cent with an average of 6.7 ± 4.3 percent S.D. Similarly, there was no significant difference in the uptake of the other nucleosides by the various groups. Fewer cells from the vitamin B_{12} deficiency group survived the 3-day PHA cultures (70 percent), but this was taken into account when making the cell lysate for enzyme assay.

Vitamin B_{12} deficiency was established by determining the level of vitamin B_{12} in serum (*10*), by the characteristic appearance of the peripheral blood and by the megaloblastic appearance of the bone marrow. Pernicious anemia was confirmed by the Schilling test (*11*). We studied two patients with pernicious anemia during relapse and during vitamin B_{12}–induced remission. The other six patients had nutritional vitamin B_{12} deficiencies. As controls, we studied four patients with pernicious anemia (during remission) and four patients with nutritional folic acid deficiencies. We based our diagnosis of folic acid deficiency on low values of serum folic acid (*12*) but normal values of vitamin B_{12}, and the presence of macrocytes and neutrophils with hypersegmented nuclei in the peripheral blood smear. We performed bone marrow aspirations in two patients and found them to be megaloblastic. There were 15 normal individuals as additional controls.

The results of the thymidylate synthetase assay are shown in Table 1. The lowest value of enzyme activity from subjects in the pernicious anemia, in remission, folic acid deficiency, and normal groups is higher than even the highest value from subjects in the vitamin B_{12} deficiency in relapse group. The difference is significant at $P < .01$ [Wilcoxon rank sum test (*13*)].

There has been no prior evidence of vitamin B_{12} participation in the thymidylate synthetase reaction (*14*); the vitamin was not detected in highly purified synthetase preparations, and its addition to such preparations did not affect their activities. We now suggest that vitamin B_{12} is involved in the synthesis of thymidylate synthetase. The exact nature of this involvement is not known. This action of vitamin B_{12} in the megaloblastic process also clarifies the relationship between folic acid and vitamin B_{12} in megaloblastic anemia. Folic acid acts as a coenzyme for thymidylate synthetase in the de novo synthesis of DNA (*15*), whereas vitamin B_{12}, as indicated in this report, is involved in the synthesis of this enzyme. Therefore, vitamin B_{12} deficiency leads to a decreased synthesis and, consequently, activity of thymidylate synthetase. This results in an accumulation of unused 5-methyltetrahydrofolic acid. This "methyl trap" phenomenon has been observed and investigated in patients with vitamin B_{12} deficiency (*16*).

Inherited defects of vitamin B_{12} metabolism which cause abnormalities in methylmalonate catabolism or in the conversion of homocysteine to methionine have not been associated with megaloblastic development (*17*), and therefore these actions of the vitamin cannot be held responsible for megaloblastosis.

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Nerve Growth Factor: Relationship to the Cyclic AMP System of Sensory Ganglia

Abstract. *Nerve growth factor and* $N^6,O^{2'}$ *dibutyryl adenosine 3′,5′-monophosphate both stimulate neurite elongation by explanted ganglia. However, the addition of nerve growth factor does not lead to increased amounts of adenosine 3′,5′-monophosphate in intact ganglia, nor does it stimulate adenylate cyclase activity in broken ganglia cells.*

Both $N^6,O^{2'}$ dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) and nerve growth factor (NGF) stimulate the in vitro elongation of neurites from embryonic sensory and fetal trigeminal ganglia (*1–3*). When certain hormones bind to their target cells there is an increase in the intracellular concentration of adenosine 3′,5′-monophosphate (cyclic AMP) and cyclic AMP is said to be the "second messenger" for those hormones (*4*). It has been suggested that since both NGF and dibutyryl cyclic AMP stimulate neurite elongation, cyclic AMP might be the second messenger for NGF (*2, 3*).

Even though dibutyryl cyclic AMP and NGF both stimulate similar mor-

Table 1. Effect of NGF on the cyclic AMP content of embryonic sensory ganglia. Each value of cyclic AMP content per ganglion is the mean and standard deviation of the number of separate experiments shown in parentheses. Each determination included in the mean for that time interval was the result obtained from triplicate cyclic AMP assays.

