

TIME SEQUENCE OF NUCLEAR PORE FORMATION IN PHYTOHEMAGGLUTININ-STIMULATED LYMPHOCYTES AND IN HeLa CELLS DURING THE CELL CYCLE

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ABSTRACT

The time sequence of nuclear pore frequency changes was determined for phytohemagglutinin (PHA)-stimulated human lymphocytes and for HeLa S-3 cells during the cell cycle. The number of nuclear pores/nucleus was calculated from the experimentally determined values of nuclear pores/ μ^2 and the nuclear surface. In the lymphocyte system the number of pores/nucleus approximately doubles during the 48 hr after PHA stimulation. The increase in pore frequency is biphasic and the first increase seems to be related to an increase in the rate of protein synthesis. The second increase in pores/nucleus appears to be correlated with the onset of DNA synthesis. In the HeLa cell system, we could also observe a biphasic change in pore formation. Nuclear pores are formed at the highest rate during the first hour after mitosis. A second increase in the rate of pore formation corresponds in time with an increase in the rate of nuclear acidic protein synthesis shortly before S phase. The total number of nuclear pores in HeLa cells doubles from ~ 2000 in G_1 to ~ 4000 at the end of the cell cycle. The doubling of the nuclear volume and the number of nuclear pores might be correlated to the doubling of DNA content. Another correspondence with the nuclear pore number in S phase is found in the number of simultaneously replicating replication sites. This number may be fortuitous but leads to the rather speculative possibility that the nuclear pore might be the site of initiation and/or replication of DNA as well as the site of nucleocytoplasmic exchange. That is, the nuclear pore complex may have multiple functions.

INTRODUCTION

It has been suggested that nucleocytoplasmic information transfer takes place through specialized openings in the nuclear envelope called nuclear pore complexes (Watson, 1959). These nuclear pores have a diameter of about 800 Å and an elaborate internal structure which has been well described (for a review see Franke, 1970). Direct experimental evidence for the transport of material

across the nuclear pore complex was obtained by Feldherr (1962, 1969) who showed colloidal gold particles crossing the nuclear pore complex at a central space much smaller than the pore diameter. Changes in pore permeability (Feldherr, 1971) or the number of nuclear pore complexes could be mechanisms for the regulation of nucleocytoplasmic transfer of macromolecules.

The first evidence of nuclear pore formation during interphase was reported by Merriam (1962) in oocytes. We have obtained structural as well as experimental evidence for nuclear pore formation by stimulating lymphocytes with phytohemagglutinin (PHA) and comparing the pore frequencies of the normal and stimulated lymphocytes (Maul et al., 1971). The observation that nuclear pores can be formed during interphase suggested that pore formation could be analyzed in terms of the cell cycle. Such an approach would allow examination of pore formation in relation to specific cell-cycle events and perhaps elucidate the function of the nuclear pore complex. In this communication we report the results of an investigation of the time sequence and rate of pore formation in PHA-stimulated lymphocytes and synchronized HeLa cells.

MATERIALS AND METHODS

Lymphocyte Culture and PHA Stimulation

Freshly drawn blood was either heparinized or defibrinated and sedimented with 3% pig skin gelatin (Coulson and Chambers, 1964). The leucocyte-rich serum was then passed through a nylon column (Cooper and Rubin, 1965), resulting in a preparation which was 99% lymphocytes with respect to other leucocytes. Samples of 1.2×10^6 lymphocytes/ml were resuspended immediately after purification in modified Eagle's medium with 15% fetal calf serum, 10 units/ml of penicillin and streptomycin, and 2 mM glutamine.

After incubation for varying times in the presence or absence of 15 μ l/ml of Difco phytohemagglutinin M (Difco Labs, Detroit, Mich.) the cells were prepared for freeze etching by incubating for 20 min at 0°C in Eagle's medium which contained 20% glycerol. After a sample was removed for nuclear size determination, the remaining cells were frozen in liquid nitrogen-cooled Freon 22. The nuclear size was determined immediately after the treatment in 20% glycerol by measuring the diameter with a Reichert phase microscope with anopteral phase optics.

Estimation of the Rate of Macromolecular Synthesis

The rates of DNA, RNA, and protein synthesis were estimated at various times by a 30 min pulse label with methyl thymidine- ^3H (2 $\mu\text{Ci/ml}$, 20 Ci/mole), 5-uridine- ^3H (2 $\mu\text{Ci/ml}$, 24 Ci/mole), or leucine- ^3H (2 $\mu\text{Ci/ml}$, 34 Ci/mole) as appropriate. All compounds were purchased from New England Nuclear Corporation, Boston, Mass. Cul-

tures were assayed in triplicate as previously described (Lieberman et al., 1971). The range of observed values was $\pm 10\%$. Results are expressed as cpm/culture and are the average of the three determinations. The uridine incorporation was not corrected for uridine kinase induction (Kay and Handmaker, 1970).

Radioautography was used to determine the number of cells in S phase. At 48 hr after stimulation with PHA, a sample was incubated with 2.0 $\mu\text{Ci/ml}$ of methyl thymidine- ^3H (sp act 6 Ci/mole, New England Nuclear Corp.) and prepared for radioautography by air-drying the washed cell suspension on an acid-washed glass slide and fixing in 100% ethanol. The glass slides were dipped in Kodak NTB (Eastman Kodak Co., Rochester, N. Y.) emulsion and exposed for 7 days. After development in Kodak D19 the cells were stained with hematoxylin-eosin and the number of labeled lymphocytes was determined by counting 1000 lymphocytes. The mitotic index was determined at 48 hr after PHA stimulation after 12 hr Colcemid treatment (0.5 $\mu\text{g/ml}$) (Ciba Pharmaceutical Co., Summit, N. J.).

Freeze Etching and Nuclear Pore Determination

The concentrated suspensions of lymphocytes were freeze etched according to Moor and Mühlethaler (1963) in a Balzer freeze-etch apparatus at -100°C . Etching time was 2 min. The exposed surface was then shadowed with platinum and carbon coated. A calibrated Zeiss 9A and an RCA EMU-4 electron microscope were used to photograph the exposed nuclear surface areas at a fixed magnification. Nuclear pore counts were made directly on the negative, using a stereo microscope. The exposed nuclear areas were determined by planimetry.

HeLa S-3 Cell Culture and Synchronization

Synchronized HeLa S-3 cells were obtained by thymidine inhibition of DNA synthesis (Nexos, 1962; Bootsma et al., 1964), followed by selective detachment of mitotic cells from 30 1-liter Blake bottles (Terasima and Tolmach, 1963; Robbins and Marcus, 1964). The detachment procedure took approximately 30 min. Time 0 was assumed to be at 15 min after we began to detach cells. We obtained about 3.5×10^8 mitotic cells at a concentration of about 4×10^6 cells/ml, in an average experiment. The number of mitotic cells in the selectively detached population was determined to be between 95 and 97%. Many of these cells were already in anaphase, telophase, or had completed cytokinesis, but daughter cells were still attached to each other by the Flemming body. The HeLa cells were kept in suspension in

Joklik-modified Eagle's Minimal Essential Medium (Grand Island Biological Co., Grand Island, N. Y.), containing 3.5% calf serum, 3.5% fetal calf serum (Flow Labs, Inc., Rockville, Md.), plus penicillin and streptomycin.

