

exception of dCTP. dATP, dTTP, and dGTP pools rose less than 2-fold between G1 and S phase. The whole-cell dCTP pool rose 10-fold while the nuclear pool rose only 2-fold.

The rates at which either radiolabeled cytidine or deoxycytidine equilibrated with the nuclear and whole-cell dCTP pools of G1 and S phase CHO cells were compared. Nuclear and whole-cell dCTP pools equilibrated at the same rate, regardless of the nucleoside used or the phase of the cell cycle. This indicates the existence of a single cellular dCTP pool. Interestingly, the deoxycytidine-derived dCTP specific activity was thirteen times larger in G1 than in S phase.

Experiments comparing the labeling kinetics of ^3H -thymidine in G1, S phase, and exponentially growing cells revealed that the S phase dTTP pool equilibrated with exogenously added thymidine faster than the G1 phase pool. The rate of equilibration in exponentially growing cells appeared to be a combination of that seen in G1 and S phases. A linear rate of ^3H -thymidine incorporation into DNA occurred at the same rate in S phase and exponentially growing cells.

DNA Precursor Compartmentation in Mammalian Cells:
Distribution and Rates of Equilibration
between Nucleus and Cytoplasm

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Janet M. Leeds

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major

Redacted for Privacy

Head of department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

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Contributions of Authors

There are three authors in the first manuscript, Chapter II, and thus I must discuss the contributions of the authors. Dr. Mary Slabaugh and I collaborated on the ribonucleotide reductase experiments. She suggested and carried out the control experiments for the ribonucleotide reductase section. Dr. Slabaugh did all the ribonucleotide reductase assays. I did all other experiments. Dr. Mathews, of course, helped to plan the experiments and discussed the results, and made suggestions for control experiments.

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DNA Precursor Compartmentation in Mammalian Cells:
Distribution and Rates of Equilibration
between Nucleus and Cytoplasm

Chapter 1: Introduction

DNA precursors, the deoxyribonucleoside triphosphates (dNTPs), are synthesized either de novo, with amino acids, CO₂, active formate, 5-D-ribose, and inorganic phosphate as building blocks, or by salvage of pre-existing nucleosides or bases that are present in the cell or in the environment of the cell. The dNTP levels are primarily regulated through allosteric interactions between the dNTPs and enzymes at pivotal points along the biosynthetic pathways. The dNTP biosynthetic pathways are presented in abbreviated form in Figure 1.1.

The first step unique to DNA precursor biosynthesis is the reduction of the four ribonucleoside diphosphates (rNDPs, see Appendices), UDP, ADP, CDP, and GDP, to their corresponding deoxyribonucleoside diphosphates, catalyzed by the enzyme ribonucleotide reductase. Ribonucleotide reductase has two distinct sites of allosteric regulation, an "activity" site and a "specificity" site. The effectors of the "activity" site are ATP, which increases the overall enzymatic activity, and dATP, which decreases the level of activity. The "specificity" site is affected

by dTTP, dGTP, and dATP. In mammalian cells there is some disagreement about the precise regulation of ribonucleotide reductase at the "specificity" site, and several different models have been proposed (Hunting and Henderson, 1983).

Once the rNDPs are converted to the corresponding dNDPs by ribonucleotide reductase, they are rapidly converted to triphosphates by nucleoside diphosphokinase, a relatively non-specific kinase activity. dUTP, which under normal circumstances is not incorporated into DNA, is quickly converted to dUMP by deoxyuridine triphosphate nucleotidohydrolase (dUTPase). Thymidylate synthase, in a reductive methylation reaction, converts the dUMP to dTMP. This dTMP is rapidly phosphorylated to dTTP and is then available for DNA synthesis. In most mammalian cells (Jackson, 1978; Nicander and Reichard, 1985) a substantial proportion of the dCMP is converted to dTMP; dCMP is deaminated to dUMP and converted to dTMP by the action of thymidylate synthase.

Salvage of nucleosides occurs through the action of nucleoside kinases: thymidine kinase, deoxycytidine kinase, deoxyadenosine kinase, and deoxyguanosine kinase. Once the dNMPs are formed through the action of the nucleoside kinases they are rapidly phosphorylated to the

triphosphate level and presumably available for DNA replication.

Normal enzymatic regulation of dNTP levels is well understood. There is, however, evidence that a higher order of regulation exists. Werner (1971) observed that radioactive thymidine was incorporated into E. coli DNA at a maximal rate before the dTTP pool had equilibrated with the label. A number of investigations on T4 phage-infected bacteria (Tomich et al., 1974; Chiu et al., 1982; Reddy et al., 1977; Mathews et al., 1979) indicated that the dNTP-synthesizing enzymes were physically juxtaposed into a complex. Mathews and Sinha (1982) found that molar concentrations of dNTPs necessary to sustain maximal rates of DNA synthesis were higher than the molar dNTP concentrations estimated from cellular dNTP pool analyses. These studies in prokaryotes led to the hypothesis that DNA precursors are channeled from a complex of biosynthetic machinery responsible for their synthesis to the DNA replication fork. This channeling allows newly synthesized dNTPs to bypass endogenous dNTP pools and create higher local dNTP concentrations at the replication fork (Mathews et al., 1979).

Evidence in eukaryotes, for the existence of the channeling of DNA precursors, or alternatively a physical compartmentation of the dNTPs, is based upon the following

lines of experimentation: (1) comparison of the labeling kinetics of acid-soluble pools and the rate at which a particular labeled nucleotide entered acid-insoluble material in both intact and permeabilized cells; (2) comparisons of the rates of incorporation into DNA of salvage-derived and de novo-derived DNA precursors; (3) co-sedimentation of the enzymes involved in DNA precursor biosynthesis and results of genetic changes in that co-sedimentation; and (4) in vivo levels of dNTP biosynthetic enzyme activity.

Baril et al. (1973) first reported that enzymes involved in dNTP biosynthesis from eukaryotes could be co-fractionated. In this particular instance the researchers found that several dNTP-synthesizing enzymes co-fractionated with a high-molecular-weight membrane fraction of hepatoma cell cultures and regenerating rat liver.

Reddy and Pardee (1980) compared the relative content of dNTP-synthesizing enzymes in quiescent and exponentially growing karyoplasts and found an increase in the relative nuclear content of dNTP biosynthetic enzymes in growing cells. More recently Noguchi, Reddy and Pardee (1983) reported that a high-molecular-weight fraction prepared by sedimentation of a nuclear lysate from S phase cells was capable of rapidly incorporating radioactivity

from rCDP into DNA. This incorporation was resistant to dilution by unlabeled dCTP and hence supports the channeling hypothesis and confirms earlier observations from studies of permeabilized cells (Reddy and Pardee, 1982). However, Spyrou and Reichard (1983) reported that the incorporated label observed by Noguchi et al. (1983) was not DNA but RNA.

Ayusawa et al. (1983) used a genetic deficiency of thymidylate synthase to probe dNTP biosynthetic enzyme-enzyme interactions. Ayusawa and co-workers stably transformed a thymidylate synthase-deficient line of mouse FM3A cells by transfecting them with human DNA containing the gene for thymidylate synthase. A multi-enzyme aggregate containing DNA polymerase alpha, thymidine kinase, and thymidylate synthase was obtained by sucrose gradient centrifugation of the wild-type parent cells. Although the thymidylate synthase-deficient and thymidylate synthase-transformed cell lines also yielded an aggregate, the human thymidylate synthase did not cofractionate with the mouse enzymes. Ayusawa and co-workers concluded that the human thymidylate synthase was incapable of the specific interactions with the other enzymes that the mouse thymidylate synthase had. Reddy (1982) reported that hydroxyurea inhibited the in vivo, but not the in vitro, activity of thymidylate synthase. Since hydroxyurea inhibits ribonucleotide reductase Reddy

interpreted his results as an indication of interactions between thymidylate synthase and ribonucleotide reductase.

The first experiments that supported the hypothesis of compartmentation of DNA precursor pools in eukaryotic cells were labeling experiments. Fridland (1973) labeled exponentially growing human lymphoblast cells (CCRF-CEM) with ^3H -deoxyuridine and ^3H -thymidine and followed the rate of radioactivity incorporation into dTTP and acid-insoluble material. Both nucleosides were incorporated into acid-insoluble material at maximum rate well before the acid-soluble pools had become equilibrated with the radioactive nucleoside. Fridland concluded that eukaryotic cells contain two non-equilibrating dNTP pools, one used for DNA replication that turns over rapidly, and a second not used for DNA replication that equilibrates more slowly with exogenous nucleosides.

Kuebbing and Werner (1975) compared the rate of incorporation of radioactive thymidine into DNA of cells whose pre-treatments had presumably led to the availability of (1) de novo-derived dTTP; (2) salvage-derived dTTP; or (3) both salvage- and de novo-derived dTTP. Their results, showing radioactive label to be incorporated into acid-insoluble material more slowly in the presence of presumably active salvage pathways, led them to postulate the existence of a replication-active

DNA precursor pool, in rapid equilibrium with salvage-derived dNTPs but in a slow equilibrium with de novo-derived dNTPs.

The results of Scott and Forsdyke (1980) contradicted the idea that salvage-derived dNTPs were in rapid equilibrium with the replication-active pool. Those researchers found, using isotope dilution techniques, that deoxyadenosine and deoxyguanosine could increase the size of their respective dNTP pools. However, when hydroxyurea, which inhibits ribonucleotide reductase, was used to inhibit exponentially growing cells, deoxyadenosine and deoxyguanosine did not restore DNA replication. Snyder (1984), who confirmed this observation, found that deoxyadenosine and deoxyguanosine were capable of overcoming a block in DNA repair biosynthesis.

A more thorough method of studying nucleotide metabolism was introduced by Maybaum, Cohen, and Sadee (1980) who, after labeling mouse S49 lymphoma cells with radioactive pyrimidine nucleosides, measured the specific activities of all the end products that had incorporated label, namely, rNTPs, dNTPs, RNA, and DNA. By measuring the radioactivity in all the end products that had passed through a given enzyme they were able to determine the in vivo activity of the enzyme. Using a comparable procedure Nicander and Reichard (1983) uncovered a discrepancy

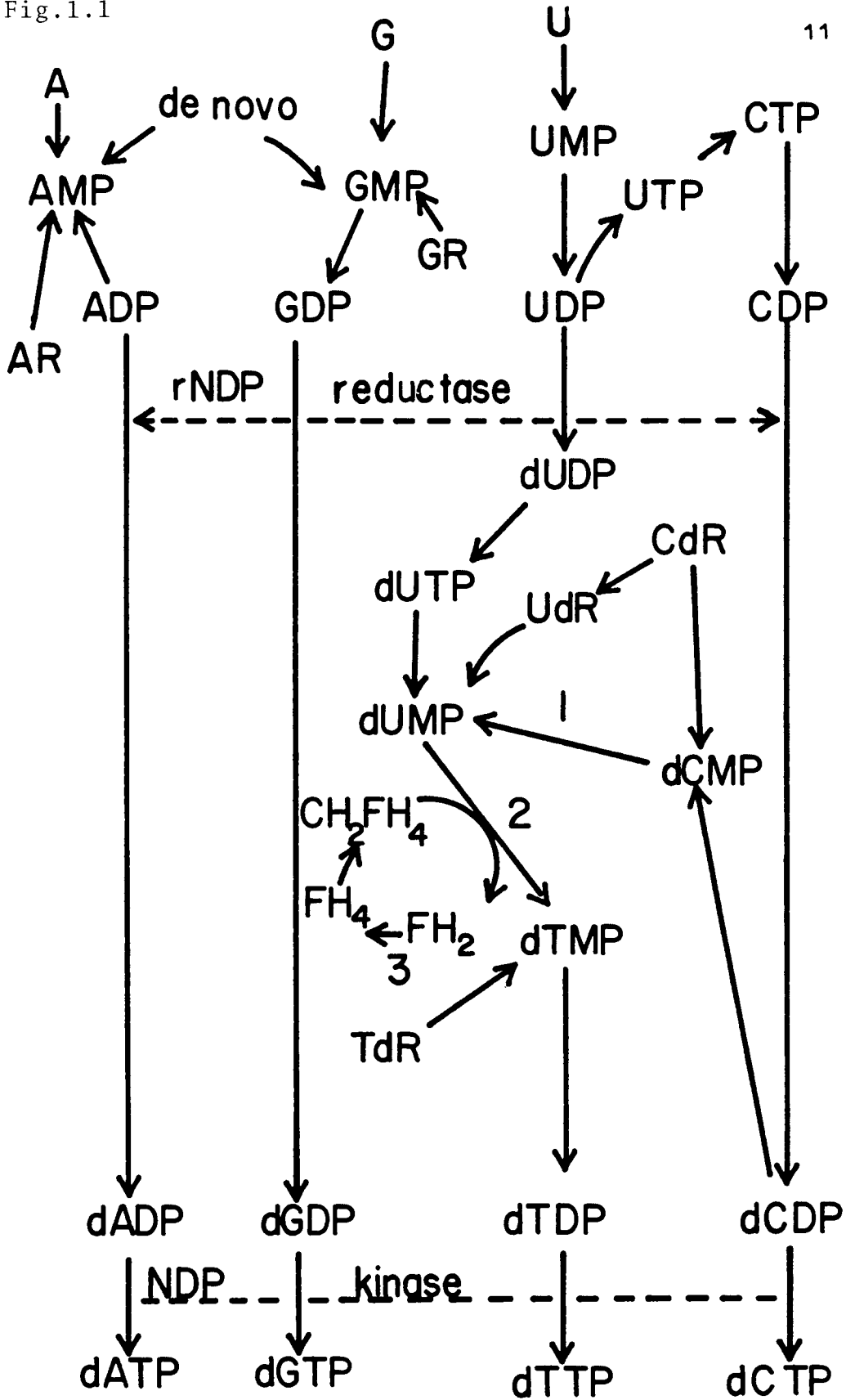
between the rate of DNA replication and the rate of label incorporated into DNA by radiolabeled deoxycytidine-derived dCTP. Apparently a substantial proportion of deoxycytidine-labeled dCTP was unavailable for DNA replication, as though it were in a separate, non-equilibrating pool. In contrast, cytidine-derived dCTP and dTTP labeled by [5-³H] thymidine were completely available for DNA replication. Although the results of these labeling experiments contradict one another insofar as whether the replication-active pool is in equilibrium with the salvage-derived dNTP pool or the de novo-derived dNTP pool, the results overall support the hypothesis that there is compartmentation of DNA precursors in eukaryotic cells. Direct evidence of this compartmentation, however, is lacking.

If there is channeling or compartmentation of DNA precursors in eukaryotes, then one can make several testable predictions. (1) DNA precursor biosynthesis, if coupled to DNA replication, should occur in the nucleus. (2) DNA precursors should have different concentrations in the nucleus and cytoplasm since the separate pools presumably do not equilibrate freely. (3) Newly synthesized dNTPs should equilibrate faster in the compartment in which they are synthesized. The experiments in this thesis were designed to test these predictions

directly and explore the nature of eukaryotic DNA precursor compartmentation.

Fig.1.1. dNTP Biosynthesis Pathways. Abbreviated pathways of DNA precursor biosynthesis in mammalian cells. rNDP reductase is ribonucleotide reductase. NDP kinase is nucleoside diphosphokinase. Enzymes represented by numbers are: 1, dCMP deaminase; 2, thymidylate synthase; and 3, dihydrofolate reductase.

Fig.1.1



Chapter 2

DNA Precursor Pools
and Ribonucleotide Reductase Activity:
Distribution between the Nucleus and Cytoplasm
of Mammalian Cells

by

Janet M. Leeds, Mary B. Slabaugh,
Christopher K. Mathews

Summary

Nuclear and whole-cell deoxynucleoside triphosphate (dNTP) pools were measured in HeLa cells at different densities and throughout the cell cycle of synchronized CHO cells. Nuclei were prepared by brief detergent (Nonidet P-40) treatment of subconfluent monolayers, a procedure that solubilizes plasma membranes but leaves nuclei intact and attached to the plastic substratum. Electron microscopic examination of monolayers treated with Nonidet P-40 revealed protruding nuclei surrounded by cytoskeletal remnants. Control experiments showed that nuclear dNTP pool sizes were stable during the time required for isolation, suggesting that redistribution of nucleotides during the isolation procedure was minimal. Examination of HeLa whole-cell and nuclear dNTP levels revealed that the nuclear proportion of each dNTP was distinct and remained constant as cell density increased. In synchronized CHO cells, all four dNTP whole-cell pools increased during S phase, with the dCTP pool size increasing most dramatically. The nuclear dCTP pool did not increase as much as the whole-cell dCTP pool during S phase, lowering the relative nuclear dCTP pool. Although the whole-cell dNTP pools decreased after 30 h of isoleucine deprivation, nuclear pools did not decrease proportionately. In summary, nuclear dNTP pools in

synchronized CHO cells maintained a relatively constant concentration throughout the cell cycle in the face of larger fluctuations in whole-cell dNTP pools. Ribonucleotide reductase activity was measured in CHO cells throughout the cell cycle, and although there was a 10-fold increase in whole-cell activity during S phase, we detected no reductase in nuclear preparations at any point in the cell cycle.

