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ROLE OF THE SULPHATE CHARGE CENTER IN IRREVERSIBLE INTERACTIONS OF HOLOTHURIN A WITH CHEMORECEPTORS*

S. L. FRIESS, R. C. DURANT, J. D. CHANLEY† and F. J. FASH

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—Further studies with the crystalline neurotoxin holothurin A and the rat phrenic nerve-diaphragm preparation (PN-D) have shown that potency and degree of irreversibility of agonistic actions are sharply dependent on the presence of the charged sulfate residue in the toxin structure. With a selectively desulfated derivative (DeH) of the natural toxin (H) to establish relative indices of potency, it has been found that H is approximately one order of magnitude more powerful than DeH in direct contractural action on the muscle, and in blockade of the directly or indirectly elicited twitch response. But strikingly, the blocking actions of DeH are largely reversible on washing, in contrast with the clear irreversibility with which the parent toxin H destroys excitability in the preparation. Additionally, the uncharged species DeH at the $1\text{–}5 \times 10^{-5}$ M level is able to afford significant elements of protection against *irreversible* destruction of twitch response normally evoked in the PN-D by the charged anion H at a 1×10^{-4} M level in the bathing medium.

In the intact mouse studied for acute toxic response on i.v. administration of DeH or H, the syndromes leading to lethality are quite similar for the two agents, except for their relative time courses. Neutral DeH is characterized by an extensive lag time in onset of its peripheral and central effects and a very prolonged interval of tissue interactions, in contrast with the very much accelerated rate at which anionic H sweeps through the same spectrum of toxic signs.

These findings are discussed in terms of the effects that anionic charge, imparted to H by virtue of its half-esterified sulfate function, may have on the strength and progress of interactions with receptor loci in neuromuscular and other tissues.

A PREVIOUS report[1] in this series dealt with selected bioactivities of the toxic principle elaborated by the Bahamian sea cucumber *Actinopyga agassizi* Selenka and designated[2] as holothurin A, with special emphasis on the irreversible facets of response blockade it produces at the cholinergic neuromuscular junction. Particular note was taken of the ability of tiny quantities of classical anticholinesterases (e.g. physostigmine, neostigmine) to protect against the *irreversible* aspects of junctional blockade induced

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† Mount Sinai Hospital.

by incubation with purified holothurin (H), as seen in protector concentration vs. protective response relationships characterized by optimum concentration levels for maximum effect, and a progressive loss of the phenomenon at supraoptimum protector concentrations. This occurrence of protection was linked to the concept that H may exert its irreversible inactivation of junctional receptor loci by virtue of its anionic charge character, with the sparing action exerted by cationic protectors (Pro^+) resulting from electrostatic interaction of charged Pro^+ and H^- entities bound in close contiguity at the receptor surface.

Now, very recent work[3] has shown that the anionic nature of H stems from the presence of a half-esterified sulfate residue $-OSO_3^-Na^+$ (Fig. 1) in major components of the crystalline mixture of steroidal glycosides constituting the natural toxin, in

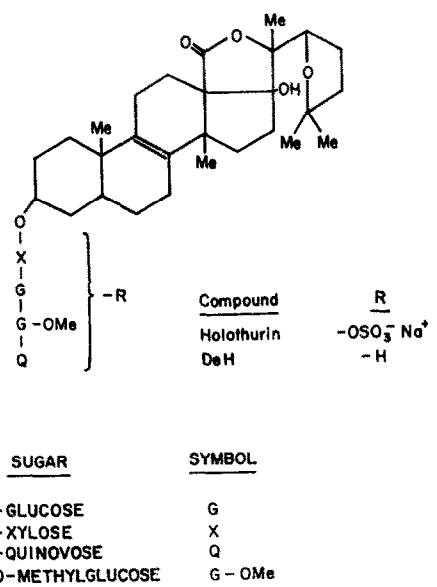

FIG. 1. Provisional structures of the major components of holothurin (H) and desulfated holothurin (DeH).

