Barbiturates Block Sodium and Potassium Conductance Increases in Voltage-Clamped Lobster Axons

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ABSTRACT Sodium pentobarbital and sodium thiopental decrease both the peak initial (Na) and late steady-state (K) currents and reduce the maximum sodium and potassium conductance increases in voltage-clamped lobster giant axons. These barbiturates also slow the rate at which the sodium conductance turns on, and shift the normalized sodium conductance vs. voltage curves in the direction of depolarization along the voltage axis. Since pentobarbital (pK_a = 8.0) blocks the action potential more effectively at pH 8.5 than at pH 6.7, the anionic form of the drug appears to be active. The data suggest that these drugs affect the axon membrane directly, rather than secondarily through effects on intermediary metabolism. It is suggested that penetration of the lipid layer of the membrane by the nonpolar portion of the barbiturate molecules may cause the decrease in membrane conductances, while electrostatic interactions involving the anionic group on the barbiturate, divalent cations, and "fixed charges" in the membrane could account for the slowing of the rate of sodium conductance turn-on and the shift of the normalized conductance curves along the voltage axis.

INTRODUCTION

It is now well-established that the local anesthetic, procaine, blocks the nerve action potential by reducing both the early transient (Na) and late steady-state (K) conductance increases in the isolated peripheral nerve fiber (Taylor, 1959; Shanes et al., 1959; and Blaustein and Goldman, 1966 *a*). Similar effects are observed in the perfused squid axon whether procaine is applied internally or externally (Narahashi et al., 1967). Alcohols also block the Na and K conductance increases in the isolated squid axon (Armstrong and Binstock, 1964; Moore et al., 1964). By contrast, tetrodotoxin (Narahashi et al., 1964; Nakamura et al., 1965 *a*; Takata et al., 1966) and saxitoxin (Kao and Nishiyama, 1965; Nakamura et al., 1965 *b*), which also block the nerve action potential, do so by selectively blocking only the early transient current.

Although barbiturates are also known to block excitation in peripheral nerve (Heinbecker and Bartley, 1940; Schoepfle, 1957), presumably by blocking the early increase in sodium conductance (Thesleff, 1956), their effects on axon membrane conductances have not heretofore been determined. The present study was therefore undertaken to test the effects of sodium pentobarbital and sodium thiopental on the voltage-clamped lobster giant axon.

The effect of pH on the action of sodium pentobarbital was also tested. In the past it has been assumed (cf. Maynert, 1965; Sharpless, 1965), on the basis of data from studies on *Arbacia* eggs (Clowes et al., 1940) and on inotropic effects in cardiac muscle (Hardman et al., 1959), that unionized barbiturate is the active form. However, with respect to determining its mechanism of

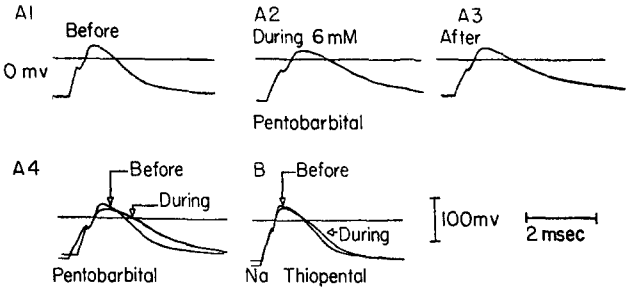

FIGURE 1. Action potentials from lobster giant axons. A, axon L-21-66, T = 6°C, pH 7.8. A 1–3, before, during, and after treatment with 6 mM sodium pentobarbital. A 4, before (A 1) and during (A 2) action potentials superimposed. B, axon L-25-66, T = 9°C, pH 7.8. Action potentials before and during treatment with 2 mM (saturated) sodium thiopental.

action at the molecular level, it is important to know whether the anionic or the unionized form of the barbiturate was active in producing nerve block.

A preliminary account of these results has been published (Blaustein, 1966).

