A Permeable Animal Cell Preparation for Studying Macromolecular Synthesis. DNA Synthesis and the Role of Deoxyribonucleotides in S Phase Initiation†

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ABSTRACT: A method for selectively permeabilizing Chinese hamster ovary cells with lysolecithin is described. These cells retained their general morphology, intact organelles; 100% of their DNA and 75% of their total protein. They synthesized protein, RNA and DNA when supplied with appropriate substrates and cofactors, many of which do not penetrate intact cells. These permeable cells will be useful for studying the roles of nonpenetrating molecules such as substrates, effectors, and inhibitors of cellular metabolic processes, including macromolecular syntheses, under relatively physiological conditions. As an initial study, some properties of DNA synthesis were investigated. DNA synthesis proceeded at an initial rate at least as high as in intact cells and continued for at least 2 h, and 20% of the genome could be replicated. The DNA was first made as small (4S) pieces that were rapidly ligated to 4–21 × 10^6 dalton DNA. Normal, semiconservative synthesis was demonstrated by showing increased density following BrdUTP incorporation. Washing the permeable cells shortened the duration of DNA synthesis; adding back the solution in which the permeable cells were prepared restored the initial ability. A soluble factor thus seems needed to allow continued DNA synthesis. The cells do not stop making DNA because of temperature-dependent deterioration or because of exhaustion of added substrates. The ability of a synchronized culture approaching S to make DNA appeared at the same time, and not earlier, in permeable cells supplied with the deoxyribonucleoside triphosphates, as in intact cells. Hence supplying these precursors cannot alone be the final event needed for initiation of the S period. A supply of deoxyribonucleotides is necessary for DNA synthesis; hydroxyurea blocked DNA synthesis by intact cells, but the capacity of these cells to synthesize DNA was restored by permeabilizing the cells and supplying deoxyribonucleotides. Ribonucleotide reductase thus does not appear to have a sufficient role for initiation of the S period, but only a necessary one.

The biochemical events responsible for initiating the S period and regulating DNA replication are poorly understood. The possibility that the supply of deoxyribonucleotides may be an important control mechanism in initiating the S period has been discussed in several recent reviews (Edenberg & Huberman, 1975; Prescott, 1976). Bjursell & Reichard (1973) and Reichard (1977) proposed that dCTP or an analogue may be important in initiating polyoma virus replication. To better examine this and other questions regarding the regulation of DNA replication, we wanted to develop a subcellular system which would allow the introduction of deoxynucleotides and other compounds into animal cells which could be highly synchronized in various periods of the cell cycle, including G_1. Ideally the subcellular system should retain the capacity to synthesize DNA semiconservatively at in vivo rates and replicate extensive amounts of the genome.

Isolated nuclei (De Pamphilis & Berg, 1975; Friedman & Mueller, 1968; Krokan et al., 1975; Krokan & Ericksen, 1977; Lynch et al., 1970; Thompson & Mueller, 1975; Tseng & Goulian, 1975) and cell lysates (Brown et al., 1977; Lazarus, 1973) have proven very useful in studying some aspects of DNA replication. But a subcellular system which is permeable to exogenous compounds and still retains a high degree of structural and physiological integrity should be better suited for studying many of the processes regulating replication.

We decided to use Chinese hamster ovary (CHO)[1] cells because they can be synchronized in various periods of the cell cycle including G_1 (Tobey & Ley, 1970, 1971; Ley & Tobey, 1970). However, we were unable to successfully permeabilize these cells using methods which have been described (see Results). We therefore developed a new method for permeabilizing CHO cells in which initial rates of DNA synthesis are as great as in vivo and 16–20% of the genome is replicated. The permeabilization procedure is presented, the permeable cells are described, and the product of DNA synthesis has been characterized.

In initial studies using this system, evidence is presented which indicates that the supply of deoxyribonucleotides alone does not seem to limit the entry of G_1 cells into S. A possible role of deoxyribonucleoside diphosphates is suggested. Cytoplasmic requirements for continued DNA synthesis were noted.

Experimental Procedure

Reagents. [*methyl*-^{3}H]TTP (10–20 Ci/mmol), [^{3}H(G)]-dATP (10–20 Ci/mmol), [4,5-^{3}H(*N*)]-L-leucine (5 Ci/mmol), [*methyl*-^{3}H]thymidine (40–60 Ci/mmol), [5,6-^{3}H]UTP (35–50 Ci/mmol), and Biofluor scintillation fluid were purchased from New England Nuclear. Nuclease-free Pronase was from Calbiochem. All other compounds were purchased from Sigma Chemical Co.

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[1] Abbreviations used: CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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Cell Culture and Synchronization. CHO cells were the generous gift of Dr. Robert Tobey. The cells were grown in suspension culture in F-10 medium containing 10% calf serum and 5% fetal calf serum with the modifications described by Tobey & Ley (1971). The cells were found to be free of mycoplasma by the method of Schneider et al. (1974). Cells were synchronized in G_1 by growth in low isoleucine medium for 36–38 h and then released from the G_1 block by resuspension in complete medium (Ley & Tobey, 1970; Tobey & Ley, 1971).

Electron Microscopy. Cells were prepared for electron microscopy by washing twice in PBS and fixed in 2% glutaraldehyde for 1 h at 0 °C. The cells were then post-fixed in osmium tetroxide, dehydrated in acetone, and embedded in epon–araldite. Electron micrographs were generously prepared by Dr. A. Krishan with a Phillips "300."

Protein Synthesis. Protein synthesis was measured essentially as described by Ochoa & Weinstein (1964). Permeable cells (see Results) and intact cells were washed with PBS and suspended in 60 mM KCl, 5 mM $Mg(OAc)_2$, 10 mM Tris-HCl (7.8), 6 mM β-mercaptoethanol. One-half milliliter 0.5 mL of 2.5 mM ATP, 0.12 mM GTP, 12 mM phosphoenolpyruvate, 40 μM [^{3}H]-L-leucine (5 Ci/mmol), 0.8 mM all other L-amino acids, and 50 μg pyruvate kinase in 60 mM KCl, 5 mM $Mg(OAc)_2$, 10 mM Tris-HCl (7.8), 6 mM β-mercaptoethanol were added to 1 mL of cells ($1–2 \times 10^8$ cells). Assays were performed at 37 °C, and 30 μL aliquots of cells in the reaction mixture were applied to Whatman 3 MM filter discs which had been soaked in 5% Cl_3CCOOH and dried. Incorporation of [^{3}H]leucine into protein was determined by the method of Mans & Novelli (1961).