Introduction

How are DNA precursors distributed within the compartments of eukaryotic cells? This question bears directly upon the extent to which DNA replication in these cells is coordinated with the synthesis of its precursors, the deoxyribonucleoside 5' triphosphates (dNTPs). In prokaryotic systems, notably T-even phage infected bacteria, there is evidence that dNTPs generated at replication sites are preferentially incorporated into replicating DNA (reviewed in Mathews, 1985). This apparently involves the action of complexes of enzymes of dNTP synthesis juxtaposed to the replication apparatus, a process that channels precursors to their sites of utilization.

If eukaryotic cells channel dNTPs to DNA in a similar fashion, then dNTP biosynthesis should occur primarily in the nucleus, and its attendant enzymes should be located

there as well. Indeed, others (Reddy and Pardee, 1980) have reported a nuclear location of several enzymes of dNTP biosynthesis, although contrary observations have also been reported (Engstrom et al., 1984).

We have approached the question by trying to measure pool sizes of DNA precursors in the major compartments of cultured mammalian cells, realizing, of course, that steady-state pool data cannot definitively answer questions of this type but can only point to answers. In previous studies (Bestwick and Mathews, 1982; Bestwick et al., 1982), Bestwick and co-workers showed that mitochondria contain physically and kinetically distinct dNTP pools. Because of the porous nature of the nuclear membrane, conventional subcellular fractionation procedures almost certainly redistribute small molecules between nuclear and cytoplasmic compartments. This problem was partly circumvented by Skoog and Bjursell (1974), who isolated Chinese hamster ovary (CHO) cells by fractionation in nonaqueous solvents. They found an increase in the relative nuclear dNTP pools in cells in S phase as compared with those in cells in G1 phase and concluded that the cell must have a mechanism to concentrate dNTPs against a gradient. Subsequently, Bestwick et al. (1982) also reported a nonrandom pattern of dNTP distribution between nuclear and cytoplasmic compartments in HeLa cells. More recently, Nicander and

Reichard (1983) used labeling kinetics to study dNTP compartmentation in 3T6 mouse fibroblasts. They concluded that the dCTP pool is compartmentalized. The dTTP pool, by contrast, appeared to be a single equilibrated pool.

Although this evidence circumstantially indicates that dNTPs are compartmentalized, we do not know whether distinct dNTP pools actually exist or under what conditions they might equilibrate. In this study, we addressed these questions by use of a technique that allows rapid isolation of nuclei from cultured cells, under conditions where redistribution of nucleotides during isolation is minimized. This technique also allowed determination of the intracellular distribution of the enzyme ribonucleotide reductase.

Materials and Methods

Reagents

Radioactively labeled deoxyribonucleoside triphosphates obtained from ICN Pharmaceuticals, Inc., Irvine, Calif., were [8-³H] dATP (27 Ci/mmol), [methyl-³H] dTTP (50 Ci/mmol), [5-³H] dCTP (30 Ci/mmol), and [8-³H] dGTP (5 Ci/mmol). Tri-N-octylamine was also obtained from ICN Pharmaceuticals. Freon-113 (1,1,2-trichlorotrifluoroethane) was obtained from Matheson Scientific, Inc., Elk Grove Village, Ill. The alternating

copolymers poly (dA-dT) and poly (dI-dC) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Unlabeled nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. Highly purified E. coli DNA polymerase I (3,700 U/mg of protein) was purchased from Worthington Diagnostics, Freehold, N.J. Propidium iodide and Hoechst H33258 were obtained from Calbiochem Behring, La Jolla, Calif. Nonidet P-40 (NP-40) was obtained from Particle Data Laboratories Ltd., Elmhurst, Ill. All other chemicals were reagent grade.

Cell Source and Growth Conditions

The CHO-K1 cells used in these experiments were obtained from Mark Meuth, Imperial Cancer Research Fund, Mill Hill Laboratories, England. HeLa cells were obtained from the American Type Culture Collection, Rockville, Md. Both cell lines were maintained in Eagle minimal essential medium with nonessential amino acids (Irvine Scientific), supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, New York) and penicillin-streptomycin (penicillin base 10,000 U/ml; streptomycin, 10,000 µg/ml, used at 5 ml/liter; GIBCO). Cells were routinely checked for mycoplasma contamination (Chen, 1977).

Isolation of nuclei

Nuclei were isolated essentially as described by Rapaport et al. (1979). The medium in a 100-mm culture dish was removed by aspiration, followed by addition of 2.5 ml of NP-40 buffer (1% NP-40, 30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 200 mM sucrose, 40 mM NaCl, 5 mM MgCl₂, 5 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetracetic acid]; pH 8.0). The buffer was removed by aspiration after 10 s, and the remaining cell contents were washed in 5.0 ml of ice-cold phosphate-buffered saline (PBS: 0.11 M NaCl, 2.0 mM KCl, 2.5 mM Na₂HPO₄, 1.2 mM KH₂PO₄; pH 7.2). The PBS was aspirated after about 5 s of washing, and the nuclei remaining on the plate were extracted for analyses of dNTP content.

dNTP analysis

Nucleotide extraction and analysis were done by methods developed in this laboratory (North et al., 1980), which in turn represent modifications of procedures in widespread use. Both whole cells and nuclei were extracted first with 60% aqueous methanol. The contents of three 100-mm culture dishes were pooled for whole-cell assays, and the contents of four dishes were pooled for nuclear assays. Each methanol extract was taken to dryness, and the contents were reextracted with 0.5 N

trichloroacetic acid, followed by neutralization with tri-N-octylamine, as described by Garret and Santi (1979). We found this second extraction with trichloroacetic acid to give less interference with the enzymatic dNTP assay than did the perchloric acid extraction described in our previous study (North et al., 1980).

Each extract was assayed for content of each of the four dNTPs by the DNA polymerase-based enzymatic assay (North et al., 1980), which measures incorporation of a limiting dNTP into an alternating copolymer template [poly (dA-dT) or poly (dI-dC)] by Escherichia coli DNA polymerase I in the presence of an excess of the labeled complementary dNTP. Each reaction mixture contained, in a total volume of 100 μ l, 45 mM Tris hydrochloride (pH 8.3), 4.5 mM $MgCl_2$, 0.9 mM 2-mercaptoethanol, 10 μ M 3H -labeled complementary dNTP (10 μ Ci/ml), 0.1 U of E. coli DNA polymerase I, 20 μ g of bovine serum albumin, and an aliquot of extract containing between 1 and 40 pmol of the unknown dNTP. After 60, 75, and 90 min of incubation (37°C), 25- μ l aliquots were spotted onto Whatman no. 3 filter paper disks. The disks were dried, washed four times with 5% trichloroacetic acid-1% sodium pyrophosphate and twice with 95% ethanol, and then dried and counted.

Cell cycle analysis

Nuclei were prepared and stained with propidium iodide, and the green fluorescence distribution was examined in an Epics V cell sorter (Coulter Electronics, Inc., Hialeah, Fla.) (Thorntwaite et al., 1981).

Cell synchronization

CHO-K1 cells were synchronized by isoleucine deprivation (Tobey, 1973). The medium was aspirated from exponentially growing cells, and each 100-mm plate was washed twice with 10 ml of PBS. Minimal essential medium lacking isoleucine and supplemented with 5% dialyzed fetal bovine serum was added to each plate. Cells were kept in this medium for 30 h, at which time isoleucine-deficient medium was removed, and fresh complete medium with 5% fetal bovine serum was added.

DNA measurements

DNA was measured by the method of Erwin et al. (1981).

Enzyme assays

Ribonucleotide reductase was assayed as described by Slabaugh et al. (1984). Lactate dehydrogenase was assayed as described by Schwert and Winer (1959). The assay of citrate synthase was performed by the method of Srere (1969).

Electron microscopy

For examination of whole cells and NP-40-prepared nuclei, CHO-K1 cells were grown on plastic disks on tissue culture plates. Nuclei were isolated as routinely done for nucleotide extraction. Samples were washed with PBS and fixed in 2.5% glutaraldehyde in 0.1 M NaH_2PO_4 (pH 7.4) for approximately 15 min at room temperature. The fixative was aspirated, and the samples were dehydrated with graded ethanol. Samples were dried to the critical point and shadowed with platinum-palladium (60:40).

Results

Evaluation of the nuclear isolation procedure

The nuclear isolation procedure used in these experiments was both rapid and quantitative. Briefly, the procedure involved aspiration of medium, 10 s of swirling the NP-40-containing buffer on the cells, aspiration of the NP-40-containing buffer, a 5 s PBS wash of the resulting nuclei on the plate, aspiration of the PBS, and addition of 60% methanol for extraction of nucleotides. The entire procedure, which required less than 30 s to complete, took place with the tissue culture plate on ice and all solutions at 4°C. To determine nuclear recovery, we measured DNA remaining on plates after treatment with NP-40-containing buffer from times varying from 10 to 60 s. Even after 60 s of exposure to the nuclear isolation buffer, all of the DNA remained on the plate, indicating quantitative nuclear recovery. We examined the stability of dNTP pools within CHO nuclei isolated by this method by varying the length of treatment with the NP-40-containing nuclear isolation buffer. Pools extracted after 10, 20, or 30 s of treatment with the nuclear isolation buffer were similar to each other, but these nuclear pools differed substantially from whole-cell pools (Fig. 2.1). In a similar experiment with HeLa cells, we found that

lengthening the PBS wash after nuclear isolation to 15 and 30 s did not reduce the size of the dNTP pools isolated (Fig. 2.2). While we cannot rule out the possibility of some dNTP leakage from nuclei within the first 10 s of NP-40 treatment, we consider this unlikely for reasons presented in the Discussion section. Therefore, our experiments suggest that the rapidly isolated nuclei have dNTP pools that are stable during the time needed for their isolation and extraction.

Examination of isolated CHO nuclei under the scanning electron microscope (Fig. 2.3) shows protruding nuclei surrounded and covered by cytoskeletal remnants that reveal the outline of the original cell. The extent of cytoplasmic contamination of NP-40-prepared nuclei was assessed by assay of lactate dehydrogenase, a soluble cytoplasmic enzyme. Less than 5% of the whole-cell activity was found associated with the nuclei in either HeLa cells or CHO-K1 cells. Twenty-five to 30% of the whole-cell level of citrate synthase, a mitochondrial matrix enzyme, remained attached to the plates after NP-40 treatment, indicating that nuclei were contaminated with mitochondria. However, mitochondrial dNTP pools are small enough (Bestwick et al., 1982) that this should not significantly affect measured nuclear pools.

HeLa whole-cell and nuclear dNTP pool size versus cell density

HeLa whole-cell and nuclear dNTP concentrations decreased as the cell density increased (Fig. 2.4). Cell cycle analysis (data not shown) revealed a similar decrease in the percentage of cells in S phase. Interestingly, changes in nuclear dNTP pool levels were similar to changes in the corresponding whole-cell dNTP pool levels; the percentage of each dNTP within the nucleus remained nearly constant between densities of 1.25×10^6 and 5.5×10^6 cells per plate. The fraction of each dNTP found in the nucleus, however, differed among the four dNTPs. For example, at all densities examined, about 20% of the dTTP and 40% of the dATP were found in the nucleus. The nuclear concentrations of dTTP and dATP were equal, however, even though the whole-cell dTTP content was 50% greater than that of dATP.

CHO cell synchronization

A representative cell cycle analysis of synchronized CHO cells is shown in Fig. 2.5. After 30 h of isoleucine starvation, approximately 90% of the cells were blocked in the G1/G0 phase of the cell cycle. Six hours after release of this block the cells were still in G1 phase. At 10 to 12 h postrelease, the cells were distributed as a single broad peak in S phase. Between 15 and 18 h after

the addition of complete medium, the cells were in the G2/M phase of the cell cycle. Nuclear dNTP values measured at this point may be underestimates of true values, as cells undergoing mitosis would have no nuclear membrane. Eighteen to 21 h after release of the isoleucine block, the cells had divided and were back in G1 phase.

dNTP pools from synchronized CHO whole cells and nuclei

We prepared nuclear and whole-cell extracts for analysis of the dNTP pools from times throughout the synchronization procedure. The results shown in Fig. 2.6 represent the average dNTP values determined in three synchronization experiments. The most striking change is the ninefold increase in the dCTP whole-cell pool between the G1- and S-phase times. This large increase is probably due to the absence of dCMP deaminase in these CHO cells as has been observed before (Skoog et al., 1973; Horder and Henderson, 1982).

The dCTP and dGTP whole-cell pools did not change for the first 6 h after the addition of complete medium to the isoleucine-starved-cells. This indicates that the dCTP and dGTP whole-cell pool levels of isoleucine-starved and G1-phase cells are similar. The dATP and dTTP whole-cell pool levels, however, increased slightly during the first 6 h after release of the isoleucine block, even though the

cells remained in G1 phase. The dATP and dTTP whole-cell pools increased only slightly from 6 to 10 h postrelease and decreased slightly at 12 h. At 15 h postrelease, the whole-cell dATP and dTTP pools rose slightly, but they decreased by 18 h, at the conclusion of S phase.

The nuclear dNTP pool levels fluctuated less than the whole-cell dNTP pools throughout the cell cycle. After 30 h of isoleucine starvation, the nuclear dNTP pools remained essentially equivalent to those measured before the isoleucine block, whereas the whole-cell pools decreased, thereby increasing the sizes of the relative nuclear pools. (Note the difference in scales for nuclear and whole-cell dNTP pool levels.) At 6 h postrelease, the relative nuclear pools were close to the values one would expect if the dNTPs were distributed proportionally to the volumes of the compartments. The relative nuclear dNTP pools in G1 phase were as follows: dATP, 30%; dTTP, 30%; dCTP, 45%, and dGTP, 40%. Skoog and Bjursell (1974) estimated that the nucleus represents 30% of the total cellular volume in G1 phase and 25% in S phase. During S phase, the nuclear dGTP, dATP, and dTTP pool sizes remained at nearly the same relative distributions between nucleus and cytoplasm, while the dCTP relative nuclear pool size decreased from 45% in G1 phase to 9% in S phase. Possible reasons for this unequal distribution will be discussed.

Ribonucleotide reductase

To investigate the subcellular location of this enzyme, we harvested whole cells and nuclei from synchronized CHO cells. After being treated with NP-40 and washed with PBS, nuclei were scraped into additional PBS and collected by low-speed centrifugation. Nuclei and whole cells were suspended in buffer and sonicated. Our measurements (Fig. 2.7) were similar to results reported by others (Cheng et al., 1977; Nordenskjold et al., 1970) in that we observed a 10-fold increase in ribonucleotide reductase activity in the extracts of S-phase cells when compared with the whole-cell extracts of G1-phase cells. At no point in the cell cycle did we find ribonucleotide reductase activity associated with rapidly isolated nuclei. The absence of reductase activity is not due to the presence of an inhibitor in nuclear extracts, because addition of nuclear extracts to the whole-cell extracts showed no inhibition of whole-cell activity. Another possibility, that the NP-40 in the nuclear isolation buffer irreversibly inactivated the enzyme, is extremely unlikely. Whole-cell extracts assayed in the presence of 1% NP-40 (the extraction concentration) gave 40% of the control activity. At a level representing that probably present after the PBS wash, namely, 0.01% NP-40, the activity was 75% of the control level. These analyses of enzyme activity complement and extend the observations of

Engstrom et al. (1984), who localized the M1 subunit to the cytoplasm by an immunocytochemical method.

Discussion

In this study, our aim was to examine dNTP compartmentation in mammalian cells. In particular, we wanted to determine the degree to which dNTP pools in the cytoplasm and nucleus exist as distinct entities. The nuclear isolation method used in these studies was both rapid and reliable, in that the dNTP pools thus isolated were relatively stable during the isolation procedure. While we cannot eliminate the possibility that extensive redistribution of dNTP pools occurs during the 10-s NP-40 treatment (i.e. leakage from the nuclei), we consider this unlikely for several reasons. (i) Random leakage of nucleoside triphosphates should yield symmetry in nuclear-to-whole-cell pool ratios, since all triphosphates should leak at comparable rates. However, as noted earlier, large asymmetries were observed; in HeLa cells, the relative nuclear dATP content was twice that of dTTP, while in CHO cells, the ratio of nuclear to whole-cell dCTP varied by fivefold during the cell cycle. (ii) The nuclear-to-whole-cell pool ratios that we observed for dNTPs were comparable to those we observed for ribonucleoside triphosphates, molecules that are undoubtedly abundant in the cytoplasm of intact cells

(data not shown). (iii) We note that the NP-40 nuclear isolation technique is capable of detecting concentration gradients where they exist. Rapaport (1980) described conditions under which nuclear ATP pools nearly doubled with respect to control values. (iv) The stability of our measured dNTP pools with respect to time of detergent treatment and washing means that if dNTP leakage is substantial within the treatment period, then there must be two distinct pools of each dNTP within the nucleus: a loosely bound pool (depleted within 10 s) and a tightly bound pool (stable for at least 30 s). While this is not impossible, Occam's razor plus the cytoplasmic localization of ribonucleotide reductase argue that most of the dNTP content lost during the NP-40 treatment does indeed represent cytoplasmic pools.

