PHARMACOLOGICAL REVIEWS Vol. 18, No. 2
 Printed in U.S.A.

TETRODOTOXIN, SAXITOXIN AND THEIR SIGNIFICANCE IN THE STUDY OF EXCITATION PHENOMENA[1]

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[1] In reviewing a topic of current interest about which new publications continue to appear, I have had to decide not to include any material appearing after October 31, 1965, except in a few instances where later publications were of great importance to some points in this article.

I. INTRODUCTION

Tetrodotoxin and *saxitoxin* are respectively the purified toxic principles responsible for tetrodon poisoning and paralytic shellfish poisoning. Both forms of poisoning in man have been known for may years. Tetrodon poisoning, also known as puffer-fish poisoning or *fugu* poisoning, occurs chiefly in countries surrounding the South China Sea, particularly in Japan. Paralytic shellfish poisoning occurs epidemically in all parts of the world, and endemically along the coasts of the Pacific northwest and the Canadian maritime provinces. Recently the toxins have been purified and, in the case of tetrodotoxin, the molecular structure has been revealed. Crystalline tetrodotoxin is the most potent of the substances that can interfere with the production of action potentials in certain common excitable cells, being some 160,000 times more potent than cocaine in blocking axonal conduction (70). In this action, tetrodotoxin is also unique in acting only on changes in sodium permeability normally associated with excitation. For these reasons, it is useful in the study of excitation phenomena.

Saxitoxin has not yet been crystallized and its structure is not known. Its biological actions are similar to those of tetrodotoxin (71), although chemically it is probably different. The similarities and contrasts have important implications in the understanding of molecular aspects of excitation. In view of the interest in these toxins both as experimental tools and as possible therapeutic agents, we will consider the actions of these toxins in whole animals as well as on isolated tissues and the potentialities and limitations of their usefulness.

Tetrodotoxin is obtained from the ovaries and eggs of several species of puffer fish of the suborder *Gymnodontes* (102, 171, 189, 192). Another toxin, at first named *tarichatoxin*, because of its origin from some California newts of the genus *taricha* (14, 70), is now known to be identical with tetrodotoxin. Because of priority, the term tetrodotoxin is used to designate both toxins except in cases where the source of the toxin is to be indicated (102). Several other terms have been used to designate tetrodotoxin: *fugu poison*, *spheroidin* (192), and *tetrodontoxin* (159). These terms are rarely seen in current literature, and the term tetrodotoxin is so widely accepted that its continued use should engender no difficulty. *Saxitoxin* is so named because it is extracted from the Alaska butterclam *Saxidomus giganteus* (150). Use of this term, however, has not received the same acceptance as that of tetrodotoxin. Other terms that have been used to designate the same substance are *clam toxin*, *mussel toxin*, and *shellfish toxin*. The first two were used to indicate the source of the material at a time when their chemical identity was not known, and the last as an inclusive term for both. The toxin, however, originates in neither the clam nor the mussel, but most probably comes from an alga, *Gonyaulax*, from which a *gonyaulax toxin* has been obtained (16). For purposes of this article the term saxitoxin is chosen because it imparts a sense of purity that the other terms do not; it is used in preference to gonyaulax toxin because there

is still some doubt that this alga is the only source of the poison in the Alaska butterclam (148).

The new names can be used to discuss various phenomena described in the older literature, because the symptomatology of poisoning after ingestion of contaminated food and the reactions produced by crude extracts of the toxins are all similar to those elicited with the purified toxins. Indeed, throughout the stages of purification of both substances, there were increases in the potency of the toxins, but no changes in the nature of the responses.

Several recent reviews are available on various aspects of tetrodotoxin or tarichatoxin (19a, 48, 102, 120, 172, 189), on saxitoxin (92, 146), and on marine animal toxins in general (143).

II. TETRODOTOXIN

A. History and clinical aspects of tetrodon poisoning

1. History. The history of tetrodotoxin poisoning is as difficult to trace as many common human ailments because it merges into the realm of folklore. One of the earliest references to the subject appeared in the first Chinese pharmacopea, *Pen-T' so Chin* (本草經, *The Book of Herbs or The Herbal*) which was usually attributed to the legendary Emperor Shun Nung (神農, 2838-2698 B.C.).[2] Of the 365 drugs included in *The Herbal*, Shun Nung, who was supposed to have tasted each one to determine its effect, classified them into three categories according to their toxicities: superior, 120 drugs, medium 120, and inferior 125 (187). Tetrodon eggs (豚卵) were classified among the medium drugs that were believed to have tonic effects but could be toxic depending on the dosage. The principal use for which tetrodon eggs were recommended was to arrest convulsive diseases (癲癎疾) (155). A detailed account on tetrodon fish was given in the last and most authoritative pharmacopea of traditional Chinese medicine, *Pen-T' so Kang Mu* (本草綱目, *Index Herbacea*, better known as *The Great Herbal*) published in 1596 A.D. Its author, *Li Shih-Chen* (李時珍) collated all drugs and recipes known up to that time, corrected errors of his predecessors, deleted duplications, filled in omissions, and added 374 new substances to the list of 1518 from earlier works (187). The reference to tetrodon fish appeared in volume 44 of this monumental work under the section "fishes without scales" (無鱗魚) (88). According to the format of the book, the account began with the proper name of tetrodon fish, followed by a series of synonyms, information from the literature, general properties of the substances, recipes, and recommended uses. The Chinese term for tetrodon, still in use today is Ho-Tun (河豚) or literally "piglet (豚) of the river (河)". Nine synonyms and popular names were given: two of these referred to the ability of the fish to distend its abdomen (吹肚魚氣包魚) and can be translated reasonably as blow fish or puffer fish, popular terms now in common use in English. One synonym, Kwei (鮭) was erroneously translated recently as salmon in a quo-

[2] In spite of the claimed early origin of the work, present day scholars of Chinese literature generally agree that it could not have been written before the first or second century B.C. The style of writing, the format, and even the use of the term Pen-T'so are all consistent with the later date.

tation, "salmon liver kills man" (102). Quoting from earlier sources, Li wrote that while the people of some regions ate tetrodon fish frequently without any deaths, the people in the south considered such fish as poisonous. This difference was believed to be due to the presence of two kinds of tetrodon fish. One of them, "light black with spots was called PeiYü (鮭魚)" and has "most abundant poison (毒最甚);" it was said that "after March Pei-Yü could not be consumed (三月後鮭魚不可食)." Li also quoted from earlier works that "tetrodon fish is found in all rivers, streams, and the sea (河豚江淮河海皆有之)," and that the "marine forms are highly poisonous; river forms, next (海中者大毒江中者次之)." The greater part of the account consisted of injunctions against the poisonous tetrodon fish, particularly its liver and eggs. It was known that the "liver and eggs have much poison (肝及子有大毒)" and it was said that "(if taken) in the mouth, (they) rot the tongue; (if taken) internally, (they) rot the guts (入口爛舌入腹爛腸)," and also that "(the poisoning), no remedy can relieve (無藥可解)." Several recipes were given detailing the cooking methods used by the inhabitants of certain regions that seemed to remove the poison. Among these was one of soaking tetrodon eggs in water overnight. Tetrodotoxin is indeed soluble in water and could be leached out, leaving the eggs less toxic. In spite of all the injunctions throughout the ages and the widespread knowledge of the poisonous nature of tetrodon fish, particularly the eggs and liver, some people "salt the eggs and marinate the testis (鹽其子糟其白)." Such practices undoubtedly led to the saying that "to throw away life, eat blowfish (捨命吃河豚)." The chief use for which tetrodon fish (except liver and eggs) was recommended was as a tonic, "to supplant inadequacies (補虛)," a recommendation which has sometimes been narrowly interpreted in the folk-ways of China and Japan to imply effectiveness in cases of sexual impotence in man.

The species of tetrodon chiefly responsible for poisoning in China has since been identified by Japanese scholars as *Tetrodon oscellatus* Osbeck (2, 165). This species migrates up various rivers in China and Korea during spawning season, a fact which can account for the Chinese term referring to the river as the source of the fish. In Japanese tetrodon fish is known phonetically as *fugu* (フグ) which is believed to be derived from the word *fukube* (フクベ), a vase with a rounded belly. The Kangi (Chinese character) designation is, however, identical to that used in Chinese, undoubtedly as a direct adoption from Chinese. This is a curious etymological phenomenon because almost all the poisonous species of tetrodon fish encountered in Japan are marine and not fresh water forms. No doubt the Japanese were equally aware of the poisonous nature of tetrodon fish, albeit their early records were strongly influenced by various Chinese writings. Archeological investigations around Nara, the ancient Japanese capital, have unearthed many bony remnants characteristic of tetrodon fish. Later, when written Japanese assumed forms quite independent of those of Chinese, numerous accounts on tetrodon poisoning appeared in poetry and other forms of literature, as they also did in Chinese (2, 165).

To the Europeans, however, tetrodon poisoning was something entirely new when they began to visit the Orient in the 17th century. Thus, Englebert Kaempfer, who served as physician to the Dutch embassy in Nagasaki from 1690 to

1693, recorded in his classic work, "A History of Japan," (68) first published in 1727, the following account:

"*Iruku* is a known Fish, called *Tenije* in the Indies. *Furube* is another Fish, not very large. The Dutch call him *Blazer*, which signifys *Blower*, because he can blow and swell himself up into the form of a round Ball. He is rank'd among the poisonous Fish, and if eat whole is said unavoidably to occasion death. There are three different sorts of it found in the Japanese seas, all in great plenty. The first sort call'd *Susumebuka* is small and seldom eat, the second is called *Mabuku*, that is true *Buku*. This the Japanese reckon a very delicate fish, and they are fond of it, but the Head, Guts, bones, and all the garbage must be thrown away, and the Flesh carefully wash'd and clean'd before it is fit to eat. And yet many People die of it, for want, as they say, of thoroughly washing and cleaning it. People that by some long and tedious sickness are grown weary of their lives, or are otherwise under miserable Circumstances, frequently chuse this poisonous Fish, instead of a knife or halter, to make away with themselves... Soldiers only and military men, are by special command of the Emporer forbid to buy and to eat this Fish. If any one dies of it, his son forfeits the succession to his father's post, which otherwise he would have been entitled to. It is sold much dearer than common fish and not eat but when fresh. The third sort is called *Kitamakura*, which signifies *North Cushion*. I could not learn the reason of this Appelation.... The poison of this sort is absolutely mortal, no washing nor cleaning will take it off. It is therefore never asked for, but by those who intend to make away with themselves..."

Peter Osbeck, a naturalist who was also physician to the Swedish East India Company, wrote about his first encounter with a tetrodon fish in the Pearl River in Canton, China, and noted that "it is forbidden under some great penalty, to be sold among other fish" (133). Similar were the experiences of European visitors to the Orient in the 18th and 19th centuries, ambassadors, naturalists, and sailors all, in finding the inhabitants of the coastal areas in India, Malaya (29), Cambodia (29), and the Philippines (60) well aware of the toxic nature of tetrodon fish. Spanish missionaries to the New World also found Mexicans in the region of Baja California fully acquainted with the fact (24, also quote in 102).

Direct European experiences of tetrodon poisoning occurred during the second circumnavigational voyage of Captain James Cook. Although the best known episode affected Captain Cook and his naturalists, J. R. and G. Forster, another incident occurred six weeks earlier on July 23, 1774, on board the same ship, H.M. S. Resolution. In the earlier incident 16 men were affected after eating 3 fish which were later identified as *Tetrodon ocellatus* and *Sparus pagrus* (40). The symptoms were varied, but the gastrointestinal symptoms described by the surgeon on board (3) were somewhat more severe than those normally expected of mild tetrodon poisoning. Nevertheless, five men who ate one fish were more severely affected than the rest, and their symptoms, numbness of limbs, flushing, and generalized muscular weakness, were consistent with tetrodon poisoning. Probably some of the victims suffered from other forms of ichthyosarcotoxism (54). There was much confusion on board the "Resolution" at the time and the possibility of *Tetrodon ocellatus* being highly poisonous was not fully realized.

Of Captain Cook's poisoning, G. Forster (39) wrote, "It (referring to the fish)

was of the genus, by Linnaeus, named *Tetrodon*, of which several species are reckoned poisonous. We hinted this circumstance to Captain Cook, especially as the ugly shape, and large head of the fish were greatly in its disfavor, but he told us he had eaten this identical sort of fish on the coast of New Holland, during his first voyage, without the least bad consequence..." Thus reassured, they had the liver (according to the Forsters) or the liver and roe (according to Cook) prepared for dinner, tasted them, became roundly poisoned, but survived to give some of the most accurate descriptions of the symptoms.

Nothing was done in the way of studying scientifically the nature and treatment of tetrodon poisoning until the end of the 19th century when Japan readily and rapidly absorbed the influence of western civilization. In 1884 Remy published the first extensive study on tetrodon poisoning in a western language, and in it he mentioned some earlier work carried on by a Dutch physician, Geerts, stationed in Japan (139). At this time the Japanese government also sent many able young men to western countries to learn the new sciences. Among them was D. Takahashi, who returned to Japan to occupy the first Chair of Pharmacology in the Imperial University in Tokyo (13). Here he commenced a series of investigations of the toxic species of tetrodon found in Japanese waters, the relative toxicities of different organs, systemic actions in various mammals, and also a beginning of chemical extraction of tetrodotoxin (160, 161, 162). In 1894, Tahara, working also in Tokyo, announced the "purification" of an extract from the eggs of spheroides which he termed *tetrodotoxin* (159). This substance had a lethal dose in mice of 7 gm/kg and was given a molecular formula of $C_{16}H_{13}NO_{16}$. Although it was far from being the crystalline tetrodotoxin as the latter is known today, it was the most potent of the various extracts, and was used not only for a series of investigations by various Japanese workers up through the 1940's (65, 66, 72, 76, 83, 117), but also clinically as a pain-relieving compound (*e.g.*, 117). In the limited clinical applications, it was found that patients suffering from the neurogenic form of Hansen's disease (leprosy) were rather resistant to the toxic effects of tetrodotoxin. This stimulated a good deal of interest among Japanese investigators to search (without much success) for the cause of the apparent immunity to tetrodotoxin.

In the 1930's and early 1940's two seemingly unrelated developments were taking place in California. Between July 16 and 18, 1927, there was a dramatic outbreak of paralytic shellfish poisoning around San Francisco involving 102 victims who had eaten local mussels. In studying the cause of this poisoning, workers at the Hooper Foundation at University of California devised an assay method for standardizing the toxicity of their clam extracts (156). The method was based on the rapidity of lethal action on a mouse following an intraperitoneal injection of the toxic extract. A roughly exponential relation was found between the speed of action and the dose, and a mouse unit was defined from this relation. The second series of developments took place 30 miles south on the campus of Stanford University. Victor Twitty, an experimental embryologist who had gone to Stanford from Yale, cast about looking for a local species of salamander to replace the eastern salamander *Amblystoma*, to which he was accustomed. He found *Taricha*

torosa (then known as *Triturus torosus*) suitable for his work, and proceeded to transplant embryonic tissues from taricha embryo into amblystoma embryo, and *vice versa*. To his surprise, when the eye vesicles (and other tissues) of taricha embryo were transplanted into amblystoma, the latter as host remained paralyzed for days (177). Through a series of ingenious transplantation experiments he concluded that taricha embryos contained a toxic substance which selectively paralyzed the nerves of amblystoma (176). Further experiments on this esoteric substance was carried on by a group of biologists at Stanford University (64, 180), who undertook, as one of their first projects, the extraction and purification of the material. In these efforts, they devised an assay method that was similar in principle to the one used in the studies on paralytic shellfish poison. By 1940, a preparation of "*tarichatoxin*" with a potency of 75 mouse units/mg was available for pharmacological studies.

