into a relative, but not absolute resistance to the feedback suppression effect of LDL; that is, to achieve any given level of enzyme suppression, about two to three times the concentration of LDL was required in heterozygotes' cells as compared with normal cells. This result can be explained by the observation that suppression of HMG CoA reductase activity is directly proportional to the absolute number of receptor sites that contain bound LDL (*4*). Since in the heterozygote the number of functional receptor sites is reduced to about 40 percent of normal, the law of mass action would require that at levels of LDL below saturation approximately a 2.5-fold higher concentration of LDL be present in the heterozygote to produce the same absolute number of occupied receptor sites and hence the same suppressive effect as in the normal cells.

If, in the body, the LDL receptor plays a physiologically important role in regulating cholesterol synthesis and LDL degradation and if these processes are controlled at a level of LDL that occupies only a fraction of the total number of receptor sites, then one would predict that heterozygotes would manifest an absolute rate of cholesterol synthesis and LDL degradation similar to that in unaffected subjects, but at the expense of a two- to threefold elevation in serum LDL. This prediction is in precise agreement with a large body of clinical and metabolic data derived from study of such patients (*5*, *6*). On the other hand, if suppression of cholesterol synthesis to normal levels and the attainment of a normal rate of LDL degradation required complete saturation of all LDL receptors present in the normal cell, then heterozygotes would be unable to generate this number of occupied receptors at any LDL level and hence would be phenotypically indistinguishable from homozygotes, a situation at variance with clinical observations (*5*).

Thus, these cell culture data offer an explanation of the biochemical mechanism by which a gene that is clinically dominant can produce a much more severe syndrome in homozygotes than in heterozygotes. The data also provide new insight into the mechanism by which a genetic abnormality in a regulatory protein may produce a metabolic defect in human cells.

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Brain Cells in Culture: Morphological Transformation by a Protein

Abstract. *One type of embryonic rat brain cell having an epithelioid morphology in the monolayer culture can be transformed by brain extract into cells having extensive processes resembling mature astrocytes. The transforming factor is a protein with a molecular weight of 350,000. A partially purified sample showed that it is active at a concentration as low as* 1×10^{-8}M. *The transforming activity is high in adult brains but low in embryonic brains and tumors of the nervous system.*

Dissociated embryonic brains when grown in a monolayer contain two major types of immature cells: the bipolar neuroblasts and the flat epithelioid cells that support the growth of the neuroblasts. The latter cell type was suspected by some (*1*, *2*) to be glial precursors. Recently, several laboratories (*3*–*4*) reported that, under certain conditions, the flat brain cells observed in vitro can undergo morphological transformation into multipolar cells resembling mature astrocytes. We have obtained the flat cells in a relatively homogeneous population and inferred that, on the basis of the nondialyzability and heat lability of the activity (*3*), one of the factors responsible for the transformation is a macromolecule found in adult brain extracts. In this report we present evidence that the transforming factor is a protein.

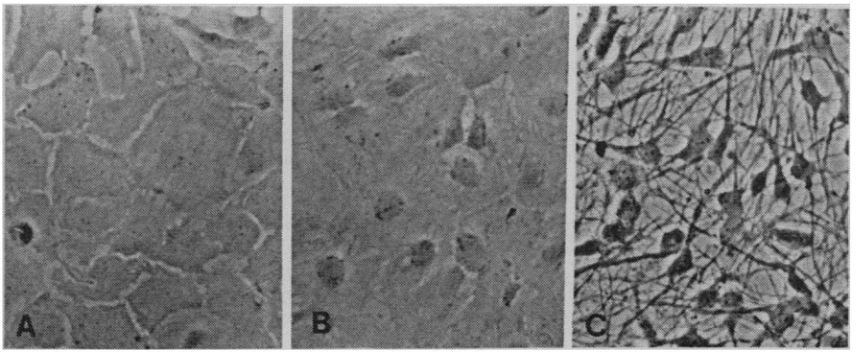

Fig. 1. Morphological transformation of embryonic brain cells in culture. (A) Cells in standard medium before testing, the morphology of which remained the same over the next few days if left undisturbed; (B) cells in the control medium for 20 hours; and (C) cells in the experimental medium for 20 hours containing brain extract. The poor contrast observed in (A) and (B) compared with (C) was due to the thinness of the cells and was not a result of poor photographic technique or interference by the media (phase contrast, × 200).

