Fractionation and Partial Characterization of Membrane Particles from *Torpedo californica* Electroplax†

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ABSTRACT: Membrane fragments derived from the electric organ of *Torpedo californica* have been fractionated by isopycnic density gradient centrifugation in sucrose and in glycerol. Separate particles containing ATPase, acetylcholinesterase, and α-bungarotoxin binding activities have been obtained. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate shows that these particles are strikingly different in protein content. The results strongly suggest that the structures of these particles are basically different, and that in an intact end plate, acetylcholine receptor molecules are localized in membrane structures different from those containing acetylcholinesterase molecules.

The isolation of synaptosomes (Hebb and Whittaker, 1958) by differential and density-gradient centrifugation has provided an excellent *in vitro* preparation for studies at the molecular level of presynaptic processes in brain tissue. In addition, postsynaptic membranes frequently are found adhering to synaptosomes (Gray and Whittaker, 1962; Whittaker and Sheridan, 1965) and although this is a source of postsynaptic material it does not represent a good *in vitro* system for functional studies of postsynaptic processes. Recently Changeux *et al.* (1969) have described the separation, by density gradient centrifugation, of acetylcholinesterase-rich and ATPase-rich particles from homogenates of *Electrophorus electricus* electroplax. Subsequent studies (Kasai and Changeux, 1971) have demonstrated that cholinergic agonists stimulate ion flux from the acetylcholinesterase particles but not from the ATPase rich fractions. Thus it appears that homogenized electroplax may provide a good *in vitro* system for molecular studies of postsynaptic processes.

Due to the greater innervation (Sheridan *et al.*, 1966) and higher concentration of acetylcholine receptors (Raftery *et al.*, 1971, and references therein) in *Torpedo* electroplax it would be expected that this tissue would be an even better source of postsynaptic particles useful for molecular studies. In this article we describe the isolation of membrane particles from *Torpedo californica* electroplax and their characterization with respect to their content of ATPase, acetylcholinesterase, and in addition acetylcholine receptor (using ^{125}I-labeled α-bungarotoxin). The results show that separate particles contain each activity and the structural and functional implications of these findings are discussed.

Materials and Methods

Materials. Torpedo californica was obtained from Pacific Biomarine Laboratories. The sucrose used was Special Enzyme grade supplied by Schwarz/Mann, and [^{3}H]acetylcholine iodide was obtained from New England Nuclear Corporation.

Reagents for polyacrylamide gel electrophoreses were all Electrophoresis Purity grade, from Bio-Rad Laboratories, except sodium dodecyl sulfate, which was Sequanal grade, from Pierce Chemical Co.

Preparation of Membrane Fragments. The electric organ of *Torpedo californica* was homogenized in a commercial Waring Blendor for 3 min at full speed with an equal volume (1 ml/g of original tissue) of 0.4 M sodium chloride–0.02 M sodium phosphate, pH 7.4. This and all subsequent steps in the preparation of membrane fragments were carried out at 3–5°. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 27,000*g* for 1.5 hr in a Sorvall RC-2B centrifuge. The pellet was resuspended in 0.5 volume of 0.02 M sodium phosphate, pH 7.4, by homogenization at low speed for 3 min and centrifuged at 39,000*g* for 1.5 hr. This step was repeated and the final pellet resuspended, the concentration of the suspension being 3.3 g of original tissue/ml. This membrane preparation was stored at 5° and used within 2 days.

Alternatively, membranes were prepared by homogenizing 19 g of electric organ in 75 ml of 0.2 M sucrose for 3 min at 40,000 rpm in a Virtis 60 homogenizer. The homogenate was then filtered through four layers of cheese cloth and used without further treatment. This method was used in an attempt to conserve as much of the acetylcholinesterase activity as possible in particulate form. The amount of particulate acetylcholinesterase activity per gram of tissue in this preparation was the same as that in 0.4 M NaCl homogenate described above.

