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SOME STRUCTURAL REQUIREMENTS UNDERLYING HOLOTHURIN A INTERACTIONS WITH SYNAPTIC CHEMORECEPTORS*

S. L. FRIESS, R. C. DURANT, J. D. CHANLEY† and T. MEZZETTI†

From the Physical Biochemistry Division of the Naval Medical Research Institute, Bethesda, Md., and the Chemistry Department, Mount Sinai Hospital, New York, N.Y., U.S.A.

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Abstract—Studies involving the interactions of the crystalline neurotoxin holothurin A with the rat phrenic nerve-diaphragm preparation have shown that an irreversible destruction of the indirectly elicited twitch response can be largely negated by preincubation of the preparation with tiny concentrations of the representative anticholinesterases physostigmine, neostigmine, or galanthamine. This protective action is afforded by concentrations of the anti-acetylcholinesterase agents distinctly below those required to elicit blockade of the tissue-localized esterase, but still in a potency sequence that is roughly proportional to their anticholinesterasic strengths *in vitro*. Further, the protection phenomenon itself is readily overridden by concentration increments of a given anti-acetylcholinesterase agent beyond its characteristic optimum value in the tissue bath, up to the point of complete disappearance of the effect.

Further experiments with purified subfractions of the total holothurin mixture have pointed to some stringent structural requirements of synaptic receptors susceptible to holothurin attack. Although a chromatographically pure component of the original mixture possesses essentially the same specific activity at the loci controlling nerve-stimulated twitch events as the mixture itself, and the loss of two sugar residues at carbon-3 affects the activity picture only moderately, the loss of the half-esterified sulfate function results in an abrupt decrease in blocking potency to virtually the zero level.

These results are interpreted in terms of possible mechanisms for interaction of holothurin moieties, degradation products, and competing agents at neuromuscular receptor loci.

THE toxic principle designated as holothurin A (Fig. 1) and isolated[1] as a crystalline mixture of steroidal glycosides from the Cuvierian gland of the Bahamian sea cucumber (*Actinopyga agassizi* Selenka) has previously been shown[2] to be a potent, irreversible inhibitor of neuromuscular function. In particular, quantitative studies of holothurin-receptor interactions at synaptic loci in the working phrenic nerve-diaphragm preparation (PN-D) from the rat have revealed a strict concentration dependence of actions that include[2] destruction of synaptic conduction and a biphasic contractural action on the striated muscle itself. The irreversibility of the first of these actions, as manifested for example in the rapid knockout of the nerve-stimulated twitch response from the muscle by a holothurin level of $1{\cdot}0 \times 10^{-4}$ M in the bathing

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† Mount Sinai Hospital.

medium and the failure of response regeneration on removal of the agent by washing, is a striking characteristic of the pattern of interaction of this agent with tissue receptors.

Accordingly, it was very germane to the issue of locating and characterizing the primary synaptic sites of attack and inactivation by holothurin that a recent set of observations[4] on competitive interactions of holothurin with the classical anticholinesterases physostigmine or neostigmine at the PN-D synapse included the key finding

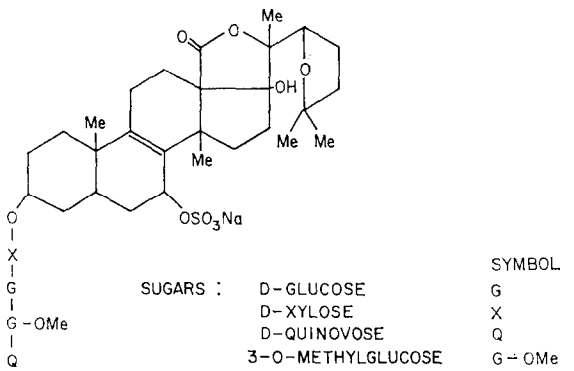


FIG. 1. Principal holothurin A component (provisional structure).[3]

that such interactions could eliminate the element of irreversibility inherent in normal holothurin attacks. In effect, physostigmine or neostigmine in carefully controlled concentration ratios with respect to holothurin, can protect the excitable synaptic loci against the destructive onslaught of holothurin$^-$ ions. The delicate concentration dependence of this protection phenomenon offered the possibility that further probing with anticholinesterasic agents of graded power and diverse mechanisms of esterase inhibition might unravel further detail on structure and function of the holothurin-sensitive chemoreceptor sites themselves.

