surface. With two nearby explants one even observes Weiss's classic two-center effect (Fig. 2, d and e), yet there is no possibility that the rubber substratum is being dehydrated (as it was never hydrated) and no indication of any other biochemical alteration.

Thus we conclude that the apparent "shrinkage" of plasma clots and other distortable substrata by outgrowing tissue cells is caused primarily, if not entirely, by the traction forces cells exert in order to propel themselves outward. But this reinterpretation of Weiss's classical observations is not at all inconsistent with his larger theory that the stretching orientation of extracellular fiber networks could serve to guide cell migrations within the embryo (*10*). Our observations imply that cell locomotion in vivo could generate large tension fields, orienting extracellular fibers over considerable distances. It would be unlike evolution not to make use of these fields to guide morphogenesis.

From time-lapse films of the distortions produced in these rubber substrata by individual crawling cells it appears that tissue cells exert traction as a shearing force in the plane of the lower surface membrane. The effect is much as if the bottom of the cell were occupied by an invisible tractor tread of some kind. Using interference reflection microscopy (*11*), we observed that the fibroblasts' contacts with the rubber substratum are distributed in the same spatial pattern as one sees when they are cultured on glass (*12*). These contacts, corresponding to the adhesions through which the tractional shear forces are exerted, are concentrated just distal to the advancing margins, mostly in a band from 5 to 25 μm rearward from this margin. This is also the region in which the compression wrinkles begin, implying that the forces are exerted beneath the cell, rather than along the advancing edge itself. Observations with a polarizing microscope revealed that the silicone rubber develops so little birefringence, even when highly stretched, that the cytoskeletal birefringence is not obscured by that of the substratum.

To measure the absolute magnitude of the shear forces exerted by individual fibroblasts we use glass microneedles whose flexibility is calibrated by hanging small paperweights on their ends. Then, using a deFonbrunne micromanipulator, the tips of these needles are pushed laterally upon the rubber until this surface is distorted to the same degree as it is by the cells. The shear force required is determined by observing the degree of lateral bending of the microneedle, and comparing this with the bending produced by the weights. Initial measurements indicate that chick heart fibroblasts exert shear forces in excess of 0.001 dyne/μm of advancing margin.

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We dedicate this report to the late Michael Abercrombie whose powerful yet kindly intellect so greatly advanced the study of cell behavior. The original idea for using flexible culture substrata developed out of a lunchtime conversation with him, although he doubted it would work. The ideas of G. Dunn and A. Middleton were also most helpful.

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"Transdifferentiation" of C6 Glial Cells in Culture

Abstract. *The activities of cyclic nucleotide phosphohydrolase, an enzyme marker for oligodendrocytes, and glutamine synthetase, an enzyme marker for astrocytes, were studied at early (21 to 26) and late (82 to 88) cell passages. The activity of cyclic nucleotide phosphohydrolase was markedly high and that of glutamine synthetase was low in the early passages, but this relation was reversed in the late passages. These findings suggest a "transdifferentiation" of C6 glial cells with passage in culture.*

Many investigators have used C6 glial cells, a rat glioma line, to study glial cell properties and function (*1*). The cell line has generally been designated as an astrocytoma (*2*). Silbert and Goldstein (*3*) reported that cells in the C6 line differentiated to cells resembling astroglia after treatment with amethopterin. In addition, a study by Bissel *et al.* (*4*) showed the induction of glial fibrillary acidic protein (GFA), an astrocyte marker, when the cells were maintained in sponge foam matrices. They suggested that C6 cells represent an undifferentiated glial cell with the potential to differentiate into astrocytes. On the other hand, 2′,3′-cyclic-nucleotide 3′-phosphohydrolase (CNP) (E.C.3.1.4.1), a marker for myelin and oligodendroglia, has been found in these cells (*5*). Likewise, the oligodendrocyte marker, glycerol phosphate dehydrogenase (GPDH) (E.C.1.1.1.8), is present and inducible in these cells (*6*).

The enzymes glutamine synthetase (GS) (E.C.6.3.1.2) and CNP have been associated with astrocytes and oligo-

Table 1. Changes in 2′,3′-cyclic-nucleotide 3′-phosphohydrolase activity in C6 glial cells of different passages.

