Interaction of Deoxycholate with the Sodium Channel of Squid Axon Membranes

CHAU H. WU, PAUL J. SIDES, and TOSHIO NARAHASHI

From the Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611, and the Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710. P. J. Sides's present address is the Department of Laboratory Medicine, Holston Valley Community Hospital, Kingsport, Tennessee 37660.

ABSTRACT Deoxycholate can react with sodium channels with a high potency. The apparent dissociation constant for the saturable binding reaction is 2 μM at 8°C, and the heat of reaction is ~−7 kcal/mol. Four independent tests with Na-free media, K-free media, tetrodotoxin, and pancuronium unequivocally indicate that it is the sodium channel that is affected by deoxycholate. Upon depolarization of the membrane, the drug modified channel exhibits a slowly activating and noninactivating sodium conductance. The kinetic pattern of the modified channel was studied by increasing deoxycholate concentration, lowering the temperature, chemical elimination of sodium inactivation, or conditioning depolarization. The slow activation of the modified channel can be represented by a single exponential function with the time constant of 1–5 ms. The modified channel is inactivated only partially with a time constant of 1 s. The reversal potential is unchanged by the drug. Observations in tail currents and the voltage dependence of activation suggest that the activation gate is actually unaffected. The apparently slow activation may reflect an interaction between deoxycholate and the sodium channel in resting state.

INTRODUCTION

Axonal membranes contain extensive regions of lipid bilayer. Considering the hydrophobic environment in which the ionic channels are embedded and the likelihood that such an environment may influence their activity (Alvarez et al., 1975; Bamberg and Läuger, 1974; Neher and Eibl, 1977), it is very likely that using detergents as perturbing probes may yield important information on the lipid-protein interaction between the channels and membrane lipids. Detergents that have been studied so far generally have an effect on ionic channels that is of low potency and selectivity. Kishimoto and Adelman (1964) studied the effects of three types of detergents on squid axons under voltage-clamp conditions. Both sodium dodecyl sulfate and cetyltrimethylammonium chloride suppress the sodium and potassium conductances irreversibly. Tween 80 (polyoxyethylene sorbitan monoooleate) decreases the sodium conductance selectively and reversibly, providing a very high concentration (100 mM) is used for a short period of time. A long exposure results in
irreversible damage to the membrane. Another nonionic detergent, Triton X-100, decreases both sodium and potassium current of *Xenopus* nodal membranes. Again, long exposure or large concentration of the detergent causes a rapid increase in membrane leak current (Brismar and Rydquist, 1978). Long-chain analogues of triethylalkylammonium salt, when applied internally, were found to block the open potassium channel (Armstrong, 1969 and 1971). These agents are similar to those just mentioned in that they do not interact with either the sodium or the potassium channel in a highly specific or selective manner.

We have found that deoxycholate (DOC) reacts with sodium channels with a high potency; the apparent dissociation constant of this reaction is 2 μM. It suppresses the peak sodium conductance while inducing a noninactivating sodium conductance. We also uncovered evidence that indicates the induction of a new kinetics of sodium channels by DOC treatment. Preliminary accounts of this investigation have been published (Sides et al., 1973; Wu et al., 1975 and 1977).

**METHODS**

**Nerve Preparations**

Giant axons of the squid *Loligo pealei* available at the Marine Biological Laboratory, Woods Hole, Mass., were used. For experiments requiring intact axons only, the isolated axons were continuously superfused with normal external solution with or without test drugs. For experiments requiring internal application of test agents, the isolated axons were internally perfused by the roller technique developed by Baker et al. (1962) and modified by Narahashi and Anderson (1967).

**Solutions**

The normal external solution had the following ionic composition (mM): Na⁺, 450; K⁺, 10; Ca²⁺, 50; and Cl⁻, 567. The pH was buffered at 8.0 by the addition of 10 mM tris(hydroxymethyl)aminomethane (Tris) or 5 mM N-2-hydroxyethylpipperazine-N’-2-ethanesulfonate (HEPES). The normal internal solution contained (mM): K⁺, 350; Na⁺, 50; F⁻, 50; glutamate, 320; phosphate, 15; and sucrose, 333. The pH was adjusted to 7.3 with phosphate buffer. The osmolalities of the external and internal solutions were in the range of 1,020-1,040 mosmol/kg. Tetramethylammonium (TMA) chloride was used to substitute for NaCl on an equivalent basis in Na-free external solution. Cesium fluoride replaced potassium glutamate in K-free internal solution. The pH was adjusted with sodium phosphate buffer in the latter solution.

**Voltage Clamp**

The method of voltage clamp was essentially the same as that described by Wu and Narahashi (1973). About 40% of series resistance was compensated for. Normally the intact axons were clamped at a holding potential of −70 mV, whereas the internally perfused axons were held at −80 mV. To isolate the sodium current, the potassium current was eliminated by the addition of 10-20 mM tetraethylammonium ions (TEA) to the internal solution or, in a few experiments, by using external and internal media free of potassium ion. Leakage current was estimated by one of the following methods. In intact axons, it was estimated from the steady-state inward currents associated with small hyperpolarizing pulses. In most of the internally perfused axons
treated with TEA, leakage currents were obtained after 300 nM tetrodotoxin (TTX) had been introduced into the external solution. Usually, correction for leakage current was made graphically during analysis of data. In some experiments, however, the leakage as well as capacitive currents were subtracted electronically from the total currents during the course of the experiment.

\[ \text{CONTROL} \]

\[ \text{DOC, 2\textmu M} \]

**RESULTS**

*Effects of DOC on Membrane Excitability*

Deoxycholate, even at micromolar concentrations, caused a dramatic broadening of the action potential, an effect that was very quickly followed by repetitive firing. Both effects occurred only at low temperature. Figure 1 A–C shows the action potentials recorded from an intact axon before drug application at 6 and 20°C. 10 min after the onset of treatment with 2 \( \mu \)M DOC at 6°C, the action potential duration was prolonged and the spike amplitude reduced (Fig. 1 D). In place of the undershoot that normally followed the spike, there was a prolonged depolarizing after-potential, which soon led to
repetitive discharges (Fig. 1E). As soon as the temperature of the preparation was raised to 20°C, the repetitive discharges quickly stopped (Fig. 1F). The onset of drug effect was slow, with a latency of 5–20 min. The resting potential was only minimally affected, the membrane being depolarized by 3–6 mV in the presence of 2 μM DOC at 6°C.

