THE INHIBITORY EFFECT OF STILBAMIDINE, CURARE AND RELATED COMPOUNDS AND ITS RELATIONSHIP TO THE ACTIVE GROUPS OF ACETYLCHOLINE ESTERASE. ACTION OF STILBAMIDINE UPON NERVE IMPULSE CONDUCTION*

by

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INTRODUCTION

Stilbamidine and the group of related chemotherapeutical agents were developed from the observation that synthaline (1:10-decane-diguanidine) has a certain curative effect on trypanosomiasis[1, 2]. It was assumed that the action of synthaline is based on its hypoglycemic effect on the host or its interference with the carbohydrate utilization by the parasite. However, no clearcut relationship exists between the parasitocidal and the hypoglycemic action of amidines[3]. Studies of the inhibitory effect on other enzyme systems[4] did not elucidate the mode of action of these drugs.

From the thorough studies by KING, LOURIE AND YORKE[5] it is evident that the presence of two polar groups containing basic nitrogen at the end of the molecule is a necessary requirement for a strong curative effect. This condition is strikingly similar to the requirement for curare action established by BARLOW AND ING[6] and by KIMURA and co-workers[7], *i.e.* the presence of two quaternary nitrogens at a distance of 13 to 15 Å. The corresponding distance for the stilbamidine molecule is about 14 Å. It appeared thus possible that both groups of compounds may have a similar mode of action and that elucidation of the underlying mechanism may reveal that the two apparently unrelated biological effects may be attributed to the same fundamental principle.

I. EFFECT OF STILBAMIDINE ON CONDUCTION

1. *Block of neuromuscular transmission*

Curare is known to block neuromuscular transmission, whereas conduction along nerve and muscle fiber is unimpaired. If a nerve-muscle-preparation is exposed to stilbamidine, the same effect may be obtained. Table I A shows the results observed with a preparation of the gastrocnemius muscle-sciatic nerve of frog (*Rana pipiens*),

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exposed to various concentrations of stilbamidine for various periods of time. The effect is reversed by washing with frog Ringer's solution; the time required is one to two days which is much longer than in the case of curare. The table shows also the concentrations and periods of time required for blocking neuromuscular transmission in the same preparation and under the same condition by *d*-tubocurarine chloride and by 1:10-decane bis(trimethylammonium)bromide (C10). However, the differences observed do not necessarily reflect different degrees of activity at the neuromuscular junction since the penetration of these compounds may vary considerably. This statement applies especially to a preparation in which the neuromuscular junction is protected by large amounts of tissue.

TABLE I

EFFECT OF STILBAMIDINE UPON NEUROMUSCULAR TRANSMISSION AND AXONAL CONDUCTION

In one series of experiments (A) gastrocnemius muscle-sciatic nerve preparations of frog were exposed to various concentrations of stilbamidine di-isethionate. At the time when neuromuscular transmission (n.m.t.) failed, conduction in nerve and muscle fiber was still present. In the second series (B), frog's sciatic nerve was exposed and conduction tested with the cathode ray oscillograph. For concentrations higher than 1 mg/ml equimolar quantity of sodium chloride was omitted from Ringer's solution. Control nerves kept for 8 days under the same conditions did not show impairment of conduction.

Concentration *M*	A	B
	Time required for block of	
	n.m.t.	conduction
a. Stilbamidine di-isethionate M.W. = 516		
$4 \cdot 10^{-2}$		45; 60 min
$2 \cdot 10^{-2}$		60; 120 min
$8 \cdot 10^{-3}$		60; 120 min
$2 \cdot 10^{-3}$	75 min	
$1 \cdot 10^{-3}$	2; 3 hours	10; 24 hours
$5 \cdot 10^{-4}$	5 hours	
$2 \cdot 10^{-4}$	4 hours	2–4 days
b. *d*-tubocurarine chloride M.W. = 327		
$8 \cdot 10^{-4}$	40 min	
$3 \cdot 10^{-4}$	60 min	
$1.5 \cdot 10^{-4}$	60– 70 min	
$6 \cdot 10^{-5}$	90–150 min	
c. 1:10-decane bis(trimethylammonium)bromide M.W. = 418		
$2.5 \cdot 10^{-3}$	30 min	
$5 \cdot 10^{-4}$	35 min	
$2.5 \cdot 10^{-4}$	75, 105 min	
$1.2 \cdot 10^{-4}$	240 min	

