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Functional acetylcholine receptor–electroplax membrane microsacs (vesicles): Purification and characterization

(*Electrophorus electricus*/density gradient/excitability/permeability)

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ABSTRACT **Kinetic analysis of the flux of sodium ions in a heterogeneous population of acetylcholine receptor-rich microsacs (vesicles) formed by membrane fragments of electroplax indicated that functional microsacs, which on average comprise only 15% of the preparation, can be filled with 190 mM sodium chloride while nonfunctional microsacs are filled by 190 mM cesium chloride. The functional microsacs have then been successfully separated from nonfunctional microsacs on the basis of their density differences with a continuous sucrose-190 mM cesium chloride density gradient. In the presence of acetylcholine analogs all the internal sodium ions in these microsacs rapidly exchange with external ions. The efflux of sodium ions follows a single exponential decay. The isolation of functional microsacs opens up at least two new avenues of investigation of the molecular mechanism of receptor-mediated processes. The first deals with the efficiency of the process, and the second with the characterization of membrane components important in this process. The conclusions reached so far are: (*i*) The efficiency of the receptor-mediated process that allows inorganic ions to equilibrate across the membranes of the microsacs can adequately account for electrophysiological results obtained with muscle and nerve cells. (*ii*) In the receptor-rich heterogeneous population of microsacs the concentration of receptor sites in functional and nonfunctional microsacs is about the same and is therefore not the only factor determining functionality. Significant differences between functional and nonfunctional microsacs have been found so far in the concentrations of acetylcholinesterase and Na^+-K^+ ATPase.**

Initiation of increased ion flow across neural membranes by the combination of acetylcholine or its congeners with the membrane-bound acetylcholine receptor plays an important role in the generation (1) and propagation of electrical signals in nerve cells (2–4). Although the acetylcholine receptor has been isolated and is being characterized in many laboratories (for a recent review see ref. 5 and also refs. 6–9), little is known, on the molecular level, about the relationship between ligand binding and receptor-mediated changes in permeability of membranes to inorganic ions. Because the receptor has no function in solution we used receptor-rich membrane vesicles (microsacs) prepared, according to the procedure of Kasai and Changeux (10), from the electric organ of *Electrophorus electricus*. They exhibit ion flux that is affected by receptor ligands *in vitro* (10). After the development of rapid and quantitative methods (11, 12), the mechanism by which receptor ligands bind to the membrane-bound receptor was studied with these membrane preparations (13). We showed that there are two types of receptor binding sites, one for activators and one for inhibitors of neural excitability (12, 14). Using a fluorescent lanthanide, terbium, to probe the calcium- and activator-binding sites of the purified receptor prepared from *Torpedo ocellata*, we obtained additional evidence (15, 16) for different receptor binding sites for activators and inhibitors. In addition, we demonstrated a half-of-the-sites relationship between inhibitors and activators (13). On the basis of kinetic data, we developed a relatively simple two-state model for the membrane-bound receptor that takes into account both equilibrium and kinetic measurements of receptor-ligand interaction (14).

Recently we investigated the kinetics of sodium-22 ion flux using the same membrane microsac preparation (17, 18). We found that sodium-22 efflux from 85% of the microsacs exhibited complex kinetics and was not affected by receptor ligands. Development of a kinetic method (17) allowed us to measure the efflux from the functional microsacs (receptor-ligand dependent flux), which comprised on average 15% of the preparation, without the measurements being obscured by efflux from nonspecific microsacs. Under these conditions the efflux follows a single exponential decay. The dependence of the rate coefficient, k_{obs}, on ligand concentration indicated a direct relationship between ligand-binding parameters, investigated by equilibrium and kinetic studies, and receptor-mediated ion flux using the same preparation. Further evidence for different receptor binding sites for activators and inhibitors was also obtained in these studies (18).

