboxylic acids exhibit very weak curare-like action in the usual conditions of the experiment. This was shown for sebacinyldicholine, containing 16 atoms between the nitrogens (26, 27), and for azelayldicholine and suberyldicholine, which contain 15 and 14 atoms between the nitrogens, respectively (150). This may be related to the rapid hydrolysis of higher dicarboxylic esters by serum cholinesterase (29, 84, 85, 100, 144).

Eserine potentiates the curare-like action of dicholinic esters of dicarboxylic acids, but in this case no correlation is observed between the rate of the enzymatic hydrolysis of this series of compounds and the increase in their blocking action under the influence of eserine (29, 77, 78, 182). Danilov (51) showed that in the atropinized cat a complete inhibition of cholinesterase by large doses of armine (*p*-nitrophenyl ester of O-ethyl-ethylphosphinic acid) or eserine increases 200-fold the blocking potency of suberyldicholine on the transmission of single impulses. The blocking potency of suberyldicholine in the cat thus becomes equal to that of stable imbretil (ED50 = 0.005 $\mu$mol/kg intravenously). In the

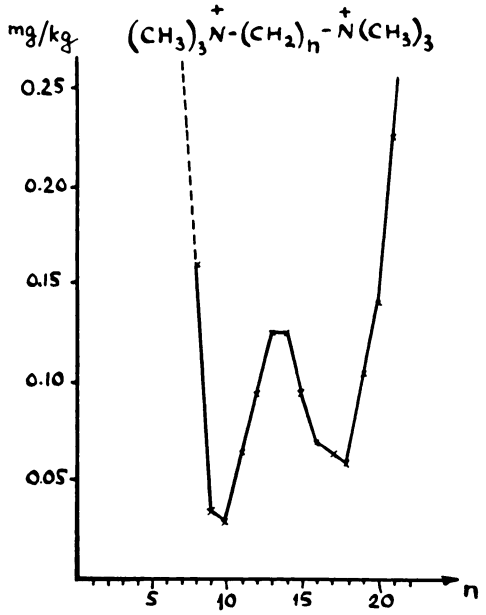



FIG. 3. Curarizing dose in the series of polymethylene-bis-trimethylammonium compounds. Cat, m.tibialis (20, 142).

rat diaphragm the blocking action of suberyldicholine also is sharply potentiated by armine. Suberyldicholine has 14 atoms between nitrogens. In these conditions sebacinyldicholine (16 atoms) also exhibits high potency (in the cat ED50 = 0.02 μmol/kg intravenously). However, the activity of adipyldicholine and pimelyldicholine (12 and 13 atoms between the nitrogens) proved to be just as high as that of suberyldicholine. These observations do not agree with the assumption that the second maximum of activity in bisquaternary compounds should be observed with 14 to 18 atoms between nitrogens. Yet the interpretation of these results is very complicated. Certain anticholinesterase agents themselves in large doses have a direct blocking effect on the receptor (81, 82, 182). In particular, a direct blocking action on the cholinoreceptors of skeletal muscles has been shown for armine (54, 89, 112, 152–154). It cannot be ruled out that large doses of anticholinesterase agents can also block nonspecific receptors (acceptors) (24, 25, 28). Besides, the anticholinesterase agents which stabilize synaptic acetylcholine can influence the blocking effect of curare-like drugs not only with high rates of stimulation but also with infrequent or single impulses (see 53).

Like all depolarizing agents, the dicholinic esters of dicarboxylic acids possess a pronounced and persistent stimulating action on amphibian and avian tonic muscles (27, 77, 78, 172). This stimulating action can give perhaps more information about the disposition of receptors on the cholinoreceptive membrane than can the blocking action. The capacity to induce contraction in the frog's rectus muscle increases with the increase in internitrogen chain length. In the absence of anticholinesterase agents, suberyldicholine is the most potent. The stimulating activity is potentiated by anticholinesterase agents in proportion to the rate of enzymatic hydrolysis of each member of this series (29, 77, 78) In the presence of neostigmine, Magazanik (see 116) obtained the following values for $D_2$ (the molar concentration of drug producing 50% of the maximal contraction) on frog rectus: succinyldicholine = $1.6 \times 10^{-6}$, adipyldicholine = $6.1 \times 10^{-8}$; pimelyldicholine = $4.3 \times 10^{-8}$; suberyldicholine = $1.5 \times 10^{-8}$; and sebacinyldicholine = $9 \times 10^{-9}$. Thus in the presence of neostigmine, sebacinyldicholine (16 atoms between the nitrogens) proved to be even more potent than suberyldicholine (14 atoms). It is significant that the compounds with 14 to 16 atoms between the nitrogens proved to be the most potent in the absence as well as in the presence of anticholinesterases. Thus with cholinomimetic effect of dicholinic esters of dicarboxylic acids it was possible to get more uniform results than with cholinolytic effects. This reflects probably the difficulties in the quantitative estimation of a blocking effect discussed above (Section I B 2). Yet only the depolarizing myorelaxants possess also a stimulant action, and this action can be quantitatively evaluated only in tonic muscles. We are obliged therefore to depend upon data concerning the relationship between the structure and the blocking action of myorelaxants.

The source of errors probably can be reduced when using stable bisquaternary compounds, but among the compounds not hydrolyzed by cholinesterases many

examples can be found which are at variance with the concept of two maxima of activity of bisquaternary myorelaxants, with 10 and with 16 atoms between the nitrogens. Some compounds with an interquaternary distance of 14 Å or 20 Å are practically inactive whereas, on the contrary, a high curare-like potency is characteristic of certain drugs with interquaternary distances other than 14 Å and 20 Å.

We shall try to examine the most important examples and to make clear if possible, some general and particular reasons for these contradictions.

### *C. Shifts of electrons in cationic heads and the distances between the positively charged groups*

*1. The role of methyl and ethyl radicals at quaternary nitrogen atoms.* A model of the trimethylammonium group is shown in figure 4. The nitrogen atom (shadowed) is in the middle, around it are 3 carbons (black), and in the outer sphere are 9 hydrogens. The radius of the sphere is 2 Å. The distribution of electronic charges in the cationic head is influenced by the inductive effect of the methyl groups. The arrows shown in (III) (see below) indicate the shift of electrons resulting from the inductive effect of methyl groups and the electron-attractive properties of the positively charged nitrogen. The positive charge of nitrogen diminishes therefore by about 15 %; +0.85 e remains on the nitrogen and +0.15 e is distributed on the surface of the sphere (IV) (84). Some examples of electron-shifts in other molecules are given for comparison. In tetramethylmethane the shift is very small, only 0.035 e (V), while in the aldehyde group (VI) it is very pronounced (0.45 e) (VI) (163).

Figure 5 gives the distribution of electric charges in trimethyl- and triethylammonium cationic heads. The minor arc corresponds to the trimethylammonium group, the major arc to the triethylammonium group. The triethylammonium radius is about 3.2 Å, or 1.2 Å greater than the trimethylammonium radius. The shadowed part is the difference between the triethylammonium and

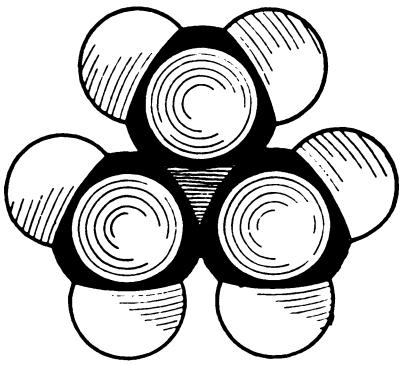


FIG. 4. Model of trimethylammonium group (explanation in text).

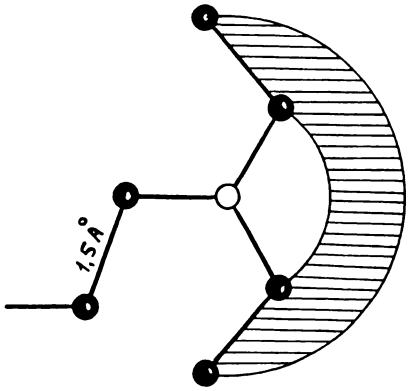
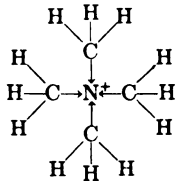
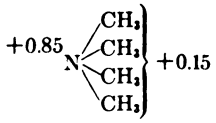


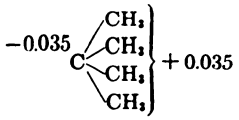

FIG. 5. Dimensions of the trimethylammonium and triethylammonium heads (explanation in text).

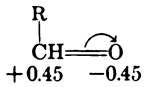
trimethylammonium hemispheres. In both cases the anionic group of the receptor can reach only the positively charged hemisphere surface (37, 49).