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2. Block of axonal conduction

In contrast to curare which has two methylated quaternary nitrogen atoms and is therefore unable to penetrate the neuronal surface membrane, in stilbamidine the cation is in equilibrium with the free base. The p_K was found to be 9.5. At p_H 7 less than 1% of the compound will be present as free base, but it is this form of the compound which is able to penetrate. It should then be expected that in contrast to curare stilbamidine may also block axonal conduction. This is confirmed by the data given in Table IB.

As may be seen from the figures, blocking of axonal conduction requires longer time of exposure and higher concentrations than the block of neuromuscular transmission. This is in agreement with previous observations in which other compounds were found to block both processes but at greatly different concentrations[8, 9]. In the case of stilbamidine this difference may be explained by the small fraction present as free base.

If exposure of the nerve to stilbamidine was interrupted before the action potential had disappeared and if the nerve was then transferred into frog Ringer's solution no late effect was observed. This makes unlikely a secondary transformation inside the nerve as responsible for the block. The long time required for the abolition of conduction may thus be attributed to the slow rate of penetration of the free base through the surface membranes.

In contrast to the reversibility of the action upon the neuromuscular transmission the block of conduction could not be reversed. This is not surprising in view of the low rate of penetration into the interior. Since the reversal of this process will require a still longer period of time and since it will thus take several days until the interior concentrations decrease to an ineffective level, secondary changes which will deteriorate the nerve must be expected.

II. EFFECT OF CURARE-LIKE COMPOUNDS ON ACETYLCHOLINE ESTERASE

Since curare and stilbamidine interfere with conduction, the question arises whether and in which way these compounds act upon the esterase-acetylcholine system. BARLOW AND ING[6] have found the inhibitory action of their aliphatic diquaternary ammonium

TABLE II

INHIBITION OF ACETYLCHOLINE ESTERASE BY MONO AND DIQUATERNARY AMMONIUM SALTS

The enzyme prepared from electric tissue (*Electrophorus electricus*) had an activity of 5 gm of acetylcholine split per ml per hour. It was used in a final dilution: 1 : 1000. The enzyme was incubated with the inhibitor for 30 min before the substrate was added (final concentration $4 \cdot 10^{-3}$ *M*). The activity was determined manometrically at 23° C. The *M* concentrations given are those which produce 50% inhibition (C_{50}).

Compound	C_{50}
a. Monoquaternary	
Tetramethylammonium iodide	$2.5 \cdot 10^{-2}$
b. Diquaternary	
d-tubocurarine chloride	$9 \cdot 10^{-4}$
Stilbamidine di-isethionate	$6.5 \cdot 10^{-5}$
1 : 10-decane bis(trimethylammonium)bromide	$2.5 \cdot 10^{-5}$

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salts upon the acetylcholine esterase of nucleus caudatus to be between 10^{-3} and 10^{-5} M. It appeared of interest to compare the inhibitory value of stilbamidine with that of curare and related compounds including compounds containing only one quaternary nitrogen. Table II gives the data obtained. It may be noted that the enzyme used is more than 1000 times as active per mg protein as the preparation obtained from nucleus caudatus. The diquaternary compounds are about 100 to 1000 times more effective as inhibitors than the monoquaternary salts.

III. RELATIONSHIP TO THE ACTIVE GROUPS OF THE ENZYME SURFACE

In previous papers[10-12] it was shown that the active surface of the enzyme protein contains two sites: (1) an anionic site which holds the positive center of substrate or inhibitor by electrostatic forces, (2) an esteratic site, combining with the polar C=O or C≡N-group of substrate or inhibitor. The quaternary ammonium salts can interact only with the anionic site. The explanation of the enormous increase in inhibitory strength from mono diquaternary compounds may be sought in terms of interactions with two anionic sites.

