[CANCER RESEARCH 35, 3181-3186, November 1975]

The Role of Adenosine Triphosphate, Chalones, and Specific Proteins in Controlling Tumor Growth Fraction[1]

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SUMMARY

During growth of Ehrlich ascites tumor cells *in vivo*, the proportion of cells in the S phase of the proliferative cell cycle decreases in a manner analogous to the decreasing growth fraction often associated with the growth of solid tumors. An examination of biochemical parameters that might regulate the growth fraction of Ehrlich ascites tumors by causing accumulation of cells in G_1-G_0 shows that (*a*) the tumor progresses from an aerobic to an anaerobic state as it approaches the plateau phase of growth, as indicated by lactate dehydrogenase content, but cellular adenosine triphosphate content remains constant; (*b*) tumor-specific growth inhibitors (chalones) are not detectable in cell-free ascites fluid from plateau-phase tumors; (*c*) electrophoretically identifiable soluble proteins isolated from tumor cells that have been exposed to labeled amino acids *in vivo* are qualitatively identical during early and late tumor growth; and (*d*) ornithine decarboxylase activity increases in a bimodal fashion in the first 10 hr after transplantation of 10^7 cells and then declines rapidly during the first few days of growth. The second (and larger) of the two ornithine decarboxylase increases coincides with the surge of cells from G_1-G_0 into S phase, suggesting that this enzyme, or the polyamines that it synthesizes, may play a role in controlling the growth fraction of this cell population.

INTRODUCTION

During tumor growth, the cells pass from a period of exponential (or quasiexponential) growth through a poorly defined transition period into plateau phase. During this process the cell cycle sometimes lengthens, at least in ascites tumors (24), and the proportion of cells in the proliferative cell cycle (the growth fraction[3]) often decreases as cells accumulate in G_1-G_0 (15, 17, 24). Although the extent of this accumulation can be manipulated by altering diets (1) or oxygen tension (30) or by transplanting a portion of the tumor (15), the factors that control cellular traffic into and out of the proliferative cycle in a growing tumor are poorly understood.

To study the mechanisms involved in control of the growth fraction, we used ascites tumors, to which both cytokinetic and biochemical techniques are easily applied. In this report we extend our earlier studies (1, 15–17) to the roles of energy supply, endogenous growth inhibitors (chalones), and specific proteins. Although the results are not definitive, they implicate protein synthesis, particularly ornithine decarboxylase synthesis, as a significant factor in growth fraction control.

MATERIALS AND METHODS

Tumor. A hyperdiploid Ehrlich ascites tumor, obtained from Dr. H. van den Brenk (Cancer Institute, Melbourne, Australia) in 1967 and carried in 8- to 12-week-old female Swiss-Webster mice (Charles River Breeding Laboratories, Wilmington, Mass.) by weekly i.p. inoculation of 10^7 cells, was used in all experiments. In some cases, cell-free ascites fluid was also obtained from plateau-phase P388 lymphocytic leukemia tumors growing in DBA/2 mice (The Jackson Laboratory, Bar Harbor, Maine) (17).

Biochemical Assays. ATP was extracted with cold 6% perchloric acid, brought to pH 7.5 with K_2CO_3, and assayed with hexokinase or firefly extract (Sigma Chemical Company, St. Louis, Mo.) (3). To collect ascites cells without exposing them to oxygen, the skin was resected, the peritoneum was punctured with a Pasteur pipet, and cells were collected and pipetted immediately into cold 6% perchloric acid. Exogenous ATP (Sigma Chemical Company) was added to 1 extract as a positive control, and results were corrected for loss of ATP during extraction. Other samples of ascites fluid were diluted for cell counting in a Model B Coulter counter.

Soluble proteins were analyzed by polyacrylamide gel electrophoresis (9) using a Canalco Model 1200 apparatus (Canal Industrial Corporation, Rockville, Md.). Twenty min after i.p. injection of labeled amino acid (New England Nuclear, Boston, Mass.), ascites tumor cells were collected, washed 3 times with cold 0.8% NaCl solution, and resuspended at 2×10^8/ml in cold distilled water. The washed cells were homogenized for 3 min with a motor-driven ground-glass homogenizer, and a concentrated solution of sucrose and salts was then added to yield a final concentration of 0.25 M KCl, 0.01 M $MgCl_2$, and 0.05 M Tris (pH 7.6). This mixture was centrifuged at $10,000 \times g$ for 10 min, and

[1] Work performed under the auspices of the United States Energy Research and Development Administration.