(III) 




(IV)

(V) 


(VI)

The distance between the remotest parts of the charged spheres of the cationic heads in bistriethylammonium compounds is therefore 2.4 Å longer than in bistrimethylammonium compounds, as if the internitrogen carbon chain became longer by two methylene groups (97).

These speculations have been confirmed by X-ray determinations of the ionic radii of crystalline bistrimethylammonium bromides and iodides. The $N^+ \ldots Br^-$ and $N^+ \ldots I^-$ distances in these salts proved to be much greater than in mineral bromides and iodides. For pentamethonium diiodide, the minimal distance $N^+ \ldots I^-$ was 4.47 Å (33). For hexa- and decamethonium dibromides it was 4.26 Å (109). For the potassium salts, $K^+ \ldots I^-$ and $K^+ \ldots Br^-$, these distances are known to be 3.49 Å and 3.28 Å respectively. The ionic radius of $I^-$ is 2.16 Å and that of $Br^-$ is 1.95 Å. Thus, the trimethylammonium ionic radius is 4.47 − 2.16 = 2.31 Å or 4.26 − 1.95 = 2.31 Å. It is a little greater than the radius of the trimethylammonium sphere, calculated from Pauling's covalent radii (about 2 Å), as could be expected. These measurements are consistent with the assumption that the anionic group of the receptor interacts with the outer surface of the cationic head of a myorelaxant.

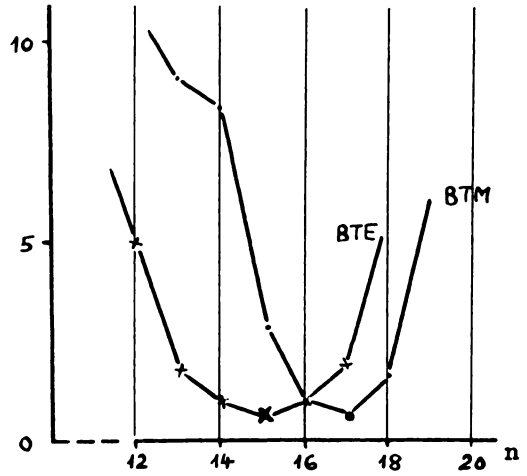



FIG. 6. Relative blocking dose in the polymethylene series of bistrimethylammonium (BTM) and bistriethylammonium (BTE) compounds. Rat diaphragm. n, number of methylene groups. On the ordinates, equipotent molar ratio relative to BTE 16 (20).

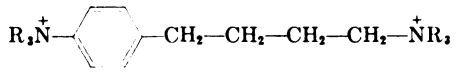
The interaction of bistriethylammonium compounds with the cholinoreceptive membrane must not be influenced by changes in the length of the polymethylene chain as sharply as in the case of bistrimethylammonium compounds, since the triethylammonium sphere is greater than the trimethylammonium sphere. The density of the electric charge is lower in the ethylated cationic head. Therefore, in the interaction with the anionic group of the receptor the Coulombic forces play a minor part, and the van der Waals forces play a greater role than in the case of trimethylammonium-head.[2] Thus the carbon chain of the most potent compound in the bistriethylammonium series should be two atoms shorter than that in the bistrimethylammonium series; also, the maximum of activity in this series should not be so steep as in the bistrimethylammonium series.

In the region of the second maximum ("C-16 structure," 20 Å) we can see that the maximum of blocking activity on the rat diaphragm in the bistriethylammonium series corresponds to the compound with a 15 C— chain, and in the bistrimethylammonium series to a 17 C— chain (20) (fig. 6). It can also be seen that in the latter series the maximum is steeper than in the former. The same relationship is true for the neuromuscular preparation of the cat, and for other tests (20). Some examples of that kind can also be found in the region of the first maximum ("C-10 structure"; 14 Å). It is very striking with some derivatives of hexamethonium, which has 6 atoms between nitrogens. With methyl radicals at the nitrogens (hexamethonium), the blocking activity in the rabbit is very weak (head-drop dose, 26 mg/kg). With ethyl radicals the potency is 26

[2] Van der Waals forces are known to play a very important part in the interaction of stimulating and blocking compounds with receptors. In particular these forces influence the type of action of myorelaxants (depolarizers or antagonists of depolarization). But in this review we will not consider these questions in detail.

times greater (head-drop dose, 1 mg/kg) (23, 114, 183, 184); in the latter case, the potency becomes approximately equal to that of octamethylen-bis-trimethylammonium (head-drop dose = 0.92 mg/kg) (142).

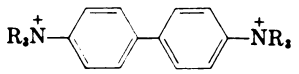
Compounds with the general formula (VII)



(VII)

have 8 atoms between nitrogens. When R = $CH_3$, the blocking potency is about 3 % of that of tubocurarine on the rat diaphragm. When R = $C_2H_5$, the potency increases 7-fold (20 % of tubocurarine activity) (184).

Compounds with the general formula (VIII)

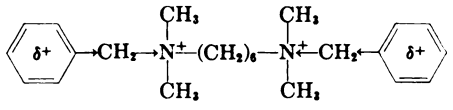


(VIII)

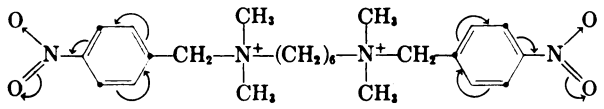
have an absolutely rigid structure and are therefore more convenient for such investigations. When R = $CH_3$, the intramuscular curarizing dose in the rat is 15 μmol/kg; when R = $C_2H_5$, the dose is 4 μmol/kg (data of Khromov-Borisov and Podlesnaja, see 97). We can suppose that in all these cases the bistrimethylammonium compounds cannot reach both anionic points of adjacent receptors at the same time. The corresponding bistriethylammonium compounds acquire this capacity.

The work of Elworthy (62) should be mentioned here. He calculated the inter-nitrogen distance for various bisammonium-polymethylene compounds from the conductivity values determined in aqueous solutions. He showed that the bis-triethylammonium compounds are somewhat longer than the corresponding bistrimethylammonium compounds. The difference was in the range of 1.3 to 2.2 Å. These findings are consistent with the above point of view.

*2. The role of easily polarizable radicals bonded with quaternary nitrogen atoms.* If one phenyl radical is introduced into each cationic head of hexamethonium (IX), the electron shift towards the nitrogens induces positive charges on the benzene carbons.



(IX)



(X)

Consequently, the positive charge on the nitrogens decreases. The distance between the 2 benzene rings, and in particular between their carbons carrying the greatest δ+ charges, is about 14 Å (10 atoms). The dibenzyl derivative (IX) is about 40 times more potent as myorelaxant than is hexamethonium (table 3). In contrast, its ganglion-blocking potency is far lower than that of hexamethonium. The introduction of easily polarizable phenyl radicals results

TABLE 3

*The influence of easily polarizable radicals at the nitrogens in hexamethonium-like compounds on the curarizing potency*

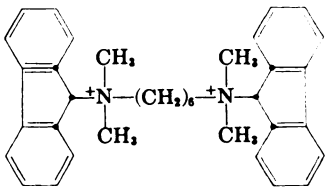
$R(CH_3)_2\overset{+}{N}—(CH_2)_6—\overset{+}{N}(CH_3)_2R$

| R | Curare-like potency. Cat. Gastrocnemius muscle. ED50 μmol/kg intravenously |
|---|---|
| $—CH_3$ | 100 |
| $—CH_2—C_6H_5$ | 2.5 |
| $—CH_2—C_6H_4—NO_2$ | 1 |

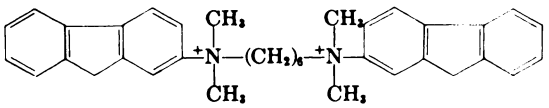
Data of A. F. Danilov (see 118, 119).

in the diminution of positive charges at the "ganglion-blocking distance" and in the appearance of $\delta+$ at the "curarizing distance." The introduction of nitro groups (X) increases the $\delta+$ charges on account of mesomeric effect. This compound proved to be about 100 times more potent than hexamethonium as a myorelaxant and nearly inactive as a ganglion-blocking agent (3, 38, 97, 118, 146, 147).

A second phenyl radical introduced in the cationic head of hexamethonium does not further increase the curare-like potency (38). But the introduction of 9-fluorenyl yields a very potent compound: hexafluorenium (XI), which is 200 to 300 times more potent than hexamethonium (23, 38) and as potent as decamethonium. The reason probably is that fluorenyl, being a whole aromatic structure, is far more polarizable than benzene.



