CONTINUOUS FLOW SYSTEM WITH IMMOBILIZED NUCLEI. A METHOD FOR KINETIC STUDIES ON RNA METABOLISM

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A method for the preparation of a column containing liver nuclei immobilized on small pieces of membrane filter is described. When a medium containing cytoplasm and the precursors is continuously passed through the column, RNA is synthesized and released at a slightly decreasing rate. The reaction can be initiated and terminated by changes in temperature, and by the addition and omission of reactants. Experiments are presented, which show that the nuclei maintain their capacity to synthesize and release RNA for more than 30 minutes, and that the material collected during the latter part of the perfusion does not originate from the gradual release of RNA synthesized during the initial minutes of incubation.

In the present report a method for the study of the biochemical activities of liver nuclei is described. A column containing the immobilized nuclei is perfused with the incubation medium. Thus, the advantages of a continuous flow system can be utilized for kinetic investigations on the synthesis and release of RNA by the subcellular particles.

Methods. Nuclei from the livers of 2-3 months male Sprague Dawley rats were prepared by the method of Widnell and Tata[1] and washed once with 0.25 M sucrose, 1 mM $MgCl_2$. Sartorius nitrocellulose membrane filters (SM 11301, pore size 8 μ) were dissected into irregularly shaped pieces of less than 0.8 mm diameter in a Thermovac Super 30 homogenizer with metal blades, washed with water, dried at 60°C in an oven and overnight in a desiccator. The following procedures were performed in the cold room. Portions of the nuclear pellet were added to the membrane filter pieces with a spatula until a pasty consistence was obtained. As a result of the uptake of the aqueous medium by the dry filter pieces, the nuclei were adsorbed to

the surface of the membrane filters, which were then suspended and sedimented in 0.25 M sucrose, 1 mM $MgCl_2$, pH 7.2. The pieces were then transferred, with the help of a funnel, into a glass column filled with the sucrose medium, care being taken to avoid the presence of air bubbles. This column (length 24 mm, inner diameter 2.9 mm) with silicone tubes (inner diameter 0.5 mm) inserted on both ends, was filled to a length of about 14 mm (volume about 90 µl). One tube of the column was connected to a Desaga peristaltic pump, the other to a drop counter. Nuclei not attached to the filter pieces were washed out by perfusion with about 300 µl of medium. The temperature was adjusted by immersion of the column into a water bath. Two "nuclei columns", a control and the experiment, were run simultaneously.

The medium consisted of 0.25 parts (v/v) of cytoplasm prepared by centrifugation of a liver homogenate (1 part liver, 6 parts 0.25 M sucrose, 1 mM $MgCl_2$) at 60 000 g for 1 hour and contained 50 mM Tris.HCl (pH 8.1), 125 mM sucrose, 5 mM $MgCl_2$, 35 mM KCl, 5 mM dithiothreitol and 6 mM KF. The concentration of the reactants was 1.25 mM ATP, 1.25 mM CTP, 1.25 mM GTP and 25 µM ^{3}H-UTP (about 20 µCi/ml medium).

To the effluent fractions and to the aliquots of the column contents, 1 mg albumin and ice cold 10% trichloroacetic acid (TCA), 1% pyrophosphate, were added, the precipitate was washed twice with ether: ethanol = 3:1 (v/v) and dried. It was dissolved in 0.1 M Tris, 0.5 M NaOH and immediately precipitated with 10% TCA, 1% pyrophosphate. The pellet was washed twice with 5% TCA, 1% pyrophosphate, twice with ether-ethanol, dried, and dissolved in 1 ml of soluene. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

At the end of the incubation period, the contents of the columns were transferred into a centrifuge tube and the membrane filter pieces were dissolved in acetone: ethanol = 3:1 (v/v). The precipitate was washed two times with acetone-ethanol and dried. A known volume of water and a few

glass beads (diameter 0.45 mm) were added and a suspension was prepared by sonification with ultra sound. Aliquots for the determination of the DNA content and of the radioactivity were taken. The DNA content was estimated by the method of Burton[2]. Incubations with pancreatic RNAse (Boehringer) were performed in 0.1 M acetate buffer, pH 5.1.

