been linked to components of this system (*12*), and changes in levels of acetylcholinesterase and of acetylcholine itself have been reported after exposure of the retina to light (*13*). The establishment of a link between these two light-sensitive enzyme systems in the retina, therefore, would be an obvious subject of further investigation.

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In vivo Inhibition of Growth of Two Hormone-Dependent Mammary Tumors by Dibutyryl Cyclic AMP

Abstract. *Growth of hormone-dependent rat mammary tumors was arrested in vivo by* $N^6,O^{2'}$*-dibutyryl cyclic adenosine 3′,5′-monophosphate. Estrogen concentration did not change, but acid ribonuclease activity and synthesis increased during treatment with the dibutyryl cyclic nucleotide, as was shown during tumor regression due to hormonal deprivation. Growth arrest, thus, appears to derive from enhanced tissue catabolism.*

Growth and morphology of a number of cultured cell lines have been reported to be influenced by adenosine 3′,5′-monophosphate (cyclic AMP) or its derivatives. Exposure of various lines of transformed cells to cyclic AMP or the dibutyryl derivative resulted in inhibition of growth rate without affecting cell viability (*1*). Concentrations of cyclic AMP were three- to fourfold higher in confluent 3T3-4 cells than in cells in the logarithmic phase of growth (*2*); higher concentrations of cyclic AMP were found in more slowly growing cells than in rapidly growing cells (*2, 3*); and addition of dibutyryl cyclic AMP decreased the rate of growth (*4*). A specific reduction of the growth rate was also obtained by exposing Reuber H35 hepatoma cells in vitro to dibutyryl cyclic AMP and other cyclic AMP analogs (*5*).

The regulatory effect of cyclic AMP on the growth of neoplastic tissues in vivo has not yet been fully investigated. Gericke and Chandra reported a partial inhibition of growth in vivo by injection of cyclic AMP directly into mouse NKL (Németh-Kellner leukemia) lymphosarcoma (*6*). Webb *et al.* (*7*) showed retarded growth of a mouse tumor in vivo by intraperitoneal injection of theophylline, which competitively inhibits cyclic AMP phosphodiesterase (*8*) and induces the accumulation of cyclic AMP within the cell (*9*).

We have tested the effect of cyclic AMP, dibutyryl cyclic AMP, and closely related substances on the growth in vivo of two hormone-dependent rat mammary tumors. One was a primary tumor induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in Sprague-Dawley female rats. The other was a transplantable tumor (MTW9) in Wistar female rats. Tumor growth was expressed as the percentage change in volume calculated from the formula, $(4/3)\pi r^3$, where r is one-half the average of the longest and shortest diameters measured by Vernier caliper daily. 3′,5′-AMP, 2′,3′-AMP, dibutyryl cyclic AMP, and 5′-AMP were obtained from Sigma Chemical; sodium butyrate was obtained from Matheson, Coleman and Bell; theophylline was obtained from Schwarz/Mann; and Delestrogen (17β-estradiol) was obtained from E. R. Squibb and Sons. Animals weighing 170 to 200 g each and bearing 2- to 3-g tumors were used at the start of experiments. Acid ribonuclease was assayed in supernatent (105,000*g* for 1 hour) of tumor hemogenates (10 percent by volume) prepared in a mixture of 0.85 percent NaCl and 0.1 percent Triton X-100 by a modification of the method of Kalnitsky *et al.* (*10*) as described (*11*). The enzyme was quantified by single radial immunodiffusion (*11*). Protein was estimated by the method of Lowry *et al.* (*12*). Estrogen levels in tumors were measured by radioimmunoassay (*13*).

Growth of the DMBA tumor was completely arrested in animals treated for 3 weeks with dibutyryl cyclic AMP, while the size of the tumors in control animals increased threefold (Fig. 1). Ovariectomy results in regression of this tumor and 17β-estradiol produces regrowth (*14*). Dibutyryl cyclic AMP also inhibited the growth in the regrowing tumors (Fig. 1). Growth of the MTW9 tumor was arrested during 3 weeks in a similar experiment in which control tumors showed a tenfold increase over their original size (Fig. 1). Cessation of dibutyryl cyclic AMP treatment restored tumor growth (Fig. 1). Although no attempt was made to determine the minimum effective dose of dibutyryl cyclic AMP, the amount injected over a 3-week period was not toxic to the animals, as evaluated by hematocrit levels, body weight, and food intake. Minimum inhibition