METHODS

The sucrose-gap technique of Julian, Moore, and Goldman (1962 *a*,*b*) has been used to voltage clamp the isolated giant axon from the circumesophageal connective of the lobster, *Homarus americanus*. The axons were bathed in artificial seawater (ASW) containing (per liter): 465 mEq Na^+, 10 mEq K^+, 50mEq Ca^{+2}, 16 mEq Mg^{+2}, 533 mEq Cl^-, and 8 mEq SO_4^{-2}, buffered with 4 mM Tris(hydroxymethyl)aminomethane HCl.

The crystalline sodium pentobarbital and sodium thiopental (without added buffer) used in this study were generously supplied by Abbott Laboratories (Chicago, Ill.).

The methods employed for the corrections for leakage currents and for computation of conductances and time parameters are described elsewhere (Blaustein and Gold-

man, 1968; and cf. Moore et al., 1966). Leakage currents were subtracted before plotting current-voltage and conductance-voltage curves.

RESULTS

1. *Effect of Barbiturates on the Action Potential. Influence of pH*

Both sodium pentobarbital and sodium thiopental reversibly reduce the amplitude of the action potential in single lobster axons (Fig. 1). They also

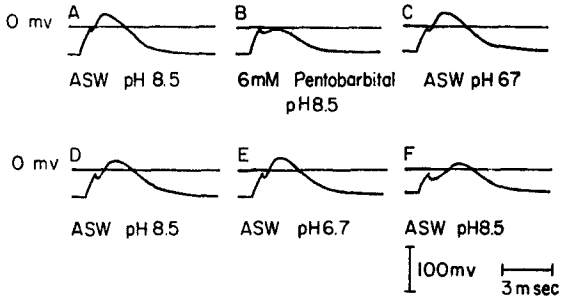

FIGURE 2. Axon L-25-66. T = 9°C. Action potentials in ASW, pH 8.5, before (A), and at the end of 3½ min in 6 mM sodium pentobarbital (B). Axon was then washed with ASW at pH 6.7 for ¾ min (C), then at pH 8.5 for 1 min (D), then at pH 6.7 for ¾ min (E), and finally, at pH 8.5 for 1½ min (F).

increase the duration of the action potential, primarily by slowing the rate of decline. This "plateau formation" during the falling phase of the action potential is best seen in the superimposed traces of Fig. 1 (A4 and B). Although the barbiturates were also found to hyperpolarize the resting membrane

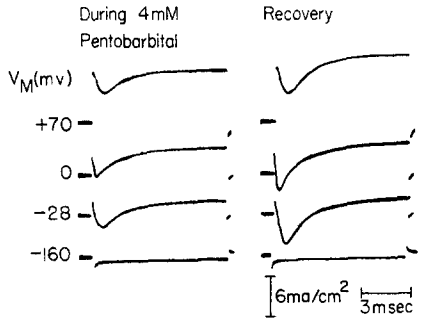

FIGURE 3. Axon L-4-66. T = 5°C. pH 7.8. Current-time curves during and after 4 mM sodium pentobarbital. Inward current is downward.

slightly (see Fig. 1), this effect is difficult to evaluate in the light of the large hyperpolarization which normally occurs under a sucrose gap (Blaustein and Goldman, 1966 *b*).

The action potentials in Fig. 2 are representative of data from three axons in which the effect of pH on the action of pentobarbital was tested. Control

