

ARGININE DEPRIVATION IN KB CELLS

I. Effect on Cell Cycle Progress

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ABSTRACT

When exponentially growing KB cells were deprived of arginine, cell multiplication ceased after 12 h but viability was maintained throughout the experimental period (42–48 h). Although tritiated thymidine ($[^3\text{H}]\text{TdR}$) incorporation into acid-insoluble material declined to 5% of the initial rate, the fraction of cells engaged in DNA synthesis, determined by autoradiography, remained constant throughout the starvation period and approximately equal to the synthesizing fraction in exponentially growing controls (40%). Continuous $[^3\text{H}]\text{TdR}$ -labeling indicated that 80% of the arginine-starved cells incorporated ^3H at some time during a 48-h deprivation period. Thus, some cells ceased DNA synthesis, whereas some initially nonsynthesizing cells initiated DNA synthesis during starvation. Flow microfluorometric profiles of the distribution of cellular DNA contents at the end of the starvation period indicated that essentially no cells had a 4c or G2 complement.

If arginine was restored after 30 h of starvation, cultures resumed active, largely asynchronous division after a 16-h lag. Autoradiographs of metaphase figures from cultures continuously labeled with $[^3\text{H}]\text{TdR}$ after restoration indicated that all cells in the culture underwent DNA synthesis before dividing.

It was concluded that the majority of cells in arginine-starved cultures are arrested in neither a normal G1 nor G2. It is proposed that for an exponential culture, i.e. from most positions in the cell cycle, inhibition of cell growth after arginine withdrawal centers on the ability of cells to complete replication of their DNA.

12 amino acids, including arginine (arg), plus glutamine are essential for mammalian cell growth in tissue culture (4). Deletion of any one of these amino acids from the culture medium leads to complete arrest of cell growth and division, and to cytopathic changes in the culture. The present study was begun as a corollary to an ongoing investigation in this laboratory of the specific inhibition of adenovirus replication observed in KB cells after arg deficiency (23, 25). The hypothesis was advanced that arg deprivation might

cause lesions in host cell functions that are crucial for virus growth as well. A promising way to look at changes in normal cellular functions is to study changes in the progress of cells through the growth cycle. This approach has been used by a number of investigators, and considerable evidence has accumulated that deficiency of various essential amino acids tends to arrest cell growth in the G1 phase of the cell cycle (2, 8, 14, 22, 28).

In this report we describe experiments which demonstrate that in the absence of exogenous

arg, the majority of the cells in an exponentially growing KB cell culture are arrested during the S-stage of the cell cycle. Further, DNA synthesis continues at a reduced rate after transfer to arg-free medium, and some initially nonsynthesizing cells are able to initiate DNA synthesis. In the accompanying paper we show that this synthesis is replicative in nature (30).

MATERIALS AND METHODS

Cells

The established human cell line, KB (5), was obtained from the American Type Culture Collection, CCL 17 (Rockville, Md.) and maintained in monolayer culture in Eagle's Minimal Essential Medium (6) containing 2× nonessential amino acids, 5% dialyzed calf serum, and 50 μg/ml gentamicin (Schering Corp., Kenilworth, N. J.). It was possible to check cells for evidence of mycoplasma contamination in most experiments by monitoring for cytoplasmic incorporation of tritiated thymidine (^3H TdR) during autoradiography (20). Less frequently, mycoplasma contamination was excluded by (a) monitoring microscopically for evidence of cytoplasmic or extracellular fluorescence in cell preparations treated with the fluorescent DNA-binding benzimidazole derivative, Hoechst 33258 (26), and (b) standard aerobic and anaerobic culture procedures (1). Periodic cultures for bacteria were also negative

Experimental Cultures

Eagle's Minimal Essential Medium with 2× nonessential amino acids and 5% calf serum was used in all experiments. Solutions of the essential amino acids were prepared from analytical grade reagents and combined at required concentrations, omitting arg (arg⁻) where designated. Calf serum was dialyzed against two successive 50-fold volumes of phosphate-buffered saline for 72 h, stored frozen, and used within 1 mo after dialysis. For experiments, approximately 1.5×10^5 trypsin-dispersed cells in 2.0 ml complete medium were dispensed to 35-mm plastic Petri dishes. Cultures were incubated at 37°C in an atmosphere of 95% air/5% CO₂ and fed with complete medium 24 h later. At the end of 36 h the cells were in the exponential phase of growth and the culture was several generations away from confluency, which is reached at $\sim 3 \times 10^6$ cells/dish (see Fig. 1). Experiments were initiated at this time. Control cultures in complete medium grew with a doubling time of 24 h. Cell cycle distributions and times, determined by standard methods (21), were: G1—50% of the population, 10 h; S—35%, 9 h; G2—10.5%, 3.5 h; M—4.5%, 1.5 h.