All of these effects were also observed in internally perfused axons treated internally with DOC. Washing reversed the effects faster in the internally perfused axons than in the intact ones.

Effects of DOC on Membrane Currents

Measurements of membrane currents in intact axons under voltage clamp conditions have revealed potent effects of DOC on sodium channels. During the treatment with 2 μM DOC at 6°C, the peak transient current in response to step depolarization was reduced to 55% of the control value. The steady-state current was decreased by 30% at low depolarizations but increased to the same extent at depolarization levels beyond the sodium equilibrium potential (E_{Na}). Such an effect on the steady-state current disappeared upon application of 300 nM TTX, resulting in an almost complete recovery of the current. The results suggest that DOC induced a residual sodium current that continued to flow during the flow of potassium current.

Direct evidence for the presence of a residual sodium current was obtained from axons internally perfused with 20 mM TEA to block the potassium current (Fig. 2). Part A of the figure is a family of sodium currents associated with 20–180-mV step depolarizations from a holding potential of −80 mV. Temperature, 8.0°C. Axon 47.

![Figure 2](image_url)

**Figure 2.** Induction of residual sodium current by DOC. Families of sodium currents before (A) and during treatment with 10 μM DOC (B) in an axon perfused internally with 20 mM TEA. Membrane potential steps are in 20-mV increments (20–180) from the holding potential of −80 mV. Temperature, 8.0°C. Axon 47.
residual sodium current demonstrates the increase in this conductance during treatment with DOC. The reversal potential was not significantly affected. The leakage currents were not increased by concentrations of DOC as high as 100 μM.

**Figure 3.** Four independent tests to determine the nature of the residual conductance induced by DOC. (A) Sodium current tracing a was recorded from an axon internally perfused with 40 μM DOC and 20 mM TEA, and tracing b during the same treatment but perfused internally and externally with Na⁺-free solutions. Both currents elicited by depolarization to 0 mV. Temperature, 5.0°C. Axon 59. (B) Current tracings a and b were elicited by depolarizations respectively to -20 mV and +80 mV in an axon perfused externally and internally with K⁻-free media, the external solutions having one-fourth of the normal Na⁺ concentration. Current tracings (c) and (d) were recorded 20 min after application of 50 μM DOC in the same media at 7.8°C. Axon 54. (C) Sodium current tracings a and b were elicited by depolarizations to 0 and 100 mV, respectively, in an axon perfused with 20 mM TEA. Current tracings c and d for identical depolarizations were obtained during treatment with 10 μM DOC. Current tracings e and f were recorded after 300 nM TTX was added to the external solution during treatment with DOC. Temperature, 5.7°C. Axon 58. (D) Current tracings a and b were recorded during the action of 50 μM DOC in response to step depolarizations from −80 mV to −20 mV and +80 mV, respectively. Current tracings c and d elicited by identical depolarizations after addition of 1 mM pancuronium to internal solution during DOC treatment. Temperature, 6.0°C. Axon 54.

**Nature of the DOC-induced Conductance**

To characterize the change in conductance induced by DOC in the Na channel, experiments were performed along the following four lines.

**Sensitivity to Sodium-Free Media.** Current tracing a in Fig. 3A was obtained in an axon treated with 40 μM DOC. Removing Na from internal
and external solutions eliminated both peak and steady-state inward currents (tracing b), suggesting that the steady-state current caused by DOC is carried by Na⁺.

**In Sensitivity to Potassium-Free Media** Current tracings a and b in Fig. 3B are inward and outward sodium currents, respectively, obtained in an axon perfused with K-free media, the external solution having one-fourth of the normal Na⁺ concentration. After treatment with DOC, the peak transient current was suppressed (tracings c and d), accompanied by an increase in the steady-state sodium current. It is clear that the DOC effect is not altered by removal of K⁺ from both phases.

**Sensitivity to TTX** Fig. 3C shows that the addition of 300 nM TTX to a DOC-treated axon eliminated both inward and outward sodium currents (tracings e and f). The alteration in conductance induced by DOC is specifically responsive to the blocking action of TTX.

**Sensitivity to Pancuronium** Pancuronium has been found to block the open sodium channel selectively from the axoplasmic side of the axon (Yeh and Narahashi, 1977). Fig. 3D shows the results of the exposure of a DOC-treated axon in K-free medium to 1 mM pancuronium internally. Current tracings a and b are records of sodium currents during the action of 50 μM DOC. After treatment with 1 mM pancuronium (c and d), the channel initially opened with the rising phase closely following the kinetics of the channel in the presence of DOC alone. However, instead of the current being maintained in either an inward or an outward direction, it declined to a very low steady-state value. This experiment supports the notion that the channels induced to open by DOC are indeed Na channels.

**Separation of Normal and DOC-modified Conductances**

The two prominent features of DOC effects on Na channels are: (a) a reduction of the peak transient conductance followed by (b) an increase in the residual conductance. The following four experiments were designed to separate in time the DOC-modified conductance from the normal one.

**High DOC Concentration** At high concentrations of DOC, such as 40 μM, the sodium current took on a pattern of activation, partial inactivation, and reactivation in sequence (Fig. 4A). The second activation, not easily discerned in the small reproduction of the traces, reaches its maximum value within 40 ms after the beginning of the step pulse at low depolarization. At high depolarization, the maximum conductance was not easily detected because Na accumulation in the periaxonal space might have distorted the current pattern. The main point is that a new type of conductance increase was induced by DOC.

