RAPID CHANGES IN NUCLEOSIDE TRANSPORT INDUCED BY GROWTH INHIBITORS

Studies with Neoplastic Mast Cells

CLIFFORD A. LINGWOOD and D. BRIAN THOMAS

From the Department of Immunology, Middlesex Hospital Medical School, London, England

ABSTRACT

Aqueous extracts of murine embryonic or uterine tissue, or [^{6}N]O$^{2\prime}$-dibutyryl 3′,5′-adenosine monophosphate (dbc-AMP) which were cytostatic for the murine mastocytoma P815Y in vitro also induced rapid changes in the incorporation of exogenous nucleosides into acid-insoluble material. However, these alterations were not a consequence of growth arrest. Different dose-response curves were obtained for cytostasis and inhibition of [^{3}H]-nucleoside incorporation, and changes in [^{3}H]thymidine uptake were detected within 15 min of treatment with the inhibitors. Also, there were differential effects of each inhibitor on the incorporation of ^{3}H-labeled thymidine, uridine, adenosine, or choline into acid-insoluble material.

INTRODUCTION

Permeability properties of the plasma membrane have been implicated in the control of cell growth in vitro (Cunningham and Pardee, 1969; Weber and Rubin, 1971). Thus, eukaryotic cells exhibit a reduced transport of certain substrates such as nucleosides or amino acids (Colby and Rubin, 1969; Plagemann et al., 1969; Kohn, 1968; Weber and Rubin, 1971; Cass, 1972) during the stationary phase of growth. Also, addition of fresh serum to confluent fibroblast cultures produces a rapid increase in the incorporation of uridine or inorganic phosphate (Cunningham and Pardee, 1969; Todaro et al., 1967) followed by a round of cell division. The stimulation of nucleoside transport is not thought to reflect changes in the *de novo* rates of DNA or RNA synthesis in all cases (Cunningham and Pardee, 1969).

Similarly, it has been suggested (Holley, 1972) that the abnormal social behavior of tumor cells is correlated with changes in membrane permeability. The rates of incorporation of amino acid analogues (Foster and Pardee, 1969; Isselbacher, 1972) and neutral sugars (Hatanaka and Gilden, 1970; Martin et al., 1971) is increased in virus-transformed as compared with normal cells.

Our attention was focused on the permeability properties of tumor cells by the finding (Thomas et al., 1973) that [^{6}N]O$^{2\prime}$-dibutyryl 3′,5′-adenosine monophosphate (dbc-AMP), a cytostatic agent, reduced the incorporation of exogenous [^{3}H]thymidine into acid-insoluble material by tumor cells in vitro under conditions where *de novo* DNA synthesis was unaffected.

In this report, it is shown that dbc-AMP and aqueous extracts of murine embryonic tissue, or of pregnant uterine tissue, produce rapid changes in the incorporation of exogenous nucleosides into acid-insoluble material by P815Y cells in vitro.

Such tissue extracts are known (Rossdale, 1973) to inhibit the growth of several murine cell lines but to stimulate the proliferation of bone marrow, granulocyte precursors, in vitro (colony stimulating factors; Metcalfe, 1970).

MATERIALS AND METHODS

Cell Culture

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

Preparation of Extracts of Embryonic (EE) and Uterine Wall (UWE) Tissue

The uteri and contents were removed from pregnant mice (outbred CDI) at 16–18 days. Embryonic tissue was separated from the uterine wall and aqueous extracts of each tissue were prepared essentially as described for the isolation of colony stimulating factor material (Rossdale, 1973). The tissue was homogenized in 5 vol of deionized water at 4°C. Streptomycin sulfate (8 mg/ml homogenate) was added and the suspension stirred for 2 h in the cold. After centrifugation (1,500 *g*, 30 min) the supernate was concentrated 5- to 10-fold by dialysis against sucrose, and active material was precipitated with 50–100% ammonium sulfate. The precipitate was redissolved in a minimum volume of deionized water, and dialyzed extensively against deionized water, followed by Fischer's culture medium. The extract was sterilized by membrane filtration and stored at −20°C.

