Proc. Nat. Acad. Sci. USA
Vol. 73, No. 1, pp. 161–164, January 1976
Cell Biology

Synthesis of acetylcholine receptors by cultured chick myotubes and denervated mouse extensor digitorum longus muscles

(*de novo* protein synthesis/metrizamide–D_2O gradients/stable isotopes)

PETER N. DEVREOTES* AND DOUGLAS M. FAMBROUGH†

* Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218; and † Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210

Communicated by Donald D. Brown, October 17, 1975

ABSTRACT **Mono-[^{125}I]iodo-α-bungarotoxin–receptor complexes extracted from chick myotube cultures as well as from adult denervated extensor digitorum longus muscles of the mouse have been banded at their buoyant density in gradients of metrizamide–deuterium oxide. When cultures or denervated adult muscles are preincubated in media containing ^{2}H- or ^{13}C-substituted amino acids under conditions of active receptor accumulation, the mono-[^{125}I]iodo-α-bungarotoxin–receptor complexes have an increased buoyant density and band in a position of higher density in the gradient relative to a marker of mono-[^{131}I]iodo-α-bungarotoxin–receptor complexes extracted from cells preincubated in normal media. It is concluded that the accumulation of receptors on the surfaces of cultured chick myotubes and on the nonsynaptic surfaces of extensor digitorum longus muscles following denervation are the result of *de novo* synthesis.**

During myogenesis acetylcholine (AcCh) receptors accumulate in the plasma membranes of myotubes and the myotube surface becomes quite sensitive to extracellular AcCh (1, 2). This accumulation results since the rate of incorporation of receptors into the plasma membrane is greater than the rate of receptor degradation (3). In developing tissue cultured chick skeletal muscle in addtion to the net increase in receptors, there is a replacement of several percent of cell surface AcCh receptors each hour (3). These observations on AcCh receptor accumulation and turnover are based upon studies using α-bungarotoxin and radioactive derivatives of α-bungarotoxin to label AcCh receptors.

While the use of α-bungarotoxin as a probe for AcCh receptors makes possible many experiments which cannot be done quantitatively by any other means and while there is good evidence that α-bungarotoxin does not significantly perturb AcCh receptor metabolism, there were several important reasons for attempting to label AcCh receptors directly with labeled amino acids. First, it was worthwhile to establish that the new receptors appearing on myotube surfaces after α-bungarotoxin blockade are, indeed, the result of *de novo* synthesis as inhibitor studies had suggested (3, 4). Second, there is evidence from kinetic analysis (and subcellular fractionation) that AcCh receptors are incorporated into some internal membrane system immediately after synthesis and that this intracellular pool of receptors can supply the cell surface with new receptors for about 2–3 hr in the absence of protein synthesis (3). This matter can be directly analyzed only if newly synthesized receptors can be labeled with metabolic precursors. Third, based largely upon the fate of ^{125}I-labeled α-bungarotoxin bound to AcCh receptors on living cells, a degradation rate of approximately 3% per hour ($t_{1/2}$ = 22 hr) has been inferred for the AcCh receptor. This rate of degradation can be directly measured by using metabolic precursors in "pulse-chase" experiments.

Abbreviation: AcCh, acetylcholine.

Unfortunately the AcCh receptor represents on the order of 1 part in 10^5 of cell protein in cultured chick skeletal muscle. Thus, complete purification of receptors (which is an essential step in confirming the degree of labeling when radioactive precursors are used) would be a very laborious undertaking and to do careful kinetic analysis would require quantitatively reproducible isolation of labeled receptors from many cell preparations. Thus, an alternative method of labeling was tried: using metabolic precursors containing stable isotopes of carbon or hydrogen, which, when incorporated into receptor protein, would yield receptors of altered buoyant density. The density-shift of newly synthesized receptors can be detected by equilibrium density gradient sedimentation without the necessity of purifying the receptors. In this report we present the density shift method for receptors and confirm that new AcCh receptors appearing in cultured chick muscle are the result of *de novo* synthesis.