**Low Temperature** Fig. 4B shows the effect of changes in temperature on kinetics of Na conductance in an axon treated with 50 μM DOC. As the temperature was gradually decreased from 20° to 7°C, the peak transient component of sodium current (measured with a step depolarization to 0 mV)
was progressively diminished. At 7°C, only a slowly activating and noninactivating conductance component remained; the amplitude of the latter was also decreased by lowering the temperature. Clearly, the activation of the two conductance components was altered in a much different manner in response to a temperature change. One could make use of this difference to unmask the slowly activating component, which was normally eclipsed by the fast transient component.

**Removal of Sodium Inactivation** From the results described above, it was apparent that the slow activation of DOC-modified component occurred at the same time as the sodium inactivation of the normal fast transient component.

**Figure 4.** Separation of normal and DOC-modified conductances. (A) High DOC concentration. Sodium current tracings a and b were recorded from an axon internally perfused with 40 μM DOC and 20 mM TEA, as elicited by depolarizations to 0 and +100 mV, respectively. Temperature, 4.9°C. Axon 59. (B) Low temperature. Sodium currents in an axon perfused externally and internally with K-free media and treated with 50 μM DOC were recorded at temperatures of 20°, 16°, 13°, 10°, and 7°C, with the currents at 20°C and 7°C indicated. Step depolarization from −80 mV to 0 mV as shown in the voltage tracing. Axon 63. (C) Removal of sodium inactivation. Sodium current tracings a and b were recorded in an axon perfused with 10 mM TEA to eliminate potassium current and pretreated with 1 mM N-bromoacetamide for 2 min internally to eliminate the normal sodium inactivation process. Current tracings c and d were obtained after the addition of 40 μM DOC to the internal solution. Square pulses of depolarization were from −80 mV to 0 mV for a and c and to +100 mV for b and d. Temperature, 11.0°C. Axon 42. (D) Sodium inactivation. Sodium currents were superimposed in a double-pulse experiment on an axon perfused internally with 20 mM TEA and 2 μM DOC. Conditioning prepulses stepped from −80 mV to −60 and −40 mV for ~120 ms before the test pulse to 0 mV. Temperature, 6.0°C. Axon 57.
component. Any method that would remove the sodium inactivation phase could conceivably clarify the manifestation of the slow component of the DOC effect. It has been shown that by subjecting axons to internal treatment with Pronase or N-bromoacetamide, the sodium inactivation process can be specifically eliminated (Armstrong et al., 1973; Oxford et al., 1978). After treatment with 1 mM N-bromoacetamide (Fig. 4C), the inward and outward sodium currents took on the pattern of one activation phase without the usual inactivation phase (tracings a and b). After the addition of 40 μM DOC (tracings c and d), the sodium conductance turned on with a fast activation phase followed by a slow one in tandem, which eventually reached the maximum conductance. The latter merges with the steady-state conductance of the control in the figure. Thus, after removal of sodium inactivation, the slow phase of DOC-induced activation could be revealed. This phase has a distinctly slower activation rate constant than the fast transient component.

**SODIUM INACTIVATION** The normally occurring process of sodium inactivation itself can be used to "filter out" the fast transient component of sodium conductance in the presence of DOC so that only the slow, persistent component is observed. After a conditioning depolarizing pulse to −40 mV, the peak transient component of sodium current associated with a test pulse to 0 mV disappeared, leaving a persistent component intact (Fig. 4D). It can be seen that the residual current without conditioning depolarization is contributed solely by the slow, persistent component of DOC effect.

**Binding of DOC to Sodium Channel: Macroscopic Estimations**

The following experiments show that the relative proportion of the two populations of sodium channels was a function of DOC concentration as well as temperature. These experiments give the apparent dissociation constant (K_D) and the heat of reaction (ΔH) and demonstrate that DOC binds reversibly to a single site of the sodium channel to form a drug receptor complex. Two independent methods commonly used in drug binding studies were employed, namely, the equilibrium and the kinetic methods.

**EQUILIBRIUM METHOD** An equilibrium dose-response curve was determined on intact axons by increasing the DOC concentration cumulatively after the effect of the previous dose had reached an equilibrium. Fig. 5A shows a dose-response curve derived from such an experiment at 8°C. The apparent dissociation constant of the drug-receptor complex was estimated in three separate experiments to be 2.2 × 10⁻⁶ M, as shown in Table I. These data were further analyzed by a Hill plot of log y/(Y_max − y) as a function of the log concentration of DOC, [DOC]. y represents the percent of disappearance of the fast transient conductance resulting from the action of DOC, and the maximum value Y_max was set at 80% to correct for the 20% contribution of slow conductance at the peak. A slope of 0.81 was obtained, indicating an almost one-to-one stoichiometry for the interaction between DOC and the binding site of the sodium channel responsible for the fast activation.
Kinetic method. DOC was applied internally to avoid the diffusion barrier of the Schwann cells and the long latent period due to the dead space of the axon chamber. We assume that the binding of DOC to the Na channel follows the following scheme:

\[ \text{DOC} + R \xrightarrow{k_1} \text{DOC} \cdot R, \]

\[ \text{DOC} \cdot R \xrightarrow{k_2} \text{DOC} + R \]

