EFFECT OF METABOLIC INHIBITORS ON
NUCLEAR PORE FORMATION
DURING THE HELA S3 CELL CYCLE

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ABSTRACT
The effect of various antimetabolites on nuclear pore formation was studied in synchronized HeLa S3 cells. The nuclear size was determined by light microscopy and the pore number per unit area of nuclear surface by the freeze-etching technique and electron microscopy. It was found that the inhibition of DNA replication or ribosomal RNA synthesis has no effect on nuclear size increase or pore formation. However, the inhibition of ATP synthesis effectively stops nuclear pore formation. Cycloheximide blocks nuclear pore formation at the same time during G1 phase of the cell cycle when nuclear size increase is blocked by high concentrations of actinomycin D. This suggests that certain proteins or other factors leading to pore formation and nuclear size increase are transcribed and synthesized at about 3-4 h after mitosis, i.e., about 1-2 h before S phase begins.

INTRODUCTION
Pore formation in the interphase nuclear envelope has been demonstrated by experimental evidence showing a doubling of nuclear pores in phytohemagglutinin-stimulated human lymphocytes (Maul et al., 1971). This finding was preceded by the report of Feldherr (1971) which suggested that changes in the complex architecture or physiology of the nuclear pore itself, rather than changes in total pore number, might be the determining factors in the regulation of nucleocytoplasmic exchange. The presence of structures undistinguishable from nuclear pores in the cytoplasm or in membranes within the nucleus, the cytoplasmic or nucleoplasmic annulate lamellae pores (Maul, 1970 a and 1970 b), as well as single pores within the rough endoplasmic reticulum (Maul, 1970 c), raised the question of pore complex functions other than facilitating selective exchange between compartments (Franke et al., 1972; Maul, 1972). We therefore embarked on a program to investigate pore complex formation in relation to cellular processes such as RNA, DNA, or protein synthesis.

Increased pore formation could be correlated to different metabolic activities in phytohemagglutinin-stimulated human lymphocytes and to specific times in the HeLa S3 cell cycle (Maul et al., 1972). In this report we describe the effect on pore formation obtained by selectively inhibiting protein, RNA, or DNA synthesis, or ATP formation.

MATERIALS AND METHODS
Synchronized HeLa S3 cells were obtained by thymidine inhibition of DNA synthesis (Xeros, 1962; Bootsma et al., 1964) followed by selectively detach-
ing mitotic cells from Blake bottles (Terasima and Tolmach, 1963; Robbins and Marcus, 1964) or by selectively detaching mitotic cells from log-phase cultures without presynchronization with excess thymidine. The synchronized HeLa cells were kept in suspension (2.5-4 × 10^5 cells/ml) in Joklik-modified Eagle's essential medium (Grand Island Biological Co., Grand Island, N.Y.) containing 5.5% calf serum, 3.5% fetal calf serum (Flow Laboratories, Rockville, Md.), plus penicillin and streptomycin. The rate of DNA replication was estimated by adding 0.2 μCi of [3H]thymidine in 0.1 ml of H2O (sp act 25 mCi/mm, New England Nuclear, Boston, Mass.) to 2 ml of cell suspension. After 30 min of incorporation at 37°C the reaction was stopped by the addition of 5 ml of ice-cold Eagle's basal salt solution. Cells were washed twice in that solution, resuspended in ice-cold 10% TCA, and collected on a 0.45-μm millipore filter (Millipore Corp., Bedford, Mass.) followed by two washes with 5 ml of 10% cold TCA. The filters were then dissolved and counted in Bray's solution using a Tri-carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The results are expressed as counts per minute per culture. Nuclear size determinations were made on 50 cells with anaportal contrast optics by measuring the long and short axis of the nuclei at a magnification of 1,000 times. Hairs were placed between slide and cover glass to prevent flattening of cells. The diameters were averaged and the nuclear surface area calculated as if the nucleus were a sphere. Cells were measured either live or one day after fixation. Cells were fixed by adding 2 ml of 1% glutaraldehyde in 0.1 M phosphate buffer to 2 ml of cell suspension in medium.

The mitotic indices of our preparation after traversing the cell cycle were determined by counting 500 cells after various lengths of a colcemid block (0.5 μg/ml). Autoradiography was performed as described (Maul et al., 1972) to monitor the number of cells actually entering S phase.

