Acetylcholine receptors were solubilized from electric organ membranes of *Torpedo californica* in mixed micelles of sodium cholate and soybean lipids. Sodium cholate, when supplemented with relatively low amounts of soybean lipids (cholate:lipid, 20:1, molar ratio), was effective in solubilizing receptors without denaturing their agonist-regulated cation channels. Another dialyzable detergent, octylglucoside, denatured the ion channel even in the presence of excess lipids.

Reassembling receptors and lipids into vesicles was achieved by cholate dialysis. About 70% of the receptors were oriented with their toxin binding sites on the external surface of the vesicles. Evidence suggests that all of the receptors in a single vesicle were oriented either right side out or inside out.

During the reassembly process about 10-fold greater lipid concentrations were required for the preservation of channel function. At lipid/protein ratios greater than 16:1 (w/w), receptors reassembled into vesicles at a constant protein/lipid ratio. These vesicles contained approximately 7% receptors by weight, 5-fold less than the native membrane. The remainder of the lipid assembled into small vesicles which did not contain receptors. At lipid/protein ratios less than 16:1 (w/w), receptors reassembled into vesicles at a constant protein/lipid ratio. These vesicles contained approximately 7% receptors by weight, 5-fold less than the native membrane. The remainder of the lipid assembled into small vesicles which did not contain receptors.

The unexpected constant acetylcholine receptor/lipid ratio in the reconstituted vesicles and the unexpected uniform orientation of the acetylcholine receptors within a vesicle are discussed in terms of the interactions occurring during the initial nucleation events of the reassembly process.

The acetylcholine receptor from *Torpedo californica* is a pentameric complex of partially homologous subunits in the mole ratio $\alpha_2\beta_2\delta$ (1-3) which in the native membrane exists as a dimer formed by a disulfide bond between the subunits (4, 5). The $\alpha$ subunits can be affinity labeled by probes for the acetylcholine binding sites (6, 7), and the agonist-regulated cation channel is contained within the $\alpha_2\beta_2\delta$ monomer (8). However, it is still not known which of the subunits contribute to the structure of the cation channel or how opening and closing of this channel is regulated.

The first reproducible reconstitution of AChRs into lipid vesicles was reported by Epstein and Racker (9), who used crude AChR-containing membrane preparations and soybean lipids. This work led to the realization that the continuous presence of lipids during solubilization of the AChR in cholate is essential for preserving the functional integrity of the channel (Refs. 10-12, except see Ref. 13). This notion permitted purification of AChRs with intact cation channels and the reconstitution of these AChRs into lipid vesicles (10, 11) and planar lipid bilayers (14). Reconstitution of functional AChRs into vesicles (13, 15, 16) or planar bilayers (17) has also been reported by several other laboratories. The role of specific lipid subclasses for the performance of the agonist-induced cation translocation has been investigated, but no absolute requirement for a specific lipid has been demonstrated (18).

In this paper we determine the conditions required for the stabilization of AChR channels in cholate-lipid solution and during the process of reincorporation into a reconstituted membrane. Surprisingly, more lipid is required to protect the AChR channel during reconstitution than in cholate solution. Another unexpected observation is that AChRs and lipids reassemble into vesicles at a fixed protein/lipid ratio which is much higher than the protein/lipid ratio in cholate-lipid solution. Addition of a large excess of lipid does not result in a reduced amount of AChRs/vesicle. These results provide insights in the mechanisms by which AChRs and lipids interact during the formation of reconstituted vesicles.

**MATERIALS AND METHODS**

Sodium cholate was purchased from Interchon (Montluçon, France), octylglucoside from Calbiochem, and crude soybean lipids (L-a-phosphatidylcholine) from Sigma. Special enzyme grade sucrose for gradient centrifugation was obtained from Schwarz/Mann and $d[1-14]C$palmitoyl-L-a-phosphatidylcholine from Amersham Radiochemical Centre. a-Bungarotoxin was purified (19) from the crude venom of *Bungarus multicinctus* obtained from Miami Serpentarium, and iodinated (20). Monoclonal antibodies against AChR (21) were generously provided by Dr. S. J. Yarows. (The Salk Institute, San Diego, CA).

**Preparation of AChR-rich Membranes—AChR-rich membranes from the electric organ of *T. californica* (Pacific Biomarine, Venice, CA) were prepared by sucrose density centrifugation, following dif-

---

*This investigation was supported by grants from the Office of Naval Research (N0014-79-C0379 to J. L. and M. M.), the National Institutes of Health (NS 11323 to J. L., EY 02064 to M. M.), the Muscular Dystrophy Association (to J. L.), and the Los Angeles Chapter of the Myasthenia Gravis Foundation (to J. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests should be addressed at The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92158.
ferential centrifugation of the initial homogenate as previously described (11). ACHe concentration was determined by radiomunnoassay (11). Protein was determined according to the method of Lowry et al. (22) using bovine serum albumin as standard.

Solubilization of ACHe-rich Membranes—ACHe-rich membranes were dissolved in 2% sodium cholate, unless otherwise indicated, in the presence of the desired concentration of crude soybean lipids in 10 mM Na phosphate buffer, pH 7.4, 100 mM NaCl. Crude soybean lipids were prepared as a 150 mg/ml stock dispersion in distilled water by sonication under argon in a Bransonic bath type sonicator. The solubilized membranes were gently shaken for the indicated periods of time at 4 °C. Undissolved material was removed either by centrifugation at 165,000 × g for 30 min or, for small volumes, by centrifugation for 15 min in an Eppendorf microfuge at 4 °C. Both procedures gave comparable ACHe concentrations in the supernatants.

