further work will be required to examine this hypothesis. There are several papers describing the presence of various antagonists of interferon action in malignant (*14*) and embryonic tissues (*15*); these antagonists are of either viral or cellular origin. Inhibitors of interferon production have been less well documented. Isaacs *et al.* (*16*) have described a "blocker" of interferon synthesis in certain crude preparations of chick interferon, and Kato and Eggers (*17*) have reported the presence of a mucopolysaccharide depressor in chick chorioallantoic fluid. Knowing that interferon synthesis depends on a subtle balance between the inducer and cellular repressors (*18*), one can envisage an excess of such repressors in leukemic cells. Another attractive hypothesis would be to ascribe the impairment of interferon synthesis to the presence of leukemia virus particles within the cells. To our knowledge, no study of the effect of particles of mouse leukemia viruses on production of interferon has yet been carried out at the cellular level. For a tumorigenic DNA virus, the Epstein-Barr virus (EBV), Swart and Young (*19*) reported the existence of an inverse relation betwen spontaneous interferon production and virus content in human cell lines derived from Burkitt's lymphoma. Their observation, however, cannot be taken as a general rule, since other workers (*20*) could not find any correlation between interferon production and infection of the cultures by EBV, in various lymphoblastoid cell lines.

Whatever mechanism will turn out to be responsible for the depression of interferon synthesis in leukemic mice, the phenomenon, at this point, has a twofold implication: (i) together with the well-documented immunodepression found in leukemia (*21*), it may contribute to the lowered resistance to viral infections (*22*); and (ii) it could be an obstacle to the efficacy of interferon inducers in therapeutic trials of human leukemias, should an inhibition of interferon production also occur in man. That this may well be the case is suggested by the work of Armstrong *et al.* (*23*), who found significantly less interferon in vesicle-fluid during herpes zoster infection, in three out of seven patients with cancer, when compared with noncancerous patients.

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Neuroblastoma: Synchronization of Neurite Outgrowth in Cultures Grown on Collagen

Abstract. *Neurite outgrowth in cultured neuroblastoma cells is inhibited in cells grown on a collagen substratum as compared to those grown on glass. Rapid, synchronized initiation of neurite outgrowth occurs after hydrolysis of the underlying collagen with collagenase. Axonated cultures exhibit an increased RNA and protein content as compared to cultures grown on collagen.*

Suspension cultures of murine neuroblastoma appear as round, undifferentiated cells; when allowed to settle onto a glass substratum these cells extend multiple processes resembling neurites. Neurite formation (axonation), which may be considered a morphologic manifestation of differentiation in these cells, is associated with the appearance of specialized neural functions: electrical and chemical excitability of membranes, generation of action potentials, and production of neurotransmitter substances (*1*).

Several laboratories have reported procedures for enhancing or synchronizing axonation. Undifferentiated cells may be grown in suspension or in vessels with plastic surfaces to which they cannot adhere, and then transferred to vessels with glass or equivalent surfaces where neurite outgrowth ensues (*2*). Other approaches involve manipulation of culture medium constituents. Neurite outgrowth is suppressed in some clones grown adherent to glass in medium containing 10 percent serum, but rapid outgrowth of neurites occurs in most cells when such medium is replaced with one devoid of serum (*3*). Addition of certain drugs [5-bromodeoxyuridine (BrdU), 5-fluorodeoxyuridine (FdU), dibutyryl cyclic adenosine monophosphate (dibutyryl cyclic AMP), and prostaglandins E_1] to medium containing serum also stimulates axonation (*4*). We report here development of a new procedure for inducing rapid, synchronized axonation, which circumvents the use of culture transfers, nonphysiologic growth conditions, and toxic drugs.

We have found that neuroblastoma cells grown on a thin layer of native collagen are round and devoid of neurites, yet are firmly adherent to the collagen substratum. Addition of purified collagenase to the culture medium

results in the rapid extension of neurites within a few hours, providing a procedure for controlled initiation of neurite outgrowth, as well as inhibition of neurite formation in adherent cells. This observation was surprising because native collagen has been employed as a supportive substratum in cell and tissue culture to enhance cellular attachment, migration, and elongation (*5*). For instance, explants of spinal cord and spinal ganglia have fared best on a collagen surface (*6*), and myoblasts are stimulated to undergo morphologic and biochemical differentiation when seeded on collagen (*7*).

