Two photoactivable derivatives of tetrodotoxin have been synthesized. Electrophysiological experiments on crab giant axons and competitive binding with \(^{3}H\)-tetrodotoxin for the tetrodotoxin receptor indicate that they are only 4.5 to 7.5 times less active than tetrodotoxin itself. These compounds give a reversible block of the sodium channel in the dark but after ultraviolet irradiation they provoke an irreversible blockade of the channel.

The sodium channel plays a central role in the generation of an action potential. Two different parts can be distinguished in the channel: (i) the selectivity filter which preferentially selects the Na\(^+\) ion and (ii) the gating system which gives to the channel the adequate kinetics of opening and closing.

Nature provides two neurotoxins, tetrodotoxin and saxitoxin (1-3), which very selectively associate with the selectivity filter of the Na\(^+\) channel (4). Both toxins are relatively small molecules (Mr = 300 to 319) carrying a charged guanidinium group which is thought to mimic the hydrated Na\(^+\) ion (5) and a complex hydrophilic moiety which probably contributes to binding of the toxin to the channel through hydrogen bond formation. Both neurotoxins reversibly associate with the Na\(^+\) channel; the complex they form with the selectivity filter is fairly stable since dissociation constants of 1 to 10 nM have been consistently found in a variety of systems (6-9).

It is evidently tempting to modify chemically these neurotoxins in order to transform them into irreversible affinity labels which will be essential in future studies of the structure and function of the sodium channel.

Attempts have been made in the past to modify chemically tetrodotoxin (10-12) with nearly no success for two reasons: (i) the tetrodotoxin molecule is extremely fragile and difficult to work with and (ii) most chemical modifications will destroy the toxic activity. It is only recently that an oxidative modification of tetrodotoxin has been shown to be nondestructive (13). Using this observation, we have been able to prepare a series of photoactivable affinity derivatives of tetrodotoxin of which we report here the synthesis and the biological activities.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following compounds were obtained from the companies indicated: 4-fluoro-3-nitroaniline, hydrazine hydrate (98%, purum), so-

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*TTX Oxidation*

Oxidation of aqueous solutions of citrate-free TTX by periodate was performed essentially as described by Tsien et al. (13). Briefly, 1 mg of citrate-free TTX (8.14 \(\mu\)mol) with \(^{3}H\)TTX in tracer amounts was dissolved in 380 \(\mu\)l of H\(_2\)O (acidified with 2 \(\mu\)l of AcOH). The oxidation was started by adding 32 \(\mu\)l (32 \(\mu\)mol) of 0.1 M aqueous NaIO\(_4\) solution and reaction was allowed to proceed for 30 min at room temperature. The reaction was followed by the decrease in absorbance at 223 nm (15). Periodate and iodate ions were eliminated from the incubation medium by precipitation in the cold with 1.6 \(\mu\)l (1.6 \(\mu\)mol) of 1 M aqueous lead(II) acetate (ice bath).

After centrifugation (2 min/2000 g) in a MSE table-top centrifuge, the clear supernatant was carefully removed and lyophilized. This step eliminated the formaldehyde produced during oxidation. The product (norTTX) could then be easily visualized on a silicon gel plate (Merck, 0.25-mm GF\(_{254}\)) by spraying with a 0.4% solution of 2,4-DNPH in 1 N HCl in water.

This procedure gave a norTTX which was directly used for the coupling reactions.

**FNAP and NAP-X Derivatives**

All synthetic work described below was done in the dark or in dim day light.

**FNAP**—It was prepared and purified from 4-fluoro-3-nitroaniline as described by Fleet et al. (16). The product was controlled by UV and IR spectroscopy and kept at \(-10^\circ\)C in the dark.

\(^{1}\) The abbreviations used are: Me\(_2\)SO, dimethyl sulfoxide; DMF, N,N-dimethylformamide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; 2,4-DNPH, 2,4-dinitrophenylhydrazine; NaCNBH\(_3\), sodium cyanoborohydride; TTX, tetrodotoxin; norTTX, \(\text{Cl}_{17}\)-nortetrodotoxin; FNAP, 1-fluoro-2-nitro-4-azidophenyl; NAP, 2-nitro-4-azidophenyl; NAP-glycinehydrazide, N-(2-nitro-4-azidophenyl)-glycinehydrazide; NAP-gly=norTTX, N-(2-nitro-4-azidophenyl)-N'- (11-nortetrodotox)ethylendiamine; TMS, trimethylsilyl.
**Photoactivable Derivatives of Tetrodotoxin**