RNA Synthesis in Permeable Cells. RNA synthesis was measured by incubating 2×10^7 washed permeable cells/mL in 35 mM Hepes (7.4), 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 150 mM sucrose, 2.5 mM ATP, 0.2 mM CTP and GTP, 0.01 mM [^{3}H]UTP (4 Ci/mmol), 500 μg/mL tRNA, and 5 μg/mL polyvinyl sulfate (to inhibit RNase) at 37 °C. At indicated times 0.2-mL aliquots were precipitated with an equal volume of cold 15% Cl_3CCOOH, 0.2 M sodium pyrophosphate followed by 3 mL of cold 5% Cl_3CCOOH, 0.2 M sodium pyrophosphate. After 10 min at 0 °C, samples were centrifuged, and the Cl_3CCOOH precipitates were washed four times with 5% Cl_3CCOOH, 0.2 M sodium pyrophosphate. The Cl_3CCOOH precipitates were dissolved in 0.1 mL of 0.1 N NaOH for 45 min at 37 °C and counted in a liquid scintillation counter with 10 mL of Biofluor.

DNA Synthesis in Permeable Cells. Maximum DNA synthesis was obtained by incubating 2×10^7 permeable cells/mL in 150 mM sucrose, 80 mM KCl, 35 mM Hepes (7.4), 5 mM potassium phosphate (7.4), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM phosphoenolpyruvate, 1.25 mM ATP, 0.1 mM CTP, GTP, and UTP, and 0.25 mM [^{3}H]TTP (0.4 Ci/mmol), dATP, dCTP, and dGTP at 37 °C. Twenty-five-microliter aliquots were applied to Whatman No. 3 filters which had been soaked in 5% Cl_3CCOOH, 0.2 M sodium pyrophosphate, and allowed to dry. The filters were then extensively washed in 5% Cl_3CCOOH followed by a 15-min wash with 75% ethanol, 25% ether, and then ether. After drying, the filters were counted in a liquid scintillation counter. All determinations were performed in duplicate.

DNA Synthesis in Intact Cells. Cells in F-10 medium ($2–5 \times 10^5$ cells/mL) were incubated with [^{3}H]thymidine (0.5 μCi/mL) at 37 °C. The cells were collected by centrifugation, washed twice with cold PBS containing 2 mM thymidine, and precipitated with cold 5% Cl_3CCOOH. After centrifugation the precipitate was dissolved in cold 0.1 N NaOH and reprecipitated with cold 10% Cl_3CCOOH, 0.2 M sodium pyrophosphate for 10 min at 0 °C. The precipitate was collected by centrifugation, dissolved in 0.1 mL of 0.1 N NaOH at 37 °C for 45 min, and counted in a liquid scintillation counter.

Alkaline Sucrose Gradients. Permeable cells were incubated in the DNA synthesis solution, which was modified as indicated in figure legends, and the DNA was then processed and applied to alkaline sucrose gradients as described by Tseng & Goulian (1975).

One hundred and thirty-five nucleotide marker DNA was prepared by restriction nuclease digestion and was the generous gift of Dr. W. Haseltine.

Density Gradient Centrifugation. Permeable CHO cells were incubated in the DNA synthesis solution containing 0.25 mM BrdUTP in place of TTP and [^{3}H]dATP (0.25 mM, 0.4 Ci/mmol) was the labeled nucleotide. After the indicated times, cells were lysed and analyzed on $CsCl_2$ gradients by the procedure of Tseng & Goulian (1975).

Normal density CHO cell DNA was prepared by incubating cells in F-10 medium containing [^{14}C]TdR (0.005 μCi/mL) for 16 h. DNA was prepared according to Tseng & Goulian (1975).

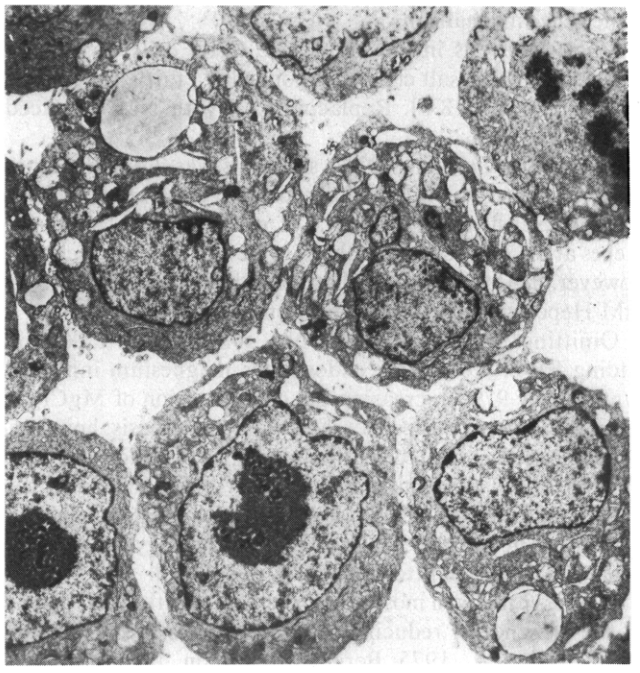
Autoradiography. Permeable and intact cells were labeled with [^{3}H]TTP (1.6 Ci/mmol) or [^{3}H]TdR (5 μCi/mL), respectively, washed twice with phosphate-buffered saline, and prepared for autoradiography essentially as described by Puck & Steffan (1963).

Protein and DNA were determined by the methods of Lowry et al. (1951) and Giles & Mayers (1965), respectively.

