Together with Eq. 2, it provides a criterion for complete separation. Two components (A and B) may be separated completely if the difference between their discharge angles ($\Theta_A - \Theta_B$) multiplied by π times disc diameter is greater than their average discharge zone length ($H_{CA} + H_{CB}/2$).

Continuous disc chromatography has inherently lower resolution than a capillary column of diameter equal to the plate-separation distance. However, plate-separation distances with this chromatography can be made less than the smallest practical capillary diameters so that the loss in resolution may be partially offset. In addition, capacity is magnitudes higher; response time is shorter, usually a fraction of the rotational period.

By making analysis a function of an angular displacement rather than a time displacement, continuous disc chromatography potentially simplifies all preparative, automatic analysis and monitoring applications of chromatography. Speed of analysis becomes less dependent on the response time of gas detectors, and different kinds of detectors can be used for each effluent species. We believe the concept can lead to a broad spectrum of new instruments and processes for separating and analyzing mixtures.

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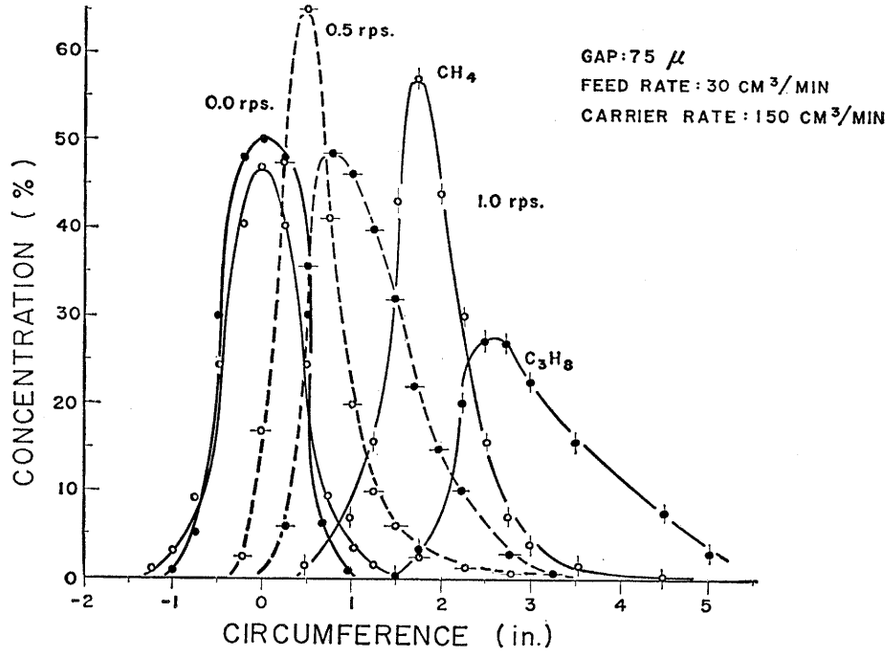

Fig. 2. Effluent concentration as a function of peripheral position for three disc-rotation speeds. Mixture of methane and propane (50 : 50) is used. High loading condition (30 cm³/min).