Reconstitution of ACHe into Lipid Vesicles—Lipid concentrations in the extract were adjusted using a sonicated dispersion of soybean lipids (150 mg/ml) in distilled water. Di[1-14C]palmitoyl-α-phosphatidylcholine was sometimes included as tracer by sonication (10-15 min) of the lipid stock dispersion added to a dry film of the radioactive lipid after evaporation of the solvent under argon. Final concentrations of the other components in the reconstitution mixture were 100 mM NaCl, 2% sodium cholate, and 10 mM Na phosphate buffer, pH 7.4. The reconstitution mixture was dialyzed for 18-19 h against 500 volumes of 100 mM NaCl, 10 mM NaN3, 10 mM Na phosphate buffer, pH 7.4, and then for the same period against 500 volumes of 145 mM sucrose, 10 mM NaN3, 10 mM Na phosphate buffer, pH 7.4.

Measurement of Carbamylcholine-induced Uptake of 22Na+—Influx of 22Na+ into vesicles was assayed at room temperature using the basic method of Gasco et al. (23) as previously described (11). All assays were done in triplicate. At time zero, 40 μl of reconstituted vesicles were added to 5 μl of 22Na+ (200 μCi/ml of stock solution, New England Nuclear) plus 5 μl of distilled water (background control) or 5 μl of carbamylcholine (10-5 M). After 10 s, 40 μl of mixture were applied to 2-ml columns of Dowex 50W-8X (purchased from Sigma, converted to the Tris form as described in (11), and washed with 3 ml of 170 mM sucrose, 3 mg/ml of bovine serum albumin immediately before use). The resin was immediately rinsed carefully with 3 ml of 175 mM sucrose, and the eluate was counted in a Nuclear Chicago model 1185 γ-counter with background correction at 38% efficiency.

The apparent equilibrium volume of the vesicles was measured in cpm/mg of lipid after incubation of a regular background control sample with 22Na+ for 48 h at 4 °C prior to passage through Dowex. Isopycnic Sucrose Gradient Centrifugation of Reconstituted Vesicles—Reconstituted vesicles were diluted with an equal volume of 100 mM NaCl, 10 mM NaN3, 10 mM Na phosphate buffer, pH 7.4, containing a trace label of 125I-aBGT (3.0 × 10-9 M) and incubated for 1-2 h at 4 °C. Samples of 0.1 ml were applied to 4.8 ml of 5-25% (w/w) linear sucrose gradients and centrifuged to equilibrium in a Beckman SW 50.1 rotor at 200,000 × g for 18 h. Fractions of 0.23 ml were collected after puncturing the tubes and counted in a γ-counter. The sucrose concentration of each fraction was determined at 20 °C with a refractometer.

RESULTS

Stability of ACHe Channels during Solubilization of the Native Membrane—It has previously been shown that the presence of supplementary soybean lipids in mixed micelles with cholate at a 1:10 (w/w) ratio can protect ACHe channels from denaturation (10, 11). These observations have recently been confirmed by other investigators (12). Under these conditions extraction of native membranes is maximal at 2% cholate and amounts to a recovery of about 68% of the initial ACHeRs in the extract (Fig. 1A). Half-maximal extraction is obtained at a concentration of about 0.8% cholate. Increasing the cholate concentration above 2% does not increase the extraction yield further, although variable amounts of additional 125I-aBGT binding activity can be obtained from the undissolved residue by treatment with 2% Triton X-100 (data not shown). When detergent concentrations above 3% cholate are used for solubilization of the membranes, a strong irreversible denaturing effect of the detergent on the channel becomes evident, even though the same 10:1 (w/w) cholate/lipid ratio is maintained (Fig. 1B).

Inclusion of low concentrations of supplementary lipids in the micellar solution does not significantly affect the extraction yield, which is constant up to protein concentrations of 6-8 mg/ml (Fig. 2). In this concentration range endogenous components of the membranes can, in the absence of supple-
Acetylcholine Receptor Reconstitution

4379

more effective than Torpedo lipids in protecting the ion channel from denaturation in cholate solution.

Replacing cholate with the structurally unrelated dialyzable detergent octylglucoside results in the irreversible denaturation of the channel, even when supplementary lipids are present in excess (Table I). [3H]-BGT binding activity is not affected by octylglucoside (data not shown). The peculiar micellar structure of cholate (reviewed in Ref. 24), as well as the presence of lipid, may therefore be important for the protection of the channel. However, we have not systematically investigated the protective effect of lipid in the presence of other detergents.