Another potential artifact was eliminated by scanning electron microscopy. Examination of a large number of nuclei showed no evidence of whole cells remaining unperturbed on the plates after treatment with the NP-40-containing nuclear isolation buffer, indicating (along with the marker enzyme data) that the pools isolated did not represent whole-cell contamination of nuclear preparations.

The results from our experiments have given us information not only on the relative distribution of dNTPs

within the cell but also the nuclear concentrations of these metabolites. Estimation of the nuclear dNTP concentrations in synchronized CHO cells showed that there is an adequate supply of dNTPs to support DNA replication. By using the values determined by Skoog and Bjursell (1974) for the volume of CHO cell nuclei during S phase, we calculate that the nuclear dNTP concentrations during S phase are the following: dCTP, 213 μM ; dATP, 104 μM ; dTTP, 88 μM ; and dGTP, 10 μM . The K_m values for dNTPs for DNA polymerase α are approximately 10 μM each (Fisher et al., 1979). Three of the four dNTPs have concentrations well above the K_m , while the fourth, dGTP, has a nuclear concentration quite close to the K_m . It is possible, then, that dGTP plays a role in regulating DNA replication through substrate limitation.

The distinctive distribution of the four dNTPs between nucleus and cytoplasm in HeLa cells, in addition to the relatively small CHO cell S-phase nuclear dCTP pool expansion compared with the corresponding whole-cell expansion, indicates that dNTPs are present at different concentrations in the two cellular compartments. However, the fact that HeLa nuclear dNTP values remained a constant proportion of the whole-cell levels at several different densities and the fact that three of the four dNTPs in synchronized CHO cells were distributed according to volume indicate that some mixing occurs between the two

compartments. From our results, we hypothesize that compartmentation of dNTPs between nucleus and cytoplasm may be the result of equilibrium concentrations established by the interactions of enzymes that are differentially distributed between the two compartments. Our model is that dNTPs are freely permeable between the two compartments but that some of the dNTP-metabolizing enzymes are located exclusively in one compartment or the other. For example, ribonucleotide reductase seems clearly now to be a cytoplasmic enzyme, whereas DNA polymerase α is located exclusively in the nucleus (Bensch et al., 1982). Other dNTP-metabolizing enzymes have not yet been clearly assigned to one compartment or the other, but work in this direction continues. Our hypothesis could explain why the dNTP concentrations are different between the two compartments, even though the dNTPs should move freely through nuclear pores. The high increase in cellular dCTP concentration during S phase, which may well result from the absence of dCMP deaminase in these cells, is not seen in the corresponding S-phase nuclear dCTP pool. The difficulty of synchronizing other cell lines by methods that do not perturb dNTP pools has kept us from extending these results. In any case, the primary site for dNTP synthesis is the cytoplasm, since that is where rNDP reductase is located. Therefore, the substrate channeling model for mammalian DNA replication (Reddy and

Pardee, 1980; Reddy and Pardee, 1982) requires modification.

In our CHO-K1 cell line, what prevents a large nuclear accumulation of dCTP? One might speculate upon the possible existence of nucleotide-catabolizing activities in the nucleus, which prevents a large imbalance of dNTPs within the nucleus. Newman and Miller (1985) have proposed that in some CHO cell lines, a dCTPase, a dCMP deaminase, and cytidine kinase are transiently induced in response to UV irradiation. Although enzyme assay data were not presented, it is intriguing to consider that a dCTPase-like activity could be stimulated by or responsive to a very large dCTP pool. Jackson (1978) and, more recently, Nicander and Reichard (1985) have found that a large proportion of the dTTP in mammalian cells is derived from CDP via dCMP deaminase. Therefore, some mechanism for dephosphorylation of dCDP must exist. Experiments to search for this type of activity are currently under way. It is unlikely that dUTPase cleaves dCTP, as all previous work with mammalian dUTPase indicates a stringent specificity (Caradonna et al., 1984).

Although others have seen large increases in dNTP pools during S phase (McCormick et al., 1981; Nordenskjold et al., 1970), the accumulations we noted, with the

exception of that of dCTP, largely mirror the increase in cell size. Although during S phase, the dNTPs are produced at greater rates than in G1 phase, their turnover rates for DNA replication increase simultaneously. In most metabolic pathways, one would not predict an accumulation of metabolites, and in fact, the concentrations of most metabolites are tightly regulated. Recent work by Nicander and Reichard (1985) shows that a substantial quantity of deoxyribonucleotides, once synthesized, are broken down and excreted by the cell. Therefore, actual dNTP concentrations may be regulated closely, and transient overproduction of dNTPs could trigger their degradation and subsequent excretion from the cell.

In summary, compartmentation of the dNTPs may be a kinetic phenomenon involving metabolite turnover rather than an active concentration of dNTPs into the nucleus. In future experiments, the emphasis will be on examining actual levels and rates of equilibration of dNTPs between the nucleus and cytoplasm.

Acknowledgements

We thank Joel Willard for running the cell sorter for cell cycle analysis and Christine Weiss for technical assistance with the electron microscopy.

This work was supported by Public Health Service training grant no. T32-GM 07774 from the National Institutes of Health and National Science Foundation research grant no. PCM-8 301748. Flow cytometric analysis was supported by Public Health Service grant no. ES00210 from the National Institute of Environmental and Health Sciences.

Fig. 2.1. Nuclear dNTP Pools vs. Time in NP-40. CHO cell nuclear dNTP pools versus time of treatment with NP-40-containing nuclear isolation buffer. Cells were treated with the NP-40-containing buffer for 10, 20, or 30 s, and the nuclear dNTPs were then extracted and quantitated.

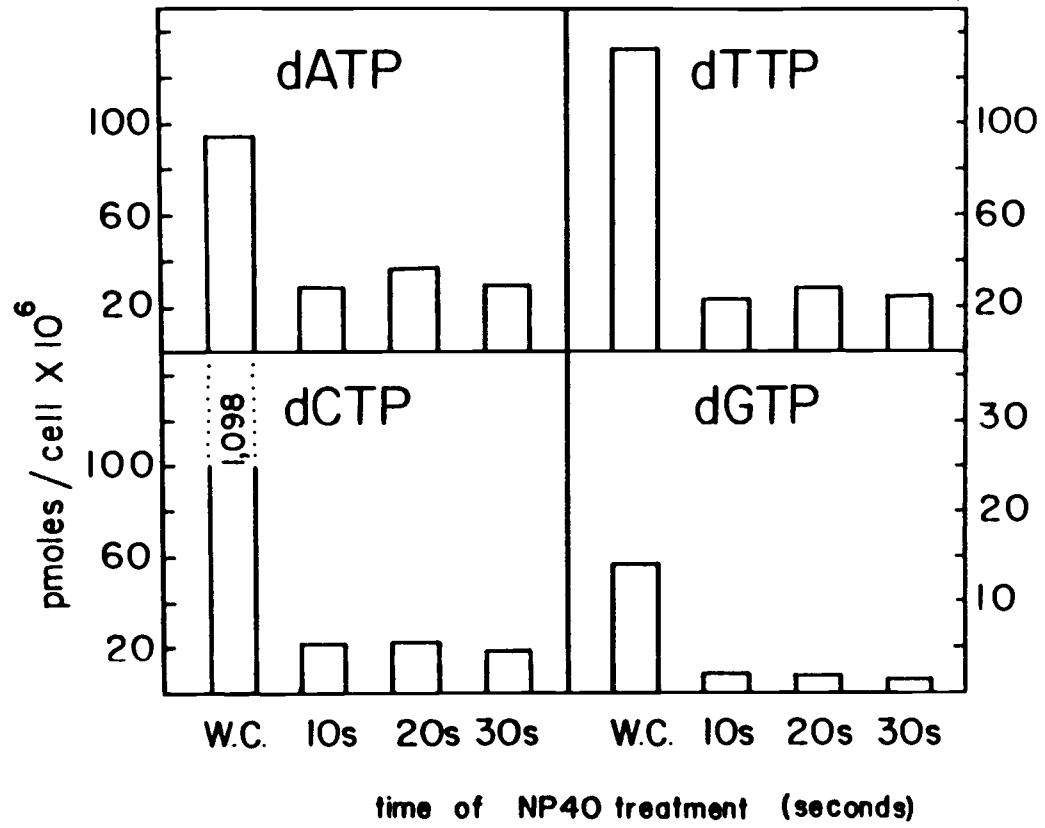


Fig. 2.1

Fig. 2.2. Nuclear dNTP Pools vs. Time of PBS Wash. HeLa cell nuclear dNTP pools versus length of PBS wash. After 10 s of NP-40 treatment, isolated HeLa nuclei were washed with ice-cold PBS for 15 or 30 s. The dNTP pools subsequently extracted and quantitated were compared with those from the nuclei washed for 5 s (the usual length of the PBS wash) and whole-cell dNTP pools.

Fig. 2.2

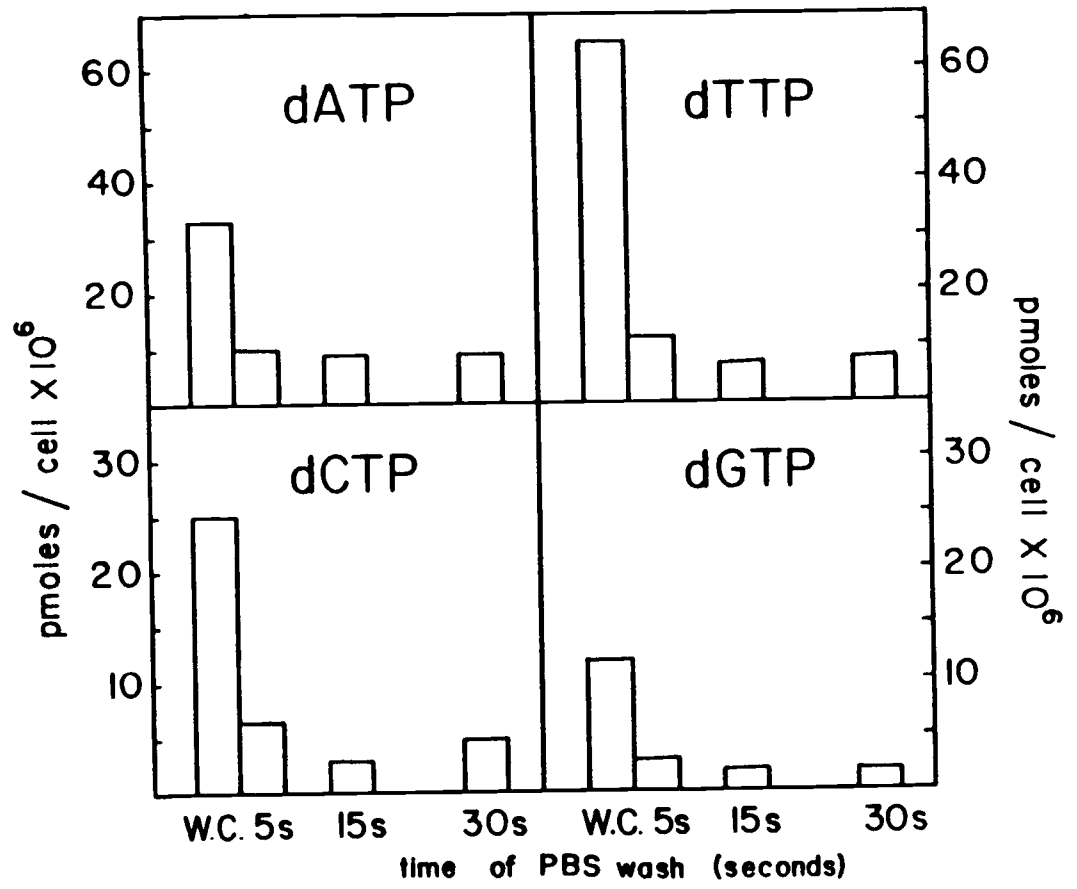


Fig. 2.3. Electron Micrograph of Whole Cell and Nucleus. Scanning electron micrograph of CHO whole cells and rapidly isolated nuclei (prepared as described in Materials and Methods). (A) Whole cell at X 6,000 magnification. (B) Nuclei at X 6,000 magnification. Bar length is 1 μ M.

Fig.2.3

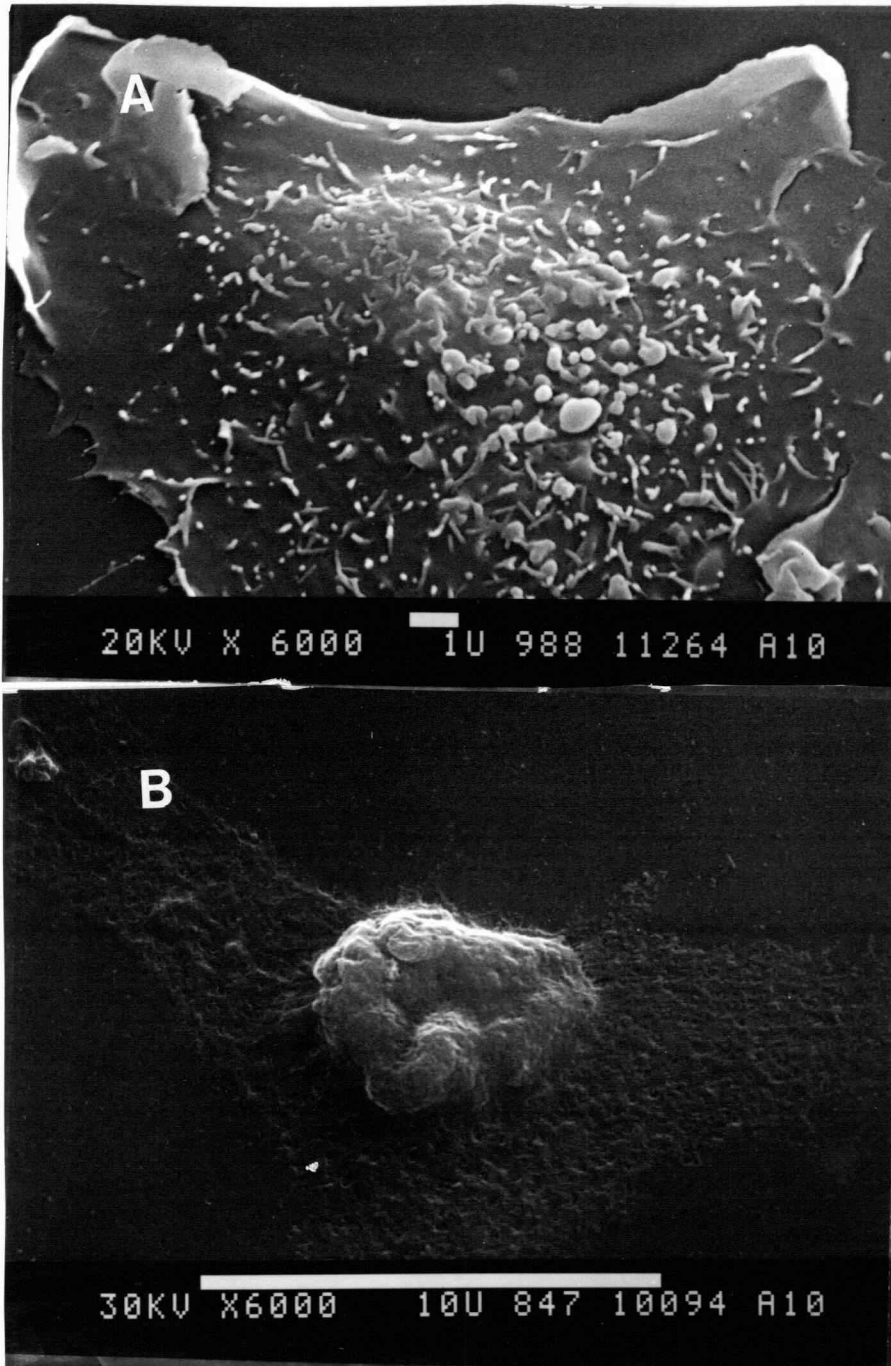


Fig. 2.4. HeLa Whole-Cell and Nuclear dNTP Pools. HeLa whole-cell (●) and nuclear (○) dNTP pools versus cell density. Pools were measured from 100-mm plates of densities between 1.25×10^6 and 5.5×10^6 cells per plate. Results are composites from three separate experiments.

