treated with thymopoietin or ubiquitin and in untreated *nu/nu* mice suffering from hepatitis. This confirms previous indications that the presence of cells bearing TL and Thy-1 is not in itself evidence of thymic epithelial function in *nu/nu* mice, because these mice have normal numbers of prothymocytes which can be induced to differentiate by nonthymic agents such as ubiquitin not only in vitro but also in vivo. In view of the diversity of agents capable of inducing T cells in the KB assay (*13, 14*), one can only speculate as to the agent or agents responsible for inducing TL^+ and Thy-1^+ cells in the *nu/nu* mice with hepatitis. Ubiquitin must normally be inaccessible to T precursor cells because, although it is present in *nu/nu* tissues (*12*), healthy *nu/nu* mice lack demonstrable numbers of TL^+ or Thy-1^+ cells. But hepatic necrosis might lead to its release in quantities sufficient to induce many T-cell precursors. In the case of bacterial infection, endotoxin (another agent that is active in the KB assay) (*14*) could be responsible.

2) The rise in CR^+ cell numbers in mice treated with thymopoietin requires consideration because thymopoietin, unlike ubiquitin, does not induce CR^+ B cells in the KB assay. However, since the number of CR^+ cells in healthy *nu/nu* mice is reduced, the effect of thymopoietin in raising their numbers in vivo may be secondary to its action in inducing newly differentiated T cells that in turn can interact with the B-cell population (*22*).

3) TL^+ cells are usually restricted to the thymus, although for reasons given above it seems probable that TL^+ cells can in some circumstances be induced at other sites. Equal numbers of TL^+ and Thy-1^+ cells were found in the spleens of healthy treated and sick untreated *nu/nu* mice. Because the phenotype $TL^+Thy\text{-}1^-$ is unknown, we assume these all to be TL^+ Thy-1^+ cells such as are normally found in the thymus, which is in keeping with results in the KB assay in vitro. The finding that lymph node cells of the same mice included many Thy-1^+ cells and few TL^+ cells implies that cells of TL^-Thy-1^+ phenotype were being generated. This is the phenotype of the medullary thymocyte and peripheral T cell; thus, in vivo induction of prothymocyte differentiation in the three groups of *nu/nu* mice gave rise to cells not only of the thymic cortical type (TL^+Thy-1^+) generated in the KB assay in vitro but also of a later TL^-Thy-1^+ maturation stage. However, this gives no indication as to the function of the induced T cells, for the TL^-Thy-1^+ phenotype is common to all functional subclasses of T cells and may characterize intermediary differentiative stages between thymocytes and functional T cells. Prothymocytes are probably scarce or absent in lymph nodes, although this needs further study; it seems likely, therefore, that the TL^-Thy-1^+ lymph node cells were maturing migrants from the spleen rather than T cells induced in situ.

4) Among the *nu/nu* mice with hepatitis were some born of *nu/nu* × *nu/nu* matings, and 2 to 17 percent of spleen cells in these progeny were Thy-1^+; the induction of these cells cannot be ascribed to passage of thymic factors from the mother.

Our studies show that healthy *nu/nu* mice of our colony had few or no cells bearing T-cell markers and that T cells could be induced in vivo in these healthy mice by thymopoietin or ubiquitin injections. Our results indicating that *nu/nu* mice suffering from overt hepatitis had many cells bearing T-cell markers emphasize that the first recognizable step in T-cell differentiation (acquisition of surface markers) in *nu/nu* mice can be related to disease rather than to thymic mechanisms. There is no need to invoke the concept of a thymus briefly functioning during embryogenesis, or maternal transfer of thymic factors. In current studies, designed in accordance with the results reported here, we do not find a perceptible background of Thy-1^+ T cells in *nu/nu* mice reared under germfree conditions, although Thy-1^+ cells can be induced in these mice by thymopoietin and ubiquitin.

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Cyclic Guanosine Monophosphate and Cellular Growth

Abstract. *The addition of serum to nongrowing cells decreases cyclic guanosine monophosphate and cyclic adenosine monophosphate levels and promotes cell growth.*

Cultured fibroblastic cells contain two cyclic nucleotides—cyclic adenosine monophosphate (AMP) and cyclic guanosine monophosphate (GMP). Cyclic AMP has the capacity to inhibit the growth of fibroblastic cells (*1*). It also regulates cell shape, motility, adhesiveness to substratum, and agglutinability by plant lectins (*1*). The role of cyclic GMP is unclear. One proposal that has received considerable attention is that cyclic GMP antagonizes the actions of cyclic AMP and promotes cellular growth (*2, 3*).