Results

Preparation of Permeable Cells. We failed to permeabilize CHO cells using methods which had been reported, as briefly described below. Toluene was used to rapidly permeabilize isolated liver cells (Hilderman & Deutscher, 1974, 1976); however, we found that, when toluene treated CHO cells were permeable to nucleotides and trypan blue, less than 10% of in vivo rates of DNA synthesis were observed. The method of Burgoyne (1972) required more than 2 h to prepare permeable cells, and CHO cells were not permeabilized effectively by this technique. Berger & Johnson (1976) permeabilized L cells in approximately 20 min by treatment with 30 mM mercaptoethanol in a hypotonic solution and obtained 60% of in vivo rates of DNA synthesis. In our hands, this procedure resulted in a mixture of permeable CHO cells and nuclei with less than 20% of in vivo rates. HeLa cells, treated with a hypotonic solution for 30 min or more, resulted in permeable cells which synthesized DNA at 50% of in vivo rates and replicated 5% of the HeLa genome (Seki et al., 1975); however, after CHO cells were exposed to this hypotonic medium, less than 30% of in vivo rates were measured. Sols (1975) permeabilized erythrocytes with digitonin after cross-linking membrane proteins with tolylene diisocyanate for 1 h; however, CHO cells permeabilized in this manner synthesized DNA at 10% of in vivo rates. Tween-80 was reported to permeabilize CHO cells in 30 min at 25 °C, and DNA replication continued at 30% of in vivo rates (Billen & Olson, 1976). In our hands we found that CHO cells were not permeable to nucleotides until placed in their hypotonic DNA synthesis assay solution at 37 °C (Billen & Olson, 1976); at this point the preparation consisted primarily of nuclei which synthesized DNA at 25% of in vivo rates.

After testing many compounds, we found lysolecithin (Sigma, type I) rapidly permeabilized CHO cells using the following procedure. Cells were collected by centrifugation, washed twice in 150 mM sucrose, 80 mM KCl, 35 mM Hepes (7.4), 5 mM potassium phosphate (7.4), 5 mM $MgCl_2$, 0.5 mM



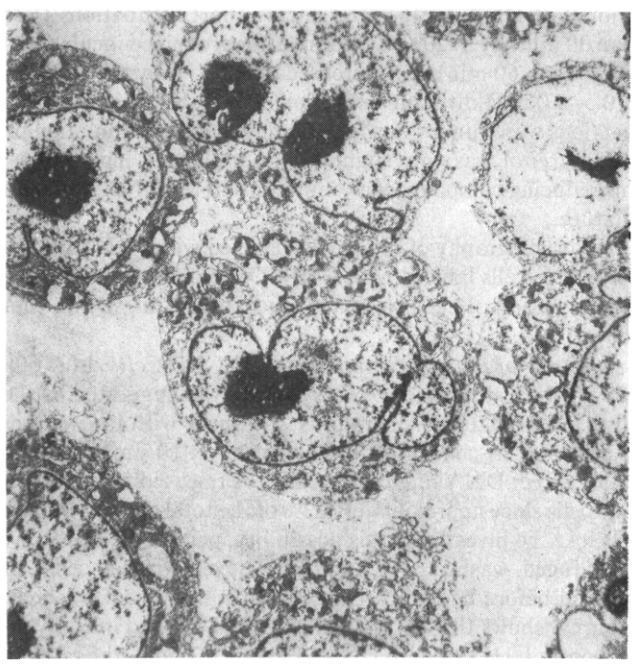


FIGURE 1: Electron micrograph of control and lysolecithin treated CHO cells. CHO cells were washed twice with solution "A"; control (A) and lysolecithin treated (B) cells were then prepared for electron microscopy as described. Electron micrographs are ~×9000.

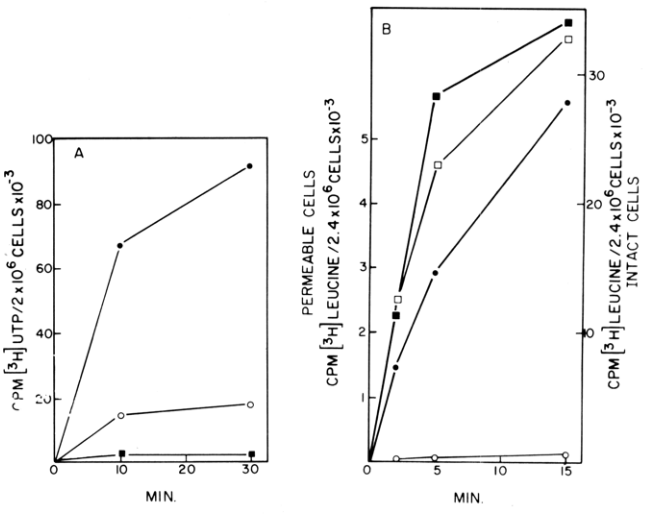


FIGURE 2: RNA and protein synthesis in permeable CHO cells. CHO cells were treated with lysolecithin and washed as described. (A) The permeable cells were incubated in the RNA synthesis mixture described, in the presence (○) and absence (●) of 2.5 mM 3′-deoxyadenosine 5′-triphosphate. Cells which were not permeabilized were incubated in the same mixture without 3′-deoxyadenosine 5′-triphosphate (■). At the indicated times, incorporation of [^{3}H]UTP into RNA was determined as described. (B) Permeable cells were incubated in the protein synthesis system described, in the presence (●) and absence (○) of ATP, GTP, and phosphoenolpyruvate. CHO cells which were not treated with lysolecithin were incubated in the same system containing 2.5 mM glucose, in the presence (□) and absence (■) of ATP, GTP, and phosphoenolpyruvate. At the indicated times, incorporation of [^{3}H]leucine into protein was determined as described.

$CaCl_2$ (solution A), suspended in cold solution A at 1×10^8 cells/mL, and $1/3$ volume of 1 mg/mL lysolecithin in solution A was added for 1 min at 4 °C. The cells were then routinely diluted into the 37 °C DNA synthesis mixture as described (not washed) or, where indicated, washed with solution A to remove any factors which were released from the cells (washed). The cells (97–100%) stained with trypan blue after they were exposed to lysolecithin at 4 °C, but only 10% of the cells were permeabilized by lysolecithin at 37 °C.