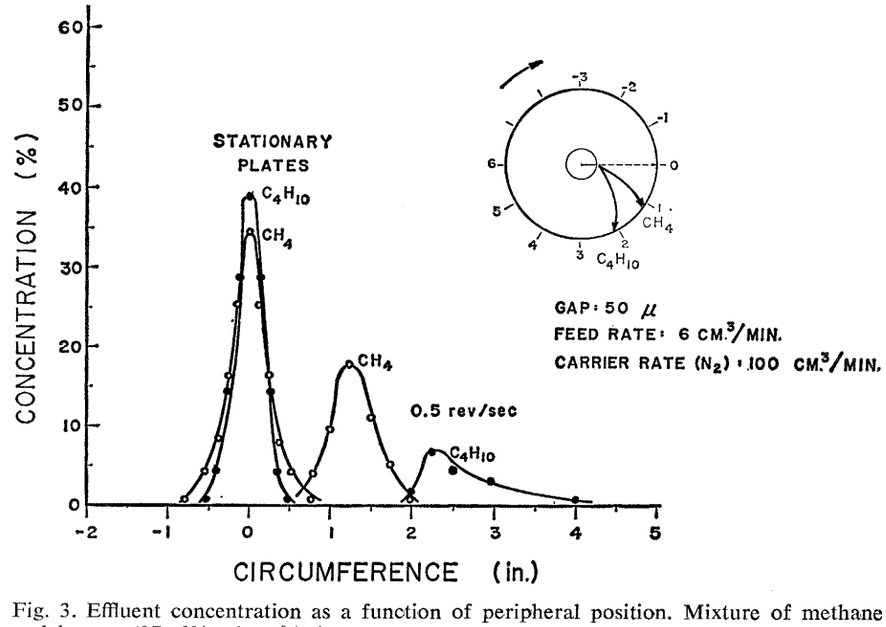

Fig. 3. Effluent concentration as a function of peripheral position. Mixture of methane and butane (37 : 63); 6 cm³/min.

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Tetrodotoxin Derivatives: Chemical Structure and Blockage of Nerve Membrane Conductance

Abstract. *The nerve-impulse-blocking actions of derivatives of tetrodotoxin have been tested on lobster and squid axons. The block produced by deoxytetrodotoxin was similar to that produced by tetrodotoxin and was probably caused by tetrodotoxin contamination. Tetrodaminotoxin and anhydrotetrodotoxin also produced a similar block but at such high concentrations that tetrodotoxin contamination cannot be ruled out. The hydroxyl group of C_4 and the hemilactal oxygen links play an important role for the nerve-blocking action.*

Tetrodotoxin (TTX), the active ingredient of the puffer fish poison, has become a useful tool in the study of electrophysiology since its blocking mechanism of nerve conduction was unveiled (*1*). The TTX blockage, which is not accompanied by any change in

resting potential, has been ascribed to the specific inhibition of the mechanism whereby the conductance of the nerve membrane is transiently increased upon depolarization (*2*). Sodium (or its substitutes in the external bathing medium) flows into the axon upon stimulation and this inflow generates the action potential.

One of the approaches to the elucidation of the mechanism of the TTX blocking action on the early transient conductance is to find out as much as possible about the structural-functional relationships of the TTX molecule. Some data on TTX derivatives were already available—data on their lethal toxicity to mice and their ability to block the conduction of frog sciatic nerve (*3, 4*). However, since the lethal action of any particular drug involves a number of factors other than the primary action at the target site, it is far from satisfactory, though not meaningless, to compare the chemical structures of the derivatives for their lethal action. Likewise, the conduction block of nerve bundles can be produced by a variety of mechanisms, so that it is still not so satisfactory to discuss the active groups of the molecule based on the data on simple conduction block.

The only significant appreciation of the structural-functional relationship of a nerve-blocking agent must come from a study of the manner of nerve blockage at the cellular or molecular level. Changes in resting potential associated with nerve blockage can be measured by intracellular microelectrodes and give some insight into the manner of nerve blockage. However, the most powerful electrophysiological approach available at present is the study of specific membrane conductances to ions by means of a voltage clamp. The present study was undertaken in order to see whether the TTX derivatives block nerve conduction in the same manner as TTX itself.

Tetrodotoxin and its four derivatives were supplied from the Sankyo Company, Tokyo, namely TTX ($C_{11}H_{17}O_8N_3$, M.W. 319), deoxytetrodotoxin ($C_{11}H_{17}O_7N_3$, M.W. 303), anhydrotetrodotoxin ($C_{11}H_{15}O_7N_3$, M.W. 301), tetrodaminotoxin ($C_{11}H_{18}O_7N_4$, M.W. 318), and tetrodonic acid ($C_{11}H_{17}O_8N_3 \cdot H_2O$, M.W. 337). The chemical structures are shown in Fig. 1. These derivatives, except tetrodonic acid, were first dissolved in a small amount of 10 percent (by volume) acetic acid solution and then diluted with the bathing medium to give desired concentrations. The final *p*H was adjusted to 7.9 by adding a small amount of tris buffer solution. For tetrodonic acid, 1 percent (by volume) hydrochloric acid was used instead of acetic acid. In any case, the test solution was prepared shortly before the experiment.

