Brit. J. Pharmacol. (1953), **8**, 327.

THE ACTION OF ACETYLCHOLINE ANTAGONISTS ON THE HEART OF *VENUS MERCENARIA**

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(RECEIVED FEBRUARY 6, 1953)

An attempt has been made to gain a better understanding of the structure-activity relationships of acetylcholine and one type of "receptor substance" by restricting attention to one suitable, isolated organ, the ventricle of the heart of the mollusc, *Venus mercenaria* L. (Welsh and Taub, 1948, 1950, 1951). It has been noted that quaternary ammonium compounds must have two or more methyl groups on a given nitrogen if they are to act like acetylcholine on this preparation. Related compounds with three groups other than methyl (e.g. ethyl or *n*-propyl) on the nitrogen are antagonistic to acetylcholine. These and other earlier observations on the requirements for blocking action led to a more extensive comparison of the relative activities of a number of known and potential acetylcholine antagonists on the Venus heart. From the results that follow it may be seen that the general pattern of action differs from that found at any of the better-known sites of action of acetylcholine in the vertebrates, such as synapses in autonomic ganglia, or neuromuscular junctions. What new light, if any, this throws on acetylcholine receptor substances will be discussed.

METHODS

The procedure used in isolating the ventricle preparation and applying the drugs was the same as previously described (Welsh and Taub, 1948). The steps followed in determining the activities of several concentrations of a given antagonist may best be set forth by reference to a given experiment. In Fig. 1 is shown a record of the blocking action of four different molar concentrations (10^{-6} M to 10^{-3} M) of 4-ketoamyltriethylammonium iodide (K-4). First, by successive trials (only the last of which is shown) a concentration of acetylcholine producing between 20 and 80% decrease in amplitude of heart beat was found. In the illustrative case 10^{-8} M acetylcholine bromide was found to produce 76% decrease in amplitude. After stopping the drum and washing until the original amplitude was regained (3–5 minutes), an amount of 4-ketoamyltriethylammonium iodide was added to produce a final concentration of 10^{-6} M in the bath. Two minutes later 10^{-8} M acetylcholine was again added. Now it was found to produce only 30% decrease in amplitude. From this it was calculated that the activity of the acetylcholine was antagonized or blocked to the extent of 61%. This assumes that repeated applications of a given concentration of acetylcholine, if separated by thorough washing, would have a constant effect, which we know is ordinarily true.

This procedure was then repeated with higher concentrations of 4-ketoamyltriethylammonium iodide up to and including 10^{-3} M, with results as indicated by Fig. 1. From 6 to 20 different heart preparations were used in determining the activity of each compound, and the results were averaged. Many compounds showed no blocking action at a concentration of 10^{-6} M, in the time they were allowed to act on the heart, therefore the tables of results include only the three higher concentrations of antagonists normally tested. With one compound, viz., "Mytolon," the antagonistic action was so much greater that a range of concentrations from 10^{-8} to 10^{-6} M was employed. Certain compounds having two or three methyl groups on the nitrogen acted like acetylcholine in low concentrations, but were antagonistic at higher concentrations. Attention will be called to these when the results are presented and discussed.

We are greatly indebted to the sources indicated for gifts of the following materials:

d-Tubocurarine chloride (crystalline), E. R. Squibb & Sons

Pentamethonium bromide, Decamethonium bromide — The Wellcome Research Laboratories

RO 2–2561, RO 2–3198, RO 1–3724/N1 — Hoffman-LaRoche, Inc.

"Banthine," G. D. Searle & Company

"Mytolon" (WIN 2747), Sterling-Winthrop Research Institute.