Accordingly, the present work dealt initially with competition experiments involving protective interactions at the synapse between holothurin and the new, reversible-type anticholinesterase galanthamine (Fig. 2). This particular agent was chosen for study

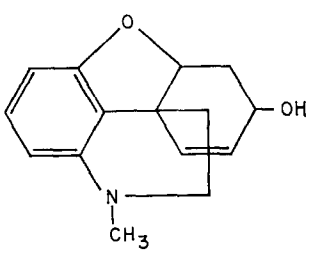


FIG. 2. Chemical structure of galanthamine.

because of its anti-acetylcholinesterase (AChE) level of potency,[5] lying intermediate between those of physostigmine and neostigmine, and also because of its structure which renders inoperable the acid-transferring AChE-inhibitory mechanism[6] attributed to the substituted carbamates physostigmine and neostigmine. The curiously cohesive results of this work, and their integration into a pattern covering the previously recorded findings on physostigmine and neostigmine protection, form the basis of

this communication. Also, some preliminary attention is directed to early findings on residual neuromuscular-blocking activities associated with components of the basic holothurin mixture, and their bearing on the nature of the chemoreceptor–glycoside interaction processes at the PN-D synapse.

EXPERIMENTAL

The holothurin A samples and their subfractions employed in this work were isolated, purified chromatographically, and characterized with respect to physicochemical properties in the laboratories of Drs. Chanley and Sobotka at the Mount Sinai Hospital in New York City. Descriptions of the formation and characterization of the degradation products will appear elsewhere.[7] All samples were stored dry and in the cold prior to use, with the exception of test amounts of crystalline holothurin deliberately kept at room temperature for extended time intervals to check thermal stability and retention of biological activity.

The rat PN-D preparation was executed uniformly and employed at pH 7·5 under isotonic recording conditions as previously described.[8] Twitch recording was continuous in time after the establishment of steady-state control twitch amplitudes, with indirect stimulus via the nerve (N-twitch) and direct stimulus via the muscle (M-twitch) spaced in pairs at a set interval of 3 sec between nerve and muscle stimuli and a 10-sec cycle on repetition.

The working stock solutions of holothurin, its degradation products, and galanthamine were prepared freshly before use from the same batches of bicarbonate Ringer's medium being used in the tissue bath, and were kept refrigerated prior to withdrawal of aliquots. In practice, after attaining uniform control twitch responses, pregassed aliquots of stock solutions of holothurin, galanthamine, or H^- fractions as required in a given experiment were added directly to the gassed tissue bath (of known total volume) at pH 7·5, and effects on twitch height and/or muscular contracture recorded continuously thereafter by the isotonic lever–kymograph system. In experiments involving competitive interactions of holothurin and galanthamine at the synapse, the agent galanthamine was added first at any desired concentration level, holothurin was next added at the $1{\cdot}0 \times 10^{-4}$ M level, and the preparation monitored to the point of extinction of the N-twitch, followed finally by the removal of the drugged bath medium and washing. Progressive measures of the effectiveness of a given pre-holothurin level of galanthamine in protecting against the normally *irreversible* blockade of N-twitch responses were then taken in the form of N-twitch amplitudes at times 5, 10, and 15 min after removal of the drugged media and washing, with stimuli at the standard rate being delivered steadily during the entire recovery period.