To gain more information about the role of cyclic GMP in cultured cells, we have measured concentrations of cyclic GMP in growing and nongrowing BALB 3T3 cells and in cells that have been stimulated to grow by the addition of serum. We find that cyclic GMP rises as cell growth slows and that the addition of serum to resting cells causes a prompt fall in both cyclic GMP and cyclic AMP. These data fail to support the proposal that cyclic GMP acts to promote cellular growth and raises the possibility that both cyclic AMP and cyclic GMP act as growth inhibitors.

BALB 3T3 cells clone B were grown in Dulbecco's modification of Eagle's medium containing either 10 percent calf serum or 5 percent calf plasma (Colorado Serum Company). The cells were propagated as described (*4*), except that the 37°C incubator was kept inside a 37°C warm room so that cells could be removed at frequent intervals without lowering their temper-

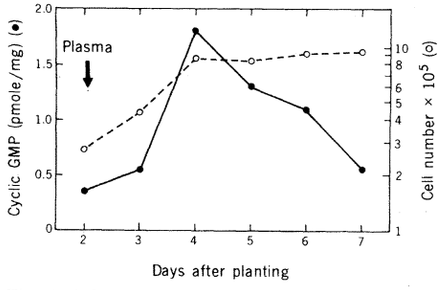
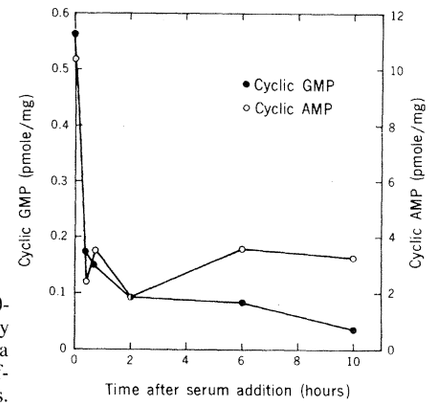


Fig. 1 (left). Cells (1×10^5) were planted on 100-mm dishes in medium with 10 percent serum on day 0; the medium was replaced with 5 percent plasma on day 2 and day 4. Fig. 2 (right). Long-term effects of serum on cyclic nucleotide concentrations. The cells were planted at 1.5×10^5 per 100-mm dish on day 0, transferred to medium containing 5 percent plasma on day 3, refed on day 5, and used on day 7. Each dish contained 0.48 mg of protein. (●) Cyclic GMP; (○) cyclic AMP.



ature. The nucleotides were extracted with 5 percent trichloroacetic acid containing cyclic [^{3}H]AMP and cyclic [^{3}H]GMP to monitor recoveries. After removal of the precipitate by centrifugation, the extracts were neutralized with 5*N* NaOH, and cyclic AMP and cyclic GMP were adsorbed on a Dowex-1–formate column (0.8 by 2.5 cm). After a washing with 10 ml of H_2O, cyclic AMP was eluted with 8 ml of 2*N* HCOOH, and cyclic GMP was eluted with 10 ml of 4*N* HCOOH. The samples were lyophilized, and the cyclic GMP samples were dissolved in 1 ml of 0.1*N* HCl and further purified on Dowex-50 columns (0.8 by 6.5 cm) and lyophilized (*4*). The cyclic AMP samples were dissolved in 50 m*M* MES buffer [2-(*N*-morpholino)ethanesulfonic acid], *p*H 6.2, and subjected to radioimmunoassay directly. The unknown cyclic GMP samples and standards were dissolved in water, succinylated as described by Cailla *et al.* (*5*), diluted eightfold, and then assayed. Radioimmunoassay reagents were obtained from Collaborative Research.

Plasma containing citrate was heated at 60°C for 60 minutes to remove fibrinogen. After centrifugation, the plasma was dialyzed for 16 hours against ten volumes of phosphate-buffered saline, which was changed two times; the dialyzed plasma was centrifuged and filtered through a 0.45-μm Millipore filter.

Kohler and Lipton (*6*) have found that BALB 3T3 cells grow poorly in medium in which plasma is used in place of serum. Therefore we propagated our cells in serum, changed them to plasma-containing medium to arrest growth, and in some cases subsequently added serum to reinitiate growth. This experimental system is convenient for manipulating cellular growth, and we used it to measure cyclic GMP in growing and nongrowing cells.