HeLa cells were prepared for freeze-etching as described previously. The incubation in 20% glycerol, however, has been reduced to 15 min. Freeze-etching was performed according to Moor and Mühlethaler (1968) in a Balzer's freeze-etch apparatus at -100°C. Etching time was 2 min. The exposed surface was then shadowed with platinum and carbon coated. A Zeiss 9A and a Hitachi Hu 12 electron microscope were used to photograph the exposed nuclear surface areas at ~10,000-fold magnification. They were calibrated before each use. Nuclear pore counts were made directly on the negative using a stereo microscope. The nuclear surface area was determined by planimetry.

Different concentrations of various antimetabolites were added at selected time points after mitosis. They will be described with the respective experiments. Experimental samples containing actinomycin D were protected from light at all times. The antimetabolites were purchased as follows: actinomycin A, cytosine arabinoside hydrochloride (Ara C) and thymidine from Sigma Chemical Co., St. Louis, Mo.; deacetylcycloheximide from Calbiochem, La Jolla, Calif.; actinomycin D (Act D) from Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y.; sodium fluoride (NaF) from VWR Scientific Div., VWR United Corp., Baltimore, Md.

The effectiveness of different concentrations of Act D and cyclohexamide was determined by incubating 2-ml samples of HeLa cells in the absence and presence of the drugs with [3H]uridine (10 μCi/ml, sp act >2 Ci/mM, New England Nuclear) for 5 min or L-[3H]leucine (1 Ci/ml, sp act 30 Ci/mM, New England Nuclear) for 60 min at 37°C. The cells were extensively washed, collected on millipore filters, washed with 10% TCA, and counted on a Tri-carb scintillation counter. The results are expressed as percent inhibition compared to control.

**RESULTS**

Because we had found an increase in the rate of nuclear pore formation which could be correlated with S phase in proliferating lymphocytes and in continuously dividing HeLa S3 cells, we investigated the effect of inhibitors of DNA replication on nuclear pore formation. The time sequence of nuclear size increase in the presence of 22.4 μg/ml thymidine or 0.1 μg/ml FUdR and 2.5 μg/ml uridine is shown in Fig. 1. It became apparent that the nuclear size is independent of DNA synthesis up to the 10th h. If the antimetabolites were washed out at 10 h after mitosis with pre-warmed medium, nuclear size increase continues in parallel with that of the control.

If one compares the number of pores per square micrometer, one finds that when DNA synthesis is blocked by the absence of TTP (FUdR block) or at excess thymidine concentrations, the pore number is equal to the control value. The total pore number (pores per square micrometer times nuclear surface), therefore, is equal in the control cells and in those where DNA synthesis is inhibited (Table I).

In a similar experiment we tested the effect of HU and Ara C on nuclear pore formation (Table II). The inhibitors were added at the time of selective detachment of mitotic cells. No effect on nuclear size can be seen at approximately 4 h.

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FIGURE 1  Nuclear size change of synchronized HeLa S3 cells in the presence of excess thymidine or FUDR. Cells were synchronized by excess thymidine followed (after traversing S and G2 phases) by selective detachment. To one of three aliquots of synchronized HeLa S3 cells thymidine was added to a concentration of 2 mM, to another one FUDR to a concentration of 0.5 mM 3 h after mitosis. Cells were removed at different times and fixed with 0.5% glutaraldehyde and measured the following day. At the 10th h after mitosis the cells containing the inhibitors were washed 2 times with fresh warm medium and further incubated at 37°C.

TABLE I

| Change in Nuclear Pore Number in the Presence of Inhibitors of DNA Synthesis in Synchronized HeLa S3 Cells |
|---------------------------------------------------------------|
| NS µm² ± SD | P/µm² ± SD | P/N | P  |
| 3-h          | 289 ± 42   | 9.8 ± 1.9 | 2826 |    |
| 10-h control | 364 ± 66   | 11.5 ± 1.2 | 4167 | 0.001 |
| 10-h FUDR    | 355 ± 67   | 11.5 ± 1.1 | 4078 | 0.35 |
| 10-h thymidine| 358 ± 57  | 11.5 ± 1.4 | 4099 | 0.40 |

Legend as in Fig. 1. Cells for nuclear pore determination were not fixed but incubated in 20% glycerol for 15 min before freezing in liquid nitrogen-cooled Freon 22. The P value of the 10-h control relates to the 3-h control, whereas the experimental values are compared to the 10-h control. P values were obtained by a modified delta test. Nuclear size (NS), pores per square micrometer (P/µm²), pores per nucleus (P/N).

after mitosis. At the 13th h after mitosis inhibition of DNA synthesis by Ara C and HU also does not seem to have an effect on nuclear pore formation, but HU increases the number of pores per square micrometer. The total nuclear pore number, however, does not increase significantly (P = 0.2). The nuclear size in the DNA-inhibited sample is again the same as the control sample.