Work on tetrodotoxin in Japan and tarichatoxin in Stanford were interrupted during the war. The next important development came in 1950 when Yokoo announced that he had crystallized a toxic substance from eggs of *Spheroides rubripes*. This substance was lethal to mice in a dose of 15 μg/Kg, and Yokoo named it *spheroidin* (192). Two years later Tsuda and Kawamura (171) published their independent work on purifying the toxic principle from the ovaries of *Spheroides rubripes*, which they named *tetrodotoxin*, a term originally used by Tahara. Spheroidin and tetrodotoxin were shown to be identical in 1956 (4) and the term tetrodotoxin became generally accepted. Renewed efforts in purifying tarichatoxin began in 1960 (14); by the summer of 1962 a crystalline preparation was obtained having a potency of 7000 mouse units/mg. Pharmacological and chemical studies on tarichatoxin indicated that it was very similar to tetrodotoxin (14, 70, 102). Definitive identification, however, could not be made until some uncertainties concerning the chemical composition of tetrodotoxin were resolved (Section II C). When this last obstacle was removed, tarichatoxin and tetrodotoxin were identified as one and the same substance (15). The structure of tetrodotoxin engaged the interest of a number of organic chemists in Japan and in the United States. In April 1964 at the IV International Symposium on the Chemistry of Natural Products in Kyoto, all participants interested in this problem, including Tsuda, Goto, Harada, Woodward and Mosher, agreed on a unique structure for tetrodotoxin. So the search for the toxic principle in tetrodon fish ended successfully after several thousand years. This new material, by its extraordinary potency and the uniqueness of action is offering new opportunities in the study of excitation phenomena.

2. Classic descriptions of poisoning. The overwhelming course of severe tetrodon poisoning can be appreciated from an account given by a Dr. Julius Hellmuth, who was surgeon on board the Dutch brig of war "Postilion" (140). On September 4, 1845, in Simon's Bay on the Cape of Good Hope, two men, a boatswain and a purser's steward who had already had the ship's lunch decided to eat a local puffer fish (identified as *Diodon oscellatus*) as an experiment even though they were aware of the poisonous nature of the fish.

"Scarcely ten minutes had elapsed (since ingestion of the fish's liver) when I

(Hellmuth) was called upon to afford medical assistance to both, and observed the following symptoms. J. Kleinhaus (the boatswain) lay between decks, and could not raise himself without the greatest exertion; his face was somewhat flushed; his eyes glistening, and pupils rather contracted; his mouth was open, and as the muscles of the pharynx were drawn together by cramp, the saliva flowed from it; the lips were tumid and somewhat blue; the forehead covered with perspiration; the pulse quick, small and intermittent. The patient was extremely uneasy and in great distress, but was still conscious . . . .The state of the patient quickly assumed a paralytic form; his eyes became fixed in one direction; his breathing became difficult, and was accompanied with dilation of the nostrils; his face became pale and covered with cold perspiration; his lips livid; his consciousness and pulse failed; his rattling respiration finally ceased. The patient died scarcely seventeen minutes after partaking of the liver of the fish. . . ." "Almost the same symptoms, following each other with equal rapidity, appeared in J. Hansen (the purser's steward); vomiting ensued before an emetic was administered to him . . . . He was still conscious, and said that he felt easier (after vomiting three times); expressing at the same time some hope; the pulse became softer; the vomiting was again repeated; but in a few moments, a single convulsive movement in the arms ensued, whereupon the pulse disappeared, and the livid tongue was protruded from between the lips. His death took place about one minute later than that of his messmate."

In milder cases of poisoning sensory and motor disturbances are not so deeply masked by severe cardiovascular derangements, and the neurological disturbances become the most prominent aspect of the symptomatology. Thus, Captain Cook (26) described his own experience as:

"Having no suspicion of its being of a poisonous nature (referring to the fish), we ordered it to be dressed for supper; but very luckily, the operation of drawing and describing took up so much time that it was too late, so that only the liver and row were dressed, of which the two Mr. Forsters and myself did but taste. About three o'clock in the morning, we found ourselves seized with an extraordinary weakness and numbness all over our limbs. I had almost lost the sense of feeling; nor could I distinguish between light and heavy bodies of such as I had strength to move, a quart pot, full of water, and a feather being the same in my hand . . . ."

In the Beaglehole edition of Cook's journal (7) the description was more detailed:

"About three or four o'clock in the morning we were seized with an extraordinary weakness in all our limbs attended with a numbness or sensation like that caused by exposing one's hands or feet to a fire after having been pinched much by frost. . . ." In addition to these, Rochas' description (p. 15 of ref. 139) "leger picotement. . . fourmillement aux extrémités" leaves little doubt that the sensory disturbances are to be characterized as parasthesia in modern terminology.

Not much can be added to these descriptions of the symptomatology, except that in physical findings made with diagnostic tools now routinely in use, low blood pressure is a constant finding in cases of moderate to severe poisoning. Circumoral parasthesia and numbness of the tongue are also early manifestations.

3. Public health problems of tetrodon poisoning. Except in Japan, tetrodon poisoning is not a public health problem. In the U. S., farsighted fishery and health specialists were, at one time, concerned with the possibility of tetrodon poisoning. Their concern was based on the observation that since World War II there has been a more frequent appearance in the eastern fish markets of blowfish which had been considered up to then as "trash fish", *i.e.*, not worthy as food (193). A second reason for their concern is the trend of turning to the sea as an increasing source of food for the world's population (55). Undoubtedly, these are both important reasons which merit the most serious considerations. Up until the present time, however, only occasional cases of poisoning by tetrodon fish have been reported in the United States (8).

Even in Japan, tetrodon poisoning cannot, in all fairness, be considered as a public health hazard. This statement is based on the observation that the number of deaths caused by tetrodon poisoning have remained quite stable at slightly more than 100 persons every year since 1886. Considering the amount of fish consumed in Japan, and the incidence of other forms of food poisoning, fatality due to tetrodon poisoning is insignificant. Tetrodon poisoning, however, is characterized by its dramatically severe course, and by frequent multiple deaths in the same household. Ogura has summarized the available statistics on tetrodon poisoning in Japan between 1886 and 1958 (119). The mortality rate of tetrodon poisoning has declined from about 80 per cent of the poisoned victims in the 19th and early 20th centuries to slightly more than 50 per cent in the years 1953–1958. The decline is attributed to both improved education of the public and improved therapeutic measures. Nevertheless, since the death rate is about the same in 1958 as in 1886, the incidence of poisoning has increased substantially in recent years. There is no explanation for this increase, but one speculation is that since tetrodon fish have become scarce in off-shore waters in Japan, the fishing fleets had to go farther out to sea to obtain such fish which may be more toxic than those living closer to the shores (Ogura, personal communication). Another possibility is that it is related to the increase in population, which more than doubled between 1891 and 1958.

Legislative control aimed at preventing tetrodon poisoning in Japan has undergone several forms from outright prohibition of sale of the fish to more realistic measures (41). These measures are within the scope of prefecture (state) laws, and since tetrodon poisoning is uncommon north of Tokyo, such measures, as they exist, are found only in the southern prefectures. At present, control measures exist in only 7 of the 46 prefectures (Tokyo, Kanagawa, Kyoto, Osaka, Hiroshima, Ehime, and Fukuoka). These laws generally take the form of licensing handlers of tetrodon fish in special restaurants. License is granted if the individual is judged knowledgeable on the species and seasonal variations in toxicity, and capable of eviscerating toxic species of fish without cutting the liver and roe. The effectiveness of these measures can be appreciated from the following illustration (119). In 1957, there were 119 episodes of poisoning involving 176 persons. Of these only 2 episodes involving 2 persons followed the eating of *fugu* fish in a licensed restaurant. One hundred and six episodes involving 155 persons were attributed to sale and preparation of fish privately by fishmongers; and 11 epi-

sodes involving 19 persons occurred among vagabonds. Of the 176 persons poisoned, 90 died; these included neither of the restaurant cases, but 81 of the group involving private transactions, and 9 of the vagabonds. From such information, it may be fair to say that the incidence of tetrodon poisoning in Japan has been brought to the lowest possible rate. To further reduce this incidence may require very drastic measures that would probably be as successful as Prohibition was in the United States.

The heat stability of tetrodotoxin has been studied by Halstead and Bunker (55), who found that after the usual commercial canning procedure much of the toxicity contained in cut whole *spheroides* fish was lost. The procedures included heating to 100° for 10 minutes and heating under pressure at 116.6°, but these steps did not inactivate completely the tetrodotoxin present. The canning process had the disadvantage of spreading the toxin from toxic organs to non-toxic tissues unless the toxic organs were first removed, a procedure which would be economically prohibitive to the industry.

B. Occurrence and distribution of tetrodotoxin

1. Species and habitat. Table 1 shows a list of animals known to contain tetrodotoxin in their tissues. As can be seen, among fishes, all of the toxic species are members of four families in the suborder *Gymnodontes*, and among amphibians, only closely related species in the family *Salamandridae*. The extensiveness of the distribution of tetrodotoxin among animals is not completely known because there are still more animals that have not been examined for the presence of the toxin than those that have been. It is known, however, that some closely related species of fish in the suborder *Gymnodontes* such as *Lactophrys tricotuis*, *Lagocephalus spadieus*, *Liosaccus cutaneous*, *Chilomycterus affinis*, and *Ostracion immaculatum* do not contain tetrodotoxin (86, 164). Among the amphibians, members of the order *Caudata* other than those of the family *Salamandridae* have no tetrodotoxin; even among the *Salamandridae*, not all members contain tetrodotoxin (181). The species that have no tetrodotoxin include *Cryptobranchus alleganiensis*, *Necturus maculosus*, *Siren lacertiae*, *Siren intermedia*, *Ambystoma tigrinum*, *Salamandriae salamandra*, *Amphiuma means*, *Batrachoseps attenuatus*, *Ensatina eschscholtzi*, *Aneides lugubris*. In the order *Salientia*, *Bufo boreas*, *Hyla cinerea*, *Rana pipiens* and *Xenopus laevis* have no tetrodotoxin. The presence of tetrodotoxin in two widely different animal forms is an interesting and perplexing phenomenon (102) which becomes even more apparent with the knowledge that the toxin is restricted to only some members of closely related species.

2. Distribution in tissues. Table 2 shows the concentration of tetrodotoxin reported for various tissues of tetrodon fish and taricha torosa. The quantities shown for tetrodon tissue are recalculated from Tani's results on the basis of the following reasoning. In his extensive study Tani (164) expressed the toxicities in an unusual unitage; 1 gram of tissue was defined as containing 1000 units of toxin, if by a standard assay procedure (Section II D 1) it was potent enough to kill 1000 grams of mice. Since the LD50 of crystalline tetrodotoxin in mice is 10 μg/kg intraperitoneally, 1000 Tani units lethal for 1 kg of mice would be roughly equiv-

TABLE 1

Animals containing tetrodotoxin and tetrodotoxin-like substances and their distribution

Species	Habitat	Reference
TETRAODONTIDAE		
Amblyrhynchotes honckenyi Block	New Caledonia, Japan, So. Africa	12
Arothron hispidus Linnaeus	Philippines, Tahiti, Hawaii, Tropical Pacific in general	12, 55
Arothron meleagris Block and Schneider	Pacific	12
Arothron nigropunctatus Block and Schneider	India, So. Pacific	12
Arothron reticularis Block and Schneider	India, Philippines, Guam, Mariannes	12
Arothron stellatus Block and Schneider	India, Japan, Hawaii, Malaysia, Red Sea	12
Spheroides alboplumbeus Richardson	Japan	12, 164
Spheroides chrysops Hilgendorf	Japan	12, 160, 164
Spheroides hamiltoni Richardson	Japan	12
Spheroides immaculatus Block and Schneider	India, New Caledonia, Philippines, China, Japan	12
Spheroides niphobles Jordan and Snyder	Japan	12, 164
Spheroides oblongus Block	India, New Caledonia, Philippines, China, Japan, Pacific	12
Spheroides oscellatus Osbeck	India, China, Japan, Yang-tze River, Nile	12, 164
Spheroides pardalis Temminck and Schlegel	Japan, China	12, 139, 160, 164
Spheroides porphyreus Temminck and Schlegel	Japan	12, 160, 164
Spheroides rubripes Temminck and Schlegel*	Japan	12, 139, 160, 164
Spheroides sceleratus Gmelin	India, Japan, Tahiti	12
Spheroides stictonotus Temminck and Schlegel	Japan	12, 160, 164
Spheroides vermicularis Temminck and Schlegel	Japan	12, 139, 160, 164
Spheroides pseudommus	Japan	164
Spheroides xanthopterus Temminck and Schlegel	Japan	12, 164
Spheroides basilewskianus	Japan	164
Spheroides maculatus Block and Schneider	Atlantic	84, 193
Spheroides annulatus	Atlantic	46
Spheroides testudineus Linnaeus	Florida, Gulf Coast	86
Tetraodon fluviatilis Hamilton and Buchanan	India, Philippines, Indochina (esp. Cambodia)	12
Tetraodon heraldii Gunther	California	12
Tetraodon lacrymatus Cuvier	Polynesia	12
Tetraodon laevigatus Linnaeus	Japan	12
Tetraodon lineatus Block	India, Japan, Nile, and East Africa	12, 139

TABLE 1—*Continued*

Species	Habitat	Reference
Tetraodon rivulatus Schlegel	Japan	12, 139, 160
Tetraodon spengleri Block	Tropical Atlantic, Senegal, Brazil	12
LAGOCEPHALUS		
Lagocephalus laevigatus	Florida	86
Lagocephalus lunaris Block	Japan	164
DIODONTIDAE		
Diodon holacanthus Linnaeus	Japan, Tahiti, Hawaii, New Caledonia	12
Diodon hystrix Linnaeus	Japan, Tahiti, Hawaii, New Caledonia	12
CANTHIGASTERIDAE		
Canthigaster bennetii Bleeker	India	12
Canthigaster cinctus Richardson	Hawaii	12
Canthigaster rivulatus Temminck and Schlegel	Japan, Hawaii	12, 164
CHILOMYCTERUS		
Chilomycterus schoepi	Florida, Gulf Coast	86
SALAMANDRIDAE		
*Taricha torosa**	California	181
Taricha torosa sierrae	California, Sierra Nevada	181
*Taricha rivularis**	North California	181
Taricha granulosa	California	181
Notophthalmus viridescens	Eastern U.S.	181
Cynops pyrrhogaster	Japan	181
Cynops ensicaudus	Ryuku Islands	181
Triturus marmoratus	Italy, Spain	181
Triturus vulgaris	Europe	181
Triturus cristatus	Europe	181
Triturus alpestris	Europe	181

* Only in these, has tetrodotoxin been identified chemically. In all others, identification was made by bioassay.

alent to 10 μg of crystalline tetrodotoxin. The order of magnitude of the concentration is probably not far wrong and the relative toxicities are certainly valid. To simplify the presentation, the data on toxicity of tissues of male fish have not been included in the table. In every case, the toxicity of a tissue from a male fish is significantly lower than that of the corresponding female tissue. Only in *S. niphobles*, *S. alboplumbeus*, and *S. pardalis* do the testes contain appreciable concentrations of toxin, and these range from $\frac{1}{40}$ to $\frac{1}{10}$ of the concentrations in the corresponding ovaries. The only exception to this sex-linked difference occurs in *L. inermis* in which the concentration of toxin in the male liver is 20 times that in the female liver. In general, the blood and muscles of tetrodon fish contain relatively little toxin.

Tani's objective in making these assays was largely oriented towards the public health point of view. He estimated the total toxin content in average fish avail-

TABLE 2

Concentration of tetrodotoxin in various tissues of tetrodon fish and taricha newt

Concentration: μg tetrodotoxin/gm fresh tissue.

Species*	Ovary	Liver	Skin	Intestines	Muscle	Blood
S. niphobles	400	1,000	40	400	4	1
S. alboplumbeus	200	1,000	20	40	4	
S. pardalis	200	1,000	100	40	1	1
S. vermicularis	400	200	100	40	4	
S. porphyreus	400	200	20	40	1	
S. oscellatus	1,000	40	20	40	<0.2	
S. basilewskianus	100	40	4	40	<0.2	
S. chrysops	40	40	20	4	<0.2	<0.2
S. pseudommus	100	10	4	2	<0.2	
S. rubripes	100	100	1	2	<0.2	<0.2
S. xanthopterus	100	40	1	4	<0.2	
S. stictonotus	20	<0.2	2	1	<0.2	
L. inermis	0.4	1	<0.2	0.4	0.4	
C. rivulatus	<2	2	40	4	<0.2	
Taricha torosa ♀	25	<0.1	25	(0.1)†	2	1
Taricha torosa ♂	<0.1‡	<0.1	80	(0.5)†	8	21

* All but the last two are toxicities of female tissues recalculated from Tani (164). See text for details. Last two are from Wakely *et al.* (181).

