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PROPERTIES OF NEURONS FROM DISSOCIATED FETAL RAT BRAIN IN SERUM-FREE CULTURE[1]

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Abstract

Because of the unknown constituents and varying composition of serum, its presence in media used in cell culture unavoidably compromises attempts to study cellular mechanisms of growth and differentiation. To overcome this, we have devised a serum-free, chemically defined medium which maintains primary cultures of fetal rat brain cells for more than 6 weeks. This medium allows expression of characteristic properties of neurons and prevents overgrowth of non-neuronal elements without use of antimitotic agents. Cells prepared and plated without exposure to serum attach in less than 20 min to poly-D-lysine substratum and begin to extend processes within 1 hr. After 2 days in culture, process-bearing cells can be divided into those with characteristic neuronal morphology, including long processes which generally branch at a distance from the perikaryon, and those having the appearance of glial cells with many short, thin processes which branch frequently near the cell body. The remaining non-neuronal cells are large and flat with few or no processes. The presence of neurons and astroglia was demonstrated by immunofluorescence detection of bound tetanus toxin as a neuron-specific surface marker, and glial fibrillary acidic protein as an astroglial marker. By the 3rd day in culture, many cells of neuronal morphology were able to generate action potentials in response to electrical stimulation. The ionic composition of the inward current changes from Ca^{2+} to predominantly Na^{+} by about 10 days in culture. The presence of synaptic vesicles and myelin was demonstrated by electron microscopy. The ability of dissociated cells from mammalian brain to grow in defined medium without serum and acquire selected properties of mature cells *in vivo* demonstrates the potential of this culture system for neurobiological studies at the cellular level.

The culture of dissociated cells has become increasingly useful for investigation of cellular functions of the mammalian central nervous system (Crain, 1976; Fischbach and Nelson, 1977). At the present time, most methods of tissue culture require the addition of serum or tissue extracts to a balanced mixture of inorganic salts, sugars, amino acids, and vitamins to provide a culture medium which allows *in vitro* growth and differentiation of cells (Waymouth, 1977). However, the addition of biological fluids to culture media, while enhancing the survival of cells, unavoidably introduces variables into the experimental system due to the unknown and inconstant composition of these fluids (Honn et al., 1975; Waymouth, 1977). This complicates and potentially compromises the study of exogenous and endogenous factors involved in the regulation of cellular differentiation (Sanford et al., 1979), including neurotransmitter biosynthesis (Patterson, 1978) and receptor expression (Jacobs and Cuatrecasas, 1981). Therefore, the need for a serum- and tissue extract-free defined medium is manifest.

Over the last several years, a number of investigators have demonstrated that serum can be completely replaced with known mixtures of nutrients, hormones, and growth factors in cultures of various cell types, including neurons (Hayashi and Sato, 1976; Bottenstein et al., 1979, 1980; Bunge et al., 1982; Sato et al., 1982). However, the use of serum-free defined medium for maintenance of dissociated primary cultures of mammalian central neurons, although successful in a number of laboratories, has included dissociation and initial incubation (up to 48 hr) in serum-containing medium (Skaper et al., 1979; Messer et al., 1980; Yavin and Yavin, 1980; Puymiral et al., 1982). The present study was carried out to determine culture conditions which would permit dis-

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sociated cells from brainstem and cerebellum of the fetal rat to survive and differentiate without exposure to serum and to characterize the expression of neuronal function under these conditions. The results of this study demonstrate that, under these conditions, neurons and glial cells survive for more than 6 weeks without overgrowth of non-neuronal elements, and that neurons undergo myelination and exhibit changes in ionic mechanisms of excitability associated with differentiated function. Preliminary reports of this work have been presented (Ahmed et al., 1982).

Materials and Methods

Cell culture. Timed 19- to 20-day pregnant Sprague-Dawley rats (King Laboratories, Oregon, WI) were anesthetized with diethyl ether and, under sterile conditions, fetuses were removed by abdominal laparotomy, weighed, and placed in an isotonic buffer solution (137 mM NaCl, 5.4 mM KCl, 0.2 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 5.5 mM glucose, and 59 mM sucrose, containing 0.02 mM phenol red, 250 μg of Fungizone, 100 mg of streptomycin, and 100,000 units of penicillin G/liter, pH 7.2), at room temperature. Whole brains were removed from fetuses weighing 3.5 gm or more and placed in fresh isotonic buffer. Under a dissecting microscope, meninges and all visible blood vessels were removed. The brainstem-cerebellum complex was isolated by transverse cuts at the level of the lateral ventricle-corona radiata and inferior to the lower medulla, and placed in fresh isotonic buffer at 10°C. Tissue pooled from 10 to 20 fetuses was minced with iris scissors into pieces small enough to pass freely through the tip of a Pasteur pipette. Minced tissue was suspended in an Ehrlenmeyer flask in 10 ml of isotonic buffer containing 0.25% trypsin (Worthington Biochemical Corp., Freehold, NJ; 180 to 220 units/mg) and incubated for 5 min at 37°C in a shaking water bath. Undissociated tissue was allowed to settle and the dissociated cells in the supernatant were carefully aspirated and added to culture medium (Table I) containing soy-

TABLE I

Complete composition of serum-free defined medium

Individual components are (A) premixed and stored at 4°C up to 3 weeks, and (B) added immediately before use.

A.	Concentration (M)	A.	Concentration (M)
Buffers		Vitamins	
*$NaHCO_3$	44.0×10^{-3}	*D-Biotin	0.10×10^{-6}
*HEPES	25.0×10^{-3}	Choline Chloride	28.7×10^{-6}
Inorganic Salts		Folic Acid	90.6×10^{-6}
NaCl	11.0×10^{-2}	i-Inositol	40.0×10^{-6}
NaH_2PO_4	1.00×10^{-3}	*DL-Lipoic Acid	5.80×10^{-6}
*KCl	25.0×10^{-3}	Nicotinamide	32.8×10^{-6}
$MgSO_4$	0.80×10^{-3}	D-Pantothenate	16.8×10^{-6}
$CaCl_2$	1.80×10^{-3}	Pyridoxal	19.5×10^{-6}
*$MgCl_2$	0.20×10^{-3}	Riboflavin	1.00×10^{-6}
Carbohydrate		Thiamine	11.9×10^{-6}
D-Glucose	25.0×10^{-3}	*Vitamin B_{12}	1.00×10^{-6}
Amino Acids		Trace Elements	
*L-Alanine	0.04×10^{-3}	*$CrCl_3 \cdot 6H_2O$	1.00×10^{-9}
L-Arginine	0.40×10^{-3}	*$CuSO_4$	1.00×10^{-9}
*L-Asparagine	0.03×10^{-3}	$FeSO_4 \cdot 7H_2O$	36.0×10^{-8}
*L-Aspartic acid	0.03×10^{-3}	*$MnSO_4$	1.00×10^{-9}
L-Cysteine	0.32×10^{-3}	*MoO_3	1.00×10^{-9}
*L-Glutamic acid	0.03×10^{-3}	*$ZnSO_4 \cdot 7H_2O$	1.00×10^{-9}
L-Glutamine	4.00×10^{-3}	Indicators and Antibiotics	
Glycine	0.40×10^{-3}	Phenol Red	15 mg/liter
L-Histidine	0.20×10^{-3}	*Fungizone	250 μg/liter
L-Isoleucine	0.80×10^{-3}	*Penicillin	1×10^5 units/liter
L-Leucine	0.80×10^{-3}	*Streptomycin	100 mg/liter
L-Lysine	0.80×10^{-3}		
L-Methionine	0.20×10^{-3}		
L-Phenylalanine	0.40×10^{-3}		
*L-Proline	0.07×10^{-3}		
L-Serine	0.40×10^{-3}		
L-Threonine	0.80×10^{-3}		
L-Tryptophan	0.08×10^{-3}		
L-Tyrosine	0.46×10^{-3}		
L-Valine	0.80×10^{-3}		

B.	Concentration (M)	B.	Concentration (M)
Hormones and Proteins		Fatty Acids	
*Insulin (bovine, 25 units/mg)	0.33×10^{-6}	*Linoleic Acid	5.40×10^{-6}
*Transferrin (human)	0.30×10^{-6}	*Linolenic Acid	3.60×10^{-6}
*Albumin (fatty acid free)	12.0×10^{-6}	Trace Elements	
Polyamines		*Na_2SeO_3	25.0×10^{-9}
*Putrescine	50.0×10^{-6}		

* Additions to DMEM.

bean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) activity equivalent to that of the trypsin. Undissociated tissue was resuspended in 10 ml of fresh 0.25% trypsin solution containing 200 μg of DNase (Worthington, 190 units/mg). After incubation for 5 min at 37°C, dissociated cells were again collected and added to medium containing soybean trypsin inhibitor. This procedure was repeated three to four times, using 10 ml of 0.25% trypsin solution without DNase, until all cells were dissociated. The combined dissociated cells were collected by centrifugation at 100 × *g* for 10 min and filtered through sterile cheesecloth. The yield was 1 to 1.5×10^8 cells/gm of tissue with a viability estimated to be greater than 98% based on trypan blue exclusion.

Plastic six-well culture dishes (Costar, Cambridge, MA), with or without 11 × 22 mm coverslips, were treated with poly-D-lysine (Sigma) for 15 to 20 hr by the method of Pettmann et al. (1979). The coated dishes were rinsed with 2 ml of a solution containing 137 mM NaCl, 2.7mM KCl, 0.4 mM NaH_2PO_4, 5.5 mM glucose, and 25 mM HEPES, pH 7.2, and stored with 1 ml of this solution until cells were ready for plating. Uncoated plates used in some experiments were rinsed and stored in the same manner. Cells were added to each well suspended in 2 ml of culture medium, at a concentration giving a final plating density of 1200 to 1800 cells/mm^2. The plates were incubated at 37°C under conditions of 10% CO_2 and saturated humidity. The medium was changed after 20 to 30 min and then every 2 days for 6 weeks or longer.