[2] Supported by NIH Biophysics Training Grant GM-00829.

[3] The term "growth fraction" is used in this paper in its most general sense. We recognize that the "non-cycling" cells in the Ehrlich ascites tumor may be delayed in G_1 rather than shunted into a biochemically distinct G_0 phase, and we use the term "recycling" to refer to the parasynchronous surge of cells into S phase after transplantation, without necessarily implying that these cells come from a true G_0 phase.

Received June 12, 1975; accepted August 11, 1975.

the supernatant was then centrifuged for another 90 min at 105,000 × g and then analyzed for protein (26). Two hundred μg of protein were applied to each polyacrylamide gel (63 × 7 mm) and subjected to electrophoresis at 4° for 130 min (4 ma/gel). Some gels were stained with Coomassie blue and destained with 7% acetic acid, and others were sliced into 2-mm pieces for counting. Each slice was placed in a scintillation vial and dissolved overnight in 0.5 ml of 0.1% SDS.[4] Six ml of Aquasol were then added, and the vials were counted in a Packard Tri-Carb scintillation counter.

LDH activity was measured in twice-washed cells resuspended in 0.8% NaCl solution (25°) at 5 × 10^5/ml and treated sonically for 30 sec. Kornberg's spectrophotometric assay (23) was used, with several dilutions of each sample. LDH isozymes were separated on polyacrylamide gels by the method of Dietz *et al.* (11); all 5 of the LDH isozymes present in homogenates of mouse kidney could be resolved by this technique.

ODC was assayed by a modification of the method of Pegg and Williams-Ashman (27), using an organic base to trap the released $^{14}CO_2$. Cells were washed twice and resuspended at 0.5 to 4 × 10^6/ml in 0.05 M phosphate buffer (pH 7.2) containing 0.1 mM EDTA. The suspension was subjected to 6 cycles of freezing and thawing and then was centrifuged at 10,000 × g for 30 min. ODC was assayed in a reaction mixture consisting of 0.5 ml of the 10,000 × g supernatant, 0.1 μmole of pyridoxal phosphate, and 0.2 μmole of DL-[1-^{14}C]ornithine monohydrochloride (2.7 mCi/mmole) in a total volume of 2 ml. Neither mercaptoethanol nor diethiothreitol was included in the reaction mixture, because preliminary experiments indicated that these agents did not alter the result and because dithiothreitol interferes with protein determinations. The assay was run with buffer blanks, because these gave the same result as blanks consisting of boiled supernatant. After incubation at 37° for 60 min, the reaction was stopped by injecting 0.5 ml of 5 N H_2SO_4 into the reaction flask. The entire polypropylene center well, together with its contents, was placed in a scintillation vial with 8 ml of Aquasol (New England Nuclear) and counted in a Packard Tri-Carb scintillation counter.

RESULTS

The Aerobic-Anaerobic Transition and Cellular ATP. Ascites tumors become hypoxic during growth, as evidenced by differences in radiosensitivity *in vivo* and *in vitro* (2) and by oxygen-electrode measurements (10). To determine when the transition from aerobic to anaerobic growth takes place in Ehrlich ascites tumors, we assayed the enzyme LDH, which is known to be elevated in anaerobic mammalian cells (6). The LDH activity of Ehrlich ascites cells increased only slightly during exponential growth (Days 0 to 4) but rose sharply during the transition period between exponential growth and plateau phase (Days 4 to 6) (Chart 1). Enzyme activity remained elevated, at 20.7 units/mg protein,