Association of AChRs with Soybean Lipid during the Formation of Reconstituted Membranes—We estimated the association of lipids with the AChR during the reconstitution process by inclusion of [3H]dipalmitoylphosphatidylethanolamine as tracer in the reconstitution mixture before dialysis and

| Solubilizing detergent | 10⁻⁴ M carbachol-induced uptake of [³¹⁴]Na⁺ | cpm/mol AChR × 10⁻¹³ ± S.E. |
|------------------------|------------------------------------------|-------------------------------|
| 2% cholate             | 5.28 ± 0.67                              |                              |
| 1% cholate/1% octyglucoside | 2.05 ± 0.54                            |                              |
| 1.5% octyglucoside     | 0                                        |                              |

Association of [³¹⁴]dipalmitoylphosphatidylethanolamine-supplemented soybean lipids with AChRs after reconstitution at different protein concentrations. AChR-rich membranes were suspended in 100 mM NaCl, 2% sodium cholate, 5 mg/ml of soybean lipid, 10 mM Na phosphate buffer, pH 7.4, and incubated for 1 h at 4 °C under gentle shaking. After centrifugation for 15 min in an Eppendorf microfuge, the extract was diluted to the desired protein concentration in 100 mM NaCl, 2% sodium cholate, 5 mg/ml of soybean lipid, 10 mM Na phosphate buffer, pH 7.4. The lipid concentration was in all samples adjusted to 25 mg/ml using a sonicated lipid dispersion containing [³¹⁴]dipalmitoylphosphatidylethanolamine. The samples were reconstituted by cholate dialysis, and AChR-associated lipid was measured by immune precipitation. The fraction of lipid in the pellet was calculated and corrected for nonspecific immune precipitation of liposomes without AChR, and lipid precipitated when the assay was conducted in the presence of 0.5% Triton X-100.
immune precipitation of the reconstituted AChRs. The results of these experiments are presented in Fig. 4 and 5. Precipitation of AChRs was complete in all samples, as measured by a > 85% precipitation of a \(^{125}\)I-a-BGT trace label. Immune precipitation of the reconstituted membranes does not, however, result in coprecipitation of all the lipid with the AChRs (Fig. 4). Previously we observed in freeze-fracture electron micrographs that reconstituted preparations contained a mixture of large (~ 0.4 \(\mu\)m) vesicles containing intramembranous particles, i.e. AChRs, and many small (mostly 0.02-0.08 \(\mu\)m) vesicles lacking such particles (11). It is evident from all these results that in the presence of excess lipid, AChRs assemble in the reconstituted membranes at a constant protein/lipid ratio, forming a vesicle population distinct from a population of non-AChR-containing liposomes. The apparent association of lipid with the AChR, according to Fig. 4, amounts to 4.3 ± 1.3 mg of lipid/mg of protein. This value, however, is most likely an underestimate, because the trapping of non-AChR-containing \(^{14}\)C-labeled vesicles in immune precipitates of AChR-containing vesicles is probably smaller than the trapping of these vesicles in immune precipitates in the absence of AChR used as background control in these experiments.

A quantitatively more reliable estimate of the composition of the AChR-containing reconstituted membranes was obtained by isopycnic sucrose gradient centrifugation. \(^{125}\)I-a-BGT trace-labeled vesicles, when centrifuged to equilibrium on a sucrose gradient, formed symmetrical peaks indicating an apparently homogeneous population of vesicles in terms of density. No AChR was pelleted in these tubes, indicating that aggregation of AChRs without lipid association was not detectable (Fig. 6). The apparent weight fraction of AChRs in the reconstituted membranes was calculated from the density of the peaks (Fig. 7). The results of these experiments are consistent with those involving immune precipitations of radiolabeled lipids in that they demonstrate an AChR-lipid association at a constant protein/lipid ratio, which at lipid concentrations > 15 mg/ml is independent of the lipid concentration. Under these conditions AChRs assemble with soybean lipids into vesicles containing an apparent weight fraction of 7% AChRs. This is a 5-fold lower packing density than in the native membrane, which contains approximately 35% AChR protein by weight (Fig. 7). When AChRs are reconstituted at 1.25 mg/ml of protein in the presence of < 15 mg/ml of soybean lipid, reconstituted membranes of greater isopycnic density are formed containing a higher apparent

![Fig. 5. Association of \(^{14}\)C-dipalmitoylphosphatidylcholine-supplemented soybean lipids with AChRs after reconstitution at different lipid concentrations. AChR-rich membranes were suspended in 100 mM NaCl, 2% sodium cholate, 5 mg/ml of soybean lipid, 10 mM Na phosphate buffer, pH 7.4, and incubated for 1 h at 4 °C under gentle shaking. After centrifugation for 15 min in an Eppendorf microfuge, aliquots of the extract were reconstituted at 1.25 mg/ml of protein in the presence of different concentrations of soybean lipid containing \(^{14}\)C-dipalmitoylphosphatidylcholine tracer. The samples were reconstituted by cholate dialysis, and AChR-associated lipid was measured as described in the legend to Fig. 4.](image-url)

![Fig. 6. Isopycnic sucrose gradient centrifugation of AChR-containing vesicles reconstituted at different soybean lipid concentrations. Reconstituted vesicles, containing 1.25 mg/ml of protein and formed at different concentrations of soybean lipid, were analyzed by isopycnic sucrose gradient centrifugation as described under "Materials and Methods." A, vesicles formed at 5 mg/ml of soybean lipid; B, vesicles formed at 10 mg/ml of soybean lipid; C, vesicles formed at 20 mg/ml of soybean lipid; and D, vesicles formed at 30 mg/ml of soybean lipid. The arrow indicates the region where pure lipid vesicles band.](image-url)
weight percentage of AChRs (Fig. 6 and 7). Centrifugation of vesicles, formed at 25 mg/ml of soybean lipid in the presence of \[^{14}C\]dipalmitylophosphatidylcholine, yields a measurement of about 9% for the apparent weight percentage of AChRs in the reconstituted vesicles (data not shown). This is in fair agreement with the value calculated from the isopycnic density (7%).

