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Ascorbic acid mediates acetylcholine receptor increase induced by brain extract on myogenic cells

(acetylcholine receptor supersensitivity/vitamin C/neural factor/trophic substances/denervation)

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ABSTRACT **Extracts of fetal calf brain cause a 3- to 5-fold increase in acetylcholine receptors (AcChoR) on cultured myogenic L_5 cells. Purification of the substance causing the major portion of this receptor increase has been completed. Ultraviolet spectral characteristics, nuclear magnetic resonance, mass spectra, and AcChoR induction by the active factor are the same as those of commercially available ascorbic acid. The biological activity of ascorbic acid is not mimicked by reducing agents with or without sulfhydryl groups. Compounds related to ascorbic acid were tested for their ability to induce AcChoR increases on L_5 cells. D-Isoascorbic acid is the only substance with identical biological activity to ascorbic acid. Dehydroascorbic acid and ascorbic acid 2-*O*-sulfate also induce AcChoR increases but with lower specific activity. These data show that ascorbic acid can play a role in regulating AcChoR expression in myogenic tissue, and the presence of ascorbic acid in the purified fraction from fetal calf brain accounts for its ability to increase AcChoR in L_5 cells.**

A major effect of coculturing rat spinal cord explants with L_6 myogenic cells is an increase in the average site density of acetylcholine receptors (AcChoR) on the differentiated myotubes. The increase in receptor site density on myotubes adjacent to the explant is greater than that occurring on myotubes a few millimeters away. This graded distribution has been taken as an indication of the release, from the explant, of a diffusible substance causing the receptor increase (1). Similar results have been obtained in cocultures derived from chicken embryos (2). In addition to the increase in receptor site density, there is also an increase in the number of AcChoR clusters in these cultures (1, 2).

Since it appears that the effects on AcChoR clustering and site density may be caused by different substances (3), in the present study we directed our attention toward the overall increase in AcChoR. This effect is of interest because changes in average AcChoR site density also occur during muscle development *in vivo* (4). Shortly after the fusion of mononucleated myoblasts, AcChoR levels increase dramatically (5, 6), and then decrease following innervation (7, 8). Denervation of adult skeletal muscle results in a return to preinnervation site densities (9–11). The purification and identification of a soluble factor capable of inducing AcChoR site density increases offers an approach to the study of *in vivo* AcChoR site density regulation and, possibly, the control of AcChoR synthesis as well.

The effects of nerve explants on AcChoR site density in myogenic tissue can be mimicked by soluble extracts of fetal rat, chicken, or calf brain (1, 12–14). We have used the increase in total AcChoR on L_5 cells caused by fetal calf brain extracts (14) as an assay for the purification of the active factor. The L_5 (46) cell line has similar characteristics to the L_6 cell line isolated by Yaffe (15). We report here the identification of the material from fetal calf brain extracts, which is largely responsible for causing the increase in AcChoR, as L-ascorbic acid. Since others have shown that L-ascorbic acid accumulates in skeletal muscle following denervation, we suggest that ascorbic acid may be involved in regulating AcChoR synthesis *in vivo*.

MATERIALS AND METHODS

Cell Culture. L_5 cells were suspended in 0.25% "pancreatin" in Dulbecco's modified Eagle's medium (DME medium). Cells were diluted to 5×10^4 cells per ml in DME medium containing 10% heat-inactivated fetal calf serum, and 0.5 ml was plated into individual wells of Falcon multiwell tissue culture plates (2.0 cm^2 = growth area). Fractions for assay were sterilized through 0.45-μm filters (Gelman) and added to triplicate wells. One dose of the sample was added per day for 2 consecutive days beginning either day 4 or 5 after plating. Cultures were assayed for α-bungarotoxin (α-BGT) binding on day 3 after the first dose. This addition schedule was previously determined to be optimal in eliciting the AcChoR response in L_5 cells (14). Fractions containing 0.1% 2-mercaptoethanol were always lyophilized prior to addition to cell cultures in order to decrease 2-mercaptoethanol concentration. Lyophilized fractions were resuspended in H_2O or H_2O containing fresh 0.05% 2-mercaptoethanol prior to assay. Volumes of fresh 0.05% 2-mercaptoethanol that were less than 15 μl did not affect cell cultures or the AcChoR response.

