Differentiation of Axon-related Schwann Cells
In Vitro. I. Ascorbic Acid Regulates Basal Lamina
Assembly and Myelin Formation

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Abstract. Rat Schwann cells cultured with dorsal root ganglion neurons in a serum-free defined medium fail to ensheathe or myelinate axons or assemble basal laminae. Replacement of defined medium with medium that contains human placental serum (HPS) and chick embryo extract (EE) results in both basal lamina and myelin formation. In the present study, the individual effects of HPS and EE on basal lamina assembly and on myelin formation by Schwann cells cultured with neurons have been examined. Some batches of HPS were unable to promote myelin formation in the absence of EE, as assessed by quantitative evaluation of cultures stained with Sudan black; such HPS also failed to promote basal lamina assembly, as assessed by immunofluorescence using antibodies against laminin, type IV collagen, and heparan sulfate proteoglycan. The addition of EE or L-ascorbic acid with such HPS led to the formation of large quantities of myelin and to the assembly of basal laminae. Pretreatment of EE with ascorbic acid oxidase abolished the EE activity, whereas trypsin did not. Other batches of HPS were found to promote both basal lamina and myelin formation in the absence of either EE or ascorbic acid. Ascorbic acid oxidase treatment or dialysis of these batches of HPS abolished their ability to promote Schwann cell differentiation, whereas the subsequent addition of ascorbic acid restored that ability. Ascorbic acid in the absence of serum was relatively ineffective in promoting either basal lamina or myelin formation. Fetal bovine serum was as effective as HPS in allowing ascorbic acid (and several analogs but not other reducing agents) to manifest its ability to promote Schwann cell differentiation. We suggest that ascorbic acid promotes Schwann cell myelin formation by enabling the Schwann cell to assemble a basal lamina, which is required for complete differentiation.

In the peripheral nervous system, the Schwann cell (SC) surrounds axons either with simple cytoplasmic ensheathment or with myelin, forming SC-axon "units" comprised of either up to 20 nonmyelinated axons or one myelinated axon per SC (for reviews see Peters et al., 1976; Thomas and Ochoa, 1984). Surrounding all SC-axon units is an endoneurial extracellular matrix, consisting of a basal lamina closely apposed to the surface of the SC and an interstitial matrix rich in collagen fibrils (Thomas and Olsson, 1984). Even during early embryogenesis SCs of developing peripheral nerves are found in contact with a basal lamina (Cravioto, 1965; Gamble, 1966; Ochoa, 1971), and the subsequent phases of SC differentiation (proliferation, axon sorting, and ensheathment and myelin formation) also occur with the SCs in intimate association with their basal lamina (Martin and Webster, 1973; Webster et al., 1973).

Tissue culture techniques that allow the purification and recombination in vitro of the different cell types of the rat peripheral nervous system have been used to study the cellular and noncellular requirements for the different phases of SC differentiation. It has been shown that the neuron regulates SC proliferation by means of an axonal surface mitogen (Salzer et al., 1980) and that the presence of neurons, but not fibroblasts, is necessary for SCs to assemble a complete extracellular matrix consisting of a basal lamina and collagen fibrils in vitro (Bunge et al., 1980; Bunge et al., 1982a), a requirement possibly mediated by neuronal regulation of SC type IV collagen expression (Carey et al., 1983). Additional, nonneuronal influences are also required for complete SC differentiation in vitro. SCs growing on axons in serum-free defined medium (DM) proliferate in response to the axonal surface mitogen, yet fail to ensheathe or myelinate the axons, even if maintained in this medium for several months. Under these conditions SCs also do not form an ultrastructurally detectable basal lamina (Moya et al., 1980), although they do accumulate some laminin on their surfaces (Cornbrooks et al., 1983). Replacement of DM with standard myelination-promoting medium, containing human placental serum (HPS) and chick embryo extract (EE), results in rapid SC differentiation, with detectable morphological changes within 24 h
(Carey and Bunge, 1981) and the occurrence of both myelin formation and basal lamina formation within 1 wk (Moya et al., 1980). This correlation between basal lamina formation and SC differentiation has led to the hypothesis that the formation of a basal lamina is a prerequisite for full SC differentiation (Bunge et al., 1986).

Such a correlation has also been observed for SCs in other situations, both in vitro and in vivo. When cultures of neurons and SCs are grown in standard myelinating medium containing the proline analog cis-hydroxyproline, which interferes with the secretion of many proteins, including collagens, by SCs (Elbridge et al., 1983), the formation of both basal lamina and myelin is disrupted (Copio and Bunge, 1980). Castanospermine, an inhibitor of N-linked oligosaccharide processing, similarly inhibits the formation of both basal lamina and myelin by SCs grown with neurons in myelinating medium, while leaving other aspects of differentiation (e.g., neuron-induced proliferation) unaffected (Ratner et al., 1986). SCs of the dystrophic mutant mouse display both ensheathment and myelinization deficits and basal lamina defects, particularly in peripheral nerve root regions (Bradley and Jenkinson, 1973; Madrid et al., 1975); the ensheathment and myelinization deficits are less marked in roots recovered from crush injury, which causes increased endoneurial extracellular matrix accumulation and a substantial improvement in the basal lamina defects (Bray et al., 1983).