In normally innervated adult skeletal muscle AcCh receptors are located almost exclusively in the region of the neuromuscular junctions, but, following denervation, AcCh receptors accumulate in extrajunctional membranes (5–7). This accumulation is dependent upon RNA and protein synthesis (8–10) and results in a many-fold increase in total muscle AcCh receptors (11). Both of these observations suggest that these receptors are synthesized *de novo* following denervation. Recently, Brockes and Hall (12) reported a 600-fold purification of AcCh receptors from rat diaphragm, demonstrated the incorporation of [^{35}S]methionine into this partially purified material, and showed that a portion of this radioactive material from denervated (but not from control) muscle behaved like AcCh receptors when subjected to velocity sedimentation in sucrose gradients and to isoelectric focusing. They concluded that the extrajunctional AcCh receptors appearing in adult muscle after denervation are the result of *de novo* synthesis. In this report we confirm this conclusion by demonstrating that AcCh receptors appearing after denervation of mouse extensor digitorum longus muscles are of greater than normal buoyant density when the denervated muscle is supplied with deuterium-labeled amino acids.

METHODS

Chemicals. The preparation of iodinated derivatives of α-bungarotoxin has been described previously (13). Ninety-eight percent ^{2}H or 80% ^{13}C-substituted amino-acid mixtures were purchased from Merck, Sharp and Dohme of Canada Ltd., Pointe Claire, Canada. The mixtures, dissolved in H_2O, were passed through Amicon UM-2 filters (Amicon Corp., Lexington, Mass.) before addition to media stocks. Metrizamide [2-(3 acetamido-5-*N*-methyl-acetamido-2,4,6-tri-iodobenzamido)-2-deoxy-D-glucose] was purchased from

Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N.Y., and deuterium oxide from ICN Chemical & Radioisotopes Division, Irvine, Calif.

Cell Culture and Media. Techniques of culture and preparation of media for both embryonic myotubes and adult muscle have been described (3, 9). F12 and F11 media were prepared as usual except that all the amino acids but asparagine, tryptophan, glutamine, and cysteine were replaced by 500 mg/liter of ^{2}H- or ^{13}C-substituted amino-acid mixture from Merck, Sharp, and Dohme. Horse serum and embryo extract were dialyzed exhaustively against Hanks' balanced salt solution. Mouse extensor digitorum longus muscles were cultured in a Trowell medium in which all amino acids were replaced by 500 mg/liter of ^{2}H-substituted amino-acid mixture.

Density Gradient Centrifugation. α-Bungarotoxin–receptor complexes were banded in spontaneously formed or preformed metrizamide–D_2O gradients. For spontaneously formed gradients, the sample in 2.0–2.5 ml of 1% Triton X-100, 10 mM Tris·HCl, pH 7.8, was layered over 3.0 ml of 60% weight/volume metrizamide–D_2O in a polycarbonate centrifuge tube. Gradients were formed by 4 days of centrifugation in a no. 40 Beckman rotor at 33,000 rpm at 20°. The shape of the gradient has been determined by measurement of refractive index and is approximately linear throughout the region where receptors band (1.3 g cm^{-3}). It has been found that preformed, shallow gradients can be maintained in metrizamide–D_2O (14) when centrifugation is carried out for shorter periods. Macromolecules can be sedimented rapidly through such shallow gradients to their density positions. Preformed gradients were prepared by forming step gradients which quickly become linear. The following solutions (0.95 ml) were poured sequentially into polyallomer tubes: 40%, 35%, 30%, 25%, 20% weight/weight metrizamide–D_2O. The sample in 1% Triton X-100, 10 mM Tris·HCl, pH 7.8, was layered in a thin band (300 μl) over the 20% step. Centrifugation was carried out at 58,000 rpm in a Beckman SW65 rotor or at 48,000 rpm in an SW50.1 rotor for 15 hr at 4°.

Preparation of α-Bungarotoxin–Receptor Complexes. *Myotubes*—Myotubes (after labeling of receptors with α-bungarotoxin) were swelled in hypotonic solution (10 mM Tris·HCl, pH 7.8) at 4° for 5 min. Cells were then scraped from the plate and homogenized in 10 mM Tris·HCl, pH 7.8, at 0° by 10 strokes of a Dounce (pestle B) homogenizer. The homogenate was centrifuged at 27,000 × g for 60 min, which sediments all the α-bungarotoxin–receptor complexes. The pellet was extracted in 1% Triton X-100, 10 mM Tris·HCl, pH 7.8, on a vortex mixer and recentrifuged at 27,000 × g for 10 min. The clear supernatant contains about 95% of the original α-bungarotoxin–receptor complexes formed on the myotube surface. These samples were transferred directly to metrizamide–D_2O gradients.