Cell passage	Day in culture	Plating cell density	Enzyme activity*		Picograms of protein per cell
			Units per milligram of protein	Units per 10^6 cells	
26	4	0.25×10^6	0.269 ± 0.041†	0.050 ± 0.004	222 ± 35
		1.00×10^6	0.320 ± 0.044	0.078 ± 0.014	200 ± 4
	10	0.25×10^6	0.618 ± 0.048	0.119 ± 0.006	181 ± 3
		1.00×10^6	0.688 ± 0.032	0.134 ± 0.008	196 ± 14
88	4	0.25×10^6	0.219 ± 0.012	0.053 ± 0.005	242 ± 21
		1.00×10^6	0.260 ± 0.021	0.044 ± 0.011	170 ± 15
	10	0.25×10^6	0.203 ± 0.031	0.033 ± 0.005	186 ± 13
		1.00×10^6	0.125 ± 0.009	0.023 ± 0.002	186 ± 13

*Units of activity represent micromoles of phosphate produced per minute, derived from a standard curve phosphate produced from 2′-adenosine monophosphate. †Mean ± standard error of the mean of four to five samples.

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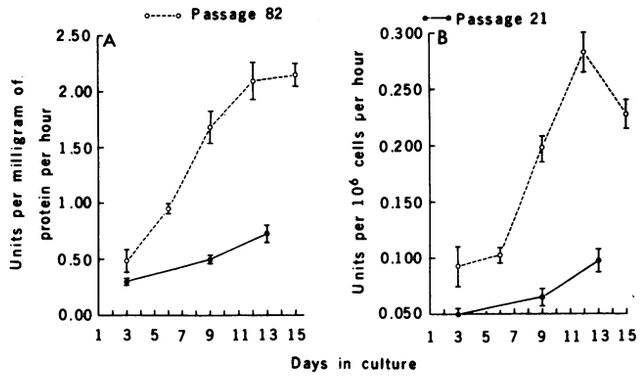

Fig. 1. Changes in glutamine synthetase activity in C6 glial cells of different passages. Activity is expressed per milligram of protein (A) and per 10^6 cells (B). Units of activity represent micromoles of γ-glutamylhydroxamic acid formed. Values are the mean ± standard error of the mean of three or four samples.

dendrocytes, respectively (*7*). We have used these two enzymes as cell markers to study C6 cells as a function of passage in culture. We report here a "transdifferentiation" of C6 cells in which oligodendrocytic characteristics are observed in early passages whereas astrocytic properties are observed in later passages. As defined by Pritchard *et al.* (*8*), transdifferentiation is the ability of a cell to lose a definitive characteristic and to acquire another feature characteristic of an alternate state.

We used C6 glial cells, 2B clone (*9*), throughout our investigation. For the CNP studies, cells of either 26 or 88 passages were plated at either 0.25×10^6 or 1.00×10^6 cells per flask (25 cm^2). For the GS studies, cells of either 21 or 82 passages were plated at 1.00×10^6 cells per flask (75 cm^2). Cells were cultured in Ham's F-12 medium containing 10 percent fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 U/ml). The cultures were maintained at 36° to 37°C in a humidified atmosphere of 7.5 percent CO_2 in air. After the appropriate number of days in culture, cells were harvested with 0.1 percent trypsin and counted with a hemacytometer. When CNP was determined, cells were frozen in a mixture of acetone and dry ice and stored at −20°C until assayed. The GS determinations were performed on nonfrozen cells immediately after harvesting.

Cyclic nucleotide phosphohydrolase was assayed by the spectrophotometric procedure of Prohaska *et al.* (*10*). For GS, cells were homogenized with a Teflon glass homogenizer in 0.05*M* imidazole buffer, *p*H 7.2, containing 0.1 percent Triton X-100. Assays were performed on portions of the homogenate according to the method of Berl (*11*), except that 19 m*M* adenosine triphosphate (ATP) was added to the assay media and the 10-minute preincubation at 60°C was omitted (*12*).

Portions of homogenates were frozen for subsequent protein determination. After extensive washing in 10 percent trichloroacetic acid, protein was assayed by the procedure of Lowry *et al.* (*13*).