Brain cells were dissociated (*5*) from 17-day Sprague-Dawley rat fetuses with trypsin in Tyrode that did not contain calcium or magnesium (*6*); the dissociated cells were seeded in Falcon plastic tissue culture flasks. The standard medium consisted of F_{10} (*7*) supplemented with 17 percent fetal calf serum. Penicillin (50 unit/ml) and streptomycin (100 μg/ml) were included. After the second passage, a homogeneous monolayer of flat epithelioid cells (*8*) was obtained (Fig. 1A). At this time morphological transformation was initiated by replacing the standard medium with 5 ml of the experimental medium consisting of four parts of F_{10}, one part of a test solution, 0.6 percent fetal calf serum, and the antibiotics mentioned above. In the control flasks where the test solution consisted of a plain solution of Tyrode, the morphology of the cells remained essentially unchanged, except that the cytoplasm became more tenuous and the nuclei more prominent (Fig. 1B). Although the borders were rugged and some fibrillary structures were present inside the cells, no distinct cell processes could be seen. For the flasks with the experimental medium the test solution was prepared as follows. Adult rat brain was homogenized (20 percent, weight to volume) in Tyrode solution, the homogenate was centrifuged at 100,000*g*, and the supernatant was dialyzed in Tyrode. In these experimental flasks, morphological transformation began within 8 hours, showing retraction of the soma and extensive outgrowth of processes (Fig. 1C). The transforming effect of the brain extract cannot be attributed to cellular toxicity because the transformed cells contained more protein than the control and could multiply normally upon subculture.