Incorporation of Radiolabeled Substrates

Cell cultures (0.8–3.0 × 10^5/ml) were incubated with [^{3}H]thymidine (5 Ci/mmol; 2 μCi/ml) or [^{3}H]-uridine (5 Ci/mmol; 2 μCi/ml) or [^{3}H]adenosine (17 Ci/mmol; 2 μCi/ml) or [^{3}H]choline chloride (0.19 Ci/mmol; 2 μCi/ml) at 37°C. After incubation, cells were washed with culture medium containing an excess (2 mM) of unlabeled substrate and ^{3}H-macromolecular material was precipitated with 5% (wt/vol) trichloroacetic acid. Precipitates were counted in a liquid scintillation spectrometer.

Cell Number

This was determined with a Coulter counter, model ZBI (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). Values for division number were computed from the ratio of the observed increase in cell number to the theoretical value for a twofold increase in cell number.

RESULTS

Inhibition of Cell Growth and [^{3}H]Nucleoside Incorporation

Growth inhibition by aqueous extracts of embryonic tissue (EE; Fig. 1 *a*) and pregnant uteri (UWE; Fig. 1 *b*) or dbc-AMP. (Fig. 1 *c*) was assayed by [^{3}H]thymidine incorporation into DNA after an 18-h incubation period or final division number at 72 h. The dose-response curves for these two parameters were significantly different. Each inhibitor altered nucleoside incorporation at concentrations which had no apparent effect on cell growth. This discrepancy was most apparent for dbc-AMP (Fig. 1 *c*); in the concentration range, 0–0.15 mM, values for final division number were equal to those of control cultures yet there was almost complete inhibition of [^{3}H]thymidine incorporation. A similar disparity in dose-response curves was found for EE (Fig. 1 *a*; at 0–1 mg/ml) or UWE (Fig. 1 *b*; at 0–0.4 mg/ml) at low concentrations and at higher concentrations, plateau values were obtained for both division number (1.7–2.0) and [^{3}H]thymidine incorporation (40–50% of control values).

In further studies, the inhibitors were used at the following concentrations: EE (2 mg/ml), UWE (1 mg/ml), dbc-AMP (0.3 mM).

Inhibition of ^{3}H-Labeled Thymidine, Uridine, Adenosine, or Choline Incorporation

Although there was a delay before growth arrest, each inhibitor produced rapid changes in the incorporation of [^{3}H]nucleosides into acid-insoluble material (Fig. 2). When P815Y cells, in logarithmic phase of growth, were treated with EE (Fig. 2 *a*) there was a simultaneous reduction in the incorporation of the three nucleosides. Differences from control cultures were apparent within 15 min and maximal at 5 h, yet nucleoside incorporation was never reduced to base-line values. Surprisingly, EE had no effect on [^{3}H]choline incorporation in the time interval 0–20 h.

In contrast, there were differences in the times required for UWE to affect the incorporation of each nucleoside (Fig. 2 *b*). Changes in [^{3}H]thymidine incorporation were evident at 30 min followed by a later reduction for [^{3}H]adenosine (3 h) or [^{3}H]uridine and [^{3}H]choline (7 h). Consistent plateau values for nucleoside incorporation, approximately equal to 40% of control cultures, were obtained in the time interval 10–25 h.

