MOLECULAR PHARMACOLOGY, 9, 360–371

On the Molecular Action of Local Anesthetics

I. The Mitochondrion as a Model Membrane System for Studying Local Anesthetic Action

CARL L. JOHNSON,[1] MARGARET A. GOLDSTEIN, AND ARNOLD SCHWARTZ[2]

Department of Cell Biophysics, Division of Myocardial Biology, Baylor College of Medicine, and Fondren-Brown Cardiovascular Research and Training Center, Methodist Hospital, Houston, Texas 77025

(Received December 19, 1972)

SUMMARY

JOHNSON, CARL L., GOLDSTEIN, MARGARET A., AND SCHWARTZ, ARNOLD: On the molecular action of local anesthetics. I. The mitochondrion as a model membrane system for studying local anesthetic action. *Mol. Pharmacol.* **9**, 360–371 (1973).

Local anesthetics inhibit valinomycin-induced potassium uptake and basic protein-induced swelling of liver mitochondria. The present results suggest that a common feature of these effects of the local anesthetics on mitochondria is a stabilization of membrane structure. In view of the proposed role of protein conformational changes in nerve impulse conduction, the results suggest that the molecular mechanism of local anesthesia may involve an inhibition of membrane conformational changes rather than a direct inhibition of transport of monovalent or divalent cations, as generally believed. A correlation exists between the activity of these drugs on mitochondria and their activity on nerve conduction blockade. The mitochondrion may therefore serve as a useful model system to study local anesthetic action at the molecular level. Structure-activity studies indicate that the major determinant of potency of these anesthetics on mitochondria is the lipid solubility of the drug.

INTRODUCTION

Local anesthetics possess the specific ability to block conduction in excitable tissues in a reversible manner. Electrophysiological measurements (1–5) on nerve and muscle suggested that these drugs exert their pharmacological effects by interfering with the transport of sodium and potassium across the cell membrane. Other studies (3, 6–8) supported the concept that anesthetics block conduction by modifying the state of intracellular or membrane-bound calcium.

Although it is widely accepted that the local anesthetics exert their pharmacological action by interacting with the cell membrane of excitable cells, study of the molecular nature of this interaction is difficult in the intact nerve or muscle. As a result, many attempts have been made to utilize other membrane systems capable of trans-

This investigation was supported by Grants HL 05925, HL 05435, HL 07906, and Contract NIH 71-2493 from the National Institutes of Health, Grant NSF GB 6895 from the National Science Foundation, and the American Heart Association, Texas Affiliate, Houston Chapter.

[1] Predoctoral Trainee of the National Institutes of Health (GM 670). This study represents partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address, Department of Pharmacology, Mt. Sinai Medical School, New York, New York 10029.

[2] Recipient of Research Career Development Award K_3 HL 11,875 from The U.S.P.H.S., National Heart and Lung Institute.

porting ions in order to examine fundamental characteristics of local anesthetic–membrane interaction.

We have previously pointed out (9) that the mitochondrion may be a useful model system upon which to study the effects of local anesthetics at the membrane level. Isolated mitochondria are capable of carrying out a number of metabolism-linked transport processes, in particular the transport of potassium. A major advantage of the mitochondrial system is that the magnitude and time course of the ion translocations are such that they can be continuously and directly monitored by means of ion-specific electrodes. In addition, the mitochondrion is capable of undergoing reversible alterations of structure which can be conveniently followed by means of spectrophotometric (light scattering) techniques.

Under the usual assay conditions mitochondria do not spontaneously catalyze net transport of potassium. However, recent studies have shown that net movements of potassium do occur in the presence of certain specific inducing agents: the "ionophore" antibiotics (10) and certain basic proteins (11).

Valinomycin and basic proteins were used in the present studies as "tools" to induce potassium transport and structural alterations in mitochondria. The purpose of these studies was twofold: (a) to characterize the effects of local anesthetics on mitochondria, and (b) to compare the activity of local anesthetics on mitochondria and their pharmacological potency on nerve conduction and thereby explore the use of the mitochondrion as a model system for studying the mechanism of local anesthetic action.

Few reports have appeared in the literature dealing with the effects of local anesthetics on mitochondria. Judah *et al.* (12) observed that the local anesthetic dibucaine diminished the loss of potassium and the uptake of hydrogen ion induced by the mitochondrial uncoupling agent 2,4-dinitrophenol. This observation was then extended by Azzi and Scarpa (13) to a number of other mitochondrial transport processes. They found that dibucaine inhibited both spontaneous and valinomycin-induced potassium extrusion (metabolism-independent transport) and valinomycin-induced potassium uptake (metabolism-dependent transport). In the latter case the inhibition by the local anesthetic appeared to be competitive with the level of potassium in the external medium. In the concentration range needed to inhibit potassium transport, no effects were observed on phosphorylation of ADP, uncoupler-stimulated respiration, or calcium transport. Higher concentrations of the local anesthetics, however, were found to inhibit calcium uptake. These authors (13) concluded that in low concentrations (on the order of 50–100 μM) dibucaine was a specific inhibitor of mitochondrial potassium transport processes.

Chance *et al.* (14) and Mela (15, 16) reexamined the effects of local anesthetics on calcium transport and reported a stimulation of calcium uptake. The reason for the discrepancy in these reports on the effects of local anesthetics on calcium transport is not clear but may be due to a difference in assay conditions, in particular, the presence or absence of phosphate in the medium. In any event, these results cast some doubt on the specificity of local anesthetic action on mitochondria.

We previously reported (9) that a variety of local anesthetics inhibit mitochondrial potassium extrusion and swelling (measured by light scattering) induced by basic proteins. Since these two effects, potassium transport and structural changes, could not be dissociated, it was not clear whether the mechanism of local anesthetic action involved a direct inhibition of cation transport or a "stabilization" of membrane structure. Preliminary studies with several local anesthetics suggested that the activity of these compounds on mitochondria paralleled their potency on nerve conduction blockade (9).

