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Marine toxins

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Throughout the world, marine toxins cause a variety of acute, subchronic, and chronic diseases in humans, as well as diseases in other mammals, fish, and birds (Table 1) (Hughes and Merson 1976; Southcott 1979; Baden 1983, ILO 1984; Sakamoto et al. 1987; Halstead 1988). The diseases in humans range from acute neurologic diseases, such as ciguatera and paralytic shellfish poisoning, to chronic dementia, as reported with domoic acid exposure. The marine toxins cause disease predominantly through the ingestion of contaminated fish and shellfish, although certain diseases are via skin contact and even by inhalation. Therefore, the food web and the bioconcentration of these toxins through the marine food web play important roles in the transmission of marine toxin diseases. These marine toxins accumulate in a range of intermediate marine hosts (i.e. the transvectors), both shellfish and fish, prior to contact with humans. Often there are additional secondary transvectors with further bioaccumulation (such as carnivore fish eating contaminated herbivores).

In addition, most of the toxins are small, nonpeptidic highly potent substances; quantities as small as < 1 mg/kg body weight can lead to illness. For example, the marine toxin ciguatoxin is estimated to be toxic for humans at a dose of 1 mg/kg body weight (ILO 1984; Miller 1991); its co-occurring toxin maitotoxin is estimated to be even more potent. The marine toxins are predominantly neurotoxins, although hemolytic substances have been identified. In general, the natural marine toxins are tasteless, odorless, and heat- and acid-stable. Therefore normal screening and food preparation procedures will not prevent intoxication if the fish or shellfish is contaminated (ILO 1984; Sims 1987; Halstead 1988; Perl et al 1990; Teitelbaum et al. 1990; Miller 1991).

The general source of these toxins, with the exception of tetrodotoxin in puffer fish and the blue-green algae cyanophytes, are the dinoflagellates and diatoms. The dinoflagellates are phylogenetically unique marine organisms. Dinoflagellates are single-celled algae-like biflagellated organisms dating back some 450 million years, with both prokaryotic and eukaryotic attributes. They can be benthic or pelagic organisms found throughout the marine world, especially in coral reefs and their surroundings (Levinton 1982; Winter et al. 1990, Miller 1991). Of the total known 2000 species of dinoflagellate, only about 20 species have been demonstrated to produce specific toxins (Steidinger and Baden 1984). Certain diatoms can also produce toxins under the proper environmental circumstances. Diatoms, like the dinoflagellates, are single-celled algae, but not flagellated and are by definition enveloped by a silica wall or frustule. Like the dinoflagellates, they occur as freely moving organisms or attached to solid surfaces.

Dinoflagellates are most notorious as the cause

TABLE 1

Intoxication syndromes caused by marine toxins consumed in seafood.

Disease	PSP	NSP	ASP	DSP	Ciguatera	Puffer Fish
Causative organism	Red tide[1] dinoflagellate	Red tide dinoflagellate	Red tide diatom	Red tide dinoflagellate	Epibenthic[2] dinoflagellate	Bacteria?
Major transvector	Shellfish	Shellfish	Shellfish	Shellfish	Fish	Fish
Geographic distribution	Temperate to tropical worldwide	Gulf of Mexico, Japan, New Zealand	Canada, NW U.S.A.	Temperate worldwide	Subtropical to tropical worldwide	Japan, worldwide
Major toxin (n)	Saxitoxin (18+)	Brevetoxin (10+)	Domoic acid (3)	Okadaic acid (4)	Ciguatoxin (8+) scaritoxin, maitotoxin	Tetrodotoxin (3+)
Neuro-mechanism	Na^+ channel blocker	Na^+ channel activator	Glutamate receptor agonist	Phosphorylase phosphatase inhibitor	Na^+, Ca^{2+} channel activators	Na^+ channel blocker
Incubation time	5–30 min	30 min to 3 h	hours	hours	hours	5–30 min
Duration	days	2 d	years	days	years	days
Acute symptoms	n,v,d **p,r**	n,v,d **b,t,p**	n,v,d,**a**,p,r	**d**, n,v	n,v.d. **t**, p	n,v,d,**p,r,↓bp**
Chronic symptoms	None	None	Amnesia	None	Paresthesias	None
Fatality rate	1–14%	0%	3%	0%	<1% (0.1–12%)	60%
Diagnosis	Clinical, mouse bioassay of food, HPLC	Clinical, mouse bioasssay of food, ELISA	Clinical, mouse bioassay of food, HPLC	Clinical, mouse bioassay, HPLC, ELISA	Clinical, mouse bioassay, immunoassay	Clinical, mouse bioassay, fluorescence
Therapy	Supportative (respiratory)	Supportive	Supportive (respiratory)	Supportive	Mannitol? TCA? supportive	Supportive (respiratory)
Prevention	Red tide and seafood surveillance, report cases	Red tide, then seafood surveillance, report cases	Seafood surveillance, report cases	Seafood surveillance, some red tide, report cases	Seafood surveillance, report cases (clusters)	Regulated food preparation, report cases

[1]Red tide refers to blooms of motile single-celled microalgae, most often dinoflagellates.
[2]Epibenthic forms live on solid surfaces or macroalgae and are inadvertently consumed during fish-grazing activities. Taken together, it is readily evident that the toxins of bloom organisms accumulate in filter-feeders, while toxins of epibenthic forms accumulate in fish.
n, nausea; v, vomiting; d, diarrhea; p, paresthesias; r, respiratory depression; b, bronchoconstriction; t, reversal of temperature sensation; a, amnesia; ↓bp, decreased blood pressure. Letters in **bold** indicate pathognomonic symptoms.
Data from Sakamoto et al. (1987) and Sims (1987).

of 'Red Tides', which are dense phytoplankton populations suddenly appearing, coloring the water red or brown. These red tides are often (but not always) associated with high concentrations of bioactive substances, the toxins, which may produce large fish and bird kills as well as human illness. Over 43 species of dinoflagellates produce bioactive substances (including toxins) often present

during the red tides. Some scientists postulate that the toxins play an ancillary role in dinoflagellate metabolism, while others believe these substances are used as a competitive advantage by the dinoflagellate against other organisms (Baden 1983; ILO 1984; Carmichael et al. 1986; Halstead 1988; Winter et al. 1990; Miller 1991). The toxins produced by marine dinoflagellates include the paralytic, the neurotoxic, and the diarrheic shellfish poisons, and the ciguatera poisons (Baden 1983). The most recent addition to the toxic marine unicellular algae are the diatoms, and certain species produce the amnesic shellfish poisons (Wright et al. 1989).

By virtue of the ecological life-style of the incriminated toxic 'microalgae', food webs dependent upon filter-feeding will tend to concentrate 'red tide' toxins Conversely, toxins from epibenthic forms tend to enter foods by initial 'grazing' of herbivorous or omnivorous fishes, with subsequent bioconcentration by predation. Hence, toxic seafood sources will either be of fish or shellfish types, depending on where and how the toxic life form is first consumed.