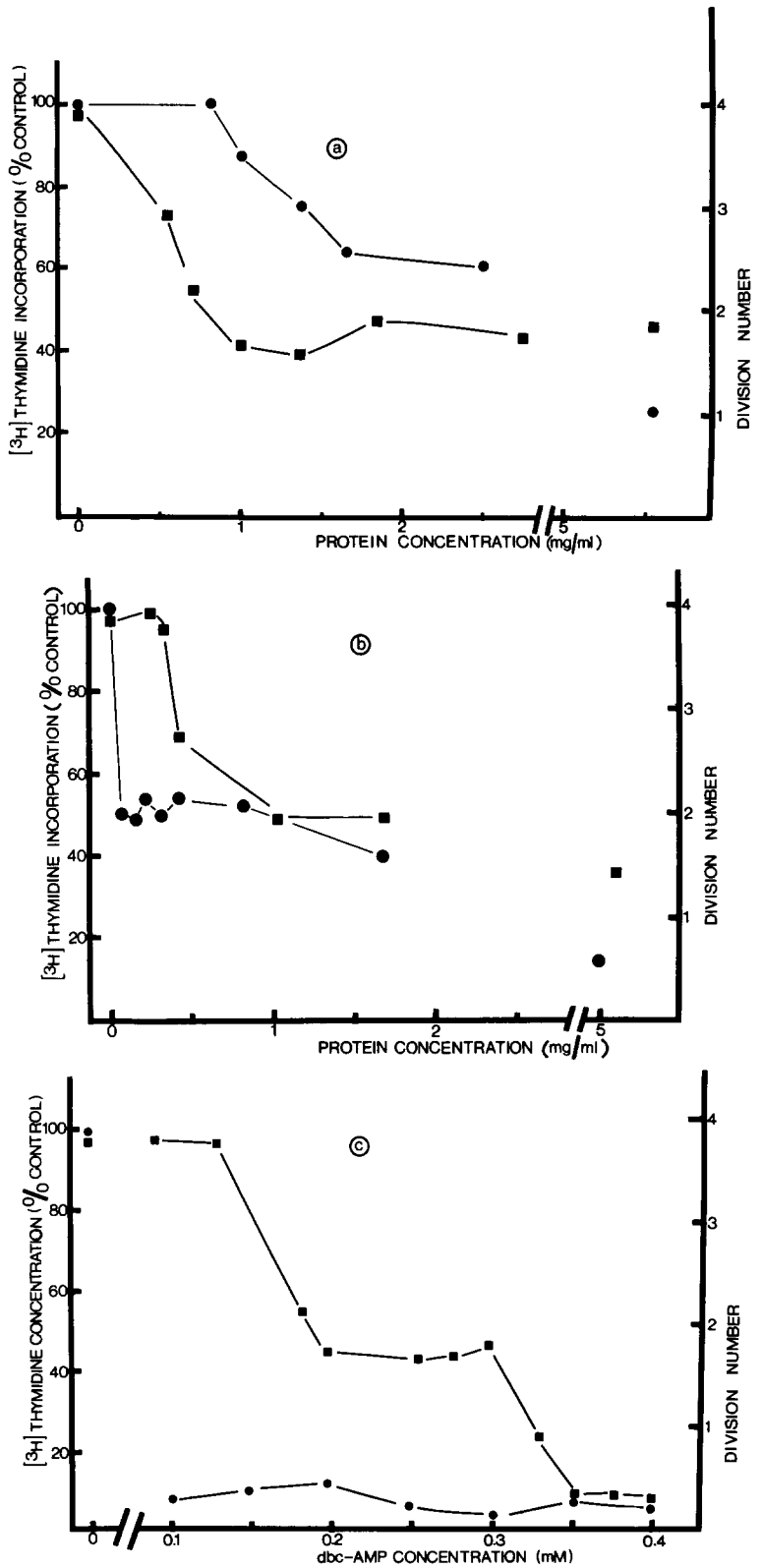

FIGURE 1 Effect of different inhibitor concentrations on asynchronous cell cultures. Exponentially growing cells (1×10^5/ml) were incubated with (*a*) EE, (*b*) UWE, (*c*) dbc-Amp; incorporation of [^{3}H]-thymidine into DNA was determined after 18 h, and final cell density at 72 h. Values for division number were computed from the relative increase in cell density. (●——●) [^{3}H]thymidine incorporation; (■——■) division number.

