Table 2. Acute intravenous toxicologic comparison of AME and IAB in dogs.

Effect	Minimal dose (mg/kg) producing indicated effect		Ratio of minimal doses AME/IAB
	AME	IAB	
Unanesthetized dogs			
Death	48	12	4
Serum urea nitrogen; increase	6	0.75	8
Renal tubular degeneration	24	1.5	16
Intravascular hemolysis	12	6	2
Hemoglobinuria	24	12	2
EKG; T-wave aberration	12	1.5	8
Serum potassium changes; increase followed by decrease	>24	6	>4
Serum glutamic pyruvic transaminase; increase	12	3	4
Bloody diarrhea	24	6	4
Anesthetized dogs			
Renal blood flow; depression	12	1.5	8
Glomerular filtration rate; depression	12	1.5	8
Intravascular hemolysis	12	6	2
Arterial pressure; elevation	6	1.5	4
Arterial pressure; depression	24	6	4
Bradycardia	24	1.5	16
EKG; T-wave aberration	24	3	8
Serum potassium; increase	6	1.5	4
Urinary potassium excretion; increase	6	1.5	4
Hematocrit; increase	12	6	2

one dog at 48 mg/kg; IAB was given to one dog each at dosages of 0.75, 1.5, 3, 6, or 12 mg/kg. Serum samples for biochemical analyses were taken 0, 4, 24, and 48 hours after dosage. Electrocardiograms and clinical signs were recorded periodically until 72 hours after dosage. At this time, nine animals were necropsied for gross and micropathologic examinations of the kidneys. In anesthetized animals, the dosages given were: AME 6, 12, or 24 mg/kg, and IAB 1.5, 3, or 6 mg/kg. The electrocardiogram (lead II), renal blood flow (electromagnetic flow probe), and carotid arterial pressure were recorded continuously for 90 minutes after injection. Blood and urine samples for biochemical analyses were taken at 15-minute intervals throughout this period. Clearance of exogenous creatinine was used as a measure of the glomerular filtration rate.

To facilitate comparisons of the two compounds, the minimal doses of each that caused changes in monitored parameters were determined. These doses and their ratios (AME/IAB) are shown in Table 2. The ratios range between 2 and 16, but usually are 4 or 8. Most importantly, the ratios for elevated serum urea nitrogen, depressed renal blood flow and glomerular filtration rate, and renal tubular degeneration are at least 8, indicating that AME is, at most, only one-eighth as nephrotoxic as IAB. Moreover, the increase in serum urea nitrogen was proportional to dose with IAB (maximal value 162 mg/100 ml), but not with AME (maximal value 46 mg/100 ml), and the probable cause of death after the high dose of AME was not nephrotoxicity, as was expected with IAB, but massive intravascular hemolysis. Hemolysis produced by disruption of the membrane structure exemplifies the mechanism of action of amphotericin B and polyene macrolide antifungal agents in general. Since the dose ratio of renal tubular degeneration to intravascular hemolysis is 2 (24/12) for AME and 0.25 (1.5/6) for IAB, it is conceivable that AME will not be nephrotoxic at therapeutic doses.

These preliminary studies indicate that AME has a significant safety advantage over IAB; however, the potential clinical worth of this new agent will be determined only after more extensive tests of efficacy, stability, and repeat-dose toxicity have been completed.

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Noradrenergic Stimulation of Cyclic Adenosine Monophosphate in Rat Purkinje Neurons: An Immunocytochemical Study

Abstract. *A specific immunofluorescent histochemical method for cyclic adenosine monophosphate was used to study rat cerebellum. After topical treatment with norepinephrine or stimulation of norepinephrine-containing afferents from locus coeruleus, there was a striking increase in the number of Purkinje cells with strong cyclic adenosine monophosphate reactivity. Other putative inhibitory transmitters had no significant effect on staining of Purkinje cells. The results provide the first histochemical support for the hypothesis that cyclic adenosine monophosphate can be generated postsynaptically in central neurons in response to noradrenergic stimuli.*

The discovery that catecholamines stimulate production of adenosine 3′,5′-monophosphate (cyclic AMP) in brain homogenates (*1*) or slices (*2*) has suggested that cyclic AMP may take part in central adrenergic neurotransmission

(*3*). Direct verification, however, of such a role will require the selective activation of central noradrenergic pathways and precise identification of the cell types [for example, neurons or glia (*4*)] involved in the catecholamine–cyclic AMP response.

