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ALTERATIONS IN BRAIN GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE LEVEL--ETC(U)
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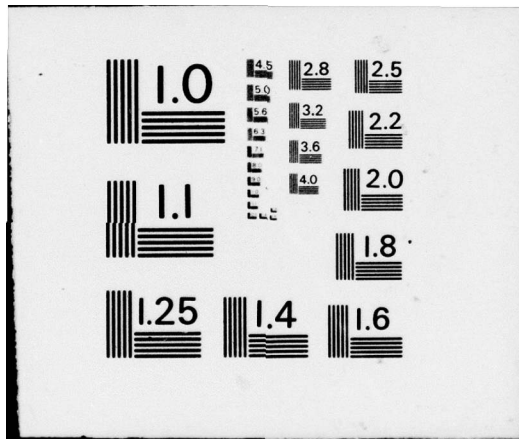
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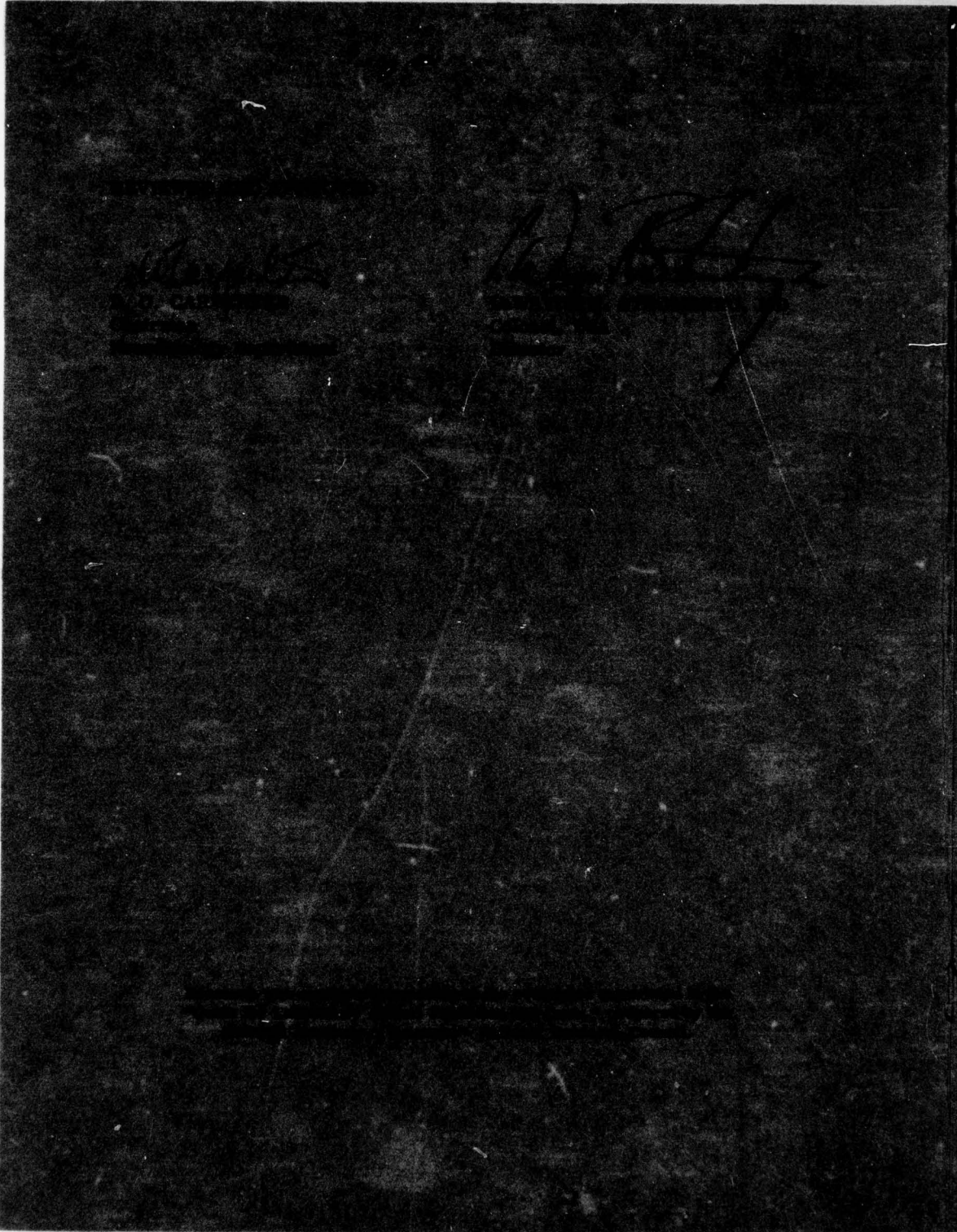