There is a nuclear size increase shortly before S phase in HeLa cells (Maul et al., 1972). We asked if this increase may be due to nucleolar growth. Therefore we blocked ribosomal synthesis with 0.5 µg/ml Act D. Preparations containing this drug were kept dark. Neither nuclear size nor nuclear pore per square micrometer was affected by this treatment (Table II). Cycloheximide at a concentration which blocked 96% of protein synthesis (0.5 µg/ml) after 15 min pre-incubation with the drug, effectively inhibited nuclear size increase, but an increase of pores per square micrometer over control values was noted, indicating the possibility that the two
Change in nuclear pore number in the presence of different antimetabolites in synchronized HeLa S3 cells. Cells were collected at mitosis after thymidine block, followed by selective detachment. Ara C (40 pg/ml) and HU (100 pg/ml) were added at 0 h; Act D (0.5 pg/ml), cycloheximide (0.5 pg/ml), antimycin A (12.5 pg/ml), and NaF (420 pg/ml) were added at 3.75 h after mitosis. 2 h later the cells were collected, fixed in 0.5% glutaraldehyde, and measured the following day. The viability was monitored by the dye exclusion test. For the test of statistical significance the total number of pores per nucleus was compared to the control except that the control at 13 h was compared to the control at 5.75 h. P values for total nuclear pore per nucleus were obtained by using a modified delta test.

Processes are not necessarily interdependent. The inhibition of ATP formation by antimycin A (125 µg/ml) and NaF (420 µg/ml) blocked both nuclear size increase and pore formation. These nuclear size increases occur at the same time in the cell cycle; however, the absolute values varied from one experiment to the next.

We then tried to determine at what time after mitosis certain inhibitors would have to be added to the cell suspension to exert an effect on the pre-S-phase nuclear size increase as measured at the 6th h after mitosis. In this particular experiment we needed only small amounts of cells and decided to omit the thymidine block of the synchronization. With this procedure we avoided any possible effect of the unbalanced growth which comes about by blocking cells at the G1-S interphase or during S phase. The change of nuclear size after mitosis was measured every half hour using the living cells. A much more pronounced plateau in the G1 phase nuclear surface area was obtained as compared to previous experiments (Fig. 2). Remeasuring the fixed cells after one day resulted in a similar curve but on the average 6% lower values were obtained. If this shrinkage has to be attributed to a difference in osmolarity of the buffer or to the fixation process has not yet been determined.

As expected (Table III), we found no change in nuclear size increase in samples where DNA synthesis was inhibited (Ara C or HU). Also, no size difference was found using low Act D concentrations at any of the times used, confirming the first result and indicating that no new ribosomal

| H after mitosis | 3.75 | 5.75 | 13 |
|---------------|------|------|----|
|               | NS µm² | NS µm² ± SD | P/µm² ± SD | P/N | NS µm² ± SD | P/µm² ± SD | P/N | % changed |
| Control       | 232   | 284 ± 33   | 10.0 ± 1.6  | 2840 | 351 ± 51  | 10.5 ± 1.5  | 3689 | >0.001 | 1.2 |
| Ara C         | 228   | 288 ± 36   | 10.0 ± 2.2  | 2880 | 355 ± 50  | 10.0 ± 1.8  | 3557 | 0.48  | 1.5 |
| HU            | 237   | 288 ± 36   | 10.0 ± 2.2  | 2880 | 350 ± 56  | 11.1 ± 1.3  | 3850 | 0.25  | 1.2 |
| Act D         | 232   | 245 ± 33   | 10.6 ± 1.4  | 2597 | >0.001    | 8.2        |
| Cycloheximide | 232   | 248 ± 30   | 9.7 ± 1.6   | 2405 | >0.001    | 8.2        |

![Figure 2](image-url)  
**Figure 2** Nuclear size change from mitosis to the beginning of S phase. Cells were selectively detached from log-phase cells without a previous thymidine block and measured immediately every 30 min unfixed and again fixed in 0.5% glutaraldehyde the following day. The nuclear size increase during the first 6 h after mitosis shows a pronounced plateau during most of the G1 phase.
TABLE III
Change in Nuclear Surface in the Presence of Metabolic Inhibitors

| Inhibitor          | H after mitosis 1-6 | 2-6 | 3-6 |
|--------------------|--------------------|-----|-----|
| Control            | 187-298            | 238-298 | 246-298 |
| Ara C              | 187-290            | 238-306 | 246-291 |
| Hu                 | 187-297            | 238-300 | 246-295 |
| Act D              | 187-276            | 238-290 | 246-299 |
| Cycloheximide      | 187-216            | 238-247 | 246-270 |
| Antimycin A + NaF  | 187-201            | 238-228 | 246-245 |