The development of an immunofluorescent histochemical technique for cyclic AMP localization (*5*) now provides a light microscopic method for detecting those nervous elements that respond to catecholamines with increases in cyclic AMP. Of the various brain regions, the cerebellum seems most advantageous for such study, since it is known to generate abundant cyclic AMP in response to catecholamines in vitro (*2*) and since a discrete noradrenergic pathway from locus coeruleus (*6*, *7*) to the cerebellar cortex can be activated (*8*). Furthermore, pharmacological and electrophysiological evidence (*8*) has suggested that the inhibition of Purkinje cell discharge produced by exogenous norepinephrine or activation of the locus coeruleus is mediated postsynaptically by cyclic AMP. We now report that noradrenergic stimulation selectively increases cyclic AMP immunofluorescence in Purkinje cells. These results provide the first histochemical demonstration of the neuronal generation of cyclic AMP in response to a specific neurohumor.

Rats were anesthetized with ¾ to 1 percent halothane (*9*, *10*) and placed in a stereotaxic apparatus. The posterior cerebellar vermis and both hemispheres were completely exposed, and the dura was removed. In stimulation experiments a fine (0.4 mm in diameter) concentric bipolar stimulating electrode was passed ventrally through the right cerebellar hemisphere into the brainstem. Stimulus parameters, histological verification of electrode placement, and the methods of recording the electrical activity of Purkinje neurons have been described (*8*). Small (15 to 30 mm^3) biopsy specimens of cerebellar vermis were immersed in liquid nitrogen immediately after application of 180 to 200 shocks at a rate of 10 per second (Fig. 1A) to the brainstem (*11*).

In pharmacological tests, drugs were dissolved in Ringer solution and topically applied to the cerebellum with cotton pledgets. Biopsies of cerebellum were then taken, as in stimulation experiments, and immersed in liquid nitrogen. Controls were obtained from intact cerebellar zones (*12*) either 5 to 20 minutes before or 10 to 30 minutes after stimulation or drug application (*13*). Cryostat sections (14 μm thick) of the cerebellar biopsies were then treated by the immunofluorescent histochemical method for cyclic AMP (*5*, *10*).

