alcohol, and 20 parts of 5 percent sodium carbonate in water (*1*). One third of the solution was removed and replaced by absolute alcohol for three successive days, and the tissue was fixed in absolute alcohol for 3 days. The specimens were then embedded in paraffin and sectioned on a Spencer rotary microtome. Staining techniques were those given in the Armed Forces Institute of Pathology manual (*9*) and the phosphotungstic acid–hematoxylin method of Lieb (*10*). A section of congested human lung was used as a control for the staining characteristics of the erythrocytes. The mixed cell agglutination reaction (MCAR) (*11*) was utilized in an attempt to identify the blood group of the red cells.

There was no evidence of pathological processes on microscopic examination. However, examination of the tissues from the thorax revealed the preservation of several large blood vessels, identified as arteries and veins by connective and elastic tissue stains. Within the veins were small groups of cells showing the typical biconcave morphology of erythrocytes (Fig. 1). These cells showed a somewhat scaphoid shape on edge. The cells stained only faintly with Masson trichrome and phosphotungstic acid–hematoxylin stains but did not reveal any differences in staining characteristics from fresh red blood cells. Two fungal stains were used; a periodic acid–Schiff stain was negative, while a number of the cells did take a methenamine silver stain to a moderate degree. The sections were tested for A, B, and H(O) isoantigens by the MCAR, and all reactions were negative. Scattered among the red cells were clumps of fragmented basophilic material, representing nuclear fragments of autolyzed polymorphonuclear leukocytes.

The intravascular location, typical shape, admixture of autolyzed white blood cells, and staining characteristics are all consistent with the interpretation that the structures are preserved red blood cells. The negative MCAR probably represents loss of antigenicity over a 2000-year period, but may be due to washing out of the antigen by the rehydration process. The only other inconsistency was the ability of some of the cells to take the silver stain for a fungus. This finding, in view of the other evidence, must be interpreted as a variation in the staining characteristics of red blood cells with mummification and rehydration. Desiccated and rehydrated fungi retain a marked avidity for this stain.

These cells are identical in appearance with those described by Giacometti and Chiarelli (*6*) and by Williams (*3*). Williams had interpreted his sections as indicating an area of hemorrhage, and the present finding confirms that diagnosis (*12*).

MICHAEL R. ZIMMERMAN
Departments of Pathology and
Anthropology, University of Pennsylvania, Philadelphia 19104

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12. I thank Dr. Louise Robbins, formerly of the Department of Anthropology, University of Kentucky, for supplying the tissue used in this study, and Dr. Israel Davidsohn of the Chicago Medical School for performing the MCAR studies. This report was presented in a symposium on skeletal biology at the meeting of the American Association of Physical Anthropologists, Lawrence, Kansas, 13 April 1972.

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Cyclic AMP Phosphodiesterase in Cloned Astrocytoma Cells: Norepinephrine Induces a Specific Enzyme Form

Abstract. *The soluble supernatant fraction of homogenates of cloned rat astrocytoma cells (line C-2A) was subjected to polyacrylamide gel electrophoresis. Two peaks of adenosine 3′,5′-monophosphate phosphodiesterase activity were found, corresponding to peaks I and IV of a similarly prepared homogenate of rat brain. Incubating cells with norepinephrine (0.3 millimolar) caused about a threefold increase in the activity of peak IV but no change in peak I. This increase was completely inhibited by prior incubation with propranolol (0.1 millimolar), a beta-adrenergic blocking agent, or with cyclohexamine (40 micromolar), a protein synthesis inhibitor. Induction of a specific phosphodiesterase form by norepinephrine suggests another feedback control mechanism whereby an organism can prevent the effects of excessive sympathetic activity.*

Adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase, the enzyme that catalyzes the hydrolysis of cyclic AMP (*1*), exists in several molecular forms (*2*, *3*). For example, using polyacrylamide gel electrophoresis we found six distinct forms of cyclic AMP phosphodiesterase in the soluble supernatant fraction of rat cerebellar homogenates (*3*). These different molecular forms of phosphodiesterase found in brain may reside in specific types of cells. Thus, we found four peaks of phosphodiesterase activity in cerebrum (designated I to IV according to their order of emergence from the electrophoresis column). However, we found only two peaks of activity in a C-2A astrocytoma cell line. These peaks had the same electrophoretic mobilities and other characteristics similar to those of peaks I and IV of rat cerebrum, and will be designated peaks I and IV. In each of two cloned neuroblastoma cell lines (N1E and N18), we found only one peak of activity corresponding to peak III of rat cerebrum (*4*).