(XI)



(XII)

The enhancement of activity by the introduction of 9-fluorenyl cannot be explained by the planar structure of this radical. The introduction of 2-fluorenyl (the planar structure remains) gives a completely inactive compound (XII). In this compound the induced $\delta+$ charges are separated by an 8-atom chain. Cavallito and co-workers (38, 39) are inclined to think that marked differences in

activity between the 9- and 2-fluorene-substituted derivatives might be related to the differences in the distribution of electrostatic field about the "onium" group. This view does not contradict the above interpretation.

Thus we can see that in some cases the appearance of $\delta+$ charges at a distance of about 14 Å contributes to high curare-like activity.

Mytolon can serve as an example of a relatively potent myorelaxant in which the induced $\delta+$ charges are separated by a 16 atom-chain (20 Å). Table 4 shows that with 12 atoms between the nitrogens, potency is low (16 % of that of tubocurarine). With 14 atoms, the potency increases 12.5 times. Mytolon itself is still more potent, *i.e.*, 5 times more potent than tubocurarine in the rabbit. In Mytolon the "formal" distance between the positive charges (between the nitrogens) corresponds to a 12-atom chain, but the induced $\delta+$ charges are separated by just 16 atoms. A further increase of potency can be attained with the introduction of electrophylic radicals ($NO_2$ or CN) into the benzene rings. Similar examples are given in table 5.

TABLE 4

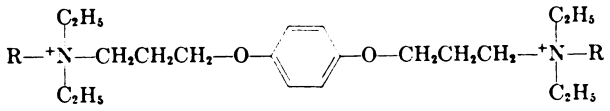
*Curare-like activity in the series of Mytolon derivatives*

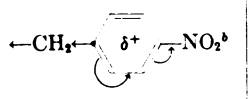
| R | Number of Atoms between Quaternary Nitrogens | Number of Atoms between Induced $\delta+$ Charges | Curare-like Activity (Tubocurarine = 1) | |
|---|---|---|---|---|
| | | | Rabbit. Head-drop dose[a] | Cat. Neuro-muscular block[b] |
| $-CH_2CH_2CH_2-{}^{+}N(C_2H_5)_2-CH_3$ | 12 | | 0.16 | |
| $-CH_2CH_2CH_2CH_2-{}^{+}N(C_2H_5)_2-CH_3$ | 14 | | 2 | |
| $-CH_2CH_2CH_2-{}^{+}N(C_2H_5)_2-CH_2-C_6H_4-\delta+$ Mytolon | 12 | 16 | 5 | 1 |
| $-CH_2CH_2CH_2-{}^{+}N(C_2H_5)_2-CH_2-C_6H_4(NO_2)-\delta+$ | 12 | 16 | | 4 |
| $-CH_2CH_2CH_2-{}^{+}N(C_2H_5)_2-CH_2-C_6H_4(CN)-\delta+$ | 12 | 16 | | 4 |
| Tubocurarine | | | 1 | 1 |

[a] 36, 39.

[b] 146, 147.

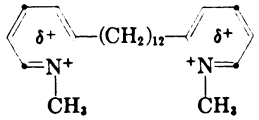
TABLE 5
*Curare-like potency in the series with general formula:*



| R | Number of Atoms between Quaternary Nitrogens | Number of Atoms between Induced δ+ Charges | Curarizing Dose in mg/kg | |
|---|---|---|---|---|
| | | | Rabbit. Head-drop dose | Cat. Neuro-muscular block |
| $-CH_3$[a] | 12 | | 1.5 | 1.0 |
| $-C_2H_5$[a] | 12 | | 0.5 | 0.5 |
|  | 12 | 16 | 0.065 | 0.075 |

[a]23.
[b]146, 147.

The high curare-like potency of the pyridine derivative (XIII)



(XIII)

(0.39 mg/kg intravenously in the rat) (23) can be understood because in the pyridine molecule, and still more in that of pyridinium salts, significant δ+ charges exist in the 2 and 4 positions. In this case the distance between the δ+ charges corresponds also to a 16-atom chain (20 Å).

Hence some cases which seemed to contradict the 10-atom and 16-atom rules, actually confirm them.

In some cases the introduction of nitrobenzyl radicals may interfere with the activity. These cases are no less significant. In decamethonium the replacement of one methyl radical at the nitrogen by a *p*-nitrobenzyl group decreases the curare-like potency on the cat muscle by a factor of 16. In succinylcholine, similar substitution decreases the potency 32 times (147). In decamethonium and in succinylcholine the internitrogen distance is already optimal (about 14 Å) and the appearance of δ+ charges at a less adequate distance can only decrease the potency.

### *D. The role of esterophilic points of cholinoreceptors in the interaction with bisquaternary myorelaxants*

It is well known (182) that a direct blocking activity is observed with some compounds that contain no cationic head at all and can react with the receptor only with their esteric group. For instance such organic phosphate compounds

as DFP, paraoxon, or armine can interact only with the esterophilic point of the receptor (54, 153–155; see also 89, 152, 182).

It is extremely interesting, therefore, to know whether the esterophilic points of the receptor can take part in the interaction with bisquaternary compounds having ester groups.

*1. "Head-to-head" and "tail-to-tail" disposition.* When studying some derivatives of imbretil as well as those of dicholinic esters of dicarboxylic acids and several other myorelaxants, Rybolovlev (151–153) suggested a scheme which could explain high curare-like potency of compounds with internitrogen chains containing 10 as well as 16 atoms, and take into account the role of esterophilic points of receptors (see also 112, 117, 118). In this scheme the mutual disposition of single receptors on the postsynaptic membrane is "head-to-head" and "tail-to-tail," where "head" means the anionic point and "tail" means the esterophilic one of the receptor (fig. 7).

The two cationic heads of a long molecule like imbretil can interact with the anionic points of neighbouring receptors confronting each other with their esterophilic points ("tail-to-tail" disposition); a shorter molecule like decamethonium interacts with the anionic points of two neighbouring receptors confronting each other with these anionic points ("head-to-head" disposition). The scheme suggests that in the first case the anionic points are separated by a distance of about 20 Å ("C-16 structure"), and in the second case by a distance of about 14 Å ("C-10 structure").

*2. Absence of esterophilic points between the anionic points in the "C-10 structure."* According to the Rybolovlev's scheme the compounds of decamethonium or succinyldicholine type can interact only with the anionic points of the "C-10 structure": there are no esterophilic groups between the anionic ones in this structure. Actually (table 6), when both ester groups in the succinyldicholine molecule are replaced by methylene groups, *i.e.*, when succinyldicholine is converted to decamethonium, the curare-like potency is not reduced (it even slightly increases probably because decamethonium is more stable). It is very significant that the reversal of ester groups does not diminish the potency, although the dipole direction is changed by 180°. If the ester groups of succinyldicholine really interacted with the esterophilic points, the attraction would be transformed into repulsion and potency would sharply decrease. Therefore the ester

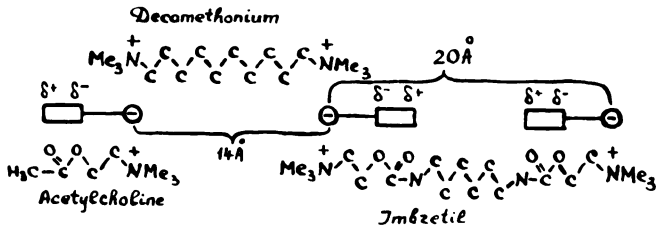



FIG. 7. Scheme of mutual disposition of cholinoreceptors on the cholinoreceptive surface of skeletal muscle fibre (112, 118, 151, 152) (explanation in text).

TABLE 6

*The role of ester groups and cationic heads of succinylcholine in its interaction with cholinoreceptors (23)*

| Structure | Curarizing Dose, Rabbit, Head-drop Dose mg/kg Intravenously |
|---|---|
| $Me_3\overset{+}{N}—C—C—O—\overset{O}{\overset{\|\|}{C}}—C—C—\overset{O}{\overset{\|\|}{C}}—O—C—C—\overset{+}{N}Me_3$ | 0.15 |
| $Me_3\overset{+}{N}—C—C—C—C—C—C—\overset{O}{\overset{\|\|}{C}}—O—C—C—\overset{+}{N}Me_3$ | 0.15 |
| $Me_3\overset{+}{N}—C—C—C—C—C—C—C—C—C—C—\overset{+}{N}Me_3$ | 0.1 |
| $Me_3\overset{+}{N}—C—C—\overset{O}{\overset{\|\|}{C}}—O—C—C—O—\overset{O}{\overset{\|\|}{C}}—C—C—\overset{+}{N}Me_3$ | 0.1 |
| $Me_3\overset{+}{N}—C—C—O—\overset{O}{\overset{\|\|}{C}}—C—C—\overset{O}{\overset{\|\|}{C}}—O—C—C—H$ | 10.0 |
| $Me_3\overset{+}{N}—C—C—C—C—C—C—C—C—C—C—H$ | 5.0 |

groups of succinyldicholine cannot play a significant part (if any) in the interaction with the "C-10 structure."[3]

On the other hand the simultaneous interaction of both anionic points of this structure with both cationic heads of the drug is very important; when one trimethylammonium group of decamethonium is replaced by an H atom, the potency diminishes 50-fold, and still more so in the case of succinyldicholine (table 6).