Fig.2.4

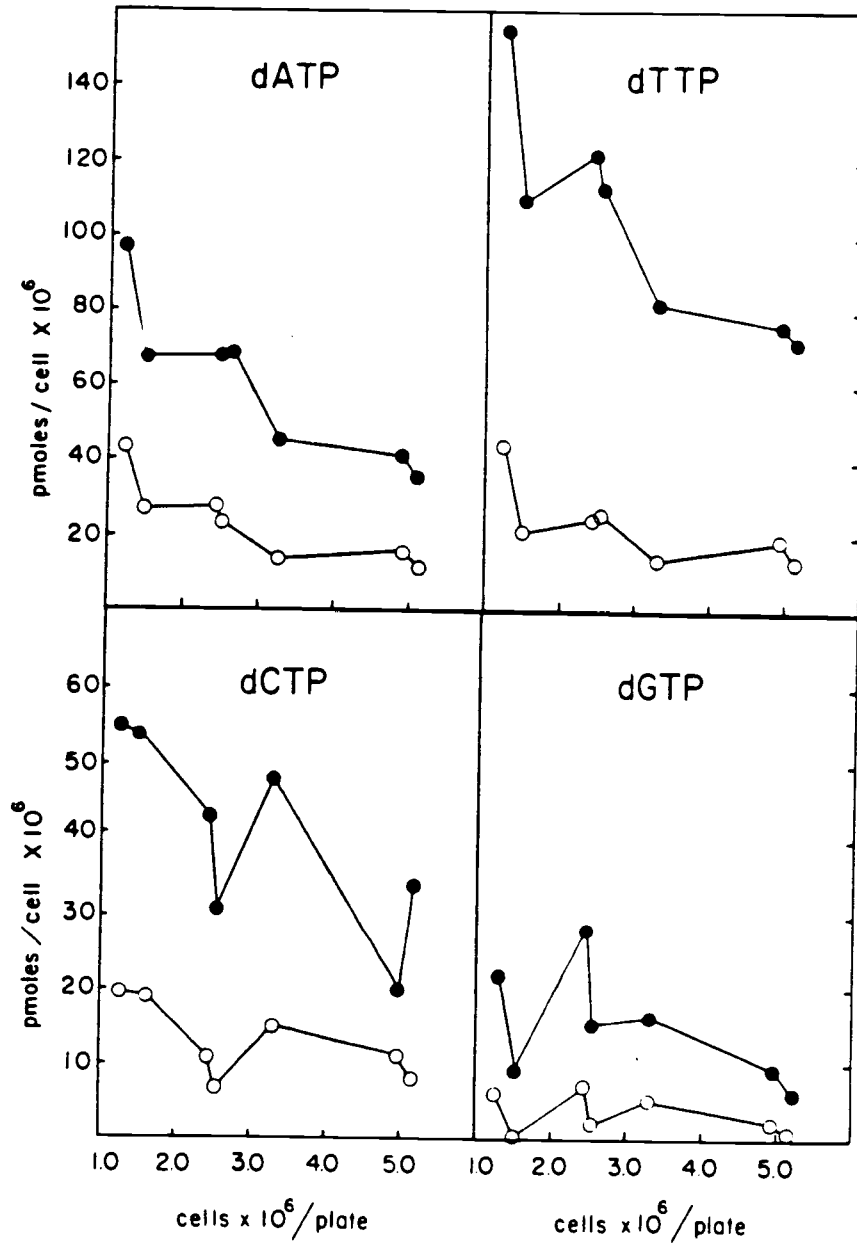


Fig. 2.5. Cell Cycle Analysis. Cell cycle analysis of synchronized CHO cells. Nuclei from synchronized CHO-K1 cells were stained with propidium iodide and examined for DNA content. (A) Cell cycle distribution after 30 h of isoleucine deprivation. (B) At 12 h after release of isoleucine block, cells are in S phase. (C) At 15 h postrelease, cells are dividing. (D) At 21 h after release, cells are back in G1 phase. In each case, 15,000 cells were counted.

Fig. 2.5

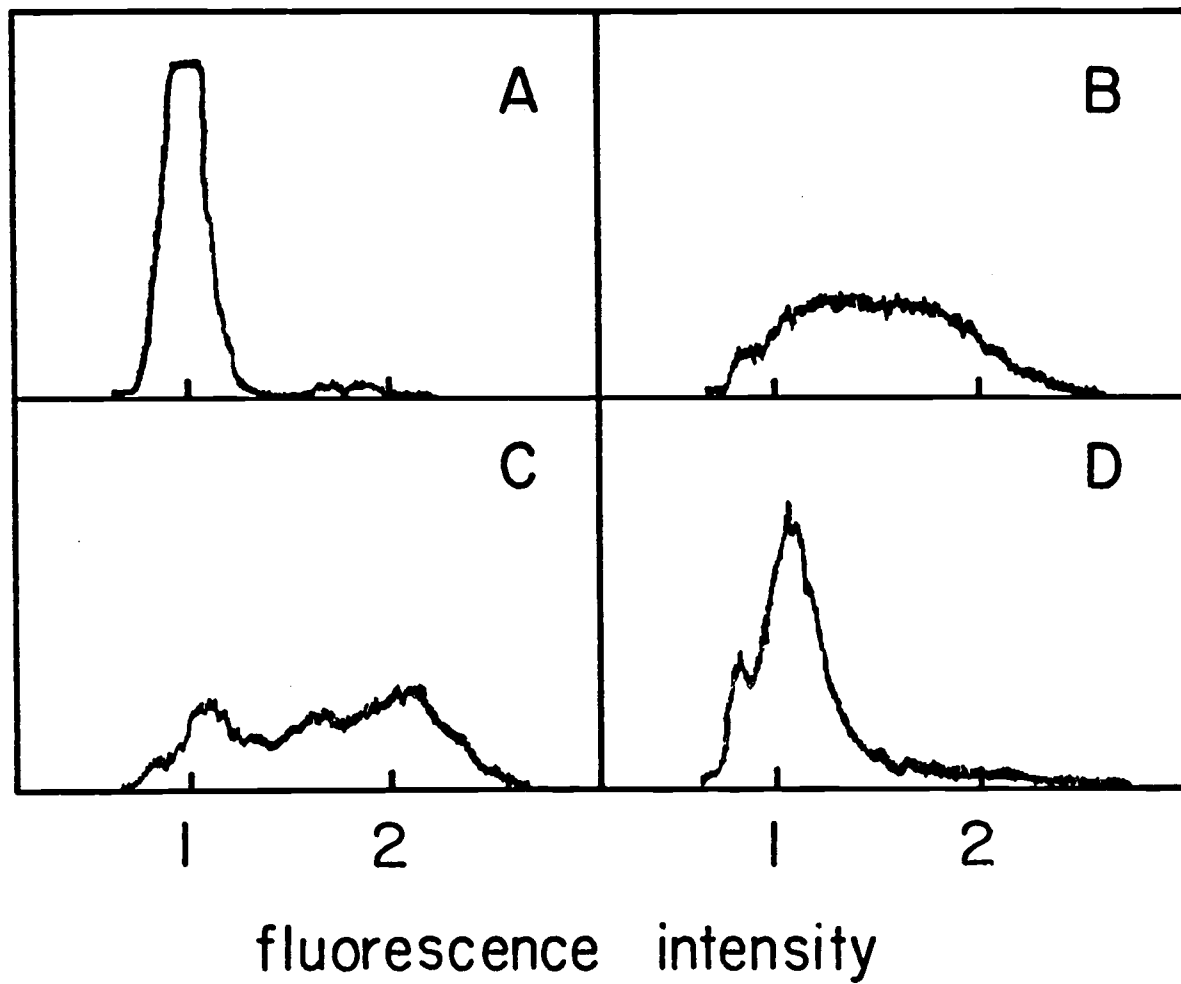
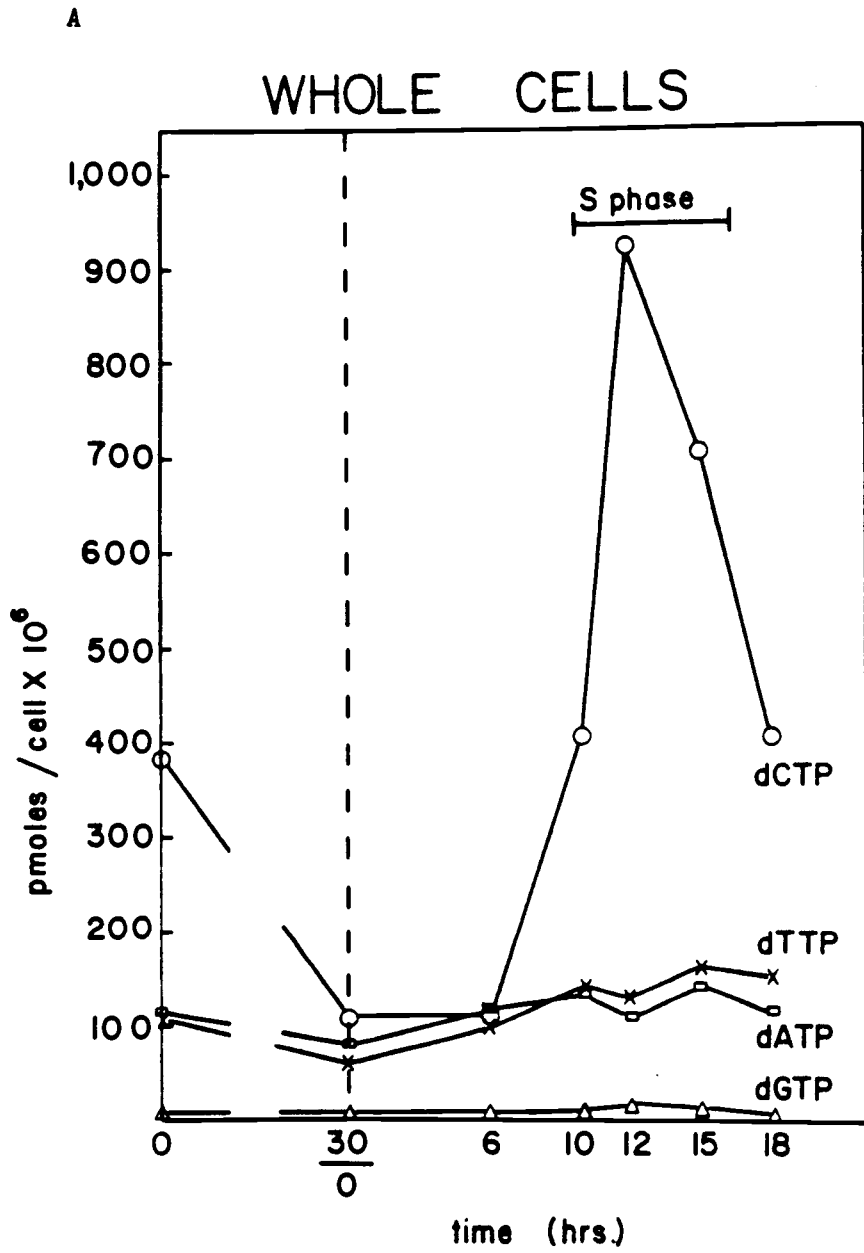


Fig. 2.6. CHO Whole-Cell and Nuclear dNTP Pools. Whole-cell (A) and nuclear (B) dNTP pools from synchronized CHO cells. Results shown here represent averages from three separate synchronizations.

Fig.2.6A



B

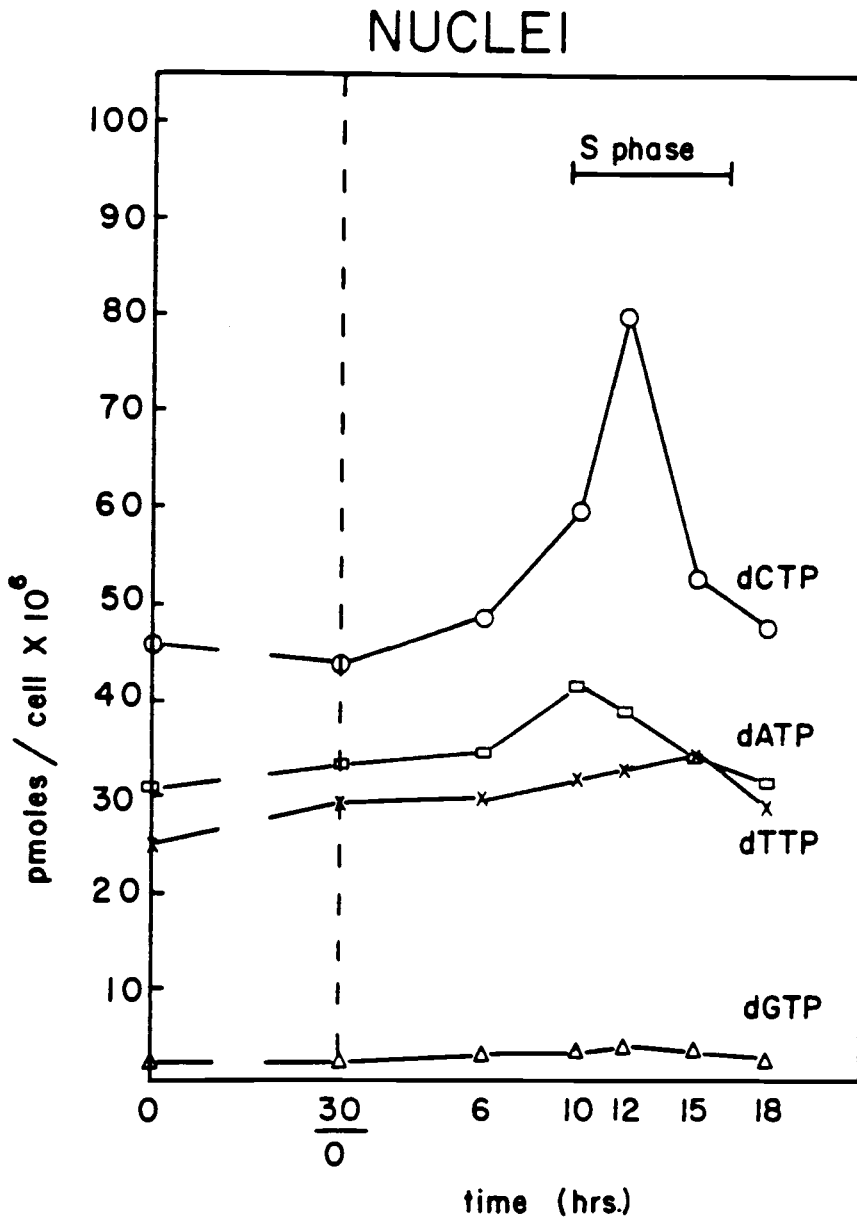
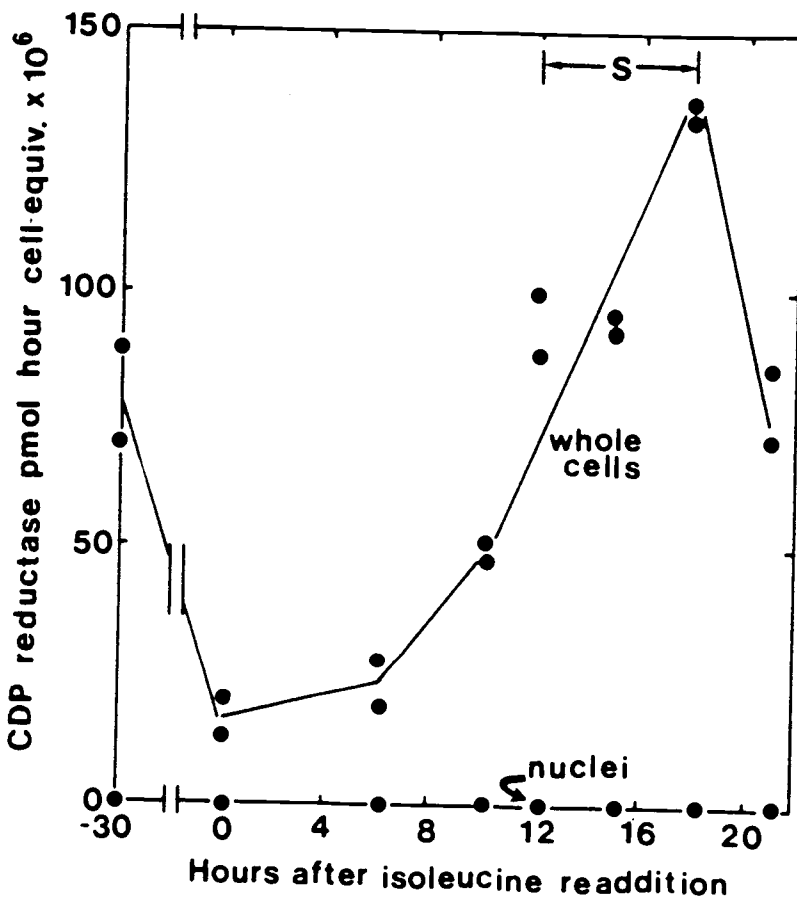


Fig. 2.7. Subcellular Location of Ribonucleotide Reductase Activity in CHO Cells. Whole-cell and nuclear levels of CDP reductase activity throughout the cell cycle of CHO cells.

Fig.2.7



Chapter 3

Cell Cycle-dependent Effects on Deoxyribonucleotide
and DNA labeling by Nucleoside Precursors in
Mammalian Cells

by

Janet M. Leeds and Christopher K. Mathews

Summary

We have compared the rates of dCTP equilibration between the nucleus and cytoplasm of synchronized CHO-K1 cells by measuring the specific activities of nuclear and whole-cell dCTP pools during the course of equilibration with [5-³H] cytidine or [5-³H] deoxycytidine. In both G1 and S phases whole-cell and nuclear specific activities were equal throughout the equilibration with either labeled nucleoside, indicating the existence of a single, rapidly equilibrated dCTP pool, equally accessible to salvage and de novo-derived dCTP. [5-³H] Cytidine-derived dCTP specific activities were nearly equivalent during G1 and S phase while [5-³H] deoxycytidine-derived dCTP specific activity was 13 times greater in G1 than in S phase cells. The rate of [5-³H] thymidine equilibration with the dTTP pool was much slower in G1 than in S phase. The rate of [5-³H] thymidine equilibration with the dTTP pool in exponentially growing cells was a combination of the rapid equilibration seen in S phase followed by a slower equilibration, similar to that seen in G1 phase. The incorporation of [5-³H] thymidine into DNA became linear at the same time in S phase and exponentially growing cells.