As part of a series of studies to determine the role of the sympathetic nervous system and other endogenous factors in controlling the intracellular concentration of cyclic nucleotides (*5*, *6*), we report here on the effect of norepinephrine on the two forms of cyclic AMP phosphodiesterase in cloned C-2A rat astrocytoma cells.

The C-2A rat astrocytoma cell line was derived by cloning rat brain glial tumors induced by the intravenous injection of *N*-nitrosomethylurea (*7*). The cells were grown to confluency in Falcon plastic tissue culture flasks in air supplemented with 10 percent CO_2; the medium was Dulbecco's modification of Eagle's medium, supplemented with 10 percent fetal calf serum, penicillin G (50 unit/ml) and streptomycin sulfate (10 μg/ml). At the initiation of each experiment, the medium was removed and replaced with fresh medium containing the

chemicals under investigation. After 6 hours of further incubation, the medium was decanted and the cultures were washed three times with an isotonic salt solution. The cells were then harvested and stored at −70°C until fractionation (usually within 24 hours). After thawing, the cells were homogenized in 0.32*M* sucrose and the homogenate was sonicated for 5 minutes and then centrifuged at 100,000*g* for 1 hour. The supernatant was than subjected to preparative polyacrylamide gel electrophoresis. Gel preparation and electrophoresis methods were as described (*3*). Phosphodiesterase activity of each fraction was measured by the method of Weiss *et al.* (*8*), with 10 μl of sample and $2 \times 10^{-4}M$ cyclic AMP as substrate in a total volume of 150 μl.

The soluble supernatant fraction of cloned C-2A rat astrocytoma cell homogenates had two distinct peaks of phosphodiesterase activity, corresponding to peaks I and IV of similar preparations from rat brain (Fig. 1). Peak I was considerably higher but narrower than peak IV; the ratio of the total activity in peak I to that in peak IV, as calculated from the area under each peak, was 1:0.9.

Figure 1 also shows that prior incubation of the astrocytoma cells with 0.3 m*M* norepinephrine for 6 hours caused a marked difference in the phosphodiesterase pattern. The phosphodiesterase activity of peak I was similar to that in the control preparation, but the activity of peak IV in the norepinephrine-treated cells was more than three times that of the control sample. The ratio of the activities of peaks I and IV was now 1:2.7.

To determine whether norepinephrine per se was activating peak IV phosphodiesterase, we added 0.3 m*M* norepinephrine to enzyme from each peak after separating the phosphodiesterases on the electrophoresis column. The catecholamine had no direct effect on phosphodiesterase activity from either peak. Apparently norepinephrine had to be incubated with the intact cells to obtain the increased activity.

We studied the influence of propranolol, the beta-adrenergic blocking agent, to determine whether norepinephrine induced this selective increase in phosphodiesterase activity through a mechanism involving beta-adrenergic receptors. Incubation of astrocytoma cells with 0.1 m*M* propranolol in the absence of norepinephrine had no effect on either of the two peaks of phosphodiesterase, and incubation of cells with propranolol and norepinephrine had no effect on peak I phosphodiesterase. However, the adrenergic blocking agent completely inhibited the increase in peak IV phosphodiesterase normally induced by norepinephrine (Fig. 1). These results suggest that the catecholamine was acting through an activation of beta-adrenergic receptors. Since the adenylate cyclase system may be an integral part of the beta-adrenergic receptor (*9, 10*) and since propranolol effectively inhibits the activation of adenylate cyclase by norepinephrine (*10*), these data suggest that norepinephrine increased the activity of one of the two molecular forms of phosphodiesterase in C-2A cells by activating the norepinephrine-sensitive adenylate cyclase system in these cells.

To determine whether the synthesis of new protein was involved in this effect of norepinephrine, we incubated the cells with cycloheximide, an agent that inhibits the translational stage of protein synthesis (*11*). Cycloheximide (40 μ*M*) alone had no significant effect on either the total phosphodiesterase activity or on the ratio of activities in peaks I and IV (the ratio was 1:1.1 for both control and cycloheximide-treated cells). As in previous experiments, norepinephrine had no effect on peak I phosphodiesterase but increased about threefold the phosphodiesterase activity of peak IV (Fig. 2). The addition of cycloheximide inhibited almost completely the increased phosphodiesterase activity induced by norepinephrine (Fig. 2). (The ratio of the activities of peaks I and IV was 1:3.6 in the presence of norepinephrine and was 1:1.4 in the presence of norepinephrine plus cycloheximide.) These results suggest that norepinephrine increased phosphodiesterase activity by inducing the synthesis of new protein.