Extensor Digitorum Longus Muscles—After labeling with α-bungarotoxin, groups of 10 extensor digitorum longus muscles were homogenized in 5 ml of 1% Triton X-100, 10 mM Tris·HCl, pH 7.8, at 0° in a ground glass homogenizer and centrifuged at 10,000 × g for 10 min. The supernates were concentrated by ultrafiltration through an Amicon XM-50 filter and were layered in thin bands on linear 5–20% weight/volume sucrose gradients where the solvent system was 1% Triton X-100, 10 mM Tris·HCl, pH 7.8. Centrifugation was carried out in a Beckman SW 50.1 rotor for 6 hr at 48,000 rpm at 20°. 10S peaks (α-bungarotoxin–receptor complexes), usually 60% of the distance through the gradient, were collected, reconcentrated on XM-50 filters, and transferred to metrizamide–D_2O gradients.

Radioisotope Counting and Analysis. Two to four drop fractions from gradients were collected into vials and mixed with 3.5 ml of scintillation cocktail (3). Samples were counted for both isotopes simultaneously on a Packard Scintillation Spectrometer. After correction for crossover between the channels, the raw data for ^{125}I and ^{131}I were plotted separately. Approximately equal activities for the two isotopes were loaded onto the density gradients so that corrections for crossover would be accurate. Smooth curves were drawn through each of the bands and the data were normalized to the peak of the smooth curve. The normalized data are plotted together in the figures.

RESULTS

Since an analysis of the kinetics of interaction of α-bungarotoxin with intact chick myotubes and of the production of acetylcholine receptors in these cultures was available (3), a strategy was devised to examine only those receptors which were likely to be newly synthesized after introduction of labeled amino-acid precursors.

Cultures of myotubes were incubated with 1 μg/ml of unlabeled α-bungarotoxin from the fourth to fifth day in culture. After 16 hr the culture medium was switched to medium containing ^{2}H- or ^{13}C-amino acids but still containing 1 μg/ml of unlabeled α-bungarotoxin. After 4 hr of of preincubation with ^{2}H- or ^{13}C-substituted amino acids, the unlabeled α-bungarotoxin was rinsed from the cultures and the cultures were incubated for an additional 2 hr in medium free of α-bungarotoxin but still containing the ^{2}H- or ^{13}C-amino acids. During the final 30 min of incubation the newly incorporated receptors were saturated with 0.2 μg/ml of mono-[^{125}I]iodo-α-bungarotoxin. Unbound α-bungarotoxin was rinsed away and the mono-[^{125}I]iodo-α-bungarotoxin–^{2}H- or ^{13}C-receptor complexes were extracted from a crude membrane pellet prepared from the myotubes (see *Methods*). This extract was mixed with a similar extract of myotubes grown in normal medium and labeled with 0.2 μg/ml of mono-[^{131}I]iodo-α-bungarotoxin for 30 min, which served as a marker for the position in the density gradient of normal α-bungarotoxin–receptor complexes. The mixture was then centrifuged through a preformed metrizamide–D_2O density gradient (see *Methods*). As shown in Fig. 1, in myotubes incubated with either ^{2}H- or ^{13}C-amino acids newly incorporated receptors have a greater buoyant density than normal receptors and band in a position of higher density in the gradient. The shift in density for ^{2}H-labeled receptors is approximately 0.006 g cm^{-3} and for ^{13}C-labeled receptors is approximately 0.0045 g cm^{-3}. Several control experiments have been performed and will be briefly described below. (*a*) The experiment in Fig. 1 illustrates the shift produced by a 6 hr incubation with ^{2}H- or ^{13}C-amino acids. Incubation for 4 hr or 12 hr produces similar shifts, while shorter incubation produces smaller shifts. (*b*) The small shift produced by a 3 hr incubation is not observed if 6 μg/ml of puromycin is included in the ^{2}H-amino acid containing incubation medium. (*c*) The shift can be observed between receptors on the same myotubes. That is, when the existing receptors are first saturated with mono-[^{125}I]iodo-α-bungarotoxin and the myotubes are subsequently incubated in ^{2}H- or ^{13}C-amino acid containing medium for 10 hr, new receptors are produced in the presence of the substituted amino acids. These receptors, labeled with mono-[^{131}I]iodo-α-bungarotoxin, are

