

Differentiation of a Teratocarcinoma Line: Preferential Development of Cholinergic Neurons

S. E. PFEIFFER, H. JAKOB, K. MIKOSHIBA, P. DUBOIS, J. L. GUENET, J.-F. NICOLAS,
J. GAILLARD, G. CHEVANCE, and F. JACOB

Departments of Molecular Genetics, Molecular Neurobiology, and Electron Microscopy, Pasteur Institute, Paris, France. Dr. Pfeiffer's present address is the Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06032, and Dr. Mikoshiba's present address is the Department of Physiology, Keio University School of Medicine, Tokyo, Japan.

ABSTRACT A line of embryonal carcinoma cells, PCC7-S, established *in vitro* from a spontaneous testicular teratocarcinoma, has been studied. Upon removing the cells from a low density monolayer culture system and permitting the cells to form aggregates in suspension, we observed a change of several physical and biochemical parameters: (a) reduction in average cell volume, (b) blockage and accumulation of cells in G₁, (c) rise in secreted protease activity, (d) rise in acetylcholinesterase and choline acetyltransferase activities, and (e) disappearance of embryonic antigen F9.

Although PCC7 aggregates did not undergo substantial morphological changes while suspended, when aggregates 4 or more days old were allowed to attach to plastic tissue culture dishes, substantial neurite outgrowth occurred over the next 1–3 d. This process was markedly enhanced by the addition to the growth medium of carboxymethylcellulose and inhibitors of DNA synthesis. Transmission electron microscopy disclosed a neurite ultrastructure consistent with that of neuronal processes. A veratridine-stimulated, tetrodotoxin-blocked sodium influx of 100 nmol/min per mg protein was also observed in these differentiated surface cultures.

This cell line is discussed in terms of its utility for the study of early events leading to a commitment to cellular differentiation, as well as for the investigation of terminal differentiation to cholinergic neurons.

Mouse teratocarcinomas are transplantable tumors that contain malignant stem cells, called embryonal carcinoma (EC), and a variety of nonmalignant differentiated cells believed to be derived from the EC corresponding to derivatives of the three germ layers (32, 43). There is now considerable morphological (33), biochemical (3), biological (6, 18, 27, 30), and serological (1) evidence documenting similarities between EC cells and "uncommitted cells" of the early embryo.

EC cell lines offer several opportunities for the study of early mouse embryogenesis. In particular, some lines are able to grow and differentiate in culture (16, 25, 28, 35). In this paper, we describe a new EC cell line, PCC7-S, which at low cell density grows as a homogeneous population of undifferentiated cells, but which upon *in vitro* growth to high cell density differentiates rapidly to several cell types, producing a particularly strong component of neuronal elements.

MATERIALS AND METHODS

Cell Culture

PCC7-S line of embryonal carcinoma cells was established by selection of a small population of cells from a homogeneous area of embryonal carcinoma cells (11). All experiments were performed on cells of passage 7–22. In some experiments a previously developed teratocarcinoma line, PCC3/A1 (28), was studied in parallel. Cell stocks were grown as monolayer cultures at low density (see Results) in plastic tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif., or Corning Glass Works, Science Products Div., Corning, N. Y.) in Eagle's Dulbecco modified medium supplemented with 15% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) (27). Growth to high density and the accompanying differentiation (Results) led to multilayering. Cells were subcultured by removing them by gentle pipetting after a 5-min incubation at 37°C in phosphate-buffered saline. Aggregates were grown in the same medium in bacteriological petri dishes. Carboxymethylcellulose obtained from Hercules Incorporated (type 7HF; Wilmington, Delaware) or from Novacel (Blanose RP195; Paris) was solubilized in water by autoclaving and mixed with an equal volume of double-strength medium before use.

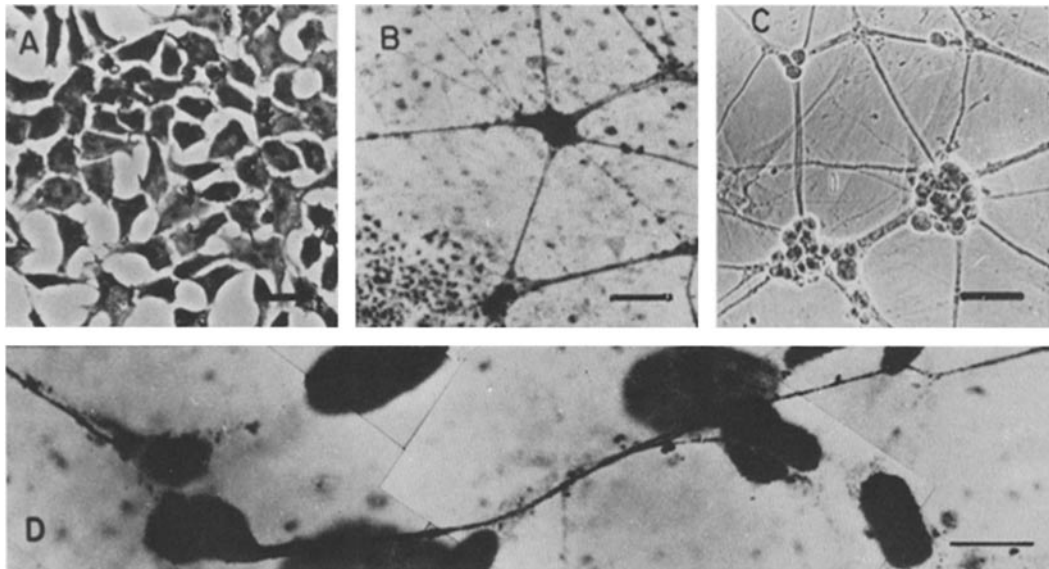


FIGURE 1 (A) EC cells of line PCC7-S in monolayer culture. Hematoxylin and eosin. Bar, 20 μm . $\times 342$. (B) Differentiated surface culture of line PCC7-S. EC were seeded into a tissue culture dish at 1.5×10^5 cells/cm² and grown for 10 d. Bodian silver impregnation. Bar, 100 μm . $\times 85$. (C) Similar to B, except cells were grown for 24 d before fixation and embedding for transmission electron microscopy (see Materials and Methods). Phase contrast. Bar, 100 μm . $\times 138$. (D) Similar to B, composite of high-power photomicrographs of individual process-bearing cells. Large non-neuronal nuclei are also stained in this photograph. Bodian silver impregnation. Bar, 10 μm . $\times 1,250$. All photographs are typical of the cultures as a whole.

Volume Measurements

Cell volumes were determined on a Coulter Counter model B (Coulter Electronics, Hialeah, Fla.) as described in the legend to Fig. 3.

