CONTINUED INITIATION OF DNA SYNTHESIS
IN ARGinine-DEPRIVED CHINeSFeR HAMSTer OVARY CeLLS

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ABSTRACT
When exponentially growing CHO cells were deprived of arginine (Arg), cell
multiplication ceased after 12 h, but initiation of DNA synthesis continued: after
48 h of starvation with continuous [3H]thymidine exposure, 85% of the popula-
tion had incorporated label, as detected autoradiographically. Consideration of
the distribution of exponential cells in the various cell cycle phases leads to a
calculation that most cells in G1 at the time that Arg was removed, as well as those
in S, engaged in some DNA synthesis during starvation. In contrast, isoleucine
(Ile)-starved cells did not initiate DNA synthesis, as has been reported by others.
Experiments with cells synchronized by mitotic selection confirmed this difference
in Arg- and Ile-deprived behavior, but also showed that cells which underwent
the mitosis → G1 transition during Arg starvation remained arrested in G1 (G0?).
The results suggest that Arg-deprived cells continue to maintain some prolifera-
tive function(s) while Ile-deprived cells do not.

The stringent requirement by mammalian cells in
tissue culture for certain amino acids has been
defined by lack of growth at suboptimal concen-
trations of any of these nutrients (2). More re-
cently, a number of studies (1, 5, 19) have shown
that cells often respond to essential amino acid
limitation by entering a quiescent state in the cell
cycle before DNA replication, i.e., that deficient
cells become arrested during G1. It has been pro-
posed (13) that control of cell proliferation, in the
normal case, resides at a critical point, the “re-
striction” or R point, which occurs early in the cell
cycle and which is sensitive to diverse suboptimal
nutritional conditions including amino acid defi-
ciences as well as serum depletion, high cell den-
sity, and other factors. Experimental evidence has
suggested that this R point is temporally (and
perhaps functionally) the same under a variety of
restrictive conditions (e.g., 9, 10, 13).

In this report, the effect of arginine (Arg) defi-
ciency on growth and the initiation of DNA syn-
thesis by Chinese hamster ovary (CHO) cells is
examined, with somewhat different conclusions.
G1 arrest following isoleucine (Ile) deficiency has
been particularly well studied in these cells (3) and
was used here for purposes of contrast.

MATERIALS AND METHODS

Cells
The established line of hamster cells, CHO (18),
was obtained from R. A. Tobey, Los Alamos, N. Mex.
Stock monolayer cultures were maintained in Ham's F-
12 medium (Nutrient mixture F-12, Grand Island Bio-
logical Co., Grand Island, N.Y.) supplemented with
10% dialyzed calf and 5% dialyzed fetal calf serum.
Freedom from Mycoplasma was demonstrated at fre-
cent intervals by the lack of cytoplasmic incorporation
of [3H]thymidine in autoradiographic experiments and
by the absence of characteristic fluorescent staining with Hoechst 33258 (16). Periodic cultures for Mycoplasma and bacteria were negative.

**Experimental Cultures**

The formula of Eagle's minimal essential medium with 2× nonessential amino acids (EM) was used in all experiments. Concentrated solutions of the essential amino acids were prepared from analytical grade reagents and combined in the medium at required concentrations, omitting either arginine (EM Arg⁻) or isoleucine (EM Ile⁻) where designated. The serum components, 10% calf and 5% fetal calf, were dialyzed against 2 50-fold vol of phosphate-buffered saline for 72 h, stored frozen, and used within 1 mo after dialysis. For experiments, 1 × 10⁵ trypsin-dispersed cells in 2.0 ml of EM were dispensed to 35 mm plastic Petri dishes. Preliminary experiments determined that such cells established exponential growth by 18 h after plating. Experiments were initiated at this time (t = 0) by discarding the medium, washing the cell sheets with warm EM lacking amino acids, then feeding the plates with complete or deficient EM as required by protocol. Control cultures in EM grew with a doubling time of 20 h. Cell cycle distributions and times, determined by standard methods (12), were:

G1-30% of the population, 5 h; S-60%, 12 h; G2-7.5%, 3.5 h; M-2.5%, 0.5 h.

**Mitotic Selection Technique**

For some experiments, synchronized populations of cells were obtained by mechanically dislodging mitotic cells from exponentially growing stock cultures according to the method of Petersen et al. (14). The cells were grown for 24 h in calcium-free EM and harvested by gently rocking the bottles containing cell sheets in a small volume of fresh medium. Approximately 4% of the total cells were dislodged in this manner and, when viewed by phase contrast microscopy, about 95% of these detached cells appeared to be in mitosis. They were immediately dispensed at 1.5 × 10⁵ cells per 35-mm dish in experimental media as designated in Results. All manipulations were carried out at 37°C.

**Cell Counts**

Cultures were dispersed with 0.025% trypsin to provide single cell suspensions which were counted in a Coulter electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.). Absence of significant cell clumping was monitored microscopically. Cell viability was tested by the exclusion of 0.2% trypan blue.