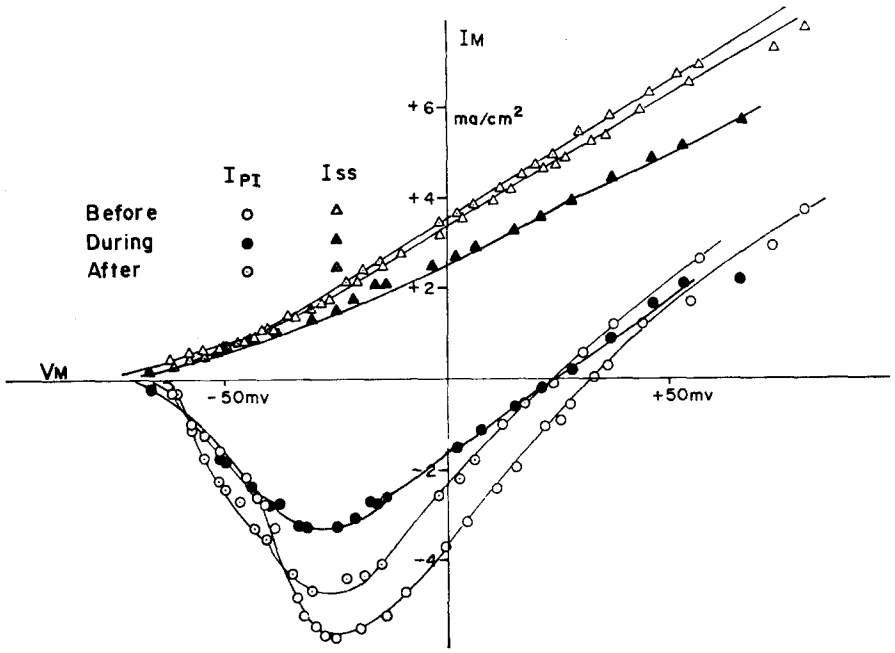

FIGURE 4. Axon L-4-66. Current-voltage relations. T = 5°C. Holding Potential = −109 mv. 4 mM sodium pentobarbital, pH 7.8. Peak initial (I_{PI}) and late steady-state (I_{ss}) current vs. voltage curves before, during, and after 4 mM sodium pentobarbital.

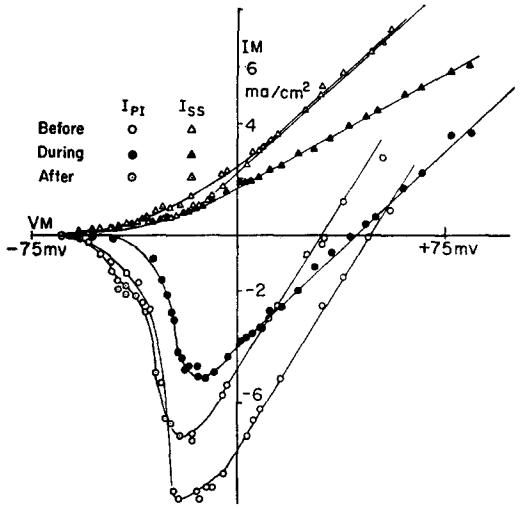

FIGURE 5. Axon L-25-66. Current-voltage relations. T = 9°C. Holding Potential = −108 mv. 2 mM (sat) sodium thiopental. pH 8.3. Peak initial (I_{PI}) and late steady-state (I_{ss}) current vs. voltage curves before, during, and after treatment with artificial seawater saturated with sodium thiopental.

studies showed that the amplitude and shape of the action potential are unaffected when the pH of the external medium is varied between pH 6.7 and 8.5. However, when the axon was loaded with pentobarbital at pH 8.5 (Fig. 2 B), so that the amplitude was markedly reduced, good recovery occurred in

barbiturate-free seawater at pH 6.7 (Fig. 2 C). When pH 8.5 seawater without barbiturate was then introduced, the amplitude decreased (Fig. 2 D), and again, the axon recovered when the pH was lowered to 6.7 (Fig. 2 E). The pK_a of sodium pentobarbital is 8.0 (Hardman et al., 1959).