Experiments were performed with mouse neuroblastoma line C-1300, clones 2-A, N-4, and N-18 (*3, 8*). Cells grown in Eagle's minimal essential medium (GIBCO) (*9*), without antibiotics, supplemented with 5 percent fetal calf serum and 5 percent calf serum in a 5 percent CO_2 atmosphere, were maintained by subculture twice weekly. Cells were monitored at each subculture, and before each experiment, for possible mycoplasma contamination, by standard agar plate cultivation (*10*), and by a newly developed detection method (*11*). Native collagen (Ethicon, bovine, 1 percent suspension in 25 percent methanol and 0.25 percent cyanoacetic acid) was diluted in 0.5 percent acetic acid to a concentration of 0.1 percent, spread evenly over an appropriate surface at a ratio of 0.1 ml/cm^2, and dried in a vacuum desiccator, containing sodium hydroxide pellets, at 25°C. Greater amounts of collagen are sometimes required for inhibition of axonation in certain neuroblastoma clones. Sterile glass cover slips coated with collagen in tissue culture dishes (5-ml capacity) were used for morphologic demonstration of axonation. Growth and radioisotope incorporation experiments, and RNA determinations, were carried out in 25-cm^2 (5-ml capacity), and 75-cm^2 (15-ml capacity) flasks (Falcon) coated with collagen. For the experiments, cells were dislodged by gentle pipetting after brief incubation with 0.04 percent ethylenediaminetetraacetic acid, and seeded at concentrations of 1.0×10^6 to 1.3×10^6 cell/ml. Neurites were quantified as those processes with lengths equal to or greater than the cell body diameter. Within 3 to 5 hours after seeding, cells become adherent to both glass and collagen surfaces, but cells grown on collagen are devoid of neurites, while those grown on glass exhibit neurites in over 85 percent of the population (Fig. 1A).

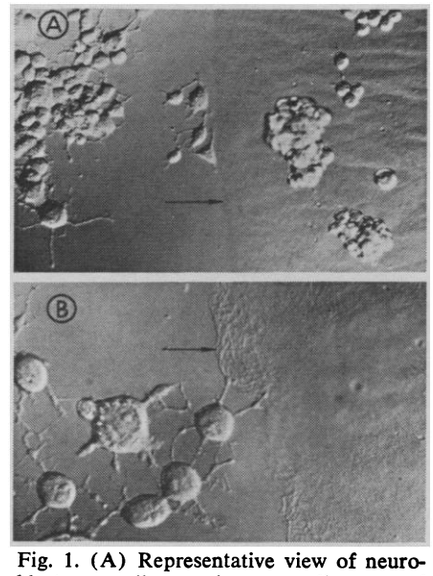

Fig. 1. (A) Representative view of neuroblastoma cells growing on collagen, and devoid of processes (right of arrow). Cells grown on glass on left exhibit abundant neurites (× 110). (B) Cells growing on glass (left) extending axons just up to the margin of collagen fibrils (arrow) (× 275).

Addition of collagenase (Worthington; about 5 unit/ml) to the medium of cells grown on collagen results in the appearance of visible neurites after 3 to 6 hours; within 16 hours the neurite population of collagenase-treated cells is equal to that of cells grown on glass. Collagenase does not enhance neurite extension when it is added to cultures grown on glass. Surfaces are coated with the minimum amount of collagen required for maximum inhibition of axonation in the appropriate cloned line, and collagenase is used at the minimum concentration required to initiate neurite extension within 3 to 6 hours. Untreated surfaces coated with collagen consist of randomly distributed collagen fibrils demonstrable with trypan blue stain, and by use of Nomarski optics (Fig. 1B). After collagenase treatment, the fibrillar matrix is no longer visible, an indication that the products of enzymatic hydrolysis are soluble.

That native collagen, rather than a contaminating protein, is an inhibitor of axonation can be further demonstrated by exposure of collagen surfaces to several other proteolytic enzymes that do not hydrolyze native collagen. Prior to inoculation with cells, cover slips coated with collagen were exposed, in medium free of serum, for 16 hours at 37°C to crystalline trypsin (0.25 percent), chymotrypsin (0.25 percent), or Pronase (1 mg/ml). After being rinsed with complete medium, cells were seeded in the usual manner. Inhibition by collagen persists in all cases, and no neurite outgrowth occurs. Collagen preparations purified by either salt or acid extraction from guinea pig skin, calf skin, rat tail tendon, or steer tendon exhibit equal capacities for inhibition of axonation, and all are susceptible to collagenase reversal. Similarly, "conditioned" medium from confluent cultures of human diploid fibroblasts (presumably containing secreted collagen) also prevents neurite outgrowth when the medium coats the surface of culture vessels. The presence of low-molecular-weight inhibitors of axonation occurring as contaminants of collagen can be ruled out by dialyzing collagen at 4°C for 24 hours against distilled water or 0.5 percent acetic acid, with no subsequent diminution of the inhibitory effect or collagenase reversal.