Changes in CNP activity in C6 glial cells of 26 and 88 passages are presented in Table 1. Enzyme activity was determined in cells at 4 and 10 days in culture, that is, logarithmic and confluent phases of growth, respectively. The CNP activity was almost the same in cells of both passages during logarithmic growth (4 days). However, at confluency (10 days) CNP activity in the cells of passage 26 was markedly higher whereas the activity in cells of passage 88 was the same or lower than in cells at 4 days in culture. At confluency there was three- to fivefold greater activity in the cells of early passage than in those of later passage. The findings were similar whether activity was expressed per milligram of protein or per 10^6 cells. The fact that protein content per cell was uniform indicates that the differences between passages reflect real differences in enzyme activity.

In contrast to CNP activity, GS activity was high in the late passage cells and low in the early passage cells (Fig. 1). As with CNP, the differences between passages at early days in culture were small. As the cells reached confluency, however, differences became marked. In cells of 82 passages the GS activity was four times higher than in cells of 22 passages. The observations were not changed by expressing activity per 10^6 cells or per milligram of protein.

Cells at 22 or 88 passages were stained by the Giemsa method and examined with light microscopy. Almost all cells of 22 passages possessed small, dark nuclei, scanty perikaryon, and relatively short, thin cytoplasmic processes (Fig. 2). In contrast, cells of 88 passages had significantly larger and paler nuclei, frequently abundant perikaryon, and considerably longer cytoplasmic processes. This cell type was never seen in early passage cultures.

The data presented in this report demonstrate that C6 glial cells have both astrocytic and oligodendrocytic glial properties as shown by the presence of specific enzyme activities, GS and CNP, respectively. Although these cells have both enzymes, the level of enzyme activity changes markedly with passage of the cells. Thus, at early passage they show predominantly oligodendrocyte-like properties whereas at later passages they are predominantly astrocyte-like.

The factors responsible for the proposed transdifferentiation from oligodendrocyte-like to astrocyte-like properties can at present only remain speculative. The fact that the cells at early and late passage were distinctly different morphologically suggests a true phenotypic alteration rather than a cell selection phenomenon. Furthermore, this morphologic evidence does not support the presence of a population of selectively differentiating glioblasts. It is possible that the transdifferentiation phenomenon may result from in vitro factors such as

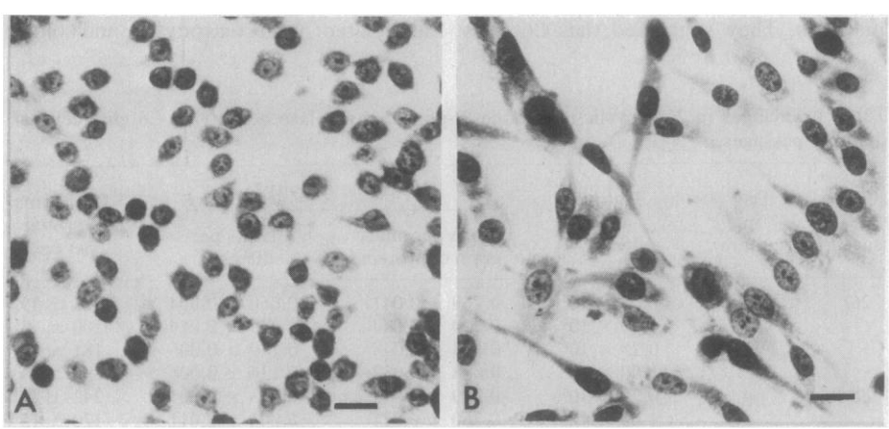

Fig. 2. Photomicrographs of Giemsa-stained C6 glial cells grown on glass cover slips. Scale bar, 20 μm. (A) Cells of passage 22. These cells possess small, round, dark nuclei, scanty cytoplasm, and very short cytoplasmic processes. (B) Cells of passage 88. Relative to the cells of passage 22, the nuclei of these cells are larger and paler, the cytoplasm is more abundant, and prominent elongated cytoplasmic processes are evident.

trypsinization for serial subcultivation (*14*). Membrane changes that are later reflected in changes in intracellular activity may be brought about by trypsinization. Exposure of cells to some substance (or substances) in either the medium or serum during subcultivation may also be a factor. Additionally, the possibility that this differentiation is a "cell aging" phenomenon must be considered. Further studies involving tumorigenicity, cytogenetics, and cloning will be required to more completely define the nature of this phenotypic alteration of C6 glial cells.