BALB 3T3 cells were planted at a density of 2000 cells per square centimeter in 100-mm dishes in a medium containing 10 percent calf serum. After 48 hours, the cells were shifted to a medium containing 5 percent plasma (Fig. 1). After transfer to this medium, cell growth continued at a reasonable rate for 2 days and then stopped. Two days after the cells were planted and just before they were transferred to plasma, the cyclic GMP concentration was 0.35 pmole/mg. The concentration rose to 1.8 pmole/mg after 2 days in plasma, and this high value correlated with the time of growth arrest. Then cyclic GMP slowly declined to 0.53 pmole/mg after 6 days.

The gradual rise in cyclic GMP concentration in these cells after transfer to plasma suggests that growth factors present in serum may be responsible for the low cyclic GMP concentrations in cells growing in serum. To test this idea, we maintained cells in 10 ml of medium containing 5 percent plasma (by volume) for 3 to 5 days; the cells were stimulated to grow by the addition of 2 ml of unheated serum (Table 1). Cyclic GMP and cyclic AMP fell promptly after the addition of serum. In experiment 1 (Table 1), cells were maintained for 3 days in plasma-containing medium, and the cell number increased from 9×10^3 to 18×10^3 per square centimeter 36 hours after 2 ml of serum was added. The levels of both nucleotides had fallen after only 10 minutes and continued to fall for up to 40 minutes; cyclic GMP fell from 2.1 to 0.45 pmole/mg, and cyclic AMP from 20.5 to 3 pmole/mg. Whether or not dexamethasone (1 μg/ml) was added to the plasma, we found that serum lowered the cyclic GMP and cyclic AMP concentrations. Before the addition of serum, the cyclic GMP varied from 0.51 to 2.1 pmole/mg and the cyclic AMP from 10.4 to 23.8 pmole/mg. The reason for the variation in these initial values is unclear, but some of the variation is related to the number of days the cells had been maintained in plasma before the addition of serum (Fig. 1). With prolonged exposure to plasma-containing medium some of the cells may have progressed to the G2 phase of the cell cycle (*7*).

The fall in cyclic GMP after serum addition persists for longer than 40 minutes. In the experiment shown in Fig. 2, the cells were studied for up to 10 hours after serum was added. The levels of cyclic GMP and cyclic AMP remained low for this entire period.

Our data indicate that cyclic GMP concentrations are relatively low in growing cells, rise when cellular growth is slowed or arrested by transfer to plasma-containing medium, and fall when cells are stimulated to grow by the addition of serum. This pattern is similar to that reported for cyclic AMP (*1*), and raises the possibility that cyclic GMP and cyclic AMP both can function to inhibit cell growth. We have observed some inhibition of the growth of 3T3 cells when they have been treated with a high concentration of 8-Br cyclic GMP (2 m*M*) (*8*). In contrast, Seifert and Rudland reported that dibutyryl cyclic GMP stimulates the incorporation of [^{3}H]thymidine into DNA in serum-starved 3T3 cells (*3*). Since the stimulation observed was small (10 to 20 percent) and no increase in cell number was reported, it seems possible that the increased labeling of DNA was due to a change in the intracellular thymidine pool and was not a reflection of enhanced cell growth.

Table 1. The effect of addition of serum on cyclic GMP and cyclic AMP concentrations (picomoles per milligram).

Experiment	Cyclic GMP: minutes after serum addition				Cyclic AMP: minutes after serum addition				Cell No. (10^3 cell/cm^2)
	0	10	20	40	Initial 0	10	20	40	
1	2.1	1.1	0.9	0.45	20.5	11	9	3	9
2*	1.6	0.55			15	7.5			9
3	0.85	0.25			18	7.5			20
4*	0.51		0.12		10.6	4.0			13
5	0.55		0.17	0.14	10.4		2.4	3.6	24
6*	1.6			0.42	23.8			9.8	13

*Dexamethasone acetate at final concentration of 1 μg/ml was added with plasma.