The other compounds used were commercial products, with the exception of 4-ketoamyltriethylammonium iodide and chloride, which were synthesized for the first time using the same general procedures

*This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

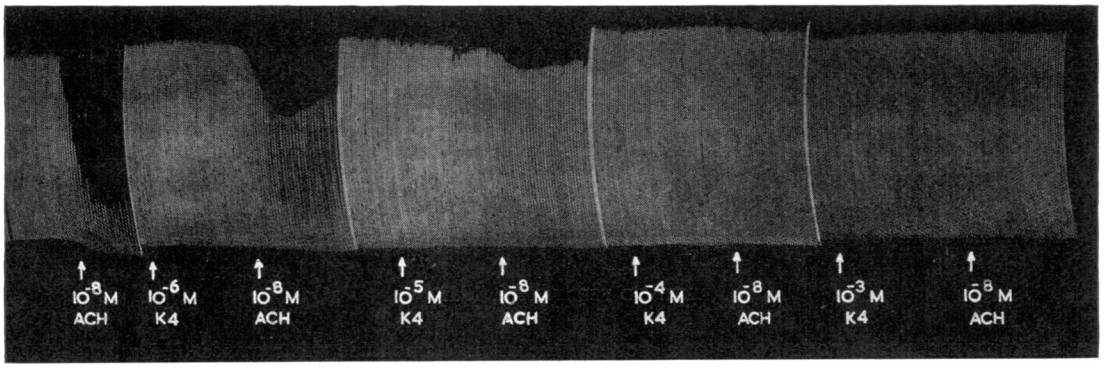

FIG. 1.—Record of the antagonism between 4-ketoamyltriethylammonium iodide (K-4) and acetylcholine bromide (Ach), using the response of the isolated ventricle of the heart of the mollusc, *Venus mercenaria*. The per cent decreases in amplitude produced by 10^{-8} M acetylcholine alone and in the presence of four different molar concentrations of K-4 are shown. From these the percentage block is calculated.

described earlier (Welsh and Taub, 1951). Ing, Kordik, and Tudor Williams (1952) also give methods for preparing certain ketone analogues of acetylcholine.

RESULTS

Table I summarizes the results that were obtained. The grouping of the compounds, although somewhat arbitrary, will facilitate discussion. A number of known acetylcholine antagonists were not tried chiefly because time limitations prevented further study.

Group A.—Included here are three of the oldest known acetylcholine blocking agents. Although acetylcholine has a negative inotropic and chronotropic action on the mollusc heart, it has frequently been observed by other workers (cf. Fredericq, 1947) that atropine fails to block the acetylcholine action as it does in the vertebrate heart. We had stated (Welsh and Taub, 1950) that atropine was completely lacking in acetylcholine blocking action on the Venus heart. However, we observed in this study that atropine was slightly antagonistic at 10^{-5} and 10^{-4} M but quite effective at 10^{-3} M. We had also failed earlier to find any consistent action of *d*-tubocurarine on the Venus heart. Now, after more extensive observation, it appears to potentiate the action of acetylcholine slightly at 10^{-5} and 10^{-4} M and to have a weak blocking action at 10^{-3} M concentration. Luduena and Brown (1952) observed a slight potentiation of acetylcholine with 3.08×10^{-4} M *d*-tubocurarine.

We had noted (Welsh and Taub, 1950) that nicotine in concentrations of 10^{-7} and 10^{-6} M had an acetylcholine-like action on the Venus heart, while at higher concentrations it blocked acetylcholine.

TABLE I

RELATIVE ACTIVITIES OF ACETYLCHOLINE BLOCKING AGENTS ON THE VENUS HEART

(Values indicate the percentage block, by the indicated concentrations of the blocking agents, of amounts of acetylcholine producing 20–80% decrease in amplitude. Each value is the average of 6 to 20 determinations on different hearts)

	10^{-5} M	10^{-4} M	10^{-3} M
Group A:			
Atropine sulphate	10	6	74
d-Tubocurarine Cl	*	*	48
Nicotine	10	60	80
Group B:			
Pentobarbital sodium	18	36	54
Phenobarbital ,,	18	50	48
Cocaine HCl	22	18	88
Tropacocaine HCl	—	18	48
Procaine HCl	0	0	64
Group C:			
Pentamethonium Br	0	0	*
Decamethonium Br	0	10	66
Group D:			
n-Octyltrimethylammonium Cl	*	*	40
Phenyltrimethylammonium I	24	60†	90†
3-Hydroxyphenyltrimethylammonium Br=RO 2–2561	[67]	[92]	[100]
3-Hydroxyphenyldimethylethylammonium Cl=RO 2–3198 or "Tensilon"	12[17]	[77]	[100]
Group E:			
"Banthine" or methantheline Br	4	54	100
4-Ketoamyltriethylammonium I	44	70	88
Phenyltriethylammonium I	40	90	100
3-Hydroxyphenyltriethylammonium I=RO 1–3724/N1	54	84	100
	10^{-8} M	10^{-7} M	10^{-6} M
"Mytolon" or WIN 2747	40	78	100

* Potentiation of the acetylcholine response or slight Ach-like action.