In the past, these marine toxin illnesses have been predominantly confined to seafood-dependent populations, often located on islands or in coastal areas, each intoxication type occurring in relatively circumscribed areas of the world. However, with increased tourism and the international fishing trade, as well as the overall increased popularity and importance of shellfish and fish in human diets, marine toxin diseases are being increasingly encountered worldwide (Halstead 1988; Lange et al. 1992). In addition, it is hypothesized that human-generated environmental changes, such as reef destruction and eutrophication, may be responsible for the apparent increased reporting in cases of human disease as well as increased incidence of red tides reported worldwide (Bagnis 1984a; Ruff 1989, Viviani 1992). There also is a body of evidence to indicate that man-induced transportation of cysts of toxic marine dinoflagellates (the seeds) or the dinoflagellates themselves occurs in 'spat' (young bivalve shellfish sold commercially to global markets for aquaculture) and ship ballast water (international regulations are now changing to require ship ballast-water purging in the open ocean prior to docking).

Until recently, the medical diagnosis of marine toxin diseases was restricted to a clinical diagnosis based on the clinical history combined with bioassays of contaminated fish or shellfish. This has hampered the accurate diagnosis, development of treatment, and the elaboration of an exact epidemiology of the extent of marine toxin diseases in human populations. In addition, the treatment for these diseases has been predominantly symptomatic and supportive, local remedies notwithstanding.

Appropriately, the majority of work in the past has emphasized the development of surveillance methods and bioassays of contaminated materials to prevent human contact with contaminated shellfish and fish. In both humans and animals, marine toxin diseases have often occurred in disease clusters, such that follow-up of individual cases has led to the discovery and prevention of new cases (Dembert et al 1981). With increasing prevalence worldwide, as well as increased scientific knowledge, new work is focusing on understanding the pharmacologic and pathologic mechanisms in humans in order to develop biomarkers for improved clinical diagnosis, prevention, and epidemiology, and improved treatment capabilities.

MOLECULAR TOXICOLOGY OF MARINE TOXINS

Specific receptor-site interaction: physiologic biomarkers of susceptibility

The naturally occurring marine toxins responsible for paralytic, neurotoxic, amnesic, and diarrheic shellfish poisoning, Fugu poisoning, and ciguatera exert their effects in the nanomole to picomole per kg body weight ranges (Yasumoto and Murata 1993). By virtue of their highly specific and potent deleterious effects on living systems, specific receptor ligands were postulated as the pharmacologically significant factor in the onset of toxicity even before the chemical identity of the toxin(s) was known (see Baden 1983 for a review). Of those toxins now known to the seafood safety regulators, four types are known to interact with specific orphan receptors located on nerve membrane glycoproteins. One type is a potent muscle enzyme inhibitor and one interacts specifically with a central nervous system neurotransmitter receptor site.

Each interacts in a highly specific ligand manner with its biological receptor site and leads to a multitude of cascade processes by which the ultimate toxicological response is manifested (Fig. 1). It follows in a human and animal susceptibility sense that those individuals, groups, strains, or races who possess the receptor, enzyme, or binding site face the greatest peril. In an ancillary sense, compromised individuals who have impaired function in one of these areas might also be considered at increased risk.

It is in this regard that molecular toxicologists are making their current greatest contribution to seafood/marine toxin safety issues. Identification and characterization of the specific toxin (ligand)/receptor interactions will provide the raw information which leads to prediction of susceptibility, identification of bound biomarkers in intoxicated individuals, and ultimate development of specific therapeutic intervention for poisoning.

Toxins as ligands for orphan receptors

Orphan receptors is a term used by toxicologists to define a specific binding site which exhibits a high affinity for exogenous xenobiotic ligands. That is to say, orphan receptors are located on essential endogenous macromolecules which bind environmental toxicants as a first step in expression of organism toxicity. The term 'orphan' refers to the

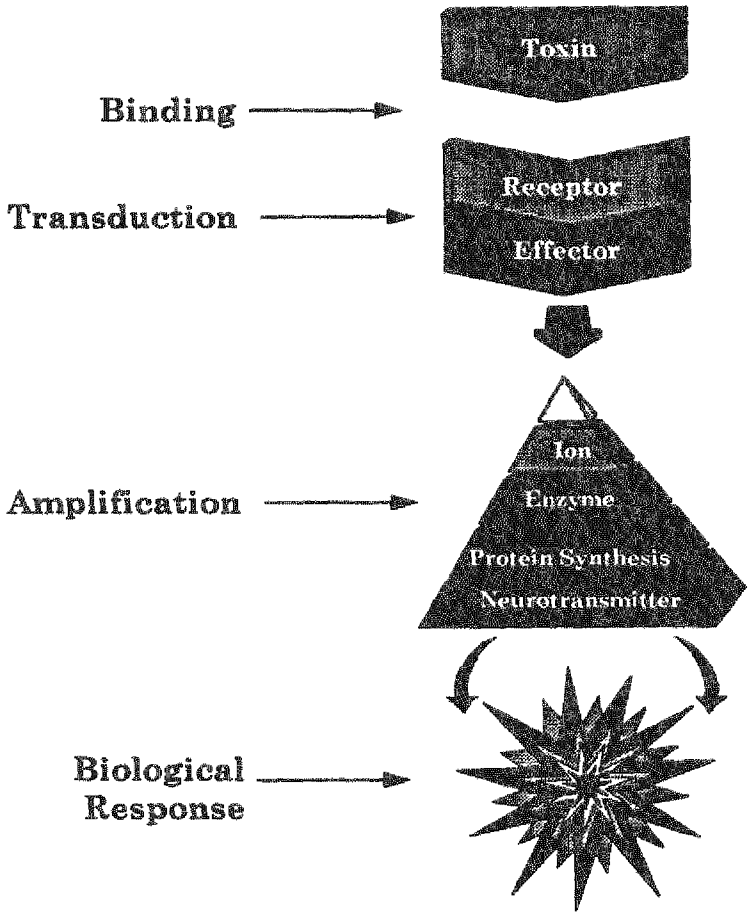


Fig. 1. Cascade of neurotoxin action on living systems. The initial step in the onset of toxicity is binding to a specific receptor Binding leads to a transduction event by coupling to its effector, which in turn leads to amplification by way of specific ion transport (or inhibition thereof), through enzyme activation or inactivation, protein synthesis, or neurotransmitter release or inhibition from release. (Adapted from Cooper et al. (1982).)

concept that, although the specific binding or receptor site may be well-defined, there is no known endogenous ligand which interacts with the site to induce the same biological response. Hence, this definition may be a fleeting one, for as soon as an endogenous ligand is identified, it is no longer an orphan receptor.