METHODS AND MATERIALS

Methods. Mitochondria were isolated from adult rat liver by the method of Schneider (17) in a medium containing 0.25 M sucrose and 1 mM EDTA buffered to pH 7.2 with Tris. The final mitochondrial pellet was suspended in the same medium to yield a protein concentration of 100

mg/ml. Protein was estimated by a biuret method (18), using bovine serum albumin as a standard.

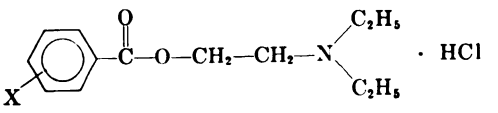
The assay of mitochondrial respiration, ion transport, and swelling was carried out simultaneously by means of a multiparameter device designed at the Johnson Research Foundation (Philadelphia). A description of this device has been published (19).

For electron microscopy, duplicate samples of mitochondria (0.6 mg of protein) were removed from the cuvette of the multiparameter device and fixed in a suspension (300 milliosmoles) of 1.5% purified glutaraldehyde in 0.05 M phosphate buffer, pH 7.3. A pellet was obtained by centrifugation and rinsed in phosphate buffer, placed in 2% osmium tetroxide for 2 hr, dehydrated with ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and with lead citrate and were examined in an RCA EMU4 electron microscope.

The assay medium used throughout these studies contained 0.25 M sucrose, 20 mM Tris, 3 mM glutamate, 3 mM malate, and 2.5 mM phosphate, adjusted to pH 7.2 with HCl. The final volume was 10 ml; the temperature was 25–26°. The mitochondrial concentration was 4.0 mg/ml. For the experiments involving valinomycin-induced potassium uptake, the potassium concentration was 1.0 mM and the amount of valinomycin was 0.05 μg/mg of mitochondrial protein. For the basic protein-induced potassium extrusion experiments, the concentration of potassium was 500 μM and the basic protein used was 10 μg of β-histone per milligram of mitochondrial protein.

Materials. The histone fraction (β-histone) used in these studies was generously donated by Dr. Harris Busch (Baylor College of Medicine, Houston). Valinomycin was purchased from Sigma Chemical Company. Nigericin was obtained from Eli Lilly and Company. The following local anesthetics were obtained from commercial sources: procaine, cocaine, tropacocaine, dibucaine, lidocaine, tetracaine, quinidine, and propranolol.

Several analogues of procaine having the general structure



were used in the present studies. They have been given the following trivial names for identification purposes: hydrocaine (X = H), chlorocaine (X = 4-Cl), fluorocaine (X = 4-F), *p*-methylcaine (X = 4-CH_3), *m*-methylcaine (X = 3-CH_3), nitrocaine (X = 4-NO_2), methoxycaine (X = 4-OCH_3), ethoxycaine (X = 4-OC_2H_5), butoxycaine (X = 4-OC_4H_9), and butylcaine (X = 4-*tert*-C_4H_9). These compounds were synthesized from benzoic acid derivatives or the corresponding acyl chlorides as previously described (20, 21).

RESULTS

Action of local anesthetics on valinomycin-induced effects. The effects of the antibiotic valinomycin on mitochondria are shown in Fig. 1. Addition of 2 μg of valinomycin caused an immediate uptake of potassium and a decrease in light scatter (representing mitochondrial swelling). Upon reaching an anaerobic state, the majority of the potassium accumulated was lost and light scattering increased. These results are essentially identical with those reported previously by other investigators (22–24). The inhibitory action of the local anesthetic butoxycaine (butoxy analogue of procaine) is shown in Fig. 2.

Action of local anesthetics on basic protein-induced effects. The effects of a basic protein, a histone fraction isolated from calf thymus nuclei, are shown in Fig. 3. Addition of 10 μg histone per milligram of mitochondrial protein caused an immediate extrusion of intramitochondrial potassium, a decrease in light scattering, and a stimulation of oxygen consumption. Upon reaching the anaerobic state, the light scattering trace leveled off.

The histone-induced effects were inhibited by the local anesthetic butoxycaine (Fig. 4).

That the light scattering changes observed in these experiments represent structural alterations in the mitochondrial inner membrane was shown by electron microscopy studies. Aliquots of the assay medium were removed from the cuvette of the multiparameter device and treated as described

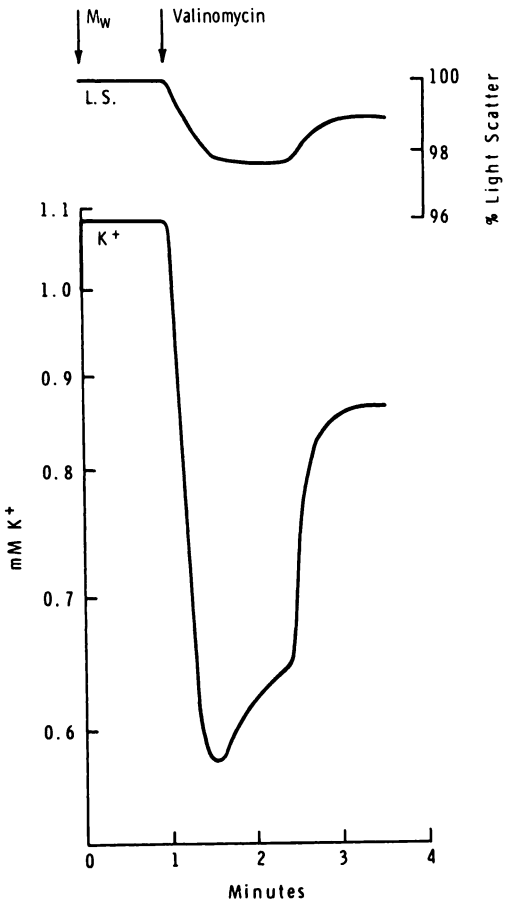


FIG. 1. *Effects of valinomycin on mitochondria*

The assay medium consisted of 0.25 M sucrose, 20 mM Tris, 3 mM glutamic acid, 3 mM malic acid, 2.5 mM phosphate, and 1.0 mM KCl, pH 7.2, at 25–26°; the final volume after addition of mitochondria was 10 ml. The additions at the arrows were 40 mg of washed mitochondrial protein (M_w) and 2 μg of valinomycin. In all experiments shown in this and subsequent figures, calibration of the K^+ electrode was carried out before and after assay; light scatter (L.S.) was calibrated qualitatively using a mitochondrial suspension.[3] A descending slope indicates a decrease in light scatter which *may* reflect swelling, although other interpretations are by no means excluded (19).[3]

