A CONVENIENT METHOD FOR ASSAY OF DNA SYNTHESIS IN SYNCHRONIZED HUMAN CELL CULTURES

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In studies of dynamic changes in cellular structure and metabolism and the experimental modification of these properties during various stages of the cell cycle, it is often desirable to determine periodically the synthesis of macromolecules in the cell. In this paper we present a simplified method for determining the rates and total amount of DNA synthesis and RNA synthesis in synchronized human cells in suspension culture. The method is also applicable to monolayer cultures of human and other mammalian cells. Comparisons have been made between the results obtained by this method and the usual radioautographic techniques. With certain modifications, the method can be extended for measuring protein synthesis.

MATERIALS AND METHODS

The cell line used in these studies was the HCAAT cell derived from HeLa (1) and adapted to growth in suspension culture. This line was isolated at the University of Michigan Medical School and was kindly provided to us by Dr. Alfred Hellman. The cells were grown at 37°C in screw cap Erlenmeyer flasks placed on a rotary shake table which turned at 100 excursions per min. In 100 ml of medium there were 81 ml of Eagle's Basal Medium in Hanks' balanced salt solution (HBSS) with 10% calf serum, 15 ml of tryptose phosphate broth (Difco Laboratories, Inc., Detroit), 3 ml of a 4% methyl cellulose solution in HBSS, and 1 ml of 10% sodium citrate in distilled water. Usually 2–3 $\times$ 10^5 cells/ml were inoculated into 200 ml of medium in a 1-liter flask.

Synchronized DNA synthesis was induced by exposing cells for 24 hr to 5-aminouracil (5-AU) at a final concentration of 3 $\times$ 10^{-3} M in the medium (2). The cells were then centrifuged, and both treated and control cells were resuspended in fresh medium at 37°C and pH 7.2–7.4. The time of 5-AU removal was referred to as time zero.

At various intervals before time zero, and usually hourly thereafter, 2-ml aliquots of cell suspension were removed from control and treated cultures. To these aliquots was added 0.2 ml of medium containing tritiated thymidine (1.9 c/mmole) or uridine (2.0 c/mmole), such that the final radioactivity of the medium was about 1 μc/ml. These samples were incubated at 37°C for 30 min and then plunged into an ice-water bath. In order to extract the tritiated DNA, the cells were lysed by immersing an ultrasonic probe (Branson Sonifier with stepped horn) into the cell suspensions and sonicating for 30 sec. 100-μl portions of each sonicate were applied to Whatman No. 3 MM filter paper discs (2.3 cm in diameter). After the sonicate had been absorbed by the filter paper, the discs were placed in 5% trichloracetic acid (TCA) at 4°C. At the conclusion of the experiment, the accumulated discs were washed as follows: three 10-min ice-cold TCA washes; three 10-min room temperature 95% ethanol washes; two brief wettings with ether. After the discs were thoroughly dry, they were placed in glass vials with 5 ml of a liquid scintillation solution which contained 4 gm of 2,5-diphenyloxazole and 0.1 gm of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene. Counting was done in a liquid scintillation spectrometer. This method is an adaptation of the DNA polymerase assay of Bollum (3).

Preliminary experiments to test the efficacy of the Bollum method as applied to our cell systems have indicated: (*a*) The wash procedure removed 98 to 99% of the unincorporated precursor.[1] (*b*) There was

[1] For example, 100-μl aliquots of a sonicate from a culture incubated for 0 min with thymidine-H^3 were placed on discs. Half of them was merely dried and counted; the other half was washed, dried, and

little or no loss of tritiated DNA from the disc. In fact, counting efficiency was enhanced by washing the disc. (*c*) In a scintillation counter whose efficiency for counting toluene-H^3 in toluene scintillation fluid was 27%, the counting efficiency of tritiated DNA in glass vials, treated as described above, was consistently 3 to 4%. Even though the efficiency is low, the HCAAT cells incorporated enough radioactive precursor, in even a 15 min pulse labeling, to demonstrate two- to fourfold differences between experimental and control cultures. (*d*) The serum protein of the medium was found to quench about 5% of counting efficiency. The protein in the sonicate of a cell culture quenched the counting efficiency to some extent, depending on the cell density. For example, cultures with 10^6 cells/ml quenched up to 15%. This variable, however, remains fairly consistent within the experiment. (*e*) Incubation of a sonicate with deoxyribonuclease for 30 min reduced the counts retained on a disc from that sonicate by 75%. This seems clearly to indicate that it was DNA which was being assayed on the disc.