† "Visceral organ."

‡ Testis in this case.

able on the Japanese market, since the severity of clinical poisoning in man depended on both the concentration of toxin and the quantity of the toxic organ ingested. Because of its small size, *S. niphobles* is only a weakly toxic species even though the concentrations of toxin in some tissues are among the highest. According to the total amounts of toxin in an average sized fish of each species, the order in decreasing toxicity is: *S. porphyreus* > *S. rubripes* > *S. pardalis* > *S. alboplumbeus* > *S. basilewskianus* > *S. vermicularis* > *S. xanthopterus* > *S. chrysops* > *S. oscellatus* > *S. pseudommus* > *S. niphobles* > *L. inermis* > *S. stictonotus* > *C. rivulatus*. It is well known in Japan that most cases of clinical poisoning are caused by the first two species, *S. porphyreus* (ma fugu) and *S. rubripes* (tora fugu).

The data on the toxicity of tissues of taricha torosa are taken directly from recent studies of Wakely, Fuhrman, Fuhrman, Fischer, and Mosher (181). The chief differences from tetrodon tissues are that the skin of taricha torosa contains the highest concentration of toxin and that the blood and muscles are relatively more toxic than those of tetrodon fish. Interestingly enough, the influence of sex appears to be just the converse: the skin and blood of male taricha torosa are among the most toxic tissues.

3. Seasonal variation. Since the ovaries contain a high concentration of tetrodotoxin, it might be expected that when the ovaries mature and grow in size, the total amount of toxin present would increase. No doubt this is an important basis

for the age-old knowledge that tetrodon fish become most poisonous as they enter the spawning season. The increase in toxicity, however, is not simply a matter of increasing total quantities of toxin. Tani's assays (164) showed clearly that the concentration of toxin per gram of ovary increased markedly with an increase in the weight of the ovaries. The concentration of toxin in the liver also increased in a parallel manner, even though the liver weight did not change appreciably. There were some species variations in the relative concentrations of toxins in the ovary and in the liver. In most cases, the hepatic concentration was lower than the ovarian concentration, but in some species the reverse was true.

There is a correlation between the incidence of human poisoning and the concentrations of tetrodotoxin in fish tissues. The incidence of poisoning in Japan is lowest in the summer and early fall months, August, September and October, and highest in the winter months, December, January and February. The toxin concentrations are also lowest in the summer and fall, and highest in the winter. Considerable species and individual variation exists in the timing of the seasonal fluctuation in toxicity. Although the incidence of human poisoning gradually declines in the spring, high concentrations of toxin can often be found in the ovaries of both *S. rubripes* and *S. porphyreus* as late as May and June (tables 4 and 8 of ref. 164). For *S. oscellatus*, which is mainly responsible for poisoning in China and Korea, the highest ovarian concentration of toxin is found in the summer, and so is the incidence of poisoning.

Until now interest in the seasonal variation of toxicity has been confined to the public health aspects of tetrodon poisoning. Actually there are also some interesting problems concerning the biosynthesis of tetrodotoxin, for these biosynthetic processes are apparently strongly influenced by processes that control sexual maturation in the female fish.

C. Chemistry

Tetrodotoxin is an amino perhydroquinazoline compound with a molecular formula of $C_{11}H_{17}N_3O_8$. It is a colorless prism which in aqueous solution is a monacidic base with a pKa at 8.5. It is only sparingly soluble in water except in a slightly acidic condition, yet at low pH it is not indefinitely stable. In alkaline conditions tetrodotoxin is readily degraded into several quinazoline compounds.

Several authoritative accounts on the isolation, purification and structural identification of tetrodotoxin have been published recently (48, 102, 172, 189). The structure of tetrodotoxin is shown in figure 1. Several unusual features are immediately apparent: an abundance of OH groups, a unique hemilactal link between two separate rings (positions 5 and 10), and a guanidinium group constituting an integral part of the molecule. The large number of OH groups, together with the highly polar nature of tetrodotoxin, leading it to retain water and other solvents, caused many difficulties in ascertaining the exact elemental content of the compound (189). It was only relatively late in the course of structural studies on tetrodotoxin that the molecular formula was changed from $C_{12}H_{19}N_3O_9$ to $C_{11}H_{17}N_3O_8$ (48). In the meantime, since direct analyses were of little avail, the structure of tetrodotoxin was established principally through arduous studies on

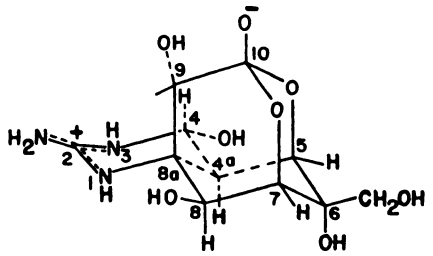

FIG. 1. Structure of tetrodotoxin (48)

a series of degradation products. The oxygen bridge between C_5 and C_{10} is unique among known organic structures. It is formed between groups that are not normally expected to react but as a result of close stereochemical apposition (189). The normally high pKa of the guanidine group of tetrodotoxin is masked by the acidic OH group at C_{10}, which has a pKa of about 8.5. The end NH_2 group enters into formation of a *zwitter ion* with one of the OH groups. Because of some uncertainties concerning the elemental contents of tetrodotoxin, Tsuda *et al.* at one time considered the possibility that tetrodotoxin might occur as a dimer of C_{22} composition. This suggestion was recently re-examined by Woodward and Gougoutas, who concluded that the monomer is the most likely form (190).

Several structural variations have been made in the molecule of tetrodotoxin (170). The biological actions of these compounds have been studied by Deguchi (personal communication), whose findings generally agree with those obtained from studies on the lethal doses of these compounds. Table 3, based on results of Tsuda *et al.* (170) show the relative lethalities of these compounds as compared to tetrodotoxin. That rather minor changes in the C_4 position results in marked alteration in lethality suggests that the OH at C_4 is of great importance for biological action. This is also shown by the low lethality of anhydrotetrodotoxin in which a second oxygen bridge was produced between C_4 and C_9. The original oxygen link between C_5 and C_{10} is essential for action since tetrodonic acid, which does not have this oxygen link, is almost completely inactive. On the basis of the similarities of action of tetrodotoxin and saxitoxin, Kao and Nishiyama (71)

TABLE 3

*Relative lethalities of tetrodotoxin and some of its derivatives**

Compound	Group on C_4	Relative Lethality
Tetrodotoxin	—OH	1.000
Anhydrotetrodotoxin	—O—	0.001
Tetrodaminotoxin	$—NH_2$	0.010
Methoxytetrodotoxin	$—OCH_3$	0.024
Ethoxytetrodotoxin	$—OC_2H_5$	0.012
Deoxytetrodotoxin	$—H_2$	0.097
Tetrodonic acid	—	0.000

* Modified from Tsuda *et al.* (171).

recently speculated that the guanidinium end of tetrodotoxin may be the working end of the molecule, and that groups close to the guanidinium moiety may be particularly important. These suggestions, made independently, appear to be consistent with the observations of Tsuda *et al.* (170).

D. Pharmacological actions

1. Methods of detection. There is no specific chemical method of determining tetrodotoxin. Several bioassay methods are in use, and they are reasonably specific when properly performed. One of these was first developed in 1941 in Stanford for studying tarichatoxin, and was used throughout 1958–1962 during the purification of tarichatoxin with very satisfactory results. The success of this method depends on the presence of a roughly exponential relation between the rapidity of death of a mouse on injection of tetrodotoxin and the dose given. As used initially the toxin was administered subcutaneously (64) to mice of 20-gram body weight; in recent years it was injected intraperitoneally (70). By appropriately diluting the concentration of toxin to produce death within 1 to 15 minutes it was possible to obtain rather reproducible estimates of the toxin given. In this method, one mouse unit (M.U.) was defined as that amount of toxin which killed a 20-gram mouse in 10 minutes. Since tarichatoxin was crystallized it has been established that 1 mg of crystalline tarichatoxin contained 7000 M.U. (102), *i.e.*, 1 M.U. is equivalent to 143 ng of crystalline toxin. As used originally, various products of chemical purification were injected directly. Recently this assay has been used to detect tetrodotoxin-like substances in animal tissues; these must first be homogenized, and the toxin extracted and concentrated (181). The accuracy of this method for detecting such natural substances was ascertained by comparison with crystalline tetrodotoxin. The sensitivity of this method for pure tetrodotoxin is 0.3 to 3 μg, or somewhat lower in the presence of interfering substances. As pointed out by Kao and Fuhrman (70) death probably results from a widespread axonal block in the body. From this consideration the exponential relation between death time and dose would appear to depend as much on the absorption and distribution of the toxin as it does on the mechanism of action. Surely, many variable factors, such as strain of animal used, the technique of injection, the volume, *etc.*, influence the result of the assay. Fortunately, by standardizing as many variable factors as possible, one can make the method surprisingly reproducible. A test of the susceptibility of several different strains of mice also showed no substantial variability within the resolution of the method (70). In some ways this method is more convenient than a method using quantal response, relying on the determination of the LD50 or some other such statistical evaluation. The reason is that the dose-response relation with tetrodotoxin is very steep and a LD50 is not easy to establish without using a substantial number of animals and doses (Section II D 2). A similar method had been used by Bartsch, Drachman, and McFarren for assaying the toxicity of central Pacific tetrodon fishes (fig. 1 in ref. 12). In their definition, however, one mouse unit was that amount of toxin which caused death 30 minutes after intraperitoneal injection. Since the time to death *vs.* dose curves are exponential, there is no simple relation-

ship between this unitage and that used by the Stanford group on tarichatoxins. If death occurred in 10 minutes, one Stanford M.U. would be equivalent to about 2.7 M.U. of Bartsch *et al.*

Since the availability of crystalline tetrodotoxin, Ogura has produced a similar graph relating the time to death after intraperitoneal injection and the dose in micrograms of crystalline tetrodotoxin (120). This graph is intended primarily for use by public health workers in Japan for estimating the toxicity of tetrodon fish.

Another type of bioassay, of which two variations are available, is based on an inhibitory action of tetrodotoxin on contractions of certain isolated smooth muscle preparations (120). The first (56, 57) involves an inhibition of a nicotine-induced contraction in isolated guinea pig ileum. In this tissue contractions can be produced by many other substances, such as acetylcholine, histamine, serotonin, substance P, and some polypeptides. Tetrodotoxin does not interfere with the contractions produced by these substances to any significant degree but it does prevent the nicotine contractions. The reason is probably that nicotine produces contractions through the ganglionic plexuses and that these are blocked by tetrodotoxin, while the other agents directly stimulate the smooth muscle cells, and these are not blocked by tetrodotoxin. The degree of inhibition of nicotine contraction is linearly related to the logarithm of the concentration of tetrodotoxin. The sensitivity of this method is 5 to 25 ng tetrodotoxin/ml in an organ bath of 30 ml.

The second variation is based on an inhibition of contractions of rat stomach elicited by vagal stimulation (122). Between the concentrations of 1 and 10 ng tetrodotoxin/ml the degree of inhibition is also a linear function of the logarithm of concentration. The sensitivity of 1 ng/ml is five times better than the assay on guinea pig ileum. In neither method is the inhibition so specific that any biological material can be tested directly. Tissue homogenates must be extracted with methanol at pH 5.8 and the residue evaporated to dryness. To this is then added 80 per cent phenol, which serves to separate tetrodotoxin from amino acids. The mixture is then passed through a cellulose column, which is subsequently washed with ether. Tetrodotoxin is eluted with water and enough 0.1 N acetic acid to bring the eluent to pH 6. Finally the eluent is concentrated by evaporation at room temperature. Recovery of tetrodotoxin by this extraction is 84 per cent (120).

The principle of these two bioassay methods devised by Ogura is ingenious, because it is based on a specific action of tetrodotoxin on the neural elements. They probably have not yet been exploited fully. For instance the absolute sensitivity of the methods probably can be increased if the volume of the organ bath is reduced. Thus if the sensitivity in some preparations is 25 ng tetrodotoxin/mg in a 30-ml bath, the absolute sensitivity is less than the best that can be obtained by the Stanford assay method (0.3 μg).

Tani assayed the toxicity of various species of tetrodon fish and their organs by still another method (164). After obtaining an extract from raw fish material by a standardized procedure, he injected 10-, 5-, and 2-fold dilutions of this ex-

tract and the undiluted extract into groups of three mice. The most dilute solution killing two out of three mice within 2 hours was used for estimating the toxicity of the raw material. The conversion is made on the basis that 1 ml of the original extract corresponded to 1 gram of raw material, which was considered as containing 1000 units if there was enough toxin to kill 1000 g of mice (50 to 66 individual mice). The essence of this method is the establishment of a dose-mortality relation statistically, and Tani was able to make an exhaustive study of the relative toxicities of tetrodon fish.

It is obvious from the foregoing discussion that there is an urgent need for a common bioassay method, convenient and sensitive, by which all investigators can express the toxicity of tetrodotoxin-containing material on a common scale. In spite of the many excellent pharmacological studies made by Japanese investigators since the very beginning of scientific work on tetrodotoxin, there has never been a uniform assay method. In much of the old work there is no way of making reasonable estimate of the concentration of toxin used. Similarly, new work will continue to bring confusion unless some uniform unitage is adopted.

2. Comparative pharmacology. For many years it has been known that tetrodotoxin can be fatal not only to man but also to dogs, cats, hogs, birds and other species (3, 26). In 1919 Ishihara (65) studied the lethal dose of tetrodotoxin in 22 species of animal. He used a Tahara toxin that was about 0.002 times as active as crystalline tetrodotoxin (*cf.* 172). Ishihara was careful to standardize all his toxin preparations so that the relative susceptibility of various animals he found remained valid. Because of the lack of a reliable bioassay method, however, there is no sure way of converting his dosages into doses of crystalline tetrodotoxin. In table 4 are listed Ishihara's findings and an approximation of the doses of crystalline tetrodotoxin estimated from present knowledge of the lethal dose in mice. There was no special predilection on the basis of phyla, and closely related species may be quite different in susceptibility. In all species affected, paralysis was the characteristic manifestation and death was usually by respiratory failure. Only in the mouse was there generalized agitation and convulsion. In dogs and cats vomiting occurred frequently.

Of all the animals Ishihara examined, vertebrates and invertebrates, warm-blooded or cold-blooded, two species were resistant to tetrodotoxin. One of these was a tetrodon fish, *Spheroides vermicularis*, from the ovaries of which the toxin used was obtained. The other, to the surprise of Ishihara, was a common Japanese salamander, *Diemictylus pyrrhogaster* Boie. This species is now classified as *Cynops pyrrhogaster*, and is known to contain a moderate amount of tarichatoxin (181). Ishihara's finding of a resistance in newts has been confirmed in recent experiments on a closely related newt of the genus *Taricha* (15). Isolated and desheathed sciatic nerves from several California taricha newts are at least 30,000 times more resistant to tetrodotoxin than are similar preparations from frogs (15). Conversely, isolated and desheathed brachial nerves from *Spheroides maculata* are about 1000 times more resistant to tarichatoxin than frog nerves (Kao and Fuhrman, unpublished). Since tetrodotoxin and tarichatoxin are identical, the cross-resistance is understandable. What is not clear, but is probably of some importance, is why these nerves are so resistant (see Section II D 5).

TABLE 4

Comparative lethality of tetrodotoxin in various animals

	Ishihara's Solution MLD*	Crystalline Tetrodotoxin MLD†
	ml/kg	*μg/kg*
Plaice (paralichthys olivaceus)	0.05	0.5
Dragonfly	0.13	1.3
Carp	0.2	2.0
Pigeon	0.27	2.7
Rat	0.27	2.7
Sparrow	0.4	4.0
Guinea pig	0.45	4.5
Frog	0.5	5
Hen	0.6	6
Rabbit	0.8	8
Mouse	0.8	8
Dog	0.9	9
Cat	1.0	10
Turtle	4.6	46
Eel	8.0	80
Toad (Bufo)	20.0	200
Snake (non-poisonous, species not given)	45.0	450

* Minimal lethal dose following subcutaneous injection (65).

† Calculated from later data (70).