Change in the nuclear surface after mitosis in the presence of different antimetabolites. Metabolic inhibitors were added at different times after mitosis to aliquots of cell suspensions. All samples were fixed at 6 h after mitosis in 0.5% glutaraldehyde and measured the following day. Fixed control samples were measured every hour after mitosis (see Fig. 2). Data in this table are from the same synchronization as the one in Fig. 2.

Blocking protein synthesis with 0.5 µg/ml of cycloheximide allows a nuclear size increase up to the G1 plateau but prevents the pre-S-phase nuclear size increase (Table III). If protein synthesis is allowed up to the 3rd h after mitosis and then blocked, we find that a small increase over the G1 plateau occurs, indicating that certain proteins synthesized at about or later than 3 h after mitosis are necessary for the pre-S-phase nuclear size increase.

The inhibition of both the ATP formation by mitochondria (antimycin A) and by the glycolytic pathway (NaF) inhibits nuclear size increase completely at any time tested (Table III).

The finding that protein synthesis at a particular time is necessary for the nuclear size increase shortly before the beginning of S phase, together with the finding that the inhibition of ribosomal RNA synthesis does not block the pre-S-phase nuclear size increase, raised the question whether the synthesis of specific messenger RNA is required for this kind of protein synthesis. The nuclear size change up to the 6th h after mitosis was therefore determined in the presence of different amounts of Act D added at different times subsequent to mitosis. In Fig. 3 the time after mitosis when the inhibitor was added is plotted versus the nuclear size by the 6th h after mitosis. The control values, i.e., the nuclear size at different times after mitosis, are indicated at the right-hand side of Fig. 3 by an asterisk and by the time after mitosis. The nuclear size rises to the G1 plateau value (3 h after mitosis) regardless of the concentration of Act D used. At 5.0 µg/ml and 2.5 µg/ml no significant rise over the G1 plateau takes place if the inhibitor is present from mitosis or from the 2nd h after mitosis on to the 5th h. If Act D is added at the 3rd h, the 4th-hr control value of nuclear size is reached and adding Act D at these high concentrations at the 4th h after mitosis has no effect, i.e., the nuclei increase to the same size as controls. If Act D is added at a concentration of 0.5 mg/ml at the time of or 1 h after mitosis, one can find a small but repeatable inhibition of nuclear size increase at the 6th h after mitosis (see also Table III). Later, addition of the inhibitor has no effect. Also, using a lower concentration (0.1 µg/ml) has no...
effect on the pre-S-phase nuclear size increase. Because of the correspondence of nuclear size increase in time if 5.0 \( \mu \text{g/ml} \) of Act D is present or 0.5 \( \mu \text{g/ml} \) cycloheximide, we assume that certain messenger RNAs for particular proteins are synthesized and will lead, after a lag of about 1 h, to the pre-S-phase nuclear size increase.

The different concentrations of Act D decreased RNA synthesis to 3\% (5 \( \mu \text{g} \)), 5.5\% (2.5 \( \mu \text{g} \)), 11\% (1.0 \( \mu \text{g} \)), 16\% (0.5 \( \mu \text{g} \)), and 65\% (0.1 \( \mu \text{g} \)) during a 5-min pulse in an exponentially growing cell population after preincubation with Act D for 4 h.

**DISCUSSION**

It has been suggested that the nuclear pore complex is responsible for the selective transport of macromolecules. Feldherr (1962) demonstrated that relatively “large objects” can pass the pore complex. In the nuclear envelope of oocytes there seems to be no barrier to the penetration of smaller solutes like sucrose (Horowitz, 1972). Other suggested functions of the pore complex include processing of RNA (Franke et al., 1972).

