ably results in greater reproductive success. However, the wide variance of the sixth within a clone suggests that different packing arrangements and growth forms of tubular bryozoan colonies result from nongenetic causes.

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2. The amplitude spectrum is calculated from automatically digitized peripheral points measured in Cartesian coordinates. Each shape is visually centered on a starburst pattern composed of 48 rays spaced at equal angles. The computer program used calculates the center of gravity of each figure and converts the Cartesian values into polar coordinates around the center of gravity.
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5. All the zooids of a colony arise from asexual budding from a single parent, thereby generating a clone.
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10. *Leptotrypella*, three species; *Atactotoechus*, two species; *Loxophragma*, and *Polycylindricus*, one species each.
11. *Leioclema*, three species; *Leptotrypella*, 13 species; *Atactotoechus*, five species; *Loxophragma*, two species.
12. We thank R. Ehrlich, J. Holman, and R. Taggart for their help.

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Adenosine 3′,5′-Monophosphate and N^6-2′-O-Dibutyryl-Adenosine 3′,5′-Monophosphate Transport in Cells

Abstract. *Incubation of Strain L cells in 0.15 millimolar adenosine 3′,5′-monophosphate (cyclic AMP) increase the intracellular level of cyclic AMP more than does incubation in dibutyryl cyclic AMP. The intracellular increase of cyclic AMP is greater with Strain L than with WI-38 cells. Similarly, cell division of Strain L cells is inhibited more by cyclic AMP. The experiments do not support the belief that dibutyryl cyclic AMP is transported better than cyclic AMP.*

In spite of numerous articles which state that N^6-2′-*O*-dibutyryl-adenosine 3′,5′-monophosphate is transported more readily than adenosine 3,′5′-monophosphate (cyclic AMP), there is as yet no direct evidence that supports this conclusion (*1*). The investigations usually cited as evidence for this statement suggest rather than establish this point (*2*). The situation is aptly described by Robison, Butcher, and Sutherland: "There is no direct evidence that the derivatives penetrate cell membranes more rapidly than cyclic AMP, although this may occur and may in some cases be the explanation for their greater potency" (*3*).

The purpose of this investigation was to obtain evidence regarding the transport of these two biologically important compounds in cell cultures.

Strain L (NCTC, clone 929) and WI-38 cells (38th or 39th passage) were used. The cells were determined to be mycoplasma-free by the method of Hayflick (*4*). The cell cultures were maintained in Eagle's minimal essential medium with Hanks' salts. Glutamine (29.2 mg/liter) was added to the medium and the *p*H was adjusted to 7.6 with 0.89*M* bicarbonate. The medium was supplemented with fetal calf serum (10 percent), neomycin (50 mg/liter), and amphotericin B (2 mg/liter).

Inhibition studies of cell cultures by cyclic AMP and dibutyryl cyclic AMP were done in Leighton tubes seeded with 25,000 cells per milliliter, total volume 2 ml. On the second and fourth days of incubation the cells were removed with 0.25 percent trypsin and counted in a Coulter counter.

Adenosine 3′,5′-monophosphate acid (Sigma, lot Nos. 90C-2080, 81C-0220, and 78B-7240), N^6-2′-*O*-dibutyryl-adenosine 3′,5′-monophosphate, monosodium salt (Sigma, lot Nos. 90C-7020 and 91C-7200), and butyric acid were dissolved directly in the culture medium and filtered through a prewashed Millipore filter (0.22 μm size). The butyric acid was redistilled, and the concentration was determined by titration. The medium containing cyclic AMP and dibutyryl cyclic AMP was used the day of preparation because of the phosphodiesterase activity in fetal calf serum.

Cells for the cyclic AMP assay were grown in T-60 flasks seeded with 50,000 cells per milliliter, total volume 20 ml. After incubation, the medium was removed and the cell layer was rinsed six times with ice cold, 0.85 percent sodium chloride. Removal of all extracellular cyclic AMP was indicated by no further decrease in cyclic AMP with additional washes. Along with each experimental T-60 flask, a control flask with no cells but containing medium and cyclic AMP was employed to determine the effectiveness of the washing procedure in removing all cyclic AMP from the glass. Because of the known binding of cyclic AMP to proteins, an additional flask of cells was used for determining the amount of protein-bound cyclic AMP. The cells were frozen and thawed repeatedly to disrupt the cell membranes, and the disrupted cells were washed six times with cold saline. The disrupted cells bound less than 1 pmole of cyclic AMP per million cells. For estimation of percent recovery, 5 ml of 0.5*N* perchloric acid in 25 percent ethanol containing tracer cyclic AMP [0.009 μc of [H^3]cyclic AMP (Amersham/Searle) per milliliter, 24.1 c/mmole] was added to the cell layer. The cells were removed from the glass by gentle scraping and centrifuged at 2000 rev/min for 15 minutes to remove the cell protein precipitate. After neutralization with KOH, the potassium perchlorate was removed by centrifugation, and the supernatant was

Table 1. Intracellular (In.) and extracellular (Ex.) concentrations of cyclic AMP and dibutyryl cyclic AMP in Strain L cells and in WI-38 cells. Values are millimolar and the average of three experiments. The molarity was calculated as millimoles of cyclic AMP per liter of packed cell volume. The cell volume was determined by microhematocrit.