**Figure 5.** Binding of DOC to sodium channel: macroscopic estimations. (A) Dose-response relation for the action of DOC in suppressing the fast transient component of sodium conductance in an intact axon, with the percent of reduction in the peak transient conductance plotted against the molar concentration of DOC applied. The curve is drawn according to the equation \( \Delta g_{Na} = 100\% \times \frac{[\text{DOC}]}{([\text{DOC}] + K_D)} \), with \( K_D \) equal to \( 2.0 \times 10^{-6} \) M. Temperature, \( 8.0^\circ \text{C} \). Axon 16. (B) Time-course of disappearance of the fast transient conductance during internal DOC treatment and its recovery upon washing. Sodium currents were elicited by constant 60 mV depolarization pulses at regular intervals. At the first arrow, 1 \( \mu \)M DOC was applied. After the steady state of drug action was reached, the axon was washed with normal internal solution. The curve was drawn by visual fitting. Temperature, \( 8^\circ \text{C} \). Axon 22.
where DOC is DOC in the aqueous phase, R is the receptor site which reacts with DOC, DOC ⋅ R is the complex formed, $k_1$ is the apparent forward rate constant, and $k_2$ is the apparent backward rate constant. By following the time-course of disappearance of the fast transient component during application of DOC and its reappearance during washing, one can estimate the apparent forward ($k_1$) and backward ($k_2$) rate constant, which determine the apparent dissociation constant ($K_D$). The main purpose of the experiment, however, was to test whether the receptor site was saturable. If DOC interacts with the receptor site according to the reversible binding scheme, it follows that the rate of drug action during introduction into the axon depends upon the drug concentration (Hill, 1909):

$$[\text{DOC} \cdot \text{R}] = [\text{DOC} \cdot \text{R}]_{\text{eq}} [1 - e^{-(k_1[\text{DOC}] + k_2)t}],$$

(2)

<table>
<thead>
<tr>
<th>Axon No.</th>
<th>[DOC] M</th>
<th>$k_1^{*}$ M⁻¹ min⁻¹</th>
<th>$k_2^{*}$ M⁻¹ min⁻¹</th>
<th>$K_D^{*}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>$3 \times 10^{-6}$</td>
<td>$1 \times 10^{-6}$</td>
<td>$0.3 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>$1 \times 10^{-7}$</td>
<td>$1 \times 10^{-4}$</td>
<td>$1.4 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>$3 \times 10^{-7}$</td>
<td>$3 \times 10^{-6}$</td>
<td>$5.0 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>2.2 $\times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>Kinetic method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>$1 \times 10^{-6}$</td>
<td>$8.70 \times 10^{4}$</td>
<td>0.13</td>
<td>$1.49 \times 10^{-6}$</td>
</tr>
<tr>
<td>1</td>
<td>$1 \times 10^{-8}$</td>
<td>$6.02 \times 10^{4}$</td>
<td>0.11</td>
<td>$1.96 \times 10^{-6}$</td>
</tr>
<tr>
<td>1</td>
<td>$1 \times 10^{-9}$</td>
<td>$5.60 \times 10^{4}$</td>
<td>0.16</td>
<td>$2.85 \times 10^{-6}$</td>
</tr>
<tr>
<td>23</td>
<td>$1 \times 10^{-5}$</td>
<td>$6.28 \times 10^{6}$</td>
<td>0.10</td>
<td>$1.43 \times 10^{-6}$</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>1.90 $\times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>Grand mean</td>
<td></td>
<td></td>
<td>(2.0±0.5) $\times 10^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>

* For $k_1$, $k_2$, and $K_D$, see Eq. 1.

where $[\text{DOC} \cdot \text{R}]$ denotes the concentration of the complex and $[\text{DOC} \cdot \text{R}]_{\text{eq}}$ the concentration at equilibrium. On the other hand, the rate of decline in drug action during washing is not a function of drug concentration:

$$[\text{DOC} \cdot \text{R}] = [\text{DOC} \cdot \text{R}]_{\text{eq}} e^{-k_D t}.$$  

(3)

Fig. 5 B shows the time-course of disappearance of the fast transient component during application of 1 μM DOC and that of its reappearance during washing, as measured by the changes in sodium currents with constant depolarizing pulses at regular intervals. The time-course of the change in the fast transient conductance shows that increasing the DOC concentration enhances the rate of drug occupancy during application (Fig. 6 A) and that it does not alter the rate of removal of occupancy during washing. The $K_D$ deduced from the two rate constants was estimated to be $1.9 \times 10^{-6}$ M (Table I).

The $K_D$ values established from the two independent methods agree remarkably well, giving a grand average of $2.0 \pm 0.5$ μM. Most importantly,
these results indicate that the DOC-receptor site on the Na channel satisfies the criteria of saturable binding.

**Influence of Temperature on DOC Effects**

Low temperatures favor the binding of DOC to the Na channel. Fig. 7A and B shows the sodium currents resulting from a step depolarization to 0 mV at two temperatures before and during application of 10 µM DOC, respectively. Clearly, the decrease in the fast transient component and the increase in the slow persistent component induced by DOC are more pronounced at 6.0°C than at 20.0°C. These results permit thermodynamic analyses.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Estimation of apparent rate constants of binding and dissociation. (A) Semilogarithmic plot of the time-course of decrease in the fast transient conductance relative to the equilibrium value, \([I(t) - I(\infty)]\). Sodium currents were elicited by constant 60-mV depolarization pulses at regular intervals and in the presence of 1 µM (solid circles) or 10 µM DOC (open circles). (B) Semilogarithmic plot of the time-course of recovery of the fast transient conductance relative to the equilibrium value, \([I(\infty) - I(t)]\). Sodium currents were elicited similarly as the axon was being washed after DOC treatment at a concentration of 1 µM (solid circles) or 10 µM (open circles). Curves drawn by least square fitting. Same axon as that in Fig. 5 B.

As tabulated in Table II, the apparent equilibrium constant obtained at 9°C is about 85% greater than that at 22.5°C, indicating a higher ratio of the population of bound channels to that of free channels at the lower temperature. The heat of reaction \(\Delta H\) for the binding reaction as calculated by the van’t Hoff equation averages \(-7,150\) cal/mol \((n = 26)\). The value of \(-7\) kcal/mol suggests that hydrogen bonding as well as ionic interaction may be involved.