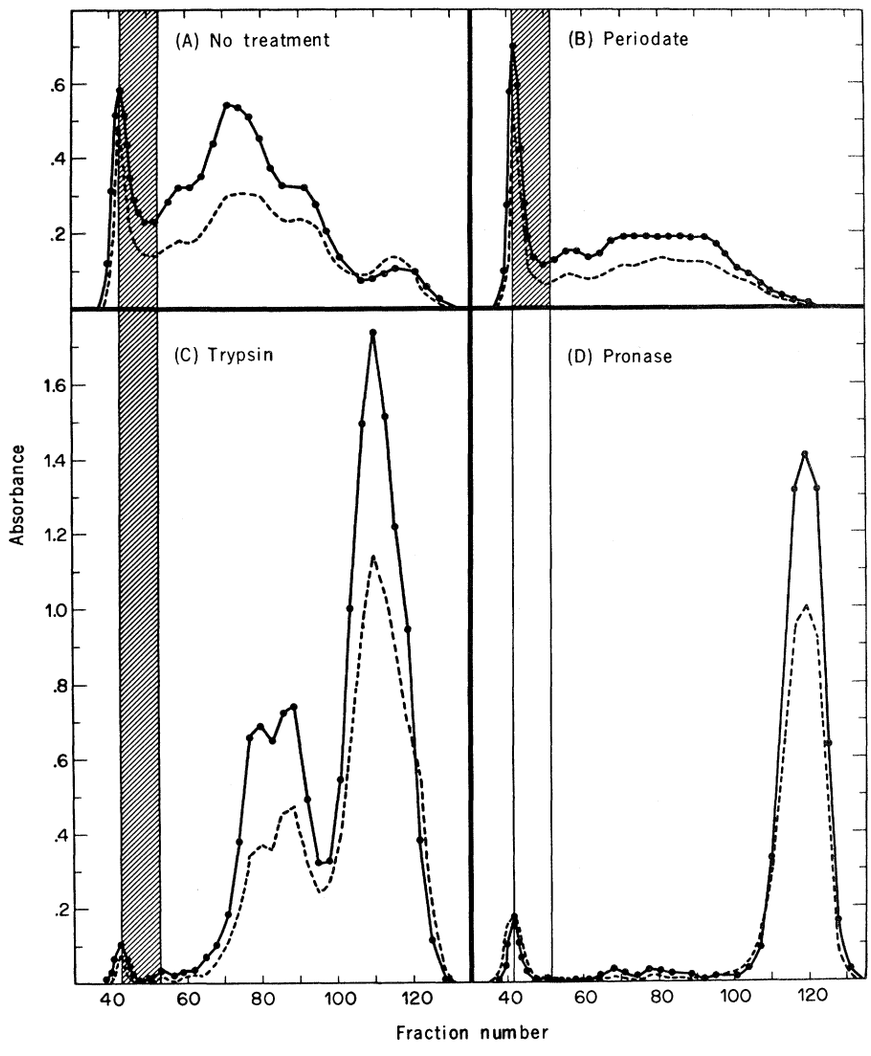

Fig. 2. Susceptibility of the transforming factor to various degradative procedures. Pig brains, 80 g (wet weight), were homogenized and extracted as described for rat brains. The exact was dialyzed against water in the cold, lyophilized, and washed with absolute ethanol at 4°C. The factor was reextracted from the ethanol-insoluble material with a Tyrode solution equivalent to one-fifth the volume of the original extract. The ethanol-washed extract, in portions of 15 ml, was subjected to one of the following treatments: (A) untreated control; (B) incubated with sodium metaperiodate (0.01*M*) at 4°C for 72 hours, and dialyzed against Tyrode; (C) incubated with trypsin (4 mg/ml) at 37°C for 3 hours and the reaction terminated by the addition of 4 mg of soybean trypsin inhibitor per milliliter; and (D) incubated with Pronase (0.1 mg/ml) at 37°C for 18 hours under aseptic conditions. The four samples were fractionated on identical Sephadex G-200 columns (2.6 by 100 cm) equilibrated with 0.02*M* tris · HCl, *p*H 7.4, containing 0.15*M* NaCl (tris-saline). Elution was conducted with the same solution at a rate of 0.4 ml/min. Fractions of 4.3 ml were collected. The solid line indicates the absorbance at 280 nm. The dashed line indicates the absorbance at 260 nm. One milliliter of every other fraction was taken and incorporated in the 5-ml experimental medium (see text) for assay of activity. Shaded areas indicate fractions whose activity resulted in more than 30 percent cellular transformation. These fractions were pooled and assayed for total recovery of activity, which was nearly complete (more than 95 percent of the control column) in the periodate and trypsin-treated samples, but was totally lost (less than 5 percent recovery) in the Pronase-treated sample. The trypsin with its inhibitor was eluted in fractions 80 to 110, whereas the Pronase activity came out in fractions 86 to 92.

We utilized this morphological change to assay the transforming factor and for monitoring the purification of this factor. The cells were scored 20 hours after addition of the extract, a time when the morphological changes attained a peak. A cell possessing at least one process longer than the diameter of the soma was counted as a positive response. At least three regions of the flask were inspected, and in each region cells in at least five different fields were estimated, covering a total of more than 500 cells. The extent of transformation per flask was expressed as the percentage of transformed cells with respect to the total cell population. Each flask was scored separately by two persons and the average taken. The results were confirmed in at least another batch of embryonic brain cells. Under the specified conditions the above brain extract (containing 6 mg of protein per milliliter or 1.2 mg of brain protein per milliliter of culture medium) usually resulted in 60 percent cellular transformation. Diluting the extract resulted in a proportionate decrease in activity, whereas an increase in the brain protein concentration led to a gradual leveling of the activity curve,

a plateau being reached at about 95 percent cellular transformation. At no time were inhibitory or lethal effects observed with concentration increase. The dose-response relation below the 60 percent point was used for quantitative comparison of activity from various samples.

The chemical nature of the transforming factor was determined by enzyme and chemical degradations. This and the subsequent experiments were carried out with pig brains as a source material because they contain as much transforming activity as the rat brains and cross-reactivity between species exists. The transforming activity was resistant to periodate and trypsin but was abolished by Pronase (Fig. 2). Under the first two conditions, the activity was retained even after column chromatography, despite big changes in the protein elution profiles. The resistance to trypsin might suggest that there are probably no arginyl or lysyl residues exposed on the outside of the protein. Its resistance to periodate oxidation does not rule out glycoprotein, but does imply that any carbohydrate moieties, if present, are probably not essential for the transforming activity. The susceptibility to Pronase is evidence that the factor is a protein. Deoxyribonuclease and ribonuclease, when incubated with the extract at 2 mg of enzyme per milliliter (37°C for 10 minutes), did not alter the transforming activity. Other protein properties of the transforming factor include its inactivation by exposure to 8*M* urea or to the extremes of *p*H. However, the transforming activity could not be reproduced by any of the commercially available proteins (*9*) tested so far. The contribution of endogenous cyclic adenosine monophosphate (AMP) which might have contaminated the dialyzed brain extract was ruled out by prior incubation with cyclic AMP phosphodiesterase. The possibility of prostaglandin interfering has also been eliminated (*3*).

