

NUCLEAR MEMBRANES OF CULTURED MAMMALIAN CELLS IN THE PERIOD PRECEDING DNA SYNTHESIS

SUSUMU KISHIMOTO, M.D., and IRVING LIEBERMAN, Ph.D.

From the Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania

ABSTRACT

When kidney cells are cultured directly from the rabbit, the nuclear membranes undergo a change that can be measured as an increase in electrophoretic mobility. The change appears to begin immediately upon culture and is maximal 2 hours later, after which the mobility remains constant at the elevated level. Actinomycin D and *p*-fluorophenylalanine, but not EDTA or ionizing radiation, suppress the increase in nuclear electrophoretic mobility. With synchronously growing L cells, no change was detected in nuclei from cells taken during various parts of the division cycle.

Before a mammalian cell can replicate its DNA, it must pass through a relatively lengthy preparatory period that culminates in the acquisition of competency to form DNA. To a large extent, studies of the changes that occur during this preparatory period have been carried out with two systems, the liver cell of the partially hepatectomized rat and the kidney cell cultured directly from the rabbit. Thus far, the two cell systems have proven themselves to be similar to a remarkable extent, differing primarily in temporal details. With both systems, quantitative and perhaps qualitative alterations in RNA and protein metabolism appear to be among the important changes of the presynthetic period (1-4).

More recently, a membrane change, measured as an increase in electrophoretic mobility, has been shown to occur in hepatic nuclei after partial hepatectomy (5). It was of interest to determine whether a similar change takes place in cultured kidney cells as they pass through the period preceding DNA synthesis. In addition, for comparative purposes, the electrokinetic properties of the nuclei of a rapidly multiplying cultured cell, the

L cell, were examined during various parts of its division cycle.

The purpose of this report is to show the evidence for an alteration in the nuclear membranes of kidney cells cultured from the animal. Just as with hepatic nuclei (5), RNA and protein synthesis are requisite for the kidney membrane change. Interestingly, no analogous electrokinetic alteration was detectable in the L cell nuclei.

MATERIALS AND METHODS

Materials

The components of the kidney culture medium have been previously described (3). The constituents of Eagle's basal medium were obtained from Microbiological Associates Inc., Bethesda, Actinomycin D was kindly provided by Dr. Elmer Alpert, Merck Sharp and Dohme Laboratories, New York, and 4-aminopteroyl-glutamic acid (Aminopterin) was a gift from Dr. S. M. Hardy, Lederle Laboratories, Pearl River, New York. *D,L-p*-fluorophenylalanine was obtained from the Nutritional Biochemicals Corporation, Cleveland, and methyl-³H-thymidine

(6000 $\mu\text{c}/\mu\text{mole}$) was supplied by the New England Nuclear Corporation, Boston.

Methods

The preparation of cultures of trypsinized rabbit kidney cortex cells has been described (3). The cells were detached from the glass surface with a rubber spatula and the nuclei were purified with 0.1 M citric acid.¹ Electrophoretic mobility measurements were made in a Northrop-Kunitz cell exactly as detailed for liver (5). Each value represents the average of 40 individual measurements, half made at the 20 per cent, and half at the 80 per cent, stationary level. The direction of the current was reversed after every 5 to 10 measurements. Moving nuclei stopped immediately when the current was turned off, indicating the absence of detectable convection currents.

The procedure used for x-radiation of the kidney cells was previously described (7).

L cell cultures were prepared in 60-mm petri dishes and kept in an incubator (37°) gassed with CO₂ at a rate just sufficient to maintain the growth medium at about pH 7.4. Each culture was initiated with 1×10^6 cells in 3 ml of Eagle's basal medium supplemented with 10 per cent calf serum. Cell growth was synchronized with aminopterin essentially as described by Rueckert and Mueller (8) for HeLa cells. Sixteen hours after implantation, to each culture was added 0.03 ml of 2×10^{-5} M aminopterin, 0.03 ml of 0.03 M glycine, and 0.02 ml of 0.01 M adenine. After incubation for an additional 16 hours, the inhibition of DNA synthesis was overcome by the addition to each dish of 0.15 ml of 10^{-4} M thymidine.

Nuclei were purified from the L cells and the electrophoretic mobility measurements were made by the procedures used for the kidney cells.