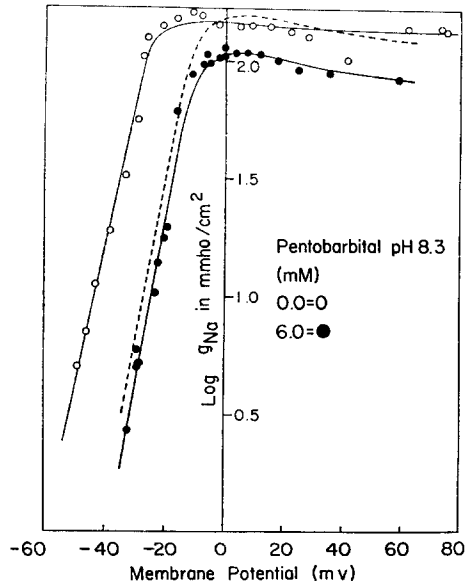

FIGURE 6. Axon L-22-66. T = 7°C. Log sodium conductance vs. membrane potential curves before and during 6 mM sodium pentobarbital. Dashed line shows normalized during curve.

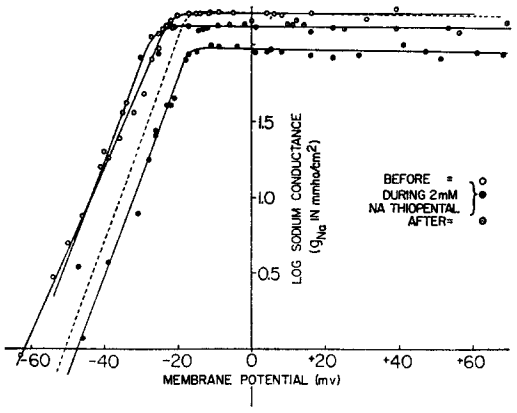

FIGURE 7. Axon L-25-66. T = 9°C. pH = 8.3. Log sodium conductance vs. membrane potential curves before, during, and after treatment with artificial seawater saturated with sodium thiopental. Dashed line is normalized during curve.

2. *Action of Barbiturates on Current-Voltage and Conductance Relations*

Both sodium pentobarbital and sodium thiopental reversibly reduce the magnitude of the early transient (Na) and late steady-state currents (K) in the voltage-clamped lobster axon. An example of these effects on the current-time curves during and after treatment with sodium pentobarbital is shown in Fig. 3. The current vs. voltage curves illustrate graphically the reversible reduction

TABLE I

EFFECTS OF BARBITURATES ON AXON MEMBRANE CONDUCTANCES

Axon	Node‖	pH	Before (B)			During drug (D)			After (A)			g^*_{Na}		g^*_K	
			g^*_{Na}‡	g^*_K§	g_{leak}	g^*_{Na}	g^*_K	g_{leak}	g^*_{Na}	g^*_K	g_{leak}	D/B¶	A/B	D/B¶	A/B
			mmho/cm²			*mmho/cm²*			*mmho/cm²*						
Sodium pentobarbital**															
L-4-66	1	8.0	78	40	11	64	37	8	—	—	—	0.82	—	0.93	—
	2	8.0	110	59	6	68	45	9	101	61	10	0.62	0.92	0.76	1.03
	3	8.0	141	60	14	78	53	16	140	63	24	0.55	0.99	0.88	1.05
L-5-66	1	8.0	179	79	4	114	59	3	141	60	4	0.64	0.79	0.88	0.76
	2	8.0	189	86	5	108	49	29	—	—	—	0.57	—	0.57	—
	3	8.0	240	77	43	202	68	39	230	79	38	0.84	0.96	0.88	1.03
L-7-66	1	8.0	154	60	13	104	42	29	137	61	26	0.68	0.89	0.70	1.02
	1′	8.0	137	61	26	112	53	27	—	—	—	0.82	—	0.87	—
L-8-66	1	8.0	122	54	18	82	44	30	—	—	—	0.67	—	0.81	—
	2	8.0	197	53	19	121	25	42	—	—	—	0.61	—	0.47	—
L-11-66	1	7.8	—	—	—	72	31	5	132	52	4	(0.54)	—	(0.60)	—
L-13-66	1	7.8	63	28	9	50	25	9	57	25	16	0.79	0.90	0.89	0.89
	2	7.8	107	45	10	76	37	11	102	39	8	0.71	0.82	0.82	0.87
L-14-66	1	8.0	—	—	—	85	38	3	139	60	2	(0.61)	—	(0.63)	—
L-17-66	1	8.5	123	58	14	90	42	14	120	59	16	0.73	0.98	0.72	1.02
L-22-66	1	8.3	148	53	13	108	28	15	—	—	—	0.73	—	0.53	—
											Average	0.68	0.91	0.74	0.96

