GROWTH INHIBITION OF MURINE TUMOR CELLS, IN VITRO, BY PUROMYCIN, [^{6}N]O$^{2'}$-DIBUTYRYL 3',5'-ADENOSINE MONOPHOSPHATE, OR ADENOSINE

Evidence of Commitment for Cell Division

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ABSTRACT

The cytostatic effects of puromycin, [^{6}N]O$^{2'}$-dibutyryl 3',5'-adenosine monophosphate, and adenosine on asynchronous and synchronous cultures of the murine mastocytoma, P815Y, have been studied. Cell growth was arrested after a minimum of one further division. A model is proposed for the inhibition of cell division in which the periods of inhibition and growth arrest are separated in time by one cell cycle.

Culture conditions unfavorable to growth such as high cell density or serum deprivation result in an accumulation of cells in the G_1 interval. Therefore it is assumed that G_1 is the sensitive period for growth control. We propose that, in some instances, growth inhibition and cell arrest do occur at G_1 but are separated in time by one cell cycle. This conclusion is based on a study of the inhibition of synchronous division of the murine mastocytoma, P815Y.

Our reasons for choice of inhibitors are as follows: cyclic adenosine 3'-5'-monophosphate (cyclic AMP) has been implicated as a mediator of growth control (Ryan and Heidrick, 1968; Burk, 1968; Otten et al., 1971; Sheppard, 1971). We selected the esterified form of cyclic AMP: [^{6}N]O$^{2'}$-dibutyryl 3',5'-adenosine monophosphate (dbc-AMP) since it is absorbed more readily by cells, in vitro (Kaukel and Hilz, 1972). Adenosine was included as a nucleoside analogue of cyclic AMP, and we were intrigued by the finding that puromycin inhibition of growth in porcine kidney cells was dependent on initial cell density (Cass, 1972).

MATERIALS AND METHODS

Cell Culture

The murine mastocytoma, P815Y, was maintained as spinner cultures in Fischer's medium supplemented with 10% horse serum.

Cell Synchrony

This was achieved by separating cells according to size, and therefore position in the cell cycle, by velocity sedimentation (Warmsley and Pasternak, 1970). Cell cultures ($2–3 \times 10^5$/ml) in logarithmic phase of growth were fractionated on an M.S.E. zonal rotor, A, (Measuring and Scientific Equipment Ltd., London) using a linear gradient of 1.5–12% Ficoll (wt/vol) dissolved in phosphate-buffered saline (PBS) with 2.5% horse serum, pH 7.35. Cell samples ($4–6 \times 10^8$) were applied to the rotor in 20 ml of 0.5% (wt/vol) Ficoll, followed by an overlay of 40 ml PBS. Centrifugation was carried out for 20 min at 600 rpm in an M.S.E. Mistral, 6L, centrifuge. Cells were exposed to Ficoll for the minimum possible time (1 h). The rotor fractions were assigned to a position in the cell cycle according to size, cell content of DNA, and ability to incorporate [^{3}H]thymidine

into DNA (Fig. 1). Cells at G_2 were arbitrarily defined by an approximate twofold increase in cell volume and DNA content.

Cells from the zonal rotor were recultured at a cell density of 0.8–1.2 × 10^5/ml and maintained at this concentration by dilution of cell cultures with increase in cell number. The degree of synchrony was determined by incorporation of [^{3}H]thymidine into DNA and estimation of division index (D.I.). Division index is the ratio of the observed increase in cell number to the theoretical value for a twofold increase in cell number; values for division index (Fig. 4, 5, 6, 7) were only computed for discrete growth plateaus.

Cell Number and Cell Volume

The values were estimated with a Coulter counter, model ZBI, as directed by the manufacturers.

Incorporation of [^{3}H]thymidine

Samples (2 ml) from the zonal rotor were centrifuged, washed once with PBS, and resuspended in culture medium. Cells (0.8–1.2 × 10^5/ml) from the rotor, or cell culture, were incubated with [^{3}H]-thymidine (2 μCi/ml) at 37°C for 30 min. The cells were washed with PBS and the DNA was precipitated with 5% (wt/vol) trichloroacetic acid. Precipitates were counted in a liquid scintillation spectrometer.

DNA assay

The DNA content of cell fractions was estimated by the diphenylamine method (Burton, 1956).