Electron microscopy of lysolecithin treated cells (Figure 1B) shows that the gross cellular morphology is maintained. The plasma membrane appears to be intact in most areas, although it is somewhat distorted. The nuclear membrane retains its double membrane integrity; and under higher magnification, both the inner and outer nuclear membranes appear unaltered. Intact nucleoli are seen in the nuclei. In the cytoplasmic area, mitochondria appear intact with condensed cristea; condensed cristea were also observed in mitochondria of control cells (Figure 1A), likely a result of handling procedures. At higher magnifications ribosomes and rough endoplasmic reticulum appear unaltered.

Intact CHO cells were found to contain 5.5 μg of DNA and 133 μg of protein per 10^6 cells. After the cells were treated with lysolecithin and washed, the amounts remaining in the cells were determined to be 5.5 and 99 μg, respectively. Retention of 100% of the DNA and 75% of the total protein supports the electron microscopy studies (Figure 1), showing that intact nuclei, mitochondria, and other structures are retained in the permeable cells.

Macromolecular Synthesis in Permeable CHO Cells. The ability of lysolecithin treated CHO cells to synthesize RNA, protein, and DNA was examined. Figure 2A shows that the permeable cells incorporated [^{3}H]UTP into acid-insoluble material when supplied with ribonucleotides. [^{3}H]Uridine was not incorporated, and cells which were not treated with lysolecithin incorporated very little [^{3}H]UTP into acid-insoluble material. 3′-Deoxyadenosine triphosphate, but not 3′-deoxyadenosine, inhibited incorporation in the permeable cells (Figure 2A), in agreement with other reports (Suhadolnik, 1970). Ninety percent of the acid-precipitable [^{3}H]UTP synthesized was converted to acid-soluble form when incubated with 0.1 N NaOH or RNase (0.1 mg/mL pancreatic RNase and 30 μg/mL T_1 RNase); Pronase (0.5 mg/mL) and DNase (0.5 mg/mL pancreatic DNase) solubilized the acid-insoluble [^{3}H]UTP less than 12%. In permeable cells the initial rate of

TABLE I: Nucleotide Requirements for DNA Synthesis in Permeable CHO Cells.[a]

	DNA synthesis (% control)
Control (TTP, dATP, dCTP, dGTP)	100
−dATP, dCTP, dGTP	11
TTP, dADP, dCDP, dGDP	115–120
TTP, dAMP, dCMP, dGMP	35–40
TTP, CDP, GDP, ADP	24
TdR, dA, dC, dG	12
−ATP	40–60
−ATP, −PEP	16
−GTP, CTP, UTP	60–70

[a] Permeable cells were prepared as described and incubated in the DNA synthesis solution with various precursors for DNA synthesis or with the indicated deletions. In the assay utilizing deoxyribonucleosides (line 5), [^{3}H]TdR (0.4 Ci/mmol) was the labeled precursor. The various DNA precursors were 0.25 mM in all cases.

RNA synthesis was calculated to be 2.5–3-fold higher than for optimum DNA synthesis (occurring at in vivo rates, see Discussion), indicating a significant rate of transcription in this system which had not been optimized for RNA synthesis.

Protein synthesis in permeable cells was dependent on the addition of ATP, GTP, and phosphoenolpyruvate (Figure 2B). The acid-insoluble [^{3}H]leucine was solublized 75% in 15 min at 37 °C with 0.5 mg/mL Pronase; but DNase (0.5 mg/mL), RNase (0.1 mg/mL pancreatic and 30 μg/mL T_1), or boiling for 15 min with 5% Cl_3CCOOH did not significantly reduce the incorporated [^{3}H]leucine. The addition of exogenous tRNA did not increase incorporation. [^{3}H]Leucine was also incorporated in cells not treated with lysolecithin; however, protein synthesis in the intact cells was not dependent on the addition of ATP, GTP, and phosphoenolpyruvate (Figure 2B). Cycloheximide (10^{-5} M) inhibited protein synthesis in both intact and permeable cells by 80–90%.

DNA Synthesis in Permeable CHO Cells. Table I shows the nucleotide requirements for DNA synthesis in permeable CHO cells. Omitting three of the dNTPs reduced DNA synthesis to 11% of control. Increasing the concentration of dNTPs to 0.5 mM or 1.0 mM did not affect DNA synthesis; however, lowering the concentration of dNTPs to 0.1 mM or 0.05 mM decreased incorporation to 84% or 60%, respectively, of control. Substituting dNDPs for three of the dNTPs consistently stimulated synthesis about 18%, whereas substituting dNMPs or rNDPs for the same dNTPs decreased DNA synthesis to 33% or 24%, respectively, of control. Hydroxyurea (1 mM) inhibited incorporation with NDPs, but not with dNMPs, dNDPs, or dNTPs, presumably by inhibiting the enzyme ribonucleotide reductase (see Reichard, 1972, 1977) which converts ribonucleotides to deoxyribonucleotides (not shown). Deoxyribonucleosides, including [^{3}H]TdR, were not efficiently utilized by the permeable cells and unlabeled TdR did not compete with [^{3}H]TTP incorporation.

Omitting exogenous ATP reduced DNA synthesis to 50% of control, and the omission of both ATP and PEP reduced synthesis to 16% of control (Table I). Omitting PEP alone decreased incorporation to 76% of control, and the addition of pyruvate kinase (4 μg/mL) did not affect DNA synthesis (not shown). The optimum ATP concentration was 1.25 mM, and increasing the concentration to 5 mM reduced synthesis to 30% of control, possibly by dissociating the replicative machinery (Thompson & Mueller, 1975). omitting GTP, CTP, and UTP reduced synthesis to about 65% of control; 1.0 mM was the optimum concentration of these rNTPs. It is not yet known if the rNTPs stimulate DNA synthesis by serving as primers for Okazaki fragments (see Edenberg & Huberman, 1975) or by some other mechanism.

DNA synthesis in the permeable CHO cells was highly dependent on the salt concentration, with a fairly sharp optimum at 80 mM KCl. Replacing KCl with NaCl reduced synthesis by 10%.