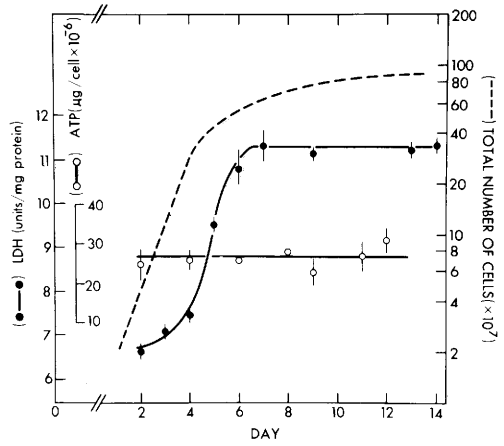

Chart 1. Lactate dehydrogenase (LDH) activity (●) and ATP content (○) of Ehrlich ascites tumor cells during growth in Swiss-Webster mice. *Points*, mean of 3 to 8 experiments; *vertical bars*, S.D., - - -, tumor growth curve. One unit of LDH is defined as the amount that causes an initial rate of oxidation of 1 μmole of NADH per min per mg of protein.

throughout plateau phase. If increased LDH activity reflects anaerobic growth in the Ehrlich ascites cell, as it does in others, this result indicates that the tumor changes from aerobic to anaerobic between late exponential growth and early plateau phase.

Only 1 LDH isozyme was detected in Ehrlich ascites cells harvested on Days 2 and 14. This isozyme corresponded to LDH-5, the MMMM isozyme typical of anaerobic growth (7, 19).

Despite the shift from aerobic to anaerobic growth, cellular ATP content remained constant throughout tumor growth (Chart 1), indicating that plateau-phase Ehrlich ascites cells did not suffer from a deficient energy supply.

Chalones and Tumor Growth Fraction. We investigated the role of chalones in the Ehrlich ascites tumor by performing 2 sets of experiments.

In the 1st set, mice bearing exponentially growing tumors (3 days after inoculation of 10^7 cells) received 1 or 2 2-ml i.p. injections of either 0.8% NaCl solution or cell-free ascites fluid collected from plateau-phase Ehrlich ascites tumors (13 day) or plateau-phase P388 tumors (7 day). The mice were divided into 2 groups: mice in 1 group were given [^{3}H]thymidine i.p. at various intervals after injection of the tumor and were killed 20 min later for autoradiographic determinations of the [^{3}H]thymidine-labeling index; mice in the other group received a single i.p. injection of Colcemid (1 mg/kg) and were killed 4 hr later for determination of the mitotic index. The results show that ascites fluid from plateau-phase tumors caused the [^{3}H]thymidine index of exponentially growing tumors to decrease by 15 to 20% after 4 hr (Chart 2). However, the injection of 0.8% NaCl solution caused an identical effect, as did fluid from P388 ascites tumors. The mitotic index, on the other hand, showed no change 4 hr after injection of ascites fluid [control, 12.7% (range, 10.2 to 16.0%); ascites fluid, 12.8% (range, 10.0 to 16.3%)].

In the 2nd set of experiments, we examined the effect of plateau-phase ascites fluid on the recycling of G_1-G_0 cells

[4] The abbreviations used are: SDS, sodium dodecyl sulfate; LDH, lactate dehydrogenase; ODC, ornithine decarboxylase.

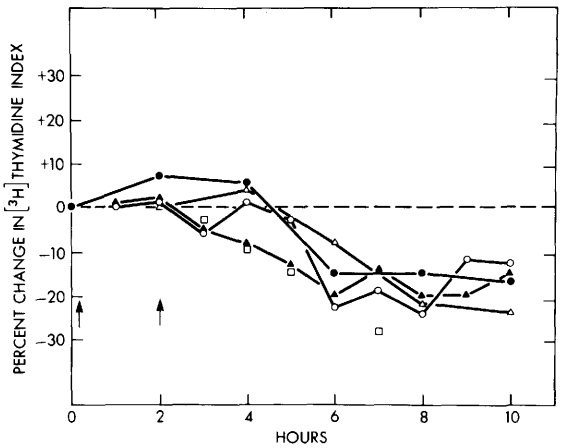

Chart 2. Effect of ascites fluid on the [^{3}H]thymidine-labeling index of exponentially growing Ehrlich ascites cells *in vivo*. Mice bearing 3-day-old tumors were given i.p. injections (2 ml each) of 0.8% NaCl solution (●, 0 time only; ▲, 0 time and at 2 hr), cell-free ascites fluid from 13-day-old Ehrlich tumors (○, 0 time only; △, 0 time and at 2 hr), or cell-free ascites fluid from 7-day-old P388 tumors (□, 0 time only). [^{3}H]thymidine was injected i.p. 20 min before sacrifice. *Points*, cells from 4 to 6 mice. Zero-time control values were between 57 and 59% in each of the 3 experiments represented here.