The complete composition of the culture medium is given in Table I. This medium is made in two stages, the first consisting of components which are stable for 3 to 4 weeks at 4°C in the dark (Table IA) and the second consisting of those components which are unstable and must be added immediately before use (Table IB). The medium can be conveniently made by addition of components (indicated in Table I by an asterisk) to Delbecco's modified Eagle's medium (DMEM; Grand Island Biological Co., Grand Island, NY, Cat. No. 430-2100). Fatty acid-free bovine serum albumin (Sigma), prepared from Cohn fraction V, was premixed in a fatty acid:albumin ratio of 0.75:1.0. Fatty acids (Sigma) were handled under a nitrogen barrier with care to prevent oxidation. All medium components were sterilized by passage through a 0.2-μm filter (Millipore Corp., Bedford, MA). Albumin and iron-free human transferrin (Sigma) were analyzed by SDS-polyacrylamide gel electrophoresis, and separated components were detected with a highly sensitive silver stain for proteins (Morrissey, 1981). As seen in Figure 1, *lanes B* and *C*, at sample loads of 0.44 μg, both albumin and transferrin appear as single bands. However, at a 20-fold greater sample load (8.75 μg), each of these shows multiple unidentified trace components (Fig. 1, *lanes D* and *E*), none of which are visible with conventional Coomassie blue staining.

In separate experiments, cells were placed in DMEM containing 10% horse serum (Grand Island Biological Co.), 10% bovine serum (Hyclone, Sterile Systems, Logan, UT), 25 mM HEPES, and 25 mM KCl, pH 7.2. The medium was changed after 30 min and then every 2 days. From day 2 on, the concentration of horse serum was reduced to 5% and bovine serum was eliminated from the medium.

Morphology and ultrastructure. Cultures were examined routinely with a Leitz Diavert microscope equipped with phase contrast optics and were photographed with Polaroid ASA-3000 film. For better visualization of cellular morphology, some cultures were stained with hematoxylin and eosin. Stained cells were examined and counted with a Leitz Orthoplan microscope and photographed with a 35-mm Vario-orthomat automatic camera using Kodak Tri-X film. For ultrastructural studies, cultures were examined with a Hitachi (Model H600) transmission electron microscope. Cells grown in plastic dishes were fixed with 2.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, stained with osmium tetroxide, dehydrated, removed with polypropylene, and embedded in Spurr's medium. Sections (0.1 μM) were post-stained with 5% uranyl acetate and lead citrate.

Indirect immunofluorescence. The presence of tetanus toxin-binding sites and glial fibrillary acidic protein (GFAP) was demonstrated by a modification of the indirect immunofluorescence procedure of Raff et al. (1979). Tetanus toxin (Commonwealth Biological Laboratory, Boston, MA) was used at a concentration of 5 μg/ml. Monoclonal antibody to tetanus toxin, rabbit antiserum to human GFAP, and fluorescein isothiocyanate-labeled goat anti-mouse IgG F $(ab')_2$ were gifts of Dr. G. Dutton. Anti-tetanus toxin was used at a dilution of 1:5 hybridoma supernatant, and GFAP was used at a dilution of 1:50. Antibody binding was visualized by the use of fluorescein-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG at a dilution of 1:50 and rhodamine-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (N. L. Cappel Laboratories, Cochranville, PA, Lot No. 10772), at a dilution of 1:100. Antibody solutions were centrifuged at 8000 × *g* for 15 min to obtain a clear supernatant. Preimmune serum and absorbed first antibody were used as controls. In additional control experiments, tetanus toxin or first antibody was omitted from the staining sequence. Coverslips were mounted with glycerol and examined with a Leitz Orthoplan microscope equipped with a PLOEM illuminator for incident light fluorescence and dichromatic beam splitter filter modules for fluorescein (K2) and rhodamine (N2).

Electrophysiology. For intracellular microelectrode recording, coverslips containing cells were removed from culture dishes and placed in a special chamber on the fixed stage of a Leitz Diavert microscope equipped with interference contrast optics. The chamber was continuously perfused at 0.1 ml/min to a depth of approximately 1 mm with DMEM plus 25 mM HEPES maintained at pH 7.2 by bubbling CO_2 in the reservoir. Temperature was maintained at 37°C by a feedback local heating circuit. A microelectrode, mounted on a piezoelectric controlled ultramanipulator (Licht Co., Englewood, CO) was connected to a high impedance pre-amplifier with a bridge balance circuit and capacity compensation (P. Getting). The micropipettes were filled with 3 M potassium acetate, pH 7.2, which had been passed through a 0.2-μm filter (Nalge Co., Rochester, NY). The microelectrode resistance in the external bath was between 40 and 60 megohms. Data were recorded on an eight-track FM tape recorder (A. R. Vetter Co., Rebersburg, PA) and were displayed on a storage oscilloscope (Tektronix, Inc., Beaverton, OR; Model 5103N) for photography.

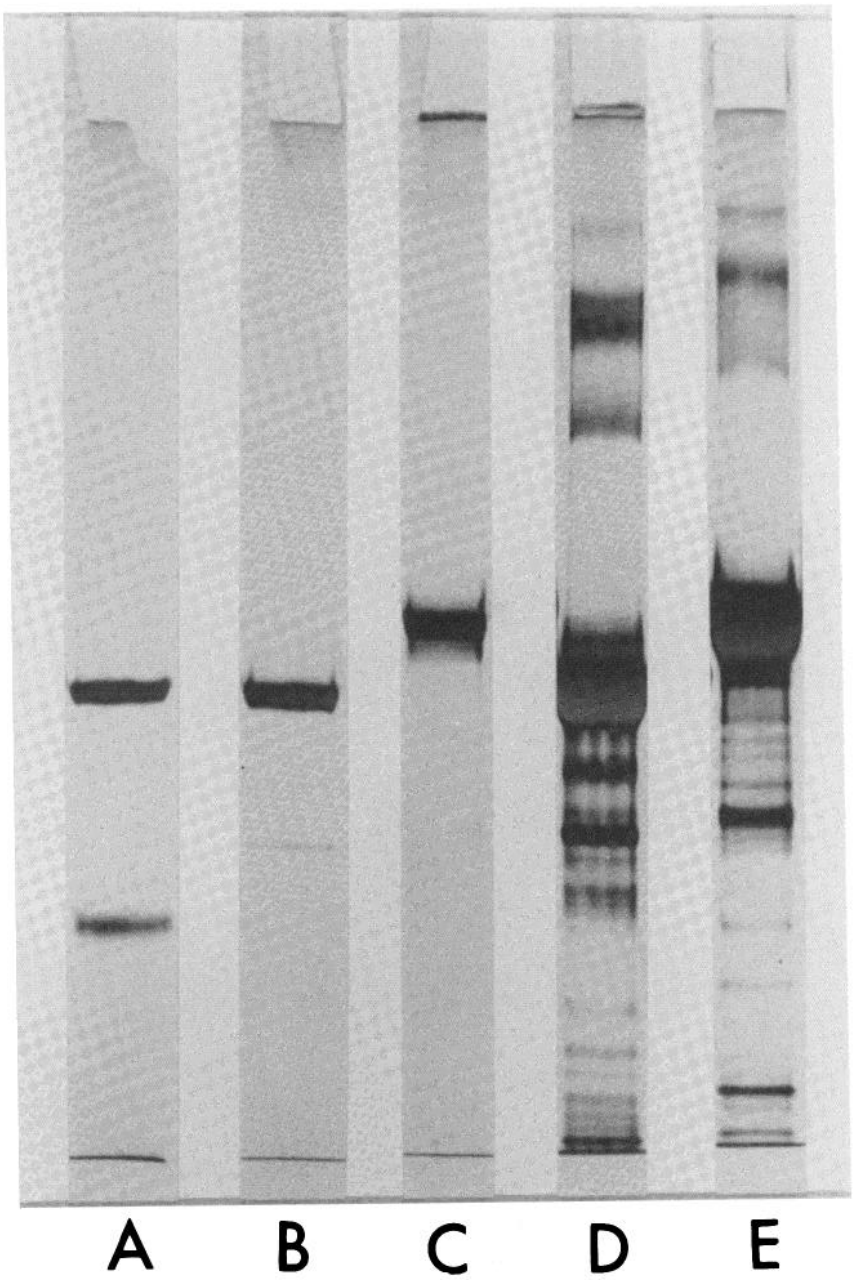

Figure 1. SDS-polyacrylamide gel electrophoresis of fatty acid-free albumin and transferrin. Samples are: *lane A*, 0.63 μg of bovine serum albumin (Cohn fraction IV) and 0.25 μg of ovalbumin; *lane B*, 0.44 μg of fatty acid-free albumin; *lane C*, 0.44 μg of human transferrin; *lane D*, 8.75 μg of fatty acid-free albumin; *lane E*, 8.75 μg of human transferrin. The gel was run at a constant current of 20 mA for 3.5 hr, immediately prefixed, then fixed and silver stained by the method of Morrissey (1981).

Results

Effects of dissociation conditions, substratum, and cell density. Small variations in the procedure used for dissociation of tissue were found to have profound effects on the survival of the cells in culture. In particular, exposure of separated cells to trypsin for the minimum possible time was found to be very important for obtaining a population of cells with high (>95%) viability. If brief (5 min) incubation with trypsin was not followed by treatment with trypsin inhibitor, even though 95 to 98% of cells were judged viable at the time of plating, they did not extend processes by 4 days in serum-free culture and died in 6 to 7 days. In order to overcome the harmful effects of residual trypsin, soybean trypsin inhibitor was added to the protocol so that dissociated cells were exposed to active enzyme for less than 5 min at 37°C. This modification resulted in healthy cultures in which cells extended processes within a few hours and survived for more than 6 weeks. Treatment with trypsin inhibitor was not necessary for cultures dissociated and

grown in serum-supplemented medium, presumably due to serum protease inhibitors and substrate competition.