RESULTS

Asynchronous Cultures

Fig. 2 shows the dose-response curves for inhibition of asynchronous cell growth. The inhibitors were cytotoxic at levels greater than those indicated by the arrow. Above these concentrations, cells showed a decrease in viability and failed to grow in fresh culture medium, in the absence of inhibitors. The following concentrations were selected for study: puromycin (0.5 μg/ml), dbc-AMP, and adenosine (0.3 mM). When added to cell cultures in logarithmic phase of growth, all inhibitors gave a similar reduction in final cell density (Fig. 3). Also, the relative increase in cell number was *independent* of initial cell density: mean values and standard error for division number being, puromycin 1.89 ± 0.02 (29), dbc-AMP 1.89 ± 0.05 (13), adenosine 1.67 ± 0.15 (12) (number of cultures tested, in parenthesis). The inhibition was reversible and cells resumed normal growth when transferred to fresh culture medium. Occasionally, cells 'escaped" inhibition at the higher cell densities (>3 × 10^5 cells/ml). The study was extended to synchronous cell cultures.

Synchronous Cultures

In preliminary work, fractions from the zonal rotor were tested for degree of synchrony on

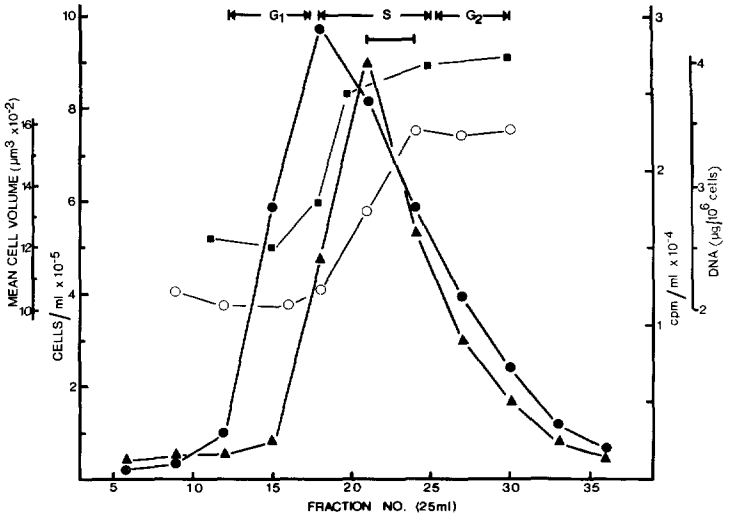
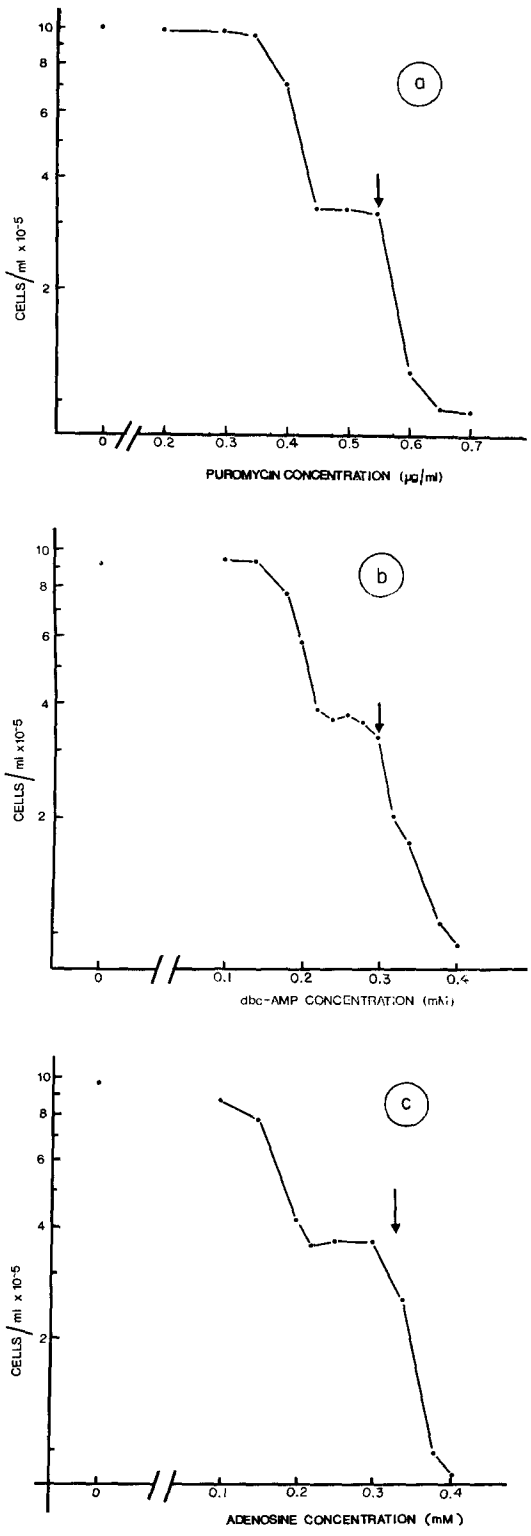