Cell Counts and Viability

Cell counts were performed on monodispersed cell suspensions with a Coulter electronic cell counter (Coul-

ter Electronics Inc., Hialeah, Fla.). Cell clumping was monitored by microscope examination of the suspensions. Cell viability was estimated by the exclusion of 0.1% trypan blue.

Tritiated Thymidine Incorporation

DNA synthesis was studied in cover slip cultures by measuring the incorporation of ^3H TdR into acid-insoluble material according to the method of Everhart (7). Cover slips were harvested, fixed with methanol-acetic acid (3:1), dehydrated in 95% and 100% ethanol, and extracted with HCl. Some cover slips were counted in a Nuclear Chicago (Nuclear-Chicago Corp., Des Plaines, Ill.) windowless gas flow counter. Subsequently, all cover slips were prepared for autoradiography by coating with NTB-3 emulsion (Eastman Kodak Co., Rochester, N. Y.), incubated for 5 days at 4°C, then developed in Dektol (Eastman Kodak Co.) and counterstained with Giemsa. Only nuclei overlaid with >16 grains were scored as positive; average nonspecific background was no more than three grains per nucleus and <0.1% of the cells showed grain counts between these values.

Preparation of Mitotic Figures

FOR OBSERVATION USING PHASE CONTRAST OPTICS: Cells were dispersed with trypsin, pelleted by centrifugation, and fixed in 50% glacial acetic acid. Mitotic cells were characterized under phase optics by the absence of a nuclear membrane and the presence of a dense mass of coiled chromatin material; 500 cells were scored for each determination of the mitotic index.

FOR AUTORADIOGRAPHY ^3H TdR and colchicine were added to cover slip cultures to give a final concentration of 0.5 μCi/ml and 1.25 μM, respectively. Samples were harvested and prepared for autoradiography as described above.

Cellular Content of DNA

The frequency distribution of the DNA content of individual cells within a given cell population was measured with flow microfluorometry (29).

Cells were washed, dispersed, fixed with ethanol-acetone, stained with ethidium bromide, and measured for fluorescence by a modification of the method of Darzynkiewicz et al. (3). The frequency distribution of fluorescence emission per cell (proportional to the DNA content) was measured for samples of 10⁴ cells with a cytofluorograph 4801 (Bio/Physics Systems, Inc., Mahopac, N. Y.) interfaced with a Nova 1220 minicomputer (Data General Corp., Southboro, Mass.). The results are expressed in a histogram representing the relative number of cells with a given DNA content.

Chemicals and Radioisotopes

[Methyl- ^3H]thymidine (sp act = 20 Ci/mM) was purchased from New England Nuclear (Boston, Mass.).

The individual L-amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo.) and colchicine from K & K Laboratories (Plainview, New York).

RESULTS

Effect of Arginine Deprivation on Cell Division and Viability

The effect of arg deprivation on cell division is shown in Fig. 1. Replicate exponentially growing cultures were fed with complete or arg⁻ medium, and the number of cells was determined at subsequent times. Cells fed with complete experimental medium proliferated with a population doubling time of 24 h. Deprived cells increased by ~20% over the 1st 12 h after arg withdrawal, and the cell number then remained constant for 72 h. This was confirmed by a study of the mitotic index which rapidly declined between 0 and 6 h; after 12 h only a rare mitotic figure (1-2/1,000 cells) could be seen. Consideration of initial cell cycle distribution (see Materials and Methods) suggests that only those cells in G₂ and M, plus 5% of the cells in late S, divided after amino acid withdrawal.