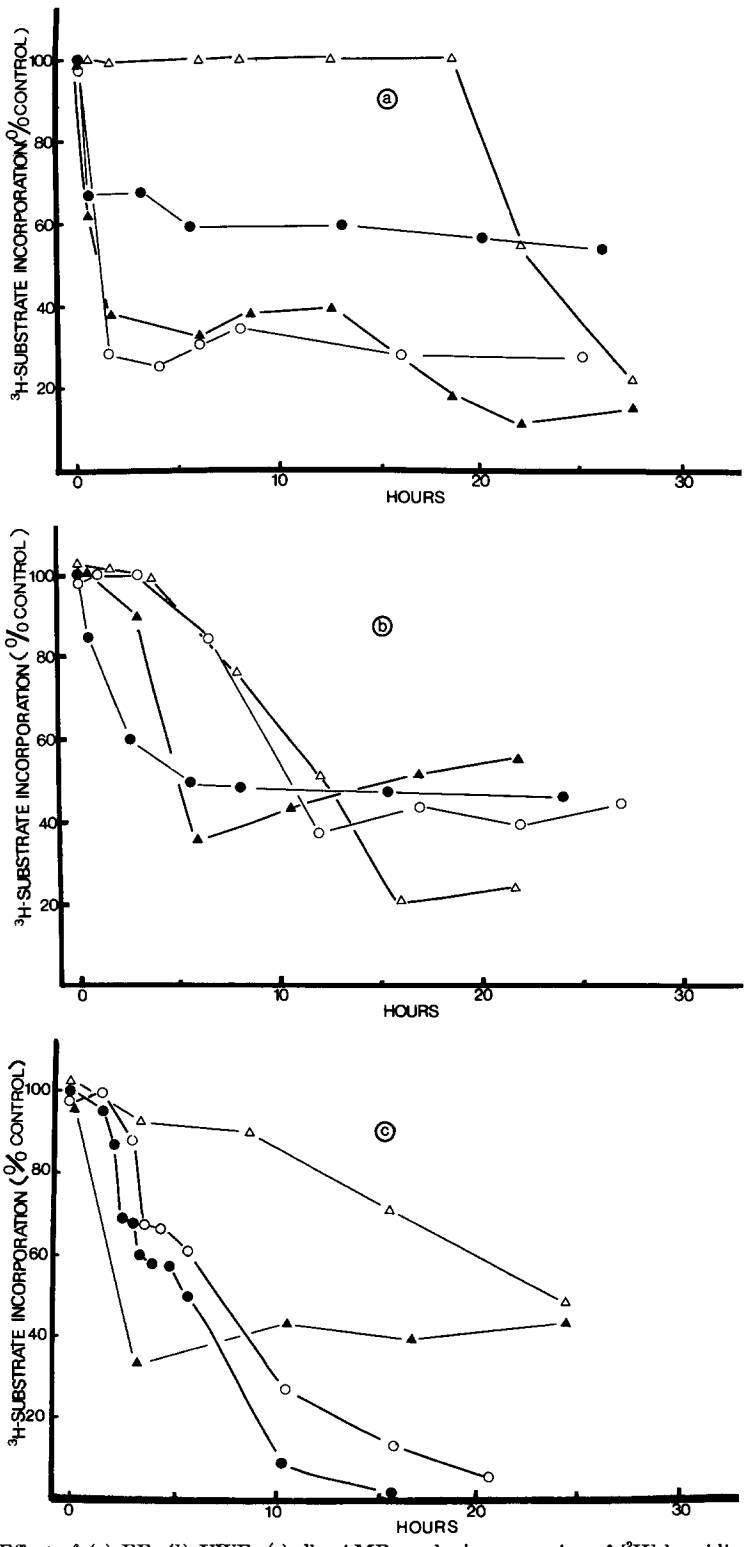

FIGURE 2 Effect of (*a*) EE, (*b*) UWE, (*c*) dbc-AMP on the incorporation of [^{3}H]thymidine (●——●), [^{3}H]uridine (○——○), [^{3}H]adenosine (▲——▲), or [^{3}H]choline (△——△) into acid-insoluble material. At the time indicated, triplicate samples (2 ml) were incubated with labeled substrate for 30 min at 37°C and the incorporation of radioactivity into acid-insoluble material determined.