Transmission Electron Microscopy

Surface cultures were fixed and embedded directly on tissue culture dishes. Using the techniques of Brinkley et al. (7) and Picart and Tixier-Vidal (31), specific regions of the culture were selected by phase-contrast microscopy, punched out with a warm stainless steel tube, reembedded, and sectioned either parallel or perpendicular to the original plane of the tissue culture dish. Fixation was done with 2% glutaraldehyde in 0.5 M phosphate-buffered saline, pH 7.4, for 1 h at room temperature followed by continued overnight fixation at 4°C. Cells were postfixated with OsO₄ before embedding in Epon. Sections were poststained with uranyl acetate before examination.

Scanning Electron Microscopy

Drops of suspended aggregates in the tissue culture medium were placed on small Millipore filters (HA; Millipore Corp., Bedford, Mass.) and incubated in a CO₂ incubator for 5 min. Alternatively, circles were punched out of plastic dishes carrying surface cultures. They were then fixed in 2% glutaraldehyde in cacodylate buffer and prepared for scanning electron microscopy by standard techniques.

Assays of Cell Surface Antigens

Cells were removed from tissue culture dishes or dissociated from aggregates by a 5-min treatment with 2 mM EDTA and 2% chick serum in phosphate-buffered saline. Cytotoxicity assays were carried out as previously described (1). Anti-F9 antiserum was prepared in syngeneic 129 male mice against whole F9 EC cells and used at 1:500. Absorbed antisera were titered on fresh F9 cells. Anti-H-2 antiserum (α -H2^b) was prepared by immunizing ASW/B10D₂ mice with B10R5 lymphocytes and used at 1:500. Absorbed antisera were titered on strain 129 lymphocytes.

Immunofluorescence

Anti-F9 antiserum described above was absorbed with PYS-2 cells (17) and used at 1:60. Anti-H-2 antiserum (obtained from T. Tanigaki) was made by immunizing rabbits with purified H-2 heavy chains eluted from mouse lymphocytes and used at 1:5.

Autoradiography

Cells were swollen in hypotonic phosphate-buffered saline for 5 min before fixation in ice-cold acetic acid-ethanol (1:3) for 15 min. A drop of cells was placed on a glass microscope slide to which they adhered and spread upon drying. The attached cells were washed in several changes of water, coated with Ilford emulsion, and stored desiccated until development in D-19.

Protease Activity

Protease activity was assayed by the fibrin-agarose overlay assay (2). Aggregates were either allowed to attach to tissue culture dishes for 4 h and overlaid with, or embedded directly into, an agarose-fibrin-plasminogen gel.

Protein Content

Protein content was assayed by the method of Lowry et al. (22).

Sodium Flux

EC cells or aggregates were grown in 35-mm tissue culture dishes. Cells were prelabeled overnight with L-³H-amino acid mixture (New England Nuclear, Boston, Mass.; NET 250) at 0.4 $\mu\text{Ci/ml}$ in the standard culture medium. The influx of sodium in response to treatment with 2×10^{-4} M veratridine (Sigma Chemical Co., St. Louis, Mo.) in the presence or absence of 10^{-7} or 10^{-6} M tetrodotoxin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; lot 101096) was measured in the presence of 5×10^{-3} M ouabain (Sigma Chemical Co.) as described by Catterall and Nirenberg (8), using ²²Na (Amersham Corp., Arlington Heights, Ill.) in 50 mM NaCl at 5 $\mu\text{Ci/ml}$. Sodium hydroxide digests were counted in a scintillation spectrometer (³H) and a gamma counter (²²Na). Corrections for variable cell loss incurred during the washing procedures were made on the basis of the tritium activities.

Acetylcholinesterase

Acetylcholinesterase (AChE) activity was assayed by the method of Ellman et al. (10) for cell homogenates with or without tetraisopropylpyrophosphoramidate (Sigma Chemical Co.) at 10^{-4} M, or by the histochemical method of Karnovsky and Roots (15), comparing the activities with acetylthiocholine and butyrylthiocholine substrates. The reliable assay sensitivity was 1 pmol/min per mg protein.

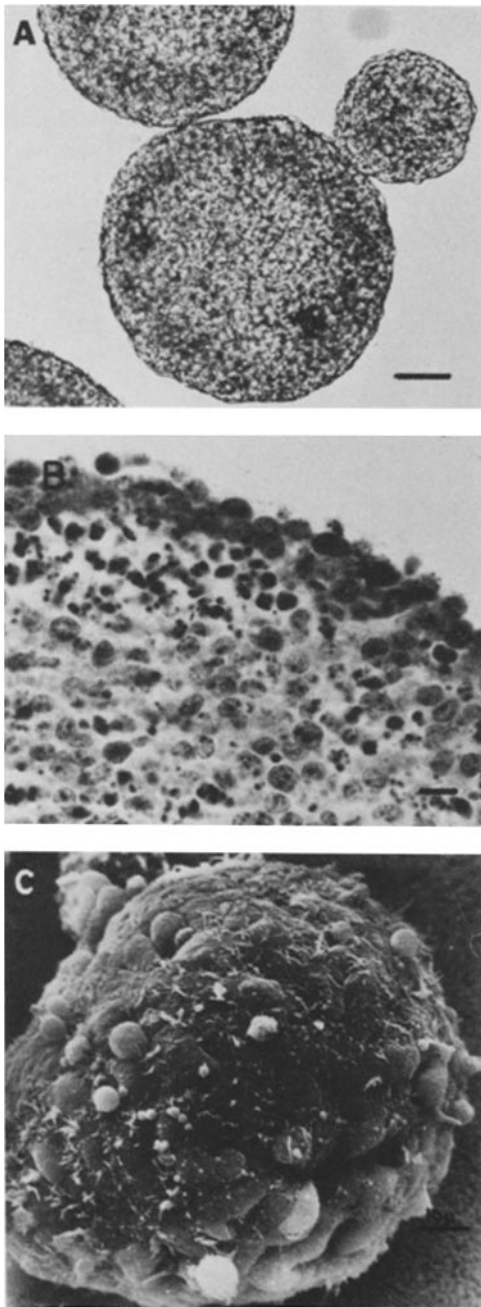


FIGURE 2 Suspended aggregates of line PCC7-S cells. EC were plated at 2.5×10^5 cells/ml (20-ml total volume) in bacteriological petri dishes and incubated for 4 d (the medium was changed after 2 d). (A) Unfixed aggregates. Bright-field optics. Bar, $100 \mu\text{m}$. $\times 83$. (B) Histological section of 4-d-old aggregate. Bouin, hematoxylin and eosin. Bar, $10 \mu\text{m}$. $\times 511$. (C) Scanning electron micrograph. Bar, $10 \mu\text{m}$. $\times 750$. All photographs portray typical aggregates.

Choline Acetyl Transferase

Choline Acetyl Transferase (CAT) activity was assayed as described by Rossier et al. (36) as modified by Fonnum (12). Each assay was done in parallel with either 0.1 mM physostigmine or AChE (Sigma Chemical Co.) at 33 U/ml, and the difference in counts per minute between the two was taken as the measure of substrate conversion to acetylcholine. The reliable assay sensitivity was 5 pmol/min per mg protein.