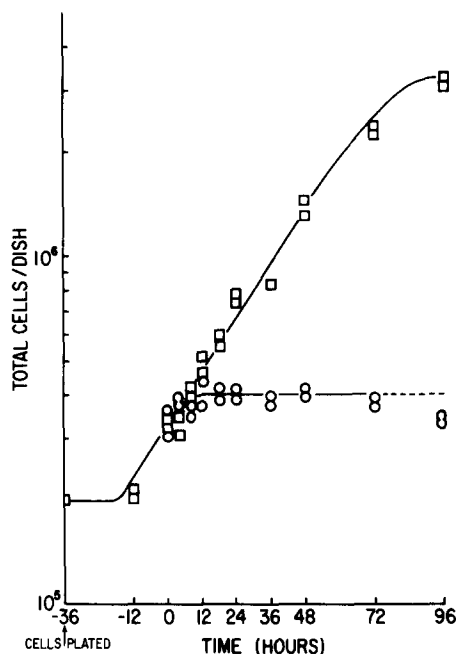


FIGURE 1 Effect of arg deprivation on KB cell growth as a function of time. Cells were plated in complete medium (at $t = -36$ h). When exponential growth was established ($t = 0$ h), all cultures were fed with arg⁻ (○) or complete medium (□). Cells from duplicate cultures were counted at the times indicated.

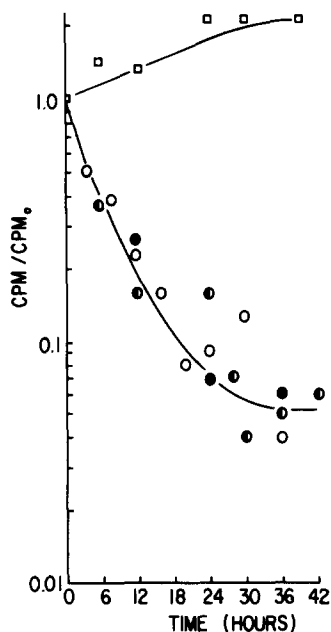


FIGURE 2 Effect of arg deprivation on the rate of incorporation of [³H]TdR in KB cells as a function of time. Cultures were pulse-labeled with [³H]TdR (0.1 μ Ci/ml) for 15 min at the times indicated. The data are expressed as fractional rates, where CPM_0 is the incorporation at $t = 0$ h and CPM is the incorporation at time, t . Incorporation at $t = 0$ h was in excess of 10,000 cpm. ○, ●, ●, arg⁻ medium (averages of duplicate samples in three experiments); □, complete medium (averages of duplicate samples in a representative experiment).

Trypan blue exclusion tests showed that cell viability remained essentially constant (~90%) for 30 h after arg withdrawal and was equal to that of controls. Viability declined slowly after 36 h and only 48% of the starved cells excluded the dye at late times after arg deprivation.

Effect of Arginine Deprivation on the Rate of DNA Synthesis

The effect of arg deprivation on the rate of DNA synthesis in cells treated as in Fig. 1 is shown in Fig. 2. The rate of [³H]TdR incorporation for 15-min intervals increased exponentially in control cultures, doubling within a single population doubling time. Experimental cultures, which were handled identically but lacked arg, showed an immediate reduction in the rate of thymidine incorporation. Incorporation declined at a steady rate for about 18 h and eventually reached a constant value at 5% of the initial rate

by 30-36 h; this rate was maintained until at least 42 h after deprivation.

Effect of Arginine Deprivation on the Fraction of Cells Synthesizing DNA

To determine the number of cells participating in the DNA synthesis observed, the fraction of cells incorporating [³H]TdR after arg withdrawal was determined. These results are presented in Fig. 3. ~35% of the control cells were synthesizing DNA at any 15-min time tested during the first 18 h of the experimental period, confirming the steady-state growth of the control culture. The decline in the number of cells in S phase thereafter probably represents a decrease in the number of cells initiating S as cells were going into stationary phase. After arg deprivation, the fraction of labeled cells increased slightly during the first 12 h, suggesting that the S phase was becoming prolonged; thereafter 40% of the cells were labeled during any 15-min interval up to 42 h after arg withdrawal. In light of the decreasing incorporation of [³H]TdR in the culture as a whole (Fig. 2), individual cells must be synthesizing DNA at progressively declining rates. Decreasing grain densities at successively later time points reflected this.

The Effect of Arginine Deprivation on the Ability of Cells to Initiate DNA Synthesis

The question of whether this constant fraction of incorporating cells represented the same cells synthesizing DNA both early and late after deprivation, or whether cells were both entering and leaving a synthetic period during this time, was addressed in the following experiment. [³H]TdR was added at the time of arg deprivation, and cultures were harvested after increasing intervals of continuous labeling. These data are presented in Fig. 4. Essentially all of the control cells underwent DNA synthesis by the end of one population doubling time. Roughly 80% of the deprived cells had also incorporated [³H]TdR into their DNA within 36 h after arg withdrawal. Because only 40% of the cells were labeled during any 15-min pulse (see Fig. 3), the results obtained after long-term exposure to [³H]TdR demonstrate that a different population of cells was synthesizing DNA late during starvation, whereas those cells that had taken up label early were no longer incorporating [³H]TdR into their DNA.