We investigated whether AChRs during the reassembly process were incorporated in the reconstituted membranes in such a way that in the same vesicle AChRs are oriented with their toxin-binding sites facing either all outward or all inward, or whether outward- and inward-facing AChRs are both present in the same vesicles. Table II shows that a monoclonal antibody, which binds with high affinity to the main immunogenic region of the Torpedo AChR on the external surface of the membrane (21), precipitated only 69 ± 8% of the total AChRs in intact reconstituted vesicles. This percentage corresponded closely with the fraction of AChRs in the preparation which was oriented toward the external surface as measured by its ability to bind \[^{3}H\]α-BGT (Table II, Ref. 11). These data suggest therefore that all AChRs which are assembled in the same vesicle during reconstitution are incorporated in the same orientation.

**Stability of AChR Channels during Incorporation into Reconstituted Membranes**—Fig. 8A demonstrates the dependence of AChR function in the reconstituted vesicles on the lipid concentration at which reconstitution is performed.

If during reconstitution at 1.25 mg of AChR protein/ml the lipid concentration is decreased below 20 mg/ml, a decrease in carbamylcholine-induced \[^{22}Na\]^+ flux/AChR results. This effect is observed in the same range of lipid concentrations at which the packing density of AChRs in the reconstituted vesicles increases (Fig. 6 and 7). An optimal condition for the

---

**Fig. 7.** Apparent composition of vesicles reconstituted at different protein/lipid ratios derived from their observed buoyant densities. AChR-containing vesicles, reconstituted at 1.25 mg/ml of protein and at different soybean lipid concentrations, were subjected to isopycnic sucrose gradient centrifugation. The apparent weight fraction of AChRs in the reconstituted vesicles was calculated from the observed densities in the peaks (Fig. 6), using the partial specific volume for AChR according to Reynolds and Karlin (1) and the observed isopycnic density for liposomes without AChR formed by cholate dialysis at 25 mg/ml of lipid in the presence of \[^{14}C\]dipalmitylophosphatidylcholine. The isopycnic density of native AChR-rich membranes was measured during the isolation of these membranes on a 32–38% (w/w) linear sucrose gradient. Values for reconstituted vesicles containing ≤ 2 mg/ml of soybean lipid were obtained by isopycnic centrifugation on a 5–35% (w/w) linear sucrose gradient in a Beckman SW 40 rotor at 255,000 × g for 18 h. AChRs were in each instance solubilized from the native membrane in a 10:1 weight ratio of cholate/lipid.

**Fig. 8.** Carbamylcholine-induced \[^{22}Na\]^+ uptake of reconstituted vesicles formed at different soybean lipid concentrations. Reconstituted vesicles were formed at different concentrations of soybean lipid, and uptake of \[^{22}Na\]^+ induced by \[^{10}M\] carbamylcholine was measured. A, all vesicles were formed at 1.25 mg/ml of protein; B, all vesicles were formed at a constant lipid/protein ratio = 20:1 (w/w); C, carbamylcholine-regulated uptake of \[^{45}Na\]^+; and O, equilibrium volume, measured after 48 h of incubation with \[^{22}Na\]^+. Data points are compiled from triplicate measurements.
Acetylcholine Receptor Reconstitution

retention of channel activity during reconstitution appears to be attained at a lipid/protein ratio of ~16 (w/w). Further addition of lipids during reconstitution has no effect, and results only in the formation of increased numbers of non-AChR-containing liposomes, as is evident from a continuous increase in total internal volume. The equilibrium volume/mg of lipid remains constant at high lipid concentrations, but increases over the range of lipid concentrations at which AChRs pack with greater density in the membranes. This most likely reflects an increase in overall internal volume due to the incorporation of a greater fraction of the lipid in larger AChR-containing vesicles rather than in the smaller liposomes (Fig. 8A).

When the lipid/protein ratio is maintained > 16 (w/w), full channel activity is retained during reconstitution at soybean lipid concentrations between 2.5-15 mg/ml, and the equilibrium volume/mg of lipid remains essentially constant (Fig. 8B). Under these conditions AChRs are inserted in the reconstituted membranes at a 7% apparent weight fraction even at lipid concentrations below 15 mg/ml (Fig. 9).

We considered the possibility that the decrease in carbamylcholine-dependent $^{22}\text{Na}^+$ influx/AChR at suboptimal lipid/protein ratios was due to equilibration of the vesicles with $^{22}\text{Na}^+$ during the response as a result of the greater packing density of AChRs rather than due to actual channel inactivation. In order to test this we titrated the carbamylcholine-dependent $^{22}\text{Na}^+$ flux response at a suboptimal lipid/protein ratio with increasing amounts of toxin. In native vesicles the packing density of AChRs is so great that equilibration limits the response, and more than 70% of the AChRs must be blocked by toxin before carbamylcholine-induced $^{22}\text{Na}^+$ uptake decreases in direct proportion with the addition of toxin (11, 25). Here we found, however, that carbamylcholine-induced $^{22}\text{Na}^+$ uptake decreased in direct proportion to blockage of toxin binding sites (Fig. 10), as is observed with reconstituted vesicles formed under optimal conditions (8, 11). This indicates that equilibration of the internal volume of the vesicles with $^{22}\text{Na}^+$ did not limit the response, but that the response was proportional to the amount of active AChR present. Extrapolation to the intercept on the abscissa in Fig. 10 yields a measurement for the fraction of the AChRs oriented with their toxin-binding sites on the external surface of the reconstituted vesicles. Both vesicles formed at 10 mg/ml of lipid, and at 25 mg/ml of lipid in the presence of 1.25 mg/ml of protein continue the same proportion of outward-oriented $[^{125}\text{I}]\alpha\text{-BGT}$ binding sites, namely 70 ± 4%. It is clear from these observations that the decrease in the carbamylcholine-regulated $^{22}\text{Na}^+$ flux response at suboptimal lipid/protein ratios is indeed due to inactivation of AChR channels during the reconstitution process rather than due to equilibration of the vesicles during the response or to an inverse orientation of AChRs caused by reconstitution at suboptimal lipid concentrations.