In this communication we report the successful separation of specific from unspecific microsacs. The procedure is based on the kinetic analysis of the flux data, which indicated conditions under which we could fill specific microsacs with sodium chloride while the unspecific microsacs were filled with denser cesium chloride. The microsacs were separated by centrifugation in a continuous sucrose-cesium chloride density gradient. The isolation of the functional microsacs allows one to determine the number of inorganic ions that can diffuse through the membrane per receptor site per unit time. This determination is important for a correlation between receptor-mediated ion flux through microsac membranes and the receptor-ligand induced changes in the electrical potential of the membranes of nerve or muscle cells. Additionally, the isolation of functional microsacs permits identification of membrane components, other than the receptor protein, which may be important in receptor ligand-controlled processes.

MATERIALS AND METHODS

Electric eels were obtained from World Wide Scientific Animals, Ardsley, N.Y. Carbamylcholine chloride and acetylthiocholine bromide were obtained from Sigma Chemical Co. Cesium chloride (99%) was the product of Alfa-Ventron, Beverly, Mass. 5,5′-Dithio-bis(2-nitrobenzoic acid) (A grade) was obtained from Calbiochem. All other chemicals were reagent grade and were obtained either from the Fisher Scientific Co. or the Mallinckrodt Chemical Co.

The concentration of acetylcholine receptor sites was determined with ^{125}I-labeled α-bungarotoxin (α-BGT) (19) ac-

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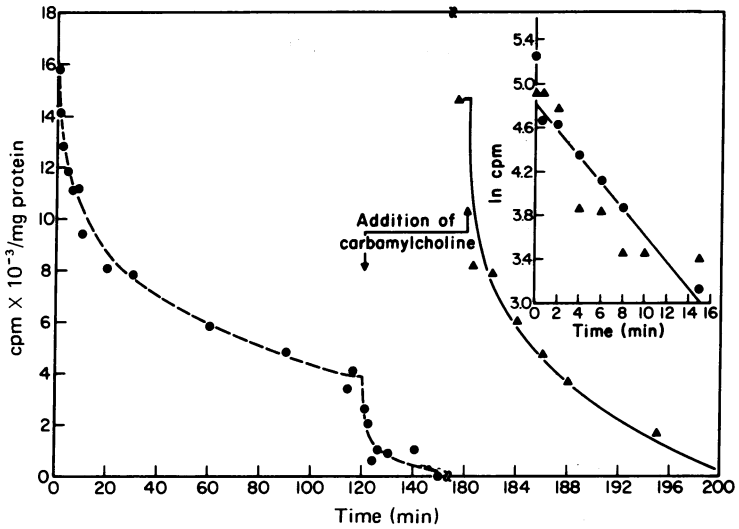

FIG. 1. $^{22}Na^+$ efflux from electroplax membrane microsacs at pH 7.0 and 4°. The microsacs were equilibrated with 1.1 M sucrose, 190 M NaCl, 0.27 μM $^{22}NaCl$ (stock solution 6.24 Ci/mg of $^{22}Na^+$) and diluted 50-fold with 1.1 M sucrose, 190 M CsCl, and 1 mM sodium phosphate buffer at pH 7.0. At the times indicated the solutions were made 1 mM in carbamylcholine. (● - - ●) Mixture of heterogeneous microsacs. (▲ — ▲) Functional microsacs isolated from the mixture. *Inset* is a first-order plot for $^{22}Na^+$ efflux in the presence of carbamylcholine. (▲) 1 mM carbamylcholine-induced efflux determined with the mixture of microsacs using a published kinetic technique for measuring only the specific efflux (17). (●) 1 mM carbamylcholine-induced flux from functional microsacs. The k_{obs} value was determined from the slope of the linear least squares fit of the experimental points obtained in both experiments; $k_{obs} = 0.12 \pm 0.01$ min^{-1}.