There was a striking similarity in the effects of dbc-AMP on [^{3}H]adenosine incorporation (Fig. 2 *c*) while [^{3}H]thymidine and [^{3}H]uridine incorporation were reduced to base-line values by 15 h, and [^{3}H]choline was only marginally reduced by 20 h.

The above changes were specific and rapid, suggesting that each inhibitor had selective effects on substrate incorporation rather than eliciting general changes in membrane transport and/or substrate phosphorylation.

In the presence of EE or UWE, the incorporation of each nucleoside was reduced to consistent plateau values and, for EE-treated cells, [^{3}H]-choline incorporation was not significantly different from control cultures. Similarly, there was a selective effect of dbc-AMP on [^{3}H]thymidine and [^{3}H]uridine incorporation.

Kinetics of Substrate Incorporation

The purpose of these experiments was to determine whether the inhibitors produced both qualitative and quantitative changes in the incorporation of radiolabeled substrates. Cell cultures were treated with EE, UWE, or dbc-AMP for 5 h and the rates of incorporation of [^{3}H]nucleosides (Figs. 3–5) or [^{3}H]choline (Fig. 6) into acid-insoluble material determined.

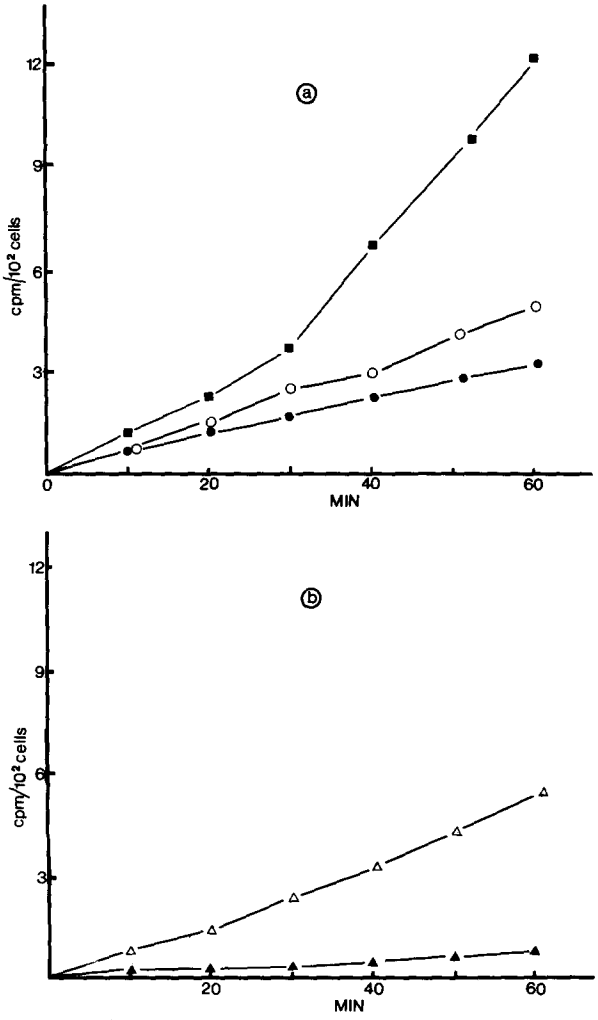

FIGURE 3 Rate of uptake of [^{3}H]thymidine into acid-insoluble material after treating cells with different inhibitors. (*a*) Control cultures (■——■), EE-treated (●——●), or UWE-treated (○——○) cultures at 5 h. (*b*) dbc-AMP-treated cultures at 5 h (△——△) and 20 h (▲——▲). Mean values for three separate determinations.

In control cultures the rates of incorporation of ^{3}H-labeled thymidine (Fig. 3 *a*), uridine (Fig. 4 *a*), adenosine (Fig. 5 *a*), or choline (Fig. 6 *a*) were biphasic with time, while in treated cultures, with the exception of [^{3}H]uridine for UWE-treated cells (Fig. 4 *a*) and [^{3}H]adenosine for dbc-AMP-treated cells (Fig. 5 *b*) the reduced rates of incorporation were approximately linear. No difference was found for the incorporation of [^{3}H]choline (Fig. 6 *a*). After treating tumor cultures with dbc-AMP for 20 h, base-line values were obtained for [^{3}H]thymidine (Fig. 3 *b*) or [^{3}H]uridine (Fig. 4 *b*) incorporation, [^{3}H]adenosine uptake (Fig. 5 *b*) was reduced, and the kinetics for [^{3}H]choline uptake (Fig. 6 *b*) were linear with respect to time.