RESULTS

Holothurin-galanthamine interactions at synaptic receptors

The qualitative effects of the alkaloid galanthamine at pH 7·5 in negating the normally irreversible destruction of N-twitch capability in the rat PN-D preparation by holothurin are strikingly akin to those previously shown[4] with the acetylcholinesterase inhibitors physostigmine and neostigmine. At low concentrations (10^{-9} to 10^{-8} M) of galanthamine added to the bathing medium before the onslaught of holothurin at the $1{\cdot}0 \times 10^{-4}$ M level, recovery of the N-twitch on washing (after

proceeding to complete N-twitch blockade in the presence of both agents) is virtually negligible. Within the galanthamine concentration range of 10^{-8} to 10^{-7} M, however, the N-twitch protection phenomenon rises to a peak level, and then falls toward zero effect as the initial galanthamine concentration is further raised beyond the optimum level. This protective effect of galanthamine vs. holothurin appears to develop progressively in time after drug removal, as illustrated in Fig. 3, which shows

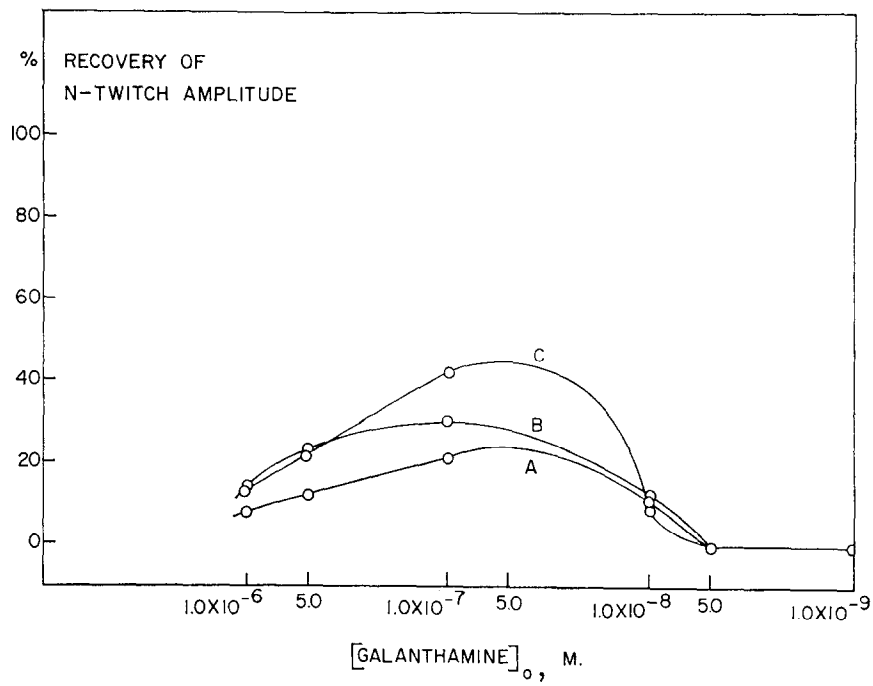


FIG. 3. Recovery of N-twitch toward control levels in the rat PN-D preparation, as a function of the incubating galanthamine concentration present in the bathing medium prior to the addition of $1{\cdot}0 \times 10^{-4}$ M holothurin. Curves A, B, and C respectively represent relative twitch responses at times 5, 10, and 15 min after washout of the drug mixtures.

that especially in the galanthamine concentration range 10^{-8} to 10^{-7} M, where the protective effect is most pronounced, the magnitude of the effect (absolute value of the ordinate) at any given prewash level of galanthamine increases measurably with the lapse of time (under constant electrical stimulus) after drug pair removal and washing. The optimum prewash concentration level of galanthamine for protection against synaptic kill by $1{\cdot}0 \times 10^{-4}$ M holothurin, roughly independent of the length of the postwash period, appears to be centered about 5×10^{-8} M. At this optimum level, the solution ratio of agonist to protector is $H : G = 1 \times 10^{-4} : 5 \times 10^{-8} = 2{,}000$.