								Sodium thiopental**							
L-6-66	1	8.0	159	83	36	56	40	29	127	69	27	0.35	0.80	0.48	0.83
L-25-66	1	8.3	158	88	15	94	54	9	127	87	9	0.59	0.80	0.61	0.99
											Average	0.47	0.80	0.55	0.91

‡ Maximum sodium conductance, calculated as $g^*_{Na} = \frac{I_{PI}}{V_M - V_{Na}}$, where I_{PI} is the corrected peak initial current; V_M is the membrane potential; and V_{Na} is the apparent sodium equilibrium potential; i.e., the positive potential at which $I_{PI} = 0$.

§ Maximum slope potassium conductance, calculated as $g^*_K = \frac{\Delta I_{ss}}{\Delta V_M}$, where I_{ss} is the corrected steady-state current.

‖ Node refers to the region of active membrane between two regions of flowing sucrose. Occasionally several such regions could be used on a single axon. Node referred to by a prime means that after washout, the same node was again tested with barbiturate.

¶ In cases in which the first voltage clamp run was made in the presence of a barbiturate (i.e., no before data were obtained), the D/B conductance ratios (in parentheses) were calculated from the washout data, as "during/after."

** Drug concentration, pentobarbital; 4.0 mM used with axon L-4-66; 6.0 mM used with other axons. Thiopental, 2.0 mM did not completely dissolve in the artificial seawater, so that a saturated solution was used. Temperatures ranged from 4° to 7°C, except L-25-66, which was at 9°C.

of Na and K currents resulting from treatment with either pentobarbital (Fig. 4) or thiopental (Fig. 5).

As expected from the decrease in early transient current, the log conductance vs. membrane potential curve during pentobarbital treatment (Fig. 6) is shifted downward so that the maximum conductance in the plateau region is considerably less than the control value. Note that when the log sodium con-

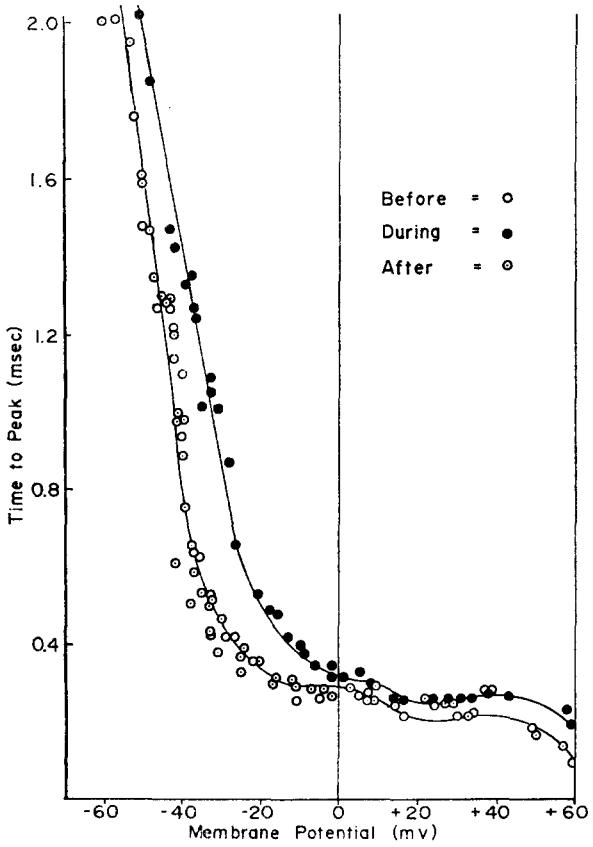

FIGURE 8. Axon L-13-66. T = 7°C. pH 7.8. 6 mM pentobarbital. Time-to-peak of early transient vs. membrane potential before, during, and after 6 mM sodium pentobarbital.