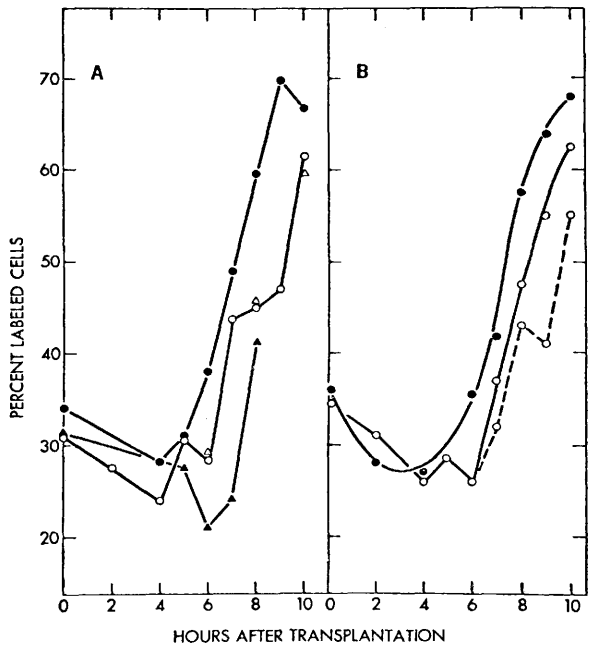

Chart 3. [^{3}H]Thymidine-labeling index of Ehrlich ascites cells in the hours after transplantation of 10^7 cells from 14-day-old plateau-phase tumors. *A*, effects of ascites fluid from plateau-phase Ehrlich tumors (○), ascites fluid from plateau-phase P388 tumors (△), inflammatory exudate fluid (▲), and 0.8% NaCl solution (●). Two ml of each fluid were injected i.p. immediately after transplantation and again 5 hr later. *B*, effects of a single injection of ascites fluid (2 ml at 0 hr) (○——○), multiple injections of ascites fluid (2 ml each at 0 and 5 hr, 1 ml each at 6, 7, and 8 hr) (○- - -○), and 0.8% NaCl solution (●).

into S phase after transplantation of 10^7 plateau-phase cells into new mice. When transplantation was followed by i.p. injection of 2 ml of cell-free ascites fluid from 14-day tumors and a 2nd injection of the same fluid 5 hr later, the rise of the [^{3}H]thymidine index was delayed slightly (Chart 3*a*). This effect was not limited to Ehrlich ascites fluid, however. Ascites fluid from plateau-phase P388 tumors gave an identical result, and inflammatory exudate collected from normal mice 3 to 4 days after i.p. implantation of small sponges was even more effective (Chart 3*a*). Increasing the number of injections of Ehrlich fluid from 1 to 5 caused a somewhat longer delay (Chart 3*b*), but this, too, was duplicated by P388 ascites fluid (data not shown).

Synthesis of Soluble Proteins during Growth. The progressive decrease in the growth fraction of Ehrlich tumors is accompanied by a decreasing rate of protein synthesis (15). To test the possibility that plateau-phase cells accumulate in G_1-G_0 because they are unable to synthesize proteins essential for DNA synthesis, we harvested Ehrlich cells from early plateau-phase tumors (Day 6) and incubated them for various periods in amino acid-free medium at 37° before transplanting them into new hosts. This treatment increased the time required for the [^{3}H]thymidine index to rise to half-maximum, 1 hr of lag being induced for every 30 min of incubation (Chart 4). Cells incubated in medium containing amino acids did not show this effect, and omission of glucose did not cause it.