The present result on the optimum concentration level of galanthamine for protection against the irreversible facets of holothurin-receptor interactions at the PN-D synapse, paired as a galanthamine-characterization parameter with an index of intrinsic anticholinesterasic strength of the alkaloid, enters into an interesting correlation with the corresponding potency parameters previously noted[4] for the protective agents physostigmine and neostigmine. This correlation is cast in the form

of a plot of C_{opt} for protection of the N-twitch (against $1{\cdot}0 \times 10^{-4}$ M holothurin) vs. C_{50} for inhibition of the AChE–ACh system (purified electric eel esterase, pH 7·4, 25·19°, $[ACh]_0 = 3{\cdot}33$ mM) at a fixed enzyme and optimum substrate level, and is shown below in Fig. 4. The C_{50} (concentration for 50% inhibition) indexes of esterase

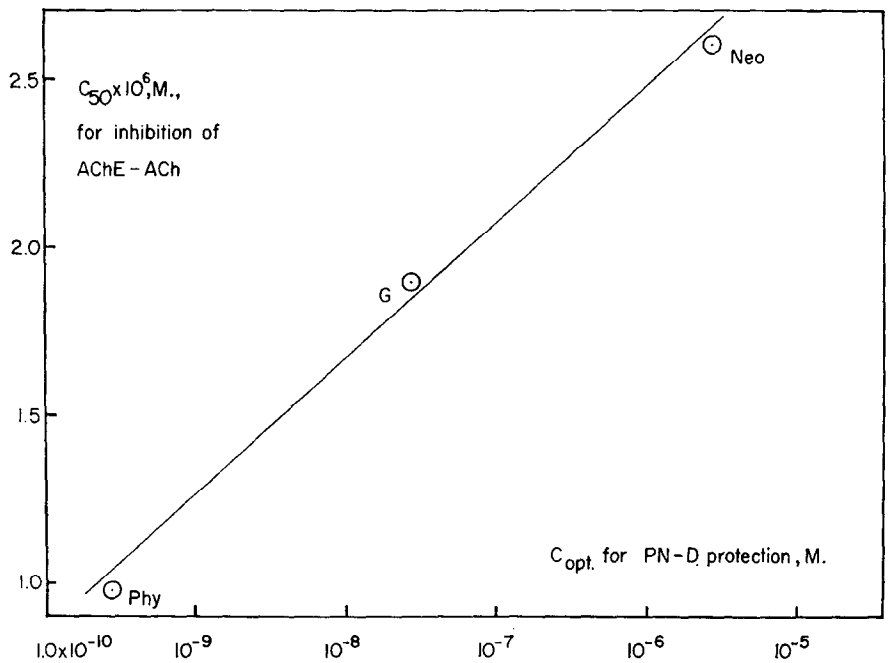


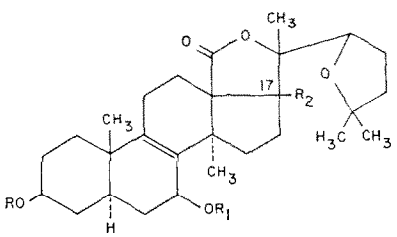
FIG. 4. Plot of C_{50} values for inhibition *in vitro* of the AChE–ACh system vs. C_{opt}. values for protection of the N-twitch (rat PN-D preparation) against irreversible inactivation by a $1{\cdot}0 \times 10^{-4}$ M level of holothurin. The points refer to physostigmine (Phy), galanthamine, (G), and neostigmine (Neo), with units of molarity on each axis.

inhibitory potency were selected for plotting rather than an enzyme-inhibitor dissociation constant parameter, in view of the recent findings of Wilson and Alexander[6] with phystostigmine and neostigmine, pointing to a multistep inhibitory mechanism as characteristic of their interactions with AChE.

From Fig. 4, it is seen that a roughly linear relationship exists between the parameters of effectiveness of the three agents phystostigmine, galanthamine, and neostigmine as protectants in warding off N-twitch destruction by holothurin, and the strength indexes of these same agents as inhibitors of the AChE–ACh system *in vitro*. The more potent the antagonism to the irreversible facets of holothurin-action (low. C_{opt} value), the more potent is the anticholinesterasic demonstration (low C_{50} value). It is of further interest to note that this parallelism exists independent of the mechanism of AChE inhibition employed by a given agent, since the substituted carbamates physostigmine and neostigmine function via an acid-transfer process[6] at the active enzymatic site, whereas galanthamine appears to be a reversible-type competitive[5] inhibitor.