Few pharmacological agents have as steep a dose-response relation as does tetrodotoxin. This peculiar property can be appreciated from the observation that while the minimal lethal dose, given intraperitoneally to 20-gram mice, is 8 μg/kg, with 12 to 14 μg/kg all the mice used in the assay procedure are killed (70). This abrupt dose-mortality response makes it difficult to determine the LD50, but the LD50 estimated graphically is 10 μg/kg in mice (70, 184). As might be expected the LD50 after oral administration is considerably higher, being 332 μg/kg for mice (144); the LD50 after subcutaneous injection is 16 μg/kg (144).

At these doses, tetrodotoxin together with saxitoxin, which has a similar LD50 (Section III D 2) are among the most potent non-protein poisons known. They are still much weaker than botulinum toxin, a protein which has a LD50 in mice of 2 pg/kg, and are about comparable to batrachotoxin, a steroid, which has a LD50 of 2 μg/kg (28). By contrast, all these are considerably more potent than the much feared sodium cyanide, which has an LD50 of 10 mg/kg. A more extensive comparison of the lethalities of a number of poisons can be found in a review by Mosher *et al.* (102). A comparison of the potencies of these toxins on the basis of molar concentration cannot be made because some of them such as botulinum toxin are not distributed evenly throughout body water. For tetrodotoxin, the assumption of such a distribution appears justifiable and the minimal lethal concentration in the body water is probably less than 100 nanomolar.

3. Absorption, distribution and fate. Tetrodotoxin is readily absorbed from the gastrointestinal tract, as evidenced by cases of poisoning in man and animals. The rapid onset of circumoral parasthesia and numbness of the tongue suggest that

absorption through the buccal mucosa probably occurs with ease. The rate of absorption from the gastrointestinal tract is probably rapid also, since in serious cases of poisoning death can occur in less than 30 minutes. From all other parenteral sites, tetrodotoxin is readily absorbed. On the rabbit cornea, in a solution at pH 7, tetrodotoxin is not as potent an anesthetic as might have been expected on the basis of its being nearly 160,000 times more potent than cocaine on isolated nerves. The simplest explanation for this is that absorption of tetrodotoxin through the cornea is limited (70). Ishihara found that application of tetrodotoxin to the rabbit cornea even in rather large doses did not cause death of the animal (65). The only other ineffective route of administration was injection into the anterior chamber of the eye (65).

There is relatively little knowledge concerning the distribution of tetrodotoxin in the body. From the rapid onset of a variety of effects involving different organs of the body, Kao and Fuhrman (70) surmised that tetrodotoxin was widely distributed in the body. Ogura (120) found that, after a single subcutaneous injection of crystalline tetrodotoxin, detectable amounts are present in the rat's kidneys, heart, liver, lungs, intestine, brain, and blood. The peak concentration in these tissues was reached in 20 minutes. The concentrations are highest in the kidneys and the whole heart and lowest in the brain and blood. Little is known about the fate of tetrodotoxin in the body. From Ogura's data (120), the half-time of disappearance of tetrodotoxin varies from about 30 minutes in the whole heart to 3 to 4 hours in the kidneys and liver. The relatively rapid appearance of high concentrations of tetrodotoxin in the kidneys and the slow disappearance from this organ may indicate that an appreciable amount of tetrodotoxin is excreted in the urine in an unchanged form.

4. Systemic actions. a. Neuromuscular systems. One of the most prominent actions of tetrodotoxin in the whole animal is a rapidly progressive and marked weakening of all voluntary muscles including the respiratory muscles. In free roaming quadruped mammals the first sign appears as a peculiar wobbling gait involving the hindlimbs; this is followed by weakening of the forelimbs (22, 39, 40, 65, 70, 104, 160, 191). This progression has led some investigators to describe an ascending type of paralysis (104) with implications that special spinal mechanisms might be involved. It is possible, however, that the apparent progression from the hindlimbs to the forelimbs is simply the result of different weight-bearing requirements of the limbs. Certainly in human cases of poisoning muscular weakness develops first in the hands and arms and then in the legs.

When neuromuscular transmission is studied by recording muscular contractions elicited by neural stimulation, a certain amount of confusion can result. In a partially blocked state, it is always possible to elicit a brief tetanic contraction by increasing the frequency of neural stimulation and usually a post-tetanic increase in the twitch height (22, 70). It is also possible to elicit a contraction by injecting concentrated acetylcholine intra-arterially (22, 70). At first sight, these reactions are very similar to those in a curare-like block, but the interrelation with other drugs is quite different. Although tetrodotoxin block is potentiated by tubocurarine or succinylcholine, it is not antagonized by neostigmine or edropho-

nium (22, 72, 79, 82) or by decamethonium (82). Moreover, when tetrodotoxin produced a complete block, increased neural stimulation neither broke the block nor caused a post-tetanic reversal, and intra-arterially injected acetylcholine was without effect (22, 70). This seemingly complex manifestation is now readily explained in the light of new knowledge on the cellular actions of tetrodotoxin. Neuromuscular transmission is interrupted by tetrodotoxin not at the end plates but on the motor axons (21, 70) and on the muscle membrane (70, 94, 114) (Section II D 4a). In a partially blocked preparation, tetanic contraction, post-tetanic reversal, and acetylcholine contraction are only manifestations of those motor units that remained functional. In complete tetrodotoxin block, no neural stimulation is effective, nor can acetylcholine depolarization of the end plate lead to excitation of the muscle membrane.

In the nerves in the intact animal, there is ample evidence that propagated action potentials are blocked. The earliest experiments in this direction, with the inductorium for stimulation and contraction of the innervated muscle as indicator, had already shown that after tetrodotoxin not only were the responses diminished, but also the threshold was increased (65, 66, 161). In some of the early studies, when stimulation of motor nerves failed to elicit contraction of the innervated muscles, direct stimulation of the muscles was still effective (65). Furthermore, by applying tetrodotoxin only to a segment of a sciatic nerve and stimulating on either side of this segment, Ishihara (65) showed that the block was only in the treated segment. These experiments are timely reminders that the general nature of tetrodotoxin action had been known since the earliest scientific investigations on this subject was started nearly 70 years ago. Axonal block *in situ* is not limited to somatic motor nerves. Disturbances to sensory nerves has been known since 1894 (161). Recently, Watanabe (182), using crystalline tetrodotoxin and direct oscillographic recording, showed that afferent impulses in the superficial peroneal and saphenous nerves of the cat's leg and in the mesenteric nerves from the intestine were reduced or abolished by tetrodotoxin in concentrations of 0.5 to 3 μg/kg intravenously. He found that less toxin was required to produce the same effect when injections were made into the femoral arteries towards the origin of the impulses than when they were made intravenously. In the saphenous nerve, afferent impulses resulting from stimulation of the skin could also be abolished by the local subcutaneous application of 0.01 to 0.05 μg of tetrodotoxin contained in 0.2 ml volumes. The impulses in the mesenteric nerves produced by instillation of acetylcholine into the intestine were not abolished until 3 μg tetrodotoxin/kg were injected intravenously (see also 178).

Aside from interruption of afferent and efferent nerve impulses to the muscle, tetrodotoxin has a direct blocking action on skeletal muscle fibers (53, 72). This has been substantiated by recent microelectrode studies in single muscle fibers (109, 114). In the animal, however, the time required to produce muscle block is usually longer than that for nerve block (65), and this difference must account for the observations that muscles can be stimulated directly with electrical stimuli (59, 65, 104, 160) or through the end plate by an intra-arterial injection of acetylcholine (22, 70). This and the subsequent progression of muscle block probably

account for some of the discrepancies in the functional states of respiratory nerves and muscles after treatment with tetrodotoxin (Section II D 4c). Slow muscles (soleus) are more easily blocked than fast muscles (tibiatis anterior, ref. 22). Tonic muscles (frog rectus abdominus) are more resistant, for contractures can be produced by acetylcholine even in the presence of 300 μM tetrodotoxin (83, 130, 154). With small doses of tetrodotoxin, which are insufficient to cause neuromuscular block, a peculiar transient increase of the maximal twitch height has been described (20, 123). Since the response is blocked by yohimbine (123) it may be due to epinephrine, which facilitates neuromuscular transmission and prolongs the active state (see references quoted in 70).

b. Cardiovascular system. A precipitous fall in arterial blood pressure is another characteristic manifestation of tetrodotoxin action. Indeed, tetrodotoxin is among the most potent agents known for producing a significant depressor effect for more than a few minutes' duration. For instance, carotid blood pressure in a cat can be reduced from 140/110 to 80/50 mm Hg in 2 to 3 minutes by an intravenous dose of 2 to 3 μg/kg, and the depressant effect can last for 30 minutes or longer. Most investigators agree that this depression is caused by alterations in the blood vessels rather than the heart. During the hypotension, heart rate is not significantly altered in the cat (70, 104); a slight sinus bradycardia is observed in rabbits, guinea pigs, rats and mice (173). This bradycardia is not prevented by vagotomy or atropinization (173). The amplitude of T-waves is increased in a way similar to that seen after administration of epinephrine, and this change was thought to be caused by reflex stimulation of the adrenal medulla rather than any specific action of tetrodotoxin on the myocardium (70). In animals dying from minimal lethal doses, the heart rate was usually regular and compression of the abdominal aorta returned the carotid blood pressure to normal or near normal levels (65, 161). With large doses (about 20 μg/kg intravenously in cats) it is possible to produce cardiac slowing and irregularities (65, 70, 103), both of which were early (161) recognized as due to conduction disturbances between the atria and the ventricles. In the toad heart, Ishihara recorded electrocardiographically a lengthening of A-V interval by as much as 25 per cent (65). More recent evidence (173) is in general agreement with these observations.

The basis of peripheral vasodilation produced by tetrodotoxin is not clear. Takahashi and Inoko (161) put much emphasis on a paralyzing action on the vasomotor center. Their evidence may be summarized as: (a) stimulation of the central ends of the vagi or the sciatic nerves that normally causes transient pressor effects became ineffective after tetrodotoxin poisoning; (b) in rabbits with transected cervical spinal cord, stimulation of the peripheral cord led to pressor effects before and after tetrodotoxin treatment. These observations were interpreted as showing that the vasomotor nerves were not significantly affected, and that the site of hypotensive action was in the medullary vasomotor center (161). Murtha *et al.* (104) and Li (87) injected solutions of crystalline tetrodotoxin into the cephalic ends of carotid arteries in cats (104) and vertebral arteries in rats (87). They observed that the onset of hypotension occurred earlier than after intravenous injection and concluded that tetrodotoxin had a central vasomotor depres-

sant effect. This mechanism of action has been so widely accepted that it appears in all Japanese textbooks dealing with tetrodon poisoning. On more thorough reflection, however, a number of questions may be raised as to the validity of the conclusion. In the recent intra-arterial injections (87, 104) into animal brains the doses used were the same as those used for intravenous injections into the body. Since the brains of cats, dogs, and rats are only about 1 to 2 per cent of the body weight, the doses of toxin injected into the head could have been some 50 to 100 times too high. The experiments of Takahashi and Inoko (161) are a little antiquated and should be repeated with modern techniques. Their failure to observe pressor effects upon stimulation of different nerves could be due to conduction block in the afferent nerves. More difficult to explain was the pressor effect they recorded after administration of tetrodotoxin on stimulating the spinal cord. Since there was a very marked fall in blood pressure after the operation (from 108 mm Hg to 18 in one rabbit; and from 160 mm Hg to 55 in the other) the toxin, which was injected subcutaneously after the operation, might not have been absorbed and distributed adequately. Thus, it may be said that there is as yet no convincing evidence pointing to any preferential action of tetrodotoxin on the vasomotor center.

On the other hand, experiments recently concluded in the author's laboratory (Kao, Suzuki, and Kleinhaus, unpublished) indicate that the medullary vasomotor center in cats and dogs is relatively unimportant for the production of hypotension by tetrodotoxin. In these experiments the circulation of the head of one animal was isolated from that of its body, and the head was perfused *via* the carotid and vertebral arteries by the systemic circulation of another animal. Tetrodotoxin in concentrations adequate to cause marked systemic hypotension in the donor animal never caused any observable systemic hypotension in the recipient. Similarly, doses of tetrodotoxin scaled down on the basis of relative weights, never produced systemic hypotension when injected directly into the recipient head. In the light of these observations, it is necessary to re-examine the evidence for a peripheral mechanism for the hypotension.

The relevant peripheral mechanisms are undoubtedly rather complex and not all the possible steps involved have been thoroughly examined. The vascular smooth muscles are probably not affected since pressor effects can be elicited with epinephrine or norepinephrine even during the most severe hypotension (65, 70, 76, 174). Nevertheless, Li (87) claimed that the vasodilation had a histamine-like component, as he was able to diminish the hypotensive effect with antihistaminics. It is not clear from his results whether the histamine-like vasodilation is due to histamine release by tetrodotoxin or to direct action of the toxin on the vascular smooth muscle. Another possible peripheral mechanism is a generalized ganglionic blockade produced by tetrodotoxin. If this is a significant mechanism in the hypotension, it must be differentiated from that of a true ganglionic blockade produced by an agent such as hexamethonium. Tetrodotoxin, in hypotensive doses, does not prevent stimulation of the adrenal medulla by high doses of acetylcholine (70), by nicotine (65), or by DMPP (1,1-dimethyl-4-phenylpiperazinium iodide, Kao, unpublished). Possibly, a ganglionic block by tetrodo-

toxin is exerted not so much at the dendrites and somata of the ganglionic cells as it is at the axons going to the effector cells. Observing the blood vessels on the conjunctiva of the rabbit eye and those on the serosal surface of dog's urinary bladder, Ishihara (65) claimed that tetrodotoxin abolished the vasoconstricting effect of neural stimulation. He took this as evidence that the vasomotor nerves were blocked, but one cannot be sure of this as no results beyond a statement was given. This conclusion is important because a block of the vasomotor nerves may be the main cause of the hypotension. While available evidence indicates that the hypotensive action of tetrodotoxin is due not to actions in the brain, heart, or vascular smooth muscle, there is no unequivocal proof, as yet, that it is due to a block of the vasomotor nerves and not to interference with spinal vasomotor control mechanisms. Release of vasomotor tone has been suggested as the cause of the hypotension (70). There is evidence that vasodilation occurs in the splanchnic areas and elsewhere. In dogs and men, priapism can follow tetrodon poisoning (40). In the splanchnic beds, there are: (a) slight increases in the weights of the viscera (125); (b) marked congestion of the viscera (63); and (c) significant increases in Evans blue space in duodenum, kidney, and spleen, increases that cannot be attributed to altered capillary permeability (121, 129).

c. Respiratory system. Although all clinical and laboratory observers are agreed that the ultimate action of tetrodotoxin on respiration is to depress it there is much controversy on how respiratory depression is produced. Some investigators are inclined towards a view that tetrodotoxin has a specific inhibitory action on the respiratory center. Evidence in support of this view is: (a) stimulation of afferent nerves which normally increases respiration became ineffective after treatment with tetrodotoxin (161); (b) during tetrodotoxin depression, central analeptics were ineffective (161); (c) upon cessation of respiration, most motor nerves (*e.g.*, sciatic, brachial, etc.) were inexcitable but the phrenic nerve was still excitable (76); (d) at death, the diaphragm was still excitable (67); and (e) extremely small amounts of tetrodotoxin applied locally to the medulla (65) or injected into the cisterna magna (11) produced prompt respiratory arrest. Other investigators are of the opinion that an important reason for respiratory embarrassment is the paralysis of respiratory nerves and muscles. Their evidence is: (a) marked increase in threshold of phrenic nerve to electrical stimulation after tetrodotoxin treatment (59, 65), (b) a decrease of the resting tension of the diaphragm during tetrodotoxin depression (74), and (c) on slow infusion of tetrodotoxin, failure of excitation in the diaphragm preceded that in the phrenic nerve (144, 183).