**Autoradiography**

Cultures were seeded in Petri dishes containing 18-mm cover slips for autoradiographic analysis of [methyl-³H]thymidine incorporation ([³H]Tdr, sp act 20 Ci/mmol, New England Nuclear, Boston, Mass.). At appropriate times, the cell-containing cover slips were removed, washed in cold buffered saline, and fixed in methanol-acetic acid, 3:1, for 10 min. Autoradiography was performed with Kodak NTB-3 photographic emulsion and Dektol developer (Eastman-Kodak, Rochester, N.Y.). The preparations were exposed for 5 days at 4°C, developed, and stained with Giemsa stain. Grain count distributions in representative preparations showed that >90% of the cells which were scored as negative were marked by <4 grains per nucleus. Only nuclei overlaid with >15 grains were scored as positive.

**RESULTS**

**Cell Growth after Removal of Arginine or Isoleucine**

When exponentially growing CHO cells were changed to either EM Arg⁻ or EM Ile⁻, the rate of cell division declined, as compared with that in cultures receiving fresh EM (Figure 1). Approximately 50% of the cells divided before the cultures came to proliferative arrest about 20 h after removal of either amino acid. Assuming an orderly, albeit slower, progress through the cell cycle stages, all the cells in G2 and mitosis and 2/3 of the cells in S at the time of amino acid withdrawal subsequently divided (see cell cycle distribution, Materials and Methods).

Viability of the starved cells (trypan blue exclusion) remained at control levels throughout the 48-h experiments; the average viability in control

![Figure 1](image-url)
cultures was 94%, in Arg-starved cultures, 94%, and in Ile-starved cells, 96%.

**DNA Synthesis after Removal of Arginine or Isoleucine**

To further analyze growth arrest in individual cells of a population randomly distributed throughout the cell cycle by autoradiographic detection of DNA synthesis was used. Exponentially growing cultures containing cover slips were refed with EM Arg-, EM Ile-, or fresh EM; [\(^{3}H\)]thymidine, 0.5 µC/ml, was added immediately and cover slips were fixed at various subsequent times. The concentration of tritium used to detect low levels of incorporation did not detectably perturb the growth rate of control cells or reduce their viability as judged by trypan blue staining at the end of the experimental period. The experiment is presented in Fig. 2. Control samples harvested after 15 min showed that about 60% of the cells had incorporated significant [\(^{3}H\)]TdR, i.e., were in S at t = 0. Continuous labeling of controls from 0 to 12 h showed that all cells engaged in DNA synthesis during that interval, as would be expected from cell cycle parameters.

Increasing intervals of continuous labeling, for 12, 24, 36, 48 h, were used to identify the cells initiating DNA synthesis in the starved cultures. Essentially no cells entered S after removal of isoleucine, as the proportion of labeled cells remained the same (~60%) throughout the 48-h experiment.

In Arg-cultures, however, the number of [\(^{3}H\)]TdR-labeled cells increased in each successive collection, indicating that cells continued to initiate DNA synthesis as late as 36-48 h after Arg withdrawal. After 48 h in EM Arg-, the proportion of [\(^{3}H\)]TdR-labeled cells had increased 25% (60 → 85%). Taking into account the increase in total cells, about 30% of the original cell population must have initiated DNA synthesis during Arg starvation, a figure equal to the proportion in G1 at t = 0.

**Progress through G1 after Removal of Arginine or Isoleucine**

Confirmation that G1 cells do initiate DNA synthesis in the absence of arginine but are not able to do so in the absence of isoleucine was sought in the following experiment. A synchronized mitotic population of cells was harvested from stock cultures and plated and the ability of such cells to proceed through G1 into S under Ile- or Arg- conditions was determined by autoradiography.

![Figure 2](image-url)

**Figure 2** [\(^{3}H\)]thymidine incorporation into individual cells after removal of arginine (Panel A), isoleucine (Panel B), and into control cells (Panel C). Exponentially growing CHO cells were fed with EM Arg-, EM Ile-, or complete EM. [\(^{3}H\)]TdR, 0.5 µC/ml, was added immediately. Coverslips were fixed after the intervals indicated, processed by autoradiography, and the proportion of labeled cells was determined. At least 500 cells in each of two or more cultures were examined. The arrow represents the proportion of labeled control cells after a 15-minute exposure to [\(^{3}H\)]TdR at t = 0.
After 5 h incubation, i.e., the time at which exponential cultures approach the G1/S boundary, [3H]TdR was added to EM Arg−, EMIle−, and EM cultures. 15 h later, at approximately the end of one normal doubling time, all cultures were fixed, processed for autoradiography, and the number of labeled cells was determined.

Two different plating protocols were used with, surprisingly, different results. The data are shown in Table I. When mitotic cells were plated directly in deficient medium (protocol 1), so that cells finished mitosis and attached to the dishes under nutritional restriction, there was no evidence of DNA synthesis in either Arg− or Ile− cultures. 94% of the fully nourished control cells were labeled as would be anticipated in approximately one cell-doubling time. Only 6% of the Arg− or Ile− cells were labeled. This value was no greater than that obtained in a 15-min [3H]TdR pulse performed on control cultures to monitor S phase contamination at the beginning of the experiment.