Holothurin degradation products and their residual neuromuscular activities

The recent work of Chanley *et al.*,[7] involving fractionation of the glycosidic mixture constituting the crystalline toxin and isolation of individual components by means of thin-layer chromatography, has furnished an interesting set of molecular probes for further study of the minimal structural elements of the total holothurin composite required to elicit ligand–receptor responses at the neuromuscular synapse. At present, preliminary information is at hand with respect to residual blocking potencies at the synapse inherent in the fractions designated as Groups A, B, and C (Fig. 5), as



Group	R	R_1
A	X—G—G—OMe—Q	SO_3Na
B	X—Q	SO_3Na
C	X—G—Q	—

FIG. 5. Structural configuration of certain fractions of holothurin.

isolated by TLC procedures[7] from the holothurin glycosidic mixture. Group A constitutes the major fraction of holothurin A, contains all four of the original sugars, and furnishes on acid hydrolysis a mixture of aglycones in which an open-chain genin predominates. Group B contains only two of the original four sugars per molecule, and its aglycone is predominately a genin possessing the cyclic ether side chain. Group C contains three of the original sugars in glycosidic linkage, lacks the sulfate residue, and furnishes an aglycone mixture composed primarily of a 17-desoxygenin along with a lesser amount of the open-chain genin found in Group A.

The results of test of Groups A, B, and C as modifying agents in receptor-mediated responses of the rat PN-D preparation, with electrically initiated twitch responses monitored for amplitude under nearly zero muscle load, are depicted in Fig. 6. All agents were tested in duplicate on separate preparations at fixed initial concentrations of $1{\cdot}0 \times 10^{-4}$ M, based on average formula weights of the structures shown. Figure 6a demonstrates that Group A, a resolved component of the original holothurin A mixture, possesses all the neuromuscular activity spectrum inherent in the full mixture at equivalent total concentration; there is seen to occur a characteristic powerful contracture (upward trace displacement) of the muscle which is partially alleviated in time, a fairly rapid and irreversible knockout of the (indirect) N-twitch response, and a slower diminution in the height of the direct M-twitch response even after washing out the agent.