**Characterization of the DOC-induced Slow Conductance**

The slow component of the sodium conductance during DOC action was characterized as follows. After the slow component was isolated from the fast
transient component by removing sodium inactivation or conditioning with depolarizing prepulses, we examined the voltage dependence, steepness of the steady-state curves, and the time constants for both activation and inactivation.

**Steady-state activation** The voltage dependence of steady-state sodium activation was measured in an axon perfused with TEA and pretreated with 1 mM N-bromoacetamide to remove potassium current and sodium inactivation. There was no shift of the curve along the voltage axis. The half-points for the control and the DOC-induced slow channel were -23.2 and -20.4 mV, respectively. The slope of the steepest region in the $m_\infty (E_m)$ curve of the slow channel was not remarkably affected, being 0.141/mV for the DOC curve as compared with 0.104/mV for the control curve (Table III).

In another axon treated with 50 μM DOC and preconditioned with a 60 mV depolarization from -80 mV before step depolarizations to various amplitudes of the test pulse, the $m_\infty$ of the steady-state component was unaffected by

### Table II

<table>
<thead>
<tr>
<th>Axon No.</th>
<th>$n$</th>
<th>$T_1$ (°C)</th>
<th>$T_2$ (°C)</th>
<th>$K_1^*$ (Kcal/mol)</th>
<th>$K_2^*$ (Kcal/mol)</th>
<th>$\Delta H^*$ (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>6</td>
<td>8.9</td>
<td>21.6</td>
<td>1.87±0.19</td>
<td>0.85±0.08</td>
<td>-10,189±1,275</td>
</tr>
<tr>
<td>27</td>
<td>4</td>
<td>9.1</td>
<td>22.6</td>
<td>2.09±0.20</td>
<td>1.25±0.04</td>
<td>-6,053±1,338</td>
</tr>
<tr>
<td>28</td>
<td>5</td>
<td>9.0</td>
<td>22.8</td>
<td>2.37±0.03</td>
<td>1.26±0.06</td>
<td>-7,596±485</td>
</tr>
<tr>
<td>29</td>
<td>5</td>
<td>9.3</td>
<td>23.1</td>
<td>2.26±0.21</td>
<td>1.23±0.05</td>
<td>-5,505±485</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>8.1</td>
<td>22.6</td>
<td>2.70±0.13</td>
<td>1.61±0.03</td>
<td>-5,824±480</td>
</tr>
</tbody>
</table>

* $K_1$ and $K_2$ are the apparent equilibrium constants of the binding reaction defined by Eq. 1 at temperatures $T_1$ and $T_2$, respectively. $\Delta H$ is calculated according to the van't Hoff equation, $\ln K_2/K_1 = (\Delta H/R) (T_2 - T_1)/T_1T_2$. The mean is $-7,146±536$. 
DOC. Both the halfpoint (−18.6 mV) and the slope (0.102/mV) are comparable to the control (Table III).

Finally, the $m_{\infty}$ was estimated from families of residual currents during DOC treatment, with the assumption that sodium inactivation did not occur for the moderate pulse duration. It gave a half-point at −19.2 mV and a slope of 0.103/mV, not appreciably different from those of the $m_{\infty}$ measured from control families of fast transient currents (Table III).

**steady-state inactivation** Since the slow component of sodium current was not inactivated by a 120-ms depolarization, to measure the inactivation of the slow component, a conditioning depolarization lasting 1 min was applied before a test depolarization to 0 mV. Fig. 8A shows the currents associated with the test pulse. The slow component was indeed inactivated.

### Table III

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>DOC-affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. $m_{\infty}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. By removal of inactivation</td>
<td>$E_m$ for 0.5</td>
<td>$E_m$ for 0.5</td>
</tr>
<tr>
<td></td>
<td>−23.2 mV</td>
<td>−20.4 mV</td>
</tr>
<tr>
<td></td>
<td>Slope</td>
<td>0.104/mV</td>
</tr>
<tr>
<td>2. By inactivation</td>
<td>$E_m$ for 0.5</td>
<td>−18.6 mV</td>
</tr>
<tr>
<td></td>
<td>Slope</td>
<td>0.102/mV</td>
</tr>
<tr>
<td>3. By family of currents</td>
<td>$E_m$ for 0.5</td>
<td>−24.6 mV</td>
</tr>
<tr>
<td></td>
<td>Slope</td>
<td>0.130/mV</td>
</tr>
<tr>
<td>B. $h_{\infty}$ (slow activation)</td>
<td>$t=1$ min</td>
<td>$E_m$ for 0.5</td>
</tr>
<tr>
<td></td>
<td>−53.4 mV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slope</td>
<td>0.135/mV</td>
</tr>
<tr>
<td>C. $\tau_m$ ($E_m=−80$ mV)</td>
<td>$\tau_{tail}$ at $t=1$ ms</td>
<td>$\tau_{tail}$ at $t=8$ ms</td>
</tr>
<tr>
<td></td>
<td>0.289 ms</td>
<td>0.106 ms</td>
</tr>
<tr>
<td></td>
<td>0.166 ms</td>
<td>0.242 ms</td>
</tr>
<tr>
<td>D. $\tau_h$</td>
<td>$E(h_{\infty}=max)$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6~1.45 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mV</td>
<td></td>
</tr>
</tbody>
</table>

However, the inactivation was incomplete; an inward current of 0.10 mA/cm² was observed at 0 mV of conditioning membrane potential (Fig. 8B). After this noninactivating portion had been subtracted from the $h_{\infty}$ curve and the remainder normalized and replotted, the half-point and the slope for the steepest region were estimated to be −53.4 mV and 0.135/mV respectively (Table III).