Response of neuroblastoma cells to growth on collagen appears to be distinct and characteristic for these cells. Cellular morphology of HeLa, human diploid fibroblasts, rabbit lens, and 3T3 cells are identical in cultures grown on collagen and on glass, with full extension of cellular processes in all cases. Collagenase treatment does not produce any significant morphologic changes in these cultures.

Several agents which affect neurite outgrowth have been tested on cultures attached to collagen. Dibutyryl cyclic AMP ($1 \times 10^{-3}M$), FdU ($1 \times 10^{-6}M$), and BrdU ($4 \times 10^{-6}M$) stimulate axonation in cultures grown on glass, yet do not override the inhibition of outgrowth in cells adherent to collagen. Furthermore, within 16 hours after the addition of collagenase to these cultures, neurite formation is equal to that in control cultures grown on glass and treated with the drugs. On the other hand, axon outgrowth does not occur if cultures grown on collagen and treated with collagenase have been incubated in the presence of spindle inhibitors, such as vinblastine ($4 \times 10^{-7}M$) or colchicine ($2 \times 10^{-7}M$). The requirement for new protein synthesis following collagenase reversal is unclear. When cultures grown on collagen are treated with cycloheximide (10 μg/ml) at the time of collagenase addition, or 3 to 24 hours prior to reversal, no flattening and only partial outgrowth of cellular processes occurs thereafter.

This simple, physiologic method for controlling axonation has no apparent deleterious effects on overall cellular

metabolism. There are no significant differences in growth rates between cells grown on collagen and cells grown on glass, nor have we noted any differences in growth patterns after collagenase treatment (Table 1). Evaluation of macromolecular synthesis in round versus axonated cells has been carried out over a 3-day period. In agreement with results from growth experiments, no significant differences in thymidine incorporation are observed between cultures grown on collagen and cultures grown on glass. However, there is a 40 to 60 percent increase in uridine incorporation in axonated cultures (Table 1) during the exponential growth phase, when the number and length of neurites is maximal. Introduction of collagenase to the medium of cultures grown on collagen results in an immediate and sustained elevation of uridine incorporation approaching 70 to 80 percent of that in control cultures grown on glass. Addition of collagenase does not induce any significant changes in uridine incorporation in cultures grown on glass. The amount of cellular ribosomal RNA (rRNA) was measured in replicate cultures grown on glass and on collagen. As seen in Table 1, glass/collagen ratios derived from the RNA content per cell, appear consistent with the uridine incorporation data, in that cells grown on glass contain 60 percent or more rRNA than companion cultures grown on collagen. Such a difference persists even when cultures enter a nongrowing state (days 4 through 7), and the amount of rRNA per cell diminishes. Furthermore, concurrent with neurite outgrowth, there is a rapid increase in rRNA in cells grown on collagen after collagenase release (not shown in Table 1). Axonated cells also contain about 20 percent more protein per cell than those in cultures grown on collagen measured throughout 7 days of growth (0.98 ng/cell versus 0.80 ng/cell on day 2).

In summary, a simple system for controlling neurite outgrowth has been defined, in which contact of cells with native collagen results in inhibition of axonation. This inhibition can be relieved by addition of collagenase to the medium, producing hydrolysis of collagen and synchronized initiation of neurite outgrowth. At least one prerequisite for axonation appears to be microtubule protein assembly. Agents that enhance neurite outgrowth (BrdU, FdU, dibutyryl cyclic AMP), do not do so in cultures grown on collagen. A significant advantage of this method is that cells grown on collagen are fully adherent, manifest normal growth patterns, and continue to do so after collagenase treatment. Axonless cells on collagen incorporate less tracer uridine during exponential growth phase, which may stem from the fact that they contain less rRNA and less protein per cell compared to axonated controls. Removal of the collagen substratum by collagenase treatment markedly reduces these disparities. It is not yet clear if this stimulation of RNA and protein synthesis in differentiated cells occurs merely to accommodate the larger volume of cells with processes, or, more significantly, for the purpose of synthesizing neural-specific products.