Introduction

Labeling studies provided the first evidence that DNA precursors, the deoxyribonucleoside triphosphates (dNTPs),

are compartmentalized, or located in separate and non-equilibrating pools in mammalian cells. Using ^3H -thymidine to label exponentially growing lymphoblasts (CCRF-CEM) Fridland (1973) found that the rate of ^3H -TdR incorporation into DNA became linear much faster than the dTTP pool reached a constant specific activity. Fridland concluded that there must be at least two non-equilibrating nucleotide pools in mammalian cells, not equally used for DNA replication.

More recent labeling studies have used radioactive nucleosides to measure the in vivo flux of metabolites through nucleoside synthesizing enzymes (Maybaum et al., 1981; Horder and Henderson, 1982; and Nicander and Reichard, 1983). Nicander and Reichard's results (1983) indicated that dNTPs, once synthesized, are not equally available for DNA replication. Their observation that deoxycytidine-derived dCTP was unavailable or not used for DNA replication was of particular interest to us because we had previously observed (Leeds et al., 1985) that only the dCTP pool was not randomly distributed between the nucleus and cytoplasm of CHO cells. To better understand this apparent compartmentation we examined the rate and level of [5- ^3H] cytidine and [5- ^3H] deoxycytidine equilibration with the nuclear and whole-cell dCTP pool in

G1 and S phase cells. We predicted, that if deoxycytidine-labeled dCTP was inaccessible or unavailable for DNA replication, then it would be excluded from the nucleus. Since cytidine-labeled dCTP was available for DNA replication, then we predicted that it would be in the nucleus as well as the cytoplasm. The results of those experiments led us to examine the rate of [5-³H] thymidine equilibration with the dTTP whole cell pool in G1, S phase, and exponentially growing cells.

Experimental Procedures

Materials and Reagents

Radioactively labeled deoxyribonucleoside triphosphates obtained from ICN Pharmaceuticals were: [8-³H] dATP (27 Ci/mmol), [methyl-³H] dTTP (50 Ci/mmol), [5-³H] dCTP (30 Ci/mmol), and [8-³H] dGTP (5 Ci/mmol). [5-³H] Deoxycytidine (22-24 Ci/mmol), [5-³H] cytidine (22-26 Ci/mmol), [5-³H] thymidine (80 Ci/mmol), [α -³²P] dATP (600 Ci/mmol), and [α -³²P] dGTP (600 Ci/mmol) were obtained from NEN, a DuPont Co. Tri-N-Octylamine was also obtained from ICN Pharmaceuticals. Freon-113 (1,1,2-trichlorotrifluoroethane) was obtained from Matheson Scientific, Inc., Elk Grove, Ill. The alternating copolymers poly (dA-dT) and poly (dI-dC) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Unlabeled nucleotides were obtained from Sigma Chemical Co., St.

Louis, Mo. Highly purified E. coli DNA polymerase I (3,700 U/mg of protein) was obtained from Worthington Diagnostics, Freehold, N.J. Propidium iodide and Hoechst H33258 were obtained from Calbiochem Behring, La Jolla, Calif. Nonidet P-40 (NP-40) was obtained from Particle Data Laboratories Ltd., Elmhurst, Ill. All other chemicals were of reagent grade.

Cell Source and Growth Conditions

The CHO-K1 cells used in these experiments were provided by Mark Meuth, Imperial Cancer Research Fund, Mill Hill Laboratories, England. The cells were maintained in Eagle minimal essential medium with nonessential amino acids (Irvine Scientific), supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, NY) and penicillin-streptomycin (penicillin base, 10,000U/ml; streptomycin, 10,000 µg/ml, used at 5 ml/liter, GIBCO). Cells were routinely checked for mycoplasma contamination (Chen, 1977).

Nuclear Isolation Procedure

Nuclei were rapidly isolated from monolayer cell culture exactly as described previously (Leeds et al., 1985). Briefly, each 100 mm plate was placed on ice, the medium was aspirated, and 2.5 ml of 1% NP-40 in buffer (30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic

acid], 200 mM sucrose, 40 mM NaCl, 5 mM EGTA [ethylene glycol-bis(β -aminoethyl ether-N,N, N',N'-tetracetic acid); pH 8.0) was added. After ten seconds of rocking the plate in the presence of the buffer, the buffer was aspirated and the plate washed for approximately five seconds in 5 ml of phosphate-buffered saline (PBS) solution. The nuclei remaining on the plates after this treatment were extracted for determination of the dNTP specific activity.

Cell Synchronization

CHO-K1 cells were synchronized using isoleucine deprivation (Tobey, 1973) as previously described (Leeds et al., 1985).

Cell Labeling Conditions

Cells were labeled essentially as described by Nicander and Reichard (1983).

A. Cytidine- and Deoxycytidine-labeled cells

Two hours before the addition of labeled cytidine or deoxycytidine the medium was reduced to 1.5 ml/plate and 0.1 ml 0.1 M HEPES, pH 7.4 was added to each plate. To begin labeling the plates were removed from the incubator, several at a time to minimize perturbations due to condition changes, and label was added directly, without dilution, to a final concentration of 0.3 μ M, and a

specific activity of between 22 and 26 Ci/mmol. Each plate was swirled and returned to the incubator. Plates were rocked several times during incubation with label to insure coverage of all cells with the incubation solution. At appropriate time points plates were removed from the incubator and whole cells and nuclei were extracted for analysis of dCTP specific activities.

B. Thymidine Labeling

Two hours before the addition of labeled thymidine the medium on each plate was reduced to 5.0 ml. To add label the plates were removed from the incubator and diluted label was added to each group of plates to a final concentration of 1 μ M and a specific activity of 2 Ci/mmol. The exact time of label addition was noted and the plates quickly rocked and replaced in the incubator. At each time point two 100-mm plates were removed from the incubator, the medium aspirated, and the cells extracted for dTTP specific activity.

Acid-Soluble Pool Measurements

The time required for acid-soluble pool equilibration with labeled exogenous nucleoside was determined by doing the experiment as outlined below. The appropriate 3 H-nucleoside, diluted with 0.1 M HEPES, pH 7.4, was added to exponentially cells on 60 mm plates where the medium

volume had been reduced two hours previously. Plates were removed at the appropriate time, the labeled medium aspirated, each plate washed with PBS twice and 0.9 ml 0.5 N trichloroacetic acid (TCA) added. The cell extract was harvested and centrifuged to remove acid-precipitable material. An aliquot of the supernatant was neutralized with tri-n-octylamine and the radioactivity of the sample determined by liquid scintillation counting.

DNA Labeling Measurements

At the same time dTTP specific activity determinations were made, two 100 mm plates were harvested for measurement of acid precipitable counts by: aspirating the medium, adding 1.5 ml of 10% TCA per plate, scraping the plates, and washing them with 0.5 ml of 10% TCA. This acid extract was incubated on ice for at least 20 minutes and the precipitate filtered onto GF/C glass fiber filters. The filters were washed several times with cold 5% TCA, dried and counted.

Specific Activity Determinations

The size and specific activities of the labeled dNTPs were simultaneously determined by a modification of the method used by Eriksson and Nicander (personal communication). Briefly, ^3H nucleoside-labeled whole cell and nuclear extracts prepared following the two-step

extraction procedure outlined by North et al. (1980) were analyzed using the enzymatic assay with the ^{32}P -dNTP complementary nucleotide in place of the ^3H -dNTP. In addition, the following changes were made in the assay: the alternating copolymer template, poly(dI-dC) or poly(dA-dT), was ethanol precipitated before use in order to remove free nucleotide contaminants; and 1 mM dAMP was added to the reaction mixture in order to limit product breakdown (Hunting and Henderson, 1981).

Results

Cell Synchronization

Time points labeled G1 were carried out between 5.5 and 6.5 hours after release of the isoleucine block and experiments with S phase cells were carried out between 12.5 and 13.5 hours after the release of the isoleucine block. Cell cycle analysis was used to confirm that the cells were in G1 phase or S phase.

Acid-Soluble Pool Equilibration

It was important to establish, before attempting to measure the specific activities of the dNTP pools labeled by ^3H -nucleosides, that the labeled nucleosides were in equilibrium with the acid soluble pool. [$5\text{-}^3\text{H}$] Deoxycytidine, [$5\text{-}^3\text{H}$] cytidine, or [$5\text{-}^3\text{H}$] thymidine was added to monolayers of exponentially growing CHO-K1 cells

at $0.3 \mu\text{M}$, a concentration low enough that it should not perturb dNTP pools (Nicander and Reichard, 1983). At 15 minute intervals duplicate plates were acid-extracted and the acid-soluble pools determined. Under these conditions both cytidine and deoxycytidine equilibrated with the acid-soluble pool within thirty minutes and remained in equilibrium for the remainder of the 75 minutes examined (see Fig. 3.1). Thymidine, however, did not reach equilibrium with the acid-soluble pool at $0.3 \mu\text{M}$ concentration. Instead the label was rapidly taken up and turned over, so that after 30 minutes incubation with label the amount of radioactivity had begun to decrease and it continued to decrease throughout the remainder of the 75 minutes the radioactivity was measured. In order to equilibrate the acid-soluble pool with $[5-^3\text{H}]$ thymidine, we increased the concentration of thymidine to $1.0 \mu\text{M}$ and increased the volume of medium on the plates to 5.0 ml. Volume or concentration increases alone did not suffice to equilibrate the labeled thymidine with the acid-soluble pool. The high rate of thymidine metabolism in this particular cell line may be due to the lack of dCMP deaminase, an enzyme which provides a substantial proportion of the dTTP in some cell lines (Jackson, 1978; Nicander and Reichard, 1985).

Cytidine and Deoxycytidine Labeling Results

Figure 3.2 shows the rates of dCTP specific activity equilibration with labeled nucleosides in the nucleus and whole cells of G1 and S phase CHO-K1 cells. In both G1 and S phase cells the [5-³H] cytidine takes about 45 minutes to reach maximum specific activity. [5-³H] Deoxycytidine is nearly equilibrated with the dCTP pool in G1 phase cells after 15 minutes of incubation with the label but in S phase this same equilibration was not completed after 60 minutes.

In all four instances there was no significant difference between the rate or level of dCTP equilibration with the labeled nucleosides between whole cells and nuclei. Although this result was contrary to what we had predicted, using this method of nuclear isolation (Leeds et al., 1985) we see what appears to be a single, equilibrated dCTP pool.

Whole Cell dCTP Specific Activities

When we directly compared the whole cell specific activities (Fig. 3.3) in G1 and S phase for each of the two labeled nucleosides, we saw a striking difference. The specific activities of the dCTP pools labeled by [5-³H] cytidine in G1 and S phases are almost the same, with the dCTP specific activity reached in S phase 38% greater

than that reached in G1 phase. The specific activity of the dCTP pool labeled by [5-³H] deoxycytidine, however, was 13 times larger in G1 than in S phase cells. Possible consequences of this large difference will be discussed.

Thymidine-Labeled Cells

We measured the time necessary for the [5-³H] thymidine to reach an equilibrium with the dTTP in whole cells in G1, S phase, and exponentially growing cells in an attempt to understand cell-cycle variations in the labeling of dNTP pools with nucleosides. In S phase cells (see Fig. 3.4, bottom panel) the dTTP pool reached maximal specific activity between 2 and 4 minutes after addition of label. The G1 phase cells, on the other hand, reached maximal specific activity more slowly than the S phase cells. It took approximately twenty minutes for the dTTP to reach maximal specific activity in G1 phase cells. The rate of dTTP pool equilibration in exponentially growing cells (Fig. 3.4, top panel) appeared to be the sum of the curves found for synchronized cells. There was an initial rapid increase in the dTTP specific activity, which correlated well with the rapid rate of equilibrium in S phase, followed by a slower, smaller increase in the specific activity, which corresponded to that fraction of cells in G1 phase cells.

[5-³H] Thymidine-Labeled DNA

The amount and rate of [5-³H] thymidine which was incorporated into DNA in S phase and exponentially growing cells over the incubation period was measured. S phase cells (Fig. 3.5A) incorporated radioactive label into DNA almost immediately at a linear rate. This is consistent with the rapid attainment of maximal dTTP specific activity in S phase cells. The exponentially growing cells (Fig. 3.5B) also incorporate radioactivity into DNA at a linear rate almost immediately, most likely reflecting the incorporation of dTTP into the DNA of replicating cells.

Discussion

Previous experiments in our laboratory (Leeds et al., 1985) suggested that the dCTP pool in the nucleus and cytoplasm might not equilibrate freely, and results from Nicander and Reichard (1983) indicated that the dCTP pools labeled by radioactive cytidine and deoxycytidine were not equally available for DNA replication. We felt these two observations might be related. To investigate this phenomenon further we synchronized CHO cells and measured the rates of dCTP pool specific activity equilibration in whole-cell and nuclear extracts using [5-³H] cytidine or [5-³H] deoxycytidine as precursors. However, the nuclear and whole cell specific activities were equal at all time

points measured in both G1 and S phase, indicating rapid and homogeneous equilibration between the two compartments. Our prediction had been that any pool unavailable for DNA replication would not be present in the nucleus. We did, however, see a large difference between the specific activities of G1 and S phase cells labeled by [5-³H] deoxycytidine. The specific activity of dCTP labeled by deoxycytidine was 13 times higher in G1 phase cells than in S phase cells. This suggests that the contribution of salvage-derived dCTP decreases relative to the de novo-derived dCTP in S phase cells. The in vivo level of deoxycytidine kinase in CHO cells has been reported to increase eight-fold in S phase cells (Hordern and Henderson, 1982). Other researchers, who used cell extracts to measure deoxycytidine kinase activity throughout the cell cycle, have reported varying degrees of changes in the activities throughout the cell cycle (Brent, 1971; Howard et al., 1974; Wan and Mak, 1978). Regardless of relative changes in enzyme activities, the relative contributions by de novo and salvage pathways to the steady state dCTP pool changes during G1 and S phase of CHO cells.

The large difference in the dCTP specific activities in G1 and S phase cells labeled by deoxycytidine made us suspect that the dCTP pool unavailable for DNA replication observed by Nicander and Reichard (1983) might simply be

the G1 phase dCTP pool. However, because the cell line that we used lacks dCMP deaminase activity, and has a large increase in the dCTP pool during S phase, it is not clear that a correlation can be drawn between our results and those of Nicander and Reichard (1983). Nonetheless, we consider the correlation likely because pools in G1 phase cells would be unavailable for DNA replication.

The results of our experiments with the labeled dCTP precursors led us to postulate that results of other labeling experiments that had been interpreted as indicating intracellular compartmentation (Fridland, 1973; Kuebbing and Werner, 1975) might actually be due to different rates and levels of labeling in G1 and S phase cells. The observation by Fridland (1973), for example, that ^3H -TdR is incorporated into DNA at full specific activity before the dTTP pool reached maximal specific activity might be explained in terms other than intracellular compartmentation if the dTTP pool in S phase cells equilibrated more rapidly than the dTTP pool in G1 phase cells. If this were the case, one would expect to see the dTTP pool continue to increase in specific activity after the rate of DNA labeling had become maximal in exponentially growing cells. When we determined the rates of dTTP pool specific activity equilibration in synchronized cells, we saw what we had predicted: a more rapid equilibration of $[5\text{-}^3\text{H}]$ TdR with the dTTP pool in S

phase cells compared to G1 phase cells. In addition, determination of the rate of [5-³H] TdR equilibration with the dTTP pool in exponentially growing cells showed a rapid equilibration, which corresponded well to the rapid equilibration of the dTTP pool in S phase cells followed by a slower equilibration, which corresponded well to the slower equilibration observed in G1 phase cells. A linear rate of tritium incorporation into DNA was attained at the same time in S phase and exponentially growing cells. This confirms the hypothesis that by labeling exponentially growing cells it could appear that DNA is labeled at a linear rate before the dTTP pool is equilibrated with [5-³H] thymidine.

The results presented here illustrate cell cycle changes in nucleotide pool equilibration that may make it difficult to interpret DNA labeling experiments in exponentially growing cells. Although the experiments were done in only one cell line, our results are capable of explaining observations from a large number of DNA labeling experiments. Scott and Forsdyke (1980) found that although the addition of deoxyguanosine and deoxyadenosine increased their respective dNTP pool sizes, these pools were unable to overcome a block in DNA replication caused by hydroxyurea inhibition. Snyder (1984) found, however, that the deoxynucleosides could overcome blocks in repair replication, which supports our

supposition that the increase in the corresponding dNTP pools is in G1 phase cells. Sadee and Nguyen (1985) did labeling experiments with guanosine and deoxyguanosine in a purine nucleoside phosphorylase (PNP)-deficient cell line, similar to the experiments with cytidine and deoxycytidine done by Reichard and Nicander (1983), that indicated that deoxyguanosine-derived dGTP was not available for DNA replication. It would be interesting to see if the same results occurred in synchronized cells.