Estimation of DNA Replication Rate

Every hour 2 ml of cell suspension were added to 0.2 μ Ci of thymidine- 14 C in 0.1 ml of H $_2$ O (sp act 25 mCi/mole, New England Nuclear Corp.). After $\frac{1}{2}$ hr of incubation at 37°C, the reaction was stopped by the addition of 5 ml of ice-cold Earle's basal salt solution. The cells were washed twice in that salt solution and then resuspended in 5 ml of ice-cold 10% trichloroacetic acid (TCA), chilled on ice for 15 min, and collected on a 0.45 μ Millipore filter (Millipore Corp., Bedford, Mass.). The precipitated cells were washed twice with 5 ml of cold 10% TCA, then the filters were dissolved and counted in Bray's solution with a Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The results are expressed as cpm/culture.

Nuclear Size Determination and Freeze Etching

Nuclear size determinations were made on 100 cells with anopteral phase optics by measuring the long and short axis of the nuclei at a magnification of 1000. Two hairs were placed between the slide and the cover glass to prevent flattening of the cells. The diameters were averaged and the nuclear surface area was calculated as if the nucleus were a sphere. Before the measurement the cells were fixed by adding 2 ml of 1% glutaraldehyde in 0.1 M phosphate buffer to 2 ml of cell suspension in medium. After 15 min fixation the cells were washed and resuspended in Hanks' balanced salt solution. The nuclear size was determined shortly thereafter, but in no case later than 1 hr. After fixation, size changes were found if the nuclei were stained with hematoxylin-eosin or if they were measured after long delays. An evaluation of these changes, i.e., individual variability of measurement, reproducibility of nuclear size measurements, possible changes during freezing, and other error determinations, will appear as a separate communication. It should be mentioned here, however, that we found a shrinkage of 4-6%, as far as nuclear surface is concerned, in 0.5% glutaraldehyde-fixed cells. As this amounts to about 2-3% change in the measured diameter, it may well be within the limits of the technique.

In order to determine how many cells had completed the cell cycle, in some experiments we accumulated the mitotic cells beginning with the 13th hr by the addition of 0.5 μ g Colcemid per ml to a separate culture of 25 ml of the HeLa cell

suspension (separated at the 13th hr) and withdrew samples at hourly intervals. The mitotic index was determined by counting 500 cells. HeLa cells were prepared for freeze etching by a procedure identical to that described for lymphocytes. Viability counts were performed by the dye exclusion test, using trypan blue at selected points before glycerol treatment, shortly after glycerol was added, and on cells of the concentrated suspension which had not been used for freezing.

RESULTS

General Remarks and Technical Considerations

Because of the high nucleocytoplasmic ratio it is difficult to accurately determine the nuclear diameter in human lymphocytes by phase contrast optics. As can be seen in Fig. 1, there is often only a less than micron-wide cytoplasmic rim around the nucleus. Therefore, our measurements of these nuclei may often be too large. The measurements become more accurate as more cytoplasm comes to surround the nucleus during blast transforma-

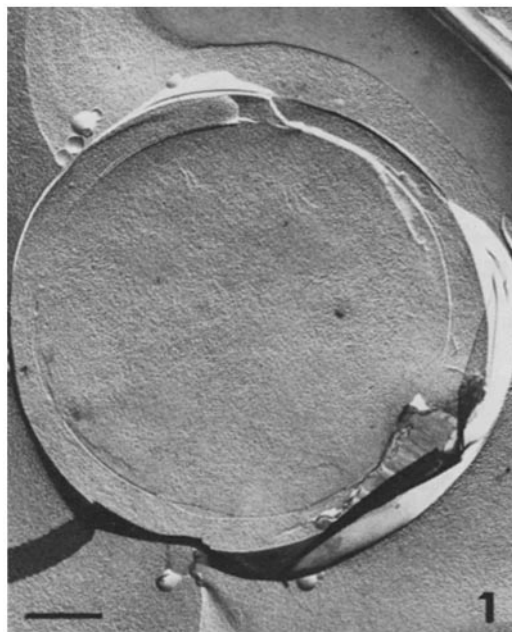


FIGURE 1 The cross fracture of a lymphocyte demonstrates the narrow cytoplasmic rim around the nucleus which makes it exceedingly difficult to measure the true diameter of the nucleus of unstimulated lymphocyte with the light microscope. Scale marker, 1 μ . \times 10,000.

tion. Slight increases in nuclear size may also be concealed by placing nuclei in size groups 0.6 or 1.0 μ apart (micrometer units).

In frozen-etched preparations not all nuclear surfaces were of the same appearance. Fig. 2 shows an exposed nuclear surface of the type which was used for our frequency study. It was present in the outer areas of a replica (fastest freezing rate) as well as in the center (slowest freezing rate). A second type of nuclear surface showed an irregular, bumpy appearance (Fig. 3). The cytoplasm of these cells also had a much coarser background. Images of both types were found side by side (Fig. 4), indicating that some condition of the cell before freezing resulted in this appearance. If one determines the pore frequency of both types of nuclear envelopes in HeLa cells, one finds that the bumpy nuclear envelope has a 10% higher pore frequency ($P \geq 0.1$). We therefore assumed that these cells were dead cells in which the turgor of the nuclei was eliminated, resulting in a shrinkage of the nuclear membrane, or was due to ice crystal formation (Maul and Maul, in preparation). This could be confirmed by using the dye exclusion test for viability. HeLa cells survive the 20% glycerol treatment well for over 2 hr where the percentage of dead cells rises from 2% in the cell suspension to only about 3–4% after 2 hr of glycerol incubation. However, if one determines the viability after the cells have been concentrated and placed on the specimen holder through a finely drawn pipette, the percentage of dead cells rises to 15% or more (up to 50%) after only 45 min in glycerol. It was found that the percentage of dead cells rises with the increase in time of close packing (after concentration) and rises significantly by dispensing them through finely drawn pipettes. Because of these considerations, we did not include the wrinkled nuclear surfaces in our results.

Changes of Nuclear Pore Frequency after PHA Stimulation of Human Lymphocytes

In order to determine the number of nuclear pores, two measurements were made. First, utilizing the replicas obtained by the freeze-etching technique, we determined the number of nuclear pores/ μ^2 . Second, we measured the nuclear surface area and then calculated the total number of pores/nucleus. In Fig. 5, the average number of pores/nucleus is plotted vs. time after stimulation. It does not change noticeably during the first 6 hr. Thereafter, one observes a rapid increase to a pla-

teau of about 500 pores/nucleus between the 24th and 36th hr after PHA stimulation. Another increase in pore number occurs between the 36th and 48th hr. In all, the average number of pores approximately doubles by 48 hr after PHA stimulation (287 vs. 671) compared to the control cells at the same time. If we compare the rate of the three major synthetic processes (protein, RNA, and DNA synthesis) with the change in pore number, we find an increase in pore number at the time when the rate of RNA synthesis increases. The curve of the rate of protein synthesis seems to correlate best, but the increase in the rate of protein synthesis occurs 4 hr before an increase in nuclear pore number. DNA synthesis begins between the 24th and 36th hr. The second increase in pore number occurs at the 36th hr after stimulation. We may therefore correlate the second increase in pore frequency with the increased DNA content or synthesis.

In order to determine how many cells are actually in S phase, we used radioautography and found that 8.0% of the cells were labeled with thymidine- ^3H at 48 hr after PHA stimulation. In contrast, at 0 hr only one out of 5000 cells was labeled, and in control lymphocytes at 48 hr after stimulation only one out of 1000 cells was labeled. After 12 hr of Colcemid treatment, only 0.2% mitotic cells were found at 48 hr after PHA stimulation.

Analysis of the Frequency Distribution of Pores/ μ^2 .