The finding that nuclear pores form during interphase and the establishment of the time sequence of pore formation (Maul et al., 1971 and 1972) allowed the examination of the mechanisms and conditions of nuclear pore formation. The control of the exchange rate was determined by Feldherr to be independent of a change in number of the nuclear pore complex and was interpreted to be due to a mechanism which worked within the highly complex pore structure. Our findings that the pore number changes rapidly shortly before and at the beginning of S phase in HeLa cells may be construed as evidence that a higher exchange rate is needed and that the nuclear membrane responds to that increase. But there is no immediate increase of RNA or protein synthesis at the time of increase in the rate of pore formation. Also, the highest number of pore complexes is present in mature (quiescent) oocytes (Scheer, 1972). From this argument and the presence of stacks of annulate lamellae or even single pore complexes in the rough endoplasmic reticulum (Maul, 1970 c), we may speculate that the pore complex has functions other than selective transport between two large compartments (see also Franke et al., 1972). We had determined that the nuclear pore number increases from a certain G\(_1\) plateau until the end of the cell cycle. Because the doubling of pore numbers seemed to correspond to the doubling of DNA, we investigated the pore formation in the absence of DNA replication in synchronized HeLa cells. The increase in pore number began shortly before S phase started. Surprisingly, there was no effect on nuclear size if excess thymidine, FUdR, Ara C, or HU were used to block DNA replication in early S phase. The nuclear size during early S phase increased at the same rate as the controls. Also, no effect on nuclear pore number was seen when DNA replication was prevented. We conclude that the inhibition of DNA replication, as such, does not reduce the number of pores to be formed nor does the mass of DNA added to the nucleus during early S phase lead to the change in nuclear size.

The same can be said about nucleolar size. It does not determine nuclear size, i.e., if its formation or growth is inhibited the nucleus attains the same size as controls. Previously it had been determined that the inhibition of ribosomal synthesis by low doses of Act D prevents cells from kidney explants (Lieberman et al., 1963) and Ehrlich ascites cells (Baserga et al., 1965) from entering S phase. The Act D sensitive step during G\(_1\) phase may also be applicable to the HeLa system. The pre-S-phase nuclear size increase then seems to be independent of the mechanism which prevents cells from synthesizing DNA if ribosomal synthesis has been inhibited during the G\(_1\) phase.

Acidic proteins have been implicated in gene activation (Teng and Hamilton, 1969; Stein and Baserga, 1971). In a previous report we mentioned the fact that the highest rate of pore formation in HeLa cells (apart from the nuclear envelope reformation) takes place at the time of the highest rate of acidic protein synthesis (Stein and Borun, 1972) shortly before the S phase. The present results indicate that nuclear size increase can be inhibited with high concentrations of Act D and cycloheximide. The time of inhibition during the G\(_1\) period is the same for both inhibitors within the limit of our time resolution (1 h). Tentatively we would like to suggest that the inhibition of synthesis of a certain protein or group of proteins, either at the transcriptional or translational level, prevents nuclear pore formation and nuclear size increase at the G\(_1\)-S transition or shortly before. It cannot be decided whether a correlation between the acidic proteins and gene activation and pore formation exists with our present technique and inhibitor study since it could be just as likely that we have inhibited some other essential protein of
the complex internal structure of pores or a membrane protein. At this time, with the present data, we cannot decide whether we inhibited pore formation or membrane synthesis which is essential for nuclear size increase or possibly observed only a secondary effect, i.e. the formation of cytoplasmic proteins necessary before nuclear membrane increase, and pore formation may be inhibited. In the study by Carlsson et al. (1973) the rate of protein uptake seemed to be directly related to the increase in nuclear size. Highfield and Dewey (1972) observed that CHO cells did not enter S phase if blocked more than 55 min before S phase with cycloheximide implicating a short-lived protein to be responsible for cells to initiate DNA replication.

The complete block of nuclear pore formation by ATP was expected because ATP is required for membrane fusion and fission processes (Jamieson and Palade, 1968). Therefore blocking nuclear pore formation by the inhibition of ATP formation served as a control for the observability of a relatively small change in pore number over a rather short time interval during the cell cycle.

Tobey and Crissman (1972) assume no unbalanced growth with HU and Ara C block of DNA replication. Also, they claim that there is no initiation of DNA synthesis taking place in the presence of the drugs because of a certain lag between removal of the drugs and resumption of DNA synthesis. We would suggest from our results using HU and Ara C, as well as FUdR and excess thymidine, that cells are in S phase but not replicating DNA or only very slowly doing so. The inhibition of DNA replication does not inhibit certain other S-phase processes. Therefore unbalanced growth has to be assumed (see also Amaldi et al., 1972). Also, the initiation of DNA synthesis cannot be checked by blocking DNA replication, the very function proving that initiation has been completed. The lag between the removal of inhibitors of DNA replication and the beginning of [H]thymidine incorporation may represent the time for the removal of the blocking agents rather than the time for any initiation process to take place as implicated in the report by Tobey and Crissman (1972).

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