The effect of various plating surfaces on cell attachment was investigated. It was found that cells attached poorly and in a spotty fashion to uncoated culture dishes. On poly-L-lysine the initially dispersed cells reorganized to form aggregates (Fig. 2*A*). These aggregates were particularly susceptible to detachment by normal handling and fluid movement during medium replacement. By contrast, on poly-D-lysine, monolayers formed in which cells remained evenly dispersed over the culture dish (Fig. 2, *B* to *F*). In addition, there was very rapid attachment of cells on poly-D-lysine, with at least 85% of cells attached in 20 min. Since most dead cells and cellular debris did not bind to the substratum during this period, washing of plates at 30 min resulted in significantly cleaner cultures. Two conditions important for the attachment of cells to poly-D-lysine-coated plates were the extensive washing of the plates to remove all free poly-D-lysine, which is toxic to cells, and the exclusion from the wash buffer of Ca^{2+} and Mg^{2+} which decrease plating efficiency.

Perhaps the most important variable in this procedure is the plating density. When cells were plated at densities of less than approximately 1000 cells/mm^2, they remained spherical and did not extend processes except in regions where average densities appeared to be greater. When cells were plated at densities between 1200 and 1800 cells/mm^2, they remained uniformly distributed throughout the plate, extended processes, and survived

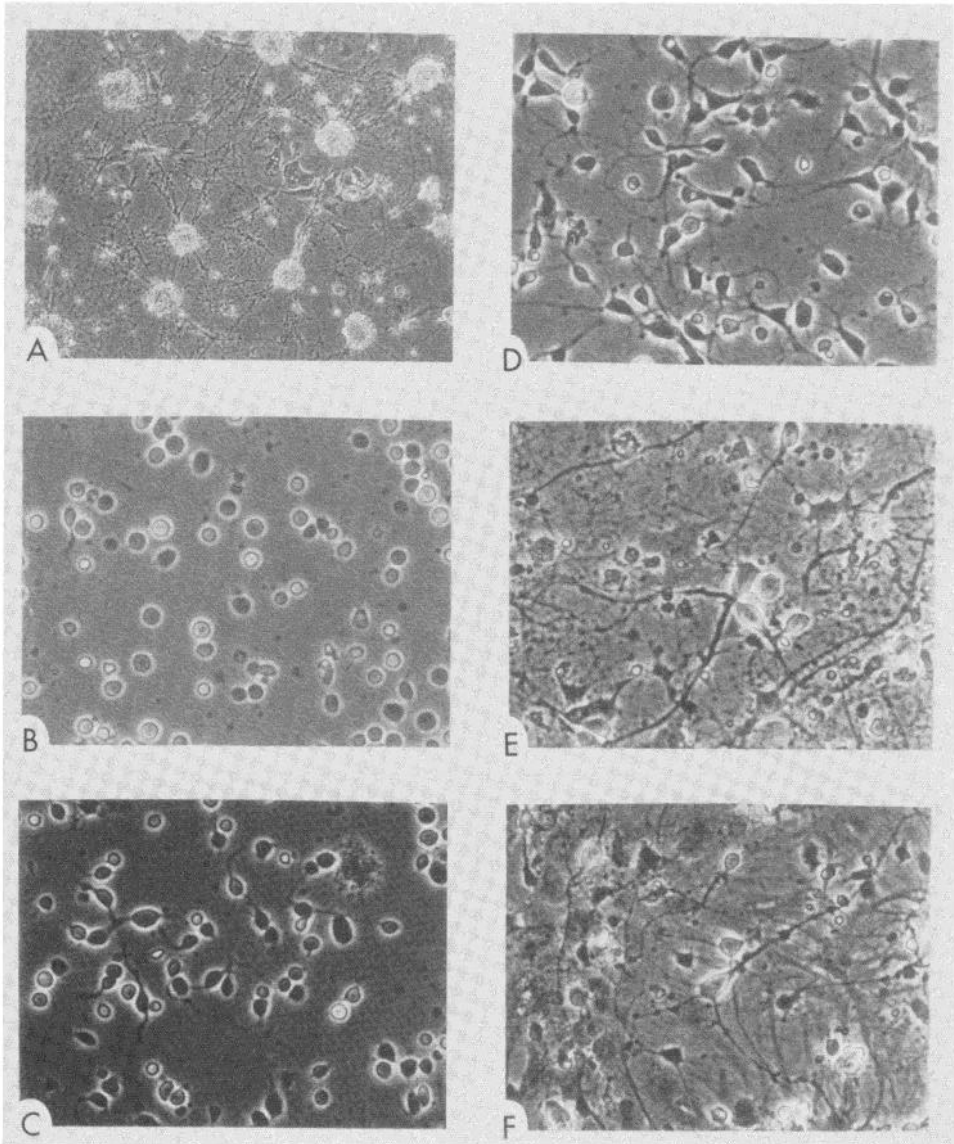

Figure 2. Effects of substratum and time on cultures of fetal rat brainstem and cerebellum in serum-free defined medium. Plating density was 1200 to 1800 cells/mm^2. Substratum was poly-L-lysine (*A*, magnification × 100) or poly-D-lysine (*B* to *F*, magnification × 320). Cells were examined by phase microscopy at: *A*, 4 days in culture; *B*, 30 min after plating; *C*, 3 hr after plating; *D*, 24 hr in culture; *E*, 13 days in culture; *F*, 40 days in culture.

for long periods of time without obvious overgrowth. When cells were plated at densities greater than 2400 cell/mm^2, survival rates were high but cell overgrowth occurred rapidly, making processes difficult or impossible to see.

Culture medium. The composition of the defined medium used in this study (Table I) was based on both previously established criteria (Waymouth et al., 1981) and experimental observations (Table II). We have found that the serum-free defined medium, N_2, developed for a neuroblastoma cell line by Bottenstein and Sato (1979), did not by itself support the culture of dissociated fetal rat brain cells. This is consistent with the observation that cultures of dissociated embryonic chick dorsal root ganglion (Bottenstein et al., 1980) and rat cerebellum (Messer et al., 1981) did not grow well in modifications of N_2 medium unless the cells were initially incubated with serum-supplemented medium.

TABLE II

Summary of effects of hormones and fatty acids on cell survival and process extension

Substances were tested on cultures plated at a density of 1500 cells/mm^2 and scored on day 4 for viability by trypan blue exclusion and process extension by phase contrast microscopy. The effect of each substance was graded, relative to cells cultured in the same medium without the addition, as: +, enhancing survival and process growth; 0, producing no significant change; or −, causing deleterious effects.

	Concentration (M)	Medium I[a]	Medium II[b]	Medium III[c]
Corticosterone	1.0×10^{-8}	0	0	0
	1.0×10^{-7}	−	−	−
Estrogen	1.0×10^{-8}	0	0	0
	1.0×10^{-7}	−	−	−
Progesterone	$0.1\text{–}1 \times 10^{-7}$	0	0	0
	2.0×10^{-7}	−	−	−
Triiodothyronine	$0.02\text{–}1.0 \times 10^{-9}$	0	0	0
Thyroxine	$0.01\text{–}1.0 \times 10^{-8}$	0	0	0
Rat Growth Hormone	$0.23\text{–}2.3 \times 10^{-9}$	0	0	0
Rat Prolactin	$0.4\text{–}4.0 \times 10^{-8}$	0	0	0
Insulin	6.7×10^{-8}	0	(NA)[d]	(NA)
	6.7×10^{-7}	+	(NA)	(NA)
Albumin	$0.28\text{–}2.8 \times 10^{-5}$	0	0	0
Transferrin	$0.6\text{–}6.0 \times 10^{-7}$	+	(NA)	(NA)
Linoleic Acid	5.4×10^{-6}	−	−	−
Linoleic Acid/ Albumin[e]	5.4×10^{-6} 7.2×10^{-6}	+	+	(NA)
Linolenic Acid	3.6×10^{-6}	−	−	−
Linolenic Acid/ Albumin[e]	3.6×10^{-6} 4.8×10^{-6}	+	+	+
Arachidonic Acid	1.7×10^{-6}	−	−	−
Arachidonic Acid/Albumin[e]	1.7×10^{-6} 2.3×10^{-6}	0	0	0
Docosahexanoic Acid/Albumin[e]	1.5×10^{-6} 2.0×10^{-6}	0	0	0
Cholesterol	$0.1\text{–}1.0 \times 10^{-8}$	−	−	−
Cholesterol/ DOPC[f]	0.3×10^{-6} 0.3×10^{-6}	−	−	−

[a] Basal medium (Table IA) with 25 μM selenium and 50 μM putrescine added.

[b] Medium I with 0.33 μM insulin and 0.3 μM transferrin added.

[c] Medium II with 5.4 μM linoleic acid premixed with 0.2 μM albumin added.

[d] (NA), not applicable.

[e] Fatty acids were premixed with fatty acid-free purified albumin.

[f] DOPC, dioleyl phosphatidylcholine.

In order to culture rat brain cells in the absence of serum, we attempted to identify specific components which are required for cell survival during dissociation, plating, and subsequent culture. The requirements for selenium and putrescine, previously demonstrated by others to be important for survival and growth of a number of cell types (Bottenstein et al., 1979), were examined in basal medium (Table IA) supplemented with insulin and transferrin alone (Table IB). At concentrations of 1 to 5 nM, selenium had no effect, whereas at concentrations from 10 to 30 nM, it enhances survival and process extension. At concentrations above 50 nM, selenium was toxic to cells. Putrescine also enhanced survival and process extension when added at concentrations between 10 and 100 μM to basal medium containing insulin, transferrin, and selenium. Ornithine and citrulline, at the same concentrations, were less effective than putrescine.

The results of screening of selected hormones and fatty acids are presented in Table II. Steroid hormones were dissolved in acetone and added to the medium in amounts not exceeding 20 μl/liter. All substances were tested at multiple concentrations on cultures plated at a density of 1500 cells/mm^2. Cultures were examined daily for process extension and scored on day 4 for viability based on trypan blue exclusion. The effect of each added substance was graded, relative to cultures grown in the same medium without the addition, as: +, enhancing survival and process growth; 0, producing no significant change; or −, causing deleterious effects. In the free form, saturated and unsaturated fatty acids were found to be without effect in concentrations less than 0.1 μM, but were toxic to cells at higher concentrations. In contrast, the addition of linoleic and linolenic acids complexed with delipidated albumin enhanced survival and process growth at 4 days and was essential for long-term survival.