**Time constants $\tau_m$ and $\tau_h$** Whereas the activation for the slow channel was very much slower than that for the normal channel, the time constant of its tail current upon repolarization, which is governed by $m$, was not appreciably different from that of the control. Combined with the absence of effect on the $m_{\infty}$ ($E_m$) curve, this suggests that the $m$ gate process was not affected by DOC.
We found that the sodium tail currents obtained in TEA were complicated by contribution from some unblocked potassium channels and distortion by the incomplete compensation of series resistance, rendering the time constant estimation inaccurate. Therefore, for more quantitative measurement of tail current time constants, we used K-free and low-Na (one quarter of normal) media to remove the potassium current and to reduce the series resistance distortion. Under these conditions, the tail currents were not prolonged by DOC (50 μM), the time constant being equal to 194 μs as against the control value of 172 μs. The tail current at the break of a very long pulse (3.8 s), which produced a sustaining residual conductance, showed normal turn-off kinetics of the m process as well (Fig. 9, inset).

The inset in Fig. 9 also compares the kinetics of the falling phase of sodium currents at two different sweep speeds after treatment with 10 μM DOC. During a 3.8-s pulse observed at the slow sweep speed, the sodium current slowly inactivates with a time constant of 0.55 s (tracing b). A similar pattern was seen at all potentials examined. Time constants of the slow inactivation
(\(\tau_\text{h}^0\)) were estimated from semilog plots of the current tracings (Table III) and are plotted against the membrane potential in Fig. 9. The smooth curve was drawn manually and reflects a voltage dependence with a maximum located at \(~50\) mV.

**Reversal Potential.** There was no detectable change in the reversal potential as estimated from the instantaneous current-voltage relationship by double pulse procedures in axons treated with \(N\)-bromoacetamide to remove sodium inactivation.

**Activation of the DOC-bound Complex**

We found that the kinetics of the activation of the DOC-modified channel could be fitted by a single exponential function:

\[
g'(t) = g_\infty (1 - e^{-t/\tau})
\]

where \(g'\) is the conductance of the slow channel as a function of time \(t\), \(g_\infty\) is the conductance of the same channel at steady state, and \(\tau\) is the time constant of activation of DOC-modified channel. \(\tau\) can be directly estimated from the conductance curve (or current tracings) by the following methods.

**Removal of Sodium Inactivation**  The axons were treated internally first with 1 mM \(N\)-bromoacetamide and then with 30 \(\mu\)M DOC. The total sodium conductance consists of two activation phases, and the kinetics of the conductance increase during the second phase can be described by the
following equation:

$$g_{Na}' = \alpha \tilde{g}_{Na} m^3 h_0 + g_s (1 - e^{-t/\tau}),$$  \hspace{1cm} (5)

where $g_{Na}'$ is the total sodium conductance during DOC treatment as a function of time ($t$), and $\alpha \tilde{g}_{Na}$ is the maximum conductance of the fast transient component which is a fraction ($\alpha$) of the control $\tilde{g}_{Na}$. The value for $\tau$ was then estimated from the semilog plot of the conductance vs. time during the second phase. The values obtained at various membrane potentials are shown as open circles in Fig. 10. The magnitudes of $\tau$ range from 1.2 to 4.6 ms, and are $\sim$1–4 times as large as those of normal $\tau_h$.

**Figure 9.** Time constant of inactivation of the DOC-induced slow conductance. Time constants of slow inactivation (ordinate) were estimated from sodium currents elicited by a long test pulse lasting 3.8 s, as shown in the inset. The abscissa shows the membrane potential of the test pulse. Curve drawn by visual fitting. (Inset) Superimposed sodium currents at two sweep speeds in response to depolarization from $-80 \text{ mV}$ to $0 \text{ mV}$. Tracing $a$ was recorded at 2 ms/cm sweep speed, with the last part of the 18-ms pulse beyond the scope display. Tracing $b$, recorded at 0.5 s/cm sweep speed, shows a prominent slow falling phase during most of the 3.8-s pulse. Temperature, 5.0°C. Axon 56.

**Sodium Inactivation** During DOC treatment, the axon was first conditioned with large depolarizations to inactivate the fast transient component and then step depolarized to various test potentials. The current associated with the test pulse showed only the slow persistent component described by Eq. 4. The time constant could be obtained from a semilog plot of the current as a function of time. The values of $\tau$ obtained by this method ranged from 0.7 to 1.8 ms, as shown by solid circles in Fig. 10. The magnitude of $\tau$ is of the same order as that of normal $\tau_h$.

**Cross Point Measurement** We measured $\tau$ directly from current tracings by taking the cross point between the sodium current obtained in the
control and in DOC treatment, as they were superimposed on the oscilloscope screen, and solving for $\tau$ analytically. The falling phase of the control sodium current ($I_{Na}$) is given by the Hodgkin-Huxley equation (Hodgkin and Huxley, 1952):

$$I_{Na} = (E_m - E_{Na}) \tilde{g}_{Na} m_\infty h_\infty e^{-t/\tau}. \quad (6)$$

The falling phase of sodium current during DOC treatment ($I'_{Na}$) is given by the equation:

$$I'_{Na} = (E_m - E_{Na}) [\alpha \tilde{g}_{Na} m_\infty h_\infty e^{-t/\tau} + g'_\infty (1 - e^{-t/\tau})]. \quad (7)$$

![Figure 10](https://example.com/figure10.png)

**Figure 10.** Time constant of the activation of DOC-bound complex. The time constants (ordinate) estimated from a total of nine axons are plotted against the membrane potential of the test pulse (abscissa). The open circles represent data taken from two axons pretreated with 1 mM N-bromoacetamide and then with 40 $\mu$M DOC internally. The solid circles represent data taken from two axons preconditioned with moderately strong depolarizations (by 60 or 80 mV) to inactivate the fast transient component and then step depolarized to various test potentials. The open triangles represent data taken from five axons, estimated by directly measuring the cross point between the sodium currents obtained in the control and in DOC as they were superimposed on the oscilloscope screen.