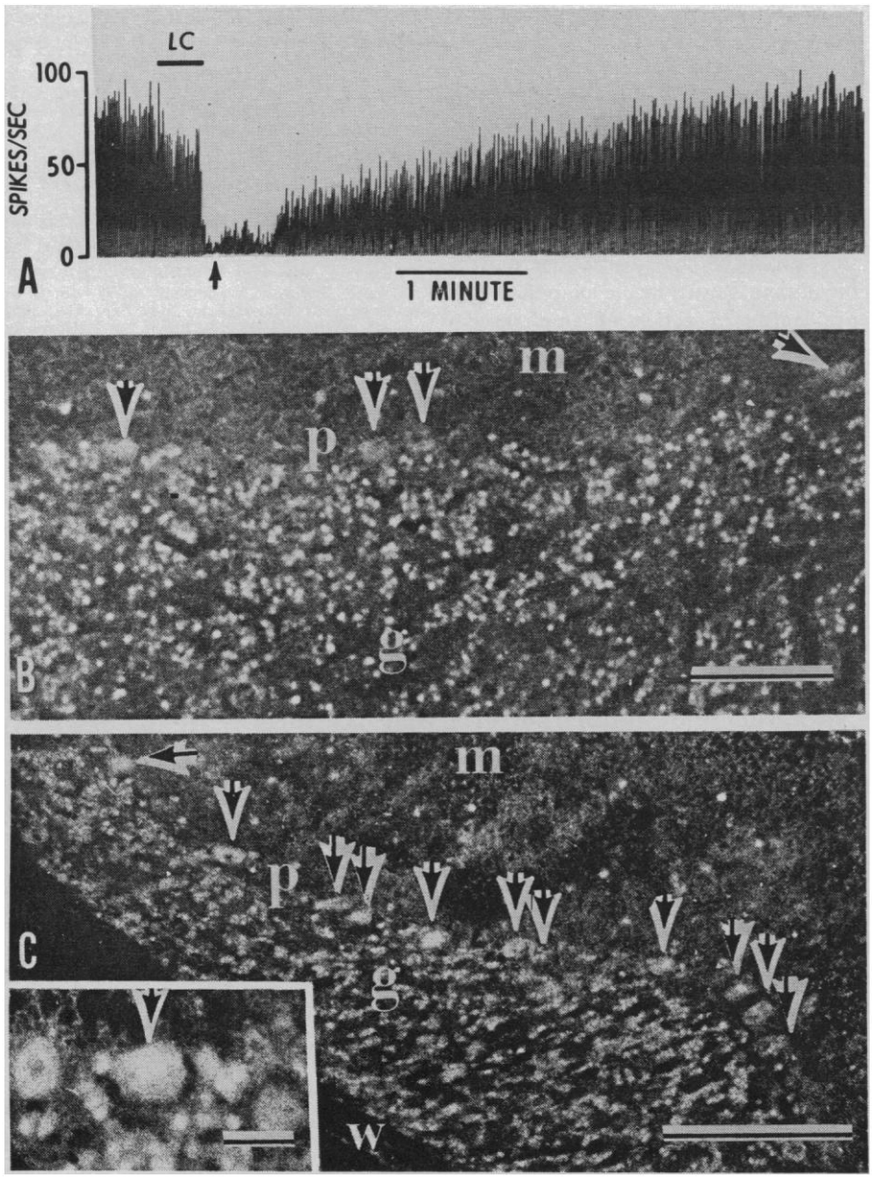

Fig. 1. Effects of electrical activation of the pathway from the locus coeruleus (*LC*) to cerebellar cortex on the spontaneous activity and the immunocytochemical staining of cyclic AMP of Purkinje neurons. (A) Inhibition of the spontaneous discharge rate of an ipsilateral Purkinje neuron recorded with an extracellular micropipette after stimulation (bar above record) of the locus coeruleus (200 stimuli; current intensity, 1 ma). The arrow indicates the approximate time after the stimulation at which the vermis was biopsied [see text and (*10*)] in a subsequent stimulation period. (B) Control dark-field fluorescence micrograph of the immunocytochemical staining for cyclic AMP of cerebellar hemisphere biopsied 20 minutes before stimulation of the locus coeruleus. Arrows indicate the few faintly reactive Purkinje cells in the Purkinje cell layer (*p*); granule cells (*g*) are also stained. There is much less fluorescence in the molecular layer (*m*). (C) Fluorescent micrograph of cerebellar vermis biopsied precisely at point indicated in (A) after stimulation of the locus coeruleus. The arrows indicate immunoreactive Purkinje cells, which appear to fluoresce brighter than reactive Purkinje cells from control biopsies. Approximately 50 percent of the Purkinje cells in this folium were reactive for cyclic AMP, while about 13 percent of the cells biopsied during the control period were reactive. There is no reactivity in the white matter (*w*). The inset shows the immunoreactivity of Purkinje cells at higher magnification; one neuron shows a nonreactive nucleus while another shows a strongly reactive nucleus. Calibrations: 250 μm for (B) and (C); inset, 25 μm.

In sections of control cerebellums, only a few (10 to 20 percent) Purkinje cells fluoresced (Figs. 1B and 2) (*14*). Granule cells showed moderate immunoreactivity, and the molecular layer (containing predominantly Purkinje dendrites and parallel fibers) showed considerably less. White matter remained entirely unreactive.

After topical application of norepinephrine (Fig. 2) or during discrete stimulation of the locus coeruleus (Figs. 1C and 2), most Purkinje cells showed marked immunoreactivity. In an effort to quantitate these changes, we counted the number of Purkinje cells showing immunoreactivity before and after treatment in many folia (*14*). Statistical analysis of the data showed that norepinephrine significantly elevated the percentage of reactive Purkinje cells (Fig. 2). To test whether general inhibition of the Purkinje cells could account for these observations, we applied γ-aminobutyric acid (GABA), a putative inhibitory transmitter for Purkinje cells (*15*), to the surface of the cerebellum. GABA had no effect on the cyclic AMP fluorescence of Purkinje cells, even in very high concentrations (Fig. 2). Other putative neurotransmitters such as serotonin, L-glycine, and histamine, all of which inhibit the spontaneous firing of Purkinje cells (*15, 16*), also had no effect on Purkinje cell staining (Fig. 2).