Binding of Iodinated α-BGT. α-BGT iodinated with ^{125}I (^{125}I-α-BGT) was prepared in our laboratory (16) and used to label cultures. Specific activities from 15 to 300 Ci/mmol were used (1 Ci = 37 GBq). Cells were labeled for 30 min with 20 nM ^{125}I-α-BGT in Tyrode's buffer containing bovine serum albumin at 2 mg/ml (buffer A). The labeled α-BGT was aspirated off, and the cells were washed in buffer A for a minimum of five 2-ml rinses over the course of 30 min. The buffer A was then aspirated, and the cells were dissolved in 1.5 ml of 2.0 M NaOH overnight. The dissolved material was harvested by inserting a folded 1.3-cm no. 2 dental roll (Johnson & Johnson) folded side up into each well and allowing a minimum of 6 min for absorption to occur. The dental rolls were then placed in gamma counter vials and assayed on a gamma counter. The total cpm bound for a given treatment was calculated as the mean of cpm bound for triplicate determinations. Nonspecific binding was calculated for each multiwell plate by preincubating triplicate wells with unlabeled 0.1 μM α-BGT for 15 min prior to assay and determining the mean cpm bound per well. Specific binding was determined by subtracting the mean nonspecific binding for the respective dish from the mean total binding for a given determination. Standard errors (SEM) for specific α-BGT binding were calculated from the square root of the sums of squares

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Abbreviations: AcChoR, acetylcholine receptor; α-BGT, α-bungarotoxin.

of the standard errors for the nonspecific and total binding.

Percentage Increase Calculation. The specific α-BGT binding for cultures receiving a particular treatment is expressed relative to controls as the percentage increase: % increase = [(Exp/Ctl) − 1] × 100, where Exp equals the mean specific cpm bound for treated cultures and Ctl equals the mean specific cpm bound for the respective untreated controls. Associated relative errors were calculated as the square root of the sums of squares of the relative error for controls and experimentals.

Active Fraction Purification. Fetal calf brain was dissected, weighed, and homogenized in 0.2 M NH_4OAc (pH 4.0; ≈0.8 gm/ml) on site at Taylor Packing (Wyalusing, PA). The homogenized tissue was extracted for 2 hr on ice and centrifuged at 22,000 × *g* for 40 min. The supernate was lyophilized, and the lyophilized material was resuspended in H_2O containing 0.01% 2-mercaptoethanol in one-quarter of the supernate volume and was centrifuged at 100,000 × *g* for 1 hr. The supernate was decanted and lyophilized. The equivalent of 10 ml of this supernate was resuspended in 6 ml of 2.0 mM NH_4OAc (pH 4.0), centrifuged at 10,000 × *g* for 15 min, and the supernate was loaded onto a 418-ml (2.6 × 78.8 cm; 60 ml/hr) Sephadex G-15 column in the same buffer. All column buffers contained 0.1% 2-mercaptoethanol and were flushed with N_2 for at least 1 hr prior to fractionation. Active fractions from all columns were flushed with N_2 for at least 10 min prior to lyophilization. Activity was eluted as a single peak at partition coefficient K_{avg} = 0.54. The active fractions were pooled, lyophilized, resuspended in 3 ml of 0.05 M NH_4OAc (pH 6.0), and loaded onto a 44-ml (1.5 × 25 cm) Bio-Rad AG1-X8 anion-exchange column. The activity was step-eluted from the column in 1.4 M NH_4OAc (pH 6.0) at 32 ml/hr. The activity was in the major UV absorbing peak (265 nm). The peak fractions were pooled and lyophilized. This pooled fraction was loaded onto a 75-ml (2 × 24 cm) Sephadex G-10 column in 1.5 ml of running buffer [2.0 mM NH_4OAc (pH 6.0); 30 ml/hr] and was eluted at K_{avg} = 0.28 as the first peak of a doublet. The active fractions were pooled and lyophilized. The lyophilized material was resuspended in 1.5 ml of 0.05 M NH_4HCO_3 (pH 8.7) and loaded onto a 37.6-ml (17.8 cm × 1.6 cm; 24 ml/hr) DEAE-Sephadex column equilibrated with the same buffer. One column volume of the running buffer was run prior to gradient elution (0.05–0.2 M; 300 ml) at 0.08 M NH_4HCO_3. The major UV absorbing peak was pooled, divided into aliquots, flushed with N_2, and lyophilized.