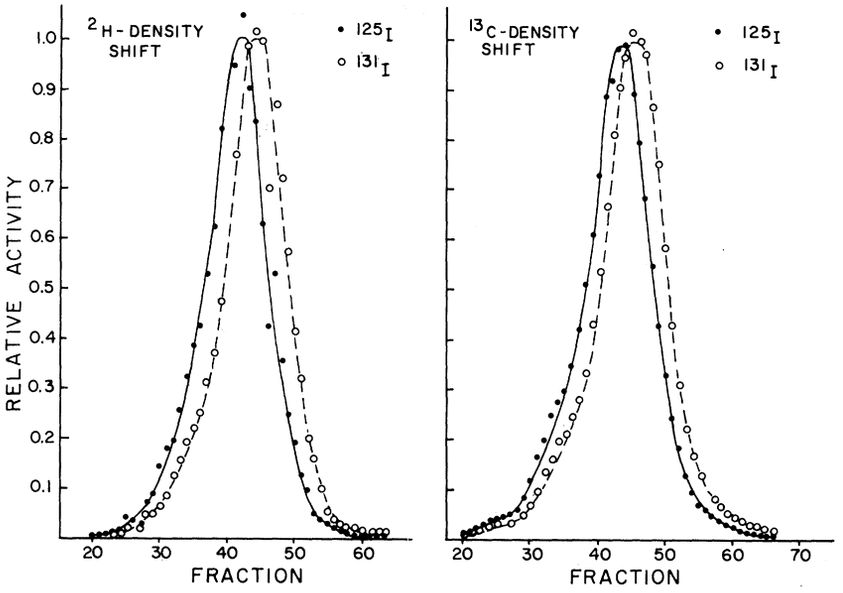

FIG. 1. Density gradient sedimentation of receptors from cultured chick myotubes. Left frame: Shift in density produced by incubation with ^{2}H-amino acids. Right frame: Shift in density produced by incubation with ^{13}C-amino acids. ● — ●, ^{125}I; ○ - - ○, ^{131}I.

shifted relative to the mono-[^{125}I]iodo-α-bungarotoxin–receptor complexes formed on the same cells prior to incubation with ^{2}H- or ^{13}C-amino acids. Experiments are not routinely performed in this manner since it is difficult to match total activity of ^{125}I and ^{131}I loaded on the gradient. (*d*) When mono-[^{125}I]iodo-α-bungarotoxin–receptor complexes and mono-[^{131}I]iodo-α-bungarotoxin–receptor complexes, each extracted from cultures grown in normal medium, are banded together no shift is observed. (*e*) Neither the rate of new receptor incorporation nor receptor degradation is affected by incubation of myotubes with ^{2}H- or ^{13}C-amino acids for at least 12 hr.

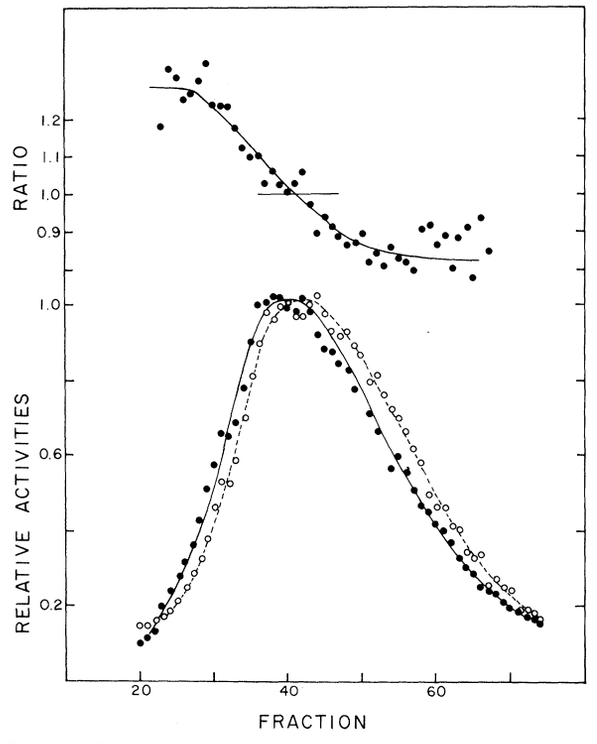

FIG. 2. Density gradient sedimentation of receptor from adult denervated extensor digitorum longus muscles of the mouse. Upper scale: Ratio of mono-[^{125}I]iodo-α-bungarotoxin–^{2}H-receptor complex activity to mono-[^{131}I]iodo-α-bungarotoxin–^{1}H-receptor complex activity. Lower scale: Shift in density produced by incubation with ^{2}H-amino acids. ● — ●, ^{125}I; ○ - - ○, ^{131}I.