probable positional attachment to a sugar residue, as indicated in Fig. 1. If this anionic charge center in the saponin molecule is indeed responsible in major part for the facet of irreversibility in blocking interactions at the junction, it should be feasible to generate a direct chemical test of the anionic requirement underlying irreversibility in attack by comparing the actions of H^- ions with those of the derivative in which desulfation has selectively removed only the anionic residue. Such a test forms the basis of the present communication, which was launched from the important experimental finding[3] that holothurin ions can be cleanly desulfated in 0·2 M methanolic HCl at 37°, yielding the neutral crystalline product 'desulfated holothurin' (DeH) in which the glycoside–genin bonds are undisturbed.

Additionally, the close structural relationship between H and DeH has also led to meaningful study of DeH as an antagonist of the irreversible actions of H at the cholinergic neuromuscular junction.

EXPERIMENTAL

Freshly chromatographed samples of holothurin A and desulfated holothurin, as prepared[3] in the Mount Sinai Hospital laboratories by Chanley and colleagues, were stored under desiccation in the cold prior to use in pharmacologic experiments. Stock solutions were kept refrigerated in the intervals between successive usage.

The rat PN-D preparation was executed uniformly and employed at pH 7·5 under isotonic recording conditions, as previously described.[4] Twitch recording was continuous 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.

Intravenous injections of DeH-saline solutions into mice followed protocols previously established[5] for acute toxicity studies employing H solutions and the same mouse strain. Solution concentrations were based on a reference injection of 0·08 ml total volume in an 18-g mouse. Male animals in the body weight range 18–22 g were used exclusively for this phase of the study. An observation interval of 12–15 hr for tabulations of lethal effects was established for DeH acting in mice, in view of its extended interval of repetitive activity.

Finally, the experiments in which DeH was studied as a potential antagonist of the irreversible aspects of PN-D inactivation produced by H followed precisely the patterns previously established[1] in probing the anti-H protection afforded by preincubation of the preparation with trace amounts of anticholinesterases. Specifically, nonagonistic concentrations of DeH in the range 1×10^{-6} to 5×10^{-5} M were preincubated with individual PN-D preparations to constancy of N- and M-twitch response amplitudes, followed by challenge in the same bath with H at the usually lethal concentration of 1×10^{-4} M. After reduction of N-twitch amplitude to near-zero level, and washing of the preparation with pre-gassed buffer medium, observations were made of the extent of recovery of N- and M-twitch responses as a function of time.

RESULTS

Comparative actions of DeH and H on neuromuscular preparations

A comparison study of the complex action spectrum displayed by DeH with respect to responses of the rat PN-D preparation, against the backdrop of the corresponding reference responses elicited by the parent agent H, proved highly instructive. First, a representative set of tracings illustrating these agonistic actions, as exerted by DeH at the $1{\cdot}0 \times 10^{-3}$ M incubation concentration and by H at the $1{\cdot}0 \times 10^{-4}$ M reference level, is shown in Fig. 2. As seen in Fig. 2, the actions of DeH largely mimic those of H, but at concentrations that must be approximately tenfold higher than those required by H for effects of nearly equal intensity. At $1{\cdot}0 \times 10^{-3}$ M in the top tracing, DeH exerts a powerful contractural action on the diaphragm (upward trace displacement), which is partially alleviated in time after washing and agent removal, coupled with a rapid blockade of both N- and M-twitch responses (shown in pairs), which is at least partially reversed with time after the washing procedure. This facet of reversibility in the DeH action pattern contrasts strongly with the corresponding phase of H interactions with the PN-D preparation at a 10-fold lower incubation level, as shown in the lower tracing of Fig. 2. The contractural action and twitch-blockade processes are qualitatively similar to those of DeH, but the twitch *recovery*

process on washing is virtually non-existent for a preparation incubated to the point of N-twitch knockout with a $1{\cdot}0 \times 10^{-4}$ M level of H.