† Some acetylcholine-like action of the compound alone followed by effective blocking action.

[ ] Values in brackets are the percentage inhibitions of heart beat produced by the compound alone. See text.

It is a more effective antagonist at 10^{-4} M than atropine or *d*-tubocurarine. Since, in addition, pilocarpine has little or no significant action on the Venus heart we had earlier suggested that the action of acetylcholine on this organ could be described as "nicotine-like" and most closely resembling that found at vertebrate autonomic ganglia. We were aware from our own results that this view required modification before it was pointed out by Luduena and Brown (1952). This will be given further attention in the discussion.

Group B. (1) Barbiturates.—The mechanism of action of the barbiturates is in dispute. One possibility that has received little attention is that they block acetylcholine, especially at certain points within the central nervous system. After recognition of the importance of the C=O group as one of the attaching groups between acetylcholine and receptor substance of the Venus heart (Welsh and Taub, 1951) the question of the significance of C=O groups in so many of the hypnotics, local anaesthetics, and anti-epileptics arose. If bis-quaternary ammonium compounds make such effective acetylcholine antagonists through the formation of double ionic linkages with acetylcholine receptor substance, might not certain compounds with two or three carbonyl groups (e.g. ureides, hydantoins, barbiturates) form multiple hydrogen bonds with receptor substance? In attempting to answer this question, we have done little more, thus far, than independently confirm the observations of Fernando (1952) that certain barbiturates do block the action of acetylcholine on the Venus heart. Fernando found sodium diethyl barbiturate and sodium phenobarbital to be moderately effective in blocking acetylcholine. We found a similar action of phenobarbital and noted, in addition, that sodium pentobarbital has about equal activity with the phenyl derivative.

Group B. (2) Cocaine, Tropacocaine, Procaine.—Thimann (1943) appears to have been the first to suggest that the actions of certain local anaesthetics such as cocaine, tropacocaine, and procaine might be accounted for on the basis of structural resemblance and competition with acetylcholine. There is evidence in the literature indicating that this is true (e.g. De Elío, 1948). These compounds react with cholinesterase (e.g. cocaine, Blaschko, Chou, and Wajda, 1947). Perlmutter (unpublished), using a variety of isolated smooth muscle preparations from invertebrates, has found (*a*) an acetylcholine-like action, (*b*) potentiation of acetylcholine through block of cholinesterase, and (*c*) acetylcholine antagonism, depending on the particular preparation and the concentration of cocaine that was applied.

On the Venus heart 10^{-5} M cocaine has a low blocking activity. Over a very narrow range (around 10^{-4} M) cocaine acts like acetylcholine, yet at the same time it is capable of antagonizing acetylcholine (Fig. 2). 10^{-3} M cocaine causes a striking increase in amplitude of heart beat partly, if not wholly, through the blocking of endogenous acetylcholine, and at this concentration is very effective as an antagonist to applied acetylcholine.

Tropacocaine acts on the heart in a manner similar to cocaine, but at 10^{-3} M concentration it is less active as an acetylcholine antagonist. Procaine, on the other hand, has no demonstrable effect on the acetylcholine response when present at concentrations of 10^{-5} and 10^{-4} M. 10^{-3} M procaine usually causes an increase in amplitude of beat and at this concentration it is about as effective as tropacocaine.