If we plot the average number of nuclear pores/ μ^2 in nuclear envelope fragments at different times after stimulation with PHA, a difference between control and stimulated cells is observable 2 hr after stimulation (Fig. 6, lower row) despite the fact that the numbers of pores/ μ^2 in control and stimulated cells are identical (Table I). At 0 hr, we have a single, narrow frequency maximum between 2.5 and 3.5 pores/ μ^2 and no, very low pore frequencies. The distribution changes considerably by 2 hr after PHA stimulation. The frequency maximum ranges now from 2 to 4 pores/ μ^2 and an additional maximum is present at 4.5–5 pores/ μ^2 . After 24, 36, and 48 hr of stimulation with PHA, we still find these two maxima.

This second maximum seems to be a consistent feature of all stimulated lymphocytes, starting with the 2nd hr after the addition of PHA. It is not present in the control sample at 48 hr. The average of this control sample is slightly larger than that at 0 hr but equal to that of the 24 hr control (Table I).

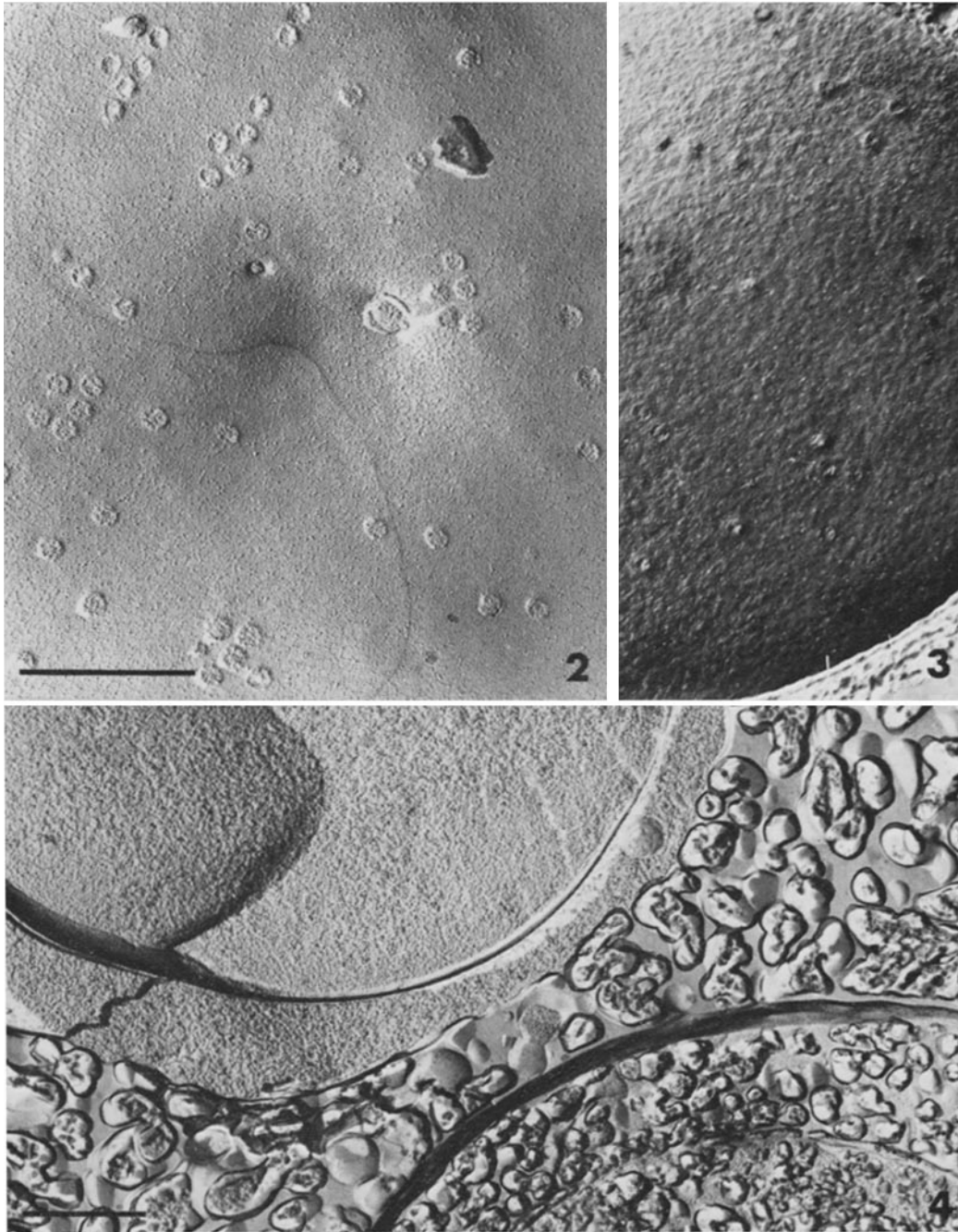


FIGURE 2 Lymphocyte nuclear envelope fragments with a smooth membrane appearance, as in this micrograph, were used in this study for the count of nuclear pores. Nuclear pores are distinct and easily recognizable. The inner nuclear membrane of this lymphocyte is one of the few found where the membrane can be seen from the cytoplasmic side of the cell. Usually concave fractures are obtained. Scale marker, 1μ . $\times 25,000$.

FIGURE 3 A number of nuclear envelope fragments in each preparation showed a wrinkled appearance and were easily distinguished from the smooth envelope pieces. These wrinkled membrane fragments were not used for counting nuclear pores. Scale marker as in Fig. 2. $\times 25,000$.

FIGURE 4 Normal and damaged cells could be found side by side on the outside of the specimen or, as in this micrograph, in the center of the specimen (area of slowest freezing). The wrinkled appearance therefore seems due to ice crystal formation in damaged cells. The presence of both cell types side by side demonstrates that we do not have an artifact of slow freezing. Scale marker, 1μ . $\times 17,000$.