The actions of DeH on the PN-D preparation have now been surveyed quantitatively over the incubation concentration range, $1{\cdot}0 \times 10^{-4}$ to $1{\cdot}0 \times 10^{-3}$ M, in which it approaches maximum effect on responses. For comparison purposes, the corresponding measures of potency of H (in its own bioeffective range of $1{\cdot}0 \times 10^{-5}$ to $1{\cdot}0 \times 10^{-4}$ M) have also been freshly obtained with preparations from the same animal stock. The results of this survey are presented in Table 1 in terms of parameters related to direct contractural actions on the muscle, the relative potencies of these agents in blockade of N- and M-twitch responses, and the extent of reversal of effects on washing.

It is seen from Table 1 that very considerable differences between intrinsic activities of H and DeH on this preparation are found as a general rule, with a clear ordering of potency in the sequence H > DeH on comparing each set of strength indices. Specifically, in assaying the relative potencies of the two agents to evoke the direct contractural action on the diaphragm muscle, comparisons are made in terms of: (1) the slope of the curve that relates mean rate of rise of the initial contracture segment of the response tracing to agonist concentration; (2) the peak contracture amplitude evoked at a fixed agonist level, relative to control M-twitch amplitude of the test preparation; and finally (3) the slope of the curve that relates peak contracture levels observed to agonist concentration. For all comparisons encompassed by these three indices of contractural potency, it is of interest that H surpasses DeH in strength by a rough factor of 5.

TABLE 1. COMPARATIVE POTENCIES OF HOLOTHURIN A AND DESULFATED HOLOTHURIN ON THE RAT PHRENIC NERVE-DIAPHRAGM PREPARATION

Response parameter	Holothurin A	Desulfated holothurin	Potency ratio (H/DeH)
Contractural action on muscle			
Mean rate of rise of initial contracture; slope of rate vs. agent concentration curve (mm/sec/M)	$14{\cdot}4 \pm 4{\cdot}6 \times 10^{2}$	$2{\cdot}36 \pm 0{\cdot}24 \times 10^{2}$	$6{\cdot}1 \pm 1{\cdot}8$
Maximum contracture level at fixed $[agent]_0 = 1{\cdot}0 \times 10^{-4}$ M (% of reference)*	150 ± 32	32 ± 2	$4{\cdot}7 \pm 0{\cdot}7$
Slope of peak contracture* vs. agent concentraton curve (%/M)	$8{\cdot}9 \pm 1{\cdot}5 \times 10^{5}$	$1{\cdot}79 \pm 0{\cdot}01 \times 10^{5}$	$5{\cdot}0 \pm 0{\cdot}8$
Twitch response blockade†			
N-twitch	partial, $1\text{–}3 \times 10^{-5}$ M	partial, up to 5×10^{-4} M	
Amplitude depression‡	full, $5 \times 10^{-5}\text{–}1 \times 10^{-4}$ M	full, $5 \times 10^{-4}\text{–}1 \times 10^{-3}$ M	10
Reversibility on wash§	poor	moderate	
M-twitch	partial, $1\text{–}5 \times 10^{-5}$ M	partial, $1\text{–}5 \times 10^{-4}$ M	
Amplitude depression‡	full, 1×10^{-4} M	full, 1×10^{-3} M	10
Reversibility on wash§	poor–moderate	good	

* Contracture magnitude relative to reference [pre-agent] M-twitch amplitude = 100%.
† Isotonic recording; N-twitch elicited by stimulus to nerve, M-twitch by direct stimulus to muscle.
‡ Depresssion of twitch amplitude: partial, >1 mm residual; full, <1 mm residual.
§ Recovery of twitch amplitude: poor, 0–<25%; moderate, ~25%; good, >25–50%.