*3. Presence of esterophilic points between the anionic points in the "C-16 structure."* Rybolovlev's scheme assumes that the drugs of the imbretil type can interact with the "C-16 structure" simultaneously at four points: two cationic heads with two anionic points and two ester groups with two esterophilic points. If this is true, the elimination of one trimethylammonium group in imbretil

[3] The question arises whether esterophilic groups are present outside of anionic ones in the "C-10 structure." Compound (XIV) is far less potent than decamethonium (11, 19)

$$CH_3—\overset{O}{\overset{\|}{C}}—O—CH_2—CH_2—\overset{CH_3}{\overset{|}{\overset{\pm}{N}}}—(CH_2)_{10}—\overset{CH_3}{\overset{|}{\overset{\pm}{N}}}—CH_2—CH_2—O—\overset{O}{\overset{\|}{C}}—CH_3 \qquad \text{(XIV)}$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad CH_3 \qquad\qquad CH_3$$

This fact does not support the above suggestion. However, the question remains unsolved. The low activity of compound (XIV) might depend on the alterations in the cationic heads, on the high rate of its enzymatic hydrolysis, on its own anticholinesterase effect, or still other factors.

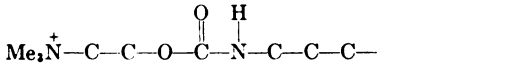
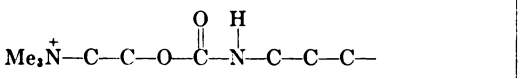
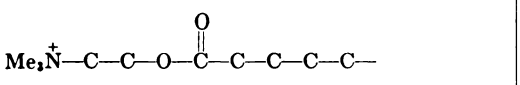
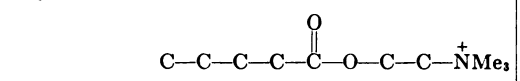
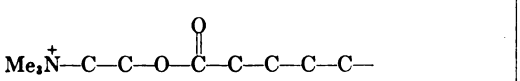
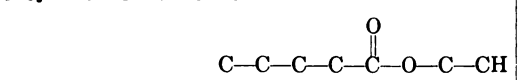
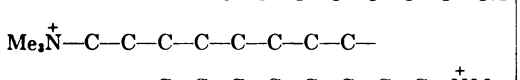
should not reduce the potency as dramatically as in the cases of decamethonium or succinyldicholine (50 to 70 times). In the case of imbretil a possibility remains to interact simultaneously with both cholinoreceptive units of the "C-16 structure"; with two points (anionic and esterophilic) of one receptor and with the esterophilic point of the other receptor. Indeed, imbretil deprived of one cationic head proved to be only 3 times less potent than imbretil itself (112, 151, 153; table 7). A similar elimination of one trimethylammonium group in sebacinyldicholine diminished the curare-like potency by a factor of only 4 to 5 (51; table 7).

In table 7, the activities of imbretil and hexadecamethylene-bis-trimethylammonium are also compared. The replacement of carbaminoyl groups in imbretil by the methylene groups decreases potency nearly 20-fold. This also confirms the assumption that the ester groups of imbretil interact with the esterophilic points of adjacent receptors in the "C-16 structure."

*4. The study of "C-16 structure" by means of compounds of the series KB-72.* For further study of the "C-16 structure" a new series of bisquaternary com-

TABLE 7

*Equieffective doses of imbretil and related compounds*

| | Rat. Phrenic-diaphragm Preparation. μmol/l | Cat. Sciatic-gastrocnemius Preparation. ED50 μmol/kg | Ref. |
|---|---|---|---|
|  | 6.3 | 0.007 | (51, 52, 112, 151, 153) |
|  | 18.0 | | (112, 151, 153) |
|  | 0.5 | 0.02[a] | (51) |
|  | ×4 | ×5 | |
|  | 2.0 | 0.1[a] | (51) |
|  | | 0.13 | (20) |
|  | | ×20 | |

[a]The animals previously received 5 mg/kg of atropine intramuscularly and 0.5 mg/kg of armine intravenously.

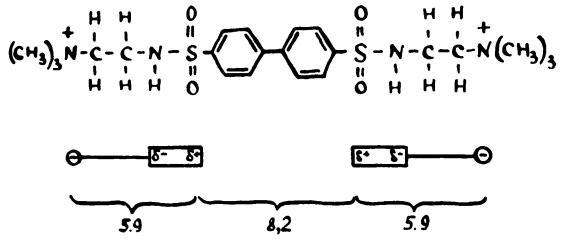



FIG. 8. Structure of KB-72 and the mutual disposition of anionic and esterophilic groups in two neighbouring receptors in the "C-16 structure" (explanation in text).

pounds (type KB-72, fig. 8) was synthesized (98). In KB-72 there are 16 atoms between the quaternary nitrogens. The middle part of the molecule (diphenyl and both sulfonamide groups) is rigid. This diminishes the possibilities of changes in the interquaternary distance, and makes this compound a more suitable tool for testing the anionic points of receptors.

The polarization of the sulfonamide groups in KB-72 is of the same type as that of the ester group in acetylcholine, but much stronger. In both cases the positions of the polarized groups with respect to the quaternary nitrogen are similar. This may contribute to the interaction with the esterophilic points of the "C-16 structures."

Indeed KB-72 proved to be a curare-like agent (52). In the cat it is as potent as succinyldicholine but its action is much more prolonged. It acts as a depolarizing blocker. Succinyldicholine potentiates its action, and tubocurarine antagonizes it. KB-72 induces a contraction of the rectus abdominis muscle of the frog and spastic paralysis in pigeons. The action of KB-72 is not antagonized by neostigmine. Besides, KB-72 possesses a considerable anticholinesterase activity.

Some derivatives of KB-72 were prepared to test the role of the sulfonamide groups in its interaction with the esterophilic points of the receptor.

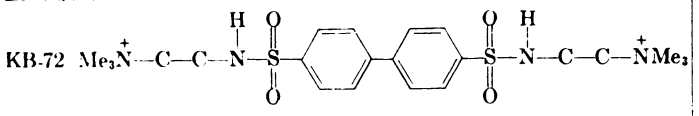
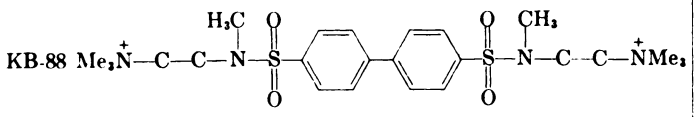
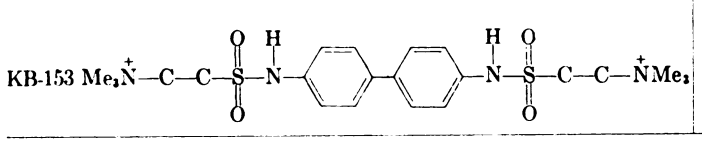
The replacement of both amide hydrogens with methyl groups (KB-88) diminishes the activity 50-fold (table 8) and changes the action to an antagonism of depolarization. It confirms the suggestion that the sulfonamide groups take part in the interaction with receptors. Probably the bulky methyl group prevents close approach of the sulfonamide group to the corresponding esterophilic point of the receptor (the dipole-dipole interaction is known to diminish inversely proportionately to the fourth power of the distance between the dipoles).

If the positions of the sulfonic and amide groups in KB-72 are reversed (KB-153), the curare-like activity disappears completely (table 8). The direction of dipoles is changed by 180° after this reversal. If a dipole-dipole attraction really exists when KB-72 interacts with the "C-16 structure," this attraction must change into repulsion in KB-153. It must be noted that in succinyldicholine a similar reversal of the carbonyl groups and ester oxygens does not cause any reduction of curare-like potency (table 6).

All the above mentioned facts favour Rybolovlev's assumption that the re-

TABLE 8

*Comparison of the curare-like potencies of some KB-72 derivatives (52)*

| | Cat. Gastrocnemius Muscle. ED50 μmol/kg Intravenously |
|---|---|
| KB-72  | 0.05 |
| | ×50 |
| KB-88  | 2.5 |
| KB-153  | Inactive |

ceptors of the endplate form two structures: "C-10" and "C-16," and that in the "C-10 structure" (14 Å) there are no esterophilic dipole groups between the anionic points, but in the "C-16 structure" (20 Å) two esterophilic groups are really present between the anionic points. These groups can undergo a dipole-dipole interaction with the corresponding groups of such myorelaxants as imbretil or KB-72.