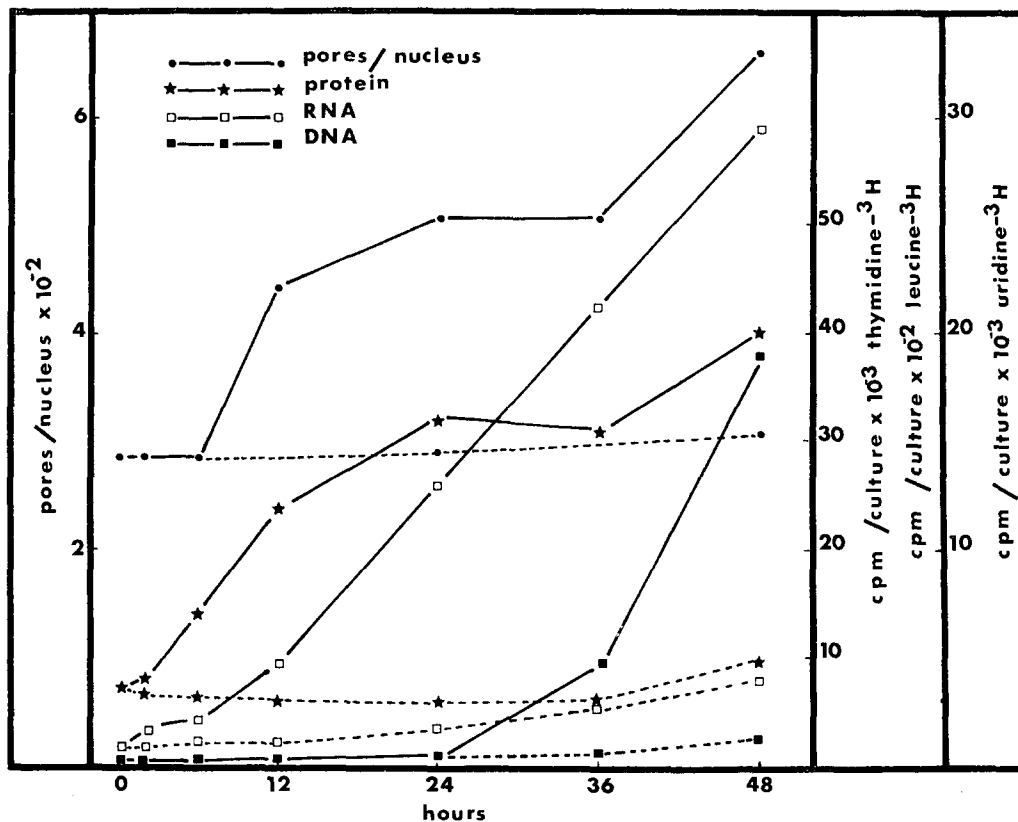


FIGURE 5 The number of nuclear pores/nucleus after stimulation of human lymphocytes with PHA is plotted as a function of time. The rates of thymidine, uridine, and leucine incorporation into DNA, RNA, and protein, respectively, are plotted on the same time scale. Control cultures are indicated by broken lines and PHA-stimulated cultures are indicated by continuous lines.

By radioautography, we had determined that 8% of the cells were in S phase. As we know that cells in S phase have a higher pore frequency than in G₁ phase (the rationale of this assumption will become evident in the results obtained from synchronized HeLa cells), we used the ~8% highest pore frequencies found to estimate the number of pores/nucleus in S phase of lymphocytes. If we take the 8% highest pore frequencies of the 48 hr sample, we find an average value of 7.7 pores/ μ^2 .

Similar results were obtained when we repeated the determination of the time sequence of pore frequency changes after stimulation with PHA (Table I). The change in number of pores/ μ^2 seemed to be reproduced up to 36 hr after stimulation; however, all of these second results were slightly lower by an average of 3.6%. The differences between the two experiments became larger after 48 hr. These differences may be explained by the fact that dif-

ferent numbers of cells were stimulated with PHA since the blood of different donors was used (Ling, 1968). The constantly lower number of pores/ μ^2 in the second experiment up to the 36th hr may be due to some factor in our technique or to an actual difference in the number/ μ^2 . As the variability around the average (3.6%) is slight, we believe that the reproducibility of the average number of pores/ μ^2 is good (Table I). The average of the 8% highest pore frequencies at the 60th hr after stimulation (Experiment 2) is 8.4 pores/ μ^2 .

Analysis of the Nuclear Size Frequencies

The precise measurement of nuclear size is difficult due to the large nucleocytoplasmic ratio in lymphocytes. With an increase in the amount of cytoplasm the measurements become more accurate, as one can distinguish more clearly the cell

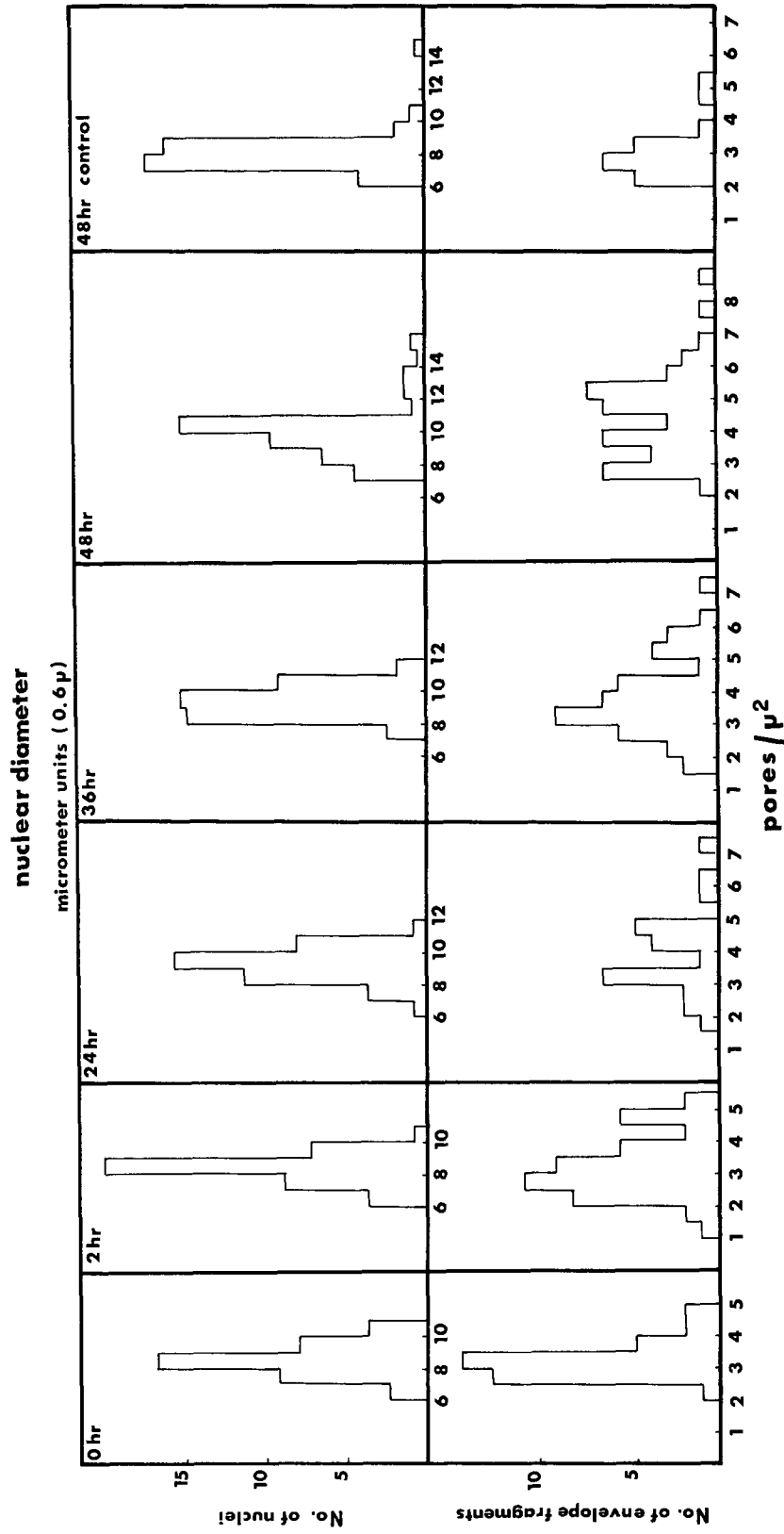


FIGURE 6 Distribution patterns of nuclear sizes and pores/ μ^2 of Experiment 1. The nuclear size distribution (upper row) is given in micrometer units (0.6 μ). The most prominent difference in distribution of pores/ μ^2 (lower row) between control and PHA-stimulated lymphocytes is the appearance of a second maximum.