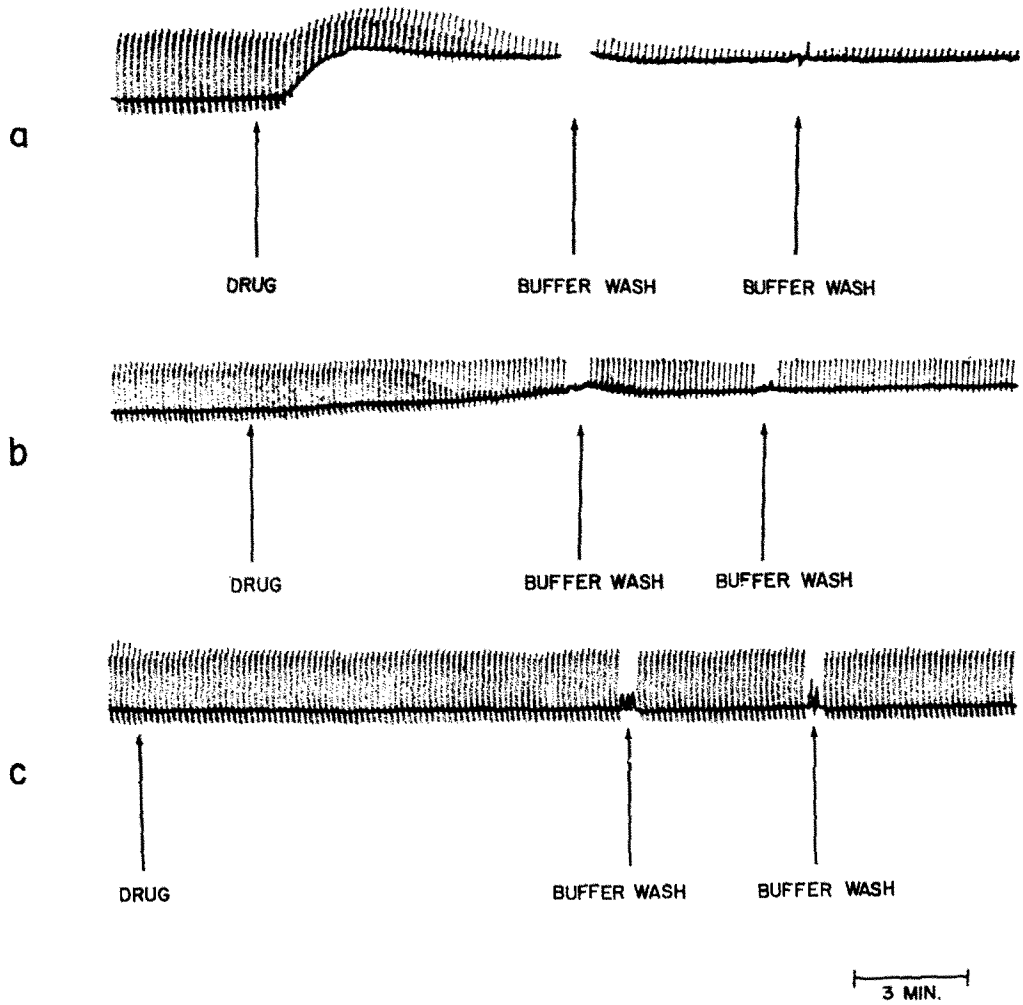


FIG. 6. Tracings of isotonic PN-D response patterns as a function of incubation with $1{\cdot}0 \times 10^{-4}$ M levels of Group A (a), Group B (b), and Group C (c). In each pair of twitch responses the N-stimulated event is recorded first.

In slight contrast with the activity pattern of Group A, the Group B fraction (Fig. 6b), which lacks two sugars of the normal complement of four found in holothurin moieties but is otherwise structurally intact, shows only a downward gradation in intensity of the major holothurin activity variables, but no radical alteration in their nature. The amplitude and time constant of the muscle contracture are altered in the direction of diminished drug potency, the N-twitch blockade is not quite so fast or irreversible as in the standard holothurin action pattern, and the potency in reduction of the M-twitch is also somewhat reduced.

But in very marked contrast to Group A or the holothurin mixture itself, in a structure resulting from degradation in which only one sugar *and the sulfate residue* are stripped from the active holothurin nucleus as in Group C (Fig. 6c), drastic

reductions in biological potency are observed. As seen in Fig. 6c, both N- and M-twitch amplitudes are virtually unaffected by the presence of Group C at the $1{\cdot}0 \times 10^{-4}$ M level. Further, the fast-rising phase of direct muscle contracture produced by holothurin is also missing in the feeble action pattern elicited from the PN-D preparation by Group C, leaving only a slowly developing contractural action that is still irreversible on washing and is reminiscent of the residual contracture produced by holothurin after decay of the initial fast phase.

Finally, it might be noted that the crystalline glycosidic mixture designated as holothurin and used freshly after column chromatographic purification displays a standard potency in its interactions with the PN-D at a reference incubating level of $1{\cdot}0 \times 10^{-4}$ M. But, on prolonged standing in the crystalline state at room temperature, a gradual deterioration in potency occurs (confirming an observation with respect to chemical characterization parameters made initially in the Mount Sinai laboratories). Specifically, two different samples of holothurin aged in separate experiments for time periods of 260 days and 147 days, respectively, required augmentations in concentration to final solution levels of $1{\cdot}99 \times 10^{-4}$ M and $1{\cdot}20 \times 10^{-4}$ M in order to achieve the initial standard responses from the PN-D preparation. These changes amount to average alterations in potency at the rate of 0·14 to 0·38% per day, as measured by requisite concentration increments.