Substances which clearly were necessary for cell survival and process outgrowth were included at the appropriate concentration in the final medium. Although substances having no demonstrable effect were not added, we recognize that some of these, particularly hormones, may have important effects on cell function not apparent under our screening conditions. In addition to substances listed in Table II, a number of vitamins, including ascorbic acid, retinal, and α-tocopherol, trace elements, purine, and pyrimidine bases and nucleosides were examined and found to have no effect.

Morphology and ultrastructure. After enzymatic dissociation, cells were uniformly dispersed without clumps. At the time of plating, cells were spherical and more than 95% were free of processes, with the remainder having processes no more than 2 times the cell diameter. Phase bright and phase dark cells were present in roughly equal numbers and both types began to extend processes within 1 hr after plating. Figure 2*B* shows a population of attached cells 30 min after plating. By 3 hr in culture, the majority of cells had extended processes up to several times the cell diameter (Fig. 2*C*), and by 24 hr in culture, additional process extension had taken place (Fig. 2*D*).

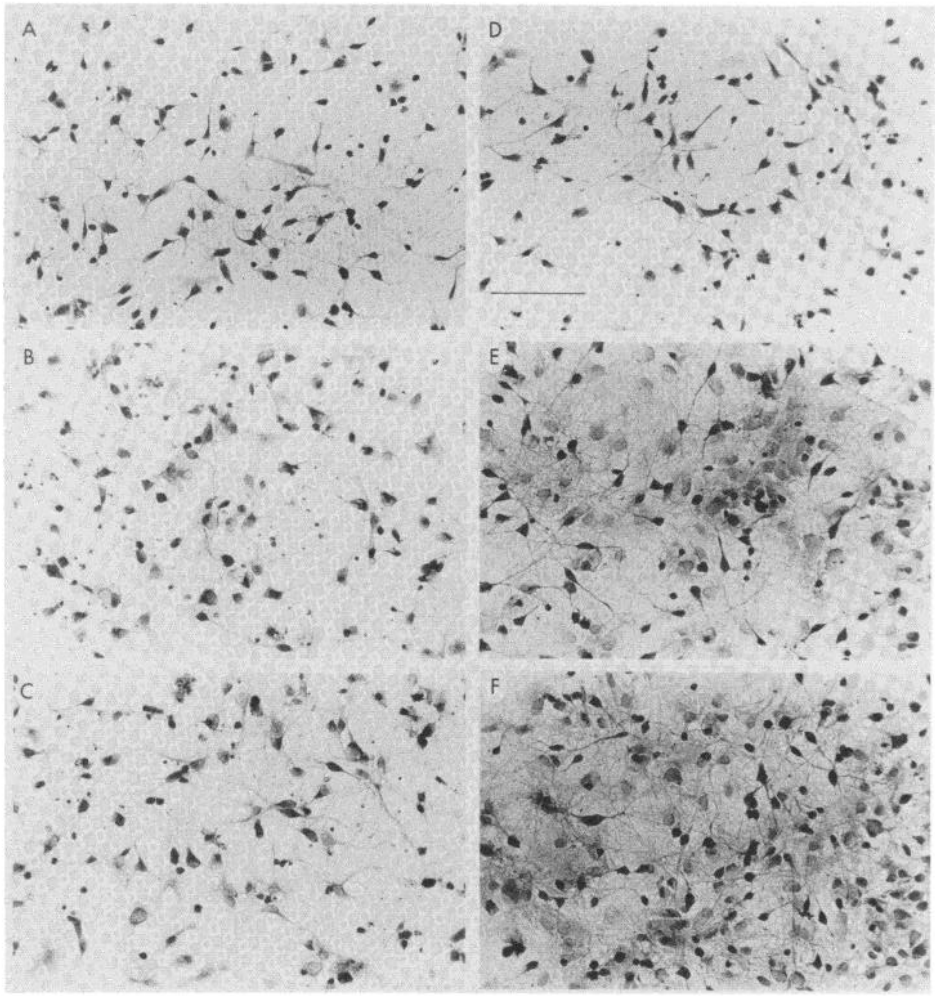

Figure 3. Comparison of cultures grown in serum-free medium (*A* to *C*) and serum-supplemented medium (*D* to *F*). Cells were cultured for 2 days (*A* and *D*), 7 days (*B* and *E*), and 14 days (*C* and *F*) in serum-free defined medium (Table I) or medium supplemented with horse serum. Cells were stained with hematoxylin and eosin. *Scale bar*, 100 μm.

After the 1st week *in vitro*, rounded, process-bearing cells could be divided into two principal groups. One group had very long processes which were usually branched at some distance from the perikaryon. These cells, seen in Figure 2*E*, are considered to be neurons (Mirsky and Thompson, 1975). Cells of the second group had many shorter, thinner processes branching near the cell body, consistent with the appearance of fibrous astroglia (Raff et al., 1979). Large flat cells were probably a mixture of protoplasmic astroglia, oligodendroglia, and fibroblasts. The cellular composition of the culture remained relatively constant up to 6 weeks (Fig. 2*F*).

In Figure 3, the cellular composition of the cultures grown for 2, 7, and 14 days in serum-free medium is compared with that of cultures grown in serum-supplemented medium. Dissociated cells were divided into two groups and plated in serum-free or serum-supplemented media at a density of 1200 cells/mm^2. On designated days, coverslips were fixed and stained. For quantitative comparison, cells were counted in 12 contiguous 0.28-mm^2 fields separated from each other by 2 mm in x and y directions. These cells were classified into two groups on the basis of appearance. One group consisted of flat cells and cells with short processes, presumed to be non-neuronal (*NN*). The second group consisted of rounded cells bearing long processes, presumably neuronal (*N*). In Figure 4*A*, the total numnber of cells at various days in culture is expressed as a percentage of the number of cells present on day 0 after the 30-min change of medium. Although there is an apparent increase in total cell numbers in serum-supplemented cultures as early as day 4, because of counting error this does not become statistically significant until after day 7. By 2 weeks in culture, there are approximately twice as many cells in serum-supplemented as in serum-free cultures.

Figure 4*B* is a plot of the ratio of the number of flat cells and cells with short processes (*NN*) to the number of rounded cells with long processes (*N*). This clearly shows that the increase in the total number of cells in serum-supplemented medium is primarily due to an increase in cells of the first type, whereas in serum-free medium the ratio of the two groups of cells remains

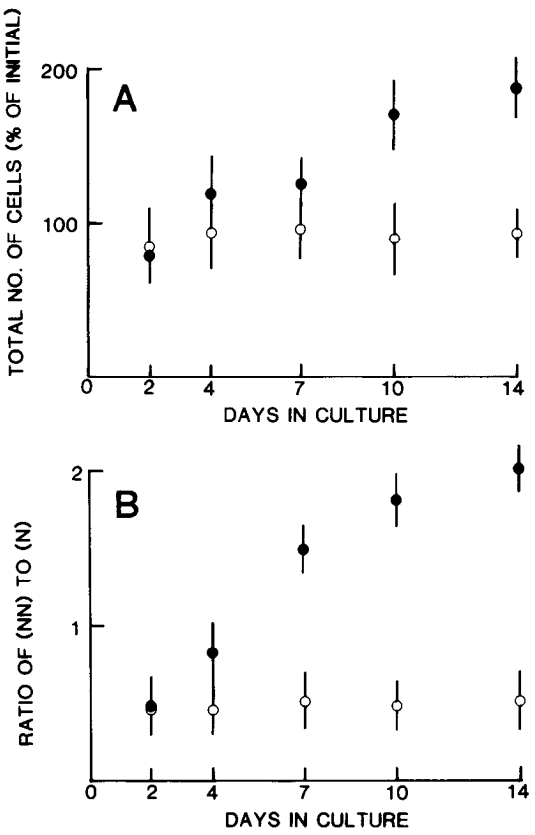

Figure 4. Number of cells in serum-free and serum-supplemented media is a function of time in culture. *A*, Total number of cells in serum-free (○) and serum-supplemented (●) cultures is expressed as a percentage of the number (1030 cells/mm^2) present on day 0 after the 30-min change of medium (mean ± SD). *B*, Ratio of the number of flat cells and cells with short processes (*NN*) to the number of rounded cells with long proceses (*N*) in serum-free (○) and serum-supplemented (●) media (mean ± SD).

relatively constant at 0.4. If the serum concentration of the medium was not reduced to 5% on day 2 (see "Materials and Methods") but kept at 20%, overgrowth of non-neuronal elements occurred very rapidly and the morphology of individual cells was difficult to determine by 4 days. This has led to the common use of antimitotic agents such as 5-fluoro-2′-deoxyuridine and arabinofuranosylcytosine to kill dividing cells, although their effect on neurons is not known. It is clear that, in the serum-free culture system, these agents are not necessary.

Electron microscopic examination of cultures grown in serum-free medium revealed the presence of synapses, including synaptic vesicles, synaptic clefts, and membrane specialization, as early as 4 days in culture. The size of these vesicles (Fig. 5*A*) ranged from 40 to 80 nm, similar to that of vesicles seen in mature neurons *in vitro* and *in vivo* (Heuser and Reese, 1977; Kim, 1980). The presence of vesicles suggests that neurons cultured in serum-free medium are capable of synthesis and storage of neurotransmitters; this is supported by studies demonstrating the synthesis of acetylcholine, γ-aminobutyric acid, serotonin, dopamine, and norepinephrine from radiolabeled precursors in these cultures (Z. Ahmed and R. Fellows, manuscript in preparation). Different stages of myelination have been demonstrated by transmission electron microscopy (Fig. 5*B*). This suggests that oligodendroglia, the myelin-forming cells of brain, are present in these cultures and are undergoing maturation (Silberberg et al., 1980).