TABLE I
Comparison of Results Obtained in Two Experiments using Human Lymphocytes

		Experiment 1					Experiment 2					% of difference P/ μ^2
		P/ $\mu^2 \pm sd$	NS	P/N	Average size	n	P/ $\mu^2 \pm sd$	NS	P/N	Average size	n	
hr			μ^2		μ^2			μ^2		μ^2		
0	Control	3.19 \pm 0.62	90.0	287	11.8	40	3.08 \pm 0.63	80.1	246	10.2	25	-3.4
2	PHA	3.20 \pm 0.93	90.5	289	11.1	50		80.7				
4	PHA							85.2				
6	PHA	3.09 \pm 1.11	91.6	283	11.7	22		87.8				
12	PHA	3.80 \pm 1.56	112.5	427	9.5	15		99.0				
24	PHA	3.92 \pm 1.28	129.3	506	12.2	25	3.75 \pm 0.64	106.2	398	11.1	45	-4.3
24	Control	3.41 \pm 0.52	87.5	298	9.9	20	3.28 \pm 0.89	75.7	248	9.6	23	-3.8
36	PHA	3.80 \pm 1.02	133.2	506	10.9	44	3.68 \pm 0.64	113.6	418	10.2	59	-3.2
48	PHA	4.47 \pm 1.42	159.5	671	12.3	44	4.21 \pm 1.48	136.7	575	10.5	22	-5.8
48	Control	3.41 \pm 1.34	90.2	307	10.2	22	3.20 \pm 0.87	84.8	271	9.1	24	-6.2
60	PHA						4.63 \pm 2.10	141.4	654	12.5	48	

The pores/ μ^2 (P/ μ^2) were obtained by freeze-etching lymphocytes, counting the pores on the negatives of nuclear envelope fragments, planimetry of the area, and dividing the number of pores by the μ^2 of the nuclear envelope fragment (sd, standard deviation). The nuclear size was calculated from the average diameter of 100 nuclei, assuming a spherical shape of the nucleus. The total number of nuclear pores/nucleus (P/N) is derived from the multiplication of the first two measured data. Also indicated are the average size (μ^2) of the envelope fragments for each sample and the number of envelope fragments (n) used for each sample. NS, nuclear size.

membrane and the nuclear membrane by phase optics (see also section on General Remarks). We find an increase in nuclear surface, beginning at the 6th hr, which plateaus between 24 and 36 hr after PHA stimulation. The nuclear size once more increases until the 48th hr, in parallel with the increase in rate of DNA and protein synthesis. At 0 hr, we find an average nuclear diameter of 5.3 μ with a normal distribution curve. At the second hr after stimulation with PHA, no change in the diameter can be measured (Table I). A shift of the distribution maximum towards the larger diameter has taken place at 24 and 36 hr, but no extremely large nuclei were encountered. A different distribution is apparent after DNA synthesis has begun at 48 hr after stimulation. There seems to be an accumulation of nuclei with a diameter between 5.6 and 6.2 μ , without a gradual drop on the side of the larger diameter. Instead, there are few cells which are larger but they are spread equally. These cells represent 11% of the total cells measured. Considering the number of nuclei measured, this figure (11%) compares favorably with the 8% nuclei found to be in the process of DNA synthesis. Also, it was found by radioautography that the larger nuclei contain the label,

not the smaller ones. If we average the diameter of the 8% largest nuclei and calculate the surface, we obtain an average surface of 228 μ^2 . If we multiply by the average of the 8% largest values for pores/ μ^2 (7.7), we calculate 1755 pores/nucleus for the S-phase lymphocytes. If the same calculation is made for the 60th hr after PHA stimulation in the second experiment, we obtain 1858 pores/nucleus for cells presumably in S phase. If one compares the nuclear diameters of the PHA-stimulated cells at 48 hr with those of the controls at the same time, one can see that a large number of nuclei (27%) have a diameter which corresponds to that of the controls. These cells may not be stimulated at all and will reduce the average of the stimulated population.

With the data presented, we may calculate the ratio of pores/nucleus, nuclear surface, and nuclear volume of control cells vs. stimulated lymphocytes or the calculated values of S-phase cells (Table II). From these ratios, it appears as if the number of nuclear pores is directly related to the nuclear volume if we compare the ratios of controls with the average PHA-stimulated cell population (pore/nucleus and volume doubled). However, the ratio of controls to S-phase cells

TABLE II
Comparison of Surface Volume and Pores/Nucleus of Control, PHA-Stimulated "Average" Cells, and S-Phase Lymphocytes of Experiments 1 and 2

	Experiment 1, 48 hr PHA stimulation		Experiment 2, 60 hr PHA stimulation	
	Control vs. average PHA-stimulated cells	Control vs. S-phase cells	Control vs. average PHA-stimulated cells	Control vs. S-phase cells
Surface	1:1.7	1:2.5	1:1.7	1:2.7
Volume	1:2.3	1:4.0	1:2.3	1:4.6
Pores/nucleus	1:2.3	1:6.1	1:2.6	1:7.5

is much larger and no correlation with nuclear volume can be found.

Nuclear Pore Frequency Changes during the HeLa Cell Cycle

Because the possibility exists that a change in the number of nuclear pores might, in some fashion, be correlated with a change in DNA content or with the initiation of DNA synthesis, as indicated by the results with stimulated lymphocytes, we used synchronized cells to study the change in nuclear pore frequency during the HeLa S-3 cell cycle. The course of the cell cycle was monitored by determining the rate of DNA synthesis at hourly intervals.

In these experiments G_1 normally lasts from mitosis until about 4–5 hr later. S phase then begins and lasts for about 9 hr, after which G_2 begins and lasts until mitosis commences about 19 hr after the last cell division. Representative results of these experiments are shown in Fig. 7 and indicate that nuclear surface area in the HeLa cell expands rapidly to $200 \mu^2$ 1 hr after mitosis, followed by a plateau level of $240 \mu^2$ 2–3.5 hr after mitosis (G_1). Just before DNA replication begins, the nuclear surface starts rapidly to increase again and finally reaches an area of about $350 \mu^2$ by 16 hr after mitosis. Since, by radioautography, only 0.6% of the cells were synthesizing DNA at 3.5 hr after mitosis, the time at which the second increase in nuclear surface began, and only 6.8% of the cells at 5 hr after mitosis (G_1 -S transition), it is probable that this second increase does precede DNA replication. Since 32% of the cells had entered mitosis by the 16th hr, it is believed that our values for pores/nucleus are a little too low. If mitosis takes about 1 hr, then 17% of the cells should have completed mitosis and be in the following G_1 phase. At the 18th hr after

mitosis an even larger number of cells must have divided, explaining the apparent decrease in nuclear pores in the first experiment at the 18th hr (Table III). During the time when the nuclear size does not increase (3 hr), we found the number of nuclear pores to be equal in all three experiments (Table III).

In one experiment, it was found that DNA replication was delayed because the temperature of the culture room had accidentally fallen to 34°C (Fig. 8). In this experiment the maximal rate of DNA replication occurred at a correspondingly later time and the plateau in the G_1 surface area also was protracted. These results clearly demonstrate that the plateau in nuclear area increase is repeatable and pronounced under these conditions and is not an error in measurement. These results also indicate that there is a temperature-sensitive step in the transition of the cells from G_1 into S phase.