The pig brains were homogenized, extracted by centrifugation, dialyzed, and washed with ethanol (Table 1). The ethanol-washed extract was then fractionated successively with Sephadex G-200 and Sepharose 4B columns, resulting in a 30-fold purification. If the ethanol-washed extract was first incubated with trypsin before it was put through the two columns, a 400-fold purification was finally achieved. The purification was only partial, since even the purest sample available showed several bands on acrylamide gel electrophoresis. Nevertheless, the final sample when used at 3 μg of protein per milliliter of medium gave the same transforming effect as did the dialyzed crude brain extract when used at 1.2 mg of protein per milliliter. Since we have assigned a molecular weight of about 350,000 to this protein factor, on the basis of the position of the activity peak in the Sepharose 4B elution profile (*10*), the effective concentration of the protein factor was estimated to be $1 \times 10^{-8} M$ or less. The persistence of the transforming activity at such a low concentration places it in the category of hormones and enzymes. However, the protein was not identical with other factors known to stimulate cell growth or differentiation, such as nerve growth factor, phytohemagglutinin, concanavalin A, wheat germ agglutinin, and pokeweed mitogen, since the same brain cells did not respond to these agents when tested at various concentrations (*11*).

How this protein brings about morphological transformation is a matter of speculation. We tend to believe that this factor modifies the membrane properties of the cells, in that an alteration in the histotypic pattern of a monolayer should reflect a change in intercellular affinity as well as the affinity between the cells and the flask surface. We also proposed (*3*) the involvement of intracellular cyclic AMP, which remains to be proved. In order to detect the role of cellular metabolism in the observed transformation, an inhibitor of protein synthesis, cycloheximide, was added to the medium. At 0.1 μg/ml, cycloheximide completely inhibited the effect of brain extract. We further observed that colchicine (0.1 μg/ml) and vinblastine (0.5 μg/ml), drugs that disrupt the assemblage of microtubules, also prevented the transformation. It therefore appears that protein synthesis as well as the formation or assemblage of microtubules (or both) might be essential for the transformation of the cultured brain cells. Cytochalasin B (5 μg/ml) has the peculiar effect of fragmenting the cytoplasm of the flat cells, indicating the predominance of microfilaments in the untransformed cells. Both microtubules and microfilaments have been reported (*12*) on electron microscopic evidence to be present in embryonic glial cells in culture.

Using the morphological assay described, we made a study on the biological distribution (*13*) of the transforming factor. The specific activity in brains of 17-day rat embryos is only one-fifth that of the adult activity. The activity detected in neuroblastoma and astrocytoma is less than one-tenth of that found in normal adult brain. Thus, the protein factor might play an important role in the maturation of the brain, and its derangement might have important bearings on such abnormal processes as brain tumor and gliosis.

Table 1. Purification of the transforming factor. Pig brains, 80 g (wet weight), were used as the starting material. Steps 1, 2 and 3 were as described in Fig. 2, except that the entire ethanol-washed extract (without periodate or enzyme treatment) was applied to a Sephadex G-200 column whose size was proportionally scaled up to 5 by 100 cm. The elution profile was identical with that in Fig. 2A. The active fractions from this column were pooled, concentrated to 10 ml, dialyzed against tris-saline, and applied to a Sepharose 4B column (2.6 by 100 cm) equilibrated with tris-saline. The column was eluted with the same buffer at 0.3 ml/min, and 4.3-ml fractions were collected. Fractions 72 to 85, where the activity appeared, were pooled. In a second experiment, also starting with 80 g of pig brain, the ethanol-washed extract was incubated with trypsin before being applied to the two columns (steps 3-T and 4-T). Aside from this trypsin treatment, the alternative procedure involving 3-T and 4-T did not differ from the first experiment. The elution profile in 3-T was similar to that in Fig. 2C. In step 4-T the activity came out in tubes 72 to 85, as in the untrypsinized sample. One unit of activity was defined as that exhibited by 1 mg of protein in the crude extract.

Step		Total volume (ml)	Protein recovered (mg)	Activity recovered (unit)	Specific activity (unit/mg)
No.	Type				
1	Extract	60	1800.0	1800	1
2	Ethanol-washed extract	300	600.0	1800	3
3	Sephadex G-200	180	54.0	1080	20
4	Sepharose 4B	60	12.0	360	30
		After trypsinization			
3-T	Sephadex G-200	180	5.4	1080	200
4-T	Sepharose 4B	60	0.9	360	400