cording to the procedure of Kohanski *et al.* (20). Protein concentrations and activities of acetylcholinesterase and Na^+-K^+ ATPase were determined by the methods of Lowry *et al.* (21), Ellman *et al.* (22), and Bonting *et al.* (23), respectively. Sucrose concentrations were measured on a Bausch and Lomb refractometer. The $^{22}Na^+$ content of the vesicles was measured by Millipore filter assay (10). The method for evaluating the efflux rate constant, k_{obs}, has been described (17). The electroplax membrane vesicles were prepared essentially as described by Kasai and Changeux (10), with one important difference. In order to obtain a narrower distribution of density, the vesicles were banded in a discontinuous sucrose density gradient between layers 0.9 and 1.2 M in sucrose. The vesicles were then equilibrated with 1.1 M sucrose, 190 mM NaCl, 0.27 μM $^{22}NaCl$ (6.24 Ci/mg of $^{22}Na^+$ stock solution) for 18 hr at 4°. They were then passed over a Sephadex G-25 (coarse) column (17 × 250 mm) equilibrated with 1.1 M sucrose, 190 mM CsCl, 1 mM sodium phosphate buffer, pH 7.0, and allowed to stand for 40 min. Four milliliters of the vesicle solution, 0.75 mg of membrane protein per ml, were placed on the bottom of a 32-ml continuous gradient. The gradients, 0.7–1.1 M sucrose containing 190 mM CsCl and 1 mM sodium phosphate buffer, pH 7.0, were made with a Buchler linear gradient maker. After centrifugation at 25,000 rpm in a Beckman SW-27 swinging bucket rotor for 2.5 hr, 2 ml fractions were taken. The isolation procedure was carried out at 4°.

RESULTS

Electron micrographs of the membrane preparation of Cartaud *et al.* (24) indicated the presence of microsacs with an average diameter of 1000 Å. Assuming that the membranes are approximately 100 Å thick, one can calculate that the density of the microsacs filled with 190 mM NaCl will differ from that of microsacs filled with 190 mM CsCl sufficiently to be separated from each other on the basis of density.

Kinetic analysis of the efflux of inorganic ions from the crude microsac preparation indicated that we could fill microsacs that exhibit receptor-mediated ion flux (functional) with a NaCl solution while those microsacs that are not affected by acetylcholine or its analogs (nonfunctional) are filled with a CsCl solution. The data (solid circles) shown in Fig. 1 were obtained when a heterogeneous mixture of microsacs were equilibrated with $^{22}NaCl$ and then diluted with a medium containing CsCl instead of $^{22}NaCl$. The radioactivity associated with the microsacs was measured at various time intervals (Fig. 1, solid circles). The first part of the efflux curve represents isotopic exchange in absence of effectors. We have shown previously that the rate coefficients associated with this exchange, and the fraction of the total $^{22}Na^+$ that is exchanged in this manner, are independent of the addition of acetylcholine analogs. Addition of carbamylcholine to the mixture after the unspecific efflux has ceased induces a rapid efflux of $^{22}Na^+$. A pertinent analysis of the exchange between internal $^{22}NaCl$ and CsCl in the external medium shown in Fig. 1 is given in Table 1. The table lists the half-times, τ, of the equilibration processes. Carbamylcholine (1 mM), an acetylcholine analog, does not affect the half-equilibration time of the nonfunctional microsacs, but does reduce the half-equilibration time of the functional microsacs by a factor of 100. Results similar to those in the table have been obtained using different inorganic ions and concentrations, and the details of the analysis have been published (17, 18).

The following protocol for separating functional from nonfunctional microsacs is based on the data in the table. (*i*) A discontinuous sucrose density gradient centrifugation to collect membrane microsacs rich in receptor sites and of similar density. The percent of functional microsacs, 20–25%, is higher than in the original preparation of Kasai and Changeux (10). (*ii*) Equilibration of microsacs with 190 mM NaCl for 18 hr, long enough for all the microsacs to have reached equilibrium (see Table 1). (*iii*) Exchange of 190 mM NaCl for 190 mM CsCl using a Sephadex G-25 column and allowing equilibration of the microsacs for 40 min before centrifugation in a sucrose-CsCl gradient. According to the data in Table 1, 35% of the nonfunctional microsacs (τ = 3 min) have equilibrated completely with CsCl in this time period, 65% of the nonfunctional microsacs (τ = 40 min) have exchanged half their NaCl with denser CsCl, while the specific microsacs (τ = 330 min) still retain most of the NaCl originally present. (*iv*) Separation of