Cellular Content of DNA

Cell-cycle arrest after arg withdrawal was also determined by flow microfluorometry, a method

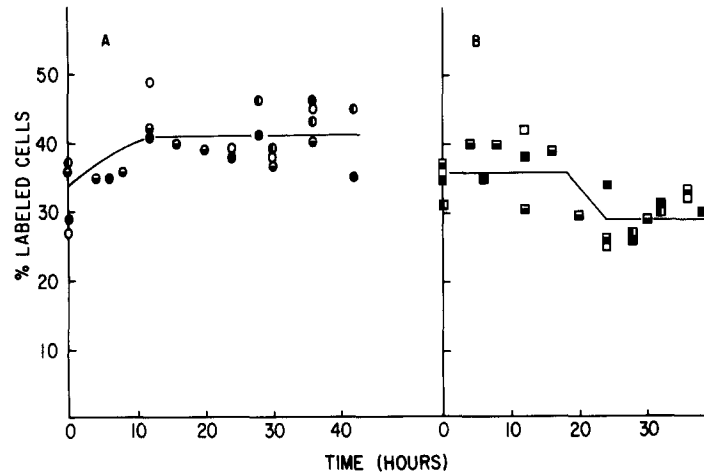


FIGURE 3 Effect of arg deprivation on the percent of KB cells engaged in DNA synthesis as a function of time. The data were obtained by scoring autoradiographs of arg⁻ (panel A) or control (panel B) cover slip cultures pulse-labeled with [³H]TdR (1.0 μ Ci/ml) for 15 min at the times indicated; 500 cells were scored for each determination. Different symbols represent averages of duplicate samples in each of four experiments.

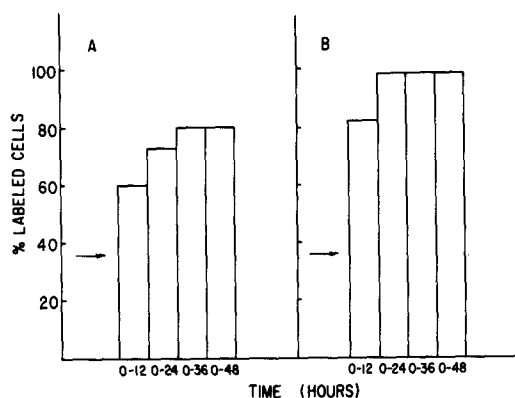


FIGURE 4 Accumulation of [³H]TdR-labeled cells during arg deprivation of KB cells. Exponentially growing cultures were fed with arg⁻ (panel A) or complete (panel B) medium. [³H]TdR (0.5 μCi/ml) was added immediately. Cover slips were fixed after the intervals indicated, processed by autoradiography, and the proportion of labeled cells determined; 500 cells were scored for each determination. The arrows represent the proportion of labeled control cells after a 15-min exposure to [³H]TdR at *t* = 0.

which identifies fractions of the population in terms of their DNA content. Although KB cells are heteroploid in karyotype, they appear to be, like other heteroploid cell lines (18), more uniform in terms of DNA content. The distribution of cellular DNA content in an exponentially growing (control) population is shown in Fig. 5B and is in reasonable agreement with the distribution deduced from cell-cycle timing (see Materials and Methods). A major peak at channel 19 (marked by a vertical line) represents cells with 2c DNA contents (G1), whereas a peak at channel 38, twice the mode of the G1 peak, represents 4c DNA contents of cells in G2 + M. Cells in S phase, having completed various amounts of DNA replication, appeared as distributed between these two peaks. The G1 peak represents ~60% of the total population.

30 h after arg deprivation (at which time thymidine incorporation had declined to minimum rate), the majority (~60%) of the growth-arrested population also registered in a peak around channel 19 (Fig. 5A). There were very few cells in channel 38 or higher, indicating that essentially no cells had a 4c complement of DNA and that the remaining 40% had DNA contents characteristic of S-phase cells. It was concluded, therefore, that cells which had ceased synthesizing had not

achieved a normal G2 DNA content at the end of the starvation period.