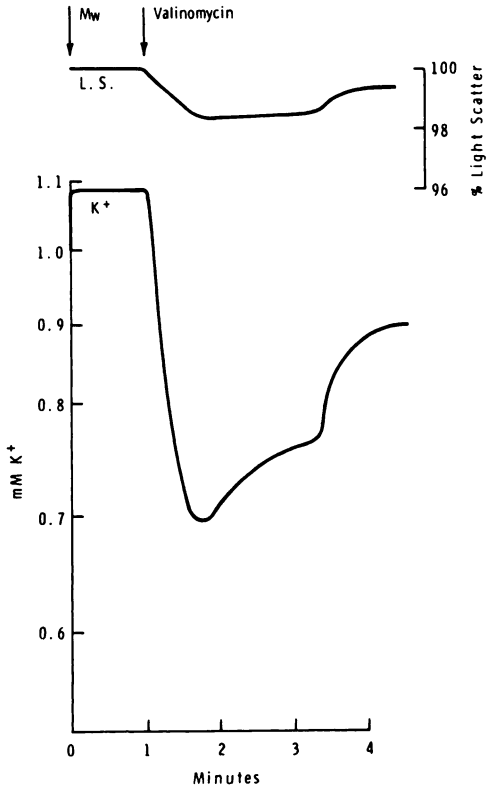


FIG. 2. *Action of butoxycaine on valinomycin-induced effects*

The medium was the same as in Fig. 1 plus 200 μM butoxycaine. The additions at the arrows were 40 mg of washed mitochondrial protein (M_w) and 2 μg of valinomycin.

under METHODS AND MATERIALS. Prior to addition of the basic protein (Fig. 5A and E), the mitochondria exhibited a typical condensed, "nonenergized" configuration characteristic of so-called state 4 mitochondria (25). When the basic protein was added and light scattering had decreased to a minimum, electron micrographs (Fig. 5B and F) showed that the inner membrane had unfolded and the matrix appeared less dense (25). Mitochondria in the presence of butoxycaine were indistinguishable from the control mitochondria (Fig. 5C and G). Addition of basic protein to the local anesthetic-treated mitochondria failed to produce ultrastructural changes (Fig. 5D and H).

In our earlier studies (11, 26) of the effects of the basic proteins on mitochondria we were unable to dissociate the extrusion of potassium from the light scattering changes. We have now obtained a dissociation of these two processes by means of the antibiotic nigericin.

As shown in Fig. 6, low concentrations of nigericin (on the order of 1 ng/mg of pro-

[3] B. C. Pressman, personal communication.

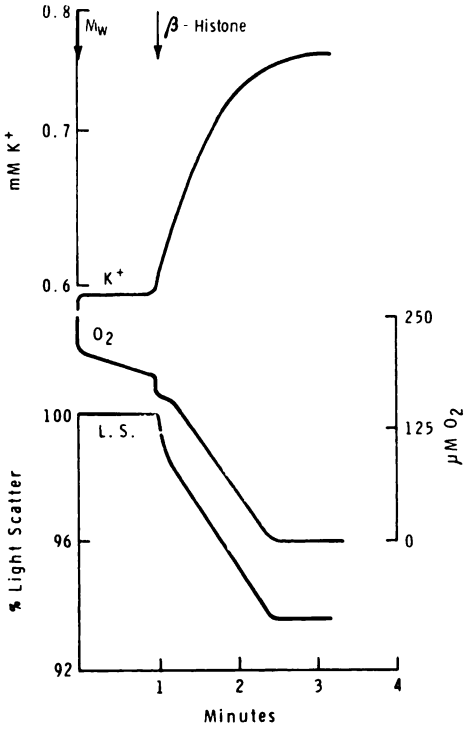


FIG. 3. *Effects of basic proteins on mitochondria*
The medium was the same as in Fig. 1 except that the initial KCl concentration was 0.5 mM. The additions at the arrows were 40 mg of mitochondrial protein (M_w) and 400 μg of β-histone.

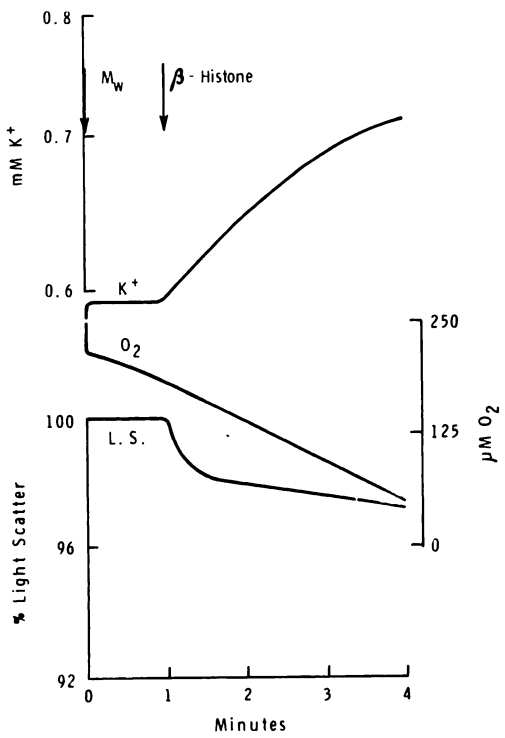


FIG. 4. *Action of butoxycaine on basic protein-induced effects*
The medium was the same as in Fig. 3 plus 200 μM butoxycaine. The additions at the arrows were 40 mg of mitochondrial protein (M_w) and 400 μg of β-histone.

tein) caused a complete loss of intramitochondrial potassium and a slight increase in light scattering. No measurable effect on respiration was observed. A large number of studies concerned with the action of nigericin on mitochondria have appeared (e.g., 24, 27–31). It is generally accepted that nigericin causes a release of potassium and an apparent contraction of mitochondria. In most cases inhibition of NAD-linked substrate oxidation has also been noted. However, this respiratory inhibition could be avoided under suitable assay conditions, namely, high substrate concentration and mild hypotonicity (30). We have also consistently observed that a complete release of potassium can be obtained in an isotonic medium of moderate substrate concentration if the concentration of nigericin is lowered to approximately 1 ng/mg of mitochondrial protein.