There are four different natural marine toxin types known which interact with ligand receptor sites located on the voltage-sensitive sodium channel (VSSC). The VSSC is composed of three nonidentical glycoprotein subunits, associated in a 1:1:1 stoichiometry: the α-subunit which is composed of four homologous domains and a total molecular weight of ca 360,000; and the β_1- and β_2-subunits of molecular weight 36,000 and 33,000, respectively (Catterall 1992). Acting in concert, these three subunits modulate the flow of sodium ions across excitable membranes by modifying allosterically in response to changes in membrane potential That VSSC activation and inactivation involves a major conformational change which

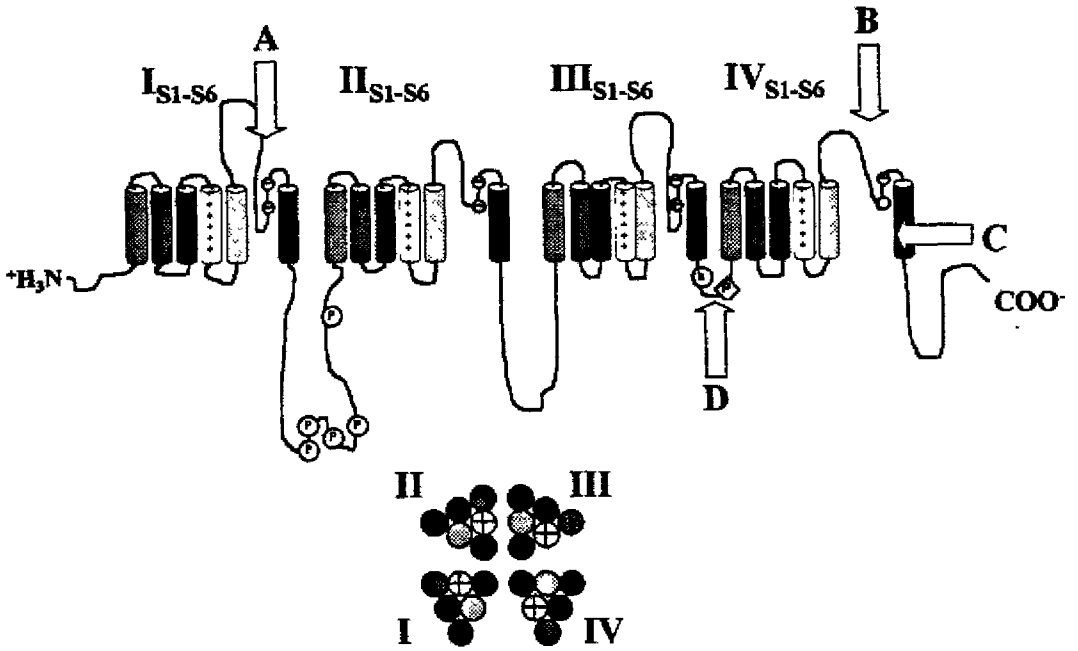


Fig. 2. Model depiction of sodium channel α-subunit primary structure and proposed orientation in excitable membranes. Illustrated in the extended figure is the α-subunit as a single polypeptide glycoprotein, which is fourfold homologous. Each homologous domain, labeled I–IV, contains six transmembrane helices (S1–S6) which alternately extend through the membrane, with short to long transhelical peptide regions. In order, S1–S3 in each domain contain a few '−'-charged amino acids, the S4 subunit is highly '+'-charged, alternatively one arginine or lysine with two hydrophobic amino acids, and the S5 and S6 subunits are hydrophobic helices. Based on models known as the 'sliding helix' model, the highly charged S4 subunits are thought to slide out of the membrane in a corkscrew-type fashion upon depolarization, revealing by allosteric modulation the ion pore necessary for sodium ion intrusion. The S4 subunit is kept in place by charge-charge complexation with the S1–S3 subunits, and either electrical stimulation or specific ligand interaction can disrupt normal ion pairing which leads to depolarization. Shown in the figure are (A) the site required for tetrodotoxin and saxitoxin binding, with at least threefold redundancy in the various domains, (B) the site of brevetoxin and ciguatoxin binding in domain IV, (C) the site of an essential methionyl residue for normal sodium channel inactivation, and (D) the inactivation loop (or IMF particle), which closes over the interior of the channel upon inactivation. A proposed organization of the α-subunit is illustrated in the lower structure of Figure 1. Although one might expect the hydrophobic subunits to be on the 'exterior' of the channel to aid in membrane insertion or solubility, and the S1–S3 in close articulation with S4 for ion pairing, no specific organization is suggested by this portion of the figure. The principle aim is to illustrate the fourfold redundancy, the ion pore site proposed as being central to the arrangement, and the S4 subunits in such a position as to offer allosteric 3-dimensional modulation upon channel activation. (Reproduced from Catterall (1992) by courtesy of the Editors of *Physiological Review*.)

occurs in response to disruption of protein-membrane charge complexes is supported by the original work of Hodgkin and Huxley (1952a-d), and by the description of 'gating' currents which represent the actual movement of charges that accompanies activation (Armstrong 1992). According to Catterall (1992), 'In all probability these charged groups are amino acids in the protein structure which are altered by conformational modification'.

Translated as a single polypeptide chain, the primary structure of the α-subunit reveals a four-fold homology, with 6 transmembrane helices labeled S1-S6 in each of the 4 domains (see the extended structure in Fig. 2) (Noda et al. 1984). When inserted into the membrane, the α-subunit arranges itself with the four homologous domains arranged in quadrants, as shown in the condensed illustration in Figure 2 (viewed from the outside towards the interior of the membrane). Presumably, the 'ion pore' itself is located in the center of the four domains.

Transmembrane helices S1, S2, and S3 are relatively negatively charged helices, S4 is highly positively charged (+++ in Fig. 2), and S5 and S6 are largely hydrophobic. The S4 transmembrane helix in each domain is particularly interesting in that every third residue is an arginine or other positively charged amino acid, the remaining residues in the series being hydrophobic (Noda et al. 1984). When arranged in a helix, computer molecular modeling reveals ε-amino functions which extend from the helix much like the treads on a circular staircase. The S4 'sliding helix model' (Catterall 1986a) is proposed as an explanation and identity of the gating or voltage sensor of the channel. Changes in membrane potential disrupt the charge pairing between '+'-charged ε-amino functions and '−' charged residues in the S1, S2, and S3 segments of the individual domains, leading to an outward twisting of the four S4s, ultimately exposing or opening the channel to sodium ion flow. S5 and S6 are thought to render the subunit's effective character as hydrophobic which aids insertion into the membrane (Catterall 1986b).

On the channel, it is the highly ordered 3-dimensional topography of the α-subunit which exhibits a profound affinity for saxitoxin and tetrodotoxin at orphan receptor Site 1 (A in Fig. 2) (Catterall et al. 1979), and for brevetoxins and ciguatoxin at orphan receptor Site 5 (B in Fig. 2) (Poli et al. 1986; Baden 1994). Binding of these ligands leads to conformational changes in the 'ion pore' component of the channel, much like membrane electrical field-induced modification. The spatial relationship of the voltage sensors of the channel to the toxin binding sites is a topic of current research activity, as are the uses of the toxins as molecular 'probes' or 'rulers' measuring devices for determining topographic distances and conformational changes which activate or block the transmembrane. In other words, the toxins modulate the allostery of the transmembrane pore by merely binding to a specific orphan receptor site. Thus, biochemistry and electrophysiology may find common ground in the study of toxin-induced channel modulation.