**Photoactivable Derivatives of Tetrodotoxin**—FNAP, 1.092 g (6 mmol), was added to 838 mg (6 mmol) of glycine ethyl ester (containing a desired amount of [3]Cglycine ethyl ester) in 11.26 ml of MeSO. After adding 2.02 ml (18 mmol) of triethylamine, the reaction was allowed to proceed at 50°C for 1 h (17). FNAP-glycine ethyl ester was purified by chromatography on Silica Gel H (Merek) using Solvent System B followed by Solvent System A. The second elution gave a 94% pure product; the yield was 64%. UV: \( \epsilon^{260} = 5.200 \), \( \epsilon^{252} = 23.250 \) (in MeOH); IR: 2.120 cm\(^{-1}\) (N\(_2\)), 1.754 cm\(^{-1}\) (ester carbonyl); NMR: \( \delta_{CH} = 8.22 \), \( \delta_{NH} = 7.81 \), \( \delta_{CH} = 7.07 \), \( \delta_{CH-CH} = 3.96 \), \( \delta_{CH-CH} = 4.23 \).

Two milliliters of NH\(_2\)NH\(_2\)-H\(_2\)O (98%) in 37.5 ml of absolute EtOH were added to 900 mg (3.4 mmol) of FNAP-glycine ethyl ester in 67.5 ml of absolute EtOH. The mixture was refluxed during 90 min. After a fold concentration, 15 ml of Solvent F (see “Materials”) was added and the strongly colored solution was chromatographed on Silica Gel G60 (Merek) using Solvent System F followed by Solvent System E. The first elution (Solvent F) elimated the rest of adduct and Solvent E eluted the desired product, NAP-glycinhydrazide. Recrystallization from DMF gave 220 mg (0.87 mmol) of pure product (yield: 95%). The \( R_f \) in Solvent System F was 0.49. UV: \( \epsilon^{465} = 5.300 \), \( \epsilon^{296} = 23.000 \) (MeOH); IR: 3.640 cm\(^{-1}\) (N\(_2\)), 2.120 cm\(^{-1}\) (N\(_2\)), 1.715 cm\(^{-1}\) (hydrazide carbonyl) in MeSO.

\( \text{C}_{12}\text{H}_{18}\text{N}_{6}\text{O}_{3} \)

Found: C 38.22, H 4.05, N 37.85, O 19.87
Calculated: C 43.24, H 4.54, N 37.82, O 14.40

**NAP-ethylenediamine—**Ethylenediamine, 0.4 ml (6 mmol), was added to 189 mg (1 mmol) of FNAP in 9 ml of MeSO. The reaction was allowed to proceed for 30 min at 50°C. MeSO was lyophilized and the product was recrystallized in a minimum of MeSO to give quantitatively the desired product (yield >95%). The amino reactivity was tested with TNBS according to Snyder and Sobocinski (18). UV: \( \epsilon^{259} = 23.500 \), \( \epsilon^{245} = 5.200 \) (in MeOH); IR: 2.12 cm\(^{-1}\) (N\(_2\)).

\( \text{C}_{10}\text{H}_{16}\text{N}_{2}\text{O} \)

Found: C 43.40, H 4.36, N 36.91, O 14.67
Calculated: C 43.04, H 4.54, N 37.02, O 14.40

**NAP-TTX Derivatives**—NAP-glycinhydrazide of norTTX—Citrate-free TTX, 4.1 mg (12.87 \( \mu \)mol), with [\(^{1}H\)TTX as tracer was solubilized with 1.18 ml of H\(_2\)O and 10 \( \mu \)l of AcOH and subjected to oxidation with 13 \( \mu \)mol (130 \( \mu \)l) of freshly prepared NaI\(_2\)O\(_4\) (0.1 M). The product had a specific radioactivity of 894 dpm/mMol. After I\(_2\)\(_2\)O\(_4\) precipitation and lyophilization, 9.4 pmol of norTTX were added to 47.1 pmol of NAP-glycinhydrazide (at 40 dpm/mMol) and the mixture was lyophilized and added to the strongly colored solution was chromatographed on Silica Gel G60 (Merek) using Solvent System F followed by Solvent System E. The first elution (Solvent F) elimated the rest of adduct and Solvent E eluted the desired product, NAP-glycinhydrazide. Recrystallization from DMF gave 220 mg (0.87 mmol) of pure product (yield: 95%). The \( R_f \) in Solvent System F was 0.49. UV: \( \epsilon^{465} = 5.300 \), \( \epsilon^{296} = 23.000 \) (MeOH); IR: 3.640 cm\(^{-1}\) (N\(_2\)), 2.120 cm\(^{-1}\) (N\(_2\)), 1.715 cm\(^{-1}\) (hydrazide carbonyl) in MeSO.