There is substantial evidence that activities of dNTP biosynthetic enzymes are cell cycle-dependent and that changes in their activities vary among cell types. Horder and Henderson (1982) measured changes in nucleotide-synthesizing enzyme activities throughout the cell cycle of both CHO and HeLa cells and found differences in the changes between the two cell lines. We propose that changes in the specific activity of a given dNTP pool throughout the cell cycle will be dependent on the particular changes in enzyme activities of the cell line examined. Maybaum et al. (1980) found that the dTTP specific activity was five times higher in G1 than in S phase while the cytidine-derived dCTP specific activity was 50% higher in S phase than in G1 phase. Our results showed that the dTTP specific activity was 50% higher in S phase than in G1 phase and the cytidine-derived dCTP specific activity was 38% higher in S phase compared to G1

phase. Although the changes in cytidine-derived dCTP specific activities are nearly equivalent, there is a substantial difference in the changes in thymidine-derived dTTP specific activities. These results lead us to hypothesize that de novo and salvage nucleoside precursors may differ in the rates and levels at which they are used to form dNTPs, dependent on changes in enzyme levels throughout the cell cycle; and these changes differ from cell line to cell line. Researchers who wish to label cells should be aware of differences in labeling of dNTP pools throughout the cell cycle.

Fig. 3.1. Acid-Soluble Pool Equilibration. Panels show acid-soluble pool equilibration with deoxycytidine (top panel), cytidine (middle panel), and thymidine (bottom panel). Conditions are described in the text.

Fig.3.1

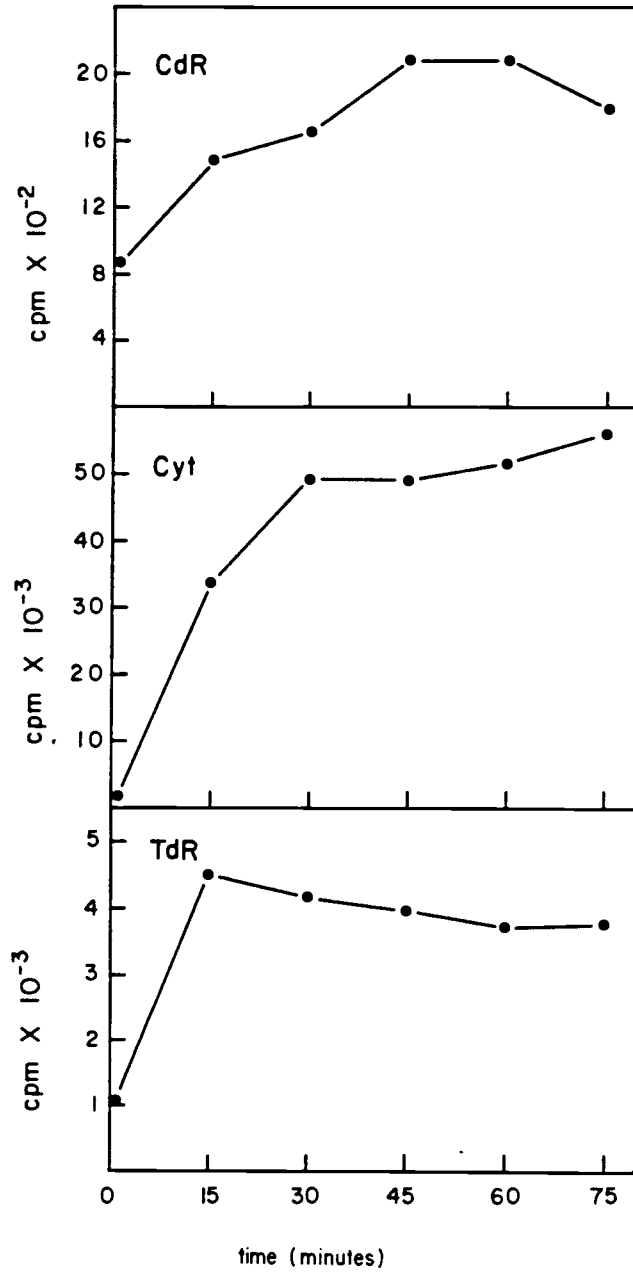


Fig. 3.2. Specific Activity Equilibrations. The rate at which nuclear (\circ, Δ) and whole-cell (\bullet, \blacktriangle) dCTP pools were labeled in G1 and S phase with either cytidine or deoxycytidine as the radioactive nucleoside precursor.

Figure 3.2 Specific Activity Equilibrations

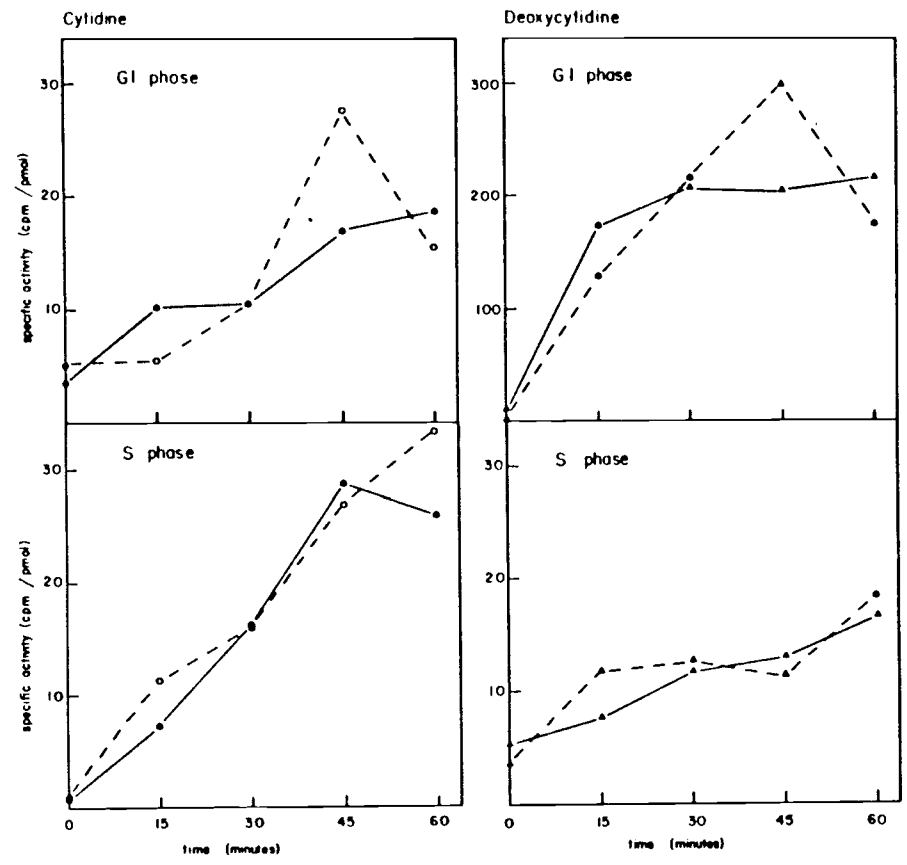


Fig. 3.3. Whole-Cell Specific Activity Equilibrations. A comparison of the rates and levels at which the whole-cell dCTP pools were labeled in G1 and S phase by either cytidine (●, ■) or deoxycytidine (●, ■).

Figure 3.3 Whole-Cell Specific Activity Equilibrations

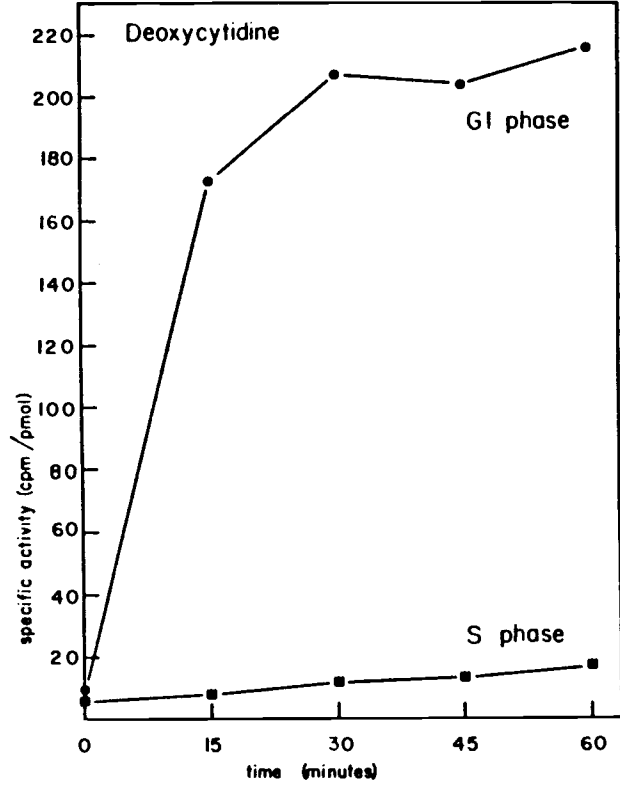
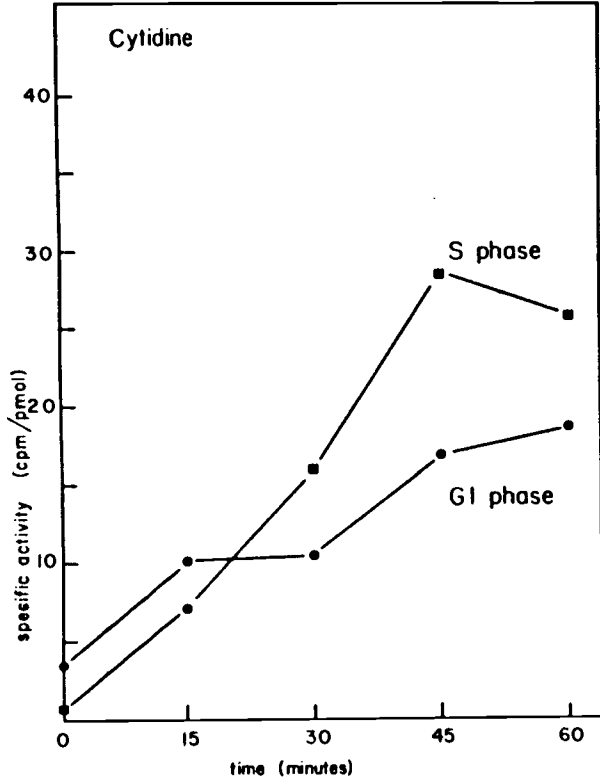


Fig. 3.4. Thymidine Specific Activity Equilibration.
Rate of [5-³H] thymidine equilibration with dTTP whole-cell pool is cell cycle dependent: (A) rate of dTTP pool specific activity equilibration in exponentially growing cells; (B) rate of equilibration in G1 and S phase cells.

Fig.3.4

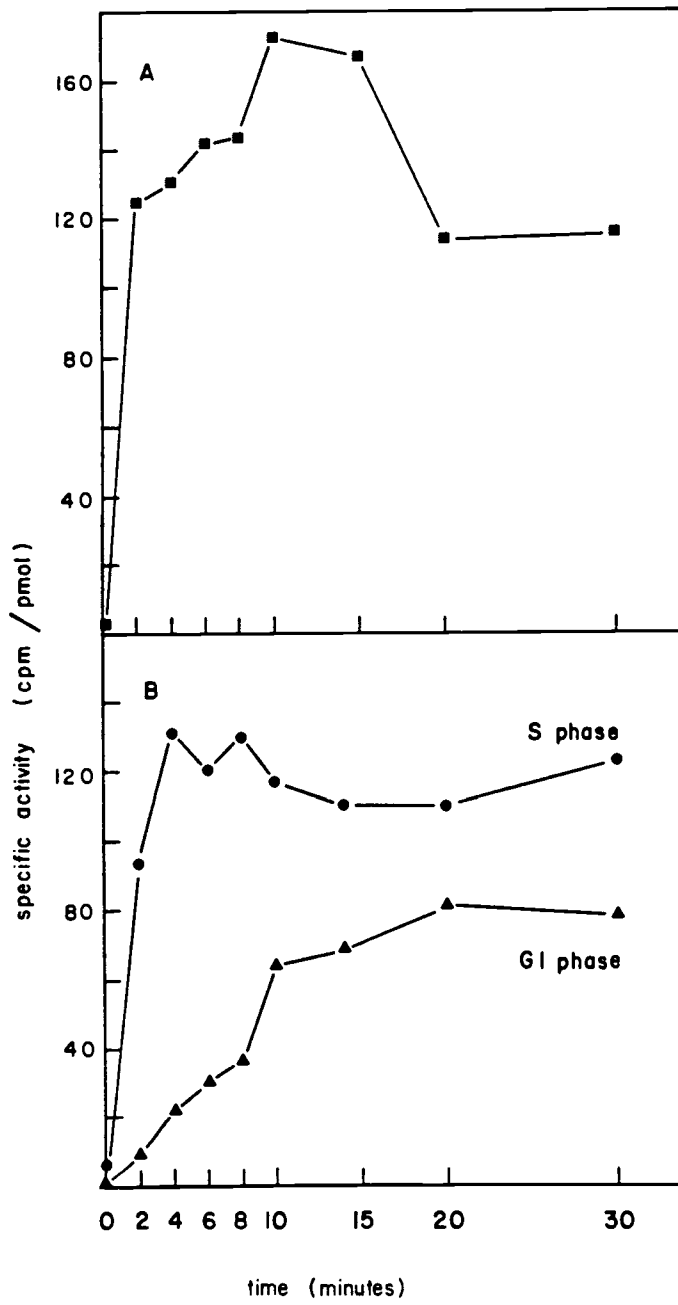
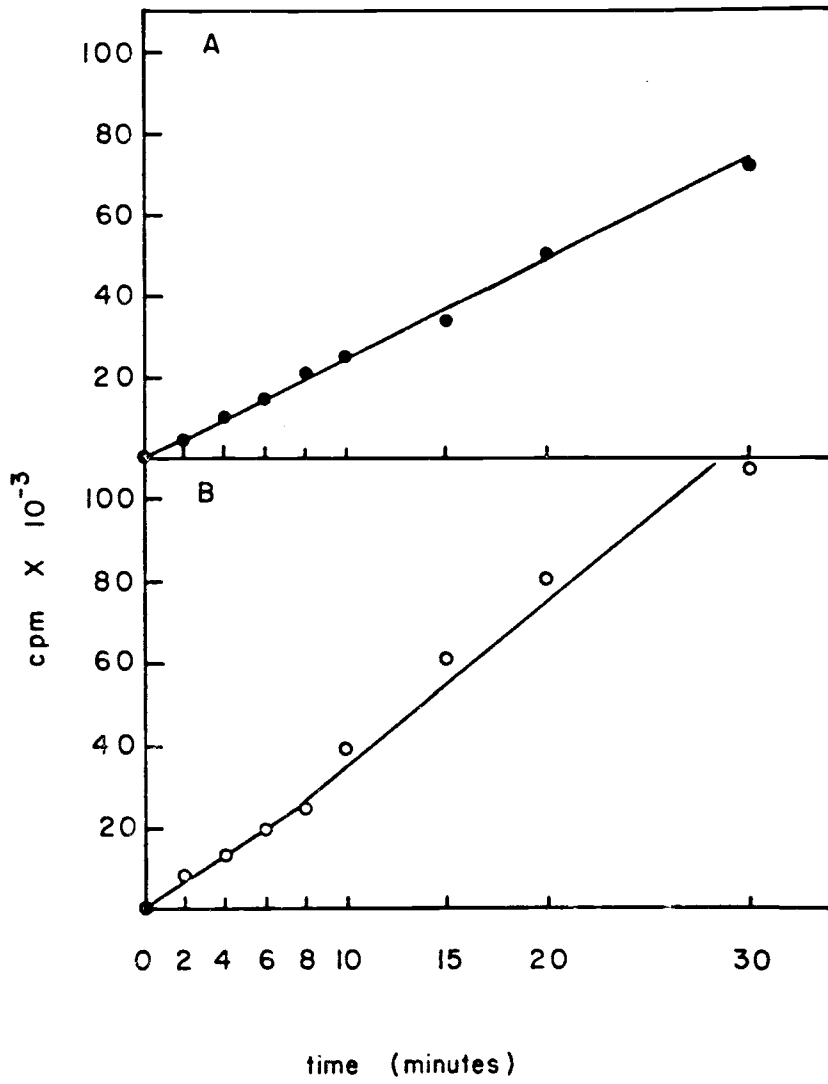


Fig. 3.5. DNA Labeling by Thymidine. Comparison of rates at which DNA labeling by [5-3H] thymidine precursor becomes linear in (A) S phase cells and (B) exponentially growing cells. (Note, absolute amount of label incorporation is somewhat different as slight difference in specific activity of thymidine may have occurred.)

Fig.3.5



Chapter 4: Discussion

The experiments presented in this thesis were done in an attempt to determine if DNA precursors are sequestered in slowly or non-equilibrating compartments in mammalian cells. The basic approach involved using a rapid nuclear isolation procedure, which minimized redistribution of metabolites, to examine the distribution and relative rate of dNTP movement between nucleus and cytoplasm. Although the experimental results indicated that some dNTPs were maintained at different steady state concentrations in the nucleus and cytoplasm, my investigations indicated for each dNTP the existence of a single, rapidly equilibrating, homogeneous pool within the cell. Interestingly, my experiments suggest an alternate explanation for many of the labeling experiments that have been interpreted as indicating compartmentation of dNTP pools in eukaryotic cells.