### *E. Aggregates of receptor molecules on the endplate; the arrangement of "C-10" and "C-16" structures in the aggregates*

When we consider the mutual receptor disposition on the endplate, two important questions arise.

*1. The disposition of adjacent receptors on neighbouring protein macromolecules.* We suppose that the two cationic heads of a myorelaxant molecule interact with two similar anionic groups of neighbouring protein macromolecules. Is this point of view in agreement with our knowledge about the dimensions of protein molecules?

The Pauling-Cory helix is a universal element of the secondary structure of biochemically active proteins. Its diameter is about 10 Å; the minimal van der Waals radii of atoms and groups attached to its surface are 1 to 2 Å (H = 1.2 Å; $CH_3$ about 2 Å). When two protein molecules are sufficiently close to each other, the distance between two identical points on their surfaces will be just about 12 to 14 Å.

*2. The uneven (nonhomogenous) distribution of the receptor molecules on the endplate surface.* In the above schemes, the question of the even or uneven distribution of the receptor molecules on the endplate surface was not touched upon. The diameter of an endplate was found to be about $8 \times 10^4$ to $18 \times 10^4$ Å and the number of receptors on it was about $3 \times 10^6$ to $4 \times 10^6$ (178). Therefore

if the distribution of receptors on the endplate were homogeneous, the distance between the adjacent units would be 35 to 90 Å. Hence even distribution is ruled out. This deduction is in accordance with the more precise figure given by Waser (180), of $2.6 \times 10^6$ receptors per endplate. It should be assumed that a few receptor molecules form certain aggregates, and in such aggregates the above mentioned "C-10" and "C-16" structures exist. The formation of aggregates (complexes, oligomers) is well known in protein chemistry (43, 127).

*3. The scheme of distribution of the receptor molecules in the aggregates (quaternary structure).* One of the possible suggestions is that the mutual disposition of single receptor subunits in an aggregate is as shown in figure 9 (97, 99, 119). An aggregate contains 4 single receptor subunits. The anionic points of these receptors are situated at the angles of the square. The esterophilic dipolar groups are lying on the diagonals. This scheme is in accordance with one of Barlow's schemes (13) (fig. 2B). The ratio 14 to 20 Å is the ratio of a side of the square to its diagonal. But Barlow's scheme does not consider the position of the esterophilic points.

The suggested scheme is in agreement with the main features of Rybolovlev's scheme too (112, 152). 1) On the "C-10" axis (the side of the square) there are no dipolar groups between the anionic ones. The myorelaxants can interact only with the anionic points of "C-10 structure." 2) On the "C-16" axis (the diagonal of the square) two esterophilic dipolar groups are situated between the anionic points; *i.e.*, the "C-16 structure" is formed by two receptor-subunits which are directed to each other with their esterophilic groups. But the scheme in figure 9 suggests the absence of esterophilic groups outside of the anionic points in the "C-10 structure."

Accordingly, the tetrameric structure of receptor protein seems likely.

*4. Symmetry of the myorelaxant molecule and the "C-10" and "C-16" structures.* One possible test of the suggested scheme is the examination of the symmetry

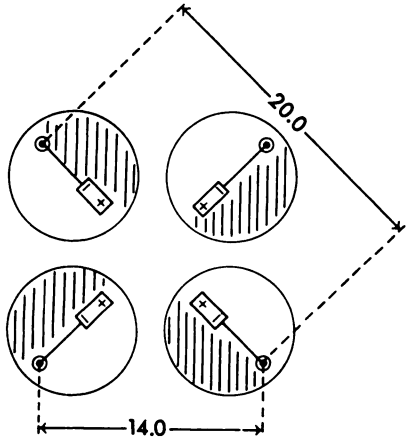
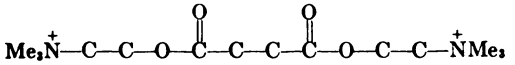
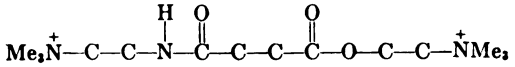
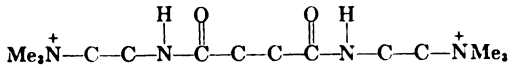
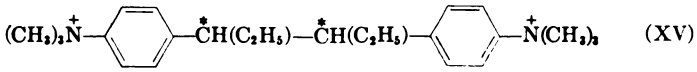



FIG. 9. Suggested tetrameric arrangement of receptor subunits (103) (explanation in text).

TABLE 9
*The role of asymmetry of molecule of myorelaxant for its action on the "C-10 structure" (23, 124)*

| | Activity in Arbitrary Units |
|---|---|
|  | 1 |
|  | 1.8 |
|  | Inactive |

of the myorelaxant. Primary and secondary structures of protein molecules are asymmetric. Therefore the "C-10 structure" (side of the square) must be asymmetric too. In order to show this asymmetry one side of each receptor subunit (fig. 9) is shaded. It is evident that a myorelaxant molecule comes into contact with different parts of two subunits forming the "C-10 structure" (the shaded part and the open one). A nonsymmetric molecule A-B can be therefore more potent than the symmetric molecules A-A or B-B (if A fits the shaded part better and B fits the open one better). Such examples are not unusual. Bovet even advanced an opinion concerning the nonsymmetric myorelaxants that ". . . il n'est pas exclue que leur structure "non-homogène" puisse représenter egalement par elle même un caractère favorable au développement de l'action curarisante" (23). Some compounds of the succinyldicholine series can serve as an example (23, 124, 125) (table 9).



(XV)

As another example we can cite the stereoisomers of paramyon (XV). The mesoisomer which consists of two different halves (*l* and *d*) is 10 times more potent than the mixture of *l* and *d* isomers (166).

The diagonal "C-16 structure," on the contrary, is a symmetric one. Therefore the myorelaxant molecule with an internitrogen distance of 20 Å and nonhomogeneous structure A-B should be less potent tha onne of its homogeneous analogues (A-A or B-B). Mediatonal and its symmetric analogue given in table 1 can to a certain extent serve as an example. The symmetric analogue is 14 times more potent than mediatonal.

### *F. Possible biological significance of the tetrameric arrangement of the cholinoreceptive protein*

The oligomeric structure probably has some advantages (perhaps in kinetics or energetics) as compared with the monomeric one. Recent study of allosteric self-regulating proteins (enzymes, haemoglobin) has shown that all of them are oligomeric (43, 127, 128). Conformational changes induced by allosteric effects

are connected with intermonomeric forces. A clue to understanding the advantages of an oligomeric structure seems to lie in the shape of the curve describing the relationship between the effect and the substrate concentration.[4] In the cases of myoglobin and haemoglobin in a lower vertebrate (the lamprey), which have a monomeric structure, the curve is a hyperbola. In the case of haemoglobin of higher vertebrates (tetrameric), the curve is sigmoid (79). It is assumed that the interaction of $O_2$ with one subunit favours by means of allosteric effect oxygen binding by other subunits.

Perhaps this positive feed-back principle can be applied to the interaction of acetylcholine with the tetrameric receptor. Katz and Thesleff (96) studied in frog's sartorius muscle the endplate depolarization with various doses of acetylcholine applied by means of a micropipette. They found a distinct S-shaped dose-effect relation. One of the possible explanations given by the authors is: "An S-shaped relation could be the result of a reaction in which two (or more) drug molecules become attached to a receptor molecule, and the efficacy of the compound increases with the number of attachments" (96, see also 40, 66, 67, 87).

It is possible that one of the advantages of the sigmoid relationship is that the minute amounts of acetylcholine released during the rest periods induce only a weak reaction in the form of miniature endplate potentials. With a nerve impulse, a simultaneous release of significant amounts of acetylcholine results in an endplate potential high enough to induce a spike. The same explanation probably can be applied to the well known cases when the receptor is sensitized to acetylcholine by very low doses of atropine or other cholinolytic agents (see 80, 90, 92). In such cases the interaction of one atropine molecule with one subunit of the tetrameric receptor may increase the affinity of other subunits for acetylcholine (compare with the Haldane paradox, when low concentrations of carbon monoxide increase the affinity of haemoglobin for $O_2$).

## II. CHANGES IN THE MUTUAL DISPOSITION OF CHOLINORECEPTORS IN THE COURSE OF EVOLUTION

All the above data, which served as the basis for discussing various arrangements of receptors, deal only with the skeletal muscles of higher vertebrates: mammals and birds. It is interesting to know whether a similar arrangement of receptors is characteristic of locomotor muscles of animals representing lower steps of evolution. Or perhaps a definite mutual disposition of single receptors has been gradually attained in the course of evolution and the presence of an oligomeric structure (as compared with a monomeric one) can be considered as a sign of higher organization. Available data on this subject are scanty.