At the cross point ($t = t_x$), the two currents are equal, and the two expressions can be equated to yield:

$$g'_\infty (1 - e^{-t_x/\tau}) = (1 - \alpha) \tilde{g}_{Na} m_\infty h_\infty e^{-t_x/\tau} \quad (8)$$

or

$$I'_\infty (1 - e^{-t_x/\tau}) = (1 - \alpha) I_0 e^{-t_x/\tau}, \quad (9)$$

where $I'_\infty = g'_\infty (E_m - E_{Na})$ is the steady-state current in DOC and $I_0 = \tilde{g}_{Na} m_\infty h_\infty (E_m - E_{Na})$ is obtained by extrapolating the falling phase of the semilog plot of control $I_{Na}$ to zero time. The fraction of the fast transient component ($\alpha$) was measured by taking the ratio of the peak transient current during DOC treatment to that of the control and multiplying the ratio by a correction factor (0.94). The correction factor was found by theoretical analysis.
on the simulated current based on the model presented below. The value for \( \tau_h \) was given by the slope of the linear falling phase in the semilog plot of the control \( I_{Na} \) and \( t_4 \) by direct measurement of time to the cross point. The time constants \( \tau \) measured from both inward and outward currents at 0 and 100 mV, respectively, are given in Fig. 10 as open triangles. They ranged from 1.8 to 4.4 ms, depending on the temperature, which ranged from 8° to 12°C.

From the combined data in Fig. 10, we could not detect any definitive pattern of voltage dependence for the time constant.

**Molecular Mechanism of DOC Action**

We shall now propose a mechanism of the DOC action that represents one of the several possible models consistent with all of our present observations. The DOC molecules redistribute themselves at the membrane-solution interface according to the partition coefficient. Some of the DOC in the membrane phase reacts with the Na channel as given by Eq. 1. All these occur while the axon is at rest. Upon depolarization, the unbound channels freely undergo activation and inactivation with normal kinetics, while the DOC-bound channels have to first partially dissociate from DOC to be able to activate. The partial dissociation mechanism is distinctly slow, so that the conductance increase is dominated by the rate-limiting dissociation process. The drug-modified channels remain open for a moderately long period of time without appreciable inactivation. Upon repolarization, however, the channel turns off with normal \( m \) kinetics, indicating that in the open conformation only the \( h \) gate is affected. Thus, we can summarize the actions of DOC at the macroscopic level in the following scheme:

\[
\text{Free, closed Na channels} \quad \text{DOC} \quad \left\{ \begin{array}{c}
K_D = 2 \mu M \\
\Delta H = -7 \text{ kcal/mol}
\end{array} \right. \quad \text{DOC-bound, closed Na channels} \\
\text{Partial dissociation} \quad \text{DOC-modified, open Na channels}
\]

The rates of binding to and dissociation from the Na channel, shown in Table I, are clearly several orders of magnitude slower than the time constant of the partial dissociation of the complex upon depolarization. We do not detect any distinct pattern of voltage dependence for \( \tau \) nor any modulation of \( \tau \) by DOC concentration (Fig. 10). Therefore, the rate constants for the partial dissociation and reassociation appear to be independent of the membrane potential.

For our calculations of Na conductance before and during DOC action, we adopted, with slight modifications, the kinetic model developed by Moore and Cox (1976) for normal sodium conductance. For the DOC-modified channel, a modified scheme was used that incorporated the partial dissociation of the bound complex as discussed above and in which the normal sodium inactivation was eliminated. Differential equations were written for all transition for normal and modified channels and integrated numerically by the Euler method, usually with steps of 10 \( \mu \)s. The voltage dependence of the rate
constants $\alpha_m$, $\beta_m$, $\alpha_h$, and $\beta_h$ were taken from Palti (1971). A $Q_{10}$ of 3 was assumed for all Hodgkin-Huxley rate constants. The amount of the DOC-bound fraction of the total population of Na channels was determined by the dose-response relationship, $[\text{DOC}]/([\text{DOC}] + 2 \times 10^{-6} \text{ M})$. All simulation programs were run on a Hewlett-Packard calculator (HP 9281) with digital X-Y plotter (HP 9864) (Hewlett-Packard Co., Palo Alto, Calif.).

Fig. 11 shows the results of simulation based on the model. The sodium current during DOC treatment was calculated by assuming that 70% of the total population is contributed by the free fraction and the remaining 30% by the DOC-modified fraction. The curve for the free fraction takes on a normal pattern of activation and inactivation, whereas that for the DOC fraction assumes a delayed activation but without inactivation. The simple addition of currents due to the two fractions give the DOC curve that answers the four criteria of the DOC-induced conductance change, namely, (a) a reduction in the peak transient current, (b) an apparent slowing of sodium inactivation, (c) an appreciable amount of residual current flowing at the end of the 7-ms pulse, and (d) a fast turn-off of the tail current.

**SODIUM INACTIVATION** Fig. 12A shows sodium currents from an axon treated with 50 $\mu$M DOC in response to a step depolarization to 0 mV with or without a conditioning prepulse to $-20$ mV. The fast rising current pattern
was obtained during the test pulse without the prepulse. With the −20 mV prepulse, only the slow persistent component remained. The two patterns of sodium current also resulted from calculation based on the model (Fig. 12 B).

**REMOVAL OF SODIUM INACTIVATION** As a final test of the model, we calculated the sodium currents to simulate the ones with 1 mM N-bromoacetamide pretreatment to eliminate the sodium inactivation. Fig. 13 A is identical to Fig. 4 C, which shows sodium currents from such a pretreated axon before and during application of 40 μM DOC. The calculated currents (Fig. 13 B) simulate the observation well in that the sodium conductance turns on with a fast activation followed by a delayed activation, which eventually merges with the steady-state conductance of the control. The simulated currents actually were predicted by use of the model and subsequently confirmed by experimental observations on the axon. The model can be used not only to account for observed phenomena but also to make predictions.