A variety of exogenous and endogenous factors have been reported to alter the activity of cyclic nucleotide phosphodiesterase. For example, the phosphodiesterase activity of rat brain increases with the age of the animal (*12, 13*). This increase in enzyme activity, however, is not uniform throughout the brain. Whereas the phosphodi-

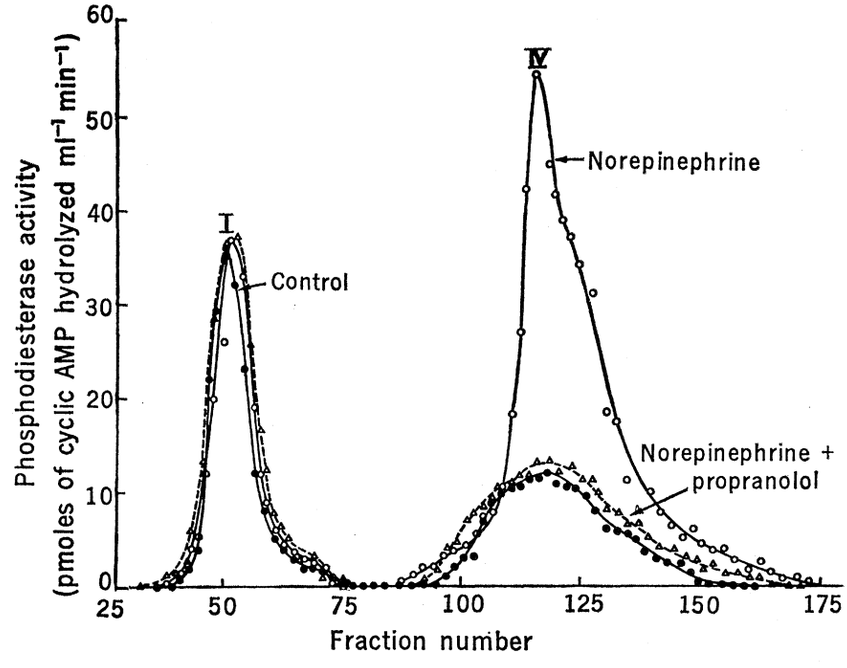

Fig. 1. Effects of norepinephrine and propranolol on the two molecular forms of phosphodiesterase of C-2A astrocytoma cells. Cells were prepared and cultured as described in the text. Norepinephrine (0.3 m*M*) and propranolol (0.1 m*M*) were added alone and in combination during the final 6 hours of incubation. The cells were then homogenized, the supernatant fraction was placed on a polyacrylamide gel electrophoresis column, and the eluted fractions were analyzed for phosphodiesterase activity. The ratio of the total phosphodiesterase activity of peak I to that of peak IV was 1:0.9 in the control culture, 1:1.1 in the propranolol-treated culture (not shown), 1:2.7 in the norepinephrine-treated culture, and 1:1.2 in the culture treated with norepinephrine and propranolol.

esterase activity of cerebrum and brain stem increases severalfold between birth and 20 days of age, the enzyme activity of cerebellum does not change (*13*). The different molecular forms of phosphodiesterase increase ontogenetically at different rates (*6, 14*).

The phosphodiesterase activity of fibroblasts increased when these cells were incubated either with prostaglandin E_1, which increases the cyclic AMP content of cells, or with dibutyryl cyclic AMP (*15*). These studies suggest that cyclic AMP can induce the synthesis of cyclic AMP phosphodiesterase. Our present results support this suggestion and show further that norepinephrine, which increases the intracellular concentration of cyclic AMP in C-2A and other astrocytoma cells (*16*), can selectively induce the formation of one of two molecular forms of phosphodiesterase in a cloned line of rat astrocytoma cells.