It is not easy to determine whether the peculiarities of a single receptor unit or the differences in the mutual disposition of receptors are responsible for various cholinoreceptive properties of different muscles.

The cholinoreceptive properties of locomotor muscles have been studied in

[4] In these cases the substrate concentration must be given not in logarithmic scale but in arithmetic progression.

comparative-physiological aspect by many authors (10, 30, 32, 48, 50, 64, 65, 157, 171, and others). From the point of view of evolutionary physiology, the cholinoreceptive properties of muscles were extensively studied in Orbeli's laboratories by Ginetsinsky, Itina, Shamarina, Voscresenskaya, and others (70, 72–76, 85, 133–135, 174–176). In these investigations all the three main methods for the study of the evolution of function have been used: the comparative-physiological method, the ontogenetic method, and the method of denervation (132). But in these studies the problem of mutual disposition of receptors was not touched upon.

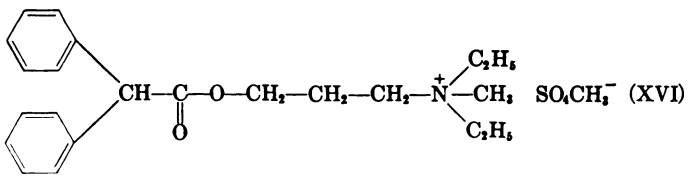
Some data, so far incomplete, concerning the changes in the mutual disposition of receptors of locomotor muscles in the course of evolution have been obtained recently with the comparative-physiological method and the method of denervation (68, 111–113, 116, 120, 149). These data deal mainly with the formation of the "C-10 structure" in development. Isolated muscles were usually used in order to reduce the influence of transportation and distribution of drugs on the results obtained. For cholinomimetic agents, dose-effect curves were obtained and the values $D_2$ were determined (*i.e.*, the dose or the concentration of drug producing 50 % of the maximum contraction). For cholinolytic agents, the $A_2$ values were determined (*i.e.*, the dose or the concentration in the presence of which the dose of acetylcholine must be doubled in order to obtain the same contraction (7, 8, 156, 158). When neostigmine by itself did not induce a contracture, the determination was performed in presence of this inhibitor.

In various species, especially in invertebrates, it is not easy to classify the cholinoreceptors as muscarinic or nicotinic (85, 112, 120, 171). Nevertheless the sensitivity to various cholinomimetic and cholinolytic agents has shown that the receptors of all the muscles studied can be considered as mainly nicotinic (112, 113, 120). The detailed characteristics of the receptors in the muscles of different species (120) is beyond the aim of this review. Here only those data will be touched upon which appear to be concerned with the formation of the oligomeric receptor structures in the course of evolution.

### *A. The "C-10 structure" in locomotor muscles of various animals*

*1. Comparative-physiological data.* In order to detect the presence of the "C-10 structure" the sensitivity of muscles to mononitrogen cholinomimetic and cholinolytic agents was compared with their sensitivity to bisquaternary drugs. The phylogenetic relations of the animals studied in this aspect are illustrated in figure 10.

Let us consider first the results obtained with cholinolytic agents. Among all the monoquaternary cholinolytic agents studied, mesphenal (XVI)




(XVI)

proved to be the most potent. Among the bisquaternary antagonists tubocurarine was the strongest.

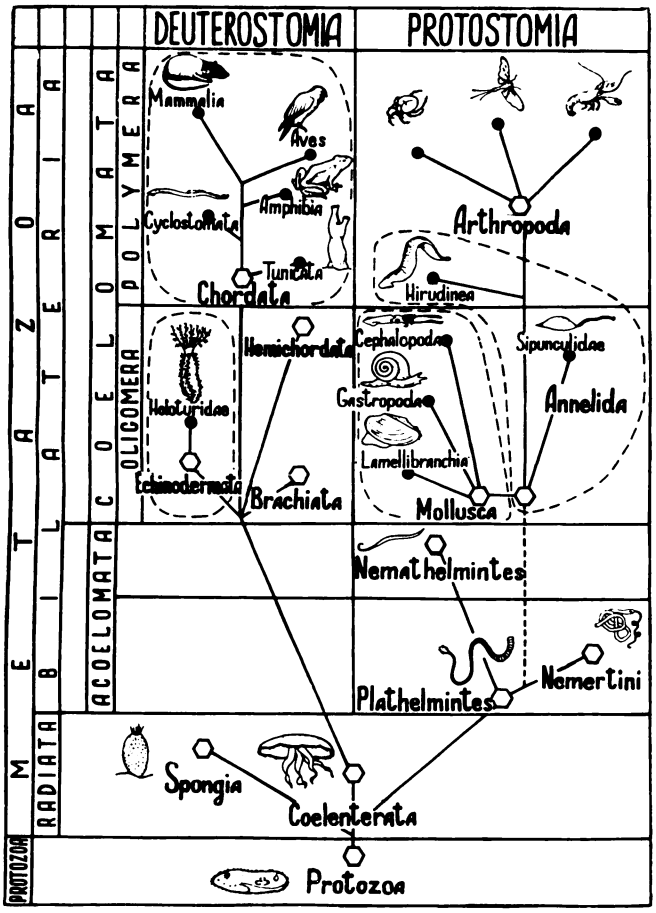



FIG. 10. Phylogenetic relationships of the animals studied [after A. V. Ivanov *et al.* (86)]. The following muscles were studied:

On the Protostomia line. In the phylum Mollusca: Bivalvia: *Mytilus edulis*, m.retractor byssus anterior; Gastropoda: *Rapana bezoar*, m.retractor radulae; Cephalopoda: *Ommatostrephes sloanei-pacificus* and *Octopus dofleini*, m.retractor infundibuli. In the phylum Annelida: Sipunculida: *Phascolosoma japonicum*, m.retractor strombii; Hirudinea: *Hirudo medicinalis*, m.dorsalis.

On the Deuterostomia line. In the phylum Echinodermata: Holothurioidea: *Cucumaria frondosa*, m.protractor; Echinoidea: *Strongylocentrotus droebachinensis*, m.retractor dentis. In the phylum Chordata, in the subtype Urochordata (Tunicata): Ascidia: *Molgula euprocta*, the muscle of body wall; in the subtype Vertebrata: Cyclostomata: *Petromyzon fluviatilis*, m.retractor linguae: Amphibia: *Rana temporaria*, m.rectus abdominis and m.sartorius. Birds: hen and pigeon, m.gastrocnemius. Mammals: rat, the diaphragm.

On each muscle equieffective doses ($A_2$) of mesphenal and tubocurarine were determined (table 10, columns 7 and 8). As could be expected, in higher vertebrates tubocurarine was several hundred times more potent than mesphenal. For instance, on the isolated rat diaphragm the dose of mesphenal which reduces the response to indirect excitation by 50% proved to be 250 times greater

TABLE 10

*Relative potency of certain mono- and bisquaternary cholinomimetic and cholinolytic agents in locomotor muscles of various animals (112, 120)*

| | Cholinomimetics | | | | | Cholinolytics | |
|---|---|---|---|---|---|---|---|
| | Acetylcholine | Relative potency (The potency of acetylcholine = 1) | | | | Tubocurarine | Relative potency (The potency of tubocurarine = 1) |
| | $D_2$ or EC50 mol/l | Tetramethylammonium | Decamethonium | Succinyldicholine | Suberyldicholine | $A_2$ mol/l | Mesphenal |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| A. Deuterostomia | | | | | | | |
| Echinodermata | | | | | | | |
| Sea urchin[a] | $8.10^{-8}$ | 0.09 | <0.001 | 0.012 | 2.3 | $4.10^{-4}$ | 100 |
| Holoturia | $3.10^{-7}$ | 0.1 | <0.001 | 0.012 | 7 | $3.10^{-6}$ | 3 |
| Chordata | | | | | | | |
| Urochordata | | | | | | | |
| Ascidia | $5.10^{-5}$ | 0.3 | <0.001 | <0.001 | | $9.10^{-6}$ | 5 |
| Vertebrata | | | | | | | |
| Cyclostomata (Lamprey) | $3.10^{-6}$ | 0.01 | <0.001 | <0.001 | 4 | $1.10^{-7}$ | 0.2 |
| Amphibia (Frog) | | | | | | | |
| Tonic muscles | $3.10^{-7}$ | 0.01 | 0.06 | 0.15 | 17 | $6.10^{-7}$ | 0.5 |
| Twitch muscles[b] | | | | | | $8.10^{-7}$ | 0.04 |
| Birds (Hen)[a] | 0.06 μmol/kg intravenously | 0.01 | 20 | | >20 | 0.6 μmol/kg intravenously | ≪0.05 (12 μmol/kg ineffective) |
| Mammals (Rat)[b] | | | | | | $8.10^{-7}$ | 0.004 |
| B. Protostomia | | | | | | | |
| Molluscs | | | | | | | |
| Bivalvia (*Mytilus ed.*) | $3.10^{-6}$ | 0.03 | <0.001 | <0.001 | <0.001 | $1.10^{-5}$ | 10 |
| Gastropoda (*Rapana*) | $7.10^{-7}$ | 0.003 | <0.001 | <0.001 | <0.001 | $3.10^{-5}$ | 27 |
| Cephalopoda | | | | | | | |
| Octopus | $1.10^{-5}$ | | | | | $4.10^{-5}$ | 40 |
| Squid | $5.10^{-6}$ | 0.1 | <0.001 | <0.001 | 1 | $2.10^{-4}$ | 285 |
| Annelida | | | | | | | |
| Sipunculida (*Phascolosoma*) | $4.10^{-7}$ | 0.01 | <0.001 | 0.003 | 0.13 | $1.10^{-5}$ | 0.25 |
| *Hirudo med.* | $1.10^{-6}$ | 0.02 | 0.1 | 0.1 | 0.33 | $8.10^{-7}$ | 0.02 |