ductance curve obtained in the presence of sodium pentobarbital is normalized (dashed line curve of Fig. 6) to the same maximum conductance level as the control, there is still an apparent shift in the curve to the right along the voltage axis, by comparison with the control. A very similar effect is obtained when the axon is treated with thiopental, as shown in Fig. 7. Here, again, the maximum sodium conductance is decreased, and when the curve is normalized (dashed line), a net shift of the curve to the right along the voltage axis is noted. The magnitude of these shifts was quite variable, and averaged about

6–7 mv (with a maximum of about 17 mv) in 18 trials on 11 axons. In two or three instances there was no significant shift, or even a slight shift to the left (e.g., compare "Before" and "During" curves in Fig. 4).

Maximum sodium and potassium conductance data for 11 axons treated with sodium pentobarbital or sodium thiopental are summarized in Table I.

3. *Effect of Barbiturates on the Rate of Sodium Conductance Turn-On*

Fig. 8 shows the effect of sodium pentobarbital on the relation between membrane potential during the step and the time from the start of the step to the peak of the early transient. Pentobarbital increases the time to reach the peak of the early transient, particularly at small, depolarizing step potentials. Thus, there appears to be a shift of the curve of several millivolts to the right along the voltage axis. This effect, which also occurs with sodium thiopental, is most probably related to a slowing of the rate of increase of the early transient conductance.

As may be deduced from the −28 and 70 mv current-time curves of Fig. 3, the rate of decrease of the early transient and the rate of rise of the late current were not markedly affected by pentobarbital. Curves of the time required for the early current to fall from the peak to one-third of the peak value, and for the late current to rise to two-thirds of its steady-state value were plotted as a function of membrane potential. These curves were either unaffected by the barbiturates, or shifted a few millivolts in the direction of depolarization, along the voltage axis.

DISCUSSION

1. *Barbiturates and the Axon Membrane Conductances*

The experiments demonstrate that the barbiturates, sodium pentobarbital and sodium thiopental, reduce the axon membrane sodium and potassium conductance increases associated with excitation. These agents also slow the rate of sodium conductance turn-on and produce a small shift of the sodium conductance vs. voltage curve along the voltage axis in the direction of depolarization. All these effects resemble the action of procaine (Shanes et al., 1959; Taylor, 1959) and certain tropine esters (Goldman and Blaustein, 1966; Blaustein, 1968) on voltage-clamped axons.

The question of how the barbiturates act to reduce the Na and K conductance increases raises two major problems. One concerns the influence of pH on barbiturate action, and the second is related to the effect of barbiturates on resting sodium inactivation.

2. *The pH Effect: Anion vs. Unionized Molecule As the Active Form*

It has been generally assumed that the unionized barbiturate molecule is the active form. This interpretation (cf. Maynert, 1965; Sharpless, 1965) has been

based on studies demonstrating that the unionized barbiturate is the active form in reducing cell division of fertilized *Arbacia* eggs (Clowes et al., 1940) and in depressing the contractility of isolated myocardium (Harman et al., 1959). However, Hardman et al. pointed out the possibility that these effects might depend upon the barbiturates acting intracellularly, and that the unionized form may be essential for penetration of the cell membrane. A more recent report from the same laboratory (Baird and Hardman, 1961), on the influence of pH on procaine action on isolated myocardium provides evidence that the unionized drug depresses contractility, but that the cationic form slows electrical conduction and increases threshold and the refractory period. Data from *Arbacia* eggs and myocardium, however, are unlikely to have direct relevance to whether the anion or the unionized barbiturate molecule is involved in the production of nerve block.