Incubation time	Cyclic AMP content per ganglion	
	Control (pmole of cyclic AMP)	NGF (pmole of cyclic AMP)
15 minutes	0.77 ± 0.17 (2)	0.77 ± 0.07 (3)
30 minutes	0.77 ± 0.20 (5)	0.70 ± 0.21 (7)
1 hour	0.71 ± 0.18 (7)	0.70 ± 0.14 (7)
3 hours	0.95 (1)	0.75 (1)
6 hours	0.46 (1)	0.42 (1)
18 hours	0.66 ± 0.01 (2)	0.69 ± 0.10 (2)

phological changes in explanted ganglia, certain differences exist. For example, NGF elicits the elongation of substantially more neurites than does dibutyryl cyclic AMP (*5*). Moreover NGF, unlike dibutyryl cyclic AMP, stimulates de novo synthesis of the microtubule subunit protein (*6*).

Robison *et al.* (*4*) have proposed that cyclic AMP may be considered a second messenger for a hormone if it satisfies the following criteria: (i) the action of the hormone is mimicked by either exogenous cyclic AMP or dibutyryl cyclic AMP, (ii) addition of the hormone leads to increased concentrations of cyclic AMP in intact tissues, (iii) the hormone activates adenylate cyclase in broken cell preparations, and (iv) the action of the hormone is potentiated by inhibitors of cyclic nucleotide phosphodiesterase. We have measured the effect of NGF upon both the cyclic AMP content and the adenylate cyclase activity of embryonic sensory ganglia. Results suggest that cyclic AMP does not mediate the action of NGF. The similar morphological effects of these two agents may in part be due to different effects on the microtubule system of sensory ganglia.

To determine the effect of NGF on the amount of cyclic AMP in ganglia, 100 dorsal root ganglia were removed from the lumbar regions of 9-day-old chick embryos. The ganglia were washed with Earle's salt solution, and then randomly divided into experimental and control groups of 25 each. Ganglia were placed in small plastic petri dishes containing 2 ml of Eagle's minimum essential medium supplemented with 1 m*M* theophylline (*6*) and the experimental groups were treated with NGF (final concentration of 3 units per milliliter of medium, isolated from male mouse submaxillary glands, Burroughs-Wellcome Laboratories). The morphologic effects of NGF were assessed by incubating ganglia that were grown on collagen-coated cover slips or plastic petri dishes (*6*) for 18 hours at 37°C. Preparations were incubated at 37°C in a CO_2-conditioned, humidified incubator. Ganglia were then homogenized in 0.5 ml of ice-cold trichloroacetic acid (10 percent), and the homogenates were cleared of debris by centrifugation at 15,000 rev/min for 5 minutes. The supernatants were further acidified with 0.1 ml of 1*N* HCl, and 10 pmole of cyclic [^{3}H]AMP (25 c/mole, New England Nuclear) was added to each sample. These samples were extracted five times with 2 ml of ether to remove the trichloroacetic acid, and the aqueous phase was lyophilized. The residue was redissolved in 200 μl of 50 m*M* sodium acetate, *p*H 4, and the cyclic AMP content of three 50-μl portions was measured (*7*). The cyclic AMP–binding protein was prepared from bovine brain (*8*) and stored at −20°C. For each experiment, a standard curve was constructed from triplicate measurements of four known samples of cyclic AMP (Sigma). Radioactivity was measured with a Packard spectrometer, and a scintillation solvent containing toluene, Triton X-100 (2 : 1), and fluors.

The NGF had virtually no effect on the cyclic AMP content of embryonic sensory ganglia (Table 1). In other cyclic AMP–hormone systems, increases in cellular cyclic AMP under hormone stimulation are far greater than indicated by the standard deviations in Table 1 (*4*). We were unable to enhance the morphological effects of suboptimal amounts of NGF by adding 0.5 m*M* theophylline to the culture medium of sensory ganglia that were grown on collagen-coated cover slips.