Density Gradient Centrifugation. Linear sucrose gradients, 60–17% (w/v), were prepared by layering 18 successively lighter sucrose solutions into a cellulose nitrate tube. Linear glycerol gradients, 100–18%, were prepared by layering 13 successively lighter glycerol solutions into cellulose nitrate tubes. Four to five milliliters of the membrane homogenates were layered on top of the preformed gradients and these were then centrifuged in a Beckman SW-25.2 rotor at 25,000 rpm for 6 hr at 3°. Fractions were collected by pumping through a capillary tube from the bottom of the gradient.

Assays. Acetylcholinesterase was assayed by the method of C. Johnson and R. Russell (unpublished), based on the selective extraction of the hydrolysis product, [^{3}H]acetic acid, into a scintillation fluid. The incubation mixture was 0.1 ml containing 2 mM [^{3}H]acetylcholine and 25 mM potassium phosphate, pH 7.0, with 10-μl aliquots of the gradient fractions added for 10 sec at 25°. The reaction was stopped by adding 0.1 ml of a solution with the composition 1.0 M monochloro-

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[1] Abbreviation used is: ATPase, adenosine triphosphatase.

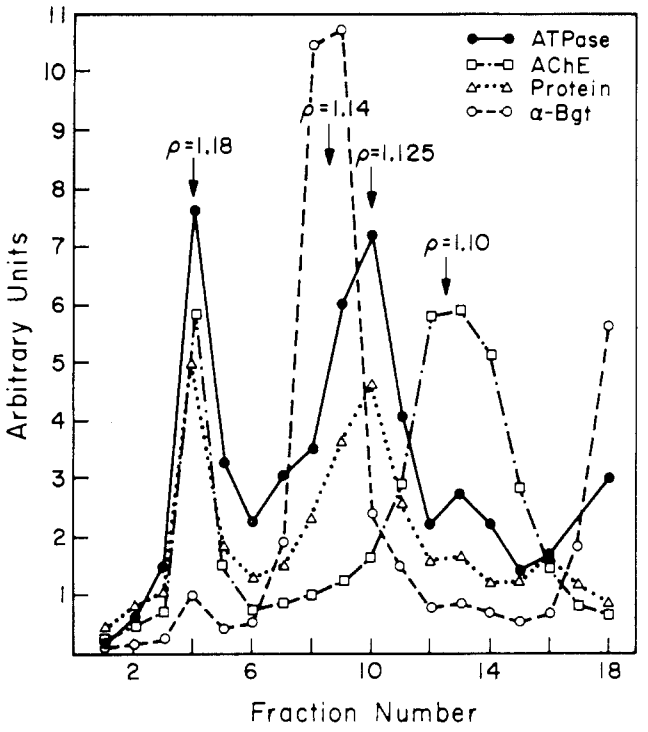

FIGURE 1: A 5-ml aliquot of a sodium chloride chromogenate (see Materials and Methods) was labeled with 8.0 μg of ^{125}I-labeled α-bungarotoxin and applied to the sucrose gradient described under Materials and Methods. The gradient was centrifuged and 3.35-ml fractions were analyzed as described under Materials and Methods. One arbitrary unit is equal to the following activities per fraction: 0.24 μg of ^{125}I-labeled α-bungarotoxin, 1.34 mg of protein, 43 μmol of acetylcholine hydrolyzed/min, and 14 μmol of ATP hydrolyzed/hr.

acetic acid, 2 M sodium chloride, and 0.5 M sodium hydroxide. [^{3}H]Acetic acid was selectively extracted from the acidic solution into 3 ml of a scintillation fluid added to the reaction vial. The composition of the scintillation fluid was 1.14 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 18.95 g of 2,5-diphenyloxazole, in 3 ml of 10% isoamyl alcohol in toluene. The vials were capped, shaken, and counted using a Packard Tri-Carb liquid scintillation spectrometer.

ATPase was assayed by the method of Bontig *et al.* (1961) and ^{125}I-labeled α-bungarotoxin, prepared by the method of Clark *et al.* (1972), was determined by liquid scintillation spectrometry. Protein concentration was measured by the method of Lowry *et al.* (1951) and polyacrylamide gel electrophoresis was performed using the method of Fairbanks *et al.* (1971). Gels were scanned using the linear transport attachment for a Gilford Model 240 spectrophotometer at 550 nm.