Our observations (Fig. 2) demonstrate that after an axon has been loaded with pentobarbital, washing with low pH seawater will restore the action potential. When the axon is then washed with high pH seawater (Fig. 2 D), the action potential amplitude is again reduced, suggesting that barbiturate is still present.

These data suggest the following interpretation: (*a*) Pentobarbital is more effective in its anionic form, since more drug is ionized at high pH. (*b*) Less drug is likely to wash out of the membrane and axoplasm at low pH, since less drug is ionized and the oil-water partition coefficient is therefore larger. (*c*) The seawater pH only affects the external medium and axon membrane since it is unlikely that changing the pH over a membrane area of 2–3 $\times$ 10^{-4} cm^2 (the area of the artificial "node" in the sucrose gap) could significantly affect the axoplasm pH. Thus, barbiturates probably affect the membrane conductances as a result of a direct action on the axon membrane, rather than indirectly through an action on (e.g.) cell metabolism.

3. *Is the Reduced Sodium Conductance a Manifestation of Increased Resting Inactivation?*

A second problem concerns the resting sodium inactivation; i.e., that portion of the sodium-carrying system which cannot undergo an increase in conductance when the axon is depolarized. This refractory condition may be removed by hyperpolarizing the fiber just prior to the depolarizing step (Hodgkin and Huxley, 1952 *a*). Schoepfle (1957) suggested that the thiopental-induced action potential block in the single frog node of Ranvier resulted from increased resting inactivation since the block was reversed by prehyperpolarization.

Although resting sodium inactivation was not directly measured, indirect evidence suggests that this was not a significant factor in the reduction of the sodium conductance increase in barbiturate-treated lobster axons. In all the

voltage clamp experiments described above (Table I), resting membrane potentials were beyond −100 mv (range: −102 to −116 mv), and clamping ("holding") potentials ranged from −105 to −116 mv. This steady hyperpolarization (lobster axon resting potentials average about −70 mv in the absence of a sucrose gap) which normally occurs under a sucrose gap (Julian et al., 1962 *a*; Blaustein and Goldman, 1966 *b*) may be expected to minimize resting sodium inactivation. Takata et al. (1966, Fig. 4) have shown that in the lobster giant axon under a sucrose gap, the resting sodium inactivation in normal artificial seawater is negligible at holding potentials greater than −100 mv.

In general, the time- and voltage-dependent parameters of Hodgkin and Huxley (1952 *b*) for activation and inactivation of the sodium conductance ("m" and "h", respectively) are affected similarily by a variety of conditions such as: (*a*) Changes in external divalent cation used (Dodge, 1961; Blaustein and Goldman, 1968); (*b*) changes in divalent cation concentration (Frankenhaeuser and Hodgkin, 1957; Blaustein and Goldman, 1968); and (*c*) changes in axoplasmic ionic strength (Moore et al., 1964 *b*; Chandler et al., 1965). In other words, the m and h parameters, plotted as a function of membrane potential, are shifted together under these conditions.

An important inconsistency in Schoepfle's results stems from his statement that the intrinsic time parameter for the inactivation process was unaffected by thiopental. To account for a 50% reduction of peak sodium conductance (cf. Table I) solely on the basis of increased resting sodium inactivation would require that the inactivation curve (cf. Takata et al., 1966, Fig. 4) be shifted by about 50–60 mv in the direction of hyperpolarization. This would in turn require that the time constant for sodium inactivation, τ_h, as a function of membrane potential, also be shifted in that direction (cf. Hodgkin and Huxley, 1952 *b*, equations 16 and 18). Insofar as the rate of decline of the early transient current may be taken as a crude measure of τ_h, no such shift was observed (see Fig. 3). The rate of sodium current decline, at a given membrane potential, did not increase at all, and may even have decreased slightly, particularly with small depolarizations.