In an attempt to better understand the connection between basal lamina formation and SC differentiation, we have investigated the individual effects of serum and chick EE supplementation to DM on basal lamina formation and on myelin formation by SCs cultured with sensory neurons. Our results demonstrate the central role of ascorbic acid in allowing full SC differentiation in culture and the remarkable linkage between basal lamina assembly and myelin formation. Preliminary reports of this work have appeared in abstract form (Elbridge et al., 1984, 1985).

Materials and Methods

Cell Culture

SC-neuron cultures were prepared using modifications of previously published methods (Bunge et al., 1983). Briefly, dorsal root ganglia from 15-d embryonic Sprague-Dawley rats were dissociated with trypsin and plated on rat tail collagen-coated Aclar coverslips housed in Aclar minidishes (Bunge and Wood, 1973). The cultures were then exposed to three 2-3-d pulses of 10^{-5} M 5-fluorodeoxyuridine in HPS-containing medium, grown in DM (see below) for 7 d, seeded with 10-20 \times 10^5 purified SCs, and maintained in DM for an additional 4 wk before initiating DM supplementation experiments.

DM Supplementation

The serum-free DM used in these experiments was essentially the N2 medium (Bottenstein and Sato, 1979) with nerve growth factor added previously (Moya et al., 1980; Bunge et al., 1982b) but with rat transferrin (10 \mu g/ml; Jackson ImmunoResearch Laboratories, Avondale, PA) instead of human transferrin, and with purified (2.5S) nerve growth factor (100 ng/ml; a gift from Dr. E. Johnson; Bocchini and Angeletti, 1969) instead of crude (7S) nerve growth factor. DM also contained bovine insulin (5 \mu g/ml, proguanil (20 nM), putrescine dihydrochloride (100 \mu M) (all from Sigma Chemical Co., St. Louis, MO), and sodium selenite (30 nM; Pfaltz & Bauer, Inc., Stamford, CT); the total protein concentration was 15.1 \mu g/ml.

Serum used for supplementation was HPS prepared from blood obtained locally or, where indicated, PBS (HyClone Laboratories, Logan, UT) which was supplied with an analysis of the concentrations of some components (i.e., serum albumin, transferrin). In experiments where dialyzed serum was used, the serum was dialyzed aseptically against three changes of 20 vol of a 1:1 mixture of Hanks' F-12 and Dulbecco's MEM (the salt base of DM) at 4°C in 36 h; nondialyzed serum was stored at 4°C for the same period of time.

Chick EE (Paul, 1970) was used at 10 or 15% (vol/vol). In some experiments, chick EE was pretreated as follows. Chick EE was incubated with different concentrations of ascorbic acid oxidase (Sigma Chemical Co.) at pH 7.0, 25°C, or with 1 mg/ml trypsin (TRL3; CooperBiomedical, Inc., Malvern, PA) at pH 8.0, 35°C, for 1 h. Chick EE ultratritrates were then obtained by centrifuging the pretreated samples in Centricon 10 microcentrators (10 K, nominal cutoff; Amicon Corp., Danvers, MA) at 5,000 g, 4°C, for 5 h, and the ultratritrates were filter sterilized and used to prepare supplemented media. Fetuin (type III, Sigma Chemical Co.; "Pederson fetuin") was made as a 20 mg/ml stock solution in DM salt base and used at a final concentration of 1 mg/ml. A fresh 5 mg/ml stock solution of L-ascorbic acid (Sigma Chemical Co.) in distilled water was made immediately before each feeding and used at 1% for a final concentration of 280 \mu M (50 mg/ml), except as otherwise noted. Similarly, 100x stock solutions of d-isoascorbic acid, L-ascorbic acid 2-sulfate, reduced glutathione (all from Sigma Chemical Co.), and dihydroxyfumaric acid (Pfaltz & Bauer, Inc.) were made in distilled water before each feeding. A 100x stock solution of dehydroascorbic acid was made before each feeding by inverting Mo-dehydroascorbic acid (Pfaltz & Bauer, Inc.) at 60°C for 1.5 min in distilled water which had been acidified with CO2 to pH 4 to obtain the water-soluble monomer. When necessary, media were filter sterilized using low-protein-binding 0.22 \mu m Millex-GV filters (Millipore Corp., Bedford, MA). Filter sterilization of DM or supplemented DM did not adversely affect the results.

Ascorbic Acid Assay

The 2, 6-dichlorophenolindophenol method of Orme et al. (1979) was used exactly as described, except p-chloromercuribenzoate was omitted after determining it had no effect on assay results. L-Ascorbic acid was used as a standard.