Results

The homogenized, unfractionated membrane preparation was assayed for ATPase, acetylcholinesterase, and α-bungarotoxin binding activities. These results, as well as the particulate protein content of the preparation, are given in Table I, normalized for 1.0 g of fresh tissue. In addition, the effect of solubilization with 1% Triton X-100 on the acetylcholinesterase and α-bungarotoxin binding activities was investigated. There was no significant difference, suggesting that no substantial amount of inside-out vesicles was formed by the homogenization in 0.4 M NaCl.

The distribution of protein, ATPase, acetylcholinesterase, and α-bungarotoxin binding activities from the 0.4 M NaCl homogenate after centrifugation in a 60–17% sucrose gradient is shown in Figure 1. In the experiment shown, approximately one-tenth of the available α-bungarotoxin binding sites were labeled. When virtually all of the toxin binding sites were labeled no significant change in the distribution of α-bungarotoxin binding activity in the gradient was observed. The most interesting characteristic of this profile is the essentially complete separation of α-bungarotoxin binding particles, banding at $p = 1.14$, from those containing acetylcholinesterase activity. The acetylcholinesterase particles band primarily at two densities, 1.18 and 1.10, although at times a shoulder appeared on the lower density peak toward the top of the gradient. ATPase particles band at densities of 1.18 and 1.125. The protein profile closely follows that of the ATPase activity.

TABLE I: Activities in Homogenized, Unfractionated Membrane Preparation.

Function	Act./g of Tissue: Membranes	Act./g of Tissue: Triton Extract
Acetylcholinesterase (mmol of acetylcholine hydrolyzed/min)	0.133	0.163
α-Bungarotoxin (μg bound)	5.0[a]	4.5[a]
ATPase (μmol of ATP hydrolyzed/hr)	300	
Protein (mg)	5.5	1.5

[a] This value varied from preparation to preparation, ranging from 5 to 11 μg/g of tissue with corresponding changes in the Triton extract.

A homogenate prepared in 0.2 M sucrose was applied to a similar gradient and the distribution of acetylcholinesterase and α-bungarotoxin binding activities after centrifugation is shown in Figure 2. Although the densities of the heavier acetylcholinesterase particles and α-bungarotoxin binding particles are somewhat greater than those from the 0.4 M NaCl homogenate, the separability of the activities is almost as complete.

The distribution of α-bungarotoxin binding and acetylcholinesterase activities in a glycerol gradient (100–18%) is shown in Figure 3. α-Bungarotoxin particles band at a density of 1.17 in a symmetric peak, and are again separated from acetylcholinesterase particles, which band at two densities, 1.23 and 1.14.

The particulate nature of the α-bungarotoxin binding and acetylcholinesterase activities from the sucrose gradients shown in Figures 1 and 2 was demonstrated by centrifugation for 1 hr at 40,000*g*. An aliquot of each of the peak fractions was diluted with 3 volumes of water and assayed before and after centrifugation. The supernatant was devoid of activity in all cases except for the α-bungarotoxin at the top of both gradients, representing free α-bungarotoxin, and the acetylcholinesterase at the top of the gradient in Figure 2, showing the presence of soluble acetylcholinesterase in these fractions. This was expected since the 0.2 M sucrose homogenate was

