

## DNA REPLICATION IN CHINESE HAMSTER OVARY CELLS MADE PERMEABLE TO NUCLEOTIDES BY TWEEN-80 TREATMENT

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Cells made permeable to small molecules have proven useful in the study of DNA synthesis and repair. In bacteria, toluene treatment provides a semi-in vitro system which allows a limited semi-conservative DNA synthesis utilizing exogenously supplied deoxyribonucleoside triphosphates (11). However, essentially all of the bacterial cells are nonviable and lose much of their cellular constituents (7, 10) as a result of the toluene treatment. Seki et al. (12) have reported the use of a hypotonic treatment to permeabilize HeLa cells to nucleotides. We report here a simple method using the detergent Tween-80 to make Chinese hamster ovary (CHO) cells permeable to nucleotides, and show that such cells will synthesize DNA semiconservatively in a reaction dependent on ATP and all four deoxyribonucleoside triphosphates. The permeabilized state is apparently reversible, since most of the cells are still viable after the treatment. Thus, we also have the prospect of studying DNA replication and repair in cell cycles after permeabilization. A preliminary report of this work has been published (3).

### MATERIALS AND METHODS

#### *Growth and Permeabilization of Cells*

CHO cells (line CHO-K1 from the American Type Culture Collection, CCL 61) were grown at 37°C in monolayer culture in Nutrient Mixture F-12 containing 10% fetal calf serum (both from Grand Island Biological Co., Grand Island, N.Y.) with 10<sup>5</sup> U/liter of penicillin and 0.1 g/liter of streptomycin. Cells for experiments were grown in Falcon plastic tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), 75 cm<sup>2</sup> area, to a density of 4–8 × 10<sup>6</sup> cells/flask. They were harvested by trypsinization (0.1% trypsin, 1:300, from Grand Island, in 0.9% NaCl; after 3 min at 37°C, the trypsin was poured off, the cells were dislodged by a sharp rap, and resuspended in 10 ml of F-12 plus serum), counted,

and collected by centrifugation at room temperature at 1,000 rpm for 5 min in 15- or 50-ml plastic centrifuge tubes. Cells to be permeabilized were resuspended in 1% Tween-80 (Sigma Chemical Co., St. Louis, Mo.) in 0.25 M sucrose at a concentration of 2.0–2.8 × 10<sup>7</sup> cells/ml. They were left at room temperature for 30 min, then centrifuged as before, and resuspended in the same volume of Tween-sucrose. This cell suspension was assayed for DNA synthesis activity as soon as possible, although the cells could be left at room temperature for 1–2 h without much loss of activity. About half the activity was lost after storage overnight at 4°C.

#### *Assay for DNA Synthesis*

The assay mixture for DNA synthesis was adopted from previous bacterial work (1, 11); we have not yet made any attempt to optimize components and concentrations for the permeabilized CHO cells. Each reaction mixture contained, in a total vol of 0.25 ml, 60 mM potassium phosphate buffer, pH 7.4, 13 mM MgSO<sub>4</sub>, 2 mM dithiothreitol, 1.3 mM ATP, 33 μM each dATP, dCTP, and dGTP, 20 μM TTP (all from Sigma), 10 μCi/ml [*methyl*-<sup>3</sup>H]TTP (New England Nuclear Corp., Boston, Mass.), and 50 or 75 μl (1.5–2.0 × 10<sup>6</sup> cells) of cell suspension. (In density shift experiments, 20 μM BrdUTP [P-L Biochemicals, Inc., Milwaukee, Wis.] was substituted for TTP.) Reaction mixtures were incubated at 37°C, and at 15-min intervals, aliquots of 25 μl were spotted on a 1 × 2-cm strip of Whatman 3MM filter paper that had previously been soaked in 10% trichloroacetic acid (TCA) and dried. At the end of the assay, all strips were washed twice for 30 min in 10% TCA, for 15 min in ethanol-diethyl ether (3:1) and in diethyl ether. They were dried, and radioactivity was determined.

### RESULTS AND DISCUSSION

In our initial attempts to permeabilize CHO cells we tried various approaches, including toluene treatment. Only after incubation with 1% Tween-80 in 0.25 M sucrose were the cells able to incorporate exogenous deoxyribonucleoside tri-

phosphates. This treatment was tried because of Kay's report (8) in which it was shown that Tween-80 increased the permeability of Ehrlich ascites carcinoma cells to dyes and metabolites without affecting their viability.