To estimate the number of L cells that were forming DNA, 10 μc of ³H-thymidine was added to the culture fluid. One hour later, the medium was replaced with Carnoy's solution. After 10 minutes, the dish was washed with 70 per cent ethanol, extensively with water, and it was then coated with Kodak Nuclear Track Emulsion, Type NTB3. After 2 days, the photographic preparation was developed and the cells, attached in the dish, were stained with Mayer's hematoxylin. Under these conditions, background grains were absent. Cells were scored as labeled or unlabeled and each value was obtained by examining 200 cells. Mitoses were estimated in the same preparations, and a mitotic cell was defined

¹ It is recognized that purification of nuclei with citric acid may result in the removal of their outer membrane (6) and that the electrokinetic changes may reflect chemical alterations limited to the inner membrane.

TABLE I
Nuclear Electrophoretic Mobility of Cultured
Kidney Cells as a Function of Time in
Culture

Culture time	Electrophoretic mobility ($\frac{\mu/\text{sec}}{\text{volt/cm}}$)			
	I	II	III	average
hrs.				
0	-0.90	-0.89	-0.88	-0.89
1	-0.94	-0.98		-0.96
2	-1.08	-1.12	-1.11	-1.10
4	-1.11	-1.14	-1.08	-1.11
80	-1.08	-1.07	-1.11	-1.09

Kidney cultures were prepared from trypsinized cells distributed in 60-mm petri dishes. Each determination was made on the nuclei from 96 culture dishes (ca 3×10^6 cells in 3 ml of growth medium per culture). The results of three separate experiments are given. For the 80-hour-old cultures, the medium was removed at 40 hours by aspiration and replaced with fresh growth medium. The cells were harvested and the electrophoretic mobilities of the purified nuclei were estimated as described in Methods.

in the conventional manner as one in which a condensation of the chromatin material had occurred. Each value was obtained by examining 1,000 cells.

RESULTS

KIDNEY CELLS

NUCLEAR ELECTROPHORETIC MOBILITY AS A FUNCTION OF TIME IN CULTURE: The rise in electrophoretic mobility of the kidney nuclei appeared to begin immediately upon culture, and a maximum was achieved 2 hours later, after which the mobility remained constant at the elevated level (Table I). As the table shows, the change was about 1.2-fold, only a little more than half of that found with liver nuclei after partial hepatectomy (5).

Table II shows the distribution of the electrophoretic velocities (seconds of time required to move 40 μ) of the individual nuclei from eight batches of kidney cells. Half the preparations were from cells harvested at zero time and the other half, 4 hours later.

EFFECT OF ACTINOMYCIN D, P-FLUOROPHENYLALANINE, EDTA, AND IONIZING RADIATION: The three chemical agents and ionizing radiation are capable of preventing passage of the kidney cells through the period preceding DNA

TABLE II
Distribution of Electrophoretic Velocities of Individual Nuclei

Electrophoretic velocity <i>sec.</i>	Time after culture							
	0				4			
	I	II	III	IV	I	II	III	IV
4.5-4.8	0	0	0	0				
4.1-4.4	0	2	1	0				
3.7-4.0	7	5	2	4	0	0	0	0
3.3-3.6	8	7	7	7	0	0	1	0
2.9-3.2	15	19	16	14	4	6	9	7
2.5-2.8	9	6	11	11	11	11	5	15
2.1-2.4	1	1	3	3	16	16	14	16
1.7-2.0	0	0	0	1	9	7	11	2
1.3-1.6	0	0	0	0	0	0	0	0
Mean velocity (sec.)	3.02	3.10	3.02	3.18	2.42	2.39	2.44	2.54
Over-all mean velocity (sec.)	3.08 ± 0.44				2.54 ± 0.36			
95 per cent confidence limit of over-all means	3.01-3.15				2.39-2.51			

Each column of zero time electrophoretic velocities represents the observations made with a different kidney preparation. The measurements made after 4 hours in culture correspond to the zero time determinations. To simplify the arithmetical calculations, electrophoretic velocities, instead of mobilities, were used to make the statistical calculations.

formation (3, 4, 7). For this reason, their ability to inhibit the nuclear membrane change was tested, and levels were used that could totally block the synthesis of DNA. The results of a typical experiment are shown in Table III. As may be seen from the table, actinomycin D and *p*-fluorophenylalanine, added to the cultures at zero time, completely suppressed the increase in nuclear electrophoretic mobility as measured after 4 or 4.5 hours of culture. EDTA and ionizing radiation, on the other hand, were without effect. The ineffectiveness of EDTA ruled out the possibility that Zn⁺⁺, essential for passage through the DNA presynthetic stage (3, 7), is required for the nuclear membrane change.