Since each enzyme molecule appears to have only one active surface[13], two situations appear possible: (a) a molecule of the di-quaternary compound may serve as a bridge between two enzyme molecules at their active surfaces. Such a combination would not explain the greater inhibitory strength of the diquaternary salts. (b) One enzyme molecule may contain more than one anionic site. The following considerations show that this picture can explain the superiority of the diquaternary compounds.

On the basis of two anionic sites in the active surface an approximate relationship between the C_{50} of tetramethylammonium and curare-like compounds can be derived. Combination of tetramethylammonium with one anionic site should have practically no effect on the reaction velocity since it essentially replaces normal enzyme molecules by others which have about half the substrate affinity. That this inhibitory effect is negligible may be seen from the following application of the MICHAELIS-MENTEN equation:

$$\frac{v}{v_{max}} = \frac{(S)}{K_M + (S)} \tag{1}$$

Since (S) is about 10 times as great as K_M, doubling K_M cannot appreciably affect the reaction velocity. Therefore, such a type of inhibitor must combine with both anionic sites and form an EI_2 compound in order to prevent the enzymatic reaction. These considerations apply only to relatively small quaternary ions, where the essential effect is confined to the occupation of the anionic site without interfering sterically with the esteratic site. Quaternary salts with larger substituents may sterically block the approach to the esteratic site and thus the EI complex may already be inactive.

The esteratic site has been previously shown to contain acid and basic groups[11]. Since the inhibitors of the type under investigation cannot interact with these groups the following equation is applicable.

$$\frac{v^\circ}{v} = 1 + \frac{(I)/K_I}{1 + (S)/K_M} \tag{2}$$

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For tetramethylammonium (A), enzyme = EH we have

$$EH + 2A \rightleftharpoons EHA_2$$

which introduces $(A)^2$ for (I) in equation (2).
For curare-like compounds (B)

$$EH + B \rightleftharpoons EHB' \rightleftharpoons EHB$$
(one site) (both sites)

at 50% inhibition

$$\frac{(A_{50})^2}{K_A} = \frac{(B_{50})}{K_B} \tag{3}$$

We now compare K_A and K_B. As a first approximation K_A and K_B are equal since they involve the formation of two bonds at the two anionic sites. But K_A also involves the approach of two positive ions to a distance of about 12 Å—introducing a factor of about 2 in the equilibrium constant.

$$K_A = 2\,K_B \tag{4}$$

$$B_{50} = \tfrac{1}{2}\,(A_{50})^2 \tag{5}$$

for tetramethylammonium $(A_{50}) = 0.025\,M$

$(B_{50}) = 3 \cdot 10^{-4}\,M$

which agrees with the experiment.

Thus the assumption of two anionic sites quantitatively explains the great superiority of curare-like compounds as compared to tetramethylammonium ion as inhibitors of acetylcholine esterase. The assumption of two anionic sites in the active surface may be used for analysing the relatively high affinity constant of the ES_2-compound of acetylcholine esterase in contrast to the corresponding very low affinity of unspecific esterases (*e.g.* liver esterase)[14, 15]. According to the picture of ZELLER AND BISSEGGOR[16], one substrate molecule in ES_2 is bound to a negative site and the second to the ester binding groups (termed "esteratic" groups in this series of papers). In their explanation the change

$$EHS + S \rightleftharpoons EHS_2$$

involves replacing a complex in which one acetylcholine molecule is bound at the anionic and esteratic sites by a supercomplex in which one molecule is bound at the anionic site and another molecule occupies the esteratic surface. The complex EHS is not the "critical" or transition state complex—it is a stable complex. It is improbable therefore that any considerable strain exists in this complex. The reaction would therefore involve only a small change in potential energy and since the entropy change is probably unfavorable, K_2 should have a value somewhat greater than 1. The experimental value of K_2 is, however, 0.032. The picture proposed becomes hereby questionable.