The controversy is due not solely to different interpretations but also to differences in observations. The action of tetrodotoxin on respiration is undoubtedly rather complex, and cannot be simply ascribed to predominant action on the medullary centers or on the peripheral neuromuscular apparatus without specifying the experimental conditions. The extraordinary sensitivity of the respiratory system to tetrodotoxin can be appreciated from the observation that very low doses of tetrodotoxin (0.04 of a lethal dose) can inhibit respiration rather markedy (65). with intravenous doses of 0.5 to 3 μg/kg (*e.g.*, in the cat) respiratory move-

ments can change, within one breath, from a normal pattern to complete arrest for several minutes (59, 66, 103, 161, 191, Kao, Suzuki and Kleinhaus, unpublished). Yet, some investigators have observed an increase in both amplitude and frequency of respiratory movements with similar doses (74, 117, 118). The reasons for such different observations are not entirely clear, but it appears that the speed of injection into the vein or the route of administration may both be rather important. With relatively slow accumulation, such as from a subcutaneous injection (67, 118) or by slow intravenous infusion (144), there appears to be only progressive depression without any initial stimulation. With rapid intravenous injection the respiratory depression is prompt and severe. Possibly these depressions are of two different types, the former being primarily peripheral and the latter, central. Sakai, Sato, and Uraguchi (144) showed that with slow intravenous infusion, action potentials in the diaphragm disappeared before those in the phrenic nerve, and that nikethamide could elicit a burst of action potentials in the phrenic nerve without causing corresponding diaphragmatic contraction. If a minimal dose is injected into the vein slowly so that there is only a small and transient vaso-depressor effect, the amplitude and frequency of the respiration are often increased (74, 117). In the cross-perfusion experiments referred to above (Section II D 4 b) when tetrodotoxin was given to the donor animal and the head of the recipient animal, respiration of the recipient was always stimulated. The minute volume of respiration of the recipient could sometimes be doubled even though the donor's respiration was completely stopped. The stimulation was thought to be caused by baro- and chemoreceptor reflexes originating from the carotid sinus region, because it lasted as long as systemic hypotension prevailed in the donor and stopped when the donor's blood pressure was recovered. These observations would suggest that central depression is not an important factor in respiratory depression in anesthetized animals.

That tetrodotoxin does have some central action on respiration has been known since 1919, when Ishihara observed an increase in respiration following the injection of tetrodotoxin into cerebral lateral ventricles in rabbits (65). Similar findings have been made recently with crystalline tetrodotoxin in chronic preparation in cats, when the total dose given was 0.2 μg (11). When the dose was 0.5 μg or more, respiratory depression and failure occurred. Respiratory failure was accompanied by decreased sensitivity to CO_2 and a development of apneustic breathing. The integrating center, however, continued responding to electrical stimulation (11).

Other actions of tetrodotoxin on the respiratory system are: (a) depression of the cough reflex (74) and (b) relaxation of bronchial muscles (74). The former was established by eliciting cough with electrical stimulation of laryngeal nerves in anesthetized dogs; after tetrodotoxin treatment, the severity of the cough produced was much attenuated. The latter finding was made by measuring the resistance to passive respiratory movement; after tetrodotoxin treatment the resistance was reduced. Since this was carried out in the rabbit with closed chest, it is conceivable that part of the change in resistance can be attributed to a relaxation of the diaphragm and costal muscles.

d. Central nervous system. Since the primary action of tetrodotoxin is to intere-

fere with excitation of nerves, it should be expected that in sufficient doses central nervous system manifestations must occur. Whether these can be considered as specific actions of tetrodotoxin on central nervous system is debatable, and no attempt will be made to summarize various observations that may fall into this category. For instance, Rech, McCarthy, and Borison (138) reported that intraventricular injections of rather large doses of tetrodotoxin in cats produced "volitional paralysis" of the peripheral musculature. However, since by the usual routes of administration, these doses of tetrodotoxin caused block of peripheral nerves and muscle without disturbing the labyrinth component of the righting reflex (128), it is not possible to place in proper perspective interpretations such as those of Rech *et al.* Also, if changes in reflex activity are studied by methods that depend much on the participation of peripheral mechanisms (*e.g.*, slowing of the knee jerk reaction, ref. 80), it would not be possible to differentiate conduction block in axons from alterations in central synaptic mechanisms. For a clear view of the actions of tetrodotoxin on some central synapses, one must employ more sophisticated neurophysiological techniques. Moreover, it is important to monitor incoming spike in order to avoid erroneous conclusions on changes in reflex activity. There are, however, several unique actions of tetrodotoxin on the central nervous system that deserve special attention.

In tetrodon poisoning in man, dogs, and cats vomiting is a frequent sign. In dogs, as little as 0.3 μg/kg intravenously or 0.7 μg/kg subutaneously or intramuscularly will consistently induce emesis (58). Tetrodotoxin is the most potent emetic agent known. Vomiting, however, is not seen in anesthetized dogs and cats, and is not evident in conscious animals when marked muscular weakness is produced by large doses of tetrodotoxin (58, 65, 161). The emetic action can be abolished by prior surgical ablation of the medullary chemotrigger receptor zone in dogs and cats (71, 58). Neither hexamethonium nor chlorpromazine antagonized the emetic action, and tetraethylammonium chloride was effective only if it was given less than 30 minutes before administration of tetrodotoxin (58).

Tetrodotoxin also causes a sustained fall in body temperature of several degrees in rabbits, dogs and cats. This phenomenon has been known since 1890 (161) and has been confirmed repeatedly (65, 66, 117, 191). Hypothermia can also be induced in cats by injecting crystalline tetrodotoxin into the lateral ventricles (11).

e. Smooth muscles and glands. The actions of tetrodotoxin on these autonomic effector cells are limited to actions on neurally elicited responses, and do not extend to responses of the effector cells to direct stimulation by pharmacological agents. In the cat's tail, piloerection produced by stimulation of the sympathetic nerve or the lumbar spinal cord was prevented after treatment with tetrodotoxin, but could still be elicited by epinephrine injected intravenously (65). In the sweat and salivary glands of the cat, tetrodotoxin blocked the secretory activity elicited by neural stimulation, but had no effect on that produced by the intravenous injections of pilocarpine or epinephrine (65). In cats and rabbits, pupillary dilation occurred after tetrodotoxin was given systemically (65, 160), into the anterior chamber of the eye (38), or subsclerally (107), or instilled on the cornea

(65). In these cases, while light reflex was impaired or abolished (38, 65) and electrical stimulation of the cervical sympathetic nerve no longer produced mydriasis (38), miosis could still be produced by corneal application of pilocarpine, physostigmine, acetylcholine, or carbamylcholine (38, 65), and mydriasis by intravenous injection of epinephrine (65). Indeed, the response to acetylcholine was reported to be greater after than before treatment with tetrodotoxin (38), a result that suggests some processes resembling denervation supersensitivity. Contractions of the cat's nictitating membrane evoked by preganglionic stimulation were diminished but not abolished by doses of tetrodotoxin up to 5 to 10 μg/kg, while contractions produced by epinephrine were unaffected (70; Kao, unpublished observations).

On visceral smooth muscles, the actions of tetrodotoxin are similar but somewhat more complex. The spontaneous phasic contractions and tone of the rabbit uterus (hormonal status not recorded) were not affected by tetrodotoxin (65, 76), nor was the stimulatory effect of epinephrine on this muscle (65). The isolated intestines of mouse, rat, and guinea pig were inhibited by tetrodotoxin, the guinea pig ileum being particularly sensitive (56). There is no information on the action of tetrodotoxin on neurally elicited responses in the intestine, but in the rat stomach tetrodotoxin blocked contractions produced by vagal stimulation (see Section II D 1) (122). In the guinea pig ileum, tetrodotoxin block resembled hexamethonium block in preventing nicotine-induced contractions (57). In the intestines of these rodents, as well as those of the rabbit, tetrodotoxin did not prevent the inhibitory action of epinephrine (65), or the stimulatory actions of acetylcholine, pilocarpine, histamine, or serotonin (56, 57, 65).

In the rabbit intestine, tetrodotoxin produces a peculiar stimulation. This effect, first seen in the *in situ* preparation, was thought to result from the hypoxia associated with the cardiovascular and respiratory effects of tetrodotoxin in the whole animal (161). While hypoxia could be a contributory cause because adequate artificial respiration prevented the stimulation by tetrodotoxin (76), it was not the only cause because the stimulation could be seen in isolated preparations also (65). The stimulation was described as an increase in the frequency and amplitude of the phasic contraction as well as an increase in "tone" (65, 76, 161). Since the overall contractile activity of the intestinal smooth muscle is largely controlled by the frequency of its spike activity (17), the increases in the amplitude and the "tone" probably resulted from summated phasic contractions produced by an increased rate of spike discharges. Microelectrode recordings from the guinea pig taenia coli actually showed that the spike frequency after treatment with tetrodotoxin might be two to three times the normal rate (169). One interesting point emerging from a consideration of these results and those on cardiovascular responses is that the various mammalian smooth muscles are quite resistant to tetrodotoxin. In the isolated rabbit intestine, for instance, tetrodotoxin up to 30 μM had no blocking action (Kao, unpublished). The significance of this resistance will be discussed further in Section II D 5 d.

In rats secretion of gastric acid was inhibited by sublethal doses of tetrodotoxin given subcutaneously or intraperitoneally. The total volume, free acid

content, and total acidity were decreased and the pepsin concentration was increased. Tests with histamine, physostigmine, adrenocorticotropic hormone (in both adrenal-intact and adrenalectomized animals), and insulin suggested that the inhibitory action of tetrodotoxin was due to its ganglionic blocking action and not to direct action on the acid-secreting cells (126).

5. Cellular actions. To appreciate fully the significance of tetrodotoxin in the study of excitation phenomena, it is necessary to have some understanding of the modern concepts of excitation (62). Numerous general reviews on this topic are available (*e.g.*, 152), and only the most pertinent aspects will be stated, to help in understanding the action of tetrodotoxin. In the resting excitable cell, the distribution of ions is such that potassium tends to move outwards and sodium inwards under passive electrochemical forces. The maintenance of such ionic gradients is accomplished through energy-requiring active transport processes that do not participate directly in the rapid events occuring during excitation. The resting membrane is relatively far more permeable to potassium than to sodium. Upon threshold *depolarization* (*i.e.*, when the resting membrane potential is reduced to a critical level), a series of rapid and self-limiting alterations takes place in the ionic permeability of the membrane. The sodium permeability increases markedly and rapidly and becomes reduced again in a very short time. After the onset of increased sodium permeability, the potassium permeability increases at a somewhat slower rate but for a longer period. Since both cations are under electrochemical forces to cross the cell membrane in directions of attaining equilibrium, changes in the permeability of the cell membrane to these ions are accompanied by movements of these ions *passively down their respective electrochemical gradients.* Thus, the initial increase of sodium permeability is accompanied by a displacement of the membrane potential towards the *sodium equilibrium potential* (E_{Na}) and the interior of the excitable cell becomes positive with respect to the exterior. Sodium permeability, however, is dependent on voltage and time. When the membrane is depolarized, sodium permeability is rapidly reduced by an unknown process which has been termed *sodium inactivation.* This process, along with the subsequent increase of potassium permeability allows the membrane to *repolarize* towards the *potassium equilibrium potential* (E_K). Thus, within a fraction of a second an electrical signal is produced in a small region of an excitable cell (*e.g.*, an axon). A change in potential difference in one region necessarily spreads to adjacent regions, which, upon threshold depolarization, undergo the same regenerative changes in permeabilities. In this manner, the *action potential* becomes *conducted* or *propagated.* With the directional movements of cations, there are measurable currents which can be employed experimentally as indications of changes in permeability. Inward sodium movement can be followed as an inward current and outward potassium movement as an outward current. Such currents can be measured, however, only when the membrane potential can be held constant, *i.e.*, under *voltage clamp* conditions. The molecular events underlying the permeability changes are unknown, but the ionic movements are generally believed to occur through channels in the cell membrane.

a. Isolated nerves. Many substances can cause conduction block in isolated nerves, either by producing a sustained depolarization, which will in turn cause sodium inactivation, or by making it difficult to initiate the regenerative changes in sodium and potassium permeabilities (*cf.* 152). Although for many years tetrodotoxin has been known to cause conduction block in nerves (65, 175), it was only recently that the block was shown to occur without depolarization (21, 31, 32, 70). These experiments were based on recording propagated action potentials in desheathed sciatic nerves of frogs, which were blocked by as little as 3 nM tetradotoxin (31, 70). With this method of recording nerve activity, it is not possible to differentiate tetrodotoxin block from that produced by cocaine or procaine because these agents also produce conduction block without depolarization.

In a voltage-clamped giant axon from the squid or lobster, however, the action of tetrodotoxin on the one hand and procaine and cocaine on the other are fundamentally different. With the latter agents, the initial inward sodium current is reduced, as is also the outward potassium current (153, 167). On the basis of such observations, it had been argued (153) that the changes in membrane permeabilities in normal excitation might not be so ion-specific as was originally proposed (62), *i.e.*, sodium and potassium ions might travel through the same membrane channels instead of through separate channels having distinct properties. With tetrodotoxin (in nanomolar concentrations), however, inward sodium current can be markedly reduced or even obliterated while the outward potassium current remains totally unaffected (47, 111, 115, 163). This finding may be of great importance for the eventual elucidation of the molecular alterations underlying the excitation phenomenon. For immediate purposes it is essential to ascertain that the reduction in inward sodium current is due chiefly, if not exclusively, to a failure of sodium permeability to increase. Direct evidence is not as readily obtained as might be expected, but the conclusion is supported by excluding other factors that may lower sodium current. The factors ruled out include a delayed onset and a faster termination of the increase in sodium current, as well as an enhanced sodium inactivation (163). Since the sodium concentration gradient could not have changed under the experimental conditions, the driving forces were the same. The only known factor remaining that would explain the action of tetrodotoxin is a reduction in the membrane permeability to sodium (163). To date, tetrodotoxin is the only substance known to have this selective action. Saxitoxin probably has a similar action (71), although definitive voltage clamp experiments have not yet been published at the time of writing.

Under voltage-clamp conditions, if a depolarizing potential greater than the sodium equilibrium potential is applied, the driving forces will cause an outward movement of sodium which can be measured as an outward sodium current. Even this outward sodium movement is impaired by nanomolar concentrations of tetrodotoxin applied to the external surface of squid or lobster giant axons (47). There is a species difference between the two axons: in the squid axon, outward movement can be completely abolished (101, 163), while in the lobster axon, it is reduced at best to about 25 per cent of the untreated preparation (47).

Thus, tetrodotoxin blocks both inwardly and outwardly directed passive sodium movements.

In squid giant axon it is possible to introduce various agents either by microinjection (19, 49) or by perfusion (5, 132). The latter, developed within recent years, has many advantages over the earlier microinjection techniques adapted for physiological studies by the present writer from classical methods of Chambers. Combining perfusion and voltage clamp techniques, it is possible to study in detail ionic movements across the squid axon membrane under many different conditions. Thus, by manipulating the compositions of internal and external media, it is possible to create a situation in which the initial response to depolarization is an *outward* movement of sodium rather than the usual inward movement. This outward sodium movement is also blocked by tetrodotoxin (101). Another unusual situation can be established in which hydrazinium, guanidinium, or hydroxylamine ions replaced all external sodium and became the charge-carrying ions responsible for inwardly directed membrane currents under voltage-clamped conditions (166). These inward currents were also blocked by tetrodotoxin in 30 nM to 3 μM concentrations. However, under another set of conditions, when initial inward current was due to hydrazinium ion and the slower outward current was due to sodium, tetrodotoxin blocked only the former and did not affect the latter (166).

Tetrodotoxin has also been introduced into the interior of the squid giant axon by perfusion. In concentrations 1000 times higher than those that block when applied to the external surface membrane (1 μM) it has no appreciable effect on either inward or outward sodium currents under voltage-clamped conditions (100, 101). Even in a concentration 10,000 times higher (10 μM), internally applied tetrodotoxin has no effect on the form of the propagated action potential (100, 101). On the other hand, there is a claim that tetrodotoxin microinjected by the Chambers-Kao technique (19) in an internal concentration of about 300 nM produced conduction block in squid giant axon (111). Since the technique of microinjection is difficult, it is possible that this claim, which is based on only two observations, is tainted with some technical flaws. In general it can be said that tetrodotoxin is effective only when applied to the exterior surface of the cellular membrane, and that in this position it can block both inward and outward movements of sodium, and possibly inward movement of other ions that are capable of replacing sodium as the current-carrying ion species. The various observations may be interpreted as showing that the molecular alterations underlying the permeability changes proceed from the outer surface toward the inner surface of the excitable membrane, and that the specificity for any cation to travel in the fast sodium channels is not rigorous as long as the cation is monovalent (for effects on divalent cation see Section II D 5 d).