In the normal experiments, the number of pores/unit area increases at a decreasing rate in the HeLa cell from a G_1 value of about eight pores/ μ^2 to a G_2 value of about 11 pores/ μ^2 . The values of pores/unit area determined at different times through the cell cycle are plotted on a logarithmic time scale in Fig. 9, and it can be seen that they fall in a straight line. We therefore analyzed our data (Experiment 3) by the method of linear regression. The regression equation is given as $y = A + B \log \text{time}$. The significance of the slope (B) was tested by the t test and found highly significant ($P \ll 0.005$). The test of linearity shows a significant F test at the 0.01 level. This means that we may have a linear fit of our values for pores/ μ^2 if expressed as a function of log time. As the logarithm of 1 is 0, we can determine the number of pores/ μ^2 at 1 hr after mitosis at the point of interception of the line with the ordinate (A). The increase of pores/

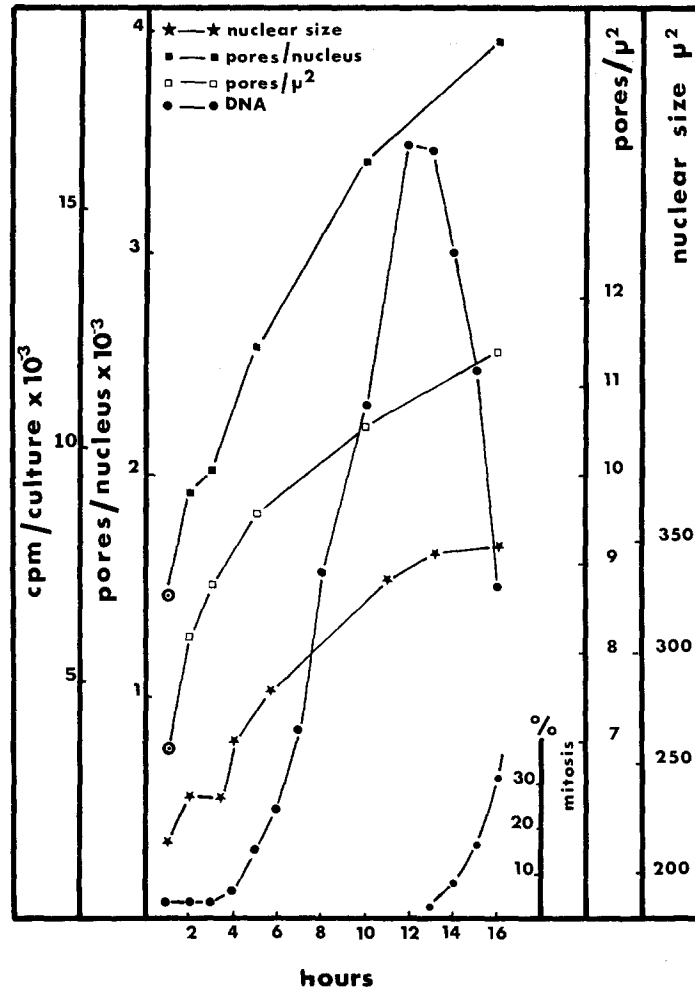


FIGURE 7 The changes in pores/μ² (open squares), nuclear surface area in μ² (stars), and pores/nucleus (solid squares) of HeLa S-3 cells are plotted with the rate of thymidine incorporation (small dots) against time in hours after mitosis (Experiment 3). The mitotic index is given in per cent from the time of Colcemid addition at the 13th hr after mitosis (lower right-hand corner). The open circles surrounding a dot at the beginning of the curve of pores/μ² and pores/nucleus at 1 hr after mitosis indicate that this is a calculated value and not experimentally determined.

nucleus can then be plotted from the 1st hr after mitosis on, as we had determined the nuclear size at the 1st hr after mitosis. The calculated values in Fig. 7 are indicated by open circles with a dot.

In general, these experiments show that the number of pores/nucleus approximately doubles from the G₁ period (~2000) to the end of the S phase (~4000). The nuclear surface increases during this time only about 1.57 times, whereas the volume nearly doubles from 332 μ³ in G₁ to 626 μ³ at the end of S phase (ratio 1:1.9). There-

fore, the increase in the number of nuclear pores seems to be correlated closely with a nuclear volume increase in HeLa cells.

We determined the rate of nuclear pore formation from two experiments in which we made observations at closely spaced time intervals (Table III). The highest rate of pore formation (25.1 pores/min) occurs between mitosis and the 1st hr. Between the 2nd and 3rd hr the rate falls to about 1.4-1.6 pores/min. The rate then increases between the 3rd and 5th hr after mitosis to about 4-6 pores/min, followed by a marked

TABLE III
Comparison of Results Obtained in Three Experiments using Synchronized HeLa Cells

Time after mitosis	Experiment 1					Experiment 2					Experiment 3					% cells S phase
	P/ $\mu^2 \pm$ SD	NS	P/N	Average size	n	P/ $\mu^2 \pm$ SD	NS	P/N	Average size	n	P/ $\mu^2 \pm$ SD	NS	P/N	Average size	n	
hr		μ^2		μ^2			μ^2		μ^2			μ^2		μ^2		
1											7.05*	214	1510*			
2						7.43 ± 1.8	248	1848	8.8	42	8.13 ± 1.4	236	1918	18.6	22	
3	7.94 ± 1.2	246	1950	9.0	15	8.00 ± 1.2	244	1954	9.3	30	8.75 ± 1.7	232	2030	22.7	15	0.6
4						8.24 ± 1.2	270	2224	10.2	30		260				
5	9.58 ± 2.0	313	3080	11.4	15	8.73 ± 1.2	298	2601	7.8	30	9.54 ± 1.9	268	2556	21.0	12	6.8
9	10.18 ± 1.8	394	4010	11.5	15											
10						8.81 ± 1.8	350	3083	8.5	32	10.56 ± 1.5	323	3410	27.6	21	78.5
13												345				
16											11.38 ± 2.1	347	3948	17.0	22	37.0
18	10.62 ± 1.5	262	2782	16.7	15											

* Value obtained by calculation as explained in the text. Legend as in Table I.

decrease to values of less than one pore/min in G₂ (Fig. 10). This indicates that the highest rates of pore formation occur in G₁ just after mitosis (nuclear envelope reformation and chromosome decondensation) and then shortly before DNA synthesis but not in S phase when the rate of DNA synthesis is maximal.

DISCUSSION

Our investigation of the nuclear pore complex was stimulated by the central role that this structure is assumed to have in nucleocytoplasmic exchange. Direct experimental evidence was provided by Feldherr (1962) that particles in the general size range of macromolecules can penetrate the nucleus through the pore complex. Two different means of control of macromolecular exchange can be imagined. First, nuclear pores could be selectively permeable to different substances and regulate nucleocytoplasmic exchange by such permeability changes. Feldherr (1971) assumed that such a mechanism exists in amoeba nuclei in which he found differing numbers of

gold particles entering the nucleus, depending on the physiological state of the cell. The different uptake of gold particles was apparently not dependent on changes in the number or size of the pores.

We investigated the second possibility, namely, that the number of pores/nucleus can increase in a controlled fashion. In a previous communication we demonstrated nuclear pore formation experimentally and on an ultrastructural level (Maul et al., 1971). It was shown that the number of nuclear pores doubles in the "average" lymphocyte stimulated for 48 hr with PHA. This experimental model was then used in the present study in an attempt to correlate nuclear pore formation with protein, RNA, and DNA synthesis.