Culture Analysis

Duplicate cultures were fed DM or various supplemented DMs for 2 wk (six feedings) and then analyzed as described below. Cultures were analyzed for basal lamina and myelin formation by cutting each coverslip in half, bisecting the area containing all of the neuronal somata, and processing one half for basal lamina and myelin formation by cutting each coverslip in half, bisecting the area containing all of the neuronal somata, and processing one half for immunocytochemistry using antibodies to basal lamina components. In this way, for each medium, two independent samples were obtained for myelin quantitation and for immunocytochemistry. Experiments were performed a minimum of two, and typically three or more, times; representative experiments are presented in the tables and figures.

Myelin Quantitation

A modification of a previously published whole mount fixation and Sudan black staining procedure (Wood, 1976) was used to enhance the contrast between darkly staining myelin segments formed by myelinating SCs and lightly staining nonmyelinating SCs. Culture halves were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed with 0.1% OsO4 in 0.1 M phosphate for 1 h at room temperature, stained with 0.5% Sudan black (Fisher Scientific Co., St. Louis, MO) in 70% ethanol for 1 h, destained, and mounted in glycerin jelly.

Quantitation of myelin formation was accomplished by counting the number of myelin-associated SC nuclei ("myelin segments") per field in 60 high power fields (area = 0.17 mm2) for culture half. The fields were arrayed at 1-mm intervals as a 6 \times 10 mm grid that covered the same area of each culture. The density of myelin in fields containing more than \approx 60 segments made accurate counting impossible; such fields were scored as 60 for calculating the average number of myelin segments per field. The values presented in Tables I-VI represent the average number of myelin segments per field for the duplicate cultures, normalized to 1 mm2. Thus at higher values the average segments per mm2 was not linearly proportional to the actual number of segments per mm2, and the maximum possible average was 350.
**Immunocytochemistry**

Rabbit antisera to mouse laminin (Timpl et al., 1979) and to the NCI domain of mouse type IV collagen (Weber et al., 1984) were gifts from Dr. R. Timpl; they were found to be monospecific by immunoprecipitation from metabolically labeled conditioned medium of myelinating SC-neuron cultures and of rat Reichert's membrane organ cultures (see Eldridge et al., 1986 for methods). Mouse monoclonal antibody B3 ascites fluid to rat basal lamina heparan sulfate proteoglycan (HSPG; Eldridge et al., 1986) was a gift from Dr. C. Cornbrooks. Affinity-purified rabbit antibody to the large, low-density basal lamina HSPG from mouse Engelbreth-Holm-Swarm sarcoma (Hassell et al., 1985) was a gift from Dr. J. Hassell. Secondary antibodies used were fluorescein conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse (Cappel Laboratories, Malvern, PA); these showed no cross-reactivity with the inappropriate species primary antibodies. Controls for staining used normal rabbit serum and mouse monoclonal antibody ascites fluid to rat antigen not produced in these cultures (antibody CI against muscle synaptic basal lamina; Sanes and Chiu, 1983). All antibody dilutions and culture rinses were done with L-15 containing 10% heat-inactivated horse serum (Gibco, Grand Island, NY), and all procedures were carried out at room temperature except where noted.

To double label two surface/extracellular antigens, live cultures were rinsed and then incubated with rabbit and mouse primary antibodies, diluted (1:100) together, for 30 min. After rinsing, the cultures were incubated with fluorescein anti-rabbit and rhodamine anti-mouse secondary antibodies, diluted (1:100) together, for 30 min. After rinsing the cultures were fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer, rinsed in 0.1 M phosphate, and mounted in glycerol/PBS containing an antibleaching agent (Citifluor Ltd., London). In some cases, where indicated, cultures were permeabilized before staining using 5% acetic acid/95% ethanol for 10 min at -20°C.

Stained cultures were photographed using Tri-X film (Eastman Kodak Co., Rochester, NY) at a nominal ASA of 1600 and developed using Rodinal developer (Agfa-Gevaert, Teterboro, NJ). To allow comparisons of staining intensity, photographs of all cultures stained with a particular primary-secondary antibody pair within an experiment were exposed for the same length of time (typically 3-15 s). Negatives of each set were then printed identically.

**Results**

The 345 SC-neuron cultures used here were generated from dissociated embryonic rat dorsal root ganglion cultures that were first treated with fluoro-deoxy-uridine to eliminate all nonneuronal cells and then seeded at low density with purified SCs. Subsequent maintenance of the cultures in DM allowed the SCs to proliferate in response to an axonal surface mitogen (Salzer et al., 1980) and homogeneously repopulate the axon network, but did not enable the SCs to proceed to complete differentiation (basal lamina formation, myelination). Supplementation of DM was begun ~4 wk after seeding the cultures with SCs, at which time a full complement of SCs had been generated. The cultures were processed for myelin quantitation and for immunocytochemical localization of basal lamina components after 2 wk in supplemented DM.