Fig. 1 *a* illustrates the effect Tween-80 preincubation on the utilization of deoxyribonucleoside triphosphates by CHO cells. The presence of 1% Tween-80 during the 30-min incubation in 0.25 M sucrose resulted in a 12-fold increase in the incorporation of [<sup>3</sup>H]dCTP into acid-insoluble material. This incorporation was dependent on the presence of ATP and all four deoxyribonucleoside triphosphates (Fig. 1*b*); omission of any of these components from the assay mixture reduced DNA synthesis. We have no data bearing on the question of nucleotide precursor pools remaining; however, residual pools of deoxyribonucleoside triphosphates could account for the remaining activity. In experiments not presented here, we showed that any one of the four deoxyribonucleoside triphos-

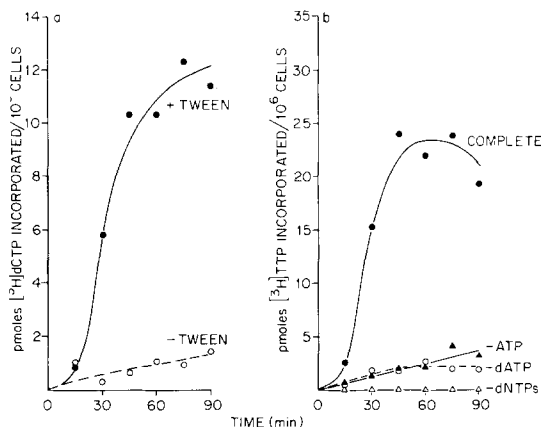


FIGURE 1 *a* Dependence of [<sup>3</sup>H]dCTP-incorporation on Tween treatment. Cells were grown and harvested as described in Materials and Methods. Half of the cells were incubated in 0.25 M sucrose for 30 min at room temperature, while the other half were incubated in 1% Tween-80 in 0.25 M sucrose. Both batches were assayed for DNA synthesis in the standard assay mixture, except that [<sup>3</sup>H]dCTP was substituted for [<sup>3</sup>H]TTP. (●—●) cells incubated in Tween-sucrose; (○---○) control cells incubated in sucrose. (*b*) Dependence of [<sup>3</sup>H]TTP incorporation on the presence of ATP and of all four deoxynucleoside triphosphates. CHO cells were grown, harvested, and permeabilized as described in Materials and Methods. They were assayed in the standard assay mixture with the following omissions: (▲—▲) -ATP; (○---○) -dATP; (Δ—Δ) -dATP, -dCTP, and -dGTP; and (●—●) complete reaction mixture.

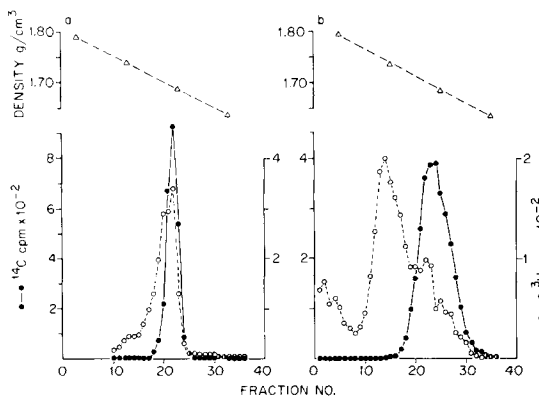


FIGURE 2 CsCl density gradient analysis of DNA synthesized in permeabilized CHO cells. Cells were labeled with 0.005  $\mu$ Ci/ml of [<sup>14</sup>C]thymidine for 66 h before harvest. They were harvested, permeabilized, and incubated for 45 min in the standard assay mixture in which BrdUTP was substituted for TTP. (The label was [<sup>3</sup>H]TTP.) The cells were centrifuged out of the reaction mixture and resuspended in 0.1% SDS in  $2 \times 10^{-4}$  M EDTA. The sample was split—one half was sonicated ( $3 \times 10$ s, Branson sonifier, Heat Systems-Utrasonics, Inc., Plainview, N.Y.) and the other half was homogenized with six strokes of a Potter homogenizer. In each case, 0.2 ml of lysate was combined with 4.5 ml of CsCl (refractive index 1.402), and centrifuged for 72 h at 29,000 rpm in an SW 50.1 rotor. Gradients were collected from the bottom onto filter paper strips, washed in 5% TCA, then alcohol, dried, and counted. (*a*) Not sonicated; (*b*) sonicated.

phates will serve as the labeled precursor, provided the other three are also present.