Despite the depletion of mitochondrial potassium, the basic protein still retained the ability to stimulate respiration and to cause "swelling" of the mitochondria (Fig. 6). It is clear, therefore, that the action of the basic proteins does not necessarily involve the transport of potassium. Rather, the efflux of potassium that occurs in the presence of the basic proteins is probably due to a rearrangement of the mitochondrial membrane structure, allowing for passive leakage of potassium down its concentration gradient. It should be emphasized, however, that the structural changes in the membrane cannot be characterized as a generalized destruction of membrane integrity. These changes are completely metabolism-dependent, requiring either electron transport or ATP utilization (26). Furthermore, mitochondria treated with basic proteins still retain the ability to

phosphorylate ADP and to accumulate potassium upon addition of valinomycin.[4] These results suggest the importance of initial histone-membrane interaction leading to significant structural alterations.

As shown in Figs. 3, 4, and 6, the light scattering changes observed with the basic proteins exhibit two phases. There is a very rapid decrease in scattering, terminating within about 10–15 sec, which has been shown to be completely metabolism-independent and is not inhibited by very large concentrations of the local anesthetics. The nature of this process is unknown, but it may reflect some aggregation of the mitochondria caused by the strongly charged basic protein. The second phase of the light scattering change is completely metabolism-dependent (being prevented by electron transport inhibitors) and is inhibited by the local anesthetics. This second phase is reasonably linear with time. As a measure of the effect of the basic protein on mitochondrial swelling, we have calculated the relative change in light scattering during the period from 20 to 80 sec after addition of the basic protein.

A dose-response curve for the inhibition of histone-induced swelling is shown in Fig. 7. The control experiment (solid circles) represents a titration of the effects of the local anesthetic butoxycaine under the conditions employed in Fig. 4. The other titration (open circles) was carried out on mitochondria that had been depleted of potassium by prior treatment with nigericin as in Fig. 6. No difference was obtained in the dose-response relationship, indicating that *the local anesthetics are not acting on a potassium transport mechanism.* Thus the original hypothesis of Azzi and Scarpa (13), that low concentrations of local anesthetics are specific inhibitors of mitochondrial potassium transport processes, does not appear to be correct. A more likely mechanism of action of the anesthetics appears to be an inhibition of the membrane conformational changes which are associated with action of both basic proteins and valinomycin, and which are reflected by light scattering changes.

[4] Unpublished observations.

It is significant that the local anesthetics do not inhibit the nigericin-induced extrusion of potassium, a process which is not dependent on energy and which is not accompanied by swelling. Thus a clear distinction can be made between the extrusion of potassium caused by the basic protein and that caused by nigericin.

Structure-activity studies on mitochondria. In an effort to gain some information as to the mechanism of the local anesthetic inhibition of the valinomycin- and basic protein-induced effects, structure-activity studies were carried out using a large number of local anesthetics or local anesthetic-like molecules.

To conserve space, dose-response curves for all the compounds considered are not shown, but some representative results are shown in Fig. 8 for valinomycin-induced potassium uptake and in Fig. 9 for histone-induced swelling.

As shown in Table 1, there is a close correlation between the relative potency of a series of local anesthetics on valinomycin-induced uptake of potassium and on histone-induced swelling. Under the conditions of these experiments the concentrations of local anesthetics required to inhibit the histone-induced effects were generally on the order of 25% of those required to inhibit the valinomycin-induced effects. However, this may be ascribed to the fact that the concentrations of local anesthetic required for half-maximal inhibition of these responses depend on the concentrations of the inducing agents. Care was taken in all these studies to maintain constant experimental conditions, in particular the concentration of valinomycin and histone and the amount of mitochondrial protein used.

It is noteworthy that the relative potency of several common local anesthetics on nerve conduction blockade (32, 33) is closely correlated with the potency of these compounds on valinomycin-induced potassium accumulation and histone-induced swelling (Table 1).

As shown in Fig. 10, a good correlation was obtained between the activity of a series of procaine analogues on mitochondria and the lipid solubility of the drug [as esti-