Mass Spectrometry. Mass spectrometry was done at the Cornell University Chemistry Department facility. Side-by-side derivatizations were carried out with 1 mg of L-ascorbic acid (ICN) in 0.3 ml of silylating mixture (Tri-Sil; Pierce) and ≈450 μg of the purified fraction in 0.1 ml of the silylating mixture; 1.8 μl of each were injected into a Finnigan 3300 GC/mass spectrometer. GC stationary phase was 3% OV101 (Alltech), and the column temperature gradient was 80–250°C in 30 min. Samples were ionized by electron impact. (Spectra obtained with chemical ionization were identical for ascorbate and the purified fraction.)

NMR Spectroscopy. ^3H NMR spectra were obtained at 300 MHz with a Bruker WM-300 spectrometer at the Cornell University Department of Chemistry. Samples were ≈0.5 mg/ml of ^2H_2O (Aldrich). Data were obtained at 27°C in the Fourier transform mode by using a *ca.* 60° pulse width and a 2.7-sec relaxation time. Data manipulation included exponential multiplication of the free induction decay, which corresponded to 0.1 Hz of line-broadening after Fourier transformation. Spectra were referenced externally to 1 mg of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) per ml of ^2H_2O. Homonuclear decoupling experiments were performed in the usual manner.

Thin-Layer Chromatography. Chromatography was performed on silica gel (Merck) coated glass plates (≈7 × 2 cm). The two solvent systems used were (*i*) 2-propanol/2-butanone/1 M HCl, 12:3:5 (vol/vol), developed with phosphomolybdic acid, and (*ii*) *sec*-butanol/formic acid/H_2O, 15:3:2 (vol/vol), developed with vanillin/sulfuric acid (1 g of vanillin per 100 ml of 50% H_2SO_4).

Protein Determination. Protein was determined by the method of Lowry *et al.* (17).

Ascorbic Acid Analogues and Other Compounds. Compounds tested for biological assay were prepared in H_2O at the concentrations indicated. Compounds and their sources are as follows: Dehydroascorbic acid (Pfaltz & Bauer; Stamford, CT); D-gulono-γ-lactone, L-ascorbic acid 2-*O*-sulfate, D-isoascorbic acid, 2-mercaptoethanol, dithiothreitol, glutathione, L-cysteine, and EDTA (Sigma); ferrous sulfate (Baker); ferric chloride and ferric ammonium sulfate (Mallinckrodt).

RESULTS

Under our assay regime, 0.2 M NH_4OAc (pH 4.0) extracts of fetal calf brain induced a maximum increase in ^{125}I-α-BGT binding on L_5 cells of 306% ± 35% (SEM; n = 14) as compared to untreated controls. Highly purified fractions from the extract (for purification scheme, see *Materials and Methods*) induced maximal AcChoR increases of 220% ± 71% (SEM; n = 3).

Amino acid analysis failed to reveal appreciable quantities of amino acids in the purified fraction. These results were confirmed by NMR spectroscopy, which revealed no significant peptide bond resonances. The purified fraction was not volatile and could not be analyzed by solid-probe mass spectrometry because of decomposition. In order to obtain a mass spectrum, we derivatized the purified fraction. Silylated derivatives were analyzed by GC/mass spectrometry. The mass spectrum observed was found to be identical to similarly derivatized ascorbic acid (Fig. 1). This suggests that the purified fraction is structurally similar to ascorbic acid. Since functional groups such as 2-*O*-sulfate (18) can be displaced by derivatization and side chain isomers might not be obvious in the mass spectra, we used ^1H NMR spectroscopy to confirm the structure of the fraction. ^1H NMR resonances in deuterium oxide were observed at 3.73, 4.00, and