These results are consistent with the finding that NGF has no effect upon the adenylate cyclase activity of broken cell preparations of dorsal root ganglia. Adenylate cyclase activity was measured in control, NGF-treated, and fluoride-stimulated ganglion preparations. We used NGF in concentrations that were 20 and 200 times that necessary to produce observable neurite outgrowth from explanted sensory ganglia. These concentrations of NGF had no effect on the adenylate cyclase activity in our preparations, although sodium fluoride produced a characteristic stimulation of enzyme activity (*4*) (Table 2).

Table 2. Effect of NGF on the adenylate cyclase activity of broken cell preparations of dorsal root ganglia. Ganglia were removed from 9-day-old chick embroys, washed three times with 50 m*M* tris(hydroxymethyl)-aminomethane (tris) · HCl, 100 m*M* sodium EDTA, *p*H 7.5, and then homogenized in 0.5 ml of the same buffer with ten strokes of a motor-driven Potter homogenizer. For each assay, 25 μl of homogenate was mixed with 25 μl of an incubation solution containing 1 μc of [α-^{32}P]adenosine triphosphate (ATP) (International Chemical and Nuclear Corporation), 25 m*M* tris · HCl, 10 m*M* $MgCl_2$, 0.1 m*M* cyclic AMP, 1.5 m*M* ATP, 5 m*M* phosphoenolpyruvate, and 40 μg per milliliter of phosphoenolpyruvate kinase. These preparations were treated either with tris · HCl buffer (control), NGF, or sodium fluoride. Samples were incubated at 37°C for 20 minutes, and the reaction was terminated by the addition of 1 ml of a solution containing 50 μg of cyclic AMP and 100 μg of ATP. Cyclic [^{3}H]AMP was also added to permit calculation of recovery of cyclic [^{32}P]-AMP. Cyclic AMP formation was determined as described by Lefkowitz *et al.* (*15*). The data are expressed as the mean and standard deviation of triplicate determinations.

Test material	Amount	Cyclic [^{32}P]-AMP formation (pmole per ganglion)
Control		4.2 ± 0.5
NGF	20 unit/ml	4.4 ± 0.6
NGF	200 unit/ml	3.9 ± 0.7
NaF	10 m*M*	31.7 ± 0.5

Several lines of evidence indicate that one way to stimulate nerve growth is to increase the availability of microtubule protein for incorporation into the lengthening axon. For example, there is a proliferation of microtubules in axoplasm of developing and regenerating nerves (*9*); developing mouse brain has a much higher content of microtubule protein than adult brain (*10*); and agents that disrupt normal microtubule structure not only prevent continued nerve growth but also initiate axon retraction (*11*).

There are at least two different mechanisms by which more microtubule polymer may be made available to support nerve growth. One is to stimulate synthesis of the microtubule subunit protein, and the other is to promote the assembly of preformed subunits. It has been suggested that cyclic AMP stimulates neurite extension by enhancing the assembly of microtubule subunits into polymer (*2, 12*). Consistent with this idea, dibutyryl cyclic AMP does not stimulate the synthesis of microtubule subunit protein in embryonic sensory ganglia (*6*). Further, the disruption of microtubules by both low temperature and colchicine appears to be antagonized by dibutyryl cyclic AMP. Thus the dibutyryl derivative may counteract these treatments by promoting the assembly of microtubule subunits into polymer (*12, 13*). NGF probably increases the cellular level of microtubule polymers by a different mechanism, since it appears to stimulate synthesis of the microtubule subunit protein (*6*).

Roisen *et al.* (*5*) have suggested that cyclic AMP is the second messenger for NGF since both agents reverse the inhibitory effects of Colcemid on neurite elongation. However, we believe that there is another interpretation of these results. Colcemid binds to the microtubule subunit, and subunits bound to colchicine (a related drug) fail to polymerize normally into microtubules (*14*). Thus neurons treated with Colcemid are depleted of subunits that can be polymerized into the microtubules necessary to support nerve

growth. One way to reverse the effects of Colcemid would be to stimulate de novo synthesis of the microtubule subunit, and thereby decrease the effect of Colcemid on the subunit pool. Another way might be to promote the polymerization of those subunits that are not bound to Colcemid. Consequently, the fact that NGF and dibutyryl cyclic AMP counteract the effects of Colcemid need not imply that both compounds act through the same mechanism.