FIG. 5A FIG. 5B

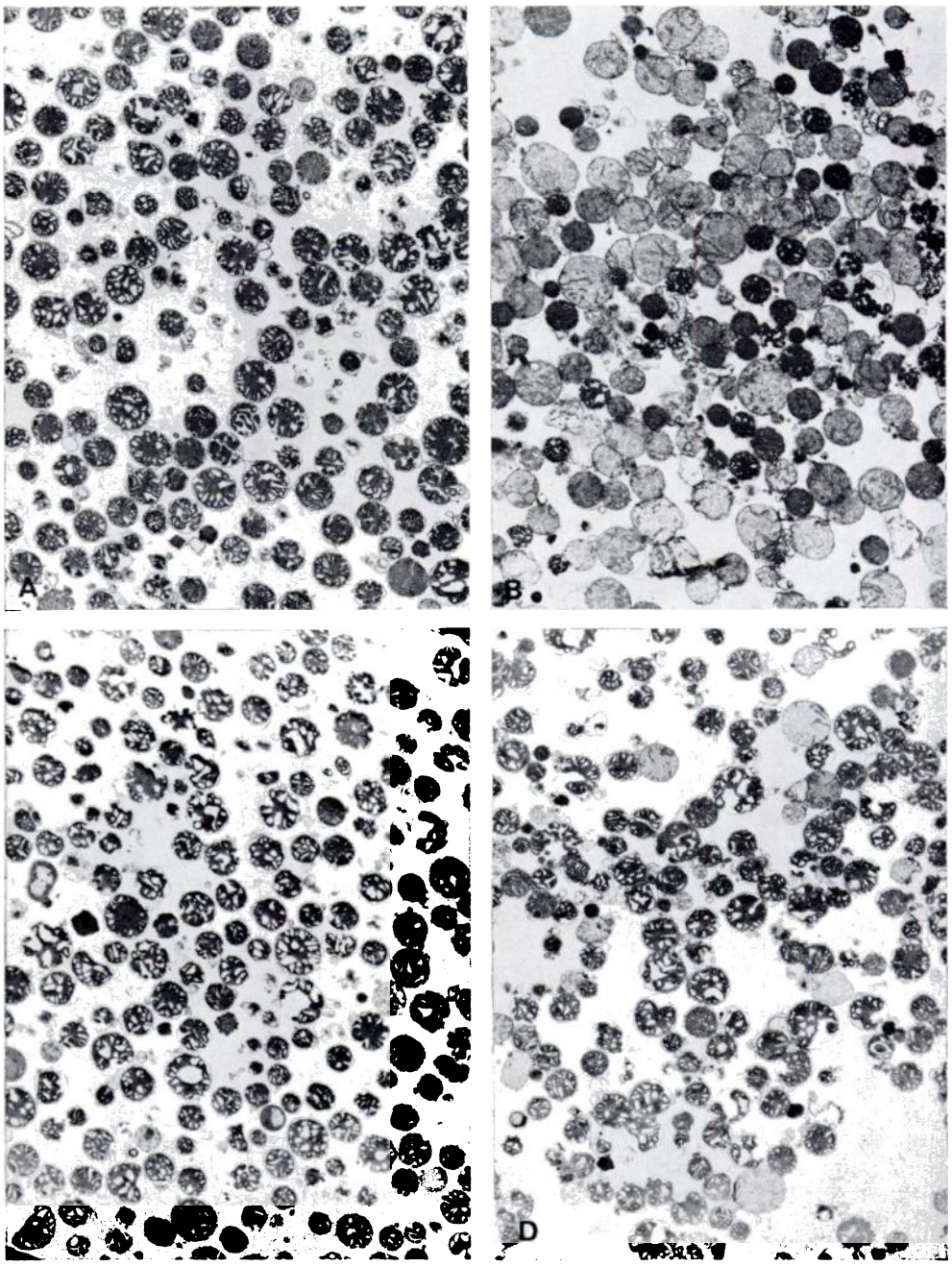


FIG. 5C FIG. 5D

FIG. 5. *Action of butoxycaine on ultrastructural effects of basic proteins*

The medium was the same as in Fig. 3. A and E. Control mitochondria before addition of basic protein. B and F. Mitochondria treated with 400 μg of β-histone. C and G. Mitochondria that had been preincubated with 200 μM butoxycaine. D and H. Mitochondria from Fig. 5C after addition of 400 μg of β-histone. A–D × 6500; E–H × 17,500.

FIG. 5E

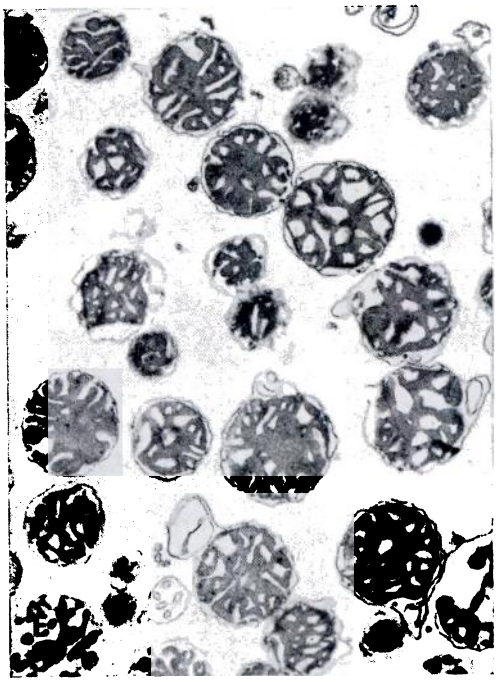
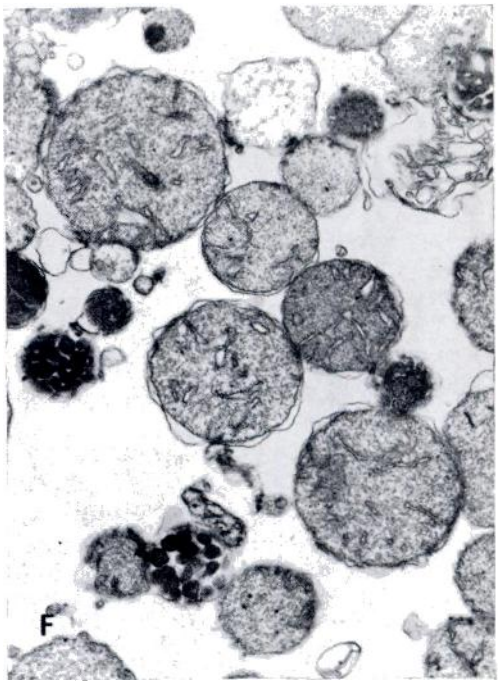