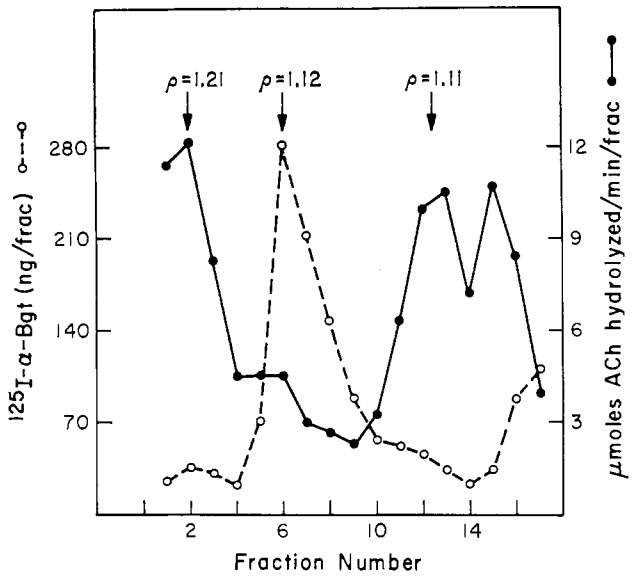

FIGURE 2: A 4.5-ml aliquot of a 0.2 M sucrose homogenate was applied to a sucrose gradient after prelabeling with 1.6 μg of ^{125}I-labeled α-bungarotoxin. The gradient was centrifuged and 3.5-ml fractions were analyzed as described under Materials and Methods.

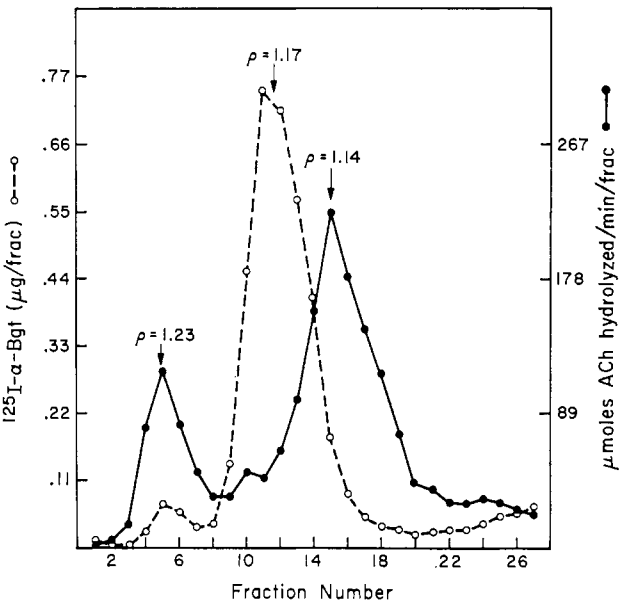

FIGURE 3: A 4-ml aliquot of the sodium chloride homogenate was prelabeled with 4.5 μg of ^{125}I-labeled α-bungarotoxin, then applied to the glycerol gradient described under Materials and Mehods. The gradients were centrifuged and 2.2-ml fractions analyzed as described under Materials and Methods.

used without prior centrifugation of membranes to separate them from soluble proteins.

In Figure 4, the densitometry traces of sodium dodecyl sulfate gels from given fractions of the sucrose gradient (Figure 1) are shown. Here again, the point of greatest interest is the difference between the protein species of the α-bungarotoxin binding particles and those of the cholinesterase and ATPase containing particles. This difference strengthens the conclusion, which can be drawn from the separability in density gradients of α-bungarotoxin toxin binding particles and those containing acetylcholinesterase activity, that the structure of these particles is basically different.

Discussion

The results obtained in this study show that membrane particles obtained by homogenization of *Torpedo californica* electric tissue can be separated, by density gradient centrifugation, into different classes, each with a differing functionality. Electroplax cells have a somewhat invaginated innervated face which the acetylcholinesterase and acetylcholine receptor molecules occupy and a larger and much more highly invaginated noninnervated face where the ATPase resides. It was not surprising then to find (Figure 1) that the major part of the membrane protein was associated with ATPase activity. However, the distinct separation obtained between particles having acetylcholinesterase activity and those bearing acetylcholine receptor molecules (as determined by specific interaction with ^{125}I-labeled α-bungarotoxin) was surprising since both activities are associated with the postsynaptic region of the innervated face of the electroplaque (Bourgeoise *et al.*, 1971). This finding obviously relates to the structure of the postsynaptic region and specifically to the relative disposition therein of acetylcholinesterase and acetylcholine receptor molecules. It is known that acetylcholinesterase may be partially solubilized into aqueous salt solutions from disrupted membranes while the acetylcholine receptor is strongly membrane bound and is solubilized only by detergents (Raftery *et al.*, 1971, and references therein). Recent experiments by Hall and Kelly (1971) have shown that treatment of mouse diaphragm with collagenase solubilizes end-plate acetylcholinesterase. They interpreted these findings to mean that the enzyme occupies an exposed position on the nerve or muscle cell surface and that it was probably not a structural component of the membrane. More recently Betz and Sakmann (1971) have suggested that the ectolemmal sheath anchors acetylcholinesterase and after dissolution of the sheath by enzymatic treatment the enzyme diffuses away from its normal location. Because of the density of acetylcholine receptors in the end-plate region of muscle (10^4 receptors/μm^2) Miledi and Potter (1971) have also suggested the possibility that acetylcholinesterase may not be located in the membrane matrix but instead in the basement membrane. However, Barnard *et al.* (1971) found a similar value ($1.2 \times 10^4/\mu m^2$) for end-plate receptor density in muscle and suggested a model involving a mosaic of receptors and acetylcholinesterase over the whole postsynaptic membrane.