An explanation of the ES_2 complex may be attempted on the basis of the model with two anionic sites. One possibility is that one substrate molecule is attached to one anionic and the esteratic site and the second substrate molecule combines only with

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the second anionic site. K_2 should then be the same as for the dissociation of one tetramethylammonium ion from EA_2. If the dissociation of both positive ions were equivalent, the dissociation constant for one ion would be the square root of the constant for both, *i.e.* $\sqrt{K_A}$. This situation is analogous to the ionization of dicarboxylic acids. There is therefore involved a statistical factor of 4 and also a potential energy factor of about 2. Thus

$$K_2 = \sqrt{8K_A} = 0.025$$

which is close to the experimental value of 0.032 for K_2. This picture appears satisfactory from the point of view of the energetics involved. The question now arises why in such a complex the molecule held at the esteratic site is not hydrolyzed. The ES complex is not the critical complex but requires activation energy to be transformed into the activated complex ES*. The second substrate molecule may interfere, *e.g.* sterically, with the transition from ES to ES*. Further investigations are necessary to test these concepts.

DISCUSSION

The localization of the action of curare and related compounds at the neuromuscular junctions has long been considered as evidence for a peculiar mechanism at these foci. The fact that acetylcholine acts exclusively at the synapse was the basis of the hypothesis of chemical mediation of nerve impulses across synaptic junctions[17]. Recent investigations, however, have shown that this hypothesis has to be abandoned[18]. The release and removal of acetylcholine are essential events in the alterations of the axonal membrane necessary for generating the currents which propagate the impulse along axons and across synaptic junctions. The axonal surface is surrounded by a structural barrier impervious to quaternary ammonium salts, such as curare, acetylcholine and prostigmine[19]. Therefore, these compounds are effective only at synaptic junctions where the postsynaptic membrane appears less protected or not all. Whereas, *e.g.*, all those inhibitors of acetylcholine esterase which block conduction such as DFP and eserine have been demonstrated to be able to penetrate into the interior, prostigmine does not penetrate and has no effect upon the nerve action potential[20].

The observations with stilbamidine offer a new and striking support for the view that the special localization of effects obtained with quaternary ammonium salts is not evidence for a different mechanism of propagation at the site of action. The localization must be ascribed to the inability of these compounds to reach the active surface inside the axon. Since stilbamidine which has a typical "curare-like" action exists at neutral pH partly as free base, it can penetrate the barrier in contrast to curare, acetylcholine and prostigmine. However, the fraction of free base being less than one per cent, higher concentrations and much longer periods of exposure are required for blocking axonal conduction.

Lourie and co-workers[2] suggested that the trypanocidal activity of the amidines, guanidines, isothioureas and amines, which are all strong bases forming neutral salts, is a property of the free bases, liberated by partial hydrolysis. In the light of the present observations it appears possible that the basic form is responsible only for the penetration, whereas the cation is the biologically active form. The possibility that stilbamidine acts upon trypanosomes by interfering with the esterase-acetylcholine system

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can only be considered if the presence of the system is demonstrated in these monocellular organisms.

However, a still more important problem of the mode of action of stilbamidine, curare and related compounds is the question whether they act upon conduction and transmission by inhibiting the enzyme or by interacting with a second protein. This question is in principle the same as that of the physiological action of acetylcholine itself. It has been proposed that the ester changes some proteins in the membrane making it more permeable to ions, whereby the ionic concentration gradient becomes effective and supplies the energy for the electric current[19]. There are several reasons which make it likely that this protein is not the enzyme protein, but that there exists a second protein member of the system. The situation may be similar to that existing in the primary event of muscular contraction where the myosin which reacts with ATP has first been assumed to be identical with ATPase, whereas more recent studies have revealed that ATPase may be separated from myosin[21, 22]. No analogous protein constituent of the conductive membrane has as yet been isolated. The question whether the effect of stilbamidine, curare and related compounds upon conduction has to be attributed to the inhibition of acetylcholine esterase or to the action upon a second protein cannot be answered at present.

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SUMMARY

Stilbamidine like curare contains two cationic nitrogens at a distance of about 14 Å. In view of this structural similarity the action of stilbamidine upon neuromuscular transmission and upon the esterase-acetylcholine system has been investigated.

1. Stilbamidine like curare blocks neuromuscular transmission.

2. In contrast to curare stilbamidine also blocks axonal conduction. Since the free base of stilbamidine is present at neutral p_H, although in a small fraction, it may penetrate the axonal surface membranes which are impervious to quaternary ammonium salts.

3. The observations add further support for the assumption that the fundamental mechanism underlying axonal conduction and synaptic transmission is the same and that the apparent discrepancies must be attributed to secondary facts as *e.g.*, the inability of certain compounds to penetrate the axonal surface membranes.