FIG. 5F



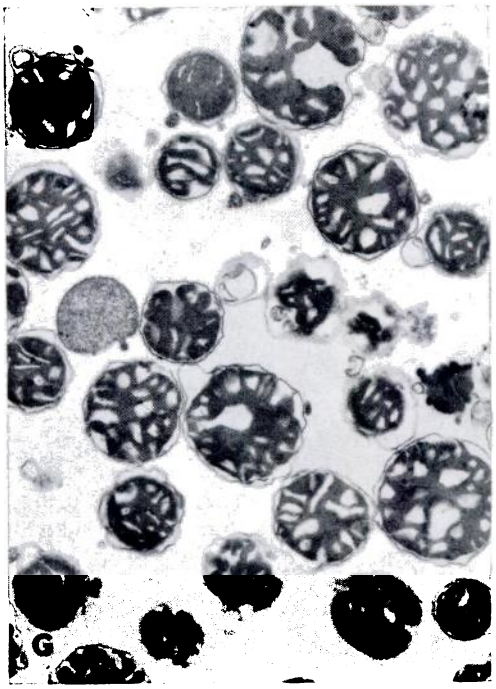


FIG. 5G

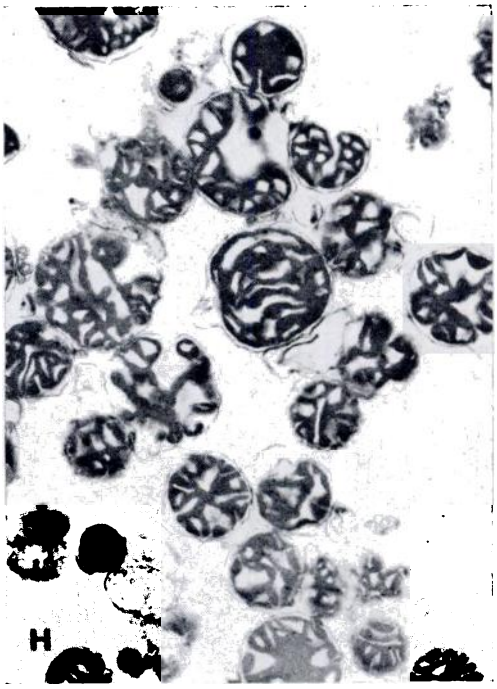


FIG. 5H

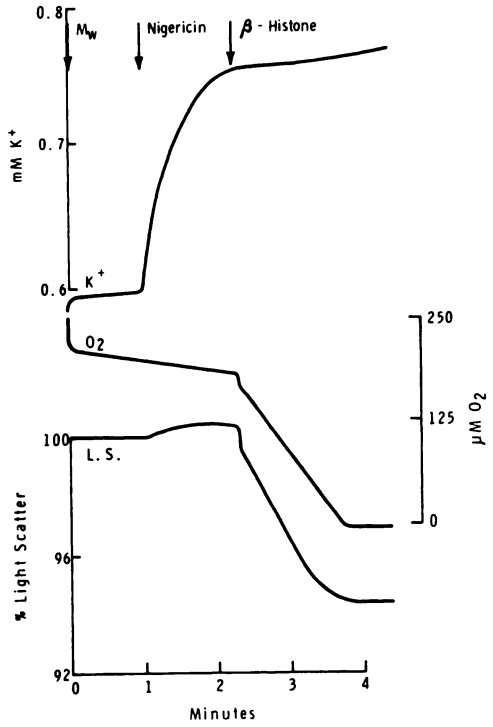


FIG. 6. *Effects of basic proteins after depletion of mitochondrial potassium by nigericin*

The medium was the same as in Fig. 3. The additions at the arrows were 40 mg of mitochondrial protein (M_w), 50 ng of nigericin, and 400 μg of β-histone.

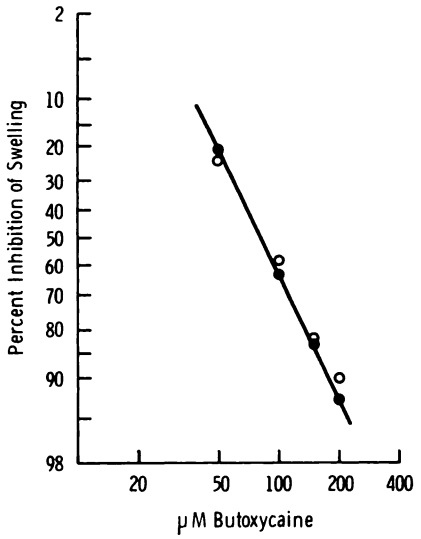


FIG. 7. *Log dose-response curve for inhibition of basic protein-induced swelling by butoxycaine*

The medium was the same as in Fig. 3. The rate of swelling was calculated as described in the text. The ordinate represents the percentage inhibition of this rate, expressed in probit units to improve the linearity of the curve. ●, without prior depletion of potassium by nigericin; ○, after depletion of intramitochondrial potassium by treatment with 50 ng of nigericin. The concentration of β-histone was 10 μg/mg of mitochondrial protein.

mated by Hansch π values (34)]. Only the parent compound, procaine, did not fit the regression well. This may have been due to an incorrect π value for the amino group. On the other hand, this group is the only strong hydrogen-bonding group within the series studied. It is possible that a second parameter is required in order to correlate derivatives capable of strong hydrogen bonding.

DISCUSSION

Local anesthetics have been shown to affect several parameters of mitochondrial function, including valinomycin-induced potassium uptake and basic protein-induced swelling. It is clear from the present studies that the extrusion of potassium is incidental to the action of the basic proteins. It is noteworthy that valinomycin-induced potassium uptake is accompanied by light scattering changes that have been shown by electron microscopy (35) to be accompanied by ultrastructural changes in the inner membrane of the mitochondrion. In the present study a similar correlation has been found for the light scattering and ultrastructural effects of basic proteins. In view of the close correlation between the inhibitory potency of local anesthetics on valinomycin-induced potassium uptake and basic protein-induced swelling, it seems probable that the action of local anesthetics on mitochondria is due to an inhibition of membrane configurational or conformational changes rather than to a direct inhibition of cation transport. It is also of significance that the extrusion of mitochondrial potassium induced by low concentrations of nigericin is *not* accompanied by swelling and is *not* inhibited by local anesthetics.

The correlation of local anesthetic activity on mitochondria and on nerve con-

duction blockade suggests that the mitochondrion might be a useful model system for testing more potent anesthetics. It is well known that lipid solubility is a major determinant of local anesthetic activity on nerves (36–42). It is of interest, therefore,

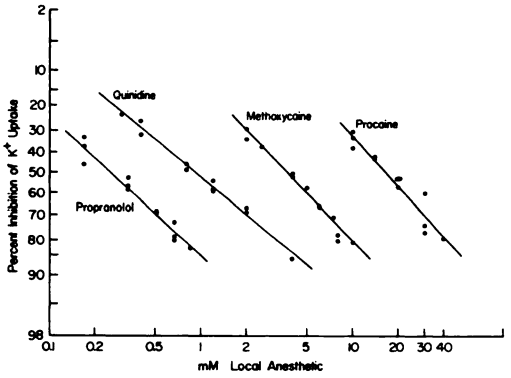


FIG. 8. *Representative log dose-response curves for action of several local anesthetics on valinomycin-induced potassium uptake*

The assay conditions are described in Fig. 1. The ordinate represents the percentage inhibition of the potassium uptake rate, expressed in probit units to improve the linearity of the curves. The millimolar concentrations of drug producing half-maximal inhibition of potassium uptake (and the standard errors) were as follows: propranolol, 2.252 ± 0.022; quinidine, 0.905 ± 0.034; methoxycaine (methoxy analogue of procaine), 3.62 ± 0.08; procaine, 18.8 ± 0.9. The concentration of valinomycin was 0.05 μg/mg of mitochondrial protein.