For experiments in which liquid scintillation counting and radioautography were done in parallel, the aliquots of cell suspension were divided after incubation with the tritiated precursor. One half of them was sonicated and placed on discs as indicated above; the other half was centrifuged, washed once in ice-cold HBSS, fixed for 20 min in 1:3 acetic acid: methanol, spread on slides, and air-dried.

Radioautographs were prepared from these slides in the usual manner, using Kodak NTB-3 nuclear track emulsion and toluidine blue staining. Cell counts were done by hemocytometer.

RESULTS

Fig. 1 shows DNA synthesis and RNA synthesis measured by incorporation of tritiated precursors (uridine-H^3 and thymidine-H^3) into TCA-insoluble material and retained on discs as described above in control and 5-AU–synchronized cells. In the control culture, previous to medium change, both DNA synthesis and RNA synthesis proceeded at rates that we have found typical for pre- and early-log phase populations of this cell line. At time zero, when the cells were centrifuged and placed in fresh medium, incorporation of both precursors was minimal. The synthetic activities remained at a low level for about 15 hr before slowly moving up toward the log phase rate.

In the presence of 5-AU, both DNA synthesis and RNA synthesis were at minimal levels. When the 5-AU was removed, a burst of DNA synthesis began immediately and continued to increase, reaching a peak at about 5 hr. This activity decreased rather sharply, reaching minimal levels again at about 7 hr. In several repeat experiments, we have found that RNA synthesis always remained at minimal levels during the first DNA synthesis after 5-AU treatment. In experiments of 30 hr duration, RNA synthesis does occur in the second S period; incorporation of uridine-H^3 follows the pattern of thymidine-H^3 incorporation, but lags behind by about 1 hr.

Fig. 2 compares DNA synthesis measured by liquid scintillation counting (*a*), as described above, and DNA synthesis measured by radioautography (*b*) in the same aliquots of cell culture. In the radioautographs, 1000 cells per point were scored, and a cell with 5 or more silver grains was considered labeled. In this case the radioautographic data are qualitative and immediately approach a maximum. Scintillation counts of the same population provide a more quantitative picture of the increase in DNA synthesis after removal of 5-AU. In both methods of measurement, however, the peak of synthesis and subsequent reduction to minimal levels in the 5-AU–treated cells is equally apparent.

No correlation was found between mean number of silver grains per interphase nucleus (25 cells scored per time point) radioautography and counts per minute per cell on the disc in either control or synchronized cells. In view of the probable heterogeneity of the cell population with regard to rate and amount of DNA synthesis, and the differences in efficiency of the two methods, the lack of correlation between these two parameters is not surprising.

Cell counts indicated that the peak of mitosis occurred at 11 to 12 hr. The degree of synchrony obtained is not the same in all experiments, as indicated by the percentage of doubling that occurs in the first mitotic period, and also by the difference between control and experimental DNA curves in different experiments. Synchrony in most experiments is between 65 and 95%. The degree of synchrony induced by 5-AU seems to be affected by cell concentration. At lower population densities, e.g. 3–4 $\times$ 10^5 cells/ml, the relative height of the first DNA synthesis peak is greater and occurs earlier: in some experiments at 3 to 4 hr after 5-AU removal. The per cent doubling of the population is greater between 10 and 12 hr, under these conditions. At 7–8 $\times$ 10^5 cells/ml,

counted. Mean count per minute of the unwashed discs was 17,490, of the washed discs, 342.