DISCUSSION

There is no evidence to suggest a causal relationship between the above changes in nucleoside incorporation and events leading to growth arrest. Each inhibitor produced rapid changes in nucleoside incorporation (Fig. 2) yet tumor growth was arrested after a further increase in division number of 1.7–2.0 (Fig. 1). Also, different dose-response curves were obtained for cytostasis and inhibition of [^{3}H]thymidine incorporation into DNA (Fig. 1).

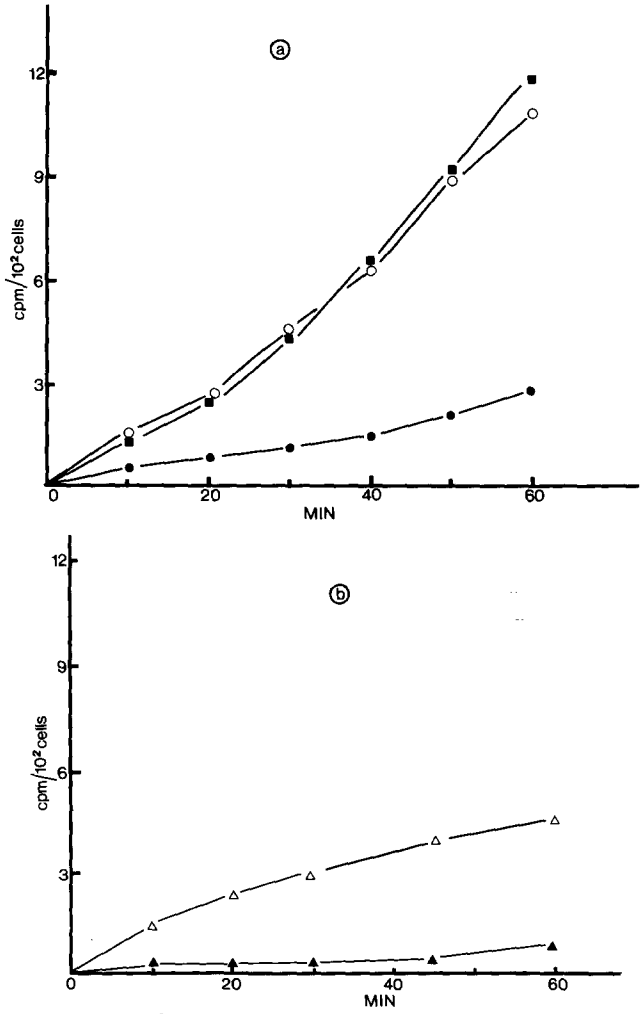

FIGURE 4 Rate of uptake of [^{3}H]uridine into acid-insoluble material after treating cells with different inhibitors. (*a*) Control cultures (■——■), EE-treated (●——●), or UWE-treated (○——○) cultures at 5 h. (*b*) dbc-AMP-treated cultures at 5 h (△——△) and 20 h (▲——▲). Mean values for three separate determinations.