Group C. Pentamethonium and Decamethonium.—Since the early reports concerning these bis-trimethylammonium polymethylene compounds (Barlow and Ing, 1948 ; Paton and Zaimis, 1948) there has been much interest in their mode of action (Paton, 1951 ; de Beer *et al.*, 1951). Decamethonium (C10) blocks at neuromuscular junctions in the vertebrates, whereas pentamethonium (C5) and the adjacent member of the series, hexamethonium (C6), block transmission at synapses in autonomic ganglia. Since we had concluded earlier that the general pharmacological pattern of the Venus heart resembled rather closely that of vertebrate autonomic ganglia, we assumed that C5

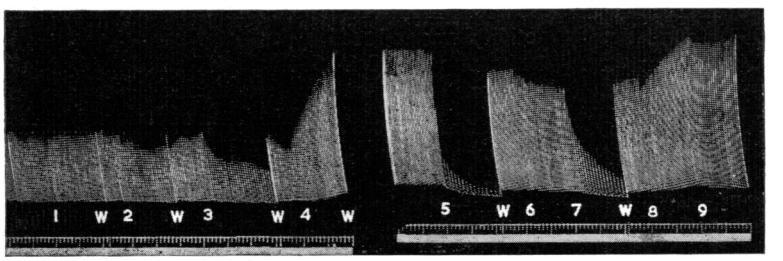

FIG. 2.—Record of the action of cocaine hydrochloride on the isolated ventricle of *Venus mercenaria*. 1 = 10^{-6} M cocaine, 2 = 10^{-5} M cocaine, 3 = 10^{-4} M cocaine, 4 = 10^{-3} M cocaine, 5 = 3×10^{-8} M acetylcholine, 6 = 10^{-4} M cocaine, 7 = 3×10^{-8} M acetylcholine, 8 = 10^{-3} M cocaine, 9 = 3×10^{-8} M acetylcholine. At W the drum was stopped and the bath flushed several times. Smallest time interval = 10 sec.

might antagonize the action of acetylcholine on the Venus heart. This proved not to be true. In fact, it was found that C5 showed no measurable effect until a concentration of 10^{-3} M was reached, at which it usually had an acetylcholine-like action and potentiated slightly the action of acetylcholine (Fig. 3A). On the other hand, C10 had generally a weak blocking action at 10^{-3} M (Fig. 3B). Like C5, it sometimes had a weak acetylcholine-like action in relatively high concentrations. With some hearts the actions of C5 and C10 were practically indistinguishable.

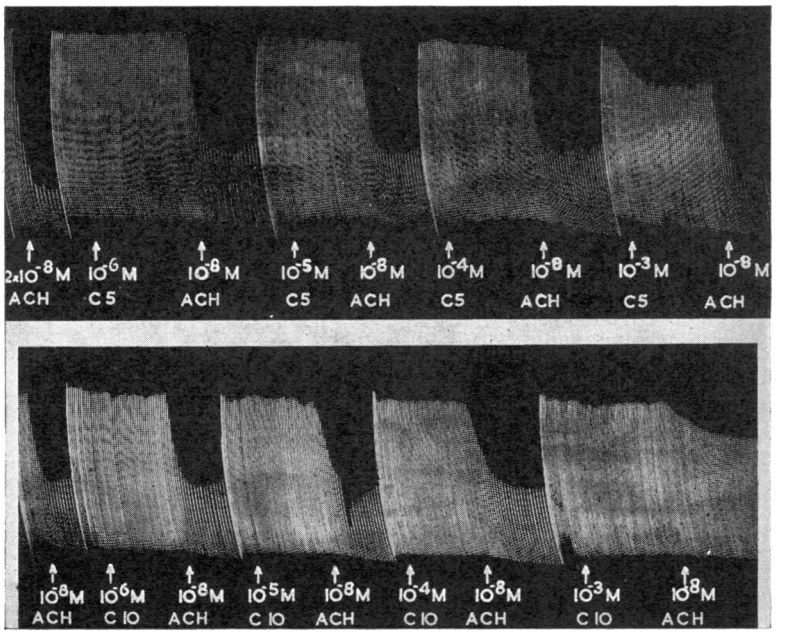

FIG. 3.—In A is shown a record of the actions of pentamethonium bromide (C5) and acetylcholine (Ach) on the isolated ventricle of *Venus mercenaria*. Note that 2×10^{-8} M acetylcholine was first used, but subsequently it was lowered to 10^{-8} M. In B is seen the effect of decamethonium bromide (C10) and acetylcholine.