**Comparison of SC-Neuron Cultures in Defined Medium and Standard Myelinating Medium**

SCs in SC-neuron cultures maintained in unsupplemented DM displayed one of two morphologies, either flattened (Fig. 1 a) or highly rounded (see Fig. 5 in Carey and Bunge, 1981), with the latter morphology associated predominantly with larger neurite fascicles. Most SCs contained prominent perinuclear granules which stained deeply with Sudan black and were particularly evident in flattened SCs (Fig. 1 a). Quantitative evaluation of the Sudan black–stained cultures revealed that myelin had not been formed in DM, as previously reported (Moya et al., 1980), except in one experiment (out of 18) where a few short myelin segments were observed.

When SC-neuron cultures were switched to standard myelinating medium (Eagle's MEM containing 25% HPS, 10% chick EE, and nerve growth factor; Moya et al., 1980), the rounded SCs rapidly elongated along the neurite fascicle and became less prominent, as described by Carey and Bunge (1981). By the end of the 2-wk supplementation period, the perinuclear granules prominent in SCs in DM were no longer detectable in most Sudan black–stained preparations (Fig. 1 b) and very substantial amounts of myelin had been formed. In eight experiments, cultures in standard myelinating medium contained an average of 280 myelin segments/mm² (SEM = 5), whereas cultures maintained in DM contained an average of 0.12 myelin segments/mm² (SEM = 0.1).

The deposition of basal lamina components by SCs cultured with neurons in DM or in standard myelinating medium was dramatically different, as assessed by indirect immunofluorescence of living, nonpermeabilized cultures. Cultures in standard myelinating medium were stained brightly with antibody to laminin in a pattern continuous along SC surfaces (Fig. 2 f), whereas cultures in DM appeared to have little or no staining when identical photo-
Figure 2. Comparison of the deposition of basal lamina components by SCs cultured with neurons in DM (a–d) or for 2 wk in standard myelinating medium (e–h) or DM supplemented with 15% dialyzed FBS plus ascorbic acid (i–l). The two left-hand columns show cultures stained in the living state with rabbit anti-laminin followed by fluorescein goat anti-rabbit, using phase-contrast (a, e, and i) and fluorescein (b, f, and j) optics. The two right-hand columns show cultures doubly stained in the living state with rabbit anti-NC1 and mouse anti-HSPG followed by fluorescein goat anti-rabbit and rhodamine goat anti-mouse, using fluorescein anti-NC1 (c, g, and k) and rhodamine anti-HSPG (d, h, and l) optics. The figures in each column were photographed and printed identically. Bar, 50 μm.

graphic exposures were compared (Fig. 2, b and f). By using exposure times five times longer than those in Fig. 2, however, it was possible to observe that the surfaces of all SCs in DM were specifically stained in a punctate pattern with laminin antibody (Fig. 3 a), as previously reported (Cornbrooks et al., 1983). Staining of SCs in DM by both mouse monoclonal antibody and affinity-purified rabbit antibody to basal lamina HSPG was similar to that of anti-laminin in that staining was punctate (Fig. 3 e) and faint in comparison with cultures grown in standard myelinating medium (compare Fig. 2, d and h). In contrast, rabbit antiserum to the NCl domain of type IV collagen did not stain any extracellular components in DM cultures (Figs. 2 c and 3 c), although the antibody intensely stained cultures grown in standard myelinating medium (Fig. 2 g).

To determine whether SCs in DM were expressing type IV collagen but not depositing it on their surfaces, cultures were permeabilized with acid/alcohol before immunofluorescence staining. As shown in Fig. 3 d, no intracellular type IV collagen was detectable, whereas both laminin (Fig. 3 b) and HSPG (Fig. 3 f) could be localized to the perinuclear region of the SC cytoplasm. Thus, while SCs grown in DM expressed both laminin and the basal lamina HSPG and accumulated small amounts of these molecules on their surfaces, they did not organize them into the linear arrangement characteristic of basal laminae of differentiated SCs in standard myelinating medium.
myelinating medium, and they failed to express or accumulate type IV collagen. These results are consistent with the observation that SCs in DM do not have an ultrastructurally detectable basal lamina (Moya et al., 1980).

**Ascorbic Acid Is a Specific Requirement for Schwann Cell Differentiation**

The mechanism by which standard myelinating medium acts to promote SC differentiation was investigated by manipulations of its complex components, HPS and chick EE. The results indicate that both ascorbic acid and a nondialyzable serum factor are essential for normal basal lamina and myelin formation by SCs.