Histology

Cells were routinely fixed overnight in Bouin's and stored in 70% ethanol until embedding, or processed directly for Bodian silver impregnation (4).

RESULTS

Origin of Line PCC7-S.

Cell line PCC7-S and its 8-azaquinine-resistant clone PCC7-S-Aza were established from a spontaneous teratocarcinoma of a male recombinant inbred mouse (129XB6). Injection of the cells into hybrid (129XB6) mice led to the frequent appearance of well-differentiated tumors. However, differentiation *in vitro* as described below gave an abundance of nervous system derivative cell types (although cells of an indeterminate cell type are also present). The karyotype with regard to chromosome number and banding pattern appeared to be normal (11).

Cultures in Tissue Culture Dishes

PCC7-S cells were routinely grown as undifferentiated EC cells at relatively low density ($0.2\text{--}3 \times 10^5$ cells/cm²) in plastic culture dishes (Fig. 1A). Under these conditions, PCC7-S cells grew with a population doubling time of ~ 10 h. If PCC7-S EC cells were allowed to attain high cell densities ($>6 \times 10^5$ cells/cm²) in surface culture, areas of multilayering soon developed, which by morphological (Fig. 1B–D) and biochemical (see below) criteria rapidly began to differentiate.

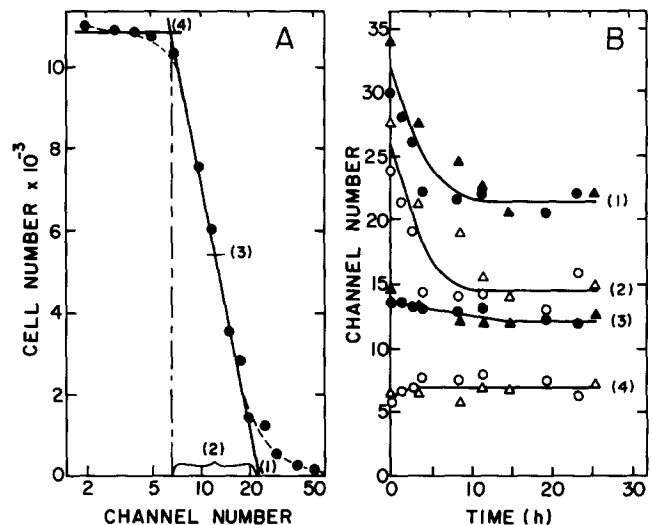


FIGURE 3 Cell volume changes in cellular aggregates. Cells were removed from monolayers (EC) or dissociated (aggregates) by gentle pipeting (possible without apparent cell loss up to ~ 36 h of aggregation) after a 3- to 5-min incubation in Ca^{2+} , Mg^{2+} -free phosphate-buffered saline. (A) The cell number was first determined over a range of decreasing discrimination widths. Low channel numbers indicate a broad width, counting both small and large cells; high channel numbers indicate a narrow width, counting only large cells. The "best" straight lines were drawn through the low channel plateau, through the rapidly falling portion of the curve, and through the high channel plateau (generally the x axis, indicating the absence of undissociated aggregates). Intersection (1) was taken as an estimate of the largest cells in the population, while (4) was used as an estimate of the smallest cells, and (3) was determined as an approximation of the average cell size. The heterogeneity in cell volumes within the population (2), was estimated by the difference between (1) and (4). (B) Cell size (1, 3, and 4) and cell volume heterogeneity (2) are plotted as a function of time after EC were replated into aggregation conditions. Two typical experiments (of several) have been plotted together (circles and triangles). Open and closed symbols serve merely to help differentiate the curves. In such experiments single samples were generally taken and assayed as a function of channel number (A) twice. Such repeated assays nearly always agreed within 5% or less.

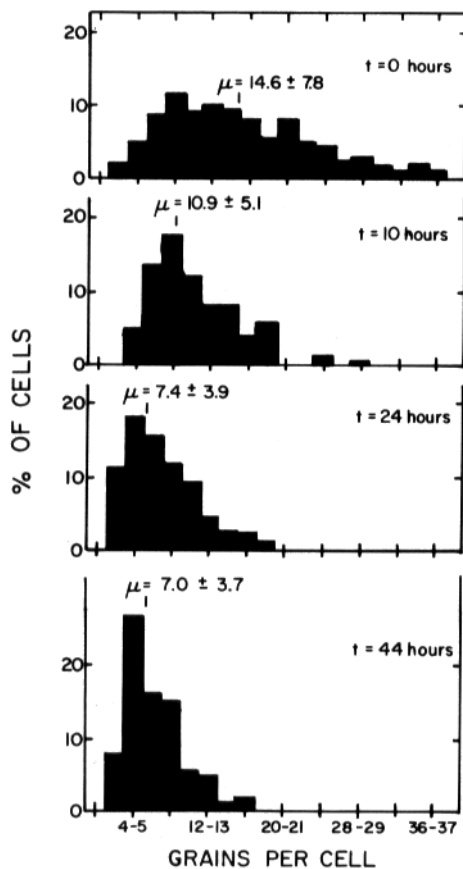


FIGURE 4 Distribution of grains per cell nucleus as a function of time after the onset of aggregation of prelabeled (^3H TdR) EC (see text for further details). A minimum of 200 cells was counted at each time point. The means and standard deviations of the means are indicated.

Aggregate Cultures

Differentiation μ in surface cultures was accompanied by substantial cell death and resultant floating debris before extensive differentiation. A more controlled method for creating high cell densities was used. EC cells were plated into bacteriological petri dishes in which the attachment of these cells proved to be minimal. Under these conditions, the EC cells quickly and synchronously began to aggregate into spheres of cells (Fig. 2), a process that was largely completed by 12 h. Most of the aggregates were 400–500 μm in diameter, although smaller and larger aggregates were present (Fig. 2A and C). Histological examination of these aggregates during the first 120 h after aggregation demonstrated little or no morphological changes in the cells, which continued to resemble EC cells throughout the aggregate (Fig. 2B). No morphological differentiation into cellular layers was observed during the first 4–7 d of aggregation, occasional pockets of necrosis could be found in sections, and the cells on the surface tended to be somewhat more densely packed relative to those in the interior.

Several parameters were studied as indices to cellular differentiation in these aggregates.

Cellular Volume

Cell volumes were measured during aggregation using a Coulter counter (Fig. 3A; see legend for details). Fig. 3B shows that although the average size of the smallest cells apparently

remained unchanged (or possibly increased slightly), within 15 h after the onset of aggregation, the average size of the largest cells decreased to $\sim 70\%$, and the spread of volumes decreased to $\sim 60\%$, of the values of these parameters observed for EC cells at the time of replating to start aggregate formation. In other words, the proportion of smaller cells increased while larger cells were essentially lost from the population. Similar results were obtained with aggregating cultures of EC line PCC3/A1.