We next examined the synthesis of specific soluble proteins in young and old tumors, following the protocol used by Frindel *et al.* (14). Two hundred μCi of [^{3}H]leucine (New England Nuclear; >98% radiochemical purity) were injected i.p. into exponentially growing (4-day-old) and plateau-phase (11-day-old) Ehrlich ascites tumors, and the cells were collected 20 min later. After being washed at least 3 times with 0.8% NaCl solution,[5] the cells were homogenized and prepared for electrophoresis (14). The results (Chart 5*a*) confirmed the observation of Frindel *et al.* (14) that 2 peaks of radioactivity appear in gels prepared from 4- and 11-day-old tumors, the older tumors containing more radioactivity than the younger in the mid-gel peak. However, when we added [^{3}H]leucine to a 105,000 × *g* supernatant prepared from unlabeled cells and subjected this material to electrophoresis, radioactivity appeared in 2 peaks identical to those observed with "labeled" cells (Chart 5*a*). Moreover, [^{3}H]leucine put on the gel alone (without protein) ran to the same position. Identical results were obtained with [^{3}H]leucine purchased from other suppliers and with [^{3}H]valine (data not shown). Other workers have reported similar results (13).

When a 105,000 × *g* supernatant was prepared from [^{3}H]leucine-labeled cells and treated with Sephadex G-200 to remove free amino acids before electrophoresis, there

[5] Electrophoretograms of proteins extracted from Ehrlich ascites tumor cells that had been washed only once contained a Coomassie blue-stainable band about half-way down the gel. This band was not present when the cells were washed 3 times before homogenization. An identical band was found in cell-free ascites fluid, and the addition of ascites fluid to thrice-washed cells, followed by a single wash, resulted in the reappearance of this band in the "cellular protein." These observations demonstrate that at least 1 protein from ascites fluid can associate with the cell membrane and be eluted during homogenization to appear in the gels as a soluble cellular protein unless the cells are washed thoroughly.

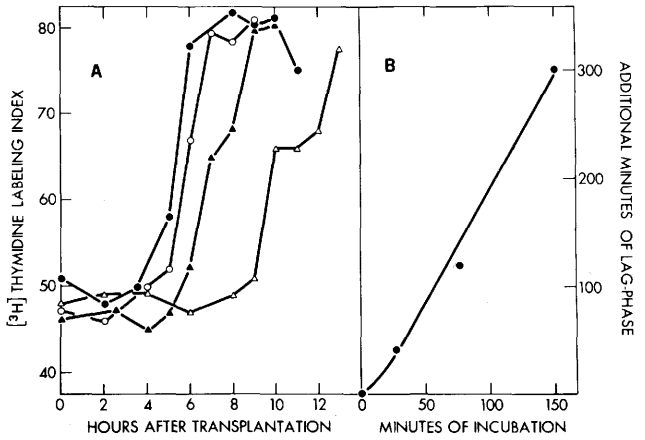

Chart 4. *A*, effect of *in vitro* incubation on the [^{3}H]thymidine-labeling index of transplanted Ehrlich ascites tumors. Cells were collected from 6-day-old tumors (early plateau phase), washed, resuspended in Eisen's medium containing 0.2 M Hepes buffer (pH 7.3), and either kept on ice or incubated at 37° with gentle rocking for various times before transplantation. ●, control, 0° for 150 min; ○, 37° for 30 min; ▲, 37° for 75 min; △, 37° for 150 min. *B*, relationship between incubation time and lag phase after transplantation. The *points* were obtained by taking the time required for the labeling curves to reach half-maximum and subtracting the control value.