As shown in Table I, SC-neuron cultures given DM supplemented with HPS at 15% (DM₁₅) for 2 wk accrued essentially no myelin. Such cultures showed little change in morphology from DM cultures and showed the same patterns of staining with basal lamina antibodies as cultures in DM; anti-NC1 did not stain, whereas anti-laminin and anti-HSPG staining was light and punctate (data not shown). The addition of EE to DM₁₅ led to substantial myelin formation by SCs (Table I) and also to the deposition of type IV collagen, laminin, and HSPG in the linear pattern characteristic of cultures in standard myelinating medium. It was found that ascorbic acid could substitute for chick EE. The addition of 50 μg/ml ascorbic acid to DM₁₅ resulted in the formation of myelin in quantities comparable to those obtained in standard myelinating medium (Table I). Furthermore, ascorbic acid, like chick EE, promoted the deposition of type IV collagen, laminin, and HSPG on the SC surface,

**Table I. Ascorbic Acid Mimics the Effects of Embryo Extract on Schwann Cell Differentiation**

| Medium                  | Myelin segments/mm² | Basal lamina |
|-------------------------|---------------------|--------------|
| DM                      | 0                   | -            |
| DM₁₅                    | 0.4                 | -            |
| DM₁₅ + EE10             | 250                 | +            |
| DM₁₅ + 50 μg/ml L-ascorbic acid | 280                 | +            |

For the experiments reported in Tables I–VI, fully repopulated SC-neuron cultures grown in DM were maintained for 2 wk in the medium indicated, and then fixed and stained with Sudan black, and myelin formation was quantitated as described in Materials and Methods. Myelin segments/mm² values in all tables are averages of duplicate cultures for each medium tested. A numerical subscript to DM (e.g., DM₁₅) indicates the percent serum; all serum-containing media in this table were made with HPS. EE followed by a number indicates the percent chick embryo extract.
II) nor the deposition of basal lamina components (Fig. 4a).

The ascorbic acid concentration dependence of basal lamina assembly and of myelin formation was investigated using DM supplemented with FBS which had been dialyzed to remove any endogenous ascorbic acid–like activity. As shown in Fig. 5, a doubling of the ascorbic acid concentration from 8.5 to 17 μM led to a >200-fold increase in the quantity of myelin formed, and led to a change from essentially no basal lamina assembly (8.5 μM; Fig. 5a) to the formation of a basal lamina (17 μM; Fig. 5b) which was very similar to that formed with the standard ascorbic acid concentration of 50 μg/ml (280 μM; Fig. 5c).

Several compounds related to ascorbic acid were tested for effects on basal lamina assembly and on myelin formation, in an attempt to determine whether it might be possible to promote one of these aspects of SC differentiation independently from the other. As shown in Fig. 5, all three ascorbic acid analogs tested were active in promoting myelin formation in DMd15, although they were considerably less active than ascorbic acid, with D-isoascorbic acid ~3–5 times less active and L-ascorbic acid 2-sulfate and dehydroascorbic acid ~20–30 times less active. All these analogs were also active in promoting basal lamina assembly at the higher concentrations that promoted myelin formation (Fig. 5, d–f), but at none of the concentrations of any analog tested were we able to obtain basal lamina formation without myelin formation, or vice versa. Also tested were two other compounds, reduced glutathione and dihydroxyfumaric acid, which share with ascorbic acid the ability to donate electrons to appropriate cellular acceptors. As shown in Fig. 5, neither compound had any myelin-promoting activity over the 1,000-fold concentration range tested, nor were they active in promoting basal lamina assembly (not shown).

Some batches of HPS were found which had the anomalous property of being effective in promoting SC differentiation in the absence of chick EE or exogenous ascorbic acid. In medium made with such HPS, both basal lamina and myelin formation were similar with or without chick EE or ascorbic acid supplements (Table IV). When such HPS was first dialyzed and then used to prepare DMd15, its ability to stimulate SC myelin formation and the deposition of basal lamina components was lost, and could be restored by the addition of either chick EE or ascorbic acid (Table IV). Furthermore, treatment of such HPS with ascorbic acid oxidase abolished its ability to promote myelination and basal lamina formation (Table IV). Finally, samples of HPS were directly resulting in patterns of antibody staining indistinguishable from those observed for cultures grown in standard myelinating medium (not shown).

Medium made with 15% dialyzed HPS (DMd15), like DMd15, promoted neither significant myelin formation (Table II) nor the deposition of basal lamina components (Fig. 4a).

Similarly, the addition of either chick EE or ascorbic acid to DM d15 was sufficient to stimulate maximal myelin formation (Table II) and the deposition of basal lamina components (Fig. 4, b and c). To confirm that ascorbic acid was the component responsible for the effects of chick EE on SC differentiation, chick EE was first treated with ascorbic acid oxidase and then passed through a 10-kD cutoff membrane, and the ultrafiltrate was tested for its ability to promote SC differentiation in DMd15. As shown in Table II, chick EE ultrafiltrate which had been treated with ascorbic acid oxidase lacked the ability to promote substantial myelin formation, whereas treatment with 1 mg/ml trypsin had little effect on such ability. In addition, whereas untreated or trypsin-treated chick EE ultrafiltrate promoted normal basal lamina formation, ascorbic acid oxidase–treated ultrafiltrate did not (Fig. 4, d–f). These results indicate that the active component of EE is ascorbic acid and that in the presence of ascorbic acid no dialyzable serum components are required for SC differentiation. In addition, they clearly illustrate the close correlation between conditions that promote basal lamina assembly and those that promote myelin formation.