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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The effect of acute and chronic ethanol treatment on guanosine 3', 5'-cyclic monophosphate (c-GMP) levels was studied in six areas of the rat brain. Rats were either gavaged with a single dose of ethanol (6 g/kg) or rendered ethanol-dependent with 11-15 g/kg per day in three to five fractions over a 4-day period. A single dose of ethanol depleted c-GMP levels in most areas of the brain studied. In the three areas in which a time course of the response was determined, the cerebellum, → over		

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INTRODUCTION

Evidence has been accumulating that suggests that the cyclic nucleotides adenosine 3',5'-cyclic monophosphate (c-AMP) and guanosine 3',5'-cyclic monophosphate (c-GMP) play an important role in synaptic transmission. In the superior cervical ganglion c-AMP levels are elevated by dopamine (DA)²⁷ and c-GMP levels by acetylcholine (ACh),²⁹ both increases being localized in postganglionic neurons.²⁶ In brain tissue, norepinephrine (NE) and DA increase c-AMP levels,^{25,28} while ACh increases c-GMP levels.^{30,32}

Acute and chronic administration of ethanol results in a number of alterations in the function of NE, DA and ACh. Although it is generally accepted that acute and short-term chronic treatment with ethanol has little or no effect on catecholamine levels in the brain,^{3,11,13,24} a number of changes in catecholamine turnover have been reported. Single doses of ethanol have a biphasic effect on catecholamine turnover. Shortly after ethanol administration NE turnover is increased, while DA turnover is either increased or unaffected.^{5,9,24,43} At later time periods both NE and DA turnover are depressed.^{24,43,44} Chronic treatment results in elevated NE turnover and decreased DA turnover.^{24,44}

Ethanol induces a biphasic response also in the cholinergic system. Low blood ethanol concentrations result in a reduction in ACh levels,^{23,45} but with high blood ethanol concentrations, ACh levels are elevated²³ and ACh release is depressed.¹⁵

Because of the apparent role of cyclic nucleotides in synaptic transmission and the ethanol-induced alterations in transmitter function, a study of the effects of ethanol on c-AMP and c-GMP is warranted. Several studies have appeared on investigations of interactions of ethanol and the c-AMP system. Single doses of ethanol have been reported to deplete c-AMP levels selectively in the cerebellum, but not in the cerebrum, pons and medulla oblongata.^{31,56} However, this depletion has become suspect because of inadequate means of preventing postmortem accumulation of c-AMP, and could not be reproduced using focused microwave irradiation to fix c-AMP levels in the brain.⁴⁶ c-AMP levels were

unaffected after 4 days of chronic ethanol treatment,⁴⁶ but were elevated after 7 days.³¹ Treatment for 9 to 14 weeks results in noradrenergic subsensitivity of the c-AMP generating system in cortical brain slices,^{19,20} but reverses to develop noradrenergic supersensitivity 3 days after withdrawal.^{18,19}

The purpose of the present investigation was to determine the effect of acute and chronic ethanol treatment on regional brain c-GMP levels. This included measurements of c-GMP in six brain areas and in ethanol-dependent animals.

METHODS

Induction of ethanol dependence. Ethanol was administered orally using a pediatric feeding tube (American Hospital Supply, Evanston, Illinois) as a 20 percent (w/v) aqueous solution. Ethanol dependence was induced by the method of Majchrowicz.³⁵ Doses of 9-15 g/kg were given to male Sprague-Dawley rats (200-300 g) in three to five fractions over a 4-day period. The animals were euthanatized on the day of withdrawal while still intoxicated or undergoing a withdrawal syndrome. In acute experiments rats were given a single dose of ethanol (6 g/kg) and euthanatized at different times thereafter.