The product of the reaction was analyzed on neutral CsCl gradients to determine whether it was DNA that had been synthesized semiconservatively or in a repair-type reaction. For these experiments, the DNA of cells was prelabeled with [<sup>14</sup>C]thymidine for several generations before harvest, and BrdUTP was substituted for TTP in the assay mixture. Fig. 2 *a* shows the profile of DNA from lysates that had been sheared by six strokes in a Potter homogenizer (Potter Instrument Co., Inc., Plainview, N.Y.). Such treatment of bacterial lysates results in DNA fragments with an average mol wt of at least  $1 \times 10^7$  daltons (2). In this case, the parental (<sup>14</sup>C-labeled) DNA banded at a density of about 1.700 g/cm<sup>3</sup> as expected for mammalian DNA. The DNA synthesized "in vitro" (<sup>3</sup>H-labeled) banded mainly at a density slightly higher than the parental DNA density, with a significant shoulder in the position of hybrid

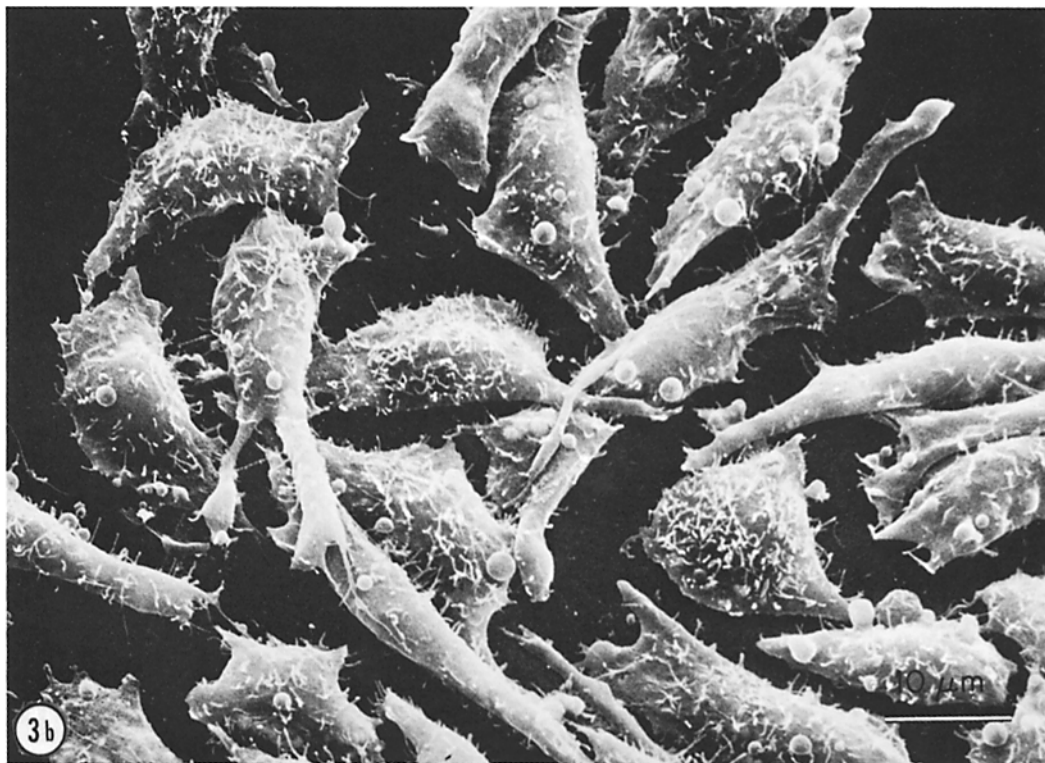
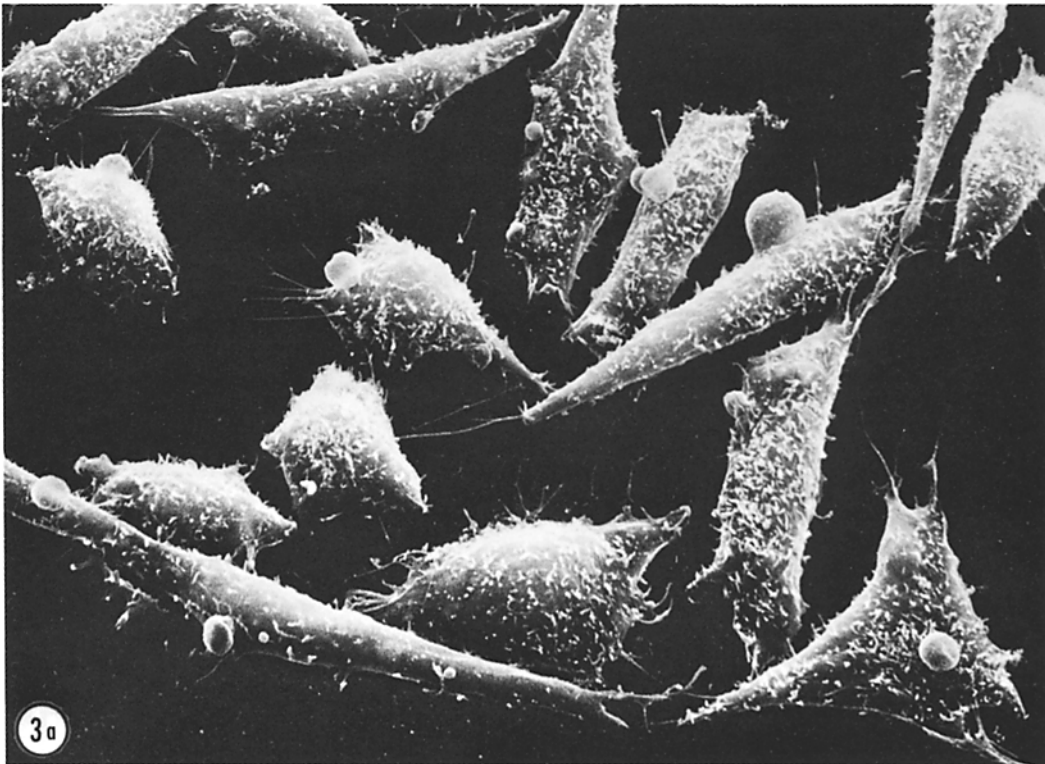