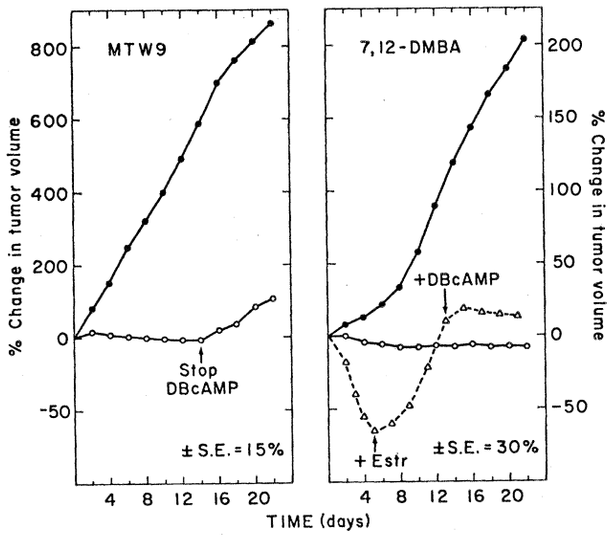

Fig. 1. Growth arrest by dibutyryl cyclic AMP (DBcAMP) of hormone - dependent rat mammary tumors. ●—●, Control, no treatment; ○—○, DBcAMP, 8 mg per 0.2 ml of 0.85 percent NaCl per day subcutaneously; △—△, ovariectomized (↑Estr, start of Delestrogen, 33 μg per 0.1 ml of sesame oil given subcutaneously every 6 days for the duration of experiment; ↓DBcAMP, start of treatment with dibutyryl cyclic AMP with 8 mg per 0.2 ml of 0.85 percent NaCl per day, subcutaneously). Values represent the average of ten tumors; S.E., standard error. All subcutaneous injections were made at a site remote from tumors.

of growth of no statistical significance was observed with 3′,5′-AMP, 2′,3′-AMP, 5′-AMP, sodium butyrate (8 mg/day per compound injected subcutaneously), or with theophylline (4 mg/day). In studies of growth arrest, animals bearing DMBA tumors in diestrus were killed and serum estrogen concentrations were compared with controls (*13*). No significant difference was found during the three estrus periods. Thus growth arrest was not due to a reduction of circulating hormones needed by the tumors for growth.

Acid ribonuclease behavior in the growth-arrested tumors suggested that dibutyryl cyclic AMP increases the rate of tissue catabolism. Activities of several lysosomal enzymes increase sharply during regression of hormone-dependent mammary tumors after ovariectomy, and activity and quantity of acid ribonuclease increase two to threefold within 6 hours after the host is deprived of proper hormonal stimulation (*11, 15*). Thus, the response of acid ribonuclease is the earliest sign of intracellular degradation during regression. A twofold increase in acid ribonuclease in both activity and quantity was also shown in tumors of animals treated with dibutyryl cyclic AMP. Precipitation of the labeled acid ribonuclease with specific antiserum to acid ribonuclease showed that the increased enzyme quantity during dibutyryl cyclic AMP treatment is due to new synthesis as indicated by the reduction of specific activity compared to that of controls treated with sodium butyrate (Table 1). New formation of acid ribonuclease resulted in an accumulation of the enzyme twice the normal quantity observed in growing tumors during a 7-day period of growth arrest (sodium butyrate treatment, Table 1). Our studies show that dibutyryl cyclic AMP consistently arrested growth of two hormone-dependent mammary tumors, most probably by enhancing the rate of tissue catabolism.

Table 1. Relative dilution of radioactivity in acid ribonuclease and total protein of MTW9 tumors after treatment with dibutyryl cyclic AMP. Uniformly labeled D-[^{14}C]glucose (150 to 200 mc/mmole; New England Nuclear) was diluted in 0.015*M* NaCl to a concentration of 1 μc/0.02 ml and continuously infused at a rate of 1 μc/hour for 6 days into the thoracic aorta of animals having two tumors of approximately equal size. After infusion was terminated, the animals remained overnight without treatment; then one tumor was removed from each animal and used for determinations at zero time. Dibutyryl cyclic AMP or sodium butyrate (as control), 8 mg/0.2 ml of 0.85 percent NaCl for a 170-g rat, was then injected subcutaneously daily, and the tumor sizes were measured at intervals. Animals were killed at times indicated, and the remaining tumors were removed. All tumors were homogenized immediately after removal in five volumes of cold 0.85 percent NaCl containing 0.1 percent Triton X-100 (*11*). The homogenates were centrifuged at 105,000*g* for 1 hour, and the supernatants were used for the determinations. The radioactivity in the protein precipitated by specific antiserum to acid ribonuclease and that in the total protein of the supernatant was determined as described (*16*). Acid ribonuclease in each tumor extract was determined by single radial immunodiffusion, with crystallized acid ribonuclease as the reference standard (*11*). Results are expressed as the number of disintegrations per minute per milligram of protein.

Time (days)	Acid ribonuclease in tumor (micrograms per gram of tumor)	Mean radioactivity			
		Acid ribonuclease		Total protein	
		Disintegrations per minute per milligram	Percent	Disintegrations per minute per milligram	Percent
		Sodium butyrate			
0	92	9210	100	1330	100
3	90	7368	80	532	40
7	88	5526	60	333	25
		Dibutyryl cyclic AMP			
0	90	8896	100	1112	100
3	155	3113	35	500	45
7	175	888	10	222	20

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