Cell Cycling

One interpretation of the cell volume data shown in Fig. 3B is that, upon aggregation, the cells accumulate in the G_1 phase of the cell cycle and cease proliferation. To test this hypothesis further, EC cells growing in monolayer culture were labeled for 10 h with ^3H thymidine (0.2 mCi/mmol, 2×10^{-5} M) before being allowed to aggregate (direct labeling of aggregates was not done to avoid uncertainties of thymidine penetration into the aggregate interior). Subsequently, samples of the aggregates were taken at several time points after the onset of aggregation, the cells were dissociated as for the volume measurements, and the dissociated cells were fixed and prepared for autoradiographic analysis of labeled cells. If the hypothesis of cycling cessation in aggregates is correct, the average number of grains per cell should initially decrease as cells undergo their final mitoses, and then should become constant. Fig. 4 shows a typical experiment in which this prediction was observed, with the grains per cell reaching a constant value by 24 h.

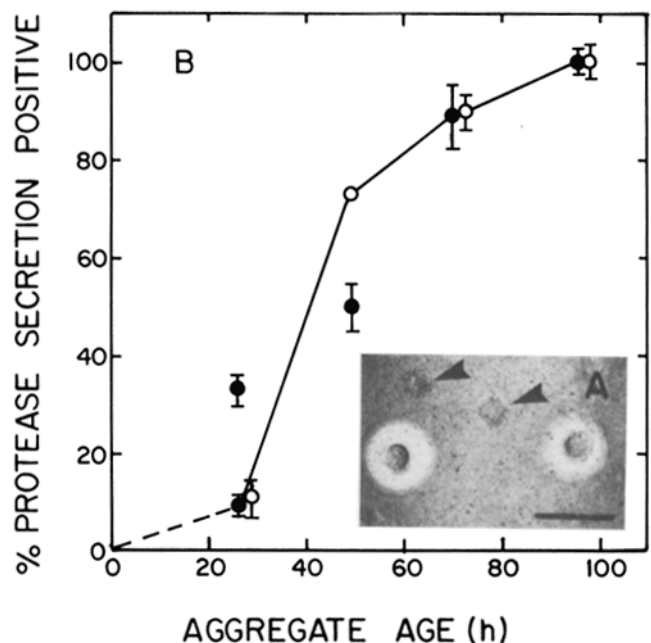


FIGURE 5 Protease activity in developing aggregates of line PCC7-S. Aggregates of various ages were either plated onto 60-mm tissue culture dishes for 4 h and then overlaid with agar containing plasminogen and fibrin, or embedded directly into the agar substrate. The plates were then incubated for 24 h before scoring for lysis rings. (A) Typical example of positive and negative (arrows) aggregates. Phase contrast. $\times 10$. Bar, 1 mm. (B) Appearance of protease-positive aggregates as a function of time after the onset of aggregation. Open and closed symbols represent results of two typical independent experiments, bars indicate the range of duplicate points.

Protease Activity

Protease activity was investigated by plating aggregates of various ages into agar containing plasminogen and fibrin. After 24 h of incubation, the percent of aggregates with clear rings of lysis around them (Fig. 5A) were scored. Fig. 5B shows that protease activity first appeared at ~24 h after the onset of aggregation, and that essentially all the aggregates were positive by 100 h. Similar experiments with line PCC3/A1 yielded comparable results (two experiments), with the onset occurring at 24 h but with only 80% of the aggregates becoming positive by 100 h. In addition, the lysis rings were much smaller.

Surface Antigens F9 and H-2

The presence of the embryonic surface antigen F9(1) and histocompatibility antigens (H-2) was assayed on EC and on cells dissociated from aggregates of various ages. By use of fluorescence-labeled antibody techniques, it was found that F9 was present on 74% of the EC cells, 48% of the cells after 3.5 d of aggregation, 12% of the cells after 4.5 days, and only 4% after 10 d (Table I). In contrast, H-2 antigens were undetectable at all stages. These data were corroborated by absorption followed by measurement of residual cytotoxicity on lymphocytes (for H-2) or F9 cells (for F9 antigen).

Morphological Differentiation of PCC7-S Aggregates after Attachment to Solid Substrates

Cell aggregates of various ages up to 6 d old were plated onto solid substrates (generally plastic tissue culture dishes) to

TABLE I
Surface Antigens on Line PCC7-S as a Function of Age

Cells tested	Antigen	
	F9	H2
	% positive cells	
Embryonal carcinoma	74	0
Aggregates, 3.5 d	48	0
Aggregates, 4.5 d	12	0
Differentiated surface culture, 10 d	4	0

which they attached very rapidly. After 24–48 h of attachment, aggregates 4 or more days old at the time of plating had begun to send out numerous processes (Fig. 6A), often on top of large flat cells. When similar aggregates were overlaid soon after attachment with medium containing 1.3% carboxymethylcellulose, process outgrowth proceeded more rapidly at rates up to 20–30 $\mu\text{m}/\text{h}$ and more extensively (Figs. 6B and 7A–F). The processes now appeared to grow out as individuals, rather than in bundles, and did not require a background of flat cells upon which to rest. The addition of 10^{-5} M cytosine arabinoside and 10^{-6} M FUdR further limited flat cell outgrowth, and enhanced neurite outgrowth. Scanning electron microscopy of the ends of these processes (Fig. 7B) revealed structures reminiscent of the growth cones seen in primary culture of neurons (e.g., reference 5). Close to the aggregates themselves could be found round, refractile process-bearing cells 5–10 μm in diameter which had migrated out of the mass of cells (Fig. 7C). The processes often had extensive arrays of varicosities along their length (Fig. 7D). The process network was invariably stained by the Bodian silver impregnation technique (Fig. 7E), and interactions (as evidenced by membrane flattening) between most processes were observed wherever they came into close opposition (Fig. 7F).