The pH for optimum DNA synthesis was 7.4 with Hepes buffer; a 25% reduction in incorporation occurred at pH 7.2 and 7.8. Substituting Tris-HCl or potassium phosphate for Hepes at pH 7.4 reduced synthesis by 26% or 53%, respectively; however, 5 mM potassium phosphate, in conjunction with 35 mM Hepes, increased incorporation about 5%.

Omitting $MgCl_2$ reduced DNA synthesis by 67%, and adding EDTA to chelate endogenous magnesium inhibited synthesis by 97%. Increasing the concentration of $MgCl_2$ to 10 mM did not significantly affect DNA synthesis; however, 20 mM $MgCl_2$ inhibited synthesis by 55%. The addition of 0.5 mM $CaCl_2$ did not significantly affect synthesis in 30 min, nor did the addition of EGTA; however, with long incubations (more than 90 min) at 37 °C, cellular morphology deteriorated when $CaCl_2$ was omitted or EGTA was added.

DNA synthesis in most broken cell preparations is assayed in the presence of reducing agents (Jazwinski & Edelman, 1976; Seki et al., 1975; Berger & Johnson, 1976; Tseng & Goulian, 1975; Krokan et al., 1975); however, dithiothreitol or mercaptoethanol (0.05–5.0 mM) consistently decreased the amount of DNA synthesized during short incubations (less than 30 min) by 5–20%. Synthesis after prolonged incubations (more than 60 min) was increased about 15% by the addition of 0.5–3.0 mM dithiothreitol (not shown). Since most of the reactions were run for less than 30 min and the stimulation by dithiothreitol was only slight during prolonged incubations, this reducing compound was omitted from the DNA synthesis mixture.

Autoradiography of intact cells labeled with [^{3}H]TdR and permeable cells labeled with [^{3}H]TTP demonstrated that the same fraction of cells were actively synthesizing DNA (not shown).

Leakage of Factors from Permeable CHO Cells. Loss of a factor which stimulates DNA synthesis was suggested by the observation that DNA synthesis was linear with the number of cells only at concentrations above 1.5×10^7/mL. Factors which affect DNA replication could be released from permeable cells since approximately 23% of the total cellular protein was lost. To investigate this possibility, permeable cells were centrifuged, washed once, and then assayed (washed cells), or assayed before being centrifuged and washed (not washed). Figure 3 shows that cells which were not washed initially incorporate [^{3}H]TTP into DNA at about 1.3 times the rate observed in washed cells. Additionally, while washed cells stopped synthesizing DNA after 45–60 min, synthesis in cells which were not washed continued for at least 2 h. When the cells were centrifuged and resuspended in the supernatant, the ability to synthesize DNA was restored to 90% of the level found in cells not centrifuged; hence the effect of washing is not primarily due to cell damage. These results suggest that a factor(s) (presumably cytoplasmic) which enhances DNA synthesis leaks out of the permeable cells. Cytoplasmic factors have been reported to stimulate DNA synthesis in isolated nuclei (Otto & Reichard, 1975; Thompson & Mueller, 1975; De Pamphilis & Berg, 1975; Thompson & McCarthy, 1971; Hershey et al., 1973; Tseng & Goulian, 1975). We are characterizing this factor(s).

The rate of DNA synthesis in permeable cells declined over a 2-h period (Figure 3); however, the following experiments

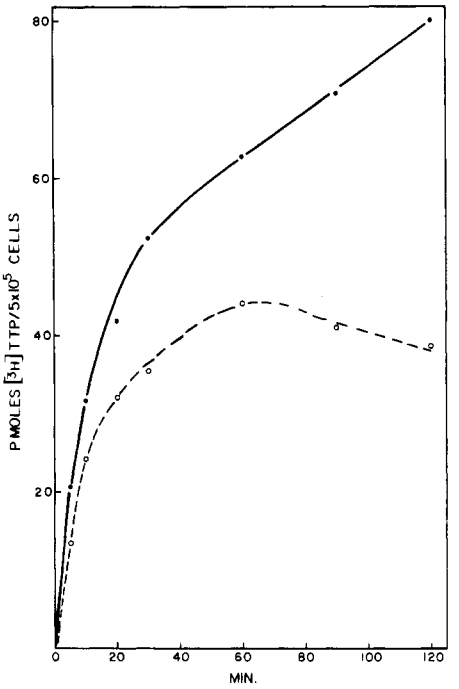


FIGURE 3: DNA synthesis before and after washing permeable CHO cells. An exponential culture of CHO cells was collected, washed two times with solution "A", and lysolecithin was added at 0 °C as described. An aliquot of the permeable cells was removed and kept at 0 °C (not washed), while the remaining permeable cells were collected by centrifugation, washed once with solution "A", and resuspended in solution "A" at the same concentration as the cells which were not washed. DNA synthesis was assayed in the cells which had been washed (○) and in the cells which were not washed (●) and is expressed as pmol of [^{3}H]TTP incorporated in DNA/5 × 10^5 cells as a function of time.

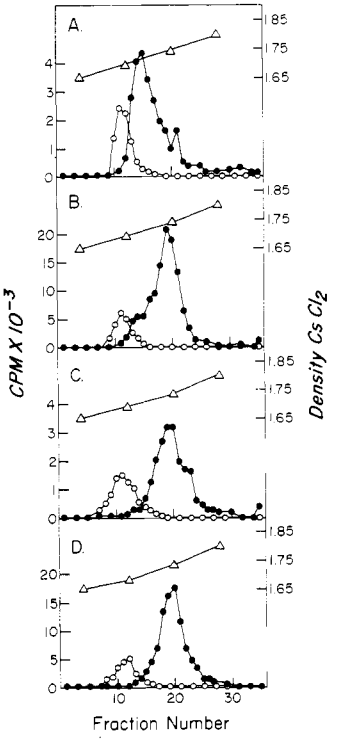


FIGURE 4: Neutral cesium chloride gradients of BrdUMP substituted DNA. Cells from an exponential culture were permeabilized and incubated in the DNA synthesis mixture containing BrdUTP in place of TTP, and [^{3}H]dATP (0.25 mM; 0.4 Ci/mmol). At the indicated times, synthesis was stopped by lysing the cells, and the DNA was analyzed on neutral $CsCl_2$ gradients as described. All operations were performed in the dark. (●—●) ^{3}H-labeled, BrdUMP-substituted DNA; (○—○) ^{14}C-labeled, unsubstituted marker DNA; (△) density, determined with a refractometer. (A) Five-minute incubation; (B) 60-min incubation; (C) 5-min incubation, sonicated 1 min at setting 3 on Branson Sonifier 185 before banding; (D) 60-min incubation, sonicated before banding.