Although we can detect an increase in leucine incorporation as early as 2 hr after stimulation with PHA, no increase in the average number of pores/nucleus is apparent until 6 hr after stimulation. However, early changes in pore/ μ^2 distribution frequencies do seem to occur. The

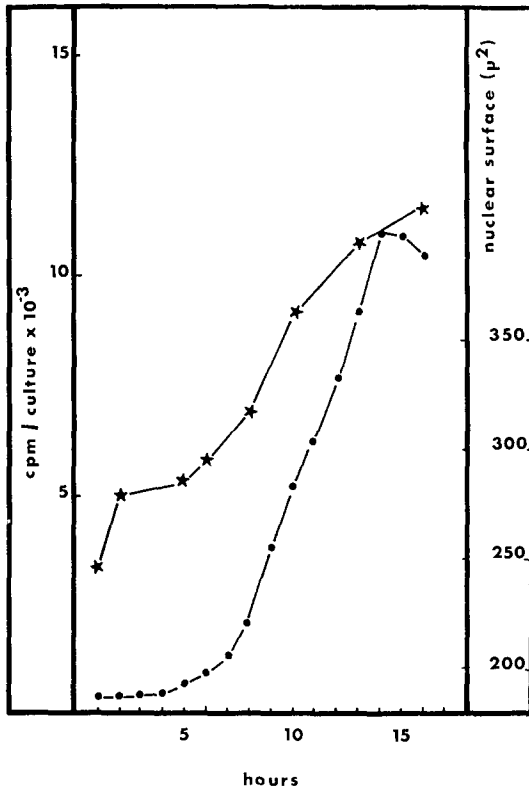


FIGURE 8 Nuclear surface change is shown (stars) in relation to the S phase of HeLa S-3 cells. The filled circles represent the rate of thymidine-³H incorporation. This experiment was done at 34°C and shows in an exaggerated way the level of the nuclear surface area during G₁.

presence of two pore frequency maxima in stimulated populations and only the original frequency maximum in the control cells makes it seem likely that only some of the lymphocytes are stimulated by PHA. There are parallel increases in pores/nucleus and leucine incorporation in PHA-stimulated lymphocytes, but the initial increase in pores/nucleus seems to lag behind the leucine incorporation by about 4 hr. However, we may not be able to determine early changes in pores/nucleus due to the inadequacy of nuclear size determinations. Also, the correlation between protein synthesis and pore formation may not reflect an intimate relationship between the two processes but may rather reflect the over-all process of stimulation.

A second increase in number of pores is apparent at the time when DNA synthesis begins.

At 48 hr we found that about 8.0% of the cells were synthesizing DNA, a figure equal to the one reported by Knight et al. (1965). Thus, it seems that this second rise is intimately associated with the onset of DNA synthesis.

We think that the data obtained by the freeze-etching technique are extremely reproducible under controlled conditions. Small differences in the data obtained at 48 hr in the two experiments are interpreted as variations in stimulation between lymphocytes of different donors. In our second lymphocyte experiment, a smaller number of cells was stimulated at 48 hr after the addition of PHA. The largest error in the determination of nuclear pores/nucleus was introduced in measuring the diameter of nuclei of control cells at early times after stimulation. Small changes in nuclear size will also go undetected by placing the nuclei in size classes of 0.6 or 1 μ. The absolute values for these cells, therefore, have to be taken with caution, but they probably are accurate within ±10%. This means, however, that we may not

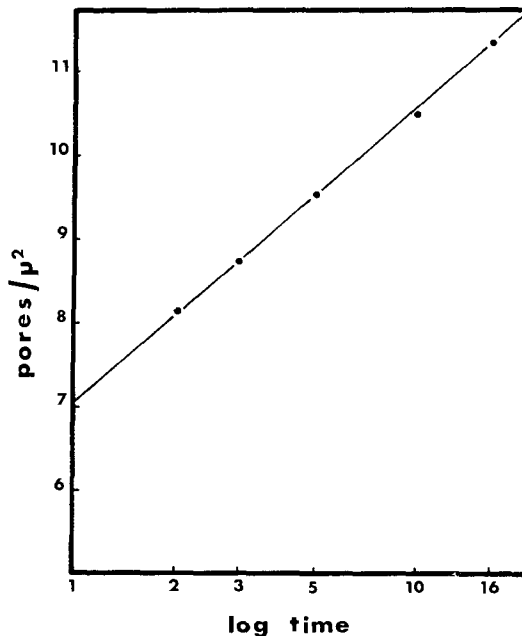


FIGURE 9 Nuclear pores/μ² (HeLa cells; Experiment 3) were plotted as a function of time (logarithmic time scale), revealing an apparently linear relationship. The line intercepts at 7.05 pores/μ². This value may then be used as the number of pores at 1 hr after mitosis. The significance of the slope as tested with the *t* test was found highly significant ($P \ll 0.005$). The test for linearity showed a significant *F* test at the 0.01 level.

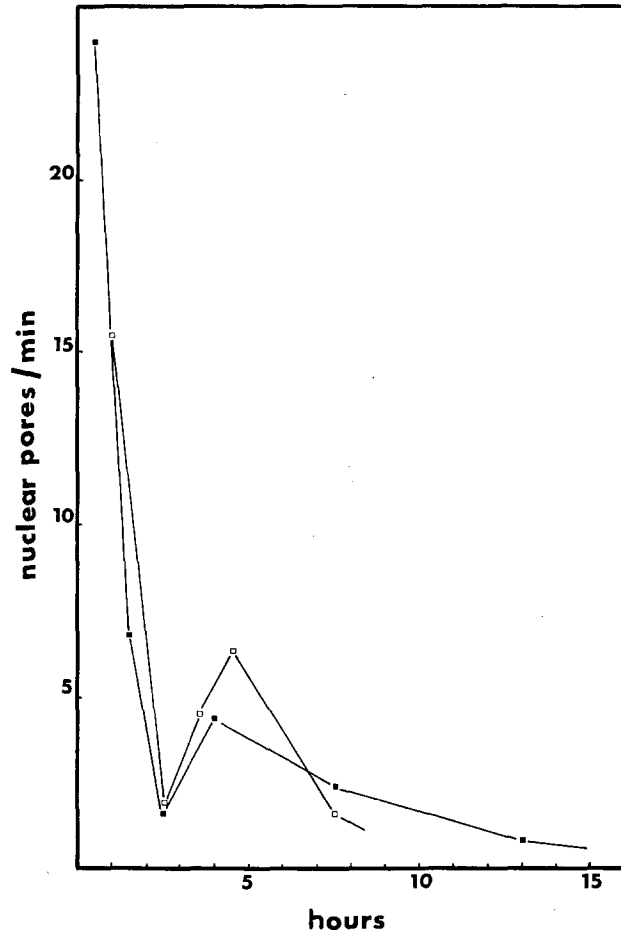


FIGURE 10 Analysis of the rate of nuclear pore formation (HeLa cells; Experiments 2 and 3) as a function of time after mitosis. The rate was calculated by dividing the difference between pores/nucleus of different time points by the number of minutes between these time points. The values obtained were then plotted between the time points. Open squares represent values of Experiment 2, closed squares of Experiment 3 in Table III. This curve shows that there is a maximum in the rate of pore formation shortly after mitosis and a second one shortly before S phase which starts between the 5th and 6th hr after mitosis.

be able to recognize the earliest increase in pore frequency.

In the hope of obtaining more information about the time sequence of pore frequency changes, we utilized synchronized HeLa cells. Our data do not correspond to those reported by Comes and Franke (1970) who found 12,000 pores/nucleus. They had isolated and negatively stained nuclear envelope pieces for their determination of the pore number/ μ^2 . The value of 13 pores/ μ^2 obtained by Fisher and Cooper (1967), using the thin-sectioning technique, is much closer to our values, but they did not estimate the number of

pores/nucleus. Very recently Scott et al. (1971) reported that the number of nuclear pores/unit area increases in Chinese hamster cells and mouse L cells during the cell cycle. However, they did not determine the nuclear size changes during the cell cycle.