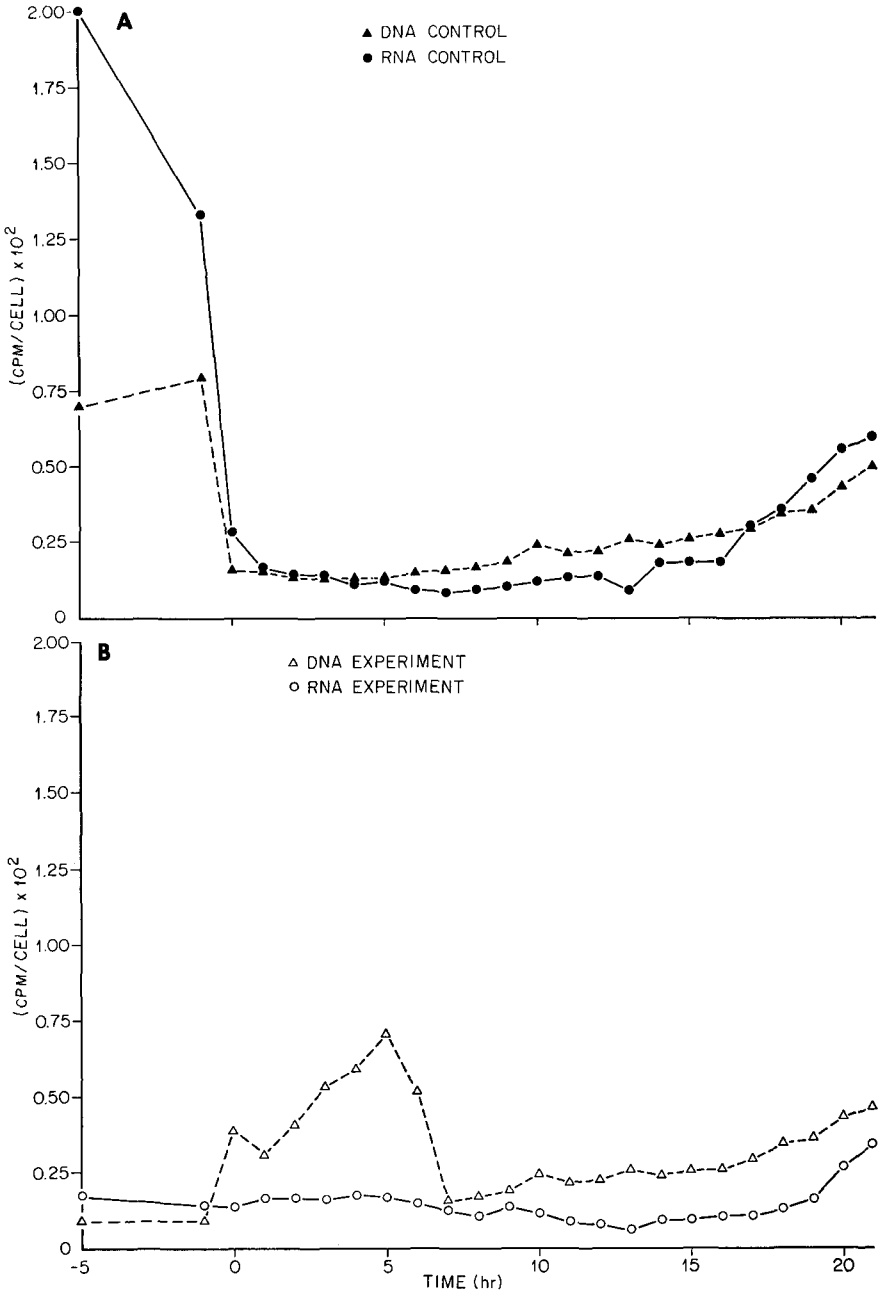

FIGURE 1 DNA synthesis and RNA synthesis in HCAAT cells. Cell density at time zero was 5–6 × 10^5 cells/ml in control and experimental cultures. Fig. 1 *a* Control. At time zero, cells were placed in fresh medium. Incorporation of both precursors (uridine-H^3 and thymidine-H^3) was minimal for 15 hr, and then gradually increased. Fig. 1 *b* 5-AU treated. No uridine-H^3 incorporation occurred during or after first DNA synthesis.