FIGURE 1 Fractionation of cells by velocity sedimentation. (●) cell number, (○) mean cell volume, (▲) [^{3}H]thymidine incorporation, (■) total DNA, |——| fractions taken for synchrony experiments.



reculture. Only cell fractions corresponding to "late S" (Fig. 1, fractions 21–24) maintained synchrony after the first division. Cells from this position in the gradient were used routinely. Their growth properties were characteristic of late S, viz. a rapid decrease in [^{3}H]thymidine incorporation and corresponding increase in cell number (Fig. 4). Adequate synchrony was observed for the duration (60–72 h) of all experiments. The adverse effects of cell contact were reduced by diluting cultures to the initial cell density (0.8–1.2 $\times$ 10^5/ml) at each cell division.

To determine the period in the cell cycle sensitive to their action inhibitors were added to synchronous cultures at time intervals corresponding to different phases of the cycle, viz. S, G_2, and G_1. There was a minimum of one further division irrespective of time of addition (Figs. 5, 6, 7). Clear-cut results were obtained with puromycin. Addition at 0–5 h (Fig. 5 *a*, *b*) corresponding to S and G_2 had no apparent effect on mitosis or the next division cycle; cells were arrested in G_1 at the third generation. Similarily, if puromycin was added at 10–15 h (Fig. 5 *c*, *d*) corresponding to late G_1 or S, no difference was found from control cultures (Fig. 4) for a further two divisions.

There was a similar effect on cell growth with dbc-AMP (Fig. 6) and adenosine (Fig. 7); cells were arrested after two further divisions. However, incorporation of [^{3}H]thymidine, in the presence of these inhibitors, was not a valid index of DNA synthesis. Addition of dbc-AMP at 0 h (Fig. 6 *a*) abolished the second [^{3}H]thymidine peak, yet cells continued to divide at a reduced rate. Assay of total DNA content of cell cultures (Fig. 6 *a*, time interval 15–30 h) confirmed an expected increase (from 0.28 μg/ml to 0.43 μg/ml) with cell number. Adenosine (Fig. 7) showed an immediate inhibition of [^{3}H]thymidine incorporation followed by a slow "recovery." Thus, increase in cell number was the only reliable measure of cell division. By this criterion, the three componunds had a similar action. These results support the view that growth inhibition and cell arrest do occur at G_1 interval, but are separated in time by one cell cycle.

FIGURE 2 Effect of different inhibitor concentrations on asynchronous cell cultures. Exponentially growing cells (1 $\times$ 10^5/ml) were incubated with (*a*) puromycin, (*b*) dbc-AMP, (*c*) adenosine for 72 h and the final cell density was determined.