Analysis for cyclic nucleotides. Rats were euthanatized by focused microwave irradiation (Litton Menumaster 70/50, modified by Medical Engineering Consultants, Lexington, Massachusetts; 1.3 kW) using a 3.5-sec exposure time. The brains were excised and dissected into the following parts: cerebellum, brain stem, thalamus, hippocampus, caudate nucleus and cerebral cortex. c-GMP was isolated and purified according to the procedure described by Mao and Guidotti³⁷ and quantitated by the competitive protein binding assay of Murad et al.⁴¹ as modified by Dinnendahl.¹⁰ Protein content was determined by the method of Lowry et al.³⁴ The recovery of c-GMP from spiked brain homogenates was 70-80 percent. All values reported here are uncorrected for recovery. Statistical comparisons of the data were made using Student's "t" test.

Guanylate cyclase activity. Rats were decapitated and the brains excised and homogenized in 10 volumes of ice-cold 0.25 M sucrose containing 20 mM Tris-Cl buffer (pH 7.4), 1 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 x g for 10 min at 4°C. The supernatant solution was recentrifuged at 105,000 x g for 1 hour at 4°C. The resulting supernate was used as the source of guanylate cyclase.

The incubation procedure follows the method of Nakazawa and Sano⁴² and was performed in a total volume of 120 μ l. The medium contained the following constituents in their final concentration: 0.4 mM [8-³H] GTP (New England Nuclear, Boston, Massachusetts), 2 mM c-GMP, 2 mM MnCl₂, 0-200 mM ethanol and an aliquot of enzyme protein, all in 200 mM Tris-Cl buffer (pH 7.7). The MnCl₂ was freshly prepared the day of the experiment to minimize its oxidation. The mixture was incubated at 30°C for 15 minutes and transferred to a boiling water bath for 2 minutes, then chilled on ice. The [³H] c-GMP was separated from [³H] GTP by absorption and ion exchange chromatography according to the method of Mao and Guidotti,³⁷ used to isolate unlabeled c-GMP from brain tissue. Removal of [³H] GTP is over 99.9 percent complete. The data were evaluated using the paired t-test with each tissue sample serving as its own control.

c-GMP phosphodiesterase activity. The source for brain phosphodiesterase activity was prepared in the same manner as for guanylate cyclase. c-GMP phosphodiesterase activity was determined by the method of Thompson and Appleman⁵⁵ as modified by Nakazawa and Sano.⁴² An aliquot of the enzyme preparation was incubated in a medium containing 0.1 mM [8-³H] c-GMP, 5 mM MgCl₂ and 4 mM 2-mercaptoethanol, all in 40 mM Tris-Cl buffer (pH 8.0) and in a final volume of 0.4 ml. After incubation at 30°C for 10 minutes, the mixture was placed in a boiling water bath for 2 minutes then chilled on ice. 0.1 ml of Cobra venom suspension (Sigma Chemical Company, St. Louis, Missouri) was added and the mixture incubated at 30°C for 10 minutes, then chilled on ice. The final product, [³H] guanosine, was separated from the other radiolabeled

material by passing the mixture through a Dowex 50W-X4 column (0.5 x 1.0 cm; 200-400 mesh). The resin was washed with 4 ml of 0.05 M Tris-Cl buffer, pH 7.4, and the [³H] guanosine eluted with 4 ml of 2 M ammonium chloride.

Blood ethanol determination. Blood was obtained after decapitation from all animals treated with ethanol. The blood was deproteinized with 3.4 percent perchloric acid, centrifuged at 1000 x g for 5 minutes and the ethanol quantitated using the Calbiochem Ethanol Stat-Pack.