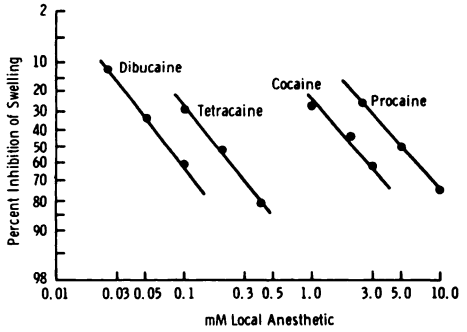


FIG. 9. *Representative log dose-response curves for action of several local anesthetics on basic protein-induced swelling*

The assay conditions are described in Fig. 3. The rate of swelling was calculated as described in the text. The ordinate represents the percentage inhibition of this rate, expressed in probit units to improve the linearity of the curves. The millimolar concentrations of drug producing half-maximal inhibition were as follows: dibucaine, 0.074; tetracaine, 0.18; cocaine, 2.1; procaine, 5.0. The concentration of β-histone was 10 μg/mg of mitochondrial protein.

that the action of local anesthetics on mitochondrial processes is also closely correlated with lipid solubility.

We propose that the primary action of local anesthetics on mitochondria is related to an inhibition of membrane configurational or conformational changes (the former refers to a rearrangement of membrane protein or lipoprotein units, the

TABLE 1

Correlation of relative potency of local anesthetics on nerve conduction blockade and on mitochondria

Anesthetic	Relative potency on nerve		Relative potency on mitochondria[c]	
	Skou (32)[a]	Truant and Takman (33)[b]	Swelling[d]	K^+ uptake[e]
Procaine	1.0	1.0	1.0 (5.0)	1.0 (19.3)
Lidocaine		3.8	1.5 (3.2)	1.8 (10.7)
Cocaine	1.8	9.6	2.4 (2.1)	2.5 (7.7)
Fluorocaine			2.9 (1.7)	2.6 (7.4)
Tropacocaine	2.1			5.5 (3.5)
Ethoxycaine			7.8 (0.64)	7.9 (2.4)
Tetracaine	460	36.5	27.8 (0.18)	29.7 (0.65)
Dibucaine	920	53.8	68.5 (0.074)	68.8 (0.28)

[a] Desheathed frog sciatic nerve (epineurium removed).
[b] Frog sciatic nerve.
[c] Values in parentheses are millimolar concentrations for 50% inhibition of swelling or K^+ uptake.
[d] Basic protein-induced swelling as in Fig. 9.
[e] Valinomycin-induced K^+ uptake as in Fig. 8.

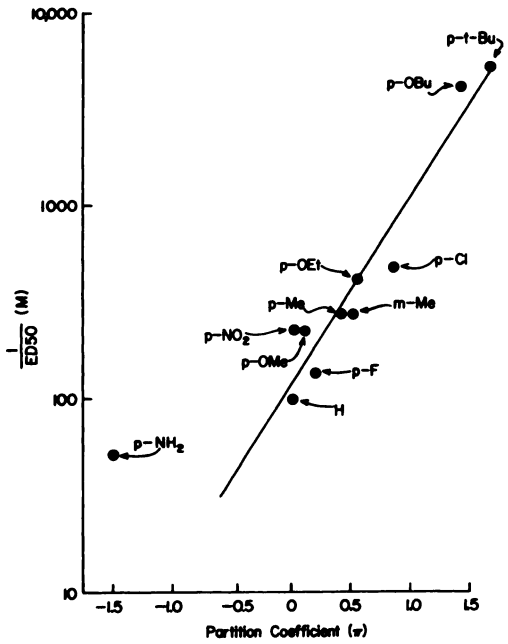
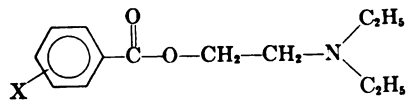


FIG. 10. *Correlation of relative potency of procaine analogues on mitochondria with partition coefficients*

The assay conditions were the same as in Fig. 1. The ordinate represents the reciprocal of the millimolar concentration of drug required for half-maximal inhibition of valinomycin-induced uptake of potassium. The abscissa represents the Hansch hydrophobic bonding constant, π, for the corresponding benzoic acid derivatives (34). The local anesthetics had the general structure



where X represents the substituent groups shown in the figure.

latter to changes in the tertiary or quaternary structure of an individual unit). Our results further suggest that there is a correlation between the potency of the local anesthetics on mitochondria and on nerve conduction. Thus it is attractive to propose that the mechanism of action of local anesthetics on nerves may be directly related to a stabilization of membrane structure rather than an inhibition of sodium, potassium, or calcium movements, as is generally believed.

It is of particular interest, therefore, that the process of conduction of the nerve impulse has been considered by several investigators to involve reversible cooperative changes in conformation of the macromolecules in the nerve membrane (43–45). Recent studies of the optical properties of nerves (46–48) support the view that some kind of conformational change does take place in the membrane during conduction. It might be predicted that local anesthetics would prevent these conformational changes, although this has apparently not been tested. Such studies would not, however, demonstrate that the primary action of the anesthetics is to block structural changes, since cation movements must accompany the conformational alterations in order to have impulse conduction. The present results appear to be the first indication that local anesthetics can prevent membrane structural changes which are not directly associated with a movement of cations.

ACKNOWLEDGMENT

We extend our appreciation to Dr. Jeanie M. Wood for carrying out several experiments and critically reviewing the manuscript.