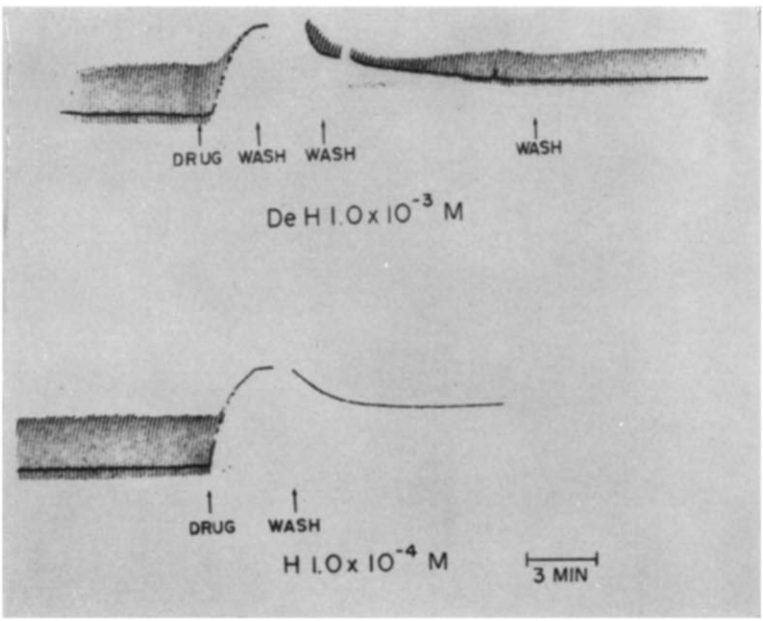

FIG. 2. Tracings of the effects of DeH ($1{\cdot}0 \times 10^{-3}$ M) and H ($1{\cdot}0 \times 10^{-4}$ M) on the rat PN-D preparation. Responses are isotonic and are recorded in pairs in the sequence N-twitch, M-twitch. Washing with pH 7·5 buffer is indicated by arrow points on the abscissa time scale.

facing page 1620

Next, the data of Table 1 on relative potencies of H and DeH in depressing twitch response via either direct or indirect stimulus pathways reveal a clear differentiation in inhibitory power in favor of H by one order of magnitude. H is highly effective in the concentration range 1×10^{-5} to 1×10^{-4} M, whereas DeH requires 10-fold higher concentrations in the range 1×10^{-4} to 1×10^{-3} M in order to achieve blockade effects of comparable magnitude. Further, whereas H produces twitch blockade that is relatively poorly reversible on washing and removal of the agent, the recovery from the effects of DeH on washing is moderately good by comparison.

Protection experiments involving H and DeH competition

In view of the observations (Table 1) that H exceeds DeH in potency of actions on the rat PN-D preparations by factors ranging from 5 to 10 and that the qualitative features of action of the two agents are quite similar in all aspects except the degree of irreversibility, it appeared feasible to test the possibility of simultaneous DeH and H interactions at the neuromuscular junction leading to protection by DeH against the irreversible aspects of twitch blockade effected by H. To this end, a series of experiments was executed in which varying concentrations of DeH (below its dosage threshold for significant degrees of blocking action) were preincubated briefly with individual working PN-D preparations, followed by addition to each tissue bath of sufficient H to achieve its $1{\cdot}0 \times 10^{-4}$ M level of high blockade potency. Then, observations were made on response behavior under combined action of the pair of agents, followed by subsequent observation of responses after agent removal by washing. The results of this series of competition-protection experiments are summarized in Table 2. The data of Table 2 clearly indicate that DeH at tissue bath concentrations in the range $1{\cdot}0\text{–}5{\cdot}0 \times 10^{-5}$ M is able to afford significant elements of protection against the irreversible onslaught of H at the $1{\cdot}0 \times 10^{-4}$ M level. Even when blockade of twitch response under combined DeH + H action has progressed to the point of diminution of the indirectly elicited (N-) twitch response to <1 mm residual amplitude, washing restored a slight degree (~20 percent) of isotonic response