Immunohistochemical identification of cell types. Tetanus toxin and GFAP have been widely used as neuronal and astroglial markers, respectively (Mirsky, 1982). The immunofluorescence staining of a 6-day-old culture for tetanus toxin is shown in Figure 6*A*. The staining pattern is typical of that reported by others for tetanus toxin immunofluorescence, with beaded staining of processes and the surface of soma (Mirsky et al., 1978). Comparison with the phase contrast image of the same field (Fig. 6*B*) indicates that staining is selective. Indirect immunocytochemistry also showed the presence of morphologically distinct types of GFAP-positive cells in these cultures (Fig. 7). These include both flat cells and cells with prominent processes, consistent with descriptions of protoplasmic and fibrous astrocytes, respectively (Raff et al., 1979).

Double staining experiments for tetanus toxin and GFAP showed that most cells stained only for one antigen, some cells stained for both, and some did not stain for either. In control experiments, fluorescence was limited to a small population of spherical cells with no processes (presumably macrophages) and cell debris. Although quantitative analysis of specific cell types requires the identification of all cells present, an estimate can be made on the basis of indirect immunofluorescence staining. This was done by counting total cells in eight to ten 0.04-mm^2 areas of each coverslip under phase contrast optics and simultaneously counting cells with fluorescein or rhodamine fluorescence in the same fields. In 6-day-old serum-free cultures of brainstem and cerebellum, approximately 40% of cells stained for tetanus toxin alone and 25% stained for GFAP alone.

Electrophysiology. Cells of both neuronal and glial morphology were impaled with microelectrodes after 2 to 12 days in culture to determine electrical excitability. The membrane resting potential (V_{rest}) of impaled cells ranged from −10 mV to −70 mV. Values in the lower range (−10 to −40 mV) were presumed to be due to membrane damage during impalement (Spitzer, 1979) and were not analyzed.

Membrane resistance was determined in cells which were able to generate action potentials by measuring the membrane voltage change (ΔV) produced by injection of hyperpolarizing currents (ΔI) of up to 0.5 nA and of 100 msec duration. The cell input resistance is defined as $\Delta V/\Delta I$. The resting membrane potential of the neuron during measurement of input resistance was maintained at −40 mV by injecting a small steady current as needed, on which the test current pulses were superimposed. Table III lists the various electrophysiological properties of neurons grown in serum-free and in serum-supplemented media. V_{rest} and input resistance of cells grown for 3 to 6 days in serum-free medium are not significantly different from those of cells grown for 10 to 12 days in serum-free or 9 to 12 days in serum-supplemented media.

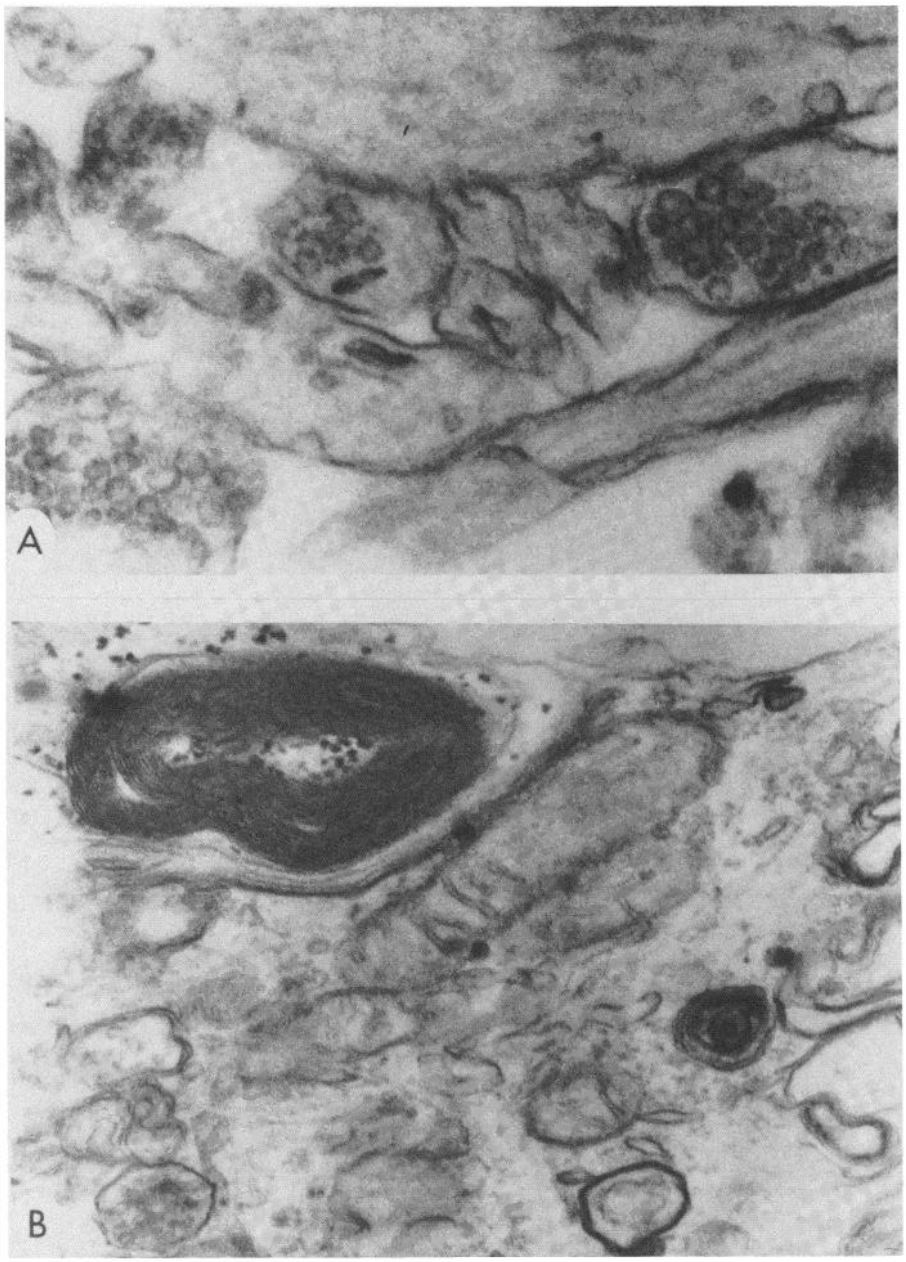

Figure 5. Synaptic morphology and myelination in cultures of fetal rat brainstem and cerebellum. *A*, Synaptic membrane specialization, vesicles, and cleft in an 8-day-old culture (magnification × 74,000); *B*, several stages of myelination visible in a 10-day-old culture (magnification × 59,000).

Cells of neuronal morphology generated action potentials in response to electrical stimulation when the V_{rest} was −40 mV or lower. Occasional cells of apparent neuronal morphology with V_{rest} between −40 mV and −60 mV did not generate action potentials in response to stimulation. It is not clear whether these were immature neurons or non-neuronal cells. Figure 8, *A* and *C*, shows action potentials recorded from neurons from separate wells of the same six-well culture dish on day 4 and day 11. In all neurons cultured for 3 to 6 days, the evoked action potentials had no positive overshoot and were of longer duration than those of 10- to 12-day-old cultures (Table III). Also, in 3- to 6-day-old cultures, evoked action potentials of a given cell showed significant variation in amplitude, duration, and onset time in response to identical suprathreshold stimulation, and after-hyperpolarization was either negligible or absent.

In five neurons examined at 3 to 6 days, the evoked action potentials were abolished when the superfusion solution contained 5 mM cobalt (Fig. 8*B*). In three separate cells, action potentials were insensitive to 5 μM tetrodotoxin (TTX). This suggests that the inward current in these neurons is carried predominantly by calcium ions.