After aggregates like those shown in Fig. 6A had been in surface culture for a week or more, bundles of processes extended from the individual aggregates, often interconnecting one or more aggregates (similar to the situation seen in Fig. 1B). These cultures were examined by electron microscopy, cutting sections either parallel or perpendicular to the plane of the culture dish. Particular attention was paid to the nature of the bundles of processes. Fig. 8A–D show a concentration of cell bodies and process bundles in (a) longitudinal section at relatively low power (Fig. 8A) and at higher power (Fig. 8B), and (b) in cross section at low (Fig. 8C) and higher (Fig. 8D) power. Fig. 8E shows a section through cell bodies, including one which displayed a well-developed Golgi apparatus at the process hillock. The processes were characterized by abundant microtubules ranging in diameters from 250 to 290 \AA and the absence of endoplasmic reticulum. Overall, the process morphology was consistent with that of rather mature neuronal axons. The cell bodies appeared generally to be somewhat less

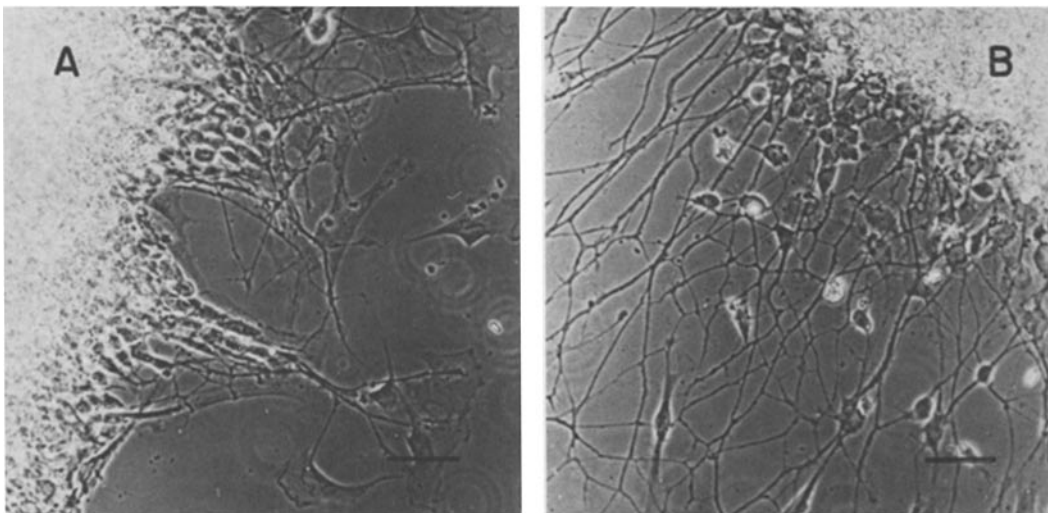


FIGURE 6 Morphological differentiation of 6-d-old aggregates of line PCC7-S after 48 h of attachment to a tissue culture dish, showing the effect of carboxymethylcellulose on process formation. (A) Phase contrast of unfixed cells in standard culture medium. (B) Same as A, but with a 1.3% carboxymethylcellulose-containing medium overlaid after the first 24 h of attachment. Bars, 50 μm . Both photographs $\times 205$. In any given experiment, 80–90% of the aggregates exhibited these appearances (see also Fig. 7).

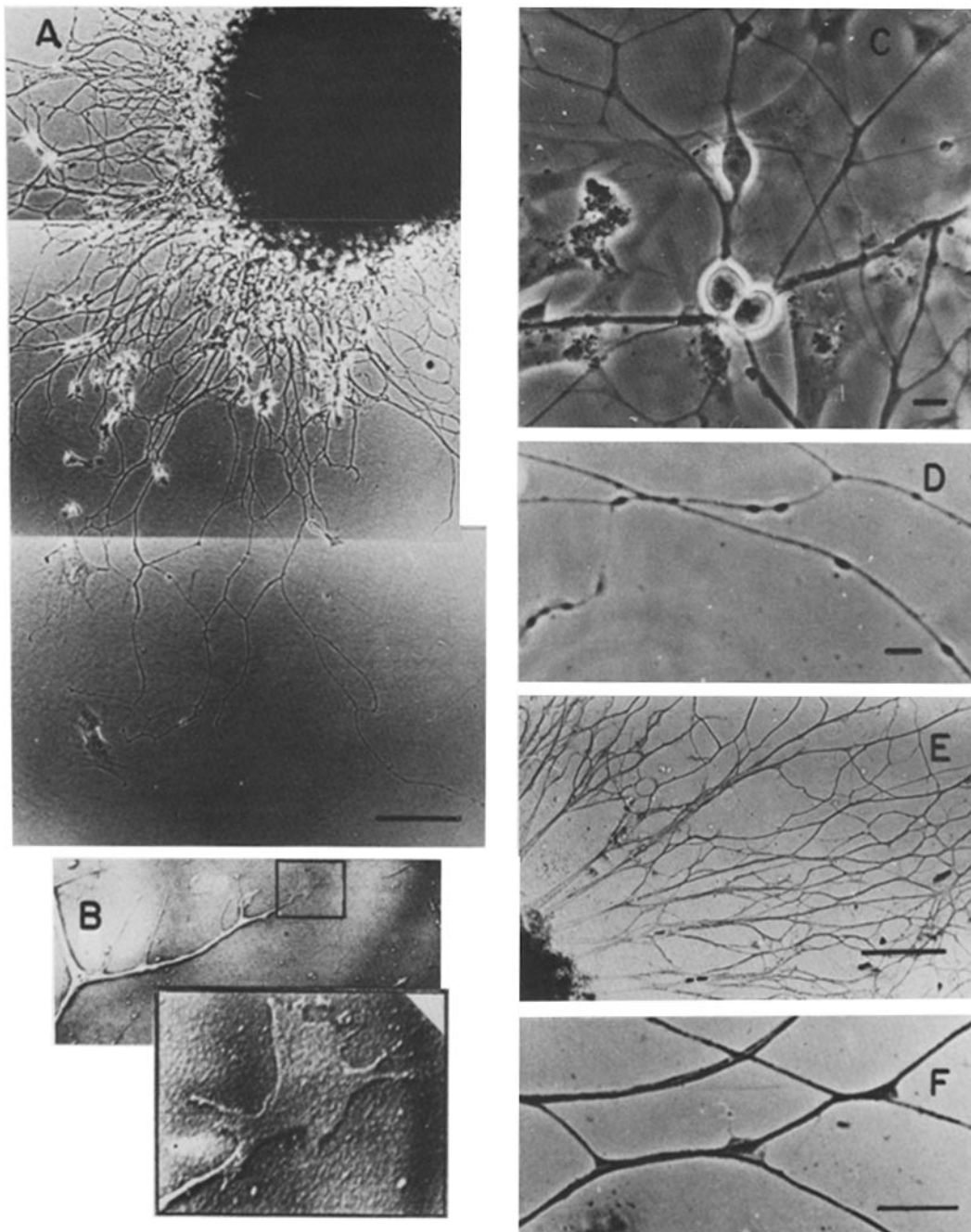


FIGURE 7 Morphological differentiation of a typical 6-d-old aggregate of line PCC7-S after 3 d of attachment to a tissue culture dish in medium containing 1.3% carboxymethylcellulose. (A) Phase contrast of unfixed cells. Bar, 100 μm . $\times 138$. (B) Scanning electron micrograph showing a commonly found presumptive growth cone at the end of a long process. $\times 600$. Inset $\times 2,700$. (C) Higher magnification of three process-bearing cells of the type routinely found near the aggregate on top of several flat cells. Phase contrast of unfixed cells. Bar, 10 μm . $\times 550$. (D) Varicosities commonly found near the ends of processes. Phase contrast of unfixed cells. Bar, 10 μm . $\times 520$. (E) Bodian silver impregnation of a similar aggregate as in A–D. Phase contrast. Bar, 100 μm . $\times 138$. (F) Same as E at higher magnification showing interaction of processes coming in contact with one another. Phase contrast. Bar, 10 μm . $\times 1,214$.

mature, with a relative paucity of rough endoplasmic reticulum, although ribosomal rosettes could be easily located. Although collections of small (40–100 nm) vesicles within processes were not uncommon (Fig. 8 B–D), and slight membrane thickenings were observed occasionally, no bona fide synapse formation was noted in attached aggregate cultures up to 10 d of age. Similarly, myelination was not observed in these relatively young cultures.