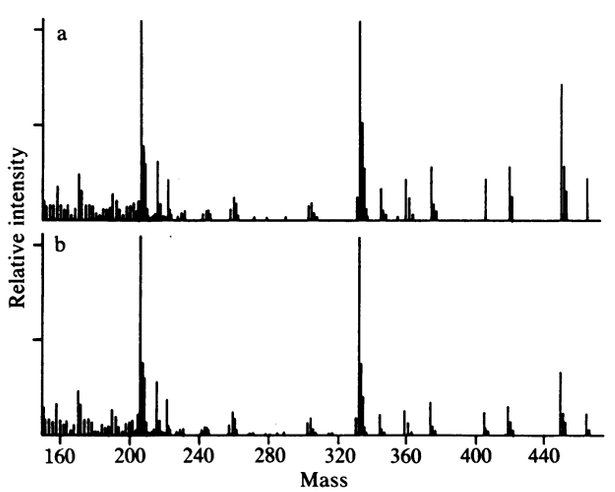

FIG. 1. Mass spectrum of the silylated purified fraction (*a*) and silylated ascorbate (*b*). Relative intensity above mass 380 is magnified ×10. These spectra agree well with published spectra for tetrasilylated ascorbate (18). For illustrative purposes, the relative intensity scales in both panels have been truncated. The peak at mass 205 in both spectra was 100%. *a*, the relative intensity at mass 332 was 62% and at mass 449 was 1.7%; in *b*, the relative intensity at mass 332 was 82% and at mass 449 was 3.5%.

4.50 ppm, corresponding to the C-6, C-5, and C-4 protons, respectively, of L-ascorbic acid at neutral pH (20). The multiplicities and coupling constants of the purified fraction correspond to the published values for L-ascorbic acid (21). We used homonuclear decoupling techniques to eliminate the possibility of ascorbic acid structural isomers with alternative side-chain configurations. These experiments confirmed the 5,6-dihydroxy-substituted side-chain structure of ascorbic acid. The purified fraction ran identically to ascorbic acid on thin-layer chromatography in two different solvent systems (R_f = 0.6) and displayed the same UV spectral properties, including a 265- to 243-nm absorption maximum shift in low-pH solution (22).

The effect of ascorbic acid on α-BGT binding was tested in our assay system and appears to be the same as the purified fraction (Fig. 2), inducing a maximal AcChoR increase of 248% ± 37% (SEM; n = 6). Estimates of the concentrations of the purified fraction using the ascorbic acid extinction coefficient led to close agreement in the dose–response curves of ascorbic acid and the purified fraction (Fig. 2). From these data we calculate a yield of 100–300 μg of ascorbate purified from 1.0 g of wet weight of fetal calf brain. At doses of ascorbic acid that induced a maximal AcChoR response, total protein levels in treated cultures were only 24% ± 2% (range; n = 4) greater than untreated controls. Under these same conditions, levels of the cytoplasmic enzyme creatine kinase remained unchanged. These results were essentially the same for unfractionated extract, the details of which will be published elsewhere. The increase in total cellular protein for both ascorbic acid and crude extracts was more than 1 order of magnitude less than the increase occurring in AcChoR. For this reason AcChoR responses were not normalized to total protein levels.

Ascorbic acid can mediate chemical reductions and chelate metal ions as well. It is possible that AcChoR increases induced by ascorbic acid could be a nonspecific response to its reducing properties. The specificity of the response was tested with a variety of reducing agents, chelators, iron compounds, and ascorbic acid analogues. The results of these experiments and the dose ranges tested are shown in Table 1. Only three of these compounds induced any AcChoR response, and each of the active substances was an ascorbic acid derivative. The dose–response curves of the active substances are given in Fig. 3. D-Isoascorbic acid, an ascorbic acid isomer, had biological activity identical to ascorbic acid. Dehydroascorbic acid and ascorbic acid 2-*O*-sulfate had specific activities 1/5th to 1/10th that of ascorbic acid. When EDTA was added simultaneously with a dose of ascorbic acid that elicits a maximal AcChoR response, the ascorbic acid effect was unchanged. It is concluded that the effect of ascorbic acid is not likely to be a nonspecific result of its properties as a chelator or reducing agent.