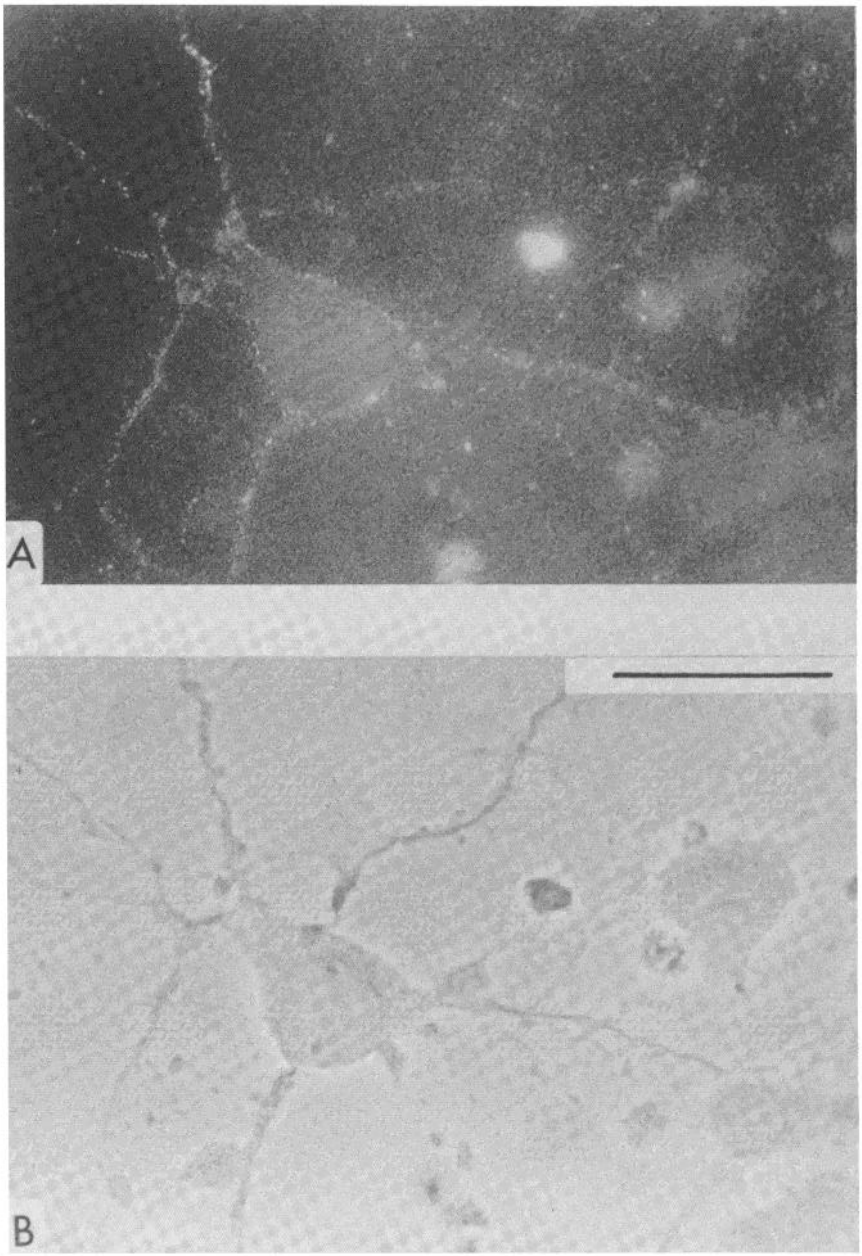

Figure 6. Indirect immunofluorescence demonstration of tetanus toxin binding to a neuron from a 6-day-old culture. Cells were incubated with tetanus toxin and monoclonal antibody to tetanus toxin, and labeled with fluorescein-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG. *A*, Surface staining of a single tetanus toxin-positive cell; *B*, phase micrograph of the same field showing staining cells. *Scale bar*, 50 μm.

In contrast, neurons from 10- to 12-day-old cultures (Fig. 8*C*) generated action potentials with positive overshoot, demonstrated after-hyperpolarization, and showed no significant changes in amplitude, duration, or onset time with identical electrical stimulation. However, the action potentials of neurons from 10- to 12-day-old cultures were substantially altered in amplitude and duration in the presence of 5 μM TTX (Fig. 8*D*), but only slightly changed by 5 mM cobalt, indicating that Na^+ current is the principal inward current in these neurons. The action potentials of 10- to 12-day-old neurons cultured in serum-free medium (Fig. 8*C*) resembled those recorded from 9- to 12-day-old brainstem-cerebellum neurons in serum-supplemented medium (Fig. 8*E*). They also resemble the action potentials recorded from central neurons from a variety of mammalian species in culture (Nelson and Peacock, 1973; Dichter, 1978; Nelson et al., 1981). None of the cells from 3- to 6-day-old or 10- to 12-day-old cultures in serum-free medium showed spontaneous electrical activity, even when contacts between

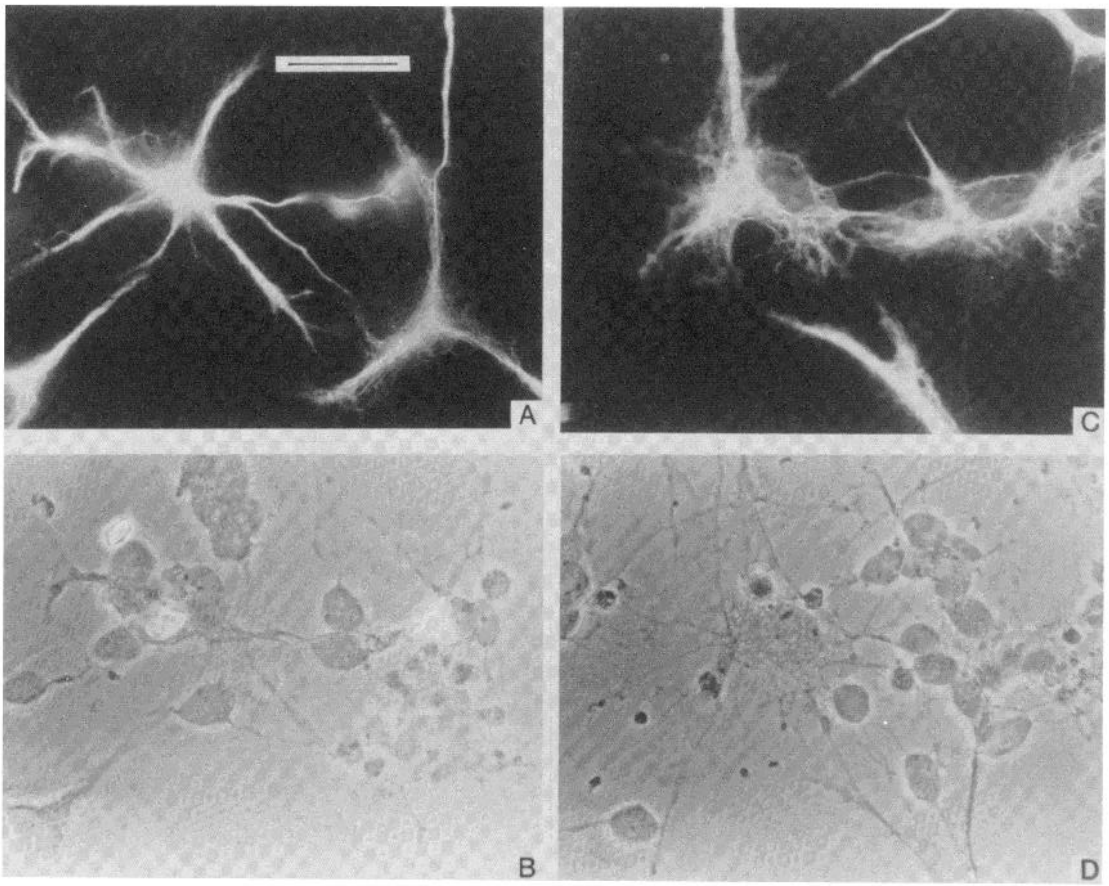

Figure 7. Demonstration of GFAP by indirect immunofluorescence in 6-day-old cultures. Cells were incubated with anti-GFAP and rhodamine-labeled $F(ab')_2$ fragment of goat anti-rabbit IgG. *A*, Cell demonstrating a staining pattern characteristic of fibrous astrocytes; *C*, cells demonstrating a fibrillary staining pattern characteristic of protoplasmic astrocytes. *B* and *D* are phase micrographs of the same areas. *Scale bar*, 50 μm.

TABLE III

Electrophysiological properties of neurons in serum-free and serum-supplemented medium

No. of Days in Culture	No. of Cells	Rest Potential	Input Resistance	Overshoot	Duration	After-Hyperpolarization
		mV	*megohms*	*mV*	*msec*	*mV*
Serum-free						
3–6	28	−58 ± 15[a]	36 ± 12	−15 ± 12	2.45 ± 0.65	<1.0
10–12	23	−55 ± 12	28 ± 14	+17 ± 5	0.67 ± 0.14	5.5 ± 1.5
Serum						
9–12	13	−61 ± 10	32 ± 9	+15 ± 4	1.0 ± 0.2	8.2 ± 3.0

[a] Data are given as mean ± SD.

adjacent cells were visible. However, spontaneous electrical activity was observed in all neurons cultured for 9 to 12 days in serum-supplemented medium (Fig. 8*F*).

Discussion

This study has demonstrated that the long-term culture of dissociated cells from fetal rat brainstem and cerebellum can be carried out in a serum-free defined medium, under conditions which permit the maintenance or expression of neuronal properties. An important feature of this system is that exposure of cells to serum is completely eliminated. In this respect, the procedure differs from previously reported methods for primary culture of central neurons in defined media (Skaper et al., 1979; Messer et al., 1980; Yavin and Yavin, 1980; Puymiral et al., 1982), which involve exposure of cells to serum initially, and, in some instances, up to 48 hr. As a result, the medium used in this study is probably more defined in a chemical sense than those developed earlier by other investigators and ourselves for the culture of mammalian brain neurons. Although ultrapure water and highly purified organic chemicals were available for the formulation of this medium, some components, notably macromolecules, are difficult if not impossible to obtain in an equally pure form. In this study we have found that even highly purified albumin and transferrin from com-