Since we wanted to avoid the use of human serum, FBS was tried as a substitute and found to have the same properties as HPS. Medium made with 15% FBS (i.e., DMd15) was unable to promote myelin formation (Table III). The addition of ascorbic acid to FBS DMd15 resulted in myelination which was quantitatively the same as in equivalent HPS-containing medium and in standard myelinating medium. Furthermore, media made with dialyzed FBS had effects identical to their counterparts made with HPS; namely, DMd15 failed to promote myelin formation (Table III) or basal lamina assembly (data not shown), whereas the effects of DMd15 plus ascorbic acid in promoting both myelin formation (Table III) and basal lamina assembly (Fig. 2, j–l) were indistinguishable from those of similar HPS-containing media. In three experiments, FBS DMd15 plus ascorbic acid promoted the formation of an average of 310 myelin segments/mm² (SEM = 15), whereas standard myelinating medium promoted an average of 280 myelin segments/mm² (SEM = 6).

**Table II. Ascorbic Acid Is the Component Responsible for the Effects of Embryo Extract on Schwann Cell Differentiation**

| Medium                              | Myelin segments/mm² | Basal lamina* |
|-------------------------------------|---------------------|---------------|
| DM                                  | 0                   | -             |
| DMd15 + EE15                         | 2.6                 | -             |
| DMd15 + 50 μg/ml L-ascorbic acid     | 250                 | +             |
| DMd15 + EE15 ultrafiltrate, no pretreatment | 6.0                 | -             |
| DMd15 + EE15 ultrafiltrate, pretreated with 0.05 U/ml ascorbic acid oxidase | 230                  | +             |

SC-neuron cultures were grown and analyzed as described in Table I. A numerical subscript to DM preceded by d (e.g., DMd15) indicates that serum dialyzed as described in Materials and Methods was used; all serum-containing media in this table were made with HPS. Pretreatment and ultrafiltration of chick EE were performed as described in Materials and Methods. Chick EE treated with 0.5 and 5 U/ml ascorbic acid oxidase stimulated the formation of 3.2 and 4.6 myelin segments/mm², respectively. Other abbreviations used are given in Table I.

* Basal lamina formation in these media is illustrated in Fig. 4.

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Figure 4. Effect of ascorbic acid oxidase and trypsin treatments on the ability of embryo extract to promote basal lamina formation by SCs grown with neurons. Myelination results for the experiment illustrated here are presented in Table II. Cultures were grown for two weeks in DM supplemented with 15% dialyzed HPS without additional additives (a), or with 15% chick EE (b), ascorbic acid (c), 15% chick EE ultrafiltrate, no pretreatment (d), 15% chick EE ultrafiltrate, pretreated with 0.05 U/ml ascorbic acid oxidase (e), or 15% chick EE ultrafiltrate, pretreated with 1 mg/ml trypsin (f). Living cultures were stained with rabbit anti-NCl followed by fluorescein goat anti-rabbit, then photographed and printed identically. Bar, 25 μm.

Table III. FBS Plus Ascorbic Acid Acts as a Differentiation-promoting Medium

| Medium                                      | Myelin segments/mm² | Basal lamina* |
|---------------------------------------------|---------------------|---------------|
| DM                                          | 0                   | -             |
| DM₁₅                                        | 0.1                 | -             |
| DM₁₅ + 50 μg/ml L-ascorbic acid             | 310                 | +             |
| DM₁₅ + 0.2 μg/ml L-ascorbic acid oxidase     | 0.2                 | -             |
| DM₁₅ + 50 μg/ml L-ascorbic acid oxidase      | 310                 | +             |

SC-neuron cultures were grown and analyzed as described in Table I. All serum-containing media in this table were made with FBS.

* Basal lamina formation in DM₁₅ + A is illustrated in Fig. 2.

assayed for ascorbic acid, and the measured value, in conjunction with the ascorbic acid–myelin dose-response curve (Fig. 5), was used to calculate the expected myelin segments/mm² value. As shown in Table V, batches of HPS which exhibited the ability to promote myelination without other additives had sufficient ascorbic acid to account for that ability.