indicated this was not due to a general deterioration of the system at 37 °C. Cells were permeabilized, divided into two samples, and incubated in the DNA synthesis mixture minus [^{3}H]TTP at 37 °C. [^{3}H]TTP was added to one sample immediately, and the other sample was preincubated at 37 °C for 30 min before [^{3}H]TTP was added. The rate of [^{3}H]TTP incorporation in the cells preincubated at 37 °C was 91% of that in the culture not preincubated. In another experiment, permeable cells were incubated in the DNA synthesis system for 60 min, and these "used" cells were separated from the "used" assay solution by centrifugation. The "used" assay solution sustained DNA synthesis in new permeable cells 85% as effectively as fresh assay solution, and the addition of fresh assay solution to the "used" permeable cells did not result in stimulation of synthesis above control levels. Degradation of nucleotides or proteins associated with replication therefore does not appear to be the primary reason for the reduction in rate of DNA synthesis at 37 °C.

Characterization of DNA Synthesized in Permeable CHO Cells. A shift in the density of DNA, resulting from incorporation of BrdUMP, has been used to distinguish replicative and repair DNA synthesis (Cooper & Hanawalt, 1972). Permeable cells were incubated in the DNA synthesis solution containing BrdUTP in place of TTP. The density of DNA synthesized in the permeable cells progressively increased above the density of normal CHO DNA during 5- and 90-min incubations (Figure 4A,B). Essentially all of the DNA made in the permeable cells banded at hybrid density after the DNA was fragmented by sonication (Figure 4C,D), as expected for semiconservative replicative synthesis; and the ^{3}H-labeled DNA banded near the position of fully substituted DNA after denaturation to single strands (unpublished observations).

DNA replication in both prokaryotes (Kornberg, 1974) and eukaryotes (Edenberg & Huberman, 1975; Prescott, 1976) is discontinuous on at least one of the parental DNA strands. To determine if DNA synthesis in the permeable cells was discontinuous, these cells were incubated for 10 s with high specific activity [^{3}H]TTP at 37 °C ("pulse"), followed by the addition of excess unlabeled TTP ("chase") for up to 5 min. At the indicated times cells were lysed and the DNA was analyzed by sedimentation through 5–20% alkaline sucrose gradients (Figure 5).

After a 10-s "pulse", more than 45% of the labeled DNA was in short pieces comigrating with 135 nucleotide marker DNA (4S) (Figure 5A). After a 1-min "chase", the short pieces were converted to approximately 4–21 × 10^6 dalton DNA (Figure 5B). During 2 and 5-min "chases", there was a large increase in both 4–21 × 10^6 dalton DNA and very large DNA at the bottom of the gradient (Figure 5C,D). The 4S DNA constituted less than 1% of the total labeled DNA after 2 and 5-min "chases". The amount of labeled DNA increased threefold during the 5-min "chase". Continued incorporation of 24-fold diluted [^{3}H]TTP during the 5-min "chase" would account for increasing the labeled DNA 2.3-fold, and the increase above 2.3-fold may be due to noninstantaneous equilibration of added TTP with [^{3}H]TTP or slightly more efficient synthesis in the presence of 300 μM TTP than with 12.5 μM TTP. This same pattern of discontinuous synthesis was observed after permeable cells had been synthesizing DNA for 70 min. The 4–21 × 10^6 dalton DNA probably represents replicon intermediates; it is approximately the right size as that

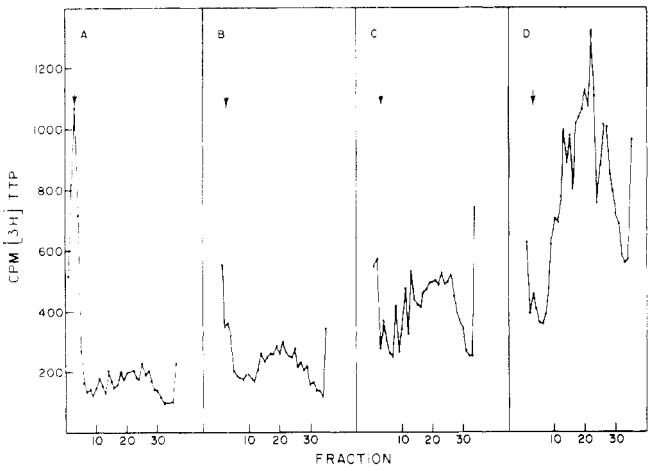


FIGURE 5: Alkaline sucrose gradients of "pulse-labeled" DNA in permeable CHO cells. Cells from an exponential culture were permeabilized and incubated in the DNA synthesis mixture which was modified to contain 12.5 μM [^{3}H]TTP (18-20 Ci/mmol) for 10 s at 37 °C. After the 10-s "pulse", reactions were made 300 μM with unlabeled TTP and allowed to incubate for 1, 2, or 5 min. Immediately after the 10-s "pulse" (A) or the 1 (B), 2 (C), or 5 (D)-min "chase", DNA synthesis was stopped with Sarkosyl lysis buffer (Tseng & Goulian, 1975); and the samples were treated with Pronase, ethanol precipitated, and run on 5-20% sucrose gradients as described in Methods. The arrow indicates the position of 135 nucleotide, ^{32}P-labeled marker DNA. Counts per minute of [^{3}H]DNA applied to each gradient were: (A) 9486; (B) 9153; (C) 14 859; (D) 31 014. Recovery of ^{3}H-labeled DNA in all gradients was greater than 85%.