AChE and CAT Activity

On the basis of the morphological changes which appeared when PCC7-S cells were grown in surface culture, AChE and CAT, two enzyme activities associated with neurotransmission, were investigated. Fig. 9A shows that AChE activity was present at low levels in EC cells and increased approximately fivefold during the first 4 d of aggregation. Subsequent plating

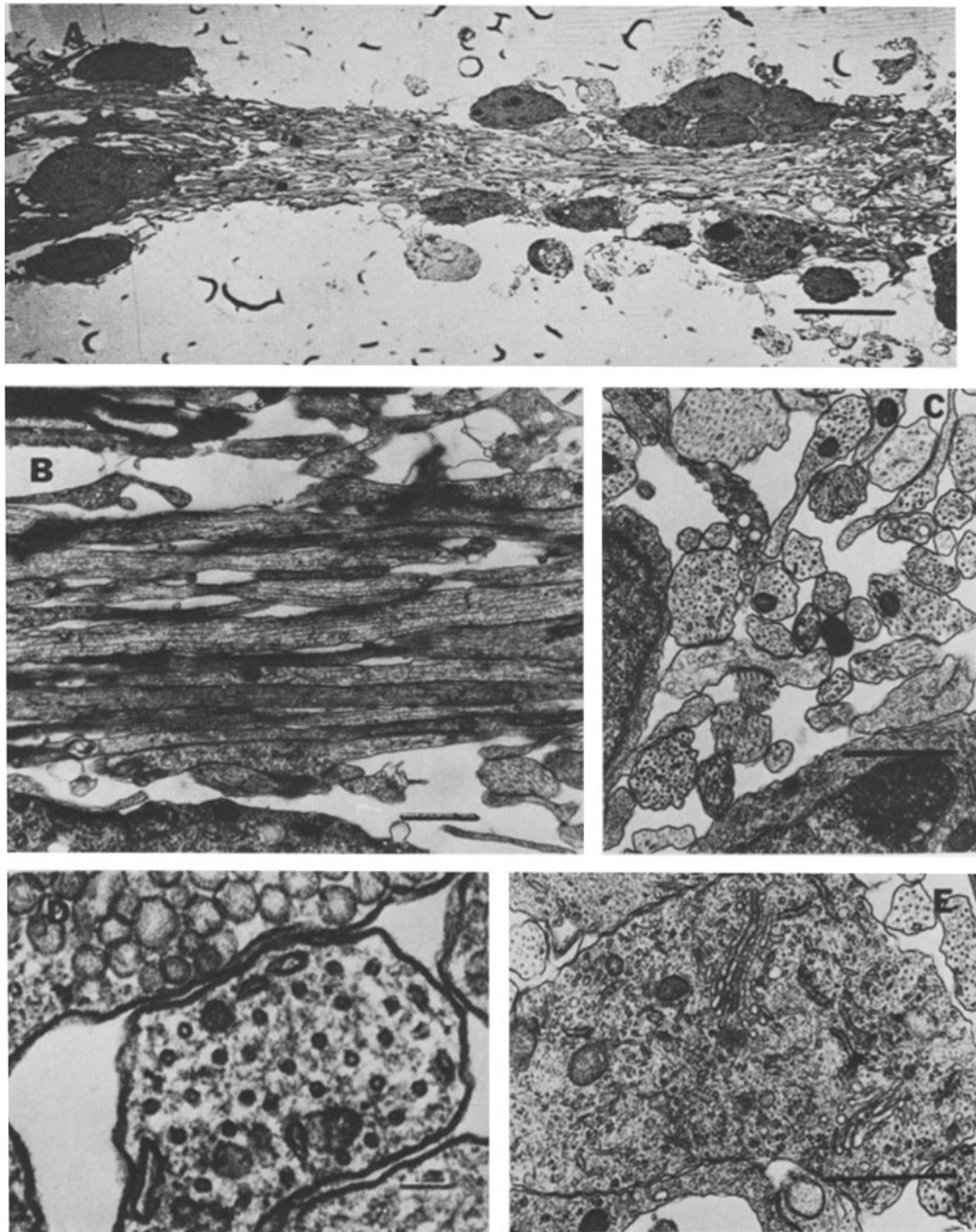


FIGURE 8 Transmission electron micrographs of attached differentiated PCC7-S aggregates. Typical 4-d-old aggregates after an additional 7 d as attached cultures. (A) Longitudinal section through neurite bundle extending between two attached aggregates. Bar, 10 μm . \times 1,600. (B) Same as A at higher magnification. Bar, 1 μm . \times 11,800. (C) Cross section of same neurites as in A and B. Bar, 1 μm . \times 18,100. (D) Same as C at higher magnification. Bar, 100 nm. \times 82,400. (E) Section through cell body from same culture. Note Golgi apparatus. Bar, 1 μm . \times 22,000.

of 4-d-old aggregates into tissue culture dishes led to a further twofold increase in the specific activity of this enzyme. Changes in the specific activity of CAT were similar although more abrupt (Fig. 9A): the specific activity of this enzyme was below detectable levels in EC cells and 2-d-old aggregates but rose sharply after 4 d of aggregation. Again, plating the aggregates into tissue culture dishes resulted in a further increase in CAT specific activity. Similar increases in the specific activity of these two enzymes were observed with time after PCC7-S EC cells were plated at high density directly into tissue culture dishes (Fig. 9B).

AChE activity was also examined by histochemical techniques. Fig. 10 shows the staining of cultures derived from the plating of 4-d-old suspension aggregates onto a tissue culture dish and incubated as a surface culture for an additional 7 d. Enzyme activity was limited to the bundles of processes and the cell bodies from which they emanate; flat background cells did not have appreciable activity (Fig. 10).

The relative effectiveness of aggregate culture vs. high density surface culture to induce differentiation was examined further using AChE and CAT activities as markers of differentiation. At various times after EC cells were plated into

aggregation cultures conditions, samples of aggregates were taken and either assayed directly for AChE and CAT activity, or first plated onto tissue culture dishes for an additional 2 d of incubation before assay (Fig. 11). Consistent with Fig. 9, enzyme activities began to rise in the aggregates after 3 and 4 d. In contrast, when aggregates were transferred to surface culture for 2 d before assay, an extra 24–48 h of total incubation time was required to attain comparable levels of enzyme activity.

