ATP POOL LEVELS IN SYNCHRONOUSLY GROWING CHINESE HAMSTER CELLS

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Many cell lines grown in tissue culture accumulate in a postmitotic, preDNA-synthetic part of the cell cycle when brought to a nonproliferating state by a variety of techniques (1-5). In our laboratory we have recently shown that a qualitatively similar, G1-blocked population of cells can be produced by growing mammalian cells in complete medium with limiting amounts of the major energy source, glucose. These results suggested that possibly energy metabolism was involved in the control of progression of cells about the cell cycle, and that this control might be ex-

pressed at the level of the available energy pools (adenosine triphosphate [ATP] levels) about the cell cycle. The G1 part of the cell cycle has already been shown to have a minimum in respiration rate in synchronized HeLa cells, and the respiration rate is maximum where the DNA synthetic rate is maximum (6). In this report, data are presented which show that the ATP level in synchronous populations of Chinese hamster cells increases by a factor of ~2 over the cell cycle, and that the concentration of ATP computed over the total cellular volume is essentially constant with a mean value of 3.01 mm ± 0.41.

MATERIALS AND METHODS

Chinese hamster cells (strain V79-379-A) were cultured and maintained in Eagle’s minimal essential medium supplemented with nonessential amino acids (MEM, F-15, purchased as a premixed powder from Grand Island Biological Co., Grand Island, N.Y.), antibiotics (penicillin, 50 IU/ml and streptomycin, 50 µg/ml), and 15% fetal bovine serum (Flow Laboratories, Rockville, Md.) At 37°C in a 5% CO₂ environment the cells had a doubling time of ~9 hr.

Synchronous populations of cells were established by a mitotic harvest technique. Routinely, 12 16-oz. bottles were used to culture monolayers of cells from which mitotic cells were to be selected. When the cultures reached the mid-exponential phase of growth the medium was decanted from the bottles and replaced with 5 ml of either serumless MEM or Puck’s saline A solution. The best synchrony was achieved by shaking the cultures for 30 sec on a reciprocating shaker table (165 cycles/min. with a stroke length of 2 inches). The bottles were shaken four at a time and the mitotic cell suspension from the first set of harvested bottles was transferred to subsequent sets to facilitate the concentrating of mitotic cells. Suspensions of mitotic cells selected in this manner showed a mitotic index of 55–75% and their volume distribution was bimodal, the mean volume of the larger cells being approximately double that of the smaller cells. Such suspensions were stored at 0°C in an ice bath until such time as equal numbers of cells were plated in 60-mm plastic Petri dishes containing 3 ml of medium prewarmed to 37°C (7). The first division of such mitotically selected populations of cells was 1.5–3.0 hr longer than the normal 9 hr doubling time. The elongation of cell cycle was expressed as an elongation of G1 phase.

The cell number per dish and the mean cell volume of the cells was determined with a Coulter counter Model B (Coulter Electronics, Chicago, Ill.) and an associated volume analyzer (Model J).

DNA synthetic rates of synchronously growing populations were determined by a pulse-labeling procedure using tritiated thymidine (TriH) (SA 18.3 Ci/m mole, Amersham-Searle Corp., Des Plains, Ill.) at a final concentration in medium of 1 µCi/ml. The pulse time was 20 min at 37°C, after which the monolayers of cells were washed once with phosphate-buffered saline and were trypsinized from the surface in 2 ml of 0.10% trypsin solution. A portion of this cell suspension was precipitated in 5% cold trichloroacetic acid (TCA). The precipitate was collected on 0.45-µm millipore filters, washed with cold TCA, dried, and counted for radioactivity in a liquid scintillation counter. The relative counts per minute per cell were used as a measure of the relative rates of DNA synthesis. The cell number, cell volume, and DNA synthetic rates were used primarily to determine the quality of synchrony in each population analyzed.

ATP pools of cells were determined from the bioluminescence of luciferin-luciferase solutions. An enzyme solution was prepared by suspending ground-up firefly tails (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 mg/ml in buffer consisting of 0.1 m N-Tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid, 0.02 m MgSO₄·7H₂O, and 0.05 m sodium arsenate at pH 7.4 (TES buffer). This suspension was centrifuged at 7000 g for 30 min at 4°C, and the supernatant was decanted and used as the source of luciferin-luciferase. Cell ATP pools were released by rapidly pipetting 0.2-ml portions of the trypsinized cell suspensions into 0.5 ml of boiling, double-distilled water. Control experiments to determine whether or not this procedure for removal of cells altered the ATP pool levels indicated that cells removed by trypsinization or mitotic selection yielded similar values, but that cells removed by scraping with a rubber policeman consistently yielded lower levels. This cell lysate was boiled for an additional 10 min to inactivate any ATPases released from the cellular debris. This treatment did not modify the concentration of ATP in control samples. 0.2-ml or 0.4-ml samples of the cell lysate were rapidly mixed with 1 ml of firefly-tail extract in a cuvette in a light-tight chamber. The cellular lysate gave similar readings whether or not the cellular debris was pelleted before analysis of the supernatant. The intensity response measured by a photomultiplier tube reached a maximum values 2 sec after mixing and then decayed with a half-life of about 40 sec. The intensity maximum was proportional to the concentration of ATP in prepared standards and was used to measure the number of moles of ATP in each lysate analyzed. Each preparation of luciferin-luciferase was calibrated with a freshly prepared set of ATP standards. The photomultiplier tube signal was amplified and recorded so that a full-scale deflection on the recorder (100 units) was equivalent to about 2 × 10⁻¹⁸ mole of ATP.
RESULTS AND DISCUSSION