The basic similarity in the electrical properties of frog node (Dodge, 1961) and lobster axon (Julian et al., 1962 *a*, *b*) suggests that phylogenetic differences are unlikely to account for the discrepancy between Schoepfle's results and those reported here. In sum, these considerations lead to the conclusion that the action of barbiturates on excitability is not primarily the result of an increase in resting sodium inactivation. Furthermore, these drugs have a number of other effects on the membrane conductances which must be entirely independent of any effect on sodium inactivation: (*a*) They reduce the maximum potassium conductance by about the same per cent as the sodium conductance (Table I); (*b*) they slow the rate of rise of the sodium conductance

(Fig. 8); and (*c*) they shift the sodium conductance curve in the depolarizing direction along the voltage axis (Figs. 6 and 7).

4. *A Possible Molecular Basis for the Action of Barbiturates*

The fact that anesthetic potency has been correlated with solubility in media with low dielectric constants for cationic (for example, "local" anesthetics) and anionic (such as barbiturates) as well as nonionic (e.g., alcohols) drugs (see Butler, 1950) suggests that all these agents reduce the maximum sodium and potassium conductances by a similar mechanism of action. These agents may, in fact, reduce the membrane conductances by dissolving in the membrane lipid bilayer, thereby increasing the transmembrane electrical resistance (Blaustein and Goldman, 1966 *c*).

In Hodgkin-Huxley (1952 *b*) terminology, this increased resistance would be represented by a decrease in $\bar{g}_{Na}$ and $\bar{g}_K$, the membrane sodium and potassium conductance constants. A decrease in these constants will lead to a reduction in action potential amplitude (Moore et al., 1964), but will not account for the prolongation of the action potential and the plateau formation on the falling phase. On the other hand, certain polyvalent cations, which apparently do not affect the maximum sodium and potassium conductances (or at least $\bar{g}_{Na}$ and $\bar{g}_K$), do cause plateau formation and a prolongation of the action potential (for example, Takahashi et al., 1958; and see Blaustein and Goldman, 1968). These cations affect the time- and voltage-dependent parameters of the sodium and potassium conductances, giving rise to shifts of the time parameter and conductance curves along the voltage axis (Blaustein and Goldman, 1968). Since the barbiturates have also been shown to affect at least some of these parameters, there exists the possibility of a common factor in the mode of action of calcium and the barbiturates. Indirect evidence suggests that calcium is bound to "fixed" negative charges in the membrane of the resting axon (cf. Frankenhaeuser, 1957; Frankenhaeuser and Hodgkin, 1957). If the lipid-soluble moiety of the barbiturate penetrates into the lipid layer of the membrane, and if the anionic form of the drug is active, this would increase the net negative charge on the external surface of the membrane, thereby favoring an increase in calcium binding to the membrane. It might be argued that fixed negative charges in the membrane would make it difficult for barbiturate molecules to enter and remain in the membrane. However, repulsion of these negatively charged drug molecules would be offset by: (*a*) calcium ions binding to the drug molecules as counterions, and (*b*) relatively strong van der Waals forces between the drug molecules and membrane lipid fatty acid chains. The latter possibility is supported by the observation that pentobarbital is approximately 50-fold more soluble in certain low dielectric constant media than the cationic anesthetic, procaine (Blaustein and Goldman, 1066 *c*).

The barbiturate effects which mimic raising of the calcium concentration might be attributable to the enhanced calcium binding, while the presence of the lipid-soluble part of the drug molecules in the lipid layer of the membrane might account for the reduced conductance (Blaustein and Goldman, 1966 *c*). In this regard it is interesting to note that another anionic, lipid-soluble compound, the detergent, sodium lauryl sulfate, also mimics some of the effects of increased calcium in the voltage-clamped axon (Kishimoto and Adelman, 1964).

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