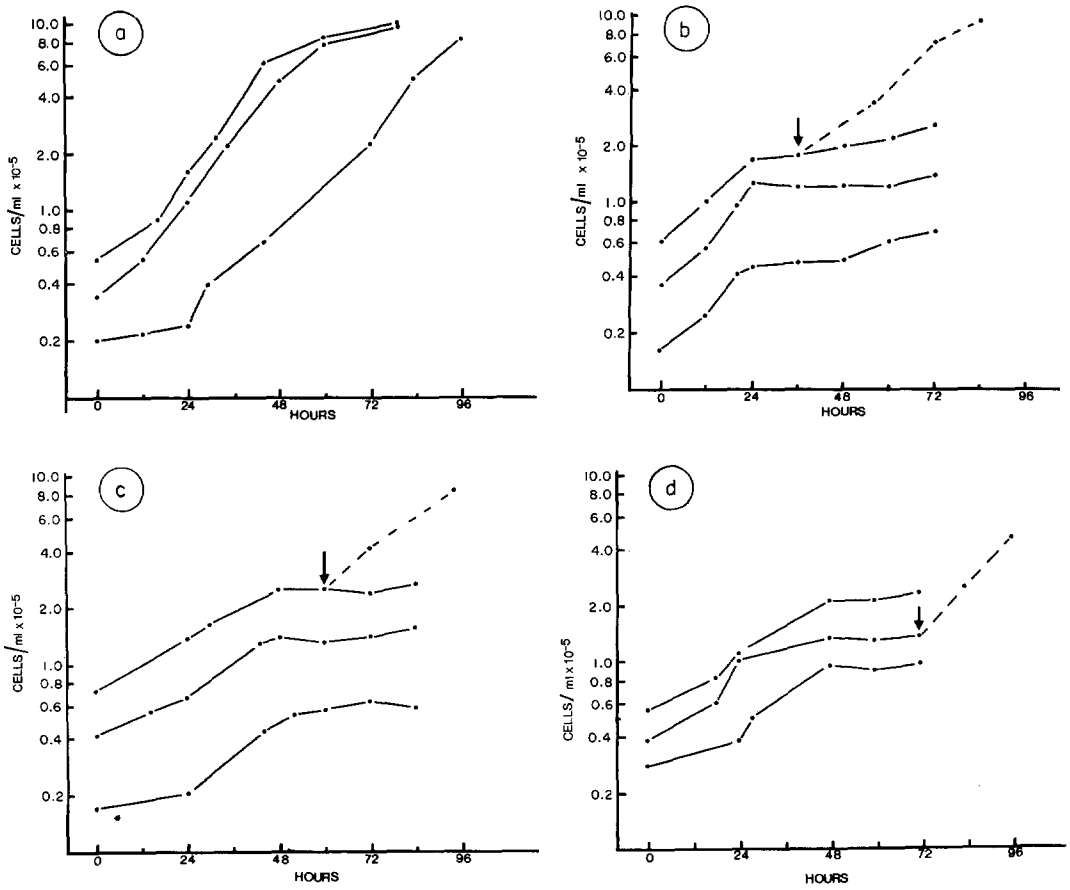


FIGURE 3 Growth inhibition of asynchronous cultures: (*a*) control, (*b*) puromycin, (*c*) dbc-AMP, (*d*) adenosine. Cells in a logarithmic phase of growth (1–4 × 10^5/ml) were centrifuged and reincubated with inhibitors at different initial cell densities. At the time indicated by the arrow, cells were centrifuged, washed, and transferred to fresh culture medium without inhibitors (●---●). Cell density was adjusted to the value at collection.

DISCUSSION

Velocity sedimentation in a zonal rotor is an efficient method for producing cell synchrony. It is not subject to artifacts (Warmsley and Pasternak, 1970) inherent in chemical methods (Studzinski and Lambert, 1969; Bergeron, 1971). Cells are viable and have a doubling time (12–14 h) which is close to values for cells in logarithmic phase of growth. The mean cell volume of rotor fractions did not show a steady increase, as reported by others (Warmsley and Pasternak, 1970). Cell size and DNA content increased in concert (Fig. 1). The finding that only fractions from the zonal rotor corresponding to late S maintained synchrony may be due to increased size resolution at this position of the gradient. Schindler and Hurni (1971), using conventional tube centrifugation, found that tumor cell fractions corresponding to G_1 showed the optimum degree of synchrony on reculture.

The results presented here raise several questions. Firstly, how do such diverse compounds exert a similar effect on cell division? Puromycin is an effective inhibitor of protein synthesis (Yarmolinsky and de la Haba, 1959). However, at the concentration selected for study, control and puromycin-treated cultures showed no significant difference in division rate or [^{3}H]thymidine incorporation. This would suggest that most biosynthetic events were normal. In contrast, dbc-

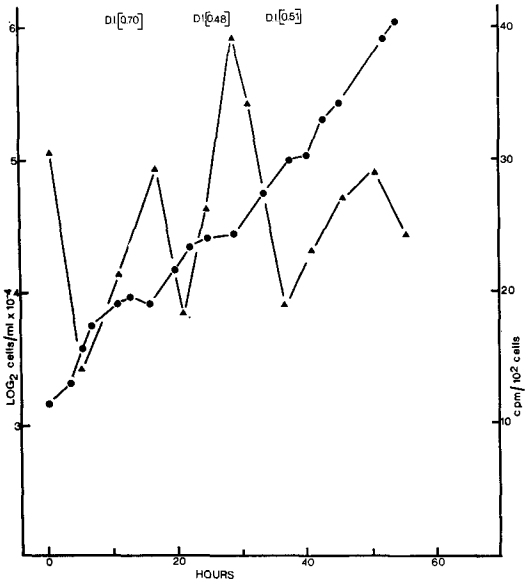


FIGURE 4 Growth of synchronous cultures. Cell samples (4 ml) were removed, incubated with [^{3}H]thymidine (2 μCi/ml) for 30 min, and the DNA was precipitated with 5% trichloroacetic acid. Increase in cell number was calculated from the dilution volume necessary to maintain a constant cell density (0.8–1.2 $\times$ 10^5/ml). D.I. is the division index which was only calculated for distinct growth plateaus. (▲) [^{3}H]thymidine incorporation, (●) cell number.