Table 1. $^{22}Na^+$ efflux from electroplax membrane microsacs at pH 7.0 and 4°

Vesicles	% Total radioactivity	Half-equilibration time (min)	Acetylcholinesterase (mmol of ATCH hydrolyzed·hr^{-1}·mg^{-1} of protein)	Na^+–K^+ ATPase (μmol of ATP hydrolyzed·hr^{-1}·mg^{-1} of protein)
Nonfunctional	26	3	1.5	9
	49	40		
Functional	25	330	0.04	<1
		3*		

The values given pertain to the data shown in Fig. 1 (dashed line). The procedure for analyzing the efflux data has been published (17). ATCH, acetylthiocholine.
* In the presence of 1 mM carbamylcholine.

the NaCl-filled functional microsacs from nonfunctional microsacs filled with denser CsCl by centrifugation in a continuous sucrose-190 mM CsCl density gradient.

The combined results of two such density gradient experiments are shown in Fig. 2. Most of the membrane protein (solid circles) is found in the denser portion of the gradient. The squares indicate the percent of sodium inside the microsacs whose flux rate is affected by carbamylcholine. This value is given by the following:

$$\frac{100 \times \left[\dfrac{\text{[inorganic ion] specific efflux}}{\text{mg protein in functional microsacs}}\right]}{\left[\dfrac{\text{[inorganic ion] specific and unspecific efflux}}{\text{mg protein in unfractionated microsac preparation}}\right]} \quad [1]$$

It can be seen from the data (solid squares) that the microsacs in the fractions that are 0.8 M in sucrose and 190 mM in CsCl comprise approximately 5% of the total membrane protein and that, by the criterion applied, this fraction contains functional microsacs only. The technique has been used routinely for 7 months and is reproducible. The purification achieved is comparable to that obtained in purification of receptor from *Torpedo marmorata* with cobra toxin affinity columns (25). An interesting result is that the receptor sites (α-bungarotoxin-binding sites) per mg of membrane protein (solid triangles) are rather uniformly distributed in the membrane preparation. There are significant differences between the functional and nonfunctional vesicles in levels of acetylcholinesterase and Na^+–K^+ ATPase. The values are listed in Table 1. In Fig. 2 the dashed line with open squares represents a control experiment in which the mixture of microsacs was equilibrated with $^{137}CsCl$ only. Although our separation procedure worked,