A Macromolecular Serum Factor Is Required to Enable Ascorbic Acid to Promote Normal Schwann Cell Differentiation

In the experiments reported above, the effects of ascorbic acid on SC differentiation were examined in the presence of serum. Additional experiments revealed that supplementation of DM with ascorbic acid in the absence of serum resulted in only partial promotion of SC differentiation. SCs responded to the ascorbic acid by tending to aggregate with one another on the surface of the axon network (Fig. 6 a); deposition of laminin, type IV collagen, and HSPG occurred in the presence of ascorbic acid alone, both around aggregated SCs and on SCs displaying apparently normal morphological differentiation (data not shown). The staining for these components was, however, considerably less intense and regular than when serum was added with the ascorbic acid. In addition, myelin formation was observed to occur in the presence of ascorbic acid alone, but quantitatively it was small and variable compared to ascorbic acid plus FBS (average for three experiments, DM = 0 myelin segments/mm² [SEM = 0], DM plus ascorbic acid = 22 [SEM = 16], and DM₁₅ plus ascorbic acid = 290 [SEM = 17]), and the myelin segments were abnormally short (Fig. 6).

We tested several proteins for their ability to promote myelination in the presence of ascorbic acid, and the results indicate that the requirement for serum with ascorbic acid is relatively specific in that it cannot be satisfied by bulk protein, although a slight enhancement of myelin formation can be obtained with several different proteins. Media made with ascorbic acid plus BSA at the concentration of total protein in FBS DM₁₅ (6.3 mg/ml), bovine transferrin at its concentration in DM₁₅, BSA plus transferrin, or rat transferrin at 10 times the concentration in DM all promoted more myelin formation than did ascorbic acid alone, but none promoted myelin formation comparable to dialyzed FBS plus ascorbic acid (Table VI). In addition, in the presence of ascorbic acid plus the proteins tested, the myelin remained abnormally short, extensive SC aggregation still occurred, and the deposition of basal lamina components was patchy. In a different experiment the addition of 45 μg/ml human plasma fibronectin with ascorbic acid failed to promote the formation of...
more myelin than ascorbic acid alone (data not shown). Previous reports (Carey, 1983; Eldridge et al., 1984) have suggested that fetuin, an FBS protein, might be an effective agent for promoting SC differentiation in the presence of ascorbic acid. We found, however, that the effect of fetuin (1 mg/ml) plus ascorbic acid was relatively small and variable (65 myelin segments/mm², SEM = 31, average of three experiments), in comparison with standard myelinating medium (300 myelin segments/mm², SEM = 11).

**Discussion**

Obtaining peripheral nervous system myelination in vitro has traditionally required the use of the complex medium additives HPS and chick EE (Murray et al., 1965; Bunge et al., 1967; Moya et al., 1980). The experiments reported here substantially explain the role of chick EE. The sole activity of chick EE important for SC myelin formation appears to be ascorbic acid; the activity was dialyzable, insensitive to trypsin, sensitive to ascorbic acid oxidase, and replaceable by ascorbic acid. The ability of some batches of HPS to stimulate myelination in the absence of other additives appears to be due to endogenous ascorbic acid; the measured ascorbic acid concentration accounted for the ability of such sera to promote myelination, and ascorbic acid oxidase treatment abolished the myelination-promoting effects of such sera.

These experiments also demonstrate that SCs require a macromolecular serum factor(s) in addition to ascorbic acid...
Table V. Serum Ascorbic Acid Concentration Correlates with Ability to Promote Myelin Formation

| Serum   | Measured concentration in DM<sub>10</sub> (µM) | Myelin segments/mm<sup>2</sup> | Expected | Observed |
|---------|---------------------------------------------|-------------------------------|----------|----------|
| HPS<sub>1</sub> | 13.1                                      | 170                           | 210      |          |
| dHPS<sub>1</sub> | 1.2                                       | <1                            | 0.1      |          |
| HPS<sub>2</sub> | 2.3                                       | <1                            | 0.5      |          |
| HPS<sub>3</sub> | 3.7                                       | <1                            | 0.6      |          |
| HPS<sub>4</sub> | 13.2                                      | 175                           | 160      |          |
| dHPS<sub>4</sub> | 0.9                                       | <1                            | 0        |          |
| FBS<sub>1</sub> | 2.1                                       | <1                            | 0.1      |          |
| FBS<sub>2</sub> | 0.6                                       | <1                            | 0.2      |          |
| dFBS<sub>2</sub> | 0.5                                       | <1                            | 0.5      |          |

SC-neuron cultures were grown and analyzed as described in Table I. A serum abbreviation preceded by d indicates that the serum had been dialyzed. Sera were assayed for ascorbic acid concentration as described in Materials and Methods. The ascorbic acid concentration achieved in DM<sub>10</sub> medium was then calculated and used, in conjunction with the ascorbic acid–myelin dose-response curve in Fig. 6, to calculate the number of myelin segments/mm<sup>2</sup> that would be expected to be formed in that medium.

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for maximum expression of their potential to myelinate axons in this culture system. Ascorbic acid in the absence of serum stimulated the formation of small quantities of myelin, but also elicited abnormal SC behavior (aggregation with other SCs). Substitution for serum with other proteins, such as BSA or transferrin, fetuin, or fibronectin failed to enable ascorbic acid to promote the formation of myelin in the quantities observed in the presence of serum, thus suggesting that the activity of serum may be due to a specific macromolecular component.