The nucleotides were obtained from the Sigma Chemical Co., St. Louis, or from Boehringer, Mannheim. The ^{3}H-UTP was a product of the New England Nuclear Corporation, Boston.

Results and discussion. In order to equilibrate the nuclei with the medium, and to obtain background values, the medium was first pumped through the column at 0°C. As the uptake of the solution, the temperature change, and the collection of the effluent occur at different places, all events in the figures are correlated to that occuring in the column.

Figures 1-3,-A show the incorporation of ^{3}H-UTP into TCA-insoluble

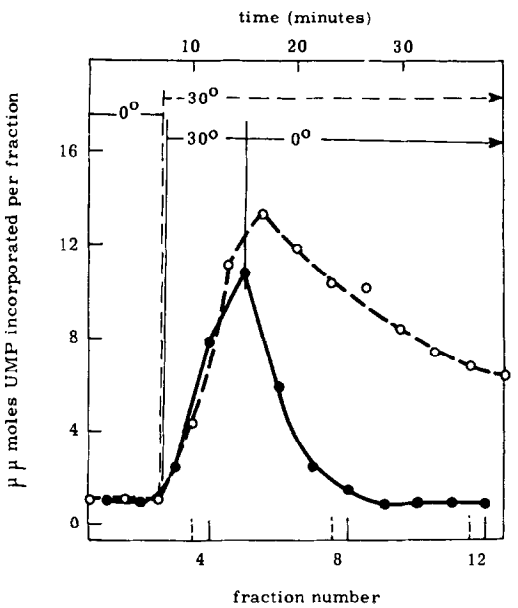

Fig. 1. Effect of temperature on the reaction. A o----o: Control. In column: 1.36 mg DNA, 85 μμ moles UMP incorporated. Released: 82 μμ moles incorporated UMP. (Total: 123 μμ moles/mg DNA). B ●——●: Experiment. 1.55 mg DNA. The temperature of the columns was changed as indicated. Fractions of 180 μl were collected.

material of the effluent fractions. The reaction was started by raising the temperature from 0° to 30°C. Immediately after the temperature change, release of labeled RNA sets in, reaching a maximum after 8-10 minutes. In the following period of incubation, the rate of the reaction gradually decreases. The decrease is probably due to the loss of RNA polymerase, as it has been reported that even at 0°C the enzyme is eluted by a sucrose medium from liver nuclei[3]. The amount of labeled product released per minute of incubation was equivalent to about 1-3% of the amount retained in the column.

The following facts indicate that the material formed is RNA: Exposure of the effluent solution to RNAse decreased the radioactivity of the TCA-insoluble material by 92-97 per cent. Omission of the reactants ATP, CTP and GTP inhibited the incorporation (Fig. 2, 3). Preliminary results obtained by density gradient centrifugation show that the labeled product is of high molecular weight.