In plating protocol 2, all cultures were established in complete EM and starvation was instituted by a medium change 3 h later, i.e., during G1. After [3H]TdR labeling from 5 to 20 h, EMIle− cultures still showed no increase in [3H]TdR labeling. In the EM Arg− cultures, however, 60% of the cells had incorporated significant thymidine.

**DISCUSSION**

Two trivial explanations of the [3H]TdR incorporation into Arg− cells must be considered. First, if a relatively larger pool of Arg than of Ile were available to or in the cells, this might delay institution of the Arg-starved phenotype. It is difficult to select a rigorous criterion other than the operational one by which to identify this starved phenotype. While the Arg− and Ile− deficient experimental media were formulated without any of the respective essential nutrient, the necessity to maintain a serum supplement, though exhaustively dialyzed, prevents complete definition of the media. Furthermore, cell uptake of trace concentrations of either amino acid is not necessarily equally efficient. With respect to arginine, the water-extractable internal pool in various cell lines is reported to be smaller on a molar basis than that of other essential amino acids including isoleucine under optimum nutritional conditions (6, 15). This small pool decreases over 80% within 15 min after deletion of external Arg (6, 21) but does remain detectable for at least several hours. Similar observations have been reported for other essential amino acid pools. Such a persisting low level of free amino acid may well derive from and must support the protein turnover but not net synthesis seen in starved cells (2). In any case, the kinetics of protein synthesis in CHO cells deprived of Arg or of Ile are essentially identical. [3H]amino acid incorporation decreases to about 30% of the initial rate within 90 min after either amino acid is withdrawn. It remains at the same substantial rate for at least 36 h under both deprivations (unpublished observation). New initiations of DNA synthesis in Arg− cells, but not in Ile− cells, are still occurring at the end of this time.

The second objection is that [3H]TdR incorporation might represent a repair process rather than replication. Indeed, some studies in the literature have assumed this to be the case (17), and the high Arg content of histones makes it plausible. While the experiments described here do not directly address this possibility, it seems unlikely that repair synthesis would occur in an orderly progression of incorporating cells, as seen in the continuous labeling experiment. Further, it might be expected that repair processes would occur in all cells and under all schedules of Arg withdrawal, neither of which was the case (See Table I). Finally, in another cell system (KB), it has been demonstrated directly that [3H]TdR incorporation

**TABLE I**

| Labeled cells* | Plating protocol 1 | Plating protocol 2 |
|----------------|--------------------|--------------------|
| Medium         | %                  | %                  |
| EM Arg−        | 6                  | 63                 |
| EM Ile−        | 6                  | 6                  |
| EM             | 94                 | 94                 |

* Coverslip cultures of cells obtained by mitotic selection were labeled with [3H]TdR (0.5 μCi/ml) from 5 to 20 hours after plating. The data were obtained by autoradiography: 500 cells were scored for each determination.

† Sample cultures were pulsed for 15 minutes with [3H]-TdR at the beginning of the experimental period. 4% of the cells incorporated label and were considered to be S contaminants of the G1 population. These cells may or may not contribute to values given.

‡ Mitotic cells were plated in the medium indicated at t = 0 hour.

§ Mitotic cells were plated in EM for attachment so that cells initiated G1 under complete nutritional conditions; cultures were refed with the medium indicated at t = 3 hours.
The cellular response to Arg deficiency observed in this report suggests an interpretation somewhat different from those which have appeared in the literature. Our data appear to support the hypothesis of a "restriction point" early in the cell cycle which is sensitive to nutritional deficiencies. However, Arg-starved cells become arrested in G1 (G0) only when the cells finish mitosis and begin a new cell cycle under deficient conditions; thus, the R point, as demonstrated by Arg deficiency, is in mitosis or early G1. It has been reported (10) that deficiency of several essential amino acids, including arginine, in 3T3 cell cultures causes arrest of cell proliferation in G1. Complete experimental details were not presented, but we propose that this would be true only of recently divided, Arg-starved cells.

Studies with other amino acids (1, 4, 10) have suggested a generalization (excluding ile) that, in media completely omitting (as here), rather than limiting, the compound in question, substantial G1 arrest does not occur. Cells appeared to be arrested in all stages of the cell cycle because of failure both to initiate and to continue DNA synthesis. The decreased rate of [3H]TdR incorporation into mass cultures of Arg-starved cells (7, 20) was consistent with that generalization. In the present experiments in which [3H]TdR incorporation into individual cells was examined, mid- to late-G1 cells, when deprived of Arg, continue slowly to become [3H]TdR labeled, i.e., to initiate DNA synthesis, for more than 36 h after Arg is withdrawn. As a result, after mitosis ceases most of the cells must accumulate in a synthetic or post-synthetic state with respect to DNA replication. Similar long-term [3H]TdR labeling values have been reported in Arg polyoma-transformed mouse cells (8). Whether, however, such cells can complete a normal round of DNA replication and enter G2 remains to be demonstrated.

All cells except recently divided G1 cells, when deprived of arginine, continue to initiate DNA synthesis, whereas isoleucine-deprived ones do not. This suggest that Ile cells lack some function(s) of the proliferative state that Arg cells continue to maintain.

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