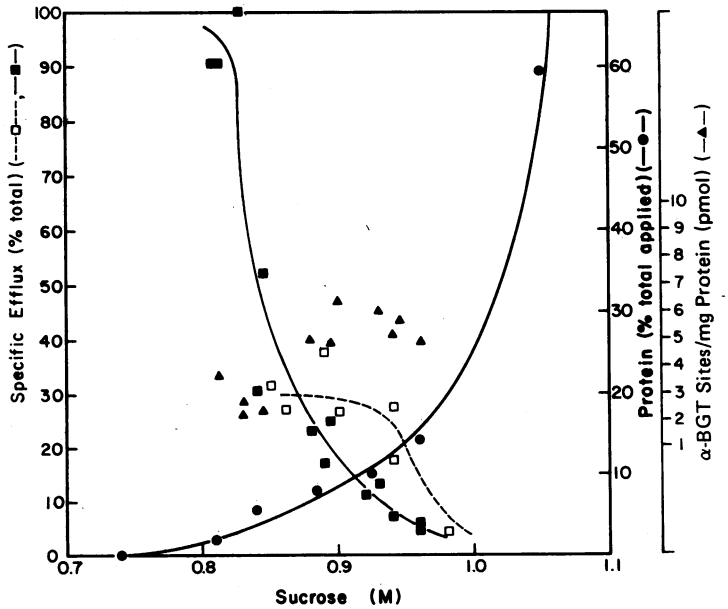

FIG. 2. Purification of functional acetylcholine receptor-rich microsacs by sucrose-190 mM cesium chloride density gradient centrifugation. Four milliliters of acetylcholine receptor-rich microsacs (0.75 mg of membrane protein per ml, 1.1 M sucrose, 190 mM CsCl, 1 mM sodium phosphate buffer at pH 7.0) were placed on the bottom of a continuous gradient (32 ml, 0.7–1.1 M sucrose containing 190 mM CsCl, 1 mM sodium phosphate buffer, at pH 7.0) and centrifuged in a Beckman SW-27 swinging bucket rotor for 2.5 hr at 4°. Two-milliliter fractions were collected and analyzed. The abscissa gives the molarity of sucrose in the fractions. The results of two experiments obtained with microsac preparations from different eels are shown. The solid lines and closed symbols represent experiments in which the functional microsacs were mainly filled with 190 mM NaCl and the nonfunctional vesicles with 190 mM CsCl. The broken line, open symbols, was a control experiment in which all microsacs were equilibrated with 190 mM CsCl. (●) % of membrane protein applied to column; (▲) pmol of α-bungarotoxin (α-BGT)-binding sites per mg of membrane protein; (■ — ■, □ - - - □) specific efflux (% total), see text (Eq. 1) for calculation.

we wanted to demonstrate that it did for the reasons we suggested. In the control experiment the microsacs were first collected on a discontinuous sucrose gradient and then equilibrated with 190 mM CsCl before they were placed on the continuous sucrose-CsCl gradient. The open squares indicate the percent of $^{137}Cs^+$ inside the microsacs whose flux rate is affected by carbamylcholine (Eq. 1). It can be seen that the functional microsacs moved as a broad band from the bottom of the gradient (1.1 M sucrose, 190 mM CsCl) into a region that was 0.84–0.92 M in sucrose. The functional CsCl-filled microsacs were therefore located in a denser region of the gradient than the functional NaCl-filled microsacs, and only a slight purification was achieved. This purification is not unexpected because the microsacs were first collected on a relatively broad discontinuous gradient.

In Fig. 1 the $^{22}Na^+$ efflux from the unfractionated microsac preparation (solid circles, dashed line) is compared with that from purified functional microsacs (solid triangles, solid line) obtained from the same membrane preparation. In this experiment the carbamylcholine-induced $^{22}Na^+$ efflux was initiated 3 hr after the equilibration with $^{22}Na^+$ was complete because the purification requires this amount of time. In this time period the efflux of $^{22}Na^+$ is negligible in the absence of carbamylcholine in the experiment with the purified functional microsacs. From the graph shown in the inset of Fig. 1, k_{obs} for $^{22}Na^+$ efflux can be calculated. This value, 0.12 ± 0.01 min^{-1}, obtained from the slope of the graph after addition of 10^{-3} M carbamylcholine, is the same for the functional vesicles in the mixture and after separation.

DISCUSSION

Correlation between receptor-mediated ion flux through microsac membranes and receptor-ligand induced changes in the electrical potential of the membranes of nerve or muscle cells requires a determination of the number of inorganic ions that pass through receptor-mediated channels per unit time (26–29). This value, E, will be referred to as the efficiency of the receptor-mediated ion channel. For purposes of discussion and insight into the process, we use the following equation to calculate E:

$$E = k_{max}\overline{N} \quad [2]$$

where $\overline{N}$ is the number of inorganic ions that pass through the membrane in the receptor-mediated process divided by the number of acetylcholine receptor sites per mg of membrane protein. We chose the parameter $\overline{N}$ because it reflects internal volume and the concentration of both inorganic ions and receptor sites. The relationship between k_{max} and k_{obs}, the observed rate coefficient for the efflux of inorganic ions from microsacs, and the concentration of receptor ligands has been given (17):