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9. Included in this list are bovine serum albumin, methylated bovine albumin, bovine serum globulin, egg albumin, egg globulin, egg phosvitin, α-casein, gelatin, fibrinogen, calf thymus histone, protamine chloride, fetuin (GIBCO), and poly-L-aspartic acid. In each instance, the final concentration of the protein tested was 1.2 mg per milliliter of medium.
10. The standard molecules for the interpolation of the molecular weight were thyroglobulin (molecular weight, 670,000), β-galactosidase (molecular weight, 500,000), and catalase (molecular weight, 250,000).
11. Nerve growth factor (Wellcome Research Laboratories) was used at 0.1 and 1 unit/ml; phytohemagglutinin (Wellcome), at 1 : 100 and 1 : 1000 dilutions of the purchased material as recommended by the manufacturer; concanavalin A, at 10 and 25 μg/ml; wheat germ agglutinin (gift of M. M. Burger), at 10, 100, and 200 μg/ml; pokeweed mitogen (GIBCO), at 25, 50, and 100 μg/ml.
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Convergent Morphological Evolution Detected by Studying Proteins of Tree Frogs in the Hyla eximia Species Group

Abstract. *Protein studies have uncovered an apparent case of convergent evolution among North American tree frogs. The species* Hyla eximia *and* Hyla regilla *are so similar in external morphology that the* "wrightorum" *subspecies is assigned by some authorities to* H. eximia *and by others to* H. regilla. *Yet microcomplement fixation experiments show that* "wrightorum" *albumin, though virtually indistinguishable from authentic* H. eximia *albumin, differs as much from* H. regilla *albumin as from albumins of species outside the genus* Hyla, *such as* Acris crepitans. *The morphological resemblance of* "wrightorum" *to* H. regilla *is thus probably due to convergence.*

Protein studies can uncover cases of convergent anatomical evolution because protein evolution and anatomical evolution proceed independently (*1–4*). We describe here a case of convergent evolution in frogs.

Although frog fossils are known from rocks 150 million years old (*5*), living frogs (order Anura) are exceedingly uniform in anatomy and way of life. Despite the many speciation events responsible for the existence today of thousands of frog species, frogs have retained much the same way of life for 150 million years (*6*). Frogs are so alike in anatomy and way of life that it is very difficult for zoologists to detect convergent evolution within the order. Nevertheless, some notable cases of convergence are established. Tree frogs, for example, have evolved several times independently from ground-dwelling frogs (*5*). This happened both in the New World, where a bufonoid stock gave rise to the tree frog family Hylidae, and in the Old World, where ranoid frogs gave rise to the tree frog family Rhacophoridae (*5*). The anatomical similarities among convergent tree frogs are so great, however, that when a distinguished zoologist was confronted not long ago with a rhacophorid, mislabeled as coming from Brazil, he misclassified it as a new hylid (*7*).

We now present evidence for a case of convergent evolution among North American tree frogs of the genus *Hyla*. These animals have been classified into several species groups (*8*) on the basis of external morphology, skin color and pattern, osteology, and mating calls of adults, as well as larval morphology and, in some cases, potential for interspecific hybridization. This report deals with the *eximia* species group. As defined by Blair (*9*) and Duellman (*10*), this group includes seven or eight species, of which we have studied the following four: *H. eximia, H. euphorbiacea, H. regilla,* and *H. cadaverina.* Our interest in this species group arose from a disagreement which has developed over the taxonomic placement of an Arizona population, referred to here as the *"wrightorum"* subspecies. Duellman (*10*) assigned *"wrightorum"* to *H. eximia,* although a quantitative study of external morphology led Jameson *et al.* (*11*) to assign it to *H. regilla.* Our protein studies indicate that the morphological resemblance of *"wrightorum"* to some subspecies of *H. regilla* is due to convergence.

We compared the serum albumins of these species as well as other North American hylids by a quantitative immunological approach. Serum or plasma samples were obtained from representatives of the frog species listed in Table 1. Albumin was purified from two of them (*"wrightorum,"* Apache County, Arizona and *H. regilla hypochondriaca,* Contra Costa County, California) by preparative polyacrylamide gel electrophoresis (*12*) and injected into groups of three or four rabbits. After a 3-month period of immunization by a published method (*2*), antiserums were collected, pooled in inverse proportion to their microcomplement fixation titers, and tested for purity by several of the methods outlined by Arnheim and Wilson (*13*). Each antiserum pool was then tested for reactivity with the unpurified albumin present in serum from each of the species listed in Table 1. Reactivity was measured by the quantitative microcomplement fixation method (*14*). The results are given in immunological distance units, which are defined elsewhere (*14–16*). Immunological distance (y) is generally related to percentage difference in amino acid sequence (x) by the equation $y \cong 5x$ (*14, 16, 17*). For the particular case of albumin, there is direct empirical evidence that each unit of immunological distance is roughly equivalent to one amino acid substitution (*18*).

We worked with albumin, not only because of considerable experience in our laboratory with the study of species