We considered the possibility that the inactivation of channels might be reversible. In order to test this we solubilized membranes formed at a suboptimal lipid/protein ratio, in 2% cholate in the presence of sufficient supplementary soybean...
lipids to allow reconstitution for a second time under optimal conditions. Table III shows that only the channel activity which survived the first reconstitution under suboptimal conditions can be recovered after a subsequent reconstitution under optimal conditions. The decrease in the carbamyl-

TABLE III
Irreversible inactivation of AChR channels by reconstitution at a suboptimal soybean lipid concentration

| Soybean lipid concentration during 1st reconstitution | 10⁻¹⁸ M carbamylcholine-induced uptake of ²²Na⁺ | 2nd reconstitution at 20 mg/ml soybean lipid |
|------------------------------------------------------|---------------------------------------------|---------------------------------------------|
| 5                                                    | 1.70 ± 0.21                                | 2.25 ± 0.50                                |
| 20                                                   | 5.27 ± 1.14                                | 5.65 ± 1.28                                |

Fig. 11. Isopycnic sucrose gradient centrifugation of vesicles reconstituted at a suboptimal soybean lipid concentration and containing irreversibly inactivated AChRs. Reconstituted vesicles containing 1.25 mg/ml of protein were formed at soybean lipid concentrations 5 mg/ml and 20 mg/ml, respectively. After assay for carbamylcholine-regulated ²²Na⁺ uptake (Table III), aliquots of each batch of vesicles were dissolved in 2% sodium cholate, 100 mM NaCl, 10 mM Na phosphate buffer, pH 7.4, supplemented with soybean lipids to give a final lipid concentration of 20 mg/ml in both aliquots. Both samples were then reconstituted by cholate dialysis for a second time by cholate dialysis, and the resulting vesicles were assayed for carbamylcholine-induced uptake of ²²Na⁺. Measured uptake of ²²Na⁺ at 10⁻¹⁸ M carbamylcholine was 7.3 ± 0.50 cpm/mol AChR for both samples after the first reconstitution (7.3 × 10⁻¹⁷ M) as well as after the second reconstitution (4.8 × 10⁻¹⁷ M).

Fig. 12. Sucrose gradient centrifugation of cholate-solubilized reconstituted vesicles. Reconstituted vesicles containing 1.25 mg/ml of protein were formed at a soybean lipid concentration of 5 mg/ml or 20 mg/ml. The reconstituted vesicles were labeled with a trace of [³⁵S]α-BGT (3.0 × 10⁻⁶ M) by overnight incubation at 4 °C, and dissolved in 100 mM NaCl, 2% sodium cholate, 5 mg/ml of soybean lipid in 10 mM Na phosphate buffer, pH 7.4. Aliquots of 50 μl were applied to 4.8 ml of 5–20% (w/w) linear sucrose gradients in 10 mM Na phosphate buffer, pH 7.4, 100 mM NaCl, 2% sodium cholate, 5 mg/ml of soybean lipids. Centrifugation was performed in a Beckman SW50.1 rotor at 300,000 × g for 5 h, fractions of 0.21 ml were collected after puncturing the tubes, and they were counted in a γ-counter. A, vesicles reconstituted at 5 mg/ml of soybean lipid; and B, vesicles reconstituted at 20 mg/ml of soybean lipid.

line-induced ²²Na⁺ flux response at suboptimal lipid/protein ratios is thus due to permanent deactivation of a fraction of the AChR channels during the reconstitution process. Isopycnic sucrose gradient centrifugation of vesicles formed first under suboptimal reconstitution conditions and subsequently under optimal conditions reveals an apparent packing density of AChRs intermediate between the isopycnic density expected under optimal conditions and that measured after the first reconstitution under suboptimal conditions (Fig. 11). We cannot, however, distinguish whether this peak represents a single population of vesicles or whether it results from the summation of equal amounts of two components, banding at 20% (w/w) and 16% (w/w) sucrose, respectively.

Denaturation of AChR channels might be accompanied by aggregation of the inactive AChRs, which could account for the relatively higher packing density of the AChRs during the second reconstitution. We tested whether aggregation of the reconstituted AChRs was detectable in cholate-lipid prior to a second reconstitution. First we dissolved the vesicles in 2% cholate and 5 mg/ml of soybean lipid, and then performed sucrose gradient centrifugation essentially as previously described (8), except that 5 mg/ml of lipid was present in the sucrose gradient. Both AChRs reconstituted under optimal as well as suboptimal conditions were predominantly present as dimers (Fig. 12, Ref. 8). Aggregates near the bottom of the gradients below the dimer peaks were not discernible. Therefore, we did not detect aggregates which persisted in cholate-lipid solution.