The experiments reported here additionally demonstrate that SC basal lamina formation has requirements identical to SC myelin formation, namely ascorbic acid and a nondialyzable serum component. Without exception, media that promoted myelin formation also promoted basal lamina formation, and vice versa. This correlation persisted through the various manipulations of sera and chick EE, and with analogs of ascorbic acid. Perhaps the most striking example of this correlation was provided by an examination of the ascorbic acid concentration dependence of these two aspects of SC differentiation, in which a doubling of the ascorbic acid concentration from 8.5 to 17 µM in the presence of dialyzed serum led to a change from the absence of basal lamina and myelin formation to the formation of both in a manner similar to that obtained in control myelinating media.

Ascorbic acid appears to be a specific requirement for SC differentiation. The concentration at which ascorbic acid was active in promoting both basal lamina and myelin formation by SCs is similar to the range over which it is active in vitro in promoting collagen production by human skin fibroblasts (Murad et al., 1983), collagen deposition by rat smooth mus-

Figure 6. Comparison of the effects of ascorbic acid in the absence (a) or presence (b) of 15% dialyzed FBS. SC-neuron cultures were grown for 2 wk in these media and then fixed and stained with Sudan black. In the absence of serum, ascorbic acid predominantly causes SCs to migrate into aggregates (curved arrows), but also promotes the formation of occasional short myelin segments (arrowheads). Bar, 50 µm.
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We hypothesize that ascorbic acid acts to promote SC myelin formation in an indirect manner, by directly enabling the SC to assemble a basal lamina around itself. Under this hypothesis a basal lamina is required for the SC to generate the plasma membrane polarity and cytoskeletal organization needed to execute the complex shape changes which myelin formation requires. This hypothesis emphasizes the epithelial nature of the differentiated SC, its membrane being functionally polarized into basal lamina- and axon-facing domains, and is based on the known influences of extracellular matrices on the polarity (Chambard et al., 1981; Montesano et al., 1983), cytoskeleton (Sugrue and Hay, 1981), and differentiation (Hadley et al., 1985) of epithelial cells.

This interpretation of our results is supported by the following evidence to be presented in the second paper in this series (Eldridge, 1986; Eldridge, C. F., M. B. Bunge, and R. P. Bunge, manuscript in preparation). (a) Oligodendrocytes, which do not form a basal lamina, show no requirement for ascorbic acid in order to myelinate axons in our culture system. (b) Exogenous basal lamina provided to SCs grown with neurons in DM is sufficient to allow the formation of myelin in quantities equal to those formed in DM supplemented with ascorbic acid plus serum, whereas exogenous interstitial extracellular matrix is not. (c) Finally, SCs grown with neurons in the absence of ascorbic acid do not secrete native, triple-helical collagen molecules, although they secrete a repertoire of other proteins very similar to those secreted by differentiated SCs, and, as shown in this paper, they express both laminin and HSPG on their surfaces.

Ascorbic acid is known to be an important participant in the posttranslational hydroxylation of collagen proline residues; hydroxyproline is essential for collagen chains to associate into triple helices, thereby forming native collagen molecules (for reviews see Prockop et al., 1976; Kivirikko and Myllyla, 1982). It is known that the interactions between type IV collagen, laminin, and HSPG (Woodley et al., 1983; Kleinman et al., 1983; Fujisawa et al., 1984), which may be important in the formation of a basal lamina, require native type IV collagen (Kleinman et al., 1983), and it may be that ascorbic acid promotes SC myelin formation by allowing the SC to assemble a basal lamina stabilized by triple-helical type IV collagen.

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Note Added in Proof: Carey and Todd (Carey, D. J., and M. S. Todd, 1987 Dev. Brain Res. 32:95–102) recently reported that BSA and fetuin were as effective as serum in enabling ascorbic acid to fully promote SC myelination, suggesting that the serum requirement is nonspecific. Our data on the effect of fetuin and BSA on myelination agree well with those of Carey and Todd, in that these proteins (in the presence of ascorbic acid) promoted the formation of 30–65 myelin segments/mm². Our positive control for myelination (85% dialyzed serum plus ascorbic acid), however, consistently yielded values of ~300 segments/mm², whereas that of Carey and Todd (10% serum plus 5% chick EE) promoted only 18 segments/mm². Given the possibility of variability in endogenous ascorbic acid levels in the undefined EE and serum components (see Table V), it seems likely that the positive control of Carey and Todd had insufficient ascorbic acid levels to promote maximal myelination. Recent preliminary experiments we have done show that dialyzed plasma-derived serum is as effective as dialyzed whole blood serum in enabling ascorbic acid to fully promote myelination, suggesting that the active factor(s) in serum is not platelet derived.
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