Sodium Channels

The development of a neurotoxin-stimulated inward sodium flux was measured in 4-d-old aggregates after they had undergone morphological differentiation for an additional 7 d in surface culture. While EC cells were unaffected, treatment of differentiated cultures with veratridine at 10^{-4} M (a drug known to stimulate sodium flux in electrically excitable cells [8]) resulted in a sodium flux of 100 nmol/min per mg protein (range 89–120 in three experiments), a 2.4-fold increase over control cultures treated with ouabain alone at 10^{-3} M. This stimulation was completely eliminated by the sodium channel blocking agent tetrodotoxin at 10^{-6} M. The magnitude of this response compares favorably with those obtained using cells with electrophysiologically documented electrically active cell membranes (8).

DISCUSSION

The data presented above demonstrate that undifferentiated EC cells of line PCC7-S are induced by high cell density, such as aggregation of cells in suspension, to undergo a rapid differentiation in vitro. While neuronal-like cells are prominent in number, other unidentified cell types appear as well both in vivo and in vitro. Unlike several other EC cell lines (e.g., reference 26), PCC7-S cells do not undergo substantial morphological changes while suspended as aggregates. No outside layer of presumptive endoderm develops, and cysts are rare or absent.

The process of aggregation leads to a rapid commitment of EC cells to differentiate, a phenomenon noted frequently enough to be considered a general principle (24, 25, 28, 40, 41). What is not clear is how aggregation leads to differentiation. From the experiments reported here, it appears that as soon as EC cells begin to aggregate, they accumulate in the G₁ phase of the cell cycle as evidenced by cell volume measurements and [³H]thymidine-labeling studies. Thus the role of aggregate formation in the induction of differentiation could be either a direct or indirect one. Unlike the case with Friend virus-transformed erythroleukemia cells, in which a decrease in volume occurs upon the onset of differentiation in addition to changes in volume associated with cell cycle progression (21), the volume reduction in PCC7-S cells upon aggregation appears to be caused by an accumulation of smaller G₁ cells.

The data presented in this paper also suggest that there is a critical minimum length of aggregation required in order for the program of differentiation to become fixed. For example, in Fig. 11, a period of at least 2 d at the high cell density afforded by aggregation in suspension was necessary for the rapid appearance of cholinergic neurotransmitter enzyme activity. Attachment of the aggregates to tissue culture dishes earlier than 2 d resulted in cell migration from the aggregate, a general loosening of the cell packing, and a reduced rate of differentiation. It is interesting to speculate that this minimum time in aggregated form could be related to the minimum time

that cells must remain in G₁ phase before an irreversible commitment to differentiation is firmly made.

Although morphological changes in aggregated cells are minimal, biochemical changes indicate that in fact these cells undergo a program of differentiation. Thus, a secreted protease activity appears, AChE and CAT activities develop, substantial neurotoxin-induced sodium influx potential arises, and the F9 antigen activity present on EC cells is lost.

The CAT specific activities (pmol/min per mg protein) we observed in differentiating PCC7-S cells are lower than those found in adult mouse and rat brain (550–2,000) (12, 34, 36, and our own assays) but comparable to those observed in fetal rat brain primary culture (100–200) (38), and higher than those

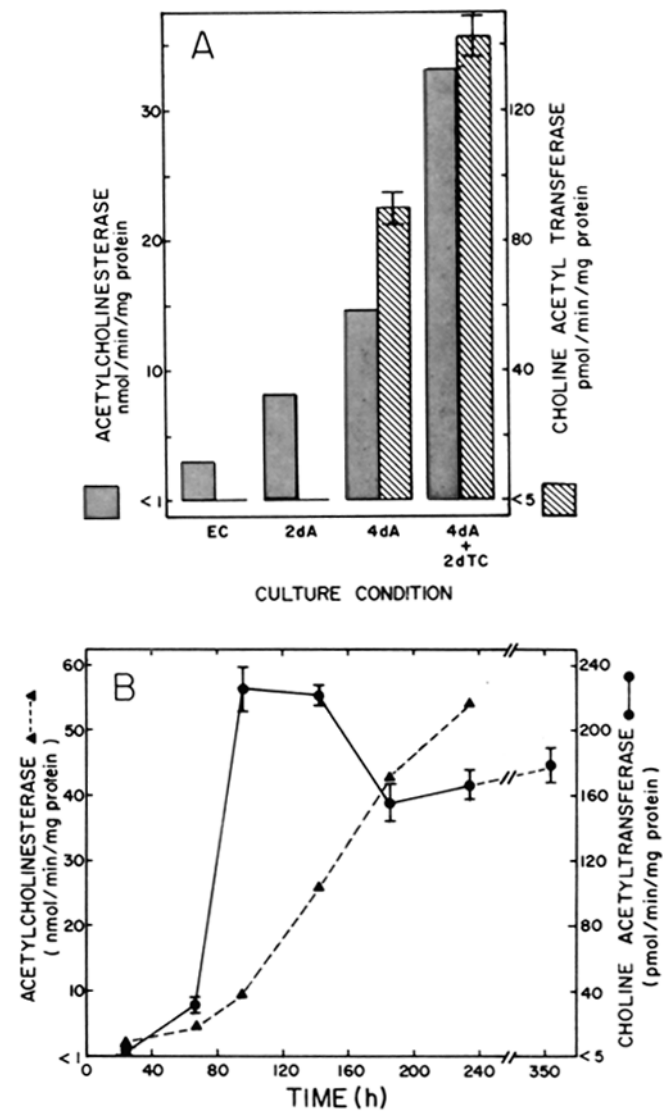


FIGURE 9 Appearance of AChE and CAT activities in line PCC7-S cells. Bars show ranges of duplicates for CAT activities. Single samples were taken for AChE for which repeated assays agreed within 5%. (A) EC; floating aggregates 2 d old (2dA) or 4 d old (4dA); and 4-d-old aggregates grown an additional 2 d as attached cultures (4dA + 2dTC) in standard culture medium. Results of one of two similar experiments are shown. (B) Differentiating surface cultures. EC were plated at an initial density of 1.6×10^5 cells/cm² and allowed to grow to high density with medium changes every 48 h (see Fig. 1). The results of a single complete experiment performed are shown; experiments with only earlier or later time points confirmed these results (data not shown).