Since ^{2}H- or ^{13}C-amino acids could be incorporated into receptors in myotube cultures, this technique was used to test whether the receptors which appear on extrajunctional surfaces of adult muscles following denervation are also the result of *de novo* synthesis. Ten mouse extensor digitorum longus muscles were removed 3 or 6 days after denervation (in separate experiments) and transferred to organ culture in a medium containing both ^{2}H-amino acids and 2 μg/ml of unlabeled α-bungarotoxin. After 3 hr of incubation unlabeled α-bungarotoxin was removed by five 15-min washes with culture medium still containing ^{2}H-amino acids. The muscles were then cultured for 16 hr in medium containing ^{2}H-amino acids. During the final hour of the incubation 2 μg/ml of mono-[^{125}I]iodo-α-bungarotoxin plus 5 mg/ml of bovine serum albumin were added to the medium. The muscles were then rinsed to remove unbound radioactivity and the mono-[^{125}I]iodo-α-bungarotoxin–^{2}H-receptor complexes were prepared as described in *Methods*. These partially purified receptors were mixed with mono-[^{131}I]iodo-α-bungarotoxin–^{1}H-receptor complexes prepared in a similar manner from denervated muscles which had not been organ cultured in ^{2}H-amino acids. Receptors in the mixed extract were banded in a spontaneously formed metrizamide gradient (see *Methods*). Fig. 2 shows the results from 6 day denervated muscles; similar results were obtained from 3 day denervated muscles. A small but significant density shift was detected; complexes from muscles incubated in ^{2}H-amino acids band at a position of higher density than normal receptors. The ratio of activities in the upper part of the graph illustrate in another way that the peaks do not coincide. The observed density shift is approximately 0.006 g cm^{-3}.

DISCUSSION

It has been demonstrated that both embryonic myotubes and denervated adult extensor digitorum longus muscles

supplied with amino acids incorporated these amino acids into the acetylcholine receptor. Newly synthesized receptors which have incorporated ^{2}H- or ^{13}C-amino acids have a slightly greater buoyant density than normal receptors when both are examined as α-bungarotoxin–receptor complexes on metrizamide–D_2O density gradients. It is concluded that the receptors appearing on the surfaces of these cells are the result of *de novo* synthesis.

It has been shown (3) that myotubes contain a pool of presynthesized receptors which can supply the surface for about 2–3 hr in the absence of new protein synthesis. This is consistent with the observation that 4–6 hr are required for the shift in receptor density produced by ^{2}H- or ^{13}C-amino acids to reach a maximum, steady-state value. The small shift observed after short incubation times may involve two components which are not resolved. That is, the peak may consist of presynthesized receptors which are not density shifted and newly synthesized receptors which are shifted. Detailed observations of the kinetics of incorporation of ^{2}H- and ^{13}C-amino acids during the first 4 hr will be presented elsewhere.

It is conceivable that the density shift is not produced by *de novo* receptor synthesis but by *de novo* synthesis of some other component (lipid, sugar, etc.) which is specifically attached to the receptor after its synthesis. Several observations make this possibility unlikely. The receptor density in metrizamide–D_2O gradients is very similar (about 1.3 g cm^{-3}) to the density (about 1.34 g cm^{-3}) estimated for the homologous nicotinic AcCh receptor of *Electrophorus* from its amino-acid composition (15). Extensive lipid or sugar modification should lower its density considerably [both carbohydrate and lipid would float on the preformed gradients used in these experiments (16)]. Furthermore, since ^{2}H and ^{13}C were supplied as amino acids, the necessary carbohydrate or lipid would have to be efficiently converted from amino acids. Since a common pathway of amino-acid degradation is through the Krebs tricarboxylic acid cycle, a significant dilution of isotope by unlabeled glucose and pyruvate (which are each in about 10-fold molar excess over amino acids in the medium) should occur at this point. Even if an efficient conversion of amino acid to lipid or carbohydrate takes place, two additional observations control for post-translational addition of ^{2}H- or ^{13}C-labeled components to new receptors. New receptors can be shifted relative to existing receptors on the same cells, and the small density shift produced by a 3 hr incubation is blocked by puromycin. In summary, post-translational addition of ^{2}H- or ^{13}C-labeled lipids or carbohydrates is a very unlikely explanation of the density shift observed in these experiments.