$$k_{obs} = k_{max}\frac{L}{L + K_D} \quad [3]$$

The purification procedure used yields, by the criterion applied, a preparation of microsacs that are essentially all functional. From the data in Fig. 1 (18×10^3 cpm from functional vesicles per mg of membrane protein) and Fig. 2 (2 pmol of α-bungarotoxin sites per mg of membrane protein) one can calculate an $\overline{N}$ value of 10^5. In comparing the efficiency of the receptor-mediated process in microsacs with that of whole cells (26–29) there are two important points that have not been considered previously: (*i*) Since the parameter $\overline{N}$ reflects internal volume, the concentration of inorganic ions, and the concentration of receptor sites, its value may be quite different for microsacs than for nerve or muscle cells. Indeed, from the data of Catterall (29) one can calculate $\overline{N}$ to be about 35 times greater in muscle cells than the highest value we obtained in microsacs. (*ii*) As long as the concentration of sodium ions that moves through receptor-controlled channels per unit time does not exceed the capacity of the channels, E is expected to be directly proportional to $\overline{N}$, and k_{max} is expected to be independent of $\overline{N}$. Therefore, differences in E values between microsacs and whole cells may simply reflect different $\overline{N}$ values rather than differences in the molecular mechanism of receptor-mediated processes.

There is good evidence that underutilization of receptor-formed channels in microsacs is the major reason for differences in E values between microsacs and muscle cells. The evidence for this is that the k_{max} value obtained with microsacs at low NaCl concentrations is sufficient to account for the data with muscle cells, which have an $\overline{N}$ value 10^3 times greater. Previous experiments with microsacs (17) indicated that k_{max} is constant when the NaCl concentration is varied 40-fold, from 5 mM to 200 mM. From the data presented here, this corresponds to a variation in $\overline{N}$ from 2.5×10^3 to 10^5. In order to see whether the k_{max} value obtained with microsacs with an $\overline{N}$ value of 2.5×10^3 also accounts for the data with muscle cells (29) with an $\overline{N}$ value of 3.5×10^6, we determined k_{max} with microsacs using the same ion concentration used in the external solutions in the muscle cell experiments (29), but with addition of 0.4 M sucrose. We obtained a k_{max} value of 0.3 min^{-1}. Using this k_{max} value we calculate E values for microsacs of 3×10^4 and for muscle cells of 1×10^6 inorganic ions per min per receptor site. This value is well within the range of values found experimentally with muscle cells, E (average) $= 3 \times 10^6$ (29). These data suggest that receptor-mediated processes have similar k_{max} values in microsacs and in muscle cells, and that k_{max} values are independent of $\overline{N}$ when these values vary by three orders of magnitude. The difference in E values between microsacs and muscle cells is, therefore, accounted for by differences in $\overline{N}$ values. There is no reason to assume that the $\overline{N}$ value for muscle cells represents a maximum value for receptor-formed channels, either on the basis of the diffusion coefficient of Na^+ or on the basis of experimentally determined E values close to 10^9 (27, 30).

Although a malfunction of receptor-formed channels in membrane microsacs has been considered (26–29), the proportionality between E and $\overline{N}$ (Eq. 2) has not been appreciated. Small microsacs specifically selected for high receptor content in the initial isolation procedure (10) are expected to have lower $\overline{N}$ values than much larger cells, leading to underutilization of receptor-formed channels and low values of E.

An additional factor that may have to be considered is that the composition of the solution on either side of the microsac membrane has a definite effect on the receptor-mediated flux of inorganic ions, and that k_{max} values higher than those we have reported may be observed.

Another important aspect of the isolation procedure is that it allows one to characterize the functional microsacs and to compare their composition to that of the nonfunctional microsacs. The observation of similar concentrations of α-bungarotoxin-binding sites in the two types of microsacs, but of significant differences in the levels of Na^+–K^+ ATPase and acetylcholinesterase, suggests that essential membrane components not measured by α-bungarotoxin may exist, which can be identified.