A. Subcellular Location of Deoxynucleotide-Synthesizing Enzymes

If DNA precursors are channeled to the site of replication in eukaryotes, then enzymes responsible for dNTP biosynthesis must also be localized in the nucleus. In an attempt to localize dNTP biosynthesis Reddy and Pardee (1980), using enucleation to rapidly separate

nuclei from whole cells, found that S phase cells contain a larger proportion of deoxynucleotide-synthesizing enzymes in the nucleus than do G1 phase cells. Mary Slabaugh and I were unable to detect any nuclear-associated ribonucleotide reductase activity (Leeds et al., 1985) when we used synchronized CHO cells and the rapid nuclear isolation technique, under conditions where all of the DNA polymerase alpha activity remained on the plates with the isolated nuclei. Engstrom et al. (1984) found, using immunocytochemical methods, that the M1 subunit of ribonucleotide reductase is located exclusively in the cytoplasmic portion of cultured mouse 3T6 cells, MDBK cells (a baby kidney cell line), and cells from several different rat tissues. Although definitive studies have not been carried out on other enzymes of nucleotide biosynthesis, the cytoplasmic location of ribonucleotide reductase, because it is the branch point for deoxyribonucleotide metabolism, suggests that dNTPs are not synthesized in the nucleus. Without a nuclear location for de novo dNTP biosynthesis it becomes difficult to envision any means of de novo and salvage-derived dNTP pool separation.

B. Subcellular Distribution of dNTPs

In order to measure the nuclear and cytoplasmic dNTP concentrations I used the rapid nuclear isolation

procedure, described in Chapter 2, to isolate nuclear dNTPs from exponentially growing HeLa cells and synchronized CHO cells. The results, presented in this thesis, illustrate the apparent mixing or equilibration of nuclear and cytoplasmic DNA precursor pools. The nuclear dNTP pools of HeLa cells, though distinct for each dNTP, largely reflect the volumes occupied by the nucleus and cytoplasm (Skoog and Bjursell, 1974). The subcellular distribution of dNTPs in CHO cells, with the exception of dCTP, seems also to be according to the volume occupied by the compartments. dCTP, on the other hand, seems, by some mechanism, to be excluded from the same expansion in the nucleus that occurs in the cytoplasm. The small differences between subcellular dNTP distribution in HeLa cells and the volume occupied by the cellular compartments can conceivably be explained by differences in enzyme activities in the two compartments that act to create differences in steady state dNTP levels. The large difference in the subcellular dCTP distribution of S phase CHO cells is more difficult to understand.

C. Rate of dNTP Equilibration between Compartments

Nicander and Reichard (1983), using labeled nucleosides to examine the question of compartmentation, compared the following: (1) the amount of label incorporated into DNA from a dNTP of a known specific

activity; and (2) the true rate of DNA synthesis. They reasoned that if the dNTPs existed in a single pool, then the label incorporated into DNA should reflect the actual rate of DNA synthesis. However, their results indicated that even after equilibration for over an hour, the deoxycytidine-labeled dCTP was not fully available for DNA replication while cytidine-labeled dCTP and thymidine-labeled dTTP were both completely available for DNA replication. If, even after a long period of equilibration with the label, deoxycytidine-derived dCTP was unavailable for DNA replication, I predicted that by following the equilibration of the subcellular dCTP specific activities in synchronized CHO cells, I would see the exclusion of deoxycytidine-labeled dCTP from the nucleus. On the other hand, cytidine-derived dCTP should be equilibrated between the nucleus and cytoplasm, since labeling kinetics had shown this to be fully available for DNA synthesis. The results of the nucleoside labeling experiments, presented in Chapter 3 of this thesis, showed no indication of any restrictions to equilibration between the nucleus and cytoplasm. An interesting observation was that the deoxycytidine-derived dCTP specific activity was 13 times higher in G1 than in S phase cells. I felt that this was potentially important since G1 phase dNTP pools would, of course, be unavailable for DNA replicative synthesis.

Nicander and Reichard (1983) obtained only one-third the true DNA synthetic rate when they calculated the rate according to the amount of deoxycytidine-derived dCTP that entered DNA. If the dCTP specific activity reached in S phase was only one-third of the dCTP specific activity of G1 phase cells when radiolabeled deoxycytidine was used as the precursor, then only one-third the measured dCTP specific activity would be available for DNA synthesis. This could explain the anomalous apparent rate of DNA synthesis. One might predict that accurate DNA synthetic rates will be obtained from studies with exponentially growing cells only when the specific activity of the radiolabeled dNTP pool in S phase is greater than or equal to the specific activity of the pool in G1 phase.

To further investigate the possibility that the phenomenon, which had been interpreted as compartmentation of nucleotides, was actually the result of cell cycle changes in nucleotide metabolism, I examined the labeling kinetics of thymidine in G1, S phase, and exponentially growing cells. I predicted that the dTTP pool would turn over, and hence equilibrate with radiolabeled thymidine, at a much faster rate in S phase than G1 phase. Exponentially growing cells would equilibrate at a rate, and to a level, which would be a combination of the rates and levels of different phases of the cell cycle. Labeling of DNA would become linear as soon as the dTTP

pool in S phase cells had equilibrated with thymidine. In fact I did observe that the S phase dTTP specific activity equilibrated with exogenous [5-³H] thymidine faster than was seen with G1 phase cells. The rate at which DNA labeling became linear was identical in S phase and exponentially growing cells even though the rates of dTTP specific activity equilibration differed. If I had measured the rate of DNA labeling and dTTP specific activity equilibration with exponentially growing cells and not synchronized cells, I would have concluded that two dTTP pools existed, one rapidly equilibrating and available for DNA replication and a second, more slowly equilibrating and not used for DNA replication.

Is it possible, then, that other experimental results which suggested separate, non-equilibrating intracellular pools were misinterpreted and actually due to the use of a non-homogeneous population of cells? In fact, the nucleoside labeling studies whose results were supportive of the compartmentation hypothesis (Fridland, 1973; Kuebbing and Werner, 1975; Nicander and Reichard, 1983; Scott and Forsdyke, 1983; and Snyder, 1984) were all carried out using exponentially growing cells and thus the potential for misinterpretation existed. Questions, however, remain as to several differences in the results from these studies. Although the results from Kuebbing and Werner (1975) indicated that salvage-derived

nucleotides were in rapid equilibrium with the replication pool and were able to by-pass the de novo pool, the results from Scott and Forsdyke (1983) and Snyder (1984) indicated that salvage-derived dNTPs were unavailable for DNA replication. Nicander and Reichard (1983) showed that salvage-derived dTTP but not salvage-derived dCTP was available for DNA replication.

These apparent differences may be resolved by using the following model. Cell cycle variations in the activities of nucleotide-metabolizing enzymes vary among cell lines and hence the relative contribution to the steady state dNTP pool by salvage or de novo-derived dNTPs depends on the cell line being examined. The specific activity obtained with a labeled nucleoside will depend on the combination of dNTP biosynthetic activities in G1 or S phase cells present in that particular cell line. Salvage-derived dNTPs would appear to be unavailable for DNA replication in instances where the specific activity of the labeled pool was higher in G1 phase than in S phase. Results would indicate that the salvage-derived pools bypass de novo-synthesized dNTP pools when cells in S phase equilibrated more rapidly than G1 phase cells with the exogenously added nucleoside. Evidence in support of this model includes the work of Horder and Henderson (1982), who measured the in vivo activities of nucleotide metabolizing enzymes throughout the cell cycle of

synchronized HeLa and CHO cells and found significant differences between the changes in activities seen in the two cell lines. Maybaum et al. (1980) found cell cycle changes in the specific activities of thymidine-labeled dTTP, which was five times higher in G1 than S phase, and cytidine-labeled dCTP, which was 50% higher in S than G1 phase. Our results with cytidine-labeled dCTP were similar to those obtained by Maybaum et al. (1980) but our results with TdR-labeled dTTP were significantly different. This provides further evidence that cell cycle variations in nucleotide synthesizing enzymes vary among cell types; however, confirmation of our model requires studies of the changes in labeling and enzyme activities throughout the cell cycle of several different cell lines.

In conclusion, the experimental results in this thesis do not support DNA precursor compartmentation in eukaryotic cells. The pivotal enzyme in de novo dNTP biosynthesis, ribonucleotide reductase, appears to be located in the cytoplasm, dNTPs are not concentrated or synthesized in the nucleus, and close examination of dNTP pools in synchronized cells reveals that pyrimidines, at least, have only one rapidly equilibrating cellular pool.

Chapter 5: Bibliography

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APPENDICES

Chapter 6: AppendicesAbbreviations Used

PEI	polyethylene imine
TLC	thin layer chromatography
MeOH	methanol
TCA	trichloroacetic acid
PCA	perchloric acid
CHO	Chinese Hamster Ovary
dNTP	deoxyribonucleoside triphosphate
rNTP	ribonucleoside triphosphate
ATP	adenosine triphosphate
CTP	cytidine triphosphate
UTP	uridine triphosphate
dTTP	deoxythymidine triphosphate
TdR	thymidine
FdUMP	5-fluorodeoxyuridylate
U	uracil
C	cytosine
A	adenine
G	guanine
T	thymine
CdR	deoxycytidine
Ar	adenosine
Gr	guanosine
UdR	deoxyuridine
AdR	deoxyadenosine
GdR	deoxyguanosine
NMP	nucleoside monophosphate
NDP	nucleoside diphosphate

Controls for dNTP enzymatic assay

The results in this thesis depended on the ability to accurately and reproducibly quantitate dNTPs. Earlier work in our laboratory (North et al., 1980) showed that nucleotide extracts prepared using a single extraction step, either 60% methanol (MeOH) or 5% perchloric acid (PCA), still contained enzymatic activities which interfered with the enzymatic dNTP assay. North et al. (1980) combined the 60% MeOH extraction with the PCA extraction to obtain a nucleotide extract that was relatively free of the interfering enzyme activities. In my hands, however, the PCA extraction caused occasional interference in the enzymatic dNTP assay.

To determine the most accurate and reproducible extraction method I prepared extracts from HeLa cells using five different extraction procedures: (1) 60% MeOH; (2) 0.5N trichloroacetic acid (TCA); (3) 0.5 N TCA followed by 60% MeOH; (4) 60% MeOH followed by 0.5N TCA; and (5) 60% MeOH followed by 0.5 N PCA. [Note: Tri-n-octylamine was used to neutralize acid extracts rather than KOH because it was a more reproducible neutralization.] The rNTP and dNTP values from the five different extracts were compared with results previously obtained in our laboratory. I also measured the level of

interfering activities present in each of the five extracts by assaying the extracts in the presence of 20 picomols of standard dNTP.

The resulting rNTP values, determined by HPLC, are shown in Table 6.1. The TCA/MeOH extraction gave very low rNTP values, indicating either triphosphate degradation or incomplete extraction. This was also evident in the corresponding dNTP values. Methanol and MeOH/TCA extractions gave the highest rNTP values and were nearly identical. The rNTP values in the TCA and MeOH/PCA extracts were also close to each other but slightly lower than the rNTP levels measured in MeOH and MeOH/TCA extracts, indicating some degradation or incomplete extraction by TCA and MeOH/PCA extraction procedures. In this study MeOH and MeOH/TCA extraction gave the highest recovery of triphosphates. HPLC analysis is a useful tool for evaluating which extraction method gives the highest recovery but cannot identify methods that inadequately remove interfering enzymatic activities; to do that one must examine values measured using the enzymatic dNTP assay.

The resulting dNTP levels are shown in Table 6.2. The results agree with those of North et al. (1980) in that the methanol extract appears to have larger pools than the other extracts. North et. al. showed that these

large values are due to enzymatic activities present in the extracts that interfere in the enzymatic assay. The TCA/MeOH extracts had lower levels of dNTPs than the other extracts. The remaining extraction methods did not differ significantly in the resulting dNTP levels.

Table 6.3 shows the dNTP values measured before and after adding 20 picomols of a standard dNTP to each of the five extracts. The results clearly show that neither MeOH nor TCA extraction allowed accurate quantitation of dNTP levels in a sample. The addition of 20 picomols of dATP to the MeOH extract added only 7.5 picomoles to the apparent dATP value. Twenty picomols of dCTP added to the MeOH extract raised the apparent dCTP value by only 5.7 picomols while the apparent dCTP value of the TCA extract increased by 6.9 picomols. Of the five different extraction methods examined MeOH/TCA most reliably measured the 20 added picomols of standard.

Before beginning extraction of nucleotides from CHO cells I compared the time course of the enzymatic assay using extracts prepared using methanol and MeOH/TCA. The results (see Fig. 6.1) show that MeOH extraction leaves activities which degrade the alternating copolymer template, poly (dA-dT). In addition, the two-step extraction procedure gave higher apparent dCTP, dTTP, and dGTP values for CHO cells, indicating that the MeOH

extracts of CHO cells contained some activity that prevented accurate determination of dNTP levels.

Nuclear Isolation Procedure

A. Results of V79 Cell Nuclear Isolation Attempt

CHO and HeLa cell nuclei were reproducibly and quantitatively isolated in these studies. This procedure, however, cannot be used with all monolayer cells. Studies with a hamster lung fibroblast cell line (V79) showed that ten seconds of incubation with the NP-40-containing nuclear isolation buffer removed 11% of the whole cell DNA. Thirty seconds of incubation in the buffer removed 27% of the whole cell DNA. Similar studies with CHO and HeLa cells did not reveal any loss of whole cell DNA. Visual inspection of the V79 cells under the phase contrast microscope revealed that a substantial proportion of nuclei did not remain attached to the plate. Thus, this nuclear isolation method may be useful in only a limited number of cell lines.

dCMP deaminase +/- cell lines

A. dCMP deaminase assay procedure (Langelier et al., 1978).

The incubation mixture contained, in a total volume of 40 μ l: 25 mM Tris·HCL, pH 8.0, 1 mM MgCl₂, 20 μ M dCTP,

1 mM dCMP, plus [5-³H]dCMP (7X 10⁵ cpm) and cell extract (100-400 µg of protein). This mixture was incubated at 37°C for 15 minutes and stopped by immersing the tubes in boiling water for 2 minutes. The solution was centrifuged to remove debris and an aliquot of the supernatant along with dCMP and dUMP markers was spotted on water-washed PEI cellulose. The TLC system used was the following: 15 minutes in 1 N acetic acid followed by 40 minutes in 0.3 M LiCl. After air-drying the chromatograms were examined under UV-light, cut up, and the dCMP and dUMP spots counted in the liquid scintillation counter.

B. Results of dCMP deaminase measurements

I had CHO-K1 cells from three different sources: Mark Meuth, Imperial Cancer Research Fund, Mill Hill Laboratories, England; Chick Newman, Battelle Laboratories, Richland, Washington; and the American Type Culture Collection (ATCC). Although all the cells were nominally the same cell line there were detectable differences. Cells provided by Mark Meuth attained the highest degree of synchrony after isoleucine deprivation when examined on the cell sorter. For that reason I used cells provided by Mark Meuth for synchronized cell pool measurements.

The unusually high dCTP pool in the synchronized CHO cells led me to examine the level of dCMP deaminase in the

CHO cells provided by Mark Meuth as well as the cells from the other sources. The results, in Table 6.4, show that the CHO cells from the ATCC as well as those obtained from Mark Meuth had no measureable dCMP deaminase activity. The CHO cells provided by Chick Newman; however, had a very small amount of dCMP deaminase activity. The level of activity in BSC-40 cells, a cell line known to have dCMP deaminase activity, was much greater.

After determining that the small amount of dCMP deaminase activity in the cells from Chick Newman was not due to contamination by mycoplasma, I investigated the possibility that the presence of dCMP deaminase might alter the dCTP pool subcellular distribution. Extracts from isolated nuclei and whole cells of the CHO cells provided by Mark Meuth and Chick Newman were compared. The proportion of dCTP in the nuclei of these exponentially growing cells (Table 6.5), although a combination of G1 and S phase pools, was 14% in CHO cells from both sources. Thus, it appears that the presence or absence of dCMP deaminase activity does not affect the subcellular distribution of dCTP in CHO cells. However, because the level of dCMP deaminase activity in the CHO cells was so low, one cannot preclude the possibility that the distribution may differ in cells with a large amount of the enzymatic activity.

Studies with Isolated Mitochondria

One project I initially spent a great deal of time on was an analysis of dNTP metabolism in mitochondria, which had been studied previously in our laboratory by Richard Bestwick. I concentrated my efforts on attempting to identify deoxynucleotide metabolizing enzymes in specific association with mitochondria.

A. Mitochondria Isolation Procedure

HeLa spinner cells were collected by centrifugation and the medium removed. The cells were resuspended in approximately six cell volumes of hypotonic buffer (10 mM Tris-HCl, pH 6.7 at 25°C, 10 mM KCl, and 0.15 mM MgCl₂). After the cell suspension was incubated on ice for five minutes the cells were lysed by Dounce homogenization (usually five or six downward strokes with the pestle). After visual inspection of the sample to check that the cells were indeed lysed, the nuclei and any unbroken cells were pelleted by low-speed centrifugation in a table top clinical centrifuge for 3 minutes. The recovered supernatant was centrifuged for 15 minutes, at 27,000 X g, to pellet mitochondria. The isolated mitochondria were resuspended in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 0.15 mM MgCl₂. Remaining nuclei and unbroken cells were removed by a second low-speed centrifugation in the clinical centrifuge. The mitochondria were again pelleted

by centrifugation at 27,000 X g for 15 minutes. At this point the isolated mitochondria were washed by resuspending and centrifuging them three times in the following buffer: 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 3 mM EDTA.