Site 1 toxins: Saxitoxin (Fig. 3) and tetrodotoxin (Fig. 4) inhibit sodium ion flow when applied to nerves, muscle, and isolated channels (Narahashi 1974). Their effects are effected from the exterior

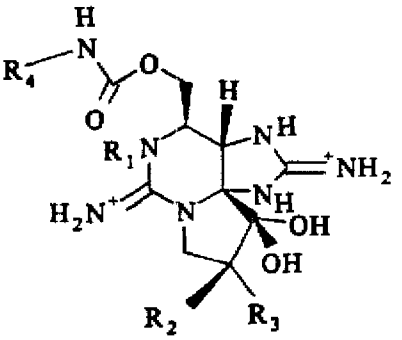


Fig 3. The structure of saxitoxin. Natural derivatives have substitutions at R1–R4. Substition moieties have considerable effects on toxicity, binding, and activity in electrophysiology. Saxitoxin and its derivatives are water-soluble and block sodium channels.

Toxin	*R1*	*R2*	*R3*	*R4*
Saxitoxin	H	H	H	H
Neosaxitoxin	OH	H	H	H
Gonyautoxin 3	H	OSO_3^-	H	H
Gonyautoxin 2	H	H	OSO_3^-	H
Gonyautoxin 4	OH	OSO_3^-	H	H
Gonyautoxin 1	OH	H	OSO_3^-	H
Gonyautoxin 5	H	H	H	SO_3^-
Gonyautoxin 6	OH	H	H	SO_3^-
EpiGonyautoxin 8	H	H	OSO_3^-	SO_3^-
Gonyautoxin 8	H	OSO_3^-	H	SO_3^-
C3	OH	H	OSO_3^-	SO_3^-
C4	OH	OSO_3^-	H	SO_3^-

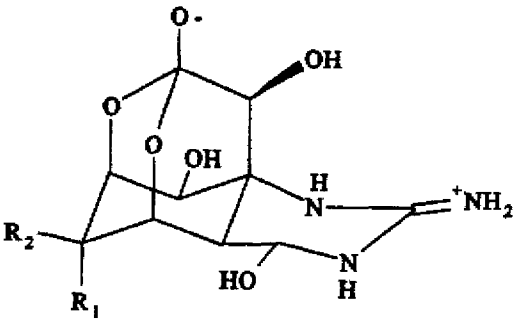


Fig. 4. The structure of tetrodotoxin. Seven different tetrodotoxin derivatives are known from a variety of marine and terrestrial sources. Like saxitoxin, tetrodotoxin blocks sodium channels. All derivatives occur on R1 and R2. On the basis of the toxicity data of the seven derivatives, a pocket-type binding site on the surface of the channel has been postulated. As in saxitoxin and its derivatives, the guanidinium functions are essential for activity and act as one point of a proposed 'three-point' fit to the active binding site.

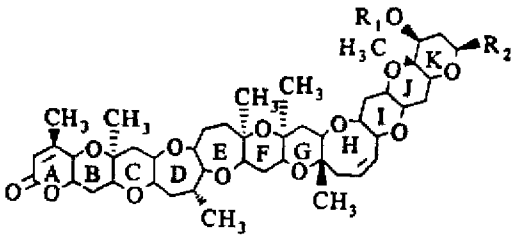
Toxin	*R1*	*R2*
Tetrodotoxin (TTX)	OH	CH_2OH
6-epi-TTX	CH_2OH	OH
11-deoxy TTX	OH	CH_3
11-oxoTTX	OH	CHO
11-norTTX-6-(R)-ol	H	OH
11-norTTX-6-(S)-ol	OH	H
Chirquitoxin	OH	$CH(OH)CH(NH_2)COOH$

of the pore, and molecular studies including sodium channel deletion mutations indicate that the specific binding site for the Site 1 toxins is located at point 'A' in Figure 2. This orphan receptor site is believed to be located near the extracellular side of the channel, an hypothesis supported by site-directed mutagenesis (reduced binding in the absence of glutamate 387 at the extracellular loop between S5 and S6 of domain 1, just outside S6 (Noda et al. 1989), by the lack of activity of saxitoxin or tetrodotoxin upon intracellular injection (Ritchie and Rogart 1977), and by several lines of photoaffinity biochemistry and chemical modification of carboxyl residues.

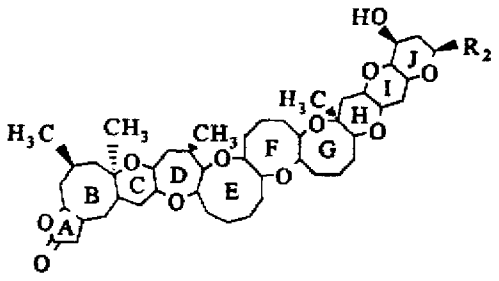
Binding is pH-dependent (Henderson et al. 1973; Ritchie and Rogart 1977), under optimum conditions each toxin binds with a dissociation constant of about 1 nMolar, and in a 1:1 stoichiometry with the subunit (Kreuger et al. 1979, J.C. Lawrence and Catterall 1981). The guanidinium character of the two toxins is considered essential for their activity, the guanidinium being viewed by the channel as transportable, but the rest of the molecule is prevented from transversing the channel based on ion charge pairing. Hence, these two toxins act as plugs (Kao 1993) or lids on the channel. It is generally agreed that at least three of the four S6 transmembrane helices act in concert to form the Site 1 binding site at or near the extracellular side of the pore and create the classical '3-point site' of attachment of ligand to active site. Thus, the Site 1 toxins will aid in the complete description of the topography surrounding sodium channel α-subunit exterior pore dimensions.

Site 5 toxins: Both brevetoxin types (Fig. 5) and ciguatoxin (Fig. 6) interact with the polyether ladder toxin binding site known as Site 5. Binding at this site causes channels to open at normal resting potential, slows normal inactivation of channels, and often results in repetitive firing in nerves. Like tetrodotoxin and saxitoxin, a specific binding site has been described (Poli 1986; Trainer et al. 1991) which exhibits a 1:1 stoichiometry with channels, and which exhibits half-maximal binding in the nanomolar concentration range. Specific labeling of Site 5 with brevetoxin photoaffinity probes on the rigid H-I-J-K ring side chain of PbTx-3 (Fig. 5 R1) revealed a domain 4-specific high affinity site located between S5 and S6 (B in Fig. 2). Its specific localization may exist in the portion of the extracellular loop which lines the interior surface of the pore itself (Trainer et al. 1991). Thus, the initial binding event in the toxicity of brevetoxins is probably binding of this rigid region to the receptor site.

Considerable molecular modeling and structure/activity work have been carried out with brevetoxin PbTx-3 (a PbTx-2-type toxin). All the polyether ladder toxins possess considerable flexibility across their lengths, contain similar rigid structural features topographically similar to the G-H-I-J ring region of PbTx-2, and carry a lactone or extant lactone electrophile on the end distal to the rigid portion. The lactone appears to be essential for activity; at least an electrophile is required (Baden et al. 1994). The overall lengths of the molecules range from 26 Å to 35 Å, and the proposed S5-S6 binding site extends approximately 6 Å into the membrane. The present conclusion is that



PbTx-2-Type



PbTx-1-Type

Fig. 5. Structure of the two different types of brevetoxins. Natural derivatives occur at R1 and R2.