We also attempted to detect aggregated AChRs in the
reconstituted membranes by cross-linking with glutaraldehyde. Reconstituted vesicles were incubated for 5 min at 4 °C with 0.4% glutaraldehyde. The reaction was quenched with an equal volume of 1 mM glycine in 10 mM Na phosphate buffer, pH 7.4, 100 mM NaCl, 2% Triton X-100, 0.5 mM 2-mercaptoethanol, and the reaction products were analyzed by sucrose gradient centrifugation as previously described (8). Noncross-

![Graph showing efficiency of conversion of AChR dimers into monomers by 2-mercaptoethanol.](image)

**FIG. 13. Efficiency of conversion of AChR dimers into monomers by 2-mercaptoethanol.** Reconstituted vesicles containing 1.25 mg/ml of protein were formed at a soybean lipid concentration of 5 mg/ml (○) or 20 mg/ml (●). The reconstituted vesicles were labeled with a trace of \(^{35}S\)H-BGT (3.0 × 10⁻⁸ M) by overnight incubation at 4 °C and dissolved in 2% Triton X-100 in the presence of the indicated concentrations of 2-mercaptoethanol. Sucrose gradient centrifugation was then performed as previously described (8). Relative amounts of dimers and monomers were measured by integration of the peaks, and the percentage of conversion of AChR dimers into monomers was calculated. AChRs subjected to suboptimal conditions, as well as control AChRs, displayed the same relative amounts of dimers (80 ± 4%) and monomers (20 ± 4%) in the absence of reducing agents. Reduction with 250 mM 2-mercaptoethanol, supplemented with 50 mM dithiothreitol, resulted in 72 ± 4% conversion of dimers into monomers for AChRs which had been exposed to low lipid conditions during reconstitution, compared to 94 ± 5% dimer to monomer conversion for control AChRs.

**FIG. 14. Schematic representation of AChR-lipid interactions during the solubilization and reconstitution processes.**

**Fig. 14.** Schematic representation of AChR-lipid interactions during the solubilization and reconstitution processes.
Acetylcholine Receptor Reconstitution

Approximate molar compositions of AChR-lipid assemblies during the solubilization-reconstitution cycle

| AChR-lipid assembly            | Lipid/protein | Cholate/protein | Colate/protein | Approximate average number of lipid annulus/\(\text{AChR}\) |
|-------------------------------|---------------|-----------------|----------------|---------------------|
| Native membrane               | ~600          | 0               | ~6-8           | N.D.                |
| Micellar solution             | ~250          | ~20             | ~1-4           | ~4000 N.D.          |
| Reconstituted membrane        | ~4000 N.D.    | N.D.            | ~20            |                     |

*The calculated values were obtained using 250,000 as the molecular weight of the AChR monomer (1) and assuming an average \(M_r\) = 750 for soybean lipid.

Calculations were based on the assumption that the intramembraneous portion of the AChR molecule can be considered as a cylinder of 30 Å diameter (29) and using 69 Å² as estimate for the area occupied by a lipid molecule (36).

N.D., not determined.

linked AChRs were detected as monomers. About 45% of the AChRs which had been exposed to suboptimal reconstitution conditions were detected as a continuous smear in the gradient below the AChR dimer peak after glutaraldehyde treatment. In contrast, only about 18% of the control AChRs (reconstituted under optimal conditions) displayed this sedimentation behavior after cross-linking under identical conditions (data not shown). This difference in cross-linking efficiency could, however, be fully accounted for by the greater packing density of AChRs reconstituted under suboptimal conditions (13% apparent weight fraction as compared to 7%, Fig. 7). Therefore, although aggregates of denatured receptors could not be readily distinguished by centrifugation in cholate-lipid, their existence in the reconstituted membranes can still not be excluded.

We observed that the disulfide bond between the \(\delta\) subunits of the AChR dimer became less susceptible to reduction once AChRs had been subjected to suboptimal reconstitution conditions. Reducing agents, such as 2-mercaptoethanol and dithiothreitol, cause an efficient conversion of AChR dimers into monomers by reduction of the disulfide bond between the \(\delta\) subunits (4, 5, 8). Fig. 13 demonstrates that this dimer to monomer conversion occurs less readily with AChRs after exposure to suboptimal lipid concentrations during reconstitution than with AChRs maintained under optimal conditions. The lower efficiency of AChR dimer reduction reflects a permanent conformational alteration of the protein, which persists after solubilization of the membranes in Triton and provides a protected configuration for the \(\delta-\delta\) disulfide bond. This is surprising, since this disulfide is not involved directly in channel function (8). However, since it must be at the periphery of adjacent AChR monomers, an alteration in its reactivity with reducing agents indicates that the association of adjacent AChR monomers is altered. This evidence, like the only partially reversible packing density of AChRs after exposure to suboptimal lipid conditions, suggests that this denaturation of the cation channel is accompanied by changes in AChR conformation that may affect its interaction with other AChRs. However, denaturation of the channel and denaturation of the area around the disulfide bond-linking monomers are probably not functionally related (8).