L CELLS

With synchronized cultures of L cells, electrophoretic measurements were made with nuclei from cells in various stages of the division cycle (Fig. 1). As the figure shows, no electrophoretic mobility differences were detected with nuclei from cultures in which most of the cells were forming DNA (-0.94), had recently completed the replication of DNA (-0.97), or were prepar-

TABLE III
Effect of Actinomycin D, *p*-Fluorophenylalanine, EDTA, and Ionizing Radiation on the Change in Electrophoretic Mobility of Nuclei from Cultured Kidney Cells

Age of culture	Addition or treatment	Electrophoretic mobility
<i>hrs.</i>		$\frac{\mu/\text{sec}}{\text{volt/cm}}$
0	None	-0.88
4	None	-1.11
4	Actinomycin D	-0.87
4.5	<i>p</i> -Fluorophenylalanine	-0.88
4	EDTA	-1.11
4	Ionizing radiation	-1.07

Kidney cultures were prepared from trypsinized cells as for Table I. Actinomycin D (0.01 $\mu\text{g/ml}$), *p*-fluorophenylalanine (0.75 $\mu\text{mole/ml}$), and EDTA (1.7 $\mu\text{moles/ml}$) were added at zero time as indicated. Exposure to x-radiation (3000 r) was also at zero time. The cells were harvested at the times indicated and the electrophoretic mobilities of the purified nuclei were estimated as described in Methods.

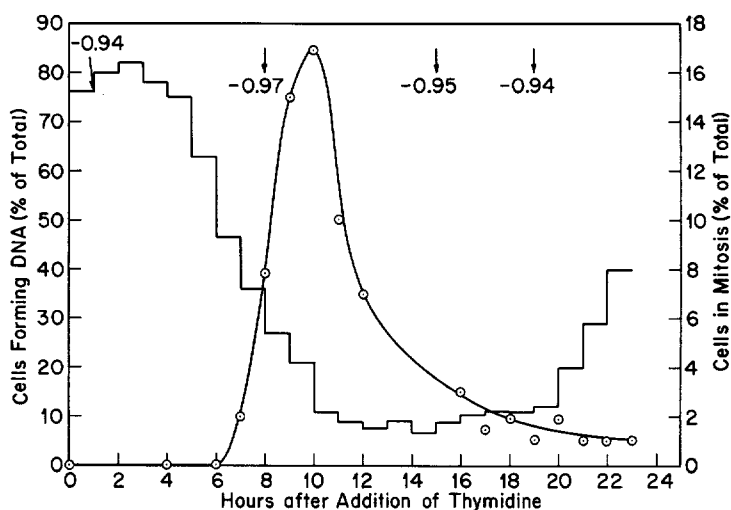


FIGURE 1 Electrophoretic mobility of L cell nuclei from cells in various stages of the division cycle. The cultures of L cells were prepared and synchronized with aminopterin as described in Methods. The time of addition of thymidine is designated as zero time. Each of the values for the number of cells forming DNA (line curve) and the number of cells in mitosis (open circles) represents the average of the results obtained with two cultures. Electrophoretic mobility measurements were each made with the nuclei from 96 culture dishes that were harvested, as shown by the arrows, 1, 8, 15, or 19 hours after DNA synthesis was initiated by the addition of thymidine. The results of these measurements, in $\frac{\mu/\text{sec}}{\text{volt/cm}}$, are shown below the arrows.

ing to re-enter the period of DNA synthesis (early = - 0.95; late = - 0.94).