Our results show that both acetylcholinesterase and receptor are membrane bound in *Torpedo californica* electroplax but that these membrane structures are distinct since they can be fractionated by density gradient centrifugation of tissue homogenates (Figure 1) and since the total membrane protein profiles of the separated particles are dissimilar (Figure 4). This renders highly unlikely a model in which enzyme and receptor molecules occupy the same membrane as a mosaic. We do not consider it likely that the acetylcholinesterase molecules are merely held at end plates by entrapment due to the ectolemmal sheath—the acetylcholinesterase-rich particles we obtain are vesicular in nature (unpublished observations with J.-P. Revel) and have the same enzymatic activity in the particulate and dissolved states (Table I), a fact inconsistent with entrapment during homogenization. However, further characterization is necessary to relate our findings to those of Hall and Kelly (1971) or Betz and Sakmann (1971).

The results presented here differ from those of Changeux *et al.* (1969) in that obviously a more complete fractionation

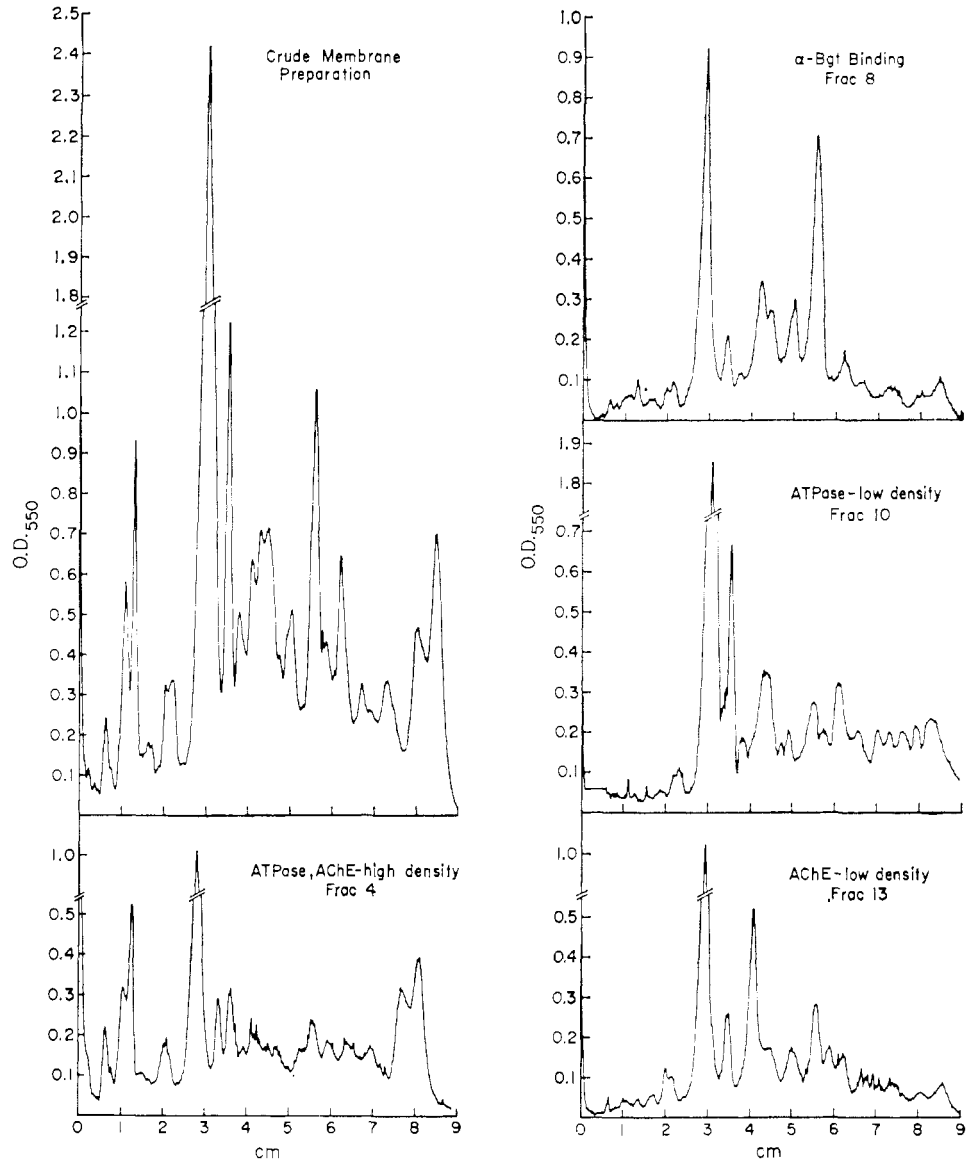

FIGURE 4: Aliquots of the indicated fractions from the sucrose gradient shown in Figure 1 and of a crude membrane preparation were dissolved in 1% sodium dodecyl sulfate and electrophoresed on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. The densitometry traces of the Coomassie Brillant Blue stained gels are shown. β-Galactosidase, ovalbumin, and myoglobin were run as standards with mobilities of 2.35, 5.2, and 7.7 cm, respectively.

has been achieved. These workers utilized step gradients, rather than continuous sucrose gradients, and did not specifically monitor acetylcholine receptor distribution. However, the demonstrated functionality of their acetylcholinesterase-rich particles by Kasai and Changeux (1971) shows that the preparation did contain acetylcholine receptors. It is our opinion that this was due to incomplete fractionation since we have also been able to obtain results similar to those reported here using *Electrophoresis electricus* electric tissue (Duguid and Raftery[2]).