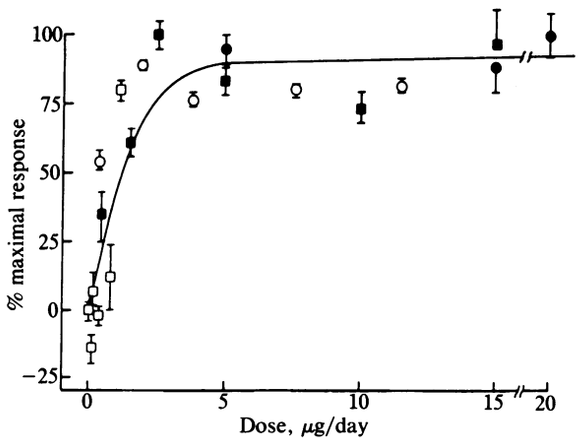

FIG. 2. Dose–response curve of ascorbic acid (●, ■) and the purified fraction (○, □). The normalized AcChoR increase is given as a function of dose. The figure is the composite data from two experiments (experiment 1, ○, ●; experiment 2, □, ■). The concentration of the purified fraction was estimated from its absorption at 265 nm by using an ascorbic acid extinction coefficient of 8.46×10^3 liter·mol^{-1}·cm^{-1}, determined in our own laboratory. The percentage of maximum response represents the normalization of specific α-BGT binding to the maximal response to ascorbic acid in each experiment, after first subtracting the respective control values. Thus, 0% increase represents no change above control levels, and 100% increase is the maximal response induced by commercial ascorbate in each experiment. The maximal increase in α-BGT binding induced by ascorbate, relative to controls (% increase), in the two experiments was: experiment 1, 209% ± 9%; experiment 2, 262% ± 14% (for calculation, see *Materials and Methods*). Each data point and associated error bars in the figure are derived from the mean and SEM of triplicate determinations of specific α-BGT binding.

DISCUSSION

L_5 cells and other myogenic cells divide and differentiate in the absence of exogenously applied ascorbic acid. We have demonstrated that serum level concentrations (23) of ascorbic acid (10 μg/ml) in the growth medium can significantly increase AcChoR levels on L_5 cells *in vitro*. This induction of AcChoR by ascorbic acid is a relatively specific effect. If it were due to a generalized growth, fusion, or survival effect of ascorbic acid on L_5 cells, then total protein and the activity of the muscle-specific cytoplasmic enzyme creatine kinase should increase similarly instead of remaining virtually unaffected.

Ascorbic acid is present at 100–500 μg/g of brain tissue (wet weight) in adult vertebrates (23). It is believed that in fetal and newborn animals the levels are somewhat higher (24–26). Thus, aqueous brain extracts contain sufficient amounts of ascorbic acid to account for their ability to induce AcChoR accumulation *in vitro*. Although the differences in the magnitudes of the responses induced by both the purified fraction and ascorbic acid are not statistically different from unfractionated extracts, it remains possible that ascorbic acid does not account for all of the activity in brain extracts. Because the means of both responses are substantially lower than that of the unfractionated extract and there is a fair amount of variability from experiment to experiment (for an example of this variation, see the percentage increases in the legend of Fig. 2), such a conclusion must await direct tests involving selective depletion of ascorbic acid from unfractionated extracts.

Using extraction procedures identical to those used for fe-

Table 1. Compounds failing to increase AcChoR levels

Class of compound	Compound	Concentration range tested*, μM
Sulfhydryl reducing agents	Cysteine	13–392
	Glutathione	11.3–340
	Dithiothreitol	11.5–346
	2-Mercaptoethanol	10–400
Non-sulfhydryl-reducing agents	Dihydroxyfumaric acid	11.3–340
	Maleic acid	114–3400
Iron salts	Ferric ammonium sulfate	0.2–60
	Ferric chloride	10–70
	Ferrous sulfate	10–70
Other	EDTA	9.2–275
	D-Gulono-γ-lactone	12.7–381

All compounds were tested according to the standard AcChoR assay regime.

*The concentration range of the compound in the medium after one dose is given.