TABLE 2. PROTECTION BY DESULFATED HOLOTHURIN AGAINST IRREVERSIBLE ACTIONS OF HOLOTHURIN ON THE RAT PN-D PREPARATION

		Blockade		Recovery			
		Relative twitch amplitude at wash time*		Relative twitch amplitude post wash†			
H (M)	DeH (M)	N-twitch (%)	M-twitch (%)	N-twitch 5 min (%)	N-twitch 10 min (%)	M-twitch 5 min (%)	M-twitch 10 min (%)
$1{\cdot}0 \times 10^{-4}$	0	0‡	12 ± 10	0	0	4 ± 4	0
$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-6}$	2‡	17	0	0	9	4
$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-5}$	5 ± 2‡	14 ± 1	11 ± 9	20 ± 5	19 ± 7	25 ± 2
$1{\cdot}0 \times 10^{-4}$	$5{\cdot}0 \times 10^{-5}$	8‡	51	30	58	65	70
$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-5}$	42 ± 4§	43 ± 3§	37 ± 9	37 ± 9	39 ± 9	38 ± 8

* As a per cent of pre-H control twitch amplitude (isotonic recording).
† As a per cent of pre-H control twitch amplitude (isotonic recording), at indicated times post wash with Ringer medium.
‡ Blockade allowed to progress till N-twitch amplitude reduced to <1 mm prior to wash.
§ Washed at this extent of blockade, observed after about 5-min incubation with H + DeH.

when DeH was initially present at the $1{\cdot}0 \times 10^{-5}$ M level, and resulted in an even greater restoration (30–70 percent) when the initial protecting level of DeH was $5{\cdot}0 \times 10^{-5}$ M.

It is of further interest (Table 2) that the depth of depression of N- and M-twitch amplitudes on incubation of the PN-D preparation with the binary mixture of $1{\cdot}0 \times 10^{-5}$ M DeH + $1{\cdot}0 \times 10^{-4}$ M H is not a unique factor in regulating the degree of recovery in twitch response met later on washing. As seen in the final entry in this table, when this particular mixture of agents is held in contact with the preparation until twitch heights have fallen to about 40 per cent of control, followed by wash, increments of recovery *above this level* are no better than in the experiments in which N- and M-twitch amplitudes were allowed to fall to 5–10 percent of control levels prior to wash and removal of the glycosidic mixture. But, the absolute values of postwash twitch-response amplitudes attainable are seen to be significantly higher in those experiments in which the combined actions of DeH + H were not allowed to progress to near extinction of twitch responses prior to agent removal.

Intravenous toxicities in mice

The clear differentiation in properties of H and DeH acting as blocking agents on a peripheral neuromuscular tissue led to the need for comparative data on actions of these agents *in vivo*, with special reference to the acute toxic syndrome that follows intravenous administration of graded dosages of each agent. This information, which was already available[5] for H acting i.v. in the NMRI strain of white mice, has been freshly verified with the present pool of animals, and can be briefly summarized by citation of an acute LD_{50} value of 9 mg/kg body wt., and a very rapid onset of toxic signs that culminate either in death within a few minutes or in full recovery within an hour. Major toxic signs included: tautness of muscles, thrashing convulsions, whole-body tremors and contortions, limbs splayed and stiff, back humped, and complex impairment of respiratory processes.

The corresponding toxicity information has now been obtained for the desulfated product DeH administered i.v. to the same mouse strain, with rather interesting results. First, the acute LD_{50} value itself ($\pm$ S.D.) for DeH was found to be $11{\cdot}0 \pm 1{\cdot}2$ mg/kg, as evaluated from dosage vs. per cent survivor relationships in which the survivor counts were made 12–15 hr post injection. Reduced to molar units, the comparative figures for acute i.v. toxicities of H and DeH in the mouse are, respectively, 7·8 μmole/kg and $10{\cdot}5 \pm 1{\cdot}1$ μmole/kg, which points to virtual equivalence in lethal potency. However, more striking than the numerical comparison itself is the dramatic extension of the interval required for DeH to develop serious toxic signs post injection, as compared with H, and also the very prolonged period during which DeH exerts its polyphasic action. For example, at DeH dosages slightly above the LD_{50} level, initial excitation immediately after injection is followed by long periods (many minutes to several hours) of torpor, and ultimately by onset of a complex sequence of signs including tonic and clonic convulsions, tail lift and curl, paralysis and splaying of rear limbs, transient respiratory blockade, and recovery to the torpor state for survivors. Elements of this toxic syndrome are repeated spontaneously in surviving animals at irregular intervals during total observation periods up to 15 hr, followed by apparent full recovery.