The degree of synchrony achieved using this mitotic selection technique is related to the initial mitotic index of the population, along with possible cell trauma resulting from mechanical selection. Panels A, B, and C of Fig. 1 show the cell kinetic data associated with the synchronous growth of a population of cells having an initial mitotic index of ~60%. The initial volume distribution was bimodal and could be resolved into two cell populations with approximately 50% of the population associated with each distribution. The mean cell volumes of the initial populations were computed to be 1255 µ³ and 2340 µ³. This suggests that, although the mitotic index of the initial population is only 60%, many of the cells selected have just newly divided and the age distribution of most cells in the initial population is at most 1 hr. This quality of synchrony is also suggested by the other cell kinetic data. It can be seen that there is a negligible increase in cell number per dish before the 12th hour after plating, and that most cells divide between the 12th and the 15th hour. Within 1 hr of plating the initial bimodal volume distribution becomes unimodal with a mean cell volume slightly larger than that of the initial distribution of smaller cells. At each hour after plating, the mean cell volume of the synchronous cells was computed as a single population distribution. As shown in Panel B, this parameter increases in a near-linear manner until cell division is detected, at which time a corresponding decrease in mean cell volume is measured. No attempt was made to separate the volume distribution into components at the first cell division, since the spread in cell age of the population is at least 4 hr by that time. Panel C shows the relative rates of DNA synthesis of this cell population as it traverses one cell cycle. These data also reflect the extent of synchrony in the cell population and are used to determine the approximate length and position of S phase within the first cell cycle.

Panel D displays the data obtained for measured ATP pool levels throughout the cell cycle. It can be seen that early in the cell cycle the amount of ATP is about $4.0 \times 10^{-14}$ mole/cell and increases to a value greater than $8.0 \times 10^{-15}$ mole/cell by early G2 phase.

If we make the assumption that the ATP is distributed uniformly over the total volume of a cell, we can compute an average molar concentration of ATP by using the mean cell volume; this is shown for three independent synchrony runs in Fig. 2. It is apparent from these data that the molar concentration of ATP within Chinese hamster cells traversing the cell cycle is extremely constant, the mean value of all determinations being $3.01 \text{ mm} \pm 0.41$. The fluctuations in ATP concentration within any one synchronous experiment are thought to represent experimental error. This is supported by the fact that the standard deviation about the mean value was smallest in runs displaying the highest degree of synchrony and vice versa.

As stated earlier, our initial interest in ATP pools of cells traversing the cell cycle was asso-
associated with the question of whether or not the progression of cells about the cell cycle was controlled by the level of the energy pool. Our data suggest that the differences in ATP pool levels throughout the cell cycle are not sufficient to be the controlling step in regulation of cell growth. This can also be demonstrated by our measurements of the ATP pools of cells during various phases of culture growth. The ATP pool level of cells in the early stationary phase of growth (contact-inhibited) which are blocked in G1 phase is almost the same as the ATP pool level of cells rapidly proliferating in the exponential phase of growth. Our measurements on Chinese hamster fibroblasts in culture agree with the ATP measurements of Colby and Edlin (8) who recently reported that the nucleotide pool levels of chick fibroblasts in growing, inhibited, and transformed conditions of growth were the same. On the other hand, the ATP pool levels of HeLa cells synchronized by blocking DNA synthesis with amethopterin appear to be greater by a factor of 100 than the pool levels measured in our experiments (Gerschenson et al., 9). Whether this difference is real or is an artifact resulting from the many differences in experimental technique between the two experiments remains to be seen.

Although the computed concentration of ATP throughout the cell volume is quite acceptable for physiological processes, the instantaneous pool is small compared to the estimated ATP required per cell division. Kilburn et al. (10) have determined that mouse LS cells, mammalian cells similar in size to Chinese hamster cells, require $4.5 \times 10^{-11}$ mole of ATP for the biosynthetic and maintenance functions of one cell cycle. Using a similar value for Chinese hamster cells, we can compute from our ATP pool measurements that the turnover time of the ATP pool in our cells is on the order of 5 sec. This value is similar to ATP pool turnover times reported for the bacterium *E. coli* (11). Certainly these considerations support the widely accepted idea that ATP is solely a molecule for the transport of metabolic energy and not a molecule for energy storage. Furthermore, the turnover time of ATP pools is so short in Chinese hamster cells compared with the duration of the cell cycle that the concept of an ATP reservoir maintaining cell progression through significant portions of the cell cycle is unfeasible.

**SUMMARY**

The ATP pool levels in synchronous populations of Chinese hamster cells growing through the first cell cycle after mitotic selection have been measured by the luciferin-luciferase method. The ATP level in early G1 phase was found to be $\sim 4.0 \times 10^{-15}$ mole/cell and increased to $\sim 8.4 \times 10^{-11}$ mole/cell for cells in G2 phase. Using the mean cell volume of the cell populations analyzed, we found the molar concentration of ATP throughout the cell cycle to be relatively constant at $3.01 \pm 0.41$. The small size of the ATP pool and the relatively minor fluctuations in concentration have led us to conclude that progression of cell growth about the cell cycle is not regulated at the level of the energy (ATP) pool.

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