AMP and adenosine extended the division time, yet the position of growth arrest was identical.

Secondly, why is the incorporation of exogenous [^{3}H]thymidine reduced in the presence of dbc-AMP or adenosine? Estimation of the DNA content of cells during the division cycle has shown that *de novo* synthesis of DNA is not affected by these inhibitors. This anomaly might be explained by inhibition of thymidine kinase activity, or altered uptake of [^{3}H]thymidine or change in intracellular pool-size of deoxyribonucleotide triphosphates. Caution should therefore be taken in the interpretation of data (Willingham et al., 1972; Froehlich and Rachmeler, 1972) for radionucleotide incorporation in the presence of these inhibitors.

It is also surprising that dbc-AMP, the derivative of a possible growth regulator, did not have a more immediate effect. In fact, the nucleoside analogue adenosine showed a similar inhibition over the same concentration range. These questions remain to be resolved.

According to current views of growth control, the period of inhibition and growth arrest are both at G_1. However, the present results indicate that the cytostatic effects of the inhibitors are manifest in the second or third cell generations, irrespective of the time of their addition. Therefore, a minimum of one further cell division is required for an apparent effect (Figs. 5, 6, 7 *a*, *b*); also, if inhibitors are introduced to cell cultures at late G_1 or S (Fig. 5, 6, 7 *c*, *d*), cells "escape" inhibition for two further divisions.

It is a matter of conjecture whether these findings are relevant to the normal events of growth control, in vivo and in vitro. There is no immediate inhibition of tumor cultures by monovalent concanavalin A (Burger and Noonan, 1970), dbc-AMP (Sheppard, 1971), interferon (Gresser et al., 1970), or pH (Ceccarini and Eagle, 1971), and the final increase in cell number exceeds that of one cell division. Also, examination of cultures by time-lapse cinematography (Martz and Steinberg, 1972) has shown that a high cell density at G_1 has no immediate effect on cell growth. Perhaps a delay of one cell cycle is a feature of growth inhibition, in vitro.

A possible criticism of the present data is that a lag period equal to, or greater than, one division cycle is required for inhibitors to reach a critical intracellular concentration and exert their effect. According to this view, it is surprising to find such consistent effects for both asynchronous and synchronous cultures. The final increase in cell number of asynchronous cultures (1.8 cell divisions) is similar for all three inhibitors in spite of different division rates (Fig. 3, *b*, *c*, *d*). Also, inhibition of synchronous division is dependent on relative position of cells in the division cycle rather than total exposure time to inhibitors (Fig. 5 *b* vs. 5 *c*; Fig. 6 *b* vs. 6 *c*; Fig. 7 *b* vs. 7 *c*). Further studies are in progress, using "pulse-exposure" to inhibitors at discrete phases of the cell cycle to clarify this point.

We wish to propose a model to account for our results (Fig. 8). The evidence in support of the scheme is as follows:

(*a*) The inhibitors are cytostatic and not cytotoxic; their effect is reversible.

(*b*) Asynchronous cultures are arrested after approximately 1.8 cell divisions, irrespective of initial cell density (Fig. 3).

(*c*) Inhibition is restricted to a discrete phase of the cell cycle viz. G_1 (or early S), yet synchronous cells undergo a further division before arrest (Figs. 5, 6, 7).