A biphasic pattern in the rate of pore formation is observed in synchronized HeLa S-3 cells. Pores are formed with the highest rate during telophase and early G_1 phase. A most interesting observation in this system is that the nuclear surface area reaches a plateau during G_1 between the 2nd and 3rd hr after mitosis. It is thought that this

size of the nucleus represents the HeLa "2C" size and that the following increase is preparatory to the imminent S phase in this continuously dividing cell line. The nuclear size increase shortly precedes the beginning of DNA replication. The initial increase in volume cannot therefore be due to an increase in the amount of DNA. However, the total volume of the nucleus doubles (1:1.9) from the plateau in G₁ to the end of S phase. Also, the number of pores/nucleus doubles during this time (1:1.9). This correlation between nuclear volume and nuclear pore number becomes even more obvious if we compare the number of nuclear pores/ μ^3 . The values for the 3rd (G₁), 5th (G₁-S), 10th (S), and 16th hr (G₂) after mitosis, respectively, are 6.1, 6.2, 6.3, and 6.3, that is, nearly equal. The values for earlier times are lower (2 hr, 5.6). If we calculate the number of nuclear pores/ μ^3 for PHA-stimulated human lymphocytes which are in S phase, we obtain the value of 5.8. The number of pores/ μ^3 in the unstimulated cells is normally 3.6, and in the "average" PHA-stimulated cell population 4.1.

The equal number of pores/ μ^3 in both cell types in S phase suggested that one might be able to correlate the number of pores/nucleus of S-phase lymphocytes with the HeLa cell values by calculating the number of pores/chromosome (46 chromosomes vs. 79 chromosomes). If this is done for S-phase cells of lymphocytes, we obtain a value of ~ 40 pores/chromosome. For HeLa cells the number of pores/chromosome is similar (44) at the middle of the S phase. A different way of comparing the total number of nuclear pores of the S-phase lymphocytes with the pore number for HeLa cells is to divide the total number of pores by 46 chromosomes and multiply by 79, the modal chromosome number of HeLa cells. The calculated pore number then would be ~ 3100 . This value fits well with the pore number in mid-S phase in HeLa cells (see Table III). The calculation above implies that S-phase cells may have the same number of nuclear pores. This could be investigated by measuring S-phase nuclei of other human cell types with varying numbers of nuclear pores/nucleus during their nonreplicative phase (G₁ or G₀).

The results presented in this paper represent a numerical analysis of pore formation during the cell cycle. Under the assumption that the total number of pores as found varies little from the

in vivo state and that the tendencies of frequency change are correct with respect to time, we may try to correlate these numbers with other experimental data or calculated values, realizing that finding such correspondence does not prove a direct relationship but may help in the formulation of concepts and experimental design.

Doubling of nuclear pores during S phase in HeLa S-3 cells corresponds to a doubling of the chromosomes. Another correlation can be found between the rate of nuclear protein synthesis and the rate of pore formation. After the initial high rate of pore formation due to nuclear envelope reformation, there is a minimum in the rate of pore formation between the 2nd and 3rd hr after mitosis. The subsequent increase in the rate of nuclear pore formation precedes histone and DNA synthesis but is concurrent with an increased synthesis and turnover of nuclear acidic proteins (Stein and Borun, 1972; Borun and Stein, 1972) within the limits of the separate time points investigated (1 hr).

Although we cannot as yet relate any cellular event directly to the increase in the rate of nuclear pore formation at the end of G₁, we may speculate that it is in a preparatory way connected to the S phase. In this connection we may mention the following numerical similarities. The number of nuclear pores in HeLa S-phase cells corresponds in magnitude to the number of replicons which have to be active simultaneously in order to replicate the DNA in a 9 hr S phase. The number of replication sites was estimated to be about 3000 (Painter et al., 1966). If the number of nuclear pores in HeLa cells and human lymphocytes is not just by chance similar to the calculated number of simultaneously replicating replication sites, we may have a first hint that a specialized membrane structure of the nucleus is involved in DNA initiation and/or replication.

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REFERENCES

- BOOTSMA, D., L. BUDKE, and O. VOS. 1964. Studies on synchronous division of tissue culture cells initiated by excess thymidine. *Exp. Cell Res.* **33**:301.
- BORUN, T. W., and G. S. STEIN. 1972. The synthesis of acidic chromosomal proteins during the cell cycle of HeLa S-3 cells. II. The kinetics of residual protein synthesis and transport. *J. Cell Biol.* **52**:308.
- COMES, P., and W. W. FRANKE. 1970. Composition, structure and function of HeLa cell nuclear envelope. I. Structural data. *Z. Zellforsch. Mikrosk. Anat.* **107**:240.
- COOPER, H. L., and A. D. RUBIN. 1965. RNA metabolism in lymphocytes stimulated by phytohemagglutinin: Initial response to phytohemagglutinin. *Blood J. Hematol.* **25**:1014.
- COULSON, A. S., and D. G. CHAMBERS. 1964. Separation of viable lymphocytes from human blood. *Lancet.* **1**:468.
- FELDHERR, C. M. 1962. The nuclear annuli as pathways for nucleocytoplasmic exchanges. *J. Cell Biol.* **14**:65.
- FELDHERR, C. M. 1969. A comparative study of nucleocytoplasmic interaction. *J. Cell Biol.* **41**:841.
- FELDHERR, C. M. 1971. Evidence for changes in nuclear permeability during different physiological states. *Tissue Cell.* **3**:1.
- FISCHER, H. W., and T. W. COOPER. 1967. Electron microscopic observation on the nuclear pores of HeLa cells. *Exp. Cell Res.* **48**:620.
- FRANKE, W. W. 1970. On the universality of the nuclear pore complex structure. *Z. Zellforsch. Mikrosk. Anat.* **105**:405.
- KAY, J. E., and S. D. HANDMAKER. 1970. Uridine incorporation and RNA synthesis during stimulation of lymphocytes by PHA. *Exp. Cell Res.* **63**:411.
- KNIGHT, S., N. R. LING, S. SELL, and C. E. OXNARD. 1965. The transformation in vitro of peripheral lymphocytes of some laboratory animals. *Immunology.* **9**:565.
- LIEBERMAN, M. W., R. N. BANEY, R. E. LEE, S. SELL, and E. FARBER. 1971. Studies on DNA repair in human lymphocytes treated with proximate carcinogens and alkylating agents. *Cancer Res.* **31**:1297.
- LING, N. R. 1968. Lymphocyte Stimulation. North Holland Publishing Co., Amsterdam.
- MAUL, G. G., J. W. PRICE, and M. W. LIEBERMAN. 1971. Formation and distribution of nuclear pore complexes in interphase. *J. Cell Biol.* **51**:405.
- MERRIAM, R. W. 1962. Some dynamic aspects of the nuclear envelope. *J. Cell Biol.* **12**:79.
- MOOR, H., and K. MÜHLETHALER. 1963. Fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17**:609.
- NEXOS, N. 1962. Deoxyriboside control and synchronization of mitosis. *Nature (Lond.)* **194**:682.
- PAINTER, R. B., D. A. JERMAN, and R. E. RASMUSSEN. 1966. A method to determine the number of DNA replicating units in cultured mammalian cells. *J. Mol. Biol.* **17**:47.
- ROBBINS, E., and P. MARCUS. 1964. Mitotically synchronized mammalian cells: A simple method of obtaining large populations. *Science (Wash. D. C.)* **144**:1152.
- SCOTT, R. E., R. L. CARTER, and W. R. KIDWELL. 1971. Structural changes in membranes of synchronized cells demonstrated by freeze-cleavage. *Nat. New Biol.* **233**:219.
- STEIN, G. S., and T. W. BORUN. 1972. The synthesis of acidic chromosomal proteins during the cell cycle of HeLa S-3 cells. I. The accelerated accumulation of acidic residual nuclear protein before the initiation of DNA replication. *J. Cell Biol.* **52**:292.
- TERASIMA, T., and L. J. TOLMACH. 1963. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res.* **30**:344.
- WATSON, M. L. 1959. Further observations on the nuclear envelope of the animal cell. *J. Biophys. Biochim. Cytol.* **6**:147.