\( \text{C}_{12}\text{H}_{18}\text{N}_{6}\text{O}_{3} \)

Found: C 38.22, H 4.05, N 37.85, O 19.87
Calculated: C 43.24, H 4.54, N 37.82, O 14.40

**Spectral Data**—UV/VIS spectra were recorded on a Beckman ACTA M VI spectrophotometer. IR and proton-NMR spectra were obtained with a Perkin-Elmer (type 477) and with a 60-MHz Varian instrument, respectively. NMR measurements were made with TMS as the internal standard.

Radioactivity was measured by liquid scintillation counting on Packard spectrometer (Type 2400) using \( \beta \)-scintillator (Tackard) as the scintillator.

**Biological Activity Measurements on Crabs**—NAP-glycinhydrazide of norTTX and its derivatives consisted of injecting 100 \( \mu \)l of a solution of toxin (1 to 10 \( \mu \)g) into a small crab of about 10 g. The time necessary to paralyze the animal was found to be inversely proportional to the concentration of the toxin. Generally, with a 1 \( \mu \)l solution of unmordified TTX, paralysis was obtained 40 to 50 s after injection.

Electrophysiology Measurements—The activity of TTX or TTX derivatives was measured by following the ability of the toxin to decrease the rate of the rising phase of the action potential. Resting and action potentials were recorded at 18°C by inserting a glass capillary microelectrode filled with 3 M KCl solution (resistance, 10 megohms). Electrical stimulation was applied at one end of the nerve with a pair of silver wire electrodes. The action potentials were then recorded directly on an oscilloscope in digital format with an Intertechnique Plurimat S (Plaisir, France) digital computer to obtain automated analysis and maximization of the dV/dt signal. The biological material used was the giant axon isolated from the circumoesophageal nerve of the crab *Carcinus maenas*. The physiological solution was artificial sea water (465 mM NaCl, 10 mM KCl, 8 mM MgCl\(_2\), 23 mM CaCl\(_2\), in 10 mM Tris-HCl (1020 mosm)) at pH 7.8.

**Photoirradiation**—For irreversible binding of the TTX derivatives to the axonal membrane, the nerve preparation was irradiated with an UV lamp (100 watts) connected to a spectroflash switching unit (Applied Photophysics, London) working with a 25-kV constant current (3 mA) DC power supply. A 10-kV discharge was routinely used. Flash time was in the millisecond range, the time between each flash being about 10 to 15 s.

The UV source was positioned at 5 cm from the nerve preparation. The irradiation was focused by a simple lens system.

**Axonal Membranes**—Axonal membranes were prepared from walking legs axon bundles of *Cancer pagurus* as previously described (8). Fraction II was used for binding assays.

**Binding Assays**—Equilibrium dialysis measurements of [\(^{3}H\)TTX (0.4 Ci/mmol) binding to its receptor were performed as previously described (8) in a 10-cell device with 900-\(\mu\)l compartments equipped with SM 11535 Sartorius membranes. Two hundred micromgrams of crab axonal membranes (Fraction II) were used in 140 \(\mu\)l of 50 mM Tris-HCl buffer at pH 7.4. Equilibrium was obtained after 2 h at 25°C.

**RESULTS AND DISCUSSION**—Critical Problems in the Synthesis of the TTX Derivatives—The routes for the synthesis of the two photoactivable affinity derivatives of TTX are represented in Fig. 1.

The first and crucial reaction is the oxidation of TTX by periodate at positions 6 and 11. It has previously been demonstrated by NMR that only the C6 and C11 carbons are oxidized (13). The periodate treatment leaves the guanidinium and the hemimethyl groups unmodified as well as the hydroxyls at carbons C4, C8, and C9 which are essential for the toxic activity (10–12). The kinetic properties and the stoichiometry of this oxidation reaction are shown in Fig. 2. Fig. 2A shows that 1 eq of IO\(_4\)^−/eq of TTX gives a rapid and quantitative oxidation of the toxin. If the oxidation is carried out with more than 1 eq of IO\(_4\)^−/eq of TTX (Fig. 2B), secondary and slower reactions occur. Products of these secondary oxidations have not been analyzed nor used for subsequent coupling procedures. However, similarly to the primary oxidation prod-
Photoactivable Derivatives of Tetrodotoxin

FIG. 1. Schematic structure of TTX, norTTX, NAP-gly=norTTX and NAP-en-norTTX.