PbTx-2-type	*PbTx-1-type*
PbTx-2, R1=H, R2=$CH_2C(=CH_2)CHO$	PbTx-1, R2=$CH_2(=CH_2)CHO$
PbTx-3, R1=H, R2=$CH_2C(=CH_2)CH_2OH$	PbTx-7, R2=$CH_2C(=CH_2)CH_2OH$
PbTx-5, R1=CH_3CO, R2=$CH_2C(=CH_2)CHO$	
PbTx-6, R1=H, R2=$CH_2C(=CH_2)CHO$,27,28 epoxide	
PbTx-8, R1=H, R2=$CH_2C(=CH_2)COCH_2Cl$	
PbTx-9, R1=H, R2=$CH_2CH(CH_3)CH_2OH$	PbTx-10, R2=$CH_2CH(CH_3)CH_2OH$

these toxins exert their effects near the inside of the membrane and have the potential to affect both activation and inactivation (Trainer et al. 1993).

The model for the activation properties of polyether toxin involves a disruption of the charge-charge pairing of the '+'-charged S4 helices with the S1, S2, and S3 helices by the toxin lactone electrophile substituting for one of the S1, S2, or S3 '−'-charges (Rein et al. 1993a). Just as electrical stimulation disrupts the charge pairing, so does toxin intercalation the ion pairing. An interesting observation (Jeglitsch et al. 1993) involves the application of brevetoxin under patch-clamp conditions to reveal 5 separate subconductance states. A possible explanation for the multiplicity of subconductance states induced by the toxin is an allosteric one, in which the four individual domains must sequentially 'flip' into a favorable conformation for channel activation (Fig. 7). Were brevetoxins to have an ion-pair disrupting effect in each of the domains (while only binding to domain 4), five subconductance states would be revealed (one normal and one subconductance state for each of the four S4 regions). Thus, these toxins may possess the ability to assist in description of which charge-charge complexes represent the first, second, and so forth in the ordered sequence of subunit allostery modulation for channel activation.

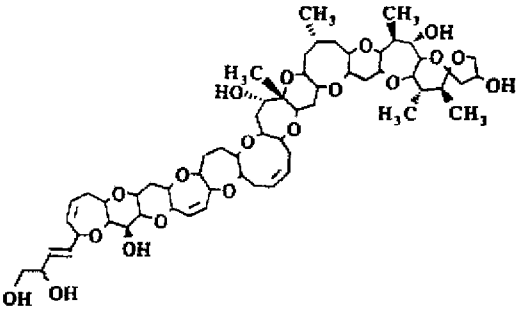


Fig. 6. Structure of ciguatoxin.

The effect of brevetoxins and ciguatoxin on inactivation has been debated for several years. Slowed inactivation is most assuredly seen in invertebrate systems (Huang et al. 1984), a result thought by some to be an artifact of the model system. Patch clamping of rat endothelial neurons has illustrated in this mammalian system that indeed brevetoxins alter the kinetics of inactivation (Jeglitsch et al. 1993).

Based on experimental evidence (Huang et al. 1984; Schreibmayer and Jeglitsch 1992; Jeglitsch et al. 1993) on the molecular mechanism and primary structure of the 'inactivation particle' (D in Fig. 2) (Catterall 1992) and on molecular modeling studies (Rein et al. 1994a,b; Yasumoto and Murata 1993), brevetoxins may indeed affect inactivation by a similar disruption of normal ion pairing during the inactivation process.

Toxins as enzyme inhibitors

The toxins responsible for diarrheic shellfish poi-

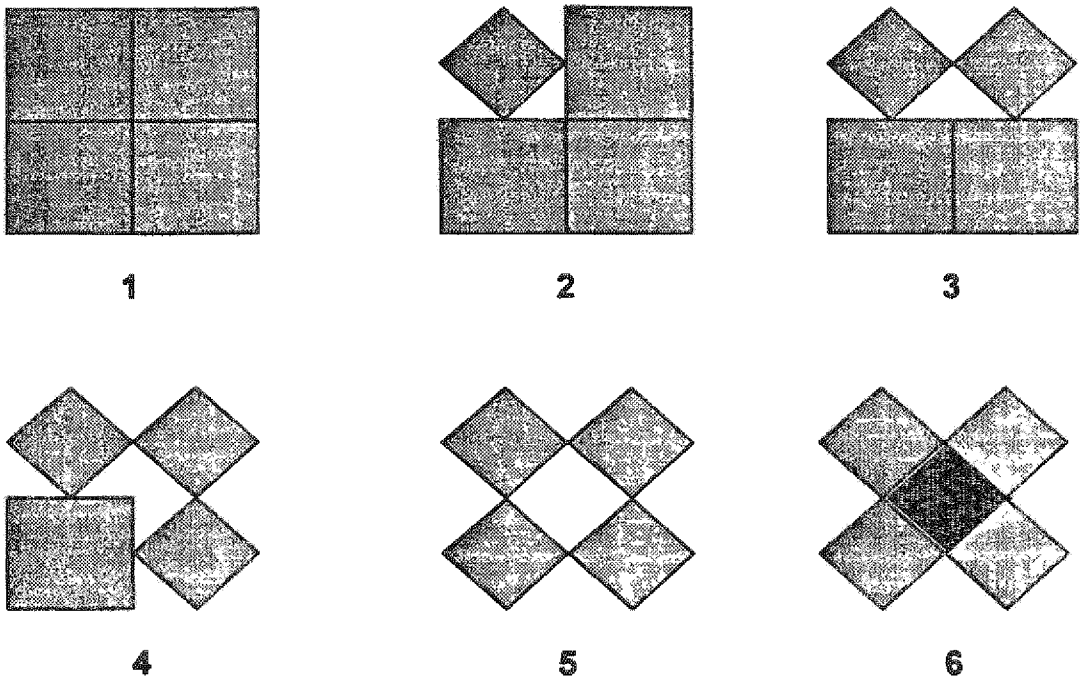


Fig. 7. A proposed model for voltage-sensitive sodium channel (VSSC) opening. VSSC is thought to undergo a number of allosteric modifications which ultimately results in the opening of the sodium channel. It is proposed that with each of 4 sequential 3-dimensional changes, sodium channels undergo transformation from closed to open according to: C(1)→C(2)→C(3)→C(4)→O(5)→I(6). If the four steps required were true allostery, each sequential modulation would increase the ease of the next modulation. Thus, the energy of activation of 4 would be less than 3 which is less than 2 which is less than 1. The polyether ladder brevetoxins and ciguatoxin interact in some manner with the portion of the channel which modulates activation. If modeling conjectures and subconductance state information are correct, these toxins could induce multiple subconductance states by interacting with each of the four domains independently, inducing one normal and four abnormal conductance states.

soning (DSP) worldwide are based on the structure of okadaic acid (Fig. 8). The toxin was first isolated from a sponge (Tachibana et al. 1981) and later described as having its biogenesis in a commensal dinoflagellate and as a principal causative organism in outbreaks of DSP (Murikami et al 1982). Numerous derivatives of okadaic acid have now been described, some of which occur in the various dinoflagellate species which have now been incriminated as well as those found in shellfish transvectors of toxin from seawater to man The R1 functionality, a proton in okadaic acid, is a common site of derivatization and several *o*-acyl fatty acid derivatives have been isolated and characterized (Murata et al. 1982). The latter derivatives appear to predominate in shellfish that concentrate the toxins. The R2 position is either a hydrogen (in okadaic acid) or a methyl group and these are known as the dinophysis toxins (Yasumoto and Murata 1993). Two other toxins, yessotoxin and the pectenotoxins, co-occur with okadaic acid derivatives but will not be discussed here due to the lack of information on human incidence of disease.