[a] Data of L. G. Magazanik (see 116).

[b] Response to nerve excitation.

than the equieffective dose of tubocurarine. In the hen, mesphenal in maximal tolerable doses (12 μmol/kg intravenously) did not reduce at all either the contraction of the gastrocnemius muscle induced by indirect stimulation, or the contraction induced by intravenous injection of acetylcholine; tubocurarine was effective in both cases in the dose of 0.6 μmol per kg.

The relationship was reversed with the muscles of the lower representatives of the deuterostomia phylogenetic line. Thus in echinodermata the acetylcholine contraction of the muscles was reduced by lower concentrations of mesphenal than of tubocurarine. In the sea urchin muscle, mesphenal proved to be 100 times more potent than tubocurarine, and in the holoturian muscle 3 times more potent. Even in Ascidia, one of the lowest representative of the chordata type (subtype urochordata), mesphenal was 5 times more potent than tubocurarine. Hence, in the locomotor muscles of invertebrates on the deuterostomia line, no signs of the "C-10 structure" could be revealed.

In all the vertebrates studied, tubocurarine was more potent than mesphenal. But in lower vertebrates the difference was not great. In the lamprey (cyclostomata) tubocurarine was only 5 times more potent than the monoquaternary cholinolytic agent. In the frog (Amphibia) a great difference was revealed between twitch and tonic fibres. With the acetylcholine contraction of m. rectus abdominis (tonic fibres), tubocurarine had only twice the potency of mesphenal. With indirect excitation of m. sartorius (faster, more differentiated twitch fibres), tubocurarine was 25 times more potent than mesphenal. In birds and mammals tubocurarine was several hundred times more potent than mesphenal, even in tonic muscles.[5]

These results invite the speculation that the "C-10 structure" was gradually formed in the evolution of Deuterostomia.

The same method was applied to the locomotor muscles of some Protostomia. In the molluscans, no signs of "C-10 structure" could be revealed in any class studied, even in Gastropoda, which possess highly differentiated muscles. In every case mesphenal was much more potent than tubocurarine in reducing the acetylcholine contraction: in Bivalvia (*Mytilus*), 10 times as active; in Gastropoda (*Rapana*), 27 times; in Cephalopoda (*Octopus* and *Ommatostrephes*), 40 and about 300 times, respectively. However, in annelids, tubocurarine proved to be more potent than the monoquaternary cholinolytic agent: in Sipunculida (*Phas-*

[5] The doses of cholinolytic agents depressing the response to indirect excitation have been determined in twitch muscles (frog sartorius, rat diaphragm) which do not respond by contraction when immersed in acetylcholine solution. Tubocurarine proved to be much more potent than mesphenal in this case. It is certainly difficult to compare these data with those obtained in tonic muscles, in which the contraction was induced by acetylcholine. However, this difference in methods probably does not alter essentially the main relationship: the increase of relative sensitivity to bisquaternary cholinolytic agents as compared to mononitrogen ones in the course of evolution. In higher vertebrates (avian tonic muscles), tubocurarine proved to be much more potent than mesphenal against acetylcholine contraction too. Besides, we must consider that tubocurarine and mesphenal were always compared in the same conditions: The equieffective doses were determined with contractions induced either by nerve stimulation or by acetylcholine.

*colosoma*) it was 4 times more potent; in Hirudinea (leech), 50 times. Hence, in some annelids signs of a well formed "C-10 structure" were revealed. The locomotor muscles have not yet been studied in this aspect with Arthropoda, because in most cases these muscles are insensitive to acetylcholine.

With cholinomimetic agents containing one or two quaternary nitrogens, it is also possible to check the formation of the "C-10 structure" in the locomotor muscles in the course of evolution (table 10, columns 2, 3, 4 and 5). High sensitivity to acetylcholine is characteristic of most of the muscles studied, including those of some of the lowest animals (*e.g.*, in the Deuterostomia line, Echinodermata). But decamethonium is quite inactive in the same representatives of Echinodermata, in Urochordata, and even in the lamprey (the lowest representative of the vertebrates). In the frog, the tonic muscles are sensitive to decamethonium but its potency is $\frac{1}{17}$ that of acetylcholine. In the tonic muscles of birds, decamethonium is 20 to 100 times more potent than acetylcholine (table 10, see also 34, 46).

In the Protostomia line, the muscles of the representatives of all the classes of molluscs studied as well as the muscles of Sipunculida (Annelida) proved to be quite insensitive to decamethonium, but in the muscle of another annelid, the leech, decamethonium induces a contraction though it is 10 times less potent than acetlycholine.

The relative insensitivity of the lowest animals to decamethonium might be explained by the absence of an ester group in its molecule. This would prevent its interaction with the esterophilic group of the receptor. But this suggestion can be ruled out if we compare the sensitivity to decamethonium and to tetramethylammonium, which can react only with the anionic point of the receptor (columns 2 and 4 in table 10). Here the results are still more demonstrative. In the Deuterostomia line, the muscles of Echinodermata, Urochordata, and Cyclostomata have some sensitivity to tetramethylammonium but are quite insensitive to decamethonium.[6] In Amphibia, decamethonium is 6 times more potent than tetramethylammonium, and in birds 2000 times more potent. Among the Deuterostomia studied, the muscles of molluscs and of the annelid, *Phascolosoma*, possess some sensitivity to tetramethylammonium but are quite insensitive to decamethonium, whereas in the leech decamethonium is 5 times more potent than tetramethylammonium.

Attention should be drawn to the lack of any signs of the "C-10 structure" in the most differentiated class of molluscs, the Cephalopoda, especially in the squid, which possesses well developed muscles.

It is possible that in the course of evolution of molluscs another variant of the arrangement of receptors developed, different from the "C-10 structure." We shall return to this question later when discussing the formation of the "C-16 structure" in the course of evolution.

The most important conclusion seems to be the appearance of the signs of

[6] As yet it is impossible to decide whether the inactivity of decamethonium is determined by the presence of the second quaternary nitrogen, by the influence of the polymethylene chain, or by both.

the "C-10 structure," which is characteristic of higher vertebrate muscles, on a certain step of evolution in both main phylogenetic lines of evolution: in the Deuterostomia line (in lower vertebrates) and in the Protostomia line (in annelids).

Another method, that of denervation, has been applied to obtain more information on this problem.

*2. The influence of denervation on the sensitivity to mono- and bisquaternary compounds.* The changes in the mutual disposition of receptors has not been as yet investigated from the ontogenetic point of view. However, it was shown in Orbeli's laboratories that the denervation of a muscle reveals some features characteristic of an earlier period of development (71, 72, 132–134). This was shown in particular for the spatial distribution of cholinoreceptors on the surface of skeletal muscle fibres in vertebrates.

By applying nicotine to various parts of frog muscles, Langley (105–108) showed that in fast muscles (*e.g.*, m. sartorius) the "receptive substance" is localized only in the neural part of the fibre. Ginetsinsky and Shamarina (1942) have shown that in the rabbit this localization of the cholinoreceptive zone is formed gradually in the course of individual development. In the newborn rabbit this zone occupies the whole surface of the muscle fibre (m. semimembranosus, m. gracilis). In the first days of postnatal life this zone becomes more and more narrow, and at the 10th day the sensitivity to acetylcholine remains only in the neural zone. After denervation, a reversed process can be observed in an adult animal (mouse, m. semimembranosus). The cholinoreceptive zone, originally limited to the neural part of the fibre, gradually spreads and on the 14th day after denervation occupies the whole fibre (71, 76). Ginetsinsky's results have been recently confirmed and refined by means of microelectrode techniques (9, 56, 94, 95, 121–123, 164).