FIG. 2. A, kinetics of periodate consumption during TTX oxidation followed by the decrease of UV absorption at 223 nm. The concentrations were 10 mM for TTX and NaIO₄, temperature, 22°C. The plateau value obtained after 20 to 30 min is due to IO₃⁻ absorption at the same wavelength. B, stoichiometry of the periodate oxidation of TTX. TTX was incubated with various amounts of sodium periodate as described under “Experimental Procedures.” Periodate consumption was then measured by the decrease of A₂₃₃ after 40 and 100 min of reaction at room temperature. Absorbance measurements were made by diluting 10 μl of the reaction mixture in 990 μl of H₂O.

uct, norTTX, they were found to retain a high biological activity.

The presence of a new and free carbonyl function in norTTX can easily be demonstrated by hydrazone formation with 2,4-DNPH (see “Experimental Procedures”).

Once norTTX is obtained, the next important step is the synthesis and the characterization of the photoactivable NAP-glycinhydrazide of norTTX (NAP-gly=norTTX). The synthesis of this molecule was carried out as described under “Methods.” The demonstration that the molecule which was synthesized was as expected was made using the tritium label of the norTTX moiety, the carbon 14 label of the NAP-glycinhydrazide moiety, and the orange color of the NAP moiety. The purification of the final product from norTTX is so crucial that it is explained in detail here under “Results.” This purification has been carried out on a cellulose-coated plate. In the first dimension (Solvent System A), the excess of NAP-glycinhydrazide and DMF are eliminated; an orange spot remained at the origin which contained the mixture of the final product and of norTTX. Because of the differences of polarity between norTTX and NAP-gly=norTTX, the polarity-sensitive chromatographic system designed by Heimer (19) and Ivany and Heimer (20) was used in the second dimension. This second dimension gave the separation diagram represented in Fig. 3. Only the orange colored substance migrating with an Rₚ of 0.48 was found to contain both ²H and ¹⁴C radiolabels; moreover, and this is of fundamental importance, it was biologically active when injected into crabs. The colored substance migrating with an Rₚ of about 0.8 was not toxic and was therefore eliminated. Under these chromatographic conditions, TTX and norTTX have a very limited migration with an Rₚ of about 0.15. All these results taken together indicate that the colored and biologically active substance with an Rₚ of 0.48 is the NAP-gly=norTTX. Rechromatography of this colored substance on a cellulose-coated plate in Solvent System D gave a single colored spot, containing both radioactive labels and being biologically active. In this last chromatographic system, only trace amounts of the tritium label corresponding to norTTX are found in the 0 to 0.32 Rₚ zone. A 1:1 ratio of the NAP-gly and norTTX moieties in the final product, the NAP-gly=norTTX molecule, is expected. Experimentally, using the ¹⁴C and ²H radiolabels, we found a value of 1.0.

The final yield in the synthesis of NAP-gly=norTTX is of the order of 10%. This relatively low yield has probably three origins: (i) the angular nature of the ketone function in norTTX, (ii) the solubility of TTX in DMF, and (iii) the limited availability of TTX. These last two facts preclude the use of large concentrations of norTTX which would be necessary to displace the equilibrium toward the formation of NAP-gly=norTTX.

NAP-gly=norTTX is perfectly stable in DMF solution when kept at high enough concentration (of the order of 10 mM). The stability of the compound is not high, probably due to hydrolysis, when it is kept in aqueous solution at high temperatures.

The specific radioactivity of [²H]norTTX was 20% lower than that of the starting [³H]TTX due to a partial loss of tritium during the oxidation reaction.
of crabs induced by TTX and TTX derivatives was used. The biological assay using the observation of spastic paralysis in aqueous solution (storage at 4°C).

reactivity toward Schiff-base formation. Starting from norTTX, the yield of synthesis of RAP-en-norTTX is of the order of 5%. In that case, the relatively low reaction are: (i) the use of perfectly anhydrous conditions and ethylenediamine is the reductive amination step in the presence of sodium cyanoborohydride. The critical points in this reaction are: (i) the use of perfectly anhydrous conditions and (ii) a long enough time of reaction (92 h). Characterization and purification of the final product, NAP-en-norTTX, were carried out as previously described for NAP-gly=norTTX. 

The important step in the synthesis of the second TTX derivative, NAP-en-norTTX (Fig. 1) from norTTX and NAP-ethylenediamine which does not favor a high reactivity toward Schiff-base formation.

NAP-en-norTTX is a very stable derivative of TTX even in aqueous solution (storage at 4°C). Biological Activity of Photoactivable Derivatives of TTX—The biological assay using the observation of spastic paralysis of crabs induced by TTX and TTX derivatives was used throughout to follow synthetic steps and purifications.