Incubation time (days)	Dibutyryl cyclic AMP (m*M*)			Cyclic AMP (m*M*)		
	In.	Ex.	In./Ex.	In.	Ex.	In./Ex.
			Strain L cells			
0		0.15			0.15	
1	0.07	.14	0.5	1.01	.07	14.4
2	.04	.12	.3	0.14	.03	4.7
3	.01	.11	.1	.09	.02	4.5
4	.005	.09	.06	.10	.02	5.0
			WI-38 cells			
0		0.15			0.15	
1	0.10	.14	0.7	0.10	.03	3.3
2	.17	.10	1.7	.15	.002	75.0
3	.06	.08	0.8	.02	.0008	25.0
4	.05	.07	.07	.02		

chromatographed on AG 1-X2, 200 to 400 mesh, and the cyclic AMP was eluted as described by Brooker (*5*). Dibutyryl cyclic AMP is converted to cyclic AMP by the method of sample preparation (recoveries range from 92 to 100 percent). For this reason, dibutyryl cyclic AMP is measured as cyclic AMP. Cell counts were done on aliquots as previously described (*6*). Cyclic AMP is determined by the saturation assay of Brown *et al.*, which employs a cyclic AMP binding protein isolated from bovine adrenals (*7*).

The extracellular level of cyclic AMP decreases much more rapidly during incubation than the extracellular level of dibutyryl cyclic AMP (Table 1). This is expected because of the resistance of dibutyryl cyclic AMP to hydrolysis by phosphodiesterase (*8*). When Strain L cells are incubated in cyclic AMP, the intracellular concentrations of cyclic AMP are higher at each day of incubation than are the intracellular concentrations of cyclic AMP resulting from incubation of the Strain L cells in dibutyryl cyclic AMP. This is particularly noticeable after 24 hours of incubation when the intracellular concentrations reach 1 m*M* as contrasted with 0.07 m*M* with dibutyryl cyclic AMP. The intracellular/extracellular ratio of cyclic AMP after incubation in cyclic AMP suggests that cyclic AMP is actively transported. Dibutyryl cyclic AMP is either not transported as readily, or it may be that it must first be hydrolyzed to cyclic AMP before it is transported.

In previous studies, we have found that WI-38 cells are not inhibited by 0.15 m*M* cyclic AMP (*9*). These cells offered an interesting comparison with Strain L cells, which are markedly inhibited by cyclic AMP. The results in Table 1, lower portion, show that WI-38 cells reduce the extracellular cyclic AMP more than they do the dibutyryl cyclic AMP. During incubation of WI-38 cells in cyclic AMP, the intracellular level of cyclic AMP never reaches the 1 m*M* level observed on the first day of incubation with Strain L cells. However, the intracellular/extracellular ratios of cyclic AMP indicate active transport of cyclic AMP but not of dibutyryl cyclic AMP.

In Table 2, it can be seen that Strain L cells are inhibited up to 60 percent by 0.15 m*M* cyclic AMP, whereas WI-38 cells are not inhibited. In addition, Strain L cells are inhibited less by dibutyryl cyclic AMP, which correlates with the lower intracellular levels achieved with dibutyryl cyclic AMP. The approximately equal inhibition of WI-38 cells by dibutyryl cyclic AMP and butyric acid suggests that dibutyryl cyclic AMP does not inhibit WI-38 cells, but that the inhibition observed is an artifact resulting from the release of butyric acid by the dibutyryl cyclic AMP.

Table 2. Percent inhibition* of WI-38 and Strain L cells by dibutyryl cyclic AMP and cyclic AMP. Each result is the average of two determinations. Concentration of cyclic AMP, dibutyryl cyclic AMP, and butyric acid in the culture medium was 0.15 millimolar.

Incubation time (days)	WI-38 cells			Strain L cells		
	Dibutyryl cyclic AMP	Cyclic AMP	Butyrate	Dibutyryl cyclic AMP	Cyclic AMP	Butyrate
2	17	0	5	15	33	5
4	20	0	22	21	60	3

* Percent inhibition $= \frac{\text{Cell count (control)} - \text{cell count (cyclic nucleotide)} \times 100}{\text{Cell count (control)}}$

The assumption that dibutyryl cyclic AMP is transported more readily than cyclic AMP is not supported by these experiments. On the contrary, in the case of the two cell lines studied, cyclic AMP is transported better than dibutyryl cyclic AMP. The high intracellular levels of cyclic AMP attained would suggest that it is actively transported. In general, there appears to be some correlation between the intracellular levels of cyclic AMP and the degree of inhibition of cell division. The endogenous level of cyclic AMP found in density-inhibited WI-38 and Strain L cells is 0.03 m*M* and 0.02 m*M* (*6*). These levels were exceeded approximately fivefold with WI-38 cells without inhibition of division, which suggests that factors other than cyclic AMP may be involved in regulation of cell division. Although butyric acid does not affect the Strain L cells, it produces a considerable inhibition of WI-38 cells, which indicates the importance of adequate controls when studying the influence of dibutyryl cyclic AMP on cell growth and division.

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Type C RNA Tumor Viruses as Determinants of Chemical Carcinogenesis: Effects of Sequence of Treatment

Abstract. *Fischer rat embryo cells were treated with 3-methylcholanthrene before or after inoculation with Rauscher murine leukemia virus. Transformation was not observed in untreated control cultures, cultures given virus or 3-methylcholanthrene alone, or cultures treated first with 3-methylcholanthrene followed by inoculation with the virus after removal of the chemical. Transformation was dependent upon the presence of Rauscher murine leukemia virus at the time of chemical treatment.*

We have reported that a number of chemical carcinogens (*1–5*) induced morphological and malignant transformation of rat embryo cultures chronically infected with murine leukemia virus (MuLV). Untreated cultures, or cultures treated with virus or chemical alone, were not transformed in our test system. As a first step in determining the mechanisms responsible for the synergistic activity of the two agents, we undertook a series of timed-sequence experiments in which low passage (below passage 50) Fischer rat embryo