RESULTS

Effect of acute ethanol treatment. A single dose of ethanol (6 g/kg) substantially reduced c-GMP levels in most areas of the brain studied 2 hours after administration (Table 1). The greatest depletion was observed in the cerebellum where c-GMP levels were down by 80 percent. In other areas that were affected c-GMP levels decreased 40-60 percent. The time course of this response was examined in the cerebellum, caudate nucleus and cerebral cortex. The maximum effect was observed 1 hour after administration of ethanol. The cerebellum again showed the greatest depletion with 95 percent of the c-GMP lost (Figure 1). Cerebral c-GMP was reduced 80 percent and striatal c-GMP 60 percent (Figure 2). c-GMP levels slowly returned to control values in all three areas as blood ethanol was eliminated.

Effect of ethanol dependence. When ethanol-dependent rats were euthanized while still intoxicated c-GMP levels were reduced in all areas of the brain (Table 1). With the exception of the cerebellum and the brain stem the magnitude of the depletion was similar to that obtained after a single dose of ethanol at similar blood ethanol concentrations. In the cerebellum and the brain stem, however, although c-GMP levels were lower than controls, the values obtained were significantly higher than found for the acutely treated animals at similar blood ethanol concentrations (2 hours) (Table 1) and higher than would be predicted from Figure 1.

Table 1. Rat Brain c-GMP Levels* in Discrete Areas After Acute and Chronic Treatment with Ethanol

	Controls	Acute Intoxicated†	Chronic Intoxicated†	Withdrawal Syndrome
Cerebellum	4.05 ± 0.141	0.47 ± 0.045‡	1.24 ± 0.056‡§	3.35 ± 0.332
Brain Stem	0.78 ± 0.091	0.31 ± 0.039‡	0.51 ± 0.083‡§	0.67 ± 0.034
Thalamus	0.16 ± 0.016	0.09 ± 0.007‡	0.10 ± 0.009‡	0.16 ± 0.020
Hippocampus	0.18 ± 0.033	0.14 ± 0.030	0.09 ± 0.015‡	0.20 ± 0.015
Caudate Nucleus	0.26 ± 0.032	0.12 ± 0.017‡	0.09 ± 0.005‡	0.23 ± 0.042
Cerebral Cortex	0.46 ± 0.067	0.21 ± 0.022‡	0.24 ± 0.010‡	0.39 ± 0.061

* c-GMP levels are expressed in pmoles/mg of protein ± S. E. Each group consisted of 5-10 rats.

† Blood ethanol levels in acute intoxicated animals were 373 ± 9.8 mg/dl, while in chronic animals blood ethanol levels were 384 ± 22.9 mg/dl.

‡ Significantly different from control (p < 0.05).

§ Significantly different from acute intoxicated (p < 0.05).

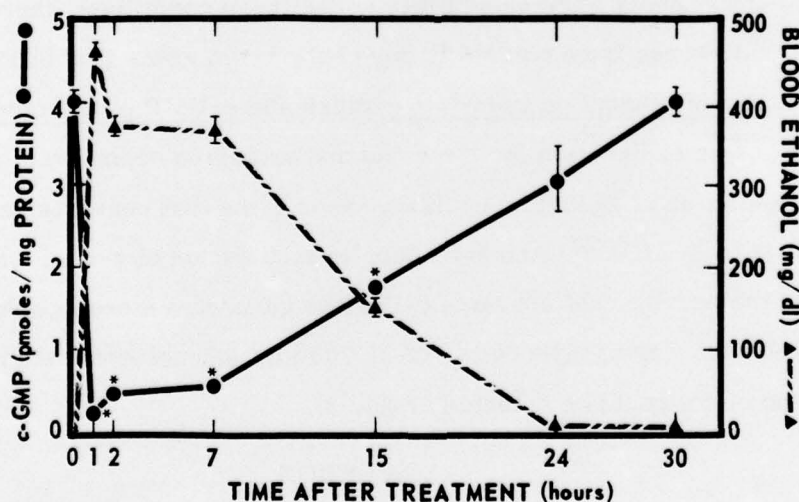


Figure 1. Cerebellar c-GMP levels after a single dose of ethanol (6 g/kg, p.o.). Each point represents the mean ± S. E. of six animals.