Our demonstration of catecholamine-induced increase in phosphodiesterase activity points to another mechanism whereby an organism can regulate the intracellular concentration of cyclic nucleotides in the face of excessive sympathetic tone. A reduction in sympathetic nerve activity causes an increase in the norepinephrine-sensitive adenylate cyclase system, and an increase in sympathetic activity causes a decrease in adenylate cyclase activity (*5, 17*). We also showed that norepinephrine may be responsible for regulating not only the activity of adenylate cyclase but also the amount of the norepinephrine-sensitive adenylate cyclase system (*18*).

On the basis of these results and those in the present report, we propose the following: Increased sympathetic nerve activity causes several changes in the adenylate cyclase–phosphodiesterase system. The immediate effect is to activate adenylate cyclase (through the release of the adrenergic neurotransmitter, norepinephrine), causing an increased intracellular concentration of cyclic AMP. However, this action is balanced by two long-term effects that are complimentary to each other—namely, a reduction in the norepinephrine-sensitive adenylate cyclase system and an increase in a specific inducible form of cyclic AMP phosphodiesterase. Accordingly, these long-term responses to increased sympathetic activity would constitute a protective mechanism which would not allow continuously high intracellular concentrations of cyclic AMP to persist during excessive and prolonged sympathetic activity.

In summary, our results show that norepinephrine causes a selective increase in one of two forms of phosphodiesterase found in the soluble supernatant fraction of rat C-2A astrocytoma cells. Moreover, this increase in phosphodiesterase activity induced by norepinephrine involves the synthesis of new protein and is a consequence of beta-adrenergic stimulation.

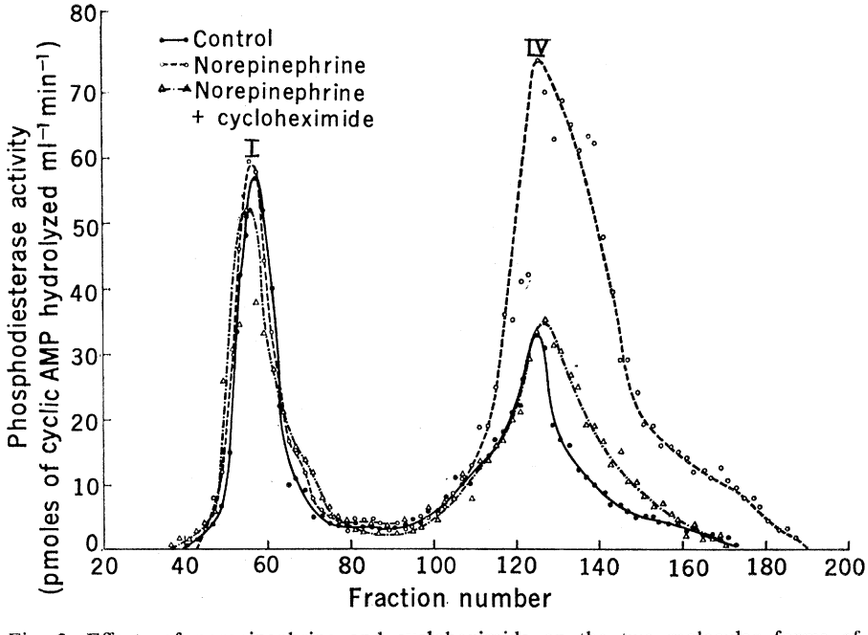

Fig. 2. Effects of norepinephrine and cycloheximide on the two molecular forms of phosphodiesterase of C-2A astrocytoma cells. Cells were prepared and cultured as described in the text. Norepinephrine (0.3 m*M*) and cycloheximide (40 μM) were added alone and in combination during the final 6 hours of incubation. The cells were homogenized, the supernatant fraction was placed on a polyacrylamide gel electrophoresis column, and the eluted fractions were analyzed for phosphodiesterase activity. The ratio of the total phosphodiesterase activity of peak I to that in peak IV was 1 : 1.1 in the control culture, 1 : 1.1 in the cycloheximide-treated culture (not shown), 1 : 3.6 in the norepinephrine-treated culture, and 1 : 1.4 in the culture treated with norepinephrine and cycloheximide.

PETKO UZUNOV
Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C. 20032
HARVEY M. SHEIN
McLean Hospital, Belmont, Massachusetts 02178
BENJAMIN WEISS*
Laboratory of Preclinical Pharmacology, National Institute of Mental Health

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* Present address: Department of Pharmacology, Medical College of Pennsylvania, Philadelphia 19129. Address reprint requests to B.W.

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