Table 1. Growth and macromolecular synthesis in cultures grown on glass and on collagen. For growth experiments, flasks were briefly incubated with 0.04 percent ethylenediaminetetraacetic acid, suspended in complete medium, and counted in a hemocytometer. Trypan blue was used to evaluate cell viability. For determination of cellular RNA, monolayers were lysed in 1 percent sodium deoxycholate in 0.01*M* tris(hydroxymethyl)aminomethane-HCl buffer, *p*H 7.6; sodium dodecyl sulfate (SDS) was added to a final concentration of 2 percent. Aliquots were analyzed for total protein content by a modified Lowry method (*13*). The lysate was layered over a 15 to 30 percent linear SDS (0.5 percent)—sucrose gradient, and centrifuged in a SW27 rotor at 20,000 rev/min for 18 hours at 25°C. Ribosomal RNA was monitored at 260 nm on a recording spectrophotometer, and expressed as the area of the leading half of the peak representing 28*S* RNA. For incorporation experiments, replicate culture flasks were exposed at indicated intervals for 30 minutes with [^{14}C]thymidine (0.1 μc/ml, 54.8 mc/mmole) or [^{14}C]uridine (0.2 μc/ml, 53 mc/mmole). Monolayers were washed with cold Hanks buffer, *p*H 7.3, precipitated with cold trichloroacetic acid, washed until neutral with Hanks buffer, and then dissolved in 2 percent SDS. The lysate was added to scintillation fluid, and counted in a liquid scintillation spectrometer. Cultures grown on glass and exposed to [^{14}C]thymidine contained 15,950 count/min on day 1; 11,140 on days 2 and 13; 541 on day 3, and those exposed to [^{14}C]uridine contained 20,366 count/min on day 1; 24,155 on day 2; and 24,410 on day 3. Cultures grown on glass contained the following cell densities ($\times 10^5$): 2.5, 4.3, 7.0, 11.0, 11.0, 11.0 on days 1, 2, 3, 4, 5, and 7, respectively. The rRNA content of cultures grown on glass on the same days, and expressed as optical density units per 1000 cells were: 0.7, 3.8, 3.7, 3.1, 2.5, 1.3. Data for rRNA content of cultures grown on glass on days 4 and 5, and cultures grown on collagen on days 3 and 5, have been estimated by interpolation. In all cases, glass/collagen ratios were calculated by dividing the appropriate experimental data for cultures grown on glass by analogous data obtained from cultures grown on collagen.

Day	Ratios in cultures grown on glass to cultures grown on collagen of			
	Growth	[^{14}C]Thymidine incorporation	rRNA	[^{14}C]Uridine incorporation
1	1.2	1.2	1.8	1.6
2	1.1	0.97	1.6	1.6
3	1.1	.96	1.7	1.4
4	1.1		1.7	
5	1.0		1.9	
7	1.0		3.6	

Although some previous studies imply that enhanced axonation is somehow related to decreased growth rate (*3*) or increased adherence (*2*), our studies do not support this concept of a simple relation between these parameters: when neuroblastoma cells grow on and adhere to collagen just as well as when cultured on glass, but are strikingly deficient in neurites. Our observation also may have relevance to certain developmental phenomena deriving from nerve cell interactions with extracellular collagen. In the embryo, for instance, collagen-like material, rich in hydroxyproline, has been found intimately associated with the developing neural tube (*12*). In addition, collagen is a ubiquitous component of the peripheral nervous system, where it serves in cooperation with glial (Schwann) cells to guide generating and regenerating nerve processes to their ultimate destination.

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Silver-Foil Psychrometer for Measuring Leaf Water Potential in situ

Abstract. *The water potential of leaves in situ can be measured without temperature control with a miniature, single-junction psychrometer constructed from silver foil and attached to the leaf with a silver-impregnated, conductive coating. The temperature of the psychrometer has been found to stay within 0.025°C of the temperature of a simulated leaf when the latter temperature was changing at a rate of 1°C per minute. Leaf water potentials can be measured with a precision of ± 1 bar, or better.*

Although the psychrometric method of measuring the water potential of leaves (chemical potential of water in leaves) in situ is theoretically sound (*1*), it is subject to errors introduced by temperature fluctuations. As a result, most leaf water potentials are measured on detached leaves in constant temperature baths (*2*). In the past, measurements of the water potential of leaves in situ also required precise temperature control (*3*). This requirement for temperature control has precluded routine measurements of the leaf water potential in situ.