**DISCUSSION**

A schematic representation of the solubilization-reassembly cycle is shown in Fig. 14. The approximate molar compositions of the different AChR-lipid assemblies at each stage of this process, calculated on the basis of our data, are presented in Table IV. By electron microscopy of the native membrane, AChRs appear as densely packed 85-Å diameter doughnuts (26, 27) which protrude as mushroom-like projections from the extracellular surface of the membrane (28, 29). We suggest that AChRs solubilized in cholate-lipid mixtures are surrounded by an annulus of lipid in the form of a bilayer, and that an annulus of cholate solubilizes this infinitesimal patch of AChR-containing membrane (Fig. 14). Mixed micelles of cholate and lipid are thought to consist of various sizes of small lipid bilayer discs surrounded by an annulus of cholate (Ref. 24, Fig. 14). We propose that during dialysis some of the cholate molecules dissociate from these bilayer discs, leaving a hydrophobic region which may then fuse with another cholate-depleted region of lipid bilayer. About 68% of the AChRs can be extracted in mixed micelles of cholate and soybean lipids. It is not clear whether the remaining unsolubilized AChRs are present in the native membranes in a different structural arrangement. A 43,000-molecular-weight protein has been suggested to associate with the cytoplasmic surface of AChRs (30). We used alkaline extraction methods to remove this protein (11, 31, 32), but this did not increase the extent of the extraction (data not shown).

The channel denaturation observed at elevated concentrations of cholate (Fig. 1B) is probably due to the concentration of lipid-free cholate micelles which displace the lipid protecting the AChR channel. Although even at 2% cholate, 2 mg/ml of lipid, cholate is present in 20-fold molar excess over the lipid (Table IV), the absolute concentration of disruptive micelle configurations probably becomes significant above 3% cholate. Heidmann et al. (12) have shown that in sodium cholate in the absence of protective lipids, the AChR is stabilized in a low affinity state for agonists, which can no longer be triggered into a slow interconversion to a high affinity state by agonists or local anesthetics. The characteristic allosteric properties of the membrane-bound AChR could be preserved by the presence of protective lipids in the micellar solution. These observations, like ours, suggest that the AChR channel must be shielded from the detergent molecules and the aqueous medium by a protective annulus of lipids as indicated in Fig. 14. Taking into account the 20-fold molar excess of cholate over lipid in the micellar solution (Table IV), it is evident that under optimal conditions (2% cholate, 2 mg/ml of soybean lipid) the binding affinity of the lipids for the AChR must be significantly higher than the binding affinity of cholate for the AChR. Irreversible denaturation of the ion channel at elevated cholate concentrations might be due to: 1) a direct interaction between cholate and specific regions of the protein; 2) denaturation of hydrophobic regions of the channel by direct contact with the aqueous environment; or 3) displacement of lipids essential to maintain a native conformation of the channel. Local anesthetic-like effects of the detergent on the channel (33, 34) could not account for this irreversible inactivation, since such effects would also take place under optimally protective conditions, because the cholate is present in 20-fold molar excess. This large excess of detergent would probably also prevent access of water to a delipidated hydrophobic protein moiety. Our data therefore favor the third possibility.

Our observation that octylglucoside denatures the channel in the presence (or initially in the absence) of supplementary lipids is at variance with a recent report by Gonzalez-Ros et al. (13). They reported reconstitution of AChRs after solubilization and purification of the AChRs in octylglucoside in the absence of supplementary lipids. The incorporation of AChRs into reconstituted lipid vesicles is well documented in this report. However, since these investigators used a high AChR concentration and a low lipid/protein ratio (~2) during their
reconstitution, the carbamylcholine-induced $^{22}Na^+$ flux which they observed may result from only a very small fraction of intact AChR channels.

Adequate data are not available yet to allow a clear description of the molecular interactions which occur during the reassembly process. We hope, however, that our data will provide a useful conceptual framework for further studies on the mechanism of AChR reconstitution. Any hypothesis concerning the molecular mechanism of vesicle formation during the reconstitution process must accommodate the following experimental observations: 1) incorporation of a large fraction of the lipid into small unilamellar liposomes which do not contain AChRs; 2) incorporation of AChRs into large unilamellar vesicles at a constant AChR/lipid ratio independent of the AChR/lipid ratio in the micellar solution, when lipid is present in excess; and 3) incorporation of AChRs in the reconstituted membranes with their extracellular portions preferentially oriented outward and with all the AChRs in a single vesicle oriented in the same direction. It is likely that AChR-AChR interactions play an important role in the reassembly process. One hypothetical mechanism which may account for our observations employs the idea of “pseudo-crystalline spherical arrays” of AChRs being formed during the nucleation stage of the reconstitution process. This hypothesis postulates that as a result of interactions between the extracellular surfaces of the AChRs, they pack into spherical arrays, in most cases (1 to 300) with their $^{125}$I-BGT binding sites oriented toward the exterior of the nascent sphere. We suppose that subsequent to the formation of such pseudo-crystalline spherical arrays of AChRs, lipid is incorporated until a surface tension is reached which allows the complex to be sealed as a stable vesicle. We suggest that under conditions of limiting lipid, vesicles would close at unusually high surface tensions which would denature a fraction of the channels. Channel inactivation during reconstitution would probably be a reversible process if it were due to limited amounts of an essential chemical factor, since AChR channels survive in micellar solution in the presence of even lower lipid concentrations. Formation of aggregates is, although not excluded, not readily demonstrable. We suggest that denaturation of the channel per se or impairment of its gating mechanism may result from unfavorable surface tension and/or electrostatic interactions during reassembly under limiting lipid conditions. This loss of channel function is probably concomitant with a substantial permanent alteration in AChR conformation reflected in the lowered susceptibility of the $\delta-\delta$ disulfide bond to reducing agents added during solubilization of the reconstituted membranes in detergent.

