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

cultures were treated with 3-methylcholanthrene (3MC) before or after inoculation with Rauscher murine leukemia virus (RLV). The virus pool had been passaged 16 times in Fischer rat embryo cells (rat-adapted). We report here that transformation is dependent on the presence of the virus at the time of treatment with 3MC. Transformation was not observed in untreated control cultures, cultures given RLV or 3MC alone, or cultures treated first with 3MC and then inoculated with RLV after the removal of 3MC. Our conclusions were drawn from the results of three separate experiments representing two different pools of Fischer rat embryos.

Replicate cultures of each cell line in 75-cm^2 Falcon flasks were placed in the following experimental groups three passages after being removed from the freezer: (i) untreated, (ii) treated with 3MC, and (iii) inoculated with RLV. In the last group, 3 hours after subculturing, the medium was decanted, and 0.5 ml of virus stock was added and allowed to adsorb to the cells at 37°C for 2 hours; growth medium was then added.

At timed intervals, some of the chemically treated cultures were inoculated with RLV, and the cultures inoculated earlier with RLV were treated with 3MC. The 3MC was diluted to 1000 μg/ml in acetone, and then further diluted in growth medium (Eagle's minimal essential medium supplemented with 10 percent fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 100 μg of streptomycin and 100 units of penicillin per milliliter) to the desired concentration. In each case, cultures were treated with 3MC 2 hours after being subcultured, and again on days 3 and 5. On day 7, the cultures were subdivided with a transfer ratio of 1 : 2 and cultured serially in the absence of 3MC. At each passage, one set of flasks was set aside to be held indefinitely without subdivision (holding series), and the other set was subdivided 1 : 2 biweekly to provide two new sets of cultures—one for holding indefinitely, and the other for subdivision (vertical series). According to our laboratory terminology, the passage level represents the approximate number of population doublings, rather than the number of physical subdivisions.

In the first experiment, cell line F2304 at subculture 8 was treated with 0.1 and 1.0 μg of 3MC, three passages (3 weeks) before or after inoculation with rat-adapted RLV. Transformed foci appeared in those cultures which had been inoculated with RLV first and then treated with 1.0 μg of 3MC three subpassages later. The foci were first noted after a 2-week holding period of the sixth vertical subpassage after 3MC treatment. Untreated control cultures, cultures treated with RLV or 3MC alone, cultures treated with 3MC followed by RLV inoculation, and cultures inoculated with RLV and later treated with 0.1 μg of 3MC all retained their normal morphology after an additional 20 subcultures (Table 1).

In the second experiment, cell line F111 at subculture 19 was treated with 0.1, 0.5, or 1.0 μg of 3MC three subpassages before or after inoculation with rat-adapted RLV. Transformed foci appeared after a 2-week holding period, five to six subpassages after treatment with 1.0 μg or 0.5 μg of 3MC in those cultures that had been inoculated with RLV three subpassages earlier. Untreated cultures, cultures treated with RLV alone, 3MC alone, 3MC followed by RLV inoculation, or RLV inoculation followed by treatment with 0.1 μg of 3MC, remained normal after 16 additional subpassages (Table 1).

In the third experiment, cell line F111 at subculture 24 was treated with 0.5 μg of 3MC 2 hours after virus inoculation, and at 1 week (one subpassage), 2 weeks, (two subpassages), and 3 weeks (three subpassages) before and after inoculation with rat-adapted RLV. Transformed foci were evident four to five subcultures after 3MC treatment in each case in which the cultures were inoculated with RLV and treated with 3MC 2 hours, 1 week, 2 weeks, or 3 weeks later. All other cultures were still normal when the experiment was terminated 14 to 16 subcultures after treatment (Table 1).

Table 1. Effects of sequence of treatment on transformation of Fischer rat embryo cells. Numbers in parentheses indicate passage numbers.

First treatment		Second treatment		Transformation	
		Cell line F2304, subculture 8			
None		None		–	(34)
3MC, 0.1 μg	(11)	None		–	(34)
3MC, 1.0 μg	(11)	None		–	(34)
RLV	(11)	None		–	(34)
3MC, 0.1 μg	(11)	RLV	(14)	–	(34)
3MC, 1.0 μg	(11)	RLV	(14)	–	(34)
RLV	(11)	3MC, 0.1 μg	(14)	–	(34)
RLV	(11)	3MC, 1.0 μg	(14)	+	(20)
		Cell line F111, subculture 19			
None		None		–	(41)
3MC, 0.1 μg	(22)	None		–	(41)
3MC, 0.5 μg	(22)	None		–	(41)
3MC, 1.0 μg	(22)	None		–	(41)
RLV	(22)	None		–	(41)
3MC, 0.1 μg	(22)	RLV	(25)	–	(41)
3MC, 0.5 μg	(22)	RLV	(25)	–	(41)
3MC, 1.0 μg	(22)	RLV	(25)	–	(41)
RLV	(22)	3MC, 0.1 μg	(25)	–	(41)
RLV	(22)	3MC, 0.5 μg	(25)	+	(31)
RLV	(22)	3MC, 1.0 μg	(25)	+	(30)
		Cell line F111, subculture 24			
None		None		–	(41)
3MC, 0.5 μg	(24)	None		–	(41)
RLV	(24)	None		–	(41)
3MC, 0.5 μg	(24)	RLV	(25)	–	(41)
3MC, 0.5 μg	(24)	RLV	(26)	–	(41)
3MC, 0.5 μg	(24)	RLV	(27)	–	(41)
RLV	(24)	3MC, 0.5 μg	(24), 2 hours	+	(29)
RLV	(24)	3MC, 0.5 μg	(25), 1 week	+	(30)
RLV	(24)	3MC, 0.5 μg	(26), 2 weeks	+	(31)
RLV	(24)	3MC, 0.5 μg	(27), 3 weeks	+	(31)

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