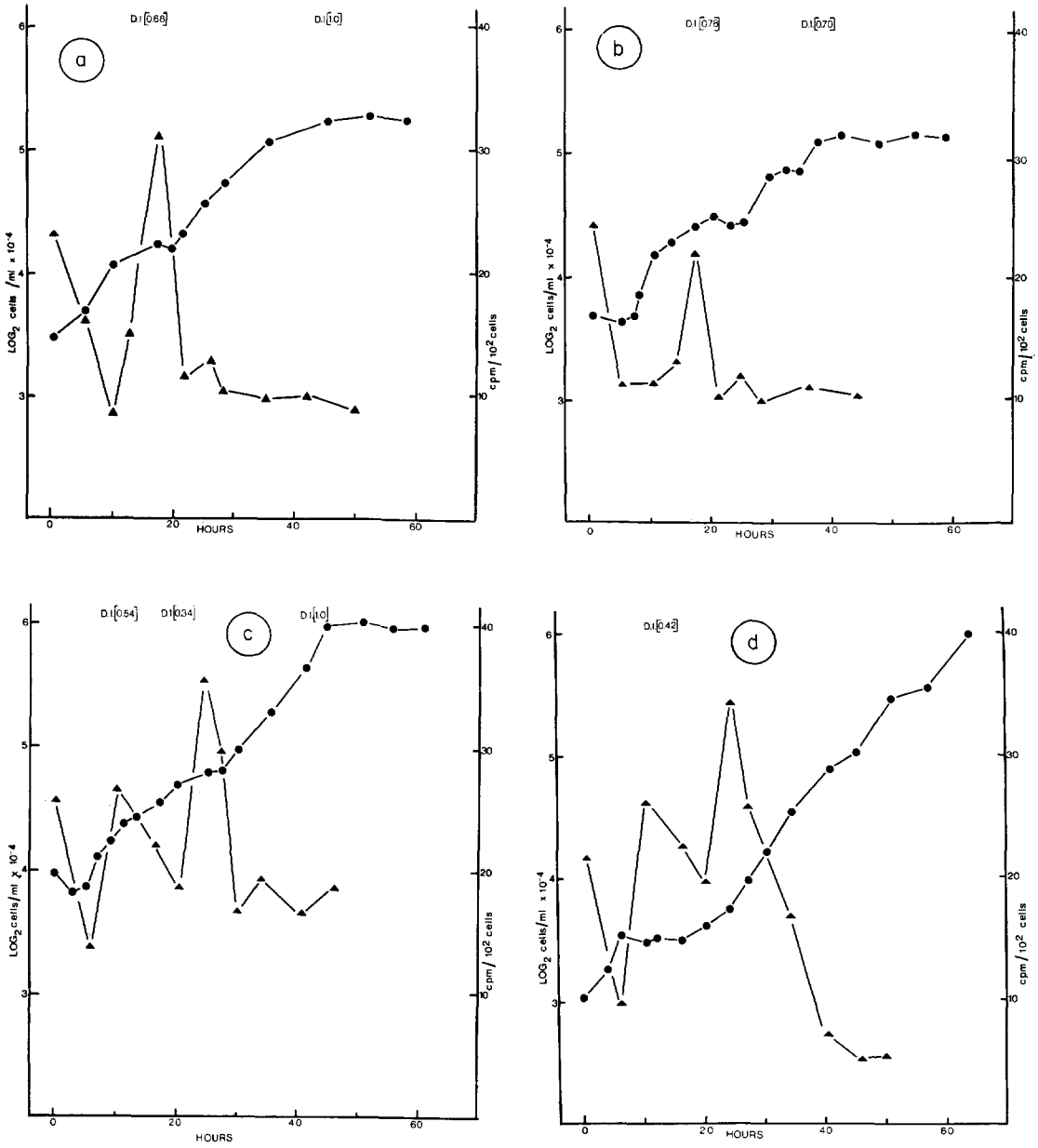


FIGURE 5 Inhibition of synchronous cultures by puromycin (0.5 μg/ml), added at (*a*) 0 h, (*b*) 5 h, (*c*) 10 h, (*d*) 15 h. (▲) [^{3}H]thymidine incorporation, (●) cell number.

Accordingly, there are two periods in the cell cycle which are sensitive to inhibition, *a* and *c*. It is assumed that an event at *a* commits daughter cells to further division. Inhibition at this point by puromycin, dbc-AMP, or adenosine diverts cells to route *b* with arrest at $G_1(G_0)$. There are, therefore, two pathways to mitosis ($S^1G^1{}_2M^1$ and SG_2M) which are distinguished by ability of *progeny cells* to divide. Restriction of commitment to period *a* implies a transition point; cells at position in the cycle beyond *a* escape inhibition for a further two divisions. This would account for