Growth Restoration in Arginine-Deprived Cells

The behavior of cells was also examined after restoration of arg; this should give information as well about stages of cell-cycle arrest. After 30 h of arg deprivation, cell proliferation was restored by the addition of arg to give a final concentration of 0.5 mM (Fig. 6). The addition of arg alone to the medium in which cells were carried during starvation unequivocally limited any changes in cell behavior to the presence or absence of that amino acid. After restitution of arg, no cell division was observed for 16 h, but a steady rate of cell increase was achieved by 24 h. Essentially, the entire culture (89%) remained viable as late as 66 h after restoration. It was concluded that after arg restoration there is considerable variation among individual cells in the time before the first division occurs and that the population behaves, therefore, in an unsynchronized manner.

The data in both Figs. 5 and 6 suggest that the cells contributing to the increase in cell number must synthesize DNA before they enter mitosis. An autoradiographic analysis of the cells entering mitosis after restoration was carried out to clarify this point. [³H]TdR was added to cultures at the time arg was restored, mitotic figures were col-

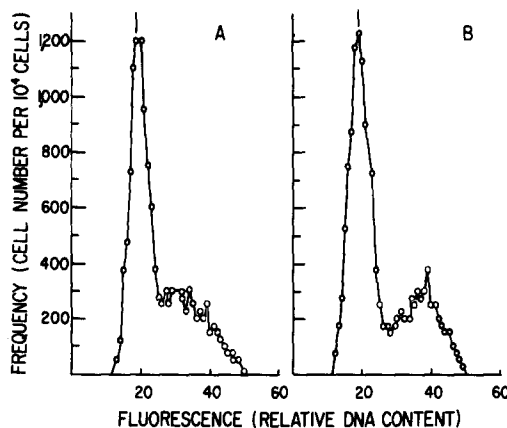


FIGURE 5 Flow microfluorometric analysis of relative DNA contents in KB cells during arg starvation. Arg-deprived (panel A) or exponentially growing (control, panel B) cultures were prepared for analysis as described in Materials and Methods. The line represents the mode of the G1 peak in panel B.

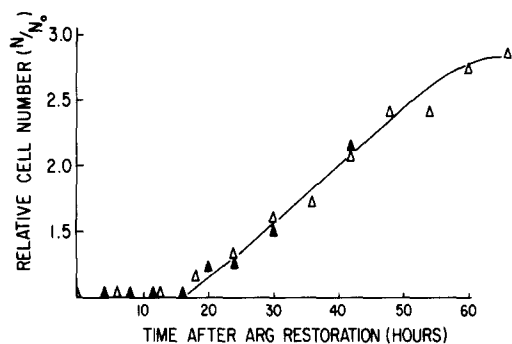


FIGURE 6 Effect of arg restoration on KB cell growth as a function of time. At $t = 0$ h arg (at a final concentration of 0.5 mM) was added to a culture of cells previously maintained for 30 h in arg-deprived medium. Cells from duplicate cultures were counted at the times indicated. Cell number is plotted by the convention N/N_0 in order to compare two experiments (Δ , \blacktriangle); N_0 is the average initial cell number and N is the average number of cells at any given point in the experiment.

TABLE I
*³H*TdR-Labeled Metaphases as a Function of Time after Arginine Restoration of KB Cells*

Hours after restoration	Metaphases counted	Labeled metaphases	Labeled metaphases %
0-6	25	0	< 4
6-12	50	46	92
12-18	100	94	94
18-24	300	298	99
24-30	500	500	100

* Cultures were maintained in [3 H]TdR (0.5 μ Ci/ml) from the time of arginine restoration ($t = 0$ h); 6-h colchicine treatments were used to collect all the mitotic figures that developed.

lected with colchicine in adjacent 6 h periods, and the percent of labeled figures was determined. As seen in Table I, essentially all metaphase figures collected between 6 and 30 h after arg reversal were labeled. Thus, all cells must undergo additional DNA synthesis before dividing.

DISCUSSION

Arg withdrawal from exponentially growing KB cells causes an immediate decline in mitotic activity and in the rate of [3 H]TdR incorporation into macromolecular components. Nevertheless, DNA synthesis continues in a significant fraction of the population because 40% of the cells are synthesizing DNA during any 15-min interval tested

throughout a 42-h starvation period. Furthermore, cells are able to initiate DNA synthesis after arg withdrawal, as judged by the fact that 80% of the cells incorporate [3 H]TdR at some time during the deprivation period. Previous workers have reported a decrease in the fraction of cells synthesizing DNA after withdrawal of arg (9). During the course of these studies, we have found that the effect of arg withdrawal on KB cells is dependent on the distribution of cells in the various phases of the growth cycle. It was noted, for example, that if cells were not actively growing at the start of the deprivation period, the number of cells incorporating [3 H]TdR in successive 15-min intervals did indeed decrease. The inconsistency between our data and those of others might be a reflection of differences in the growing state, and therefore cell cycle distribution, of stocks used to initiate experiments. As others have observed (2, 27), the response to amino acid withdrawal depends on cell type, the individual amino acid, and the cultural conditions.