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1. Fatt, P. & Katz, B. (1951) *J. Physiol. (London)* **115,** 320–370.
2. Nachmansohn, D. (1955) in *Harvey Lectures 1953–1954* (Academic Press, New York), pp. 57–99.
3. Nachmansohn, D. (1974) in *Biochemistry of Sensory Functions,* ed. Jaenicke, L. (Springer-Verlag, Berlin), pp. 431–464.
4. Nachmansohn, D. & Neumann, E. (1975) in *Chemical and Molecular Basis of Nerve Activity* (Academic Press, New York).
5. Eldefrawi, M. E., Eldefrawi, A. T. & Shamoo, A. E. (1975) *Ann. N.Y. Acad. Sci.* **264,** 183–302.
6. Changeux, J.-P., Bendetti, L., Bourgeois, J. P., Bresson, A., Cartaud, J., Devaux, P., Grünhagen, H., Moreau, M., Popot, J.-L., Sobel, A. & Weber, M. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 211–230.
7. Karlin, A., Weill, C. L., McNamee, M. G. & Valderrama, R. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 203–210.
8. Maelicke, A. & Reich, E. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 231–235.
9. Raftery, M. A., Vandlen, R. L., Reed, K. L. & Lee, T. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 193–202.
10. Kasai, M. & Changeux, J.-P. (1971) *J. Membr. Biol.* **6,** 1–23.
11. Donner, D. B., Fu, J.-j. L. & Hess, G. P. (1976) *Anal. Biochem.* **75,** 454–463.
12. Bulger, J. E. & Hess, G. P. (1973) *Biochem. Biophys. Res. Commun.* **54,** 677–684.
13. Fu, J.-j. L., Donner, D. B. & Hess, G. P. (1974) *Biochem. Biophys. Res. Commun.* **60,** 1072–1080.
14. Hess, G. P., Bulger, J. E., Fu, J.-j. L., Hindy, E. F. & Silberstein, R. J. (1975) *Biochem. Biophys. Res. Commun.* **64,** 1018–1027.
15. Rübsamen, H., Hess, G. P., Eldefrawi, A. T. & Eldefrawi, M. E. (1976) *Biochem. Biophys. Res. Commun.* **68,** 56–63.
16. Rübsamen, H., Montgomery, M., Hess, G. P., Eldefrawi, A. T. & Eldefrawi, M. E. (1976) *Biochem. Biophys. Res. Commun.* **70,** 1020–1027.
17. Hess, G. P., Andrews, J. P., Struve, G. E. & Coombs, S. E. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4371–4375.
18. Hess, G. P., Andrews, J. P. & Struve, G. E. (1976) *Biochem. Biophys. Res. Commun.* **69,** 830–837.
19. Lee, C. Y. (1972) *Annu. Rev. Pharmacol.* **12,** 265–286.
20. Kohanski, R. A., Andrews, J. P., Eldefrawi, M. E. & Hess, G. P. *Anal. Biochem.*, in press.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
22. Ellman, G. L., Courtney, K. D., Andrews, V., Jr. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95.
23. Bonting, S., Simon, K. A. & Hawkins, N. M. (1961) *Arch. Biochem. Biophys.* **95,** 416–423.
24. Cartaud, J., Benedetti, E. L., Kasai, M. & Changeux, J.-P. (1971) *J. Membr. Biol.* **6,** 81–88.
25. Eldefrawi, M. E. & Eldefrawi, A. T. (1973) *Arch. Biochem. Biophys.* **159,** 362–373.
26. Kasai, M. & Changeux, J.-P. (1971) *J. Membr. Biol.* **6,** 58–80.
27. Katz, B. & Miledi, R. (1972) *J. Physiol. (London)* **224,** 665–699.
28. Rang, H. P. (1974) *Q. Rev. Biophys.* **7,** 283–399.
29. Catterall, W. A. (1975) *J. Biol. Chem.* **250,** 1776–1781.
30. Anderson, C. R. & Stevens, C. F. (1973) *J. Physiol. (London)* **235,** 655–691.