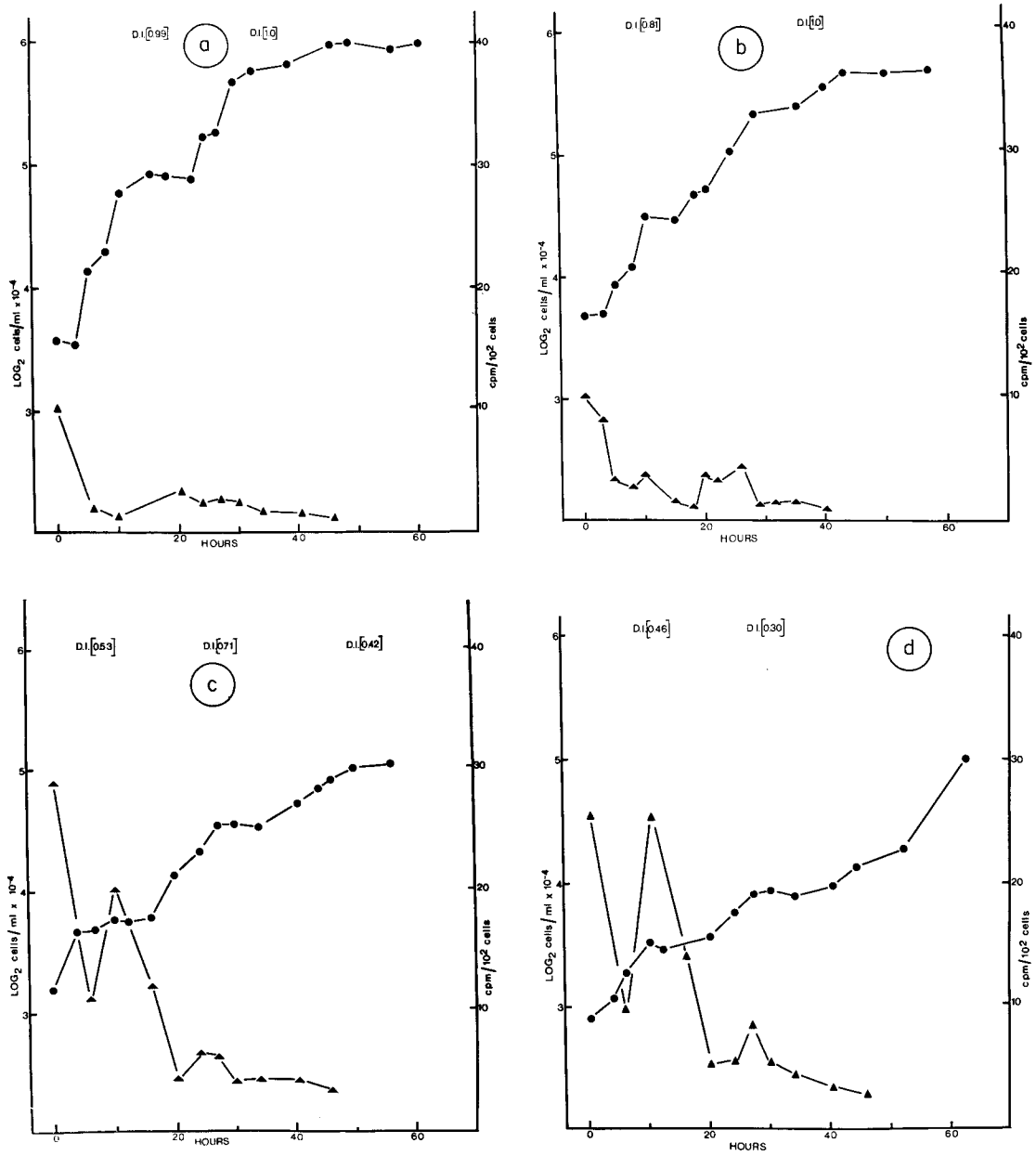


FIGURE 6 Inhibition of synchronous cultures by dbc-AMP (0.3 mM), added at (*a*) 0 h, (*b*) 5 h, (*c*) 10 h, (*d*) 15 h. (▲) [^{3}H]thymidine incorporation, (●) cell number.

the odd value of 1.67–1.89 divisions (Fig. 3) for inhibition of asynchronous cultures, and also suggests that commitment is an early event at G_1.

The scheme is consistent with a previous model for antigenic variation with cell division (Thomas, 1971). Changes in cell surface expression of the carbohydrate moieties: α-galactosyl 1 → 3 galactose and α-fucosyl 1 → 2 galactose in synchronous tumor cultures indicated a branch point at mitosis leading to growth arrest or further division at G_1. Thus, ability of cells to divide was determined in the preceding generation. It