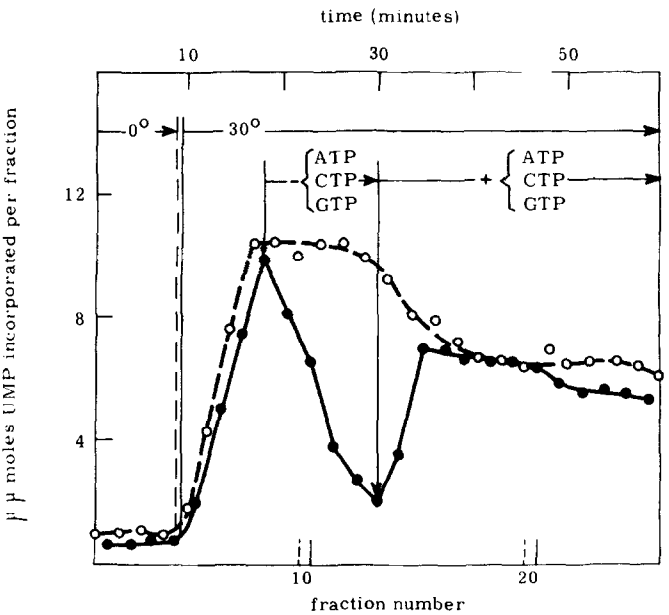

Fig. 2. Inhibition of the reaction by the omission of reactants. A o----o: Control. In column: 1.04 mg DNA, 148 μμ moles UMP incorporated. B ●——●: Experiment. 1.01 mg DNA. ATP, CTP and GTP were omitted and added to the perfusion medium as indicated. Fractions of 120 μl were collected.

Simultaneously with the experiment of Fig. 1 and under otherwise similar conditions, a conventional incubation was performed in glass tubes. After about 10 minutes, the rate of UMP incorporation decreased markedly, and after 40 minutes of incubation, only 16 $\mu\mu$ moles UMP were incorporated per mg of DNA, compared to the 8-fold amount incorporated in the nuclei plus effluent of the column (123 $\mu\mu$ moles/mg DNA).

In the experiment B of Fig. 1 the temperature of the column was again reduced to 0°C. The concentration of the labeled product decreased in the following fractions.

The reaction in Fig. 2-B was interrupted and reinitiated by omission and addition of the reactants ATP, CTP and GTP. In another experiment the reaction was started by the addition of ATP 23 minutes after raising the temperature (Fig. 3-B). In both cases rates comparable to the controls were reached.

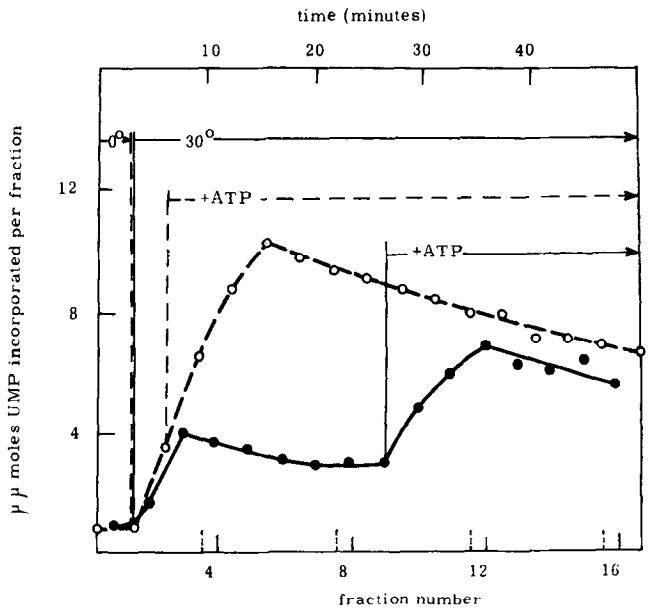

Fig. 3. Initiation of the reaction by ATP. A o----o: Control. In column 0.90 mg DNA. 225 $\mu\mu$ moles UMP incorporated. B ●——●: Experiment. 0.91 mg DNA. ATP had been omitted from the incubation medium, and was added as indicated. Fractions of 180 μl were collected.

The results indicate: (a) that the nuclei maintain their capacity to incorporate UMP into RNA even after a prolonged incubation period, and (b) that that part of the newly synthesized RNA leaving the column is released during a limited period of time and does not "smear out" gradually. Thus, the curves do not show the gradual release of RNA synthesized during the first minutes of incubation. Under appropriate conditions, the synthesis and the release of RNA may be differentiated.

The technique may also be applicable to kinetic studies on the biochemical activities of other cellular and subcellular systems.

References

1. Widnell, C.C., and Tata, J.R., Biochem. J. 92, 313 (1964).
2. Burton, K., Biochem. J. 62, 315 (1956).
3. Liao, S., Sagher, D., and Fang, S., Nature 220, 1336 (1968).