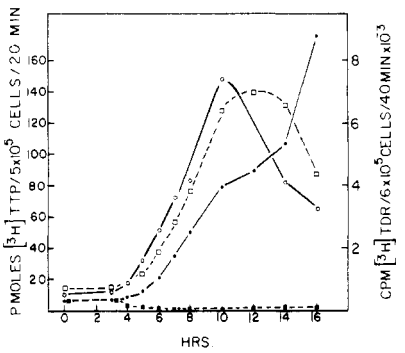


FIGURE 6: DNA synthesis in synchronized CHO cells. Duplicate cultures of CHO cells were synchronized in G_1 by isoleucine starvation (Ley & Tobey, 1970; Tobey & Ley, 1971). At zero time, one culture was resuspended into complete medium (control), and the other culture was resuspended into medium containing 1 mM hydroxyurea (HU). At the indicated times, aliquots of cells from both cultures were pulsed with [^{3}H]TdR (0.5 μCi/mL) for 40 min or permeabilized and incubated in the DNA synthesis solution for 20 min. DNA synthesis in permeable cells is pmol of [^{3}H]TTP incorporated per 5×10^5 cells per 20 min, and DNA synthesis in intact cells is counts per minute of [^{3}H]TdR/6×10^5 cells per 40 min $\times 10^{-3}$. Intact control cells (□); intact cells + HU (■); permeable control cells (○); permeable cells + HU (●).

TABLE II: Subcellular Localization of DNA Synthesized in Permeable Cells.[a]

Fraction	pmol of TTP/10^7 cells
G_1 nuclear	57
G_1 cytoplasmic	8
S nuclear	1940
S cytoplasmic	43

[a] CHO cells were synchronized in G_1 as described (Ley and Tobey, 1970; Tobey & Ley, 1971). Aliquots of the cultures were then permeabilized 3 h (G_1) or 12 h (S) after release into complete medium (see Figure 6). After permeabilization the cells were incubated in the DNA synthesis mixture for 30 min at 37 °C. The cells were then placed in an ice bath and made 0.5% with Triton X-100. After 5 min the plasma membrane and cytosol components were solubilized as determined with a phase contrast microscope. The nuclei were collected by centrifugation at 600g for 5 min. The supernatant (cytoplasm fraction) was removed and the nuclei were resuspended in a cold solution which was the same composition as the supernatant. [^{3}H]TTP incorporated into DNA was then determined in the nuclear and cytoplasmic (supernatant) fractions. A background for each fraction was determined by placing permeable cells in the DNA synthesis mixture at 0 °C, immediately adding Triton X-100 to 0.5% for 5 min at 0 °C and separating the nuclear and cytoplasmic fractions by centrifugation. The 0 °C background value was subtracted from each cytoplasmic and nuclear fraction in G_1 and S cells incubated at 37 °C.

expected for average interfork distances of 15 μM or about 15×10^6 daltons (Huberman & Riggs, 1968).

To determine the extent to which nonnuclear DNA synthesis may have contributed to the measured incorporation, the permeabilized cells were treated with Triton X-100 after incubation in the DNA synthesis mixture. Triton X-100 is known (Blobel & Potter, 1966) to solubilize the plasma membrane and the outer nuclear membrane of animal cells, so that mitochondria would be released or lysed. The nuclei were then collected by centrifugation; any acid-insoluble counts remaining in the supernatant would be due to nonnuclear (mitochondrial) DNA synthesis or leakage of nuclear DNA. The 0.5% Triton X-100 was found to completely solubilize the plasma membrane of permeable cells, leaving intact, clean nuclei. Table II shows nonnuclear DNA synthesis in permeabilized cells contributed very little to the total incorporation; and very little, if any, labeled DNA leaked from the nucleus.

Role of Deoxyribonucleotides in Initiating S Phase. Permeable cells from synchronized cultures were used to determine if a low supply of deoxyribonucleotides was limiting the entrance of G_1 cells into S. An inadequate supply of substrates could be overcome by permeabilizing cells and providing the nucleotides; the permeable cells would then enter the S period before intact cells if only the supply of nucleotides was limiting replication. Cells were arrested in G_1 by isoleucine starvation and then allowed to complete G_1 by resuspension in complete medium, enter S and divide (Ley & Tobey, 1970; Tobey & Ley, 1971). At various times after release from G_1, DNA synthesis in intact (by [^{3}H]TdR incorporation) and permeable (by [^{3}H]TTP incorporation) cells was determined. Figure 6 shows the incorporation of both TdR and TTP in control cultures was very low for the first 4 h, and then DNA replication in both intact and permeabilized cells increased at the same time and at comparable rates. The fraction of intact and permeable cells labeled with [^{3}H]TdR and [^{3}H]TTP, respectively, was determined by autoradiography to be the same during these experiments. In many similar experiments permeable cells never began to synthesize DNA before intact cells. Identical results were obtained when synchronized baby hamster kidney cells (BHK) were permeabilized by a different procedure (unpublished experiments), demonstrating that this result was not an artifact of the lysolecithin treatment.

We also wanted to know if inhibiting the synthesis of dNTPs prevented cells from becoming competent to replicate DNA. Hydroxyurea inhibits ribonucleotide reductase (see Reichard, 1972, 1977) and decreases dNTP levels (Skoog & Bjursell, 1974; Walters et al., 1973). Figure 6 shows the effect of this drug on DNA synthesis in permeable and intact cells from synchronized cultures. Hydroxyurea (1 mM) effectively suppressed [^{3}H]TdR incorporation in intact cells. Supplying dNTPs to permeable cells from hydroxyurea treated cultures

overcame the block on DNA synthesis (Figure 6), and hydroxyurea (1–10 mM) did not affect [^{3}H]TTP incorporation in these permeable cells. Increased DNA synthesis was observed at the same time in permeable cells from both control and hydroxyurea treated cultures. The elevated rate of DNA replication observed in permeable cells from cultures treated with hydroxyurea for 16 h (Figure 6) is likely due to accumulation of the cells very near the G_1/S boundary (Ley & Tobey, 1970; Tobey & Ley, 1971; Hamlin & Pardee, 1976), 95% of which replicate DNA when permeabilized; however, in the control culture there is no time at which all cells are in S, due to a loss of synchrony.