Given the extent of nonexchangeable ^{2}H substitution in amino acids (determined by Merck, Sharp and Dohme) supplied in the medium and the approximate amino-acid composition of the receptor (15), the magnitude of the density shift that would be produced if the receptor was assembled entirely from these amino acids is estimated to be about 4.9%. The density shifts observed are about 0.5%, about 10% of the theoretical maximum. Since the incubation media we have employed contain other carbon sources from which amino acids can be synthesized, amino acids supplied in the medium are likely diluted by pools of amino acids in the cells.

The great advantage of labeling membrane proteins through amino-acid precursors substituted with stable isotopes rather than with radioactive tracers is that purification of the protein is unnecessary. In this method the protein is identified by a specific marker (such as enzyme activity or ligand binding) and the presence of labeled isotope is detected by a change in a physical property (the density) of the protein. In principle, the technique used here and described by Hüttermann and Guntermann (ref. 14, see also ref. 17) can be used to study the metabolism of any identifiable protein.

We would like to thank Drs. Leaf Huang and Saul Roseman for critical review of the manuscript and Dr. Aileen Ritchie for helpful discussions. We also wish to thank Huei-Wen Chen for excellent technical assistance. Publication costs were covered by National Institutes of Health Training Grant P10 6614.10.

1. Fambrough, D. M. & Rash, J. E. (1971) "Development of acetylcholine sensitivity during myogenesis," *Dev. Biol.* **26,** 55–68.
2. Fischbach, G. D. (1970) "Synaptic potentials recorded in cell cultures of nerve and muscle," *Science* **169,** 1331–1333.
3. Devreotes, P. N. & Fambrough, D. M. (1975) "Acetylcholine receptor turnover in membranes of developing muscle fibers," *J. Cell Biol.* **65,** 335–358.
4. Paglin, S. & Prives, J. (1975) "Regulation of elaboration of cholinergic receptor, acetylcholinesterase and Na^+,K^+-ATPase during differentiation of embryonic chick skeletal muscle *in vitro*," (Abstract) *EMBO Muscle Workshop*, Rehovot, Israel.
5. del Castillo, J. & Katz, B. (1955) "On the localization of acetylcholine receptors," *J. Physiol. (London)* **128,** 157–181.
6. Miledi, R. (1960) "Junctional and extrajunctional acetylcholine receptors in skeletal muscle fibers," *J. Physiol. (London)* **151,** 24.
7. Axelsson, J. & Thesleff, S. (1959) "A study of supersensitivity in denervated mammalian skeletal muscle," *J. Physiol. (London)* **147,** 178–193.
8. Chang, C. C. & Tung, L. H. (1974) "Inhibition by actinomycin D of the generation of acetylcholine receptors induced by denervation in skeletal muscle," *Eur. J. Pharmacol.* **26,** 38.
9. Fambrough, D. M. (1970) "Acetylcholine sensitivity of muscle fiber membranes; mechanism of regulation by motoneurons," *Science* **168,** 372–373.
10. Grampp, W., Harris, J. B. & Thesleff, S. (1972) "Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis," *J. Physiol. (London)* **221,** 743–754.
11. Miledi, R. & Potter, L. T. (1971) "Acetylcholine receptors in muscle fibers," *Nature* **233,** 599–603.
12. Brockes, J. & Hall, Z. (1975) "Synthesis of acetylcholine receptor by denervated rat diaphragm muscle," *Proc. Nat. Acad. Sci. USA* **72,** 1368–1372.
13. Hartzell, H. C. & Fambrough, D. M. (1973) "Production of acetylcholine receptors and incorporation into membranes of developing muscle fibers," *Dev. Biol.* **30,** 153–165.
14. Hüttermann, A. & Guntermann, V. (1975) "Metrizamide in D_2O—A novel solute for the separation of labeled and unlabeled proteins by density gradient centrifugation," *Anal. Biochem.* **64,** 360–366.
15. Meunier, J. C., Sealock, R., Olsen, R. & Changeux, J. P. (1974) "Purification and properties of the cholinergic receptor protein from *Electrophorus electricus* electric tissue," *Eur. J. Biochem.* **45,** 371–394.
16. Rickwood, D. & Birnie, G. D. (1973) "Metrizamide, a new density-gradient medium," *FEBS Lett.* **50,** 102–110.
17. Filner, P. & Varner, J. E. (1967) "A test for *de novo* synthesis of enzymes: Density labeling with H_2O^{18} of barley α-amylase induced by gibberellic acid," *Proc. Nat. Acad. Sci. USA* **58,** 1520–1526.