Other isolated nerve preparations that have been shown to be sensitive to tetrodotoxin action are isolated single nodes of Ranvier in frog sciatic nerves (31, 32), cat mesenteric nerves (90, 182), and crayfish nerves (90, 127).

b. Skeletal muscle, neuromuscular junction, and electroplates. The first intimation of a selective action of tetrodotoxin on the sodium permeability of an

excitable membrane was made by Narahashi, Deguchi, Urakawa and Okubo (114). By studying the current-voltage relationship (not under voltage-clamped conditions) in isolated frog sartorius fibers, they found that the threshold to excitation by depolarizing pulses was considerably elevated after treatment with 300 nM tetrodotoxin. Eventually, they were able to pass large outward currents (depolarizing) without eliciting spikes. Yet, under these conditions the effective resting membrane resistance, which is determined chiefly by potassium and chloride conductances (61), remains unchanged from untreated preparations. Moreover, delayed rectification, a manifestation of increased potassium permeability upon depolarization, was also unaltered. This blocking action on spike production in muscles is the basis of many of the changes observed in whole animals as well as in man summarized in earlier sections. Similar results were obtained by Nakajima, Iwasaki, and Obata (109). It is not known whether the contractile machinery and the excitation-contraction coupling mechanism are affected by tetrodotoxin. Qualitatively, they are probably not, because local depolarizations produced by an internal microelectrode are accompanied by microscopically visible local contractions (33, 114).

At the neuromuscular junction, the action of tetrodotoxin is complex because it acts on both the axon and the muscle membrane. As indicated above (Section II D 4 a) unless proper techniques are employed, erroneous conclusions can result. On single end plates of frog as well as guinea pig studied with intracellular microelectrodes, tetrodotoxin does not cause depolarization (33, 42, 43, 131). The end plate potential elicited by neural stimulation becomes rapidly reduced in 30 to 100 nM concentrations of tetrodotoxin (42). Along with this, the acetylcholine output produced by tetanic stimulation of motor nerves is either markedly reduced or completely inhibited (20, 38). The end plate membrane, however, remains entirely responsive to acetylcholine arriving in any of the following ways: on release in the normal spontaneous quantal manner from the nerve terminal, manifested as miniature end plate potentials (42, 43); applied microelectrophoretically to the end plate (73); placed in the bathing solution (43); or given by close intra-arterial injection (22, 70). These observations all point to the conclusion that the sites of tetrodotoxin block of neuromuscular transmission are on the axon and the muscle membranes and not on the chemosensitive end plate receptors. This is a welcome addition to the other lines of evidence that conduction in nerve and muscle membranes is fundamentally different from transmission across synapses. The usual evidence depends on selective action of various agents on the end plate, whereas tetrodotoxin now provides evidence of the reverse nature (73). Also of some importance is the fact that for the first time an agent is available that can block the electrically excitable portions but not the chemosensitive portions of the neuromuscular junction. While much elegant evidence has been obtained (18), the usual procedure of studying transmission involves the use of agents and procedures (*e.g.*, tubocurarine and high Mg^{2+}) which can interfere with one or another of the processes involved in chemical transmission (142). With tetrodotoxin, in combination with microelectrophoretic techniques of applying drugs, it should be possible to re-examine the normal

transmissional processes and the responses of the end plate to certain drugs. Evidence is already accumulating from such experiments that the various processes revealed in studies with small amounts of tubocurarine are essentially correct (73). Use of tetrodotoxin also offers a new approach to study of the role of the nerve terminal in normal transmission and in certain pharmacological responses (142).

The electroplates of the electric eel, *Electrophorus electricus*, are analogus to skeletal muscle fibers. Tetrodotoxin blocks spike production in these cells (31, 32) and this block is due to a specific reduction in the initial inward current (110). Acetylcholine and carbamylcholine can produce depolarization and increase potassium efflux in electroplates that have been otherwise blocked by tetrodoxin (quoted in ref. 32). These responses are due most probably to the postjunctional chemosensitive areas which, in the electroplates, are spread throughout the innervated surface of the electroplates.

c. Cardiac and smooth muscles. Cardiac muscle cells are fairly resistant to tetrodotoxin. Most investigators have found little effect on the form of the action potential of various isolated cardiac muscles. By recording the maximum rate of rise of the spike, a measure of the inward sodium current, Hagiwara and Nakajima (52) recently showed that in frog ventricular fibers, tetrodotoxin (150 nM) reduced it to 17 per cent of the rate of untreated preparations, even though the amplitude of the overshoot was reduced by only a few millivolts. This concentration is equivalent to a dose of about 20 μg/kg in a whole animal if tetrodotoxin is assumed to be equally distributed throughout body water. At this dose tetrodotoxin is known to cause cardiac depression (Section II D 4 b). From available information it might be expected that the conducting tissues of the heart might be more susceptible to the action of tetrodotoxin.

Smooth muscle cells of the guinea pig taenia coli are not blocked by tetrodotoxin in concentrations up to about 3 μM, and the frequency of their spontaneous spike discharges is significantly increased by tetrodotoxin (169). This observation has been used to support the view that the spike-generating mechanism in taenia coli is not dependent on sodium permeability changes (169). Although the observation is interesting, the conclusion is too sweeping and debatable (Section II D 5 d).

An interesting warning should be sounded for those investigators who may be interested in studying the action of tetrodotoxin on cells that may be resistant. The commercially available tetrodotoxin preparation (Sankyo) contains 500 μg of sodium citrate for each 100 μg tetrodotoxin. When 1 ml of water is added to the powdered preparation, a solution with a pH of 4.5 is obtained. If this stock solution is diluted to contain nanomolar concentration of tetrodotoxin, no difficulty is encountered. When a high concentration of tetrodotoxin is used (*e.g.*, 100 μg/ml) enough citrate can be present to remove some ionized calcium from solution. As is now realized by the authors themselves, this is the reason for a claim that in high concentrations, tetrodotoxin depleted calcium in some smooth muscles (36).

d. Resistance of taricha and tetrodon nerves and other tissues. As indicated above

(Section II D 2) taricha newts and tetrodon fish are resistant to the lethal action of tetrodotoxin (65, 102). The immunity is due chiefly to a remarkable resistance of the nerves and muscles of these animals to tetrodotoxin. Thus, in isolated desheathed sciatic nerve of *Taricha torosa*, 90 μM tetrodotoxin (30,000 times higher than the minimal blocking concentration for frog sciatic nerve) had no effect on the form of the propagated compound action potential (15). At 270 μM, there was a slight block that could be readily reversed (Kao and Fuhrman, unpublished). Similarly, on isolated desheated brachial nerves of *Spheroides maculatus*, partial block was obtained only when the concentration of tetrodotoxin was increased to 3 μM. On both types of nerves, however, the compound action potentials were promptly decreased when external sodium was replaced by choline or Tris (Kao and Fuhrman, unpublished). On muscle fibers of a Japanese tetrodon, similar resistance of the spike to tetrodotoxin and its susceptibility to removal of external sodium have been observed (Kuriyama, personal communication). Whatever the basis of the extraordinary resistance is, it is certainly not that these tissues do not depend on external sodium ions to carry the inward current.

Several sensory receptor cells are apparently resistant to tetrodotoxin. In the crayfish stretch receptor (90, 108) and the Pacinian corpuscle (108, 133a), the generator potentials are little affected while the spike responses in their axons are blocked. In the guinea pig cochlea, iontophoretic application of tetrodotoxin in the region of the hair cells abolished axonal impulses without affecting cochlear microphonic potentials (71a). Although there is little doubt that tetrodotoxin can differentially affect the generator and spike potentials, some of the evidence provided for this action is of dubious validity (69). In a careful study of the generator potential in the Pacinian corpuscle in which recordings were taken at an oil-saline interface immediately at the junction of a Pacinian corpuscle with its axon, Ozeki and Sato (133a) showed that tetrodotoxin in 300 nM blocked 40 per cent of the generator potential and left 60 per cent unaffected. Yet, from recordings made 2 mm away on the axon of a Pacinian corpuscle, Lowenstein, Terzuolo and Washizu (90) claimed that tetrodotoxin as high as 30 μM had no effect on the generator potential. Since the recording conditions of the latter investigators could not prevent tetrodotoxin from blocking conducted impulses in the axon, it is doubtful that their claim of having blocked spike responses without affecting generator potential has any validity. Moreover, if 300 nM tetrodotoxin can affect a substantial part of the generator potential (133a), it is strange that 30 μM should have no effect on it (90).

The giant plant cell, *Nitella*, possesses a spike-producing mechanism that appears to be dependent on efflux of internal chloride ion, like that in *Chara* (44). These spikes are not blocked by tetrodotoxin (77). The giant muscle fiber of the barnacle has a complex spike-generating mechanism that is dependent on calcium ions as current carriers (51). These spikes are also unaffected by tetrodotoxin (52). Crayfish muscles, which also have a calcium-dependent spike mechanism, are also resistant to tetrodotoxin.

e. Active cation transport, metabolism, and enzyme systems. In frog skin, tetro-

dotoxin in concentrations up to 25 μM added to the inside, the outside, or both sides, had no effect on the potential difference or short-circuit current; this indicates that active sodium transport is totally unaffected (70). There is no known work on the effect of tetrodotoxin on the Na-K activated ATPase system. Oxygen consumption of frog nerves (70) and slices of rat brain and liver (113, 180) were unaffected by tetrodotoxin, as were the extra oxygen uptake in rat brain caused by increased external potassium (113). Aldolase efflux from muscle cells of rat diaphragm immersed in media containing 5 mM or 67 mM K^+ was unaffected by tetrodotoxin (124). Oxidation of pyruvate and glutamate in homogenates of brain and liver were unaffected (113). Cytochrome oxidase was said by one group to be inhibited (81) and by another to be unaffected (113). There is also an unconfirmed report that prior injection of cytochrome *c* protected mice from the lethal action of tetrodotoxin (112). There is universal agreement that the cholinesterase system is unaffected or only slightly affected even in concentrations of tetrodotoxin up to 30 μM (31, 32, 38, 70, 106). This is interesting because there is a widely circulated hypothesis that conduction in axons, like transmission across some synapses, is fundamentally an acetylcholine-cholinesterase reaction.

III. SAXITOXIN

A. History and clinical aspects of paralytic shellfish poisoning

1. History and classic description of poisoning. Food poisoning caused by shellfish is not uncommon, but the vast majority of the cases are either of the gastrointestinal variety owing to bacterial or other forms of contamination or of the hypersensitivity variety (97). Paralytic shellfish poisoning is a distinct clinical entity in which neurological symptoms outweigh all other manifestations. An admirable account of the early historical aspects of paralytic shellfish poisoning can be found in a paper by Meyer, Sommers, and Schoenholtz (98) which itself has become a milestone in the literature on this topic. These authors recounted an age-old custom among certain coastal tribes of Indians of Alaska, particularly the Poma, who "place sentries on watch for Kal-ko-o (mussel poison)." "Luminescence of the waves, which appeared rarely and then only during very hot weather, caused shellfishing to be forbidden for two days; those eating shellfish caught at such times suffered sickness and death." An early direct European experience with paralytic shellfish poisoning occurred during the second of three exploratory voyages made by Captain George Vancouver to the coast of the Pacific northwest. On June 15, 1793, two boatfuls of men were sent to explore what is now known as Mathieson's Channel in present day British Columbia. The channel formed "some little bays on the southern side." "In one of these they stopped to breakfast, where finding some mussels, a few of the people ate them roasted; as had been their usual practice when any of these fish were met with; about nine o'clock they proceeded in very rainy unpleasant weather down the south-westerly channel, and about one landed for the purpose of dining. Mr. Johnstone was now informed by Mr. Barrie, that soon after they had quitted the cove, where they had breakfasted, several of his crew who had eaten of the

mussels were seized with a numbness about their faces and extremities; their whole bodies were very shortly affected in the same manner, attended with sickness and giddiness. . . . Mr. Johnstone entertained no doubt of the cause from which this evil had arisen, and having no medical assistance within his reach, ordered warm water to be immediately got ready, in the hope that by copiously drinking, the offending matter might have been removed. (John) Carter attracted nearly the whole of their attention, in devising every means to afford him relief . . . ; but all their efforts at length proved ineffectual, and being unable to swallow the warm water, the poor fellow expired about half an hour after he was landed. His death was so tranquil, that it was some little time before they could be perfectly certain of his dissolution. . . . This very unexpected and unfortunate circumstance detained the boats about three hours; when, having taken the corpse on board, . . . they continued their route, in very rainy unpleasant weather, down the south-west channel, until they stopped in a bay for the night, where they buried the dead body. To this bay I gave the name of Carter's Bay, after this poor unfortunate fellow; it is situated in latitude 52° 48′ longitude 231° 42′; and to distinguish the fatal spot where the mussels were eaten, I have called it Poison Cove, and the branch leading to it Mussel Channel" (179).

Unlike tetrodon poisoning, which has a definite pattern of seasonal variation, paralytic shellfish poisoning occurs sporadically and in an entirely unpredictable manner. For this reason, even though epidemics had occurred in Leith, Wilhelmshaven, Santa Cruz, and other places in the 19th and early 20th century, the true cause of the poisoning eluded detection until the most severe epidemic occurred around San Francisco in 1927. There, between July 16 and 18, 102 persons became ill in a characteristic manner after eating mussels collected in localities extending 50 miles north and south of Golden Gate. With the collaboration of the California State Department of Health and the State Fish and Game Commission, scientists at the Hooper Foundation, University of California, began investigating the cause of the epidemic almost immediately. In their first report on the results of the investigations, Meyer, Sommer and Schoenholz (98) speculated on the possibility that "the food (certain dinoflagellates) may be one of the factors responsible" for a transient metabolic disease of shellfish which then became toxic to man. They accordingly initiated a study on the food of mussels, including a detailed plankton count of the water, along with periodic checks of toxicity of shellfish. During a period of strong toxicity in 1932 (157), "a new species of dinoflagellate (*Gonyaulax catenella*) was discovered in large numbers which aroused immediate suspicion," and soon "increased to such numbers that the influence of all other species could safely be disregarded and the connection between plankton and shellfish poison proved with a few conclusive experiments."

The nature of the poison was soon traced directly to the newly discovered dinoflagellate rather than to some intermediary disease in the shellfish. One strong piece of evidence obtained at that time was that non-toxic shellfish kept in the laboratory could be made highly toxic upon feeding them *Gonyaulax ca-*

tenella (157). Results of recent investigations amply support this conclusion; *e.g.*, 500,000 mouse units of toxin were centrifuged from 5,000 liters of sea water that contained *Gonyaulax catenella* (141), and from axenic cultures of *Gonyaulax catenella* was obtained a toxin which is chromatographically identical with purified saxitoxin (16). Another most important contribution by Sommer and Meyer was the establishment of a reliable and reproducible bioassay method (156) which, with slight modifications in recent years, is still the standard assaying procedure (92). In retrospect, this bioassay method contributed greatly to the progress of investigations on paralytic shellfish poison, for it provided a reproducible yardstick for comparing different batches of material that became available in a sporadic and unpredictable manner. It also served as a stimulus to the establishment of a similar assay method for tarichatoxin (Section II D 1).

Epidemics of paralytic shellfish poisoning were largely confined to the coast of California and the Pacific northwest until 1945, when an epidemic broke out in the Bay of Fundy along the shores of Quebec and New Brunswick. Since then there have been repeated outbreaks along these and the Pacific shores (92). The causative organism in the Bay of Fundy was established by Needler as *Gonyaulax tamarensis* (116). Because of the unpredictable and insidious appearance of toxic shellfish, concerted efforts were made by the federal governments of United States and Canada as well as various state agencies in the affected localities to monitor the toxicity of shellfish taken in these regions. This practice produced some most rewarding scientific by-products; through large-scale combined efforts of several chemical laboratories, purified toxic principles were obtained from mussel (*Mytilus californianus*) and clam (*Saxidomus giganteus*) in 1955 (147).