It would appear that C5 and C10 both have weak acetylcholine-like actions on the Venus heart which may be attributed to the presence of $(CH_3)_3N^+$-groups in each (cf. Welsh and Taub, 1950), but that the 10 carbon chain in decamethonium made this compound a slightly effective antagonist at 10^{-3} M. Luduena and Brown (1952) found decamethonium to be inactive on the Venus heart at a concentration of 2.39×10^{-4} M.

Group D. Octyl- and Phenyl-trimethyl-ammonium Compounds.—In this group are three trimethylammonium compounds and one dimethylethyl compound whose activities as potential acetylcholine antagonists are to be compared with the compounds in Group E. The first of these, *n*-octyltrimethylammonium, was found to have a weak acetylcholine-like action or to potentiate acetylcholine at 10^{-5} and 10^{-4} M concentrations, while at 10^{-3} M it had a moderate blocking action. Phenyltrimethylammonium iodide proved to be quite an active antagonist at all three of these concentrations. At 10^{-4} and 10^{-3} M it also had some acetylcholine-like activity.

The anticurare actions of 3-hydroxyphenyltrimethylammonium bromide (RO 2-2561), 3-hydroxyphenyldimethylethylammonium chloride (RO 2-3198 or "Tensilon"), and related phenolic salts were reported by Randall and Lehmann (1950), Randall (1950), and Wescoe and Riker (1951). It was of interest to find that RO 2-2561 and RO 2-3198 each had high acetylcholine-like activity on the Venus heart. Except in the case of 10^{-5} M tensilon it was not possible to determine the potential acetylcholine antagonism in the same manner as was done for the other compounds, hence the values appearing in brackets in Table I give the per cent decrease in amplitude of heart beat produced by these compounds.

A comparison of the activity of the phenyltrimethylammonium compound with that of the 3-hydroxyphenyltrimethylammonium shows that the latter has a more powerful acetylcholine-like action. This could be due to differences in solubility and adsorptive properties of these two substances. Randall (1950) observed that anticurare activity was reduced by a factor of five when the OH group of RO 2-2561 was shifted to the 2- or 4-positions, therefore the OH group in the 3-position appears to give a configurational superiority to the molecule in terms of anticurare action.

Group E. Di- and Tri-ethylammonium Compounds.—We had earlier noted the acetylcholine blocking action on the Venus heart of quaternary

ammonium compounds with three or four alkyl groups such as ethyl or *n*-propyl. The averaged data of Table II in Welsh and Taub (1950) can be recalculated to express the acetylcholine blocking action of 10^{-4} M concentrations of several *n*-alkyltriethylammonium compounds so that they may be compared with the compounds of Group E, Table I of this paper. Thus the blocking action of 10^{-4} M methyltriethylammonium salt is found to be 75% ; *n*-propyltriethylammonium, 78% ; *n*-amyltriethylammonium, 55% ; acetoxyethyltriethylammonium, 50% ; *n*-octyltriethylammonium, 93%. An interesting point to note is that the acetoxyethyltriethylammonium analogue of acetylcholine was the least effective of these five compounds, suggesting that, other things remaining constant, some departure from the acetoxyethyl-grouping favours antagonistic action on the Venus heart.

Whereas the addition of the 3-hydroxy-group increased the acetylcholine-like activity of the phenyltrimethylammonium salt, it is interesting that no marked difference was found in the antagonistic actions of phenyl- and 3-hydroxyphenyltriethylammonium salts.

Mytolon.—After the work thus far reported had been completed and we had concluded that the compounds of Group E represented the most effective acetylcholine antagonists for the Venus heart, there appeared the paper by Luduena and Brown (1952). From the results of their study it was apparent that there were far more effective anti-acetylcholine compounds for the Venus heart than any we had thus far tested.