The behavior noted above for DeH in the NMRI strain of white mice differs

markedly from that of H, chiefly in respect to lag time in onset and in the extended duration of the action, rather than in any qualitative features of the toxic action pattern itself.

DISCUSSION

The present body of results on relative potencies of the negatively charged H^- species and its neutral desulfated derivative DeH, acting as agonists or competitors or both in peripheral neuromuscular tissues and in intact mice, furnishes some instructive insights on receptor interactions with these steroidal glycosides. First, the comparative actions of H^- and DeH in blockade of the rat PN-D preparation point to a very significant role of H^- negative charge character in processes that govern the intensity of responses resulting from holothurin interactions at cholinergic receptors, and in regulating the degree of irreversibility of those processes that lead to loss of excitability. In the excised PN-D preparation, H^- is of the order of 5–10 times more potent than DeH in its direct contractural power on the muscle and in its ability to block twitch response to either direct or indirect stimulation. Further, the charged species H^- displays a far higher degree of irreversibility (see Fig. 2) in its blockade of contractility in the preparation than does its neutral analog DeH. These observations, coupled with the finding that sufficiently high levels of neutral DeH can protect the PN-D preparation from irreversible destruction of excitability induced by the onslaught of charged H^- species, are compatible with a model of glycoside–chemoreceptor interactions at the myoneural junction in which both H^- and DeH molecules can reach and trigger the same receptor sites to overt response, but with differing degrees of efficacy. In this model, H^- and DeH can compete for binding at a limited number of sites per synaptic unit, with each act of DeH binding amounting to an essentially reversible association-blocking process, but with the binding of H^- species to receptor culminating in destruction of tissue excitability.

This model can provide a comprehensive (but not necessarily unique) framework for correlation of all available observations on holothurin blocking interactions with the PN-D preparation. Specifically, in previously employed[1] notation, either H^- or DeH species would interact at F_1 sites in essentially irreversible and reversible blocking modes, respectively, and competition between the two species could result in the DeH sparing actions observed. Further, the nearness of F_1 sites to acetylcholinesterase-like E_1 sites in the model could account[1] for the observation that (+)-charged anticholinesterases of the type Pro^+, bound to E_1 loci and interacting electrostatically with adjacent H^- species bound at F_1 sites, protect against the irreversible facets of inactivation normally effected by H^- at its binding points.

Now, a major question resides in the nature of possible sitic interactions at F_1 which could afford irreversible blockade on binding of the anionic agent H^-, but reversible blocking action at diminished efficiency under the influence of the neutral agent DeH. Inspection of molecular models of the two species as per the structures of Fig. 1 (assembled from Corey-Pauling-Koltun atomic models) offers at least some beginning clues to the structural features underlying this dual differentiation in potency and reversibility of agonistic action at the cholinergic neuromuscular junction. First, both H^- and DeH contain as elements in common a neutral steroid moiety that is structurally rigid and roughly cylindrical, and a flexible carbohydrate chain of four sugars pendant from an end face of the cylinder. Further, and unexpectedly, a discrete

element of cylindrical surface in the steroid moiety, as pictured schematically in Fig. 3, turns out to possess relatively high surface density in neighboring oxygen atoms, brought about by spatial proximity in the model of the two oxygen atoms from the lactone ring, the tertiary hydroxyl group, and the oxygen atom in the tetrahydrofuran ring. But H^- differs from DeH significantly in its possession of a formal locus of negative charge ($-OSO_3^-$) at some point in the sugar chain.[3]

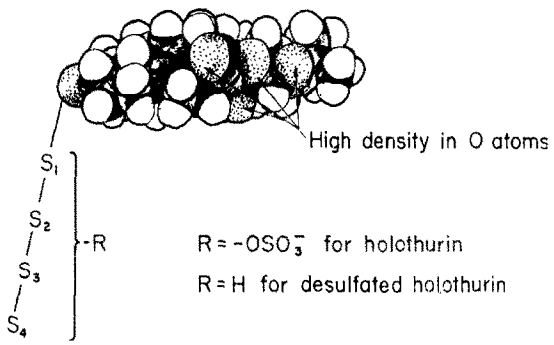

FIG. 3. Steric and structural features of H^- and DeH molecular species. The steroid moiety is based on Corey-Pauling-Koltun atomic models.