Microelectrode experiments. The giant axons in the circumesophageal connective of the lobster *Homarus americanus* were used as material. A conventional glass micropipette filled with 3*M* KCl was inserted in an axon, and measurements were made on the resting potential, the propagated action potential, and its maximum rate of rise. The following artificial sea water was used as the bathing medium (millimolar): Na^+, 468; K^+, 10; Ca^{2+}, 25; Mg^{2+}, 8; Cl^-, 533; SO_4^{2-}, 4; HCO_3^-, 3; *p*H 7.9. The experiments were done at room temperature (21° to 23°C).

Tetrodotoxin, deoxytetrodotoxin, anhydrotetrodotoxin, and tetrodaminotoxin blocked the action potential of the lobster axons without changing the resting potential. The effect was completely reversible upon washing unless the preparation was exposed to the test solution for a long period of time (over some 10 minutes). However, the time course of recovery was much slower (approximately 5 to 20 times) than that of blockage. The threshold concentration was different between these derivatives. Judging from the time for blockage, derivative concentrations equivalent to $3 \times 10^{-8}M$ TTX (which was near blockage threshold) were: $2 \times 10^{-7}M$ for deoxytetrodotoxin, $2 \times 10^{-6}M$ for tetrodaminotoxin, and $3 \times 10^{-6}M$ for anhydrotetrodotoxin (Table 1).

Tetrodonic acid was totally ineffective on the resting and action potentials at a concentration of $1 \times 10^{-4}M$ for a period of up to 35 minutes (Table 1).

Voltage-clamp experiments. The giant axons of the squid *Loligo pealii*, available at Marine Biological Laboratory, Woods Hole, Massachusetts, were used. The sucrose-gap voltage-clamp technique was essentially the same as that described (*5*) and modified (*2*) previously. Natural sea water was used as the bathing medium. The experiments were done at 7° to 9°C.

The three effective derivatives and TTX blocked the early transient membrane currents associated with step depolarizations, without affecting the late steady-state membrane currents, in voltage-clamped squid axons. The effect of all of the derivatives was indistinguishable from that of TTX itself aside from different threshold concentrations. An example of membrane current recordings is shown in Fig. 2, in which the current recordings were superimposed for a period of 4.5 minutes after application of $2 \times 10^{-5}M$ anhydrotetrodotoxin. The early transient inward current (downward deflection) was