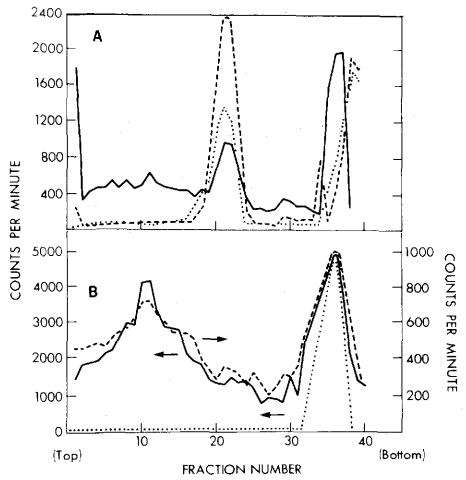

Chart 5. Distribution of radioactivity in polyacrylamide gels prepared from a 105,000 × *g* supernatant of Ehrlich ascites tumor cells labeled for 20 min *in vivo*. *A*, distribution of [^{3}H]leucine. Isotope (200 μCi in 0.2 ml) was injected i.p. and polyacrylamide gel electrophoresis was performed as described in "Materials and Methods." ——, 4-day-old cells; – – –, 11-day-old cells; · · · ·, [^{3}H]leucine (0.3 μCi) added to the supernatant from unlabeled cells. Each fraction was 2 mm; gels were run at 4 ma/gel for 130 min. *B*, distribution of [^{3}H]phenylalanine. In this experiment, the decreased rate of protein synthesis by old tumors was partially compensated for by injecting 100 μCi of [^{3}H]phenylalanine into 4-day-old tumors and 400 μCi into 11-day-old tumors; the counts obtained are presented on separate scales to permit direct comparison of the distributions of radioactivity in young and old tumor cells. ——, 4-day-old tumors; – – –, 11-day-old tumors; · · · ·, [^{3}H]phenylalanine added to the supernatant from unlabeled cells.

were no discrete peaks of radioactivity in the mid-gel region and no differences between young and old tumors. Taken together, these results indicate that the "soluble protein" described by Frindel *et al.* (14) was free leucine rather than leucine-labeled protein. That old tumors contained more of this material than young ones (Chart 5*a*) (14) is probably explained by the fact that old tumors synthesize protein more slowly than young ones (15), thereby leaving more [^{3}H]leucine in the intracellular pool to be extracted into the soluble protein fraction.

We then repeated the experiment using [^{3}H]phenylalanine as the tracer, compensating for the lower rate of protein synthesis in old tumors by injecting them with 4 times the amount of labeled precursor used for young tumors. Phenylalanine alone ran as a single peak, and the gel patterns from young and old tumors injected with this precursor were essentially identical (Chart 5*b*), as were those in the water-insoluble, SDS-soluble proteins (data not shown). SDS-polyacrylamide electrophoresis (12) of the 105,000 × *g* supernatant also disclosed no differences between cells harvested on Days 2, 4, 7, 11, and 14. All contained at least 4 major components (with molecular weights of 84,000, 52,000, 44,000, and 23,000) and multiple minor bands.

ODC Activity during Growth. The activity of the polyamine-synthesizing enzyme ODC changed dramatically during growth of the Ehrlich tumor (Chart 6). The enzyme was barely detectable in late plateau-phase tumors, but transplantation of 10^7 cells from these tumors into new mice was followed by a rapid and large increase in ODC activity within hours (Chart 6). By 2 hr after transplantation, ODC had increased several-fold, and a peak occurred at 5 hr, just before the [^{3}H]thymidine index began to rise. By 8 hr, ODC activity had fallen by a factor of 2 but then rose a 2nd time,

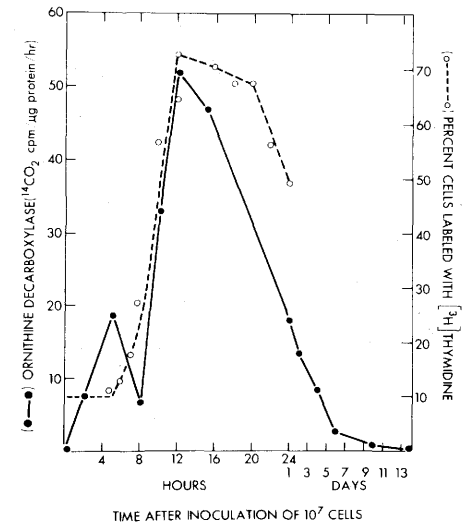

Chart 6. ODC activity and [^{3}H]thymidine-labeling index of plateau-phase Ehrlich ascites tumor cells collected at various times during growth *in vivo* after i.p. inoculation of 10^7 plateau-phase (14-day-old) cells. Cells from 2 to 4 mice were pooled for each assay, and all determinations were done in duplicate. The *ODC curve* represents data from a single experiment; similar results were obtained in repeat experiments, in which the peak at 5 hr varied between 12.8 and 20.4 cpm of $^{14}CO_2$ released per μg of protein per hr of incubation, the average activity at 8 hr was 58% of the 5-hr value, and the average activity at 12 hr was 297% of the 5-hr value. ●, ODC activity; ○, [^{3}H]thymidine-labeling index.

beginning at 9 to 10 hr. This 2nd increase paralleled the increase in the [^{3}H]thymidine index and reached its maximum at 12 hr, when the [^{3}H]thymidine index also peaked (Chart 6). ODC activity then decreased exponentially over the next 4.5 days, with a half-time of 7 hr, whereas the [^{3}H]thymidine index plateaued for approximately 1 S phase and then fell also.