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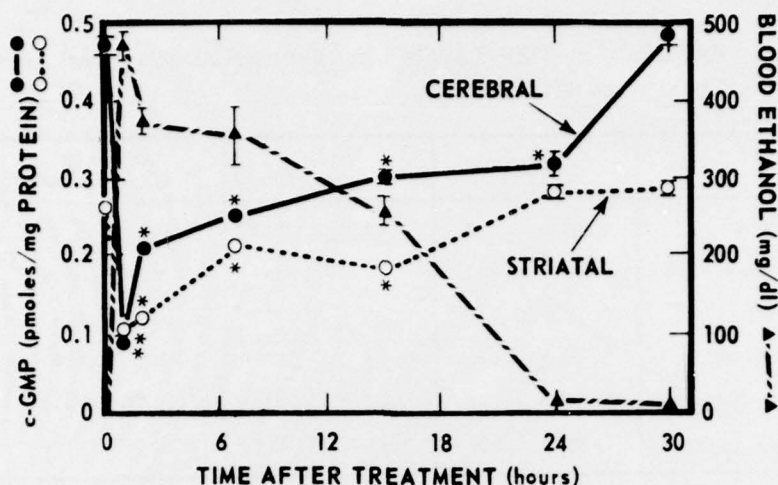


Figure 2. Cerebral and striatal c-GMP levels after a single dose of ethanol (6 g/kg, p.o.). Each point represents the mean \pm S. E. of six animals.

When blood ethanol was eliminated from ethanol-dependent rats, a withdrawal syndrome developed exhibiting signs of hyperexcitability, tremors, rigidity and convulsions similar to those described previously in the rat.³⁵ When c-GMP levels were determined under these conditions, there was no significant difference from control in any of the brain areas (Table 1).

Effect of ethanol on guanylate cyclase and c-GMP phosphodiesterase activity. One explanation for the rapid and extensive depletion of c-GMP could be the inhibition of guanylate cyclase, the enzyme that converts guanosine triphosphate to c-GMP.²¹ Another could be stimulation of c-GMP phosphodiesterase, the enzyme that converts c-GMP to guanosine monophosphate. When either enzyme was assayed with 100 or 200 mM ethanol under standard conditions, no effect could be detected (Table 2).

DISCUSSION

The results of this study clearly show that the presence of ethanol in the body is associated with a rapid and extensive depletion of c-GMP all over the brain, both after acute and chronic treatment. This effect can be observed at

Table 2. Guanylate Cyclase and c-GMP Phosphodiesterase Activity in the Presence of Ethanol In Vitro*

	Guanylate Cyclase (pmol/mg protein/min \pm S. E.)	Phosphodiesterase (nmol/mg protein/min \pm S. E.)
Control	12.9 \pm 0.22	4.2 \pm 0.24
Ethanol		
100 mM	11.8 \pm 1.22	4.3 \pm 0.33
200 mM	13.0 \pm 1.60	4.3 \pm 0.37

* Each group consisted of 7-8 animals.

low blood ethanol concentrations and is significant at concentrations encountered after moderate drinking of alcoholic beverages (50-150 mg/dl), suggesting a role of c-GMP depletion in ethanol intoxication.

Ethanol intoxication is expressed by two general responses: sedation and ataxia. Sedation is the first sign of intoxication observed and is presumably related to depression of cortical neurons and the reticular formation. Ataxia, on the other hand results from dysfunction of the cerebellum.⁴ c-GMP has been reported to be associated with excitatory responses in the brain.^{50,52} Therefore, alterations in c-GMP might be at least a biochemical concomitant of ethanol intoxication.

Evidence has shown that the mesencephalic reticular formation is responsible for arousal^{40,47} and that the reticular formation is quite sensitive to depression by ethanol.²² Because of the high concentration of c-GMP in the brain stem and its substantial depletion after ethanol administration, a disruption of brain stem function by ethanol should be considered.

The specific cellular location of c-GMP in the brain stem is unknown. However, because c-GMP has been implicated in cholinergic transmission,^{29,30,32,52} there may be a correlation between the reported effects of ethanol on ACh release and ethanol-induced c-GMP depletion. Ethanol reduces ACh release as much as 60 percent in a dose-dependent manner in both

the cerebral cortex and the mesencephalic reticular formation.¹⁵ If cholinergic activity is a modulator of c-GMP levels in the brain stem, the decreased ACh-receptor interaction could result in a decline in c-GMP.