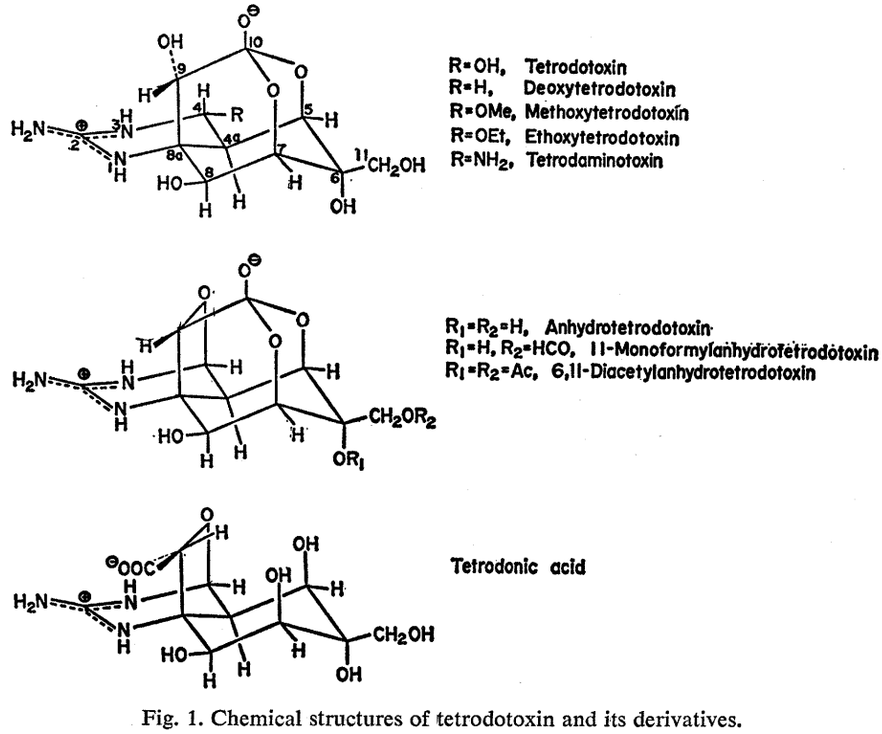

Fig. 1. Chemical structures of tetrodotoxin and its derivatives.

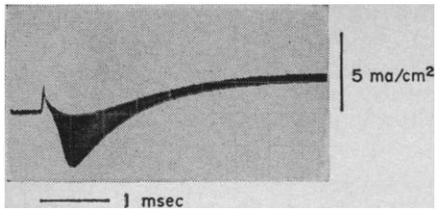

Fig. 2. Changes in the membrane current associated with repeated 80-mv step depolarizations from the holding membrane potential of −100 mv for a period of 4.5 minutes after application of $2 \times 10^{-5}M$ anhydrotetrodotoxin under voltage-clamp conditions. Downward deflections indicate inward membrane currents. Superimposed records. Note the drastic decrease in the early transient current with little change in the late steady-state current (preparation 7-16-66).

progressively suppressed during the course of anhydrotetrodotoxin application, while the late steady-state outward current (upward deflection) remained almost unchanged.

Tetrodonic acid had no notable effect on the early transient current. The effect on the late steady-state current was somewhat questionable, because in two experiments the current was slightly depressed after a long exposure (20 minutes), whereas in the other two experiments the current was kept in the normal range.

Since we have obtained evidence that three active TTX derivatives block nerve excitability by the same mechanism as TTX itself, we are now in position to discuss the relationship between the chemical structure and activity. Table 1 gives a summary of the relative effectiveness of TTX derivatives on toxicity and conduction block. The present results with action and resting potential studies on the lobster axons are in agreement with those obtained with the frog sciatic nerve (*4*).

Before discussion of the structure-activity relationship, some comments are needed on the possible contaminations of the derivatives with TTX itself. Although the derivative samples were examined for their purity at Sankyo Company by various chemical and physical methods, such as the infrared spectrum and nuclear magnetic resonance (NMR) spectrum, and were found to contain no detectable TTX contamination, there remained the possibility that a trace of TTX which was beyond the sensitivity of the available assay methods might be present in some of the derivatives (*4*).

Because contamination with TTX is critically important in drawing any conclusion on the structure-activity relationship, owing to the unusually low threshold concentration of TTX to block nerve conduction, H. S. Mosher and L. J. Durham (Stanford University) offered to test our samples of three active derivatives on their most sensitive 100-mc NMR equipment. Their analysis revealed that the deoxytetrodotoxin sample contains as much as 15 percent TTX (*6*). The amount of TTX contamination is more than enough to account for the nerve blocking action of the deoxytetrodotoxin sample, which showed about 15 percent of the toxicity of TTX. The other two active derivatives required 70 to 100 times the concentration of TTX for equivalent blocking action. It is possible that their activity is also due to a contamination of about 1 percent of TTX. However, it is not possible to reliably demonstrate this level by the presently available techniques of analysis, and the question of the actual toxicity of tetrodaminotoxin and anhydrotetrodotoxin must remain unresolved.

Although we originally thought that we were observing a gradual diminution of the TTX toxicity with structural changes, as indicated in Table 1, the NMR results force reconsideration. We may presently conclude that:

1) Opening the hemilactal link to give tetrodonic acid eliminates toxicity to below a measurable level.

2) A reduction at C_4 probably eliminates all toxicity because there is more than enough TTX contamination to account for all activity of deoxytetrodotoxin.