DISCUSSION

The present results on parameters characterizing the functional interactions of synaptic receptors with selected subunits of the total assembly of holothurin structures add some interesting insights into the nature and spatial disposition of these tissue-localized receptors. First, at the structural level of intact holothurin species, the finding (Fig. 4) that anticholinesterases of diverse types are able to effect a specific protective action against the *irreversible* features of holothurin-knockout of the N-twitch, despite the transient depression of that twitch response to zero amplitude, offers a prime bearing on the character of the holothurin-denaturable sites. These protective effects of physostigmine, galanthamine, and neostigmine as anti-holothurin agents are in rough proportion to the AChE-inhibitory potencies of the three agents, and yet are clearly operable at incubating concentration levels that are in each case at least two orders of magnitude *lower* than those respective threshold concentrations at which typical synaptic anticholinesterasic actions are observed. Therefore, it would appear that each of the three agents exerts its protective actions either at synaptic loci, which are AChE-like in their binding properties, or at a special fraction of true AChE loci which is in itself too low a proportion of the total population of esterase sites to evoke an anti-AChE syndrome when filled. If these particular sites are given the designation $E_1, E_2, \ldots E_n$ for each synapse affected, then the next step in a reasonable rationale to account for the protection phenomenon would involve a binding of holothurin species at a set of contiguous sites $F_1, F_2, \ldots F_n$ with the following necessary characteristics: (1) binding of a holothurin moiety at an F site must lead to an increment of blockade of the N-twitch; and (2) E and F sites are in sufficiently proximate spatial contiguity that a protective species bound at E can prevent an *irreversible* receptor surface transformation from occurring at the instigation of holothurin bound to its site F. Further, it is probable that $E_1 \ldots E_n$ and $F_1 \ldots F_n$ are very near

neighbours at the synapse but not identical, in view of the previous finding[9] that holothurin species possess no intrinsic anticholinesterasic power *in vitro* and therefore would not logically be expected to show any appreciable binding affinity for AChE-like sites on tissue surfaces.

This rationale in explanation of the protective actions of physostigmine, galanthamine, and neostigmine is in accord with the observed proportionality to anti-AChE potencies, since binding of these species at $E_1 \ldots E_n$ sites should correlate linearly with bulk binding constants at catalytic sites on the enzyme. Further, the rationale imputes a very precise geometric relationship between respective *Ex* and *Fx* sites on the synaptic receptor surface governing the N-twitch response and, since the peak protection levels approach 75–90% at optimum concentrations of the agents cited, it is probable that a 1 : 1 ratio of protector-filled *E* sites and holothurin-filled *F* sites is nearly achievable. In such an event, the receptor interaction picture involving bound protector (Pro^+) and H^- ionic species might well be of the form of Fig. 7 in which

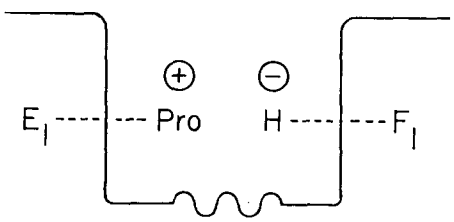


FIG. 7. Postulated interaction of holothurin with receptor.

the binding of negatively charged H^- species to *F* loci, which is normally accompanied by irreversible disruption (surface denaturation) of *F* functionality, is effectively offset by the simultaneous influence exerted by the $E \ldots Pro^+$ doublet. This influence could be directly attributable, in the geometry pictured, to electrostatic attraction between bound Pro^+ (quaternary, or monoprotonated at pH 7·4) and bound H^- ions leading to weak ion pairing and therefore a corresponding diminution in the strength of the $H^- \ldots F_1$ interaction presumptively underlying any surface alteration at F_1. Also, a sufficient degree of total receptor surface deformation to bring Pro^+ and H^- into proper juxtaposition (for the electrostatic attraction leading to the "sparing" action at F_1) could even be initiated by the attraction between Pro^+ and H^- during their initial acts of binding at the receptor surface; such an induction into proper conformation for maximal effect would be closely akin to the recent demonstrations[10] of Koshland *et al.* relating to the effects of surface-bound modifying agents in altering the catalytic topography of an enzyme into a configuration better suited for substrate binding, and ultimate chemical reaction.