Accordingly, it becomes attractive to consider that both species H^- and DeH can bind to F_1 sites by virtue of at least two major structural elements held in common, i.e. by interactions involving the patch of high unshared electron density (high oxygen atom localization) in the steroid moiety, and by the extended polysaccharide chain. The former would lend itself to electrostatic binding interaction with electrophilic groups at the F_1 surface (e.g. charged groups such as $-NR_3^+$), and the latter might well provide affinity to junctional membrane structure that is polysaccharidic (or mucopolysaccharidic) in character. In this bivalent framework of glycoside–site interactions, the imposition of a single charging residue $-OSO_3^-$ into the sugar chain of DeH to produce H^- could lead to such disruptive interactions with tissue as: (1) interference with effective surface binding by negative charge repulsion and possible attendant alteration of receptor surface configuration, if the sugar chain of H^- binds at a polynegative, acidic mucopolysaccharide unit on the surface; or (2) perturbation of polysaccharide–lipid phase relationships in a multicomponent postsynaptic membrane, presumably by virtue of selective solubility of the charged sugar chain in a polysaccharide element of an oriented array[6] of layers in nerve-like membranes. If such interactions are real, they should also occur (although perhaps at lower levels of specificity or efficacy) on PN-D attack by simpler model compounds prepared by sulfation of plant-derived steroidal glycosides, such as saponin or digitonin. Model substances of this type are in preparation for future test.

Some importance may also be attached to certain comparative aspects of H^- and DeH action spectra in the intact mouse. Particularly striking is the contrast between H^- and DeH with respect to time course of their respective toxic syndromes; DeH shows very marked delay in onset of signs and a very marked prolongation of the convulsive, paralytic state, as compared with rapidly acting H^-. Since these agents

produce effects in the mouse that are otherwise qualitatively identical, and since they are virtually equipotent, it is quite likely that they are exerting their primary actions at particular central and peripheral receptors which are either very similar or identical for the two glycosides. In this event, the differentiation in time courses for the two agents could well reflect significant differences in total residence times at receptor loci, as influenced by anionic character of H^- vs. neutrality of DeH. The anion H^- could conceivably share in the relatively facile permeability that characterizes the passage of small ions[7] (e.g. Cl^-, Br^-) through membrane "pores" and thereby arrive at central nervous system and peripheral receptor loci with a speed characterizing the observed fast rate of onslaught and kill in the mouse. In contrast, neutral DeH might well experience greater difficulty and time lag in membrane permeation to susceptible receptor sites, perhaps by virtue of selective compatibility with and sequestration in neutral lipid or polysaccharide phases of tissues, and display accordingly the observed lag period in evolving its toxic syndrome in the mouse. And of course, the possibility also exists that DeH may undergo a time-consuming metabolic alteration in the mouse, e.g. sulfation, before it becomes biologically active at the CNS level.

Finally, it is worthy of note that the greater potency and lethality of H^- relative to DeH in the rat PN-D preparation may be related to charge in a rather unique way. A latent "pore" in the postsynaptic membrane underlying a given nerve terminal, as detailed in a model suggested by Waser,[8] could well be the locus of F_1 sites responsive to H^- or DeH. In this event, penetration of such a pore by directed head-first entry of the steroid moiety of H^- could conceivably be controlled in depth by electrostatic interaction of the $-OSO_3^-$ charge center on the glycosidic tail with surface-localized $-NR_3^+$ groups in membrane phospholipids. Such an interaction could control the depth of penetration and time course of contact with F_1 sites permitted to H^-, but of course would be inoperative in the event of 'pore' penetration by the weaker neutral species DeH.

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