FIGURE 3 Scanning electron micrographs of control (*a*) and Tween-treated (*b*) CHO cells. Cells were grown on cover slips in Falcon plastic Petri dishes. Half of the cover slips were removed to another Petri dish containing Tween-sucrose for 30 min. All cover slips were then rinsed with Hanks' balanced salt solution and fixed.  $\times 1700$ .

DNA (one parental strand, one BrdUrd-substituted strand) (13). When the preparation was sonicated, a treatment which results in fragments of bacterial DNA of average size less than  $1 \times 10^6$  daltons, most of the tritium-labeled material banded at the hybrid density. These results indicate that the product of the in vitro reaction is largely DNA that has been synthesized semiconservatively, since repair synthesis would have resulted in regions of BrdUrd substitution too short to give the observed shift in the density of this sonicated DNA.

Autoradiographs were made of two portions of cells from the same passage. Cells in the control flask were incubated in fresh medium at 37°C containing 1  $\mu$ Ci/ml of [<sup>3</sup>H]thymidine for 45 min, harvested, and portions were spread on slides. Cells in the other flask were harvested, treated with Tween-80, incubated at 37°C in the assay mixture for 45 min, and spread on slides. The autoradiographs showed grains over the nuclei of 55% of the control cells and of 56% of the Tween-permeabilized cells. These results are best interpreted as showing that only cells in S phase at time of treatment are active in the assay mixture.

Scanning electron micrographs (Fig. 3) showed no gross morphological differences between Tween-treated and control cells. In experiments in which cells were prelabeled with [<sup>14</sup>C]thymidine for several generations before permeabilization, no degradation of the parental DNA occurred either during the Tween treatment or during the subsequent incubation in the assay mixture (data not shown). Viability was tested in two separate plating experiments. Cells treated with Tween for 30 min formed 82% and 84% as many colonies as did control cells incubated in 0.25 M sucrose without Tween. Thus, the viability of the majority of the CHO cells is not affected by the permeabilization procedure.