Figure 2 also demonstrates that the great enhancement of Purkinje cell reactivity seen with direct stimulation of locus coeruleus is not found during sham stimulation of normal rats (*13*), or when catecholamine-containing nerves have been selectively destroyed by prior treatment with intracisternal 6-hydroxydopamine (*17*). Furthermore, when placement of the stimulating electrode is as little as 0.5 mm outside the area of the locus coeruleus, Purkinje cell reactivity approaches that of the controls. The appearance of the granule cell layer after stimulation of brainstem or cerebellar white matter, or after topical application of norepinephrine remained unchanged from that of the controls.

The immunohistochemical staining method provides a means of localizing cyclic AMP at the cellular level (*5, 10*). In our studies [see also (*10*)], Purkinje neurons were the only cerebellar elements observed to increase their cyclic AMP content. This contrasts with reports of catecholamine stimulation of cyclic AMP formation in glial tumors in tissue cultures (*4*). Although we did not observe an increase in fluorescence in elements other than Purkinje cells, because of the limitations of the light microscopic technique we cannot exclude the possibility of a change in fluorescence in the fine processes of neuroglia. In addition, our fixation techniques may not be adequate to preserve stimulated cyclic AMP in all cell types, and the magnitude of possible change in other cell types may be below the limits of detection by the immunofluorescence technique.

Our data now provide direct support for the theory that norepinephrine is the neurotransmitter involved in the inhibition of Purkinje cells produced by electrical stimulation of the nucleus locus coeruleus (*8*) since only norepinephrine and locus coeruleus stimulation evoked a rise in Purkinje cell cyclic AMP, while other inhibitory agents had no effect. Therefore, inhibition of Purkinje cell discharge is not sufficient in itself to account for the enhanced neuronal generation of cyclic AMP.

Previously we have hypothesized, on the basis of indirect electrophysiological and pharmacological experiments (*8, 18*), and on the basis of reported biochemical studies in vitro (*2*), that the inhibitory response of Purkinje cells to stimulation of the noradrenergic pathway may be mediated by the postsynaptic generation of cyclic AMP. However, this proposal could not be directly substantiated until a cytological method was available for estimating cyclic AMP concentrations in single Purkinje neurons. The immunofluorescent histochemical technique now provides evidence that the cyclic AMP of Purkinje cells increases in response to norepinephrine or stimulation of the locus coeruleus, directly supporting the theory that the inhibitory noradrenergic response is mediated by cyclic AMP. Therefore, while the noradrenergic projection to cerebellar cortex is far less prominent than the classical myelinated afferent pathways, activation of this projection may elicit profound effects on the entire population of Purkinje neurons, through the biochemical amplification provided by the catecholamine-sensitive adenylate

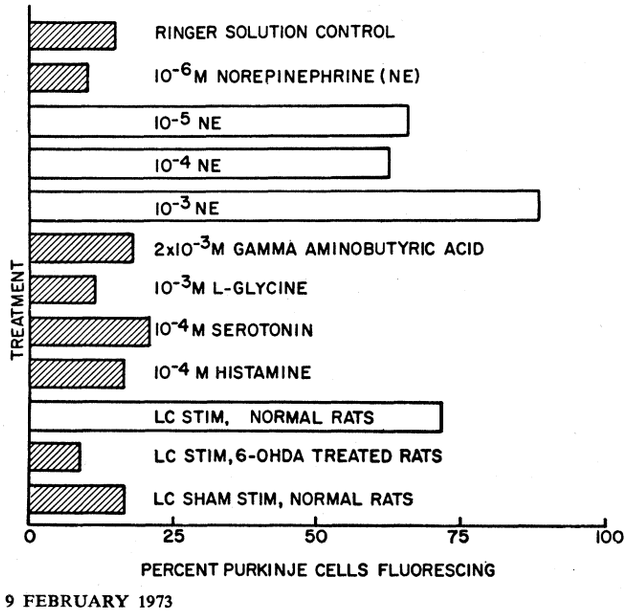

Fig. 2. Effects of pharmacological and electrophysiological stimuli on the immunoreactivity of cerebellar Purkinje cells for cyclic AMP. Solutions were applied by cotton pledget to the exposed cerebellar vermis for 10 minutes and then frozen biopsies were taken for immunocytochemical staining. Results are expressed as the mean percent of reactive Purkinje cells, which was determined by dividing the number of reactive cells by the total number of Purkinje cells in individual folia. The percentages of each sampled folia were averaged for groups of three to eight folia in at least two separate experiments to provide standard deviations. More than 200 cells were counted for each condition. Hatched bars indicate values that are not significantly different from Ringer solution controls ($P > .05$; Student's t-test); clear bars indicate values significantly greater than control values ($P \ll .001$); *LC Stim*, electrical stimulation of ipsilateral locus coeruleus; *6-OHDA*, 6-hydroxydopamine.