The AChR represents an archetype for other neurotransmitter and hormone receptors and is in fact the first neurotransmitter receptor to be incorporated into model membranes. Stabilization of the tetrodotoxin-binding component of the sodium channel from *Electrophorus electricus* by mixed lipid-detergent micelles has been reported by Agnew et al. (35). We believe that establishing optimal conditions for the stability of the AChR channel in mixed micellar solution, and a careful investigation of the molecular interactions occurring during the reassembly process may be of general use for future studies on other transmembrane proteins.

Acknowledgments—We are indebted to Brett Einarson and Drs. S. J. Tzartos and W. J. Guilclic for valuable suggestions and helpful discussions. We thank Vernita Hudson for technical assistance.

REFERENCES

1. Reynolds, J. A., and Karlin, A. (1978) Biochemistry 17, 2035–2038
2. Lindstrom, J., Merlie, J., and Yogeewaran, G. (1979) Biochemistry 18, 4465–4470
3. Raftery, M. A., Hunkapiller, M. W., Strader, C. D., and Hood, L. E. (1980) Science 208, 1454–1457
4. Chang, H. W., and Bock, E. (1977) Biochemistry 16, 4513–4520
5. Hamilton, S. L., McLaughlin, M., and Karlin, A. (1979) Biochemistry 18, 155–163
6. Weill, C. L., McNamee, M. G., and Karlin, A. (1974) Biochem. Biophys. Res. Commun. 61, 997–1003
7. Damle, V. N., McLaughlin, M., and Karlin, A. (1978) Biochem. Biophys. Res. Commun. 84, 845–851
8. Anholt, R., Lindstrom, J., and Montal, M. (1980) Eur. J. Biochem. 109, 481–487
9. Epstein, M., and Racker, E. (1978) J. Biol. Chem. 253, 6690–6662
10. Huganir, R. L., Schell, M. A., and Racker, E. (1979) FEBS Lett. 108, 155–160
11. Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osamo, M., and Montal, M. (1980) J. Biol. Chem. 255, 8340–8350
12. Heidmann, T., Sobel, A., Popot, J.-L., and Changeux, J.-P. (1980) Eur. J. Biochem. 110, 35–55
13. Gonzalez-Ros, J. M., Paraschos, A., and Martinez-Carrion, M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1796–1800
14. Nelson, N., Anholt, R., Lindstrom, J., and Montal, M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3057–3061
15. Wu, W. C. S., and Raftery, M. A. (1979) Biochem. Biophys. Res. Commun. 89, 26–35
16. Changeux, J.-P., Heidmann, T., Popot, J.-L., and Sobel, A. (1979) FEBS Lett. 105, 181–187
17. Schindler, H., and Quast, U. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3052–3056
18. Killian, P. L., Dunlap, C. R., Mueller, P., Schell, M. A., Huganir, R. L., and Racker, E. (1980) Biochem. Biophys. Res. Commun. 93, 409–414
19. Ravdin, P., and Berg, D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2072–2076
20. Lindstrom, J., Lennon, V., Seybold, M., and Whittington, S. (1976) Ann. N. Y. Acad. Sci. 274, 254–274
21. Tzartos, S. J., and Lindstrom, J. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 755–759
Acetylcholine Receptor Reconstitution

22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
23. Gasco, O. D., Knowles, A. F., Shertz, H. G., Suolinna, E. M., and Racker, E. (1976) Annual. Biochem. 72, 57–65
24. Carey, M. C., and Small, D. M. (1970) Am. J. Med. 49, 590–608
25. Moore, H. F., Hartig, P. R., and Raftery, M. A. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6285–6289
26. Cartaud, J., Benedetti, E. L., Cohen, J. B., Meunier, J. D., and Changeux, J.-P. (1973) FEBS Lett. 33, 109–113
27. Heuser, J. E., and Salpeter, S. R. (1979) J. Cell Biol. 82, 150–173
28. Klymkowsky, M. W., and Stroud, R. M. (1979) J. Mol. Biol. 128, 219–334
29. Wise, D. S., Karlin, A., and Schoenborn, B. P. (1979) Biophys. J. 28, 473–486
30. Wennogle, L. P., and Changeux, J.-P. (1980) Eur. J. Biochem. 106, 381–393
31. Neubig, R. R., Krodel, E. K., Boyd, N. D., and Cohen, J. B. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 690–694
32. Moore, H.-P. H., Hartig, P. R., Wu, W. C.-S., and Raftery, M. A. (1979) Biochem. Biophys. Res. Commun. 88, 735–743
33. Sugiyama, H., and Changeux, J.-P. (1975) Eur. J. Biochem. 55, 505–515
34. Briley, M. S., and Changeux, J.-P. (1978) Eur. J. Biochem. 84, 429–439
35. Agnew, W. S., Levinson, S. R., Brabson, J. S., and Raftery, M. A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2606–2610
36. Shah, D. O., and Schulman, J. H. (1967) J. Colloid Interface Sci. 25, 107–119
37. Karlsson, E., Arnberg, H., and Eaker, D. (1971) Eur. J. Biochem. 21, 1–16