In comparing the protein profile of the unfractionated membrane preparation with that of the α-bungarotoxin binding fraction (Figure 4) it is obvious that a great reduction in complexity has been obtained and that only a few major protein components are present in the purified particles. The major component is still that seen in the unfractionated particles (mobility ~3 cm) but the other large protein component (mobility ~5.5 cm) has been enriched to a high degree. We believe that this material represents the acetylcholine receptor due to (1) its enrichment relative to the starting material, (2) the agreement of this enrichment factor with the increased capability of this material to bind ^{125}I-labeled α-bungarotoxin (× 6–7), (3) the molecular weight of this component relative to standards in sodium dodecyl sulfate–gel electrophoresis (~4 × 10^4 apparent molecular weight, in agreement with a recent estimate of the subunit molecular weight of an acetylcholine receptor from *Electrophorus electricus* electroplax by Reiter *et al.*, 1972), and (4) based on similar gel electrophoreis patterns of highly purified preparations of acetylcholine receptor from the same tissue used here (Schmidt and Raftery, 1973).

These acetylcholine receptor rich particles should represent an excellent *in vitro* system for studies at the molecular level of postsynaptic events and it is hoped that in the future such studies will contribute to our understanding of chemical events during synaptic transmission.

[2] Duguid, J. R., and Raftery, M. A., submitted for publication to *Arch. Biochem. Biophys.*

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References

Barnard, E. A., Wieckowski, J., and Chiu, T. H. (1971), *Nature (London) 234*, 207.

Betz, W., and Sakmann, B. (1971), *Nature (London), New Biol. 232*, 94.

Bontig, S. L., Simon, K. A., and Hawkins, N. M. (1961), *Arch. Biochem. Biophys. 95*, 416.