One way of studying the TTX receptor in excitable membranes is to titrate the receptor with [3H]TTX prepared from TTX using the Wilzbach technique (14). Fig. 4a shows this binding obtained with the Fraction II of purified axonal membranes of crabs (Cancer pagurus) (8). Fig. 4b shows the competition between [3H]TTX and NAP-gly=norTTX in equilibrium dialysis experiments carried out with the same axonal membranes. NAP-gly=norTTX completely inhibits [3H]TTX binding. Half-inhibition of [3H]TTX binding occurred at a NAP-gly=norTTX concentration (Kd) of 0.76 μM. Under the conditions of this experiment in which displacement of [3H]TTX by NAP-gly=norTTX was carried out from saturated receptors, the true dissociation constant of the complex formed between NAP-gly=norTTX and the TTX receptor, Kd(NAP-gly=norTTX) can be given by

\[ K_d(\text{NAP-gly=norTTX}) = K_d(\text{TTX}) \times \frac{2 \times [\text{NAP-gly}]}{2 \times [\text{[3H]TTX}] - [\text{TTX}]}. \]

where Kd(TTX) is the true dissociation constant of native TTX, Kd(NAP-gly=norTTX) is the total concentration of NAP-gly=norTTX necessary to displace 50% of the maximal [3H]TTX binding, Rb is the TTX receptor concentration, and [3H]TTX is the total concentration of [3H]TTX used. Taking the value of Kd(TTX) = 3 nM obtained in Fig. 4a, the calculations indicate that Kd(NAP-gly=norTTX) = 20 nM.

A quantitative analysis of the reversible blocking of the Na+ channel by TTX derivatives in the dark is presented in Fig. 5. In the biological assay that was used which concerns the crab giant axon, the half-maximal inhibitory effect of native TTX is observed at a concentration of 16 nM. The dose-response curves obtained with norTTX, NAP-gly=norTTX and NAP-en-norTTX are very similar, nearly superimposable. The half-maximum inhibitory effect of the Na+ channel obtained with these molecules is observed at 75, 120, and 97 nM, respectively, i.e., at a concentration only 4.7 to 7.5 times higher than for TTX. This limited diminution in affinity is in agreement with that found from direct binding experiment with NAP-gly=norTTX shown in Fig. 4. The Hill coefficient for the blocking action of TTX derivatives is similar to that found for TTX.

The introduction of an azido group in the TTX derivatives,
NAP-gly-norTTX and NAP-en-norTTX, should permit irreversible cross-linking of TTX to its receptor after UV irradiation. The derivatives which have been prepared fulfilled this objective as shown in Figs. 6 and 7. Fig. 6 shows that norTTX gives a perfectly reversible inhibition of the Na⁺ channel in the dark. The same reversibility was observed even when preparation was irradiated with UV flashes. NAP-gly-norTTX-induced blocking of the Na⁺ channel is reversible in the dark. UV irradiation provokes the irreversible association of the toxin with its receptor and permits a nearly complete block of the channel under the concentrations which have been used. The reactions can be represented as follows:

\[ R + \text{NAP-gly-norTTX} \rightarrow R-\text{NAP-gly-norTTX} \]

where \( R \) is the TTX receptor, \( R-\text{NAP-gly-norTTX} \) is the irreversible (covalent) association of the TTX derivative to its receptor. Experiments in Fig. 6 have been carried out under conditions where nearly 50% of the TTX receptor is in the form of \( R-\text{NAP-gly-norTTX} \) in the dark. UV irradiation, as expected, provoked covalent labeling of the receptor and displaced the equilibrium towards the formation of \( R-\text{NAP-gly-norTTX} \).

Similar kinds of experiments are described in Fig. 7 with NAP-en-norTTX. In this typical series of experiments, NAP-en-norTTX (100 nm, Fig. 7A) is shown to reversibly block the sodium channel. Application of NAP-en-norTTX (100 nm, Fig. 7A') followed by a short period of UV irradiation gives a partial irreversible block of the sodium channel; successive reapplications of the derivative (Fig. 7, A'' and A''') followed by series of UV flashes lead to an irreversible block of nearly 90% of the sodium channels.

**CONCLUSION**

This paper confirms that position 6 in TTX can be used for chemical modifications without loss of the biological activity of the toxin. It also describes the properties of photoactivable derivatives of TTX which will be probably very useful to...
reach a better understanding of the structure-function properties of the TTX binding component in excitable membranes, i.e. the selectivity filter of the Na⁺ channel.

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