The cerebellum functions as a regulator of body movement as well as posture and tonus. The Purkinje cell, a major cell type in the cerebellum, assists in this regulation by providing inhibitory input to other areas of the brain. Since insufficient excitatory input to Purkinje cells can lead to ataxia⁴ and since Purkinje cells are quite sensitive to inhibition by ethanol,¹⁴ cerebellar dysfunction might be the mechanism of action in ethanol-induced ataxia.

c-GMP is found in its highest concentrations in the cerebellum³⁷ and appears to be localized, on the basis of studies using mutant mice deficient in different cell types, predominately in Purkinje cells.³⁹ If c-GMP is also an intermediate in Purkinje cell activation, the depletion of c-GMP by ethanol would further support a role of the cerebellum in ethanol-induced ataxia. Our data do not indicate that either inhibition of guanylate cyclase or stimulation of c-GMP phosphodiesterase is responsible for the observed c-GMP depletion. In the cerebellum Purkinje cells are consistently excited by glutamate,^{7,50} which also stimulates c-GMP accumulation in cerebellar brain slices.¹⁶ Ethanol has been reported to reduce glutamate levels in the cerebellum by 15-20 percent.⁵⁴ Although this effect is small, the result could be an inhibition of glutamate release, which can be produced *in vitro* with high concentrations of ethanol.⁶ Therefore, the ethanol-induced cerebellar depletion could possibly be explained, at least in part, by alterations in the glutamate system.

Calcium is known to be an important modulator of nervous activity.¹ During depolarization calcium is taken up into brain slices^{8,51} and synaptosomes.⁵³ Recent studies of the properties of guanylate cyclase have suggested that calcium may regulate guanylate cyclase activity.⁴² Calcium was shown to stimulate guanylate cyclase sixfold in the presence of low concentrations of manganese. Also, the ability of depolarizing agents to induce c-GMP accumulation in cerebellar brain slices is dependent on calcium.¹⁷ If calcium influx is important in

regulatory c-GMP levels, its inhibition could result in a reduction in c-GMP. Ethanol has been reported to block inward calcium currents in *Aplysia* neurons² and enhance calcium binding to erythrocyte ghost membranes.⁴⁸ If ethanol restricts calcium flux, one result might be a reduction in c-GMP levels.

Chronic use of many depressant drugs can lead to the development of tolerance. If a biochemical change occurs along with the intoxication produced by a single dose of the drug, chronic administration should produce not only behavioral tolerance, but also biochemical tolerance, if the parameter under consideration is to be related to intoxication. We found in our experiments that chronic administration of ethanol over 4 days produced significantly less depletion of c-GMP in the cerebellum and brain stem than found after a single dose at equivalent blood ethanol concentrations. Since a significant degree of tolerance is attained after this treatment regimen,³⁶ the reduced intoxicating properties of a given blood ethanol concentration might be related to the diminished ability of ethanol to deplete c-GMP.

Alterations in the c-GMP system may be related to some of the aspects of the ethanol withdrawal syndrome. As discussed earlier, inhibition of Purkinje cell activity can lead to ataxia. There is also evidence that hyperactivity of Purkinje cells can result in a tremulous state. Harmaline produces tremor by stimulating climbing fibers which synapse on Purkinje cells³³ in a manner resembling activation of climbing fibers.¹² Harmaline also increases cerebellar c-GMP levels,³⁸ presumably in Purkinje cells. Benzodiazepines antagonize harmaline-induced tremors and c-GMP accumulation.³⁸ These drugs have also been used in the treatment of the ethanol withdrawal syndrome in man⁴⁹ and will antagonize the tremors observed in ethanol-dependent rats (Majchrowicz and Hunt, unpublished observation). Although we found no alterations in c-GMP levels during the withdrawal syndrome, the likely reduction of c-GMP throughout the 4-day period of induction of physical dependence might activate some compensatory mechanism to overcome the depressant effect of ethanol.

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