B. Determination of Mitochondrial Recovery and Purity

Citrate synthase, a mitochondrial matrix enzyme, was used to determine the recovery of intact mitochondria. The assay procedure was the same used by Srere (1969). Lactate dehydrogenase, a soluble cytoplasmic enzyme, was used to determine soluble cytoplasmic contamination and was assayed as described by Schwert and Winer (1959).

The original criteria used by Richard Bestwick (Richard K. Bestwick, Ph. D. Thesis, Oregon State University, 1982) for determination of mitochondrial recovery was to measure the level of cytochrome c oxidase activity. Cytochrome c oxidase is an inner membrane associated activity. A comparison of the recovery of cytochrome c oxidase with that of citrate synthase revealed that the mitochondrial isolation procedure used by R. Bestwick, in my hands, did not yield intact mitochondria. That is, the mitochondria as isolated had 99% of the whole cell level of cytochrome c oxidase activity but only 3% of the citrate synthase activity. By using the mitochondria isolation procedure from the lab of

Guessepi Attardi (personal communication) I recovered identical proportions of the whole cell cytochrome c oxidase and citrate synthase activity in the mitochondrial fraction. The "Attardi" procedure was routinely used for isolation of intact mitochondria.

C. Thymidylate Synthase Assay Procedure

This assay procedure was taken from the thesis of Richard Mosher (Ph. D. Thesis, University of Arizona, 1978). Three milligrams of dl,L-tetrahydrofolate was added to a solution consisting of 1 ml of 1 M Tris-HCl, pH 7.4, 8.5 ml double distilled H₂O, and 22 ul of a 37% solution of formaldehyde. The tube was placed in a sonicating water bath until the FH₄ completely dissolved. Seventy ul of 2-mercaptoethanol as well as 0.5 ml of 1.0 M NaF were then added to the solution. Enough [5-³H] dUMP was added to a 1.5 ml centrifuge tube to give the undiluted reaction mixture a specific activity of 40 cpm/picomol and the solution taken to dryness under nitrogen gas. The thymidylate synthase assay mixture was added to the tube with enough 10 mM dUMP stock solution to give a final dUMP concentration of 50 μM. The assay mixture was added to the cell or mitochondrial extract in a 1:1 ratio and incubated for 15 and 30 minutes at 37°C. At the end of the incubation period an equal volume of 15% charcoal (Norite A) in 4% PCA was added to each tube. The

samples were vortexed at room temperature and spun for 3-4 minutes in a microfuge. An aliquot of the supernatant was counted in the liquid scintillation counter to determine the radioactivity.

D. Results of Thymidylate Synthase Measurements

Using the Attardi lab mitochondrial isolation procedure, I routinely recovered 1.4% of the whole cell level of thymidylate synthase with the mitochondrial fraction (see Table 6.6). Subtracting the level of cytoplasmic contamination (0.4%) from the recovery of thymidylate synthase associated with the mitochondria left about 1% of the total cellular activity associated with the mitochondria. In an effort to determine whether this activity was non-specifically bound to the mitochondrial membrane, I washed (resuspended and pelleted) the mitochondrial in a buffer made 20 mM $MgCl_2$. This removed only a small amount of the thymidylate synthase activity. In a separate experiment isolated mitochondria were divided into four equal samples and subjected to the following treatments. One sample was sonicated to break open the mitochondria and debris pelleted by brief centrifugation. (In all cases the supernatant was assayed for thymidylate synthase activity.) A second sample was sonicated but the debris was not removed. The third sample was not sonicated but the mitochondria were

subjected to centrifugation. The fourth sample was neither sonicated nor spun to pellet debris. The results (Table 6.7) show that the sample, as typically prepared, gave the highest level of activity. The sonicated but not spun sample gave the next highest level of activity. Supernatant from unsonicated mitochondria that had been pelleted had very little activity. However, unsonicated mitochondrial in suspension had nearly the same level of activity as the sonicated sample. The samples were all sensitive to inhibition by 5-fluorodeoxyuridylate (FdUMP). The fact that the unsonicated, unspun sample had measureable thymidylate synthase activity was surprising because rat liver mitochondria (Cybulski and Fisher, 1981) do not take up reduced folates.

The results do not localize the thymidylate synthase activity to the inside of the inner mitochondrial membrane or the mitochondrial matrix. The result showing thymidylate synthase activity in unlysed intact mitochondria could be due to uptake of reduced folates by HeLa cell mitochondria, the specific association of thymidylate synthase activity with the outside of the inner mitochondrial membrane, or the nonspecific association of thymidylate synthetase with the isolated mitochondria. The association of the enzyme activity with the mitochondria is quite reproducible but at this time

there is no feasible way to further characterize the specificity of the association.

D. Attempts to Measure Dihydrofolate Reductase Activity

My aim in studying isolated mitochondria was to determine the source of mitochondrial DNA precursors. One possibility was that dNTP synthesizing enzymes were specifically located in the mitochondria. At the same time I was measuring levels of thymidylate synthase associated with the isolated mitochondria I was attempting to determine if there was any measureable dihydrofolate reductase. Since rat liver mitochondria had been shown incapable of taking up reduced folates (Cybulski and Fisher, 1981) while Chinese hamster ovary cells had a large amount of serine hydroxymethyltransferase (Taylor and Hanna, 1982), an enzyme which requires a reduced folate cofactor, and yeast had been shown to contain mitochondrion-associated dihydrofolate reductase (Zelikson and Luzzati, 1977), it seemed reasonable to assume that mammalian mitochondria had this enzyme activity. However the available assays for DHFR were not sensitive enough to measure the expected level of activity. By predicting that the DHFR activity, if present, might be no more than 1% of the whole cell activity, I knew the sensitivity needed in an assay. The conventional spectrophotometric assay for DHFR was definitely not sensitive enough to meet

my needs. I tried two different approaches to developing a more sensitive DHFR assay.

The first attempt at developing a more sensitive DHFR assay was to set up a "coupled assay" using the thymidylate synthase assay. That is, the radiometric assay of ^3H -release after methylation of dUMP could become dependent on DHFR activity by the replacement of FH_4 with FH_2 and the inclusion of NADPH in the reaction mixture. I believed that since I could measure thymidylate synthase activity associated with the mitochondria I ought to be able to measure activities coupled with this assay. The apparent activity of the coupled assay was only 60% of the thymidylate synthase activity from an equal amount of cell extract. The coupled activity was inhibited 88% by $0.5 \mu\text{M}$ Methotrexate, a potent inhibitor of DHFR activity, while the thymidylate synthase activity was only inhibited 23%. The level of mitochondrial activity, however, was too close to background to be considered significant.

My second attempt at developing a more sensitive assay for DHFR was to modify a direct radiometric assay described by Rothenberg et al. (1980). Those researchers used ^3H -folic acid, which they reduced in vitro to FH_2 with sodium dithionite. After incubation with DHFR or cell extract the radioactive substrate was separated from the FH_4 produced by co-precipitation of remaining folic

acid and FH_2 with zinc sulfate. Presumably the only remaining radioactivity in the supernatant was FH_4 . After optimizing conditions for the reduction of folic acid to FH_2 I attempted to work out conditions for the radiometric DHFR assay. In addition to the precipitation technique for separation of substrate and product, I used thin-layer chromatography (Scott, 1980). Preliminary assays using the precipitation method with whole cell extracts resulted in a background of over 5,000 cpm while the whole cell activity after 10 minutes incubation left 18,000 cpm in the supernatant, including background. This activity did increase with time and was inhibited by methotrexate. Unfortunately, if mitochondria had 1% of the whole cell DHFR activity an assay of mitochondria would have only resulted in roughly 150 cpm over the 5000 cpm background. Although the thin-layer chromatographic separation of the products and substrates from the reaction from the reaction worked well, the assay was not nearly sensitive enough to detect predicted levels of mitochondria associated activity.

E. Attempt to measure mitochondrion-associated ribonucleotide reductase activity

Mary Slabaugh assayed whole-cell and mitochondria extracts, which I had prepared, for ribonucleotide reductase activity. We measured approximately 3% of the

whole-cell activity associated with the mitochondria. Although 1.0 mM dATP inhibited 96% of the whole-cell activity, the mitochondrion-associated activity was stimulated nearly two-fold. Because of the high concentration of mitochondria, approximately 10^8 cell-equivalents per ml, we decided to remove the endogenous nucleotides from the protein by an ammonium sulfate precipitation step. Cytoplasmic and mitochondrial fractions were subjected to 45% ammonium sulfate saturation, the precipitate collected, dissolved, and dialyzed. Less than 1% of the whole-cell level of ribonucleotide reductase activity was recovered from the mitochondrial ammonium sulfate precipitate, less than the recovery of lactate dehydrogenase, the determinant of cytoplasmic contamination. However, the mitochondrion-associated ribonucleotide reductase activity was still stimulated two-fold by 1 mM dATP. No further experiments were conducted.

Table 6.1 rNTP Values from Five Extraction Methods.
 rNTP values obtained using five different extraction
 methods compared to results previously obtained in this
 laboratory.

Method	rNTP Values (nmols/10 ⁶ cells)			
	ATP	UTP	GTP	CTP
Previous Result	3.12	1.57	0.37	0.67
MeOH	27.1	11.2	2.2	6.4
TCA	15.5	8.3	1.8	4.8
TCA/MeOH	1.4	0.7	0	0
MeOH/TCA	26.2	10.5	3.8	5.7
MeOH/PCA	21.3	8.3	1.8	4.8

Table 6.2 dNTP Values from Five Extraction Methods. dNTP levels obtained using five different extraction procedures compared to values previously obtained in this laboratory.

Method	dNTP Values (pmols/10 ⁶ cells)			
	dATP	dTTP	dCTP	dGTP
Previous result	130	95	20	8
MeOH	>350	311	265	36
TCA	92	161	129	10
TCA/MeOH	60	86	99	2
MeOH/TCA	135	224	96	13
MeOH/PCA	92	159	137	38

Table 6.3 Standards Added to Extracts. Increase in measured dNTP values after addition of 20 pmols of standard dNTP to extract.

Method	dNTP Values (pmoles/sample)			
	dATP	dTTP	dCTP	dGTP
MeOH	71.2	9.8	26.5	2.3
+ 20 pmols	78.7	33.0	32.2	24.7
increase	7.5	23.2	5.7	22.4
TCA	6.9	9.7	12.3	0.4
+ 20 pmols	26.3	33.5	18.6	16.9
increase	19.4	23.8	6.9	16.5
TCA/MeOH	4.8	5.2	7.9	0.6
+ 20 pmols	26.5	27.6	40.0	21.7
increase	21.7	22.4	32.2	21.1
MeOH/TCA	9.8	17.1	8.0	1.1
+ 20 pmols	28.8	36.8	27.6	18.8
increase	19.0	19.7	19.4	17.7
MeOH/PCA	7.3	12.3	7.9	2.6
+ 20 pmols	27.1	35.8	55.6	25.3
increase	19.8	23.5	47.7	22.7

Fig.6.1. Extraction Methods Compared. Comparison of MeOH (O) and MeOH/TCA (●) extraction methods on resulting CHO cell dNTP pools.

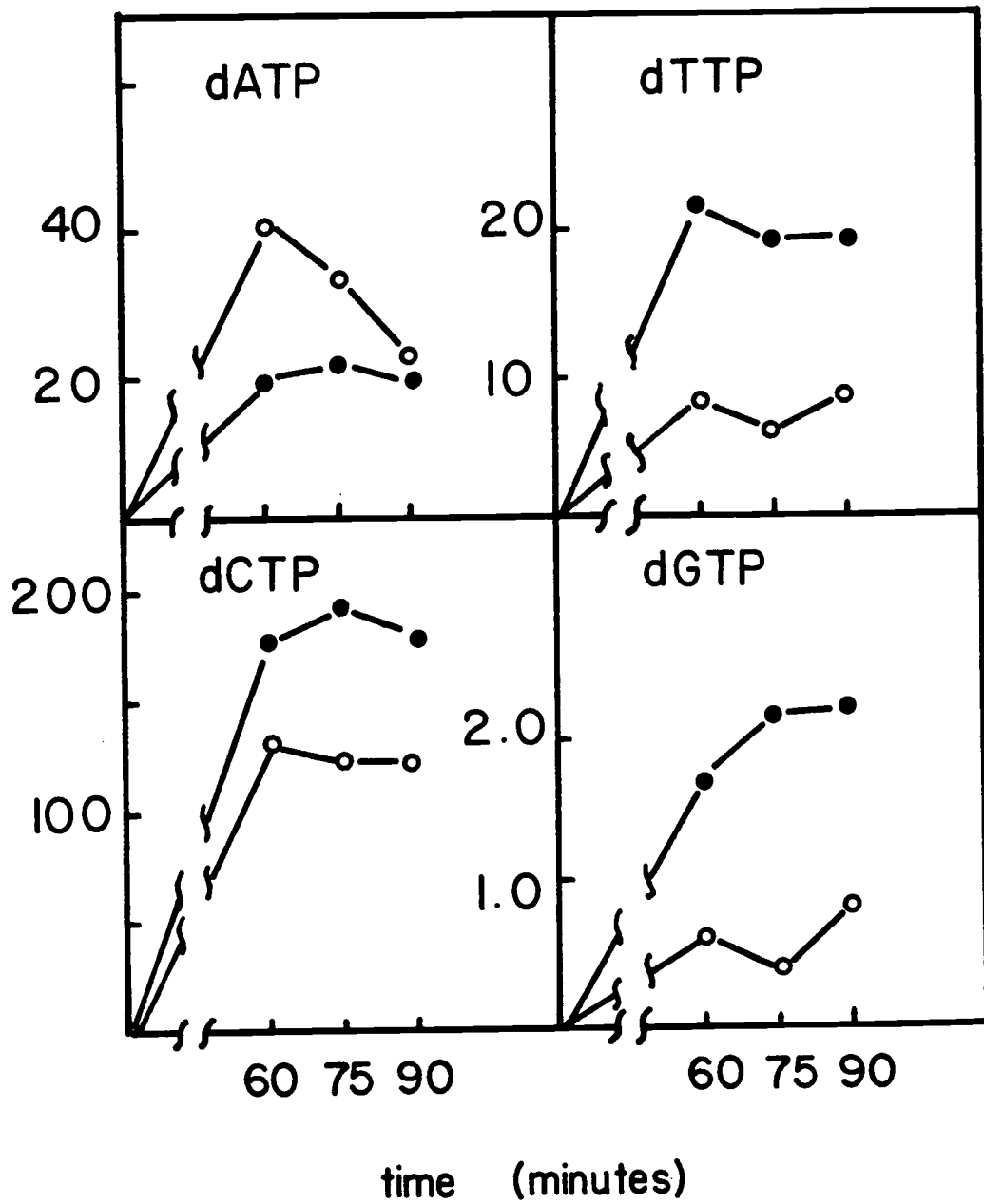


Table 6.4 dCMP Deaminase Activity. Comparison of dCMP deaminase activity among CHO cells from three different sources and BSC-40 positive control.

<u>Time</u> (minutes)	<u>dCMP Deaminase Activity</u> (cpm/spot-blank)			
	<u>Cell Type</u>		<u>CHO-K1</u>	<u>ATCC</u>
	BSC-40	M.M.	<u>C.N.</u>	
0	326	148	90	0
15	4938	36	616	0
30	7685	46	999	0

Table 6.5 CHO Cell dCTP Pool Distribution. Comparison of whole cell/nuclear dCTP pool distribution in exponentially growing CHO cells from Mark Meuth and Chick Newman.

<u>dCTP Pool Distribution</u>		
<u>Sample</u>	<u>pmols/10⁶ cell-equivalents</u>	<u>% Nuclear</u>
Whole Cells		
-Mark Meuth	357	
Nuclei	53	14.8
Whole Cells		
-Chick Newman	773	
Nuclei	111	14.0

Table 6.6 Mitochondrial Thymidylate Synthase Activity. Typical activity from whole cell extracts and isolated mitochondria and activity in the presence of 0.5 uM FdUMP.

Mitochondrial Thymidylate Synthetase

<u>Sample</u>	<u>10⁶ $\frac{\text{pmols/minute}}{\text{cell-equivalents}}$</u>	<u>% Whole Cell</u>
Whole Cells	30.9	
+ FdUMP	0.80	
Mitochondria	0.43	1.4
+FdUMP	0.05	

Table 6.7 Thymidylate Synthase Activity after Four Different Treatments. Thymidylate synthase activity of mitochondria treated in four different ways.

treatment	<u>Thymidylate synthetase</u>	
	<u>pmols/min</u>	
	<u>10⁶ cell-equivalents</u>	
1. Sonicated, Spun	0.43	
2. Sonicated, Unspun	0.38	
3. Unsonicated, Spun	0.07	
4. Unsonicated, Unspun	0.34	