The rise in cyclic AMP in NRK fibroblasts undergoing density dependent inhibition of growth has been found to be associated with an increase in adenylate cyclase activity (*9*). The rise in cyclic GMP in BALB 3T3 cells is likewise associated with increased guanylate cyclase activity (*10*), and this rise could be responsible for the increase in cyclic GMP. We have also measured cyclic GMP and guanylate cyclase activity in transformed NRK and transformed BALB 3T3 cells growing in serum-containing medium. Many transformed 3T3 and NRK cells show greatly diminished guanylate cyclase activity (*10*). Also, NRK cells transformed by the Kirsten, Moloney, or Harvey strains of murine sarcoma virus have very low cyclic GMP (*10*). Thus transformed cells can grow rapidly with diminished cyclic GMP levels. Seifert and Rudland reported that the addition of serum to resting serum-starved 3T3 cells causes a transient rise in cyclic GMP (*3*). We have failed to detect any rise in cyclic GMP after addition of serum to cells maintained in plasma. More recently Moens *et al.* reported that cyclic GMP levels do not rise in Swiss 3T3 cells undergoing density dependent inhibition of growth in serum (*11*). Our results cannot be directly compared with those of Seifert and Rudland and Moens *et al.* since there are differences in experimental design and in methodology. Some of these are as follows. (i) They washed their cells prior to extraction; we do not wash cells to prevent possible changes in cyclic nucleotide levels due to this manipulation. (ii) They used direct radioimmunoassay without succinylation; we have been unable to reliably measure cyclic GMP levels without the enhanced sensitivity and specificity of succinylation. (iii) Moens *et al.* used a different strain of 3T3 cells. (iv) Moens *et al.* allowed cells to undergo contact inhibition of growth in serum, whereas we used plasma that is deficient in some of the growth factors found in serum (*5*).

All in all, our data are inconsistent with the proposal that cyclic GMP promotes growth and opposes the action of cyclic AMP (*2, 3*). The role of cyclic GMP in cultured fibroblastic cells remains obscure.

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Independence of "On" and "Off" Responses of Retinal Ganglion Cells

Abstract. *Recordings of action potentials from retinal ganglion cells that are stimulated repetitively demonstrate two properties: (i) variability introduced during the stimulus is not evident in the response that occurs at stimulus offset and (ii) variability in the* ON *response shows a different temporal structure than variability in the dark. Our findings demonstrate that these responses are generated independently.*

There are two responses to every visual stimulus: one after a light is introduced and one after it is withdrawn. During the time that the stimulus is present, the firing rate of a ganglion cell may be either higher or lower than its firing rate was in the dark. After stimulus offset the firing rate is again modified, usually in the direction opposite to the response during the stimulus (*1*). While the ON response is generally considered to reflect the cell's response to the stimulus, there is some controversy about the origin and significance of the OFF response (*2, 3*). Many researchers tacitly assume it to be a reflection of the same process that generates the ON response, and group the two together for analysis (*4*). We have found that the ON and OFF responses are manifestations of two independent processes; in addition, there are separate ON mechanisms that correspond to different parts of the receptive field.

If the same stimulus is repeated on a regular schedule, the number of spikes (action potentials) that are elicited varies from presentation to presentation; we have examined the statistical properties of this variability and its correlation with the variability that is exhibited by the maintained discharge. It is important to realize that these statistics are derived from total numbers of spikes and are not necessarily related to the statistics derived from the variability of interspike intervals.

Table 1. Correlation matrix for 39 stimulus presentations to the center of the receptive field of an off-center unit. The stimulus was a 0.562-mm diameter spot. Correlations are between all pairs of lists of the numbers of spikes in each of the 1-second bins. The mean and variance (σ^2) of the number of spikes in each bin are also presented.

List	1	2	3	4	5
1	1.00	.86	.09	.84	.85
2		1.00	.04	.82	.87
3			1.00	.03	−.11
4				1.00	.80
5					1.00
	Number of spikes in each bin				
Mean	38.7	38.7	24.1	65.6	46.4
σ^2	13.7	13.9	8.9	88.8	42.7

We used platinum-iridium microelectrodes to make extracellular recordings of action potentials from the isolated retinas of goldfish (*Carassius auratus*) (*5*). Data consisted of the total number of action potentials that occurred in each of five successive 1-second bins. (A bin is an interval of time during which spikes are counted.) The stimulus was a flash of deep red monochromatic light (680 nm or longer) coincident with the third bin. This regimen was repeated every 30 seconds and generated five lists, each consisting of the total number of spikes in the corresponding bin for each of at least 30 repetitions of the same stimulus. Lists 1 and 2 correspond to firing before the stimulus and represent the maintained discharge; list 3 is the ON response; and lists 4 and 5 are OFF responses.

We computed a matrix of the correlations between all pairs of lists; a typical matrix (Table 1) was derived for stimulation to the center of an off-center cell (*6*). These correlations express relations between the changes about the means of each of the lists. The most striking feature of this matrix is that there is a uniformly high correlation between any two lists in which there was no stimulus, and a low correlation between list 3 (that included the stimulus) and any other. The failure of the OFF response to show a lower correlation with maintained firing cannot simply be ascribed to a lack of response at OFF; the absolute magnitude of the OFF response (measured as the difference from maintained) in this case is larger than that of the ON response (see means in Table 1).