The clinical manifestations of paralytic shellfish poisoning are nearly identical to those of tetrodon poisoning. They are best summarized in the words of Meyer, Sommer, and Schoenholtz (98):

"The symptoms of poisoning are primarily peripheral paralyses which may vary from a slight tingling and numbness about the lips to a complete loss of power in the muscles of the extremities and neck, and to death by respiratory failure. In a moderately severe case the tingling, stinging sensation around the lips, gums, and tongue would develop from five to thirty minutes after the consumption of the mussels. This was regularly followed by numbness or a prickly feeling in the finger tips and toes, and within four to six hours the same sensation would progress to the arms, legs and neck, so that voluntary movements, as for example, raising of the head, were made only with great difficulty ... this ataxic weakness and stiffness of locomotion was accompanied by a peculiar feeling of lightness. Some patients declared that they felt as if they were floating or could fly. Even heavy objects appeared to them very light ...."

With one possible exception, the physical findings are also essentially the same as those found in tetrodon poisoning. This exception is that in all published clinical descriptions of paralytic shellfish poisoning (*e.g.*, 10, 45, 98, 145, 168) there is no mention of the blood pressure of the victims. Seven (151) recorded normal blood pressures in two patients at a time when they were already re-

covering from the poisoning. To the present reviewer, it is inconceivable that such an obvious oversight could be made by practitioners in all parts of the world; more likely this omission results from the absence of any significant changes in blood pressure. If this is a correct surmise then it is one of the few points of difference between the two forms of poisoning.

2. Public health problems. Simply considering the number of cases involved, paralytic shellfish poisoning cannot be classified as a major threat to public health. Since 1793, only some 600 people are known to have been so poisoned. However, because of the stability of saxitoxin, particularly its ability to withstand heating, its threat extends far beyond regional confines. The governments of United States and Canada therefore exercise rigid control over interstate shipment of fresh or canned shellfish. An excellent and authoritative review on this and related aspects of paralytic shellfish poisoning was published recently (92). As the result of extensive collaboration, a toxicity equivalent to 80 μg of purified saxitoxin per 100 grams of raw mussel meat was accepted as the quarantine level in a cooperative certification program (92). By and large the control measures have prevented the spread of paralytic shellfish poisoning. One unfortunate outcome, however, is that the shellfish industry of Alaska has been all but destroyed because the Alaska butterclam is continuously toxic in all seasons. Within recent years, small epidemics have still occurred in affected localities, chiefly among picknickers engaged in sport shellfishing. Such poisoning usually results from failure to heed official warnings and injunctions against the collection of contaminated shellfish (95).

B. Occurrence and distribution of saxitoxin

1. Geographic and species factors. Between 1793 and 1958 epidemics of paralytic shellfish poisoning have been reported from the northwest Pacific coast, the northeast Atlantic Coast, England, Wales, Scotland, Ireland, Norway, Germany, France, Belgium, South Africa, and New Zealand (92). There is no published report of any epidemic from the Mediterranean and Red Sea areas, and only tenuous and suggestive cases from the Gulf of Mexico. What factors are responsible for these geographic distributions are not clear, but they may not be as mysterious as might be thought. In the Gulf of Mexico around Galveston, Texas, for instance, the only shellfish normally collected is the local oyster, and this is not harvested during the summer months when paralytic shellfish poisoning is most likely to occur (25). Cases of poisoning have followed consumption of shellfish taken either from open waters or from relatively sheltered areas, and in some instances even from brackish water (156). Although most clinical cases of poisoning were associated with eating mussels and clams that are customarily used for food in the affected localities, apparently no shellfish is immune from becoming toxic. The various species known to be capable of containing saxitoxin are: *Mytilus californianus*, *M. edulis* (black or blue mussel), *Saxidomus nuttalii* (Washington clam), *Schizothaerus nuttalii* (horse neck clam), *Paphia staminea* (quahaug, little neck clam), *Siliqua patula* (razor clam), *Pholadidea penita* (rock clam), *Tivela staltorum* (pismo clam), *Macoma*, *Modiolus demissus* (horse mussel),

Mya arenaria (soft shell clam), *Spisula solidissima* (bar clam), *Saxidomus giganteus* (butterclam), *Crassostrea gigas* (cultivated Pacific oyster), and *Pecten grandis* (used as scallop) (116, 135, 156). Even a sand crab, *Emitera analoga* was found to be toxic (156).

All these species are described from the Pacific and Atlantic coasts of North America. Only in these two areas has the toxicity been traced directly to gonyaulax dinoflagellates. On the Pacific coast, the toxic dinoflagellate is *Gonyaulax catanella* (157); on the Atlantic coast, it is *Gonyaulax tamarensis* (116, 134). In no other area has the toxicity been traced unequivocally to any similar organism. Koch (78) implicated *Pyrodinium phoneus* as the causative agent in a Belgian outbreak of paralytic shellfish poison. In a recently published note Ray and Aldrich (137) implied that *Gymnodinium breve* might produce a toxin capable of causing paralytic shellfish poisoning. Their description of the symptomatology, however, did not permit a proper evaluation. A toxin from cultures of *Gymodinium veneficum* (1) has different biological actions from those of saxitoxin. No matter how strongly suggestive it may be, the mere existence of a correlation between the abundance of *Gonyaulax* and the toxicity of shellfish is not a definite proof of a causal relationship between the two. In the cases of *G. catenella* and *G. tamarensis*, however, toxic substances similar to or identical with saxitoxin have been extracted from the dinoflagellates (6, 135, 141). The converse situation has frequently been observed, *i.e.*, a substantial toxicity in shellfish need not necessarily be preceded or accompanied by the presence of large numbers of *Gonyaulax* organisms in the water. This discrepancy has been attributed to possible difficulties in preserving for examination dinoflagellates in collected water samples (116, 157), but the explanation is probably inadequate in some situations. For instance, in the case of *Saxidomus giganteus*, which often remain toxic throughout a greater part of the year, repeated examinations of water samples by different investigators have never shown any significant quantities of *Gonyaulax* (148). Another possible reason was suggested by Needler (116), who observed some similar discrepancy for bivalves in the Bay of Fundy. She suggested the possibility that *Gonyaulax* under adverse conditions encysted into spores, which, being denser, sank to the ocean bottom where they escaped collection but remained available as food to the shellfish. The main reason that shellfish become highly toxic can be found in their manner of feeding. An average mussel (*Mytilus*) filters 19 liters of water in one feeding (157), and an average butterclam filters 40 liters a day (148). By straining the *Gonyaulax* organisms out of the water, shellfish are an efficient apparatus for concentrating the toxic principle. In *Mytilus*, the toxic dinoflagellate is digested, and the toxin is present chiefly in the hepatopancreas, which contains about 95 per cent of the total toxin present. The toxin is then lost by some unknown mechanism. By following the toxicity of mussels fed *Gonyaulax* under laboratory conditions, Sommer and Whedon (157) estimated that the half time for loss of toxicity from a mussel is about 10 days. In *Saxidomus giganteus* about 70 per cent of the total toxicity is found in the syphon, and seasonal variations in toxicity of butterclam have been attributed to fluctuations in the toxicity of this organ (148). In the open this long-lasting

toxicity could be partly caused by continued availability of the toxic food. It is known, however, that in clams kept in the laboratory away from known sources of toxin, the syphon can still retain toxicity for many months (147). Thus, it appears that a genuine difference exists in the way in which different molluscs handle the toxin.

2. Ecological factors. Whereas a clear causal relation has been established between the toxicity of shellfish and the blooming of *Gonyaulax* dinoflagellates, little is known about the factors that trigger and influence *Gonyaulax* blooming. By blooming is meant the explosive multiplication of *Gonyaulax* which is usually necessary to ultimately produce toxicity in shellfish. In the Bay of Fundy, hydrographical, meteorological, and ecological factors have been examined that may influence the growth and disappearance of *Gonyaulax*. Needler (116) found that *Gonyaulax tamarensis* can multiply when the summer water surface temperature exceeds 10°C. Prakash and Medcof (135) confirmed this, and showed further than while a direct correlation exists between surface water temperature and toxicity of shellfish on a month-to-month comparison, the temperature from year to year did not necessarily affect the toxicity of shellfish. They found an inverse relationship between the water surface temperature in the preceding winter and shellfish toxicity the following summer. The meaning of this relation is not immediately clear but Prakash and Medcof (135) suggested that it might influence the encystment and excystment of *Gonyaulax*. Other factors which effect *Gonyaulax* growth are the amount of river discharge into the bay, the salinity, and the amount of sunshine. As a phytoflagellate, *Gonyaulax* is dependent on photosynthesis as the chief form of metabolism. A statistically significant direct correlation exists between the total hours of sunshine in the month of May and shellfish toxicity in June (135). The influence of river discharge is complex. A large amount of spring river discharge tends to lower the salinity in Passamaquoddy Bay, a condition which is associated with low shellfish toxicity subsequently. The discharges from some rivers carry large amounts of silt and this reduces the depth to which sunlight can penetrate. Low salinity has been suggested recently as a contributing cause for the rarity of *Gonyaulax* blooms in the Gulf of Mexico (137). Another factor that may be of some importance is the role of sewage contamination. In a rare instance of a bloom of *Gonyaulax* in the Gulf of Mexico, around Galveston, there was evidence of a direct correlation between the number of *Gonyaulax* in water samples collected in an area and the amount of sewage contamination (25). Much remains to be learned of the factors that trigger *Gonyaulax* into blooms, and the various species of *Gonyaulax* that may produce saxitoxin.

Given the right temperature, proper amount of light, and other favorable conditions *Gonyaulax* can multiply so rapidly that in a few days' time the surface of the ocean becomes discolored with a characteristic rusty-brown appearance. This discoloration begins to appear when there are about one million organisms in a liter of sea water. When the organisms increase to 20 to 40 million per liter, the surface water becomes brightly red in the day and brilliantly luminescent at night (141). Such discolored patches of sea are the "red-tides" or "red-waters"

that give the warning to the Poma sentries in Alaska. Not all "red-tides," however, are due to *Gonyaulax*, as many other organisms can produce similar blooms. The peak of a bloom lasts a few days and then, almost as mysteriously as it appeared, it disappears. At the ebb there may be only an occasional *Gonyaulax* in the water or none at all. Shellfish in the vicinity of the bloom or in bays that receive tidal washes from the contaminated water remain toxic for about 2 weeks. During the decline of one bloom in the Bay of Fundy, Needler (116) found large numbers of a ciliate which she recognized as *Favella ehrenbergii*, ingesting and digesting *Gonyaulax tamarensis*. Prakash and Medcof (135) made similar observations, and though they did not find any *F. ehrenbergii*, they were satisfied that various species of *Favella* were the natural predators that selectively fed on *Gonyaulax*. This may well be an important mechanism for terminating a bloom.

C. Chemistry

Schantz (146) has written a detailed summary of the methods of extraction, purification, and chemical properties of the poison obtained from several shellfish sources. The work is based on a large scale co-operative study among several laboratories which all had similar sources of raw material. These were: California mussels (*M. californianus*, *M. edulis*), whole or the hepatopancreas; Alaska butterclam (*Saxidomus giganteus*), usually only the syphon, and Bay of Fundy scallops (116, 149). By all indications, the chemical, physical, and biological properties of the poisons from mussel and clam are very similar, if not identical. The poison from scallop behaves somewhat differently during chromatographic purification, but this difference may be due to the presence of some impurities. By various criteria of chromatographic, electrophoretic, and countercurrent distribution methods, the poison from mussel and clam is at least 95 per cent and possibly 100 per cent pure (6, 99, 149). The greatest difficulty in complete purification appears to be the separation of several non-toxic bases that are closely related to the toxin. Although dried preparations of the poison often appear crystalline, there is as yet no successful attempt at crystallization from solution. In the best form available, saxitoxin has a potency of about 5500 mouse units/mg of dry toxin (see Section III D 1 for definition of mouse unit).

Saxitoxin is a basic substance that readily forms salts with mineral acids. The dihydrochloride salt is readily soluble in water, moderately so in methanol and ethanol, and insoluble in all lipid solvents. This compound had been given a molecular formula of $C_{10}H_{17}N_7O_4 \cdot 2HCl$. Recent studies on the structure of saxitoxin (136a) seem to cast doubt on this formula. By sequential oxidation and reduction, saxitoxin can be converted to a compound, $C_9H_{12}N_6O_2$, which contains all but one each of the original complement of carbon and nitrogen atoms. The missing C and N are accounted for by the formation of carbon dioxide and ammonia. Hence, the original compound may be $C_{10}H_{15}N_7O_4$, not $C_{10}H_{17}N_7O_4$. The compound $C_9H_{12}N_6O_2$ has been proved by degradation and synthesis to be 2-imino-8-amino-6-methyl-3-β-carboxyethylpurine. An oxygen substituent is believed to be located on the 6-methyl group in the original toxin. Thus, saxitoxin

would appear to be a reduced form of this degradation product carrying a substituent which is readily hydrolyzed to carbon dioxide and ammonia. The toxicity of saxitoxin is apparently closely dependent on an unsaturated bond that can be readily reduced (146). Saxitoxin dihydrochloride can be catalytically reduced in hydrogen to a dihydro compound ($C_{10}H_{19}N_7O_4 \cdot 2HCl$ by the older formula) which is not toxic. One mole of hydrogen is taken up for each mole of toxin, and the loss of toxicity varies directly with the extent of hydrogenation.

To the biologist the significance of the new information on the possible structure of saxitoxin is that the toxin most probably has a perhydropurine nucleus in which are incorporated two guanidinium moieties. Mindful of the structure of tetrodotoxin, one would find it difficult not to seriously consider the importance of cyclic guanidinium compounds in excitation. As has been pointed out (71), the importance may lie in the simultaneous presence of some other reactive groups close to the guanidinium moiety. In the case of saxitoxin, this view brings attention to the groups at position 6 (C with substituents) and positions 1, 2, and 3 (guanidinium).

D. Pharmacological actions

1. Methods of detection. Only one bioassay method is in use for the quantitative estimation of saxitoxin and fortunately there is general agreement concerning its use. This is largely the result of the need (and work) of fishery and health authorities in Canada and the United States for a standardized quarantine procedure to monitor the toxicity of shellfish. The method is based on the procedure of Sommer and Meyer (156) in which an extract of suspected material was made by a standardized procedure, and a standardized volume was injected intraperitoneally into mice. The time between injection and death is an exponential function of the amount of toxin injected. Sommer and Meyer (156) determined corrections to be allowed for mice differing in weight from the standard 20 grams. Toxicity was expressed in mouse units (M.U.); one M.U. was defined as the amount of toxin that caused death in 15 minutes. It was soon discovered that in different laboratories the assay procedure could produce results that differed by 60 to 70 per cent. Two of the important reasons for such differences were: (a) differences in tolerance of various species of mice used, and (b) different methods of extraction (96, 156). These differences were reduced when rather pure saxitoxin became available and was adopted as reference standard (147). Other factors that still influence the result of the assays are: (a) sex, female mice being slightly but significantly more sensitive than male mice, (b) concentration of sodium chloride in the toxin preparation, the higher the salt concentration, the less potent being the toxin (147, 158, 186), and (c) pH, the toxin being less active at pH 6 to 7 than at pH 2 to 3 (185).

The official method of analysis for saxitoxin as adopted by the Association of Official Agricultural Chemists can be found in a review by McFarren *et al.* (92). Details of the procedure for extracting raw shellfish have been published (92, 147, 156). Some of the precautions that must be observed in carrying out an assay should be repeated here, because readers may have occasion to check on

the potency of samples of saxitoxin: (a) One can use either the relation between mouse units and median death time or a quantal response method (LD50). (b) The toxin solution injected should be diluted in water, not salt solutions. (c) The pH of the solution injected should be between 2 and 4. (d) The concentration of toxin should be adjusted so that death occurs within 5 to 7 minutes after injection. (e) The mice used should weigh between 19 and 21 grams and not over 23 grams. Another most important step is establishing a mouse unit *vs.* median death time relation for the strain of mice employed. In the method adopted by the association of Official Agricultural Chemist, a CF (conversion factor) value must be determined to convert mouse units into absolute weight of pure toxin. This practice is necessary because the mouse unit-death time relation employed is based on a strain of mouse (156) that may not be available in other laboratories. In this table death time is estimated to the nearest 5 seconds, and the result can be quickly read off in mouse units. For those not engaged in the routine analysis of suspect shellfish it would probably be simpler to establish one curve for amount of toxin *vs.* death time with a sample of pure saxitoxin. Since this relationship is adequately described by a single exponential term (146, 147), plotting death time against the logarithm of dose should yield a straight line that could be established by assaying as few as three doses. From such a curve intermediary doses may be obtained by interpolation with errors no greater than those of the more involved procedure.