REFERENCES

1. A. M. Shanes, W. H. Freygang, H. Grundfest, and E. Amatniek, *J. Gen. Physiol.* **42**, 793–802 (1959).
2. R. E. Taylor, *Amer. J. Physiol.* **196**, 1071–1078 (1959).
3. M. P. Blaustein and D. E. Goldman, *Fed. Proc.* **24**, 584 (1965).
4. L. Hurwitz, F. Battle, and G. B. Weiss, *J. Gen. Physiol.* **46**, 315–332 (1962).
5. G. B. Weiss, R. E. Coalson, and L. Hurwitz, *Amer. J. Physiol.* **200**, 789–793 (1961).
6. S. Weidmann, *J. Physiol. (London)* **129**, 568–582 (1955).
7. M. B. Feinstein, *J. Gen. Physiol.* **47**, 151–172 (1963).
8. M. B. Feinstein, *J. Pharmacol. Exp. Ther.* **152**, 516–524 (1966).
9. C. L. Johnson and A. Schwartz, *J. Pharmacol. Exp. Ther.* **167**, 365–373 (1969).
10. C. Moore and B. C. Pressman, *Biochem. Biophys. Res. Commun.* **15**, 562–567 (1964).
11. C. L. Johnson, C. M. Mauritzen, W. C. Starbuck, and A. Schwartz, *Biochemistry* **6**, 1121–1127 (1967).
12. J. D. Judah, A. E. M. McLean, K. Ahmed, and

G. S. Christie, *Biochim. Biophys. Acta* **94**, 441–451 (1965).
13. A. Azzi and A. Scarpa, *Biochim. Biophys. Acta* **135**, 1087–1088 (1967).
14. B. Chance, L. Mela, and E. J. Harris, *Fed. Proc.* **27**, 902–906 (1968).
15. L. Mela, *Arch. Biochem. Biophys.* **123**, 286–293 (1968).
16. L. Mela, *Biochemistry* **8**, 2481–2486 (1969).
17. W. C. Schneider, *J. Biol. Chem.* **176**, 259–266 (1948).
18. A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.* **177**, 751–766 (1949).
19. B. C. Pressman, *Ann. N. Y. Acad. Sci.* **148**, 285–287 (1968).
20. H. Vanderhaeghe, *J. Pharm. Pharmacol.* **6**, 55–59 (1954).
21. H. Vanderhaeghe, P. Kolosy, and M. Claesen, *J. Pharm. Pharmacol.* **6**, 119–126 (1954).
22. B. C. Pressman, *Proc. Nat. Acad. Sci. U. S. A.* **53**, 1076–1083 (1965).
23. E. J. Harris, G. Catlin, and B. C. Pressman, *Biochemistry* **6**, 1360–1369 (1967).
24. H. Lardy, *Fed. Proc.* **27**, 1278–1282 (1968).
25. M. A. Goldstein, C. L. Johnson, and A. Schwartz, *J. Cell Biol.* **55**, 88a (1972).
26. C. L. Johnson, J. Orò, and A. Schwartz, *Arch. Biochem. Biophys.* **131**, 310–315 (1969).
27. S. N. Graven, S. Estrada-O., and H. A. Lardy, *Proc. Nat. Acad. Sci. U. S. A.* **56**, 654–658 (1966).
28. S. Estrada-O., S. N. Graven, and H. A. Lardy, *Fed. Proc.* **26**, 610 (1967).
29. H. A. Lardy, S. N. Graven, and S. Estrada-O., *Fed. Proc.* **26**, 1355–1360 (1967).
30. B. C. Pressman, E. J. Harris, W. S. Jagger, and J. H. Johnson, *Proc. Nat. Acad. Sci. U. S. A.* **58**, 1949–1956 (1967).
31. S. N. Graven, H. A. Lardy, and S. Estrada-O., *Biochemistry* **6**, 365–371 (1967).
32. J. C. Skou, *Acta Pharmacol. Toxicol.* **10**, 281–291 (1954).
33. A. P. Truant and B. Takman, *in* "Drill's Pharmacology in Medicine" (DiPalma, J. R., ed.), p. 133. McGraw-Hill, New York, 1965.
34. T. Fujita, J. Iwasa, and C. Hansch, *J. Amer. Chem. Soc.* **86**, 5175–5180 (1964).
35. L. Packer, J. M. Wrigglesworth, P. A. G. Fortes, and B. C. Pressman, *J. Cell Biol.* **29**, 382–391 (1968).
36. X. Perlia, *Pharm. Acta Helv.* **42**, 517–533 (1967).
37. J. Büchi, X. Perlia, and R. Portmann, *Arzneimittel-Forschung* **18**, 610–616 (1968).
38. J. Büchi and X. Perlia, *Arzneimittel-Forschung* **12**, 626–630 (1962).
39. J. C. Skou, *Acta Pharmacol. Toxicol.* **10**, 325–337 (1954).
40. J. C. Skou, *Biochim. Biophys. Acta* **30**, 625–629 (1958).
41. N. Lofgren, "Studies on Local Anesthetics. Xylocaine, a New Anesthetic." Haeggstrom, Stockholm, 1948.
42. J. Büchi, O. Meyer, and X. Perlia, *Arzneimittel-Forschung* **17**, 1491–1500 (1967).
43. J.-P. Changeux, J. Thiéry, Y. Tung, and C. Kittel, *Proc. Nat. Acad. Sci. U. S. A.* **57**, 335–341 (1967).
44. A. L. Lehninger, *Proc. Nat. Acad. Sci. U. S. A.* **60**, 1069–1080 (1968).
45. I. Tasaki, "Nerve Excitation. A Macromolecular Approach." Thomas, Springfield, Ill., 1968.
46. L. B. Cohen and R. D. Keynes, *J. Physiol. (London)* **194**, 85P–86P (1968).
47. I. Tasaki, L. Carnay, R. Sandlin, and A. Watanabe, *Science* **163**, 683–685 (1969).
48. I. Tasaki, A. Watanabe, R. Sandlin, and L. Carnay, *Proc. Nat. Acad. Sci. U. S. A.* **61**, 883–888 (1968).