**DISCUSSION**

Since the introduction of bile salts to biochemical preparative procedures, especially in solubilizing and purifying electron transfer components of mitochondria, the methodology of fractionation of membrane proteins using bile salts has been developed to a high degree of sophistication. Yet, virtually nothing concrete has been known about the interaction of bile salts with membrane or about the mechanisms of solubilization of membrane lipopro-
teins until very recently (Helenius and Simons, 1975; Maddy and Dunn, 1976; Tzagoloff and Penefsky, 1970). Among bile salts, deoxycholate enjoys particular popularity as a detergent for the extraction of various membrane proteins. Of particular interest to electrophysiologists is its application to the extraction of α-bungarotoxin receptor (Changeux et al., 1971) and (Na⁺ + K⁺)-adenosine triphosphatase (Skou, 1965; Jørgensen, 1974) and to the dissociation of tetrodotoxin-receptor complex (Henderson and Wang, 1972).

Only very recently has the nature of lipid-protein interaction between DOC and membrane proteins begun to be characterized (Helenius and Simmons, 1972; Makino et al., 1973; Robinson and Tanford, 1975; Tanford et al., 1974; see also reviews by Tanford [1973] and Tanford and Reynolds [1976]). These studies conclude that DOC binds to sites of membrane proteins previously associated with membrane lipids and that membrane proteins solubilized by DOC have a relatively high probability of retaining their native conformations.

The molecular conformation of deoxycholate is unique among the natural and synthetic detergents (Hofman and Small, 1967). The polar carboxylic group is some distance from the steroid nucleus. The side chains of the body are so arranged that the hydrophilic area faces one way while the lipophilic

---

**Figure 13.** Comparison of sodium currents without normal sodium inactivation as derived from axon and kinetic model. (A) Superimposed inward and outward sodium currents before and after treatment with 40 μM DOC in an axon pretreated with 1 mM N-bromoacetamide to eliminate sodium inactivation process. Square depolarizing pulses were from −80 to 0 and +100 mV. See legend to Fig. 4 C for further details. Temperature, 11.0°C. Axon 42. (B) Simulated inward and outward sodium currents for control and DOC-treated axon with sodium inactivation deleted from the kinetic scheme. The maximum conductance for the DOC-bound channel was set at 30% of total conductance; holding potential and repolarization were −80 mV; test pulse was 0 and +100 mV; and temperature was 8.0°C. In addition, $g_{Na}$ was scaled down by a factor of 0.35 in both the control and DOC-treated axon to mimic the attenuation in conductance normally seen in axons pretreated with N-bromoacetamide.
area faces the other. Deoxycholate may be considered a planar amphiphile, in contrast to typical detergents that are polar amphiphiles (Hofmann and Mekhjian, 1973).

The lipophilic area is presumably required for its initial penetration and incorporation into the membrane lipid phase. However, several lines of indirect evidence suggest that the hydrophobic area is not as important as the hydrophilic area in the binding reaction. Deoxycholate has been found (Makino et al., 1973; Tanford, 1973) to bind to the amphiphilic binding sites of native serum albumin with a binding constant on the order of $10^6$ liters/mol, an affinity roughly equivalent to that of decanoate. Because much of the free energy of association at these sites can be ascribed to the carboxylic head group of DOC, these authors conclude that the hydrophobic portions of these sites have no special affinity for the steroid ring.

Steroids lacking the hydrophilic groups have been found to be inactive on axonal membranes. Henkin et al. (1973) applied methylprednisolone and 2-methylfluorohydrocortisone to squid giant axons and found no alteration of the sodium or potassium conductance. In contrast, the effects of a steroid chemically related to DOC were similar to those of DOC on the squid axon membrane. In testing the effects of hydroxydione (the sodium salt of 21-hydroxyprogremadiolene succinate) on rat peripheral nerves, Meinardi (1961) reported that this substance suppressed the spike amplitude and increased the duration of action potential while prolonging the refractory period. Although only a few studies on the action of steroids on the peripheral nerves have been conducted, the results are consistent with the notion that the hydrophilic groups appear to be the active moieties of the DOC molecule in binding to sodium channel.

Because the transfer of hydrocarbons from an aqueous to a nonpolar environment is endothermic, it is expected that hydrophobic bonds should become more stable as the temperature is increased (Némethy and Scheraga, 1962). This is true of micelle formation inasmuch as it essentially results from hydrophobic bonding, which is indicated by the decrease in the critical micellar concentration with rising temperature for a number of bile salts and other typical anionic detergents (Small, 1971; Carey and Small, 1972). The negative value of $\Delta H$ for the binding reaction of DOC to sodium channel indicates that it is exothermic, the bound state being more stable in colder temperature. This thermodynamic consideration suggests that the participation of hydrophobic bonds plays only a minimal role in the binding reaction.

Since low temperatures favor the DOC binding reaction, more channels are modified, and the population of free channels is reduced. As a consequence, the rising phase of the action potential is slowed and the peak amplitude is reduced. The maintained sodium current contributed by the modified channels, which open slowly, is responsible for the depolarizing after-potential. If the depolarizing after-potential is more positive than the critical depolarization (threshold potential), repetitive discharges will ensue. Thus, the increased tendency to repetitive discharges at low temperatures can be accounted for by the dose-dependent induction of modified channels with altered kinetics.
We are grateful to Kendall Powers and Denise Ring for their help in data analyses and Pat Burks, La Verne Brown, and Sasi Kumar for their secretarial assistance.

P. J. Sides was supported during the initial phase of this work by the Grass Fellowship in Neurophysiology from the Grass Foundation. This work was supported by grant NS 14144 from the National Institutes of Health to T. Narahashi and by a grant-in-aid from the Muscular Dystrophy Association and Alfred P. Sloan grant from the Southern Regional Education Board to C. H. Wu.

Received for publication 22 February 1978.

REFERENCES