Discussion

Confidence in using any subcellular system to study the processes regulating DNA replication depends on establishing that the normal replicative processes are retained, and on showing that these processes occur at rates approximating those in vivo. In this permeable cell system, DNA was synthesized in a semiconservative, discontinuous manner dependent on the cell cycle (Figures 4–6), demonstrating that normal replicative processes were retained; the following calculations indicate these processes occurred at in vivo rates. The initial rate of DNA synthesis in permeable cells from an exponential culture was 70–80 pmol of TTP incorporated per 10^7 cells per min (Figure 3); or about 280–320 pmol of total nucleotides per 10^7 cells per min. The rate of DNA synthesis in intact cells was determined in two ways: (1) The generation time of these CHO cells is 18 h in our hands; exponential cultures therefore replicate 55 μg of DNA per 10^7 cells per 18 h or about 170 pmol of total nucleotides per 10^7 cells per min. (2) An exponential culture of CHO cells was suspended in F-10 medium containing dialyzed serum and 0.2 μg/mL fluorodeoxyuridine to inhibit thymidylate synthetase (Blakley, 1969). [^{3}H]TdR (2 μCi/mL; 3 μM) was added, and the rate of incorporation was determined to be 58 pmol of TdR per 10^7 cells per min, or 230 pmol of total nucleotides per 10^7 cells per min. The two values for the rate of DNA synthesis in intact cells give comparable rates (within 26%). The initial rate of DNA synthesis in the permeable CHO cell system is at least as great as that in intact cells. Most other subcellular systems are considerably less active in DNA synthesis (10–30% of in vivo rates with 1–2% of the genome replicated (Tseng & Goulian, 1975; Billen & Olson, 1976; Krokan et al., 1975); however, Thompson & Mueller (1975), Hershey et al. (1973), Berger & Johnson (1976), and Seki et al. (1975) reported 50–65% of in vivo rates with 5% of the genome replicated.

To determine the fraction of the cell DNA replicated, 10^7 cells must replicate 55 μg of DNA or about 183 nmol of total nucleotides in one generation. By autoradiography 20% of the permeable cells in Figure 3 were synthesizing DNA; 1.1×10^5 cells incorporated 80 pmol of TTP in 2 h or 29 nmol of total nucleotides/10^7 cells. This represents replication of 16% of the genome, and in other experiments calculations indicated 20% of the genome was replicated.

The possibility that dNDPs may be the preferred or direct substrates for DNA synthesis was raised by the observation that synthesis with these substrates was consistently 18–20% higher than with dNTPs (Table I). Similar results have been obtained with BHK cells permeabilized by a different procedure. Purified DNA polymerases utilize dNTPs in DNA synthesis (see Kornberg, 1974); however, Miller & Wells (1971) indicated a nucleoside diphosphokinase may be associated with the DNA replicase complex, in which case dNDPs could be preferentially utilized in vivo. Kinetic studies have failed to determine whether dNTPs or dNDPs are the precursors for DNA synthesis in intact cells (Rubinow & Yen, 1972). Although the dNDPs could first be converted to dNTPs in the permeable cells, we found (unpublished observation) that synthesis with dNDPs is increased 15–20% above that with dNTPs at both 5 and 90 min with 0.25 mM (saturating) and 0.05 mM (half-saturating) substrates. Phosphorylation of dNDPs to dNTPs would still not explain the 15–20% stimulation above dNTPs at saturating levels. Other explanations, such as dNDPs entering the nucleus more readily than dNTPs, are also possible. We are investigating these possibilities.

The permeable cells provided a means of investigating the role of deoxyribonucleotides in regulating DNA replication. Deoxyribonucleotides are certainly required for replication; and because the activities of many enzymes involved in their synthesis are low in G_1 and increase when cells enter S (see Prescott, 1976), the supply of these substrates could limit entrance into S. Ribonucleotide reductase is the rate-limiting enzyme for dNTP formation, and Elford (1972) and Reichard (1972, 1977) have discussed the importance of this enzyme in regulating DNA synthesis. However, Walters et al. (1973) found a small increase in dNTP pools just before S and concluded that availability of precursors did not limit entrance into S. Whether this small increase was localized in the cytoplasm (Skoog & Bjursell, 1974) and therefore unavailable for nuclear replication was not determined. Because a low concentration of dNTPs could still limit entrance into S and dNDPs may be the preferred substrates in vivo, we supplied permeable cells from synchronized cultures with more than enough substrates to replicate the entire genome (greater than 150 times intracellular concentrations). Permeable cells supplied with dNTPs (Figure 6) or dNDPs (unpublished observations) did not begin to synthesize DNA before intact cells; therefore some factor other than (or in addition to) the general synthesis of substrates limits the entrance of G_1 cells into S.

Furthermore, inhibiting ribonucleotide reductase activity and decreasing dNTP pools with hydroxyurea (Reichard, 1972, 1977; Walters et al., 1973; Skoog & Bjursell, 1974; our unpublished observations) inhibited DNA synthesis in intact cells approaching S, but did not prevent the same cells from synthesizing DNA when they were permeabilized and supplied with substrates (Figure 6). Production of dNTPs was therefore not necessary for making cells competent to replicate DNA. Our findings do not eliminate the possibility that dCTP or another nucleotide is required in addition to some other component which limits entry into S.

Our conclusion that the supply of substrates does not limit entrance into S is subject to the assumptions that deoxyribonucleotides are not completely excluded from the nucleus of G_1 (but not S) cells, and that the processes involved in initiating DNA replication are not preferentially disrupted by the permeabilization process. However, permeable G_1 cells can use exogenous dNTPs to repair DNA after treatment with bleomycin (unpublished observation), indicating that dNTPs can efficiently enter their nucleus. A selective disruption of initiation processes seems somewhat unlikely because identical results were obtained with synchronized BHK cells permeabilized by a different procedure, and the lysolecithin permeabilized cells initiate Okazaki fragments, synthesize DNA at in vivo rates, and replicate a large fraction of the genome. We are attempting to answer this question directly.

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