Unlike the toxins described earlier that exhibit intraperitoneal animal lethalities in the fractions of micrograms per kilogram body weight range, the DSP toxins more approximate 200–500 g/kg body weight. The most profound effects of okadaic acid appear to be smooth muscle effects, which in 1982 were traced to modification of protein phosphorylation (Shibata et al. 1982). This is reflected in the prolonged contraction of smooth muscle in arteries (Shibata et al. 1982) and is due to an inhibition of myosin light chain phosphatase (Takai et al. 1987). Cohen and others have since shown that

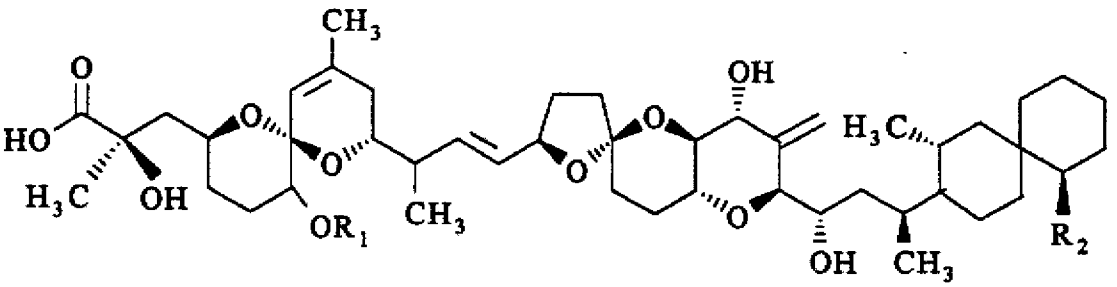


Fig. 8. The structure of okadaic acid. The basic molecule can be derivatized at R1 or R2.

Toxin	*R1*	*R2*
Okadaic acid	H	H
Dinophysis toxin 1	H	CH_3
Dinophysis toxin 3	Fatty acid esters	CH_3

okadaic acid and its derivatives are potent inhibitors of protein phosphatase 1 (PP1) and protein phosphatase 2a (PP2A) (Cohen 1989; Haystead et al. 1989). These two enzymes are essentially ubiquitous in mammalian cells and serve numerous regulatory roles in enzyme activation and inactivation, in cascade systems, transport across cell membranes, and in intermediary metabolism. Thus, an exogenous compound which can so systematically and completely deregulate more than 20 phosphoproteins would be expected to play havoc among public health circles. In fact, there is also considerable literature which points to okadaic acid as a potent non-TPA type tumor promoter, again a finding not unexpected owing to its disruption of vital cellular regulation (Fujiki et al. 1988).

The exact mechanism by which okadaic acid inhibits protein phosphatases is thought to relate to the potential role of the toxin for the dinoflagellate itself, that of regulating protein phosphorylation. There is some mounting evidence that okadaic acid is in fact a phosphatase regulator in *Prorocentrum* species which assists the cells in scavenging organic phosphate (Kinoshita, personal communication). There is much sequence homology in the catalytic subunits of protein phosphatases (Bernt et al. 1987), and so it is not surprising to see effects on both PP1 and PP2a. However, the inhibition is not at the substrate binding site, for okadaic acid inhibition exhibits noncompetitive or mixed inhibition patterns with respect to substrate.

It is possible, however, that the protein phosphatase inhibitor exerts its activity via the regulatory subunit binding site on PP1 and PP2a. To decide if this is the case, competition studies employing both the regulatory subunit (normally a peptide in mammalian systems) of the PP1 or PP2a and okadaic acid should be conducted. In all likelihood, competitive binding at the regulatory subunit binding site will be observed. The reason then for its exquisite selectivity for PP1 and PP2a is that okadaic acid is a regulator of protein phosphatases in some systems naturally, and when it comes in contact with mammalian protein phosphatases, it seeks to regulate and inactivate the phosphatase by combining with a regulatory subunit binding site.

Exactly how okadaic acid binds and what the molecular topography of the molecule must be for potent inhibitory activity is not known with certainty. However, there is currently much interest in microcystin peptides, and molecular modeling of okadaic acid reveals some cyclized forms which appear as predominant conformers. The future of okadaic acid research may provide clues to exactly how protein phosphatases are regulated in mammals, and it may provide some interesting information on how lower life forms have evolved homologous mechanisms for regulating the phosphorylation and dephosphorylation of their cellular proteins.

Toxins as neurotransmitter agonists

The final (known) toxin is domoic acid (Fig.9), an excitatory dicarboxylic amino acid similar to kainic acid (Baden and Trainer 1993). Like kainic acid, domoic acid competes for glutamate recep-

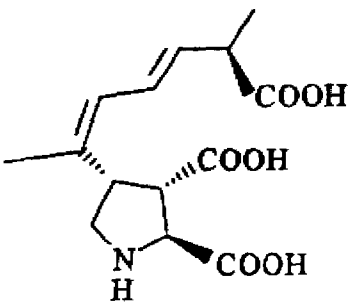


Fig. 9. Structure of domoic acid.

tors in the CNS. Application of, or exposure to, domoic acid leads to persistent and debilitating necrosis of the hippocampal region due to high affinity for kainate receptors. All of the effects of domoic acid mimic kainic acid Kainate and domoic acid are purely competitive in binding activity; the latter exhibits a 3–5-fold greater affinity over the former (Angst and Williams 1987), and in some receptor systems in vivo domoic acid exhibited a 20-fold greater potency than did kainate (Debonnel et al. 1989).

Domoic acid has been recognized as a public health threat only since 1987 and there is still a great deal to learn about its molecular mechanism. That this toxin binds to the kainate receptor subtype of glutamate receptor is reassuring in a research sense for a great deal is known about the kainate receptor in CNS. Rapid progress in complete characterization can be expected in this area.

EPIDEMIOLOGY, DIAGNOSIS, AND MANAGEMENT OF MARINE TOXIN EXPOSURE

Paralytic shellfish poisoning (PSP)

Background and epidemiology. PSP is a marine toxin disease with both gastrointestinal and neurologic symptoms reported worldwide. It is caused predominantly by the consumption of contaminated shellfish (Southcott 1979; Baden 1983; Halstead and Schantz 1984; ILO 1984; Shimizu 1984; Carmichael et al. 1986; Saunders 1987; Halstead 1988; Viviani 1992, Kao 1993).