For measuring leaf water potential it is essential that either the psychrometer chamber be at the temperature of the leaf, or the temperature difference between the two be known. Rawlins and Dalton (*4*) met this requirement for soil psychrometers used in situ by embedding the psychrometer within the sample. Wiebe *et al.* (*5*) used this same technique satisfactorily for measuring the water potential of stems, but it is not possible at present to embed a psychrometer within a thin leaf.

Two publications describe psychrometers designed to overcome this temperature problem. Calissendorf (*6*) added a second set of thermocouple junctions to measure the temperature difference between the leaf and the psychrometer chamber. Neumann and Thurtell (*7*) clamped the leaf between metal heat sinks to assure uniform temperature. In this report, we describe a leaf psychrometer that overcomes temperature-induced errors in water potential measurements by being small enough and constructed of metal with sufficiently high thermal conductivity to follow the normal temperature fluctuations of an exposed leaf.

The psychrometer, illustrated in Fig. 1, consists of a silver-foil (*8*) disk, 50 μm thick and 13 mm in diameter, in which an indentation 1 mm deep and 5 mm in diameter has been formed to serve as the psychrometer chamber.

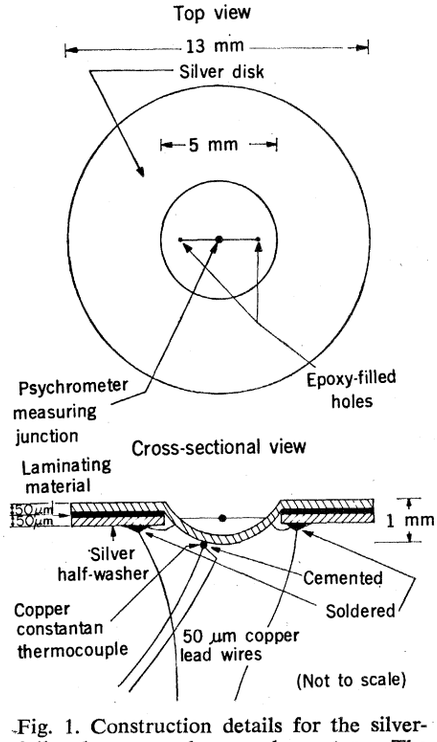

Fig. 1. Construction details for the silver-foil thermocouple psychrometer. The measuring thermocouple is constructed from 25-μm Chromel and constantan wire. The junction is approximately 0.2 mm in diameter (*14*).

The indentation is formed by placing the silver disk on a lead surface, positioning a steel die over it, and tapping the die with a hammer. To the back of the washer-shaped lip of the psychrometer, two half-washers of silver foil 50 μm thick are bonded with thermosetting, electrical insulating adhesive film (*9*). The film permits good thermal contact between the half-washers and the psychrometer chamber while serving as an electrical insulator. Each half-washer serves as a reference junction for the pair of thermocouple and lead wires soldered to it. Two small holes are drilled through the psychrometer chamber opposite each other for passage of the thermocouple wires. The holes are sealed with epoxy and each thermocouple wire is positioned in the center of the hole before the epoxy solidifies. A copper-constantan thermocouple is cemented to the back of the psychrometer chamber for monitoring the chamber temperature.

The psychrometer, insulated on the back with about 2.5 cm of foamed plastic, is cemented to a leaf by coating the lip of the psychrometer, which is 4 mm wide, with a silver-impregnated, water-based conductive coating (*10*), and pressing it against the abaxial side of the leaf. Tests conducted by Hoffman and Herkelrath (*11*) showed that the coating is not visibly harmful to leaves and remains attached for several weeks. Subsequent tests on citrus leaves, which have no stomates in the adaxial epidermis, also showed no visible damage. To prevent the silver coating from shorting out the psychrometer by coming in contact with the half-washer reference junctions, the edge of the psychrometer is coated with an epoxy sealant. Until the coating dries, the psychrometer is supported and a small weight is placed on top of the leaf to prevent its curling. The conductive coating can be removed from the psychrometer without damage with solvent (*12*).

The capability of the psychrometer to follow changes in simulated leaf temperature was measured in the laboratory by attaching a psychrometer, well insulated on its back side, to a copper plate with the conductive coating. The copper plate simulated a leaf of uniform temperature. The temperature of the plate was changed by heating or cooling a copper rod soldered to it. A thermocouple, attached to the plate, monitored the plate temperature. The temperature of the psychrometer chamber was monitored with both the