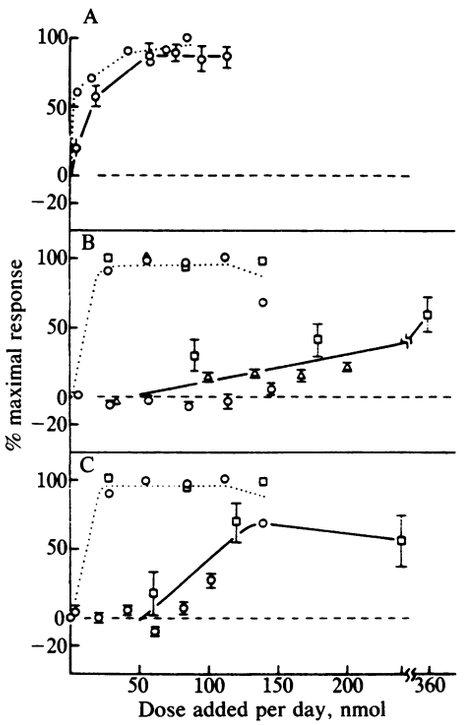

FIG. 3. Dose–response curves of ascorbic acid analogues. The normalized AcChoR increase is given as a function of dose. (*A*) D-Isoascorbic acid. (*B*) Dehydroascorbic acid. (*C*) Ascorbic acid 2-*O*-sulfate. Data were from a number of experiments normalized as in Fig. 2. The dotted lines and symbols without error bars represent the responses to ascorbic acid. The differently shaped symbols are data points corresponding to data from separate experiments. (For purposes of comparison to Fig. 2; 100 nmol of ascorbic acid is equivalent to 17.6 μg.)

tal brain, we have assayed homogenates of fetal calf liver, spleen, and kidney (unpublished results). No ability to induce AcChoR accumulation was detected in any of these tissues. Others have found similar tissue specificity for AcChoR-inducing activity (1, 12). Ascorbic acid is, however, present in the liver and spleen at levels equals to or exceeding those of brain (23), and extracts of these tissues might be expected to induce ascorbic acid-like effects. It is significant that others have reported similar differences between liver extracts and ascorbic acid when added to myogenic cells *in vitro*. Kalcheim *et al.* (27) have found that, although both ascorbic acid and fetal brain extracts induce a doubling of the number of AcChoR clusters on myotubes, liver extracts do not show this effect. Taken together, these findings suggest the presence of substances in the crude extracts that affect the stability, extraction, or biological activity of ascorbate. Such substances may be differentially distributed among the tissues we have studied so that the biological activity merely appears to be brain specific.

Our initial attempts at purification of the AcChoR-inducing activity from fetal calf brain by a number of methods led to low and inconsistent recovery of activity. We attempted to further purify some of these low-activity fractions with poor results. This result is explained by the fact that ascorbic acid is susceptible to rapid oxidation. The rate is inversely related to concentration with a $t_{1/2}$ at 0.6 mM = <4 hr (28). When 0.1% 2-mercaptoethanol, a reducing agent, was introduced into all steps of the purification, activity comparable to that of the crude extract was consistently recovered as a single peak. Under these conditions we observed no other fractions with comparable activity.

Buc-Caron *et al.* (29), using substantially different assay conditions with reducing agents only in their extraction buffer, reported AcChoR synthesis-promoting activity from embryonic chicken brain is likely to be due to one or more small peptides. This activity was retained on reversed-phase HPLC in trifluoroacetic acid and was eluted in acetonitrile. Neither ascorbic acid (30) nor our activity was appreciably retained on reversed-phase HPLC in the absence of ion-pairing agents. Thus, it is unlikely that ascorbic acid would be present in the partially purified fraction described by Buc-Caron *et al.*

The AcChoR increase is specific to ascorbic acid and some closely related analogues. None of the reducing agents tested affected AcChoR levels. These results imply that the biological activity of ascorbic acid is not likely to be the result of the reduction of some medium component, such as a specific metal ion. Furthermore, addition of reduced iron resulted in no AcChoR increase. Ascorbic acid also can chelate ions such as Ca^{2+} and Mg^{2+} (28). EDTA chelates these cations as well but did not induce an AcChoR increase, indicating that the removal of an ion by chelation does not explain the biological activity of ascorbic acid.