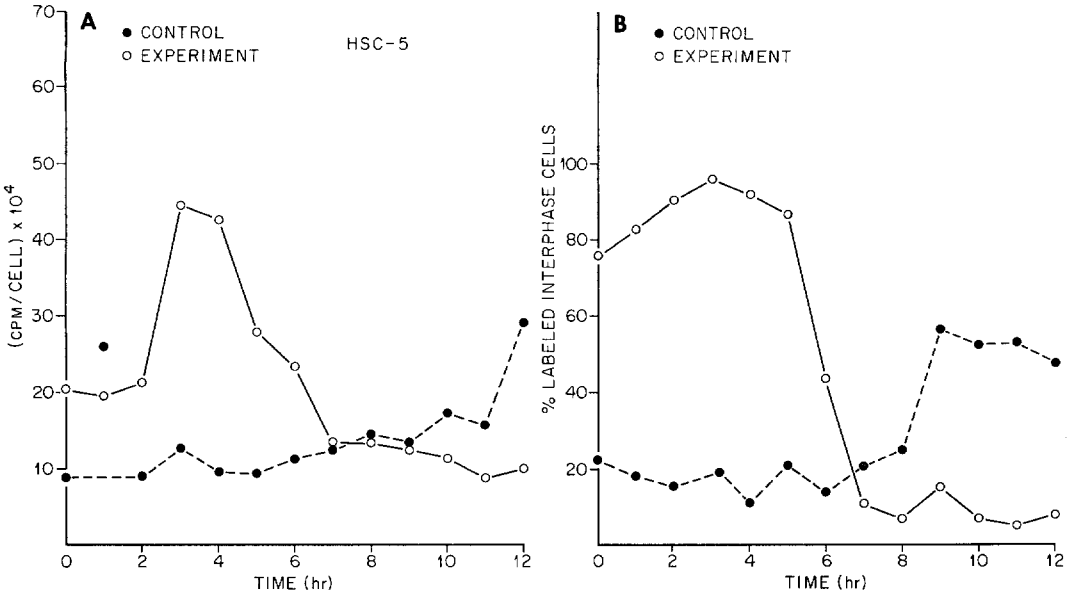

FIGURE 2 DNA synthesis in control and 5-AU-synchronized HCAAT cells. Cell density at time zero was 3–4 × 10^5 cells/ml in control and experimental cultures. Isotope, thymidine-H^3. Fig. 2 *a* Measurement by liquid scintillation spectrometry. Fig. 2 *b* Measurement by radioautography. Nuclei with 5 or more silver grains were considered labeled. Background was 3 to 4 silver grains per nucleus area.

the peak of DNA synthesis occurs at 5 to 6 hr, and the difference in thymidine-H^3 incorporation between synchronized and control cells is not so pronounced.

DISCUSSION

The method described here for measuring the incorporation of H^3-precursors into acid-insoluble material is convenient, rapid, and accurate; and the data are available in the time it takes to wash and count the discs. In time-series experiments, washing and counting the first several discs will indicate how the experiment is progressing and whether it is worth continuing experiments which are invalid owing to some undetected preparative error.

The method described here is equally useful with monolayer cultures. The cells can be removed from the surface of the culture vessel and lysed simultaneously by brief sonication. Results obtained with 5-AU–treated Chinese hamster cells in monolayer have been essentially similar to the results reported here with suspension cultures (4).

The incorporation of H^3-amino acids into cell protein can also be followed by the disc method; a modified wash procedure for this purpose has been described (5). It is, however, difficult to get much label into cell protein in a pulse label of even 2 or 3 hr; this is probably due to the large cell pool of amino acids (6) and to the amino acids in the medium. At present we are testing the effects, on incorporation of isotope into cell protein, of brief amino acid starvation and subsequent incubation with medium in which two essential amino acids are provided only in the tritiated form.

The primary effect of 5-AU in mammalian cells is not known. It is clear, however, that both DNA synthesis and RNA synthesis are minimal during 5-AU treatment, and that the DNA biosynthetic system recovers immediately upon removal of the analogue. If we assume that the same is true for RNA biosynthesis, then it is interesting to speculate that it may be necessary to replicate the DNA of the inhibited cell first before any transcription of messenger RNA and other RNA can occur. Failure to incorporate uridine-H^3 during the first S period may also be due to the 5-AU treatment itself, possibly by a flooding of the uridine pool with the analogue. Experiments to determine this and other aspects of 5-AU action are in progress.

SUMMARY

A simplified method is described by which DNA synthesis and RNA synthesis can be studied in

tissue culture systems. The method consists of incubation of the cells with tritiated precursors, sonication of the cells, application of an aliquot of sonicate to a filter paper disc which is washed with cold trichloracetic acid. The wash procedure removes unincorporated precursors and precipitates the macromolecules on the disc. The discs are counted by liquid scintillation spectrometry. Results obtained by use of this method in a study of 5-aminouracil–synchronized human cells in suspension culture are presented. The method is applicable to any study of incorporation of radioactive precursors into macromolecules in tissue culture systems.

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