We have shown here that CHO cells can be permeabilized to nucleotides by incubation in 1% Tween-80 in 0.25 M sucrose. Such cells carry out limited semiconservative, ATP-dependent synthesis of DNA. The cells are apparently not damaged by the treatment, and the results of the autoradiography suggest that DNA synthesis after permeabilization continues in those cells that were replicating their DNA before the treatment. If we suppose that during the assay no initiation of replicon replication can occur (synthesis only on those replicons already initiated), then we can calculate the amount of DNA synthesis expected during the

assay, using estimates of replicon size and rate of replication from the literature. If the average replicon is 40  $\mu$ m long (4, 6), and a mammalian cell contains  $1.6 \times 10^6$   $\mu$ g of DNA, then there are about 40,000 replicons/cell. If S phase takes about 6 h to complete in CHO cells (13), and the rate of replication fork movement is about 1  $\mu$ m/min (5), then about 2,500 replicons must be replicating bidirectionally at any time in those cells in S phase. According to the model of Maaløe and Hanawalt (9) for bacterial DNA replicating at a constant rate, those replicons that are active in vitro should increase the amount of their DNA by a factor of 0.39. Converting from micrometers to daltons to picomoles, and taking CHO DNA to be 30% TMP and 55% of the cells in S phase, we calculate that  $10^6$  cells should incorporate 60–70 pmol of TTP. We observed incorporation of about 20 pmol of TTP/ $10^6$  cells. For the approximations used, these values are in reasonably good agreement.

In preliminary experiments, we have shown that permeabilized CHO cells will also incorporate [<sup>3</sup>H]UTP into acid-insoluble material, and that this incorporation requires the presence of the other three ribonucleoside triphosphates. We plan also to examine protein synthesis in these cells and the possible interdependence of DNA, RNA, and protein synthesis as well.

## SUMMARY

We have developed a method for permeabilizing CHO cells to nucleotides under conditions which allow most cells to remain viable. Permeabilized cells can carry out ATP-dependent, semiconservative synthesis of DNA. The data are consistent with the continuation of DNA synthesis in those cells in S phase at the time of treatment, possibly limited to completion of replicon synthesis without new initiations.

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## REFERENCES

1. BILLEN, D., G. R. HELLERMANN, and D. R. STALLIONS. 1975. Role for deoxyribonucleic acid

- ligase in deoxyribonucleic acid polymerase I-dependent repair synthesis in toluene-treated *Escherichia coli*. *J. Bacteriol.* **124**:585-588.
2. BILLEN, D., R. HEWITT, and G. JORGENSEN. 1965. X-ray induced perturbations in the replication of the bacterial chromosome. *Biochim. Biophys. Acta.* **103**:440-454.
  3. BILLEN, D., and A. C. OLSON. 1975. DNA metabolism in Tween-80 permeabilized Chinese hamster ovary cells. *J. Cell. Biol.* **67**(2, Pt. 2):32 a. (Abstr.).
  4. GAUTSCHI, J. R., R. M. KERN, and R. B. PAINTER. 1973. Modification of replicon operation in HeLa cells by 2,4-dinitrophenol. *J. Mol. Biol.* **80**:393-403.
  5. HAND, R., and I. TAMM. 1973. DNA replication: direction and rate of chain growth in mammalian cells. *J. Cell Biol.* **58**:410-418.
  6. HUBERMAN, J. A., and A. D. RIGGS. 1968. On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol.* **32**:327-341.
  7. JACKSON, R. W., and J. A. DE MOSS. 1965. Effects of toluene on *Escherichia coli*. *J. Bacteriol.* **90**:1420-1425.
  8. KAY, E. R. M. 1965. The effects of Tween-80 on the *in vitro* metabolism of cells of the Ehrlich-Lettré Ascites carcinoma. *Cancer Res.* **25**:764-768.
  9. MAALØE, O., and P. C. HANAWALT. 1961. Thymine deficiency and the normal DNA replication cycle. I. *J. Mol. Biol.* **3**:144-155.
  10. MOSES, R. E., J. L. CAMPBELL, R. A. FLEISCHMAN, and C. C. RICHARDSON. 1971. Enzymatic mechanisms in DNA replication. In Proceedings of the Miami Winter Symposia, vol. 2. D. W. Ribbons, J. Woessner, and J. Schultz, editors. Elsevier Publishing Co., Inc., New York. 48-66.
  11. MOSES, R. E., and C. C. RICHARDSON. 1970. Replication and repair of DNA in cells of *Escherichia coli* treated with toluene. *Proc. Natl. Acad. Sci. (U. S. A.)* **67**:674-681.
  12. SEKI, S., M. LEMAHIEU, and G. C. MUELLER. 1975. A permeable cell system for studying DNA replication in synchronized HeLa cells. *Biochim. Biophys. Acta.* **378**:333-343.
  13. TAYLOR, J. H., T. M. MYERS, and H. L. CUNNINGHAM. 1971. Programmed synthesis of deoxyribonucleic acid during the cell cycle. *In Vitro.* **6**:309-321.