3) Substitution of NH_2 for an OH group at C_4 or the formation of an additional oxygen link between C_4 and C_9 reduces toxicity by at least 100 fold. It is possible that the reduction in toxicity was much more, if the observed activity was also due to TTX contamination.

It is very likely that the guanidinium group of the TTX molecule plays a key role, as suggested by Kao and Nishiyama (*7*) for saxitoxin, because guanidine can pass through the squid nerve membrane to produce action potentials under certain conditions (*8*). The receptor on the nerve can be visualized as the gate that controls the early transient channel of the nerve membrane and that is located on the external surface of the nerve membrane (*9*). The hydroxyl group on C_4 has a great influence on the positive charge of the guanidinium group. A reduction at this position might drastically change the electron profile there, affecting the charge of the guanidinium group. This may be the main cause of the activity loss of deoxytetrodotoxin. Opening the hemilactal link abolishes the nerve-blocking activity, as we have seen with tetrodonic acid, and this may be attributed to the transfer of negative charge closer to the positive charge on the guanidinium group.

Table 1. Comparison of tetrodotoxin and its derivatives.

Tetrodotoxin and its derivatives	LD_{50} for mice* (μg/kg)		Minimum lethal dose for mice (μg/kg)†		Blockage* of frog sciatic nerve (*M*)	Blockage of lobster nerve (*M*)
	Intravenous	Oral	Intravenous	Intraperitoneal		
Tetrodotoxin	9.0	435	8.22‡		1.6×10^{-8}	3×10^{-8}
Deoxytetrodotoxin	41.7	2,700	84.5		1.2×10^{-7}	2×10^{-7}
Methoxytetrodotoxin	341	12,700	341		4.5×10^{-7}	
Ethoxytetrodotoxin	692	25,700		692	9.6×10^{-7}	
Tetrodaminotoxin	528	26,100	841		1.7×10^{-6}	2×10^{-6}
11-Monoformylanhydrotetrodotoxin	1,380	13,600		3,000	1.8×10^{-6}	
Anhydrotetrodotoxin	1,250	16,900	4,140		2.8×10^{-6}	3×10^{-6}
6,11-Diacetylanhydrotetrodotoxin	96,700			> 50,000	3.6×10^{-5}	
Tetrodonic acid	68,700		> 300,000		$> 5.9 \times 10^{-4}$	$> 1 \times 10^{-4}$

*After Deguchi (*4*). †After Tsuda *et al.* (*3*). ‡LD_{50}.

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3 March 1967

Lactic Dehydrogenase and Metabolism of Human Leukocytes in vitro

Abstract. *During transformation and division of lymphocytes in culture, the lactic dehydrogenase isozymes migrate increasingly toward the cathode. With extension of the time in culture, the mitotic index declines, and the isozyme pattern reverts to dominance of those bands that move toward the anode, despite the cellular tendency to anaerobic metabolism. These findings suggest that synthesis of the more slowly migrating lactic dehydrogenase isozymes in this system is related to mitotic activity, and not to the aerobic or anaerobic conditions of cell culture.*

Primary cultures of human leukocytes provide a rapidly dividing and metabolically active tissue for the investigation of isozyme synthesis. Under stimulation with phytohemagglutinin (PHA), leukocytes in short-term, 72-hour cultures undergo an orderly series of morphological changes, beginning with degeneration of the granulocytes, and followed by transformation and mitosis of lymphocytes (*1*). There is an early increase in RNA synthesis (*2*) and in the phosphorylation and dephosphorylation of nuclear proteins (*3*). Together with the fact that the rate of protein synthesis is increased after stimulation with PHA, these findings have been interpreted as indicative of gene activation before or during mitosis (*3*).

Genetic control of the synthesis of the heart (H) and muscle (M) polypeptides of lactic dehydrogenase (LDH) is now well established (*4*). It has also been suggested that the bands of LDH that migrate towards the cathode are associated with conditions of relative anoxia and anaerobic metabolism, while the isozymes on the anode side predominate under conditions of higher oxygen tension and aerobic cellular metabolism (*5*). In our study of leukocytes stimulated with PHA, the relationship between transformation and cell division and the activity of a specific isozyme system was examined. During 168 hours of culture, sequential observations were also made on the energy metabolism of the cells.