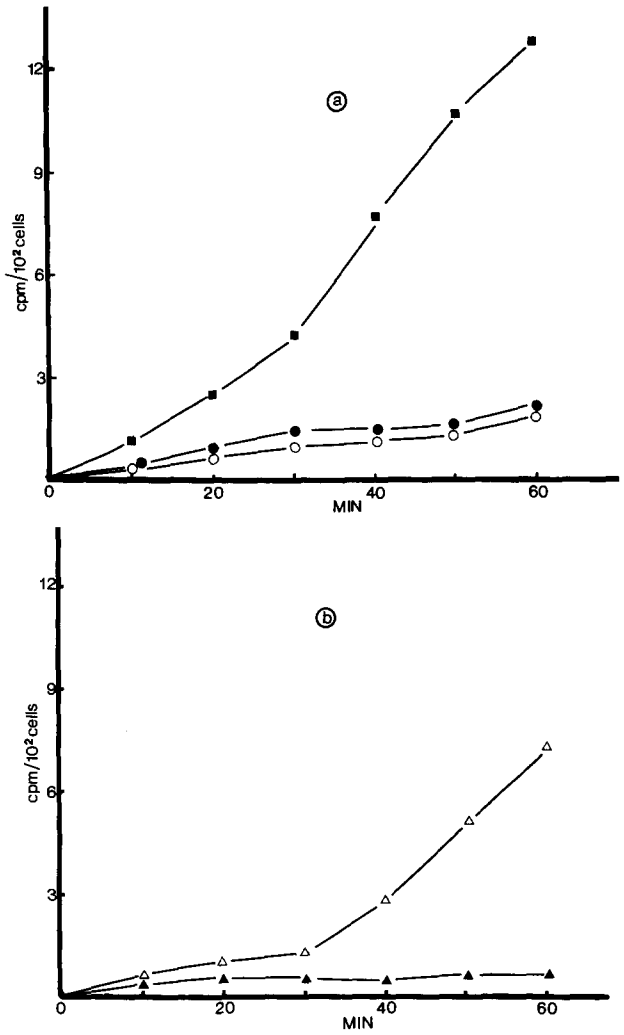

FIGURE 5 Rate of uptake of [^{3}H]adenosine into acid-insoluble material after treating cells with different inhibitors. (*a*) Control cultures (■——■), EE-treated (●——●), or UWE-treated (○——○) cultures at 5 h. (*b*) dbc-AMP-treated cultures at 5 h (△——△) and 20 h (▲——▲). Mean values for three separate determinations.

Yet it is a remarkable coincidence that each growth inhibitor had similar and specific effects on membrane transport (or phosphorylation). After treating asynchronous cultures for 5 h with EE or UWE, the kinetics for the incorporation of [^{3}H]thymidine (Fig. 3) or [^{3}H]adenosine (Fig. 5) were changed from normal biphasic modes to linear rates of incorporation, while the incorporation of [^{3}H]choline (Fig. 6) did not differ from cultures.

Similar changes were found after brief treatment (5 h) of tumor cells with dbc-AMP. The rates of incorporation of [^{3}H]thymidine (Fig. 3 *b*) and [^{3}H]uridine (Fig. 4 *b*) were also linear with time, that of [^{3}H]adenosine (Fig. 5 *b*) was quantitatively reduced, and the incorporation of [^{3}H]choline (Fig. 6 *b*) into phospholipid was unaltered. However, with longer incubation periods (20 h) the incorporation of [^{3}H]thymidine (Fig. 3 *b*) and [^{3}H]-uridine (Fig. 4 *b*) were completely inhibited, [^{3}H]-adenosine (Fig. 5 *b*) was reduced, and the rate of incorporation of [^{3}H]choline (Fig. 6 *b*) was linear with time.

There has been no attempt to estimate the activity of nucleoside kinases in cell-free extracts of the P815Y tumor after treatment with the above