Certain amino- and quaternary ammonium alkylamino-benzoquinone derivatives were first shown to be active curare-like agents by Cavallito, Soria,

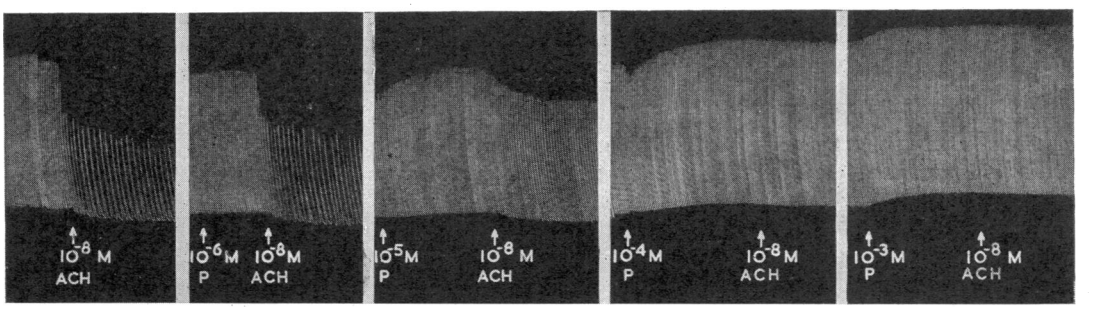

FIG. 4.—Record of the antagonism of the inhibitor action of acetylcholine (Ach) by phenyltriethylammonium bromide (P). Isolated ventricle of *Venus mercenaria*.

Banthine (β-diethylaminoethyl-9-xanthene carboxylate methobromide), a blocking agent at autonomic ganglia, was found to be less active, at the two lower concentrations, than the other compounds in Group E.

All four compounds markedly increased the amplitude of heart beat at one or more of the concentrations employed (Fig. 4). This was in contrast to the compounds of Group D. It was earlier suggested that this results from the blocking of endogenous acetylcholine, of which there is an appreciable amount in the heart. In order to obtain an estimate of the acetylcholine content of the Venus heart extractions and assays were made. Three determinations gave values equivalent to 0.47, 0.50, 0.7 μg. acetylcholine per gramme of wet weight of ventricle. For comparison the acetylcholine content of nerve ganglia of *Venus mercenaria* was found to be of the order of 5 μg./g. and that of adductor muscle 0.1 μg./g.

and Hoppe (1950). Of these compounds, the 2:5-bis(3′ - diethylaminopropylamino) - benzoquinone bis-benzyl chloride (WIN 2747 or mytolon chloride) was further studied by Hoppe (1950). This was shown to be the most effective curare-like member of the series. Luduena and Brown (1952) found mytolon an extremely active acetylcholine antagonist on the Venus heart. This is surprising, since it compares so favourably with decamethonium and *d*-tubocurarine as a neuromuscular blocking agent in vertebrates, yet neither of these has a significant acetylcholine-blocking action on the Venus heart except at a high concentration (10^{-3} M). We have now studied the effect of mytolon on the Venus heart and can affirm that it is in a distinctly different class from the other compounds we had earlier tested.

Using the same procedure employed with other agents, we find that 10^{-6} M mytolon completely blocks a dose of acetylcholine that would by itself

produce between 20 and 80% decrease in amplitude of heart beat. 10^{-8} M mytolon was found to block the action of such an amount of acetylcholine by about one-half.

Luduena and Brown had found that a concentration of 5.4×10^{-8} M mytolon reduced by 50% the inhibitory effect of a 5.5×10^{-8} M concentration of acetylcholine. They calculate that from two-thirds to three-fourths of the molecules of a given dose of acetylcholine are blocked by the same molar concentration of mytolon. We found, with different hearts, that the molecular blocking ratio of mytolon to acetylcholine varied from 2:1 to 5:1. We had earlier (Welsh and Taub, 1950) reported that from 1,500 to 3,000 molecules of tetraethylammonium were required to block one molecule of acetylcholine on the Venus heart. They found mytolon to be 980 times as effective as tetraethylammonium bromide on the Venus heart. Our quantitative data on mytolon, based on relatively few separate determinations, therefore agree very well with those of Luduena and Brown. They also found that maximal blocking activity required a benzyl and two ethyl groups on each of the nitrogens. The substitution of methyl for ethyl groups on the quaternary nitrogens (=WIN 3280) reduced the activity by one-third.