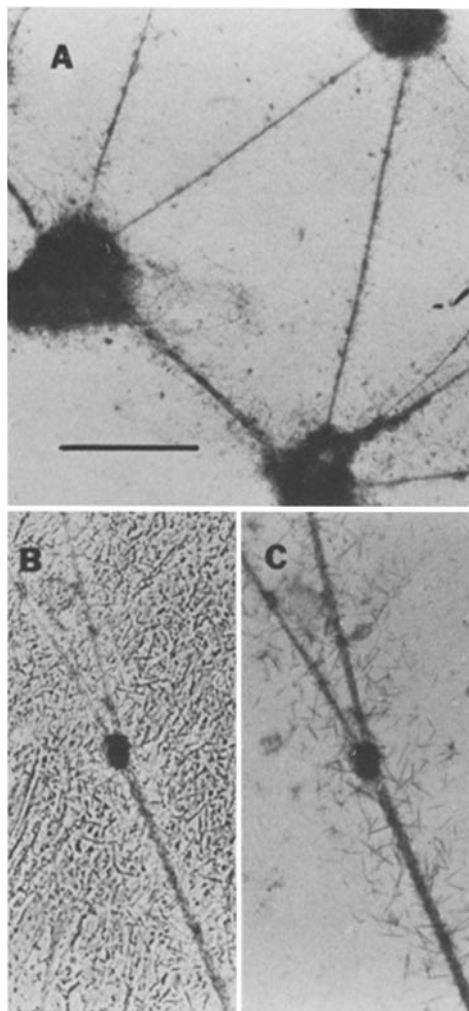


FIGURE 10 Histochemical demonstration of AChE activity in differentiated PCC7-S cells growing as high density attached surface cultures for 10 d after subculture. (A) Bright-field optics; typical result, showing stained cell body aggregates and processes passing between such aggregates. (B) Phase-contrast and (C) bright-field optics of another field, demonstrating the absence of enzyme activity in the flat background cells (seen only in phase-contrast view). Bar, 100 μ m. All photographs \times 215.

reported for most neuronal tumor cell lines (2–77, but 490 for line NS-20) (see reference 34) and for teratocarcinoma cells in culture derived from tumor OTT6050 (0.4: calculated from 1.2 nmol/h per g wet weight) (29). The AChE specific activities (nmol/min per mg protein) we observed in PCC7-S are comparable to those found for adult rodent brain (42–70) (34, and our own assays), higher than those observed in fetal rat brain primary cultures (2) (see reference 39), lower than those reported for many neuronal tumor cell lines (105–256) (see reference 34), and substantially higher than those seen in OTT6050 teratocarcinoma cultures (1–10: calculated from 0.01 Δ OD₅₅₀/min per mg protein using an extinction coefficient of 1.36×10^7 [20] and 18.5–42.1 nmol/h per g wet weight [29], respectively).

The presence of the F9 antigen(s) on PCC7-S EC cells is considered to be diagnostic for true embryonal carcinoma cell characteristics, as this antigen has been found on all other teratocarcinoma EC cell lines tested, as well as male germ line cells and mouse embryos up to the morula stage (13, 14). What

is perhaps surprising in view of studies with other EC cell lines derived from other tumors is the absence of H-2 antigens on otherwise differentiated PCC7-S cells, as H-2 antigenicity has generally been observed to increase as F9 antigenicity decreases (13, 14). PCC7-S cells do acquire the H-Y male antigen upon differentiation, an antigen apparently associated with β_2 -microglobulin (11).

Upon plating 4- to 7-d-old aggregates onto a solid substrate to which they could attach, substantial morphological changes occur. The most pronounced of these changes is the rapid appearance of numerous processes which by electron microscopy resemble rather mature neuronal processes. This outgrowth of processes is enhanced by the presence in the medium of carboxymethylcellulose. Whether this additive acts in a purely protective manner through increased medium viscosity, by altering charge interactions between the developing processes and the tissue culture dish, or via the presence of impurities in the polymer preparation that act as neurite growth factors, is not known. These observations have recently been extended to neuroblastoma cells growing in vitro (19). Certainly the importance of neurite-substrate interactions in neurite development is well known (37, 46), and methylcellulose

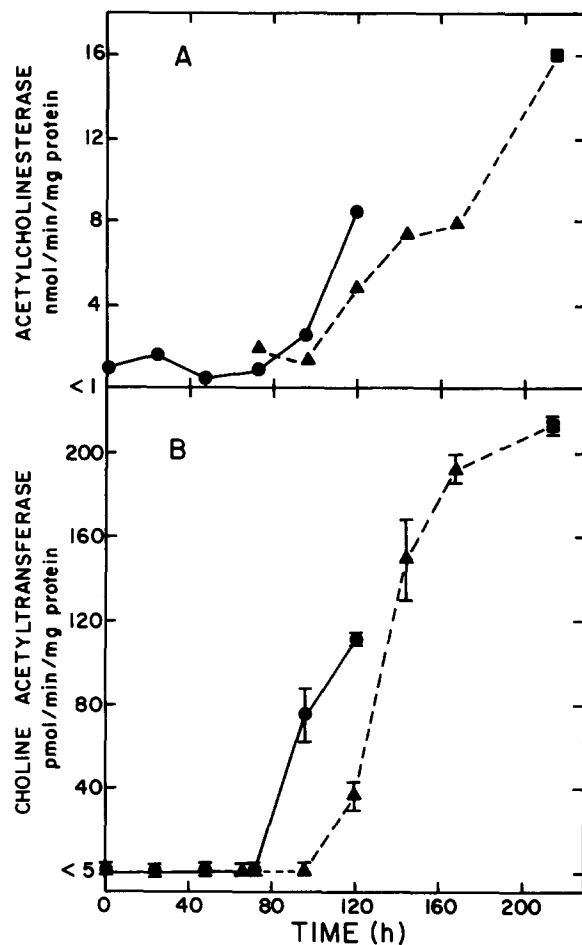


FIGURE 11 Appearance of AChE and CAT activities in developing aggregates of PCC7 cells. Cells were grown as floating aggregates and assayed either directly at various times after plating the cells into aggregation conditions (circles), or after an additional 2 d (triangles) or 4 d (squares) of incubation as attached aggregates in tissue culture dishes. One of two similar experiments are presented. Bars indicate ranges of duplicates for CAT. Single samples were taken for AChE, for which repeated assays agreed within 5%.

has been used as a routine supplement in culture media for sympathetic ganglia cultures (23).

The combination of the data on neurotransmitter enzyme activities, neurite ultrastructure, and sodium transport characteristics in response to certain neurotoxins leads us to conclude that the process-bearing cells are in fact well-differentiated cholinergic neurons. Neural tissue is frequently found in teratocarcinomas, and nonspecific stimuli such as necrosis can induce its formation from EC cells (42). Levine and co-workers have described the differentiation of neural and muscle cells in a teratocarcinoma (20, 44), and Herman and co-workers (29, 45) have analyzed another teratocarcinoma for several differentiated parameters characteristic of the nervous system. Damjanov et al. (9) have described a teratocarcinoma that through transplantation developed into a tumor consisting only of EC cells and neural tissues in various developmental stages. PCC7-S is not much restricted when grown in vivo as a tumor. However, when grown in vitro, at least as relatively short-term cultures, it is partially restricted or directed towards neuroectoderm.

This cell line is expected to prove useful for both an analysis of the events leading to a commitment to differentiate, as well as the processes of terminal differentiation of neurons, particularly as regards neurite outgrowth.

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