PSP was first recognized by Vancouver (1801) in the Pacific Northwest of North America in 1793 with the report of one death. Further outbreaks were reported as food poisoning in the 18th century from Europe, North America, Japan, South Africa, and New Zealand. Red tides were even reported in the ancient Mediterranean in the writings of Homer (Iliad), Tacitus, and early European navigators (Bower et al. 1981; Halstead 1988). In the past decade, reports of red tides and PSP cases have occurred with increasing frequency throughout the world, not only in endemic areas but also from countries importing from Malaysia, Solomon Islands, Tasmania, Philippines, Thailand, Brunei Darussalam, South America, as well as the European Atlantic coast (Hermes and Villoso 1983; Mee et al. 1986; Eason and Harding 1987; Rodrigue et al. 1990. Saldate Castaneda et al. 1991; Viviani 1992). Worldwide, about 1600 cases of PSP are estimated to occur each year (Halstead and Schantz 1984). More than 24 deaths occurred out of the 905 cases reported from 1969 to 1983 (ILO 1984). It is not clear if this apparent increase in the incidence of PSP is due to increased reporting or to an actual increase in incidence. However, Kao (1993) believes that the clinical manifestations of PSP are so characteristic that this increase in incidence is real.

Dinoflagellates (mainly the species in genus *Gonyaulax*, recently reclassified to *Alexandrium*) are the source of the PSP marine toxins. These unicellular dinoflagellates develop algal blooms throughout the world for unknown reasons, although a variety of factors have been studied, including change in weather, upwellings, temperature, turbulence, salinity, and transparency. In addition, new theories are examining the importance of hypertrophication of terrestrial origin in the development of toxic algal blooms; for example, possibly due to aquaculture pollution, there was an emergence of red tides in the Faroe Islands in 1984 (Viviani 1992). Of note, these red tides can be toxic or not, again for unknown reasons. Significant epidemics of PSP can occur in humans in the absence of a known red tide (Rodrigue et al. 1990).

These dinoflagellates produce at least 12 toxins which are heat- and acid-stable tetrahydropurines. Shellfish, once having accumulated the toxins, metabolize them to as many as 6 additional potent materials and possess the enzymology to interconvert toxins from one derivative to another. Saxitoxin was the first characterized and the best understood; the others (including neosaxitoxin,

gonyautoxin I. gonyautoxin III, decarbamoyl saxitoxin; less toxic are gonyautoxin II, IV, V-VIII, and sulfocarbamoyl gonyautoxin I, IV) have been divided into three groups predominantly based on chemical substitutions: carbamate, N-sulfocarbamoyl, and decarbamoyl components. In general, they have similar chemical properties to saxitoxin. However, this multiplicity of toxins associated with PSP makes accurate detection and quantification difficult. Although PSP is an illness which occurs worldwide, there is complete heterogeneity in terms of toxin composition and saxitoxin is not always the major constituent (Baden 1983).

The major transvector for PSP are the bivalve molluscs (mussels, clams, oysters, with the Alaskan butterclam having the highest concentrations) (Sommer and Meyer 1937). PSP toxins are also found in certain crabs and snails which feed on coral reef seaweed. The transvectors accumulate the toxins via feeding in their digestive organs and soft tissues, apparently without harm to the transvectors.

Humans, birds, and fish can all be affected by PSP toxins. Herbivorous zooplankton is the primary transvector which can in turn transmit the toxin to fish and possibly other marine creatures which consume zooplankton (Baden 1983). The usual route for humans is the consumption of raw or cooked contaminated shellfish. There has been only one case of human contamination through consumption of fish and birds which had died from PSP-contaminated shellfish ingestion In this case, which occurred in Indonesia, the whole fish was consumed including the viscera which could have been contaminated with PSP from shellfish consumption (MacLean and White 1989; Viviani 1992).

The overall mortality is reported to be 1–10% for people with clinical PSP, with death 1–12 hours postingestion; the mortality rate does depend on the availability of emergency medical care. In a PSP outbreak on the Pacific coast of Guatemala in July-August 1987 in 187 people, there were 26 fatalities (14%), the case fatality being highest in young children (50%) (Rodrigue et al. 1990).

Neural mechanism Saxitoxin is the most well known of the PSP-associated toxins. It is a heat-stable neurotoxin. In mice, the saxitoxin LD_{50} parenterally is 3–10 μg/kg body weight and orally 263 μg/kg body weight (death within minutes of respiratory failure). Humans are the most sensitive to saxitoxin; the oral dose for death is 1–4 mg (5000 to 20,000 mouse units) depending upon the age and physical condition of the patient (Wiberg and Stephenson 1960; Shimizu 1984; Winter et al. 1990). It is rapidly absorbed through the gastrointestinal tract and excreted in the urine.

Experimental animal work has shown that saxitoxin and its analogues cause predominantly peripheral neurologic intoxication, unless directly injected into the CNS, especially with respect to respiratory paralysis (Borison et al. 1980a,b; Baden 1983; Winter et al. 1990). Saxitoxin inhibits the temporary permeability of Na^+ ions by binding tightly to a receptor site on the outside surface of the membrane very close to the external orifice of the sodium channel. In fact, neurophysiologic studies using saxitoxin as a probe helped to show that Na^+ and K^+ act independently with separate membrane channels A blocking agent reduces the number of conducting Na^+ channels by occupying some site near the outer opening in a 1:1 high-af - finity specific receptor binding. This prevents sodium ions from passing through the membranes of nerve cells, thus interfering with the transmission of signals along the nerves. The resulting widespread blockade prevents impulse generation in peripheral nerves and skeletal muscles (Murtha 1960; Evans 1967; Schwartz 1971; Narahashi et al. 1972; Adams et al. 1976; Bower et al. 1981; Kao 1983; Winter et al. 1990; Viviani 1992).

Saxitoxin has a direct effect on skeletal muscle by blocking the muscle action potential without depolarizing cells; it abolishes peripheral nerve conduction but with no curare-like action at the neuromuscular junction. Direct cardiac effects are minimal and transient, therefore there is little or no hypotension (Murtha 1960; Evans 1967; Schwartz 1971; Narahashi 1972; Adams et al. 1976; Bower et al. 1981; Kao 1983; Winter et al. 1990; Viviani 1992).

Saxitoxin and tetrodotoxin (the etiologic agent of puffer fish poisoning; see below) are very similar in terms of their clinical effects and they cannot be distinguished easily by traditional mouse bioassay. Saxitoxin and tetrodotoxin both block or close the passage of sodium ions through the channel. Sax-

itoxin binds at a sodium channel receptor reversibly and in a concentration-dependent fashion; it is specific even at low concentrations (10^{-9} M). When saxitoxin is mixed in small amounts with many classes of anesthetics, the anesthetic action is greatly enhanced in a multiplicative fashion; this allows for decreased doses of anesthetic for the same effect. Saxitoxin and tetrodotoxin have both been important in the characterization of sodium channels in myelinated and unmyelinated nerve membranes, and in the study of related diseases such as multiple sclerosis (MS); both block the inward current of sodium ions at equally low concentrations of 10^{-7}–10^{-9} M and occupy the same receptor sites at the sodium channel although with different chemical structures (Shimizu 1984; Schantz and Johnson 1992).