Some of the other compounds tested were chosen because they had more specific similarities to ascorbic acid. Like ascorbic acid, dihydroxyfumaric acid is a nonsulfhydryl reducing agent, and has reactivity similar to that of ascorbic acid in a number of other chemical reactions (31), but it did not induce an increase in AcChoR. L-Gulono-γ-lactone is a structural analogue to ascorbic acid lacking only the ring double bond. Likewise, it had no activity in the AcChoR assay. The compounds that did show biological activity were dehydroascorbic acid, ascorbic acid 2-*O*-sulfate, and D-isoascorbic acid. Each of these molecules has been shown by others to have biological activity equivalent to ascorbic acid, at least in some systems (32–34). Although D-isoascorbic acid does not compete well with L-ascorbic acid for tissue uptake *in vitro* (23), it has been shown to be an effective antiscorbutic agent on its own (33). D-Isoascorbic acid also mimics the biological activity of ascorbic acid on fibroblasts *in vitro* (34). The antiscorbutic effects of ascorbic acid 2-*O*-sulfate and dehydroascorbic acid have been attributed to the existence of cellular systems or enzymes capable of converting them to ascorbic acid (31–33, 35–37). Thus, the activity of these compounds in our assay system is consistent with ascorbic acid being a specific effector of AcChoR increases on L_5 cells.

The molecular mechanism by which ascorbic acid affects the accumulation of AcChoR is not known. Ascorbic acid could have a direct effect on any of a number of steps in the AcChoR biosynthetic pathway. These include effects on synthesis, degradation, processing, packaging, or transport. Alternatively, ascorbic acid may act indirectly, affecting other cellular processes, which in turn affect AcChoR. One such well-known ascorbate-sensitive process is collagen synthesis. Ascorbate is a cofactor in the post-translational hydroxylation of collagen (38). Kalcheim *et al.* (27) have reported that, in rat primary muscle cell cultures, along with the induction of AcChoR clustering, ascorbic acid stimulates the synthesis of collagen. It is possible that the induction of AcChoR accumulation that we have observed was a secondary result of a stimulation of collagen synthesis or hydroxylation. Other indirect mechanisms aside from collagen effects are also possible because ascorbate is involved in a number of additional intracellular processes including lipid metabolism (39), peptide amidation (40), and neurotransmitter synthesis (41, 42). Ascorbic acid also has been implicated in specific muscle metabolic states (43) and inhibits calcium uptake by purified mitochondria (44).

Ascorbic acid levels in skeletal muscle tissue *in vivo* are generally quite low [<50 μg/gm of tissue (wet weight) (23)]. In rat skeletal muscle, ascorbic acid levels have been shown

to increase by 25% within the first 36 hr after denervation, leading to a doubling of ascorbic acid levels by day 5 (45). Similar results have been obtained in frogs (19) and in guinea pigs (unpublished results). This change occurs concomitantly with a dramatic increase in AcChoR synthesis (9, 10). In view of these observations, our results suggest ascorbic acid uptake may possibly be a causative agent in denervation-induced AcChoR synthesis in skeletal muscle.

The authors acknowledge that the identification of the purified fraction was facilitated by a personal communication from Dr. Z. Vogel to M. Salpeter, which prompted the comparison to commercial ascorbic acid. We also thank Kris Holley for her help with tissue culture and other aspects of this work; Irving Shen for his contribution to the purification as well as creatine kinase assays; Guy Monteleone for assistance and advice concerning NMR spectroscopy; Dr. Jim Gloer for help in the derivatization and organic chemistry of the purified fraction; and Dr. Miriam Salpeter, Dr. Ron Harris-Warrick, and Tami Levitt-Gilmour for helpful discussions. This work was supported in part by National Institutes of Health Grant NS14679 and National Institutes of Health Predoctoral Training Grant GM07469 and was submitted to Cornell University as a portion of D.K.'s dissertation in partial fulfillment of the requirements for his doctorate.

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