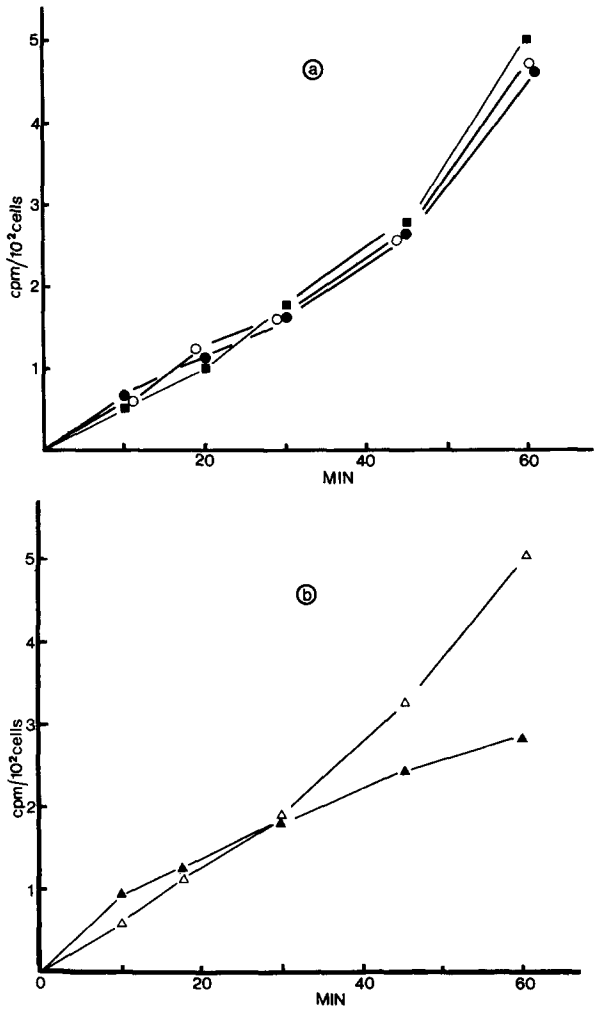

FIGURE 6 Rate of uptake of [^{3}H]choline into acid-insoluble material after treating cells with different inhibitors. (*a*) Control cultures (■——■), EE-treated (●——●), or UWE-treated (○——○) cultures at 5 h. (*b*) dbc-AMP-treated cultures at 5 h (△——△) and 20 h (▲——▲). Mean values for three separate determinations.

inhibitors; it remains to be shown whether membrane transport or intracellular phosphorylation are primarily affected.

Similar effects of dbc-AMP on nucleoside transport have been reported for other cell lines in vitro (Hauschka et al., 1972; Kram et al., 1973), and Hauschka et al. (1972) have suggested that dbc-AMP has two separate effects on nucleoside incorporation: an initial effect on the cell membrane or membrane-bound carriers and a later inhibition of intracellular thymidine kinase. The present results would support such a view.

It is reasonable to consider that there are two possible mechanisms for the uptake of thymidine, uridine, or adenosine: a specific carrier mechanism which can be blocked by EE, UWE, or dbc-AMP, and an alternative pathway whereby nucleosides diffuse across the cell membrane and are phosphorylated intracellularly by a second nucleoside kinase. Perhaps the most convincing evidence for carrier-mediated transport of nucleosides is provided by the isolation of "transport mutants." After treatment of Chinese hamster cells with high activity [^{3}H]thymidine (Breslow and Goldsby,

1969) mutants were isolated which failed to incorporate exogenous [^{3}H]thymidine into DNA in spite of normal intracellular levels of thymidine kinase. The defect was attributed to alterations at the cell membrane. In addition, two forms of thymidine kinase have been found in tumor cells (Okuda et al., 1972).

Certain analogies can be drawn between the effects of EE, UWE, and dbc-AMP on P815Y cells and those obtained by adding fresh serum to confluent 3T3 cells (Todaro et al., 1967; Cunningham and Pardee, 1969). In each case there is a rapid change in nucleoside incorporation: serum stimulates uptake and incorporation is changed from linear to biphasic rates (Cunningham and Pardee, 1969). EE, UWE, and dbc-AMP inhibit nucleoside uptake and there is a change from biphasic to linear rates of incorporation (Figs. 3–5). Also, stimulation and inhibition are independent of *de novo* DNA and RNA synthesis although cell growth is ultimately affected.

EE and UWE are relatively crude extracts which were prepared according to the procedure (Rossdale, 1973) for the isolation of colony stimulating factor(s). Further fractionation of each extract is necessary to determine whether inhibition of tumor cell growth and nucleoside incorporation, or the stimulation of bone marrow granulocyte precursors in vitro are due to a single component.

There is no nutritional requirement for thymidine, uridine, and adenosine in vitro, and growth arrest is not necessarily a consequence of altered nucleoside transport yet a further study of these events may provide an insight into the role of the cell membrane in growth regulation.

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