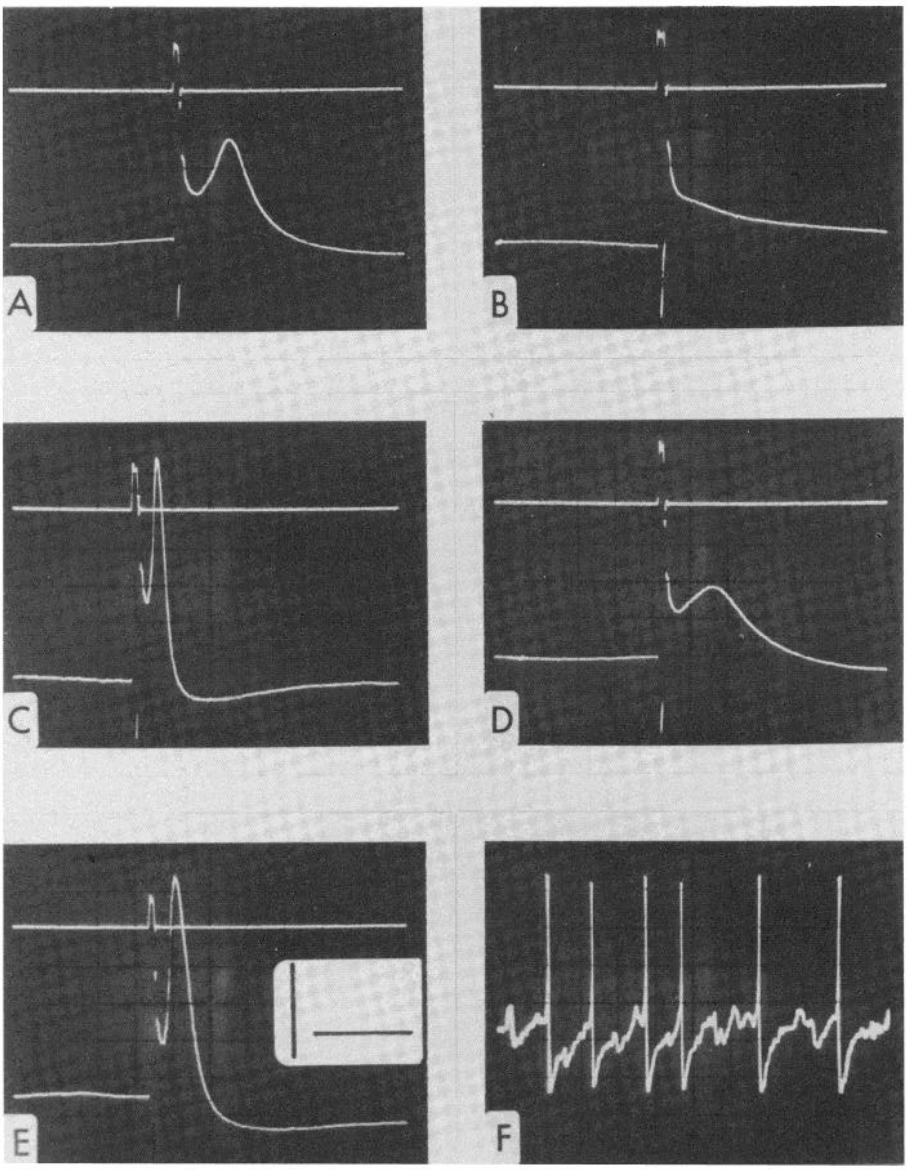

Figure 8. Intracellular microelectrode recording of action potentials from neurons cultured in serum-free and serum-supplemented medium. Evoked action potentials (1/sec) are shown for typical neurons cultured for (*A*) 4 days, or (*C*) 11 days in serum-free defined medium, and (*E*) 9 days in serum-supplemented medium. Spontaneous action potentials recorded from a single neuron cultured in serum-supplemented medium for 9 days are shown in *F*. Calibration is 40 mV, 1.0 nA, and 5 msec (*A* to *E*) or 50 msec (*F*). *B*, Elimination of the action potential in the 4-day-old neuron in the presence of 5 mM cobalt (same cell as in *A*). *D*, Effect of 5 μM TTX on the evoked action potential of an 11-day-old neuron (same cell as in *C*).

mercial sources can be shown by ultrasensitive analytical techniques to have multiple trace contaminants which are usually overlooked. Although albumin with these contaminants did not enhance cell survival or process growth (Table II), they remain as a possible source of biologically active trace materials. As a result, the development of a "defined" serum-free medium is, and may always be, an evolving process tempered by practical considerations. Part of this evolution will also be dependent upon a better understanding of the specific factors required by cells for normal function.

A number of investigators (Bottenstein et al., 1980; Messer et al., 1981) have suggested that initial incubation of enzymatically dissociated cells in a serum-containing medium was necessary to inactivate residual proteolytic enzymes and to maximize cellular attachment to the substratum. We have been able to eliminate these requirements for serum in the medium by treating dissociated cells with trypsin inhibitor at a concentration which inactivates residual trypsin and by using poly-D-lysine as a substratum for cell attachment. Although poly-L-lysine and poly-D-lysine are equally effective in

facilitating attachment, cells grown on poly-L-lysine become less firmly attached and tend to form aggregates with time in culture. One possibility is that lysine-specific proteases associated with one or more types of cell are capable of digesting the natural isomer poly-L-lysine, but not poly-D-lysine, and thus disrupt the attachment matrix.

The composition of the basal defined medium given in Table IA is similar to that of medium N_2 (Bottenstein and Sato, 1979), with several significant modifications. Hypoxanthine, thymidine, sodium pyruvate, and free linoleic acid are not included; the concentrations of the trace elements iron, copper, and zinc are greatly reduced; and manganese, chromium, and molybdenum are added because of their role in cellular metabolism (Jeejeebhoy et al., 1977; Underwood, 1977). A high concentration of potassium ion, used by some investigators (Lasher and Zagon, 1972) but not by others (Dichter, 1978), appears to be important, at least in the first 4 days of culture. At low concentrations of potassium (4.3 mM), survival of neurons was poor and overgrowth of non-neuronal cells occurred early.

As reported by others (Bottenstein and Sato, 1979), there is a fixed requirement for selenium, transferrin, insulin, and a diamine in this system. Transferrin is an essential component of the system, probably as a carrier for iron (Barnes and Sato, 1980). Insulin has been considered to be essential for serum-free culture of most cell types and is necessary for culture of brainstem and cerebellum cells in our system. Although insulin immunoreactivity and specific binding of ^{125}I-insulin have been reported in mammalian brain *in situ* (Havrankova et al., 1978) and *in vitro* (Weyhenmeyer and Fellows, 1983), it is not clear whether the actual requirement is for insulin per se or for an as yet uncharacterized, endogenous insulin-like growth factor. Among the diamines investigated, putrescine was found to be more effective for long-term culture than ornithine or citrulline. Although progesterone has been reported to be an essential component of other serum-free culture systems, we have not found it to be necessary.

Additional components essential for long-term survival of cells are polyunsaturated fatty acids of the n-3 and n-6 classes, which are not synthesized by mammalian cells. As indicated in Table II, a combination of linoleic acid (n-6) and linolenic acid (n-3) supports cultured cells better than either alone. It is not clear whether these are needed to meet metabolic requirements or for the synthesis of complex lipids. Since free fatty acids are not transported across the cell membrane and have detergent-like effects which are toxic to cells, they were complexed with highly purified albumin (fatty acid free) to facilitate cellular uptake (Rockwell et al., 1980). Although albumin is known for its role as a carrier of many biologically active molecules, the albumin used in this study did not by itself support the survival of cultures. This leads us to conclude that its essential role in this system is as a carrier for fatty acids. In this respect, it functions in a manner similar to transferrin.

An overriding factor in the survival of cells and the growth of processes in this medium is the cell density at the time of plating. Our studies indicate that there is a critical threshold below which cell survival and process growth are severely limited. Increasing survival above this level suggests the possibility that soluble factors elaborated into the medium by some if not all cells have trophic effects and are necessary for long-term survival. This hypothesis is supported by our preliminary findings that conditioned medium from high density cultures will allow survival and growth of cultures plated well below the threshold density (Z. Ahmed, P. S. Walker, and R. E. Fellows, unpublished observations) and anticipates the isolation of endogenous brain growth factors (Barde et al., 1982).

Quantitative data on the number of cells in serum-free and serum-supplemented cultures and the grouping of these cells into neuronal and non-neuronal categories provide several interesting observations. After the medium change at 30 min, approximately 86% of plated cells are attached in both serum-free and serum-supplemented cultures. Although the number of cells remains essentially constant in serum-free cultures for at least 2 weeks, the number increases from day 4 on in serum-supplemented cultures and is double that of serum-free cultures by the end of the 2nd week. This increase is due to non-neuronal elements. Since these cells complicate, if not prevent, investigation of neuronal function, a traditional solution has been to use metabolic poisons to kill dividing cells regardless of their effects on neurons. A clear advantage of the serum-free system is the ability to do away with this strategy and thus study neural mechanisms in cells not exposed to mitotic inhibitors.

The presence of neurons, astroglia, and oligodendroglia as the major constituents of these cultures has been established by a number of different criteria. Three lines of evidence indicate the presence of neurons in these cultures: (*1*) the binding of tetanus toxin, (*2*) the occurrence of synapses and neurotransmitter vesicles, and (*3*) the ability to generate action potentials. The presence of astroglia has been demonstrated by positive staining for GFAP (Fig. 6). Although we have not identified oligodendroglia directly, their presence is indicated by electron microscopic evidence of myelin formation. Whereas the binding of tetanus toxin and immunocytochemical staining of GFAP have been considered to be specific markers for neurons and astroglia, respectively (Mirsky, 1982), we have observed the presence of both markers in about 20% of cells. This may represent such close intercalation of neurons and astrocytes that both markers appear to be located on the same cell. Alternately, this may represent some degree of nonspecificity in the expression of either tetanus toxin binding or GFAP synthesis, or it may be a methodological artifact.

The changes in electrical excitability seen in this study (Fig. 8, *A* and *C*) are suggestive of the developmental changes observed in other systems and are not due to differences in passive membrane properties. Neurons that were able to generate action potentials had mean resting membrane potentials and mean input resistances of −55 to −61 mV and 28 to 36 megohms, respectively. No significant differences were observed between 3- to 6-day- and 10- to 12-day-old cultures or between cultures in serum-free and serum-supplemented media. These values for resting membrane potential and input resist-

ance are similar to those reported by others for cultured mammalian brain neurons (Nelson and Peacock, 1973; Dichter, 1978).

The differences observed in action potential parameters of 3- to 6-day- and 10- to 12-day-old cultures are due to differences in the ionic mechanisms of the inward current. The elimination of action potentials in neurons of 3- to 6-day cultures by 5 mM cobalt and the lack of effect of 5 μM TTX indicate that at this stage the inward current is carried primarily by Ca^{2+} ions. The dramatic reduction in action potential amplitude of 10- to 12-day neurons in the presence of 5 μM TTX, coupled with only a slight reduction in amplitude with 5 mM cobalt, indicates that, by this time, Na^+ ion has become the dominant inward current carrier. This shift from Ca^{2+}-dependent to predominantly Na^+-dependent action potentials is similar to that reported as a developmental change in amphibian neurons (Spitzer, 1979), chick muscle (Kano, 1975), and regenerating cockroach giant axon (Meiri et al., 1981). The action potentials of neurons in serum-free and serum-supplemented cultures are very similar. However, an unexplained feature of neurons in serum-free medium is that they do not have spontaneous action potentials or synaptic activity, whereas those cultured in serum-supplemented medium demonstrate both. This was a consistent observation, even though there were no apparent differences in neuronal morphology or contacts with adjacent cells. At present we have not determined whether the phenomenon is due to a general alteration in membrane property, to selective membrane damage as yet undefined, or to a nonfunctional synaptic mechanism. Higgins and Burton (1982) have demonstrated the formation of electrotonic synapses in fetal rat sympathetic neurons maintained in a serum-free medium, whereas neurons maintained in serum-supplemented medium rarely formed synapses. Thus, in rat superior cervical ganglion neurons, synaptogenesis appears to proceed differently in the presence and absence of serum. It is possible that, in rat brain neurons, serum-free medium prevents the formation of functional synapses even though most of the components of a chemical synapse are present. Formation of active synapses among mammalian central neurons in serum-supplemented culture has been reported (Nelson et al., 1981).