DISCUSSION

Many factors may act to prevent mammalian cells from moving into S phase from G_1-G_0 (21). In the complex situation of a tumor growing *in vivo*, it is exceedingly difficult to discover which microenvironmental factors (*e.g.*, deprivation of critical nutrients, decreased oxygen tension, or the presence of growth-inhibiting substances) are responsible for decreasing the growth fraction, and it is even more difficult to determine the biochemical mechanisms by which such factors act. As a result, one is often limited to inferences drawn from the temporal relationships between biochemical and cytokinetic events. Although this limitation applies to this study, several conclusions seem warranted.

First, it is clear that the Ehrlich tumor does not suffer a gross deficit of ATP during growth, even though it changes from aerobic to anaerobic between the 4th and 6th days (Chart 1). Since the [^{3}H]thymidine-labeling index declines linearly over virtually the entire period of growth (15), there is no clear relationship between ATP supply and the growth fraction.

Second, in contrast to the results obtained by Bichel (4, 5) with other tumors, ascites fluid from plateau-phase Ehrlich tumors does not contain a specific chalone capable of depressing the growth fraction. Although injection of ascites fluid into exponentially growing or freshly transplanted tumors did change the [^{3}H]thymidine-labeling index, other proteinaceous fluids, and indeed sometimes a 0.8% NaCl solution alone, had identical effects (Charts 2 and 3). The report that ascites fluid chalones must be activated by adrenalin and hydrocortisone to be effective (8) does not seem relevant to our experiments, because we have no reason to believe that our animals were deficient in these substances.

Third, and most important, our data suggest that changes in protein synthesis may play a role in regulating tumor growth fractions. Although young and old Ehrlich tumors had the same amounts of electrophoretically identifiable soluble proteins (Chart 5), the regulating enzyme ODC varied markedly with tumor age, increasing in a bimodal fashion in the hours after transplantation and then declining to a very low level in the days after the 2nd peak (Chart 6). The increased ODC activity shortly after transplantation is consistent with the observation that a variety of G_0 cells synthesize ODC soon after they are stimulated to enter the proliferative cell cycle (18, 20, 22, 28), although a causal relationship between increased ODC and increased growth fraction has not been formally established in any of these cases. Indeed, in the Ehrlich tumor, it is possible that the 2nd rise in ODC activity may reflect, rather than cause, the movement of cells into S phase. Russell and Stambrook (29) reported recently that synchronized Chinese hamster V79 cells contain much higher ODC levels in S phase than in G_1, and if this is also true for Ehrlich cells, the temporal coincidence between the 2nd ODC increase and the increased [^{3}H]thymidine index (Chart 6) may be trivial rather than causal.

Perhaps of more immediate interest is the 1st ODC increase, which occurred well in advance of the increase in the [^{3}H]thymidine index (Chart 6). If this increase has a causal relationship to recycling, the most likely link between the two would be the synthesis of the polyamines, putrescine, spermine, and spermidine. Several reports suggest a relationship between polyamine synthesis (or content) and DNA synthesis, the most recent being plateau-phase WI38 cells that are stimulated to reenter the proliferative cell cycle by fresh medium (18). In this model (and others), putrescine synthesis follows closely behind an initial increase in ODC activity. The implication is that this polyamine somehow "turns on" or stabilizes the metabolic events that lead to DNA synthesis. Because we have observed a peak in putrescine content in transplanted Ehrlich tumors approximately coincident with the 1st ODC peak and just before increased DNA synthesis (C. R. Kehe, unpublished data), our working hypothesis is that this polyamine may represent an important control mechanism for growth fraction control in this system. Although this is the most reasonable hypothesis, other possibilities exist [for example, synthesis of specific "G_1 proteins," which may be required before a cell can move from G_1 into S (25)], and further work is required to determine its validity.

ACKNOWLEDGMENTS

We thank Dr. N. S. Ling for helping with the G-200 column analysis and the molecular weight determinations, and I. Ewing for technical assistance.

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