4. Monoquaternary ammonium salts such as tetramethylammonium iodide have an inhibitory effect upon acetylcholine esterase. The effect is 100 to 1000 times greater in the case of the diquaternary salts.

5. The striking increase of inhibitory power of the diquaternary salts have been analyzed. It is interpreted as an indication that two anionic sites may be present in the active surface of acetylcholine esterase.

6. On the basis of this assumption the sharp optimum of the activity-pS curve of acetylcholine esterase caused by the ready formation of an ES_2-complex has been discussed.

RÉSUMÉ

La stilbamidine contient comme le curare deux azotes cationoïdes à une distance d'environ 14 Å. En vue de cette similitude structurale nous avons examiné l'action de la stilbamidine sur la transmission neuromusculaire et sur le système estérase-acétylcholine.

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1. La stilbamidine, de même que le curare, bloque la transmission neuromusculaire.

2. Contrairement au curare la stilbamidine bloque aussi la conduction le long du cordon nerveux. Comme à p_H neutre une partie de la stilbamidine se trouve sous forme de base libre, elle peut pénétrer à travers les membranes de la surface du cordon nerveux qui sont imperméables aux sels d'ammonium quaternaires.

3. Nos observations soutiennent l'hypothèse que le mécanisme fondamental de la conduction le long du cordon et de la transmission synaptique soit le même et que les divergences apparentes soient dues à des faits secondaires tels que l'impossibilité pour certains composés de pénétrer les membranes de la surface du cordon nerveux.

4. Des sels d'ammonium monoquaternaires tel que l'iodure de tétraméthylammonium inhibent l'acétylcholine-estérase. Cet effet est 100 à 1000 fois plus grand pour le cas des sels diquaternaires.

5. Nous avons analysé l'augmentation frappante du pouvoir inhibiteur des sels diquaternaires. Elle semble indiquer la présence de deux centres anionoïdes dans la surface active de l'acétylcholine-estérase.

6. En nous basant sur cette hypothèse nous avons discuté l'optimum aigu de la courbe activité-pS de l'acétylcholine-estérase causé par la formation rapide d'un complexe ES_4.

ZUSAMMENFASSUNG

Stilbamidin enthält wie Curare zwei kationische Stickstoffatome deren Abstand ungefähr 14 Å beträgt. Im Hinblick auf diese strukturelle Ähnlichkeit haben wir diese Wirkung von Stilbamidin auf die Übertragung Nerv → Muskel und auf das System Esterase-Acetylcholin untersucht.

1. Stilbamidin blockiert ebenso wie Curare die Übertragung Nerv → Muskel.

2. Zum Unterschied von Curare blockiert Stilbamidin auch die Übertragung langs des Nervenstranges. Da Stilbamidin bei neutralem p_H in geringer Menge als freie Base vorliegt, kann es durch die Membranen der Nervoberfläche eindringen, welche für quaternäre Ammoniumsalze undurchlässig sind.

3. Diese Beobachtungen unterstützen die Annahme, dass der grundlegende Mechanismus der Übertragung langs des Nervenstranges und der synaptischen Übertragung derselbe sei und dass die scheinbaren Unterschiede auf sekundäre Tatsachen, wie z.B. die Unmöglichkeit für gewisse Verbindungen die Membranen der Nervoberfläche zu durchdringen, zurückgeführt werden müssen.

4. Monoquaternäre Ammoniumsalze wie Tetramethylammoniumjodid hemmen die Acetylcholin-esterase. Für diquaternäre Salze ist die Wirkung 100 bis 1000 Mal stärker.

5. Die auffallende Zunahme der Hemmwirkung bei den diquaternären Salzen wurde untersucht. Sie wird als ein Anzeichen dafür angesehen, dass sich in der aktiven Oberfläche der Acetylcholin-esterase zwei anionische Zentren befinden.

6. Unter Zugrundelegung von dieser Annahme wurde das scharfe Optimum der Aktivität-pS Kurve der Acetylcholin-esterase erörtert, welches durch die rasche Bildung eines ES_2-Komplexes verursacht wird.

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