Although several aspects of this culture system are under continuing investigation, the system as described here is clearly capable of supporting long-term *in vitro* survival and function of fetal rat brainstem and cerebellum cells under serum-free conditions. Since neurons, astroglia, and oligodendroglia appear to undergo differentiation in this system, it should prove particularly useful for the study of environmental and cellular factors regulating brain cell development and function.

References

Ahmed, Z., P. S. Walker, and R. E. Fellows (1982) Primary culture of cerebrum, cerebellum and brainstem in completely defined medium. Fed. Proc. *41:* 1754.

Barde, Y. -A., D. Edgar, and H. Thoenen (1982) Purification of a new neurotropic factor from mammalian brain. EMBO J. *1:* 544–553.

Barnes, D., and G. H. Sato (1980) Serum-free cell culture: A unifying approach. Cell *22:* 649–655.

Bottenstein, J., I. Hayashi, S. Hutchings, H. Masui, J. Mather, D. McClure, S. Ohasa, A. Rizzino, G. Sato, G. Serrero, R. Wolfe, and R. Wu (1979) The growth of cells in serum-free hormone-supplemented media. Methods Enzymol. *45:* 94–109.

Bottenstein, J. E., and G. H. Sato (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. (U. S. A.) *76:* 514–517.

Bottenstein, J. E., S. D. Skaper, S. S. Varon, and G. H. Sato (1980) Selective survival of neurons from chick embryo sensory ganglionic dissociates utilizing serum-free supplemented media. Exp. Cell Res. *125:* 183–190.

Bunge, R. P., M. B. Bunge, D. J. Carey, C. Cornbrooks, D. Higgins, M. I. Johnson, D. C. Kleinschmidt, P. M. Wood, L. Iacovitti, and F. Moya (1982)Functional expression in primary nerve tissue cultures maintained in defined medium. Cold Spring Harbor Conf. Cell Proliferation *9:* 1017–1032.

Crain, S. M. (1976) *Neurophysiologic Studies in Tissue Culture*, Raven Press, New York.

Dichter, M. A. (1978) Rat cortical neurons in cell culture: Culture methods, cell morphology, electrophysiology, and synapse formation. Brain Res. *149:* 279–293.

Fischbach, G. D., and P. G. Nelson (1977) Cell culture in neurobiology. In *Handbook of Physiology*, Sect. 1: *The Nervous System*, E. R. Kandel, vol. ed., Vol. 1, pp. 719–774, American Physiological Society, Bethesda, MD.

Havrankova, J., D. Schmechel, J. Roth, and M. Brownstein (1978) Identification of insulin in rat brain. Proc. Natl. Acad. Sci. (U. S. A.) *75:* 5737–5741.

Hayashi, I., and G. H. Sato (1976) Replacement of serum by hormones permits growth of cells in defined medium. Nature (Lond.) *259:* 132–134.

Heuser, J. E., and T. S. Reese (1977) Structure of the synapse. In *Handbook of Physiology*, Sect. 1: *The Nervous System*, E. R. Kandel, vol. ed., Vol. 1, pp. 261–294, American Physiological Society, Bethesda, MD.

Higgins, D., and H. Burton (1982) Electrotonic synapses are formed by fetal rat sympathetic neurons maintained in a chemically-defined culture medium. Neuroscience *7:* 2241–2253.

Honn, K. V., J. A. Singley, and W. Chavin (1975) Fetal bovine serum: A multivariate standard. Proc. Soc. Exp. Biol. Med. *149:* 344–347.

Jacobs, S., and P. Cuatrecasas (1981) Insulin receptor: Structure and function. Endocr. Rev. *2:* 251–263.

Jeejeebhoy, K. N., R. C. Chu, E. B. Marliss, G. R. Greenberg, and A. Bruce-Robertson (1977) Chromium deficiency, glucose intolerance, and neuropathy reversed by chromium supplementation in a patient receiving long-term parenteral nutrition. Am. J. Clin. Nutr. *30:* 531–538.

Kano, M. (1975) Development of excitability in embryonic chick skeletal muscle cells. J. Cell. Comp. Physiol. *86:* 503–510.

Kim, S. U. (1980) Tissue culture analysis of synaptogenesis. In *Tissue Culture in Neurobiology*, E. Giacobini, A. Vernadakis, and A. Shahar, eds., pp. 237–252, Raven Press, New York.

Lasher, R. S., and I. S. Zagon (1972) The effect of potassium on neuronal differentiation in cultures of dissociated newborn rat cerebellum. Brain Res. *41:* 482–488.

Meiri, H., M. E. Spira, and I. Parnas (1981) Membrane conductance and action potential of a regenerating axonal tip. Science *211:* 709–712.

Messer, A., P. Maskin, and J. E. Mazurkiewicz (1980) Effects of using a chemically defined medium for primary rat monolayer cerebellar cultures: Morphology, GABA uptake, and kainic acid sensitivity. Brain Res. *184:* 243–247.

Messer, A., J. E. Mazurkiewicz, and P. Maskin (1981) Growth

of dissociated rat cerebellar cells using serum-free supplemented media and varied transferrin concentrations. Cell. Mol. Neurobiol. *1:* 99–114.

Mirsky, R. (1982) The use of antibodies to define and study major cell types in the central and peripheral nervous system. In *Neuroimmunology*, J. Brockes, ed., pp. 141–182, Plenum Press, New York.

Mirsky, R., and E. J. Thompson (1975) Thy-1 (theta) antigen on the surface of morphologically distinct brain cell types. Cell *4:* 95–101.

Mirsky, R., L. M. B. Wendon, P. Black, C. Stolkin, and D. Bray (1978) Tetanus toxin: A cell surface marker for neuron in culture. Brain Res. *148:* 251–259.

Morrissey, J. H. (1981) Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal. Biochem. *117:* 307–310.

Nelson, P. G., and J. H. Peacock (1973) Electrical activity in dissociated cell cultures from fetal mouse cerebellum. Brain Res. *61:* 163–174.

Nelson, P. G., E. A. Neale, and R. L. Macdonald (1981) Electrophysiological and structural studies of neurons in dissociated cell cultures of central nervous system. In *Excitable Cells in Tissue Culture*, P. G. Nelson and M. Lieberman, eds., pp. 39–80, Plenum Press, New York.

Patterson, P. H. (1978) Environmental determination of autonomic neurotransmitter functions. Annu. Rev. Neurosci. *1:* 1–17.

Pettmann, B., J. C. Louis, and M. Sensenbrenner (1979) Morphological and biochemical maturation of neurons cultured in the absence of glial cells. Nature (Lond.) *281:* 378–380.

Puymiral, J., C. Loudes, A. Faivre-Bauman, A. Tixier-Vidal, and J. M. Bourre (1982) Expression of neuronal functions by mouse fetal hypothalamic cells cultured in hormonally defined medium. Cold Spring Harbor Conf. Cell Proliferation *9:* 1033–1051.

Raff, M. C., K. L. Fields, S. -I. Hakomori, R. Mirsky, R. M. Pruss, and J. Winter (1979) Cell type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. Brain Res. *174:* 283–308.

Rockwell, G. A., D. McClure, and G. H. Sato (1980) The growth requirements of SV40 virus-transformed Balb/c-3T3 cells in serum-free monolayer culture. J. Cell. Physiol. *103:* 323–331.

Sanford, K. K., R. Prashad, S. L. Handleman, F. M. Price, R. R. Gantt, and V. J. Evans (1979) Serum-induced chromosome damage and neoplastic transformation of mouse cells *in vitro*. In Vitro *15:* 488–496.

Sato, G. H., A. B. Pardee, and D. A. Sirbasku, eds. (1982) Growth of Cells in Hormonally Defined Media. Cold Spring Harbor Conf. Cell Proliferation, Vol. 9.

Silberberg, D. H., S. H. Dorfman, N. Latovitzki, and L. H. Younkin (1980) Oligodendrocyte differentiation in myelinating cultures. In Tissue Culture in Neurobiology. E. Giacobini, A. Vernadakis, and A. Shahar, eds., pp. 489–500, Raven Press, New York.

Skaper, S. D., R. Adler, and S. S. Varon (1979) A procedure for purifying neuron-like cells in cultures from central nervous tissue with a defined medium. Dev. Neurosci. *2:* 233–237.

Spitzer, N. C. (1979) Ion channels in development. Annu. Rev. Neurosci. *2:* 363–397.

Underwood, E. J. (1977) *Trace Elements in Human and Animal Nutrition*, Ed. 3, pp. 14–264, Academic Press, Inc., New York.

Waymouth, C. (1977) Nutritional requirements of cells in culture, with special reference to neural cells. In *Cell, Tissue and Organ Cultures in Neurobiology*, S. Fedoroff and L. Hertz, eds., pp. 631–648, Academic Press, Inc., New York.

Waymouth, C., R. G. Ham, and P. J. Chapple (1981) *The Growth Requirements of Vertebrate Cells In Vitro*, Cambridge University Press, New York.

Weyhenmeyer, J. A., and R. E. Fellows (1983) Presence of immunoreactive insulin in neurons cultured from fetal rat brain. Cell Mol. Neurobiol. *3:* 81–86.

Yavin, Z., and E. Yavin (1980) Survival and maturation of cerebral neurons on poly(L-lysine) surfaces in the absence of serum. Dev. Biol. *75:* 454–459.