DISCUSSION

Among the features of the period preceding DNA replication that are shared by liver and kidney cells are (1) an increased rate of metabolism of RNA (1-4); (2) the synthesis of essential RNA and protein (1-4); (3) an obligate requirement for Zn^{++} (9,3); (4) rises, at about the time of DNA synthesis, in the specific activities of enzymes involved in DNA formation (10, 11, 7); and (5) a sensitivity to ionizing radiation (12,7). An additional shared property can now be added, a nuclear membrane change that occurs early in the DNA presynthetic period. Although the cause, the chemical nature, and the effects of these nuclear changes are not yet understood, it seems probable that they will also prove to be alike for both the liver and kidney cells.

The absence of a similar membrane change in the presynthetic period of a rapidly multiplying cell, the L cell, is not surprising. These cells may lack many of the controls that regulate entry of liver and kidney cells into the period of DNA

formation. Thus, only minor changes in DNA polymerase activity were found in L cells during their division cycle (13, 14) in contrast to the striking increases exhibited by liver (10) and kidney cells (7). Again, doses of ionizing radiation that have little effect on the essential processes of the DNA presynthetic period of the L cell (15) completely block the entry of liver (12) or kidney cells (7) into the stage of DNA replication.

Two final points related to the nuclear electrophoretic measurements warrant some consideration. First, it should be emphasized that only the net charge of the nuclear surface determines its electrokinetic properties. The size of the nucleus, its shape, and the nature of its contents should not influence its electrophoretic mobility (16). Second, the use of citric acid to prepare the nuclei raises the question of whether the final products are encased in a single or a double membrane. Although this point has been investigated with liver nuclei (their outer membrane is removed by citric acid (6, 17)), no information is available about kidney or L cell nuclei.

This investigation was supported by a research grant from the National Institutes of Health, United States Public Health Service. The senior author is on leave from the Third Department of Internal Medicine, Osaka University Medical School, Osaka, Japan.

The authors wish to thank Dr. Lincoln J. Gerende of the Department of Preventive Medicine, University of Pittsburgh, for his help in making the statistical calculations.

Received for publication, April 23, 1964.

REFERENCES

1. FUJIOKA, M., KOGA, M., and LIEBERMAN, I., *J. Biol. Chem.*, 1963, **238**, 3401.
2. TSUKADA, K., and LIEBERMAN, I., *J. Biol. Chem.*, 1964, **239**, 2952.
3. LIEBERMAN, I., and OVE, P., *J. Biol. Chem.*, 1962, **237**, 1634.
4. LIEBERMAN, I., ABRAMS, R., and OVE, P., *J. Biol. Chem.*, 1963, **238**, 2141.
5. KISHIMOTO, S., and LIEBERMAN, I., *J. Cell Biol.*, 1964, **23**, 511.
6. GURR, M. I., FINEAN, J. B., and HAWTHORNE, J. N., *Biochim. et Biophysica Acta*, 1963, **70**, 406.
7. LIEBERMAN, I., ABRAMS, R., HUNT, N., and OVE, P., *J. Biol. Chem.*, 1963, **238**, 3955.
8. RUECKERT, R. R., and MUELLER, G. C., *Cancer Research*, 1960, **20**, 1584.
9. FUJIOKA, M., and LIEBERMAN, I., *J. Biol. Chem.*, 1964, **239**, 1164.
10. BOLLUM, F. J., and POTTER, V. R., *Cancer Research*, 1959, **19**, 561.
11. MALEY, F., and MALEY, G. F., *J. Biol. Chem.*, 1960, **235**, 2968.
12. BOLLUM, F. J., ANDEREGG, J. W., McELYA, A. B., and POTTER, V. R., *Cancer Research*, 1960, **20**, 138.
13. LITTLEFIELD, J. W., MCGOVERN, A. P., and MARGESON, K. B., *Proc. Nat. Acad. Sci.*, 1963, **49**, 102.
14. GOLD, M., and HELLEINER, C. W., *Biochim. et Biophysica Acta*, 1964, **80**, 193.
15. WHITMORE, G. F., STANNERS, C. P., TILL, J. E., and GULYAS, S., *Biochim. et Biophysica Acta*, 1961, **47**, 66.
16. ABRAMSON, H. A., MOYER, L. S., and GORIN, M. H., *Electrophoresis of Proteins and the Chemistry of Cell Surfaces*, Reinhold Publishing Corporation, New York, 1942, 14.
17. RUBY, J. R., Ph.D. thesis, University of Pittsburgh, 1963.