The evidence presented above indicates that NGF does not alter intracellular cyclic AMP levels, that it does not stimulate adenyl cyclase activity, and that its morphological effects are not enhanced by theophylline. Thus, we infer that cyclic AMP does not mediate the action of NGF.

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Gamma-Aminobutyric Acid Antagonism in Visual Cortex: Different Effects on Simple, Complex, and Hypercomplex Neurons

Abstract. *Intravenous bicuculline was used to examine how removing gamma-aminobutyric acid–mediated inhibition affects the visual response properties of single cortical neurons. Simple neurons were depressed and complex neurons showed increase in the vigor and range of responses. Hypercomplex cells were no longer inhibited by elongated stimuli. The results are consistent with present evidence concerning the origin and distribution of inhibitory connections within the cortex.*

A number of lines of evidence implicate γ-aminobutyric acid (GABA) as an inhibitory transmitter in the mammalian central nervous system. This evidence is based on the distribution and synthesis of this amino acid in various brain regions (*1*), its release from nervous tissue in relation to the activity of inhibitory neurons (*2*), and the observation that microiontophoretically applied GABA hyperpolarizes neurons in the brainstem (*3*) and cerebral cortex (*4*) by membrane mechanisms which are indistinguishable from those occurring during synaptic inhibition.

The most recent line of evidence comes from the finding that the plant alkaloid bicuculline, which causes epileptiform convulsions of cortical origin, is a potent and specific antagonist of GABA-induced inhibition (*5*). Bicuculline has no effect on the strychnine-sensitive inhibition thought to be mediated at other sites, particularly in the spinal cord, by the second putative inhibitory transmitter, glycine (*6*).

We sought to examine the role played by GABA in the cat's primary visual cortex, where the functional characteristics of single neurons are known in some detail (*7, 8*) and where inhibitory mechanisms have been proposed to account for some of these characteristics (*9*). Since intravenously administered bicuculline has been shown to be a potent and reversible antagonist of GABA in the cerebral cortex, we used this method to examine how removal of GABA-induced inhibition affects the highly specific responses of visual cortical neurons to patterned visual stimuli. Bicuculline produced dramatic effects on neurons with complex fields, including marked increases in spontaneous and evoked activity, loss of specificity for the orientation or direction of a moving line, and the appearance of plottable "on" and "off" areas like those of incoming lateral geniculate fibers. Hypercomplex cells, whose response to a line is normally inhibited if the length exceeds some optimum (*8*), lost their inhibitory end zones. Simple cells, whose fields can be mapped into "on" or "off" areas with flashing stimuli, showed only mild effects, such as a depression of responsiveness or a slight shift in preferred orientation.

Single neurons were studied in the striate cortex (area 17) of ten normal adult cats weighing between 2 and 3 kg. The preparation was routine (*7*). Initial surgery was carried out under Fluothane, and anesthesia was maintained during recording with nitrous oxide. Eye movements were prevented by a continuous intravenous infusion of Flaxedil and *d*-tubocurarine (*10*). Stimuli were held by the arm of an X-Y recorder in the object plane of an overhead projector which focused them on a screen 57 cm from the animal. The X-Y recorder could be controlled by hand for initial receptive field plotting or by a computer that presented a series of sweeps across the receptive field in chosen directions and stored all spike data for later analysis.

Before administering bicuculline to each cell we tried to collect as many data as possible about its functional characteristics. Receptive field plots with flashing spots and lines and averaged responses to various directions of movement, orientation, and line lengths were obtained and usually enabled a cell to be classified according to Hubel and Wiesel's (*8*) three categories, simple, complex, and hypercomplex. After categorization of the cell, an intravenous dose of bicuculline (0.2 mg/kg) was slowly given and all tests were repeated until the cell's properties had returned to control or until the cell was lost (*11*).

All cells studied were affected by the drug. Based on our observations of 13 simple, 7 complex, and 2 hypercomplex cells, these effects appear to be consistent within a given class of cell, and reversible (45 minutes to 1 hour). Examples are shown in Fig. 1.

Simple cells were the least affected. Evoked activity was depressed, and in