In Orbeli's laboratories some qualitative changes in cholinoreception after denervation have also been revealed. The sensitivity of denervated muscle resembled that of the muscles at lower stages of development (1, 71, 85, 174). The use of this method has shown (table 11) that after chronic denervation of skeletal muscles in mammals (rat diaphragm) and in birds (gastrocnemius muscle of the hen) the difference in their sensitivity to mesphenal (a mononitrogen cholinolytic agent) and to tubocurarine sharply decreased. In normal rat diaphragm tubocurarine is 250 times more potent than mesphenal in blocking responses to indirect excitation, but in the denervated muscle the difference was only 8 times for antagonizing acetylcholine-induced contraction. In the normal hen gastrocnemius, mesphenal does not antagonize acetylcholine-induced contraction at all, but after denervation tubocurarine is only 4 times more potent than mesphenal.

In normal tonic muscles of the hen, the intravenous injection of cholinomimetic agents induces a contraction. The effect of denervation on the relative sensitivity to mono- and bisquaternary cholinomimetics also has been studied. In normal muscle decamethonium proved to be 20 times more potent than acetylcholine, but after denervation it was only 6 times more potent. In normal

TABLE 11

*Relative potencies of certain mono- and bisquaternary cholinomimetic and cholinolytic agents in normal and denervated muscles of birds and mammals*

| | Cholinomimetic | | | Cholinolytic | |
|---|---|---|---|---|---|
| | | Activity of acetylcholine = 1 | | | (Potency of acetylcholine = 1) |
| | $D_2$ or ED50 acetylcholine | Tetramethyl – ammonium | Decamethonium | $A_2$ Tubocurarine | Mesphenal |
| Hen gastrocnemius muscle | | | | | |
| Normal | 0.06 μmol/kg intravenously | 0.01 | 20 | 0.6 μmol/kg intravenously | $\ll 0.04$[a] |
| Denervated | 0.018 μmol/kg intravenously | 0.04 | 6 | 0.6 μmol/kg intravenously | 0.25 |
| Rat diaphragm | | | | | |
| Normal[b] | | | | $8.10^{-7}$ mol/l | 0.004 |
| Denervated | $7.10^{-6}$ mol/l | 0.02 | 0.015 | $2.10^{-7}$ mol/l | 0.125 |

Data of L. G. Magazanik (112, 116, 120).

[a] 12 μmol/kg mesphenal ineffective.

[b] Response to nerve excitation.

muscle decamethonium was 2000 times more potent than tetramethylammonium, but after denervation only 150 times. Thus it seems that after denervation the "C-10 structure" plays a less important part than in a normally innervated muscle. Some authors (9, 22, 87, 123, 136) could not find any qualitative difference between the receptors in the neural part of a normal muscle fibre and those induced in distal parts of the fibre after denervation, but they did not study the relative sensitivity to mono- and bisquaternary compounds before and after denervation. The same is true for the numerous investigations of muscle cholinoreceptors during ontogenesis (see for example 56–58).

The comparative physiological method as well as the method of denervation (tables 10 and 11) allow us to suppose that in the course of evolution a regular arrangement of receptor units was gradually achieved, a gradual transition from the monomeric structure to the oligomeric one.

### *B. The formation of the "C-10 structure" and the "C-16 structure" in the course of evolution*

Certain data seem to indicate that the "C-16 structure" (more precisely "C-14 to C-18 structure") appeared in the course of evolution earlier than the "C-10 structure." At any rate, the sensitivity to the cholinomimetic action of suberyldicholine and sebacinyldicholine (respectively, 14 and 16 atoms between the nitrogens) appeared much earlier in the course of evolution than the sensitivity to decamethonium and succinyldicholine (10 atoms between the nitrogens) (see columns 4, 5, and 6 in table 10). Thus in the Deuterostomia line the high

sensitivity of the locomotor muscles to decamethonium and succinyldicholine appears only at the level of Amphibia, whereas the muscles of Echinodermata, Urochordata, and Cyclostomata are either quite insensitive to mimetics with 10 atoms between the nitrogens or have a very low sensitivity to them. At the same time the sensitivity to suberyldicholine proved to be very high in the muscles of all the Deuterostomia studied, even in Echinodermata. In the Deuterostomia line the relative sensitivity to decamethonium and to suberyldicholine (table 10, columns 4 and 6) varies very significantly. The muscles of all the invertebrates studied, and even those of Cyclostomata, are quite insensitive to decamethonium but are highly sensitive to suberyldicholine. The muscles of Amphibia are sensitive to decamethonium, but the sensitivity to suberyldicholine is about 300 times higher. In birds, the mimetic effect of both decamethonium and suberyldicholine is very strong (110). In the Protostomia line the muscles of Bivalvia and Gastropoda (relatively low classes of the mollusc type) have shown no sensitivity either to decamethonium or to suberyldicholine. The much more differentiated muscles of the squid (a cephalopod mollusc) also exhibit no sensitivity to decamethonium and succinyldicholine but possess a high sensitivity to suberyldicholine. In annelids it can be seen that the muscles of Sipunculida are nearly insensitive to succinylcholine and decamethonium but possess a relatively pronounced sensitivity to suberyldicholine. The muscles of the leech possess a considerable sensitivity to decamethonium and succinylcholine and are still more sensitive to suberyldicholine.

These data suggest that in the course of evolution the dimeric "C16 structure" was formed first and the "C-10 structure" was added later. Moreover, it is possible that the "C-10 structure" first appeared only with the formation of a tetrameric receptor, and a separate dimeric "C-10 structure" did not exist at all.

It is difficult to conceive that the two dimeric structures ("C-10" and "C-16") could exist on the cholinoreceptive surface independently. If it were so, it would be difficult to get a complete block of neuromuscular transmission with either C-10 or C-16 bisquaternary compounds. Indeed, the available data yield no examples of high sensitivity to mimetics with 10 atoms between nitrogens and of low sensitivity to those with 16 atoms. Possibly, the "C-16 structure" is in general more universal, more widespread than the "C-10 structure." Thus the receptors of the neurons of autonomic ganglia and of the cells of the adrenal medulla are highly sensitive to the stimulating action of suberyldicholine, aselayldicholine (150), and sebacinyldicholine (27), which have 14 to 16 atoms between nitrogens (see also 29). Hexadecamethylene-bis-trimethylammonium is 5 times more potent than hexamethonium in blocking the cat sympathetic ganglia (20).

Thus, in the polymethylene-bis-trimethylammonium series two maxima of activity have been revealed: in skeletal muscle fibres, a maximum with 10 and 16 methylene groups, and in autonomic ganglionic neurons a maximum with 6 and 16 methylene groups. In other words the "C-16 structure" has been detected in muscles as well as in autonomic ganglionic neurons.

Little information is available about the brain neurons of higher vertebrates.

Ganglion-blocking compounds with 5 ot 6 atoms between nitrogens proved to have very high antinicotinic potency in mice and rats on intraventricular injection (190, 191). The sensitivity of brain receptors to compounds with 16 atoms between nitrogens has not yet been studied.

Certain preliminary data indicate that the "C-16 structure" can be observed in neurons at very low stages of evolution. For example, the giant neurons of a mollusc, *Planorbis* (Gastropoda), proved to be highly sensitive to suberyldicholine and KB-72 (14 and 16 atoms between nitrogens), but far less sensitive to succinyldicholine, decamethonium, and hexamethonium (177, 192).

The authors are fully aware that the data discussed above are incomplete and fragmentary. Therefore the hypothesis suggested here is tentative. The cholinoreceptive proteins in muscles of animals representing various steps of evolution differ certainly not only in their more complex structures, but in their primary structures as well. The aggregation of monomeric cholinoreceptors into oligomeric complexes probably requires certain alterations of their primary structure.

A direct verification of the above schemes concerning the proposed dimeric and tetrameric arrangement of cholinoreceptive protein would be possible only after the isolation of this protein and the application of chemical and biochemical methods to the analysis of its structure. This subject has been investigated (42, 131, 169) but as yet the results cannot be used for the solution of the problem. So far the chemicopharmacological method remains the most fruitful one for the investigation of the receptor structure. This method is especially valuable because it provides a means of testing any scheme of the arrangement of receptors by tailoring new synthetic compounds to fit the receptors, and because it gives to the chemist and the pharmacologist an incentive to further investigations.

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