Bourgeoise, J. P., Tsuji, S., Boquet, P., Pillot, J., Ryter, A., and Changeux, J.-P. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16*, 92.

Changeux, J.-P., Gautron, J., Israel, M., and Podleski, T. (1969), *C. R. Acad. Sci., Ser. D 269*, 1788.

Clark, D. G., Macmurchie, D. D., Elliott, E., Wolcott, R. G., Landel, A. M., and Raftery, M. A. (1972), *Biochemistry 11*, 1663.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10*, 2606.

Gray, E. G., and Whittaker, V. P. (1962), *J. Anat. 96*, 79.

Hall, Z. W., and Kelly, R. B. (1971), *Nature (London), New Biol. 232*, 62.

Hebb, C. O., and Whittaker, V. P. (1958), *J. Physiol. 142*, 187.

Kasai, M., and Changeux, J.-P. (1971), *J. Membrane Biol. 6*, 1.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Miledi, R., and Potter, L. (1971), *Nature (London) 233*, 599.

Raftery, M. A., Schmidt, J., Clark, D. G., and Wolcott, R. G. (1971), *Biochem. Biophys. Res. Commun. 45*, 1622.

Reiter, M. J., Cowburn, D. A., Prives, J. M., and Karlin, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1168.

Schmidt, J., and Raftery, M. A. (1973), *Biochemistry 12*, 852.

Sheridan, H. N., Whittaker, V. P., and Israel, M. (1966), *Z. Zellforsch. Mikrosk. Anat. 74*, 291.

Whittaker, V. P., and Sheridan, M. N. (1965), *J. Neurochem. 12*, 363.

Studies on the Topography of the Fat Cell Plasma Membrane†

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ABSTRACT: Purified plasma membranes from isolated fat cells are composed of at least 13 major peptide components including two major glycopeptides, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. These glycopeptides with apparent molecular weights of 94,000 and 78,000 apparently account for a substantial amount of the total membrane protein. Membrane components which are exposed on the exterior cell surface were determined by catalytic iodination of intact cells with lactoperoxidase in the presence of H_2O_2 and $Na^{125}I$. Following iodination of fat cells only 6% of the total ^{125}I incorporated into isolated membrane fractions was found associated with mitochondrial membranes, indicating little or no penetration of lactoperoxidase into fat cells. Gel electrophoresis of the isolated plasma membranes derived from catalytically iodinated intact fat cells revealed that essentially all the label was associated with the glycopeptide of 94,000 molecular weight and, to a lesser extent, the lighter glycopeptide. In contrast, exposure of isolated plasma membranes to this labeling procedure resulted in greater total incorporation of radioactivity into the membrane and a more uniform iodination of all membrane peptides. These studies indicate that the two major membrane glycopeptides represent most of the protein on the exterior of the isolated fat cell surface. Procedures used to isolate the plasma membrane fraction from these cells apparently severely disrupt the highly organized structure of the intact cell surface which may account, at least in part, for the relative insensitivity of isolated membranes to effects of insulin and other hormones.

Many of the hormones which regulate adipose cell functions are thought to interact with these cells at the level of the plasma membrane. Direct evidence for this concept was provided in the cases of insulin (Cuatrecasas, 1969) and growth hormone (Hecht *et al.*, 1972) by the demonstration that covalent linkage of these hormones to Sepharose beads larger than the fat cells does not prohibit their physiological actions. Understanding the molecular events involved in these hormone–plasma membrane interactions will probably require a detailed knowledge of the membrane components and their organization. We recently initiated studies on the fat cell plasma membrane and found that major peptide components could be reproducibly resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Czech and Lynn, 1973a). Further characterization of some of these components is in progress.

The aim of the present studies was to obtain information about the spatial arrangement of the peptide and glycopeptide constituents of the fat cell surface. We have employed the lactoperoxidase iodination procedure which has been success-

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received April 16, 1973.* This work was supported in part by grants from the National Science Foundation (GB-17440) and from the National Institutes of Occupational Safety and Health (5-R01-OH-0032-02). This paper is the second of a series which describe our studies on the fat cell plasma membrane.

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