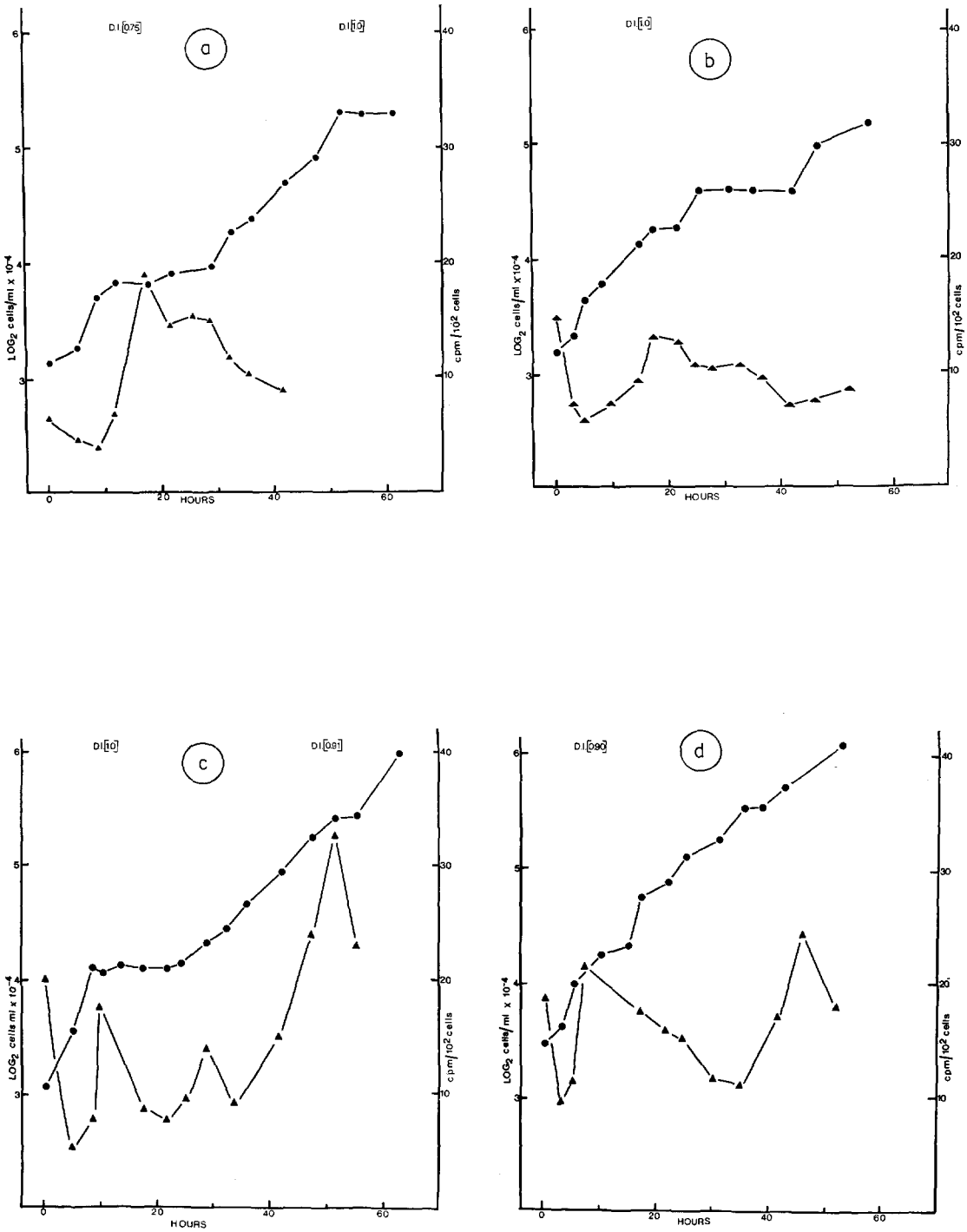


FIGURE 7 Inhibition of synchronous cultures by adenosine (0.3 mM) added at (*a*) 0 h, (*b*) 5 h, (*c*) 10 h, (*d*) 15 h. No further increase in cell number was observed for *a* and *b* in the time interval 60–70 h. (▲) [^{3}H]thymidine, (●) cell number.

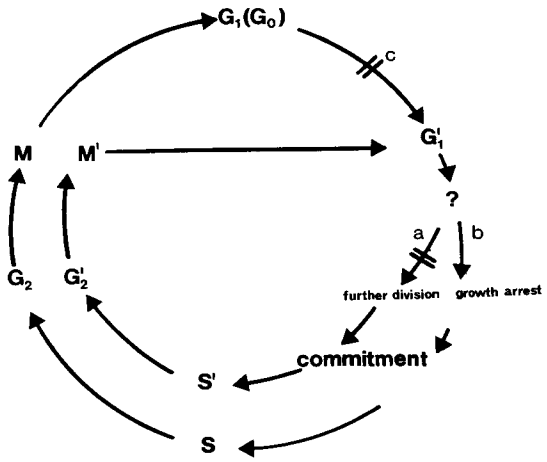


FIGURE 8 Model for inhibition of cell division, in vitro.

remains to be determined whether the model is relevant to "normal mechanisms" of growth control.

This work was supported by grants from the Medical Research Council and Cancer Research Campaign of Great Britain.

Received for publication 6 October 1972, and in revised form 14 November 1972.

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