Discussion

The aim of this study was to acquire data from which we might make certain further deductions concerning the acetylcholine receptor substance of the Venus heart. We are still following the hypothesis put forth by one of us (Welsh, 1948) that acetylcholine receptor substances may be a family of enzymes for which acetylcholine serves as a coenzyme. While in many respects the pharmacology of the Venus heart resembles that of vertebrate autonomic ganglia it differs in some important respects. Most striking is the failure of pentamethonium to block acetylcholine on the Venus heart, whereas mytolon, which has little action at synapses in autonomic ganglia, is the most effective acetylcholine antagonist yet found for this organ.

We have shown earlier (Welsh and Taub, 1950, 1951) that the carbonyl group is an important combining group between acetylcholine and the receptor substance of the Venus heart. This was especially clear when the relative activities of 2-, 3-, and 4-ketoamyltrimethylammonium salts were determined. In the vertebrates these substances are primarily nicotine-like in their actions (Ing, Kordik, and Tudor Williams, 1952). Recently many workers (see Aeschlimann, editor, 1951), in accounting for the curare-like properties of a large number of new neuromuscular blocking agents, have placed much emphasis on the effectiveness of compounds containing two quaternary ammonium groups separated by a distance of approximately 14 Å. The belief, expressed or implied, is that this allows two ionic linkages to be formed between the nitrogens and correspondingly spaced acidic groups in the receptor molecule. A given number of bis-quaternary molecules could be more effective than twice as many corresponding half molecules, owing to the greater effectiveness of an anchor at both ends of the bis-compound.

It now seems logical to ask whether two or more carbonyl groups appropriately spaced in a molecule may not provide a double anchor keeping a molecule linked by more than one bond to points on a protein molecule where two molecules of acetylcholine might normally become attached. It has been suggested earlier in this paper that the acetylcholine blocking action of the barbiturates might be accounted for in this way. Decamethonium and *d*-tubocurarine are conspicuously poor acetylcholine antagonists on the Venus heart, as we have seen. In attempting to account for the far greater effectiveness of mytolon attention is drawn to the presence of two C=O groups in the benzoquinone ring, each of which might combine with a group in the receptor molecule. This would make possible two ionic linkages and two other bonds between mytolon and the receptor molecule of the Venus heart. No other feature would seem to account so adequately for the great superiority of mytolon as an antagonist to acetylcholine on the Venus heart. One might picture mytolon, and related compounds, lying more or less flat on the cell surface, attached by the two cationic groups (approx. 14 Å apart) to negatively charged groups in the receptor molecule and by the two carbonyl groups to approximately spaced OH- or NH_2-groups, as in semiquinone compounds.

Although this study adds only a small amount toward a fuller understanding of acetylcholine receptor substance, it confirms earlier views that the reaction between acetylcholine and cellular constituents is one in which molecular configuration is of great importance. The precise organization of groups (possibly amino-acid side-chains) within the receptor molecule may account in a large measure for the particular pharmacological pattern seen in a given organ or acetylcholine-sensitive region. Further attention should be paid to the possibility that such substances as the hydantoins and succinimides which have useful central nervous actions, as in the treatment of epilepsy,

are acting by blocking acetylcholine in the central nervous system.

Summary

1. The nature of the acetylcholine receptor substance of the heart of the lamellibranch mollusc, *Venus mercenaria*, was further explored by determining the relative activities of a number of acetylcholine antagonists.

2. Earlier work had indicated that the general pattern of response of this organ to acetylcholine analogues and to blocking agents was rather similar to that of vertebrate autonomic ganglia. Present results bring out important differences, e.g., the ineffectiveness of pentamethonium as an antagonist and the remarkable activity of " Mytolon."

3. The acetylcholine blocking action of certain substances having two carbonyl groups, such as the barbiturates and mytolon, leads to the suggestion that these groups may attach themselves simultaneously to the acetylcholine receptor molecule. This could account for the action of the large group of substances having two or more carbonyl groups that are useful as analgesics, hypnotics, and anticonvulsants.

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