De Vellis and associates (*15*) have commonly used RG C6 cells of less than 25 passages for their studies of steroid hormone induction of GPDH, another enzyme marker for oligodendrocytes. It would be of interest to know whether GPDH is present at later cell passages.

The finding of marked GS activity as a factor of passage of cells may explain why little GS was detected previously by Nicklas and Browning (*16*). They reported activities that were 50- to 100-fold lower than those found in whole rat brain. In the present study, the activity of cells at 82 passages and 15 days in culture (2.137 ± 0.096) is almost as high as activities found in whole rat brain (2.940 ± 0.105). To a minor extent the present higher values may also be due to methodology. The higher amount of ATP used in our assay media results in approximately 40 percent increased activity; trypsinization of the cells causes a twofold increase in activity. Finally, this study strongly emphasizes the large amounts of GS present in C6 cells and furthermore stresses the importance of reporting passage when describing properties of C6 glial cells.

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Differential Avoidance of Mimetic Salamanders by Free-Ranging Birds

Abstract. *Members of a free-ranging avian community avoided the mimetic morph of the salamander* Plethodon cinereus *significantly more often than a nonmimetic morph when offered with the model eft stage of* Notophthalmus viridescens *and the palatable salamander* Desmognathus ochrophaeus. *This is apparently the first demonstration of the efficacy of mimetic coloration of salamanders to uncaged birds.*

Although the efficacy of mimicry in salamanders has been demonstrated with caged birds, there have been no attempts to demonstrate the advantage of mimetic coloration to free-ranging birds (*1*). Attempts have been made to assess the value of mimetic coloration of moths and butterflies to wild bird communities by releasing and retrieving individuals painted to resemble palatable or unpalatable species (*2*). None of these studies included the use of unaltered mimics or restricted predation to birds, which are generally assumed to be responsible for the evolution of mimetic colorations.

In our study, we tested the survival value of a presumed mimetic color morph of the polymorphic salamander *Plethodon cinereus*. Certain populations of *P. cinereus* have two color morphs: a red-striped morph, which is present in most portions of the species' range, and an all-red morph, which is nearly uniformly red-orange (*3*). This all-red morph is thought to be a mimic of the eft stage of *Notophthalmus viridescens* that is toxic, aposematic, and a model for other mimic salamanders (*1, 4*).

In order to test the survival value of natural mimics to natural avian communities we have developed an experimental design exposing unaltered salamanders to predation only by birds. Single salamanders were placed in 50 trays 30 cm square with an aluminum wall 10 cm tall; the top of the aluminum was bent inward, forming a 3-cm lip to prevent the escape of the salamanders (*5*). Each tray was nailed to a vertical section of pine log, 60 cm in length and 15 to 20 cm in diameter. Each tray contained four dead maple or oak leaves (or both) from the adjacent deciduous forest; the leaves were soaked with water just prior to each trial. The trays were placed in three rows of 18, 17, and 15 trays; the rows and trays within a row were 10 m apart. The rows of trays were positioned between rows of trees in a 50-year-old pine plantation.

The distribution of salamanders within this grid was randomized from a hypothetical community of a million animals made up of 30 percent *N. viridescens* efts, 40 percent *Desmognathus ochrophaeus*, 24 percent striped *P. cinereus*, and 6 percent all-red *P. cinereus*; a computer generated random orders of contact for 50 individuals from this community. In each trial, an independent random distribution of salamanders was used and the actual percentages varied slightly from the hypothetical community as follows: *N. viridescens*, 29.4 percent; *D. ochrophaeus*, 41.4 percent; striped *P. cinereus*, 23.3 percent; and all-red *P. cinereus*, 5.8 percent (*6*). The *D. ochrophaeus* were offered as alternate, palatable prey because mimicry is effective only when an alternate food source is available (*7*).

Initially each trial lasted for 4 hours (5 to 9 a.m. and 5 to 9 p.m.); the predation rate during five 4-hour trials was 75.1 percent of all edible salamanders (*D. ochrophaeus* and *P. cinereus*). Because of this high predation rate, the length of

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