Now, the simple ionic mechanism pictured for sparing of the *irreversible* facets of $H^- \ldots F$ interaction must obviously involve just enough contribution from $Pro^+ \ldots H^-$ pairing to negate the pervasive destruction of F_n loci by H^-, but not enough to cause disruption of the primary adsorption of H^- species to $F_1 \ldots F_n$ loci leading to the observed *reversible* blockade of the N-twitch in the presence of Pro^+ and H^-. Further, if this interacting couplet mechanism is to have general validity, it must also accommodate the further finding with all three agents physostigmine, galanthamine,

and neostigmine that the protection phenomenon is itself inhibited, as the initial concentration of a given "protector" in the bathing medium is further increased beyond its characteristic optimum level. Such an accommodation is indeed found in a logical extension of a previously postulated[4] rationale, based on the concept that excessive loading of $E_1 \ldots E_n$ sites by Pro^+ species leads to a deformation of the $E_1 \ldots E_n$-containing surface via the electrostatic repulsion of contiguously bound Pro^+ species (Fig. 8), and therefore a disruption in the E_n-to-F_n spatial order potentially responsible for the observed protection.

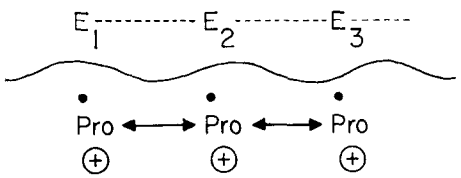


FIG. 8. Postulated scheme of inhibition of the protection phenomenon.

Next, the present findings with respect to the residual neuromuscular activities displayed by the fraction Group A of the total holothurin mixture, and the partial degradation products Groups B and C, add some interesting information on the structural requirements of the holothurin-denaturable synaptic sites. The fact that Group A on a weight basis displays essentially the same blockade and contractural properties as the total mixture from which it is separated, rather infers that all components of that mixture are virtually equivalent in specific activity at the PN-D synapse, with no inert diluents in significant quantity. Such an inference is also reasonable from the standpoint that the holothurin mixture originates from several genins of closely related structure. Further, the findings with Groups B and C that loss of two sugar residues from the glycosidic array at carbon-3 (Group B) of the steroid nucleus results in only a modest decrease in intensity of the major holothurin activities, whereas loss of the sulfate group and only one sugar (Group C) leads to a dramatic decrease in the ability to depress the N-twitch response, point to the vital role played by the negatively charged sulfate group of holothurin species in binding to synaptic sites controlling the N-twitch. It may well be that these sites are cationic in character (F sites), and respond with a large increment of binding power to the negative charge center in holothurin species, and with relatively smaller increments to variations in the basic molecular architecture such as the number of sugar residues in glycosidic linkage, the fine structure of the genin nucleus, and the configuration of the side chain attached to carbon-17.

This apparent essentiality of a sulfate residue attached to the holothurin-genin nucleus for maximal interaction potency at synaptic sites offers a possible clue to the molecular processes underlying the observed thermal instability of holothurin crystals. Viewed as a half-esterified sulfuric acid derivative, each holothurin species might well be susceptible to thermal ester cracking involving the elimination of hydrogen and $-OSO_3Na$ fragments, in a standard 1,2-elimination reaction characterized by its own specific rate constant, with the total of such processes for all holothurin species in the mixture adding up to a measurable alteration in total ester content in time, and

consequently to the observed rate of change in biological potency. This possibility has not been subjected to experimental test, as yet, for ultimate proof by isolation of the probable elimination product $HOSO_3Na$.

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