Clinical presentation. Ingestion of molluscs contaminated with PSP results in the following clinical picture (Bower et al. 1981; Kao 1993). Five to 30 minutes from consumption, there is slight perioral tingling progressing to numbness which spreads to face and neck in moderate cases. In severe cases, these symptoms spread to the extremities with incoordination and respiratory difficulty. There are medullary disturbances in severe cases evidenced by difficulty in swallowing, sense of throat constriction, speech incoherence or complete loss of speech, as well as brainstem dysfunction. Within 2–12 hours, in very severe cases, there is complete paralysis and death from respiratory failure in absence of ventilatory support. After 12 hours, regardless of severity, victims start to recover gradually and are without any residual symptoms within a few days (Bower et al. 1981; ILO 1984; Halstead 1988).

In one patient with acute bulbar and respiratory paralysis following ingestion of saxitoxin-contaminated clams, serial electrophysiologic observation showed prolonged distal motor and sensory latencies, slowed conduction velocities, and moderately diminished amplitudes at the outset. There was a return to normal over 5 days. This illustrates the incomplete reversible sodium channel blockade caused by saxitoxin and other PSP toxins (Long et al 1990).

Other symptoms include headache, dizziness, nausea, vomiting, rapid pain, and anuria. There is no loss of consciousness and reportedly the reflexes are unaltered except maybe pupillary size and temporary loss of sight. As opposed to tetrodotoxin poisoning, there is rarely significant hypotension. Symptomatology is essentially identical for Pacific and Atlantic cases, although gastrointestinal symptoms may be more prominent in the Atlantic ones (ILO 1984; Halstead 1988).

Lactic acidosis of unexplained origin has been found in experimental animals administered saxitoxin (Franz and LeClaire 1989). Because the toxin interferes with respiratory function, this acidosis could not be compensated for naturally by hyperventilation. Lactic acidosis has also been seen in human cases of PSP (Kao 1993).

In an outbreak of PSP in southern Taiwan, 3 of 5 patients had significant increases of creatine kinase levels without correlation with the severity of poisoning, this abnormality resolved completely with recovery (Cheng et al. 1991). There are no reports of chronic disease associated even with severe cases of PSP except for some residual headaches, memory loss, and fatigue for up to 2 weeks reported in a recent epidemic in Guatemala (Rodrigue et al. 1990).

The overall mortality was about 8.5–9.5% in two large series (Meyer 1953; Ayres and Cullum 1978). The Guatemalan 1987 outbreak on the Pacific coast had a case fatality rate of 14%, which was even higher in young children (50%). It is possible that children may be more sensitive to PSP toxins than adults (Rodrigue et al. 1990). In addition, the access to emergency medical services in acute cases is crucial to the prognosis.

The differential diagnosis of this clinical scenario of an acute gastrointestinal illness with recent shellfish ingestion would be bacterial or viral gastroenteritis. The neurologic manifestations are more consistent with poisoning by other marine toxins such as neurotoxic shellfish poisoning (NSP) and puffer fish poisoning, or even recent organophosphate pesticide poisoning.

Diagnosis. The clinical scenario is the primary method of diagnosis initially. Recent shellfish ingestion, often but not always associated with known red tide, and an acute gastrointestinal illness with neurologic symptoms are part of the classic presentation. It is imperative to obtain samples

of contaminated tissues and the source of the shellfish.

As mentioned above, each PSP epidemic is associated with different mixtures of PSP toxins; this complicates the laboratory analysis of contaminated tissues. The mouse bioassay (time to death) of food extract is the recommended diagnostic method (Sommer and Meyer 1937; Association of Official Analytical Chemists 1980), but it cannot distinguish between tetrodotoxin and other PSP toxins. The oral dose for death in humans is 1–4 mg (5000 to 20,000 mouse units) depending upon the age and physical condition of the patient. A mouse unit (MU) is defined as the minimum amount needed to cause the death of an 18–22-g white mouse in 15 minutes (Wiberg and Stephenson 1960; Shimizu 1984; Winter et al. 1990).

Radioimmunoassay and indirect enzyme-linked immunoabsorbent assay (ELISA) have been developed for saxitoxin but not all PSP toxins (Carlson et al 1984). High performance liquid chromatography (HPLC) analysis for all the PSP toxins has been developed with good correlation with mouse bioassay in terms of quantification (Sullivan et al. 1983; Halstead 1988).

Management and treatment. In general, supportive measures are the basis of treatment, especially ventilatory support in severe cases. In animals, artificial respiration is the most effective treatment. Without supportive treatment, up to 75% of severely affected persons die within 12 hours.

When the ingestion of contaminated food is recent, gut decontamination by gastric lavage and administration of activated charcoal or dilute bicarbonate solution is recommended. Care must be taken concerning aspiration in the neurologically compromised patient. Anticurare drugs were ineffective, while DL-amphetamine (benzedrine) was most effective in aiding the artificial respiration and decreasing the recovery period. Use of anticholinesterase agents is not recommended and could actually be harmful (Murtha 1960; Bower et al. 1981; ILO 1984, Halstead 1988; Brown and Shepherd 1992; Kao 1993).

The lactic acidosis of unknown origin can be treated by assisted ventilation, fluid therapy, and periodic monitoring of the blood pH. It is possible that fluid therapy will also assist in the renal excretion of toxin (Kao 1993).

Many endemic areas have traditionally used local treatments with variable success. In the Philippines, a drink of coconut and brown sugar is administered; administration of this drink to mice shows that these ingredients may contain active detoxification substances (Viviani 1992).

Recent work by Kaufman et al. (1991) has focused on the development of a therapeutic antiserum, although this is complicated by the wide range of PSP toxins. A tetrodotoxin-formaldehyde-keyhole limpet hemocyanin conjugate was used to immunize a rabbit for the production of antitoxin antiserum which cross-reacted against tetrodotoxin and saxitoxin. In a quantitative in-vitro assay, it was able to protect cells in a dose-dependent manner from the effects of either toxin and the antiserum was able to passively protect mice challenged in vivo with either toxin Finally, hybridomas producing monoclonal antibodies against toxin were obtained from the spleens of mice immunized with the same conjugate (Kaufman et al. 1991, Viviani 1992).

Obviously, the most effective form of PSP prevention is to eliminate human contact with contaminated shellfish and other transvectors. Surveillance and closure of commercial shellfish beds by monitoring the amount of PSP using the mouse assay are common practice throughout the world. For example, in the USA, PSP levels in edible shellfish exceeding 800 µg/kg by mouse assay means that commercial beds will be closed until they are monitored below this level; this action level is more than 10 times lower than the lowest level associated with human outbreaks (Anon 1965, ILO 1984). Furthermore, there is active monitoring of algal blooms in fish and bird kills.

Ozonation can remove low levels of toxins from soft-shell clams, but not if the clams have retained the toxin for long periods of time; some industrial canning processes may lead to a decrease in PSP concentration (Halstead 1988; Viviani 1992). Biological controls such as using parasitic dinoflagellates to attack the red tide (for example *Amoebophrya ceratii* parasitizes a variety of dinoflagellates responsible for PSP) have been considered (Viviani 1992).