The accuracy of the bioassay method has been checked by 11 Canadian and United States laboratories in a collaborating survey (92). When bioassays were carried out on non-toxic mussels to which pure saxitoxin had been added, the estimates were lower by 15 to 40 per cent. The larger discrepancy occurred at low toxin levels because of a greater interference by salt. In spite of the errors, the results were reproducible from laboratory to laboratory. Since the purpose of the assay from a public health viewpoint is to determine the minimum safe level of toxin in shellfish, no harm is done if an adequate margin of safety is allowed. The sensitivity of the bioassay is about 160 ng (minimal lethal dose of 8 μg/kg in 20-gram mice). The inherent error in the bioassay method, however, has prompted the search for a suitable chemical method for determining saxitoxin. One method proposed by McFarren, Schantz, Campbell, and Lewis (93) depends on a positive Jaffe color reaction given by saxitoxin. The procedure was carried out on the toxin after it was first absorbed onto a cation exchange resin column and then eluted. This method tends to overestimate the amount of toxin present.

2. Comparative pharmacology. In contrast to studies made with tetrodotoxin, little is known about the relative susceptibility of different animals to saxitoxin. The LD50's on oral administration were determined in 8 species (188). Converted from the original values in mouse units, they are (as micrograms of pure toxin per kilogram of body weight) pigeon 91, guinea pig 135, rabbit 181, dog 181, rat 192, cat 254, mouse 382, monkey 364 to 727. In frogs, the signs of poisoning are like those in mammals, although the frog appears to be more resistant to the lethal effects of saxitoxin (136).

The LD50 of saxitoxin for mice in intraperitoneal injection is 10 μg/kg; the

oral LD50 is 263 μg/kg (147, 185). In rats, the intraperitoneal LD50 is 10.5 μg/kg, but the oral LD50 is considerably higher, 531 μg/kg (184). Age is important in influencing the susceptibility of rats to saxitoxin; in the newborn rat, the intraperitoneal LD50 is 5 μg/kg and the oral LD50, 72 μg/kg, as compared to the respective values above for adult rats. Prior experience with sublethal doses apparently increases the tolerance of rats to the lethal action of saxitoxin, for the oral LD50 can be nearly doubled after rats are fed single sublethal doses two weeks before testing (92).

The lethal dose for man is not known, although the problem is of some interest to public health workers. On the basis of lethal dose in animals and on the assumption of a homogeneous distribution in body water, Kao and Nishiyama (71) estimatd the intravenous lethal dose to be 0.4 mg. This has little practical significance aside from setting a lower limit of the lethal dose. In an interesting clinical study, Meyer (97) reported three cases of paralytic shellfish poisoning in which the number of mussels eaten by each victim was estimated from the empty shells left and the toxicity of the remaining uneaten mussels was assayed. From these, he estimated that the oral lethal dose for man was about 40,000 mouse units. This value has been quoted by Kao and Nishiyama (71) as corresponding to 8 mg, but this conversion is in error because Meyer's mouse unit is based on an extraction procedure using acid alcohol, not the acid aqueous extraction adopted by the Official Association of Agricultural Chemists. Death in man has followed ingestion of the equivalent of as little as 1 mg of saxitoxin (168).

3. Absorption, distribution and fate. From clinical and experimental observations, it can be deduced that saxitoxin is readily absorbed from the gastrointestinal tract. Bond and Medcof (10) describe the case of a young man who experienced "a pins-and-needles feeling in his lower lip and tongue while chewing the belly portion of a single raw clam." Although this instance does not indicate the degree of absorption through the buccal mucosa, it does show clearly that a sufficient amount is absorbed through the mucous membrane to produce local effects. Evidence that absorption can take place in small intestines was given by Prinzmetal, Sommer and Leake (136), who produced characteristic symptoms and death in a rabbit by injecting a crude toxin preparation into a tied segment of ileum. The degree of absorption through the gastrointestinal tract is not known, but it probably is not complete since a large difference exists between the lethal doses on oral and intraperitoneal administration. The distribution of saxitoxin is unknown, but reasoning from the rapid and wide-spread actions in mammals, Kao and Nishiyama (71) assumed that it is distributed throughout body water. Little is known about the elimination of saxitoxin. Prinzmetal, Sommer and Leake (136) assayed the toxin content of the urine of a dog that had been given 100 mouse units of a crude toxin preparation (route unspecified), and found 40 mouse units in the urine in 2 hours. This observation suggests that urinary excretion may be the chief route of elimination.

4. Systemic actions. Neuromuscular systems. The actions of saxitoxin on neuromuscular systems are like those of tetrodotoxin (Section II D 4 a), consisting of rapidly developing weakness of muscular contractions produced by neural

stimulation (9, 27, 34, 35, 37, 71, 75, 103). As in the case of tetrodotoxin, from studies of mechanical or electrical responses of whole muscles, the action of saxitoxin has been erroneously compared to that of curare. Actually the block of neuromuscular transmission is in the motor axon and on the muscle membrane while the end plate is relatively unaffected (see below).

There is some controversy over the susceptibility of different muscles of the cat to block by saxitoxin. Evans (35) felt that the respiratory muscles are more readily blocked than are the limb muscles, but Kao and Nishiyama (71) thought that it was just the reverse. This difference in viewpoint probably cannot be resolved until some more carefully controlled comparative studies are made.

Cardiovascular system. A marked hypotensive effect is usually described (25, 75, 104, 105, 136). It occurs with as little as 2 μg/kg in anesthetized dogs and cats, but smaller doses (0.75 μg/kg) that still produce muscular weakness may not cause any fall in blood pressure (71). This is an important difference between the actions of saxitoxin and tetrodotoxin (see also Section III A 1). With the latter, any dose sufficient to produce muscular weakness causes hypotension. The action of saxitoxin on the heart is minimal, and appears only with large doses (7 to 20 μg/kg). As with tetrodotoxin, there is disturbance of conduction chiefly at the atrio-ventricular node and to a lesser extent in the atrial and ventricular muscles (71, 75, 104, 136). In cross-perfusion experiments, saxitoxin had the same effects as tetrodotoxin (Section II D 4 b): applied to the donor's body and the recipient's head, it lowered blood pressure in the donor's, but not in the recipient's systemic circulation (Kao, Suzuki, and Kleinhaus, unpublished observations). The hypotension produced by saxitoxin, therefore, results from action elsewhere than in the heart or the brain. It is accompanied by vasodilation in the splanchnic area, as Kellaway was able to record plethysmographically increases in the volumes of an intestinal loop and of the spleen, but not of a leg (75). It cannot be attributed to direct action on the vascular smooth muscle, because pressor responses could be elicited by intravenous injections of epinephrine and norepinephrine (71). So it seems that, like tetrodotoxin, saxitoxin may cause a release of vasomotor tone.

Respiratory system. Clinically as well as experimentally, respiratory depression is the most prominent and most serious action of saxitoxin. As in the case of tetrodotoxin, the mechanism of respiratory depression is not clear. Most investigators adhere to a belief that saxitoxin has profound effect on the respiratory center, and that this is the most important action leading to respiratory depression. Kellaway (75) and, recently, Evans (35) showed that impulse discharges in phrenic nerves and in intercostal nerves not only remained but actually increased at a time when diaphragmatic movement ceased. Unfortunately in neither study can one decide whether the recorded action potentials were afferent or efferent. Hence, it is not possible to state from these studies that respiratory failure was due to peripheral paralysis. In the cross-perfusion experiments, the action of saxitoxin on respiration is similar to that of tetrodotoxin (Section II D 4 c): a fall in systemic pressure of the donor, reflected in the recipient's head, usually caused a transient increase in the rate and amplitude of respiration, probably through a chemoreceptor reflex.

Other actions. Clinically, disturbance of proprioception is a constant finding and some victims are reported to miss objects they reach for (92). Possibly, some interference with afferent stretch receptor impulses is responsible as with tetrodotoxin, but there may also be some interference with cerebellar function.

On isolated uterine and intestinal smooth muscles saxitoxin apparently has no action (136).

5. Cellular actions. Isolated nerves. The cellular actions of saxitoxin have not been studied as extensively as those of tetrodotoxin. On isolated desheathed frog sciatic nerve (71) and on isolated nodes of Ranvier (31, 32) saxitoxin blocked propagated spikes without depolarization. The minimum blocking concentration was 3 nM (71). Higher concentrations produced block more quickly and the rate of recovery was slower. In the blocking action, there are two possible differences from tetrodotoxin that must be studied further experimentally to see whether they are genuine observations. First, a block by saxitoxin may be more readily reversible than one by tetrodotoxin. The difference is manifested not in the rate of recovery after block by low concentrations of the toxins, but in the reversibility following block by higher concentrations in frog, squid, and lobster nerves. Often block by 300 nM concentration or higher of tetrodotoxin cannot be reversed (70, 163), but with saxitoxin, block in isolated nodes of frog nerve caused by concentrations as high as 300 μM could be reversed (32). Second, with low concentrations of saxitoxin there was often a transient increase in the spike amplitude. This change can be seen in figure 3 of a paper by Kao and Nishiyama (71), but, since that record was taken on bundles of nerve fiber, not much could be made of the increase. A similar transient change, however, has been observed on single nodes of Ranvier (32). At present, there is no explanation for these changes, since the resting potential remained the same. At the time of writing, there have been no published voltage clamp experiments with saxitoxin. On tetrodon and taricha nerves, saxitoxin is almost as potent as it is on frog nerve, an observation which further indicates that saxitoxin is different from tetrodotoxin (Kao and Fuhrman, unpublished).

Skeletal muscle, end-plate, and electroplates. On skeletal muscles and on the electroplates of the electric eel, saxitoxin, like tetrodotoxin, blocks spikes without depolarization (31, 32, 71). In frog sartorius muscle fibers, analysis of the steady-state current-voltage relationship of the excitable membrane showed that saxitoxin up to 300 nM had no action on effective membrane resistance, delayed rectification, or anomalous rectification (71). From these findings it was concluded that saxitoxin did not affect the potassium and chloride conductances of the excitable membrane nor did it interfere with the increase in potassium conductance that normally followed depolarization. Since spike generation was blocked in these concentrations, the final conclusion was that saxitoxin, like tetrodotoxin, interfered specifically with the initial increase of sodium permeability (71). Although this conclusion is probably correct, it must be confirmed by voltage-clamp analysis, which alone can show definitively the dynamic aspects of current-voltage relation of the excitable membrane.

The amplitude of the end plate potential is reduced by saxitoxin (9). Recently with intracellular microelectrodes, this observation was confirmed. The reduction

is due to a block of spike conduction in the motor axons and not to marked changes in the responsiveness of end-plate receptors (71). The evidence for this conclusion is similar to that for tetrodotoxin, *viz.*, when neuromuscular transmission was completely blocked, the end plate could still be depolarized by applied acetylcholine or by spontaneously released acetylcholine made manifest as miniature end plate potentials (71). In some instances a transient increase in their frequency was observed. Whether this is a stimulation of the nerve ending by saxitoxin remains to be studied. This effect has not been reported for tetrodotoxin. Furthermore, in contrast to tetrodotoxin, after long immersion in saxitoxin, the amplitudes of miniature end plate potentials were somewhat smaller (71). Whether this is due to a change in the responsiveness of the end plate or to a change in the quanta of acetylcholine released from the nerve endings (18) must also await further experiments.

On the electroplates of electric eel, saxitoxin in 300 nM concentrations inhibits spike generation on direct stimulation (32). At the time of writing the action on current-voltage relationship is not clear. Acetylcholine and carbamylcholine can produce depolarization and increase in potassium efflux when spike generation is blocked by saxitoxin (32). As in the case of tetrodotoxin (Section II D 5 b), these phenomena are probably to be attributed to continued responsiveness of the postjunctional membrane.

Other systems. There is little concrete information on the effects or the lack of effects of saxitoxin on active cation transport or on cholinesterases. Dettbarn *et al.* (31, 32). in discussing their electrophysiological observations, mentioned that the cholinesterase system was unaffected by saxitoxin. In a preliminary note, Clapham and Evans (23) claimed to have shown a decrease in the resting sodium fluxes across frog sartorius muscle membrane. Their data are too scant to permit a proper evaluation, but the claim, if proved, would be the first piece of evidence to show that resting as well as active sodium channels in an excitable membrane can be blocked by saxitoxin. The resistance of mussels and clams to saxitoxin is an interesting problem that has not been studied at all. Some of the clams contain as much as 50,000 mouse units of toxin without any apparent ill effect. Could this immunity be due to a calcium-dependent membrane as in the barnacle (51)?

IV. CONCLUDING REMARKS

From the available evidence there is little doubt that tetrodotoxin and saxitoxin are chemically distinct, and that their biological actions are very similar. The chief point of chemical similarity is the presence of guanidinium groups in both toxins; and guanidinium compounds are known to have interesting actions on excitable membrane (30, 85, 89, 91). From these considerations, it was suggested recently that the guanidinium groups may enter the sodium channels in the excitable membrane, and that some adjacent groups on the molecule may form some bonds with certain membrane structures to close off these channels for sodium movement (71). Although this notion is only speculative and may prove to be erroneous, it illustrates the potential usefulness of these toxins in

helping to elucidate the molecular structures of some very important loci in the excitable membranes and possibly also the alterations in these loci that form the basis of excitation. In this direction, therefore, tetrodotoxin and saxitoxin are of great significance in the study of excitation phenomena, for they are the first substances that promise to take us beyond the level of "homogeneity and heterogeneity of excitable membranes," into more concrete thinking about a hitherto unaccessible area of study. From a practical standpoint, these toxins are also useful in eliminating all-or-none responses, and leaving chemosensitive response available for detailed study. For instance, normal transmissional processes and responses of these two drugs may be re-examined without the use of agents and procedures that can affect the chemoreceptors, and the role of the nerve terminal in these processes can be studied with a new tool. Also, for some studies on those excitable membranes that undergo changes in sodium permeability, it is now possible to eliminate the spike with minute amounts of tetrodotoxin or saxitoxin. Use of these agents obviates difficulties created by special solutions of markedly altered ionic compositions or ionic strengths that have been used heretofore. As with many other good tools, however, caution should accompany their use lest the observations lead to absurd deductions. Tetrodotoxin and saxitoxin are not preternatural substances that will quickly and painlessly sort out those excitable membranes that undergo changes in sodium permeability from those that do not. The resistance of taricha and tetrodon nerves to tetrodotoxin and the dependence of their spikes on the presence of external sodium are the clearest examples that immunity to tetrodotoxin alone is no indication whatsoever of the nature of the ionic mechanism in any excitable cell. Lastly, since interest in these toxins originated out of human sufferings, let us hope that the disclosure of the chemical nature of the toxins will soon lead to new drugs for the relief of human sufferings.

Acknowledgment. In the preparation of this article, I have enjoyed the friendly help and advice of many people. Foremost among these is Prof. F. A. Fuhrman of Stanford University who introduced me to the fascinating world of toxinology, and who has always been most generous in sharing his enthusiasm and knowledge in this area. Prof. T. Suzuki and Dr. A. Nishiyama of Tohoku University gave most handsomely of their time and effort in collecting the extensive Japanese literature, and helped patiently with the interpretation of many subtle details. Prof. M. Goto and Dr. I. Tani of Kyushu University, Dr. Y. Ogura of Chiba University, Prof. K. Tsuda of Tokyo University and Drs. R. Sakai and T. Deguchi of Sankyo Company were all most generous in supplying me with useful information. Prof. H. S. Mosher of Stanford University, and Dr. J. W. Moore of Duke University provided many helpful discussions. Dr. F. F. Kao, of State University of New York Downstate Medical Center, kindly lent me his collection of Chinese medical books. I also owe a debt to Mrs. R. C. R. Kao, Mrs. Helen Kovacs, and Miss S. Tanner for helping with the references and Miss Joan Miles for help in typing. The unpublished work from my laboratory was partly supported by a grant from the United States Public Health Service, National Institute of Child Health and Human Development HD-378.

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