We propose the following interpretation of the labeling experiments described in this paper. (a) The 20% fraction of the cell population that does not incorporate [3 H]TdR represents the fraction that divides after the amino acid has been withdrawn; these cells become arrested in G1 (G0). (b) The majority of the cells which were in S at $t = 0$ h gradually stop synthesizing DNA and move into a "postsynthetic" period. (c) Cells that were in G1 at $t = 0$ h sequentially initiate DNA synthesis during the starvation period and are the cells that are synthesizing DNA at late times after arg is withdrawn.

The ordered progression of cells into and out of DNA synthesis suggests that these cells may be undergoing S. In the accompanying paper (30) we show that the incorporation of [3 H]TdR into DNA represents replicative synthesis. One might have expected, therefore, as has been suggested in previous reports (for example, see 12), that cells which leave S have progressed to G2. However, the flow microfluorometer (FMF) profile seen at $t = 30$ h shows that essentially no cells register as having a 4c complement of DNA. Moreover, the observations (a) that after arg is restored, all cells incorporate [3 H]TdR before division, and (b) that cell number does not increase for at least 16 h after addition of arg, are both consistent with the idea that few, if any, cells were in a normal G2 period 30 h after withdrawal of arg. Therefore, cells must initiate

and/or continue DNA synthesis for a time during deprivation but stop before duplicating their entire complement of DNA. The 16-h interval observed between arg restoration and the resumption of mitosis approximates that occupied by S + G2 + M in an actively growing culture of KB cells. Therefore, cells that did incorporate thymidine during starvation are either positioned at a very early point in S or must undergo some sort of temporal setback before resuming normal cell cycle traverse.

A seeming problem with the FMF profile of starved cells is that at the end of a 30-h starvation period 60% of the cells register as possessing a G1 DNA content, although some 80% of the cells have incorporated [³H]TdR into their DNA. Other workers have noted that early S is a time of low net DNA synthesis and that this period may be mistaken for G1 if methods other than autoradiography, i.e., total [³H]TdR incorporation or FMF analysis, are used (17). It is not unreasonable to suppose that arg-deficient KB cells that initiate DNA synthesis late during starvation accumulate only a small amount of additional DNA, because 12 h after withdrawal of arg, the rate of DNA synthesis is only one-tenth to one-twentieth of prestarvation rate. In any case, the FMF data obtained in this study lend emphasis to the critical necessity for evaluating FMF patterns in conjunction with data obtained by more conventional techniques. On the other hand, the use of autoradiography alone does not distinguish between cells that have successfully completed S and those that have not. Thus, failure to use more than one technique for cell cycle analysis may result in misleading conclusions.

We have recently reported (31) that Chinese hamster ovary cells deprived of arg also continue to initiate DNA synthesis for at least 40 h after withdrawal of the amino acid; isoleucine-starved cultures are arrested in G1, as shown by others (28). Data in the present study are consistent with results in the Chinese hamster ovary cell system; i.e., cells in G1 at the beginning of the deprivation period retain the capacity to enter S.

At least two different protein synthetic processes are involved in the initiation and continuation of S. Continuation, but not initiation of DNA synthesis, can occur to a limited extent in the absence of isoleucine or leucine (7). Inhibition of protein synthesis with puromycin or cycloheximide also selectively blocks entry into S but does not affect continuation of synthesis already begun

(10, 11, 13, 15, 16, 19). Thus it has been suggested that any reduction in the rate of protein synthesis inhibits initiation of DNA synthesis. However, arg-deprived KB cells in which, as one would expect, protein synthesis is reduced (24; our unpublished observations), are able to enter S, and, indeed, continuation of DNA synthesis seems more sensitive to the absence of arg than does initiation of DNA synthesis.

These studies have shown that, although withdrawal of exogenous arg from an exponentially growing KB cell culture does not arrest cells at one unique point in the growth cycle (for example, the G1 restriction point [22]), inhibition of cell cycle progress in the majority of cells centers on their ability to continue replicating their DNA. This poses the interesting question of why some cells cease S without completing a full complement of DNA, whereas other cells initiate S and begin replicating their DNA. Future studies will, therefore use the technique of arg deprivation to study the mechanism involved in DNA synthesis and traverse of the S phase.

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