Human leukocytes from normal volunteers were cultured, under stimulation of phytohemagglutinin, in tissue-culture medium 199 with a volume of fetal bovine serum equal to that of the cell suspension. From 6×10^6 to 8×10^6 cells were inoculated into each culture, and cultures were incubated at 37°C for 24, 48, 72, 96, or 168 hours. Cells were harvested, washed three times in isotonic saline, and homogenized in 0.5 ml saline, at room temperature, at 2500 rev/min, with a Teflon tissue homogenizer.

Analyses of glucose, pyruvic and lactic acids, and α-ketoglutaric acid were performed on samples of the medium. Electrophoresis on agar gel, and determinations of total lactic dehydrogenase activity and total protein were performed on cell homogenates. Five microliters of cell homogenate were used for each electrophoresis. The concentration of glucose was determined with a modification of Folin's micromethod (*6*); that of pyruvic acid, according to Friedmann and Haugen (*7*); and that of lactic acid, according to Huckabee (*8*). Determinations of the amounts of α-ketoglutarate were done on the basis of Shimizu's quantitative technique for extraction of its 2,4-dinitrophenylhydrazine derivative into xylol (*9*). The assay for the total activity of lactic dehydrogenase was adapted for cell homogenates from the procedure originally described by Wroblewski for reduction of pyruvic acid in the presence of reduced nicotinamide-adenine dinucleotide (NAD) (*10*). Determinations of total protein were done according to the Lowry technique (*11*). Electrophoresis of cell homogenates was carried out on agar gel at 4°C with sodium lactate as the substrate, NAD as the coenzyme, and phenazine methosulfate as the electron carrier. A purple band was produced at the sites of LDH activity, by reduction of nitro blue tetrazolium to formazan. The following results are derived from studies of 95 cultures from 16 subjects.

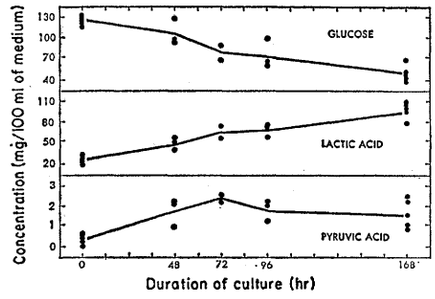

Fig. 1. Glucose and lactic and pyruvic acids in the medium of leukocyte cultures. Each point represents a single determination from a culture assayed at the indicated time. The curves are derived from plots of the mean values obtained.

In the first 72 hours of culture, the concentrations of pyruvic and lactic acids rose, while that of glucose in the medium fell. Continued culture of these leukocytes resulted in a further increase in the concentration of lactic acid, with a precipitous decline in available glucose in the medium by 168 hours (Fig. 1). The concentration of pyruvic acid, however, appeared to reach a plateau, after increasing in the first 72 hours of culture.

In all cultures, total activity of LDH had increased by at least 100 percent by 72 hours of culture, the time of maximum mitotic activity, with mean values ranging from 1130 Wroblewski units per milliliter of cell homogenate before culture, to 2450 units at 72 hours, and 2200 units at 168 hours. Values for total protein fell and stabilized, during the first 72 hours of culture, from a mean of 3.0 mg/100 ml of homogenate before culture, to 1.9 mg/100 ml of medium at 72 hours. This decrease in total protein early in culture reflected degeneration of the granulocytes, the largest cell population inoculated into culture. Thus, the specific activity (Fig. 2) defined as the number of units of LDH per milligram of protein rose most significantly before mitosis and during active mitosis.

The shift in isozyme pattern normally seen is shown in Fig. 3, with the increase in LDH-3 and LDH-4 at 72 hours indicating a decrease in H-subunit activity and an increase in the activity of M subunits (*12*). In the first