cyclase. An immunohistochemical survey of the entire brain will be required to determine if other central adrenergic responses are also accompanied by elevations in neuronal cyclic AMP.

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14. As the immunocytochemical staining method is now applied, we could not objectively evaluate gradations of staining intensity when comparing different experimental specimens. Therefore, all unequivocally reactive neurons were grouped together and the results were expressed as a percentage of the total number of Purkinje neurons visualized within the section. In the results of Fig. 2, since the response is evaluated only in terms of a single all-or-none detection threshold, rather than by graded measurement, it is not surprising that $10^{-6}M$ norepinephrine and control values are equivalent, and that at 10^{-5}, 10^{-4}, and $10^{-3}M$ norepinephrine, cell counts are similar. A dose-dependent rise would be unlikely if a homogeneous population of Purkinje cell receptor content (*8*) and distribution exists from cell to cell. Granule cells appear to be maximally reactive even in the control conditions and it is difficult to determine whether the reactive granule cells are qualitatively changed by any of the experimental conditions studied.
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Selenium: Biochemical Role as a Component of Glutathione Peroxidase

Abstract. *When hemolyzates from erythrocytes of selenium-deficient rats were incubated in vitro in the presence of ascorbate or H_2O_2, added glutathione failed to protect the hemoglobin from oxidative damage. This occurred because the erythrocytes were practically devoid of glutathione-peroxidase activity. Extensively purified preparations of glutathione peroxidase contained a large part of the ^{75}Se of erythrocytes labeled in vivo. Many of the nutritional effects of selenium can be explained by its role in glutathione peroxidase.*

Although the nutritional importance of selenium and its relation to vitamin E are well known (*1*), definition of a specific biochemical role for selenium has so far proved elusive. We present evidence here for a role of Se in glutathione (GSH) peroxidase (glutathione: H_2O_2 oxidoreductase, E.C. 1.11.1.9; $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$) which provides further evidence for the essentiality of Se for animals and for one of its biochemical functions, and provides a plausible explanation for its relation to vitamin E.

Earlier, we found that dietary Se helped prevent oxidative damage to rat erythrocytes incubated in vitro, as evidenced by decreased hemolysis and decreased hemoglobin oxidation (*2*). These effects of dietary Se were dependent on the addition of glucose in vitro, and the well-known protection against hemolysis and hemoglobin oxidation afforded by glucose (*3*) was virtually absent in erythrocytes from rats deficient in Se. A related dietary inhibitor of oxidative damage, α-tocopherol (vitamin E), protected against hemolysis whether or not glucose was present, but did not protect against hemoglobin oxidation. This result demonstrated that the effect of Se was specific and distinct from that of vitamin E (*2*).

Protection by glucose against oxidative damage to red blood cells has been attributed to the maintenance of the intracellular concentration of reduced glutathione (GSH) through the combined actions of the enzymes, glucose-6-phosphate dehydrogenase [which generates reduced nicotinamide adenine dinucleotide phosphate (NADPH)] and glutathione reductase [which generates GSH from NADPH and oxidized glutathione (GSSG)] (*3*). At least in part, GSH acts by destroying hydrogen peroxide and fatty acid hydroperoxides through reactions catalyzed by GSH peroxidase (*3, 4*). In our earlier work (*2*) we found that the GSH concentration was higher in Se-deficient than in Se-adequate erythrocytes and that it was as effectively maintained during incubation in vitro. This suggested that the defect in Se deficiency was not in the maintenance of GSH but rather in the utilization of GSH in protecting the cell (*2*). We therefore focused on a possible role for Se in the GSH protection against hemoglobin oxidation and ultimately on the enzyme (GSH peroxidase).

All experiments were performed with